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REVIEW

MODERN INDUSTRIAL AND PHARMACOLOGICAL APPLICATIONS OF INDIGO DYE AND ITS DERIVATIVES – A REVIEW

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Abstract: Plant sources, chemical properties, bioactivities, as well as the synthesis of indigo dye and its derivatives, are reviewed in this paper. These compounds were chosen because of their significant benefits and scope of application as both coloring agents in the textile industry and as pharmacologically active natural products. Their use in traditional chinese medicine (TCM) has directed the attention of European researchers and medical doctors alike. The preparation of indigoferous plants – *Indigo naturalis* is currently about to be introduced into the European Pharmacopoeia.

Keywords: indigo dye, traditional chinese medicine, indigoferous plants, indirubin, *Indigo naturalis*

Dyes have been used throughout history for various esthetic and later practical applications. Primarily used as coloring agents in the cotton and textile industries to dye materials, their importance and our understanding of these substances has grown, leading to their increased use across industries and science alike.

Up to the turn of the nineteenth century, all coloring agents were obtained from natural sources, originating from plants, lichens, insects and molluscs. Following the industrial era of the 1800's, natural dyes began to be chemically synthesized (1).

Currently, dyes are used widely and play an important role in modern electronics, from the printing industry, where dyes are used in electrophotographics (laser prints and photocopies), to medical applications, where dyes may be used to cure diseases and treat ailments (2). Probably the oldest and most famous dye is indigo, which has an intense dark blue color. The name 'indigo" is derived from a Greek word '*indikón*" meaning 'Indian". It can be traced back to ancient Asian civilizations, and further west across Europe particularly in ancient Greece and Rome. At that time, dyes were imported from the Indian subcontinent as a highly valuable commodity. Since the 'middle ages", indigo has been imported across Europe in large quantities making it readily available.

Indigo remains the main component of *Indigo naturalis*. Indigo derivatives have different shades and colors: yellow, green, brown and violet. Together with indirubin (red) and their derivatives, they are the constituents of a preparation called *Indigo naturalis*.

In Chinese medicine, it is commonly used as a heat remover to treat various ailments. The preparation known as 'Qing Dai" may be produced from indigo plants like *Polygonum tinctorium* Ait., *Isatis tinctoria* Ait. and *Baphicacanthus cusia* Brem. Currently, its synthesis is predominantly chemical to cater for its large demand (3).

PLANT SOURCES

One particular species of indigo plant is *Polygonum tinctorium* Ait., an annual perennial from the Polygonaceae family. The species" habitat resides predominantly in China and India. Its Latin name is derived from the type of knotweed (*Polygonum*) and recently it has been classified as a species which belongs to the *Persicaria* genus (4). The species can reach up to 80 cm in height. Its stem usually has a reddish color and is characterized by leaves which are narrowed at the base and are embedded on stalks. This plant is distinguishable by its pink or red flowers with white perianths. These

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flowers are grouped together in the form of short, thick ears, forming a loose flower panicle. In addition, this plant has compact, egg-shaped bundles in leaf axils and a fruit which forms a shiny nut (5).

Baphicacanthus Cusi (Nees) Bremek., syn. *Strobilanthes Cusi* (Nees) is another plant species from which indigo is extracted. Kuntze is a perennial plant reaching 60 cm in height. It grows in clay and wet soils and is tolerant to different soil pH levels. The species blooms well in either partial or complete shade and has oval-shaped leaves and hermaphrodite flowers (6).

Isatis indigotica Fort. is a species, commonly known as 'Woad", whose habitat is found in steppe areas of the south-eastern Europe, the Caucasus and Asia Minor. Today, this species can be found in wild crops across almost all of Europe and in parts of Algeria and Morocco (7).

This species can reach a height of 50–140 cm and is distinguishable by its characteristically bluish color. The lower stem leaves are oval shaped and are located on stalks, while the upper leaves are seated and cover the stem. *Isatis indigotica* Fort. is characterized by many yellow flowers gathered in inflorescences. Its petals are almost two times longer than the sepals, the cup has four sepals and the four-lobed crown contains six stamens and one pistil. Fruits form in single pods of up to 2.5 cm in length. Its color transitions from a dark shade to black violet, while it ripens (8).

The species can often be found growing in chalk soils and around cliffed areas. Hot days and warm night temperatures, together with humid conditions accelerate the degradation and extraction processes. In tropical climates, freshly harvested plants are kept in water until the degradation and extraction of indigo water has occurred.

The cultivation of indigoferous plants was also introduced into Europe and Japan. Here, harvested leaves are dried before being combined with water. Then, the leaves are composted in order to increase the concentration of the compound (9).

INDIGO AND ITS DERIVATIVES

Dyes are colored substances, which have a chemical affinity to the substrate to which they are applied. They appear colored, because they absorb some wavelengths of light from the spectrum better than others.

Pure indigo is only slightly soluble in water, making it ideal for use as a pigment. Originally, its use as a dye arose due to the reduction reaction. White indigo is formed as a result of indigo undergoing the reduction reaction. White indigo's properties offer a better solublility than the one of the original substance, facilitating the dyeing of clothing and other textile materials. Its reduced form may be re-oxidized into substances of intense deep blue color, as a result of being left in contact with the open air (10).

Indigo production decreased sharply in the mid-twentieth century only to rise again as a result of indigo being used as a colorant of denim. The demand for indigo increased to more than twenty tons in 2003. Indigo may also be mixed with a variety of other substances, resulting in a chemical reaction to obtain coloring pigments with green, blue and violet shades (11).

A pharmacologically important isomer of blue indigo, present in the indigoferous plants is indirubin. It is red in color, however it is not used in the textile industry, just like the former compound. There are also other colorants derived from the other compounds, which constitute the pigment of different colors (12).

Tyrian purple – 6,6-dibromoindigo, is a naturally occurring substance isolated from old sea shells. Currently, its use remains highly limited.

5,7,5", 7"-Tetrabromo-derived indigo (blue „Vat Blue 4B") an analogue of bisulfonic acid (cyan „Blue Saxon") is used to dye textiles blue.

Dye mixtures are also produced, which contain indigo as a constituent, for example indigo in the form of anthrone – in „Vat Blue 8" (13).

Chemical characteristic of indigo

The substance has a dark blue color referred to the last visible shade of blue in the spectrum before the transition into purple. The attributed color index for indigo is C. I. Pigment Blue 66 I C. I. 7300.

Chemical properties

Indigo is a dark blue crystalline powder that sublimes between a temperature of 390 and 392°C. This alkaloid is insoluble in water, alcohol and diethyl ether, but is soluble in DMSO, chloroform, nitrobenzene and concentrated sulfuric acid. Indigo has the chemical formula of $C_{16}H_{10}N_2O_2$ and a molecular weight of 262.26. The molecule absorbs light in the orange part of the spectrum (λ_{max} = 613 nm).

This compound has its deep color thanks to the conjugation of double bonds which are adjacent to each other. Thus, the molecule has a planar structure (14).

Natural synthesis

Polygonum tinctorium Ait. is characterized by the presence of a large amount of β-indoxyl-D-glu-

coside – an indican, which is a precursor of indigo in this species. Indican hydrolyzes only at relatively low pH levels or in the presence of the primary β-glucosidase. The natural β-glucosidase is naturally present in chloroplasts (15).

The β-glucosidase is immediately hydrolyzed to β-D-glucose and indoxyl during the mustication of leaves. Following the reaction, a spontaneous conversion of free indoxyl to natural indigo dye takes place. It is activated by oxidation in the open air (16) (Fig. 1).

Chemical synthesis

Considering its economic importance, indigo can be chemically synthesized by various methods. The Baeyer-Drewson synthesis process, published in 1882, was found impractical. The first useful method was created by Pfleger in 1901. In this process, N-phenylglycine is added to the molten mixture of sodium hydroxide, potassium hydroxide, and sodium amide. This extremely delicate alloy forms indoxyl. Indoxyl is then oxidized in the open air to form indigo (Fig. 1). There are a number of

The structural formula of indigo:

Synthesis of indigo in natural samples:

Synthesis of indigo by Heumann:

Synthesis of indigo by Pfleger:

Figure 1. Indigo structure and its syntheses (10)

variations to this method. One such alternative and more cost-effective synthetic route was discovered by Heumann in 1897. This process involves heating N-(2-carboxyphenyl)-glycine with sodium hydroxide to 200°C in a noble gas atmosphere. This leads to the formation of indoxyl-2-carboxylic acid, which easily undergoes decarboxylation to form indoxyl. Subsequently, the indoxyl oxidizes itself to form indigo (Fig. 1). This method is simpler than the technique discovered by Pfleger, however, the precursors used in the synthesis of indigo by Heumann are more expensive than those used in Pfleger's method (17).

Chemical characteristic of indirubin

Indirubin is a red dye and an isomer of blue indigo and brown isoindygo. After indigo, it is the second major component of the *Indigo naturalis*.

Chemical properties

Indirubin has an intense red color, which arises from the spontaneous dimerization reaction between colorless precursors of the compound – indoxyl and isatin (18).

Similarly to indigo, indirubin is only slightly soluble. The chemical formula of this compound is: $C_{16}H_{10}N_2O_2$ and it has a molecular weight of 262.26.

Natural synthesis

Plant species such as *Polygonum tinctorium* Ait., *Baphicacanthus Cusi* (Nees) Bremek, *Isatis indigotica* Fort. and Mediterranean snails of the genus *Murex,* contain natural precursors of indirubin. Those precursors produce three isomers or their derivatives.

Compounds containing indigoid bonds do not occur naturally in the pure forms of the above mentioned species. They are products of either an enzymatic reaction of dead material or an acid hydrolysis under the influence of air oxidation.

Indirubin can also be formed by either the action of certain bacteria at so called metabolic indican or an indoxyl sulfate. This is a consequense of disorders in tryptophan degradation. Then, this amino acid is present in larger amounts than normal in urine, which can lead to the diagnosis that patient has kidney disease. In such cases, the urine will exhibit a purple-blue color.

Those derivatives of indoxyl with a free hydroxyl group, as well as the derivatives of isatin are intermediates in the process of the formation of indigoids (19).

Chemical synthesis

Initially, indirubin was obtained as a by-product of the synthesis of indigo. In 1870, Baeyer and Emmerling produced this compound in different proportions by heating isatin, phosphorus trichloride and acetyl chloride in a closed glass tube after adding phosphorus. This resulted in an aqueous solution being obtained (20). Indirubin can also be synthesized in the reaction of sulfur 2-chloro-3H-

Structural formula of indirubin:

Chemical synthesis of indirubin from indoxyl and isatin

Figure 2. Structure and chemical synthesis of indirubin (34)

indol-3-one with ammonium sulfide or white phosphorus. Then, one of two methods can be used to obtain the dye. The first is to obtain an aqueous solution utilizing the Baeyer reaction. The second uses zinc dust in a glacial acetic acid to give the dye as a side product (21).

Indirubin was synthesized for the first time as a desired product in 1881, where indirubin was formed during a synthesis of isatin sodium carbonate from an ethanolic solution of indoxyl. This synthesis was first conducted by Baeyer (22).

Qualitative composition of indigoferous plants

Apart from being used in the process of dyeing materials, especially denim, indigo finds its main medicinal application as being used as *Indigo naturalis*. As mentioned, indigo is the most important constituent of this preparation. It is derived from plant species containing indigo. The production of *Indigo naturalis* involves a 100-day-long fermentation of leaves (carefully separated from their stems) from indigo plants. The fermented leaves along with an alkali such as limestone, wood-ash lye and wheat bran are transferred into an indigo vat. Wheat bran is used as food for the fermentation bacteria and the remaining constituents" purpose is to keep the pH of the indigo vat at the value of 10–13. In such conditions, the fermentation process may be sustained and the indigo dye can be extracted easily from the aqueous solution (23). The composition of *Indigo naturalis* consists mainly of the colorants indigo, indirubin and their derivatives.

Depending on the plant species from which the substances are extracted, *Indigo naturalis* contains 5–8% of indigo and 0.05–0.4% of indirubin (24). The Chinese Pharmacopoeia requires the minimum content of 0.13% indirubin (and 2.0% of indigo).

The composition of indigo containing plant species differs, showing the presence of compounds of different origins. These are listed below:

- *Baphicacanthus cusia* (Nees) Bremek: indigo, indirubin, indobrown, indoyellow, isoindigo, triphtrantin;
- *Polygonum tinctorium* Ait.: indigo, indirubin, indican, qingdainon, triphtrantin and other compounds: N-phenyl-2-naphthylamine, lacerol, isatan B, β-sitosterols;
- *Isatis tinctoria* L.: indigo, indirubin, qingdainon, triphtrantin, n-nonacosane (25, 26).

In addition to these organic compounds, *Indigo naturalis* also contains a small quantity of inorganic substances, a small amount of starch and reducing sugars (25).

The content of indigo in commercial products remains within the range of 1.29–3.12%. There is also a difference in the content of limestone used in a titration process, during the *Indigo naturalis* production. The content of lime varies from 6.85 to 49.59%, which results in the increase of total ash required – in the preparation it ranges between 26.64–71.75%. These significant differences in the content of calcium and magnesium carbonates are the result of the variable traditions and practices used in the production process (9).

PHARMACOLOGICAL APPLICATIONS

'Qing Dai" – a Chinese name for *Indigo naturalis*, has a salty taste and cooling properties. It affects the functions of the liver, which results in the elimination of toxic heat from the body and a drop of blood's temperature. Moreover, it relieves convulsions. Accordingly, it is indicated in the increased incidence of epidemic disease, hemoptysis associated with increased temperature, bleeding from the nose, chest pain, mouth ulcers, mumps, inflammation of the throat and larynx, and childrens" convulsions (27).

Orally, 'Qing Dai" is usually used in combination with other herbs from TCM for the following ailments: sun stroke, convulsions associated with epilepsy, cough, chest pain, hemoptysis, phlegm and childrens" convulsions (28).

It is forbidden to administer 'Qing Dai" orally to pregnant women. A potential side effect of using the medicine is hay fever.

'Qing Dai" can be used either alone or together with other herbs of Chinese medicine in order to treat: sore throat, eczema, psoriasis, saliva gland, ulcers in the mouth and gingivitis (28).

Indirubin

Due to the low stability of the color, indirubin is rarely used in dyeing of textiles (9).

It has been identified that this compound provides antitumor benefits within the cancer cells of animals. According to studies, indirubin achieves this antitumor effect through the inhibition of DNA synthesis in the tumor cells, while not significantly affecting the inhibition of protein synthesis (29).

Indirubin can form a tertiary compound with the DNA strand and DNA polymerase and thereby block its synthesis. Subsequently, the growth of cancer cells is inhibited (14). It was also discovered that indirubin affects myeloid cells in patients with cancer (12).

In China, indirubin is used in conjunction with other substances of plant origin, for the treatment of chronic myeloid leukemia (CML). It is considered that *Indigo naturalis* is responsible for the anti-leukemia effect. A more detailed analysis has shown that this result was achieved mainly due to the indirubin concentration in the amount of 0.05–0.3% of ‘Qing Dai” (30).

Indirubin was also identified in the dried parts of plants from the species *Orchidaceae Calanthe* R. Br. This species in TCM is considered as a substance which can be used to treat inflammations and bacterial infections (1).

Results suggest that a complex interaction of several mechanisms is the basis of the anti-cancer effect of indirubin. The main mechanism called cyclin-dependent kinases (CDK) involves the inhibition of enzymes, which represents a crucial role in the late phase of the division cycle. Therefore, indirubin and some of its derivatives, block the enzyme complex-dependent kinases called CDK1/cyclin B and CDK5/p25 (31).

Indirubin also affects the immune system and has been shown that its long-term use leads to an increased cellular immunity in patients with disorders of this type. It has also been shown to improve the condition of the impaired humoral immunity in the same group of patients (32, 33).

CONCLUSIONS

Derivatives of indigo and the *Indigo naturalis* preparation containing indigo dye, have been commonly used in the treatment of various diseases, such as fevers, different kinds of inflammations, or carcinomas. Their pharmacological effects were noticed and adopted by the Chinese into traditional chinese medicine hundreds of years ago. The discovered pharmacological properties confirm the importance of natural dyes in current medical treatment strategies and not only solely for their use in the process of textile dyeing. The current review recommends the collection of all published knowledge currently available on this topic.

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APPLICATION OF CPC AND RELATED METHODS FOR THE ISOLATION OF NATURAL SUBSTANCES – A REVIEW

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Abstract: A review of research on the isolation of various alkaloids from plant material by centrifugal partition chromatography (CPC) and related preparative techniques was made, in order to provide various conditions for separation of these important plant derived secondary metabolites. First of all, the construction of the CPC apparatus was presented as well as the principle of isolation of natural products with its help, and then the influence of operating apparatus parameters on the separation efficiency. Finally, a review of the alkaloids separation conditions was made, specifying used parameters and best solvent system.

Keywords: alkaloid separation, CPC separation, centrifugal partition chromatography, counter current chromatography

Centrifugal partition chromatography (CPC) is a type of counter-current chromatography (CCC), and the advanced form of liquid chromatography (LC) (1).

CPC was developed by Yoichiro Ito in 1964 and since then it is being used for the separation and purification of compounds (mainly of herbal origin). It remains an analytical preparative liquid – liquid technique, that does not require solid sorbent, but only two solvents that are immiscible two phases – stationary and mobile (2).

Typical CPC device is a group of cable channels combined in a cascade, located in the cartridges forming circles around the rotor. When the rotor is in motion, system is subjected to continuous centrifugal force and (by the action of pump) the mobile phase flows through the stationary phase. Due to the state of two liquid phases, their roles can be reversed – ascending mode (when the mobile phase is the lighter phase) or descending mode (when the mobile phase is heavier phase) may be selected. During the flow of one phase against the other, a process of elution (percolation) of the substance from one phase to another, and the process of retention of the substance in the stationary phase (3) are taking place. Substances are separated between the mobile and the stationary phase based on differences in the partition coefficients (4).

CPC compared to other modern chromatographic techniques, is a technique well suited to large scale separation (3), and capable of being used for the separation of substances with a wide range of polarities (4).

Selection of isolation parameters. The choice of solvent system, flow rate and rotation speed

The choice of solvents remains an important stage in the preparation of the analysis. Distribution of the substances dissolved in the sample between the two liquid phases, on the basis of the partition coefficient values, is essential for the CPC separation (2). The compounds contained in the test material should be soluble in both mobile and stationary phases. The most efficient solvent is a combination of such, at which the distribution ratio of a chosen compound will range around 1 (preferably) or within the values of 0.2–5. After mixing the two phases should be separated quickly – preferably within 30 s (5).

Retention of active compounds depends largely on the partition coefficient and the volume of each phase. Following equation shows this relationship:

$$Vr = Vm + P\ Vs \qquad (1)$$

where Vr – retention volume, Vm – volume of the mobile phase, P – partition coefficient, Vs – volume of the stationary phase.

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Retention of the substance should not be too high – not to let the substance remain too long on the column, nor too low for it not to be washed out immediately. For this reason, it is important to select a solvent suitable to maintain the partition coefficient and hence the corresponding retention (6). Solvents are chosen also on the basis of other parameters, that is the difference in density between the two liquid phases, on the viscosity of the liquid, or on the interfacial tension. To determine the appropriate solvents, thin layer chromatography (TLC) method is often used. It may be done by developing the sample on a silica plate with solvents. The solvents that give retention factor in the range R_f = 0.4–0.6 are best suited for CPC analysis (7).

The column is a place where substances are separated. Centrifugal force is necessary to keep the stationary phase on place, while the mobile phase flows through. Thus, the stationary phase is stable only when the device rotor sets the column in motion, and is creates centrifugal forces (6).

In addition to the appropriate solvents selection, it is also important to set the right flow rate and speed of rotation of the rotor in the CPC apparatus. The impact of these factors on the distribution of the substance was conducted by Matsuda et al. (8). It appeared that low rotation of the rotor and high flow rate can result in insufficient retention. The results also showed that the flow rate reduction improves peak resolution and increased the number of theoretical plates. It was concluded that the higher flow rate increased the minimum value of the rotor speed, which allowed for adequate resolution (8).

CPC apparatus

There are two types of liquid-liquid chromatography columns: hydrostatic and hydrodynamic ones. On the primary column of the first type (hydrostatic), the mobile phase passed through the stationary phase only by gravity (DCCC – Droplet Counter Current Chromatography), however, it requires long elution time (1). Modern hydrostatic counter-current known as CPC (centrifugal partition chromatography) enables to speed up the process. CPC unlike DCCC produces a centrifugal force of the rotor, which allows faster movement of the mobile phase through the stationary phase. This gives a wider range of solvents that can be used in this method (7). CPC displaced DCCC method because it is much more efficient (9).

The most widely used techniques are two slightly different types of CPC apparatus. One is produced *inter ali*a by Pharma-Tech Research Corp., and the second by Sanki Engineering Ltd. (2). The second in its original form had a column separator in the form of rolls arranged around the rotor connected by narrow tubes. Column is filled with the stationary phase through which the mobile phase is passed as a stream thanks to the rotation of the rotor (5). The other components of the device were: solvent pump, valve regulating the flow, the dispenser of the sample, a detector and a recording device (2). Diagram of such a device is presented in Figure 1.

Advantages of CPC

CPC as the analytical technique is much more advantageous than other types of chromatography

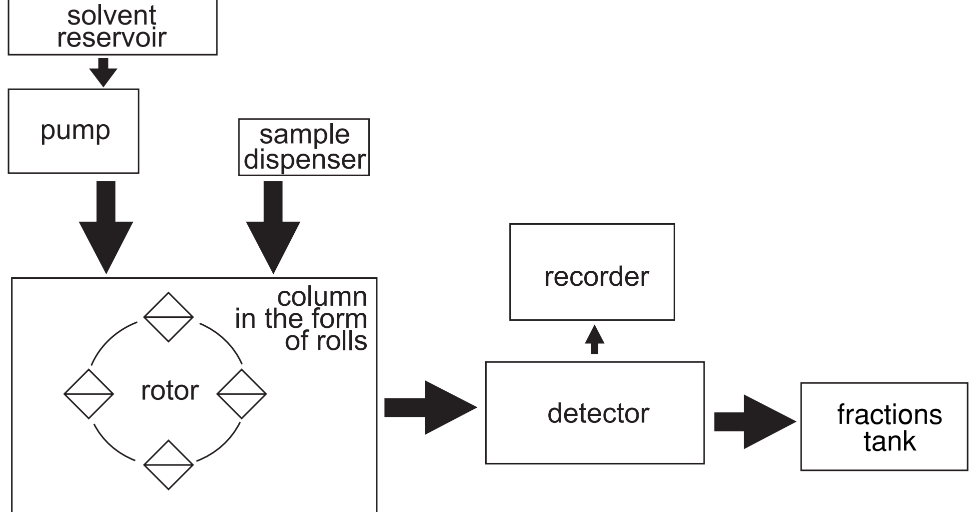

Figure 1. Diagram of CPC device

Table 1. Parameters of alkaloids isolation.

Type of alkaloids	Used phases stationary / mobile	Parameters: Speed of rotation / flow rate
Ergoline alkaloids lysergol, chanoclawine (10)	methyl *tert*-butyl ether, acetonitrile, water (4 : 1 : 5 v/v/v)	1250 rpm 3 mL/min
Indole alkaloids vindoline, vindolinine, catharanthine and vincaleukoblastine (11)	methyl *tert*-butyl ether, acetonitrile, water (4 : 1 : 5 v/v/v)	800 rpm 3 mL/min
Isoquinoline alkaloids palmatine, jatrorrhizine, columbamine, pseudocolumbamine (12)	dichloromethane, methanol, water (12 : 4 : 9 v/v/v)	700 rpm 9 mL/min (first stage) 3 mL/min (second stage)
Isoquinoline alkaloids palmatine, berberine, worenine, epiberbine, coptisine, jatrorrhizine (13)	n-hexane, ethyl acetate, methanol, water (2 : 5 : 2 : 5 v/v/v/v)	850 rpm 2 mL / min and then 650 rpm 5 mL / min
protopine, tetrahydropalmitine, bicuculline (14)	methyl *tert*-butyl ether, acetonitrile, water (2 : 2 : 3 v/v/v) + triethylamine and hydrochloric acid	800 rpm 2 mL / min
huperzine A , huperzine B (15)	N-heptane, ethyl acetate, n-propanol, water (5 : 15 : 35 : 45 v/v/v/v)	1400 rpm 6 mL / min
michellemine B (16)	chloroform, methanol, 0.5% hydrobromic acid	300 rpm 15 mL / min
Tropane alkaloids darlirgine (17)	(5 : 5 : 3 v/v/v) chloroform, methanol, water (13 : 7 : 8 v/v/v)	No data

such as high performance liquid chromatography (HPLC). In contrast to the HPLC, CPC gives complete recovery of the sample introduced. There is no denaturation of the molecules, no irreversible absorption on the solid absorbent and the solvent consumption is very low (7). The main advantage of this technique is due to significant reduction of analysis costs, allowing the use of this method on a larger scale. CPC has a high capacity. Relatively large volume of stationary phase makes the separation of substances effective (4).

Survey on using isolated alkaloids CPC

CPC is an analytical method used increasingly for the separation and purification of individual substances from raw plant material. Below an overview of research aimed at different groups of alkaloids separated using centrifugal partition chromatography is presented. Due to the similarity of CCC to the CPC method, several examples of the isolated alkaloids will be also presented by this method. Summary of methods used for the isolation of alkaloids is presented in Table 1.

The first example of the preparation of the alkaloid purification with CPC is a separation of clavine alkaloids from methanolic extract of *Ipomoea muricata* L. seeds. The separation of these substances was not easy because of their sensitivity to temperature and light, however, Mauri and Srivastava coped with this problem by using a modified CPC method, which separated the substances with an electric charge depending on the pH value. They managed to separate the two alkaloids: lysergol and chanoclavine from each other. CPC apparatus used for the separation contained 1320 cells (total column capacity of 200 mL) with a modification of rotation speed from 200 to 2000 rpm. As the stationary and the mobile phases used methyl *tert*-butyl ether, acetonitrile and water in a ratio (4 : 1 : 5 v/v/v) were used. Separation process was performed in the descending mode under the rotor speed of 1250 rpm and at the flow rate of 3 mL/min. From 4 grams of dry extract, 210 mg lysergol (97% purity) and 182 mg chanoclavine (79% purity) were obtained (10).

Renault and co-workers (11) implemented pH-zone refining mode in their separation for the

purification of indole alkaloids from the crude alkaloid extract from plant *Catharanthus roseus* (L.) Titer to obtain four alkaloids: vindoline, vindolinine, catharanthine and vincaleukoblastine. The study used a apparatus HPCPC with 2136 channels, and the total capacity of the column 250 mL. As the stationary and mobile phases the following solvents were used: methyl *tert*-butyl ether, acetonitrile and water (4 : 1 : 5 v/v/v) (the lower – the water phase was acidified). During the run, the apparatus was adjusted to 800 rpm and the flow of 3 mL/min. Attempts were made on four different amounts of the injected substance, starting from 0.8 g through 1.6, 2.4 to 7 g. After injections, the smallest mass of the sample received only two purified substances: catharanthine and vindoline. All four alkaloids were isolated, when mass of the sample was increased. Analyses were performed in the ascending mode. The following purity was obtained for the individual substances: vindoline (about 43% purity), catharanthine (about 55% purity), vincaleukoblastine (about 70% purity) (11).

Another group of alkaloids, which has been successfully separated using the technique of high-performance CPC was a group of isoquinoline alkaloids. From methanolic extract of *Enantia chlorantha* stem bark, four alkaloids were obtained: palmatine, jatrorrhizine, columbamine and pseudocolumbamine, using the following solvent system: dichloromethane, methanol and water (48 : 16 : 36 v/v/v). The aqueous phase was used as the stationary phase and organic one as the mobile phase, to isolate each of the alkaloids. Six hundert milligrams of palmatine was isolated (purity above 95%) and a mixture of the other three alkaloids was received and submitted to further separation. In the second phase, the mixture of jatrorrhizine, columbamine and pseudocolumbamine was separated, and two modifications of the above system were used. In the first one, addition of potassium perchlorate was performed and in the second system, the alkaline sodium hydroxide was added. Separation was performed as follows: in the first phase, with the addition of $KClO_4$, the rotation speed was set at 700 rpm and the flow rate at 9 mL/min in the descending mode, then, using the solvent system alkalized with sodium hydroxide, at 700 rpm and the flow rate of 3 mL/min. Jatrorrhizine and columbamine were washed out by 1100 mL of mobile phase in descending mode, and pseudocolumbamine was washed only after introduction of the dual mode by 200 mL of the mobile phase. Sixteen milligrams of jatrorrhizine, 13 mg of columbamine and 16 mg of pseudocolumbamine were received, all with a purity exceeding 95% (12).

To obtain alkaloids from the same group also the CCC technique was used. As the stationary and mobile phases, a mixture of n-hexane/ethyl acetate/methanol/water (2 : 5 : 2 : 5 v/v/v/v) at a flow rate of 2 mL/min and a rotation speed of 850 rpm were used. After 2.5 h of separation, the flow was accelerated to a speed of 5 mL/min and rotation speed was reduced to 650 rpm. The process yielded 4.7 mg of palmatine (purity 98.5%), 7.1 mg of berberine (purity 94.1%), 0.8 mg of worenine (purity 90.4%), 1.5 mg of epiberbine (purity 95.5%), 1.9 mg of coptisine (purity 88.4%) and 0.6 mg of jatrorrhizine (purity 91.1%) from 20 mg of dry extract obtained from ethanolic extract of *Coptis chinensis* Franch. rhizome (13).

The next group of alkaloids was separated using a CPC from 3.1 g sample of evaporated to dryness ethanolic extract of rhizome *Corydalis decumbens* (Thunb.) Pers. For separation, a mixture of methyl *tert*-butyl ether, acetonitrile and water was used in proportions of 2 : 2 : 3 by volume. To the top organic phase triethylamine (5–10 mM) was added, and to the lower aqueous phase hydrochloric acid (5–10 mM) was added. The mobile phase was pumped into the column at 2 mL/min while the column rotated at 800 rpm. Protopine (495 mg), tetrahydropalmityne (626 mg) and bicuculline (423 mg) were obtained. Each of the compounds was over 93% pure (14).

The pH-zone refining centrifugal partition chromatography technique was used to separate the alkaloids of the club moss *Huperzia serrata* (Thunb. ex Murray) Trevis. pH zone refining mode was used as a better modification of displacement method. n-Heptane/ethyl acetate/n-propanol/water (5 : 15 : 35 : 45 v/v/v/v) were used as solvent system. Triethylamine (8 mM) and methanesulfonic acid (6 mM) were also added to the solvent system. The pH of the upper and the lower phase was adjusted to pH 10 by addition of ammonia solution. Alkaloids were injected in the form of salts by lowering the pH to 2. The flow rate during the injection of the sample was increased gradually from 2 to 6 mL/min. Rotation speed of the column was set to 1600 rpm. The amount of 0.4 g of dry plant extract gave 9 mg huperzine A and 7 mg of pure huperzine B (15).

CPC technique was used as one of the three stages of receiving alkaloid michelemine from the dichloromethane and methanol extract from leaves and twigs of the liana *Ancistrocladus korupensis* D.W. Thomas & Gereau. Optimization of the alkaloids isolation from the above raw material was performed, and as the best solvent, mixture of chloroform – methanol – 0.5% hydrobromic acid (5 : 5 : 3

v/v/v) was selected. Separation was carried out initially at a flow rate of 16 mL/min and a rotation speed of 400 rpm, however, a reduction in these values, respectively, to 15 mL/min and 300 rpm significantly improved resolution. During the entire three-step process, about 0.5 g of pure michelemine B was obtained from about 8–9 g of a crude mixture of alkaloids (16).

The next example illustrates the use of the method for the CCC separation of tropane alkaloids from dichloromethane and methanol extract from the bark of *Darlingia darlingiana* (F. Muell.) L.A.S. Johnson. With 100 g of the extract from the bark, 74 mg of darlirgine were received. Solvent system was used as a mixture of chloroform – methanol – water (13 : 7 : 8 v/v/v) (17).

SUMMARY

CPC technique is cheap and efficient separation method, and often superior to other chromatographic techniques. However, in order to obtain the expected results, the appropriate analysis parameters must be chosen: from the choice of biphasic mixtures, the flow rate of the mobile phase up to the rotation speed of the rotor. In the present work, a few studies on the separation of alkaloids from plant material were shown. The most commonly used solvent system for alkaloids was a system containing: methyl *tert*-butyl ether, acetonitrile, water. Flow rates ranged from 2 mL/min, usually 6 mL/min up to 15 mL/min. The rotor speed values ranged from 300 to 1400 rpm.

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G PROTEIN-COUPLED RECEPTORS: ABNORMALITIES IN SIGNAL TRANSMISSION, DISEASE STATES AND PHARMACOTHERAPY

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Abstract: The aim of this review is to present the research results and draw new conclusions about the impact of alterations in the signal transmission through the G protein-coupled receptors (GPCRs) on the formation of diseases and drug therapy. GPCR family is the largest and the most diverse group of membrane receptors. They transmit signals into the cell by interaction with different ligands, which include, *inter alia*, hormones, neurotransmitters, and photons. GPCRs are responsible for the proper conduction of many physiological processes such as vision, intercellular communication, the neuronal transmission, hormonal signaling and are involved in many pathological processes. They are also point on the binding pathway of multiple drugs. They are targets of nearly one third of the drugs at the current pharmaceutical market. The genes encoding GPCRs represent about 4% of the human genome. Mutations that occur in them are associated with a broad spectrum of diseases of diverse etiology. As a mutations result, there is a change in receptor activity (GPCR become inactive, overactive, or constitutively active), in the process of ligand binding and signal transduction. Changes in the GPCRs functioning can cause diseases such as retinitis pigmentosa (rhodopsin mutations), nephrogenic diabetes insipidus (vasopressin receptor mutations), obesity (melanocortin receptor mutations). Many mutational changes in genes encoding GPCR can change drug therapy of already existed diseases: heart failure (adrenergic receptors), asthma (cysteinyl leukotriene receptors). Studies concerning the structure and function of genetically modified GPCRs allow to get know a variety of mechanisms of its action, which in turn can contribute to broaden the knowledge on the etiology and pharmacotherapy of many currently incurable diseases.

Keywords: G protein- coupled receptor, pharmacotherapy

Abbreviations: AC – adenylate cyclase, ADH – autosomal dominant hypocalcemia; AVPR2 – arginine vasopressin receptor 2, β-Arr – β-arrestin, CAM – constitutively active mutants, CaSR – calcium-sensoring receptor, CysLT – cysteinyl leukotrienes, FHH – familial hypocalciuric hypercalcemia, GPCR – G protein-coupled receptor, GRK – G protein-coupled receptor kinase, LT – leukotriene, MC4R – melanocortin-4 receptor, NDI – nephrogenic diabetes insipidus, PLC – phospholipase C, PTH – parathormon, RP – retinitis pigmentosa, SNP – single nucleotide polymorphism

G protein-coupled receptors (GPCRs) represent the largest family of membrane proteins that mediate in the cellular responses process, passing the signal into the cell. They participate in signaling cascades of hormones, neurotransmitters. GPCRs are also important in many physiological processes, play a key role in the vision and the sensing of taste and smell (1). They are also the target for a large group of drugs.

GPCRs have been discovered in 1971 by Martin Rodbell, while scientist studied the formation of cyclic adenosine monophosphate (cAMP) under the influence of glucagon in plasmatic membranes. For the discovery of G-proteins, Martin Rodbell and Alfred Gilman were awarded the Nobel Prize in Physiology or Medicine in 1994. Studies on the GPCR genes sequence revealed the existence of about 800 types of receptors in this family (2). Despite of their large number, the crystal structures are available for less than 20 unique GPCRs of the rhodopsin-like class (3). These findings contributed to the knowledge on the activation mechanism of these receptors. Disorders in GPCR signal transduction are the result of multiple mutations in genes encoding these receptors. Knowledge about the consequences of mutations in the genes encoding the GPCR is particularly important

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due to the fact that, GPCRs are point on the binding pathway for many pharmacologically active substances. Each discovered mutation and its effects, provide important information about the mechanism of the receptor action (4). The purpose of this review was to present research results and new findings concerning the effects of GPCR mutations on the formation of disturbances in cell signaling processes, their importance for the development of diseases and related pharmacotherapy.

Structure and function of receptors coupled with G protein

Receptors located in the cytoplasmatic membrane can be classified based on the number of structures permeating through the membrane on: the ion channels, receptors with tyrosine kinase activity and GPCRs (5). GPCRs have seven hydrophobic transmembrane domains of the α-helix structure, and therefore they have an alternate name – the seven transmembrane receptors (6). GPCRs are known to be very versatile receptors to extracellular signals as diverse as biogenic amines, purines, nucleic acid derivatives, proteins and peptides, odoriferous substances, pheromones, calcium ions, and even photons (6). GPCRs represent a large family of proteins that control many physiological processes and they interact with about 70% drugs used. One of GPCR classifications defined the five family of receptors: rhodopsin-like (family A), secretin-like (family B), glutamate (family C), adhesion receptors and frizzled/taste2 receptors (7). The first family A is the largest of them, also known as a class of rhodopsin like receptors, which comprises nearly 90% of all GPCRs. According to the list created by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification, receptors from family A are encoded by 273 genes including 89, which are so-called orphan receptors, for which ligands are not known. Structure of receptors belonging to this family is similar to the rhodopsin receptor. This is the first known structure of GPCR. The other members of the family A include β1 and β2 adrenergic, opioid, histamine, and dopamine receptors. The second family of GPCRs – secretin-like receptors are encoded by 48 genes. The third family of receptors has a structure similar to metabotropic receptors (encoded by 22 genes). Group called frizzled/taste2 is encoded by 11 genes. They are most closely related to the second family of GPCR (8, 9).

GPCR signal transfer occurs through the activation of heterotrimeric G protein (composed of α, β, and γ subunits) binding guanosine-5'-triphosphate (GTP). This protein, depending on the type of signal, transmits information to the cell through its ability to activate or inhibit a variety of proteins and effector enzymes (10). Subunit α (Gα), depending on the receptor activity (active/inactive), binds guanosine-5'-diphosphate (GDP), or GTP. Ligand interaction with GPCR activates it and causes conformational change of G protein. GTP displaces GDP linked to the α subunit and Gα is disconnected from the Gβγ complex. Active Gα subunit interacts with multiple effectors such as calcium ions, adenylate cyclase (AC), phospholipase C (PLC) and protein kinases (10). Although the β and γ subunits are synthesized separately, they form a biologically inseparable complex Gβγ. Through acetyl and prenyl group it is anchored in the cell membrane, and may regulate the activity of ion channels, PLC, and many other mediators (5).

G-protein type is determined by the type of α subunit. Based on the properties and amino acid similarity in this subunit, four types of G protein can be distinguished: a) Gs protein (stimulating) – activates AC, which is responsible for the conversion of adenosine-5'-triphosphate (ATP) to 3'5'-cyclic adenosine monophosphate (cAMP); b) Gi protein (inhibitory) – inactivates AC by reducing the synthesis of 3'5'-cAMP; c) Gq protein – activate PLC, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-tri-4',5'-phosphate (IP_3) and diacylglycerol (DAG); d) $G_{12}/_{13}$ proteins – activate Rho protein (Ras homologous), belonging to a small Ras proteins family (10).

The interaction of ligand with one molecule of GPCR, can activate many G proteins and creates number of second messengers and causes signal amplification. Consequently, one attached molecule can induce a strong physiological response. Appropriate signal amplification depends on the type of G protein involved, the specific characteristics of the receptor and the presence of other proteins, enhancing or extinguishing the signal. A single amino acid substitution in the receptor, can cause a dramatic increase or loss of function, what can cause pathological dysregulation of signal transduction (4). As the result of ligand attachment to the receptor, there is a change in the GPCR conformation and activation of G protein. Gα and Gβγ free subunits transmit the signal through their effectors (E1 and E2, respectively) into the cell and trigger a physiological response. Then, Gα-GTPase hydrolyzes Gα-GTP to Gα-GDP and the phosphoric residue (Pi). This leads to the re-bounding of Gα and Gβγ subunits, GDP connection to Gα and this leads to G protein inactivation (4, 11).

There is a process of desensitization, internalization and renewal of GPCR for the receptor to be again ready to ligand binding. Receptor desensitization protects cell against a constant supply of the signal and prevents uncontrolled stimulation of the receptor. There are two main patterns of desensitization: a) homologous desensitization – associated with an agonist interaction with receptor, specific kinase phosphorylation, and β-arrestin (β-Arr) binding, which inhibits the signal path; b) heterologous desensitization – is independent of agonist and affects less GPCR. In this process, protein kinase A and protein kinase C are involved (4). A key role in the regulation of GPCR desensitization play G protein-coupled receptor-specific kinases (GRK). Receptor upon agonist binding is susceptible to incorporation of kinases, that phosphorylate serine and threonine residues in the C-proximal end of the peptide chain. It should be noted that this process takes place only when the receptor is linked to the ligand. Then, phosphorylated receptor binds to the β-Arr, what blocks G protein-mediated signaling and targets receptors for internalization, and redirects signaling to alternative G protein-independent pathways. The complex receptor-β-Arr is internalized into the cell, in the form of clathrin-coated endosomes (4).

GRK family consists of seven kinases which, on the basis of sequence homology and gene structure, can be divided into three subfamilies: a) GRK1 subfamily – consists of rhodopsin kinase and kinase 7 (GRK7); b) GRK2 subfamily – consists of 2 and 3 kinase β-adrenergic receptor; c) GRK4 subfamily – consists of kinases 4, 5, 6 (12). GRK mutations cause inappropriate desensitization of receptors, resulting in increased activity of GPCRs. For example, kinases from GRK1 subfamily are involved in the pathophysiology of harmful mutations connected with rhodopsin acting, which are related with many diseases associated with hereditary retinal disorders (4).

GPCRs signaling pathway consists of many complex processes, which also involve many regulatory proteins and enzymes. Point mutations of genes encoding GPCRs can cause structural changes in their structure, intracellular interactions, increasing the flexibility of proteins, resulting in a change in the primary activity of receptors (13). Modification of the processes resulting from mutations at each level of signal transduction, may contribute to the pathological changes in a number of communication and may generate molecular phenotypes of numerous diseases, which have a great influence on drug efficacy.

Characteristics of selected GPCR – mutations, pathology and pharmacology

Impaired signaling of various GPCRs is the cause of many congenital and acquired diseases. They are caused by number of mutations, changing the structure and function of receptors (14). These disorders affect GPCR activity, reinforcing the function of receptors (gain-of-function) or loss (loss-of-function). Mutations of the first type are generally acquired, while the second type are mostly inherited. To date, more than 600 mutations detected are deactivating and about 100 mutations activate GPCR, and they are responsible for the formation of more than 30 different diseases. Not every mutation in GPCR initiates the formation of the disease. Mutation can affect pharmacotherapy by changing receptor response under the drug attachment.

The most common types of mutations that occur in the GPCR, are missense and nonsense mutations, small deletions and insertions, that may change the reading frame. GPCR dysfunction due to these different kinds of mutations can be classified depending on which level of receptor maturation they occur. On this basis, four classes of damages have been distinguished (15):

a) the first class – partial or complete deletions and mutations in the gene promoter;
b) the second class – mutations affect mRNA stability, translation, post-translational modifications (nonsense and missense mutations, insertions, deletions, mutations in exons splicing sites) and connected with secretion from the endoplasmic reticulum;
c) the third class – mutations that cause changes in the structure of the receptor and affect the adequate structure formation in the Golgi apparatus. Altered protein is targeted for degradation in the endosome;
d) the fourth class – interfere receptor signaling through changes in the ligand binding domain.

GPCRs are the target for various groups of drugs. Diseases in which drugs action is directed on GPCRs may include cardiovascular, endocrine, may be associated with vision disorders, maintenance of energy homeostasis, coagulation and immune system, and many others (4). This review characterizes some receptors, which impaired function resulting from mutations has led to variety of disease states.

Rhodopsin is a receptor belonging to the family A (rhodopsin like receptors), which is responsible for vision process. It is located in specialized cells of the retina – rod cells. All members of this family are activated by small ligands, such as bio-

genic amines and nucleotides, and rhodopsin is activated by photons. The action of the light is converted into an electrical signal sent to the brain (15). Rhodopsin is the first receptor of GPCRs family, for which high-resolution structure was obtained by the crystallography. Rhodopsin is different from other GPCRs in that it is constantly connected with the inverse agonist – 11-cis-retinal, which maintains the receptor in an inactive state. Photon absorption affects the configuration change of 11-cis-retinal to trans-retinal, and thus the conversion of rhodopsin to its active form – metarhodopsin II. Consequently, transducin (G protein) coupled with rhodopsin is activated by the exchange of GDP to GTP in the α subunit and then initiates a phototransduction cascade (16, 17).

Rhodopsin mutations lead to diseases associated with impaired vision. Twenty percent of them are point mutations that cause improper folding, transport or processing of the receptor (15). Mutations that cause the diseases are often nonsense mutations that lead to a single amino acid substitution in the peptide chain of the receptor. Taking into account the effects of mutations on rhodopsin receptor activity, they can be divided into two groups: a) mutations leading to an increase of receptor activity by creating constitutively active mutants (CAM). These mutant receptors are still capable of activation, even in the absence of exposure to the ligand; b) mutations leading to decreased receptor activity due to changes taking place in the phosphorylation process. The consequence of the most of these mutations is the development of the disease. Most of the known rhodopsin mutations are constitutively active rhodopsin mutants. CAM has modified binding site of an inverse agonist, 11-cis-retinal, and therefore mutated receptor is not inhibited. As a result, there is a dysfunction of rods, resulting in impairment of perception of light in the dark. Constitutively active mutants were first discovered in a severe, progressive disease that is retinitis pigmentosa (RP) (17). RP defines a group of heterogeneous inherited disorders associated with changes and loss of retinal cells. RP is reported to be approximately 1 : 4000 people (18). Mutations in the gene encoding rhodopsin, leading to the formation of CAM, are in positions: Thr4Lys, Asn15Ser, Thr17Met, Pro23His, Pro23Leu, Gln28His, Glu113Gln and Lys296Glu (4). Classic RP begins with the problems in adaptation to the dark vision that during adolescence goes into night blindness (nyctalopia). The next stage of the disease is the progressive loss of peripheral vision in the early years of adulthood. As the disease progresses, there is a total loss of the peripheral vision with the possibility of occurrence of a tunnel vision, and usually to 60 years of age there is a loss of central vision. The symptoms are the result of a progressive retinal dystrophies with reduction of two types of photoreceptors: – rods, which enable vision in black and white in low light intensity; – cone cells, which are responsible for color vision. Degeneration of both types of photoreceptors, occurs in the process of apoptosis. RP is a disease damaging the visual perception, but there are cases in which the disease is associated with other disorders. There are about 30 syndromes co-existing with RP. These include (18):

- Usher syndrome – RP is associated with loss of hearing. There are 3 types of this syndrome. In the first one, hearing loss can be very large and it manifests at birth. Balance difficulties can also occur. In the second type of disease, hearing loss can be moderate or mild and does not increase in time. At last, the third type, hearing loss occurs gradually during adolescence. Usher syndrome is a result of mutations in at least 11 genes;
- Bardet-Biedl syndrome – in this syndrome RP is associated with other disorders such as obesity, hypogonadism, renal failure, or mental retardation. Ten genes have been identified whose mutations are responsible for 70% of RP cases.

Currently, there are no drugs for selective RP pharmacotherapy. There are many different methods to prevent the progressive loss of vision. These are: – vitamins A and E supplementation – the daily dose of 4.5 mg of retinyl palmitate may delay blindness by up to 10 years; – docosahexaenoic acid supplementation, which belongs to the group of ω-3 acids. In the membranes in which rhodopsin is located, there is significant amount of docosahexaenoic acid; – oxygen therapy – in normal conditions, retinal photoreceptors have high oxygen consumption. It is assumed that the supply of oxygen to the retina, can partially rescue photoreceptors and they are able to carry out the necessary metabolic processes (18).

More methods, which are still in research domain are: – gene therapy; – transplantation of retinal cells; – modification of apoptosis. The last method concerns the use of calcium channel inhibitors such as diltiazem and nilvadipine, having an impact on apoptosis. Analyzing the process of photoreceptor cell death, it was found that it was associated with higher concentration of calcium ions. In studies conducted in mice suffering from RP, it was found that D-cis-diltiazem, by blocking the calcium channels in the photoreceptor cells, causes a reduction in its degeneration in examined mice. Properties and action of nilvadipine prove to

be better than these of diltiazem. Nilvadipine is a hydrophobic drug, making it easier to go to the central nervous system and retina. The result of nilvadipine injection in mice was the reduction of calcium ions concentration in the photoreceptors cells, including individuals that D-cis-diltiazem was not acted on. An additional advantage of nilvadipine is the highest antioxidant potential among calcium channel inhibitors, so it can fight against the products of oxidative stress associated with photoreceptor cell death in RP. To date, the study concerning the effect of nilvadipine on the course of RP was performed only in mice and in a small group of people. It was also noted, that the combination therapy of D-cis-diltiazem, taurine and vitamin E has a beneficial effect on improving the patients vision. Studies concerning the effects of calcium channel inhibitors on apoptosis are still conducted (19). Recently, a new method was described, that will help in the prevention of patients predisposed to developing RP. Treatment consists in placing in the conjunctival sac drops of a mixture of insulin with growth factor, which helps in the recovery of Muller cells. It is a glial type of cells in the retina, that protect photoreceptor cells against excess of glutamate and free radicals. Their protective effect concerns rods mainly, which come from the same progenitor cells as the Muller cells. Research on this method of treatment offers hope to achieve a simple treatment (20).

Another group of disorders associated with rhodopsin are mutations in the GRK encoding genes. An example is the Oguchi disease. It is a rare disease involving disturbances of vision at dark, at night and in situations, where there is a little access to light (scotopic vision). The cause of this disease is a mutation in the gene encoding the GRK1 or arestin, and in result, there is a lack of rhodopsin phosphorylation. In patients, who have this mutation, receptor constantly activated by light, constantly activates transducin. This process occurs until the entire amount of 11-cis-retinal is transformed into 11-trans-retinal. The consequence of this transition is the lack of sensitivity to light, which continues until there is a renewal of 11-cis-retinal. In patients, rhodopsin regeneration takes more than two hours, after which the rods again reach their full sensitivity to light. Rhodopsin kinase is in both, the rods and cones, but in Oguchi disease phosphorylation occurs only in the rods (21). *In vitro* studies have shown that deletion in the 5th exon in the gene encoding GRK1, is a null mutation, which abolishes the enzymatic activity of the kinase. In other research, *in vivo*, there was no phosphorylation of rhodopsin. Based on both studies, we can say that desensitization is one of the key processes determining the proper functioning of rhodopsin receptors (4).

α-Adrenergic receptors (α_1, α_2) and β (β_1, β_2, β_3) in combination with endogenous catecholamines (adrenaline and noradrenaline) regulate the activity of the sympathetic nervous system. They are also the point on the binding pathway of many drugs (agonists or antagonists) for the treatment of diseases such as heart failure, asthma, or obesity (4). Many types of adrenergic receptor polymorphisms, which consists of a single nucleotide substitution in the gene sequence encoding the receptor protein (SNP, single nucleotide polymorphism), are known (22). Polymorphisms caused by mutations in the gene promoter can cause changes in the expression of receptor; mutations in coding region can cause changes in binding with ligand and/or G-protein and interfere signal transduction regulation. For example, β_1 receptor variant (arginine at 389 is converted to glycine) and α_2 receptor variant (asparagine at 251 is converted to lysine). These mutations cause receptor dysfunction in the intracellular signaling process and as a result occurs an increase in the function of second messengers. Depending on the type of polymorphism, a variant can have a major impact on the course of the disease, or only a potential risk of its development. Typically, formation of polymorphic receptor gene is not the cause of the disease. Changed receptor often is associated with some disease and mainly affects receptor response to medicines used in the treatment of a specific disease (4).

Among the β-adrenergic receptors, there are three types of receptors: β_1, β_2 and β_3, which are distributed in many organs. The first (β_1 receptor) is located mainly in the heart, where it influences the pulse rate and myocardial contractility, and in the kidneys, in which it directs the release of renin. Therefore, the β_1-AR stimulation leads to the activation of the renin-angiotensin-aldosterone system (RAA). β_2 Receptors are widely distributed in the lungs, and on the surface of smooth muscle cells of blood vessels. As response to stimulation by endogenous ligands of the sympathetic nervous system, the widening of blood vessels (vasodilation) occurs. Thus β_2-AR plays an important role in blood pressure regulation. β_3 Receptors with β_2 receptors are also found in the heart. All three types of receptors play an important role in the pathophysiology of cardiovascular diseases including hypertension, stable and unstable coronary artery disease (angina pectoris), myocardial infarction, ventricular and

supraventricular arrhythmias and chronic heart failure. β Receptors are also a point of capture of many commonly used drugs as these used in the treatment of bronchial asthma ($β_2$ agonists), and diseases of the cardiovascular system ($β_1$ antagonists) (22).

The most common **polymorphic variants of the $β_1$-adrenergic receptor** are the results of a single amino acid replacement at positions 49 and 389. At position 49 in the N-terminal part of the polypeptide chain of the receptor, serine is substituted by glycine and at position 389 in the proximal part of the C-terminus of the peptide chain, arginine is substituted by glycine. In studies carried on hamsters fibroblasts, there has been shown that substitution of the glycine at position 49 in the receptor resulted in a decrease in the number and density of receptors on the cell surface as a result of its down regulation.

Various polymorphisms within the $β_1$ receptor may affect the body's response to medications. The difference in the individual's response in humans with a mutated form of this protein is particularly important in hypertension, coronary heart disease and heart failure. Therefore, polymorphism Arg389Gly intensifies response to drugs, which are $β_1$-adrenergic receptor agonists as well as to antagonists for these receptors. It was shown that treatment with dobutamine or adrenaline has better effect on function of the heart of people with Arg389Gly polymorphism, who underwent coronary artery bypass grafting, in relation to persons having a Gly389Arg polymorphism. A similar result was obtained in studies concerning the effect of $β_1$-AR antagonists on the lowering of blood pressure in healthy subjects and in patients with hypertension and chronic heart failure. It turned out that in patients with a mutation Arg389Gly, a better response to medications was achieved than in patients with Gly389Arg polymorphism. On the basis of this result, it was assumed that persons with Arg389Gly polymorphism in the gene encoding the $β_1$-AR respond better to treatment with agonists and antagonists of these receptors, in comparison to those with receptor not changed. Therefore, it is suggested that, in order to quicker obtain the favorable therapeutic effects, administration of the higher dose of such drugs to persons without polymorphism Arg389Gly is recommended (22).

In the case of **$β_2$-AR, polymorphic variants**, positions 16, 27 and 164 of the polypeptide chain of receptor are involved. These mutations have been observed in diseases such as hypertension, asthma, obesity, and certain immune disorders. As in the case of $β_1$-AR, the presence of polymorphic variants may affect the body's response to a given drug. At the mutation site 16, there is a change from arginine to glycine. Such variant is often associated with elevated level of immunoglobulin E in asthma (4). In addition, those who had Arg16Gly polymorphism, exhibit lower response to $β_2$-AR agonists in relation to Gly16Arg variant. The receptor with polymorphism at position 16 of the peptide chain, under the influence of long exposure to the agonist is down regulated. Another variant is based on SNP at 27 position, which was converted from glutamine to glycine. As in the case of Gly16Arg, this receptor is also down regulated (14). The third $β_2$-AR polymorphism results from threonine substitution for isoleucine at 164 position. Despite the fact, that it occurs rarely, it has a significant impact on the function of the receptor. Binding to the G protein and the affinity of $β_2$-AR to the ligand are reduced. It has also been shown that it causes disturbances in the process of vasodilation, thus contributing to an increase in blood pressure, the frequency of hypertension and other cardiovascular diseases. The study concerning Thr164Ile polymorphism of $β_2$-AR receptor on 66,770 patients were conducted. In the group of study there were Danish men and women of Caucasian origin. It was demonstrated that the presence of Thr164Ile is associated with increased blood pressure and the other above-mentioned changes in the cardiovascular system in women (23). Thr164Ile polymorphic variant may occur in patients suffering from heart failure and affects response of $β_2$-AR to agonists. In the context of these results, those who had Thr164Ile polymorphism can be classified into a group of patients for whom there is a need to establish individual therapy treatment of cardiac failure, for example, by increasing the dose of the drug (4). Understanding of the $β_2$-AR polymorphic variants makes possible to determine their potential impact on the development of the disease and is useful in predicting the response to medications used during treatment.

Heart failure is a disease with a high mortality. The main reasons for its occurrence are: coronary heart disease, hypertension and heart attack. As a result of the above diseases, there is an insufficient blood flow and myocardial ischemia, and necrosis of the tissue. The heart is increasingly attenuated, and the contractions are ineffective. The organism trying to compensate heart disturbances activates systems stimulating heart to work. Mechanisms compensating ineffective myocardial work are: activation of the sympathetic nervous system, stimulation of the RAA system and vasopressin synthesis. As a result of the activation of mechanisms described, there is an increase in cardiac workload,

which leads to the development of heart failure. In the next step, there are changes in the structure of the myocardium. One of them is an abnormal left ventricular hypertrophy, which causes the change of the heart geometry and its dysfunction. In addition, there are risk factors that can facilitate the incidence of heart failure. They can be divided into physiological and genetic. The first group includes age, female gender, and diabetes. The second group includes, among others, changes in the expression of genes encoding the receptors that can influence the activation of signal transduction and transcriptional and translational factors (22). The presence of polymorphic variants of β-adrenergic receptor and their impact on the development of cardiovascular disease are shown in Table 1. In the heart, there are two AR main receptors – β_1 and α_2, and to a lesser amount, β_2 and β_3 receptors. Activation of both types of receptor (β_1 and β_2) causes stimulation of protein Gs and activation of AC, which causes an increase of cAMP concentration. The effect of this signaling cascade is an increase of frequency, strength and speed of contraction of the heart muscle and increase of the relaxation phase of the muscle fibers. β_1-AR is considered to have cardiotoxic properties due to the fact, that its continuous stimulation causes apoptosis of cardiomyocytes. In contrast, β_2 receptor is considered to be cardioprotective.

This receptor may stimulate both Gs and Gi proteins, thereby it can activate two signaling paths. As a result of Gi protein stimulation, kinase Akt is activated, which has anti-apoptotic properties (22, 24). In heart failure, there is a constant stimulation of β_1 receptors by noradrenaline leading to its down

Table 1. Polymorphic variants of β-adrenergic receptors and their influence on the pathogenesis of cardiovascular disease (22, 23).

Receptor	Polymorphism	Signal change	Clinical observations
β_1	Ser49Gly	Gly49 – increase of the susceptibility of receptor on down regulation	Gly49 – reduced risk of deepening failure, heart transplantation or death
	Gly389Arg	Arg389 – binds stronger Gs protein – receptor more sensitive to stimulation with agonist and antagonist	Homozygotes Arg389 – better treatment effects of β-blockers in the form of an increase in left ventricular ejection fraction than Gly389, an increase the risk of arrhythmia. Homozygotes **Gly389** with heart failure – lower oxygen consumption during exercise
β_2	Thr164Ile	Ile164 – reduced affinity for catecholamines, reduced binding of Gs protein and weaker activation of adenylate cyclase Thr164 – increased sensitivity to stimulation of the receptor with agonist	Ile164 – there is an increase in blood pressure, the risk of coronary heart disease
	Arg16Gly	Gly16 – increased susceptibility to agonist-induced receptor down regulation	No apparent effect on the pathophysiology of diseases of the cardiovascular system
	Gln27Glu	Glu27 – increased resistance to agonist-induced receptor down regulation	

regulation. In extreme cases, it may lead to the disappearance of up to 50% of β_1 receptors. Using during long period of β_1-receptor antagonist can restore the original state of these receptors. As a result of continuous stimulation of β_2 receptors, there is not its down regulation but there is an increase of the inhibitory Gi protein concentration. In consequence, there is a weakening of the response to continuous stimulation of the sympathetic nervous system (22).

Melanocortin-4 receptor

More and more people around the world suffer from the problem of obesity. The causes of this civilization disease are lifestyle changes, diet and genetic factors. Significant influence on the development of obesity has a mutation in the gene encoding the melanocortin-4 receptor (MC4R). MC4R participates in regulations relevant to the proper functioning of the body's processes, it is responsible for controlling hunger and satiety. Receptor belongs to the family A, rhodopsin-like GPCRs. It is small, has only 332 amino acids. The receptor may be associated with three types of G proteins: Gs, Gi/o or Gq. Its stimulation results in a further activation of signal transmission by the relay of the second messengers (cAMP), which affects the increase of intracellular calcium ion concentration. In classical signal transduction through the receptor, protein Gs is stimulated (25). MC4R is located in the paraventricular nucleus in the hypothalamus, where it is a part of the melanocortin signaling of appetite and energy balance. MC4R modulation depending on the attached ligand can have opposite effects. After binding with α-melanocortin, receptor stimulates protein Gs, which activates AC, and then executes a further signal transduction cascade. The effect of α-melanocortin interaction with MC4R is mute the center of hunger and stimulation of satiety center. After a meal, a person endowed with genetically modified MC4R do not feel satiety (26). On the contrary, when inverse agonist – agouti-related protein is connected to the receptor, satiety disappears and hunger center is triggered. *In vitro*, MC4R connected with the agouti-related protein shows a continuous inhibition on the hunger center (27). MC4R mutations are the most common cause of monogenic obesity, usually autosomal dominant. It is estimated that such mutations occur with a frequency of 1–6% in children and adults with severe cases of obesity. On the basis of more than 50 types of mutations described so far, three main pathomechanisms of receptor acting were distinguished (26): a) impaired activation of the receptor after ligand interaction; b) decreased expression of receptor on the cell membrane; c) reduced constitutive activity of the receptor. The first group of disorders is determined by mutations in the gene encoding MC4R, changing receptor agonist response. After the interaction of the receptor with α-melanocortin, there is no answer or it is limited and this could happened because of the reduced receptor affinity for agonists or weak signal transduction. Disorders classified in the second group are caused by mutations, that cause the reduction of expression of the receptor on the cell surface. It was demonstrated that 80% of children with severe obesity have showed a partial or complete intracellular retention of the MC4 receptor. In the last group of receptor disorders, there are mutations that result in a loss of constitutive activity of the receptor. It has been shown that the continuous sending satiety signal provided by the constitutive activity of the receptor is required to maintain the energy balance of the body. Mutations that cause this kind of disruption in the functioning of the receptor are the most common defects observed in obese individuals.

Obesity is mainly based on the excessive accumulation of body fat, which substantially affects the conditions of life. Severity of obesity can be measured by the scale of body mass index (BMI). BMI is body mass divided by height in meters to the second power. This index helps to identify individuals who have overweight (BMI = 25) and obesity (BMI = 30) (28). Carried studies have shown that obesity results from environmental and genetics factors. The first of them is disturbed homeostasis between the energy supplied with food and energy spent on physical activity. As a result, people have a positive energy balance, resulting in the deposition of energy reserves in the body fat. Genetic factors constitute 50–90% of the causes of obesity (29). There are 3 types of inherited obesity): a) multigenic obesity – is the most common, but so far it is the least known; b) obesity, which is a part of syndrome – is rare, obesity is one of the phenotypic characteristics of the disease, for example, in Prader and Willi syndrome it is accompanied with hyperphagia and behavioral disorders; c) monogenic obesity – it is rare, but is important in understanding the mechanism of appetite regulation (30). Mutations in the gene encoding MC4R are the most common cause of monogenic obesity. Frequency of monogenic obesity, determined by mutations in the gene MC4 was analyzed within 500 people with severe obesity which started in childhood, and specific phenotype of disease was set up. It has been shown that mutations in genes encoding MC4R occurs in 5.8% of the studied population. Higher growth and higher bone

density was demonstrated in persons with mutations in the MC4 receptor than in obese persons without this mutation (25).

Due to the fact that obesity is a global problem and is classified as a chronic disease, there are many studies on its pharmacotherapy. One of the therapeutic options are drugs binding to the MC4 receptor. Currently, a number of clinical studies are conducted on substances that can be used as medicaments for the treatment of obesity. MC4 receptor agonists must fulfill several requirements. Due to the fact that the receptors are in the brain, such drugs should have a good penetration of the blood-brain barrier. In addition, they must be strictly selective for the MC4 receptor, because the group of melanocortin receptors show a high degree of homology to each other (26, 27). Research is also conducted on drugs that can be used in the treatment of disorders associated with mutations in genes encoding a second class of receptors. Due to the retention of the receptor in the cell, potential drug would have a mechanism of action such as pharmacoperons (25). Universal and often used method in the treatment of obesity caused by excessive eating or genetic factors is increased physical activity. Regular exercise can cause increased energy consumption, cause lose weight and prevent obesity.

Mutations in the arginine vasopressin receptor 2

Vasopressin (antidiuretic hormone) is a hormone released from the pituitary in hypovolemia or hypernatremia. Its function is to control the water reabsorption in the kidney collecting duct cells. Vasopressin interacts with arginine vasopressin receptor 2 (AVPR2), which is located in the membrane of cells of the distal convoluted tubule and

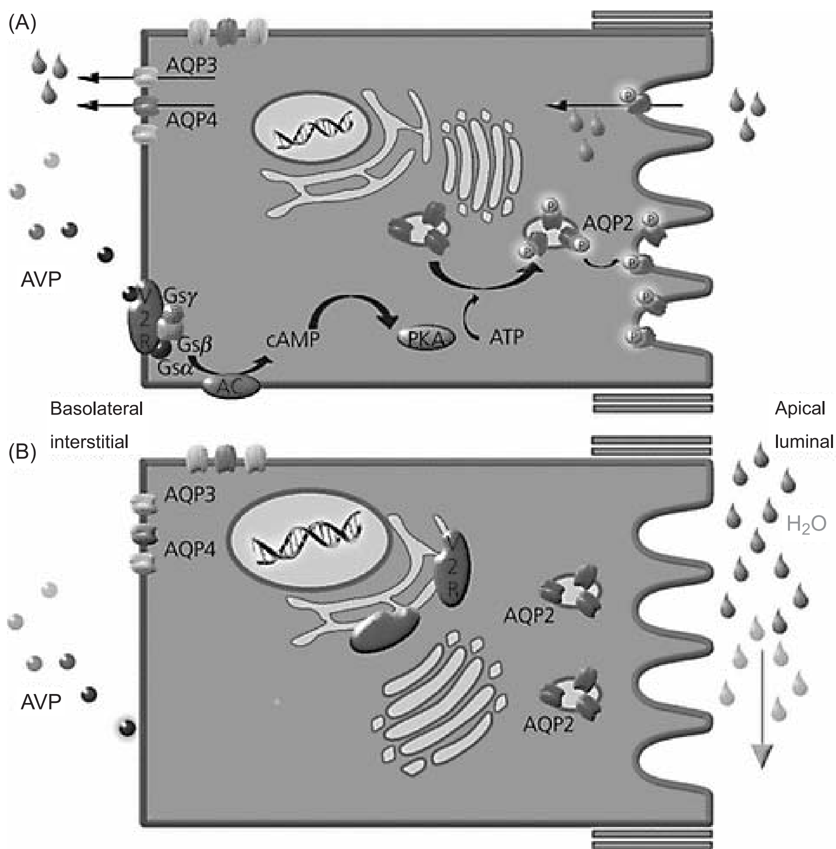

Figure 1. The mechanism of water transport in the collecting duct principal cells in a healthy individual (**A**) and a patient with NDI (**B**). AVP – arginine vasopressin; V2R – vasopressin receptor 2; AC – adenylate cyclase; cAMP – cyclic adenosine monophosphate; PKA –protein kinase A; ATP – adenosine-5'-triphosphate; AQP2 – aquaporin 2; AQP3 – aquaporin 3; AQP4 – aquaporin 4 (33). The description in the text

collecting ducts. This receptor belongs to the family B of GPCR, for which characteristic is the detection of large particles and a long N-terminal domain. The interaction of vasopressin with receptor results in the activation of specific proteins that are responsible for the transport of water into the cells, aquaporin 2. They are tetrameric proteins that form in the cell membrane of the renal tubule channels with diameter corresponding to water molecule (31). In the inactive state, aquaporin 2 is arranged in vesicles inside the cell. After connection to the vasopressin receptor, aquaporin 2 moves from the alveoli to the cell membrane, where they form a transverse channels, increasing membrane permeability to water (31). Currently, 221 mutations are known in the gene encoding AVPR2, that cause nephrogenic diabetes insipidus (NDI), and 21 mutations that do not initiate this disease (23). All mutations can be divided into 15 types within the 4 classes, taking into account the impact of specific disorders on the several steps of receptor maturation process. The most common type of mutations that occur in AVPR2 and the most important in terms of initiating NDI are missense mutations. These belong to the second class of disorders and constitute up to 48% of all mutations occurring in AVPR2 (31). Transcellular water transport is running properly in the body thanks to AVPR2. By the vasopressin connection with AVPR2 in the basal-lateral membrane, Gα subunit is disconnected from the trimeric G protein, then it comes to activation of AC and increase of cAMP levels. Second messenger activates protein kinase A, which stimulates aquaporin type 2 to move to the basement membrane of the follicle cells. In this way, it is possible to move the water through the membrane, from the tubular lumen to the main cell, and transcellular transport, then with aquaporin 3 and 4, the flow to the interstitial nephron. After obtaining a suitable concentration of urine, vasopressin detaches from receptor, and aquaporins 2 are endocytosed or are excreted with the urine (32). The most common disorder arising from mutation of a receptor AVPR2 is NDI. In the people with NDI, there is disruption of the receptor maturation. AVPR2 is permanently attached to the endoplasmic reticulum and cannot be connected to vasopressin in the basal-lateral membrane of the principal cell tubular nephron. In consequence, no water resorption leads to polyuria (33). The process is illustrated in Figure 1. The proper conduction of the mechanism of water transition from collecting duct to the interstitial space is responsible for maintaining the body's water balance and blood pressure regulation. Patients with NDI are unable to concentrate urine and they produce its large quantities. Depending on the initial cause, there are two the most occurring types of diabetes insipidus (32): – central diabetes insipidus – is associated with synthesis or secretion of antidiuretic hormone from the pituitary; NDI – is caused by insensitivity to the renal tubular vaso-

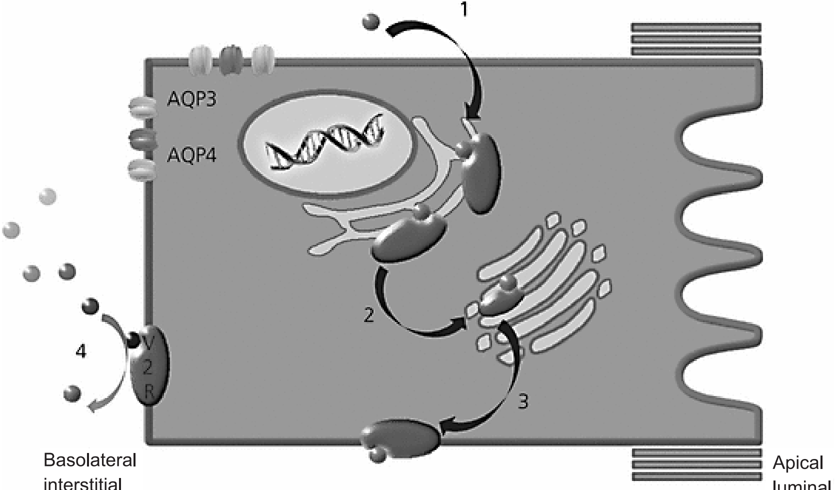

Figure 2. The mechanism of action of the antagonist (pharmacoperons) on the formation of AVPR2. (1) Antagonist passage through the cell membrane into the cytoplasm, an interaction with the mutant receptor in the endoplasmic reticulum (ER) and stabilization of the structure. (2) Escape of receptor from the ER. (3) Aging in the Golgi and location in a cell membrane. (4) High levels of vasopressin displaces antagonist from receptor and activates it

pressin. In these patients, the urine cannot been concentrated despite normal hormone level in the blood. NDI may be inherited or acquired. In almost 90% of cases is inherited and passed on as a recessive disorder linked to the X chromosome. Mutations in the gene encoding AVPR2 usually cause the loss of receptor function. In such cases, the water resorption is disturbed in renal collecting tubule, due to the interference signal of AVPR2 dependent on it expression, and their transport to the main cell membrane (34). The remaining 10% are mostly autosomal recessive mutations or less dominant. These are mutations in the gene encoding aquaporin 2, which result in the molecules that are not displaced from the cell membrane vesicles to the collecting tubule. There are also pathological conditions, which promote NDI formation, which include hypokalemia, hypercalcemia, low-protein diet and lithium therapy (31). Characteristic symptoms occurring in patients with NDI are, *inter alia*, polydipsia, polyuria (from 15 to 20 liters of diluted urine per day) and nocturnal urination (nocturia) (32). In newborns with NDI may occur disorders in eating, difficulties in putting on weight and symptoms of dehydration, which include dryness and loss of elasticity of the skin and dark circles under the eyes. In the case of giving up treatment, among young people there is a disturbance in the development and reduction of growth. Probably, this is due to the problems of nutrition, excessive water intake or recurrent episodes of dehydration. In addition, electrolyte abnormalities such as hypernatremia or hiperchloremia and constant dehydration can cause permanent brain damage, mental retardation and problems with growth and development (33).

The patients with NDI, which undergo therapy, can live to adulthood. In children, quick diagnose and beginning of the treatment can prevent the expansion of mental retardation. The treatment is based primarily on the administration to patient a large amounts of water to prevent dehydration. This is a hindrance in daily life, because the constant fluid intake and an inability to concentrate urine is associated with the need for frequent urination. In addition, elderly patients may have difficulty in the sensation of thirst or in maintaining urine. The use of low-sodium diet, thiazide diuretics and indomethacin are the other methods by which partial reduction of urine amount is possible (32). The expectancy for improving the treatment of NDI, there is a therapy with new class of drugs called pharmacological chaperones or pharmacoperons. In the endoplasmic reticulum (ER), which is the site of synthesis, formation and transport of proteins, there are chaperone proteins. Their role is to join and stabilize newly formed proteins, preventing their reaction with other peptides and assistance for transport to the other places in the cell. The pharmacoperons allow for proper folding and the inherent spatial structure of the protein and receptor transport from the ER to the plasma membrane. It is important that, when attached to new proteins, chaperones do not affect their functions. Mutated protein, which failed to pass the checks in the ER, cannot leave the endoplasmic reticulum and is degraded. The pharmacoperons are small lipophilic compounds, which penetrate through the membrane into the cell and bind to the structurally modified, as a result of mutations, proteins. These proteins have impaired spatial structure, they accumulate in the endoplasmic reticulum and are degraded, what can cause the disease. The pharmacoperons allow proper folding and achieving proper spatial structure of proteins and receptor transport to the ER membrane cell. This mechanism is presented in Figure 2. Yhe pharmacoperons interact selectively with mutated receptor and allow for escape of proteins from ER according to proper process of protein synthesis. The structure of pharmacoperons affect its efficiency, which determines the selectivity of the target protein, severity of the damage and the location of the mutation in the protein (for example, the mutation should not occur in the part of the gene responsible for encoding the part of protein interacting with ligand) (35). Examples of pharmacoperons for AVPR2 are its antagonists SR121463, SR49059, OPC41061 and OPC31260. So far, there was only one clinical trial in which five patients were treated with an antagonist SR49059 (Relcovaptan). As a result of the experiment, decreased levels of daily urine were observed. Vaptans class of drugs (for example Tolvaptan) are used in the treatment of hypernatremia. Their use in the treatment of NDI is still in the research phase (33).

Calcium-sensoring receptor (CaSR)

This receptor belongs to the C family G-protein coupled receptors. Extracellular domain binding calcium ions and other cations is characteristic for this family. CaSR is located on the surface of parathyroid cells in hormone-secreting glands, which are responsible for maintaining levels of calcium in the extracellular fluid and blood serum. A large number of receptors are present on the surface of kidney, they are less in the bone and intestine. CaSR is very sensitive to calcium ions, and therefore can effectively regulate the level of its concentration in the extracellular fluid and blood. If the

concentration of calcium ions is less than the physiological level, receptor stimulates parathyroid cells to secrete parathyroid hormone (PTH). This hormone enhances calcium ions level, by affecting the bone structure, the glomerular reabsorption from the initial and calcitriol synthesis in the small intestine (36).

Rare mutations in the CaSR encoding gene, contribute to the formation of disorders manifested by hypocalcemia and hypercalcemia (14). Mutations causing loss of CaSR function occur in familial hypocalciuric hypercalcemia (FHH) and in neonatal severe hyperparathyroidism. The incidence of both diseases is < 1/10000. Mutations causing increase of CaSR function occur in autosomal dominant hypocalcemia (ADH). In FHH, there is a loss of function of the receptor as a result of mutations and their effect is a reduced CaSR sensitivity to calcium ions. In most cases, in families suffering from FHH, the level of calcium ions is gently increased, but this does not require an increase of PTH level above physiological. Patients with FHH, in contrast to patients with primary hyperparathyroidism, have not severe symptoms of hypercalcemia such as the formation of kidney stones and disorders of the skeletal system. In most patients, there are no symptoms, and if they are, they have a gentle nature. These include dizziness, anxiety, feeling faint, tartar, muscle pain and weak memory. In the FHH treatment calcimimetics are used, which are modulators of CaSR. The action of this class of drugs is to increase the sensitivity of the receptor to calcium ions, which in turn leads to increased signal transduction. Research concerning the effects of cinacalcet (drug used to treat people who have drug-resistant hypercalcemia caused by mutations in the CaSR gene) were conducted. Among four people with FHH three of them were cognates. The drug was administered at a dose of 30 mg to 60 mg once a day for three months. The result of the study was an amelioration of well-being of patients and improvement in calcium homeostasis, which was maintained up to three years without increasing the dose. In three related patients, complete disappearance of the symptoms of hypercalcemia has been shown. None of the patients experienced adverse reactions. During cinacalcet therapy, bone mineral density was not improved (37). Mutations in the CaSR encoding gene, which increase the receptor activity, have been identified among patients with ADH. Factor, initiating the ADH creation, is constitutively active mutation in the gene encoding CaSR, which reduces the EC_{50}. This value represents the concentration needed to cause 50% of the maximum response to the agonist. The result of activating mutation in the calcium receptor is its increased sensitivity to calcium ions. Therefore, the receptor does not respond properly to the reduced concentration of calcium ions in serum and does not stimulate secretion of parathyroid hormone (17). Patients with the inherited form of hypocalcemia in most cases have no symptoms of the disease. Children during fever may have seizures and be sensitive. Patients usually have mild or moderate form of hypocalcemia with abnormal serum PTH level and a partial or complete hypercalciuria with increased urinary calcium excretion, despite its low concentration in serum blood. Supplementation with calcium and vitamin D is one of the ADH treatment. Is followed until the patient reaches a suitable serum calcium level and gets rid of the symptoms of the disease. People with ADH who have an increased secretion of calcium, can have an impaired kidney function and the formation of kidney stones. In this case, it is often required to monitor urinary excretion and administration of the drug from thiazide diuretics group, which can reduce the concentration of calcium in the urine (36).

Cysteinyl leukotrienes receptor

Leukotrienes (LT) define a group of biologically active molecules with lipid structure. One of the subgroups are cysteinyl leukotrienes (CysLT), which include leukotrienes C4 (LTC4), D4 (LTD4) and E4 (LTE4). They are important mediators of inflammation and allergic reactions in the course of diseases such as asthma and allergic rhinitis. CysLT interact with two types of receptors: CysLT1 and CysLT2. The cysteinyl leukotrienes are synthesized in the 5-lipoxygenase pathway from arachidonic acid, which is a component of the phospholipids of cell membranes. The source of leukotrienes are mainly mast cells, eosinophils, basophils, and macrophages. They exert their effects through reactions with specific membrane receptors belonging to the comprehensive GPCR family. They cause bronchoconstriction and vasodilation. As a result of increased vascular permeability and exudation of macromolecules, swelling of the tissue is also formed. Mutations in genes encoding leukotriene receptors play an important role in the pathophysiology of asthma and inflammation and warrants the creation of atopic asthma. Atopic asthma is a chronic, inflammatory disease, that is characterized by bronchial hypersensitivity to allergens. After an exposure occurs bronchospasm and reduction of air flow,which results in difficulty in breathing (38, 39). Polymorphisms of CysLT1 and CysLT2 receptors are not the main cause of the disease, but in most

cases it accompanied. Their presence also affects the course of the mechanism of action of drugs, thereby constituting an important reference point for the further search for more effective pharmacologically active substances (38, 40). CysLT1 receptors are the point of capture of "classical" antagonists (montelukast, zafirlukast, pranlukast, pobilukast and MK571), while in the case of CysLT2 receptors, signal transduction is not inhibited by mentioned antagonists. The only common antagonist of both groups is BAYu9773. CysLT1 and CysLT2 are homologous only in 38% to each other. CysLT1 occurs widely in leukocytes, spinal cord, less in the lungs, pancreas, small intestine, and in a small extent in other organs. In asthma, CysLT1 receptors are widely distributed in most inflammatory cells, and their number is significantly increased. CysLT2, due to the fact that there is not known its selective antagonist, it was not as well studied as CysLT1. CysLT2 widely occurs in the heart, in the pulmonary veins and in various parts of the brain. To a lesser extent, it is located in the spinal cord, kidneys and other organs (39). Signal transduction after leukotriene interaction with receptor is mediated by G protein. Both CysLT1 and CysLT2 are linked to protein Gq. Different amounts of CysLT released from mast cells and macrophages, and the transmitted signal strength affect the severity of asthma. In the treatment of atopic asthma blocking leukotrienes were used primarily (41). After administration of selective antagonists of leukotriene receptor such as montelukast, pranlukast, in most of patients CysLT1 receptor inhibition has occurred. The rest of patients who did not exhibited any pharmacological effects following administration of drugs, could have mutational changes that reduced receptor functions. CysLT2 receptor is also an important target in the treatment of asthma. In many cases, it forms, together with CysLT1 receptor, a highly active dimers with unique properties. Patients unresponsive to CysLT1 blockers, could be treated with drugs directed at CysLT2. Currently, only BAYu9773 operates antagonistically to both types of receptors, in that partially as an agonist on CysLT2. Research on CysLT1 and CysLT2 receptor variants allowed to determine the way in which they influence the response after joining agonist and their association with asthma. CysLT2 receptor has at least four polymorphic variants. Met201Wal variant causes partial inactivation of receptor, and Arg292Gly/Arg315Lys variant increases its activity compared to the wild-type of receptor. Probably, the part of the ligand binding site was changed, what causes an increased activity of agonist connected with Arg292Gly/Arg315Lys receptor variant and reduced agonist potential for Met201Wal. Paradoxically, only Met201Wal variant was observed in people with atopy or asthma. Therefore, it is uncertain whether the reduction in the sensitivity of the receptor to the ligand (as compared to the wild type) has a protective effect in lung diseases. These considerations are the subject of ongoing studies to determine risk factors for atopic asthma (4).

Neurotransmitter receptors

Antipsychotics bind to multiple receptors belonging to the family of GPCR. These are dopaminergic, serotonergic, muscarinic and opioids receptors. Dopamine is a major neurotransmitter in the central nervous system. It participates in neuroregulation, is responsible for motor activity, secretion of hormones and emotional states. There are five types of dopaminergic receptors, which are divided in terms of structure and pharmacological properties on the two groups: D1, which includes the D1 and D5 receptors and D2 receptors including the D2, D3 and D4 ones. Drugs for Parkinson's disease are dopaminergic receptors agonists, and in schizophrenia they are their antagonists. In contrast to the first group, the second one has a lot of polymorphic variations, changing effect of antipsychotic medications. An example is bromocriptine, which antagonist acting to D4 receptor is two times weaker than to the D2 receptor.

The opposite situation occurs in the case of clozapine, the activity of which is twofold higher to D4 than when connected to D2 or D3 receptors. Intensive studies on dopamine receptors have shown that a group of mutations in genes encoding D2 receptors is associated with mental disorders. A large number of medicinal substances has the point where the drug first associates in the serotonergic system. These include drugs acting as antidepressants and antipsychotically. However, there is often a resistance to some drugs, for example to clozapine, in the case of Cys22Ser mutation in the gene encoding 5-hydroxytryptamine receptor 2C (5-HT_{2C}). Altered response to a drug also occurs when there is a mutation Cys23Ser in $5HT_{2C}$ and Gly22Ser in $5HT_{1A}$. Pharmacogenetics research on serotonin receptors may contribute to the improvement of pharmacotherapy of disease associated with this system.

Opioid receptors are also in the interests of researchers, because their stimulation is associated with the development of addiction. An example is the receptor having several polymorphic variants, such as Asn40Asp, Asn152Asp, His260Arg,

His265Arg and Ser268Pro, that may change the effects of opioids. Receptors with Asn40Asp and Asn152Asp have four times greater affinity for the β-endorphins as compared to the wild type receptor. When the Ser268Pro mutation occurs, receptor desensitization process is reduced, which in turn makes that they are often in the active state. This may contribute to increased predisposition to addiction of persons with the Ser268Pro mutation (4, 28).

SUMMARY

GPCRs are the largest family of membrane receptors binding to many, different ligands, which include, *inter alia*, hormones, neurotransmitters, and photons. GPCRs are responsible for the proper conduction of a number of processes such as vision, intercellular communication, neural transmission and hormonal signaling. A characteristic feature of this family is their binding with heterotrimeric G protein, which upon activation changes its conformation. Depending on the type of protein G attached to GPCR, there is a cascade of different secondary messengers transmitting a signal into the cell. Through desensitization, inactivation and internalization, there is a controlled process of extinction of the transmitted signal (11). GPCRs control many physiological processes, and are also involved in many pathological processes. They also interact with a large group of drugs *inter alia* in the treatment of heart failure, asthma, or of renal diabetes insipidus (23, 31, 41, 43). Mutations, which occur at different levels of receptor maturation, initiate changes in receptor activity (inactive, overactive, or constitutively active GPCR), signaling processes and expression in the cell membrane. They lead to the diseases of different etiologies (4). Rhodopsin is one of the first GPCR for which a structure and mechanism of activation has been known. This receptor is responsible for the process of seeing. Some of mutations in genes encoding rhodopsin causes: formation of CAM, decrease of receptor activity or disturbance in the correct folding, transport and processing of the receptor protein. Retinitis pigmentosa (RP) is a complex disease characterized by progressive blindness resulting from the apoptosis of rod cells. One of the reasons of RP occurrence are mutations giving rise to a CAM. Constantly, research searching for an effective method of pharmacotherapy RP are conducted (42). Genetic disorders that cause loss of rhodopsin function result *inter alia* in Oguchi disease (4, 16). Receptor sensitive to calcium ions – CaSR – regulates its level in blood serum by stimulating parathyroid to the secretion of parathyroid hormone or inhibition of it secretion. FHH is a genetic disease, which arised as the result of a mutation that reduces the sensitivity of the CaSR to calcium. Studies on the use of calcimimetics in the treatment of FHH are conducted. Mutations that increase receptor activity led to a CAM and are the cause of autosomal dominant hypocalcemia (17). The most commonly occurring mutational changes within adrenergic receptors are SNP mutations leading to changes in the protein, and more specifically in its expression, interaction with a ligand, binding to a G protein and the regulation of signal transduction. Polymorphic variants can initiate disease or be a potential risk to their development. Examples of such diseases are hypertension, asthma and cardiac failure. In addition, as a result of the SNP, a change in the actions of many drugs may occur (4). Mutations in the gene coding for AVPR2, that reduce receptor activity, are the cause of NDI. It is characterized by abnormal process of collecting and concentrate urine in the kidney tubules. Currently, studies are being conducted on the use of specific compounds known as pharmacoperones in NDI therapy (33).

Melanocortin 4 receptor (MC4R) controls hunger and satiety center. Mutations in the gene encoding MC4R cause monogenic obesity in children and adults. In the majority, these are missense mutations, which in different ways influence the activity of the receptor (26).

Cysteinyl leukotriene receptors, by responding to inflammatory mediators, play an important role in allergic reactions. Mutations in the genes encoding the CysLT1 receptor and the CysLT2 cause polymorphs in these receptors, which affect the treatment of atopic asthma (4).

Dopamine, serotonin and opioid receptors are also important in pharmacotherapy. Mutations in the genes encoding these receptors cause many changes in the course and treatment of mental illness (4). Studies on the structure and function of genetically modified GPCRs allow to know a variety of mechanisms of action, which in turn can contribute to better knowledge on the etiology and pharmacotherapy of many currently incurable diseases.

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ANALYSIS

PREPARATION AND RADIOCHEMICAL CONTROL OF ^{99m}Tc LABELED BLOOD POOL AGENT FOR *IN VIVO* LABELLING OF THE RED BLOOD CELLS

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Abstract: Our aim was to prepare cheap blood pool imaging kits by simplified method to overcome the burden on purchase department of MINAR, Nishtar Hospital, Multan, Pakistan. Secondarily, prompt supply of kits should save the time of patient during transportation. A total of 24 subjects selected for this study were equally divided into two groups. Mixture of stannous chloride and sodium pyrophosphate solution at pH 7 was injected to these subjects. Various concentrations (ranging from 200 to 800 µg) of stannous chloride dihydrate were injected to group one, followed by intravenous administration of technetium-99m (^{99m}Tc) pertechnetate at 30 min interval in 12 subjects. Labeling percentage of each sample was calculated afterwards followed by imaging under γ camera. Each parameter was tested on three different patients and average of these three was calculated. In second set of experiments done on group two the same procedure was repeated in another 12 subjects, while keeping the concentration of Sn PYP constant at 400 µg. In this case, ^{99m}Tc was administered at different time intervals in different subjects ranging from 15 to 120 min (15, 30, 60 and 120 min) followed by calculation of labeling percentage and imaging under γ camera. In group one, average percentage values of binding of red blood cells with ^{99m}Tc were 23.24, 84.88, 83.78 and 60.33% for concentrations of 200, 400, 600 and 800 µg, respectively. In group two, average percentage binging values of 22.26, 84.36, 55.54 and 28.67% were calculated at time intervals of 15, 30, 60 and 120 min, respectively. It is concluded from the results that the best blood pool imaging under γ camera was observed for the concentration of 400 µg and the time interval of 30 min. The maximum percentage binding of red blood cells with ^{99m}Tc was calculated at concentration of 400 µg after 30 min interval that also correlated with imaging results.

Keywords: radiochemical control, ^{99m}Tc labeled blood pool agent, *in vivo* labeling, percentage binding of red blood cells with ^{99m}Tc

Technetium-99m (^{99m}Tc) labeled red blood cells (TlRBC) have become the radiopharmaceutial of choice for blood pool scintigraphy because of the convenience of *in vivo* labelling procedure of Paval et al. (1). As the clinical use expanded to include studies of intermittent GIT bleeding, variable amount of gastric, urinary and colonic activities were seen with red blood cells (RBC) labeled *in vivo* (2). The non-imaging procedures used in past have met with limited success, but in radionuclide imaging, such as ^{99m}Tc sulfur colloid and ^{99m}Tc labeled red blood cells have wider applications. Both radiopharmaceutials provide similar information and their sensitivity appears to be significantly higher than that of angiography for detection of lower GIT bleeding (3). A similar modification by Bauer et al. was shown to improve image quality (4). Bearn et al. labeled red blood cells with ^{99m}Tc for *in vivo* study but the results showed higher bone affinity in experimental subjects (5).

^{99m}Tc RBC scintigraphy can play a useful role in the preoperative localization of unexplained gas-

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trointestinal bleeding in hospital with nuclear medicine facilities. Blood pool scintigraphy is routinely done at all nuclear medicine centres of Pakistan Atomic Energy Commission using imported blood pool agent in the form of kits, which are quite expensive, i.e., approximately US $ 25/kit (containing only 5 vials).

This study was done in an attempt to replace high cost radiopharmaceuticals with the locally produced kits to save high foreign exchange. We developed *in vivo* labeling of RBC with ^{99m}Tc for blood pool scintigraphy with excellent clinical results. The calculated cost for the local preparation is not more than US $ 5/per kit having 5 vials (total cost includes cost of chemicals, staff salaries and related expenditure etc.). This technique was evaluated in term of variation in the concentration of stannous chloride and the time interval between stannous chloride and ^{99m}Tc injection, radiochemical binding and its safety.

MATERIALS AND METHODS

Materials

Technetium-99m and stannous chloride solution were obtained from Gamma Chemicals, Karachi, Pakistan. They prepared two solutions, i.e., solution A consisted of Na pyrophosphate solution (20 mg/10 mL saline) which acts as chelator, while solution B consisted of stannous chloride solution (20 mg/5 mL HCl). Then, the solution A (1 mL) was mixed with the solution B (0.25 mL) and the obtained total volume was 1.25 mL. Its pH was adjusted to 7.3 with NaOH. Total volume was brought up to 4 mL by saline and injected to patient. After it, 20 mCi (^{99m}Tc) was injected to the patient. Then, blood samples of patients were taken to check binding to RBCs after specific time intervals and total activity was recorded. Then, plasma and RBCs were separated. Now again, activity in plasma and RBCs was measured. RBCs activity was calculated as percentage binding of total injected activity. Asuming possible binding mechanism, chelator ruptures the RBC membrane and then ^{99m}Tc binds to protein in RBC hemoglobin. Binding can be calculated in terms of percentage of total activity injected (activity found in plasma + activity found in RBCs or hematocrit).

METHODS

This prospective study was carried out at Multan Institute of Nuclear Medicine and Radiotherapy (MINAR). Twenty four healthy volunteers (14 males and 10 females) participated in this study. An informed consent was obtained from all volunteers. The subjects were divided into two groups and each group was further divided into four subgroups each with three volunteers.

Appropriate volume of prepared stannous chloride solution (3 μg/kg body weight) at pH 7 was injected intravenously to male and female volunteers, six each. After 15 min, ^{99m}Tc was withdrawn from ^{99m}Tc sterile generator and its activity was checked by radioisotope calibrator. ^{99m}Tc was injected intravenously to all 12 volunteers as 0.1 mCi/kg body weight. After 15 min in each experiment, 5 mL of blood sample was obtained from each volunteer in a disposable syringe. Red blood cells and plasma were separated by centrifugation at 3000 rpm for 15 min in a centrifuge tube. Then, the packed red blood cells were washed with normal saline. Plasma and red blood cells were transferred into separate γ counting vials and counted in γ well counter. Then, the percentage labeling of each sample was calculated. Similarly, the labeling efficiency of red blood cell was determined after 30, 60 and 120 min of injection of ^{99m}Tc.

In group 1, the effect of concentration of stannous chloride was studied while keeping the time between stannous chloride and ^{99m}Tc injections constant. In this group, different concentrations of stannous chloride solution were given to each subgroup (200, 400, 600 and 800 μg to subgroup 1a, 1b, 1c and 1d, respectively) and average percentage binding of red blood cells with ^{99m}Tc of each subgroup was calculated after fixed time interval i.e., 30 min. In group 2, the effect of time between stannous chloride solution injection and ^{99m}Tc injection was studied while keeping the concentration of stannous chloride solution constant. In this group, average percentage binding of each subgroup was calculated at the following intervals: (15, 30, 60 and 120 min for subgroups 2a, 2b, 2c and 2d, respectively).

Clinical study

Blood pool images of all 24 patients were acquired using single headed Siemens Orbiter Gamma Camera interfaced with ICON computer system. The clinical procedure used for this study is outlined as follows: firstly, stannous pyrophosphate was intravenously injected, followed by intravenous injection of ^{99m}Tc-pertechnetate, then static imagings of brain, heart, great vessels, aortic bifurcation and lower extremities were obtained.

Table 1. Percentage binding of RBCs with ^{99m}Tc for various concentrations of Sn-PYP.

Subgroup	Concentration of Sn-PYP (µg)	Percentage binding of ^{99m}Tc with red blood cells (%)			Average percentage binding (%)
		1	2	3	
1(a)	200	22.64	23.68	23.40	23.24
1(b)	400	85.92	83.52	85.20	84.88
1(c)	600	84.80	83.62	82.92	83.78
1(d)	800	60.36	61.30	59.33	60.33

Table 2. Red blood cells binding with ^{99m}Tc for various time intervals between Sn-PYP and ^{99m}Tc injections.

Subgroup	Time interval between Sn-PYP and ^{99m}Tc injections (min)	Percentage binding of ^{99m}Tc with red blood cells (%)			Average percentage binding (%)
		1	2	3	
2(a)	15	21.52	22.91	22.35	22.26
2(b)	30	86.86	84.44	81.78	84.36
2(c)	60	54.37	54.38	57.87	55.54
2(d)	120	29.31	27.33	29.37	28.67

RESULTS AND DISCUSSION

The study was performed on a total of 24 subjects. Mean age was 36 years (standard deviation = 11.3 years) and range was 19–58 years. Percentage binding of ^{99m}Tc with red blood cells (%) was studied in both groups and the results of group 1 are given in Table 1. The result showed that maximum binding percentage (84.88%) is calculated at the concentration of 400 µg. Table 2 exhibits the results of group 2. The result showed that maximum binding percentage (84.36%) is calculated at a time interval of 30 min between the injection of stannous chloride solution and ^{99m}Tc. Best blood pool images were obtained at the concentration of 400 µg of Sn-PYP and at the lag time of 3 min between Sn-PYP injection and ^{99m}Tc-pertechnetate injection.

In the present study, 400 µg was found to be the minimum concentration of stannous chloride that resulted in satisfactory RBC labeling. This dose is less than the previously reported dose for the same procedure [6]. The resulting scan was also of good quality without any significant artifacts. It was also observed that RBC labeling with ^{99m}Tc depends upon the time interval between inactive stannous chloride injection and ^{99m}Tc. A time interval of 30 min was selected, based on the dual consideration that this time interval provided better red blood labeling than longer time interval, and also because 30 min is very convenient interval for the patient and staff in busy nuclear medicine department. A shorter interval of 15 min has not provided good quality image as stannous chloride ions take some time to penetrate into the red blood cells. If this time is significantly less than 30 min, the penetration of stannous chloride into red blood cells becomes insufficient and hence labeling efficiency also decreases. If the concentration of stannous chloride or time interval is increased or decreased beyond the above limits, the percentage of bound fraction of technetium decreases in red blood cells as is evident from the images and statistics provided. The results of this study suggest that ^{99m}Tc-labeled RBC by *in vivo* technique is a superior blood pool imaging agent compared to any other preparations.

CONCLUSION

The technique is described for in-house production of blood pool agent for nuclear medicine studies. This methodology produces low-cost kit

that can be used in any study requiring labeling of ^{99m}Tc with red blood cells.

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STABILITY-INDICATING HPLC METHOD FOR THE DETERMINATION OF CEFQUINOME SULFATE

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Abstract: A novel and sensitive stability-indicating RP-HPLC method for the quantitative determination of cefquinome sulfate has been developed. Chromatographic separation and quantitative determination were performed using a high-performance liquid chromatograph with UV detection. As the stationary phase a LiChroCART RP-18 column (5 μm particle size, 125 mm × 4 mm, Merck, Darmstadt, Germany) was used. The mobile phase consisted of 10 volumes of acetonitrile and 90 volumes of an 0.02 M phosphate buffer (pH = 7.0). The flow rate of the mobile phase was 1.0 mL/min. The eluents were monitored by a UV-VIS detector at 268 nm. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. Significant degradation was found under basic, oxidizing stress and UV light. The developed method was validated with respect to linearity, accuracy, precision and robustness.

Keywords: cefquinome sulfate, degradation, RP-HPLC, stability-indicating, validation

Cefquinome sulfate is a veterinary, parenteral, fourth-generation cephalosporin. A significant enhancement of activity and an extension of the antibacterial spectrum were achieved by the introduction of a methoxyimino–aminothiazolyl moiety into the acyl side chain of cephalosporins, which made them resistant to inactivation by β-lactamases (1–3).

Fourth-generation cephalosporins have a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Enterobacteriaceae* (4–6). Those compounds are also easily transported across the blood-brain barrier (7–14). Fourth-generation cephalosporins are used to treat infections of the urinary tract, lungs, skin and soft tissues as well as in post-operative prophylaxis (7, 15).

Cefquinome sulfate is an aminothiazolyl cephalosporin with a broad spectrum of activity against the majority of strains found in animal infections (16) such as *Actinobacillus spp., Haemophilus spp., Clostridium spp., Corynebacterium, Erysipelothrix rhusiopathiae, Proteus spp., Salmonella spp., Streptococcus spp., Pasteurella spp., Staphylococcus spp., Pseudomonas aeruginosa, Escherichia coli* and *Enterobacteriaceae*. Cefquinome sulfate is often applied in the treatment of *meningitis-mastitis-agalactiae* (MMA) (17, 18).

Pharmaceutical dosage forms should be stable during preparation, administration and action. ß-Lactam antibiotics (penam analogues, cephalosporins and carbapenems) are susceptible to degradation both in aqueous solutions (19–27) and in the solid state (28–38). Determination of cephem analogs is the result of different physical and chemical factors activity.

The guidelines of the International Conference on Harmonization (ICH) require the development of stability-indicating assay methods (SIAMs) appropriate for the determination of drugs after stability analysis (Q1A-R2) (39).

During stress tests, the effect of temperature and air humidity should be determined in solid state. For solutions, the effect of temperature, light, oxidizing agent, buffer pH and infusion liquid need to be analyzed. The impact of biochemical processes on the formation of metabolites has to be considered as well. Finally, the chemical structure and toxicity of principal degradation products, impurities and metabolite(s) should be established.

The aim of this work was to develop and validate an HPLC method with UV detection suitable

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for the identification, determination, and stability study of cefquinome sulfate.

EXPERIMENTAL

Chemicals

Cefquinome sulfate was obtained from Bepharm Pharmaceuticals, China. All other chemicals and solvents (acetonitrile, disodium hydrogen phosphate, orthophosphoric acid) were obtained from Merck KGaA (Germany) and were of analytical grade. High-quality pure water was prepared using an Exil SA 67120 purification system (Millipore, Molsheim, France). Acetanilide 98.5% grade (Sigma-Aldrich, USA) was used as an internal standard.

Equipment

The LC system used for method development, forced degradation studies and method validation was from Shimadzu (Japan) and was composed of LC-6A pump, C-R6A CHROMATOPAC interface and UV-Vis Shimadzu SPD-6AV detector. The Rheodyne injection valve had 50 µL volume. Photostability studies were carried out using a Suntest CPS$^+$ device (Atlas®) with a Solar ID65 filter, USA. Thermal stability studies were performed in a Wamed KBC – 125W heat chamber, Poland.

Chromatography

The chromatographic column used was LiChroCART 125 mm × 4 mm (5 µm), No. 019297 (Merck, Darmstadt, Germany). The mobile phase consisted of 10 volumes of acetonitrile and 90 volumes of an 0.02 M phosphate buffer (pH = 7.0) and was also used as a diluent. The flow rate of the mobile phase was 1.0 mL/min. The eluents were monitored by a UV-VIS detector at 268 nm. The injection volume was 50 µL.

Preparation of standard solutions

Fifty milligrams of cefquinome was accurately weighed into 25 mL volumetric flask, dissolved and diluted to 25.0 mL with the mobile phase (standard solution).

Twenty milligrams of acetanilide was accurately weighed into a 100 mL volumetric flask, dissolved and diluted to 100.0 mL volume with acetonitrile (internal standard solution).

Specificity/application of stress (forced degradation study)

The degradation of cefquinome sulfate in aqueous solutions was studied at 313 K in hydrochloric acid (0.1 M) and in sodium hydroxide (0.1 M). The ionic strength of all solutions was adjusted to 0.5 M with a solution of sodium chloride (4.0 M). Degradation was initiated by dissolving an accurately weighed 5.0 mg of cefquinome sulfate in 25.0 mL of the solution equilibrated to 313 K in a stoppered flask. At specified time intervals, samples of the solutions were withdrawn and instantly cooled with a mixture of water and ice.

In order to perform oxidative degradation, 5.0 mg of cefquinome sulfate was accurately weighed and dissolved in 5.0 mL of the mobile phase, to which 20.0 mL of a 3% H_2O_2 solution was added and kept at room temperature for 20 min. Samples of reaction solutions were withdrawn and instantly cooled with an ice/water mixture.

Thermal degradation was involved weighing 5.0 mg of cefquinome sulfate into a 5 mL vial and placing it in a heat chamber at 373 K (RH = 0%). After 1 week the vial was removed, cooled to room temperature and the content was dissolved in the mobile phase. The so obtained solution was transferred into measuring flasks and diluted with the mobile phase to 25.0 mL.

UV degradation was conducted by weighing 5.0 mg of cefquinome sulfate and exposing it to sunlight for 48 h (1.2×10^{-6} lux/h. The samples were dissolved and diluted with the mobile phase to 25.0 mL.

Method validation

The HPLC method was validated with respect to specificity, linearity, precision, accuracy and robustness, according to the ICH guidelines (38).

Precision

The precision of the method was determined by injecting six samples 20 mg/mL in triplicate on the same day. The %RDS area of cefquinome was calculated.

Sensitivity

LOD (limit of detection) = $3.3\sigma/S$ and LOQ (limit of quantitation) = $10\sigma/S$, (σ = the standard deviation of the response, S = the slope of the calibration curve) were determined from the regression equation for cefquinome sulfate.

Linearity and range

Method linearity was evaluated in the concentration range 0.034 – 0.1 mg/mL (50 – 150% of the nominal concentration of cefquinome sulfate during the degradation studies). Samples of each solution were injected three times and each series comprised 6 experimental points.

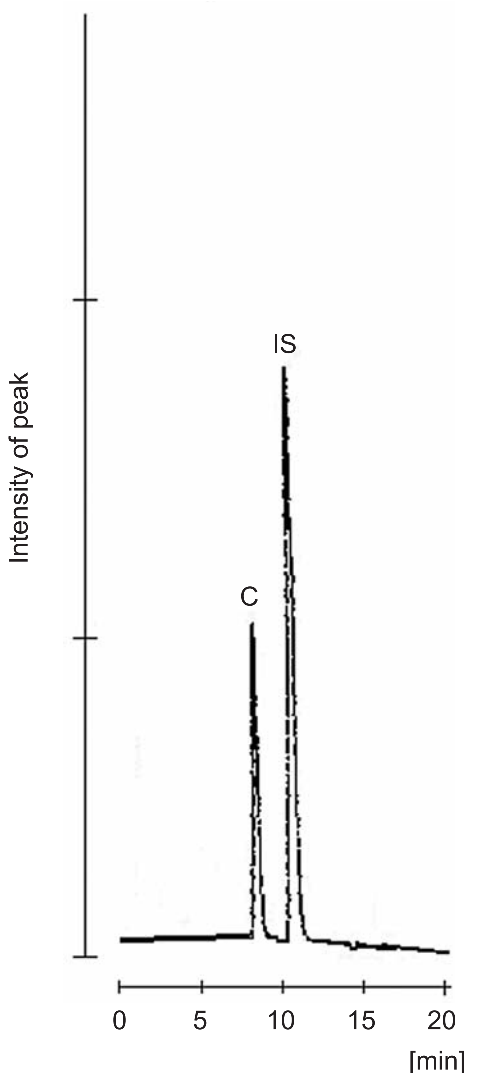

Figure 1. The HPLC chromatogram of a blank sample of cefquinome sulfate (C – cefquinome sulfate, IS – internal standard)

Accuracy

The accuracy of the method was determined by recovering cefquinome sulfate from the placebo. At three levels (80, 100 and 120%) of the nominal concentration of cefquinome sulfate during the degradation studies, three samples were prepared for each recovery level. The solutions were analyzed and the percentage of recovery was calculated.

Robustness

To determine the robustness of the method, the experimental conditions were changed and the purity and shape of the peak was evaluated. The following parameters were altered: the composition of the mobile phase (content of acetonitrile 5% and 20%), the mobile phase flow rate (flow rate 0.8 and 1.4 mL/min), wavelength of absorption (258 nm), temperature (20 and 30°C), the pH of the phosphate buffer (6 and 8). The influence of each parameter on the retention time, resolution, area and peak shape was evaluated (Table 1).

Solution stability and mobile phase stability

The solution stability of cefquinome sulfate in the assay was examined by leaving the test solutions in tightly capped volumetric flasks at room temperature and at 4°C for 24 h. The samples were assayed against a freshly prepared standard solution. The stability of the mobile phase was evaluated by determining the samples against a freshly prepared reference standard solutions at 0 and 24 h.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

A satisfying resolution of cefquinome sulfate and its degradation products formed under various

Table 1. Results of robustness studies.

Parameter	Retention time [min]	Area of peak	Shape of peak	Purity [%]
Optimal	8.54	946484	High, sym.	100.00
ACN = 5%	8.16	943662	High, asym.	100.00
ACN = 20%	1.68	934631	Asym.	95.68
pH = 6	1.69	940015	High, sym.	100.00
pH = 8	8.86	941895	High, asym.	100.00
f = 0.8 mL/min	10.5	1141293	High, asym.	100.00
f = 1.4 mL/min	5.84	929714	High asym.	100.00
λ = 258 nm	8.08	937399	High, asym.	100.00
T = 20°C	8.67	934385	High, sym.	99.99
T = 30°C	7.62	1028480	High, asym.	99.94

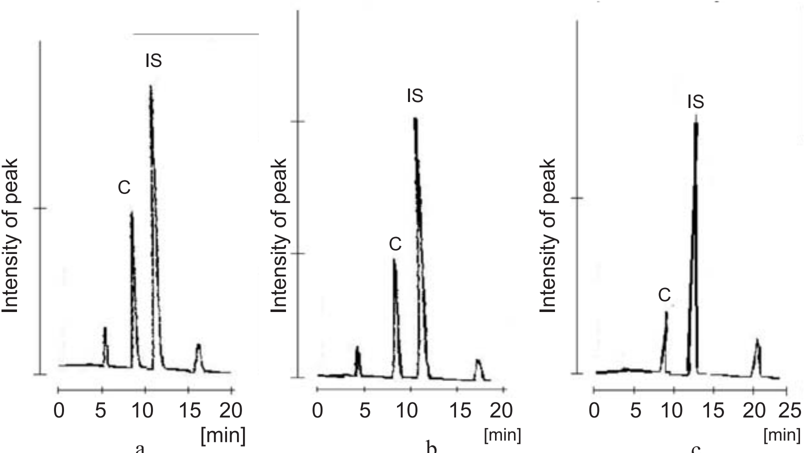

Figure 2. Chromatograms for cefquinome sulfate (C) after basic hydrolysis (a), sunlight (b) and xidizing stress (c), IS – internal standard.

Table 2. Precision studies (n = 6).

A_{cefq}/A_{is}	
0.322	SD = 0.003 RDS = 0.95%
0.315	
0.315	
0.318	
0.321	
0.320	

A = peak area, cefq = cefquinome sulfate, is = internal standard

stress conditions was achieved when the stressed samples were analyzed by using an RP C-18 column and a mobile phase composed of 10 volumes of acetonitrile and 90 volumes of phosphate buffer (pH 7). Detection was carried out at 268 nm. The mobile phase flow rate was 1.0 mL/min. The retention time of cefquinome sulfate was 8.54 min, and the internal standard (acetanilide) 10.65 min (Fig. 1). The purity of the sample peak was 100%.

Method validation

The method was validated for parameters such as specificity, linearity, precision, accuracy and robustness.

Photodiode array detection was used to demonstrate the specificity of the method and to evaluate the homogeneity of the cefquinome sulfate peak. The peak purity values were more than 98.5% at 268 nm, what proves that the degradantion products did not interfere with the main peak.

The linearity of the method was determined in a range 50–150% of the assay concentration. The calibration plots were linear in the concentration range 0.03 – 0.1 mg/mL (n = 13, r = 0.9963) The calibration curves were described by the equation y = ac + b, y = (4.816 ± 0.252)c, b = 0.014 ± 0.018. The b values were not statistically significant.

The intra-day and inter-day precision values were calculated for concentration 6.67×10^{-2} mg/mL of cefquinome sulfate (Table 2). The RDS value was 0.95% and proved that the method was precise.

The recovery test was performed at three levels (80, 100 and 120%) of the nominal concentration of cefquinome sulfate .The recovery values ranged from 99.10 to 101.31% for each concentration, which proved that the method was accurate (Table 3).

Under the chromatographic conditions applied, the LD of cefquinome was 6.14 10^{-3} mg/mL and the QL was 18.61×10^{-3} mg/mL. The robustness of the procedure was evaluated by changing the composition of the mobile phase, its flow rate (0.8–1.2 mL/min), temperature (20–30°C (±1°C)) and pH (6.0–8.0). The effect of those changes on retention time, peak resolution, shape and area was evaluated. It was found that those alternations did not effect those parameters. Modifications of the mobile phase composition (organic to inorganic component ratio) resulted in significant changes in peak retention time.

Results of forced degradation experiments

Cefquinome sulfate, similarly to other cephalosporins, is vulnerable to degradation under

Table 3. Recovery studies (n = 3).

Spiked concentration (mg/mL)	Measured concentration ± SD (mg/mL)	Recovery (%)
0.0534 (~80%)	0.054 ± 0.002	101.31
0.0667 (~100%)	0.066 ± 0.001	99.10
0.0804 (~120%)	0.080 ± 0.001	99.38

Table 4. Results of forced degradation studies.

Stress conditions and time studies	Degradation [%]	Peak purity [%]
Acidic 0.1M HCl/313K/90 min	8.2 9	8.87
Basic 0.1M NaOH/313K/3.5 min	75.0	99.56
Oxidizing 3% H_2O_2/298K/20 min	92.4	99.76
Thermal /373K/ 1 week	18.4	98.65
Sunlight 48 h	25.2	100.0

the influence of physical and chemical factors. It was observed that 8–93% of cefquinome sulfate was degredated during acidic hydrolysis, basic hydrolysis, oxidation, UV irradiation and exposure to an increased temperature (Table 4).

CONCLUSIONS

This HPLC method for assay cefquionome sulfate was successfully developed and validated for its intended purpose. The method was shown to be specific, linear, precise, accurate and robust. Cefquinome sulfate was found to be very susceptible to basic hydrolysis, oxidizing and photolysis (Fig. 2).

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DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF MEROPENEM AND POTASSIUM CLAVULANATE

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Abstract: A stability-indicating LC assay method was developed and validated for a simultaneous determination of meropenem and potassium clavulanate in the presence of degradation products formed during acid-base hydrolysis, oxidation and thermolysis. The isocratic RP-HPLC method was developed with a LiChrospher RP-18 (250 mm × 4.6 mm, 5 µm) column and gradient elution of 12 mmol/L ammonium acetate and acetonitrile. The flow rate of the mobile phase was 1.0 mL/min, the detection wavelength 220 nm and the temperature 303 K. The method was validated with regard to linearity, accuracy, precision, selectivity and robustness, and was applied successfully for the determination of meropenem and potassium clavulanate separately as well as jointly in pharmaceutical formulations.

Keywords: liquid chromatography**,** meropenem, clavulanate potassium

Meropenem, a β-lactam antibiotic and a member of the carbapenem group (Fig. 1), has a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria (1–4). Similarly to other carbapenems, meropenem is often used as a last resort in the treatment of many complicated bacterial infections. Potassium clavulanate (Fig. 2) is a β-lactamase inhibitor which shows a relevant post-antibacterial effect when administered together with amoxicillin. β-Lactam antibiotics are known to be ineffective against *Mycobacterium tuberculosis*, as they are rapidly hydrolyzed by the chromosomally encoded *blaC* gene product.

Recent *in vitro* and *in vivo* studies have indicated the effectiveness of connecting meropenem and potassium clavulanate in the treatment of *Mycobacterium tuberculosis*, including its most resistant strains (5–7).

The significant instability of meropenem and potassium clavulanate, associated with the presence of 4,5 fused β-lactam and heterocyclic rings, determines the process of preparing and the storage of formulations containing β-lactam analogs (8, 9). Several studies investigated the chemical instability of meropenem in aqueous solutions (10) and in the solid state (11). Depending on affecting factors, degradants of different chemical structures are formed. Similarly to β-lactam analogs, potassium clavulanate is also chemically unstable. Its degradation products have been observed to have a catalytic effect on the rate of degradation of clavulanic acid

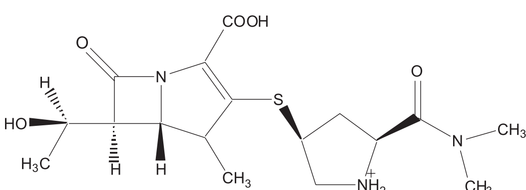

Figure 1. Chemical structure of meropenem

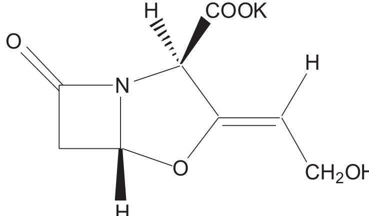

Figure 2. Chemical structure of potassium clavulanate

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and to be determined by pH and the presence of ions (12).

Since the majority of the side effects of β-lactam antibiotics are caused by their degradation products, it is vital to improve analytical methods for the determination of β-lactam analogues. An optimal method is expected to separate and determine the substance to be examined in the presence of related products such as in-process impurities, degradation products and metabolites (13). The current guidelines of the International Conference on Harmonization (ICH) require the development of stability-indicating assay methods (SIAMs) suitable for the determination of drugs based on the analysis of stability test samples (Q1A–R2) (14). It is equally important to develop an appropriate analytical method to assess changes in drug concentrations in intravenous solutions. Furthermore, in the case of labile drugs and those for frequent administration, it is essential to establish whether they are suitable to be mixed in one infusion flow in order to preclude their interaction. Also, it is necessary to develop a selective method for determining their concentration changes in the presence of their degradation products.

Until now, no chromatographic methods for a simultaneous determination of meropenem and potassium clavulanate are available in the literature. The aim of this work was to develop and validate an HPLC method with UV detection suitable for a simultaneous determination of meropenem and potassium clavulanate in the presence of their degradation products.

EXPERIMENTAL

Materials

Meropenem (purity > 98%) and potassium clavulanate were obtained from CHEMOS (Germany). Potassium clavulanate was diluted in ratio 1 : 1 by 50% silicon dioxide and contained less than 2% of impurities.

All other chemicals and solvents were obtained from Merck KGaA (Germany) and were of analytical grade. High quality pure water was prepared by using the Millipore purification system, model Exil SA 67120 (France).

Instrumentation

The HPLC system (Dionex UltiMate 3000) consisted of quaternary pump (LPG-3400RS), an autosampler (WPS-3000TRS), a column oven (TCC-3000RS) and diode array detector (DAD-3000). Separations were performed on a LiChrospher RP-18 column, 5 µm particle size, 250 mm × 4 mm (Merck, Germany). The mobile phase consisted of a mixture of solution A (12 mmol/L ammonium acetate) and solution B (acetonitrile) with an initial composition of 4% solution B. From t_0 to $t_{4\ min}$ the concentration of solution B was constant and equal to 4%, then it was changed linearly to reach 25% at $t_{12\ min}$, after which the column was re-equilibrated to starting conditions for 5 min. The flow rate of the mobile phase was 1.0 mL/min. The wavelength of the DAD detector was set at 220 nm. The column and autosampler tray were set at 303 K and 278 K, respectively.

Validation method

The HPLC method was validated according to the International Conference on Harmonization Guidelines (14). It comprised parameters such as selectivity, linearity, precision, accuracy and robustness.

Procedure for forced degradation study of meropenem and potassium clavulanate

Degradation in aqueous solutions

The degradation of meropenem and clavulanate potassium in aqueous solutions was studied at 293 K in hydrochloric acid (0.1 mol/L) and in sodium hydroxide (0.1 mol/L). Degradation was initiated by dissolving an accurately weighed 10.0 mg of meropenem and 10.0 mg potassium clavulanate in 25.0 mL of the solution equilibrated to 293 K in stoppered flasks. At specified times, samples of the reaction solutions (1.0 mL) were instantly neutralized and cooled with a mixture of ice and water. The degradation in condition of an oxidation was initiated by dissolving an accurately weighed 10.0 mg of meropenem and 10.0 mg potassium clavulanate in 25.0 mL of 3% H_2O_2 solution equilibrated to 293 K in stoppered flasks. At specified times, samples of the reaction solutions (1.0 mL) were instantly cooled with a mixture of ice and water.

Degradation in the solid state

Ten milligrams samples of meropenem and potassium clavulanate were weighed into glass vials. In order to achieve the degradation of meropenem and potassium clavulanate in the solid state, their samples were put in the heat chambers at 373 K at RH = 0% and 313 K at RH ≈ 76.4%. At specified time intervals, determined by the rate of degradation the vials were removed, cooled to room temperature and their contents were dissolved in water. The obtained solutions were quantitatively

transferred into measuring flasks and diluted with water to 25.0 mL.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The main objective was to develop an HPLC-UV method for the determination of meropenem and potassium clavulanate in the presence of degradation products.

The mobile phase consisted of 12 mmol/L ammonium acetate and acetonitrile (96 : 4 v/v) with isocratic elution. The stationary phase used was a LiChrospher RP-18 column. Although the detection wavelength for meropenem was 300 nm, the absorption maximum of the products of its degradation was at 220 nm, which was also the wavelength at which the absorption maximum of clavulanate potassium occurred. The so-designed mobile phase was proposed for a simultaneous determination of meropenem and potassium clavulanate. However, in order to separate potassium clavulanate from the degradation products of meropenem it was necessary to use gradient elution. By applying an experimentally obtained HPLC gradient program (from t_0 to $t_{4\,min}$ the concentration of solution B was constant and equal 4%, then it was changed linearly to reach 25% at $t_{12\,min}$) it was possible to achieve a satisfying separation of clavulanate potassium (retention time 2.933 min.), meropenem (10.213 min.) and their degradation products (2.073, 4.530 and 11.447 min.) (Fig. 3).

Method validation

The selectivity was examined for non-degraded and degraded samples (the solutions of meropenem and potassium clavulanate after stress conditions: acid and base hydrolysis at room temperature, oxidation H_2O_2 and thermal degradation at 313 K and 373 K). The HPLC method for determination of meropenem and potassium clavulanate was found of selective in the presence of degradation products as shown in the pictures (Fig. 3). Peaks were symmetrical and clearly separated from each other (Fig. 3). Photodiode array detection was used as an evidence of the specificity of the method and to evaluate the homogeneity of meropenem and potassium clavulanate peaks. The calibration plots were linear in the following concentration range 40 – 480 mg/L, n = 7, r = 0.9999 (meropenem), 80–480 mg/L, n = 6, r = 0.9996 (potassium clavulanate). The calibration curve was described by the equation y = ax; y = (0.1257 ± 0.005)x (meropenem) y = (0.1513 ± 0.0044)x (potassium clavulanate). The b value, calculated from equation y = a*x* + b, was insignificant because it was lower than the critical value $t_b = b/S_b$. Statistical analysis using Mandel's fitting test confirmed that linear model is preferred over quadratic regression for the calibration curves. The samples of each solution were injected three times and each series comprised 7 experimental points. Precision of the assay was determined in relation to repeatability (intra-day) and intermediate precision (inter-day) for six samples. Intermediate precision was studied comparing the assays performed on two different

Table 1. Precision data (n = 6) for meropenem and clavulanate potassium.

Analyte	Spiked concentration (mg/L)	Measured concentration ± SD (mg/L)	RSD (%)
Intra-day precision			
Potassium clavulanate	320.00	320.84 ± 4.58	1.36
	400.00	393.87 ± 5.57	1.35
	480.00	464.89 ± 7.49	1.53
Meropenem	320.00	321.27 ± 1.44	0.43
	400.00	395.96 ± 1.54	0.37
	480.00	472.50 ± 3.60	0.66
Inter-day precision			
Potassium clavulanate	320.00	317.32 ± 9.83	3.19
	400.00	389.98 ± 11.05	2.91
	480.00	462.02 ± 9.71	2.16
Meropenem	320.00	319.89 ± 3.74	1.20
	400.00	393.96 ± 4.33	1.13
	480.00	470.64 ± 5.61	1.14

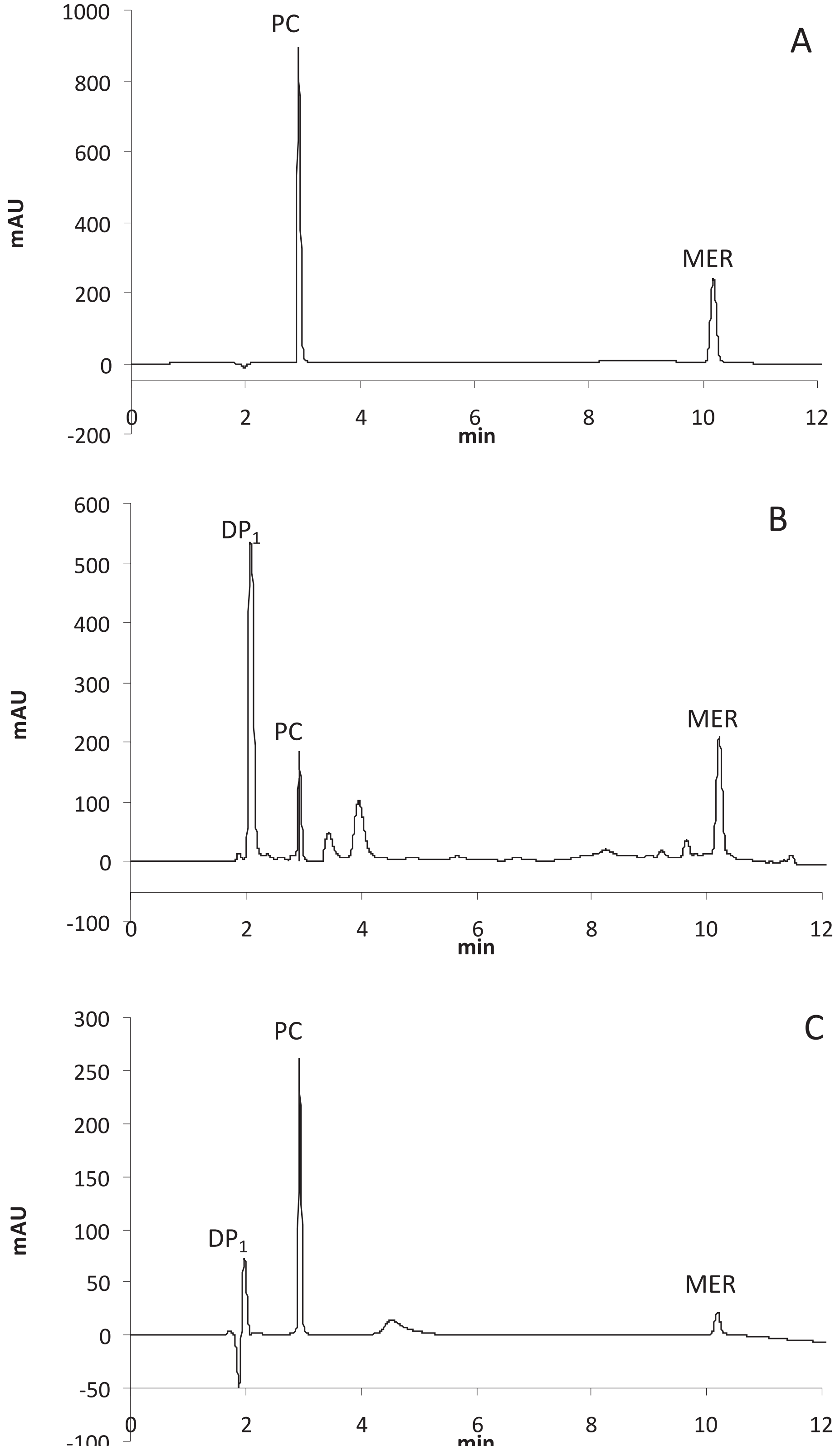

Figure 3. Chromatogram of blank sample of meropenem (MER) and potassium clavulanate (PC) (**A**); chromatogram of meropenem and potassium clavulanate during oxidative forced degradation study (**B**); chromatogram of meropenem and potassium clavulanate during alkali forced degradation study (**C**); DP_1 – meropenem degradation product with an open β-lactam ring

Table 2. Accuracy data (n = 6) for meropenem and potassium clavulanate.

Analyte	Spiked concentration (mg/L)	Measured concentration ± SD (mg/L)	RSD (%)
Recovery studies			
Potassium clavulanate	320.0 (~ 80%) 400.0 (~ 100%) 480.0 (~ 120%)	307.67 ±1.57 375.67 ± 1.87 440.47 ±1.84	96.15 93.92 91.76
Meropenem	320.0 (~ 80%) 400.0 (~ 100%) 480.0 (~ 120%)	316.80 ± 0.54 390.44 ± 0.91 462.67 ± 1.81	99.00 97.61 96.39

Table 3. Results of forced degradation studies.

Stress conditions and time studies	Degradation CP [%]	Degradation MER [%]
Acidic /0.1 mol/L HCl 293 K/ 53 min	23.63	22.73
Alkali /0.1 mol/L NaOH 293 K/ 1 min	98.82	89.86
Oxidizing /3% H_2O_2/293 K/ 115 min	82.55	70.09
Thermal /373 K RH ≈ 0%/ 3 days	69.23	57.91
Thermal /313 K RH ≈ 76.4%/ 28 days	78.25	68.12

CP = potassium clavulanate; MER = meropenem

days. The intra-day and inter-day precision values of measured concentration of meropenem and potassium clavulanate, as calculated for 80, 100 and 120% levels their of initial concentrations, the RSD values were 0.37 and 3.19%, respectively, demonstrating that the method was precise for both of analyzed substances (Table 1). The accuracy of the method was determined by recovering meropenem and potassium clavulanate from placebo. The recovery test was performed at three levels 80%, 100% and 120% of the nominal concentration of meropenem and potassium clavulanate during degradation studies. Six samples were prepared for each recovery level. Values of RSD for meropenem and potassium clavulanate indicate that excipients do not have effect or interference on the determination of tested analytes (Table 2). The LOD and LOQ parameters were determined from the regression equations of meropenem and potassium clavulanate, respectively: LOD = 3.3 S_y/a, LOQ = 10 S_y/a; where S_y is a standard error and a is the slope of the corresponding calibration curve. Under applied chromatographic conditions, the LOD and LOQ of meropenem were 2.57, 7.78 mg/L and of potassium clavulanate 14.51, 43.98 mg/L, respectively.

The robustness of the procedure was evaluated after changing the following parameters: the composition of the mobile phase (concentration of ammonium acetate in the range 12 ± 2 mmol/L), the pH of mobile phase in the range 7.15 ± 0.05, initial and final concentration of acetonitrile in the mobile phase in the range 4 ± 1 and 25 ± 1%, respectively, time of a gradient increase in the range 8 ± 1 min, flow rate in the range 1.0 ± 0.1 mL/min, wavelength of absorption 220 ± 3 nm, temperature 303 ± 2 K. No significant changes in separation, retention time, area and asymmetry of peak were observed when tested parameters were modified.

Results of forced degradation experiments

A comparison of the results of the kinetic studies carried out separately for the potassium clavulante and meropenem under the same degradation conditions with the results obtained for their mixture indicated the absence of any mutual catalytic effect or any impact of their degradation products.

The degradation of potassium clavulanate and meropenem was observed during stress studies in solutions (acidic and basic hydrolysis, oxidation) and in the solid state at an increased relative air

humidity (RH ≈ 76.5%) and in dry air (RH = 0%). No degradation was noted in the solid state during photolysis. Potassium clavulante was found to be the most prone to degradation under oxidizing conditions (3% H_2O_2, RT), whereas meropenem under basic conditions (0.01 M NaOH, RT). Under the influence of an acidic medium (0.1 M HCl, RT) the degradation of meropenem and potassium clavulanate was comparable (approx. 40% after 3 min). The results of forced degradation in various media were summarized in Table 3. The chromatograms of solutions obtained after forced degradation are shown in Figure 3. No peaks of degradation products of potassium clavulanate occurred on the chromatograms of degraded samples. The lack of substituents containing π-bond system chromophores appeared to prevent the application of an LC method with a PDA detector for recording peaks originating from the degradation products of potassium clavulanate. Although during the degradation of meropenem a product with an open β-lactam ring (DP_1) (retention time of 2.15 min) was formed, the presence of potassium clavulanate did not lead to the appearance of any other degradation products.

CONCLUSIONS

The linear gradient RP-LC method for a simultaneous analysis of meropenem and potassium clavulanate in intravenous solutions or in their potential pharmaceutical preparations was developed. Selectivity, accuracy and precision and short run time make this method useful for routine analysis.

Acknowledgment

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STABILITY OF NEW ANTICONVULSANT DERIVATIVES OF PICOLINIC, NICOTINIC, CYCLOCARBOXYLIC ACIDS IN BODY FLUIDS AND TISSUES

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Abstract: The stability of new compounds with established anticonvulsant activity: picolinic acid 4-pyridylmethylamide (Pic-4-PMA), cyclopentanecarboxylic acid benzylamide (Cpc-BZA), cycloheptanecarboxylic acid benzylamide (Chc-BZA), picolinic acid 2-fluoro-3-trifluoromethylbenzylamide (Pic-2F-3TFM-BZA), 2-chloronicotinic acid benzylamide (2-Cl-Na-BZA), 6-chloronicotinic acid benzylamide (6-Cl-Na-BZA) and 6-trifluoromethylnicotinic acid benzylamide (6-TFM-Na-BZA) in homogenates of body organs and in body fluids was determined after incubation. It was found that three compounds were stable against enzymes present in body fluids and organs and two were found to decompose in liver and kidney homogenates and two decomposed only in liver homogenate.

Keywords: picolinic, nicotinic and cyclocarboxylic acid derivatives, anticonvulsant activity

In the search for new anticonvulsants, picolinic acid benzylamide (Pic-BZA) was previously synthesized. It is a strong antagonist of excitatory amino acid receptor but of short action time (1). Searching for equally effective anticonvulsant but more stable, the basic structure Pic-BZA was modified and the new amide derivatives of some heterocyclic and cyclic acids were synthesized (2, 3). Their stability (Pic-BZA, Na-BZA) was tested. In our previous papers it was reported that Pic-BZA decomposed in liver and kidney homogenates and it was a first-order reaction relative to substance concentration (the relationship ln c *versus* time was linear). The half-time was 1.25 h and 5.73 h for the liver and kidney, respectively (4). Several derivatives with modified structure of Pic-BZA were synthesized (5). Picolinic acid benzylamide was substituted with CH_3 and F in various positions of the ring (Pic-2-F-BZA, Pic-3-F-BZA, Pic-4-F-BZA, Pic-2-Me-BZA) to prolong the stability. Also these compounds decomposed in liver and kidney homogenates. The half-life in liver for Pic-BZA was 0.5 h, for Pic-2-F-BZA 1.5 h and over 3 h for Pic-3-F-BZA and Pic-4-F-BZA. The decomposition in kidney homogenate was much slower. Na-BZA described in paper (4) was stable in all body fluids and organ homogenates but the time of anticonvulsant action was not so long, thus some new derivatives were synthesized. Those were derivatives of Na-BZA (2-Cl-Na-BZA and 6-Cl-Na-BZA) and some benzylamides of acids containing 5- or 7-member rings in the structure (Cpc-BZA and Chc-BZA). Also benzylamide of picolinic acid was substituted with pyridylmethylamide (Pic-4-PMA) (6). All newly synthesized compounds were evaluated in the Anticonvulsant Screening Program (ASP) of Antiepileptic Drug Development Program (ADDP) of NIH, USA.

The stability of all compounds was examined in body fluids and organ homogenates. The concentrations of derivatives were measured at different time points during incubation. The HPLC method was developed and used to determine the concentration of all derivatives isolated from biological material by liquid-liquid extraction.

EXPERIMENTAL

Apparatus and chromatographic conditions

A Shimadzu HPLC apparatus that consisted of an LC-10AT pump and SPD-10A spectrophotometer was used with Chroma computer recorder (POL-

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Pic-4-PMA Chc-BZA Cpc-BZA Pic-2F-3TFM-BZA

2-Cl-Na-BZA 6-Cl-Na-BZA 6-TFM-Na-BZA

Figure 1. Chemical structures of new synthesized compounds

Table 1. Parameters of chromatographic determination and validation procedure of determined compounds.

Compound	Mobile phase (v/v)	λ nm	Internal standard %	Recovery %	CV	r
Pic-4-PMA	MeOH–H_2O –triethylamine 70 : 130 : 0.02	262	Na-BZA	98.11 ± 5.41	5.45 ± 1.83	0.9958
Chc-BZA	MeOH–H_2O 40 : 60	210	Pic-2-Me-BZA	99.13 ± 3.23	6.28 ± 1.09	0.9996
Cpc-BZA	MeOH-H_2O 60 : 40	210	–	97.84 ± 3.79	6.40 ± 1.34	1.0000
Pic-2F-3TFM-BZA	MeOH–H_2O 62.5:32.5	265	Pic-3F-BZA	98.30 ± 4.38	3.88 ± 3.78	0.9996
2-Cl-Na-BZA	MeOH–H_2O 50 : 50	265	2-Cl-iNa-BZA	100.88 ± 4.04	5.70 ± 2.56	0.9990
6-Cl-Na-BZA	MeOH–H_2O 60 : 40	262	Chlordiazepoxide	100.29 ± 1.51	3.66 ± 3.02	0.9998
6-TFM-Na-BZA	MeOH–H_2O 60 : 40	262	2-Me-Pic-BZA	98.76 ± 3.34	4.54 ± 2.91	0.9990

LAB, Poland) and the Chromax 2001 software (POL-LAB, Poland).

The separation was carried out in the reversed phase system with a Beckman Ultrasphere ODS column (150 mm × 4.6 mm). The flow rate was 1 mL/min. The mobile phases, wavelengths and internal standards are presented in Table 1.

Compounds

Cpc-BZA, Chc-BZA, Pic-2F-3TFM-BZA, Pic-4-PMA, 2-Cl-Na-BZA, 6-Cl-Na-BZA and 6-TFM-Na-BZA and internal standards were synthesised in the Department of Drug Chemistry, Warsaw Medical University. Chemical structures are presented in Figure 1.

Preparation of solution of compounds 1–7

Stock standard solutions of determined compound and internal standards were prepared by dissolving each compound in methanol (0.1 mg/mL). The final working concentration for the examined substances and internal standards was 10 µg/mL. The liquid-liquid extraction method was used for all biological material.

Validation of analytical procedures

The method of Cpc-BZA, Chc-BZA, Pic-2F-3TFM-BZA, Pic-4-PMA, 2-Cl-Na-BZA, 6-Cl-Na-BZA and 6-TFM-Na-BZA determination was validated. Standard deviations, recovery, accuracy and linearity of the analytical procedure are presented in Table 1.

Table 2. Stability of Cpc-BZA, Chc-BZA, Pic-2F-3TFM-BZA, Pic-4-PMA, 2-Cl-Na-BZA, 6-Cl-Na-BZA and 6-TFM-Na-BZA.

Compound	Stability	
	Kidney homogenate	Liver homogenate
Pic-4-PMA	stable	stable
Chc-BZA	a first-order reaction relative to substance concentration $t_{0.5}$ = 142.5 min	a first-order reaction relative to substance concentration $t_{0.5}$ = 96.6 min
Cpc-BZA	a first-order reaction relative to substance concentration $t_{0.5}$ = 36.5 h	a first-order reaction relative to substance concentration $t_{0.5}$ = 20.4 h
Pic-2F-3TFM-BZA	stable	a first-order reaction relative to substance concentration $t_{0.5}$ = 231 min
2-Cl-Na-BZA	stable	stable
6-Cl-Na-BZA	stable	stable
6-TFM-Na-BZA	stable	stable

Determination of Cpc-BZA, Chc-BZA, Pic-2F-3TFM-BZA, Pic-4-PMA, 2-Cl-Na-BZA, 6-Cl-Na-BZA and 6-TFM-Na-BZA in gastric and intestinal juice

Five milligrams of each compound was dissolved in 1 mL of ethanol and 49 mL of freshly prepared gastric or intestinal juice (USP). The solutions were incubated at 37°C and the samples were collected at the time points 0, 30, 60, 90, 120, 150, 180 min. The so-obtained results are presented in Table 2.

Determination of Cpc-BZA, Chc-BZA, Pic-2F-3TFM-BZA, Pic-4-PMA, 2-Cl-Na-BZA, 6-Cl-Na-BZA and 6-TFM-Na-BZA in pork liver, kidney, brain and lung homogenates

The homogenates of body organs (40%) in 0.1 mole/L TRIS solution (pH = 8.4) were prepared. Two milliliters of the homogenate was spiked with 20 µg of each compound and incubated at 37°C. The samples were collected at the time points 0, 30, 60, 90, 120, 150, 180 min. Results are presented in Table 2.

RESULTS AND DISCUSSION

The stability study of seven amide derivatives in body fluids and organs homogenates showed that Chc-BZA and Cpc-BZA only decomposed in liver and kidney homogenates and Pic-2F-3TFM-BZA decomposed only in liver homogenate, while nicotinic acid benzylamides and Pic-4-PMA were resistant to enzymes present in all tested tissues. Decomposition in liver and kidney homogenates is a first-order reaction relative to substance concentration because the relationship ln c *versus* time is linear. For Pic-BZA, the basic structure represented class I of ASP (Anticonvulsant Screening Program). All modification of its structure decreased the anticonvulsant activity to II or III class but significantly prolonged time of action. Even for Chc-BZA and 1-Cpc-BZA, which decomposed in liver and kidney homogenates and Pic-2F-3TFM-BZA, which decomposed in liver homogenates, the half-life time was relatively long (e.g., more than 36 h for 1-Cpc-BZA in kidney homogenates). Introduction of nicotinic acid instead of picolinic acid (2-Cl-Na-BZA, Chc-BZA, Cpc-BZA) and replacement of picolinic acid benzylamide to picolinic acid pyridylmethylamide decreased anticonvulsant activity mostly to III class but made these compounds resistant to enzymes presented in all tested tissues. Based on obtained data we conclude that duration of action was prolonged but the activity was reduced.

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ANALYSIS OF COMPOUNDS WITH PHYTOESTROGENIC ACTIVITY IN DIETARY SUPPLEMENTS WITH USE OF HPTLC-DENSITOMETRY METHOD

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Abstract: Soy (*Glycine max* L., Fabaceae) and soy products are becoming more popular because of their low toxicity and therapeutic effects. Soy possesses antioxidant, anti-inflammatory and anti-allergic properties, however, the most important is its estrogenic activity associated with occurrence of phytoestrogens. Isoflavones with phytoestrogenic effects were determined in four commercially available soya formulations. Analyses were performed with the use of high performance thin-layer chromatography (HPTLC) combined with densitometry. The compounds were extracted, hydrolyzed in order to obtain aglycone forms and separated on HPTLC silica gel 60 F_{254} plates with the use of mobile phase consisting of chloroform – ethyl acetate – formic acid 4 : 6 : 0.1 (v/v/v). After drying, the spots on the plates were determined in absorbance/reflectance mode at a wavelength of 260 nm using a computer-controlled densitometer Desaga CD 60.

Keywords: phytoestrogenes, isoflavones, daidzein, genistein, densitometry

Recently, dietary supplements, particularly derived from plants have gained an increasing popularity as an alternative treatment for their low toxicity and therapeutic effects. Soy (*Glycine max* L., Fabaceae) and soy products are a good example of these types of natural preparations. They are commonly used in the treatment of cardiovascular diseases, menopausal complaints, some types of cancer and for the prevention of osteoporosis (1, 2). Traditional diet based on soy may be responsible for the low breast cancer cases in the region of Asia (3, 4). Soy is a rich source of many valuable compounds including proteins, lipids, saponins, trypsin inhibitors, fiber, phytic acid; however, components with estrogenic activity such as isoflavones and cumestrol are the most important from the point of view of health benefits as alternative to the synthetic estrogen receptor modulators commonly used in hormone therapy (4). There are a lot of various preparations of soy available on the Polish market. The most of them are classified as dietary supplements and not as drugs. Therefore, manufacturers are not obliged to confirm quantities of the active ingredients in the product. Concentration of compounds in plants strongly depends on genotypes, growing seasons and environmental effects. For example, total isoflavones amount in soy samples can ranged from 140 to 748 µg/g and from 258 to 1137 µg/g before and after hydrolysis, respectively (5).These results suggest that quality control of natural products is a necessary process.

The most widely employed method for analysis of isoflavones is HPLC (6–8) but it is time consuming and requires large amounts of solvents during the whole chromatographic run.

On the other hand, HPTLC has been an inexpensive and environmental friendly tool for the phytochemical assessment of plant extracts and herbal drug formulations.

The aim of the present work was to elaborate chromatographic conditions for the quantification of the major constituents with phytoestrogenic effects such as isoflavones in soya formulations with use of HPTLC method and comparison of their contents in the most popular commercially available products.

EXPERIMENTAL

Materials and chemicals

Four different preparations of *Glycine max*: Menoplant Soy-a 40+ (ASA Sp. z o.o), Soya meno (Medana Pharma, Terpol Group S.A.), Menostop

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(Hasco-Lek) and Soyfem (Biofarm) were purchased from a local pharmacy. Isoflavones standards: genistein and daidzein were supplied by Sigma-Aldrich (Germany).

All solvents and reagents were at least pro analysis grade from Polish Reagents (POCh, Gliwice, Poland). HPTLC plates were from Merck (Darmstadt, Germany).

Standards and samples preparation

Ten tablets or capsules of each preparation were accurately weighted. An equivalent of one tablet or content of one capsule (Sample 1: Menoplant Soy-a 40+ = 0.3918 g; Sample 2: Soya meno = 0.4613 g; Sample 3: Menostop = 0.2769 g; and Sample 4: Soyfem = 0.3351 g) was twice extracted with methanol (2 × 50 mL) within 30 min. at room temperature in an ultrasonic bath. The obtained extracts were combined and concentrated to 25 mL.

Hydrolysis condition

A volume of 0.8 mL of 1.0 mol/L hydrochloric acid was added to 10 mL of each extract and made up with methanol to 25 mL in volumetric flask. Hydrolysis was conducted during 2 h at 37°C. Before application, all samples were neutralized.

A stock solutions at concentration of 100 mg/mL and 25 mg/mL for daidzein and genistein, respectively, were prepared in methanol.

Chromatography

Chromatography was performed on 20 cm × 10 cm HPTLC silica gel 60 F_{254} plates. The plates were washed with methanol and dried in a stream of hot air before use. Volumes of 2, 4, 6, 8, 10, 12 mL of standard solution of daidzein; 3, 6, 9, 12, 15, 18 mL of standard solution of genistein; 6 mL of samples no 2, 4; 10 of sample no. 1 and 16 mL of sample no. 3 were spotted as 5 mm bands using an automatic applicator Desaga AS 30 (Heidelberg, Germany) under nitrogen at 2.5 atm (track distance: 9 mm, distance from the left edge: 13 mm).

The plates were developed with the mixture of chloroform – ethyl acetate – formic acid 4 : 6 : 0.1 (v/v/v) to a distance of 80 mm in chromatographic chamber DS (Chromdes, Lublin), previously saturated with vapors of the mobile phase. After drying in the stream of warm air, the plates were analyzed by densitometric scanning (Desaga CD-60,

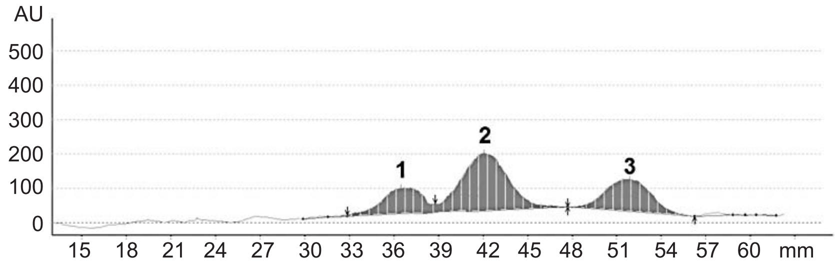

Figure 1. The example of densitogram of hydrolyzed extract from Sample 1: Menoplant Soy-a 40+ obtained at λ = 260 nm; 1 – glycitein, 2 – daidzein, 3 – genistein

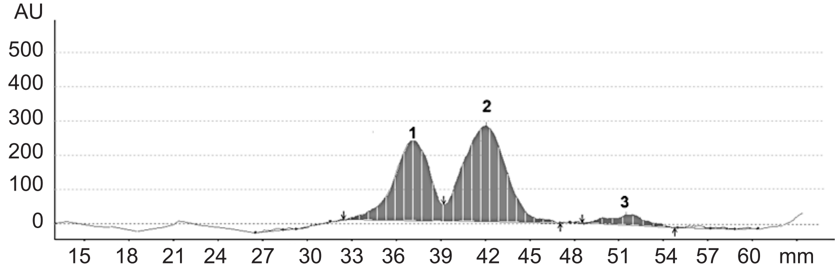

Figure 2. The example of densitogram of hydrolyzed extract from Sample 2: Soya meno obtained at λ = 260 nm; 1 – glycitein, 2 – daidzein, 3 – genistein

Table 1. Summary of validation data for determination of isoflavones.

Validation parameter	Genistein	Daidzein
Linearity range	75–450 ng/spot	200–1200 ng/spot
Regression equation	1861x – 14.6	1240x + 115
Correlation coefficient (R^2)	0.9969	0.9990
Limit of detection (LOD)	9.8 ng/spot	14.2 ng/spot
Limit of quantification (LOQ)	29.8 ng/spot	43.1 ng/spot
Precision (% RSD)	2.5–4.5%	2.7–4.7%
Recovery (%)	98.7%	99.2%

Table 2. The comparison of isoflavones contents in pharmaceutical products.

Pharmaceutical preparation	Genistein [mg/g] ± SD	Daidzein [mg/g] ± SD	Glycitein [mg/g] ± SD	Total amount average [mg/g]
Menoplant Soy-a 40+	5.56 ± 0.17	9.84 ± 0.34	3.45 ± 0.10	18.84
Soya meno	1.70 ± 0.07	13.85 ± 0.40	10.30 ± 0.33	25.85
Menostop	1.10 ± 0.05	4.74 ± 0.22	4.24 ± 0.11	10.07
Soyfem	17.00 ± 0.43	20.32 ± 0.55	14.08 ± 0.35	51.40

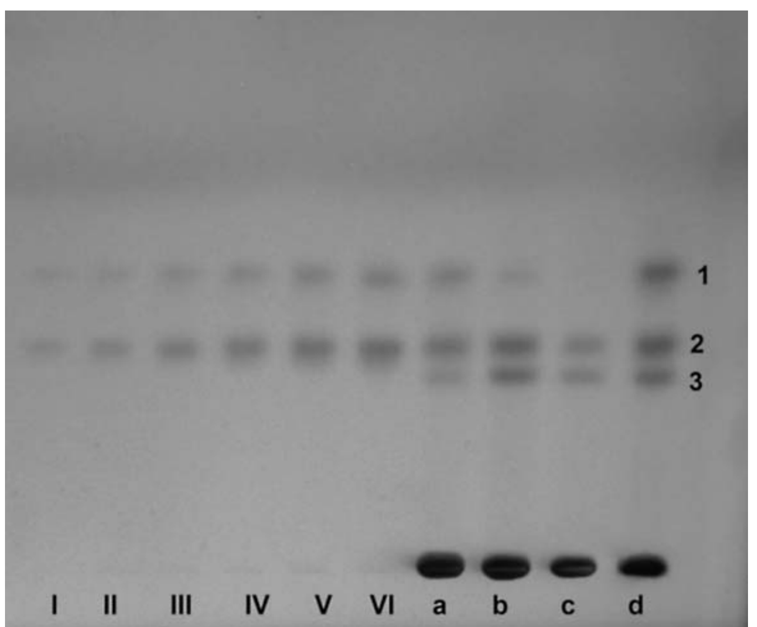

Figure 3. The photograph of HPTLC plate: I – VI calibration curve; a – Menoplant Soy-a 40+, b – Soya meno, c – Menostop, d – Soyfem; 1 – genistein, 2 – daidzein, 3 – glycitein

Heidelberg, Germany) in absorbance/reflectance mode at λ = 260 nm.

RESULTS AND DISCUSSION

Positive health effects documented for *Glycine max* preparations including alleviating menopausal symptoms in women (4, 9) are attributed to the presence of natural compounds such as glycitein, genistein and daidzein with estrogenic properties (10). Additionally, these isoflavones stimulate osteoblastic bone resorption and protect against osteoporosis (5).

β-Glucosides and acetate or malonyl esters are the predominant forms of isoflavones present in soy

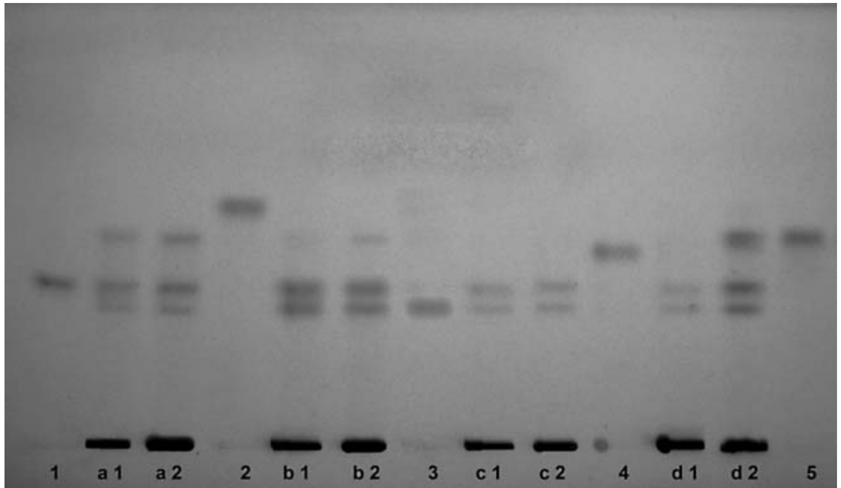

Figure 4. The photograph of HPTLC plate: 1 – daidzein, 2 – biochanin A, 3 – glycitein, 4 –coumestrol, 5 – genistein; a – Menoplant Soy-a 40+ (a1 – before, a2 – after hydrolysis), b – Soya meno (b1 – before, b2 – after hydrolysis), c – Menostop (c1 – before, c2 – after hydrolysis), d – Soyfem (d1 – before, d2 – after hydrolysis)

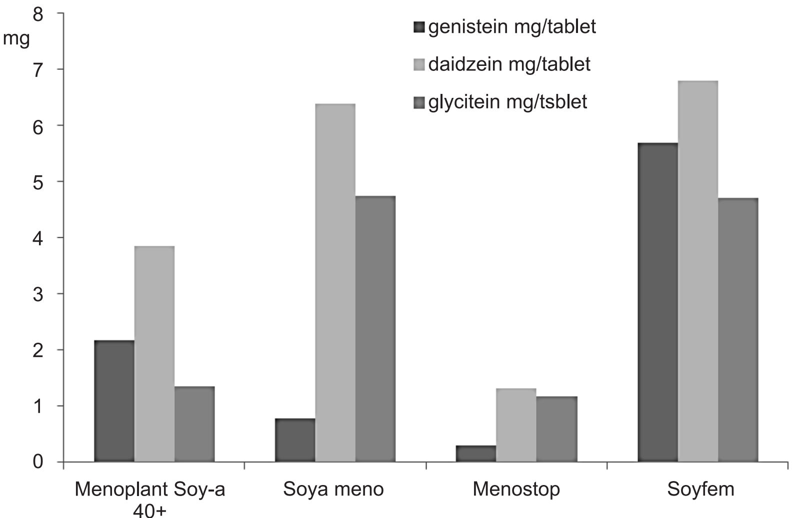

Figure 5. Comparison of isoflavones contents in soy formulations

products, however, free aglycones formed during enzymatic hydrolysis are the bioactive forms (11–13); thus, our studies focus on determination of isoflavones after acid hydrolysis. In our investigations, the hydrolysis parameters such as acidity (pH = 1.5), temperature (T = 37°C) and time (t = 2 h) were similar to physiological conditions of gastric fluid.

Usually, total amount of isoflavones is given by the manufacturers; however, the concentration of genistein, daidzein and glycitein is especially important because of their estrogenic activity. Thus, in our investigation particular attention on these compounds was paid.

In preliminary chromatographic investigations, the mobile phase composition was optimized. The best results were obtained for the mixture of chloroform – ethyl acetate – formic acid 4 : 6 : 0.1 (v/v/v). The bands of analyzed compounds were dense, compact and well separated from the accompanying components (Figs. 1, 2).

The identification was done on the basis of the R_f values. The purity of the peaks in the sample was

ascertained by comparison of absorption spectra with those obtained from the standards. The example of chromatographic plates with standards at different concentrations and analyzed samples is presented in Figure 3.

The method was validated for linearity, precision and accuracy. A calibration plot was established by analysis of standard solution at six different concentrations in the ranges: 0.20–1.2 µg/spot for daidzein and 0.08–0.45 µg/spot for genistein. The amount of glycitein was calculated on the basis of calibration parameters for daidzein as a reference compound because of the high prices and low availability of glycitein standard. The mean peak areas (n = 5) were taken for the construction of calibration curve. The data were analyzed by linear regression least square model and showed a good linear relationship over the tested range.

The accuracy of the method was established by performing recovery experiments at two different levels. Known amounts of genistein (3 and 6 mg) and daidzein (3.5 and 7 mg) were added to the extract before hydrolysis and analyzed as described in Experimental section. The recovery was calculated on the basis of differences between the amount added and quantified. The average recovery was 98.7% for genistein and 99.2% for daidzein. The validation data are summarized in Table 1.

Various concentrations of daidzein, genistein and glycitein were observed in all tested preparations (Table 2). The highest total amount of selected isoflavones (51.40 mg/g) was determined in Soyfem, which is registered as a drug. In this case, the obtained results are close to the total amount of isoflavones given by manufacturer. For the dietary supplements, determined amount of daidzein, genistein and glycitein are much lower than declared isoflavones content. This fact can be explained by occurrence in these preparations the other group of isoflavones or their derivatives, which are not hydrolyzed under condition described in Experimental section. The lowest amount, both of glycitein, genistein and daidzein was quantified in Menostop. It was also noticed that only in the case of Soyfem concentration of aglycones increased significantly after acidic hydrolysis. The differences between extracts before and after hydrolysis are presented in Figure 4.

The comparison of contents of the investigated compounds in one tablet/capsules is given in Figure 5.

The described HPTLC-densitometry method is simple, low cost and fast technique and can be used for routine control of herbal preparation containing isoflavones with phytoestrogenic activity.

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DRUG BIOCHEMISTRY

INVESTIGATION OF THE INFLUENCE OF VANADIUM COMPOUNDS TREATMENT IN NZO MICE MODEL – PRELIMINARY STUDY

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Abstract: New Zealand obese mice (NZO) are characterized by symptoms similar to human metabolic syndrome. Vanadium in different investigations showed anti-diabetic activity but until now an NZO mice model has not been tested with this element. The aim of this study was to investigate anti-diabetic activity of three vanadium compounds ($VOSO_4$, $VO(mal)_2$ and $Na(VO(O_2)_2bpy)\times 8H_2O$) in the NZO model. Metabolic syndrome was induced by special diet (1.5% of cholesterol and 15% of saturated fatty acids) during 8 weeks. In the next 5 weeks, the tested vanadium compounds were administered once daily, in a dose of 0.063 mmol/kg of body mass. At the end of the experiment, glucose, cholesterol, triglycerides and alanine transaminase were measured in the serum. The obtained results showed that the glucose level was decreased nearly to the healthy NZO mice in comparison to the NZO mice with metabolic syndrome. In all groups on the diet with cholesterol, the level of this parameter was statistically higher in comparison to the group without cholesterol addition. Vanadium treatment in a dose 0.063 mmol/kg of body mass does not influence cholesterol, triglycerides and alanine transaminase activity.

Keywords: New Zealand obese mice, vanadium, biochemical parameters

Metabolic syndrome (MS) is one of the most important problems in developing countries. This syndrome is associated with five principal conditions: large waistline, high triglyceride level, low HDL level, high blood pressure, high fasting blood sugar. Minimum three from the five risk factors mentioned above are the basis for recognizing this illness (1–3). MS increases risk of diabetes type 2 about five times and cardiovascular disease about two times in comparison with patients without MS (4). Diabetes, especially type 2, and cardiovascular diseases are among the main challenges for modern medicine and health care system not only due to the problem of treatment but also due to the number of cases, later multi-organs complication, as well as age and lifestyle of patients (5, 6). Prevention is one of the methods, which can significantly decrease the risk of MS and the associated health problems such as diabetes or cardiovascular diseases (7–10). Proper nutrition in both quality and quantity, an appropriate amount of physical activity are among the most important factors, which determine the likelihood of this syndrome. For the testing of new therapies or new medicines an appropriate animal model is necessary. One of them is New Zealand obese (NZO) mice. These mice can be used as a polygemic model of obesity, insulin resistance and hyperinsulinemia (11, 12). Especially fatty diet has an influence on the development of diabetes in these mice (13). Vanadium in different compounds has been tested as a potential anti-diabetic agent for about 30 years (14–17). In these investigations, organic compounds showed more interesting anti-diabetic activity than inorganic compounds (18, 19). Similar observation was reported on toxicity of this metal (20, 21). Some of organic complexes were

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tested also in a human study (15, 16) with positive results. Nowadays, some groups of researchers have synthesized novel organic compounds of vanadium, which can have less toxic effect with higher antidiabetic activity. However, for a better quality of investigation, it is necessary to have a good animal model to study the influence of vanadium on maintaining a proper glucose level. Diabetes type 2 is evidently associated with MS. Proper prevention is very important in diabetes development and it can postpone the moment to start a medicine treatment. Vanadium organic ligands can be useful potential agent in this period and also with other drugs treatment during diabetes.

MATERIALS AND METHODS

Vanadium compounds

1. $VOSO_4 \times H_2O$ – this compound of vanadium was purchased from Sigma.

2. $VO(mal)_2$ was prepared in the Faculty of Chemistry of the Jagiellonian University. The complex of $VO(mal)_2$ was prepared during synthesis under argon. To 5 mL of a hot aqueous solution containing 12 mmol of $VOSO_4 \times 5H_2O$ was added dropwise a hot solution of maltol 25 mmol in 25 mL of water. The pH of reaction mixture was adjusted to ca. 8.5 by addition of 2 M NaOH. The resulting mixture was refluxed with stirring for about 4 h and after cooling to room temperature the resulting green precipitate was filtered off, washed with cold water, and dried *in vacuo* at the room temperature.

3. $Na(VO(O_2)_2bpy) \times 8H_2O$ was also prepared in the Faculty of Chemistry of the Jagiellonian University, using 10 mmol of $NaVO_3$, which was dissolved in molar excess of 10% H_2O_2. The mixture contained molar ratio of H_2O_2 to vanadium 1 : 3. To the obtained solution cooled in the ice bath, 20 mL of ethanol solution containing 10 mmol of 2,2'-bipyridine was added. Temperature of the reaction mixture did not exceed 10°C during the synthesis. Afterwards, 50 mL of cooled ethanol was added. The solid phase was filtered and washed with 10 mL of cold ethanol. The synthesized vanadium complex was dried in the air, in a dark place.

Purity of these two compounds was confirmed by elemental analysis and infrared spectroscopy.

Animals

Fifty male NZO mice and twenty white CD1 mice, five weeks old, body mass 28–33 g were divided into seven groups of 10 animals (five per cage) as follows: – control NZO with standard diet (CN), control white CD1 mice strain with standard diet (CW), control NZO with fatty diet (FN), control white CD1 with fatty diet (FW) and three groups of NZO mice with tested vanadium compounds and fatty diet (V1FN, V2FN, V3FN). MS was induced during eight weeks by special diet containing 15% of saturated fats (lard) and 1.5% of cholesterol. The control group had standard diet. By the next five weeks, the investigated vanadium compounds were administered once daily by gavage in a dose of 0.063 mmol/kg of body mass in the volume of 10 mL/kg of body mass. During the vanadium administration, a composition of diet for all animals was not changed. Mice had all the time free access to water and feed. Day/night cycle was 12 h (7 a.m. – 19 p.m), temperature 22°C and humidity about 55 ± 10%. After thirteen weeks of the experiment, the animals were anesthetized (thiopental 60 mg/kg) and blood from abdominal aorta was taken, and after centrifugation (2500 rpm at 4°C), the serum obtained for biochemical parameters was collected and frozen at –80°C until analysis.

Biochemical analysis

All biochemical parameters were measured in serum using the standard Alize apparatus and standard kits for glucose, cholesterol, triglycerides, ALT, AST, ALP and uric acid (from Biomérieux), and it was controlled using Normal Control Serum – Seronorm and Pathological Control Serum – Pathonorm. The apparatus parameters used in these analyses were recommended by the manufacturer.

Statistical analysis

Statistical analysis of the obtained results was performed using Statistica 9 and GraphPad Prism software.

RESULTS

Glucose

Addition of saturated fats and cholesterol to diet of the investigated animals evidently increases statistically significantly glucose level (Fig. 1) in serum especially in NZO mice ($p < 0.001$, group FN). Also in white mice CD1 (FW group) an increase of this parameter was observed but was not significant ($p = 0.09$). Addition of all tested vanadium compounds decreases glucose level nearly to healthy NZO mice in comparison to NZO mice with fatty diet. This evident, visual decrease of glucose level in serum of tested animals with vanadium compounds treatment was not statistically significant because in different groups the scatter of indi-

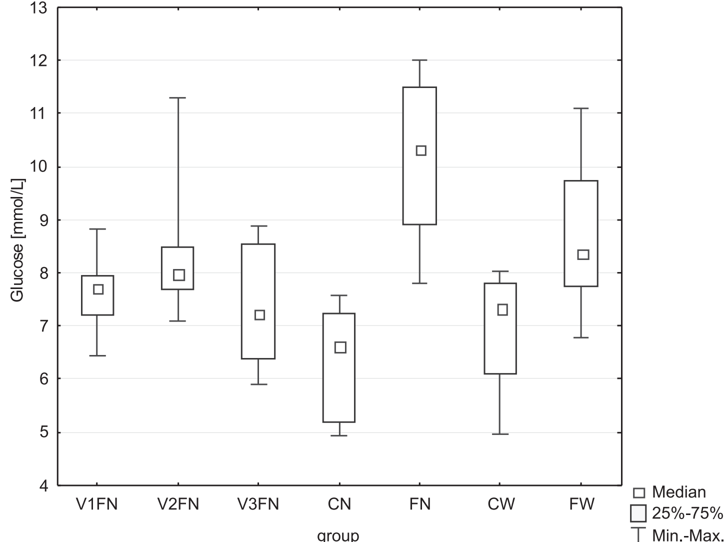

Figure 1. Glucose level in different groups of mice (V1FN, V2FN, V3FN – three NZO mice groups with tested vanadium compounds and fatty diet; CN – control NZO with standard diet; FN – control NZO with fatty diet; CW – control white CD1 mice strain with standard diet; FW – control white CD1 with fatty diet)

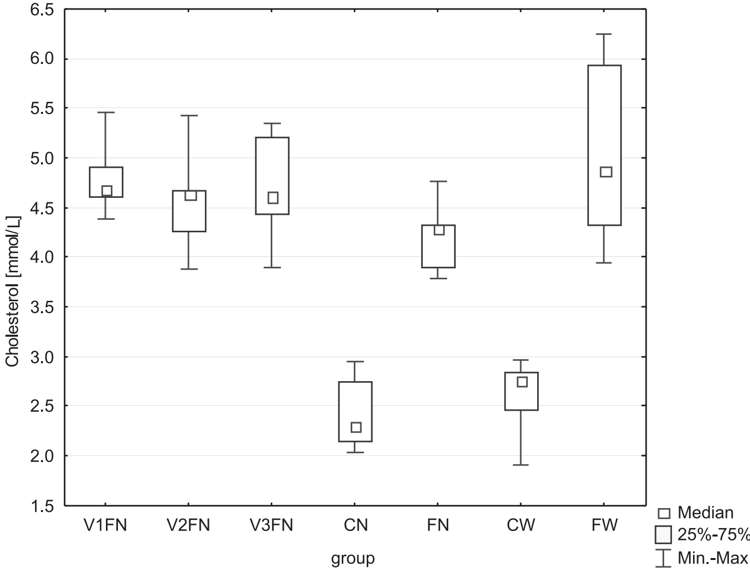

Figure 2. Cholesterol level in different groups of mice (V1FN, V2FN, V3FN – three NZO mice groups with tested vanadium compounds and fatty diet; CN – control NZO with standard diet; FN – control NZO with fatty diet; CW – control white CD1 mice strain with standard diet; FW – control white CD1 with fatty diet)

vidual results was large. However, this trend is interesting as the basis for future investigations of antidiabetic activity of vanadium treatment in the MS model.

Cholesterol

The diet rich in saturated fatty acids and cholesterol statistically increases the cholesterol level (Fig. 2) in all animal groups with this addition, in

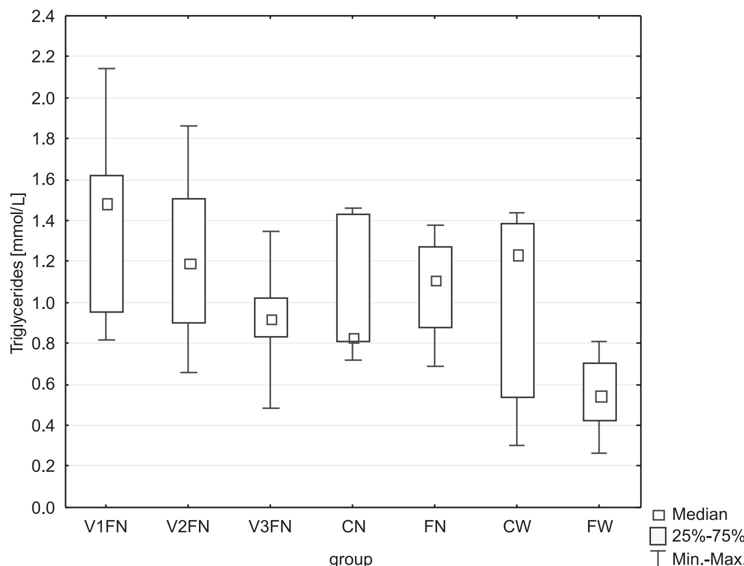

Figure 3. Triglycerides level in different groups of mice (V1FN, V2FN, V3FN – three NZO mice groups with tested vanadium compounds and fatty diet; CN – control NZO with standard diet; FN – control NZO with fatty diet; CW – control white CD1 mice strain with standard diet; FW – control white CD1 with fatty diet)

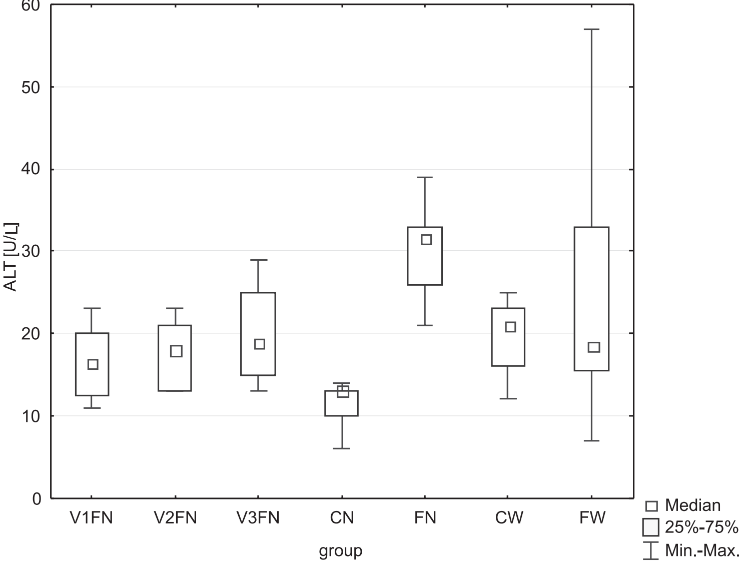

Figure 4. Alanine transaminase activity in different groups of mice (V1FN, V2FN, V3FN – three NZO mice groups with tested vanadium compounds and fatty diet; CN – control NZO with standard diet; FN – control NZO with fatty diet; CW – control white CD1 mice strain with standard diet; FW – control white CD1 with fatty diet)

comparison to the animals with standard feed ($p < 0.05$). The cholesterol level was about two times higher in the animals with modified diet (about 4.5 mmol/L *vs*. about 2.5 mmol/L) in comparison to the animals with standard diet (CN and CW groups). Between the CW and CN mice groups with standard feed, differences were not observed. Vanadium treatment in NZO mice with fatty diet (groups V1FN, V2FN, V3FN) showed not statistical increase (about 20%) of the cholesterol level in comparison to NZO mice with fatty diet (FN group).

Triglycerides

The triglycerides level was similar in all groups of animals (Fig. 3). Only one statistically significant difference was observed between CD1 mice with fatty diet in the FW group and all NZO mice with fatty diet and vanadium treatment in the V1FN, V2FN, V3FN groups. An influence of saturated fatty acids on triglycerides level decrease in white CD1 strain mice (FW group) was also observed. This can suggest a different response associated with the strain of mice used in the experiment.

Alanine transaminase (ALT)

The activity of ALT (Fig. 4) was statistically higher in the NZO mice (FN) with fatty diet in comparison to the control NZO mice (CN). An addition of vanadium compounds to the NZO mice with fatty diet decreased the activity of this enzyme nearly to the level of the CN group. It was not significant but the trend was spectacular. Between the CW and FW groups there were no differences in the ALT activity but a difference between the strains of mice was observed. The ALT activity in the CW mice was higher in comparison to the CN group.

Aspartate transaminase (AST), alkaline phosphatase (ALP), and uric acid

As for the other investigated parameters, namely aspartate transaminase (AST), alkaline phosphatase (ALP), and uric acid, differences between groups were not observed (Tab. 1). The observed very large variability between animals in investigated groups preclude any valid interpretation of the data.

DISCUSSION

Diabetes type 2 is associated with insulin resistance and relative insulin deficiency. Usually, it is associated with the age of patients and other diseases. Proper prevention, such as healthy diet and physical exercise, can frequently significantly delay the start of drug therapy (22). For prevention, vanadium compounds, which showed antidiabetic activity in different scientific researches (15–21, 23–25) can be used. In animal models, vanadium was usually used in fully developed diabetes, both types 1 and 2. Using this microelement in the early stages of the diabetes development is interesting from the scientific and therapeutic point of view. MS in an animal model can be a suitable point for this study. In the New Zealand obese (NZO) mice with special fatty diet insulin resistance, hyperglycemia and abdominal obesity increase more quickly than in the NZO mice with standard diet (26). This relationship is very useful for the study of an initial stage of diabetes type 2. Incorrect diet rich in cholesterol and saturated fatty acids is frequently one of key factors of MS and subsequently diabetes type 2. Till now, the NZO mice's MS and vanadium compounds were not examined together. The obtained results of the glucose level confirmed statistical influence of fatty diet on the level of this parameter and they are consistent with the work of other authors (27–29). In both CD1 and NZO mice after fatty diet, the glucose level was higher in comparison to these mice with standard diet. An increase was more spectacular in the case of the NZO mice. Unfortunately, to the best of our knowledge for this moment, the comparison

Table 1. Activity of aspartate transaminase (AST), alkaline phosphatase (ALP) and uric acid level in serum in different groups of animals.

Group	AST [U/L]	ALP [U/L]	Uric acid [mmol/L]
V1FN	80 ± 15	26 ± 4	1.13 ± 0.50
V2FN	72 ± 13	27 ± 5	1.03 ± 0.48
V3FN	76 ± 10	28 ± 5	1.22 ± 0.58
CN	83 ± 14	23 ± 5	1.14 ± 0.45
FN	86 ± 20	24 ± 4	1.55 ± 0.74
CW	87 ± 20	26 ± 5	1.06 ± 0.33
FW	85 ± 16	29 ± 6	1.20 ± 0.41

V1FN, V2FN, V3FN – three NZO mice groups with tested vanadium compounds and fatty diet; CN – control NZO with standard diet; FN – control NZO with fatty diet, CW – control white CD1 mice strain with standard diet, FW – control white CD1 with fatty diet.

of influence of high fatty diet on the glucose level in both mice strain is not presented in the Pubmed base. A higher level of glucose in the NZO mice with high fat diet confirms the findings of other researchers concerning the usefulness of this model in the MS and diabetes study. All vanadium compounds (V1, V2 and V3) used in the present investigation showed an anti-diabetic – but not significant – effect. The results obtained for the glucose level after the vanadium treatment showed that a very small administered dose of different compounds (0.063 mmol/kg of body mass or 3.21 mg V/kg of body mass had anti-diabetic activity and was one of smaller doses used in vanadium anti-diabetic investigations. The used dose was chosen as 1/20 LD_{50} for $Na(VO(O_2)_2bpy) \times 8\ H_2O$ obtained in other investigation with streptozotocin-diabetic rats (30). For all three vanadium compounds, the same dose was used to observe potential differences of these compounds. Higher doses of the selected vanadium compounds can probably give more interesting statistical differences. It should be noted that the present work is the first study of this animal model with vanadium compounds and, therefore, it cannot be compared to other similar studies. In the work of Xie at al. (31) an administered dose was 10 and 20 mg V/kg, in the work of Yanadarg et al., an administered dose was 0.2 mmol/kg (32), in the work of Gao et al. an administered dose was 0.1, 0.2 and 0.4 mmol/kg (33). Using a small dose of vanadium can eliminate the toxic effect of this metal reported sometimes by other authors (20, 21). This suggests that the NZO mice model of MS with vanadium treatment can be useful in pre-diabetes and early stage of diabetes investigations. The reaction of the glucose level after vanadium treatment in the NZO mice model is interesting for the future studies of new vanadium compounds and other anti-diabetic substances. The choice of a suitable dose of investigated compounds is a very important issue, which can be investigated in the future studies.

The second observation is an important influence of diet on the cholesterol level. High fat diet with 1.5% of cholesterol significantly increases this parameter in the group of animals with fat and cholesterol addition. The results are similar to the work of Irwin et al. and Zhou et al. (34, 35). These authors report that high fat diet increases statistically the total cholesterol level. In our work, diet which contained additionally 1.5% of cholesterol increased of total cholesterol in mice blood higher (about 4.0–4.7 mmol/L) than in the case of solely high fat diet in the work of Irwin and Zhou (3.5–4.1 mmol/L). In the CD1 mice, an increase of total cholesterol was higher but not significant in comparison to the NZO mice after high fat diet. This result can speak about genetic differences between both mice strains for high fat diet treatment. Also in this case, to the best of our knowledge for this moment, a comparison of influence of high fat diet on the cholesterol level in both mice strains is not presented in the Pubmed base. Treatment of vanadium in NZO mice with high fat diet minimally increases the cholesterol level – about 0.5 mmol/L – in the blood in comparison to vanadium not treated mice with high fat diet. Some works reported a lowering effect of vanadium for the cholesterol level (36–39). This small increase of the cholesterol level in blood in our case can be associated with a different model of animals or with changes of proportion of the HDL and LDL level. If it is lowering of LDL fraction, it is interesting action of vanadium in this model. If it is associated with lowering HDL fraction, this organism response to vanadium treatment can be dangerous. The work of Ramachandran et al. (40) presents an increase of the HDL level in STZ rats after vanadium treatment. Probably in our case there is also the same mechanism but a response to this question can be given by further investigation, extended to a full lipid profile as well as all cholesterol fractions and triglycerides. Triglycerides are one of the parameters, which has an important role in cardiovascular disease and diabetes development. High fat diet used in the present experiment does not influence this parameter and the obtained results are similar to the work of other authors (41–44).

The activity of alanine transaminase in animal blood after vanadium administration was lower in comparison to vanadium not treated animals. This can suggest that the doses of vanadium, which were used did not have toxic effects and do not influence the liver function. The activity of ALT was investigated by Pepato et al. (45). These authors observed an increase of the activity of ALT but vanadium doses used in the investigation were about 10 times higher that in our experiment.

As for the other investigated parameters, namely aspartate transaminase (AST), alkaline phosphatase (ALP) and uric acid, differences between groups were not observed. This can suggest that the used dose of vanadium compounds does not have negative effects on the examined parameters and can be used in higher doses in similar future investigations.

The obtained results demonstrated the potential of the NZO mice model in anti-diabetic investigations of vanadium compounds in the starting stage of MS and, consequently, diabetes. The present

work was a preliminary study to find a possible preventive action by vanadium compounds in the development of MS and diabetes. For a better understanding of the interaction of the NZO mice model and vanadium treatment, it is necessary to make also other investigations in blood, especially such as glucose – insulin tolerance test, plasma insulin level, different doses of vanadium compounds, insulin sensitivity. Other interesting areas are to investigate blood pressure, atherosclerotic lesions in aorta and oxidative changes in organs. Potential use of vanadium compounds in MS treatment may delay the time of the application of drugs, which stimulate insulin secretion. It is very important for elderly patients because insulin application in the form of injections is burdensome for them.

CONCLUSION

The obtained results suggest an interesting biological activity of vanadium compounds in very small doses in the MS model. This shows a possibility of using vanadium compounds as an anti-diabetic agent, especially in an early stage of diabetes development.

Also the NZO mice model can be useful in the diabetes development study.

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NATURAL DRUGS

ANTIOXIDANT AND ANXIOLYTIC ACTIVITIES OF *CRATAEGUS NIGRA* WALD. ET KIT. BERRIES

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Abstract: Hawthorn has been present for a long time in traditional medicine as antihypertensive, hypolipidemic, anti-inflammatory, gastroprotective, antimicrobial agent. Hawthorn can be used for the cure of stress, nervousness but there is no published paper about actions of *Crataegus nigra* Wald. et Kit. fruits. The present study was carried out to test free-radical-scavenging and anxiolytic activity of *C. nigra* fruits. DPPH (α,α-diphenyl-β-picrylhydrazyl) assay was used to measure antioxidant activity. BHT, BHA, PG, quercetin and rutin were used as standards. The total amount of phenolic compounds, procyanidins, and flavonoids in the extracts, was determined spectrophotometrically. Results were expressed as equivalents of gallic acid, cyanidin chloride and quercetin equivalents, respectively. LC-MS/MS was used for identification and quantification of phenolic composition. The anxiety effect, expressed as the difference in time spent in the open and closed arms, was measured and compared between groups. Phenolic compound content of *Crataegus nigra* fruits was 72.7 mg/g. Yield of total flavonoid aglycones was 0.115 mg/g. Procyanidins were 5.6 mg/g. DPPH radical-scavenging capacity of the extracts showed linear concentration dependency, IC_{50} value were 27.33 μg/mL. Anxiolytic effect was observed. Species *Crataegus nigra* fruits hydroalcoholic extract showed antioxidant and anxiolytic activity.

Keywords: *Crataegus nigra*, anxiolytic effect, DPPH, total phenols, flavonoids

In traditional medicine leaves, flowers, and berries of species of genus *Crataegus* have been used as astringent, antispasmodic and diuretic agents. Hawthorn products are introduced as an alternative treatment for hypertension, angina, arrhythmia. Furthermore, they can be used to treat indigestion, diarrhea, abdominal pain, hyperlipidemia (1). Although the main application is for cardiovascular disease, hawthorn fruits have also been used as a medicament against stress, nervousness, sleep disorders in traditional medicine (2, 3). It was shown that hawthorn preparations are safe and well tolerated by patients (4). The most common adverse side-effects are vertigo, gastrointestinal pains, headache and migraine. There were no reports of drug interactions. Hawthorn fruits, leaves, and flowers contain: amines, flavonoids (vitexin, vitexin-2”-*O*-rhamnoside, chlorogenic acid, hyperoside, quercetin, isoquercitrin, rutin, etc.), procyanidins, organic acids, tannins, and triterpene derivatives (1, 5, 6). Some flavonoids, procyanidins, tannins, chlorogenic acid in various plants containing these constituents have anxiolytic and sedative activities (7–10). There are no published studies examining CNS activities or anxiolytic actions of *C. nigra* fruits, although the evidence of usage in the traditional medicine of hawthorn fruits do exist for the cure of stress, nervousness and sleep disorders. The presented study evaluated possible effects of *C. nigra* fruits extract on the CNS. There is a number of medicinally active phytochemicals that have been isolated from hawthorn, with most of the data generated in studies of those species that are native to Europe and Asia.

EXPERIMENTAL

Extract preparation

Crataegus nigra Wald. et Kit. was collected from autochthonous sources. *C. nigra* is located on

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flooded plains, alluvial terrain near major rivers. In Serbia, it is prevalent near the Danube and Sava, where it is common and abundant (11). Pannonian hawthorn (*Crataegus nigra* Wald. et Kit.) is a shrub or low tree. Mature fruit is black, almost round in shape, glossy and soft. The voucher specimens (the number BEOU 16406) were deposited in the Department of Biology and Ecology, Faculty of Sciences, University of Kragujevac and botanical garden of Department of Biology, Faculty of Sciences, University of Belgrade.

Species *C. nigra* was collected and separated. The fruit was collected in the fall, September of 2010. It was taken in the vicinity of Beocin, Serbia. The collected material was dried under the shade.

Extraction was performed in the Soxhlet extractor. Ninety grams of dried and chopped herbs and 500 mL of 80% ethanol has been used for extraction. The powder fruits have been extracted for 12 h at 80°C. Dry extracts were obtained by rotating vacuum evaporators (RV05 basic IKA, Germany) at 40°C, under reduced pressure (12).

Determination of phenols

Determination of the total phenolics content was carried out according to the standard method of Singleton et al. (13), customized for 96-well microplates (14). In this study, Folin-Ciocalteu reagent (FC) (Fisher Scientific, UK), anhydrous Na_2CO_3 (Analytika, Czech Republic), and gallic acid (Sigma Aldrich, Germany) as standard have been used. We examined the following various extract concentrations: 0.5, 0.25, 0.125 and 0.063 mg/mL. Gallic acid (100–0.063 µg/mL), was used as a standard for plotting a calibration curve. Thirty microliters of each extract or standard solution, was added to 150 µL of 0.1 mol/L FC reagent and mixed with 120 µL of sodium carbonate (7.5%) after 6 min. Absorbance at 760 nm was measured after 120 min. The content of total phenol compounds was expressed as mg of gallic acid equivalents (GAE) per gram of dry extract weight.

Prepared extract was diluted in mobile phase (0.05% HCOOH : MeOH, 1:1, v/v) to final concentrations of 20 mg/mL as well as 2 mg/mL, and analyzed by high-performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). For the purpose of quantification, a serial dilutions of 45 reference standard mixtures were prepared in 1.5 ng/mL to 25 µg/mL range.

Separation was achieved using Agilent Technologies 1200 Series liquid chromatograph coupled with Agilent Technologies 6410A Triple Quad mass selective detector with electrospray ion source (ESI). Five microliters of extract/standard was injected, and compounds were resolved on a Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm, 1.8 µm) column, set at 50°C. Mobile phase, consisting of 0.05 % HCOOH and MeOH, was delivered in gradient mode (0 min 30% B, 6 min 70%, 9–12 min 100%, post time 3 min), at 1 mL/min flow rate. Eluted compounds were detected in dynamic SRM mode.

Obtained results were analyzed using MassHunter Workstation Software – Qualitative Analysis (B.03.01). For each compound, a calibration curve (MRM peak area *vs.* concentration) was plotted.

Content of procyanidins

The content of procyanidins was calculated by standard method described in European Pharmacopoeia 6.0 and expressed as equivalent of cyanidin chloride (15). Butanol (BuOH) (POCh, Poland) and cyanidin-chloride (Carl Roth, Germany) were used. The investigated extract was hydrolyzed under reflux by an EtOH/HCl mixture. Procyanidins were separated with BuOH from the aqueous layer. The absorbance was measured at 550 nm by spectrophotometer (Cecil CE 2021) (16).

Content of flavonoids

The content of flavonoids was calculated by aluminum chloride colorimetric method (17) adapted for 96-well microplates. $AlCl_3 \times 6H_2O$, $CH_3COONa \times 3H_2O$ (Centrohem, Serbia), quercetin (Sigma-Aldrich Germany) and methanol (MeOH) (J.T. Baker, USA) were used. Investigated extract was prepared in concentrations of 10.0, 5.0, 2.5, 1.25 and 0.625 mg/mL, quercetin was used as a standard. Thirty microliters of extract or standard was diluted by 90 µL of methanol and 6 µL of 10% aluminum chloride, 6 µL of 1 mol/L sodium acetate, and 170 µL of distilled water were added. Absorbance at 415 nm was measured after 30 min. All samples were made in triplicate, and the mean values of flavonoid content were expressed as milligrams of quercetin equivalents per gram of dry extract weight calculated according to the standard calibration curve.

Evaluation of antioxidative activity. DPPH assay

DPPH scavenging effect of plant extract was carried out according to the method of Soler-Rivas et al. (18). It was adapted for 96-well microplates (14, 18). The following materials were used: DPPH (α,α-diphenyl-β-picrylhydrazyl) (Fluka, Switzerland), BHT (butylated hydroxytoluene) (Alfa Aesar, USA), BHA (butylated hydroxyanisole) (Merck,

Germany), quercetin (Sigma-Aldrich, Germany), rutin (Fluka, Switzerland), PG (propyl gallate) (Alfa Aesar, USA), DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Germany). Ten microliters of investigated extract solutions, in series of seven concentrations of double dilution in DMSO, (5.0–0.078 mg/mL), was added to 100 μL of 90 μmol/L DPPH solution in methanol, and the mixture was diluted with 190 μL of methanol. As a control, the exact amount of extract was substituted with DMSO. Absorption at 515 nm was measured by the microplate reader (Multiskan Spectrum, Thermo Corporation) after 60 min. As a positive control, synthetic antioxidant BHT, BHA, PG, quercetin and rutin were used. The radical-scavenging capacity (RSC) was calculated by the equation:

$$RSC = 100 - (A_{average} - A_{corr}) / A_{control} \times 100$$

where $A_{average}$ = average absorbance of the probes for a given concentration sample level; A_{corr} = correction of extract absorbance (with no reagents); $A_{control}$ = absorbance of the DPPH radical (with no extract). The extract concentration inducing 50% of DPPH radicals inhibition (IC_{50}), was calculated from the RSC concentration curve.

Experimental anxiety effect model

Anxiety effect of investigated extract was evaluated by experimental anxiety on elevated plus-maze (EPM) test. The source of anxiety was EPM arms height. Treated animals received researched extract, while control animals received distilled water or diazepam, 30 min prior to their setting on the EPM. The anxiety effect, expressed as the difference in time spent in the open and closed arms (19, 20), was measured and compared between groups.

We used diazepam (Bensedin[R] Galenika, Serbia; IUPAC name: 7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3*H*)-one) (1 mg/kg), normal saline (Hemofarm, Serbia). Female and male BALB/c mice 5–6 weeks old, 20–22 g, (purchased from the Military Medical Academy Belgrade, Serbia) were used in this study. They were kept in environmentally controlled conditions (22°C, 12h light-dark cycle), with free access to standard pellet diet and water. Animals were kept in metal cages. Prior to the experiment, they fasted for 15 h and acclimatization to the test environment lasted 2 h before the experiment. All of the animal procedures were approved by the Ethics Committee of Medical Faculty, Kragujevac, which complies with the National Institutes of Health guidelines for treatment of laboratory animals.

Animals were divided into 6 groups of 8 animals – 4 groups were treated with different concentration of examined ethanolic extracts of *C. nigra* (10, 100, 300, 600 mg/kg). Two groups served as control, receiving vehicle (normal saline) and diazepam (1 mg/kg), in order to control and measure anxiety effect. Treated groups received extracts intraperitoneally. Anxiety was induced by arm height. The apparatus consisted of two open and two closed arms.The each arm was wooden, two of them were closed and black and other were open and white. The whole maze was lift to a height of 60 cm above floor level (21). Testing was carried out in a quiet room and a stifled light. To start experiment, mice were settled on the open arm in the center of the maze. The time spent in the open and closed arms was noted through 5-min test period.

Experimental ketamine-induced sleeping time (hypnotic effect)

We used diazepam (Hemofarm, Serbia) as a standard drug, saline (Hemofarm, Serbia), ketamine (Laboratorio Sanderson, Chile; IUPAC name: (RS)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone) (100 mg/kg). Animals were divided into 4 groups of 8 animals – two groups were treated with different concentration of researched ethanolic extracts of *C. nigra* (300 and 600 mg/kg). Two groups served as control, receiving vehicle and diazepam (1 mg/kg), in order to control and measure hypnotic effect, which was induced by ketamine (100 mg/kg). The time from the loss to regaining the righting reflex was taken as the duration of sleep (22). Mice were considered awake in the moment they could stand on all four paws. The hypnotic effect, expressed as the difference in duration of sleep, was measured and compared between groups (23).

Statistics

The results are expressed as the mean ± standard error of measurement (SEM). The data were normally distributed. The one-way analysis of variance (one-way ANOVA) followed by Bonferroni *post hoc* test were used for statistical analysis. The probability of null hypothesis lower than 0.05 ($p < 0.05$) was considered to be an indicator of statistically significant difference among experimental groups. All calculations were made by statistical software SPSS version 18.

RESULTS

Quantitative phytochemical analysis of major compounds found in the berries of *C. nigra* is presented in Table 1.

Table 1. Quantitative phytochemical analysis of ethanolic extract of *C. nigra* berries (Cn-B) (the mean value ± SD of three measurements).

Active constituents	Cn-B
Total phenolics (mg of GA[a]/g)	72.7 ± 16
Flavonoid content (mg of K[b]/g)	1.88 ± 1
Flavonoid aglycones content (mg/g)	0.115 ± 5.5
Procyanidins content (mg of C[c]/g)	5.6 ± 0.76

[a]GA – gallic acid, [b]K – quercetin, [c]C – cyanidin chloride

Quantitative and qualitative analyses of individual compounds found in berries of *C. nigra* are presented in Table 2 and Figure 1.

Examined extracts of *C. nigra* have shown DPPH free-radical-scavenging activity. When the extract of *C. nigra* berries was applied in the concentration range of 166.7–2.6 µg/mL, its DPPH free-radical-scavenging activity varied from 95.3 to 3.0% (IC_{50} value 27.33 ± 0.369 µg/mL). Results of DPPH assay performed on *C. nigra* extract are presented in Table 3.

Elevated plus maze test

The elevated plus-maze is one of the most frequently used models for testing anxiolytic activities (19). Hydroalcoholic extract of *C. nigra* significantly increased the total time spent in the open arms, according to the control at doses of 300 and 600 mg/kg. The most expressed effect of *C. nigra* was generated at the dose of 300 mg/kg. In this case, the total time spent in the open arms was 3.25 ± 0.15 min (195 ± 8.83 s) (65 %) compared with the control 1.05 ± 0.15 min (63.12 ± 9.15 s) (21%). Diazepam was also proven to show significantly higher effect in comparison with the control. Extract of *C. nigra* has not shown significantly greater effect than diazepam control, although it does have the greatest effect of all the doses tested (Table 4).

Ketamine sleeping time test

The mice in control group, who were treated with saline, showed total sleep time 1542 ± 78 s. Diazepam group significantly increased total sleeping time by 146% compared with the control. Also, *C. nigra* extract at doses of 300 and 600 mg/kg significantly increased total sleeping time, by 138% and 135%, respectively, compared with the saline control, but not with the diazepam control (Table 5).

DISCUSSION AND CONCLUSION

Hawthorn has been present for a long time in traditional medicine of many nations. Accordingly, in many pharmacopoeias in Germany, France, China, some species of hawthorn are officially listed (24). Numerous standardized hawthorn preparations (tablets, drops), which are sold in pharmacies, are being used for heart disease treatment. In Serbia, any herbalist dealing with collecting and selling medicinal herbs can offer teas of leaves, flowers and fruits of different hawthorn species. Due to the great popularity of the plant in traditional medicine of all nations, numerous studies have been derived and done on different species of hawthorn in order to prove pharmacological effectiveness. It was proven that different hawthorn species have antihypertensive (25), hypolipidemic (26), anti-inflammatory, gastro-protective, antimicrobial (27) activity. *C. nigra* is a widely spread plant in a part of Serbia and Hungary (11). In traditional Serbian medicine it was intensively used for the same purpose as other hawthorn species, however, for this particular species no pharmacological research has been conducted or published so far. This study has dealt with chemical characteristics of fruits and has shown their antioxidant and anxiolytic effects.

Hydroalcoholic extract of *C. nigra* in doses of 300 and 600 mg/kg has led to the significant increase of time that the mice spent in the open arms and to the decrease of time spent in closed arms of elevated plus-maze. For most of the plants that have anxiolytic effect, a hypnotic one has been proven as well (28–31). In this study, *C. nigra* in doses of 300 and 600 mg/kg has shown a significant prolongation of sleeping time compared to placebo. Thus, this hawthorn species presumably also has both hypnotic and anxiolytic effect.

Chemically analyzing *C. nigra* extract, rutin, kaempferol, quercetin, hyperoside, epicatechin were identified as the main compounds. In the previously published studies, it had been confirmed that flavonoids, flavonol quercetin, kaempferol aglycones are responsible for anxiolytic effects of some plants (32–34). In the extract of *C. nigra* the authors have examined the presence of these compounds and confirmed it in similar concentrations.

Table 2. Quantitative and qualitative analysis of individual compounds found in berries (Cn-B).

Compound name[a]	t_r^b (min)	t_r^c (min)	Quantification (mg/kg)
			Cn-B
Chinic acid	0.48	0.44	475
Protocatechuic acid	0.76	0.72	385
5-O-caffeocholic acid	0.80	0.76	172
Epicatechin	0.97	0.92	105
Hyperoside	2.14	2.07	164
Rutoside	2.17	2.10	868
Quercetin-3-O-glucoside	2.22	2.15	408
Kaempferol-3-O-glucoside	2.80	2.72	5.10

[a] The numbers refer to compounds signed on the HPLC spectrum. [b] Retention times of the standards. [c] Retention times of the compounds identified in the extract.

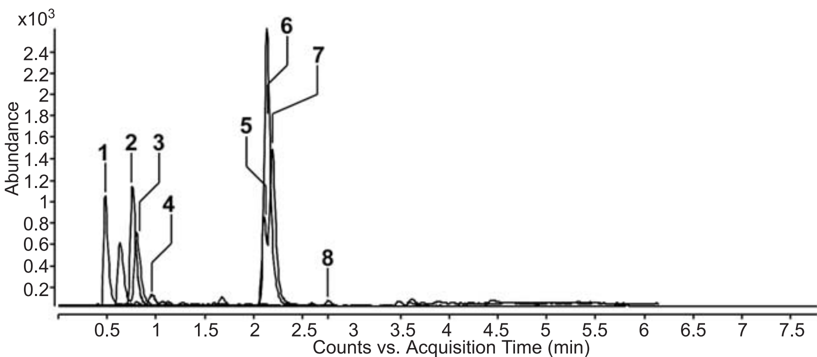

Figure 1. HPLC chromatogram of *C. nigra* berries ethanol extract: 1 – chinic acid, 2 – protocatechuic acid, 3 – 5-O-caffeocholic acid, 4 – epicatechin, 5 – hyperoside, 6 – rutin, 7 – quercetin-3-O-glucoside, 8 – kaempferol-3-O-glucoside

Table 3. DPPH free-radical-scavenging activity of the *C. nigra* extract (Cn-B).

Sample	IC_{50} (μg/mL)[a]
Cn-B	27.33 ± 0.369
BHT	11.65 ± 1.144
BHA	1.57 ± 0.093
Quercetin	0.41 ± 0.169
Rutin	1.42 ± 0.173
PG	0.62 ± 0.030

[a] The mean value ± SD of three measurements

However, many authors believe that the certain pharmacological activity of the plants is in fact the consequence of the present combination of chemical compounds in a plant. According to that, the anxiolytic effect of *C. nigra* is probably the consequence of the blend of all the compounds present in it.

The antioxidant activity in this study has been examined with DPPH method. The compounds that have antioxidant characteristics can reduce the DPPH radical. In fact, they are hydrogen donors and can convey DPPH radical into the neutral DPPHH form (35). This study has shown that the *C. nigra* fruit extract has a free-radical scavenging activity. This activity is lower than with BHA, BHT, quercetin and rutin, which were used like positive controls (EC_{50} values of 27.33, 11.65, 1.57, 0.41 μg/mL, respectively). However, in the previously conducted studies, it had been established that the compounds that realize EC_{50} with the concentration less than 50 μg/mL are active antioxidants (36). Thus, based on this, we can say that the *C. nigra* extract has the active antioxidant activity.

Table 4. Anxiolytic effect of *C. nigra* fruits.

	Time spent (s)	
	Open arm*	Closed arm*
Control	63.125 ± 9.15	236.875 ± 8.56
Diazepam 1 mg/kg	145.62 ± 14.24	54.37 ± 14.24
Extract *C. nigra* 10 mg/kg	77.25 ± 17.35	212.25 ± 22.44
Extract *C. nigra* 100 mg/kg	107 ± 11.04	193 ± 11.04
Extract *C. nigra* 300 mg/kg	95 ± 8.83	105 ± 8.83
Extract *C. nigra* 600 mg/kg	157 ± 21.68	143 ± 21.68

Numbers represent the mean ± SEM of the groups (n = 8). * $p < 0.05$ compared to saline (ANOVA followed by Bonferroni test).

Table 5. Sedative effect of *C. nigra* fruits on ketamine-induced sleep in mice.

	Sleeping time (s)*
Control	1542.5 ± 77.75
Diazepam 1 mg/kg	2250.62 ± 32.17
Extract *C. nigra* 300 mg/kg	2133 ± 58.38
Extract *C. nigra* 600 mg/kg	2086 ± 104.55

Numbers represent the mean ± SEM of the groups (n = 8).
* $p < 0.05$ compared to saline (ANOVA followed by Bonferroni test).

It has already been shown that some other hawthorn species have antioxidant activity and compared to those data *C. nigra* has shown somewhat greater effect. The blend of *C. monogina* and *C. oxyacantha* has shown EC_{50} at the extract concentration of 52.04 µg/mL (27). C. *pinnatifida* at the concentration of 100 µg/mL shows EC_{84} (37). According to the studies (37), some plants used like familiar antioxidants have shown less DPPH activity than *C. nigra*. However, there are many plants which are more potent antioxidants (38). Apart from hawthorn's antioxidant activity there are numerous evidence of its very significant and diverse pharmacological activity. In *C. monogyna* it has been established that phenol potency (phenolics) goes in the following order: quercetin > B2 procyanidin > epicatechin, while the two flavonol glycosides, hyperoside and rutin, are somewhat less effective (39). Generally, epicatechin and procyanidin B2 flavonoids are the most effective, and hyperoside and rutin follow them. This study has shown that the content of all the phenols and procyanidins in dried *C. nigra* fruits is higher than the same content in *C. monogina*. Also, the total content of phenol and procyanidins in *C. monogina* dried fruits is smaller than their content in *C. monogina* dried tops and flowers (39). Thus, chemical composition of all *C. nigra* parts should be examined as well.

Since it is known that procyanidins, flavones and flavonoids are bearers of antioxidant activity, we believe that the presence of these polyphenolic compounds in the *C. nigra* extract is responsible for this extract's feature.

Benzodiazepines are mostly used in anxiety treatment today. However, they can have significant side effects, especially if they are used at the same time as depressors of CNS, leading to development of psychophysical addiction with time. This is the reason why herb preparations with anxiolytic effect are given more attention, while side effects are expected to be minimal or none. This study showed that *C. nigra* has anxiolytic and hypnotic activity. Yet it is to be shown in the future the extent to which these preparations are effective in replacing or reducing anxiolytics. Antioxidant activity of *C. nigra*, which is alongside with numerous known antioxidants, gives special significance to it.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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PHARMACEUTICAL TECHNOLOGY

IN VITRO EVALUATION OF TRANSDERMAL PATCHES OF FLURBIPROFEN WITH ETHYL CELLULOSE

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Abstract: This study was aimed to determine effects of penetration enhancers and plasticizers on drug release from rationally designed formulations of flurbiprofen based transdermal drug delivery system. Matrix type transdermal patches were formulated with ethyl cellulose (EC) as a polymer by using plate casting method. The plasticizers such as propylene glycol (PG) and dibutyl phthalate (DBP), and enhancers such as Span 20, Tween 20, sodium lauryl sulfate (SLS), isopropyl myristate (IPM) and ethanol (EtOH) were formulated in different concentrations in the patches. Such different combinations of polymer with various enhancers and plasticizers in patches were evaluated for their effect on the physicochemical properties and drug release behavior of flurbiprofen. The drug release study was carried out by the paddle-over-disk method and permeation of drug was performed by Franz diffusion cell using rabbit skin. Patches having ethanol with ethyl cellulose showed more uniformity in the physical properties while the smoothness and clarity of patches containing sodium lauryl sulfate were not satisfactory. The drug release from patches followed Higuchi and Korsmeyer-Pappas model while maximum drug release was obtained by isopropyl myristate (903 µg). It was concluded that the patches having ethyl cellulose with isopropyl myristate and propylene glycol are more useful for transdermal patches of flurbiprofen.

Keywords: transdermal patches, ethyl cellulose, flurbiprofen, kinetic models, permeation enhancers

Certain side effects like ulceration, nausea and vomiting are reported for non-steroidal anti-inflammatory drugs (NSAIDs) if they are administered by oral route of administration. To exempt these kinds of side effects, such drugs required to be administered by some alternative routes of administration. Transdermal patches have shown potential for administration of such drugs, as they transfer the drug into general circulation through the skin and hence, bypass the gastrointestinal tract. Moreover, constant plasma levels are achieved by administering the drug through transdermal route (1). The selection of transdermal drug delivery system (TDDS) is advantageous as it can maximize the rate of transfer of drug into systemic circulation and can also reduce its time of stay in the skin tissues if properly formulated with some permeation enhancers. The metabolism of drug, that is primary factor in oral delivery system, is markedly minimized by administering the drug through skin. For efficient transdermal drug delivery system, the drug must be able to penetrate the skin barrier and reach the target site. Transdermal patches are also responsible for the sustained release of drug through skin into the blood stream (2). Hence, NSAIDs patches not only remove above mentioned side effects but also improve the patient compliance, avoid first pass effect and maintain a controlled release of drug (3). Flurbiprofen is a chiral non-steroidal anti-inflammatory drug (NSAID) having comparative efficacy with other NSAIDs like ibuprofen and diclofenac. It possesses anti-inflammatory, analgesic and antipyretic activity but can also be employed in the treatment of rheumatoid arthritis, vernal keratocon-

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juctivitus and ocular gingivitis (4). Recent studies have proved its role in the inhibition of colon tumor. Various investigators are attracted towards the quantification of flurbiprofen in the body fluids (5).

It's of remarkable value that the polymers are being used in different roles for developing various drug delivery systems, e.g., ethyl cellulose is widely used as a rate retarding polymer in TDDS due to its lipophilicity that does not allow it to be dissolved by the diffusion medium (6). The enhancers are primarily used to assist the absorption of the penetrant through the skin. Literature reveals the role of various chemical agents as permeation enhancers like Span 20 (7), Tween 20 (8), IPM (9), SLS (10) and ethyl alcohol (11).

The major objectives of this study is to prepare and evaluate various batches of flurbiprofen patches by using different combinations of polymer and enhancers as well as compare the efficiency of various enhancers. It was designed to explore the flurbiprofen interaction with matrix excepients in TDDS, mathematical modeling and critical evaluation of drug release kinetics. Moreover, the influence of unilaminated matrix formulations of flurbiprofen on various characteristics such as physical appearance, weight uniformity test, thickness variation test, folding endurance and percent flatness were also evaluated.

EXPERIMENTAL

Materials

Flurbiprofen was a gift sample by Hamaz Pharmaceuticals, Pakistan. Other materials were purchased as: ethyl cellulose (~5.1 cps) from Sigma Chemicals, USA; Tween 20, Span 20, sodium lauryl sulfate and toluene from BDH, UK; isopropyl myristate from Panreac Quimica, USA; polyvinyl alcohol from Sigma Chemicals, Japan; ethanol, propylene glycol, dibutyl phthalate, chloroform, potassium dihydrogen orthophosphate and sodium hydroxide pellets from Merck, Germany.

Preparation of backing membrane

Polyvinyl alcohol (PVA) is being used for the preparation of backing membrane (7). Four percent w/v solution of PVA was continuously stirred with double distilled water in conical flask on the hot plate magnetic stirrer at 800 rpm for 2 h at 80°C. After cooling, the solution, degassing of the PVA solution was done for 3 min using ultrasonic bath (JenKen PS-08A 1.3L) at 30°C for at least 10 min. Then, 15 mL of the prepared solution was poured in the glass Petri dishes having an area of approximately 61 cm^2 and finally dried in open air for 24 h.

Development of unilaminated matrix patches

The unilaminated transdermal patches were prepared by solvent evaporation technique (12). Hundred milliliters of the solvent (toluene and chloroform (1 : 1, v/v) for ethyl cellulose) was taken in conical flask in which measured amounts of polymer, enhancer and plasticizer were added gradually and allowed to dissolve. At the end, flurbiprofen was dispersed in the matrix solution.

Twenty milliliters of the above solution was poured in Petri dish, which already had backing membrane. The solution was dried at room temperature by using inverted funnel to avoid rapid evaporation. When the solvent was completely evaporated, the dried patch was removed from Petri dish and wrapped in the aluminum foil. Wrapped film was labeled and stored in cool container. The dried films were cut with the diameter of 1.54 cm^2. About 10–12 patches were obtained from one Petri dish.

Weight uniformity test

The weight uniformity of randomly selected patches from each formulation was checked by digital weighing balance in triplicate. Every triplicate gave uniformity in weight and the average value was similar to an individual patch. So the mean value is zero in almost all the formulations and the patches showed minimum deviation in weight (13).

Thickness variation test

For variation in thickness, micrometer screw gauge was used. For all formulations, a single patch was checked at three different places and the mean value was used to elaborate the variation in the thickness (14).

Folding endurance

The folding endurance is the number of folds which are required to cleave the matrix film (15). The test clarifies the efficiency of plasticizer in the each patch. The value was determined by repeatedly folding each patch at the same axis until it cleaved. The number of times a film could be folded at the same axis without breaking will give the value of folding endurance (16).

Percent flatness

The percentage flatness was determined by cutting the transdermal film into three strips; two strips from either sides or one at the centre. The length of each strip was calculated after constriction in the films. Minimum constriction in the films resulted in maximum flatness (17).

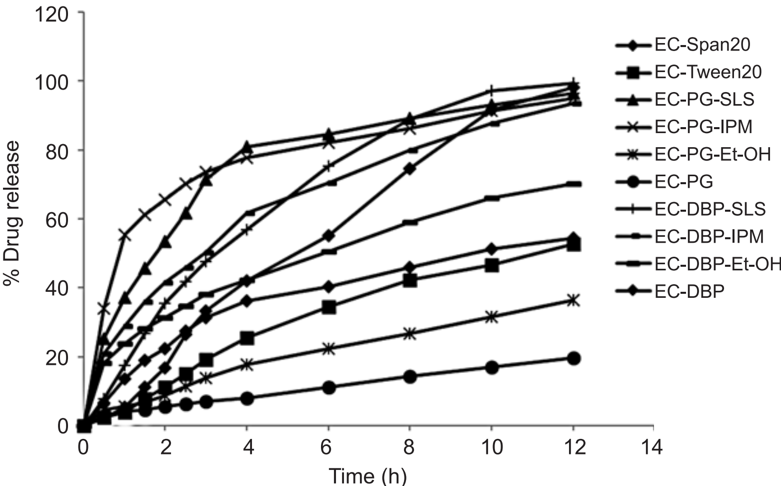

Figure 1. Effect of various enhancers (Span 20, Tween 20, SLS, IPM and EtOH) on the release profile of flurbiprofen patches using dibutyl phthalate (DBP) and propylene glycol (PG) as plasticizers

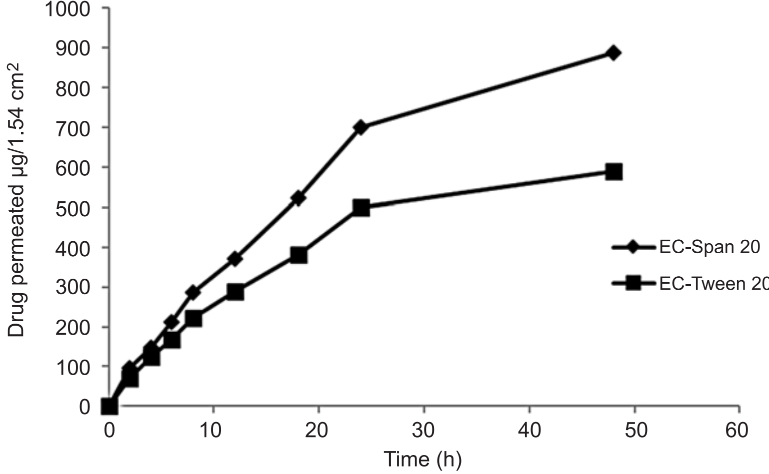

Figure 2. Effect of Span 20 and Tween 20 on the permeability profile of flurbiprofen patches

Drug content uniformity test

Drug content uniformity was evaluated for the confirmation that the drug is distributed evenly in the glass Petri dish. To calculate the uniformity of drug in patches; the patches without drug were also formulated and considered as blank. Each patch was put into conical flask containing 50 mL of dichloromethane (DCM), covered with aluminium foil to avoid evaporation of solvent and stirred continuously at 600 rpm at hot plate magnetic stirrer at 30°C for 24 h. The entrapped air bubbles were removed by sonication for 15 min. The solution was then filtered through filter paper. After a suitable dilution (up to 200 times), the solutions were analyzed on double beam UV-Vis spectrophotometer (Irmeco-U2020, Germany) at the wavelength of 247 nm for flurbiprofen.

***In vitro* drug release studies**

The dissolution test was performed by using paddle over disk method (USP apparatus 5). Due to the unavailability of commercial patch retainer or

Table 1. Formulation of transdermal flurbiprofen patches using EC with various enhancers (1 : 1) and plasticizers.

Formulation ingredients	Formulations									
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10
EC (mg)	600	600	600	600	600	600	600	600	600	600
Span 20 (mg)	600	–	–	–	–	–	–	–	–	–
Tween 20 (mg)	–	600	–	–	–	–	–	–	–	–
IPM (mg)	–	–	600	–	–	–	–	600	–	–
Ethanol (mg)	–	–	–	600	–	–	–	–	600	–
SLS (mg)	–	–	–	–	600	–	–	–	–	600
PG (mg)	–	–	210	210	210	210	–	–	–	–
DBP (mg)	–	–	–	–	–	–	210	210	210	210
Flurbiprofen (mg)	50	50	50	50	50	50	50	50	50	50
Chloroform (mL)	50	50	50	50	50	50	50	50	50	50
Toluene (mL)	50	50	50	50	50	50	50	50	50	50

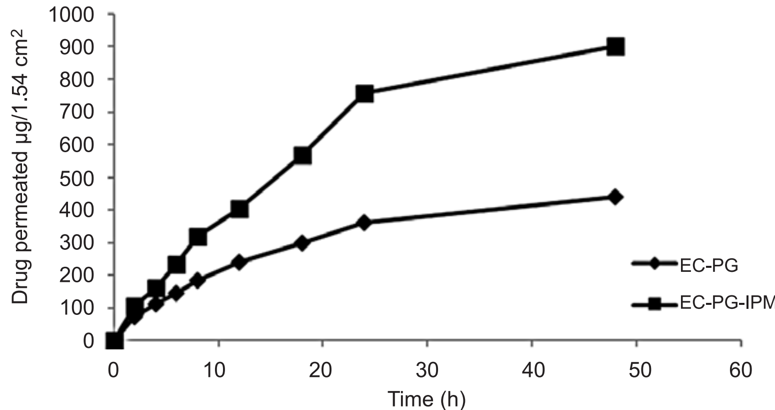

Figure 3. Effect of isopropyl myristate (IPM) and propylene glycol (PG) on the permeability profile of flurbiprofen patches

sandwich patch holder, the disk assembly was prepared by using watch glass (3 inches in diameter), 120 μm mesh stainless steel net and plastic coated stainless steel clips (18).

The patch of an area of 1.54 cm^2 having 1.22 mg of drug was placed against the watch glass and retained in position with the stainless steel mesh by using stainless steel clips. The disk assembly was designed in such a way that it could hold the system flat with the release surface of patch facing upward and parallel to the bottom of the paddle blade. Moreover, the disk assembly also minimized the "dead" volume between the patch holder and the bottom of the dissolution vessel. For dissolution studies, vessels were filled with 500 mL of phosphate buffer solution (PBS pH 7.4) maintained at 32 ± 0.5^{o}C. The disk assemblies holding patches were placed at the bottom of vessels with the release surfaces facing upward and were centered using a glass rod. The stirring speed was set at 50 rpm. Samples of about 5 mL each were collected at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h with an automated fraction collector (Pharma Test, Germany) after filtering through Millipore filters. The withdrawn sample volume was replaced with equivalent fresh volume of media already maintained at 32 ± 0.5^{o}C. Measured amount of samples were analyzed at λ 247 nm using a UV-Vis spectrophotometer (Irmeco U2020, Germany). A calibration curve showing the measured absorbance of known concentrations of flurbiprofen was constructed to measure the amount of flurbiprofen released from withdrawn samples at

Table 2. Physicochemical properties of flurbiprofen transdermal patches.

Formulation Code	Patch	Smoothness	Clarity	Overall appearance	Weight variation (g) ± SD	Thickness variation (mm) ± SD	Folding endurance	Flatness %	% Content ± SD
Q1	EC-Span 20	+	+	Satisfied	0.066 ± 0.00	0.32 ± 0.00	143	99.99	96.53 ± 0.012
Q2	EC-Tween 20	+	+	Satisfied	0.053 ± 0.00	0.27 ± 0.00	129	99.96	97.66 ± 0.006
Q3	EC-PG-IPM	+	+	Satisfied	0.06 1± 0.00	0.35 ± 0.00	119	99.89	97.02 ± 0.02
Q4	EC-PG-EtOH	++	++	Satisfied	0.05 ± 0.00	0.23 ± 0.00	101	99.93	98.21 ± 0.015
Q5	EC-PG-SLS	+	×	Unsatisfied	0.059 ± 0.01	0.29 ± 0.01	97	99.91	96.77 ± 0.020
Q6	EC-PG-No enhancer	++	++	Satisfied	0.048 ± 0.00	0.22 ± 0.00	88	99.94	98.81 ± 0.006
Q7	EC-DBP-No enhancer	++	++	Satisfied	0.046 ± 0.00	0.23 ± 0.00	94	99.93	100.71 ± 0.006
Q8	EC-DBP-IPM	+	+	Satisfied	0.067 ± 0.00	0.35 ± 0.00	131	99.92	98.98 ± 0.006
Q9	EC-DBP- EtOH	++	+++	Satisfied	0.049 ± 0.00	0.25 ± 0.00	113	99.9	101.13 ± 0.025
Q10	EC-DBP-SLS	+	×	Unsatisfied	0.066 ± 0.00	0.32 ± 0.00	99	99.91	100.35 ± 0.015

(+) level of satisfaction, (×) level of dissatisfaction, (SD) standard deviation

specified time intervals as shown in Figure 5. All the test samples were run in 5 different vessels and average values of absorbance were taken, which were later correlated with calibration curve to analyze the amount of drug released. The drug release constants and correlation coefficient (r^2) were obtained by applying zero order (19), first order (20), Higuchi (21), Korsmeyer-Pappas (22) and Hixson-Crowell models (23).

***In vitro* permeation study across the rabbit skin**

In vitro permeation study was performed only on the formulations having satisfactory physicochemical characteristics and showed maximum amount of drug release during *in vitro* dissolution test. The reason for such a selection was based on the assumption that the formulations, which have shown maximum amount of drug release during *in vitro* evaluation, will eventually permeate maximum amount of drug through rabbit skin. The formulations containing Span 20, Tween 20, IPM and EtOH as enhancers were studied using Franz diffusion cell (24).

For permeation study, Franz diffusion cell with an area of 1.76 cm^2 was used while rabbit skin was used as a permeation medium. Phosphate buffer solution (pH 7.4) was filled in the receptor compartment up to 12 mL while temperature was maintained at 37 ± 1°C by circulating water at constant temperature in the outer jacket of the receiver compartment. The temperature had to be set at 37 ± 1°C in order to produce the temperature reading of the formulation in the receptor compartment at 32 ± 1°C. Actually, the loss of heat occurs in the plastic tubes that connect the Franz cell with the thermostatic water bath. The rabbit skin membrane was carefully placed over the open end of the receptor compartment and the patch of an area of 1.54 cm^2 was placed over the membrane. The glass disk, i.e., the donor compartment was placed over receptor compartment and both compartments were kept in position with the help of the stainless steel clamp. To avoid evaporation, the junction of the two compartments was wrapped with adhesive tape. The whole assembly was kept on magnetic stirrer and the receptor fluid was kept stirring continuously during test by using magnetic bars at speed of 600 rpm. Samples (1 mL) were with-

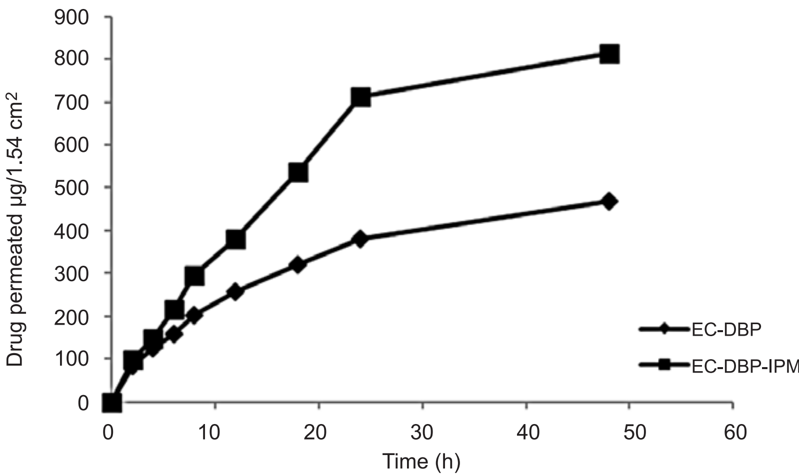

Figure 4. Effect of isopropyl myristate (IPM) and dibutyl phathale (DBP) on the permeation of drug through rabbit skin

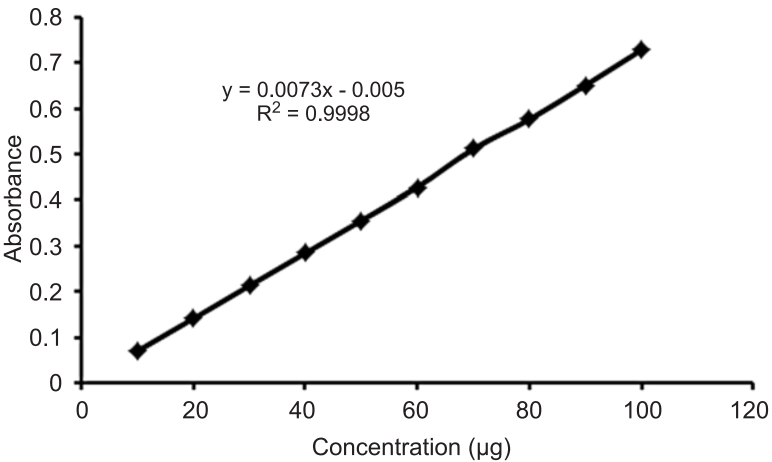

Figure 5. Calibration curve prepared to measure the amount of flurbiprofen released from withdrawn samples at specified time intervals

drawn at 0, 2, 4, 6, 8, 10, 12, 18, 24 and 48 h and replaced by an equal volume of receptor fluid at each sampling time. All the samples were analyzed for the drug contents on UV-Vis spectrophotometer at λ of 247 nm (25).

RESULTS AND DISCUSSION

Physicochemical evaluation

Regarding smoothness and clarity, all the formulations were found satisfactory except the formulation containing SLS (patches Q5 and Q10). All the films were found to be uniform in weight and thickness variation, with low standard deviation (SD) values. The folding endurance ranges from 88 (patch Q6) to 143 (Q1) and percent flatness were also satisfactory. Experimental findings for physical appearance, weight variation, thickness variation, folding endurance and percent flatness have been presented in Table 2.

Drug contents

Table 2 shows the results for the drug content uniformity in percentage. It is evident from the

Table 3. The drug release constants and correlation coefficient (r_*) obtained from various models for various formulations.

Formulation Code	Zero order		1st order		Higuchi model		Korsmeyer-Peppas		Hixon-Crowell	
	k_0	r^2	k_1	r^2	k_H	r^2	n	r^2	k_HC	r^2
Q1	8.912	0.9812	0.151	0.9354	23.821	0.8319	0.908	0.9863	0.044	0.9672
Q2	4.906	0.9544	0.066	0.9906	13.313	0.8714	0.808	0.982	0.02	0.9847
Q3	11.041	–0.5069	0.397	0.9661	32.82	0.8138	0.353	0.9342	0.109	0.9117
Q4	11.075	–3.2477	0.534	0.6979	33.743	0.1351	0.240	0.9399	0.121	0.3605
Q5	3.334	0.9268	0.04	0.9686	9.17	0.9209	0.727	0.9944	0.013	0.9573
Q6	1.769	0.9125	0.019	0.935	4.881	0.9275	0.708	0.9914	0.006	0.9281
Q7	10.282	0.8034	0.229	0.9852	28.831	0.9495	0.622	0.978	0.064	0.9984
Q8	9.798	0.364	0.241	0.9552	28.306	0.9901	0.461	0.9955	0.067	0.8933
Q9	7.278	0.2796	0.131	0.7638	21.059	0.9864	0.444	0.9982	0.037	0.6466
Q10	5.676	0.5362	0.086	0.8074	16.284	0.9739	0.505	0.974	0.025	0.7326

results that there is no significant difference in the drug content uniformity. The range for drug contents is 96.52% (patch Q1) to 100.71% (patch Q7). The test indicates that the drug is distributed uniformly in the patches developed by plate casting method.

***In-vitro* drug release studies**

All the prepared transdermal matrix patches were subjected to dissolution test and the data obtained for drug release were plotted as percentage drug *versus* time in hours. The formulations Q1 and Q2 do not contain plasticizers as Span 20 and Tween 20 both act as platicizer too. The amount of drug release from all the formulations are 98, 52, 95, 36, 95, 20, 54, 94, 70 and 98% for the formulations Q1, Q2, Q3, Q4, Q5, Q6, Q7, Q8, Q9 and Q10, respectively, at the end of 12 h. The formulations containing Span 20 and SLS as enhancers showed maximum release. The release data (0–12 h) were fitted to different kinetic models in order to determine the effect of all the enhancers on the release kinetics. The drug release constants and correlation coefficient (r^2) obtained from zero order, first order, Higuchi, Korsmeyer-Peppas and Hixon-Crowell models fitted for transdermal patches have been shown in Table 3. It is apparent that the release of drug from transdermal patches Q7, Q8, Q9 and Q10 followed Higuchi model as the values of coefficient of correlation (r^2) are most linear for these formulations. It also suggested that the predominant release mechanism from these patches is diffusion. The diffusional coefficient 'n' values obtained from Korsmeyer-Peppas model show a combination of fickian and non fickian release mechanisms from the patches that confirms the diffusion as well as erosion of the patch surface. Thus, as a whole, it can be said that the release of drug from patches is slow, controlled and followed diffusion mechanism (26).

***In vitro* permeation studies**

The cumulative amount of the drug permeated (Qn) for the selected formulations have been mentioned in Table 4. The increased permeation caused the increased amount of drug in receptor compartment. The results clearly show that maximum amount of drug is released by samples containing EC, PG, IPM, whereas the minimum amount is released by the samples containing EC and PG. Detailed discussion of these factors is given as follows.

Effect of Span 20 and Tween 20 on permeation of drug through rabbit skin

The result indicates that the rapid release of drug occurs when the patch is in a good contact with the rabbit skin. Span 20 (enhancer) releases more amount of drug in receptor compartment as compared to Tween 20 as well as from the patches in which no enhancer was included. This is mainly due to the increased receptor-solvent permeation caused by marked partitioning of drug from matrix to the solvent, which then dragged it to the receptor cell membrane (27). Table 4 and Figure 2 show that Span 20 increases the rate of permeation of drug. Span 20 permeates 888 µg of drug from the rabbit

Table 4. Cumulative amount of drug release (µg/1.54 cm^2) by various enhancers permeated through rabbit skin.

Time (h)	Cumulative amount of flurbiprofen µg/1.54 cm^2					
	EC-Span 20	EC-Tween 20	EC-PG	EC-PG-IPM	EC-DBP	EC-DBP-IPM
0	0	0	0	0	0	0
2	96	69	73	105	84	98
4	147	124	111	162	124	146
6	211	167	145	235	159	216
8	286	221	184	320	202	296
12	372	289	239	403	257	381
18	522	382	298	568	321	537
24	698	500	360	759	381	713
48	888	589	439	903	467	814

skin while patch containing Tween 20 permeates 589 µg of drug. This amount of drug release by way of permeation by both Span 20 and Tween 20 is less than the amount released from *in vitro* drug release profile during dissolution study. The formulations which are without plasticizers (Span 20, Tween 20) are self-operating as plasticizers. The results show 25% enhancement in the permeation of drug due to Span 20 (7).

Effect of IPM and PG on the permeation of drug through rabbit skin

The formulations containing EC as polymer and IPM as enhancer with and without PG as plasticizer were studied for the permeation of drug. The results show that in the presence of IPM, increased amount of drug was released as compared to the patch which did not contain IPM. Table 4 and Figure 3 clearly show the amount of drug permeated through rabbit skin. IPM is able to release 903 µg of drug as compared to 439 µg of drug released in the absence of IPM. This amount of drug release by IPM and PG is less than the amount released during *in vitro* drug release. IPM acts as a fluidizer of intercellular lipids and affects the lipid-rich phase in the stratum corneum, so decreases its barrier function (28). The increased amount of drug released by IPM can also be displaced by its intermediary polar nature that caused its penetration into the polar portion of stratum corneum. The increased subdivision of drug in both phases by the use of IPM cause the maximum amount of drug partitioning in the skin as well as in the dissolution medium (29). Therefore, IPM doubles the release of drug when combined with EC and PG.

Effect of IPM and DBP on the permeation of drug through rabbit skin

When IPM was studied with another plasticizer DBP with EC as polymer, the results indicated that 814 µg of drug is released in the presence of IPM and DBP as compared to 467 µg by the formulation which contain only DBP. This amount of drug release by IPM and DBP is less than the amount released during *in-vitro* drug release profile during dissolution study. IPM acts as a conveyer for the drug to permeate through skin barrier and DBP (plasticizer) diffuses and softens the polymer particles by reducing polymer-polymer bonding such as hydrogen bonding and forms its own bonds with the polymer lattice that promotes the latex coalescence and film formation. This results in the decreased strength of polymer and allows IPM to transport the drug through this softened film. So the physicochemical properties of a patch may vary with this effect (30, 31).

CONCLUSION

The present study suggests that ethyl cellulose releases the drug more effectively in almost all the formulations containing five different enhancers and two plasticizers. The patch having EC, PG and IPM shows maximum amount of drug permeated through rabbit skin membrane. Patches having no enhancer also show increased permeability in the presence of plasticizer (DBP), which also acts as permeability enhancer, whereas the release of drug from patches is controlled and followed diffusion mechanism. On the bases of aforementioned discussion it can be concluded that the patches having ethyl cellulose

with isopropyl myristate and propylene glycol are more useful for transdermal patches of flurbiprofen.

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PERMEATION STUDY THROUGH BACTERIAL CELLULOSE MEMBRANE

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Abstract: The objective of this study was to fabricate topical formulations of diclofenac diethylamine (DD) using isopropyl myristate (IPM) and isopropyl palmitate (IPP) as permeation enhancers. Franz cell and bacterial cellulose were used as analytical instrument and diffusion membrane, respectively. Permeation enhancers exhibited significant effect on the permeation characteristics of DD. It was concluded from the results that improved permeation of DD was observed when IPP was used as enhancer.

Keywords: bacterial cellulose, permeation, transdermal drug delivery

Transdermal drug delivery systems (TDDS) are widely being studied as an excellent substitute to deliver drugs with enhanced bioavailability (1). However, large number of active pharmaceutical substances faces trouble during crossing the intact skin (2). Thus, it is the need of time to focus our attention for overcoming diminished drug permeability *via* skin (3). There are two decisive factors in the development of TDDS including the achievement of sufficient flux athwart the skin and the reduction in lag time during skin permeation. Various approached have been introduced to overcome these issues like the addition of chemical skin enhancers into the formulation (4). Some prominently used enhancers are propylene glycol (5), isopropyl alcohol (IPA) (6), isopropyl myristate (IPM) (7) and isopropyl palmitate (IPP) (8).

It has been studied that a drug molecule passes through many barriers during its traveling from skin surface to systemic circulation. These different barriers are stratum corneum, viable epidermis and dermis. The skin is the largest body organ and is rich in blood capillaries (blood flow rate of 0.05 mL/min/cm of skin). However, skin temperature is needed to be controlled to deliver drug molecules through skin and the removal of waste products. Sink conditions are provided by this blood pool in the proximity of skin for the diffusion of drug substances during percutaneous absorption (9, 10).

Diclofenac diethylamine (DD) being an excellent non-steroidal anti-inflammatory drug is preferred for using in the treatment of painful circumstances (11). Besides, its use in the development of experimental and clinical medicines is very limited up to now, particularly in the fabrication of topical formulations. However, no study is available in the literature showing its permeation studies across bacterial cellulose based artificial skin.

Bacterial cellulose, an extremely uncontaminated cellulose substrate, is developed as a distended membrane by numerous bacteria, particularly from the *Gluconacetobacter* genera. It possesses many novel physico-mechanical characteristics depending upon its structure and elevated limpidness, which is responsible for the initiation of its use in the tissue engineering to regenerate the

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injured tissues like skin. Hovewer, its use in drug delivery system development is very limited. Based on its many versatile properties resembling to skin, it can act as an artificial skin and can perform excellent membrane in the permeation studies using Franz cells (12).

Thus, present study was performed to investigate the usefulness of such formulations, having different permeation enhancers, for transdermal delivery across bacterial cellulose. The effect of incorporation of skin permeation enhancers like IPM and IPP on the *in vitro* permeation was investigated.

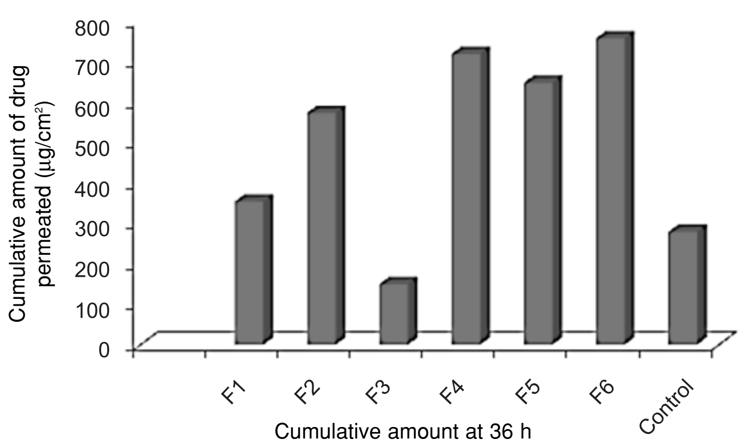
MATERIALS AND METHODS

Materials

Diclofenac diethylamine (DD) was gifted by Abbott Pharmaceuticals, Karachi, Pakistan. All analytical grade chemicals were purchased through local sources from Merck, Germany.

Formulation development

To fabricate matrix based topical formulations, eudragit RL-100 (3 g) and PVP K-30 (20 g) was separately dissolved in ethanol (16.25 g), and then both the solutions are mixed followed by the incorporation of DD (5 g) (drug solution). Another solution of ethanol (16.25 g) in WFI (water for injection, 22 g) was prepared following by the mixing of HPMC (40 g). The resulting solution was then mixed with drug solution. In this way, six different formulations were designed, each containing single enhancer in a quantity specified in the brackets, i.e., F1 (IPM 1.5 g), F2 (IPP 1.5 g), F3 (IPM 3 g), F4 (IPP 3 g), F5 (IPM 4.5 g) and F6 (IPP 4.5 g). A control for-



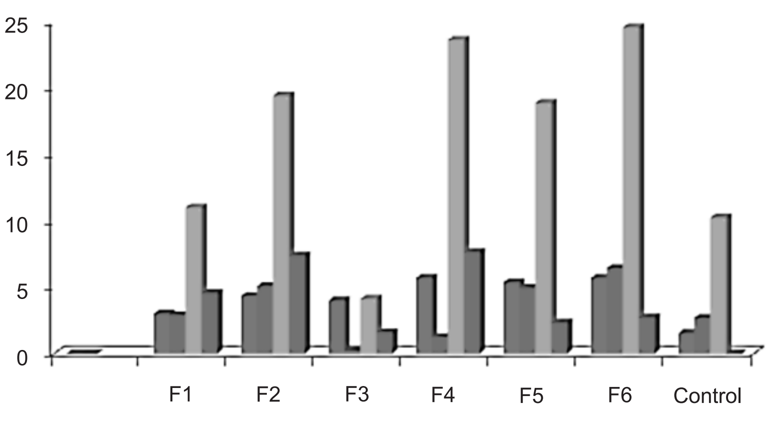


Figure 1. Permeation parameters of diclofenac diethylamine across bacterial cellulose from matrix formulations

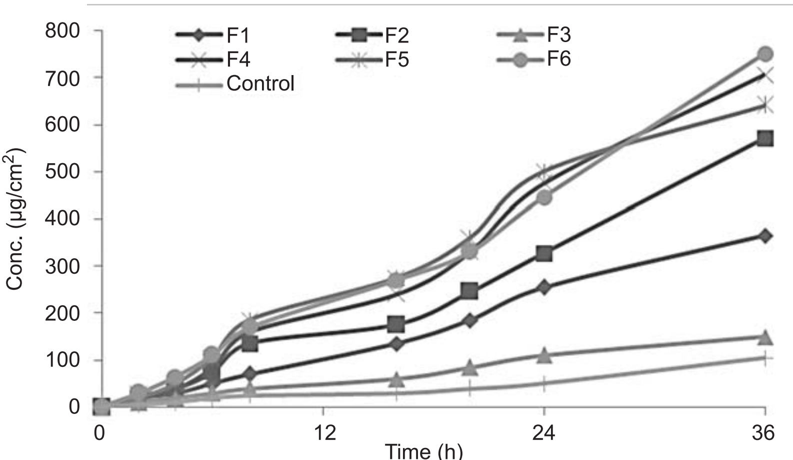


Figure 2. Influence of permeation enhancers (IPM and IPP) on the permeation of diclofenac diethylamine from various matrix formulations across artificial skin as compared to the control formulation

mulation was also formulated using no permeation enhancer.

Permeation studies and calculation of permeation parameters

Permeation studies were conducted using bacterial cellulose (0.81 cm^2) mounted on Franz cells (Emmay, Pakistan). Two grams of each formulation (equivalent to 20, 100 and 200 mg of DD) was placed on a side exposed to donor booth. The receptor compartment contained normal saline at 37 ± 0.5°C stirred continuously using a thermostatically controlled shaker. Samples were taken at predetermined time points and were replaced with the fresh medium. After filtration using disposable filters (Millipore, USA), the withdrawn samples were then analyzed for DD using UV spectrophotometer (UV 1601, Shimadzu-Japan) at 263 nm (13). A perfect sink condition was maintained throughout the experiment.

To draw permeation curve, cumulative amount (μg/cm^2) of drug permeated through membrane was plotted *versu*s time. Straight line slope of the permeation curve and its corresponding *x*-intercept was used to evaluate the steady state flux (J_{ss}) and lag time (t_L) of DD, respectively. The permeability coefficients (K_p) and diffusion coefficients (D) were determined as (14):

$$D = h^2/6\ t_L \quad (1)$$

$$K_p = J_{ss}/C_s \quad (2)$$

where, h and C_s represent bacterial cellulose membrane thickness (0.81 cm) and the initial drug concentration in the donor compartment, respectively.

Enhancing factor (EF) was calculated by the following way (14):

$$EE = CP / CU \quad (3)$$

where, CP and CU are cumulative permeated amount of DD of a formulation and cumulative permeated amount of DD of control formulation, respectively.

The enhancing ratio (ER_{flux}) was assessed by the following way (14, 15):

$$ER_{flux} = SS / SP \quad (4)$$

where, SS and SP are steady-state permeation rate of a formulation and steady state permeation rate of control formulation, respectively.

RESULTS AND DISCUSSION

Drug permeation data from various formulations as compared to the control (having no permeation enhancer) are shown in Figures 1 and 2. After 36 h study, there was a higher cumulative amount of permeated drug from all the prepared formulations containing enhancers (except F3) as compared to that of the control (Fig. 1). A previous study (4) has presented that the cumulative amount of permeated diclofenac diethylamine *via* bacterial cellulose was 7.40% and 6.71% after 23 h from gelly microemulsion and liquid microemulsion, respectively as compared to 7.46% from F5 after a permeation study of 24 h. The steady-state flux was 23.59 ± 0.12 μg/cm^2 h and 6.09 ± 0.03 μg/cm^2 h for F5 and F3, respectively. The previous study (14) has presented that the gel containing IPM with carbopol 900 base permeated a flux of 9.30 ± 0.49 μg/cm^2 h. Another

study (16) reported that the microemulsion allowed a flux of 117.89 µg/cm^2 h for diclofenac diethylamine *via* regenerated cellulose membrane.

Figures 1 and 2 show the influence of addition of enhancers, i.e., IPM and IPP, on the DD permeation from the prepared matrix formulations as compared to the control. After a permeation study of 36 h, the permeated cumulative amounts of DD were 717.73 ± 55 µg/cm^2 and 149.78 ± 8.02 µg/cm^2 for F5 and F3 matrix formulation, respectively. It has been stated in a previous study that IPP, which is a fatty acid ester type enhancers, interrelate essentially with the lipids present in cells. The increased permeated cumulative amount of drug could be due to the increase in the breakdown of lipid bilayer of stratum corneum (17).

The F4 formulation (EF value of 7.59) exhibited the highest increase in DD permeation followed by the formulation F2 (EF value 7.40) in comparison to the control formulation (EF value 0.40). It has been proposed that permeation of many drugs through stratum corneum is an essential pathway for drug transport *via* intercellular route (18). The present study also showed that the rate of DD permeation (ER_{flux}) from all the matrix formulations prepared with permeation enhancers was elevated as compared to the control formulation. It has been stated in the previous study that the value of ER_{flux} was higher (7.53) from F4 in comparison to 4.01 ± 2.604 for 5% limonene in horses (18). The obtained value of diffusion coefficients for F2, F4, F6 and control were 4.34 ± 0.39 cm^2/s × 10^{-4}, 5.69 ± 0.39 cm^2/s × 10^{-4}, 5.88 ± 0.43 cm^2/s × 10^{-4} and 1.99 ± 0.40 cm^2/s × 10^{-4}, correspondingly. This elevation in the permeation can be attributed to the jumping of drug molecules into the lipid bilayer resulting in its rotation, vibration and translocation, which cause the development of microcavities. It ultimately increases the free volume vacant for drug dispersion, which is minimal along with the interface of lipid bilayer membrane when no permeation enhancer is used (19). This whole study and discussion explored that the permeation enhancers are crucial for the improvement of drug permeation rate. These results are finally supported by the value of RPR > 1 for all fabricated formulations that permeation enhancers are crucial for efficient drug transportation through bacterial cellulose membrane.

CONCLUSION

The results elaborate that the addition of skin penetration enhancer into the formulations elevated the permeation rate of the drug in comparison to the control formulation. The F6 was found as the most efficient formulation based on its higher steady state flux, permeability coefficient and diffusion coefficient with a decrease in lag time of DD permeation in comparison to the control formulation.

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IDENTIFICATION OF PHASES OF VARIOUS OIL, SURFACTANT/ CO-SURFACTANTS AND WATER SYSTEM BY TERNARY PHASE DIAGRAM

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Abstract: The objective of this study was to select appropriate surfactants or blends of surfactants and oil to study the ternary phase diagram behavior and identify various phases obtained from the oil and surfactant/surfactant mixture combinations of different HLB. The phases include conventional emulsion, gel/ viscous and transparent/translucent microemulsion. Pseudoternary phase diagrams of water, oil and S/S_{mix} of various HLB values range of 9.65–15 were constructed by using water titration method at room temperature. Visual analysis, conductivity and dye dilution test (methylene blue) were performed after each addition and mixing of water, to identify phases as microemulsion, o/w or w/o emulsion (turbid/milky) and transparent gel/ turbid viscous. High gel or viscous area was obtained with Tween 80 and surfactant mixture of Tween 80 and Span 80 with all oils. The results indicated that non-ionic surfactants and PG of different HLB values exhibited different pseudoternary phase diagram characteristics but no microemulsions originated from mineral and olive oils. The w/o emulsion occupied a large area in the ternary phase triangle when HLB value of the surfactant/S_{mix} decreased. The o/w emulsion area was large with increasing HLB value of surfactant/S_{mix}.

Key words: Pseudoternary phase diagram, nonionic surfactants, emulsion

Generally, emulsions, micro or nanoemulsions, are dispersed systems of different ratios of oil, surfactant(s) and aqueous phase. The different phases, their behavior and changes in volume fraction of different phases of the system can be checked by using pseudoternary phase diagram. A system consisting of water, oil, surfactant (or surfactants mixture) with various phases may be depicted on a phase tetrahedron whose apexes, respectively, present the pure components. The phase behavior can easily report on pseudoternary triangles. Apparently, a fixed (weight or volume) ratio must be selected for any two of the components and one of the triangle vertices presents 100% of the binary mixture. Multicomponent phase diagrams are generally based on constant ratios of surfactant to water or co-surfactant-to-surfactant (1).

Oil/lipid based formulations have been developed in the past by using phase diagrams. To identify regions like o/w microemulsion or nanoemulsion, coarse emulsions and gel/viscous by dissolving specific oil/surfactant/surfactant mixture ratio upon dilution of water can be obtained by using phase diagram. The past research studies showed the importance of phase diagram for developing oil based drug delivery systems, their identification and characterization (2–9).

For making a productive formulation, study of phases obtained from several combinations of oil, surfactant/surfactant and co-surfactant mixture, water and their behavior is required. Numerous oils and surfactants are considered as satisfactory food grade materials or also being used in the pharmaceutical industry (10). The selection and choice of surfactant and co-surfactant is of great importance. Generally non-ionic surfactants are chosen because of their good cutaneous tolerance, lower irritation potential and toxicity (11). Ionic surfactants are used rarely in special cases (12). Non-ionic surfactants (13, 14), short and medium chain alcohols (15, 16), alkanoic acids, alkanediols and alkyl amines (17) can function as co-surfactants that can reduce surface tension and increase the flexibility of the interfacial film.

The aim of this study was to identify and select the surfactant or optimal blends of surfactants and oil using HLB values, and then, investigate the pseudoternary phase diagram behavior of mixtures of these surfactants with different oil and water. The pseudoternary phase diagrams were construct-

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ed in order to identify the types of dispersion systems formed by the mixtures at different concentrations of their components. Our interest at this time is in viscous or gel phase. The systems composed of Tween 80, water, four oils such as isopropyl myristate (IPM), eucalyptus oil (EO), olive oil (OO) and mineral oil (MO) and co-surfactants i.e., propylene glycol (PG), Span 80, Span 20 were selected for screening. Molecular structure and HLB value of the surfactants are presented in Table 1. All these surfactants, co-surfactant and oils are generally accepted as safe, nonirritant and nontoxic for use in a number of pharmaceutical, cosmetic, and food products (18).

EXPERIMENTAL

Materials

Eucalyptus oil and olive oil were purchased from Jiaxing, Sunlong Industrial and Trading Co. Ltd., China. Mineral oil was purchased from Moksha Life Style Products, New Delhi, India. Isopropyl myristate was purchased from Derifats Chemicals Sdn. Bhd., Malaysia. Propylene glycol, Tween 80, Span 20 and Span 80 were purchased from Sigma-Aldrich, USA. Other reagents or chemicals used were of analytical grade. The materials were used as received.

METHODS

Construction of ternary phase diagram

The oils employed were olive oil (OO), mineral oil (MO), isopropyl myristate (IPM) and eucalyptus oil (EO). The physico-chemical properties of the oils are presented in Table 2. The molecular volumes (v) of the oils were calculated as:

$$v = 1.66\ (Mr/d) \qquad (19)$$

where Mr represents the relative molecular mass (in g/mol) of the oil and d is the density (in g/cm^3). The values of Mr and d were taken from the manufacturer's specifications.

The pseudoternary phase diagrams consisting of oil, water and surfactant/surfactant and co-surfactant mixture of different HLB values were constructed using water titration method. The ratio of surfactant to co-surfactant was fixed at 1 : 1 on the weight basis. Each oil was mixed with surfactant or surfactant and co-surfactant mixture at ratios of 0.5 : 9.5, 1.0 : 9.0, 1.5 : 8.5, 2.0 : 8.0, 2.5 : 8.5, 3.0 : 7.0, 3.5 : 6.5, 4.0 : 6.0, 4.5 : 5.5, 5.0 : 5.0, 6.0 : 4.0, 7.0 : 3.0, 8.0 : 2.0 and 9.0 : 1.0 (w/w). Four types of surfactant (Tween 80, Tween 80 and Span 20, Tween 80 and Span 80, Tween 80 and propylene glycol) were used. Distilled water was added in increments of 100 µL by micropipette at room temperature (26 ± 2°C) to the oil and surfactant or surfactant and co-

Table 1. Selected surfactants for screening.

Name	HLB	Molecular structure
Tween 80 (polyoxyethylene sorbitan monooleate)	15	a+b+c+d = 20 Polysorbate 80
Span 20 sorbitan laurate (sorbitan monododecanoate)	8.6	Sorbitan monolaureate
Span 80 sorbitan monooleate (sorbitan (Z)-mono-9-octadecenoate	4.3	Sorbitan monooleate
Propylene glycol	4.45	OH | OH-CH_2-CH-CH_3-OH

Table 2. Chemical structures, relative molecular masses (Mr), density (*d*) (at 20°C) and calculated molecular volumes (*v*) of oils investigated.

Oil	Chemical structure	Mr (g/mol)	d (g/cm³)	*v* (cm³/mol)
Isopropyl myristate	H_3C, H_3C, O, O, CH_3	270.45	0.854	525.7
Eucalyptus oil	O	154.25	0.913	280.45
Mineral oil	As specified in USP	–	0.875	–
Olive oil	As specified by manufacturer	–	0.909	–

Table 3. Percentage of IPM, Tween 80 and PG or water using 1.0 : 9.0 oil and surfactant or surfactant and co-surfactant ratio.

IPM (µL)	Tween 80 & PG (µL)	Water* (µL)	Total volume (µL)	IPM (%)	Tween 80/PG (%)	Water (%)
100	900	100	1100	9.09	81.81	9.09
100	900	200	1200	8.33	75.00	16.66
100	900	300	1300	7.69	69.23	23.07
100	900	400	1400	7.14	64.28	28.57
100	900	500	1500	6.66	60.00	33.33
100	900	600	1600	6.25	56.25	37.5
100	900	700	1700	5.88	52.94	41.17
100	900	800	1800	5.55	50.00	44.44
100	900	900	1900	5.26	47.36	47.36
100	900	1000	2000	5.00	45.00	50.00
100	900	1100	2100	4.76	42.85	52.38
100	900	1200	2200	4.54	40.90	54.54
100	900	1500	2500	4.0	36.00	60.00
100	900	2000	3000	3.33	30.00	66.66
100	900	5000	6000	1.66	15.00	83.33
100	900	10000	11000	0.90	8.18	90.90

*The amount of water is varied to provide a water concentration in the range of 9% to 90% of total volume.

surfactant mixture until 90.90% w/w. The samples were vigorously mixed with a vortex mixer for 2 min and kept at room temperature (26 ± 2°C) for 24 h to reach equilibrium before the next addition of water. The percentages of IPM, Tween 80 and PG and water for oil and surfactant or surfactant and co-surfactant at ratio of 1.0 : 9.0 are presented in Table 3.

Visual inspection

Visual inspection was made after each addition of water to the oil and surfactant or surfactant and co-surfactant mixture. The samples were identified as microemulsions when they appear as transparent/translucent and easily flowable liquid. No attempt was made to distinguish between oil-in-water, water-in-oil or bicontinuous type microemul-

sions. The samples were identified as emulsions when they appeared as milky or turbid liquids. The samples were identified as gel when they did not show a change in the meniscus after tilting to an angle of 90°. All these categories were plotted on a triangular graph as ternary or pseudoternary phase diagram using Chemix School 3.51 software, Arne Standnes USA.

Physical appearance of emulsions

The visual qualities of the emulsion mixtures were noted as turbid or milky liquids.

Electrical conductivity test

A pair of electrodes (Sanwa YX-360TRD Multitester, Japan) connected to an electric bulb was dipped into an emulsion. If the emulsion is o/w type, the electric bulb glows.

Staining test/dye-solubility test

Water soluble dye, methylene blue solution of 10 µL was added to the emulsion. If the continuous phase is water (o/w emulsion), the dye will dissolve uniformly throughout the system. If the continuous phase is oil (w/o emulsion), the dye will remain as cluster on the surface of the system (20).

RESULTS AND DISCUSSION

A wide variety of structures and phases can be formed by mixing oil, water and surfactants in different ratios. Molecular and structural examinations, concentration of surfactants and other ingredients can expose the existence of microemulsions, typical emulsions, crystalline and lamellar structures depending on the ratio of the components. Recognition of different phases and structures can be achieved by simple visual inspection of their physical appearance (e.g., microemulsions (ME) are transparent/translucent, emulsions are nontransparent and phases separate after a while; and gels (21, 22). The appearance of ME, emulsion and gel is presented in Figure 1.

It is important to find the chemical type of surfactant which best matches that of the oil, because the chain length compatibility of a surfactant and oil is critical for the formation of emulsion systems. Surfactant type plays a major role in determining the rheological properties and droplets size of the systems (9). Choice of surfactant is crucial to obtain the desired formulation. Each oil and surfactant has a specific HLB value. The HLB of the selected surfactant or surfactant and co-surfactant that matches the HLB of the selected oil provides the lowest interface tension between the oil and water phases. The HLB of the selected surfactant(s) reflects the stability of the system and can be obtained when the HLBs of the surfactant and oil are similar (8). The stability of emulsions is improved if a combination of surfactants is used because solubilization reaches the maximum and the smallest particles are formed when the hydrophilic-lipophilic balance of a surfactant is optimal in a given oil-water system. Likewise, a type of oil, whether it is triglyceride form or long chain hydrocarbon, can change the physical properties of the systems.

Figure 1. a – Transparent ME, b – turbid emulsion, c – gel (left to right)

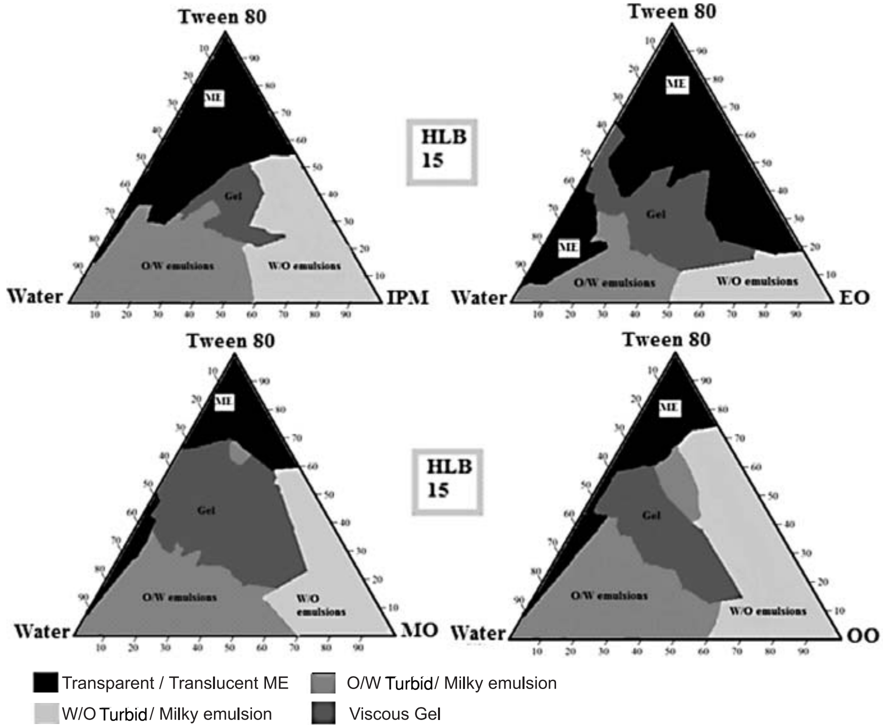

Figure 2. Ternary phase diagrams of isopropyl myristate (IPM), eucalyptus oil (EO), mineral oil (MO) and olive oil (OO) with Tween 80 and water

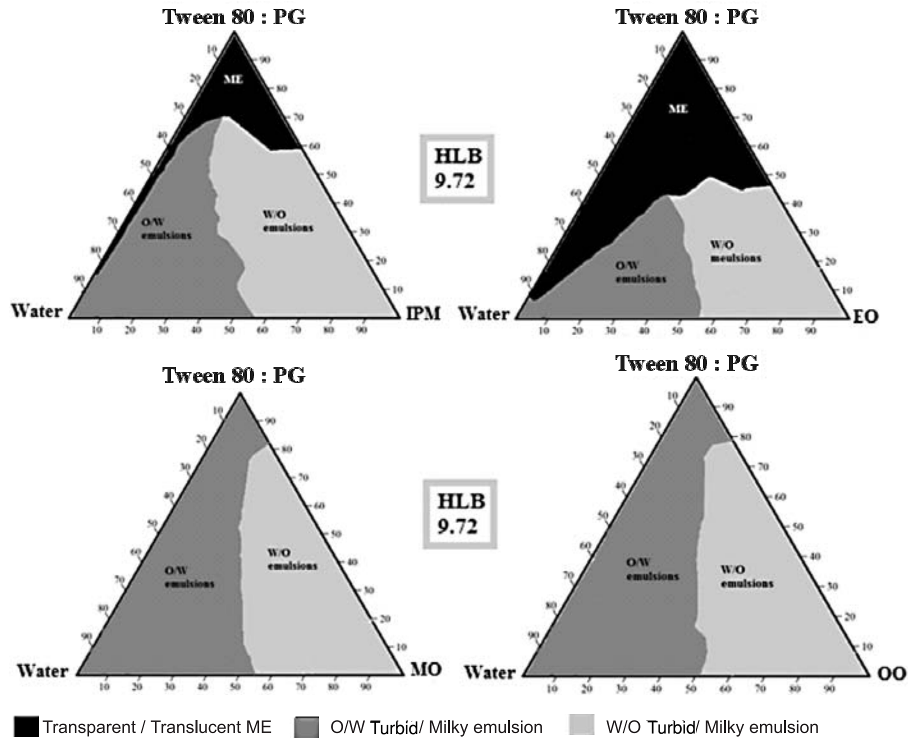

Figure 3. Ternary phase diagrams of isopropyl myristate (IPM), eucalyptus oil (EO) and olive oil (OO) with Tween 80 : Propylene glycol (PG) (Smix 1 : 1), and water

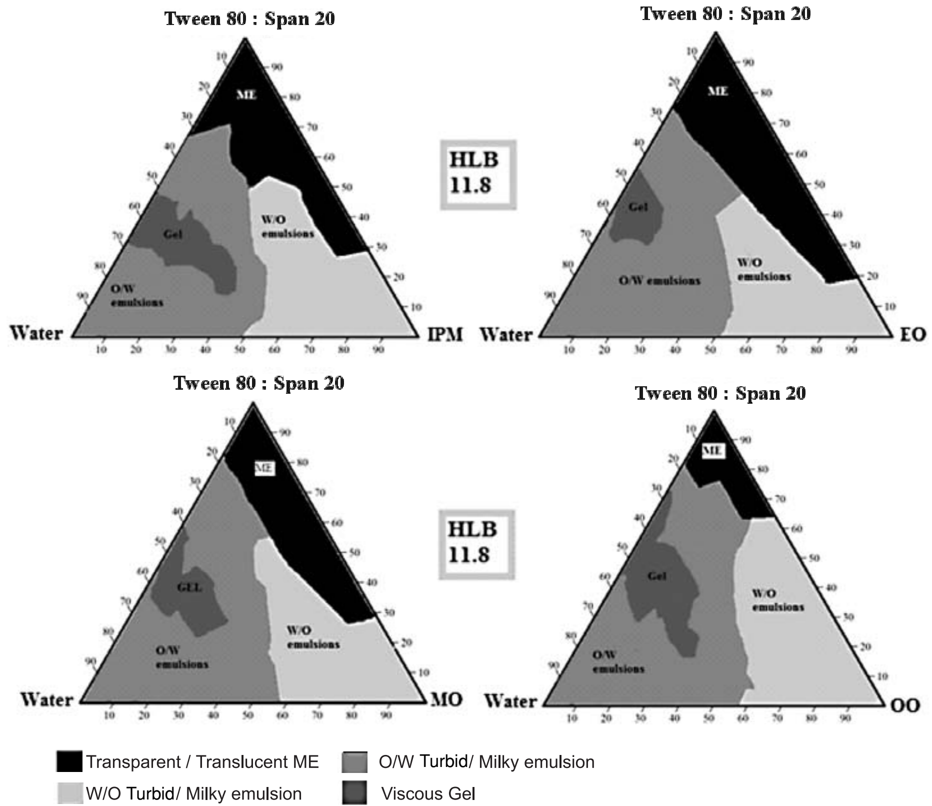

Figure 4. Ternary phase diagrams of Isopropyl myristate (IPM), Eucalyptus oil (EO), Mineral oil (MO) and Olive oil (OO) with Tween 80: Span 20 (Smix 1:1) and water

Non-ionic surfactants in general, Tween and Span in particular are safe agents for all biological tissues in general and for skin specifically (23, 24). These non-ionic emulsifiers are compatible with various ingredients used in the preparation of emulsions and are not affected by pH. A combination of lipophilic and hydrophilic non-ionic surfactants is able to build highly structured emulsions.

Tween 80 is a derivative of Span 80, but is hydrophilic in nature because the hydroxyl groups on the sorbitan ring are replaced and substituted with bulky polyoxyethylene groups. This substitution makes Tween 80 more soluble in water, so it tends to form oil-in-water emulsions. Span 80 is a viscous, lipophilic, emulsifying liquid agent, which tends to form water-in-oil emulsions.

Polyols such as propylene glycol (PG) and short-chain alcohols are known to act as co-surfactants. Hence, it is presumed that a considerable part of PG is incorporated into the surfactant layer and will increase the interfacial fluidity, and the other part of PG will decrease the polarity of the water because PG is mainly soluble in water. PG is one of the least hydrophilic simple polyols, that is soluble in water but practically non-soluble in the oil phase (25). In comparison with other alcohols, PG is relatively tolerable by the skin.

Different combinations of oil, surfactants and water when mixed together produce either typical emulsion or microemulsion in the form of o/w or w/o. Bicontinuous system can also be formed in case of microemulsion system where each phase of water and oil is found as a continuous phase. Pseudoternary phase diagram construction is the best way to study all types of formulations that can originate from mixing of surfactants, water and oil. This study is performed to predict the optimized compositions of surfactants, oil and water in the development of ME, emulsion or gel.

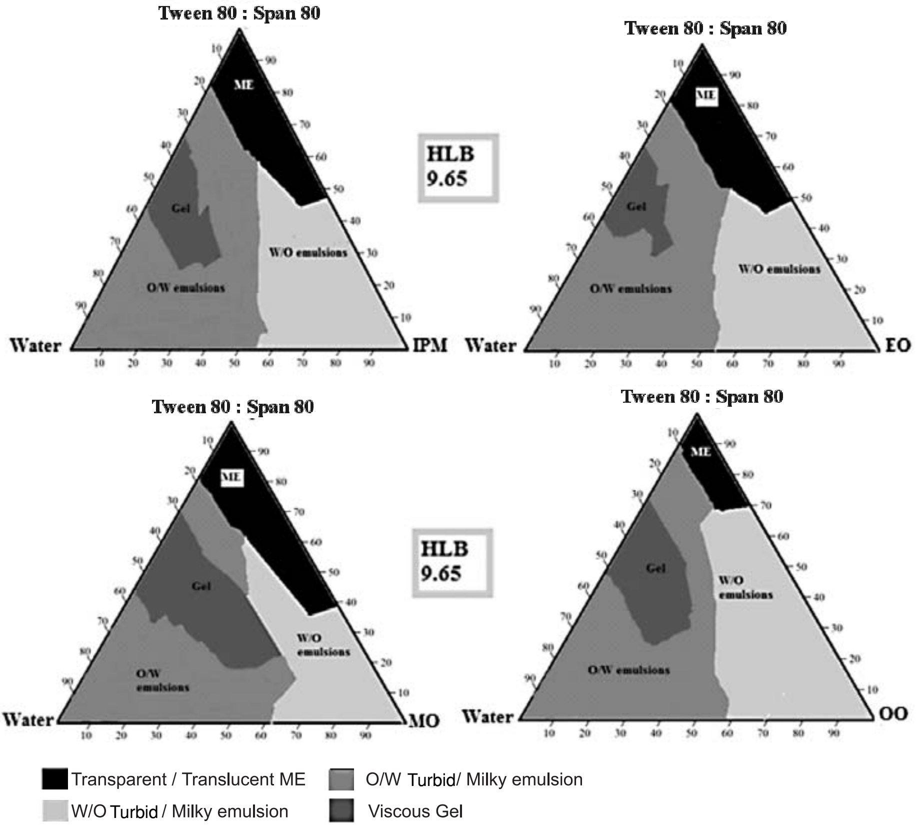

Figure 5. Ternary phase diagrams of isopropyl myristate (IPM), eucalyptus oil (EO), mineral oil (MO) and olive oil (OO) with Tween 80 : Span 80 (Smix 1 : 1) and water

Figures 2 to 5 are the phase diagrams of mixture of IPM, eucalyptus oil, mineral oil, olive oil, water and surfactant or their mixtures with HLB values of 15.00, 9.72, 11.80 and 9.65, respectively. A large area of transparent/clear solution was formed in the oil rich regions. The ME areas were found to be attached to the borders of the diagrams where water and oil ratio was low and the surfactant ratio was high. All figures showed the presence of transparent ME area except Figures 3c and 3d, representing the diagrams of mineral oil and olive oil with surfactant mixtures of Tween 80 : PG and water with HLB value of 178,758 9.72. Attempts to obtain microemulsions using mineral oil and olive oil were not successful. Mineral oil is a mixture of high molecular weight hydrocarbons. Olive oil contains predominantly long-chain triglycerides of oleic acid. Thus, the molecular weight of mineral oil and olive oil is most probably too high to assist in the formation of microemulsion (26). No liquid crystalline structure was observed.

Incorporation of co-surfactant like PG increased the maximum amount of incorporated water in the oil-surfactant system with the microemulsion zone being increased compared to the surfactant system (Fig. 3b). The presence of PG abolished the region of the gel from the phase diagrams (Fig. 3) compared to the surfactant system (Fig. 2). Breaking of the gel was reported after introduction of short chain alcohol with up to 4 carbon atoms (21).

W/O emulsion occupied the lower right region of the triangle phase diagram (oil rich region). On the other hand, o/w emulsion occupied the middle and left regions (water rich regions) of the triangle phase diagram. The formation of w/o emulsion or o/w emulsion depends on the composition of the emulsifier and its solubility in the oil and water (27). The phase diagrams obtained also showed that areas of w/o dispersions decreased as the HLB value of the surfactant system increased. This is in accor-

dance with the theory of HLB of surfactant, which stated that the types of surfactant used has more tendencies to produce the emulsion type in which it is more soluble in the external phase (9). Tween 80 : Span 80 at an HLB of 9.65 had the tendency to form a large area of w/o emulsion as shown in Figure 4. Similarly, a larger o/w area was formed by the mixture of Tween 80 : Span 20 at a higher HLB value of 11.8 (Fig. 4). Tween 80 formed large oil-in-water emulsion areas compared with water-in-oil emulsion on the oil-water axis (Fig. 1) due to higher HLB value of 15 of Tween 80, which promoted the formation of o/w emulsion (28). O/W and w/o emulsions have been used for a long time in the pharmaceutical industry and cosmetic industry (29, 30).

It can be observed in Figures 2, 4 and 5 that the gel/viscous area was formed when the water content in the system was in the range of 25 to 60%. It was found that water content below 25% was insufficient to hydrate the polyoxyethylene groups, which are critical for the swelling of surfactant chains to demonstrate the gel structure. When the water content was above 60%, the distance between the polyoxyethylene groups increased and destabilized the gel structure resulting in breaking of the swelled gel (31).

Phase diagrams containing Tween 80 (Fig. 2) and those containing surfactant and co-surfactant of Tween 80 (HLB 15) and Span 80 (HLB 4.3) (Fig. 5) exhibited higher gel area compared to Tween 80 and Span 20 (HLB 8.6) (Fig. 4). The results showed that water and surfactant and co-surfactant mixture of Tween 80 and Span 80 having HLB of 9.65 formed larger gel area than that of mixture of Tween 80 and Span 20 having HLB value of 11.8. The results indicated that a mixture of two non-ionic surfactants with big difference in HLB values between them might be able to produce stable preparations. This may be due to the fact that surfactants with very low HLB value dissolved in oil phase and the surfactant with high HLB value dissolved in the water that enable them to function together well enough to exert stronger effect than surfactant and co-surfactant mixture having closer HLB values (8). The results indicated that Tween 80 alone and surfactant mixture of Tween 80 and Span 80 would be better than surfactant mixture of Tween 80 and Span 20 for forming emulsions with gel properties. Since the Tween 80 and Span 20 showed small viscous area, this surfactant combination was excluded from further study.

CONCLUSION

This phase diagram study provides valuable information on the role that structure of the surfactant, co-surfactant, and oil plays in determining the properties of the system at any composition. It was observed that a proper mixture of surfactants is achieved when surfactants at lower and higher HLB are blended. When the difference between the hydrophilic and lipophilic surfactants will be greater, the better would be the coverage by mixtures at the interface. Tween 80 alone and surfactant mixture of Tween 80 and Span 80 produced high viscous or gel area. The interesting results obtained with surfactant mixture of Tween 80 and PG, that supported the formation of ME and turbid emulsion phases but no gel phase was appeared with this combination in all oils. Except Tween 80 and PG combination, the phase diagrams containing other nonionic surfactant mixture and Tween 80 alone, showed the appearance of different phases with oils. This study will be very useful in formulating a delivery system in pharmaceutical industry as well as in cosmetics and personal care products.

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OPTIMIZATION AND EVALUATION OF CLARITHROMYCIN FLOATING TABLETS USING EXPERIMENTAL MIXTURE DESIGN

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Abstract: The purpose of the study was to prepare and evaluate clarithromycin (CLA) floating tablets using experimental mixture design for treatment of *Helicobacter pylori* provided by prolonged gastric residence time and controlled plasma level. Ten different formulations were generated based on different molecular weight of hypromellose (HPMC K100, K4M, K15M) by using simplex lattice design (a sub-class of mixture design) with Minitab 16® software. Sodium bicarbonate and anhydrous citric acid were used as gas generating agents. Tablets were prepared by wet granulation technique. All of the process variables were fixed. Results of cumulative drug release at 8th h (CDR 8th) were statistically analyzed to get optimized formulation (OF). Optimized formulation, which gave floating lag time lower than 15 s and total floating time more than 10 h, was analyzed and compared with target for CDR 8th (80%). A good agreement was shown between predicted and actual values of CDR 8th with a variation lower than 1%. The activity of clarithromycin contained optimized formula against *H. pylori* were quantified using well diffusion agar assay. Diameters of inhibition zones *vs.* log10 clarithromycin concentrations were plotted in order to obtain a standard curve and clarithromycin activity.

Keywords: clarithromycin, floating tablets, modified release, experimental mixture design, *H. pylori*

The oral route is the most preferred form of drug administration for systemic action, having a high degree of patient compliance (1) and ease of use. However, oral route has several physiological difficulties such as variable nature of gastric emptying system, inability to localize the drug delivery system (DDS) in desired regions of the gastro-intestinal system (GIS), shorter residence time and incomplete drug release of DDS (2). As a result of above factors, a shorter residence time and incomplete drug release leads to unpredictable bioavailability of DDS. Thus, control of the placement for DDS in a specific part of GIS according to absorption window may increase gastric residence time and bioavailability of DDS by preventing drug release from reaching desired absorption site of GIS (3). There have been several systems developed by researchers to increase the retention time of a DDS in the stomach (2). The examples of these are floating systems (4), swelling and expanding systems (5, 6), modified-shape systems (5–7), high density systems (8), and other delayed gastric emptying devices (2, 9). The floating drug delivery system is used commonly among these delivery systems.

Helicobacter pylori was discovered in 1984 (10) and then it has been considered to be the most frequent bacterial infection worldwide to cause ulcer disease, gastric cancer and MALT-lymphoma (11). Complete eradication can't be achieved due to insufficient amount of antibiotics in gastric mucosa and short residence time of DDS's (12). Thus, eradication of *H. pylori* requires high concentration of antibiotics in gastric mucosa and availability of DDS in stomach for longer durations.

CLA is a semi-synthetic antimicrobial macrolide antibiotic, which was discovered and patented by Taisho Pharmaceutical Co. Ltd. Japan in 1980 (12). CLA is the most preferred molecule, due to the lowest MIC value, proper pharmacokinetic properties and high efficiency on monotherapy of *H. pylori* (12). Moreover, CLA is stable in gastric environment and pH, and further (13), it has short half-life (14). Above properties of CLA make it a proper candidate for modified release drug delivery systems for the treatment of *H. pylori*.

Traditional drug development strategies require more runs of experiments, time and money to achieve desired product quality while providing

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Table 1. Composition of formulations tested.

Formulation Code	CLA (mg)	HPMC K100 (mg)	HPMC 4M (mg)	HPMC K15M (mg)	PVP K30 (mg)	$NaHCO_3$ (mg)	Citric acid (mg)	Cellulose, microcrystalline PH 101 (mg)	Talc (mg)	Magnesium stearate (mg)	Total (mg)
F 1	250	143.00	-	-	20	98	13	109	10	7	650
F 2	250	23.83	95.33	23.83	20	98	13	109	10	7	650
F 3	250	95.33	23.83	23.83	20	98	13	109	10	7	650
F 4	250	71.50	71.50	-	20	98	13	109	10	7	650
F 5	250	23.83	23.83	95.33	20	98	13	109	10	7	650
F 6	250	-	143.00	-	20	98	13	109	10	7	650
F 7	250	71.50	-	71.50	20	98	13	109	10	7	650
F 8	250	47.67	47.67	47.67	20	98	13	109	10	7	650
F 9	250	-	-	143.00	20	98	13	109	10	7	650
F 10	250	-	71.50	71.50	20	98	13	109	10	7	650
OF	250	91.13	31.36	20.51	20	98	13	109	10	7	650

less process and product understanding. In pharmaceutical field, it's possible to evaluate the influence of process inputs on finished product quality by 'design of experiment' (DOE), which is also expressed as 'experimental design'. By performing adequate literature search and risk assessments, well-designed experiments provide helpful insight, reliable results and understanding of cause-effect relationship, with fewer runs than haphazard and unplanned experiments. In general, fractional factorial and central composite designs are used preferred to evaluate the influence of the process variables as temperature, pressure, time etc. on production properties. Another main type of DOE application concerns the preparation and modification of mixtures (15). The mixture design is considerable for investigation of relative proportions of components in pharmaceutical formulation development (16). In this study, experimental mixture design was used to determine the optimum relative proportions of polymers in formulation.

The aim of this study was to develop a floating drug delivery system containing CLA. To optimize the amount of different grades of polymers and to achieve desired dissolution ratio at 8th h, experimental design was performed by mixture design methodology, which has not been widely used for pharmaceutical formulation development. The physical and analytical characteristics of formulations were evaluated.

MATERIALS AND METHODS

CLA, cellulose microcrystalline PH 101, povidone K30, sodium bicarbonate, anhydrous citric acid, talc and magnesium stearate were donated by Deva Holding, Turkey. HPMC (hypromellose) K100, HPMC K15M and HPMC K4M were donated by Colorcon, Turkey. Folin Ciocalteu's phenol reagent was purchased from Merck, Turkey. *Helicobacter pylori* ATCC 43504 was purchased from DSMZ, Germany. Mueller Hinton agar (KKMHA) was donated by Ant Teknik and Organik Laboratuvarlarý, Turkey. All chemicals were of analytical grade.

Preparation of CLA tablets

CLA, cellulose microcrystalline PH 101, povidone K30, sodium bicarbonate and different grades of HPMC were mixed in V-shaped mixer for 20 min and sieved through 0.5 mm sieve. All ingredients were mixed and granulated with adequate amount of ethyl alcohol in laboratory scale

high shear mixer (MicroGral™, Belgium). Wet mass was dried in oven at 55°C until obtaining final loss of drying (LOD) value of 2–4%, using moisture analyzer, Mettler Toledo (Switzerland), at 105°C. Dried granules were sieved through 0.841 mm sieve, mixed with anhydrous citric acid, and then mixed with talc and magnesium stearate for further 3 min in V-shaped mixer. Final mixture was compressed with Piccola rotary press machine (England) using 11.9 mm round-biconvex punches. The final crushing strength values of tablets were obtained in the range of 80–95 N.

Experimental design

The total amount of three different grades of HPMC (K100, K4M and K15M) was fixed at 143 mg (22%) in the formulations. Simplex lattice design (augmented, degree 2) was used to generate and arrange the amount of dependent variables (K100, K4M and K15M) in formulations by Minitab 16® software (LEADTOOLS © 1991–2004, LEAD Technologies, Inc., USA.). The target was set as 80% CDR at 8th h due to the dissolution study result of marketed product URCLAR, which was reported by Patel et al. (14). Ten different design points and the formulations are described in Table 1 and Figure 1. After getting results, the data were fitted into Minitab 16® software and statistically analyzed using analysis of variance (ANOVA). The statistical model was validated by preparation and dissolution analysis of optimized formulation (OF), which was generated by Response Optimizer tool of the software.

Measurement of tablet properties

The weight variations of tablets were determined according to the PhEur. The diametrical tablet crushing strength was evaluated using a tablet hardness tester (Erweka, Germany). Tablet diameter and thickness were measured using a digital micrometer with a sensitivity of 0.01 mm (Bestool-Kanon, Japan)

***In vitro* buoyancy studies**

In vitro buoyancy studies were performed according to method described by Gambhire et al. (17). Briefly, tablets of each formulation were kept in 100 mL beaker containing 0.1 mol/L HCl. The time taken for tablet to rise to surface was determined and reported as floating lag time (FLT). The total floating time (TFT) was reported by determining the constantly remaining time of the tablet on the medium surface.

***In vitro* dissolution studies**

USP Dissolution Test Apparatus Type II was used for *in vitro* dissolution tests. The dissolution test was performed using 500 mL of 0.1 M HCl, at 37°C and 100 rpm. At various time intervals, a sample of 5 mL was withdrawn and replaced with equal volume of fresh medium (n = 3). The samples were filtered through 0.45 μm filters. Two mL of samples were diluted up to 10 mL with 2 mL of Folin-Ciocalteu's phenol reagent (diluted 1 : 2 with distilled water), 2 mL of 20% sodium carbonate solution and 0.1 M HCl and mixed properly. The absorbances of the samples were measured at 760 nm using Lambda 25 UV/Vis double-beam spectrophotometer (Perkin-Elmer, USA). The drug concentrations in the samples were calculated by using standard calibration curve (14).

Kinetic mechanism of drug release

The release mechanisms of CLA from floating tablets were determined by calculating the correlation coefficients after application of release data to Korsmeyer-Peppas equation (18).

Well diffusion agar assay of optimized formulation

H. pylori ATCC 43504 was incubated in Mueller Hinton agar (KKMHA) including 5% of sheep blood at 37°C during 3 days under micro-aerophilic conditions. Bacteria suspension was prepared in physiological saline according to Mc Farland 2 opacity after incubation. Hundred μL of suspension was spread over the KKMHA medium, which was guided for inoculation. Hundred μL of dissolution samples, which were withdrawn at 1st, 2nd, 4th, 6th and 8th h and filtered through 0.45 μm filters, were inoculated into guides in KKMHA medium. Inoculated mediums were incubated at 37°C during 3 days under micro-aerophilic conditions. Diameters of inhibition zones *vs.* log10 CLA concentrations were plotted in order to obtain a standard curve. The inhibition zones were measured by caliper and the concentration of the samples were calculated by using standard calibration curve. Results were presented as the average value of 6 samples, which were studied in different two separate days (3 samples per day) (19–21).

RESULTS AND DISCUSSION

Weight variations, hardness, thickness, floating lag time (FLT), and total floating time (TFT) were evaluated and are reported in Table 2. Target

Table 2. Physical evaluation results of formulations tested.

Formulation	Weigh variation (average weigh; mg; % RSD)	Hardness (N; average)	Thickness (mm)	FLT (s)	TFT (h)	Mass integrity at 8th h
F 1	648; 0.36	84	6.870 ± 0.020	max. 13	> 4	-
F 2	649; 0.43	85	6.930 ± 0.040	max. 5	> 8	+
F 3	651; 0.38	86	6.865 ± 0.075	max. 6	> 8	+
F 4	651; 0.40	86	6.915 ± 0.025	max. 7	> 8	+
F 5	651; 0.48	86	6.965 ± 0.015	max. 9	> 8	+
F 6	652; 0.43	87	6.935 ± 0.015	max. 8	> 8	+
F 7	652; 0.47	88	6.990 ± 0.020	max. 9	> 8	+
F 8	649; 0.32	86	6.895 ± 0.025	max. 13	> 8	+
F 9	652; 0.41	87	6.895 ± 0.015	max. 6	> 8	+
F 10	651; 0.35	91	6.895 ± 0.035	max. 15	> 8	+
OF	651; 0.49	81	6.935 ± 0.015	max. 12	> 8	+

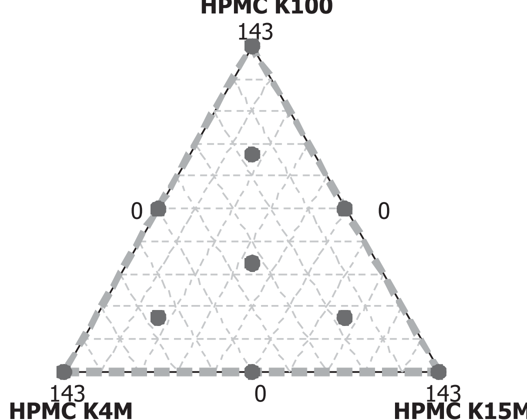

Figure 1, Simplex design plots in amounts

for average weight was set at 650 mg for all formulations. As reported in Table 2, average tablet weights were varied between 648 and 652 mg. All of the formulations proved good uniformity with % RSD lower than 0.5 when pharmacopeia limits were considered as ± 5%.

A study to determine the influence of factors on dissolution of hydrophilic polymer matrix system was performed by Saeio et al. (22). In that study, it was shown that increasing hardness values caused a decrease in porosity of polymer matrices and increasing compaction values had a high ability to retard water penetration into the core of tablet. The dissolution was slowed as a result of retarded water penetration. As the same with the mentioned study, the average hardness of the formulations was kept in the range of 81 and 91 N in our study as shown in Table 2. The hardness and tablet weights were almost similar for all of the formulations tested. Thus, no effect was considered for these physical properties between formulations to cause any variation of their dissolution behavior.

It is mandatory to use gas generating agents and hydrophilic polymers in formulation to achieve short FLT and long TFT. The buoyancy and floating are two expressions explained as reduction of the density lower than gastric fluid as a result of gas generation in dissolution media and the entrapment of generated gas by gel forming layer (by hydrated polymers). Shorter FLT is maintained by gas generating agent, and longer buoyancy (TFT) is maintained by hydrophilic polymers. In this study, sodium bicarbonate and anhydrous citric acid were used as gas generating agents. Gambhire et al. (17) reported that an increase of sodium bicarbonate decreases (alone as gas generating agent) floating lag time and excessive amounts of sodium bicarbonate causes pore formation in the tablet surface, which led to rapid hydration of polymer and rapid drug release from the system. It was also reported that 10% sodium bicarbonate concentration was essential to achieve FLT of 4 to 5 min (17). On the other hand, reduction in FLT was reported by Danki et al. (23) with the addition of citric acid to the formulation with FLT values of 24, 20 and 15 s.

According to above studies, the concentration of sodium bicarbonate and citric acid were kept constant as 15.08% and 2.00%, respectively. The FLTs and TFTs of all formulations were observed

as maximum 15 s and more than 8 h, respectively, which are reported in Table 2. The FLT of optimized formula was observed as 12 s (Table 2, Figure 2). Only F1 was disintegrated and lost mass integrity after 4th h (Table 2). It was our assumption that F1 only contained 22% of HPMC K100 concentration and its molecular weight and viscosity were not able to provide system integrity due to higher swelling property.

Similar FLT and TFT results were observed for all formulations except for F1. The results were complied with Danki et al. (23). Short FLT and long TFT obtained in this study can be explained as follows. First, immediate generation of CO_2 occurs from gas generating agent with the interaction of dissolution media and generated CO_2 is entrapped by swollen polymer matrices. Then, as a result of expansion of polymer matrix, tablet density decreases lower than that of the dissolution media and this phenomenon leads to longer TFTs.

The objective of the study was to design a formulation whose dissolution rate was 80% at 8th h. HPMC K100, HPMC K4M and HPMC K15M were used as release modifying agents to determine the ideal concentration of different grades of polymers in desired formulation. The maximum polymer level was set at 22% for all formulations. *In vitro* dissolution studies were performed on ten different formulations. Comparative dissolution data and dissolution profile graphics are shown in Table 3 and Figure 3.

Formulation F1 disintegrated within 4 h and showed maximum CDR at 6th h (see Table 2 and 3). It can be explained that low viscosity of HPMC K100 couldn't provide a robust gel layer and sustain the generated CO_2 for longer times due to earlier polymer chain relaxation than that of HPMC K4M and HPMC K15M. The rest of formulations kept their integrity until 8 h.

When we compare the release profiles of F1, F6 and F9 (Table 3, Figure 3) at the maximum concentration of polymers alone, the increase of molecular weight of the polymer decreases the CDR at all sampling times. Contrary to this phenomenon, similar results were obtained from CDR 1st h and CDR 2nd h of F7, F9, and F10 (3.5% ± 16 RSD and 12.0% ± 3.5 for F7; 3.6% ± 19.7 RSD and 11.7% ± 2.7 RSD for F9; 3.8% ± 15.6 RSD and 10.2% ± 7.2 RSD for F10, respectively) (Table 3). It was

Table 3. Dissolution results of formulations tested.

Formulation	Composition (mg)			Cumulative drug release (%; n = 3)				
	K100	K4M	K15M	1st hour ± %RSD	2nd hour ± %RSD	4th hour ± %RSD	6th hour ± %RSD	8th hour ± %RSD
F 1	143.00	-	-	14.8 ± 4.6	49.4 ± 6.0	82.8 ± 1.6	108.5 ± 2.5	108.1 ± 1.6
F 2	23.83	95.33	23.83	5.8 ± 12.3	13.9 ± 8.8	36.7 ± 3.2	59.4 ± 6.6	70.3 ± 7.2
F 3	95.33	23.83	23.83	7.9 ± 18.0	22.4 ± 6.2	43.7 ± 3.1	68.0 ± 3.8	82.5 ± 3.5
F 4	71.50	71.50	-	9.0 ± 19.0	16.9 ± 7.1	42.7 ± 4.9	62.9 ± 3.3	76.2 ± 2.3
F 5	23.83	23.83	95.33	4.0 ± 18.1	10.2 ± 12.6	32.4 ± 10.2	46.7 ± 7.8	62.0 ± 5.5
F 6	-	143.00	-	5.4 ± 10.6	11.7 ± 9.0	35.6 ± 6.8	56.9 ± 5.9	65.3 ± 4.3
F 7	71.50	-	71.50	3.5 ± 16.0	12.0 ± 3.5	38.4 ± 4.5	66.4 ± 2.7	76.6 ± 2.5
F 8	47.67	47.67	47.67	4.9 ± 11.5	12.4 ± 10.4	35.6 ± 6.2	62.3 ± 4.0	71.5 ± 2.9
F 9	-	-	143.00	3.6 ± 19.7	11.7 ± 2.7	27.2 ± 3.0	48.1 ± 6.7	53.0 ± 5.8
F 10	-	71.50	71.50	3.8 ± 15.6	10.2 ± 7.2	30.1 ± 6.6	53.0 ± 3.9	57.2 ± 2.8
OF	91.13	31.36	20.51	8.1 ± 14.2	23.4 ± 5.7	42.8 ± 5.9	61.6 ± 5.9	79.4 ± 7.9

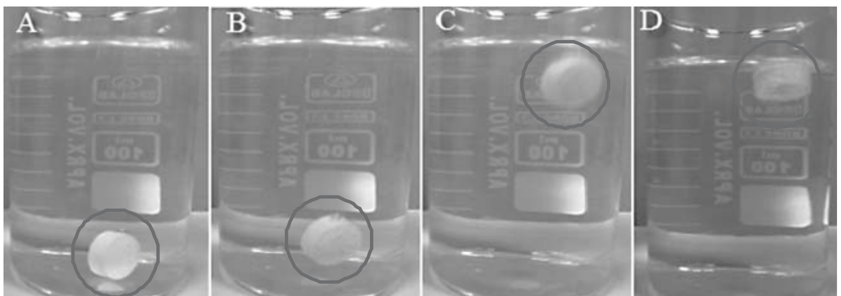

Figure 2. Photographs of optimized formulation in 0.1 M HCl (A: 0; B:4th s; C: 9th s; D:12th s)

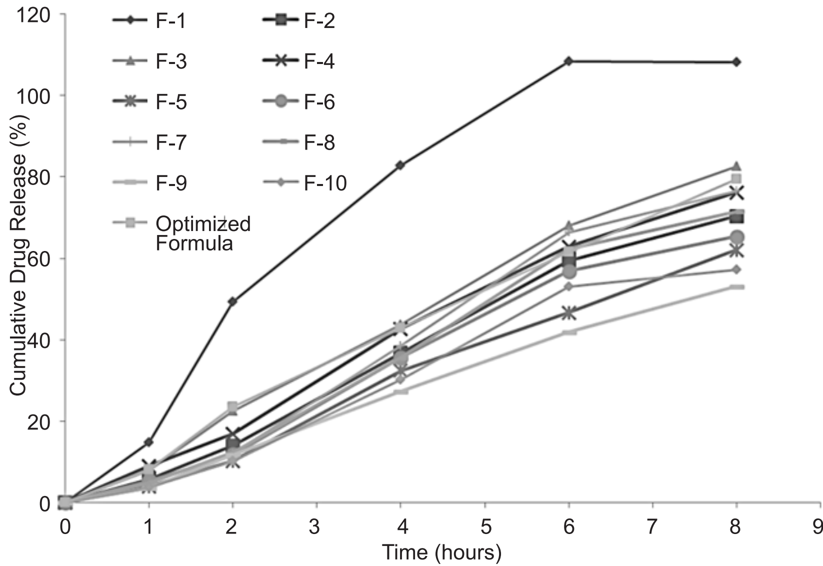

Figure 3. Comparison of *in vitro* dissolution profiles of formulations tested

observed that an increase of HPMC K15M concentration in polymer blend didn't decrease the CDR 1st h and CDR 2nd h significantly. This can be explained by less polymer-solvent interaction and polymer chain relaxation of HPMC K15M due to high molecular weight at first and second hours. The obtained results were complied with Patel et al. (14). In their study, unexpectedly, the dissolution of the system was increased by the enhancement of HPMC K15M instead of HPMC K4M. The increase of dissolution ratio was explained as a result of delaying on unwinding of the polymer chains and reduction of the gelling rate of the system due to higher molecular weight and lower flexibility of HPMC K15M than that of HPMC K4M.

Formulation F5, F9, and F10, which had higher molecular weight polymers than that of the other formulations, showed 62% for F5; 53% for F9; and 57% for F10 release of CLA, respectively, at 8th h (Table 3, Figure 3). HPMC K15M was found to be more effective for retarding the drug release of CLA from DDSs than other polymers at later dissolution stages. An increase of high molecular weight polymer in the blend decreased the drug release from DDS. Higher polymer concentrations with higher molecular weight and viscosity increased the gel formation and diffusion pathway of drug at later stages and this resulted in a reduction of drug release.

Formulations F2, F3, F4 and F8 showed 70.3, 82.5, 76.5 and 71.5% CDR at 8th h, respectively, as presented in Table 3. The highest drug release was detected for F3, which had the highest HPMC K100 amount as 95.33 mg (Table 3). By the way, the formulation F2 showed the lowest drug release, which had lowest HPMC K100 amount as 23.83 mg (Table

3). It was detected that an increase of relative proportion of HPMC K100 amount in the total polymer mixture caused enhancement in CDR 8th.

Drug release kinetics depends on several factors such as swelling rate, rate of penetration of water through the matrix, rate of dissolution of the drug, rate of diffusion of the drug through the swelled material and erosion of the matrix. Obviously, some of the above processes take place simultaneously (24). The release data of different formulations were analyzed using the linear regression to determine the release mechanism according to Korsmeyer-Peppas equation presented below (25):

$$Mt / M\infty = K \cdot t^n$$

where Mt / M8 is a fraction of drug released at time t, k is the release rate constant and n is the release exponent. The "n" value is used to characterize different release for cylindrical shaped matrices.

In this model, the value of n characterizes the release mechanism of drug shown as follows; 0.45 = n corresponds to a Fickian diffusion mechanism, $0.45 < n < 0.89$ to non-Fickian transport, n = 0.89 to Case II (relaxational) transport, and $n > 0.89$ to super case II transport (26, 27).

To study the release kinetics, data obtained from *in vitro* drug release studies were plotted as log cumulative percentage drug release *versus* log time; "*n*" values for all formulations were found to be higher than 0.89, which are presented in Table 4. The release exponent data indicated that all of the formulations showed super case II transport.

Mixture experiments are a special class of response surface experiments in which the product under investigation is made up of several components or ingredients. Designs for these experiments are useful because many product design and development activities in industrial situations involve formulations or mixtures (16). Minitab provides simplex centroid, simplex lattice, and extreme vertices designs for mixture experiments. Specifically, extreme vertices design was used when components had upper and lower limits. There were no constraints for the total amount of polymer mixture and augmented, degree 2 simplex lattice design had adequate coverage of the experimental region of interest as shown schematically in Figure 1. The total amount of three different grades of HPMC (K100, K4M and K15M) was fixed at 143 mg (22%) in the formulations. HPMC K100, HPMC K4M and HPMC K15 M (given as X_1, X_2, and X_3, respectively) were chosen as inter-dependent variables. Each point in Figure 1 indicates a formulation with different amounts of polymers. For example, the tips of the triangle represent the maximum amounts per grade of polymers and the center point represents the mixture of equally blended amounts of polymers. Cumulative drug release at 8th h (CDR 8th) was chosen as dependent variable (Y = response). The release results were statistically analyzed by using stepwise-analyze method as a function of Minitab

Table 4. Results of model fitting of drug release.

Formulation	Korsmeyer – Peppas	
	R^2	n
F 1	0.917	0.947
F 2	0.993	1.243
F 3	0.987	1.118
F 4	0.993	1.074
F 5	0.991	1.358
F 6	0.988	1.271
F 7	0.986	1.529
F 8	0.992	1.349
F 9	0.986	1.289
F 10	0.987	1.374
OF	0.982	1.068

Table 5. Summary of regression analyses for response (CDR 8th).

Terms	Coefficients	SE Coefficients	t	p	VIF
HPMC K100	108.11	2.118	*	*	2.057
HPMC K4M	64.11	1.922	*	*	1.694
HPMC K15M	56.19	1.845	*	*	1.561
HPMC K100 × HPMC K4M	-35.31	9.536	-3.70	0.021	1.982
HPMC K100 × HPMC K15M	-19.95	9.579	-2.08	0.106	2.000
HPMC K100 × HPMC K4M × HPMC K15M	-73.22	30.049	-2.44	0.071	1.21

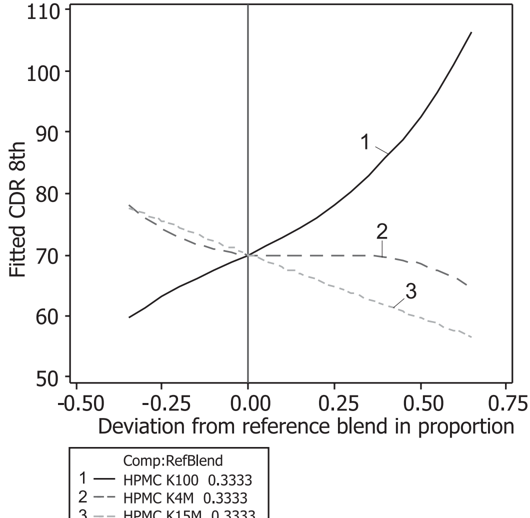

Figure 4. Cox response trace plot indicating the influence of the polymers on CDR 8th

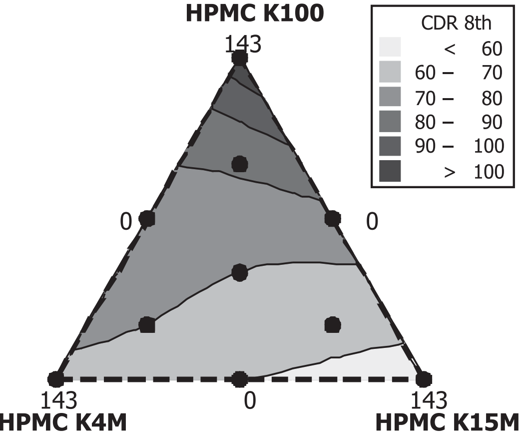

Figure 5. Mixture contour plot indicating the influence of the polymers on CDR 8th

16® software. The obtained model equation, unexplained variation (S), R-square (R-sq) and R-square adjusted (R-Sq adj.) given by Minitab 16® software are shown below:

Y (CDR 8th) = 108.11 × HPMC K100 + 64.11 × HPMC K4M + 56.19 × HPMC K4M – 35.31 × HPMC K100 × HPMC K4M – 19.95 × HPMC K100 × HPMC K15M – 73.22 × HPMC K100 × HPMC K4M × HPMC K15M

S = 2.1453; R-sq = 99.09%; R-sq (adj.) =: 97.94%

Both R-sq and Rs (adj.) values indicate that the model fits the data well. R-sq is generally considered as minimum 70% (28), however, in our study, we obtained R-sq as 99.09% which meant 99.09% of the total variation could be explained by the model and the model was reliable. Unexplained variation of the model was 2.1453 and this was low enough.

It was found that the interaction of HPMC K100 and HPMC K4M was statistically significant for CDR 8th because the *p*-value of the interaction of HPMC K100 and HPMC K4M was lower than 0.05, shown in Table 5 (16, 29).

VIF indicates the extent to which multicollinearity (correlation among predictors) is present in a regression analysis; n regression, multicollinearity refers to predictors that are correlated with other predictors. Moderate multicollinearity may not be problematic. However, severe multicollinearity is problematic because it can increase the variance of the regression coefficients, making them unstable and difficult to interpret (16).

Variance inflation factors (VIF) measure how much the variance of the estimated regression coefficients are inflated as compared to when the predictor variables are not linearly related. The following guideline is used to interpret the VIF (16):

For VIF = 1 predictors are not correlated, for 1< VIF <5 are moderately correlated and for VIF > 5 to10 they are highly correlated.

As presented in Table 5, VIF values of all factors were obtained below 5 (2.057 for HPMC K100M, 1.694 for HPMC K4M, 1.561 for HPMC K15M, 1.982 for interaction of HPMC K100M and HPMC K4M, 2.000 for interaction of HPMC K100M and HPMC K15M and 1.211 for interaction of three components). It signifies that all factors showed moderate multicollinearity, which was not considered to increase the variance of regression coefficients. The standard error of coefficient (SE coef.) is the standard deviation of the estimate of a regression coefficient. It measures how precisely the data can estimate the coefficient's unknown value. Its value is always positive, and smaller values indicate a more precise estimate (16); 't-value' measures the difference between an observed statistic and its hypothesized population parameter in units of standard error. A t-test compares this observed "t-value" to a critical value on the t-distribution with (n – 1) degrees of freedom, to determine whether the difference between the estimated and hypothesized values of the population parameter is statistically significant (16). Correspondences of t-values were presented as p-values, which indicate whether the factor is significant or not.

Table 6. Target limit, upper limit, lower limit, and desirability results to reach target response for CDR 8th.

Formulation	Composition		Composite desirability	Target (CDR 8 th)	Predicted value	Actual value	Variation
Optimized formula	K100	91.13%	0.9889%	80%	9.98%	79.4%	0.58%
	K4 M	31.36%					
	K15 M	20.51%					

Table 7. Comparative dissolution results of optimized formula using well diffusion agar assay and spectrophotometric method.

Analytical method	Cumulative drug release (%; n = 6)				
	1st hour ± %RSD	2nd hour ± %RSD	4th hour ± %RSD	6th hour ± %RSD	8th hour ± %RSD
Well diffusion agar method	6.3 ± 7.74	12.3 ± 3.95	22.1 ± 1.69	41.5 ± 0.54	76.3 ± 1.99
Spectrophotometric method	8.1 ± 14.2	23.4 ± 5.7	42.8 ± 5.9	61.6 ± 5.9	79.4 ± 7.9

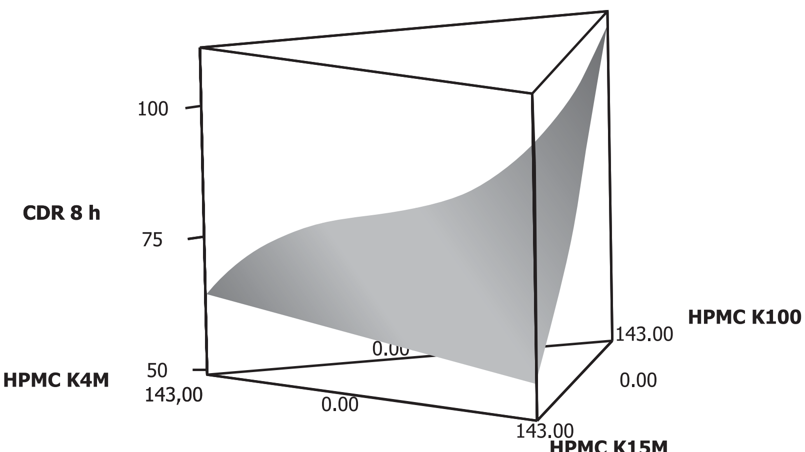

Figure 6. Mixture surface plot indicating the influence of the polymers on CDR 8th

The coefficients of three interdependent variables were 108.11 for HPMC K100, 64.11 for HPMC K4M, and 56.19 for HPMC K15M indicating that HPMC K100 had the highest impact on dissolution. Negative coefficients indicated that two components were antagonistic towards one another. That meant that the mean acceptance score of mixture was lower than that of the one, which was obtained by calculating the simple mean of two acceptance scores (16).

Cox response trace plot (Fig. 4) shows the effect of each polymer on response reference to relative blend. If relative proportion of HPMC K100 (curve no 1, Fig. 4) increases in the mixture (and the other polymer proportions decrease), the CDR 8th increases. HPMC K100 had the steepest response trace and showed greatest effect on dissolution. If relative proportion of HPMC K4M (curve no. 2, Fig. 4) was increased in the mixture (and the other polymer proportions were decreased), the CDR 8th slightly decreased, remained horizontal and again decreased. The horizontal part of the trace for HPMC K4M indicated that there was no significant effect on response between these concentrations. HPMC K4M which had the shortest response trace was used with smaller ranges. If relative proportion of HPMC K15M (curve no. 3, Fig. 4) was increased in the mixture (and the other polymer proportions were decreased), the CDR 8th linearly decreased. According to Cox response trace plot (Fig. 4) it

could be concluded that while HPMC K100 had positive effect on CDR 8th, HPMC K4M and HPMC K15M had negative effect on CDR 8th.

The mixture contour plot, shown in Figure 5, provided a two-dimensional view where all points had the same response in the same shade regions connected with contour lines (Fig. 5). Mixture surface plot, shown in Figure 6, provided a three-dimensional view supplying a clearer picture of the surface. The area with highest release was located on the HPMC K100 edge of the plots both in mixture surface and contour plots (Figs. 5, 6). It was easy to visualize that CDR 8th was at peak value when mixture contained maximal amount of HPMC K100 and, in addition, CDR 8th had the lowest value when mixture contained maximal amounts of HPMC K15M. According to the obtained model (mentioned as an equation) from the statistical analysis, the optimized formulation, which was considered to have the best values of polymers, was generated by design optimizer tool of Minitab 16® software. In design optimizer, to obtain the target response for CDR 8th, desirability was considered to be close to '1'. The target limit, upper limit, lower limit, and desirability were set as 79.98%, 82%, 78% and 0.9889, respectively, in design optimizer tool (Table 6). Optimized formulation was evaluated to validate the ability of the model and so the CDR 8th value, which was presented as actual value in Table 6 (79.43%), was compared with predicted value 79.98%. The variation was found lower than 1.0% as shown in Table 6. A good agreement was shown between predicted and actual values of CDR 8th. By this way the release profile of the optimized formula was verified.

Since CLA is an antibiotic, inhibition activity of CLA has to be shown against *H. pylori*. The CDR results of withdrawn samples at various times intervals were calculated fitting the measured inhibition zone diameters in calibration curve. CDR results of optimized formulation, which were determined by well diffusion agar assay and comparison of average CDR values between spectrophotometric and well diffusion agar assay are presented in Table 7. The antimicrobial effectiveness of the CLA contained DDS was proved by the inhibition zones in mediums. It was observed that a slight difference occurred for CDR at the 1st and 8th h. In addition, a significant difference occurred for CDR of the 2nd, 4th and 6th h. This difference can be explained by logarithmical transformation of concentrations and as a result of the fact that even minor measurement differences of inhibition zones determined by calipers had a huge impact on calculations.

CONCLUSION

A promising formulation of gastro-resistant effervescent floating drug delivery system of clarithromycin could be developed by mixture design methodology using gel forming agents (hypromellose), gas generating agents, sodium bicarbonate and citric acid. Different grades of HPMC (K100, K4M and K15M) provide reduction on release ratio of the CLA. The values of 15.08% of sodium bicarbonate and 2% of citric acid were found to be sufficient to provide FLT lower than 30 s and TFT more than 8 h. Desired dissolution ratio was achieved generating a suitable formulation using statistical software Minitab 16®. The antibiotic efficiency of optimized formula was proved by well diffusion agar assay as well.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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PHARMACOLOGY

EFFECTS OF ANTIDEPRESSANTS AND SOYBEAN ASSOCIATION IN DEPRESSIVE MENOPAUSAL WOMEN

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Abstract: Depression in menopausal women has been widely described for many years ago, and is related to hormonal decrease, mainly estrogens. The use of soy has been proposed as a possible coadjutant alternative to treat menopausal depressive disorder. In the present pilot clinical trial the effect of soybean, antidepressants and the association of soybean with antidepressants was studied in 40 depressive menopausal women for three months. Patients were divided in four groups of 10 women: fluoxetine (10 mg), soybean (100 mg), sertraline (50 mg), and sertraline (50 mg) plus soybean (100 mg). The Hamilton and Zung Depression Scales were used to measure the treatment effects. Values at the beginning and at the end of the study were compared. In all cases a significant difference was observed when the treated groups were compared *vs.* their untreated situation in both scales ($p < 0.001$). When a comparison between pre- minus post-treatment Zung scale scores was done, the effect induced by the association of sertraline and soybean was significantly higher than the other groups ($p < 0.05$). These effects were also seen using the Hamilton scale scores, showing significant differences between the association *vs.* soybean ($p < 0.05$) and setraline ($p < 0.05$) groups, but not *vs.* fluoxetine group. We conclude that soybean has an antidepressant effect *per se*, and the association of soybean and antidepressants increases their effects.

Keywords: menopause, depressive disorder, soybean, antidepressant, serotonin receptor inhibitor antidepressants

Menopause is a word developed from Greek roots, meaning cessation (pauses) and month (men), designing the interruption of the cyclic monthly blood flow named menstruation, corresponding to the end of fertility. This state is retrospectively fixed, after the absence of menstruation for 12 consecutive months. Technically, it is due to the loss of ovulation, and is linked to structural and functional modifications in the reproductive axis (1).

In menopause, several features valued by society, such as youth and the ability to procreate, disappear. The loss of these features can be considered psychological factors, which may predispose a woman to a depressive disorder, and the hormonal changes may contribute to these disorders, suggesting a multifactorial etiology (2). Menopausal women are at an increased risk of developing osteoporosis (3), cardiovascular disease (4) and depressive disorders (5, 6). Actually, depressive disorders and menopause are closely related (2). Due to the fact that these modifications in hormonal levels are conditioning the psychological symptoms, it could be considered that present symptoms could be included in psychotical group in the schneiderian criteria (7). The ethiological treatment could be the hormone replaces treatment (8), but the risk of breast and endometrium cancer related to hormone replace treatment has been reported, and natural products have been suggested as a potential alternative (9).

In previous reports, the effects of treating menopause with soybean have been a matter of dis-

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cussion (9). The purpose of this study is to compare the effect of selective serotonin receptor inhibitor antidepressants (SSRI) alone, soybean alone and combination of SSRI antidepressants with soybean.

SUBJECTS AND METHODS

The sample selection was carried out with the informed consent and commitment of psychiatric patients who have been diagnosed with depression, at ages of 45 to 55, in menopause state, willing to participate in the study. Patients were referred to the psychiatric consultation from other medical specialties consultations including endocrinology, gynecology and cardiology in which depression was observed. Forty patients were chosen and were randomly divided into four groups; each group consisted of 10 patients. Because of the ethical requirements, they were informed about the experimental conditions, but not any detail was suministered about medication, blinding to them the probe.

Two depression scales were used: the Zung Self-Rating Scale (10) and the Hamilton Rating Scale for Depression (HAMD) (11). Data are referred in comparison to their corresponding percentualized baselines (means considered as 100%). This 100% corresponded to a mean of baselines of 62.50 points for the Zung scale and 19.70 for the Hamilton scale.

A pilot prospective longitudinal clinical study was performed, in which 40 menopausal depressive women between 45 to 55 years old, treated in private psychiatry practice in Santo Domingo, Dominican Republic, were included. The study was conducted during three months. Psychological tests were administered at the beginning and at the end of the three months period, for all 40 women throughout the year, which aimed to compare the scores and evaluate the results. Women were randomly assigned to 4 groups of 10 patients each. The first group received fluoxetine (10 mg). The second group received soy (100 mg daily, soy isoflavones concentrate, 50 mg, GNC Laboratories, USA). The third group received sertraline (50 mg daily). The fourth group received soy (100 mg) and sertraline (50 mg). No differences were observed between groups in age, body weight or estrogen levels. In a few cases, a low dose of hypnotics was occasionally used (alprazolam, 0.5 mg or mesazolam, 1 mg). During the three months period, patients were observed every 21 days, evaluating their treatment courses. At the end of the study, the same scales were repeated.

RESULTS

A statistical difference was observed in the treatment effects of fluoxetine (10 mg), soybean, sertraline (50 mg), and sertraline (50 mg) plus soybean on Zung (top) and Hamilton (bottom) scales evaluating pre-treatment and post-treatment scores. The four groups were compared *vs.* their previous pre-treatment scores. In the case of the Zung depression scale, ANOVA 1 showed significant intergroup differences ($F_{7,79} = 24.06$, $p < 0.0001$), and Newman-Keuls test revealed significant differences between pre- and post-treatment in all groups ($p < 0.001$). The Hamilton Depression Scale also showed statistical differences between groups (ANOVA 1, $F_{7,79} = 31.73$, $p < 0.0001$), and statistically significant differences were observed between pre- and post-treatment conditions in all groups (Newman-Keuls, $p < 0.001$).

The difference levels comparison (pre- *versus* post-treatment scores) induced by fluoxetine (10 mg), soybean, sertraline (50 mg), and sertraline (50 mg) plus soybean on Zung (top) and Hamilton (bottom) scales showed statistical differences. The differences in the scores of the four groups of ten patients each were compared. In the case of Zung depression scale, ANOVA 1 showed significant intergroup differences ($F_{3,39} = 3.911$, $p < 0.005$), and Newman-Keuls revealed significant differences between sertraline (50 mg) plus soybean and the other groups (Newman-Keuls, $p < 0.05$). The Hamilton Depression Scale also showed statistical differences between groups (ANOVA 1, $F_{3,39} = 3.716$, $p < 0.005$), and statistically significant differences were observed between sertraline (50 mg) plus soybean and soybean and sertraline (50 mg) groups (Newman-Keuls, $p < 0.05$).

DISCUSSION

Present findings show an important treatment effect in all groups. Significant difference between pre-treatment and post-treatment scores were observed in all groups using both scales (Fig. 1). The fact that low doses had a very significant effect suggests a very sensitive population in these conditions. It must be noted that one scale was evaluated by the professional and the other by the patients. Responses coincided and were statistically significant ($p < 0.001$). It is a strong argument about the reliability of present conditions. In all cases a clear effect was observed after treatment.

When different levels (pre- minus post-treatment scores) were compared, the association of ser-

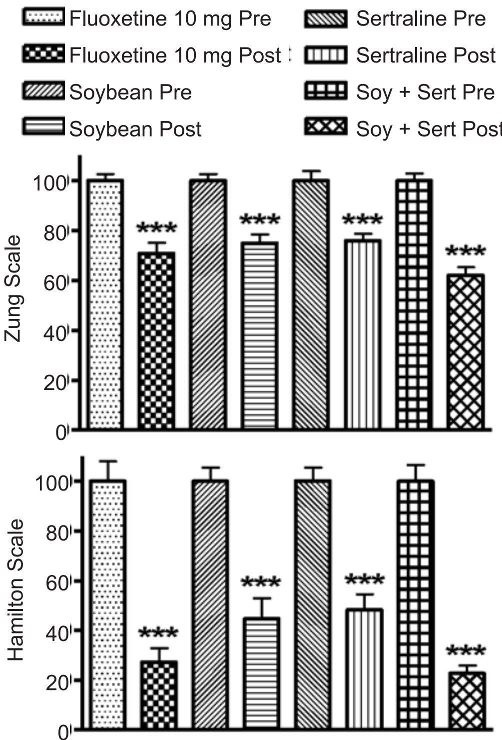

Figure 1. Comparison of treatment effects of fluoxetine (10 mg), soybean, sertraline (50 mg), and sertraline (50 mg) plus soybean on Zung (top) and Hamilton (bottom) scales evaluating pre-treatment and post-treatment scores. Four groups of 10 patients compared each one were compared *vs.* their previous pre-treatment scores. In the case of Zung depression scale, ANOVA 1 showed significant intergroup differences ($F_{7,79} = 24.06$, $p < 0.0001$), and Newman-Keuls revealed significant differences between pre- and post-treatment in all cases ($p < 0.001$). The Hamilton Depression Scale showed also statistical differences between groups (ANOVA 1, $F_{7,79} = 31.73$, $p < 0.0001$), and statistically significant differences were observed between pre- and post- treatment conditions in all cases (Newman-Keuls, $p < 0.001$). Data are presented as the mean ± standard error of the mean (SEM). *** = $p < 0.001$

traline and soybean showed a significant difference with the other groups in the Zung scale (Fig. 2, top), which is auto administered. This fact provides an idea that the subjective perception of improvement was very clear, showing a very positive treatment effect impression with the association of soybean and sertraline. It could be considered as a potentiation phenomenon.

The scores observed in the Hamilton scale (Fig. 2, bottom) showed a clear difference between soybean alone and sertraline alone *vs.* their coadministration. It also strongly suggests a potentiation phenomenon. The fact that the difference with fluoxetine group did not reach significance could be suggesting a lower potency difference with sertraline in this group of patients. However, patients'

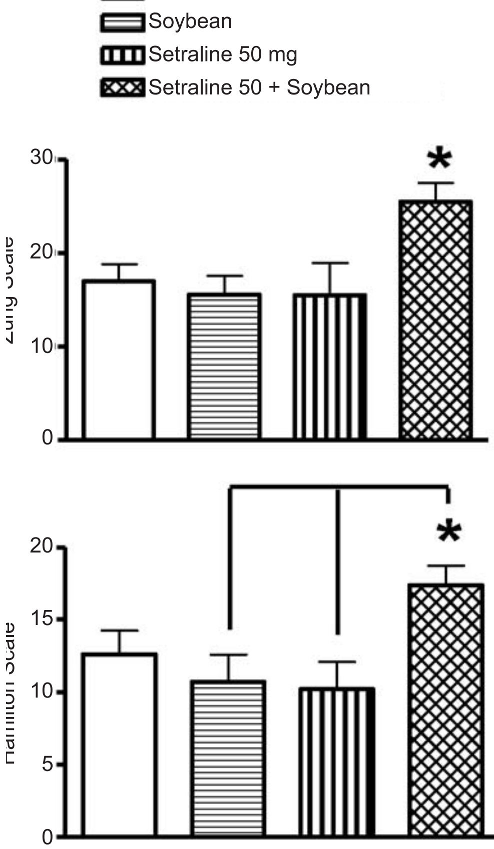

Figure 2. Comparison of the difference levels (pre- minus post-treatment scores) induced by fluoxetine (10 mg), soybean, sertraline (50 mg), and sertraline (50 mg) plus soybean on Zung (top) and Hamilton (bottom) scales. The score differences of the four groups of 10 patients each one were compared. In the case of Zung depression scale, ANOVA 1 showed significant intergroup differences ($F_{3,39} = 3.911$, $p < 0.005$), and Newman-Keuls revealed significant differences between sertraline (50 mg) plus soybean and the other groups (Newman-Keuls, $p < 0.05$). The Hamilton Depression Scale showed also statistical differences between groups (ANOVA 1, $F_{3,39} = 3.716$, $p < 0.005$), and statistically significant differences were observed between sertraline (50 mg) plus soybean and soybean and sertraline (50 mg) groups (Newman-Keuls, $p < 0.05$). Data are presented as the mean ± standard error of the mean (SEM). * = $p < 0.05$

perception (Zung scale) maintains the idea that a significant difference actually exists.

The action of antidepressant drugs is not immediate, and a latency period is necessary to induce changes that involve integrated mechanisms, including noradrenergic and serotoninergic transmission (12–14). The final effect appears to be mediated by a decrease in the number of β-adrenergic receptors and activity, measured by norepinephrine (NE) mediated stimulation of adenylate cyclase

(15). This effect appears to be induced by an increase in the synaptic concentration of NE due to a decrease of á2-receptor sensitivity. It has been postulated that this decremental sensitivity modification of α2 adrenergic autoreceptors is the first change induced by antidepressant action (14, 16). The simultaneous administration of antidepressants and α2 antagonists decrease the treatment latency in experimental approaches (14).

The role of serotoninergic neurons appears to be related to a tonic inhibition exerted on noradrenergic neurons (13). Imipramine has recognition sites on serotoninergic neurons, and desipramine in noradrenergic sites (13). However, desipramine is a metabolite of imipramine (17). The antidepressant drugs here used are all SSRIs, acting all through serotonin neurons, and a combination of antidepressants appears to be more effective than the separate exclusive use of them (18).

The temporal sequence of changes strongly suggests the involvement of integrated mechanisms, mainly noradrenergic and serotonergic (13). The antidepressant drugs here used in the study are all SSRIs, acting all through serotonin neurons, and a combination of antidepressants appears to be more effective than the separate exclusive use of them (19). Soybean extract appears to be potentiating the effect of antidepressants here used.

As previously stated, depressive disorders in menopause have been largely clinically observed (2), and hormones decrease induces important symptoms (20). Menopausal symptoms improve after hormone replacement, with the risk of relapse after cessation of hormone replacement therapy (21, 22). Clinically, it has been reported that antidepressants alone do not ensure success in treatment of depresive disorders in menopausic women (2). Estradiol alone (transdermal estradiol replacement) has a significant antidepressive effect in perimenopausic depression (23). Recently, the effect of addition of raloxifene, a selective estrogen receptor modulator (SERM) to SSRI gave satisfactory effects inducing complete remission in a postmenopausal depressive disorder (24).

The use of some treatment alternatives to hormone replacement therapy for hot flashes in breast cancer survivors has been proposed (25, 26), including soy phytoestrogens (20, 27). Soy and social stress affect serotonin neurotransmission in primates (19). The use of soy-derived isoflavones has been proposed as a protective factor against depression starting from basic translational approaches (28). A relevant stimulatory effect of phytoestrogens on noradrenaline and serotonin transporters activity has been reported (29). The effect of glutamatergic stimulation on sexual behaviors in rats appears to be driven by an induction of central adrenergic receptor prevalence modifications exerted by sexual hormones (30–35), similar to those induced by antidepressants. In fact, it has been reported that ovarian steroids induce modifications in noradrenergic and serotoninergic receptors in the rat brain (36, 37). Furhermore, estradiol has shown a synergistic antidepressant effect with fluoxetine in animal studies of experimental depression (38).

Some side effects have been reported related to soy administration, mainly thyroid dysfunctions like goiter in infants (39). However, in adult menopausal women the soybean administration seems not to affect thyroid function (39). In adults, the most relevant problem is soy allergy (40), but benefits for treating menopausal depression, as it has been shown here, outweighs side effects of soybean administration.

In the present clinical schedule, SSRI antidepressants are acting on serotoninergic transmission. The action of soybean, as homologous equivalent of hormonal replacement, could be influencing noradrenergic transmission, the final pathway. Since SSRI antidepressants are acting on serotoninergic system and the soybean active principles could be acting in noradrenergic system, the potentiation here observed could be explained by a synergistic action of both treatments. Present results give more evidences regarding the action of hormones and menopausic depressive disorders.

We conclude that the administration of soybean could enhance the response to SSRI antidepressants in menopausal women, and that soybean could act as an interesting alternative to estrogens in the treatment of mood disorders during menopause.

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GENERAL

CONSUMPTION OF OSTEOANABOLIC DRUGS AND STRONTIUM RANELATE IN THE TREATMENT OF OSTEOPOROSIS IN THE CZECH REPUBLIC IN 2005–2011

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Abstract: Anti-osteoporosis drugs with osteoanabolic (teriparatide, intact parathormone) and dual (strontium ranelate) mechanism of action are currently available for the treatment of postmenopausal, glucocorticoid induced or male osteoporosis in the Czech Republic (CZ). These expensive drugs are subjects of special prescribing limitations (2nd line treatments). The objective was to analyze trends in consumption of osteoanabolic and dual drugs in the treatment of osteoporosis since their introduction onto the market in CZ (2005–2011). The prescription-based database of the General Health Insurance Company of the Czech Republic that covers approximately 60% of the Czech population was used as the data source. An insured person with a recorded prescription for teriparatide (TRPD), intact parathormone (iPTH) or strontium ranelate (SR) in the period of interest was defined as a patient; 271 (224), 77 (75) and 5930 (5545) patients (women) treated with TRPD, iPTH and SR in 2011, respectively, were identified. The median age of patients on TRPD and SR ranged from 71 to 74 years in 2006–2011. The number of patients treated with TRPD between 2009 and 2011 has been stable, while in iPTH the number increased 2.8 times in the same time period. The number of patients treated with SR has been steadily rising since its introduction in 2005. SR was prescribed most often by physicians specialized in internal medicine (42%) and rheumatology (25%). Male patients accounted for 6% of the SR consumers in 2011. The consumption of dual and osteoanabolic drugs has been rapidly increasing. Consumption rates in men (both absolute and relative) have been increasing but still remain relatively low.

Keywords: drug consumption, drug prescription, osteoporosis, strontium ranelate, teriparatide, intact parathormone, osteoanabolic drugs, prescription-based database

Prevalence of osteoporosis (OP) has been significantly increasing together with aging of the population. In the Czech Republic (CZ), OP affects more than 400,000 women and 200,000 men. Prevalence of OP in CZ female and male population aged 50 or more amounts to 20.4% and 6%, respectively. Expenditures related to OP form an essential part of health-care budgets due to rising costs associated with low-trauma fractures. The age-standardized hip fracture rates (100,000/year) in CZ in 2010 was 374 for women and 211 for men. Incidence of hospitalization for hip fractures more than doubled in CZ from 1981 to 2009. During 1997–2007, the average year-to-year increase in the number of hip fractures in CZ has been estimated as 5.9% (1–3). Despite advances in diagnosis and therapy (pharmacotherapy in particular), inadequate treatment or undertreatment of the vast majority of patients suffering from OP still remains a concern.

In CZ, where there is virtually no private health care market, conditions of drug reimbursement play a decisive role in treatment algorithms. The so-called prescription and indication restrictions are now determined for each drug individually, with regard to, among other issues, pharmaco-economic aspects of treatment. Still, there is a lack of clear information on how great parts of the public funds' costs for pharmacotherapy in OP are consumed by different groups of patients. There are also missing data quantifying drug prescription and its trends over time. Calculation of costs associated with OP in CZ was last published in 1998 (4).

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Considering pharmacotherapy, the major burden is represented by new costly drugs that should be strictly regulated with respect to limited public health insurance budgets. In CZ, drugs with osteoanabolic and dual effect are authorized for use in postmenopausal, male and glucocorticoid-induced osteoporosis (Table 1) but due to their reimbursement conditions, they are reserved as a second-line treatment and/or for patients with severe osteoporosis (Table 2) as well (5, 6).

Good decision practices of regulatory authorities are subject to (and depend on) targeted analyses and assessment of relevant health-economic data based both on the total cost of the disease and also

Table 1. Authorized indications of teriparatide, intact parathormone and strontium ranelate in the Czech Republic (CZ) in 2010 (13).

Drug (brand name, marketing authorization holder)	Year of marketing authorization (launch) in EU (in CZ)		Current indications
Teriparatide (Forsteo, Eli Lilly)	2003	(2005)	postmenopausal, glucocorticoid-induced and male osteoporosis
Intact parathormone (Preotact, Nycomed)	2006	(2007)	postmenopausal osteoporosis
Strontium ranelate (Protelos, Servier)	2004	(2005)	postmenopausal and male osteoporosis

Table 2. Reimbursement criteria (restrictions) of teriparatide, intact parathormone and strontium ranelate in the Czech Republic (CZ) in 2011 (13).

<table>
<tr><th rowspan="2">Drug</th><th rowspan="2">Prescription restrictions</th><th colspan="2">Indication restrictions</th></tr>
<tr><th>Criteria</th><th>T-score</th></tr>
<tr><td rowspan="3">Teriparatide</td><td rowspan="3">Centres only*
Indication committee approval</td><td>Postmenopausal osteoporosis
failure of well documented antiresorptive therapy (2 years at least) + 2 vertebral fractures at least</td><td>≤ –3.0 SD
(lumbar spine)</td></tr>
<tr><td>Glucocorticoid-induced osteoporosis
= 5 mg/day prednisone or equivalent for at least 6 months</td><td>≤ –2.5 SD
(lumbar spine)</td></tr>
<tr><td>Male osteoporosis
failure of well documented antiresorptive therapy (2 years at least) + 2 vertebral fractures at least</td><td>≤ –3.0 SD
(lumbar spine)</td></tr>
<tr><td>Intact parathormone</td><td>Centres only*
Indication committee approval</td><td>Failure of well documented antiresorptive therapy (2 years at least) + 2 vertebral fractures at least</td><td>≤ –3.0 SD in lumbar spine</td></tr>
<tr><td>Strontium ranelate</td><td>Selected specialists only**</td><td>Patient should present with
a) fracture, or
b) contraindication of bisphosphonates or raloxifene, or
c) intolerance or serious adverse effects on antiresorptives</td><td>≤ –2.5 SD
(lumbar spine, proximal femur or potentially distal radius)</td></tr>
</table>

*12 centres in CZ are allowed to prescribe parathormones. **Physicians trained in rheumatology, orthopedics, traumatology, internal medicine, gynecology, endocrinology; treatment ≥ 2 years only if termination of bone loss is clearly demonstrated; prescription may be delegated for 1 year (prolonged only after reference assessment of the above mentioned specialist).

Table 3. Teriparatide consumption in years 2005–2011.

Year	Total number of patients (N)	Age: median (5–95% percentile)	Females (%)
2005	1	81	100.0
2006	79	73.0 (56.1–82.0)	100.0
2007	152	73.0 (55.5–82.7)	100.0
2008	237	73.6 (55.1–83.7)	96.2
2009	333	74.3 (53.5–84.3)	93.1
2010	312	70.6 (48.3–84.4)	89.8
2011	271	69.5 (39.4-85.2)	82.7

Table 4. Intact parathormone consumption in years 2005–2011.

Year	Total number of patients (N)	Age: median (5–95% percentile)	Females (%)
2009	28	76.8 (64.9–86.5)	100.0
2010	64	76.9 (64.6–87.0)	98.4
2011	77	75.4 (62.0–84.7)	97.4

*intact parathormone has been available in the Czech Republic since 2009

Table 5. Strontium ranelate consumption in years 2005–2011.

Year	Total number of patients (N)	Age: median (5–95% percentile)	Females (%)
2005	598	70.0 (53.9–82.0)	97.8
2006	2223	71.0 (53.0–83.0)	98.5
2007	3004	77.1 (60.0–89.0)	95.1
2008	3655	75.9 (59.3–88.6)	93.5
2009	4845	75.4 (57.8–88.0)	93.2
2010	5497	74.4 (57.6–87.3)	93.7
2011	5930	73.4 (56.8–86.5)	93.5

structure of the expenditures. Our ultimate goal is to calculate costs related to OP treatment. The objective of this preliminary analysis was to analyze trends in consumption of osteoanabolic (teriparatide and intact parathormone) and dual (strontium ranelate) drugs in the treatment of osteoporosis since their introduction on the market in CZ (2005–2010).

METHODS

Retrospective observational study based on prescription refills data was performed. Health insurance is compulsory under Czech law. Prescription-based database of the largest health insurance company of CZ, General Health Insurance Company of the Czech Republic (GHIC CR) that covers approximately 65% of the Czech population over 40 years, was analyzed.

An insured person with a recorded at least one package of teriparatide (TRPD), intact parathormone (iPTH) or strontium ranelate (SR) in the year of interest was defined as a patient. Time period from 2005 (launch of TRPD and SR in CZ) to 2011 was analyzed. Only anonymous population data provided directly by GHIC CR, already in an aggregated form, were analyzed, therefore, approval of the scientific ethical committee was not necessary.

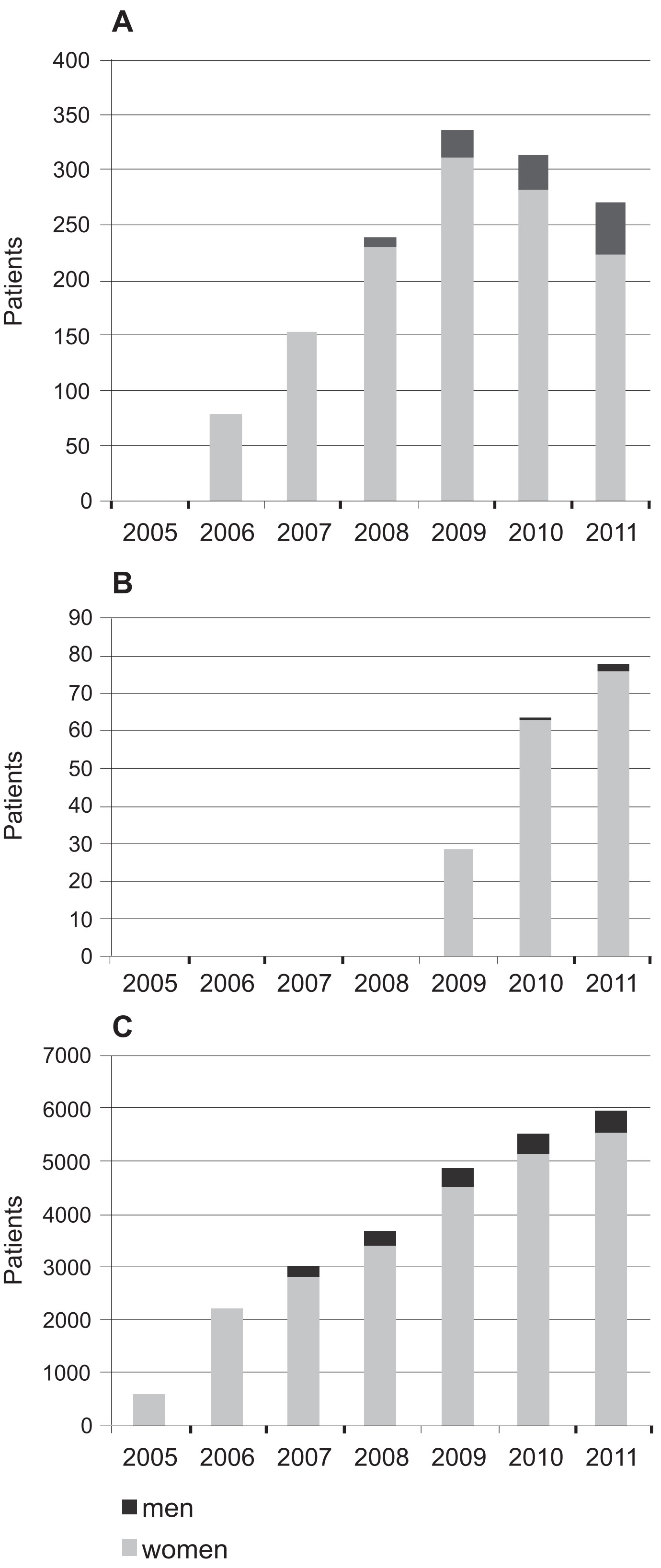

Chart 1. A – Teriparatide. B – Intact parathormone and C – Strontium ranelate consumption in General Health Insurance Company of the Czech Republic

Table 6. Osteoanabolics – cumulative consumption per patient in years 2008–2011.

	Patients (N)	Years	Monthly packages: mean (SD)
Teriparatide	351	2008-2011	16.3 (5.3)
Intact parathormone	28	2009-2011	16.5 (6.7)

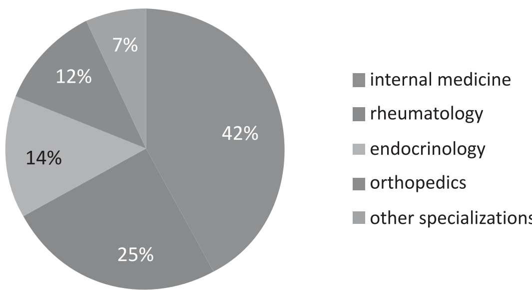

Chart 2. Specializations of doctors prescribing strontium ranelate in General Health Insurance Company of the Czech Republic (2011)

The data collected included age, gender of patient, specialization of the prescriber, time of prescription, trade name, strength and number of packages and price.

RESULTS

Since launch in CZ, the number of patients treated with osteoanabolics and SR has generally been increasing (see Tables 3–5 and Chart 1) in the observed period. The consumption of the two osteoanabolics restricted to specialized centres, however, has been relatively stable since 2009. The overall proportion of men treated with TRPD and SR increased from 2005 to 2011.

Table 6 shows the cumulative consumption per individual patient per therapy course with osteoanabolics.

Chart 2 shows the structure of specializations of physicians who prescribed SR in 2011. This structure of SR prescribers was similar during the whole observed period.

Average differences between the retail prices and reimbursement price (i.e., potential patients' co-payment) were also tracked; the highest co-payment recorded was 72 CZK (around 2.5 EUR) on a monthly package of SR in autumn 2009, which represents 6% of its retail price. For the rest of the analyzed time period all three drugs had co-payments lower or none.

DISCUSSION

The expenditures on osteoanabolic and dual drugs have been rapidly increasing since their introduction on the market in CZ. All three drugs (TRPD, iPTH, and SR) are part of the Czech OP treatment guidelines, all are reimbursed from the public health insurance funds and none of them have any other indications besides OP. There has also been no competition on the market in terms of generic or biosimilar drugs. The present population analysis of OP drug consumption is among the first in CZ.

The increasing trend of osteoanabolics (TRPD and iPTH) consumption seems to reach a plateau in 2009 (consumption rate in 2010 similar to that in 2009). Also an increase in the proportion of men treated with TRPD and SR has been observed. This is in line with the extension of indications for these two drugs and reflects greater emphasis placed on male OP in the last years. Increasing numbers of male patients were found also in our previous study that evaluated bisphosphonate consumption in 2002–2006 (7).

In accordance with the reimbursement criteria, namely "prescription restrictions", outpatient internists, followed by rheumatologists, endocrinologists and orthopedic surgeons contributed most significantly to the prescription of SR. Osteoanabolics (TRPD and iPTH), drugs administered parenterally, are very expensive treatment options; their single daily dose is on average 10 times more expensive than SR. Osteoanabolics also may be prescribed only in specialized centers (12 selected osteocenters in CZ). These aspects probably account for the fact that their use is not very widespread and their consumption reached the plateau relatively soon.

In our previous study we identified that annually around 40 thousand patients of GHIC CR (e.g., 40.397 in 2005) were treated with oral bisphosphonates alendronate and risedronate indicated only for OP. As much as 98% of the patients were women (7). Confronting this with the estimates on the overall OP Czech population of 400,000 women (adjusted to the GHIC CR market share of 65% in CZ) led

us to the estimate that only around 15% of potential OP patients (women) are treated with bisphosphonates in CZ. However, according to the national guideline, the first line intervention is calcium and cholecalciferol supplementation, which is available and reimbursed without any prescription conditions. Considering the three osteoanabolic and dual drugs (iPTH, TRPD, and SR) analyzed in the present study, their indication criteria position them in the next therapeutic line clearly after bisphosphonates (and raloxifen): they are indicated (and reimbursed) in case of contraindication, well-documented intolerance or treatment failure (development of osteoporotic fracture in the course of treatment) of antiresorptives. Therefore, it is not unexpected that the observed patient numbers for SR, iPTH and TRPD are significantly lower compared to the previously mentioned bisphosphonates. Even though SR has not appeared to reach its consumption peak yet, in 2011 (the highest number so far) around 6 thousand patients were treated, which is a mere 2% of the overall estimate on OP female population. Given the patient numbers of the two specialized centers only-resctricted osteoanabolics (TRPD and iPTH), their relative proportion in the overall OP treatment is even much lower than that of SR.

The two osteoanabolics (TRPD and iPTH) have a specific: unlike most, if not all, other drugs used in chronic conditions such as OP, these drugs have clearly stated the length of the therapy in their Summaries of Product Characteristics (SmPC): it is 24 months in both cases. This defined period, originally 18 months (until February 2009), is not as restrictive in the case of SmPC of parathormone as in that of teriparatide. Nevertheless, this therapy length for both drugs is naturally reflected also in the official Czech OP treatment guidelines (5, 6). The health insurance accounting in CZ does not enforce specifying the precise date of treatment administration by the providers; however, both drugs were charged for a monthly package in a regular interval for each patient. Detailed analysis (not shown) revealed that the cumulative accounting (i.e., a number of monthly packages) clearly corresponds and agrees well with the duration of treatment. Currently, we are not aware of any other study evaluating the real-world therapy length. Our data showing the average over 16 monthly packages per individual therapy course suggest the therapy is either shorter or less intensive than according to the SmPCs. The reasons could be the non-adherence (non-compliance) of the physician to the SmPC and guideline, and the non-adherence of the patient to the treatment schedule. Also responsible may be for instance patient's acute new or exacerbated comorbidity that precludes further OP treatment either due to its seriousness (such as life-threatening disease or event) or due to the appearance of new contraindications for the ongoing OP treatment making it to be stopped. Also the real-world tolerability of the drugs certainly may play a role in shortening the treatment course from the optimal as described in the guidelines and SmPCs. Therefore, our finding should be interpreted with caution as the underlying reasons may be multiple and we do not have data on their relative importance. Certainly, it is a topic for longer follow-up and further research.

A potential barrier in access to a new drug could be a significant co-payment paid by patients which makes the drug less attractive. However, this is not the case for any of the three drugs, as they (TRPD, iPTH and SR) have been virtually fully reimbursed already since their launch on the market in CZ. Another barrier, which is common for all new original drugs, may be prescription budget limits placed upon the physicians by the insurance companies. This "limit" essentially means that within each specialization, any single physician's spending (on prescription drugs separately) is benchmarked within a period (year) with all other doctors, as well as his/her spending is compared with the same doctor's spending in different years. Then, those physicians spending overly (often due to prescribing new expensive drugs) are penalized. These mechanisms are complex and regulated by many factors, starting from Czech Ministry of Health's annual decrees, which reflect the economic situation of the health care insurance budgets, and ending in the individual financial contracts between doctors and health insurance companies. Even though these budget restrictions are certainly important and worth mentioning in OP treatment, their deeper analysis, due to their complexity, individuality and irregularity in the long term, extends beyond the scope of our study.

The age of patients treated with TRPD and SR was stable in the observed period (median 71–74 years). In comparison, a study carried out in Slovak population showed that treatment with SR or bisphosphonates was most frequently started in 72 years-aged patients in 2009 (8). In our analysis, we analyzed the consumption of SR from its very introduction onto the market in CZ. In the first year, when all the patients were newly treated with SR, their median age was 70 years. In the following years it increased a little, but the data suggest that the treatment with SR is started at a similar age as in the Slovak population, i.e., in the patient's early seventies. The Slovak analysis reported the age when

treatment was started, studied only female population and calculated data for SR together with bisphosphonates. These facts make precise and valid comparisons impossible.

GHIC CR currently manages health insurance for more than 60% of the insured people (65% in people over 40 years) in CZ and has a major impact on drug policy as well as pricing and reimbursement of various health services including new medical technologies (9). At present, GHIC CR introduces the principles of pharmacoeconomics and health technology assessment into evaluation of medical devices and together with the Ministry of Health (Prague) and the State Institute for Drug Control (Prague) into the assessment and appraisal of pharmaceuticals. The database is considered to be precise, because the data originate in pharmacies claiming drug reimbursement, which is a process regularly audited. Patients can be dispensed their prescribed medicine in any pharmacy in CZ. The data from this database are valid for the pharmacoepidemiological research (10).

Population studies using prescription-based databases have several well-known limitations such as changing of health insurer and medication not captured in the database (11, 12), however, these aspects do not seem relevant to our pilot analysis as switching health insurer is negligible in the higher-age population and also due to the origin of the data in pharmacies being economically interested in reporting their claims completely and precisely to the insurance fund. Also analyzing only part of the population insured by a single company may lead to some bias depending namely on the age and illness structure of the group. However, this may not be the case here, because of the large market share of GHIC CR representing around 60% of the Czech population, and even more in the higher age group with OP concerned in the present study. The relative share of patients according to their age and their gender in GHIC CR has been stable over the years. GHIC CR also has contracts with virtually all health care providers including all specialized centers. Therefore, we believe that the analyzed population is a good representative sample of the overall OP population and their pharmacological treatment, which can be safely extrapolated to virtually all Czech population.

In conclusion, the consumption of osteoanabolic and dual drugs has been rapidly increasing during 2005-2011. Prescribing of expensive osteoanabolic drugs in selected centers based on Indication committee approval is probably well regulated in the last years to avoid extensive consumption growth. Consumption of SR, which is prescribed by specialist doctors with no ties to the approval process, has a tendency to a permanent increase. Consumption rates in men have been increasing but remain still relatively low. Subsequent updated analysis of consumption of other osteoporosis drugs (antiresorptives in particular), along with the calculation of the relevant treatment costs should be undertaken to provide a further, more detailed and economic insight into current OP management.

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A QUESTIONNAIRE BASED SURVEY STUDY FOR THE EVALUATION OF KNOWLEDGE OF PAKISTANI UNIVERSITY TEACHERS REGARDING THEIR AWARENESS ABOUT IBUPROFEN AS AN OVER THE COUNTER ANALGESIC

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Abstract: In recent time, due to convenient availability of number of over the counter (OTC) drugs, patients are able to treat minor ailments by themselves. The self-medicated regimen has lead to certain health problems in all age groups irrespective of their professions. People are usually unaware about the safe use of NSAIDs (non-steroidal anti-inflammatory drugs) and currently there is no study carried out in COMSATS Institute of Information Technology (CIIT), Abbottabad, regarding the choice of faculty members for NSAIDs to relieve pain and their knowledge about its safety and use. A questionnaire based survey was carried out to collect data about the choice of CIIT faculty for a specific NSAID and their cognition related to ibuprofen. Two hundred fifty faculty members (comprising of 53 pharmacy faculty members and 197 faculty members who belonged to other departments) of which 87 were females, took part in this study. Average age of participants was 34.86 ± 9.02 years. Ibuprofen was the drug of choice NSAID among the participants. Four percent participants experienced pain almost every day. Analgesia was the well known indication for ibuprofen (31%) by both the groups and in general more educated and younger participants showed better apprehension related to indications. Sixty one percent participants comprising of non-pharmacy faculty were unaware of any undesirable effects and 79% (comprising of 72% pharmacists and 5% non-pharmacists) were affirmative that ibuprofen had no adverse effects. Fifteen percent participants of department other than pharmacy were not aware of any interactions of ibuprofen. 34% of participants (comprising of 32% non-pharmacists and 2% pharmacists) entrusted their physician for an analgesic. Regardless that many participants suffered from pain almost every day and their drug of choice would be ibuprofen, they had inadequate information related to the safety and use of ibuprofen.

Keywords: ibuprofen, self-medication, knowledge, survey, over the counter

It has been detailed in a previous study that 38% participants consumed OTC drugs. It was observed that many of them used a non-prescription drug (at most one) along with other prescribed medicines. Although OTC oral pain-killers show potential side effects, yet they are most popularly used by a majority of population (1, 2). A cohort-survey analysis conducted among these people indicated higher risk for ulcers compared to non-users of NSAIDs and the risk for ulcers increased with the increased use of NSAIDs. Bleeding as a result of peptic ulcer is commonly caused by intake of NSAIDs. Forty three percent patients used NSAIDs most frequently for muscles and skeletal pain. Elderly people are more prone to damage and adverse effects caused by OTC NSAIDs due to multiple drug regimen and alterations in the pharmacokinetics of drugs due to their age (1–4).

In Pakistan, ibuprofen is manufactured under different brand names, by different pharmaceutical

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manufacturers and is dispensed as an OTC drug as well as a prescription drug. Various ibuprofen strengths for oral use are available. Oral dose of ibuprofen is available in strengths of 200 mg and 400 mg. For children, ibuprofen suspension is available and for topical use it is available as creams and gels. Ibuprofen should not be used by an adult for more than a week without physician's advice (5).

In a previous research on OTC ibuprofen, respondents were asked about their use of medicines that contained ibuprofen as an OTC drug. Among them, most of the respondents had been repeatedly using ibuprofen. One third of the people who used ibuprofen for the first time consulted a physician prior using it (6). Another study (7) focused on the level of information of those who bought OTC ibuprofen. The statistical data of study showed that 38% people who used ibuprofen, never asked any information about it. For those who used it for the first time, their information regarding its undesirable effects and contraindications was 28% compared to that of repeated users of ibuprofen, who were one half of the total. In spite of a lack of information about ibuprofen, a majority of users in both the groups indicated to have sufficient information about ibuprofen.

COMSATS Institute of Information Technology (CIIT), besides its principal campus at Islamabad, has 9 other fully functional campuses in different cities of Pakistan including that at Abbottabad. CIIT has 17 departments and 6 research centers. CIIT ranks first among the IT universities of Pakistan, sixth in research and productivity and ninth among all the higher education institutes of Pakistan recognized by HEC. CIIT Abbottabad was established in 2001 with student strength of 121. Now it has student strength of 5560 with 15 undergraduate programs and a total of 463 faculty members (8).

The objective of this survey was to highlight the preference of faculty members in CIIT Abbottabad for a particular OTC drug along with their knowledge about its safety, contraindications and adverse effects for ibuprofen in particular.

EXPERIMENTAL

We carried out a cross-sectional survey study in the COMSATS Institute of Information and Technology, Abbottabad (CIIT) in Pakistan. The data were collected from all faculty members of all the departments including Pharmacy, Computer Sciences, Environmental Sciences, Earth Sciences, Humanity, Chemistry, Geology, Power Engineering, Electrical Engineering, Civil Engineering, Computer Engineering, and Electrical Engineering. We requested all faculty members to participate but only 250 persons (comprising of 53 pharmacy faculty members and 197 faculty members who belonged to other departments) agreed to participate in this survey study, which was to get an idea about awareness of people regarding use of ibuprofen as an analgesic. In this survey study, the age group of participants was 30 to 39 years. We requested them to fill the questionnaires. The study was completed within 3 weeks very effectively. We also interviewed the faculty members about ibuprofen and its self administration (9). There were 18 questions consisting of three sections including socio-demographic and economic data (age, gender, education, and income), pain frequency and localization information about ibuprofen (Table 1). We also asked about whether they knew about its adverse effects, contraindications and sources of information. There were 7 closed questions and 11 open ended questions (1). We did not ask about their residence. This questionnaire provided an opportunity to the respondents to share their awareness about ibuprofen without any hesitation and they were also allowed to choose multiple answers.

Data analysis

Mean and standard deviation were used to provide a rapid access to metric variables. Education and knowledge included in ordinal data was identified using Kendall's regression, which is used for correlation. The results are presented in a combined form as there were no significant differences among all departments' participants (10).

RESULTS AND DISCUSSION

We studied about 250 faculty members (comprising 53 pharmacy faculty members and 197 faculty members who belonged to other departments). Most (65%) of them were males, while 35% were females. The mean age for both was 34.86 ± 9.02 years. The other sample characteristics are summarized in Table 2. Six percent pharmacy professionals and 4% faculty of other departments stated they suffered from pain nearly every day and 38% of participants (of which 16% were pharmacists) almost never experienced pain (Table 3). The head and back were the most common sites of pain, i.e., 24% and 19%, respectively. When we asked how they manage pain, 34% of respondents (comprising 32% non-pharmacists) stated that they visited a physician and bought the prescribed medicine. Other 38% (all were pharmacists) reported they had been purchas-

Table 1. Questionnaire used in this survey study.

No.	Parameters and their choices				
1	Sex				
	Man	Woman			
2	What is your age?				
3	Your highest level of education?				
	Primary	Middle	High	12 years	14 years
	16 years	M.S./M. phil.	Ph.D.		
4	What is your net monthly income (Rupees)?				
	0–5000	5000–9999	0,000–14,999	15,000–19,999	Over 20,000
5	Are you taking health related lectures? (Multiple answers)				
	No	Yes (Physician)	Yes (Pharmacist)	Other, what?	
6	Are you suffering from any pain?				
	Almost never	Less than once a month	About once a month	Several times a month but not every week	At least one day a week	Almost every day
7	For what pain you ever worry? (Multiple answers)				
	Head	Back	Crosses	Joints	Muscles	Teeth
	Abdominal	Other, what?			
8	When treating pain usually (multiple answers)				
	Ask your doctor to prescribe you something	Buy yourself an OTC drug without prescription	Heal by themselves with herbs, etc.	Heals by your relatives / friends / acquaintances	Alternative to what?	
9	OTC medication for pain (without prescription) can be bought from a pharmacy/medical store on the advice of: (multiple answers)				
	Doctor	Pharmacist	Known by television, radio, magazine or newspaper	Other, what?		
10	If you buy an OTC drug for pain (without a prescription), you get advice about its use from: (multiple answers)				
	Doctor	Pharmacist	Read the leaflet	You ask relatives / friends / acquaintances	Alternative to what?	
11	Which OTC drug you prefer to treat pain? (Multiple answers)				
	Panadol	Valetol	Brufen (Ibuprofen)	Voltral	Nalgesin	Aspirin
12	Ibuprofen is a major component of dosage regimen for: (multiple answers)				
	Pain	Fever	Inflammation	Sleeping	Of something else, what?	
13	If you purchase medicine containing ibuprofen (Brufen) your pharmacist asks you what other medications you are taking:				
	Always	Sometimes	Never	Another way, what	Cannot buy medication containing ibuprofen	
14	If you purchase medicine containing ibuprofen (Brufen), pharmacist tells you: (multiple answers)				
	Maximum daily dose	For use with / after meals	To drinking sufficient amount of water	About drugs which should not be combined with ibuprofen	About how to store the drug	About the cost of medication
	About nothing	Of something else, what?				
15	Medications containing ibuprofen:				
	No side effects	Have very few side effects	Can have serious side effects	Not know		
16	If you know any side effects or any medication or illness in which the ibuprofen should not be used, you come to know about it through: (multiple answers)				
	From doctor	From pharmacist	Through television, magazines	From relatives / friends / buddies	Otherwise, how?	I do not know
17	Do you know any side effect of ibuprofen (Ibalgin, Brufen, Apo-Ibuprofen, Ibumax)?				
	No, I donot know	Yes, I know; what?				
18	Medications containing ibuprofen should not be used by people receiving: (multiple answers)				
	Antidepressants	Blood pressure	Blood thinners	Medicines for diabetes	I do not know any of the options	Other option, what?

ing medicine from a pharmacy on their own behalf. Regarding the active ingredient in the pain medication, participants that preferred medicines containing ibuprofen were 31% (comprising 10% pharmacists), followed by Panadol® 46% (comprising 6% pharmacists) and aspirin 15% (comprising 3% pharmacists). The drug ibuprofen was chiefly regarded as the analgesic. Only small number of people (33% with 31% pharmacists) know the fact that ibuprofen has other uses besides relieving pain; it is also used as antipyretic and anti-inflammatory drug. About 7% of non-pharmacists respondents believed that it was hypnotic drug, whereas 55% of non-pharmacists respondents didn't have any idea about the drug, how and when to use it. There was a correlation between the clinical uses of ibuprofen and the level of education. Correspondingly, 40% young adults (from all departments) had more information about the use of ibuprofen.

The respondent's knowledge about adverse effects and drug interactions was mainly deficient. Most participants said that they had no knowledge about the side effects of drug (ibuprofen). About 79% of participants (non-pharmacists only) reported that ibuprofen had no adverse effects whereas 21% (comprising 5% non-pharmacist) had knowledge about the potential side effects of the drug. Elderly participants regarded the adverse effects of the drug to be less important than the younger subjects. Few subjects reported particular side effects (GI upset), likewise, there was deficient information about interaction of ibuprofen with other drugs like the interaction involved with medicines for high blood pressure and blood thinners.

While purchasing OTC (over the counter) drugs, subjects reported that they mainly followed physician's advice. Subjects that relied on pharmacist's recommendation were 18% (comprising 5% non pharmacists), 38% of subjects (comprising 34% non-pharmacists) said that physician was a reliable source of information about OTC drugs and 65% of participants (all were non-pharmacists) did not had pharmacist's counseling. There were no other effects of demographic factors on the results. The majority of respondents relied on physician's advice regarding knowledge about side effects and contraindications. The other sources of information were print/electronic media and relatives/friends. Only 25% of subjects (comprising 20% pharmacists) mentioned pharmacist as a source of information.

Our study concluded that most of the people suffered from headache and back pain, i.e., 24% and 19%, respectively. About 34% of people consulted

Table 2. Socio-demographic and economic features of respondents.

Characteristics	Limits	Total respondents		Pharmacy faculty		Faculty other than pharmacy	
		Number of cases	Percentage	Number of cases	Percentage	Number of cases	Percentage
Age (Years)	20–29	75	30	10	20	65	33
	30–39	108	43	28	56	80	41
	40–49	50	20	10	20	40	20
	> 50	17	7	5	10	12	6
Gender	Male	163	65	41	82	122	62
	Female	87	35	12	24	75	38
Education	14 years	0	0	0	0	0	0
	16 years	45	18	8	16	37	19
	M.S./M. Phil.	128	51	34	68	94	48
	Ph.D.	77	31	11	22	66	34
Income (Pakistani Rupees)	0–5000	0	0	0	0	0	0
	5000–9999	0	0	0	0	0	0
	10,000–14,999	0	0	0	0	0	0
	15,000–19,999	0	0	0	0	0	0
	> 20,000	250	100	250	100	250	100

Table 3. Number of subjects who reported pain.

Self reported pain frequency	Number of cases		
	Total respondents	Pharmacy faculty	Faculty other than pharmacy
Almost never	22	16	22
Less than once a month	20	29	20
About once a month	21	20	21
Several times a month/not every week	26	18	26
At least one day a week	8	11	8
Almost every day	4	6	4

physician for their problems as they think that physician can provide them the most reliable information about their relevant problem, adverse reactions of drug they are using and the risk factors. Thirty eight percent of respondents reported that they had been purchasing medicine from pharmacy. A majority of the faculty members preferred Panadol (46%) and ibuprofen (31%) to relieve the pain. People in our study did not have much knowledge about ibuprofen, its use, its adverse effects and consequently the risk factors. Faculty members of middle age had more knowledge about our relevant drug than the older members, while more side effects seemed to have occurred in the elder population of the faculty.

This study reflects the same results as other pain related studies carried out in different parts of the world. As reported by rural health study published recently, people above 50 years experienced pain. This study (14) shows that people suffering from chronic pain of back and joints were about 70–83%. Almost all people under study experienced acute pain. Most of the respondents of old age complained about their chronic pain and most often of back pain, joint pain and leg pain (15). Similarly, another study (16) conducted about 10 years ago in a few European countries showed that people ranging in 40–60 years of age experience chronic pain as compared to other people. There are some views of self medication study encouraged by a NGO in Australia (9); the topic was how much pharmacy clients know about ibuprofen and mostly females below 50 were studied. Large fraction did not give correct answers, as they do not have knowledge about ibuprofen use, its adverse effects, etc. Most of them do not even bother to study the leaflets. The same case occurred in Jordan (8), where patients do not have the basic information about the non-steroidal anti-inflammatory drugs and also about their side effects.

Usually elder people suffer with several diseases at one time; hence administering 5–6 medicines at a time. If such people use ibuprofen (NSAID's) for pain relief, it can be harmful for them because their body undergoes some pathological changes, so in this way they are unaware of the severe adverse effects related to NSAID's (1). Such patients can suffer from hemorrhage if they are taking antiplatelets, corticosteroids, anticoagulants and SSRI's (selective serotonin reuptake inhibitors). Moreover, patients whose kidneys are not working properly, have experienced cardiac failure, or are taking any medicine that reduces the fluid overload; there is high risk that such patients will experience kidney failure (17, 18).

Our study revealed that almost 34% of people visit a physician for obtaining analgesics. About 38% just directly go to the pharmacy for buying the medicine. The main drawback here is that neither physician nor attendant on pharmacy considers the important patient factors, hence very fatal results are often seen. This is associated with 39% of the cases under study. In this way, patient remains unaware of the severe side effects of ibuprofen, as ibuprofen and Panadol are mainly used as an OTC drugs for pain medication.

The monthly income of each faculty member was above 20,000 Pakistani rupees. In our survey, we did not get much information about the duration of pain, so we can not statistically interpret that how much people suffer from acute and chronic pain. However, we consider that chronic pain was the most problematic pain for the faculty members. Moreover, self study just gives us an idea about the presence or absence of pain, not about its intensity. In our study, there were about 18% pharmacy faculty members that had previous knowledge of health care and about analgesics, the rest including engi-

neering, management sciences, environmental sciences, developmental studies, geography and earth sciences faculty members were unaware of the basic knowledge regarding analgesics.

LIMITATION

The limitation of this survey based study is its small sample size. However, such study involving large sample size can be carried out.

CONCLUSION

It is concluded that a majority of the faculty members (61%) were lacking basic information about the OTC drugs and also they consider ibuprofen as the safest drug having no potential side effects. Thus, there is a need that such people should take health lectures, so that they gain useful information regarding the possible reactions of drugs with food and other drugs, side effects on health and the risk factors.

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SHORT COMMUNICATION

DOCKING OF THIOPURINE DERIVATIVES TO HUMAN SERUM ALBUMIN AND BINDING SITE ANALYSIS WITH MOLEGRO VIRTUAL DOCKER

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Keywords: 6-mercaptopurine, sulfur purine derivatives, human serum albumin, molecular docking

The replacement of the oxygen by sulfur atom at 6-position of guanine and hypoxanthine produced the compounds which are antimetabolites of nucleic acid purines (1). These thiopurine analogues have anticancer (6-mercaptopurine, 6-thioguanine) and immunosuppressive (azathioprine) activities. 6-Mercaptopurine (6-MP) is used for remission induction and in the maintenance therapy of acute lymphatic leukemia, and usually in combination with other drugs. 6-MP is also used to treat autoimmune diseases, such as inflammatory bowel diseases and rheumatoid arthritis (2–4).

Human serum albumin (HSA) is a major protein component of blood plasma and due to its endogenous and exogenous ligand binding properties, plays an important role in the transporting and distribution of numerous pharmaceutical compounds, mainly as a complex, to various organs and tissues within human organism. HSA consists of three homologous domains (I, II and III) and each domain is formed by two subdomains (A and B) (5). The X-ray analysis of different ligand–HSA complexes showed the existence of two binding sites in the II and III domains, which have cavities formed mostly of hydrophobic and positively charged residues and in which a very wide range of compounds may be accomodated (5, 6). The principal binding regions of albumin are located in subdomain IIA (the warfarin–azapropazone binding site, site I) and in subdomain IIIA (indole–benzodiazepine binding site, site II) (7, 8). Albumin may bind the acidic, basic and neutral molecules of drugs. The subdomain IIIA of albumin exhibits the primary binding activity, whereas subdomain IIA seems to be more specialized. Bulky heterocyclic compounds with a negative charge bind primarily to site I, whereas site II is preferred by aromatic carboxylates and anesthetics. Most drugs bind with the amino acids of target binding site in a reversible manner by means of weak chemical bonds. The several types of intermolecular bonding interaction such as hydrophobic, electrostatic, ionic and hydrogen bonds differ in their bond strengths. The number and types of these interactions depend on the structure of the drug and functional groups that are present in the drug (9).

The study of the interactions between albumin and drugs are important in pharmacology and clinical medicine, and also in research and design of new compounds. Numerous analytical methods including, among others, equilibrium dialysis, various spectroscopic, chromatographic and electrophoretic methods (10–13) are conventionally and most commonly used for investigation of the HSA–drug binding process. Additionally, the computational simulation by molecular docking procedure may be used to have more information about the specificity of the binding site and for the prediction of ligand-protein interactions. The *in silico* method will be used as an alternative and complement to *in vitro* methods and may give details of these molecular interactions, which would not be possible in solution studies (14–16). The studies by molecular docking are important, not only from a theoretical viewpoint, to explain the relationship between the structure of ligand and the function of protein but also in terms of practical applications, as they allow interpretation of the transporting process and therapeutic effectiveness of drugs.

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The previous results of computational docking study demonstrated that 6-MP may bind to the active site I located in subdomain IIA of the HSA structure (17). In order to obtain more information on how important is chemical structure of 6-MP for its interaction with albumin, a series of the sulfur purine derivatives was docked to site I of HSA and the results for this group of compounds were compared with those for 6-MP with HSA.

EXPERIMENTAL

Protein and ligand structures

The X-ray structure of HSA (PDB ID: 1AO6) (18) was downloaded from Protein Data Bank (PDB). The chemistry of the protein was corrected for missing hydrogen consistent with protonation states at pH 7.4, the His, Arg and Lys were in protonation state, while Asp and Glu were deprotonated; the protein was treated as a rigid body and the polar hydrogen atoms of the protein were added. The crystallographic water molecules were removed from the protein. The two dimensional (2D) structures of ligands were obtained using the ChemBioDraw computer program (19). 2D to three dimensional (3D) representations were converted by the use of ChemBio3D (20) software and then, these were energetically minimized by using method implemented in the same software, and saved as MDL moleFile (*.mol). The chemical 2D structures of all ligands are shown in Figure 1.

Molecular docking

Molecular docking procedure was performed using the Molegro Virtual Docker (MVD) program

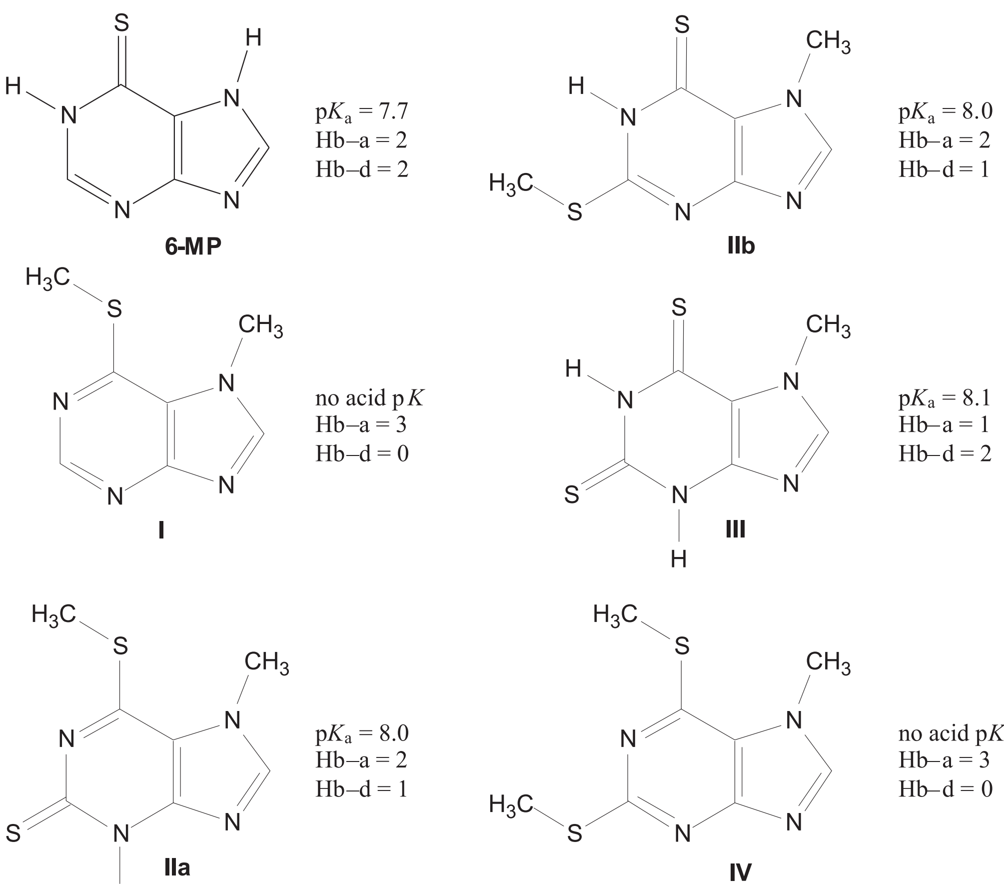

Figure 1. Chemical structures of the investigated thiopurines, their acid dissociation constants (pK_a) values and number of potential hydrogen bond donors (Hb–d) and acceptor (Hb–a) groups (pK_a values and number of hydrogen bond donors and acceptor were calculated with the use of the computer program (27). Structures of compounds were taken from data published in (28)

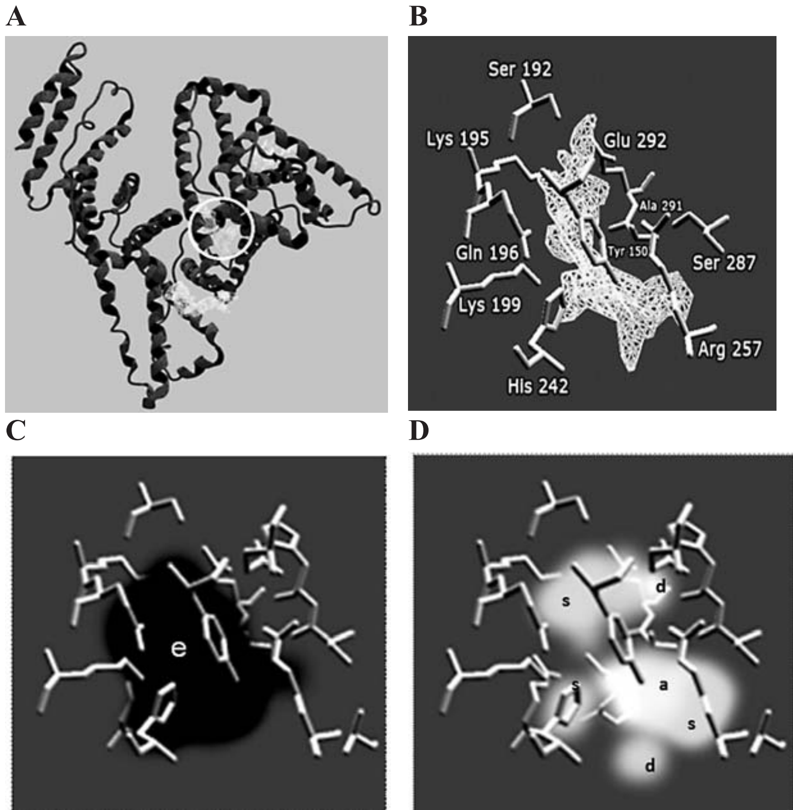

Figure 2. **A** – Ribbon model of HSA (HSA from PDB ID: 1AO6) with detected binding site for 6-MP in subdomain IIA (the site is marked with a white circle). Images **B**, **C** and **D** present the enlarged area corresponding to the 6-MP binding site in subdomain IIa; **B** – the close-up of detected site with a hydrophobic (Ala), hydrophilic uncharged (Ser, Gln, Tyr) and hydrophilic positively (Arg, Lys, His) or negatively (Glu) charged amino acid residues, according to the hydropathy index (29), capable of interacting with 6-MP (only residues around 6 Å of the docked 6-MP were taken into account); **C** – e-region shows the electrostatic potential of albumin in the binding site; **D** – s-region shows the spot where it is favorable to place non-polar atoms of the ligand, a-region shows the spot where it is favorable to place a ligand atom capable of accepting a hydrogen bond i.e., spot near donor in the protein, d-region shows the spots for heavy atoms in the ligand that are able to donate a hydrogen to hydrogen bond

(16, 21). The identification of the cavity with the potential binding site for ligands in subdomain IIA in HSA crystal structure was performed automatically using the grid-based cavity prediction algorithm. The residues close to cavity were minimized. During the minimization only torsion angles in the side chains were modified, all other properties (including bond lengths and backbone atom positions) were held fixed. The 3D structures of ligands were imported to MVD as *.mol.

During the docking simulation the backbone was kept rigid, but the torsional angles in the side chains of amino acids close to the detected cavity were allowed to change. The following steps were applied during the docking simulation: the ligands were docked with the softened potentials. At this point the receptor was kept rigid at its default conformation. After each ligand was docked, the side chains chosen for minimization were minimized with respect to the found pose. After repositioning the side chains, the ligand was energy-minimized. The repositioning of the side chains and minimization of the ligand were performed using the standard non-softened potentials. All flexible torsions in the ligand were set rigid during docking because the complexity of the docking search can be significantly reduced if the number of torsions that are set flexible during the docking run is lowered.

First, to obtain the 6-MP–HSA complexes, the 6-MP molecule as neutral and monoanionic forms, were docked individually to the cavity. For each complex, 10 independent runs were conducted, each of these runs was returning to a single final solution (pose). The resulting conformations were clustered and only the negative lowest-energy representation from each cluster was returned when the docking

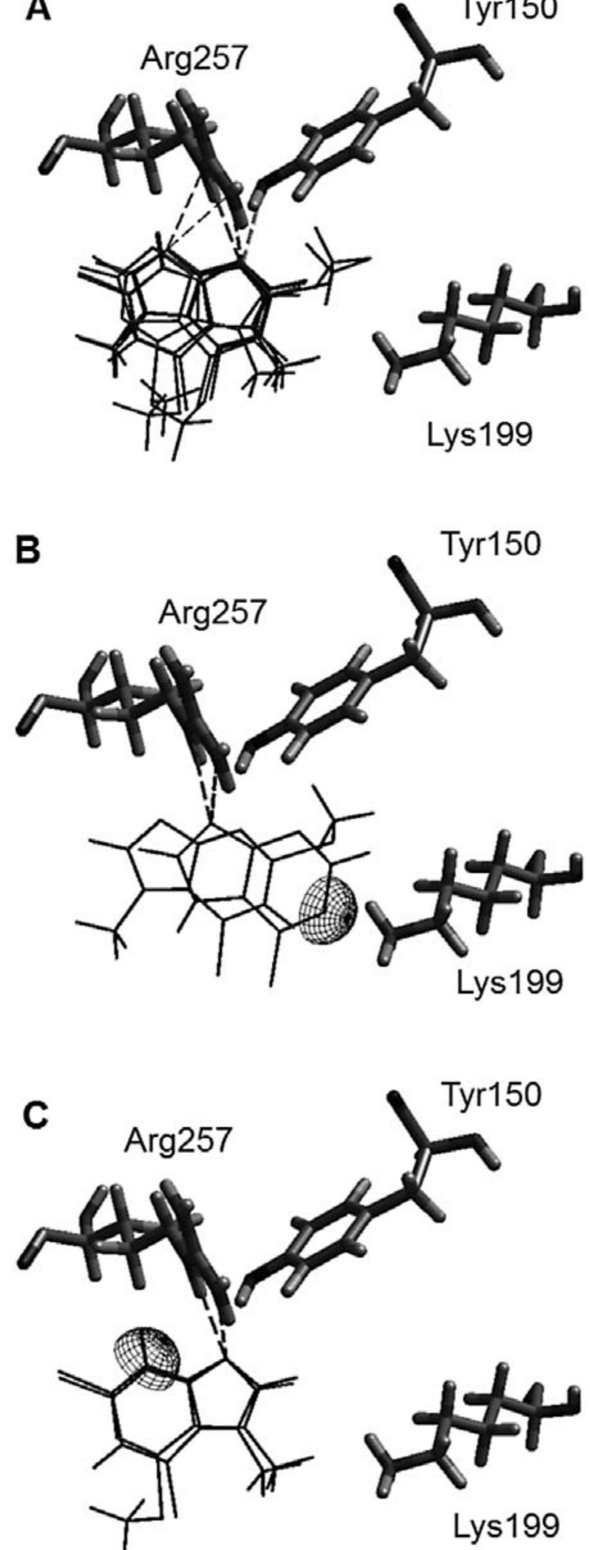

Figure 3. Overlaped docking poses of a neutral forms of compounds I–IV and 6-MP (**A**), anionic forms of IIb and 6-MP (**B**), anionic forms of IIa and III (**C**). The amino acid residues are shown in stick representation, the poses of compounds I–IV and 6-MP are shown in thin stick. Hydrogen bonding interactions are shown in dashed lines, electrostatic interaction is visualized as partial sphere oriented in the direction of the interaction

run was completed; the similar poses were removed keeping the best-scoring one. The cluster of ten poses was sorted in order of the MolDock Score. In order to increase the accuracy of the ranked order of the poses, the weighted reranking scores (Rerank Score) were used to evaluate the poses. For analysis, one pose with the lowest value of Rerank Score was selected as the best solution for each complex. Next, the same procedure was followed in order to obtain the thiopurine–HSA complexes. The compounds I and IV (basic compounds) were docked as neutral forms. The compounds IIa, IIb and III as well as 6-MP are acidic compounds. Their dissociation constants, p*K*, are about 8.0 (Fig. 1) and in aqueous solution at pH 7.4 may exist as a mixture of the neutral and monoanionic forms. Therefore, the structures of IIa, IIb and III were docked as neutral and anionic forms.

Before docking of the ligands into the HSA structure, the MVD docking protocols were validated using the two crystal structures of RWF–HSA and PhB–HSA complexes as previously described (17).

RESULTS AND DISCUSSION

The 6-MP is a purine derivative, containing four nitrogen atoms and double-bonded sulfur atom as a side group in the 6 position of the pyrimidine ring (C(6)=S). The overall purine has the potential to interact with binding site through hydrophobic interaction, while the individual N atoms present in the structure could interact by hydrogen or ionic interaction. In the molecule of 6-MP the N(3) and N(9) atoms have one lone pair of electrons and can act as an acceptor for hydrogen bonds, and N(1)H and N(7)H groups can act as a donor for hydrogen bonds, and N(1)H can act as an ionic center if it is deprotonated. Although sulfur atom is electronegative, it is a weak H–bond acceptor. Its lone pairs are in third–shell orbitals that are larger and more diffuse. This means that the orbitals concerned interact less efficiently with the small 1s orbitals of hydrogen atoms (9, 22, 23).

The binding region in subdomain IIA of HSA (namely Sudlow`s site I) is formed as a pocket with the inside wall being formed by amino acids hydrophobic side chains (Ala, Leu, Phe, Trp, Val, Ile). The interior of the pocket is predominantly apolar but contains two cluster of polar residues, an inner one towards the bottom of the pocket (Tyr150, His242, Arg257) and an outer cluster at the pocket composed by positively charged residues (Lys195, Lys199, Arg218 and Arg222) (24, 25).

The molecular docking simulation using the MVD program was previously employed to investi-

Table 1. Interaction energies between HSA and compounds I–IV and 6-MP at the binding site I on HSA

Ligand	Interaction energy (arbitrary unit)				
	E-Total	Steric	H-Bond	Electro	E-Intra
Neutral form					
6-MP	–63.8	–59.5	–8.0	0.0	3.7
I	–64.2	–64.7	–7.8	0.0	8.3
IIa	–80.0	–78.6	–7.3	0.0	5.9
IIb	–73.4	–72.6	–7.7	0.0	6.9
III	–73.4	–72.8	–7.3	0.0	6.7
IV	–78.3	–77.0	–7.3	0.0	6.0
Anionic form					
6-MP	–69.8	–47.0	–8.7	–17.8	3.7
IIa	–77.6	–66.1	–4.3	–13.2	6.0
IIb	–81.3	–66.9	–9.0	–12.3	6.9
III	–74.6	–63.0	–6.3	–12.0	6.7

The energy is not normalized in chemical units and should only be used to compare the results within one results set. E-Total is the total energy (the sum of internal ligand energies, protein interaction energies and soft penalties), H-Bond is the hydrogen bonding energy between protein and ligand. Steric is the steric interaction energy between protein and ligand. Electro is the sum of short-range ($r < 4.5$ Å) and long-range ($r > 4.5$ Å) electrostatic protein–ligand interaction energy, E-Intra is the total internal MolDock Score energy of the pose (21)

gation of the binding mode of 6-MP with HSA and the location of the 6-MP binding site in the HSA structure and structural characteristics of this site were described (17). The binding site for 6-MP found to be in subdomain IIA is presented in Figure 2A. Hydrophobic, hydrophilic as well as positively charged residues present in this site (Fig. 2B) were able to participate in electrostatic interaction (Fig. 2C), hydrogen bonding and steric interaction (Fig. 2D) with 6-MP.

To obtain the complexes of compounds I–IV and 6-MP with albumin, docking of these thio derivatives into binding cavity of HSA was performed. The final solutions of this procedure are presented in Table 1 and in Figure 3. Total binding energy between selected conformation of each ligand and HSA, and components of this energy determined from the MVD results are listed in Table 1. The results were interpreted on the assumption that the more negative are the values of predicted binding energy, the more thermodynamically favorable is binding energy (26). The estimated E-Total binding energy for all the ligands was strongly dependent on steric interactions. Hydrogen bonds and electrostatic interaction energies accounted for markedly smaller proportion of total binding energy. It can be noticed in case of all the thiopurine derivative–HSA complexes that the values of E-Total energy were larger than those for 6-MP and resulted mainly from differences between steric energy. It was also observed, that binding energy between 6-MP and compounds I–IV and HSA depends on the compounds' ionization state, and was stronger for anionic form than for unionized molecules.

The best poses of 6-MP and compounds I–IV docked to site I are shown in Figure 3. Comparing the interactions between HSA and compounds I–IV with those for the 6-MP, it was found that the investigated compounds had the same amino acids residues involved in the interaction as 6-MP. The preferred poses conformation of neutral forms of compounds I–IV were positioned to form the hydrogen bonds with Arg257 and Tyr150 (Fig. 3A) and only N(3) and N(9) atoms of 6-MP and molecules of compounds I–IV were involved in hydrogen bond interactions (as acceptors). The sulfur atom was not found to form hydrogen bonding. However, the negatively charged N(1) atom of anionic forms of 6-MP and compound IIb was bonded by a salt bridge to positively charged ε-amino group of Lys199 (Fig. 3B), while the negatively charged N(3) atom of anionic forms of compound IIa and III was bonded by a salt bridge to positively charged guanidinium group of Arg257 (Fig. 3C).

The results of molecular docking showed that despite differences in molecular structures, the investigated neutral compounds with 2- or 2,6-purinethione structure (weak acids) and with methylthio substituents in the 2- or 2,6-positions of purine nucleus (weak bases) (Fig. 1) did not differ in hydrogen bonding interaction pattern. However, for compounds with the purinethiones structure, the positions of double bonded sulfur atom seems to be important for the mode of ionic bonding interaction with HSA.

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