It is a common medical knowledge that three mycobacterial infectious agents account for the majority of the cases encountered. These are: *M. Tuberculosis* (TB), *M. Leprae* and *M. Avium* complex (MAC). *M. Tuberculosis* infects currently about one third of the global population, mostly in less developed countries, resulting in about 8 million cases of active disease and taking 2.5 million human lives annually. Claiming *M. Tuberculosis* the greatest single cause of mortality connected with infectious disease worldwide could be fully justified [1].

Several approaches as for tuberculosis treatment have been exercised. With the advent and availability of effective anti-mycobacterial drugs in the 1940’s and 1950’s, fundamental principles were rapidly appreciated. The cornerstone of the treatment for most mycobacterial infections was and still is the use of two or more drugs simultaneously (multi-drug therapy) for a prolonged period of time. This is necessitated by the frequency of emergence of resistance to a single agent and the relatively long replication time. Second principle tells us that while choosing adequate drug therapy, host factors such as rest, nutrition and surgery were of no additional importance. The deemphasis of host factors in therapy of tuberculosis was appropriate in the era when effective drugs were available [2].

Rifampicin is the drug most widely used in the treatment of tuberculosis and other infectious diseases. Taking into account its chemical structure, rifampicin can be classified as a member of rifamycin class of antibiotics. Due to their properties it was straightforwardly categorized as one of the first line „superbly-effective” anti-tuberculosis agents. Like other macrocyclic antibiotics the use of rifampicin can create potential side-effects of hepatotoxicity, allergic rashes, appetite loss, nausea or immunological disturbances [3]. Therefore, developing a sensitive, accurate and reproducible analytical method for tracing rifampicin action in pharmaceutical preparations, testing its purity and determining its contents in biological fluids appears to be a dire must. Especially useful is the knowledge of the serum drug level which helps at adjusting the proper dose to ensure therapy efficacy and avoid possible risk of toxicity.

Numerous methods for the analysis of rifampicin in biological fluids (plasma, serum, urine, etc.) have been previously elaborated. Most commonly described procedures to determine rifampicin in the presence of a complex biological matrix include mi-
crobiological assays [4,5], HPTLC [6-10], HPLC thermo-spray mass spectrometry [11] and other HPLC methods [12-15]. It has to be stressed, however, that most of these require relatively large volumes of the sample. Additionally, some of these methods need sample clean-up. Matrix effect elimination in most describes cases is connected with complex and very long extraction procedures, which greatly increase analysis time [16-21]. The recent publications concerning HPLC analysis of rifampicin were published by Bhavika et al. [22] and Kumar et al. [23]. However, these HPLC methods aims were mainly to standardize the procedure for the determination of rifampicin and its metabolite desacetyl rifampicin in plasma and urine.

Regarding the above, this paper focuses on an accurate, reproducible, sensitive and fast gradient HPLC method for assay of rifampicin in pharmaceutical complex preparation and human serum samples.

EXPERIMENTAL

Chemicals and biological samples

Rifampicin-3 (4-methylpiperazinylimino-methyl) rifamycin standard was supplied by Sigma (St. Louis, MO, USA). 100 capsules of Rifamazid (Polfa, Warsaw, Poland) containing 300 mg of rifampicin and 150 mg of isoniazid were purchased commercially. Analytical grade solvents were supplied by Merck (Darmstadt, Germany) and POCh (Gliwice, Poland).

Biological samples of plasma were all prepared in hospital laboratory. Serum samples from tuberculosis patients receiving Rifamazid were collected 2.5 h after the injection.

Plasma samples were stored at -10°C in polyvinyl containers and defrosted just before the analysis.

Standard solutions

Calibration solutions of rifampicin in the range of 5.0-15.0 mg ml⁻¹ in methanol were prepared by weighing the appropriate amount of standard. Working standard solutions of rifampicin (concentration ranging from 100-300 µg ml⁻¹ were obtained by diluting the stock solutions with methanol). Standard solutions were stored at 4°C protected from light.

Calibration standards for serum were prepared by adding different volumes of rifampicin working standard solutions to the drug-free serum. Serum calibration standard for rifampicin was prepared at the concentration of 1-15 µg ml⁻¹ on the required day.

Figure 1a. HPLC separation of isoniazid (1) and rifampicin (2). Conditions: see Experimental part.
Chromatographic system

HPLC analyses were conducted using Merck, Hitachi LaChrom system equipped with L-7100 pump and a diode-array detector. System management and data acquisition were accomplished with a HP personal computer. Separation was achieved with Zorbax C_18 reversed-phase column (250 mm x 4.6 mm id., 5 µm particle size) (Merck, Germany). The mobile phase consisted of water-methanol at a flow rate of 1 ml/min. Chromatography was carried out at room temperature and the eluate was monitored at 333.6 nm.

Gradient elution was carried out with a mobile phase of methanol (A) and water (B). Gradient profile was 5% A (v/v) for 5 minutes, then a linear gradient went up to 100% B at 15 minutes. Under the chromatographic conditions described the retention time for rifampicin was 12.86 min.

SAMPLE PREPARATION PROCEDURES

Pharmaceutical preparation

For determination of rifampicin in pharmaceutical preparation one capsule of Rifamazid containing 150 mg of isoniazid and 300 mg of rifampicin was transferred to a standard 25 ml volumetric flask, dissolved and diluted to the mark with methanol. 1.5 ml of this solution was further diluted to 100 ml with the same dilution medium. Final concentrations of rifampicin and isoniazid were 0.18 mg ml^{-1} and 0.09 mg ml^{-1}, respectively. The amounts of the investigated substances were computed by external standard quantification method.
Solid-phase extraction procedure

A system with adjustable vacuum Baker SPE-12G was used for solid-phase extraction. Procedure for the extraction of rifampicin from serum with RP-18 bonded phase sorbent was as follows: the SPE cartridge was conditioned with 1.5 ml acetonitrile followed by 4 ml water at a flow rate of 2 ml/min. 0.75 ml serum sample was applied to the SPE cartridge at a flow rate of 1 ml/min. The cartridge was then washed with 4.5 ml of deionized water and then by 0.5 ml of acetonitrile.

RESULT AND DISCUSSION

Figure 1a and Figure 1b show typical chromatograms obtained when 20 µl of serum extract containing 6 µg ml⁻¹ of rifampicin and 20 µl of sample containing isoniazid (often used in complex pharma-
ceutical formulation as a second component) and rifampicin at concentration of 0.075 mg ml⁻¹ and 0.15 mg ml⁻¹, respectively, were analyzed as described above. The rifampicin peaks were clearly separated from other potentially interfering substances. The separation gives good resolution in a total analysis time of less than 15 min. The obtained separation factor of rifampicin from isoniazid was 5.74 and expresses satisfactory selectivity of conditions applied. The purity of the separated rifampicin peak was confirmed by taking UV absorption spectra at the start, middle, and end of the peak. The spectra overlapped completely (Figure 2). Additionally, the peak of rifampicin from the sample was confirmed by comparing the UV absorption spectrum of the peak from the standard with the corresponding peak from the sample (Figure 3). The proposed chromatographic system consisting of C-18 column and a simple mobile phase: water: methanol performed in gradient elution technique was found to be appropriate for the analysis of rifampicin either in pharmaceutical preparations or in biological fluids. The retention time for rifampicin was 12.8 min. under the assay conditions described. Peak symmetry was good and band spreading minimal.

By employing such optimal conditions, 5-point calibration plots were built in order to evaluate the linearity between peak area and concentration and additionally the method sensitivity for determination of rifampicin in pharmaceutical preparation and human serum. The obtained plots indicate good linearity ranging from 0.1 mg ml⁻¹ to 0.3 mg ml⁻¹ for drug content in Rifamazid and from 1 µg ml⁻¹ to 3 µg ml⁻¹ for serum, with correlation coefficients in both cases higher than 0.98 (Figure 4). All calibration curves were done in triplicate. The results of the linear regression analysis can be expressed by the following equation for pharmaceutical preparation and for serum, respectively: \( y = 2E+07 (\pm 0.48 \ SD, n=3) \times x - 54208 (\pm 3.64 \ SD, n=3), \) \( y = 18.32 (\pm 3.2 \ SD, n=3) \times x - 3.6 (\pm 2.56 \ SD, n=3). \)

The method developed was then applied for the analysis of real samples, namely for pharmaceutical formulation and the human serum. As for studying the accuracy, reproducibility and precision of the proposed method, recovery experiments were carried out. The recovery of the added standard was found at three levels and was repeated three times. A plot of the average amount of drug found vs. amount of the added one was constructed and the percentage of recovery was calculated. Table 1 and Table 2 summarize statistic parameters of the accomplished measurements pertaining to accuracy, precision and recovery studies of rifampicin determined in Rifamazid capsules and serum.

The results showed good correlation between the amount found and the one declared. 300.47 mg of rifampicin was found with respect to the expected 300 mg.

Student’s t-tests for 95% confidence interval were lower than the tabulated critical value of t for five degrees of freedom in single tail test and equaled 1.95 and 1.63 for both experiments carried out. It means that the average values of quantity analysis obtained don’t differ significantly from the real content of rifampicin in formulation and serum investigated.

The serum rifampicin concentrations found in this study at 2.5 h varied from 6.9 µg ml⁻¹ to 8.3 µg ml⁻¹ (Table 3). This finding is much higher than amounts found by M. Y. Khuhawar et al. [14], Seth et al. [24] or Sadeg et al. [25], smaller than 50 µg ml⁻¹ found by I. Calleja et al. [15] and comparable to a range of 0.3-7.1 µg ml⁻¹ reported by T. Delahunty et al [26]. With no doubt the differentiation of the obtained values is connected with single day dose of drug taken by individual patient and the time when blood was taken after the last dose, also the method of drug administration seems to be important. There was no significant difference in the mean rifampicin concentrations between the patients in this preliminary study. More extensive studies are being considered which will explore differences in rifampicin concentrations in plasma of a bigger group of patients suffering not only from tuberculosis but also different organ dysfunctions.

**REFERENCES**


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