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Determination of counter-ions in synthetic peptides by ion chromatography, capillary isotachophoresis and capillary electrophoresis

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The utility of three various analytical techniques [ion chromatography (IC), capillary electrophoresis (CE) and isotachophoresis (ITP)] was tested in the determination of counter-ions in synthetic peptides. The analyzed ions were acetates, trifluoroacetates and chlorides. IC provided the best results; CE, except limit of detection and limit of quantification, exhibited the comparable results. ITP was classified as the less useful because of the problem with the determination of the chloride ions. Nevertheless, all the three techniques were able to analyze trifluoroacetates and acetates ions with satisfactory results. Except analytical methods, three procedures using hydrochloric acid (HCl) (at two different concentrations) and acetic acid as sample solvents processed by lyophilization were tested. It has been found that the lyophilization not only by HCl but also by acetic acid is a simple and cheap procedure for removal of toxic trifluoroacetic counter-ions from peptides on satisfactory levels. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide; counter-ion; trifluoroacetate; acetate; chloride; ion chromatography; capillary electrophoresis; isotachophorese

Introduction

Testing of the purity of a pharmaceutical drug substance is one of the most important steps in the drug development process. Because many drug substances are manufactured in the salt form, the counter-ion needs to be determined as part of the testing.

Peptides have a wide range of applications, for instance, as pharmaceuticals and therapeutic agents, and synthetic peptides are currently in great demand. The need for these substances and the technological advances in peptide synthesis are on the rise with a rapid growth of newly discovered peptides and proteins. Synthetic peptides usually contain counter-ions such as acetate, chloride or trifluoroacetate as a result of their postsynthetic cleavage, purification and preparation method based on lyophilization. In addition to these two physiologically tolerable anions, hazardous trifluoroacetic acid (TFA) ones used in the synthesis and/or purification procedures may occur in the preparations of peptides. Furthermore, acetic acid (AcOH) and TFA can be found in synthetic peptide samples as impurities [1–8]. The efficiency of a synthetic peptide is diminished in the presence of a large amount of inactive species, including the counter-ions and impurities. Formulations prepared for biological evaluation or therapeutic purposes are based on the peptide content, and hence, it is crucial that their chemical composition be evaluated before the formulation process.

Trifluoroacetic acid is commonly used as a chemical reagent to remove both residual protecting groups during purification of peptides and also as an additive to the mobile phase in a reversed-phase chromatography. Preparative high-performance liquid chromatography (HPLC) using reversed-phase techniques is frequently performed to purify synthetic peptides, leading to the formation of their trifluoroacetates. TFA is characterized as a corrosive and toxic reagent. Because of its toxicity, it can affect the biological activity of synthetic peptides [9]. It is desirable to determine the residual TFA^- and to monitor its removal to reduce toxicity caused by TFA remaining in the final product. With regard to peptide acetates, the presence of acetic acid is unlikely to cause any adverse effects. However, it can reduce the biological potency of a peptide. Therefore, development of reliable and efficient analytical methods for determining residues of these counter-ions in synthetic peptide samples is mandatory. To determine counter-ions and/or other impurities in drug substances, we used analytical techniques such as HPLC with various detectors, ion chromatography (IC) and capillary electrophoresis (CE) [1,10–15]. However, there is still a strong need to develop tools and methods that are both reliable and easy to use in a common peptide laboratory. Moreover, there are not too many studies on the analysis and quantification of various counter-ions in peptides [1,16].

Ion chromatography is a relatively fast, accurate and cheap technique allowing simultaneous analysis of chlorides, acetates and TFA in peptides. On the other hand, isotachophoresis (ITP) is a technique not so widely used in the peptide analysis. However, it is an alternative in peptide sample analysis. Along with CE, this technique belongs to electromigration methods in which the movement of particles is caused by outer electric field. In ITP, in contrast to CE, two buffer solutions are used. The sample is placed between a leading electrolyte (LE) with a higher mobility

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than that of the sample and a terminating electrolyte (TE) with a lower mobility. Thus, ITP is a separation technique of ions (cations or anions) in the electric field using appropriate electrolytes that are formed into spheres of descending mobility moving with a constant speed. CE is based on electrophoretic phenomena – migration of ions in the electric field. Separation of the molecules is possible because of their different migration speed in a capillary. The detection is achieved by indirect mode by using separation buffer with chromophore. Analytes (anions) are UV transparent and give negative peaks as a difference between them and the background. In this sort of detection, the limit of quantification (LOQ) values for chlorides, acetates and trifluoroacetates presents 0.5, 0.9, and 11.3 μg/ml, respectively [6,9,17].

In this work, we compare the efficiency of using IC, ITP and CE in the determination of the content of counter-ions (chlorides, acetates and trifluoroacetates) in three synthetic peptides: temporin A (TA), pexiganan and a lipopeptide Palm-KK-NH_2. Moreover, we test various acid solutions used in the lyophilization as purification processes (replacement of counter-ions) of the compounds.

Materials and Methods

Reagents and Chemicals

Sulfuric acid, sodium acetate, sodium bicarbonate and *N*,*N*-dimethylformamide and dichloromethane, anhydrous sodium carbonate, hydrochloric acid (HCl), acetic acid, diethyl ether, palmitic acid, *N*-ethyldiisopropylamine, *N*,*N*-diisopropylcarbodiimide, triisopropylsilane (TIS), *N*-hydroxybenzotriazole and TFA of spectrophotometric grade were purchased from Sigma-Aldrich (St. Louis, USA); amino acids and resins were purchased from Iris Biotech GmbH (Marktredwitz, Germany); and all of them were of analytical reagent grade. The HPLC-grade acetonitrile was from JT Baker (Holland). The Anion Analysis Kit was purchased from Beckman Coulter (Fullerton, CA, USA); the deionized water was a self-made product prepared at the Inorganic Chemistry Department, with conductivity below 4.3 μS/cm^3 by Hydrolab deionization system (Gdansk, Poland).

Peptides

The peptides, TA, pexiganan (PEX) and a lipopeptide Palm-KK-NH_2 (PALM), presented in Table 1, were synthesized automatically in a microwave peptide synthesizer *(CEM, USA)* by the solid-phase method using the 9-fluorenylmethoxycarbonyl chemistry (Fmoc) [18]. The peptides were cleaved from the solid support by TFA in the presence of water (2.5%), TIS (2.5%) as scavengers. The cleaved peptides were precipitated with diethyl ether. The peptides were purified by preparative HPLC using Macherey-Nagel Nucleosil 100–7 C18 HD column (16× 250 mm, C18, 7 μm). The resulting fractions of purity greater than 95–98% were tested by reversed-phase HPLC (Macherey-Nagel Nucleodur 100-5 C18ec column, 150× 4.6 mm i.d., 5 μm) and thin layer chromatography for lipopeptide. The peptides were also analyzed by martix assisted laser desorption ionization - time-of-flight (MALDI-TOF) mass spectrometry.

Sample Preparation

Equal quantities of a selected peptide (1 mg) were placed in test tubes. To each sample, 1 ml of acetic or HCl acid (1 or 0.1 mol/l HCl) was added and left overnight to freeze dry. Each sample was prepared in triplicate. After the first lyophilization, aliquots of the samples were taken to determine the levels of counter-ions by IC, ITP and CE. The remaining volumes of the samples were again diluted with 1 ml of acetic acid or in 1 ml of water (in the case of peptides of the first lyophilization from HCl). Dry samples were diluted in distilled water before analysis.

Analytical Procedure for Counter-ions

For the suppressed chromatographic analyses, the LC 10AT vp vacuum pump [Shimadzu Ltd. (Kyoto, Japan)] with a syringe-loading manual sample injector with a 100-μl sample loop [Rheodyne (Rohnert Park, USA)], the 732 IC conductometric detector [Metrohm (Herisau, Switzerland)] and the 833 IC MSM suppressor (Metrohm) were used. The column (250× 4 mm i.d., 5 μm) was a Metrosepp A Supp 5 (Metrohm); a polyvinyl alcohol-based packing with quaternary ammonium groups was dedicated to work with chemical suppression. All chromatographic data were recorded by an *IC Net 2.3* Metrohm software during experiments. The mobile phase consisted of 3.2 mmol/L sodium carbonate and 1.0 mmol/L sodium hydrogen carbonate with 20 % (v/v) of acetonitrile. The analyses were performed in isocratic mode at ambient temperature at a flow rate of 0.6 ml min^{-1}, and the elution profiles were monitored by conductivity detector. The separation column was equilibrated with the mobile phase until baseline stabilization has been achieved, at which point, the sample injections (10 μl) were made.

The electrophoretic analyzer, Ita Chrom EA 101 (Merck, Germany), ready to work in the isotachophoretic mode was used in the experiments. The preseparation capillary column, 90 mm × 0.8 mm i.d., and the analytical capillary column, 210 mm × 0.3 mm i.d. (160 mm to the detector), were made of fluorinated ethylene–propylene polymer. The instrument was equipped with two conductivity detectors (in the first and second column), and additionally, the analytical column was fitted with a UV detector. The ITP WIN 2.21 software was used for isotachophoretic data analysis. For capillary isotachophoretic analysis of anions, systems of LEs consisting of a 10 mmol/L L-histidine/10 mmol/L L-histidine monohydrochloride solution and TEs consisting of a 5 mmol/L L-histidine/5 mmol/L glutamic acid solution were used. All solutions were prepared daily, and stock solutions were stored in high density poliethylene flasks. Both water for washing the system and the electrolytes were filtered and degassed using a water pump and an ultrasonic bath. The current in the first column was 250 μA, and the

Table 1. List of the peptides used in this study

Peptide	Abbreviation	Sequence	Relative molecular mass M_r	Net charge of peptide
Lipopeptide	PALM	Palm-KK-NH_2	510.99	+2
Temporin A	TA	FLPLIGRVLSGIL-NH_2	1395.50	+2
Pexiganan	PEX	GIGKFLKKAKKFGKAFVKILKK-NH_2	2474.95	+10

current in the analytical column was 50 μA. For all experiments, conductivity detection was used. The sample volume was 30 μl in all cases.

Electrophoregrams were performed on Beckman P/ace System 5500 Apparatus with P/ace Station Software Ver. 1.0 (Beckman Coulter). The CE apparatus was equipped with a UV detector with a 254-nm filter. The capillaries were provided by Beckman Coulter and had 75-μm i.d. and 60.2 cm of total length (50.0 cm from injection site to the detector).

Before analysis, the capillary was flushed every day by Na conditioner, rinsing solution and anion coating. The conditioning was made at 28.5 kV for 10 min. Before sample analysis, the capillary was rinsed first with the anion coating solution and then with the anion separation buffer. The pressure injection (0.5 psi) of the sample (8 s) and water reinjection (2 s) was followed by separation at 28.5 kV in 7 min with a 1-min voltage ramp. Vials were replaced after every eight runs or after every 24-h storage inside the apparatus. The capillary was rinsed with Na conditioner and the rinsing solution after each run. The CE apparatus worked in the negative polarity mode, and the indirect UV detection was used at 254 nm and a data rate of 5 Hz.

Statistical Procedure

All the data were tested by Dixon's *Q* method for rejection of the outliers. The Shapiro–Wilk test was carried out to prove that the data samples fell in the normal distribution population. To compare the methods, we used the one-way ANOVA to test the equivalences of the mean values. To estimate the mathematical differences between the methods, we constructed regression graphs (scatter plots). IC, as a reference technique, was compared with CE and ITP techniques. The concentrations used in statistical tests were μg/ml.

Results and Discussion

Validation

Specificity and selectivity

The chromatograms, electrophoregrams and isotachophoregrams of standard solutions were compared with those derived from peptide samples analysis. No interference peaks were detected. Relative standard deviations of the retention times of the ions were %RSD ≤ 0.2 and %RSD ≤ 2.4 for the IC and CE, respectively, which were satisfactory values for both methods. ITP was a nonspecific method for chloride ion determination because of the presence of the chlorides in the buffer.

Linearity

The linearity was examined by five-point calibration curves in case of IC and CE over the concentration range of 1.5–300 and 10–300 μg/ml, respectively, which covered the concentration ranges of the ions found in the peptide samples. In both methods, the correlation coefficient was 0.999, and the intercept of regression equation was statistically insignificant ($p > 0.05$).

Limits of detection and quantification

The limit of detection (LOD) and LOQ were measured for all ions determined by three methods at 3 : 1 and 10 : 1 signal/noise ratios. The values for the chloride, acetate and trifluoroacetate ions measured by the three methods are shown in Table 2.

Table 2. Validation parameters for the analytical techniques used to determine the counter-ions

ION	LOD (μmol/ml)	LOQ (μmol/ml)	Area (%RSD)	Retention time (%RSD)	Linearity
Ion chromatography					
Cl^-	0.04	0.09	0.9	0.1	0.999
CF_3COO^-	0.06	0.13	1.1	0.2	0.999
CH_3COO^-	0.06	0.12	1.5	0.1	0.999
Capillary electrophoresis					
Cl^-	0.07	0.29	1.5	1.4	0.999
CF_3COO^-	0.07	0.29	2.2	2.4	0.999
CH_3COO^-	0.07	0.29	3.2	2.0	0.999
Isotachophoresis					
Cl^-	n.a.	n.a.	n.a.	n.a.	n.a.
CF_3COO^-	0.10	0.20	2.5	n.a.	n.a.
CH_3COO^-	0.10	0.20	2.7	n.a.	n.a.

LOD, limit of detection; LOQ, limit of quantification; n.a., not analyzed.

Comparison of the Techniques

To estimate the comparability of the methods, the equivalence of the means was calculated by one-way ANOVA [19,20]. The ANOVA results showed no significant differences between the means (of the ion concentrations in all peptide samples) measured by the three methods ($p > p_0$, $0.08 > 0.05$). Moreover, a comparison of the methods using the *t*-test gave the same results.

A scatter plot analysis was another test to statistically compare the methods. The concentration of the ions measured by CE was plotted against that determined in the same peptide samples by IC (Figure 1). Similar procedure was applied for ITP and IC (Figure 2). IC was chosen as a reference method owing to excellent validation parameters and worldwide recommendation as analytical tool for ion determination. The LOQ values for chlorides and acetates were lower in comparison with literature data; however, for trifluoroacetates, we have obtained slightly better values.

The scatter plots in both cases showed a very high correlation between the techniques with determination coefficient $R^2 > 0.95$. Moreover, the intercept in both cases was statistically insignificant. This indicates that any differences (e.g. IC vs CE) were due to random errors.

One of the aims of our study was to compare the three techniques of determination of the counter-ions in peptides. All these techniques proved their specificity, linearity and precision on a satisfactory level, although IC was characterized by the best LOQ values. Table 3 presents the concentration of chlorides, acetates and TFA in PEX, TA and PALM.

The determination of impurities was conducted on samples taken just after purification by HPLC (called 'crude peptide') and after each of the lyophilization steps. Knowing the peptide molar concentration and peptide nominal charge, we have estimated the minimal counter-ion concentration. For the PALM, TA and PEX, there were 3.9, 1.43 and 4.04 μmol/ml, respectively.

Determination of the Counter-ions by Ion Chromatography

Exemplary chromatogram of analyzed counter-ions is presented in Figure 3. The highest contents of the counter-ions were found in PEX, whereas in TA and PALM, they were on a comparable level. This is due to a higher number of interaction sites in PEX than in the other two peptides. In the case of the lyophilization

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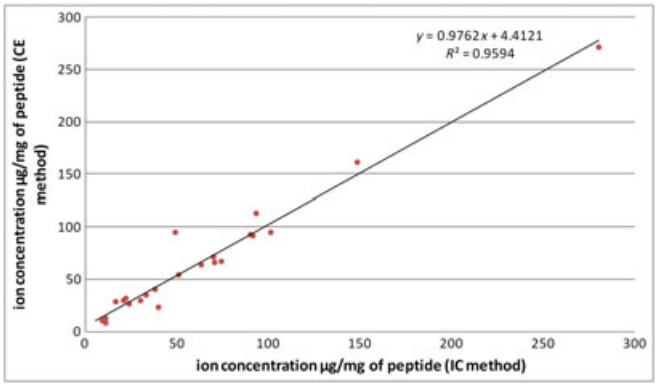

Figure 1. Scatter plot of correlation between methods IC vs CE.

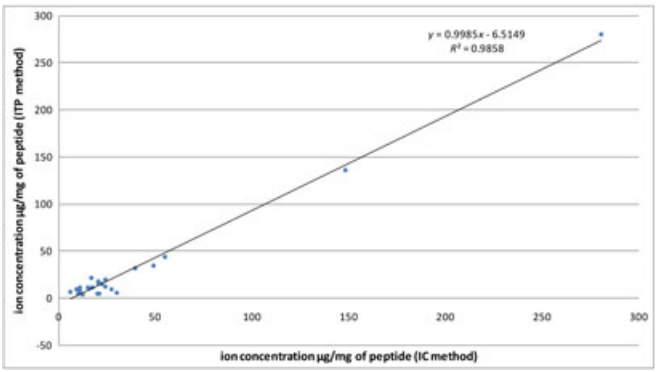

Figure 2. Scatter plot of correlation between methods IC vs ITP.

from the HCl, the analyzed ions were only chlorides and TFA. In the three peptides, a higher content of chlorides was found in samples lyophilized from 1 mol/l HCl, and at the same time, removal of trifluoroacetates was also higher (as compared with lyophilization from 0.1 mol/l HCl).

Application of acetic acid gives, apart from residuals of Cl^- and TFA^-, also acetate ions. The chloride content was usually lower than that in HCl lyophilization (except for TA where it was higher), but the TFA^- content was comparable in both procedures of freeze drying. The concentration of acetates increased during the second level, and the highest was observed in PEX peptide.

The excess of the total concentration of counter-ions was observed in the case of TA and PEX. That may indicate that removal of counter-ions by freeze drying was not so efficient. However, the level of counter-ions was decreasing with the next step of the lyophilization.

Determination of Counter-ions by Isotachophoresis

The ITP technique has one disadvantage as far as the analyzed peptide samples are concerned. Namely, most of the LEs are based on chloride salts, thus making impossible precise determination of this anion in our samples. For this reason, the anion was not analyzed in the tested peptide. However, there were attempts to determine chloride ions using different LEs [21–24].

The contents of trifluoroacetates in crude peptides in PEX, TA and PALM were 2.48, 0.30 and 1.20 µM/ml, respectively. The acetate levels were below LOQ for PALM and PEX, 0.20 µmol/ml for TA. Lyophilization from acetic acid reduced TFA^- to 0.14 µmol/ml in PEX, 0 in TA and 0.19 µmol/ml in PALM. The acetate contents were 0.19, 0.08 and 0.10 µmol/ml, respectively.

The use of HCl reduced the TFA^- level ranging from 0.09 to 0.04 µM/ml. As mentioned previously, the chloride ions were not determined by this technique.

Determination of Counter-ions by Capillary Electrophoresis

In the case of freeze drying from the 0.1 mol/l HCl solution, the content of chlorides drops in the second step in each peptide. On the other hand, lyophilization from 1 mol/l HCl increased the chloride content. Determination of TFA^- by CE showed complete disappearance of this ion just after the first step in all the peptides.

The use of acetic acid resulted finally in enhanced amounts of acetates in comparison with HCl lyophilization. The acetate ions were determined by CE only in PEX and only after the first level of freeze drying. Moreover, except the acetate ions, the TFA^- content was not entirely reduced (with the exception of TA where it was complete). In both the PEX and PALM samples, the concentration of trifluoroacetates was 0.20 and 0.27 µM/ml, respectively. However, the excess of the counter-ions above the minimal counter-ion concentration was only observed for one step of freeze drying in TA peptide. In comparison with the IC method, it may be caused by the lesser LOQ of CE. Exemplary electrophoregram is presented in Figure 4.

Table 3. Concentration of the counter-ions in peptide samples (μM/ml)

Peptide	Ion	Crude (μmol/ml)	0.1 mol/l HCl, 1st lyop (μmol/ml)	0.1 mol/l HCl, 2nd lyop (μmol/ml)	1 mol/l HCl, 1st lyop (μmol/ml)	1 mol/l HCl, 2nd lyop (μmol/ml)	AcOH, 1st lyop (μmol/ml)	AcOH, 2nd lyop (μmol/ml)
Ion chromatography								
PALM	Cl^-	0.32	2.61	2.04	0.97	0.62	2.65	2.15
	CF_3COO^-	1.31	0.18	0.15	0.14	0.11	0.22	0.15
	CH_3COO^-	—	—	0.16	—	—	0.34	0.51
TA	Cl^-	0.32	1.16	0.88	2.03	1.65	1.10	0.65
	CF_3COO^-	0.43	0.18	0.13	0.10	0.09	0.26	0.12
	CH_3COO^-	0.41	0.24	0.19	0.10	0.10	0.19	0.36
PEX	Cl^-	1.74	3.91	2.93	4.72	2.70	1.48	1.83
	CF_3COO^-	2.48	0.35	0.24	0.19	0.09	0.49	0.18
	CH_3COO^-	—	—	—	—	—	—	—
Capillary electrophoresis								
PALM	Cl^-	0.27	2.78	1.97	1.08	0.88	2.74	2.46
	CF_3COO^-	1.47	—	—	—	—	0.24	0.27
	CH_3COO^-	—	—	0.16	—	—	—	—
TA	Cl^-	0.26	2.20	0.88	0.65	0.78	1.19	0.94
	CF_3COO^-	0.87	—	—	—	—	—	—
	CH_3COO^-	—	0.16	0.23	—	—	—	—
PEX	Cl^-	—	3.10	2.84	0.92	3.37	1.61	1.91
	CF_3COO^-	2.39	—	—	—	—	0.56	0.20
	CH_3COO^-	—	—	—	—	—	0.23	—
Isotachophoresis								
PALM	Cl^-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	CF_3COO^-	1.20	0.14	0.10	0.09	0.04	0.18	0.19
	CH_3COO^-	—	—	0.16	—	—	0.08	0.10
TA	Cl^-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	CF_3COO^-	0.30	0.16	0.10	0.10	0.09	0	0
	CH_3COO^-	0.20	—	—	0.10	0.10	0.14	0.08
PEX	Cl^-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	CF_3COO^-	2.48	0.28	0.09	0.13	0.04	0.39	0.14
	CH_3COO^-	—	—	—	—	—	0.07	0.19

lyop., lyophilization; PALM, Palm-KK-NH2; TA, Temporin A; PEX, pexiganan; n.a., not analyzed; "—", below limit of quantification.

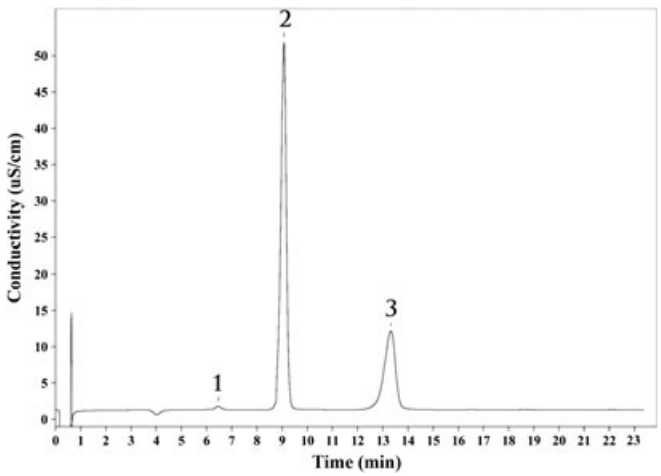

Figure 3. Chromatogram of PEX counter-ions after the first lyophilization from acetic acid: (1) acetate, (2) chloride and (3) trifluoroacetate.

Lyophilization Solutions and Number of Processes

Determination of the selected counter-ions indicated that impurity levels can soar up to 15% of the total mass of the crude peptide. Thus, it is mandatory to synthesize and release the final Active Pharmaceutical Ingriedient with ions, which either are biologically inactive or suppress the potency of a compound in a limited way. Using a stronger acid than TFA (pKa ~ 0) such as HCl (pKa ~ −7) [1] reversed the dissociation of TFA into acidic form and enabled removing the TFA^- counter-ion by freeze

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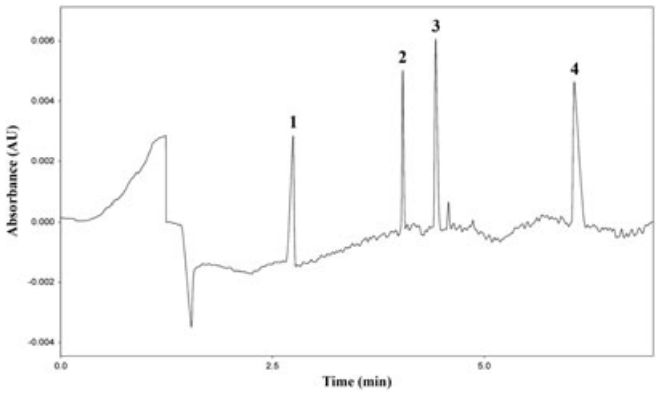

Figure 4. Electrophoregram of PEX counter-ions after the first lyophilization from acetic acid: (1) chloride, (2) trifluoroacetate, (3) acetate and (4) Internal Standard.

drying. However, the low pH might be destructive to the peptidic structure, and for this reason, we used a weaker acetic acid (pKa 4.5). Long-term use of HCl may also cause some disturbance in the integrity of the freeze dryer. However, in modern equipment, which is mostly built from resistant materials, it should not be a concern. During the analysis of the peptide samples, some interesting observations were made. Namely, the use of the two-step lyophilization procedure with 1 mol/l HCl turned out to be the most efficient technique of replacing the toxic trifluoroacetic ion by biocompatible ions. In the case of Palm-KK-NH_2, TA and pexiganan, the exchange percentage of TFA^- by Cl^- ion was 91.8%, 79.6%, and 96.4%, respectively (when using the IC method). The use of the 0.1 mol/l HCl gave comparable results. The results obtained by the procedure with acetic acid gave the exchange percentage of 88.0%, 73.0% and 92.8%, respectively (the IC method), after the second lyophilization.

Conclusion

In our work, we have used three analytical techniques to determine counter-ions in synthetic peptides. The best results were obtained by IC. CE exhibited similar specificity, linearity and precision; however, LOQ and LOD values were less satisfactory as compared with those of IC. ITP has one disadvantage that makes it less suitable to analyze counter-ions: It is the problem with the determination of the chloride ions. Nevertheless, all the three techniques were able to analyze trifluoroacetates and acetates ions with satisfactory results. From the statistical point of view, there was an acceptable agreement between the results obtained by the presented methods.

Apart from choosing the best analytical method, we also studied the influence of the freeze-drying processes on the replacement of counter-ions in peptides. Three procedures using HCl (at two various concentrations) and acetic acid as a sample solvent processed by lyophilization were tested. The results indicate that lyophilization using acetic acid as well as HCl as sample solvent is a simple and cheap method for satisfactory removal of toxic trifluoroacetic counter-ions from the peptides. In fact, all the solvents can be used, unless HCl does not cleave the peptidic bond. To minimize the possibility of bond cleavage, the concentration of HCl can be reduced without dramatic decrease in counter-ion exchange efficiency. However, the replacement of the TFA^- was not complete, and some amounts of this ion were present. Moreover, mostly due to the large amount of the Cl^-, the excess of the total concentration of ions was observed.

Further studies are needed to develop techniques for simultaneous determination of the peptide and counter-ions determination, for instance, with the use of charged aerosol and/or evaporative light scattering type detectors. The comparison of different techniques (i.e. ion exchange vs freeze drying) of replacing counter-ions during the synthesis and purification of peptides is also needed. Appropriate combination of the best analytical method with proper purification processes is crucial for the synthesis of bioactive compounds.

Another important issue is to assess the influence of the total excess of counter-ions on the biological activity of the peptide.

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