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ANALYSIS OF BIOLOGICALLY ACTIVE PEPTIDES USING TWO-DIMENSIONAL HPLC-CE

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Abstract: High-performance liquid chromatography (HPLC) is a technique most frequently used for the assessment of biologically active peptides. Owing to the ionic character of these compounds, they may also be separated and assayed using capillary electrophoresis (CE), which offers very high efficiency, short analysis time and low consumption of reagents, and is used increasingly more often. The paper describes the combination of HPLC and CE in order to increase the efficiency of the separation of complex mixture of peptides (active substance and its related impurities). The developed two-dimensional HPLC-CE technique was employed for the analysis of the impurities of octreotide, a cyclic octapeptide used in therapy. Because distinct separation mechanisms are used, the two-dimensional technique ensures higher separation efficiency and a more comprehensive impurity profile of the medicinal product than either of the techniques used separately.

Keywords: octreotide, related impurities, two-dimensional HPLC-CE separation, dynamic pH-junction sample stacking

Biologically active peptides used in therapy are most often produced using biosynthetic methods or by sequential chemical synthesis. Due to the starting biological material or multi-stage manufacturing methods, the formulations are a source of a number of issues related to appropriate quality assurance. The monitoring of any impurities is an important factor for the assurance of safety of use of such medicinal products. Considering their structure, similar to the active substance, the analysis of impurities requires specific and selective analytical methods (1).

Octreotide, a cyclic octapeptide used as a synthetic somatostatin analogue, is one of many peptides used in therapy. Octreotide is manufactured by sequential extension of a peptide chain; subsequently, it is purified using chromatography (2). It is used in medicinal products as acetate salt. The dosage form is solution for injection which contains various doses of octreotide acetate.

As a consequence of the coexistence of the peptide and acetate ions in solution, there is a tendency to form ester or amide links with –OH and $–NH_2$ functional groups available in the octreotide molecule. Additionally, the amino acids in the peptide chain may undergo spontaneous enantiomerization; in the case of octreotide, it occurs mainly at Thr^6 and Cys^7. However, the main degradation process, which occurs during the storage of the medicinal product, is the elimination of the C-terminal alcohol and the formation of [des-$Thr\text{-}ol^8$]-octreotide. The identified impurities found in the octreotide acetate formulation are shown in Table 1. HPLC is the technique used most often so far for the assessment of quality of biologically active peptides (3, 4). Because of their ionic character, they may also be separated and assayed using capillary electrophoresis (CE) (5, 6), a technique used increasingly more often. Capillary electrophoresis is an analytical technique with major advantages, such as short analysis time and low consumption of reagents and samples. It has been used for a number of years for the analysis of proteins, peptides and DNA fragments (5, 7, 8).

Along with capillary electrophoresis, the two-dimensional LC-CE technique for the analysis of peptide compounds should be mentioned; it uses first chromatographic and subsequently electrophoretic separation of the test sample. It ensures higher separation efficiency when analyzing com-

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Table 1. Octreotide and its known impurities.

Substance	Structure	RRT
Octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol	1.00
[D-allo-Thr^{6}]-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—D-allo-Thr—Cys—Thr-ol	0.74
[hydroxymethyl-D-Trp^{4}]-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol CH_2OH	0.92
[des-Thr-ol^{8}]-cystinol-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys-ol	0.95
[des-Thr-ol^{8}]-Cys^{7}-amid-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys-amid	0.97
[des-Thr-ol^{8}]-octreotide main degradation product	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys	1.06
[2-7 trisulfide]-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol S	1.16
[D-Cys^{2}]-octreotide	H—D-Phe—D-Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol	1.36
[1-acetyl-Thr-ol^{8}]-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol $CO\text{-}CH_3$	1.36
[N-acetyl-D-Phe^{1}]-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol $CO\text{-}CH_3$	1.80
[Nε-acetyl-Lys^{5}]-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol $CO\text{-}CH_3$	2.06
[des-Thr-ol^{8}]-D-Cys^{7}-octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—D-Cys	–
[2-7-SH]-octreotide reduced octreotide	H—D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol	–

RRT – relative retention time

plex mixtures of compounds which have similar structures, such as related impurities, than either of the techniques used separately (9–11). The combination of HPLC and CE as a two-dimensional technique is interesting given that distinct separation mechanisms are used. Therefore, a two-dimensional profile of the sample is obtained, which ensures selective identification of respective components.

This paper is an attempt to characterize impurities in biologically active peptides used in therapy with octreotide, a cyclic octapeptide, as the example, using two-dimensional HPLC-CE analysis.

EXPERIMENTAL

Materials and Solutions

Tetramethylammonium hydroxide (TMAH), triethanolamine (TEA), L-cystine as internal standard, 2-ethoxyethanol (Sigma-Aldrich); ortho-phosphoric acid 85% (BDH); trifluoroacetic acid (TFA) (Applichem); 1.0 and 0.2 M aqueous sodium hydroxide solution prepared from ready-to-use *fixanal* ampoules, acetic acid 99.5% (POCh); ninhydrin (Merck); Dragendorf reagent prepared according to Ph. Eur.; isopropanol (IPA), methanol (MeOH), acetonitrile (ACN) all HPLC gradient grade, (LabScan); Nano-Sil C18 10 x 10 cm TLC plates, Chromabond C18 ec 3 mL SPE-cartridges (Macherey-Nagel).

Samples and reference substances

Sandostatin (0.2 mg/mL) multidose vials, 0.5 mg/mL octreotide acetate reference standard (Novartis Pharma GmbH, Germany); phenol – according to the British Pharmacopoeia specification (Sigma Aldrich); 2.8 mg/mL aqueous solution.

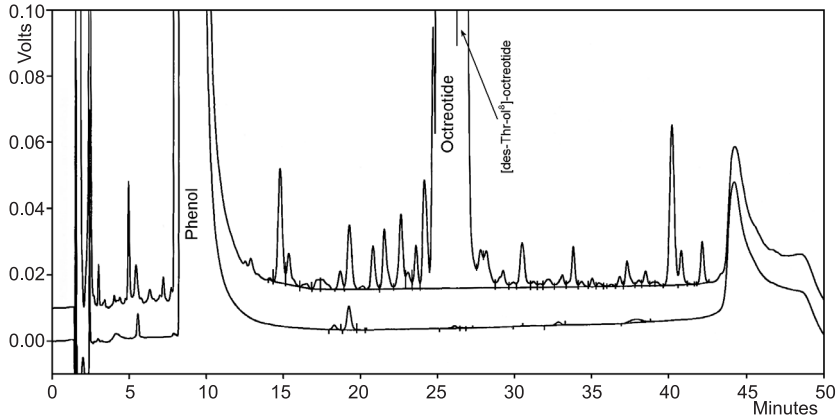

Figure 1. Overlapped chromatograms of Sandostatin 0.5 mg/mL medicinal product and phenol reference solution

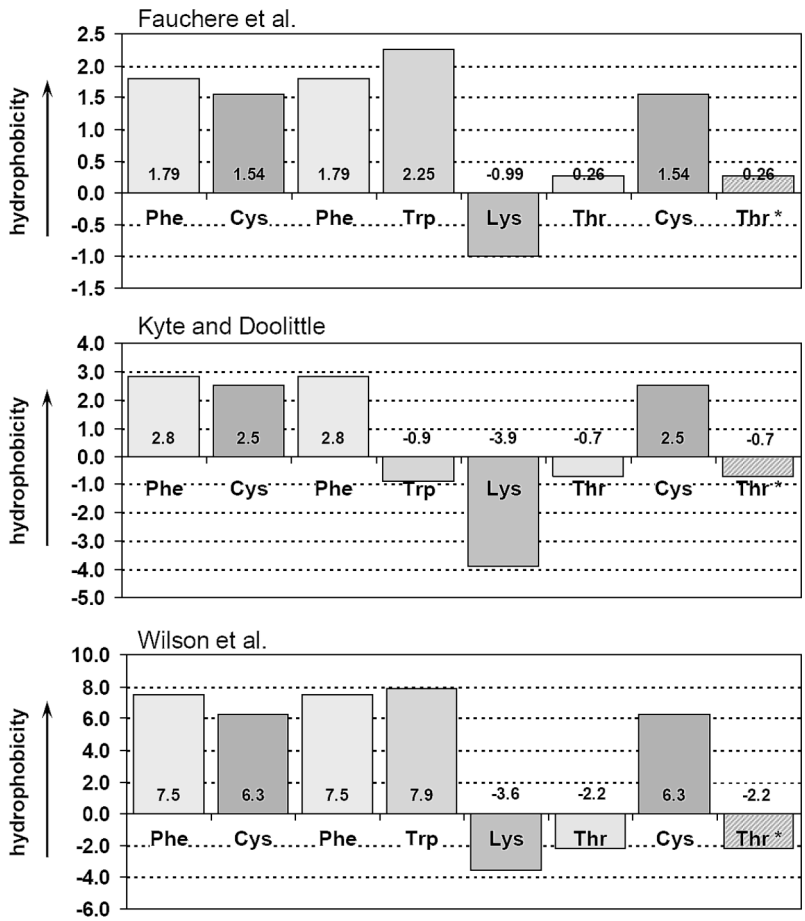

Figure 2. Related hydrophobicity of octreotide constituted amino acids according to various authors (12–14). Thr* – hydrophobicity of the last threoninol (Thr-ol) is expected to be higher than Thr because of lack of –COOH moiety

Table 2. The effect of incubation time in 37°C on the impuritiy level in medicinal product containing octreotide 0.2 mg/mL.

Degradation product	RRT	Percentage of peak area [%]				Increase between sample t_0 and t_3 [percentage points]
		Sample t_0	Sample t_1	Sample t_2	Sample t_3	
[D-allo-Thr^6]-octreotide	0.74	-	0.01	0.01	0.07	0.07
[hydroxymethyl-D-Trp^4] -octreotide	0.92	0.06	0.17	0.27	0.30	0.24
[des-Thr-ol^8]-cystinol -octreotide	0.95	0.09	0.10	0.10	0.17	0.08
[des-Thr-ol^8]-Cys^7-amid -octreotide	0.97	0.41	0.44	0.45	0.47	0.06
[des-Thr-ol^8]-octreotide	1.06	5.29	6.71	7.89	8.39	3.10
[2,6,7-trisulfide]-octreotide	1.16	0.13	0.29	0.29	0.36	0.23
[D-Cys^2]-octreotide + [1-acetyl-Thr-ol^8]-octreotide	1.36	0.06	0.09	0.10	0.14	0.08
[Nε-acetyl-Lys^5]-octreotide	1.80	0.06	0.16	0.24	nd	> 0.18

nd – no data, peaks were overlapped by rising gradient baseline

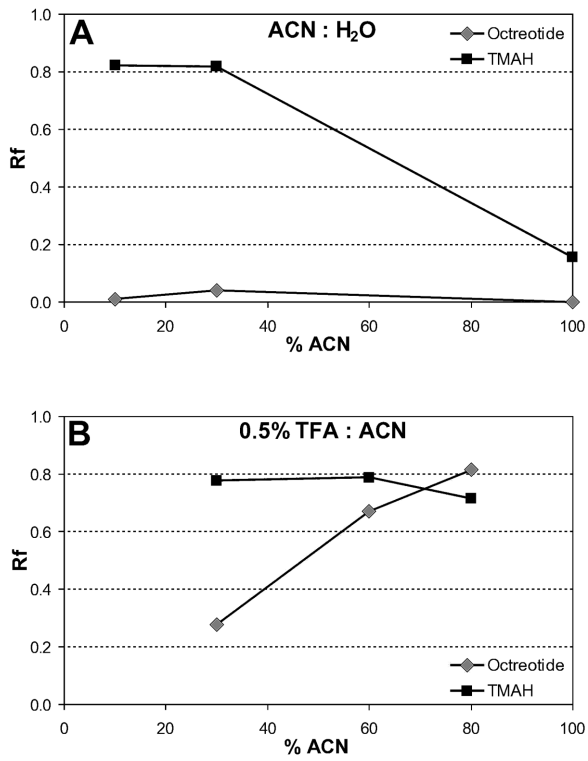

Figure 3. Retention of octreotide and TMAH on C18 TLC plates depended on the composition of mobile phase: (**A**) mobile phase ACN : H_2O; (**B**) mobile phase 0.5% TFA : ACN

In order to determine degradation products of the active substance, which are formed as a result of elevated temperature, the medicinal product was stored in the incubator at 37°C for 4, 8 and 12 weeks. The test samples were labelled as follows:

t_0 – medicinal product before incubation, previously stored in a refrigerator;

t_1 – medicinal product stored in an incubator for 4 weeks;

Table 3. Evaluation of SPE recovery for the individual peaks visible on electropherograms. The analysis includes only the peaks with a relative area of more than 0.05% of total corrected area of electropherogram.

Peak	Percentage of corrected peak area [%]	
	control sample (no SPE)	sample pretreated with SPE
1	0.11	0.06
2	0.19	0.24
3	0.16	0.22
4	0.12	0.07
5	0.64	0.53
6	0.69	0.73
[des-Thr-ol^{8}]-octreotide	6.98	6.96
8	0.33	0.36
Octreotide	**88.74**	**88.49**
10	0.27	0.44
11	0.10	0.20
12	0.15	0.11
13	0.62	0.72
14	0.70	0.50
15	0.12	0.29
16	0.07	0.08

Table 4. The effect of various injection modes on peak parameters – assumption for [des-Thr-ol^{8}]-octreotide peak.

Peak parameter	**10 psi·s vacuum injection**	**30 psi·s vacuum injection**	**30 psi·s injection with dynamic pH-junction sample stacking**
Peak width [min]	0.098	0.293	0.190
Peak symmetry	1.255	1.364	0.920
Peak efficiency (N)	135 500	29 870	77 130
Peak hight [μV]	804	2 350	2 498
Normalized area [arbitrary unit]	559	3 183	2 385

t_2 – medicinal product stored in an incubator for 8 weeks;
t_3 – medicinal product stored in an incubator for 12 weeks.

HPLC method

The testing was carried out using a Shimadzu liquid chromatograph equipped with two LC-10AT pumps, SCL-10A vp control system, SIL-10 vp autosampler, 10AV vp UV-VIS detector, DGU-14A degaser and CLASS-vp software (ver. 5.3).
Chromatographic conditions:
Column: Nucleosil 120-5 C18 250 × 4 mm (Macherey-Nagel)
Temperature: 25°C
UV detection: 210 nm
Injection volume: for analytical purposes: 100 μL
for fraction collection: 500 mL
Mobile phase A: 25 mM pH 2.0 TMAH with 10% ACN
Mobile phase B: 25 mM pH 2.0 TMAH with 60% ACN Flow rate: 2 mL/min

Time programme:	Time (min)	% of phase B
	0	10
	15	20
	45	45
	50	10
	55	STOP

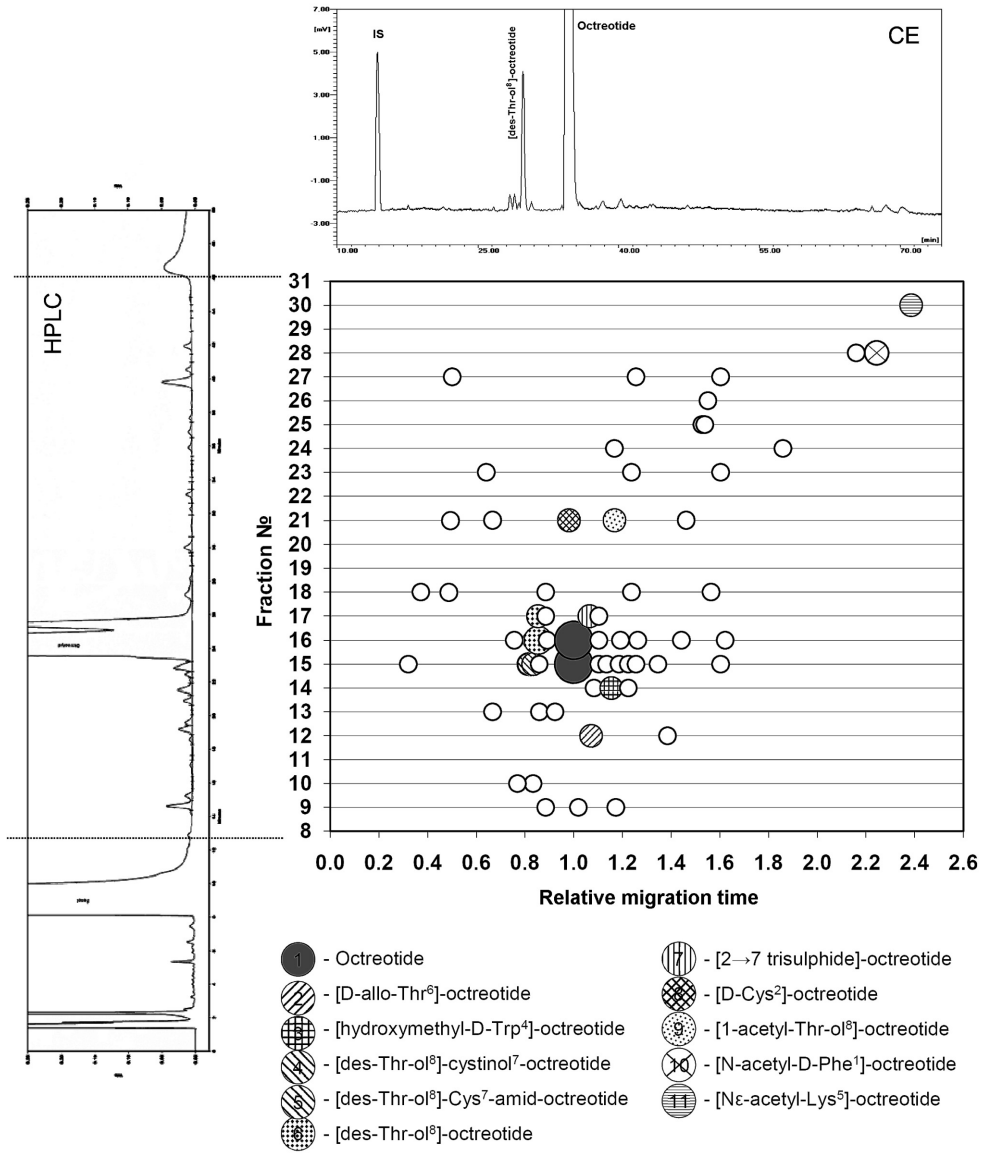

Figure 4. Two dimentional HPLC-CE separation of octreotide and related substances. Each circle represents a peak distinguished on the electropherogram of fractions. The biggest circle corresponds to octreotide present in two adjacent fractions. Circles with pattern indicate defined octreotide-related impurities

Fraction collection

After chromatographic separation, the eluted fractions were collected using a Gilson 203 B collector. The fractions were collected at 1.5 min intervals, starting from 2 min of each separation. Thirty-two fractions were collected from each sample, from 500 mL of the test solution, each being applied on the column three times.

TLC method

In order to set up the conditions for fractions preparation by solid phase extraction (SPE), a number of TLC experiments were carried out. Bands of 0.2 mg/mL octreotide solution (4 mL) were applied on a TLC RP18 (Macherey Nagel) plate using a TLC automatic sampler (Camag Deutschland, Berlin) and HPLC mobile phase solution which contained 25 mM TMAH (2 µL). The plate was developed in the selected mobile phase to a height of approx. 9 cm and dried on air. Octreotide spots were developed for 10 min at 120°C, previously sprayed with 0.2% ninhydrin solution in 2-ethoxyethanol:acetic acid (99:1 v/v)mixture and, subsequently, TMAH spots were developed using the Dragendorf reagent.

Solid phase extraction

The purification and concentration of fractions was carried out using SPE C18 cartridges. The columns were conditioned subsequently by 2.5 mL of ACN, 2.5 mL of 50% ACN in water and 4.0 mL of water. Each fraction was neutralized by a stoichiometric amount of 1 M NaOH solution (approx. 450 µL of 1 M NaOH per 9 mL of collected fraction) and applied on the SPE column. The solution was passed through the sorbent at a rate of approx. 40–60 drops/min. In order to remove TMAH (HPLC mobile phase ingredient) from the sample, the columns were additionally washed twice with 2 mL of H_2O and dried in air stream for several minutes. Subsequently, 2 mL of ACN was passed through the bed to remove phenol (preservative in the medicinal product) and its degradation products and dried in air stream again. Peptides were eluted from the column using three 0.5 mL portions of 0.1% TFA solution in 80% ACN. The resulting eluates were collected and evaporated to approx. 0.5 mL in inert gas stream at 20°C. This preparation of fraction samples yields three times as high analyte concentration with respect to the HPLC-tested solution, assuming 100% recovery.

CE method

The testing was carried out using BioFocus 2000 (Bio-Rad) with a UV detector (200–365 nm) and a silica capillary (50/46.4 cm, 50 µm ID, Beckman) thermostated at 20°C. A new capillary was prepared for electrophoresis by washing separately with isopropanol (2 min), 1 M NaOH (2 min), deionized water (2 min), separation buffer (2 min). Conditioning with the separation buffer was carried out before each separation (50 s).

The separation was carried out in the following separation buffer: 200 mM TFA brought to pH 1.8 using TEA with 10% methanol at 20 kV (positive polarization); the signal was recorded at 210 nm. The samples were applied hydrodynamically for 5 and 10 psi·s unless otherwise stated.

Pretreated and evaporated fraction samples were used as follows: 10 mL of the internal standard (IS) solution (1 mg/mL L-cystine solution) and 20 µL of 0.2 M NaOH was added to 150 mL of the eluate to obtain a pH of approx. 9–10 (pH > pI of octreotide). The resulting sample was analyzsed by electrophoresis. A sequential injection was applied: 30 psi·s of the alkalized sample and 5 psi·s of the separation buffer. The volume of the applied sample was approx. 60 nL, equivalent to approx. 3 cm of zone length (approx. 6.5% of capillary volume).

RESULTS AND DISCUSSION

Results of HPLC method

Two peaks of retention time: ~8.3 min and ~23.6 min occupied the highest percentage area of the chromatogram for the medicinal product. Comparison with the chromatogram of octreotide reference solution (0.5 mg/mL) confirmed that the peptide is visible as a peak with longer retention time. Apart from the two main peaks, additional ones were seen in the chromatograms from which, based on the assessment of relative retention times (RRT), octreotide impurities were identified (Table 1). In addition, the injection of 2.8 mg/mL aqueous phenol solution allowed the identification of the preservative and other phenol-related peaks (Fig. 1).

Following the quantitative analysis of impurities, the percentage of degradation products was found to increase with sample storage time. The highest increase was noted for the main degradation product, [des-Thr-ol^8]-octreotide (by more than 3 percentage points). Two other impurities with an increase of approx. 0.24 percentage point, are: [hydroxymethyl-D-Trp^4]-octreotide and [2-7 trisulfide]-octreotide. The effect of medicinal product storage time at 37°C on octreotide degradation is shown in Table 2. As expected, the highest amount of impurities was found in medicinal product samples stored for 3 months at 37°C (t_3 samples).

About 27 peaks of known and unknown by-products of octreotide can be identified on the chromatogram of the medicinal product stored for 3 months at 37°C. Some of the peaks are poorly resolved (e.g., [D-Cys^2]-octreotide and [1-acetyl-Thr-ol^8]-octreotide); there are also peaks overlapped with the signal derived from phenol (Fig. 1). Hence, it was suspected that the actual amount of related substances may be higher than shown on the chromatogram. To further illustrate the impurity profile of octreotide, two-dimentional HPLC-CE analysis was performed. For this purpose, fractions were collected and pretreated for electrophoretic analysis.

Sample clean-up and preconcentration

Before electrophoretic analysis, it was necessary to remove ionic components of mobile phase (TMAH and phosphoric acid) and to reduce sample volume, thus increasing the signal for second dimension separation. Particularly TMAH, a quaternary ammonium compound, had to be removed because it could adversely affect inner capillary wall and reduce or even reverse electroosmotic flow.

In order to select the optimum sample purification and concentration technique, octreotide amino

acid composition was analyzed with respect to its hydrophobicity. Figure 2 compares relative amino acid hydrophobicity according to hydrophobicity scales developed independently by several authors (12–14). Despite differences between respective scales, the prevalence of hydrophobic amino acids is noted in the octreotide molecule. Therefore, SPE using a C18-type stationary phase was selected as probably most suitable for this purpose.

The development of SPE conditions was started with initial trials using TLC RP C18 plates. The effect of the content of the organic modifier in the ACN : H_2O mobile phase on octreotide and TMAH retention was checked (Fig. 3A). Octreotide was found to be retained by the stationary phase very efficiently in such conditions, with its retention time not being much different with increased ACN concentration. However, TMAH has higher Rf values at lower organic modifier content. This experiment proved that a RP C18-type sorbent may be used to separate octreotide from TMAH from an aqueous solution with neutral pH and various ACN content. Successively, the retention of the compounds tested was verified for mobile phase of various ACN percentage in 0.5% TFA solution (Fig. 3B). Octreotide indicated decreased retention with higher ACN concentration in the mobile phase. Furthermore, it was also verified whether TFA concentration reduced to 0.1% (for 80% ACN) hinders octreotide elution from the C18 sorbent. Rf value of the peptide decreased from 0.815 to 0.769 in those conditions, which proves that 0.1% TFA in 80% ACN may be sufficient for efficient octreotide elution from the SPE column.

As a result of TLC experiments, optimum conditions for octreotide separation from mobile phase and its pre-concentration in a small amount of an appropriate eluent could be established.

SPE recovery

In order to verify the efficiency of the new SPE method for the purification and concentration of test peptides, the procedure described above was applied to HPLC mobile phase samples (blank sample), mobile phase containing 5 mg of phenol and mobile phase spiked with 0.2 mg/mL Sandostatin in an amount corresponding to 0.1, 0.2 and 0.3 mg of octreotide.

Electropherograms recorded for the blank and phenol samples subjected to SPE did not reveal any additional peaks which could interfere with the signal observed for the test peptide.

Comparison of electropherograms of spiked octreotide samples subjected to the SPE in relation to the control samples (octreotide solution of the same concentration without SPE) showed a similar profile of impurity peaks. It was found that the percentage of respective individual peaks in the SPE sample was consistent with the control sample (Table 3). A statistical evaluation with the paired *t*-test showed that the difference between the data groups was statistically insignificant ($p > 0.9999$). The mean recovery for the SPE method was 102% (RSD = 4.5%; n = 3).

Results of the CE method

Samples of HPLC fractions purified and concentrated with SPE were subsequently analyzed by capillary electrophoresis. Trifluoroacetic acid (TFA), a hydrophobic reagent which forms ion pairs and ensures efficient separation of peptides with similar structures (10, 15, 16), was used for the preparation of CE separation buffer.

The selected electrophoresis conditions ensure selective separation of compounds which form cations in the background electrolyte, like peptides do. Phenol does not form cations in BGE and it does not migrate towards the cathode. Hence, phenol was expected not to interfere with the analysis of octreotide and its impurities. This was confirmed by electrophoresis of phenol solution and no peak was observed in the electropherogram even for 90 min. However, in order to maintain an identical procedure for samples purification, an additional step of ACN wash was added during SPE. This was meant to remove phenol and its derivatives. However, it was proved that even in case of any possible incomplete phenol removal from the samples, its presence does not interfere with peptide electrophoretic analysis.

During the electrophoretic analysis of SPE samples, special attention was paid to the sample application, allowing for adequate amplification of the analytes. In this regard, a dynamic pH-junction sample stacking was applied – an increased injection time of sample of pH much different from that of the separation buffer (17–19), completed with the introduction of a short BGE plug into the capillary. Peptides at a pH higher than their isoelectric point (pI of octreotide ~9.1 (20)) form anions and migrate towards the anode accumulating in the back of the sample zone. The ions encounter a barrier there (low pH buffer) and, therefore, their anode migration is suddenly stopped. Peptides are focused at the phase interface and next, when contacting with BGE of pH lower than pI, they become positively charged and start to migrate towards the detector. This procedure of sample application made it possible to increase

signal by approx. three times without unwanted band broadening due to increased injection volume (Table 4). In addition, this ensures better peak symmetry and system efficiency.

Due to the migration time shift, which is typical in capillary electrophoresis, direct comparison of successive electropherograms may contribute to certain problems of signal interpretation. Therefore, L-cystine was used as the internal standard in order to compensate for migration time changes of the analytes. When electrophoretic separation of all the collected fractions was completed, the resulting electropherograms were compared and a two-dimensional image of the test sample was obtained (Fig. 4). Therefore, the CE data could be analyzed in relation to the HPLC data, and thus impurities of known chromatographic retention were identified in the electropherogram.

Based on the electropherograms, the elution of octreotide-related peptides in the HPLC method was found to begin at approx. 14 min. Most peaks were visible in fractions collected between 21.5 and 28 min of chromatographic separation. Analysis of electropherograms of the fractions ensured identification of five of the known octreotide impurities: [des-Thr-ol^{8}]-cystinol-octreotide; [des-Thr-ol^{8}]-Cys^{7}-amide-octreotide; [des-Thr-ol^{8}]-octreotide, [hydroxymethyl-D-Trp^{4}]-octreotide and [2-7 trisulfide]-octreotide. The identification of the first two impurities was not completely unambiguous, because the compounds were eluted in single fraction (no. 15) and they appeared in the electropherogram as two adjacent peaks with similar intensities. Furthermore, the electropherogram showed a number of other peaks of related impurities. The identification of [N-acetyl-D-Phe^{1}]-octreotide and [Nε-acetyl-Lys^{5}]-octreotide was the most difficult, because their migration time was very long (approx. 70 min).

Electropherograms of fractions no. 9, 18, 24 and 27 showed additional peaks whose number and height corresponded to the chromatographic signal. For fractions no. 3, 19, 20 and 26 no peaks were recorded in the electropherograms, which would correspond to peaks with retention time of approx. 5, 30 and 40 min in the chromatogram. Considering the selectivity of the electrophoretic system towards cations, we suppose that the peaks are related to phenol.

Due to the distinct separation mechanism (molecule hydrophobicity is the main factor responsible for retention, while migration depends on charge to mass ratio), the combination of both techniques yields data which confirm the quite complex composition of the test sample. Owing to two-dimensional HPLC-CE analysis, the presence of much more compounds could be established than using either method separately.

CONCLUSIONS

The two-dimensional HPLC-CE analysis allowed identification of octreotide-related peaks visible in the electropherograms and also provided a more comprehensive profile of the impurities in the medicinal product than either of the techniques used separately.

The comparison of chromatographic and electrophoretic data proved that the electrophoretic method is more selective towards octreotide impurities present in higher amounts than the HPLC method.

The presence of the excipient, such as phenol, does not interfere with the CE separation, thus it ensures more reliable results of purity assessment of the test sample.

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