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Clinical analysis of sampatrilat, a combined renal endopeptidase and angiotensin-converting enzyme inhibitor I: Assay in plasma of human volunteers by atmospheric-pressure ionisation mass-spectrometry following derivatisation with BF_3–methanol

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Abstract

Sampatrilat is a dual inhibitor of angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) under development for the treatment of hypertension and congestive heart failure. In order to support the early clinical development (with oral administration and an expected low bioavailability), a sensitive and selective assay was required. A method for plasma was developed and validated employing HPLC–APCI–MS–MS. The plasma samples were extracted on solid-phase extraction cartridges, derivatised with BF_3–methanol, diluted, extracted again and then subjected to HPLC–APCI–MS–MS. Derivatisation was necessary because the two carboxyl group in the molecule prevented efficient ionisation in the heated nebuliser source. The calibration range was from 0.5 to 20 ng ml^{-1} and the lower limit of quantification was 0.5 ng ml^{-1}. Imprecision and inaccuracy were determined on three separate occasions at three concentrations (0.5, 5 and 20 ng ml^{-1}) and shown to be lower than 10% in every case. © 1998 Elsevier Science B.V.

Keywords: Sampatrilat; UK-81,252; derivatisation; atmospheric-pressure ionisation mass-spectrometry; clinical bioanalysis; plasma

1. Introduction

(S,S,S) - N - {1 - [2 - carboxy - 3 - (N^2 - mesyllysyl - amino)propyl] - 1 - cyclopentylcarbonyl}tyrosine, UK-81,252, Fig. 1, sampatrilat) is a compound under development for hypertension and congestive heart failure, being a dual enzyme inhibitor having activities directed against angiotensin-converting enzyme and neutral endopeptidase [1]. To support the clinical development programme, a sensitive and selective plasma assay was judged to be required, as the compound was expected to exhibit low bioavailability. For pre-clinical stud-

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ies, a bioassay had been developed, based on the inhibition properties of sampatrilat against rat renal endopeptidase (Cussans, N.J., Department of Drug Metabolism, Pfizer Central Research: personal communication (1993)). However, the lower limit of quantification (LLOQ) of this bioassay was approximately 10 ng ml^{-1} and preliminary studies in man showed this to be inadequate. Thus there was an urgent need for a more sensitive assay, probably sub-nanogram per millilitre, to continue to support clinical development. Alternative approaches such as HPLC with electrochemical detection, HPLC-fluorescence with pre-column derivatisation and HPLC-atmospheric-pressure-ionisation mass spectrometry (APCI–MS–MS) were investigated as well as raising antibodies for an immunoassay approach. Initially we found it impossible to obtain good ionisation using the heated nebuliser source but found that methylation of the carboxyl groups (using boron trifluoride/methanol) promoted ionisation. Thus we rapidly developed and validated an assay involving APCI–MS–MS of the derivatised compound at a sensitivity of 0.5 ng ml^{-1}, relying on our in-house experience of applying APCI–MS–MS to bioanalytical problems [2]. This method was used in early clinical studies and although of adequate sensitivity it had a low throughput. This method is described in this paper, together with a later method developed using the ionspray source. The method was also used to cross-validate an immunoassay which we simultaneously developed [3].

2. Experimental

2.1. Solvents and chemicals

Sampatrilat (Fig. 1), UK-79,942 (Fig. 2) and other UK-compounds were supplied by Compound Control, Pfizer Central Research, Sandwich, UK. All reagents used were of analytical grade or better. Human plasma was either outdated stock from the Blood Transfusion Service or was collected locally from volunteers in lithium–heparin tubes and transferred after centrifugation to vials for storage at −20°C.

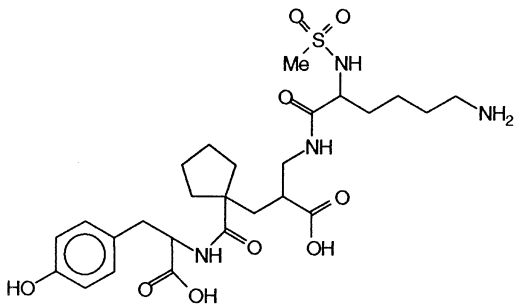

Fig. 1. Structure of sampatrilat.

Methanol (205 Super Purity HPLC grade) and acetonitrile (195 HPLC grade) were from Romil Chemicals, Loughborough, Leicestershire; water (18.2 MΩ) was obtained from a Milli-Q-plus (Millipore–Waters, Watford, Herts, UK) system. BF_3-methanol (12 wt.%) was obtained from Aldrich Chemical Company (Gillingham, Dorset, UK).

2.2. HPLC and mass spectrometry

Mobile phase was 0.02 M ammonium acetate, 0.005 M triethylamine in 80/20 v/v methanol/water, pumped at 1 ml min^{-1}. The mobile phase was filtered twice through a 50 mm diameter glass-fibre filter under vacuum. The column was a Perkin–Elmer 3 μm C18 30 × 4.6 mm (3 × 3CR C18) and the detector was a Perkin–Elmer Sciex API III atmospheric-pressure-ionisation triple quadrupole mass spectrometer with an APCI heated nebuliser sample ionisation system. Data

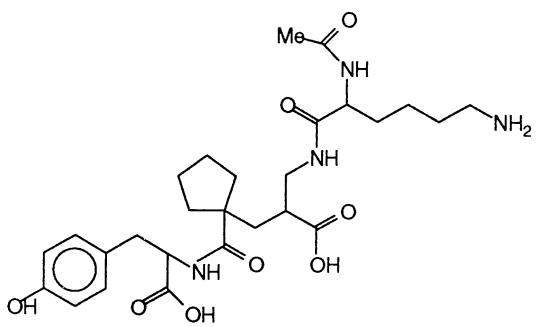

Fig. 2. Structure of UK-79,942.

were collected and analysed using the programm MacQuan 1.1.2 (Sciex) with automatic determination of peak area. Mass-spectrometric conditions were as follows: the heated nebuliser was operated at 500°C in a positive ion atmospheric-pressure chemical ionisation mode. Multiple reaction monitoring was used with argon as collision gas, and the parent/daughter transitions were m/z 613.1 to m/z 211.4 (sampatrilat methyl ester) and m/z 597.1 to m/z 211.4 (UK-79,942 methyl ester, internal standard) dwelling on each transition for 100 ms. Data were acquired for 1 min; both compounds eluted at 0.5 min. The resolution for Q1 at m/z 613.1 and m/z 597.1 was 2.2 a.m.u. peak width at half height and Q3 resolution at m/z 211.4 was 2.0 a.m.u. peak width at half height.

For determination of the time-course of the derivatisation reaction, HPLC was performed using an ESA Coulochem electrochemical detector (ESA Analytical Ltd., St. Ives, Cambridgeshire, UK) with the analytical electrode set at 700 mV. The column was of Spherisorb S5C6 (250×4.6 mm) (Hichrom Ltd., Reading, Berkshire, UK) and the eluent was 0.02 M sodium phosphate buffer pH 2.25 containing 2.5 mM octane sulphonic acid and 35% acetonitrile pumped at 1 ml min^{-1}. Data were collected and integrated using the VG Multichrom chromatography data management system.

2.3. Procedure

Plasma samples from clinical trials were prepared from blood collected in lithium–heparin tubes after centrifugation. Samples were frozen at -20°C until analysed. Samples for analysis by HPLC–APCI–MS–MS were defrosted at room temperature for 1 h and briefly vortex mixed. The calibration line, quality control samples (two each at 1, 5 and 15 ng ml^{-1}) and unknowns were extracted, derivatised, re-extracted and quantified as follows. One ml of each was transferred to tapered polypropylene 10 ml tubes, 20 μl of aqueous internal standard solution added (20 ng UK-79,942: Fig. 2) together with 1 ml phosphate buffer pH7 with vortex mixing. These were then loaded individually onto conditioned (1 ml methanol followed by 1 ml phosphate buffer pH7) Bond–Elut Certify II cartridges under light vacuum. The cartridges were washed with 1 ml phosphate buffer pH7 followed by 1 ml 15% methanol 0.1% TFA, dried by application of vacuum and eluted into 10 ml polypropylene tapered tubes with 1 ml 80% methanol 0.1% TFA. The latter were briefly centrifuged to bring all the eluate to the bottom of the tubes and then evaporated to dryness at 37°C under a stream of nitrogen. When completely dry, 1 ml BF_3-methanol was added using an Eppendorf dispenser with a 12.5 ml syringe, the tubes were capped tightly and incubated at 65°C for 30 min. After uncapping, 2 ml water was added and the solutions evaporated to approximately 1 ml under a stream of nitrogen at 65°C. This strongly acidic solution was then loaded onto preconditioned (1 ml methanol followed by 1 ml water) Bond–Elut C18 cartridges using light vacuum. The tubes were washed out with 1 ml water, which was added to the cartridges. These were then washed with three 1 ml aliquots of water and eluted into TBME-rinsed polypropylene tapered tubes with two 1 ml aliquots of methanol. The latter were then briefly centrifuged and evaporated to dryness at 37°C under a stream of nitrogen. HPLC injection solution (0.02 M ammonium acetate, 0.005 M triethylamine in methanol/water 50/50 v/v: 100 ml) was added with vortex mixing and after centrifugation this was transferred to 200 μl crimp-top autosampler vials. The capped vials were then centrifuged at 10,000 rpm for 5 min, transferred to the autosampler and the solution injected (80 μl) onto the HPLC system as described above. Method validation was carried out on three separate occasions by analysing seven replicates of three concentrations (0.5, 5.0 and 20.0 ng ml^{-1}) of sampatrilat with a calibration line, the replicates being diluted from the same stock solution as the calibration line. The lower limit of quantification was defined as the lowest concentration at which the replicates gave inaccuracy and imz precision of better than 20%.

3. Results and Discussion

Early results from preliminary clinical studies indicated that use of the bioassay, measuring inhibition of rat renal endopeptidase, had inadequate sensitivity in man, even though it had been used successfully for animal pharmacokinetic studies (Cussans, N.J., Department of Drug Metabolism, Pfizer Central Research: personal communication (1993)). Thus we explored a number of analytical techniques for the analysis of sampatrilat in human plasma, initially investigating solid-phase extraction on a range of phases to separate analyte from the plasma matrix. These ranged from C2 to C18 and Certify II, the latter giving quantitative recovery as judged by use of tritiated sampatrilat and clean extracts as judged by HPLC. These extracts were then analysed by either HPLC with electrochemical detection or by column-switching HPLC with fluorescence detection following pre-column derivatisation with 9-fluorenylmethylcarbonyl chloride (FMOC–Cl). Both these methods were capable of intrinsic limits of detection of circa 100 pg or less (data not shown), but neither was sufficiently robust to enable validation at 0.5–1 ng ml^{-1}. Having access to the Sciex API III mass spectrometer which had been used successfully in our department for the quantitative analysis of several analytes including abanoquil at 10 pg ml^{-1} blood [3] led us to exploit the mass discriminatory power and sensitivity of HPLC–APCI–MS–MS to facilitate rapid development of an assay for sampatrilat at sub ng ml^{-1} levels. Preliminary work in APCI mode led us to believe that sampatrilat in the free acid form could not be detected with sufficient sensitivity. For reasons that are not well understood such compounds with carboxylic acid moieties in their structure do not ionise well at low concentrations even in negative ion mode. This may be due to ion suppression from components in the liquid matrix or lack of volatility or thermal stability. Carboxylic acid esters are known to ionise better in positive ion mode [4] and this was confirmed for sampatrilat by investigation of the diester analogues and the dimethyl ester derivative. Using BF_3-methanol as a methylating agent [5] we found that the dimethyl ester could be produced quantitatively from sampatrilat in analytical samples. Diazomethane was another possible reagent but we considered this to be too hazardous and cumbersome for routine analysis. The detection limit for dimethyl sampatrilat was approximately 100 pg.

3.1. APCI–MS–MS assay

Derivatisation with BF_3-methanol and subsequent dilution with water leads to the production of a very strongly acidic solution. It was necessary to remove this acid and we found that solid-phase extraction on C18 Bond–Elut cartridges was ideal for this purpose. The derivatised sampatrilat could be easily eluted from the cartridges using methanol, which was easy to evaporate to dryness with a stream of nitrogen. Thus we were able rapidly to develop a method for the analysis of sampatrilat in human plasma based on our earlier experience with solid-phase extraction using Certify II cartridges, followed by methylation of the dry extracts, and by a further solid-phase extraction and chromatography with mass-spectrometric detection using multiple reaction monitoring. We used an internal standard, UK-79,942, that was a close analogue and quantified sampatrilat using peak area ratios of analyte to internal standard. Investigation of the time-course of the reaction using HPLC with electrochemical detection for the parent and the mono- and di-methylated compounds (Fig. 3) showed that the reaction was $>90\%$ complete after 30 min at 65°C.

The mass-spectrum obtained at Q1 for the derivatised sampatrilat showed a single ion at $m/z = 613$ due to the protonated molecular ion of the dimethyl ester and $m/z = 597$ for dimethyl UK-79,942. By selecting these parents and scanning for daughter ions in Q3 after collision with argon in Q2, both dimethyl sampatrilat and dimethyl-UK-79,942 gave the same daughter ion of $m/z = 211$ suitable for quantification. Fig. 4 shows the mass spectrum at Q3 for dimethyl sampatrilat. Thus we selected the parent ions of $m/z = 613$ and 597 and daughter ions of $mz = 211$ to quantify sampatrilat and UK-79,942 respectively. Peak area ratios of sampatrilat: UK-79,942 were used to generate calibration lines. Typical chromatograms obtained for derivatised blank

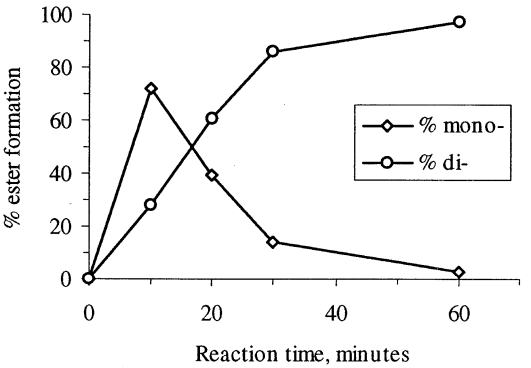

Fig. 3. Time Course of the methylation of sampatrilat at 65°C. Sampatrilat (20 ng) was derivatised using BF_3-methanol as described in the text for up to 60 min at 65°C. After solid-phase extraction of the derivatives, the dry extracts were dissolved in mobile phase (0.02 M sodium phosphate buffer pH 2.25 containing 2.5 mM octane sulphonic acid and 35% acetonitrile) and injected directly onto the analytical column.

plasma extracts and extracts containing sampatrilat are shown in Fig. 5 and a calibration line is shown in Fig. 6.

3.2. Inaccuracy and imprecision of the method

On three separate occasions, blank human plasma was spiked as described above to provide a calibration line between 0.5 and 20 ng ml^{-1}. Inaccuracy and imprecision of the method was determined by spiking seven one-ml blank plasma aliquots with sampatrilat at each concentration of

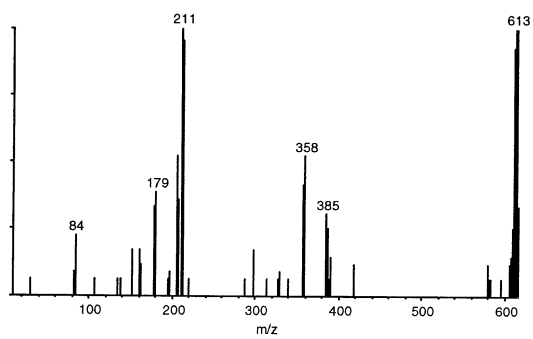

Fig. 4. Daughter ion mass spectrum of dimethylated sampatrilat (MH^+ 613) obtained at Q3 on the Sciex API III mass spectrometer.

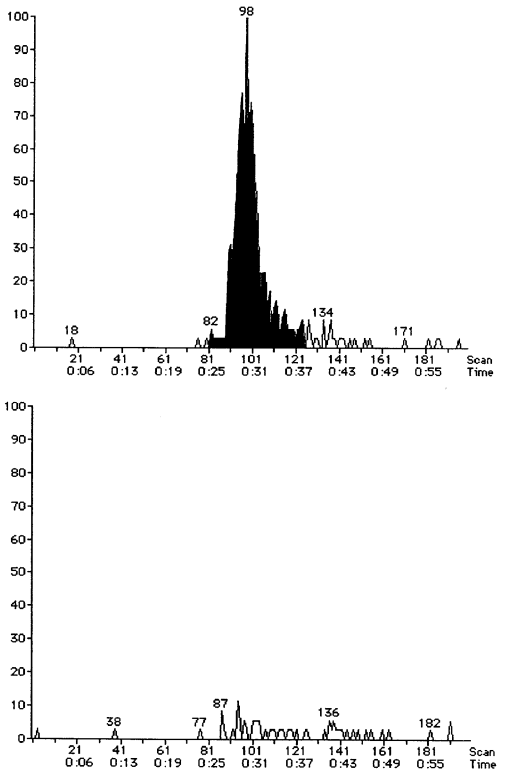

Fig. 5. HPLC–APCI–MS–MS chromatograms of spiked (1 ng ml^{-1}; above) and blank (below) plasma extracted and derivatised using BF_3-methanol.

0.5, 5 and 20 ng ml^{-1} using the same working solutions. The results obtained are as shown in Table 1; as can be seen, inaccuracy and imprecision at all three levels were better than 10% on all three occasions.

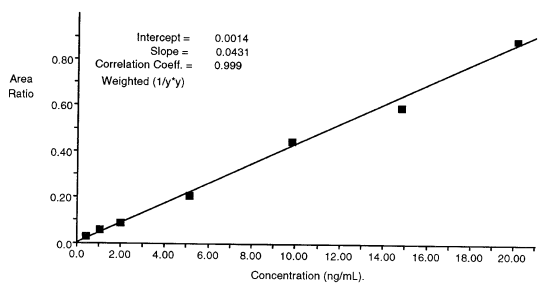

Fig. 6. Calibration line for sampatrilat from plasma after extraction and derivatisation and analysis by HPLC–APCI–MS–MS.

Table 1
Inaccuracy and imprecision of the HPLC–APCI–MS–MS method for sampatrilat on three separate occasions

Batch	Concentration spiked (ng ml^{-1})	Mean concentration measured (ng ml^{-1}) ($\pm$SD)	n	% inaccuracy	% imprecision
1	0.5	0.54 (0.03)	7	8.24	6.32
	5	4.69 (0.31)	7	−6.15	6.53
	20	20.48 (1.43)	7	2.38	7.01
2	0.5	0.49 (0.04)	7	−2.12	7.98
	5	5.20 (0.34)	7	4.02	6.46
	20	20.12 (0.88)	7	0.61	4.39
3	0.5	0.52 (0.05)	7	4.22	9.30
	5	4.78 (0.48)	7	−4.31	9.96
	20	20.32 (0.36)	7	1.60	1.78

This assay was used to assay samples from the early clinical studies with sampatrilat, but suffered from low throughput and the necessity of a double extraction. Physical constraints of the apparatus together with the length of the procedure limited sample throughput to 36 samples (including quality controls and calibrants) for each batch. Since two days were required to complete the assay, the speed of analysis available on the mass-spectrometers was not fully utilised. For these reasons we undertook further assay development using the Certify II extraction method with an ionspray interface for the API III mass-spectrometer and obtained efficient ionisation of native sampatrilat in negative ion mode. The ionspray interface is not heated and thus has the advantage for non-volatile compounds. Using a low flow rate of 200 μl min^{-1} with a mobile phase of methanol/water with 2 mM ammonium acetate and the ten-fold lower concentration of 0.5 mM triethylamine, we validated this method with no internal standard: the data are shown in Table 2. Inaccuracy and imprecision were within our accepted criteria for both batches shown. At the lowest level (0.5 ng ml^{-1}) the imprecision was less good than the derivatisation assay, but still better than 15%. Use of a deuterated internal standard (deuterated sampatrilat) would be expected to improve these figures considerably. This method was capable of a throughput of 70 to 80 samples per day, thus more than doubling that of the derivatisation method. We did not, however, need to use this method for the analysis of clinical samples since the very high throughput immunoassay described in the following paper [3] was developed and validated in time to be used for all further studies.

3.3. Stability

Stability of sampatrilat in plasma QCs and clinical samples was assessed using the APCI–LC–MS assay. The compound was shown to be stable for at least 6 months in frozen Blood Transfusion Service plasma, at least one month in clinical samples, and for 24 h at 37°C.

4. Conclusions

We have developed and validated an analytical method for sampatrilat using BF_3-methanol as a derivatisation agent to methylate the carboxyl groups and facilitate ionisation in the API mass spectrometer. This involves two solid-phase extractions and a derivatisation step, with three evaporation stages; it requires two days to complete and was limited by the apparatus available to less than 30 clinical samples in each batch. Thus a maximum of 120 samples could be assayed in a week. This assay, however, sufficed to support the first two clinical studies in man and was robust, reliable and had a LLOQ of 0.5 ng ml^{-1}. The derivatisation APCI–MS–MS assay was se-

Table 2
Inaccuracy and imprecision of the APCI–Electrospray–MS–MS method for sampatrilat on two separate occasions

Batch	Concentration spiked (ng ml^{-1})	Mean concentration measured (ng ml^{-1}) ($\pm$SD)	n	% inaccuracy	% imprecision
1	0.5	0.51 (0.05)	7	1.4	10.5
	7.5	7.28 (0.31)	6	−2.9	4.2
	20	19.46 (0.51)	7	−2.7	2.6
2	0.5	0.57 (0.08)	6	14.7	13.0
	7.5	7.22 (0.30)	7	−3.8	4.1
	20	18.66 (0.77)	7	−6.7	4.1

lective and sensitive and was also used to cross-validate the immunoassay [3] which showed no positive or negative bias with respect to this selective physical method.

Acknowledgements

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