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Determination of residual trifluoroacetate in protein purification buffers and peptide preparations by ion chromatography

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Abstract

Trifluoroacetate (TFA) is commonly used in a variety of pharmaceutical applications. Because of its toxic nature, it is important to reliably measure the effective removal of TFA. We developed an ion chromatography (IC) method to determine the concentration of residual TFA in samples found in the pharmaceutical industry. A high-capacity anion-exchange column was used to separate trace trifluoroacetate from an excess of chloride, phosphate, and other anions without the need for sample preparation. TFA was detected by suppressed conductivity. A method with four KOH eluent step changes was optimized and reproducibly executed using automated generation of the KOH eluent. We used this method to determine TFA in the following samples: a phosphate-buffered saline (PBS), an acetate-buffered saline containing protein, and a commercial peptide. The method detection limits for TFA in these samples were all less than 90 ng/ml.

Keywords: Trifluoroacetate; Peptides

1. Introduction

Trifluoroacetate (TFA) is commonly used during the purification of pharmaceutical and biotechnology products. For example, TFA is used with an acetonitrile gradient on a preparative reversed phase HPLC column to purify synthetic peptides [1]. Because TFA is toxic, its removal must be reliably measured in products intended for preclinical or clinical applications.

Ion-exchange chromatography with conductivity detection [ion chromatography (IC)] is a sensitive and reliable technique for determining TFA. TFA was determined in two buffers used in a recombinant protein recovery process [2] and in commercial peptides [3,4]. These assays used either an IonPac AS4A (250 × 4 mm) or an IonPac AS14 (250 × 4 mm) anion-exchange column with an isocratic carbonate/bicarbonate eluent. These publications reported method detection limits for TFA of 300 ng/ml.

Hydroxide eluents have several advantages over carbonate/bicarbonate-based eluents for ion chromatography [5]. Gradient elution with a hydroxide eluent and continuous eluent suppression allows separation of a very wide range of anionic analytes in a single run. Gradient separations with carbonate-based eluents are difficult and rarely used. The suppressed background conductivity of a hydroxide eluent system is lower than a carbonate-based eluent and yields lower method detection limits.

Fernando and coworkers demonstrated that an IonPac AS11-HC anion-exchange column with a hydroxide-based eluent could be used to determine TFA in a phosphate-buffered saline (PBS) [6]. Their assay validated TFA removal from the PBS. The IonPac AS11-HC's high-capacity (290 μeq./column (250 × 4 mm) column) was ideal for separating TFA from excess concentrations of chloride and phosphate [7]. The authors used an eluent generator to produce a reproducible KOH eluent gradient. TFA could be determined down to 50 ng/ml after reduction of the matrix chloride concentration using an OnGuard Ag pretreatment cartridge (containing silver high-capacity cation-exchange resin).

Recent technological advances have resulted in reagent-free ion chromatography (RFIC). This technology requires only a clean source of deionized water to generate the eluent and regenerants used for IC analysis [5]. For anion analysis, the RFIC system prepares potassium hydroxide eluent that is free of contaminating carbonate anion. A continuously regenerated trap column removes anionic contaminants from the source water and does not require

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Table 1
Chromatographic conditions

Eluent	Potassium hydroxide (EG50 as the source)
Temperature (°C)	30
EG50 offset volume (μl)	0
Eluent flow rate (ml/min)	1.0
Detection	Suppressed conductivity, ASRS-ULTRA recycle mode

Method program

Time (min)	Valve	EG50 concentration (mM)	ASRS current (mA)	Comments
0.00	Load	22.0	80	Load sample loop, 80 mA ASRS current setting acquisition ON
6.00	Inject	22.0	80	
6.01	Inject	28.0	80	Step to 28 mM KOH
12.00	Inject	28.0	80	
12.01	Inject	50.0	80	Step to 50 mM KOH for cleanup
14.00	Inject	50.0	124	Step to 124 mA ASRS current
15.00	Inject	50.0	124	
15.01	Inject	22.0	124	Step back to 22 mM KOH
17.00	Inject	22.0	80	Step back to 80 mA ASRS current
20.00	Inject	22.0	80	Acquisition OFF

regeneration. After separation on the anion-exchange column, an anion self-regenerating suppressor automatically suppresses the eluent and the sample anions are detected by suppressed conductivity. Methods developed with RFIC can be accurately transferred to other systems and laboratories. The goal of this investigation was to improve the determination of TFA in samples of interest to the pharmaceutical and biotechnology industries by using RFIC with the IonPac AS18 anion-exchange column. This is a high-capacity anion-exchange column that has a different selectivity for TFA and common inorganic anions compared to the IonPac AS11-HC.

2. Experimental[1]

2.1. Chromatographic system

All analyses were performed on a Dionex (Sunnyvale, CA, USA) ICS-2500 ion chromatograph. The system consists of a GP50 gradient pump, CD25A conductivity detector, a LC30 liquid chromatography oven, EG50 eluent generator, and AS50 autosampler. A personal computer equipped with Dionex Chromeleon chromatography software was used for data acquisition and instrument control.

All columns used in this study were manufactured by Dionex. For the analytical separation, an IonPac AG18 anion-exchange guard column (50 × 4 mm) and IonPac AS18 (250 × 4 mm) analytical column were used. A 4 mm anion self-regenerating ultra suppressor (ASRS) from Dionex operated in the recycle mode was used to reduce the conductivity of the eluent. The tubing used to connect the chromatographic components was 0.010 in. i.d. (0.125 mm) polyether ether ketone (PEEK). The Dionex continuously regenerated anion trap column (CR-ATC) was used to remove anionic contaminants from the eluent. Table 1 lists the chromatographic conditions for the method.

The packing material for the IonPac AG18 and AS18 is composed of a highly cross-linked core with an anion-exchange latex attached to the surface. The resin consists of polyvinylbenzyl ammonium polymer 55% cross-linked with divinylbenzene. The substrate for the AG18 is a microporous 13 μm-diameter bead with a pore size less than 1 Å. The substrate for the AS18 is a super macroporous 7.5 μm-diameter bead with a pore size of 2000 Å. The surface anion-exchange layer consists of 65 nm-diameter particles bonded to the substrate.

2.2. Chemicals, solutions and samples

Reagent grade chemicals were used for preparation of the TFA standards and buffers. Deionized water with a specific resistance of 18 MΩ-cm or greater from a deionized water purification system was used to prepare all eluents, reagents, and standards. A stock standard solution of 1000 mg/l trifluoroaceate was prepared with reagent grade sodium trifluoroacetate from Fisher Scientific (Pittsburgh, PA, USA).

The following samples were prepared: a PBS solution, a buffer used in a recombinant protein recovery process, and a commercial peptide. PBS, pH 7.4 was prepared with 1.06 mM potassium phosphate, monobasic (KH_2PO_4),

[1] Reagent-Free is a trademark and IonPac, Chromeleon, and ASRS are registered trademarks of the Dionex Corporation.

155.17 mM sodium chloride, and 2.96 mM sodium phosphate, dibasic 7-hydrate ($Na_2HPO_4{\cdot}7H_2O$) using reagent grade salts from Fisher [6]. A protein purification buffer was prepared with 0.1 M acetic acid, 0.25 M sodium chloride, 0.01% polyoxyethylenesorbitan monolaurate (Tween-20) (Sigma–Aldrich, St. Louis, MO, USA), and 1 mg/ml bovine serum albumin (Fluka, Buchs, Switzerland) [2]. A solution of a 40 μg/ml angiotensin was prepared using the acetate salt of human angiotensin II from Sigma–Aldrich.

3. Results and discussion

3.1. Method optimization

An anion-exchange column for monitoring residual TFA in high-ionic-strength pharmaceutical buffers should ideally have two characteristics. The column should have a sufficient capacity for the highly ionic matrix and should separate TFA from the matrix anions that are present at high concentrations. The IonPac AS18 column has both of these characteristics. The 4 mm AS18 column has an anion-exchange capacity of 285 μeq. and is an excellent match for the target application. TFA is well resolved from the early eluting anions when using the AS18 column under optimized conditions.

The best separation of TFA from the anions in the samples evaluated in this report was achieved with a series of eluent concentration step changes. The method began with an initial eluent concentration of 22 mM KOH to elute weakly retained ions such as fluoride, acetate, and formate. An eluent step change to 28 mM KOH at 6 min was used to elute trifluoroacetate. After TFA elution, the eluent stepped to 50 mM KOH at 12 min to clean the column of any highly retained matrix anions. Afterwards the eluent was stepped back to 22 mM KOH to reequilibrate the column for the next injection. This optimized eluent step change method was quickly developed by using RFIC. RFIC allowed us to program a number of isocratic methods and linear gradient eluent methods to evaluate a variety of eluent conditions. Without RFIC we would have had to prepare different eluent concentrations in order to achieve ideal eluent proportioning for proper method evaluation. Reproducibility problems in eluent step change methods that are usually associated with variable delay volumes were avoided because the EG50 is after the pump and before the column. These methods can be readily transferred to other systems and other laboratories because the delay volume is minimal and easily controlled. Separations were performed at 30 °C to provide the best retention time reproducibility. Fig. 1 shows the separation of an anion standard that includes TFA using our optimized method. Detection sensitivity was optimized by adjusting the current supplied to the ASRS with respect to the eluent concentration. These adjustments were made based on values provided by the chromatography software and current changes were delayed 2 min relative to the eluent change to account for the time required for the new eluent to reach the ASRS.

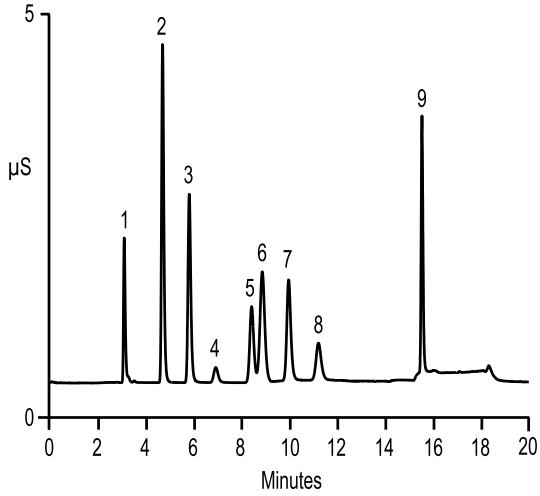

Fig. 1. Anion standard with TFA in deionized water. Peaks: (1) fluoride (2 mg/l); (2) chloride (4 mg/l); (3) nitrite (10 mg/l); (4) carbonate; (5) bromide (10 mg/l); (6) sulfate (10 mg/l); (7) nitrate (10 mg/l); (8) trifluoroacetate (10 mg/l); (9) phosphate (10 mg/l). Sample volume: 5 μl; for chromatographic conditions see Table 1.

3.2. Method performance

This method was applied to the determination of TFA in the following samples: PBS [6], a buffer used in a recombinant protein recovery process [2], and a commercial peptide [3,4]. Fig. 2 shows the determination of 300 ng/ml of TFA in PBS using a 100 μl injection with an overlay of unspiked PBS. This separation is challenging because TFA elutes between an excess of matrix anions. TFA (peak 2) is baseline resolved and is separated by more than 4 min from the end of the excess chloride peak and separated by more than 2 min from the beginning of the phosphate peak.

The retention time (t_R) for TFA was affected by the matrix and to a lesser extent the injection volume of the sample. This explains the difference between the t_R for a 5 μl injection in deionized water (11.2 min, Fig. 1) and the t_R for a 100 μl injection in PBS (12.7 min, Fig. 2). By spiking increasing amounts of TFA in each sample matrix we were able to unambiguously identify TFA in all samples. Also, because TFA was the only analyte of interest we used the chromatography software to limit the integration window so that automated TFA peak area quantification was unaffected by the other analytes.

A calibration curve was obtained using TFA standards at 100, 300, and 1000 ng/ml prepared in PBS. Three replicate injections were made at each concentration level. A plot of TFA peak area versus amount injected yielded a linear response with a coefficient of determination (r^2) of 0.9979. The method detection limit (MDL) was estimated to be 100 ng/ml TFA in PBS by measuring a TFA peak three-times higher that the background noise (S/N = 3). An

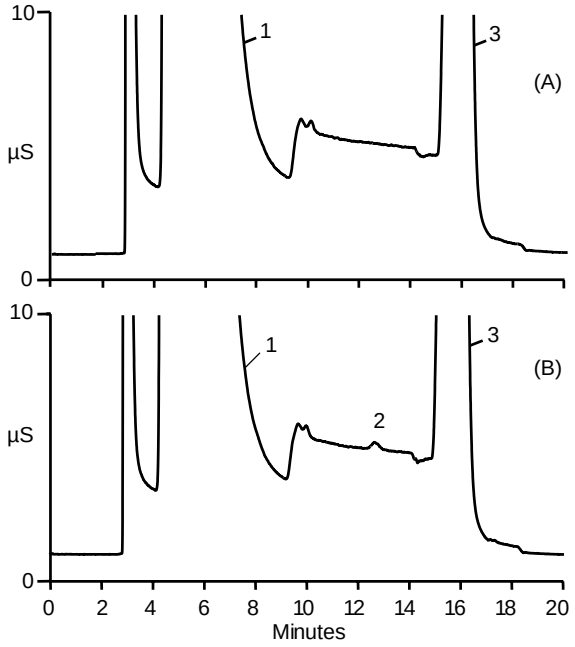

Fig. 2. Determination of trace TFA in phosphate-buffered saline: (A) unspiked; (B) 300 ng/ml trifluoroacetate. Sample: 1.06 mM potassium phosphate, monobasic (KH_2PO_4), 155.17 mM sodium chloride, and 2.96 mM sodium phosphate, dibasic 7-hydrate ($Na_2HPO_4{\cdot}7H_2O$). Peaks: (1) chloride; (2) trifluoroacetate; (3) phosphate. Sample volume: 100 μl; for chromatographic conditions, see Table 1.

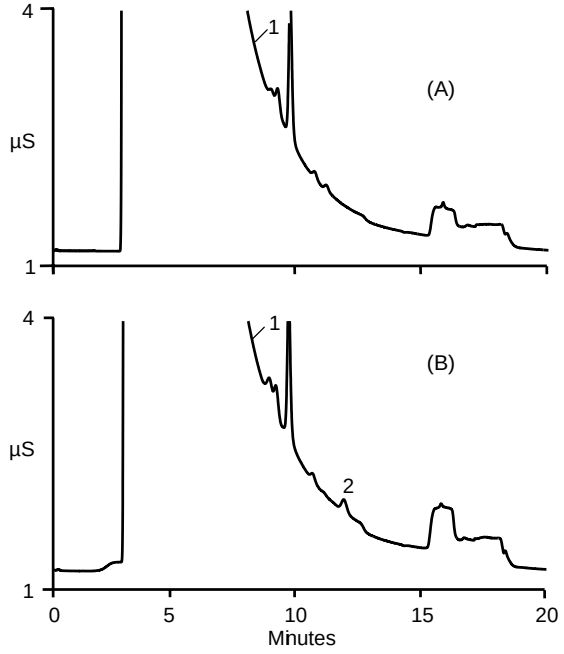

Fig. 3. Determination of trace TFA in protein buffer: (A) unspiked and (B) spiked with 300 ng/ml trifluoroacetate. Sample: protein buffer containing 0.1 M acetic acid, 0.25 M sodium chloride, 0.01% Tween-20, pH 3, and 1 mg/ml bovine serum albumin; Peaks: (1) acetate/chloride; (2) trifluoroacetate. Sample volume: 50 μl; for chromatographic conditions, see Table 1.

MDL for TFA was calculated using the standard deviation for seven replicate injections of 100 ng/ml TFA in the PBS [8]. The standard deviation was multiplied by the Student's *t*-value for the 99.5% confidence limit. The method detection limit for TFA was calculated to be 86 ng/ml in the PBS matrix under these conditions. Recovery of TFA for a 300 ng/ml spike in PBS was 98.7% for (6) replicate injections (293 ± 2.7 ng/ml). The retention time of TFA was 12.6 ± 0.021 min with an R.S.D. of 0.17%. These results compare favorably with the work by Fernando et al. using the IonPac AS11-HC column [6]. They reported an MDL of 10 ng/ml for TFA in PBS after reduction of the matrix chloride concentration using an OnGuard Ag pretreatment cartridge. We report an MDL of 86 ng/ml for TFA in PBS without a sample preparation step.

We also used this method to determine TFA in a buffer used in a recombinant protein recovery process. This buffer contained 0.1 M acetic acid, 0.25 M sodium chloride, and 0.01% polyoxyethylenesorbitan monolaurate (Tween-20) [2]. We added 1 mg/ml bovine serum albumin to simulate the presence of a negatively charged protein. The method was optimized using a 50 μl injection to give the best sensitivity for the determination of TFA in the buffer. Recovery of TFA for a 300 ng/ml spike in this buffer was 98% for five replicate injections (296 ± 19 ng/ml) based on a calibration curve prepared in the matrix. The retention time of TFA was 12.7 ± 0.021 min with an R.S.D. of 0.17%. Kabakoff and coworkers reported a MDL of 300 ng/ml for TFA in this sample with a 10 μl injection using a 250 × 4 mm IonPac AS14 column (65 μeq./column capacity) with a carbonate-based eluent [2]. By using a higher injection volume (50 μl) with the higher capacity IonPac AS18 column we were able to achieve an MDL of 36 ng/ml for TFA. Fig. 3 shows a chromatogram for a 300 ng/ml spike in this buffer with an overlay of the unspiked buffer. This method was also successfully used to determine TFA in another buffer used in a recombinant protein recovery process that contained 0.1 M Tris pH 7.4, 0.14 M sodium chloride and 0.01% Tween-20 as well 1 mg/ml bovine serum albumin [9].

We also applied this method to the analysis of a commercial peptide preparation. A solution of human angiotensin II protein was prepared at 40 μg/ml in deionized water and found to contain no detectable TFA. Complete recovery of a spike of 100 ng/ml of TFA confirmed that the method was suitable for determining TFA in this sample. The chromatogram of this sample (Fig. 4) shows a large acetate peak, which is expected because this peptide is prepared as an acetate salt, and trace amounts of chloride and sulfate. Due to the lower amount of contaminant anions compared to the other samples analyzed in this paper, a faster separation should be possible for this sample. The calibration results and calculated method detection limit for TFA in the human angiotensin II protein solution as well as the two pharmaceutical buffers are summarized in Table 2. The lowest quantifiable TFA concentration is estimated to be three to

Table 2
Calibration results for the determination of TFA

Sample	Data points	r^2	Slope	Intercept	Dynamic range (ng/ml)	Method detection limit (MDL) (ng/ml)[a]	Standard used to calculate MDL (ng/ml)
Phosphate-buffered saline	9	0.9979	3270 ± 134	28.3 ± 24	100–1000	86	100
Protein buffer	9	0.9997	7630 ± 109	43.4 ± 8.21	100–1000	36	100
Commercial peptide	9	0.9997	5850 ± 88.6	2.85 ± 2.74	30–300	4	10

[a] MDL = (S.D.) × (t_s) 99.5%, where (t_s) is for a 99.5% single sided Student's *t*-test distribution for $n = 7$.

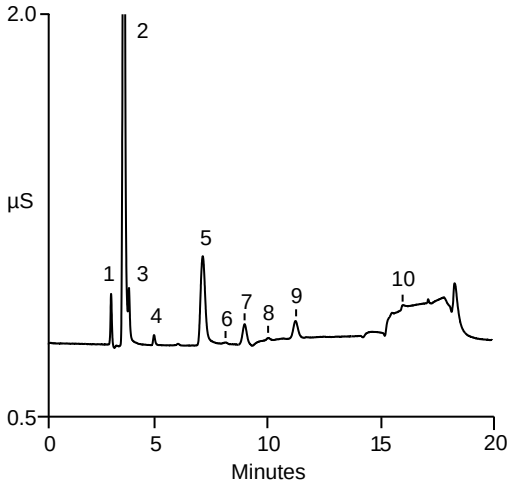

Fig. 4. Determination of trace TFA in commercial peptide. Sample: 40 μg/ml angiotensin II spiked with 100 ng/ml TFA. Peaks: (1) system peak; (2) acetate; (3) formate; (4) chloride (15 ng/ml); (5) carbonate; (6) unidentified; (7) sulfate (140 ng/ml); (8) nitrate (14 ng/ml); (9) trifluoroacetate; (10) phosphate (25 ng/ml). Sample volume: 50 μl; for chromatographic conditions, see Table 1.

five times greater than the lowest detectable concentration reported in Table 2.

4. Conclusion

RFIC was applied to the determination of residual TFA in pharmaceutical applications. The high-capacity IonPac AS18 anion-exchange column with a series of eluent step changes separated TFA from an excess of chloride, phosphate, and other anions. On-line generation of potassium hydroxide eluent allowed for improved sensitivity and method reproducibility without sample preparation. The technique was successfully applied to the analysis of PBS, a buffer used in a recombinant protein recovery process, and a commercial peptide.

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