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Development and validation of an assay method for the determination of trifluoroacetic acid in a cyclosporin-like drug

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ABSTRACT

An improved method for the assay of trifluoroacetic acid (TFA) in a cyclosporin-like drug substance is presented, based on ion chromatography with suppressed conductivity detection. Column fouling by the drug molecule is avoided by use of a sample preparation method in which the drug substance is precipitated at alkaline pH whilst the TFA remains in solution. The new method requires a smaller sample mass than a previous method based on headspace-GC-FID whilst achieving an improvement in sensitivity. During validation, the method's performance was found to be consistent with usual acceptance criteria, and the method was found to be robust in routine use.

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1. Introduction

SCY-502635 (Fig. 1) is a novel non-immunosuppressive cyclosporin-based analogue under investigation for the treatment of chronic human hepatitis C infection (HCV; [1,2]). It is similar in structure to cyclosporin A; the differences arise through modification of the residues at the 3 and 4 positions of the undecapeptide ring.

Trifluoroacetic acid (TFA) is used as a reagent in the chromatographic purification of SCY-502635. The concentration of TFA in peptides is generally controlled at 1–10 mg/g [3], and the specification chosen for TFA in SCY-502635 is currently not more than 1 mg/g.

Previous studies concerning the assay of TFA in a variety of sample matrices have used headspace-GC [4], capillary electrophoresis [5] and, more commonly, ion chromatography [6–8]. Initially, a headspace-GC-FID method was developed and validated to control TFA in SCY-502635. This involved dissolving approximately 200 mg of drug substance with methanol, in the presence of sulphuric acid, to convert the TFA present to the more volatile methyl ester (boiling points for TFA and its methyl ester are 72 °C and 43 °C, respectively; [4]). The limit of quantitation (LOQ) of this headspace-GC method was 0.05 mg/g. Although this degree of sensitivity was considered adequate for quality control testing (assuming a 1.0 mg/g specification), a lower limit of quantitation was desired for process monitoring and optimisation. It was also considered desirable, given the relatively high cost of synthesis and purification, to use a smaller sample mass for the TFA assay.

With these considerations in mind, ion chromatography was investigated as a possible alternative to GC. LOQ values reported in previous ion chromatography studies ranged from 0.4 ng to 10 ng TFA on-column. It is not possible to derive equivalent sensitivity data from the capillary electrophoresis study owing to the practical difficulty in measuring the injection volume, but the authors reported a concentration-based LOQ value of 1.2 μg/mL.

SCY-502635 is poorly water-soluble in acidic solution and practically insoluble at alkaline pH (see Table 1). It is, however, extremely soluble in methanol. Ion chromatography columns are prone to fouling by strongly-retained organic sample components [9,10], and it is important, in the interests of method robustness, either to limit the amount of these materials injected onto the column or to remove them from the column between sample injections using an organic eluent. The latter approach, however, has the disadvantage of extending the cycle time between injections owing to the need to re-equilibrate the column with aqueous mobile phase following the solvent rinse step. The Dionex AS11-HC ion

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Table 1
Solubility of SCY-502635 at different pH values.

pH	Solubility (mg/mL)
1	103.66
3	40.59
5	12.03
7.5	1.2
8.6	0.62

exchange column was selected for this study owing to its high ion exchange capacity (290 μeq), which is ideal for the retention of singly-charged organic species, and its compatibility with organic solvents.

2. Materials and methods

2.1. Reagents

All reagents used were of analytical grade or better. Sodium trifluoroacetate of certified purity was supplied by Sigma–Aldrich Schweiz (Fluka Analytical), Buchs (St. Gallen), Switzerland. This reagent is hygroscopic and was stored in a desiccator when not in use. Carbonate-free sodium hydroxide (50% w/w aqueous solution) was purchased from the same supplier. Methanol (HPLC gradient grade) was supplied by Mallinckrodt Baker (Deventer, The Netherlands). Ultra-pure water of 18 MΩ resistivity or better was produced as required using a Purelab Option reverse-osmosis unit coupled to a Maxima water treatment system (Elga Process Water, Marlow, UK). Polyvinylidene fluoride (PVDF) membrane syringe filters (13 mm diameter, 0.2 μm porosity) were supplied by Whatman International (Maidstone, UK). A 19 mM solution of sodium hydroxide was prepared by dilution of a 50% (w/w) solution with ultra-pure water. Standards and spiking solutions were prepared by making suitable dilutions of an aqueous standard of sodium trifluoroacetate with ultra-pure water, the concentration of these solution being expressed in terms of the free acid.

2.2. Equipment

The ion chromatograph used for this study consisted of a GP50 quaternary gradient pump, AS50 autosampler, LC30 chromatography oven and an ED50 electrochemical detector (Dionex Limited, Camberley, UK). Eluents were degassed on-line using a series 200 vacuum degasser (Perkin-Elmer, Seer Green, UK) and hydroxide-containing mobile phase was maintained under nitrogen at approximately 3 psi to prevent ingress of atmospheric carbon dioxide. The autosampler was equipped with a 25 μL injection loop and the detector was fitted with a conductivity flow-cell. The background conductivity of the column effluent was reduced and the analyte signal enhanced using an ASRS 300 self-regenerating suppressor (Dionex, Camberley, UK) operated in recycle mode

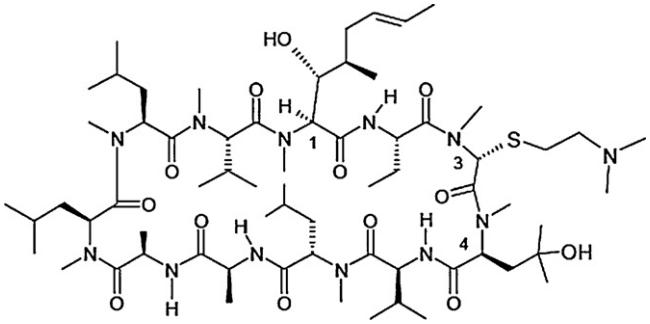

Fig. 1. SCY-502635.

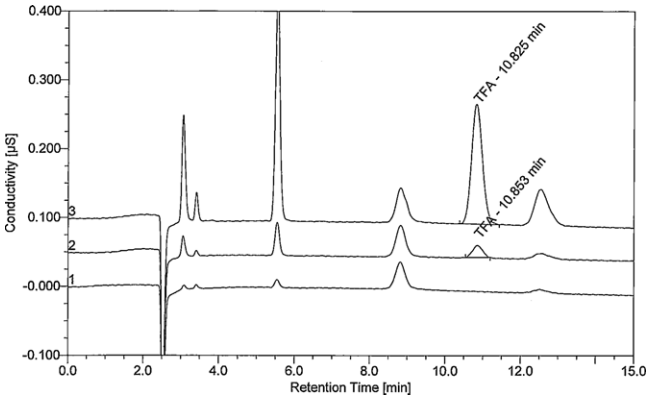

Fig. 2. Overlay chromatogram showing a reagent blank (bottom), 0.1 μg/mL TFA standard (middle) and 1.0 μg/mL TFA standard (top). The TFA peak elutes at approximately 10.8 min.

using a current of 50 mA. The column used for the study was an IonPac AS11-HC hydroxide-selective anion exchange column (4 mm × 250 mm; Dionex Part No. 52960) with a matching guard column (4 mm × 50 mm, Part No. 52962).

2.3. Method development

2.3.1. Chromatographic conditions

Initial method conditions were assessed using 19 mM sodium hydroxide as the eluent at a flow rate of 1.0 mL/min. This eluent concentration was selected based on a previous study using the same type of ion chromatography column [6]. The column, conductivity flow-cell and suppressor were mounted in the chromatography oven and maintained at 30 °C. The injection volume used was 25 μL, achieved by over-filling the injector loop. Sensitivity was evaluated by injecting standard solutions containing 0.1 μg/mL and 1.0 μg/mL TFA; these concentrations were close to the anticipated LOQ.

2.3.2. Sample preparation

The aim of sample preparation was to quantitatively recover TFA from the drug substance and to introduce the sample to the chromatographic system in such a way that robustness was not compromised. It was decided to try and avoid column clean-up between injections using an organic eluent since this would increase the time between sample injections. As has already been noted, SCY-502635 is virtually insoluble in alkaline aqueous solution (0.62 mg/mL) but readily soluble in methanol (>130 mg/mL). A sample preparation method was therefore evaluated based on complete dissolution of the sample in methanol followed immediately by dilution in mobile phase (19 mM sodium hydroxide) to remove SCY-502635 by precipitation. This approach also has the advantage of maximising the solubility and minimising the fugacity of TFA through the use of an alkaline sample diluent. Based on the instrument response from 0.1 μg/mL and 1.0 μg/mL chromatograms (Fig. 2), it was thought that dilution of a 50 mg sample of drug substance to a final volume of 25 mL would yield adequate sensitivity. Under these conditions, 1 mg/g TFA in the drug substance would produce a TFA concentration in the final extract of 2.0 μg/mL.

Four 50 mg aliquots of SCY-502635 were used to evaluate this sample preparation approach. Two of the samples were spiked with 50 μg TFA, equivalent to a concentration of 1 mg/g in the drug substance. All four samples were then dissolved in 1.0 mL methanol and 25.0 mL 19 mM sodium hydroxide was added. On addition of the hydroxide solution, a dense white precipitate was formed, which was removed by filtration through a 0.2 μm PVDF syringe filter. The potential for the filter to leach interfering ions

into the sample was evaluated by filtering and testing a portion of a reagent blank solution. The same relative volume of methanol was added to calibration standard solutions in order to match their composition and nominal concentration with sample solutions.

2.4. Validation

The method was validated with respect to linearity, specificity, accuracy, method precision (repeatability) and the stability of analytical solutions. Sensitivity data were derived from the values obtained during method development. Linearity was evaluated by injecting solutions containing 0.1 μg/mL, 0.8 μg/mL, 1.6 μg/mL, 2.4 μg/mL, 3.2 μg/mL and 4.0 μg/mL TFA. Specificity was determined by overlaying an unspiked SCY-502635 sample chromatogram with a reagent blank and a calibration standard. Accuracy was evaluated by recording the recovery values of samples spiked at TFA concentrations of 0.5 mg/g and 1.0 mg/g ($n = 3$). Method precision was determined by calculating the relative standard deviation of the TFA peak area response ($n = 6$) in samples spiked with TFA at 1.0 mg/g. The stability of a spiked sample was evaluated by re-injecting the sample after storage at ambient conditions for 24 h and recording the difference in assay result obtained. Chromatographic robustness was evaluated by comparing the quality of chromatography for initial use of the column and after 265 injections of standards and samples had been made.

3. Results

3.1. Method development

An overlay chromatogram of a reagent blank with 0.1 μg/mL TFA and 1.0 μg/mL TFA solutions is shown in Fig. 2. The signal:noise ratio of the TFA peak in the 0.1 μg/mL chromatogram is 20.6:1, equivalent to a LOQ of 0.05 μg/mL (1.25 ng TFA on-column), defined as the amount of TFA producing a peak with a signal:noise ratio of 10:1 [11]. The retention time of TFA is approximately 11 min and the symmetry factor [12] of the TFA peak is 1.02 and 1.10 in the 0.1 μg/mL and 1.0 μg/mL chromatograms, respectively.

The mean TFA recovery value from spiked samples filtered through 0.2 μm PVDF syringe filters was 107%, and no peaks were observed in filtered blank solutions at the retention time of TFA.

3.2. Validation

The square of the correlation coefficient (R^2) of the linearity plot was 0.9900 and the y-intercept value was equivalent to −8.1% of the response at a TFA concentration of 2.0 μg/mL (the concentration equivalent to the specification limit). The overlay specificity chromatogram is shown in Fig. 3. The concentration of the calibration standard chromatogram shown here is equivalent to a TFA concentration in SCY-502635 of 1.0 mg/g. Mean recovery values from accuracy studies were 106.7% and 97.7% at 0.5 mg/g and 1.0 mg/g, respectively, and the coefficient of variation derived from samples run for precision evaluation was 1.4% ($n = 6$). Re-analysis of the sample extract selected for solution stability measurement produced a result that had increased by 1.7% relative to the original value. This difference is considered to be adequately small. An overlay chromatogram showing standard injections from the first and most recent use of the ion chromatography column is at Fig. 4.

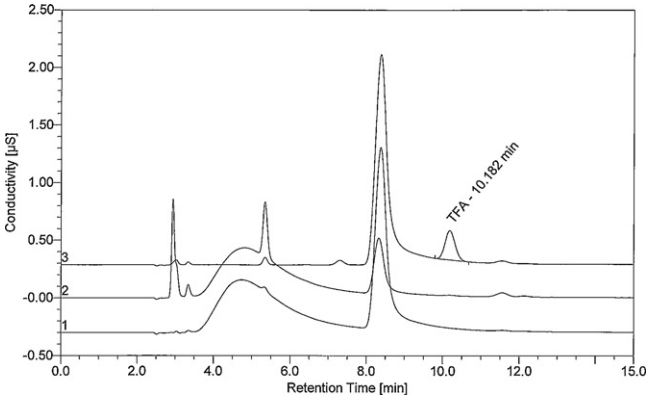

Fig. 3. Specificity overlay chromatogram. Reagent blank (bottom), unspiked SCY-502635 sample (middle) and 2.0 μg/mL TFA standard (top). TFA elutes at approximately 10.1 min.

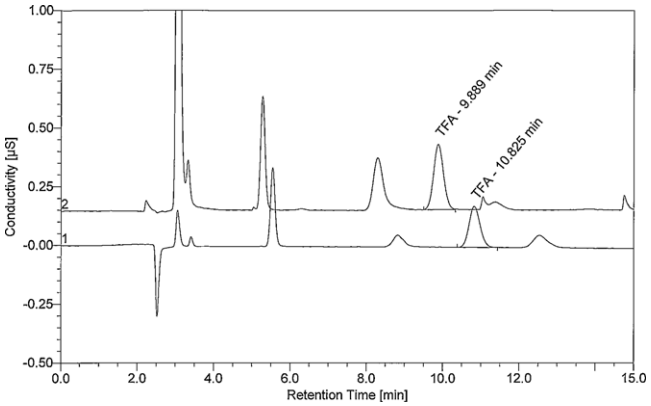

Fig. 4. Overlay chromatograms from standard injections run at the start of method development (bottom; 1.0 μg/mL) and from the most recent use of the column (top; 2.0 μg/mL).

4. Discussion

The method described here achieves a two-fold improvement in sensitivity compared to the headspace-GC method used previously, with a four-fold reduction in the amount of sample used. A further advantage of using alkaline, rather than acidic, conditions for sample preparation is the reduction in potential fugitive losses of TFA. TFA is a weak acid (pK_a 0.3) with a boiling point of 72 °C [13]. In its undissociated form a risk of loss by evaporation exists. As the

Table 2
Summary of method validation data.

Parameter	Typical limits[a]	Result
Linearity		
Correlation coefficient (R^2)[b]	>0.96	0.9900
y-Intercept relative to response at specification limit	±15.0%	−8.1%
Accuracy		
Mean recovery (0.5 mg/g TFA)	90.0–110.0%	106.7%
Mean recovery (1.0 mg/g TFA)	90.0–110.0%	97.7%
Precision		
Method precision at 1.0 mg/g TFA	RSD ≤5.0%	RSD 1.4%
Sensitivity		
Limit of quantitation	≤500 μg/g	25 μg/g
Stability of analytical solutions		
% change in concentration[c]	±10.0%	1.7%

[a] Typical limits are taken from Ref. [15].
[b] The reference value was given in terms of R and has been rendered as R^2 for consistency.
[c] Evaluated after 24 h at ambient temperature.

sodium salt, however, it is involatile (melting point 205–207 °C; [14]).

The sample preparation method described here is straightforward and generally applicable to peptides with a similar solubility profile. The method performed robustly without the need for a column clean-up step between injections or time-consuming sample pre-treatment. The retention time of TFA decreased by 8.6% during use of the method on the same column, and it is clear from Fig. 4 that the quality of the separation, in terms of peak shape and resolution, is still perfectly acceptable.

The results of method validation are consistent with typical acceptance criteria for this type of assay [15]. A comparison of experimental data from this study with typical limits is shown in Table 2.

5. Conclusions

The method described in this study is more sensitive and requires a smaller sample mass compared to the method used previously. The method's performance is adequate to control TFA as an impurity in the drug substance. The sample preparation technique employed succeeds in separating TFA from the drug molecule; this is necessary because of the poor solubility of the drug in the mobile phase and the importance of avoiding contamination of the ion chromatography column with strongly-adsorbed organic species. This method of sample preparation has the potential to be applied generally to other drug molecules of a similar nature.

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