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Short Communication

Elimination and exchange of trifluoroacetate counter-ion from cationic peptides: a critical evaluation of different approaches

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Abstract: Most synthesized peptides are nowadays produced using solid-phase procedures. Due to cleavage and purification conditions, they are mainly obtained in the presence of trifluoroacetic acid (TFA) and, for cationic peptides, as trifluoroacetate (TF-acetate) salts. However, TF-acetate interferes with physicochemical characterizations using infrared spectroscopy and might significantly affect the *in vivo* studies. Thus, TF-acetate exchange by another counter-ion is often required. Up to now, the classical procedure has consisted of freeze-drying the peptide several times in the presence of an excess of a stronger acid than TFA (pKa $\sim$0): generally HCl (pKa $= -7$). This approach means that working at pH <1 can induce peptide degradation. We therefore tested three different approaches to exchange the tightly bound TF-acetate counter-ion from the dicationic octapeptide lanreotide: (i) reverse-phase HPLC, (ii) ion-exchange resin, and (iii) deprotonation/reprotonation cycle of the amino groups. The first two approaches allow the partial to almost complete exchange of the TF-acetate counter-ion by another ion from an acid weaker than TFA, such as acetic acid (pKa $= 4.5$), and the third requires a basic solution that permits the complete removal of TF-acetate counter-ion. The efficiency of these three procedures was tested and compared by using different analytical techniques such as ^{19}F-NMR, ^{1}H-NMR and attenuated total reflectance Fourier transformed infrared spectroscopy (ATR FT-IR). We also show that ATR-IR can be used to monitor the TFA removal. The counter-ion exchange procedures described in this study are easy to carry out, fast, harmless and reproducible. Moreover, two of them offer the very interesting possibility of exchanging the initial TF-acetate by any other counter-ion. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ion exchange; TFA removal; solid-phase peptide synthesis; lanreotide; FT-IR spectroscopy; ^{19}F-NMR; ^{1}H-NMR; RP-HPLC

INTRODUCTION

In the past few decades, the field of peptide synthesis has been in full expansion. Solid-phase peptide synthesis (SPPS) introduced by Merrifield [1] is the most commonly used method in industry as well as in academic laboratories. Since the earlier developments of SPPS, many commercial resins have been developed to permit optimal trifluoroacetic acid (TFA) cleavage and deprotection. Therefore, the peptides after purification are very often in TFA excess and, for cationic peptides, are obtained as trifluoroacetate (TF-acetate) salts [2].

In all cases, TFA needs to be removed due to its drawbacks related to several physicochemical characterizations and biological studies. The presence of TFA can change the behavior of the peptide [3] or modify its conformation [4,5]. Moreover, it has been shown that even small amounts of TFA present in commercial peptides alter the accuracy of *in vivo* studies [6]. Finally, the presence of TFA and TF-acetate counter-ion hampers physicochemical characterizations using FT-IR spectroscopy [7–9].

Infrared spectroscopy is very convenient and widely used to study the whole protein and peptide secondary structure. In particular, attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (FT-IR) characterizes the conformational changes of peptides and proteins in solution under different experimental conditions [10,11]. Furthermore, it is a nondestructive technique applicable to experiments in all solvents (aqueous or not), using very small amounts of product (less than 0.1 mg). However, TFA as well as TF-acetate give rise to a strong IR absorption band at 1673 cm^{-1} [8,9] and overlap the amide I mode between 1600 and 1700 cm^{-1}. This prevents the analysis of this spectral region, which contains information on the peptide/protein secondary structure.

Even though the excess TFA is easy to remove by a classic freeze-drying method, it is more challenging to remove the TF-acetate counter-ion, which strongly interacts with the positive charges of cationic peptides.

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Hitherto, the most convenient procedure to replace the TF-acetate counter-ion by another counter-ion has been mixing the peptide with an excess of a stronger acid than TFA, followed by a freeze-drying step [12]. A stronger acid than TFA is able to reprotonate the TF-acetate into its free acid form, which is then easily removed by freeze-drying [13]. However, this approach has essentially two drawbacks: (i) the very acidic solution can chemically damage the peptides, and (ii) the very restrictive choice of possible counter-ions, i.e. the counter-ions that are associated with strong acids (e.g. HCl, H_2SO_4, HF, HNO_3).

The TF-acetate exchange was tested on the dicationic octapeptide lanreotide [14] synthesized in our laboratory by SPPS using an Fmoc strategy. Three different approaches were investigated: (i) RP-HPLC using acetic acid in the mobile phase, (ii) ion-exchange resin loaded with diluted acetic acid, and (iii) deprotonation/reprotonation cycle of lanreotide positive charges ($-NH_3^+$). The removal of TF-acetate counter-ions and its exchange by acetate were followed by quantitative ^{19}F-NMR and ^{1}H-NMR, respectively. Finally, we compared the ATR FT-IR spectra of lanreotide before and after the counter-ion exchange procedures.

MATERIALS AND METHODS

Materials and Sample Preparation

Chemicals. All solvents (reagent grade) were from Sigma-Aldrich (St. Louis, MO, USA). N^{α}-Fmoc protected amino acids were from Bachem (Weil am Rhein, Germany), the resin for peptide synthesis was from Novabiochem (Merck Bioscience, Schwalbach, Germany). The column for peptide purification was a Thermo HyPURITY C18 250 × 21.2 mm (Waltham, MA, USA). The column for peptide characterization was a XBridge C18 3 µm (4.6 × 100 mm) from Waters. The anion resin (AG1-X8, quaternary ammonium) was from BioRad (Hercules, CA, USA).

Deionized water (18 MΩ) was obtained using a Millipore Milli-Q Plus resin exchanger (Billerica, MA, USA). Lanreotide-acetate (MW = 1216 Da, purity >95% and acetate-to-peptide ratio = 2.5) was a gift from Ipsen-Pharma (Paris, France).

Lanreotide-trifluoroacetate synthesis. Lanreotide-TF-acetate was prepared in batches using a standard Fmoc protocol on rink-amide-4-methylbenzylhydrylamine (MBHA) resins. After peptide assembling, the disulfide bridge was produced by three iodine treatments and subsequent washings. Cleavage from the solid support was performed by treatment with a TFA/triisopropylsilane/water (95 : 3 : 2) mixture for 4 h at room temperature. The peptide was freeze dried and purified by RP-HPLC using a HyPURITY C18 column (250 × 21.2 mm) and eluted with an isocratic mixture of acetonitrile/water/TFA (80 : 20 : 0.1%). Peptide purity and integrity were assigned by ^{1}H-NMR and LC-MS.

Freeze-drying. Samples in water were frozen using liquid nitrogen and connected overnight to a Christ Alpha 2–4 LD plus freeze-drying system.

EXCHANGE PROCEDURES

High-Performance Liquid Chromatography (HPLC)

HPLC exchange was performed on a Waters 2545 apparatus, the column was Thermo HyPURITY C18 21.2 × 250 mm and the detection was performed by diode array (Waters 2996). Sampling and fraction recovery were done using a Waters 2767 Sample Manager.

A solution consisting of 10 mg of peptide as TF-acetate salt, 400 µl of water and 100 µl of acetonitrile was injected into an RP-HPLC system using a standard isocratic eluent consisting of water/acetonitrile 8 : 2 containing 1% acetic acid as ion pairing agent. The retention time was 7.75 min at a flow rate of 20 ml/min. The lanreotide-TF-acetate salt was thus in the presence of 196 equiv of acetic acid during the chromatographic run. The resulting fractions were pooled, concentrated to 30 ml under vacuum and freeze dried before analysis.

Resin-Ion Exchange

To conduct the exchange from TF-acetate to acetate, a strong anion resin was chosen (AG1-X8, quaternary ammonium). In order to ensure a total counter ion exchange, a high excess of 245 mg of this resin was prepared as follows: the resin was successively washed three times with 1.6 N acetic acid (10 ml) and three times with 0.16 N acetic acid (10 ml). The peptide solution (19.9 mg in 4 ml of water) was then poured on the resin. After 1 h of rotary stirring, the peptide-resin suspension was filtered and the liquid phase recovered. The resin was washed twice with 1 ml of water, the solutions were pooled and the peptide was isolated after freeze-drying. The pH was monitored during the process. The initial pH of the peptide TFA salt in pure water was 6.5, and the pH of the resin beads in 4 ml of pure water was 3.1, which can be considered as a mild pH.

Deprotonation/Reprotonation Procedure

The lanreotide-TF-acetate salt was dissolved in deionized water to a final concentration of 15 mg/ml at 4 °C. Lanreotide contains two positive charges carried by the NH_3^+-terminus and the lysine side chain with pKa values of about 8.5 and 9.5, respectively. In solution, these two positive charges are neutralized by two negative charges provided by the COO^- groups of the TF-acetate counter-ions. An NaOH solution (100 mM) was slowly added to bring the pH to 11 and the mixture was incubated for 10 min at 4 °C. Under these conditions the peptide is insoluble and precipitates. The peptide suspension was then centrifuged at 4 °C for 5 min, 2500 g using a 5810 R Eppendorf centrifuge (Hamburg, Germany). The supernatant, which contains the sodium TF-acetate salt, was removed. The pellet was successively re-suspended in 6 volumes of deionized water

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and centrifuged in order to remove all traces of sodium TF-acetate salt. The pellet, which contains the neutral form of the peptide, was finally suspended in a few milliliters of water and freeze dried. The neutral peptide powder was dissolved in a solution containing 2 equivalents of acetic acid to yield an acetate-to-peptide molar ratio of about 2.

ANALYTICAL METHODS

Nuclear Magnetic Resonance

NMR was conducted on a Bruker Avance 400 MHz spectrometer. ^{1}H- and ^{19}F-NMR were conducted in D_2O (400 µl). ^{19}F spectra were recorded at an analysis frequency of 376.4 MHz using a single pulse sequence. Spectral window was 7.5 kHz, and $\pi/2$ pulse width was 19.5 µs. Relaxation time was 2 s, acquisition time 0.87 s and 16 scans were recorded for each analysis. Internal standards were dichloroacetic acid and trifluoroethanol for ^{1}H- and ^{19}F-NMR, respectively. Chemical shifts are expressed in ppm and the coupling constants in Hertz.

^{1}H-NMR. NMR tubes were prepared by accurate weighing of the freeze-dried peptide powder and 20 mg of a previously prepared dichloroacetic acid solution (65.6 µmol/g). The peptide content was determined by comparing the areas of the dichloroacetic acid having typical single resonance at 6 ppm and one of the tyrosine 'doublet' resonances around 6.7 ppm. The peptide recovery was determined by comparing the peptide content before and after the experiment.

^{19}F NMR. The TFA spectrum shows a typical single resonance at −75.7 ppm and the TFE spectrum a triplet resonance at −76.8 ppm for TFE (J^{HF} couplings). The ratio between the areas of the two signals directly indicates the TF-acetate content of the solution.

ATR FT-IR Spectroscopy

ATR FT-IR spectra were recorded at 20 °C using a Bruker IFS 66 spectrophotometer (Karlsruhe, Germany) equipped with a 45°N Znse ATR attachment, which was continuously purged with dry N_2 gas. Ten microlitres of the peptide samples were deposited on the ATR crystal; 30 scans were averaged at a resolution of 4 cm^{-1}. The peptide concentration used for the ATR FT-IR measurements was 100 mg/ml in pure water. The infrared absorption spectrum of the peptide was extracted from the raw data by subtracting the water spectrum taken the same day from the peptide solution spectrum. Analysis was done using OPUS software 4.2 (Bruker optic).

RESULTS AND DISCUSSION

Lanreotide-acetate (NH_2-D-2-Nal-cyclo-[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr-$CONH_2 \cdot 2CH_3CO_2H$) is an octapeptide with a buffering capacity around pH 6 in water. At this pH the lanreotide is predominantly in its protonated dicationic form (NH_3^+-terminus and NH_3^+ of the lysine side chain). It was synthesized using standard SPPS and obtained as the TF-acetate salt (see Materials and Methods).

Evaluation Procedure

The different techniques investigated in this article (RP-HPLC, resin-ion exchange and deprotonation/reprotonation of amine groups) were evaluated using different key parameters, i.e. TF-acetate/peptide and acetate/peptide molar ratios and peptide recovery (Table 1). Quantitative ^{1}H-NMR was used to determine both peptide recovery and final acetate/peptide molar ratio. Quantitative ^{19}F-NMR was used to determine the TF-acetate/peptide molar ratio. These measurements were systematically performed on the starting material (lanreotide-TF-acetate salt) and after each procedure tested in this study. A successful exchange should result in only trace amounts of TF-acetate and optimal peptide recovery. All the exchange procedures were tested on the same batch of TF-acetate-lanreotide.

Figure 1 shows the ^{19}F-NMR spectra of the starting TF-acetate-lanreotide (Figure 1(a)) and the ^{19}F-NMR spectra of the peptide after HPLC (Figure 1(b)),

Table 1 TF-acetate/peptide ratios, acetate/peptide molar ratios and peptide recovery before and after each exchange procedure[a]

Counter-ion exchange procedure	TF-acetate/peptide molar ratio	Acetate/peptide molar ratio	Peptide recovery %
Characteristics of the initial peptide	4.5 : 1	0	
HPLC	1.6 : 1	4 : 1	~80
Ion-exchange resin	0.14 : 1	5.3 : 1	>95
Deprotonation/reprotonation cycle	<0.05 : 1	2 : 1	>95

[a] Values were determined by quantitative ^{1}H- and ^{19}F-NMR.

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ion-exchange resin (Figure 1(c)) and deprotonation/reprotonation (Figure 1(d)) procedures. Integration of the TF-acetate signal gives a TF-acetate to peptide molar ratio of 4.5 for the starting peptide (Figure 1(a) and Table 1). Comparison of the peptide ^{19}F-NMR spectra obtained after the three procedures shows that the concentration of TF-acetate is reduced by a factor of about 3 after HPLC procedure, while for the other two procedures the elimination is almost complete (reduction factor >30 for the ion-exchange resin and reduction factor >100 for the deprotonation/reprotonation cycle method). The new counter-ion, i.e. acetate was quantified by ^{1}H-NMR (Table 1). Only the deprotonation/reprotonation procedure allows control of the quantity of acetic acid used for the neutralization (see Materials and Methods). In the other procedures, a large excess of acid is used either in the eluent or for the preparation of the resin. Therefore, if one wants as little acetate as possible in the final product, both HPLC and ion-exchange resin procedures require an additional dialysis step before freeze-drying. The three procedures give acceptable (80% for HPLC approaches) to very good (>95% for ion exchange and deprotonation/reprotonation approaches) peptide recovery.

In conclusion, the HPLC approach seems less efficient than the other two in terms of TF-acetate removal and peptide recovery. Depending on the peptide studied, one can choose between ion-exchange resin and deprotonation/reprotonation procedures. The resin approach uses low pH (~3) but mild acidic conditions and is thus suitable for base-sensitive sequences. On the other hand, for peptides tolerating mild basic condition (pH ~ 11) without degradation, the deprotonation/reprotonation procedure allows addition of a specified quantity of counter ion, thereby preventing any acid excess in the final compound.

As a general rule, these two last procedures can be extended to a wide choice of counter-ions. Resins can be bought or prepared with several different anions and any acid can be used for the reprotonation of the neutral peptide.

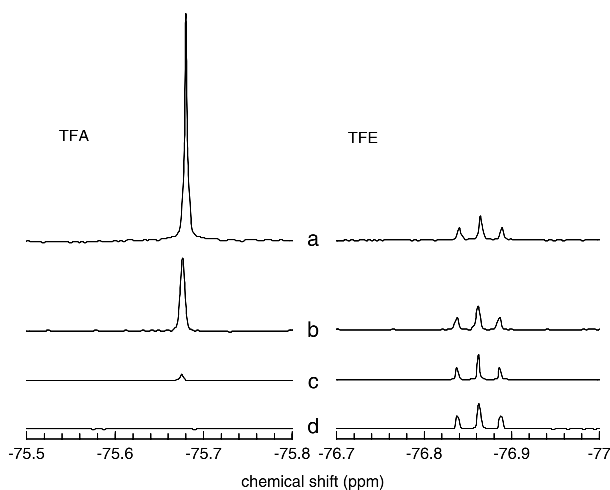

Figure 1 Solution ^{19}F NMR spectrum of Lanreotide as TFA salt in D_2O (400 ml) at 3 mM with internal reference of TFE (1 : 1 with peptide) before any ion exchange (a); after HPLC with acetic acid (b); after resin-ion exchange (c); and after deprotonation/reprotonation cycle (d). Triplet signals arise from the J^{HF} couplings of the three fluorine atoms in TFE.

ATR FT-IR Characterization After Exchange with Different Counter-Ions

Figure 2 compares the ATR FT-IR spectra recorded for the initial lanreotide-TF-acetate salt (Figure 2(e)) and the peptide recovered after the HPLC procedure, the resin-ion exchange and the deprotonation/reprotonation cycle (Figure 2(a), (b) and (c) respectively). We also compared these spectra with the commercially available lanreotide-acetate salt (Figure 2(d)) and the sodium TF-acetate (Figure 2(f)) spectra. We focused on two regions of the ATR FT-IR spectra: the region of the C–F stretching mode (1110–1220 cm^{-1}) [15] and the amide I region (1550–1750 cm^{-1}).

Concerning the C–F stretching mode, as expected TF-acetate presents two major absorption peaks centered at 1147 and 1202 cm^{-1} (Figure 2(f)). By contrast, the lanreotide-acetate spectrum in this region shows only weak absorption peaks centered at 1150 and

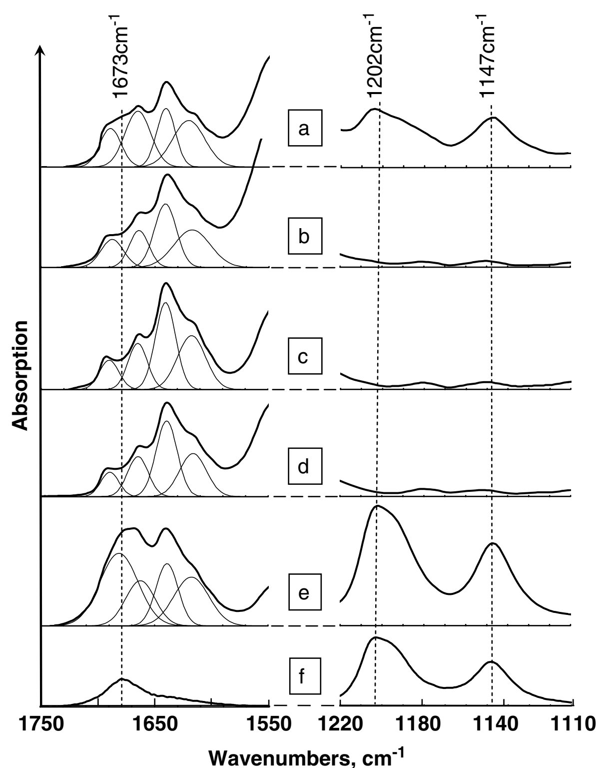

Figure 2 ATR FT-IR spectra of lanreotide (10%) after the RP-HPLC elution (a), lanreotide (10%) after the resin-ion exchange procedure (b), lanreotide (10%) after the precipitation procedure (c), commercial lanreotide-acetate (10%) (d), lanreotide-TF-acetate (10%) (e), and TF-acetate (f).

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1180 cm^{-1}, which correspond essentially to the C–N stretching of the peptide skeleton and aromatic modes (Figure 2(d)). Therefore, spectra recorded in this region are used to check the efficiency of TF-acetate removal. Comparison of the ATR FT-IR spectra of the lanreotide obtained after the three TF-acetate removal procedures clearly shows that after the ion-exchange resin and the deprotonation/reprotonation cycle the remaining amount of TF-acetate is not detected (Figure 2(b) and (c)), whereas after HPLC some TF-acetate are still present in solution (Figure 2(a)). The amount was evaluated by comparing the area of the peak at 1202 cm^{-1} after HPLC procedure to that determined from a TF-acetate spectrum at a precise concentration. We found a TF-acetate/peptide ratio of about 1.4 (±0.1), whereas the initial lanreotide-TF-acetate/peptide ratio was about 4 (±0.5). These results are in full agreement with the ^{19}F-NMR analysis (Table 1). Therefore, ATR FT-IR spectra in the region between 1110 and 1220 cm^{-1} can be used to estimate TF-acetate removal.

The amide I spectral region (1600–1700 cm^{-1}) mainly corresponds to the absorption of the carbonyl and more precisely to the C=O stretching mode of the peptide backbone. This region contains crucial information about the secondary structures adopted by the peptide. The absorption wave number depends on the conformation of the H-bond in which the carbonyl groups are involved and can be analyzed in terms of antiparallel β-sheet, β-turn, α-helix, and random-coil conformation [7,8,16]. The number of backbone carbonyls of commercial 10% lanreotide (w/w) involved in H-bonds in different conformations has been previously estimated after decomposition of the amide I band into four major peaks [17]. Comparison of the area of each peak to the total area of the amide I band was used to determine the number of carbonyls involved in the different types of H-bonds. This previous work shows that, for 10% acetate peptide, three carbonyls were in an antiparallel β-sheet (1618 and 1689 cm^{-1}), three in a β-turn (1663 cm^{-1}), and two in a random coil (1639 cm^{-1}). By using the same approach we found the same results for the conformation of the commercially available acetate peptide (Figure 2(d) and Table 2).

The shape of the amide I absorption spectrum is strongly affected by the presence of TF-acetate salt, as shown by comparing the spectrum of lanreotide-TF-acetate (Figure 2(e)) and lanreotide-acetate salts (Figure 2(d)). The spectrum of sodium TF-acetate shows a strong absorption band at 1673 cm^{-1} (Figure 2(f)) [8] corresponding to the carbonyl of TF-acetate counter-ion. Not surprisingly, decomposition of the amide I spectra leads to erroneous interpretation of the conformation of lanreotide in terms of secondary structure (Table 2). As a consequence, the decomposition of the amide I band spectrum of the peptide that still contains some TF-acetate after HPLC procedure results in an overestimation of the antiparallel β-sheet content of the sample, as for the initial TF-acetate salt of lanreotide (Table 2). Interestingly, decomposition of the amide I region recorded for the peptide recovered after the ion-exchange resin gave slightly different results compared with the commercial acetate salt of lanreotide, yet almost all the TF-acetate has been eliminated. Decomposition leads to an overestimation of the number of carbonyls involved in H-bonding in the antiparallel β-sheet conformation (see Table 2, lanreotide after ion-exchange resin). This spectral misinterpretation is due to the large excess of acetate present after this procedure, as already evidenced by ^{1}H-NMR (see Table 1, ratio). The acetate carbonyl absorbs in the region of the amide II, i.e. between 1500 and 1600 cm^{-1} (data not shown). The excess acetate and TF-acetate that remain after HPLC procedure increases absorption in the amide II region and distorts the shape of the amide I region, leading to erroneous estimation of the peptide conformation (Table 2). This underlines the necessity to dialyze the peptide solutions obtained after resin-ion exchange and HPLC procedures before freeze-drying if ATR-IR is chosen for further characterization.

Spectral decomposition of the amide I band of the peptide recovered after deprotonation/reprotonation gives an estimation of the number of backbone carbonyls involved in H-bonds in different conformations

Table 2 Number of carbonyls per lanreotide molecule involved in different H-bond conformations, calculated as described in 2004 by Valery C. and coworkers [17]

Samples	Number of carbonyls involved in β-sheet	Number of carbonyls involved in β-turn	Number of carbonyls involved in random coil
Commercial lanreotide-acetate salt	**3**	**3**	**2**
Lanreotide -TF-acetate salt	5	1.5	1.5
Lanreotide after RP-HPLC	**5**	**2**	**1**
Lanreotide after resin-ion exchange	4	2	2
Lanreotide after deprotonation/reprotonation cycle	**3**	**3**	**2**

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similar to that obtained for the commercial peptide (Table 2). For the two lanreotide-acetate salts, the acetate-to-peptide ratio provided by Ipsen-Pharma was similar to the ratio we measured after our TF-acetate/acetate exchange procedure.

In conclusion, ATR FT-IR allows semiquantitative checking of the elimination of TF-acetate as well as TFA.

CONCLUSION

In this study, we have shown that RP-HPLC is not an efficient method for exchanging a peptide-TF-acetate salt for an acetate salt. We have described two fast, efficient and reproducible protocols to remove TF-acetate counter-ions. These two methods were monitored by both ^{19}F-NMR and ^{1}H-NMR spectroscopy. More than 95% TF-acetate counter-ions were exchanged with acetate counter-ion. The resin-ion exchange procedure is performed in mildly acidic conditions, while the precipitation procedure is conducted in basic conditions: one of the two procedures can be used depending on the peptide sensitivity. Thereby, these two complementary approaches offer a wider choice in terms of counter-ion in comparison with earlier procedures using strong acids as HCl. These protocols have already been used successfully to exchange TF-acetate counter-ions from synthetic lanreotide with 11 other very different counter-ions (for example, terephthalate, camphosulfonate, benzoate, picrate, methane sulfonate, paratoluene sulfonate, butyrate, pentanoate), thus allowing the study of the influence of the counter-ions on the physicochemical behavior of the peptide in solution.

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