Analysis of residual trifluoroacetic acid in a phosphate-buffered saline matrix by ion chromatography with suppressed conductivity detection

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Abstract

As part of the formulation of a cell-based pharmaceutical product, cells were harvested from mice and incubated in a cocktail containing cell culture media and high levels of trifluoroacetic acid (TFA). The cells were washed with a phosphate-buffered saline solution to remove residual cell culture media and other reagents before the cells were infused back into the mice from which they originated. Because of the potentially toxic nature of the TFA, the cells were washed multiple times and the final wash was monitored for residual TFA in order to demonstrate the efficient removal of the reagent before the cell product could be reintroduced into the test animal. This report describes the method that was developed incorporating anion-exchange chromatography with suppressed conductivity detection for the analysis of residual TFA (down to 50 ng/ml) in the presence of high concentrations of phosphate and chloride interferences. The ultimate sensitivity of the method was improved by selectively removing halide anions using a silver cartridge before sample analysis. The method proved to be rugged and reproducible enough to be validated and used to monitor residual TFA levels in cell washes in support of an acute toxicological study. Results demonstrating the method's sensitivity, selectivity, precision and linearity were reported.   © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Trifluoroacetic acid (TFA) is used as a reagent in an incubation media to help solubilize various reagents in the production of a cell-based biopharmaceutical product at Pharmacia Research and Development. During formulation, reagents contained in a cell culture media are incubated with cells harvested from mice being used as a toxicological test species. After incubation, the cells are washed with a solution of phosphate-buffered saline (PBS) solution to remove TFA, residual reagents and

unwanted endogenous material before the cells are reintroduced into the test animals. Due to the toxic nature of TFA, its concentration in the final cell wash was monitored to ensure its removal before the cell-based product could be used in the acute toxicology study.

Various methods have been reported for the determination of TFA in pharmaceutical analysis. These include studies involving ion-selective electrodes [1], gas chromatography [2–9], spectrometry [10,11], isotachophoresis [12,13], reversed-phase high-performance liquid chromatography [14] and even the determination of TFA by ion chromatography using suppressed conductivity detection [15–

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17]. However, there are no reports available in the literature for the analysis of TFA in high ionic strength PBS media. Of the above-mentioned techniques, ion chromatography is considered to be a relatively convenient and sensitive technique for the quantitative determination of TFA, and easily lends itself as an automated technique for multi-sample analysis using little, or no, sample preparation.

This paper describes an anion-exchange separation using a polymeric high-capacity AS-11 HC anion-exchange column and suppressed conductivity detection to determine residual TFA concentration. The desired eluent (KOH) concentrations necessary for gradient separation were easily generated on-line by using an eluent generator and chromatography software. The ultimate sensitivity of the method was accomplished by selectively removing chlorides in the PBS matrix using silver cartridges (OnGuard-Ag) for sample clean up. Although this work was stimulated by the need for a simple and reliable method for the determination of low levels of TFA in PBS, this approach is also potentially suitable for the determination of TFA in other systems containing high levels of chloride anion as well. The method presented here has been validated and used to support an acute toxicological study for the determination of trace levels of TFA (50 ng/ml) in PBS washes of a biologically derived cell-based biopharmaceutical product.

2. Experimental

2.1. Chemicals and reagents

Deionized water with specific resistance of 17.8 MΩ cm obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare the stock standards, eluents and final rinsing of glassware.

Trifluoroacetic acid, sodium salt (>99.5%), used in stock standard preparation was purchased from Fluka (Milwaukee, WI, USA). Ammonium nitrate (99.999%), sodium hydrogenphosphate (99.995%) and potassium chloride (99+%) were obtained from Aldrich (Milwaukee, WI, USA) and were used for the preparation of marker standards. Phosphate-buffered saline (pH 7.4, Gibco-BRL) and Opti-Mem I

(reduced serum medium, Gibco-BRL) was purchased from Life Technologies (Frederick, MD, USA). Opti-Mem I in PBS (0.1%, v/v) was used as a diluent in the preparation of working standards in order to mimic the matrix of actual cell wash samples to be submitted for analysis.

2.2. Instrumentation

All experiments were carried out with a DX-500 ion chromatograph (Dionex, Sunnyvale, CA, USA). The system consisted of a quaternary gradient pump (GP40) with automated membrane eluent degassing capability, a chromatographic oven (LC25), an electrochemical detector (ED40), an autosampler (AS40) and an eluent generator (EG40). A 24-port Visiprep DL solid-phase extraction (SPE) vacuum manifold (Supelco, Bellefonte, PA, USA) was used to simultaneously process up to 24 OnGuard-Ag cartridges. A personal computer equipped with Dionex PeakNet chromatography software (5.1) was used for instrument control. Data collection and data processing was performed with the use of the VAX-based data acquisition system (INGRAD) available in our laboratory.

2.3. Sample/standard preparation

Samples of the final PBS cell washes received from Pharmacia Safety Evaluation group, were kept at −80°C until analyzed. Before analysis, sample tubes were thawed in water at room temperature and processed as described below.

The Visiprep DL SPE workstation was set up per vendor guidelines. Ten-milliliter plastic syringes were attached to two luer compatible OnGuard-Ag cartridges in series, which were connected to the luer tapers on the SPE workstation. Each OnGuard-Ag cartridges was conditioned with approximately 10 ml of Milli-Q water by gently pulling vacuum through the cartridges at a flow-rate of <2.0 ml/min. Five milliliters of each sample or standard were placed into the 10 ml plastic syringe and the vacuum slowly increased to start the flow of sample flow through the ion-exchange bed. The first 2 ml of eluent were discarded, the second 2 ml were collected into an autosampler vial for sample analysis and the remain-

ing volume was discarded. Each sample was prepared and analyzed in duplicate.

The standard stock solution of 100 μg/ml trifluoroacetate was prepared by weighing 12.1 mg of trifluoroacetic acid, sodium salt into a 100-ml volumetric flask. The stock standard was dissolved and diluted to volume with Milli-Q water. All other system suitability standards and other related working standards were prepared by appropriate dilutions of the stock standard using the modified PBS media (0.1% Opti-Mem I in PBS) as the diluent to mimic the real sample matrix. All blanks and standards prepared in this manner were subjected to OnGuard-Ag cartridge treatment to remove or reduce the chloride interference before injection.

In addition, to demonstrate that the OnGuard-Ag cartridges do not remove TFA, two sets of recovery standards (5–0.05 μg/ml) were prepared by spiking with the stock TFA standard. In one set, the diluent is the modified PBS in preparation of recovery standards. These standards were then subjected to cartridge treatment (post-treated PBS) and transferred to auto sampler vials. In preparation of the diluent for other set of standards, 50–100 ml of the modified PBS matrix were subjected to OnGuard-Ag cartridge treatment procedure. The collected effluent was then used as the diluent for preparation of recovery standards (pre-treated PBS). The standards prepared in this manner were directly transferred to auto sampler vials.

2.4. Chromatographic conditions

The columns used in this study were manufactured by Dionex. For anion separations (chloride, nitrate, phosphate and trifluoroacetate) an IonPac AS11-HC (250×4 mm) analytical column and IonPac AG11-HC (50×4 mm) guard column were utilized with potassium hydroxide gradient (15–80 mM) with the use of EG40 eluent generator with PeakNet chromatography software. To achieve the separation, the KOH gradient profile was initially 15 mM for 13 min, linearly increased to 80 mM over 0.1 min, held at 80 mM for 2.9 min to elute any strongly retained anions. The concentration was linearly decreased to 15 mM over 0.1 min, and the column was allowed to re-equilibrate at the 15 mM KOH concentration for 3.9 min. The eluent flow-rate was 1.0 ml/min and

the analysis time was 20 min. For the AS11-HC separation, an anion trap column (ATC-1) was used in the eluent line prior to the injection valve to prevent eluent contaminants from causing spurious peaks during gradient chromatography. Injections (100 μl) were made via the auto sampler utilizing 0.5 ml polypropylene vials equipped with 20-μm filter caps (PolyVials, Dionex). The ED40 utilized a Dionex DS3 thermally controlled conductivity cell which was set at an output range of 20 μS. Post-column eluent suppression was accomplished with an Anion Self-Regenerating Suppressor (ASRS-Ultra, 4 mm) in the recycle mode with the ASRS current set at 300 mA. Sample preparation devices containing Ag-form resins were OnGuard brand (Dionex). The devices each contain 0.95 g of 16% crosslinked, hydrated resin with an ion-exchange capacity of 2.5 mequiv./g. The OnGuard-Ag cartridge contains a silver form, high-capacity, strong acid cation-exchange resin.

3. Results and discussion

3.1. Development/optimization of the ion chromatography method

Preliminary development work for assessing the method was designed to understand matrix interference and the method performance characteristics. Since the real samples were not available at the time of development work, sample matrices were made as close as possible to the real samples. For this reason, all TFA standards were prepared in PBS solutions, which were spiked with reduced serum medium (Opti-Mem 1) as described earlier. Due to the complex nature of the sample matrix, a mobile phase gradient (15–80 mM hydroxide) was chosen to remove any highly retained anions (PO_4^{3-}) that could potentially interfere with the rest of the injection sequence and to demonstrate the method specificity. A representative separation of anions present in the preliminary mock sample is shown in Fig. 1. Due to high chloride levels, a high-capacity anion-exchange column was chosen. The increased column capacity allowed more concentrated samples to be injected without column overloading and subsequent peak
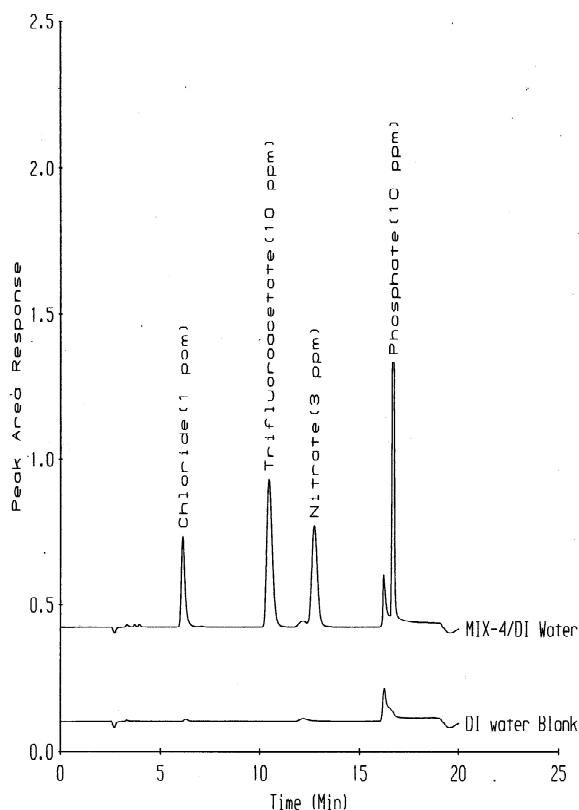
Fig. 1. Chromatogram of mixture of representative inorganic anion standards prepared in Milli-Q water to demonstrate the method specificity.

50 ng/ml and a limit of detection (LOD) of 10 ng/ml could be obtained. This corresponds to a LOQ of 5 ng and a LOD of 1 ng TFA injected on-column. The actual LOD and LOQ of the method, however, was established during sample analysis and method validation, and due to the extremely high concentration of chloride in the matrix, as well as the elution of chloride prior to the peak of interest, the TFA analysis down to low μg/ml levels was not possible without first decreasing the size of the chloride peak in the ion chromatogram.

To reduce the chloride peak intensity and maintain the desired LOQ, an OnGuard-Ag cartridge was used to selectively remove or reduce the chloride interference from the sample prior to analysis. The OnGuard-Ag sample pretreatment cartridge, having a capacity of 2.0–2.5 mequiv./cartridge, contains a silver, high-capacity, strong acid cation-exchange resin. For this work, two OnGuard-Ag cartridges connected in series were adequate for reducing the chloride interference for PBS sample sizes of 5 ml.

To demonstrate that the OnGuard-Ag cartridges do not remove TFA, results from TFA spiked standards in pre-treated PBS and post-treated matrices were compared. The recovery data obtained by comparing the TFA peak areas in pre-treated and post-treated spiking standards were listed in Table 1. The data suggest that there is no evidence that TFA is absorbed or removed with the use of OnGuard-Ag cartridges. The recoveries of TFA at 0.05 μg/ml (100±15%) and at 5–1 μg/ml (100±2%) are acceptable for our study. Fig. 2 shows the effect of the OnGuard-Ag cartridge on selectively reducing

broadening. Furthermore, the higher-capacity column allowed the injection volume to be increased to 100 μl so that the desired limit of quantitation (LOQ) of

Table 1
Recovery data from pre- and post-treated (OnGuard-Ag) TFA standards in PBS

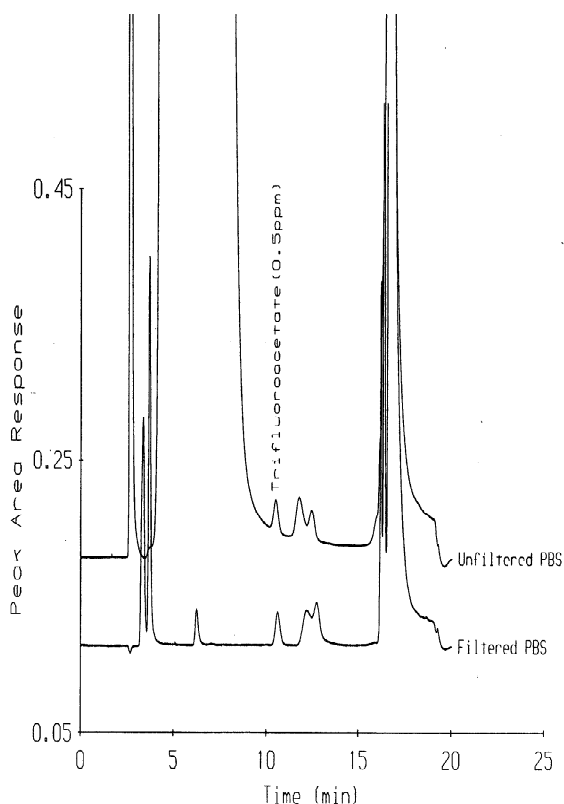| Spiked TFA level (μg/ml) (approximate)	Peak area (post-treated PBS) mean ($n=3$)	Peak area (pre-treated PBS) mean ($n=3$)	Recovery (%)
5	0.07897997 RSD=0.18%	0.07933427 RSD=0.63%	99.6
1	0.01490223 RSD=0.24%	0.01477450 RSD=0.21%	100.9
0.5	0.00714850 RSD=1.27%	0.00747550 RSD=0.34%	95.6
0.1	0.001527067 RSD=2.87%	0.001452567 RSD=2.81%	105.1
0.05	0.000857533 RSD=2.0%	0.000806133 RSD=3.9%	106.4

Fig. 2. Chromatograms of TFA standards (1 μg/ml) prepared in PBS media with and without treating with OnGuard-Ag cartridges to demonstrate the effect of chloride removal.

the chloride interferences without affecting the TFA peak of interest.

3.2. Validation of the ion chromatography method

The objective of this study is to validate the ion chromatography method for the determination of residual TFA in PBS media. The validation, based on internal guidelines consistent with International Conference of Harmonization (ICH) guidelines, was carried out on the ion chromatography (IC) method. The area responses for the peak of interest was collected and evaluated. Due to unavailability of authentic blank samples in the quantities required, the validation was performed with a simulated blank media [PBS media fortified with Opti-Mem I (reduced serum medium)]. All the blanks, standards and samples used for the validation study were subjected

to cartridge treatment procedure as described previously. The validation addressed the method linearity, specificity, accuracy, precision, stability and sensitivity. The experimental design of the validation consisted of a 2-day/one-analyst/three replicates per run. For each run of the validation, system suitability standards were evaluated as defined by the method.

The system suitability standards consisting of 0.05 μg/ml TFA were injected throughout the sample run. The relative standard deviation of peak area responses for six replicate injections on both days was <6.0%, which was well within the previously established system suitability criteria of ≤20%. Stability of the TFA standards (at 1 μg/ml) were monitored during the validation experiments over an 8–10-h period. There was no significant change in TFA peak area response over the validation runs. A percent relative standard deviation (RSD) of <2% was achieved which is well below the system suitability criteria of ≤10%.

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Linearity was evaluated by TFA standards prepared over the range of 5–0.05 μg/ml using the standard concentrations of 5, 1, 0.5, 0.1 and 0.05 (μg/ml) (Run 1 only). Fig. 3 shows the chromatograms for the standards from 0.01 to 5 μg/ml TFA. Regression analysis was performed using STDCRV (R&D statistical software package) to assess any significant bias over the linearity range using multipoint calibration (full fit analysis). The correlation coefficient of 0.9999 was obtained for full fit analysis and 0.9991 was obtained for single point analysis at the 1-μg/ml level. In addition, single point analysis at 1 μg/ml was performed to see whether single point can be used to quantitate TFA in the samples without significant biases across the range (Table 2).

The biases over the range 0.05–5 μg/ml were <20% for 0.1 ppm and greater levels for single point analysis at 1 μg/ml. The target bias at 0.05 μg/ml level was <40% at single point analysis at 1 μg/ml per protocol. The bias at the 0.05-μg/ml level is high than the target and when quantified against a single point (1 μg/ml) standard, thus the values at the 0.05 μg/ml (LOQ) region are highly inflated.
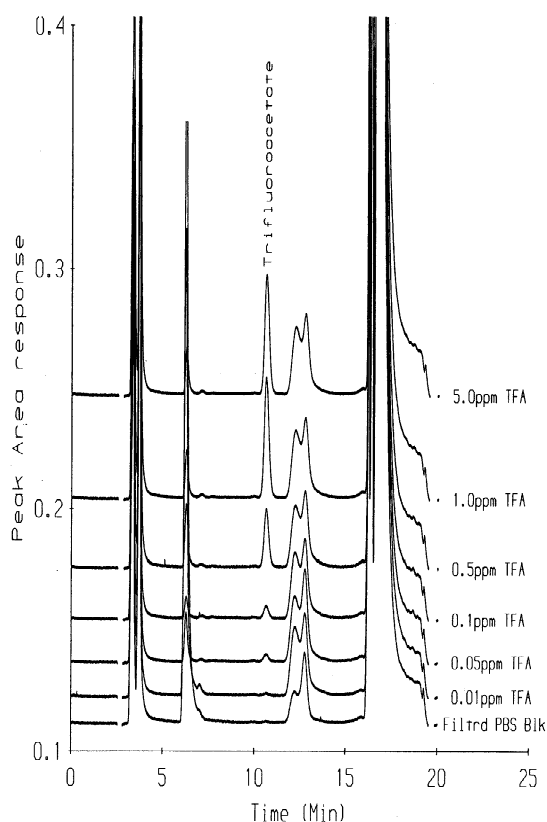
Fig. 3. Chromatograms of TFA linear standards (0.01–5 μg/ml) in post-treated (OnGuard-Ag) PBS media to demonstrate method's linearity and sensitivity.

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. The selectivity was established during the development of

Table 2
Percent Bias-D data from linear regression analysis for accuracy

Concentration (μg/ml)	Peak area response	% (Bias-D)	
		Full fit analysis	Single point analysis (1 μg/ml)
0.051	0.0013677	+68.0	+69.2
0.10	0.0018479	+12.7	+14.3
0.51	0.0079468	−4.60	−1.71
1.0	0.0161708	−3.17	+1.42E-14
5.1	0.0837962	+0.16	+3.64

experiments. The mixture of potential anions was injected and adequate resolution ($R_s > 3$) between TFA and the closest eluting nitrate peak was observed. Since the resolution between the critical pair was not an issue and there appeared to be no coeluting interferences present, robustness experiments were not performed.

The accuracy of an analytical method expresses the closeness of the analytical results to the true value and can be calculated from the recovery standards. Accuracy was calculated from the spiked recovery standard samples prepared at two different levels (0.05 and 1 μg/ml). Spiked recovery samples were quantitated for TFA using a single point (1 μg/ml) external standard. The percent recovery at 1 μg/ml is 100±20% and at 0.05 μg/ml is 100±40%, well within the target criteria.

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision was calculated from the spiked accuracy standard samples prepared at two different levels (0.05 and 1 μg/ml). The percent recoveries at each level for Run 1 were compared with Run 2. The reproducibility and intermediate precision were measured using VAL-SUM (R&D statistical software package) and are summarized in Table 3. Target criteria for RSD for both repeatability and intermediate precision at the 1 μg/ml level ≤10% and at the 0.05 μg/ml level ≤20% were determined.

The limit of quantitation of the method is defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The target accuracy of 0.05 μg/ml level was ≤40% and the total precision at the 0.05-μg/ml level was ≤40%. Evaluation of total precision (17.8%) and the mean accuracy (129.9%) data indicate that the LOQ of 0.05 μg/ml is acceptable, which is the lower linearity level for TFA. However, the linearity data suggested that bias at the 0.05-μg/ml level based on single point analysis was 69.2%, which was higher than the target of ≤40%. Therefore, LOQ is best supported at the 0.1-μg/ml level.

The limit of detection is the lowest concentration of analyte in a sample that can be detected under the

Table 3
Statistical data summary for precision and accuracy for 0.05 and 1 ppm levels

Study level (μg/ml)	Recovery RSD (%)	Between run RSD (%)	Repeatability (within run) RSD (%)	Intermediate (total) RSD (%)
0.05	129.9	15.12	9.47	17.84
1	97.2	4.00	0.55	4.04

stated experimental conditions. The lowest concentration, which satisfies the signal-to-noise 3:1 criteria has been defined as the LOD. The LOD for the method was determined for TFA by serial dilution of the TFA linear standards up to 0.025 and 0.01 μg/ml. Signal-to-noise ratio was measured manually and approximate values of 2–4 were typical for 0.01-μg/ml levels, which were considered appropriate for the method. Signal-to-noise ratios are dependent on the total background conductivity at the time of equilibration. This was typically <3 μS, which can be achieved only with high-quality deionized water with resistance of 17.8 MΩ cm or better.

3.3. Validation summary

The method is suitably accurate, precise, selective and sensitive for its intended use. The method can be used to quantitate TFA in the range of 0.1–5 μg/ml using a single point (1 μg/ml) standard with adequate precision and accuracy using peak area responses. The single point 1-μg/ml standard prepared in PBS matrix was stable during a typical run period and the precision at the 0.05-μg/ml level was within the target criteria and can be used as sensitivity/system suitability standard for the method. Linearity and accuracy data suggest that a LOQ of

0.1 μg/ml is best supported by the method rather than that of 0.05 μg/ml due to the larger than 40% error observed at the lower concentration. The method is sensitive for detecting at least 10 ng/ml (0.01 μg/ml) levels of TFA. To achieve this, the total background conductivity at the time of equilibration should be less than 3 μS. Therefore, it
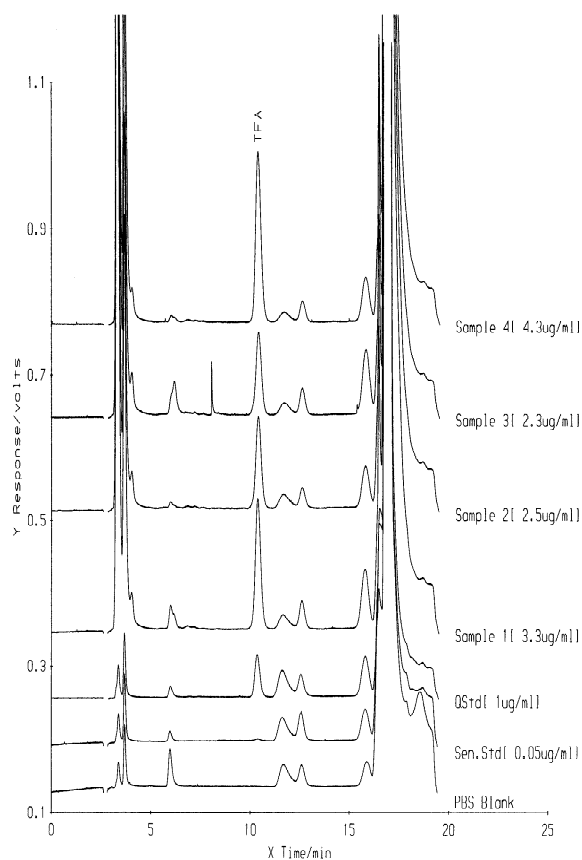

Fig. 4. Chromatograms of the final cell washers submitted for TFA analysis in comparison with quantitation and sensitivity standards prepared per method.

Table 4
Analysis of final sample washers for TFA per method

Sample identity wash # 3	TFA levels (μg/ml)	Average TFA levels (μg/ml)
Sample 1	3.31	3.3
	3.23	
Sample 2	2.38	2.5
	2.67	
Sample 3	2.15	2.3
	2.38	
Sample 4	4.43	4.3
	4.22	

is recommended that in order to achieve the ultimate LOD the water used for analysis should have a resistance of at least 17.8 MΩ cm to obtain the desired background conductance.

3.4. Sample analysis

Sample tubes containing the final PBS cell washes were received from Pharmacia, Metabolism and Safety Evaluation group, and kept at −80°C until analyzed. Before analysis, the sample tubes were thawed in warm water until reaching room temperature at which point they were prepared and analyzed according to the procedure outlined above. Due to limited sample volume (approx. 1 ml), sample pretreatment with OnGuard-Ag cartridges was scaled down using one cartridge. The results of the samples which were quantitated against 1-μg/ml TFA standards injected in bracketing fashion throughout the injection sequence are listed in Table 4. Each sample showed good reproducibility for duplicate analysis and ranged from 2.1 to 4.5 μg/ml. The stack plot of chromatograms for the samples is shown in Fig. 4.

4. Conclusions

An ion chromatographic method is described for the determination of TFA using gradient elution with suppressed conductivity detection in PBS media. The method has been validated and successfully applied to final PBS cell washes generated in bio-pharmaceutical process for monitoring the residual levels of TFA.

References

[1] N. Ishibashi, A. Jyo, Microchem. J. 18 (1973) 220.
[2] C.E. Wujcik, T.M. Cahill, J.N. Seiber, Anal. Chem. 70 (1998) 4074.
[3] D.A. Both, M. Jemal, J. Chromatogr. 596 (1992) 85.
[4] L.D. Gruenke, L.A. Waskell, Biomed. Environ. Mass Spectrom. 17 (1988) 471.
[5] R.M. Maiorino, A.J. Grandolfi, I.G. Sipes, J. Anal. Toxicol. 4 (1980) 250.
[6] L. Witte, H. Nau, J. Fuhrhop, A. Doenicke, B. Grote, J. Chromatogr. 143 (1977) 329.
[7] D. Karashima, S. Takahashi, A. Shigematsu, T. Furukawa, T. Kuhara, I. Matsumoto, Biomed. Mass Spectrom. 3 (1976) 41.
[8] D. Karashima, A. Shigematsu, T. Furukawa, T. Nagayoshi, I. Matsumoto, J. Chromatogr. 130 (1977) 77.
[9] H. Wark, J. Earl, D.D. Chau, J. Overton, J. Anaesth. 64 (1990) 474.
[10] K. Gossler, K.H. Schaller, H.G. Essing, Fresenius Z. Anal. Chem. 279 (1976) 112.
[11] M.J. Cousins, J.H. Sharp, G.K. Gourlay, J.F. Adams, W.D. Haynes, R. Whitehead, Anaesth. Int. Care 7 (1979) 9.
[12] M. Morio, K. Fujii, R. Takiyama, F. Chikasue, H. Kikuchi, L. Ribaric, Anesthesiology 53 (1980) 56.
[13] P.S.L. Jansen, J.W. van Nispen, J. Chromatogr. 287 (1984) 166.
[14] M. Imbenotte, A. Brice, F. Erb, J.M. Haguenoer, Talanta 31 (1984) 147.
[15] D.C. Haskins, E.D. Kharasch, J. Chromatogr. B 692 (1997) 413.
[16] N. Simonadez, J. Chromatogr. 634 (1993) 125.
[17] R. Kawaguchi, K. Fujii, M. Morio, O. Yuge, M.D. Hossain, J. Med. Sci. 38 (1989) 27.