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Clinical analysis of sampatrilat, a combined renal endopeptidase and angiotensin-converting enzyme inhibitor II: Assay in the plasma and urine of human volunteers by dissociation enhanced lanthanide fluorescence immunoassay (DELFIA)

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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. An HPLC-atmospheric-pressure chemical ionisation mass-spectrometric (HPLC-APCI-MS-MS) assay had been already validated (R.F. Venn et al., J. Pharm. Biomed. Anal., in press), but due to its low throughput an alternative method was sought. As the molecule is peptide-like and not metabolised, we believed the immunoassay approach was appropriate. Thus we developed an immunoassay for the compound using time-resolved fluorescence as an end-point (DELFIA®) with lower limits of quantification of 0.2 ng ml^{-1} for the plasma assay and 5 ng ml^{-1} for the assay in urine. This assay is a 96-well plate based competitive immunoassay; the end-point is the determination of a (non-radioactive) europium label by time-resolved fluorimetry. Sampatrilat is labelled with chelated europium via isothiocyanate chemistry. The advantage of this assay is its extremely high throughput, allowing rapid analysis of many thousands of samples. The DELFIA method was successfully cross-validated with the HPLC-APCI-MS-MS method. © 1998 Elsevier Science B.V.

Keywords: Sampatrilat; UK-81 252; DELFIA; Time-resolved fluorescence; Immunoassay; Clinical bioanalysis; Plasma; Urine

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1. Introduction

(*S*,*S*,*S*)-*N*-{1-[2-carboxy-3-(N2-mesyllysylamino)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 ([2] and Cussans, NJ, Department of Drug Metabolism, Pfizer Central Research: personal communication (1993)). To support the clinical development programme, a sensitive and selective plasma assay was required. Initially an assay involving APCI-MS-MS of the derivatised compound was developed and validated. This was rapidly developed to a sensitivity of 0.5 ng ml^{-1} [1]. This method was used in early clinical studies but although of adequate sensitivity, it had a low throughput. Thus, ultimately, a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) method was also developed and validated for the determination of sampatrilat in human plasma and urine. The lower limit of quantification of the plasma assay was 0.2 ng ml^{-1}, whereas for urine we validated the method to 5 ng ml^{-1}. The validated dynamic range of the assay is greater than two orders of magnitude, the method is robust and using the 96-well format for batch-analysis a high throughput was achieved which adequately supports the current clinical programme.

The DELFIA system relies on the fluorescence of lanthanide chelates as an endpoint [3,4]. For sampatrilat, a 96-well system competitive immunoassay was set up by using plates coated with anti-rabbit antibody (IgG). Diluted rabbit antiserum to sampatrilat (rAS) binds to the coated anti-rabbit IgG. Sampatrilat in standards, quality control (QC) samples and unknowns competes with europium-labelled sampatrilat for the rAS binding sites. After equilibrium is reached, the plates are washed, and bound label is released from the plates with a low pH solution which forms a new chelate of the lanthanide in a different micellar environment, greatly enhancing the fluorescence yield on UV irradiation. This chelate (usually a β-diketone) absorbs UV light and transfers the absorbed energy by inter-system crossing and intramolecular transfer to the lanthanide. In particular, the excited micellar ion emits long-lived fluorescence with a very large effective Stoke's shift (e.g. λ_{exc} 340 nm, λ_{em} 614 nm) which can be distinguished from background interference which is much shorter-lived. Each well is read for 1 s (1000 separate readings) and a 96-well plate can be read in less than 2 min. This is very advantageous compared to conventional radioimmunoassay (RIA). The dynamic range in a DELFIA system is also large (up to 10^6 for a non-competitive assay), and the system has potential advantages over conventional enzyme immunoassay (EIA) techniques in better sensitivity, lower interference, greater dynamic range and ease of use.

This paper describes the validated DELFIA methods and presents cross-validation data with the mass-spectrometric method.

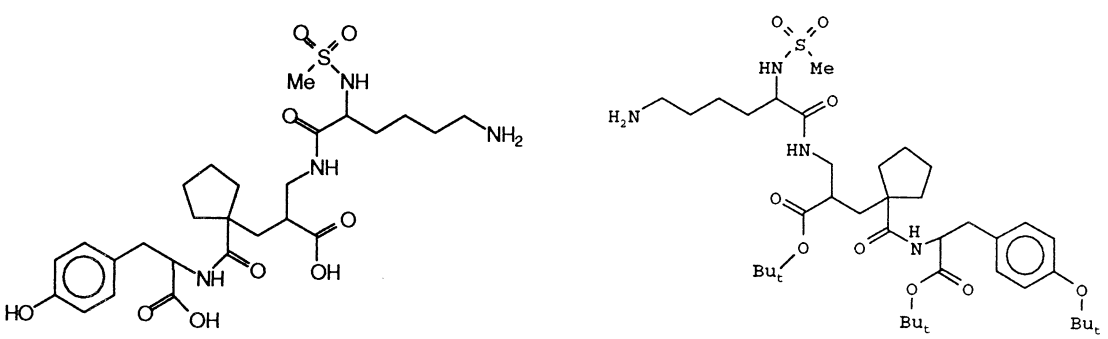

Fig. 1. Structure of sampatrilat and of UK-100 637.

2. Experimental

2.1. Solvents and chemicals

Sampatrilat and UK-100 637 (Fig. 1) and other UK-compounds were supplied by Compound Control, Pfizer Central Research, Sandwich, UK. All reagents used were of analytical grade or better. Europium-labelled sampatrilat was prepared by Wallac Oy, Turku, Finland (see below) and bovine serum albumin, heavy-metal-reduced, 75 mg ml^{-1} was obtained from the same source. Rabbit L anti-sampatrilat antiserum, bleed date 29:3:93 was obtained by King's Diagnostics, Bournemouth (see below). Human plasma was collected locally from volunteers in lithium-heparin tubes and transferred after centrifugation to vials for storage at −20°C. Plasma samples from clinical trials were obtained by centrifugation of blood collected in tubes containing lithium heparin. Samples were transferred to polypropylene tubes and frozen at −20°C until analysed. Water (18.2 MΩ) was obtained from a Milli-Q-plus (Millipore-Waters, Watford, Herts, UK) system.

Sampatrilat was labelled with a europium chelate by Dr Heikki Mikola, Wallac Oy, Turku, Finland by reaction with the isothiocyanate (Fig. 2) and purified by HPLC. Fractions were collected and stored at 4°C and have been stable for more than 2 years under these conditions.

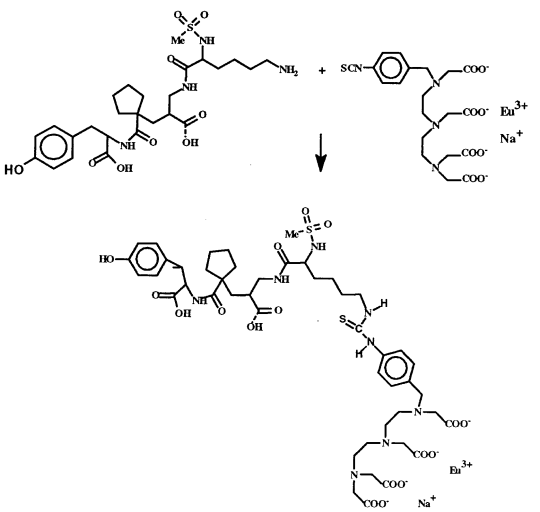

Fig. 2. Reaction scheme for the labelling of sampatrilat with europium-labelled chelate.

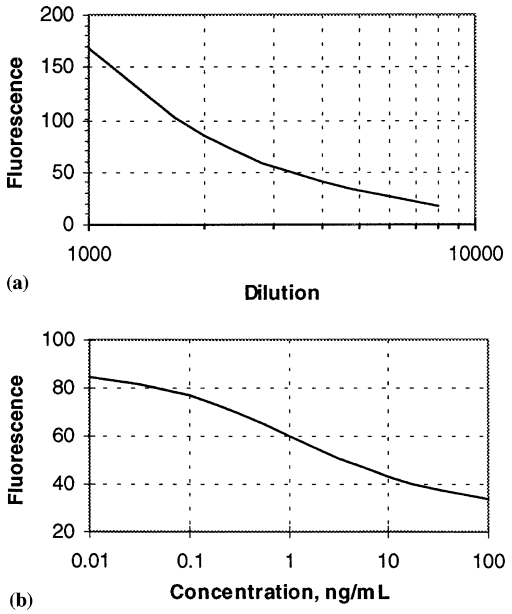

Fig. 3. Antibody titre and concentration dependence for sheep antiserum (Sheep 1914). Sampatrilat-BSA conjugate was coated onto 96-well polystyrene plates and sheep antiserum 1914 was diluted appropriately (A) or at a final dilution of antiserum of 1:2000 increasing concentrations of sampatrilat in buffer (50 μl) were added (B). The plates were incubated at room temperature for 2 h, followed by addition of europium-labelled Protein G (1:200 dilution) and incubation for a further hour. The plate was then washed, enhanced with 200 μl 'Enhance' solution and counted.

2.2. Apparatus

The platewasher, plateshaker, DELFIA Research Fluorometer 1234, DELFIA assay buffer, DELFIA platewash concentrate, DELFIA enhance solution and anti-rabbit (goat) IgG microtitration strips (plates of 8×12 wells) were obtained from Wallac EG&G Berthold UK, Milton Keynes, UK. An IBM-compatible microcomputer was used to collect data from the fluorometer which were subsequently analysed using the programme MultiCalc (Wallac, UK).

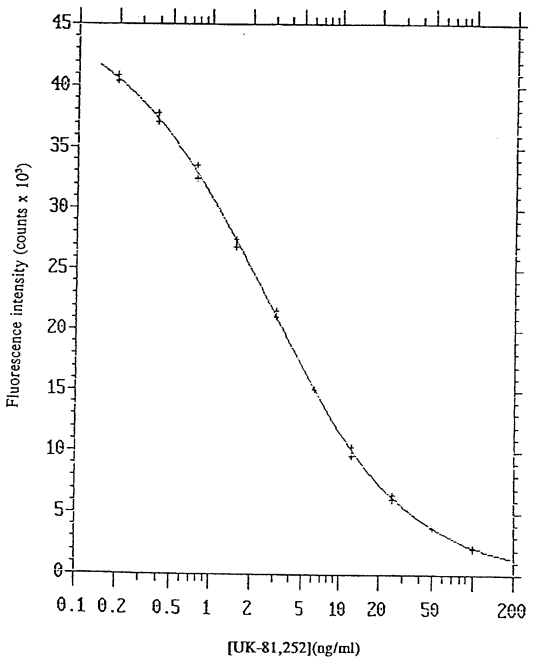

Fig. 4. Typical DELFIA calibration curve for sampatrilat over the range 195 pg ml^{-1} to 100 ng ml^{-1} from the programme MultiCalc. The ordinate is sampatrilat concentration (ng ml^{-1}); the abscissa is fluorescence counts $\times 10^{-3}$.

2.3. Antibodies

Anti-sampatrilat antibodies were raised in rabbits by coupling sampatrilat to PPD (pneumococcal polysaccharide derivative) via carbodiimide chemistry and using this conjugate to prime and then boost the animals. Sampatrilat (10 mg) and PPD (20 mg) or BSA (20 mg) were dissolved in distilled water (0.5 ml) and a solution 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (10 mg, 0.5 ml) was added dropwise with mixing. The reaction mixture was acidified with 50 μl 0.1 M HCl and the reaction allowed to proceed in the dark at room temperature for 18 h. The resulting conjugate was dialysed against 0.9 g/l NaCl (2×5 l) and the solution stored frozen in 1 ml aliquots containing 2 mg PPD or BSA conjugate. Six rabbits were given 1 ml of an emulsion of PPD conjugate (1 mg in 0.5 ml) and Freund's complete (incomplete on subsequent boosts) adjuvant (0.5 ml) by intramuscular and subcutaneous injections at six sites on each of six rabbits. The rabbits were then boosted at monthly intervals and bleeds obtained 10 days after each boost. Terminal bleeds were obtained after a final boost 21 months after initial immunisation. Antibody titre and specificity were assessed using either plates coated with sampatrilat-BSA conjugate and europium-labelled protein G to determine binding of rabbit anti-sampatrilat antibodies, or goat-anti-rabbit coated plates to bind the anti-sampatrilat antibodies which then bound europium-labelled sampatrilat.

2.4. Procedure

Solutions used were Tris–HCl buffer solution (0.05 M, pH 7.75), Tris–HCl–BSA buffer solution (0.05 M, pH 7.75, 75 mg ml^{-1} heavy-metal-free BSA), DELFIA assay buffer and wash concentrate. Stock sampatrilat solution (approx.

Table 1
Back-calculated calibration curve concentrations for the DELE IA assay on three different occasions using a 5-parameter logistic unweighted algorithm

Concentration (ng ml^{-1})										
Theoretical	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20
Found 1	98.7	51.4	26.4	12.7	6.24	2.95	1.60	0.81	0.38	0.20
Difference, %	−1.26	2.7	5.74	1.57	−0.20	−5.66	2.65	3.09	−3.39	0.95
Found 2	86.8	56.4	27.1	12.1	6.26	3.15	1.47	0.82	0.41	0.19
Difference, %	−13.2	12.9	8.4	−3.4	0.1	0.8	−5.6	4.2	3.9	−4.6
Found 3	103.9	49.0	24.7	12.5	6.34	3.13	1.53	0.81	0.39	0.20
Difference, %	3.9	−2.0	−1.0	−0.2	1.4	0.1	−2.4	3.0	−1.4	0.0

Table 2
Inaccuracy and imprecision for the manual DELFIA method for sampatrilat in human plasma

Batch	Concentration spiked (ng ml^{-1})	Mean concentration measured (ng ml^{-1}) ($\pm$S.D.)	n	% Inaccuracy	% Imprecision
1	0.195	0.195 (0.01)	7	0.2	6.7
	3.125	3.09 (0.11)	7	−1.0	3.7
	50	48.31 (0.77)	7	−3.4	1.6
2	0.195	0.21 (0.02)	7	8.1	10.0
	3.125	3.21 (0.09)	7	2.8	2.7
	50	49.92 (0.75)	7	−0.2	1.5
3	0.195	0.22 (0.03)	7	16.1	14.1
	3.125	3.08 (0.15)	7	−1.4	4.8
	50	53.15 (1.75)	7	6.3	3.3
4[a]	0.195	0.16 (0.02)	7	−17.6	12.9
	3.125	2.93 (0.12)	7	−6.3	4.2
	50	50.58 (2.83)	7	1.2	5.6

[a] An 8-way pipette was used to add label and antibody.

200 μg ml^{-1}) was stored at approximately 4°C and was stable for 3 months. The working solution of sampatrilat at 10 μg ml^{-1} was prepared daily. The stock solution of europium labelled sampatrilat (1:1000) was made by diluting 20 μl labelled sampatrilat (an HPLC fraction from Wallac Oy) to 20 ml with Tris–HCl buffer pH 7.75 0.9% NaCl, 0.2 g l^{-1} sodium azide, 0.1% BSA and was stored at approximately 4°C. This solution was stable for at least three months. The working label solution (1:2 000 000) in DELFIA assay buffer was prepared daily. The stock solution of anti-sampatrilat antiserum (1:100, rabbit L, bleed date 29/3/93) was prepared in Tris-HC1 buffer pH 7.75 0.9% NaC1 0.2 g l^{-1} sodium azide and stored at approximately 4°C. Working antibody solution (1:10 000) was prepared daily in DELFIA assay buffer. The assay was carried out as follows: the goat-anti-rabbit coated plates were washed twice using the plate-wash with 300 μl DELFIA wash buffer with a 10-s soak after each well fill: 50 μl of sample, standard or quality-control sample was then added to each well. The calibration samples were made by serial dilution. Samples were assayed in duplicate. Europium-labelled sampatrilat solution was then added (100 μl), followed by antiserum solution (100 μl). The final antibody dilution was 1:25 000. The plates were then incubated at room temperature for 2 h on the plateshaker (low speed). After incubation, wells were washed six times and 'Enhance' solution (200 μl) was added to each well. The plates were then shaken at room temperature for 5 min and then counted using the DELFIA fluorimeter. Each well was counted for 1 s at 1000 Hz and the data processed using the programme 'Multicalc'. Curve fitting was iterative using a five-parameter logistic fit. Plasma or urine samples, calibrants, QCs, label and antibody solutions were all added manually to the wells of the plate. We also used a Packard 104DT 4-way robotic sampler with disposable tips to dispense all samples and solutions in order to increase throughput further. Plasma was dispensed by pre-wetting the tips, and tips were changed between each sample. In the case of the urine assay, the calibration curve was as described above but diluted in water, and all urine sample were diluted ten-fold.

Method validation was carried out in three separate batches by analysing replicate samples at 0.195, 3.125 and 50.0 ng ml^{-1} ($n = 7$) diluted from the same working solution as that for the calibration line. The lowest concentration giving acceptable imprecision and inaccuracy ($\pm$20%

Table 3
Inaccuracy and imprecision for the automated DELFIA method for sampatrilat in human plasma

Batch	Concentration spiked (ng ml^{-1})	Mean concentration measured (ng ml^{-1}) ($\pm$S.D.)	n	% Inaccuracy	% Imprecision
1	0.195	0.20 (0.02)	7	1.4	8.1
	3.125	3.20 (0.09)	7	2.4	2.9
	25	23.1 (0.30)	7	−7.5	1.3
2	0.195	0.18 (0.02)	7	−6.5	13.0
	3.125	3.08 (0.04)	7	−1.5	1.4
	25	24.9 (0.42)	7	0.4	1.7
3	0.195	0.18 (0.02)	7	−6.3	11.8
	3.125	3.02 (0.09)	7	−3.5	3.0
	25	25.4 (0.91)	7	1.6	3.6

each) was defined as the lower limit of quantification. The MultiCalc programme generated a minimum detectable concentration for each batch which was always lower than the lowest calibration point.

3. Results and discussion

Our initial strategy for raising antisera to sampatrilat in sheep by coupling the tertiary-butyl-protected hapten UK-100 637 via the lysine-like ε-amino group using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC) to BSA or keyhole limpet haemocyanin failed to generate any antisera as judged by binding of tritiated sampatrilat using the dextran-coated charcoal method of separation at various dilutions. We could find no binding of drug at all. Further immunisations were carried out using native sampatrilat as the starting material for conjugation but still no binding was observed. We considered this surprising, since we expected the compound to be immunogenic. As a result, we also tested these bleeds with a DELFIA system in a configuration such that sampatrilat-BSA conjugate was bound onto 96-well assay plates. Any antibody binding to the immobilised sampatrilat was detected using europium-labelled protein G. We then found that there were in fact anti-sampatrilat antibodies present in these sheep bleeds. Subsequent experiments showed that the binding was specific for sampatrilat and could be displaced by increasing concentrations of free sampatrilat in the assay. Fig. 3 shows typical data obtained for one of the sheep antisera. The reason that we did not find any binding of tritiated sampatrilat to sheep antisera when using the dextran-coated charcoal assay remains unclear, but we assume that the affinity of the antibody-sampatrilat interaction was very low and that the charcoal stripped the bound sampatrilat from the antibodies before centrifugal separation occurred. We did not subsequently develop and validate a DELFIA method using sheep antisera because the rabbit antisera were superior, giving both a higher titre (1:50 000) and greater sensitivity.

3.1. DELFIA in plasma

It would have been possible to develop and validate an immunoassay for sampatrilat using the sheep antisera as described above with a europium-labelled Protein-G endpoint. This however was not as attractive a proposition as the final configuration of the assay using europium-labelled sampatrilat. The main reasons for this were the low titre of the sheep antisera, that quality-controlled plates coated with goat anti-rabbit antisera were commercially available (thus we did not have to coat our own plates with sampatrilat-BSA conjugate) and the increased sensitivity available. Once high-titre rabbit antisera and the europium-labelled sampatrilat were

Table 4
Analysis by DELFIA of three different samples from a clinical trial to assess parallelism of the method. The samples were assayed undiluted, and diluted by 2-, 4- and 8-fold with blank human plasma

Sample	Plasma dilution	Concentration, ng ml^{-1}		
		Found	Calculated	% Difference
A	neat	7.52		
	2x	3.80	3.76	+1.1
	4x	1.89	1.88	+0.5
	8x	0.80	0.94	−14.9
B	neat	12.14		
	2x	5.96	6.07	−1.8
	4x	2.64	3.04	−13.2
	8x	1.33	1.52	−12.5
C	neat	17.75		
	2x	7.48	8.88	−15.8
	4x	4.05	4.44	−8.8
	8x	2.01	2.22	−9.5

available, the assay as described was very quickly validated to a lower limit of quantification (LLOQ) of 0.2 ng ml^{-1}. Later work showed that using a disequilibrium format for the assay (in which antibody was added 1 h prior to addition of label) the assay was capable of achieving a LLOQ of 0.05 ng ml^{-1} (data not shown).

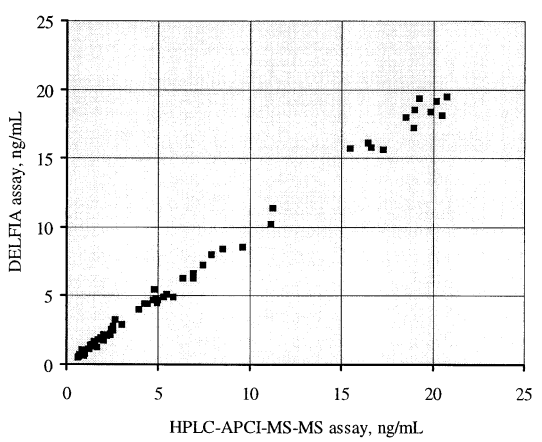

Fig. 5. Cross-validation of the DELFIA Assay for sampatrilat with the HPLC-APCI-MS-MS Method. Samples from volunteers were analysed for sampatrilat by DELFIA and HPLC-APCI-MS-MS on the same day as detailed in the text. The individual concentrations obtained by each method were plotted against each other.

A typical standard curve for the method is shown in Fig. 4 and back-calculated calibration data are shown in Table 1. The typical range of fluorescence counts was from circa 80 000 at B_0 to 5000 at 100 ng ml^{-1}, with typical differences between duplicates of less than 5% over the the entire calibration range.

3.1.1. Inaccuracy and imprecision for the assay

3.1.1.1. Manual method. Inaccuracy and imprecision were assessed by analysing 7 replicate samples at each of three concentrations in duplicate as described above. The three concentrations of drug were prepared from the same stock standard as the one used to prepare the calibration curves. A fourth validation was carried out using 8-way multi-channel pipettes: all the data is shown in Table 2.

3.1.1.2. Automated method. Similarly, we assessed the inaccuracy and imprecision of the assay when carried out using a Packard Multiprobe 104DT automatic sample handling system. This is a four-way instrument that uses disposable tips to dispense samples; we used a new tip for each sample, standard or QC thus minimising the possibility of

Table 5
Inaccuracy and imprecision for the automated DELFIA method for sampatrilat in human urine

Batch	Concentration spiked (ng ml^{-1})	Dilution factor	Mean concentration measured (ng ml^{-1}) ($\pm$S.D.)	n	% Inaccuracy	% Imprecision
1	5.00	10	0.42 (0.03)	7	−16.2	8.1
	31.3	10	3.24 (0.11)	7	3.5	3.3
	250	10	22.7 (0.61)	7	−9.0	2.7
	2000	100	18.0 (1.49)	7	−9.8	8.3
2	5.00	10	0.41 (0.05)	7	−17.2	12.6
	31.3	10	2.97 (0.25)	7	−5.0	8.3
	250	10	21.3 (0.94)	7	−14.6	4.4
	2000	100	18.4 (2.43)	7	−7.8	13.2
3	5.00	10	0.52 (0.07)	7	4.9	13.6
	31.3	10	3.45 (0.21)	7	10.1	8.7
	250	10	25.3 (2.30)	7	1.1	9.1
	2000	100	19.1 (0.92)	7	−4.3	4.8

cross-contamination. For the addition of label and antibody solution, new tips were used after each half-plate. The validation data obtained using the automated method is shown in Table 3. As can be seen from the data, the automated method offered no further improvement in the precision of the assay. Its very considerable advantage was that the throughput could be increased from three to eight plates per day while simultaneously freeing the analyst for other duties.

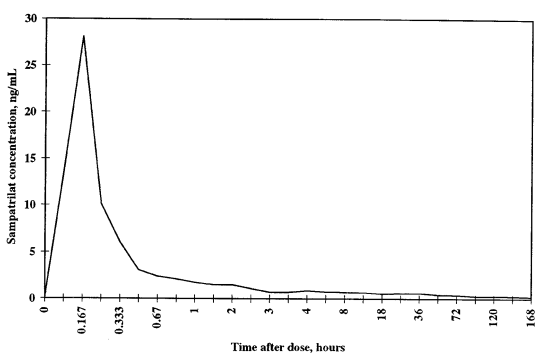

Fig. 6. Concentrations of sampatrilat in a volunteer following a single intravenous dose of 0.5 mg. Samples of blood (2 ml) were taken into heparinised tubes and centrifuged. The plasma was frozen and transported to the analytical laboratory. Analysis of the samples in duplicate by DELFIA for sampatrilat were carried out as detailed in the text. Note that the ordinate is not a linear scale.

3.1.2. Sensitivity

We defined the lower limit of quantification as the lowest calibration point with acceptable precision, i.e. 195 pg ml^{-1} in human plasma. Similarly, we set the upper limit to 50 ng ml^{-1} initially, but later experience led us to reduce this to 25 ng ml^{-1} since we did not need the extended range. Validation at 100 ng ml^{-1} was not attempted. The 100 ng ml^{-1} calibrant was used to assist calculation of the standard curve. We found that with the program MultiCalc the five-parameter logistic fit algorithm, unweighted, normally calculated the best standard curve as judged by the back-calculated values obtained from the standard curve.

3.1.3. Matrix effects

A selection of sixteen different control plasma samples was analysed to check for interference from endogenous compounds. No interference was seen. QCs and blanks made up in Blood Transfusion Service plasma showed interference in the form of spuriously high concentrations of sampatrilat being reported. This is probably due to the presence of EDTA in the plasma which would strip the Eu^{3+} from the labelled sampatri

lat, reducing the counts bound to the plate producing spuriously high concentration values.

3.1.4. Cross-reactivity

There are no known metabolites of sampatrilat to test for cross-reactivity. However, as can be seen from the cross-validation data (below), the selective mass-spectrometric method [1] showed extremely good correlation with the DELFIA method which leads us to believe that the latter is as selective as the physical method. The correlation coefficient between the two methods was 0.995. We tested a number of possible co-administered drugs in the assay to check for potential cross-reactivity, but again found no evidence for interference from e.g. captopril, enalopril, nifedipine, propranolol and paracetamol.

3.1.5. Parallelism

Clinical samples containing high (circa 10–20 ng ml^{-1}) levels of sampatrilat were serially diluted 2-, 4- and 8-fold with blank human plasma to establish the parallelism of the assay. The results are shown in Table 4, which demonstrates that the differences between found and expected values were within acceptable limits, further confirming that the assay was suitable for the analysis of samples from volunteers in clinical trials.

3.1.6. Cross-validation

The performance of the DELFIA assay was cross-validated with the APCI-LC-MS assay [1]. Six sets of plasma samples from volunteers in a multiple-dose study were assayed by both methods on the same day. The mean differences for each profile were less than 20%. A scatter graph of the data is shown in Fig. 5. The correlation was 0.995, and the slope of the line was 0.95.

3.1.7. Stability

Stability of sampatrilat in plasma QCs and clinical samples was assessed using the APCI-LCMS 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. Sampatrilat QC samples were also stable for six months in Li-heparin plasma as assessed by the DELFIA method.

3.2. DELFIA in urine

We also validated the DELFIA for analysis of sampatrilat in human urine. We found that some urine matrices gave spuriously high blank concentrations for sampatrilat when using the assay exactly as for plasma (50 μl sample) and thus it was necessary to dilute each sample tenfold with water for assay. We used water to dilute the standard curve in this case and as can be seen from the imprecision and inaccuracy data (Table 5) the LLOQ was set to 5 ng ml^{-1}. Three different batches of urine were used for these validation experiments.

4. Conclusions

We have developed and validated a fast, reliable, high throughput immunoassay for sampatrilat in human plasma and urine that is suitable for the determination of the compound in samples generated during the clinical development programme. The method uses a europium-labelled analogue of sampatrilat with time-resolved fluorescence as an endpoint. The major advantages of time-resolved fluorescence competitive immunoassays are that the samples are read very quickly (1 s per sample as opposed to several min/sample for RIA) thus increasing throughput and allowing rapid feedback during assay development and minimal disposal problems. The assay is capable of accurate quantification of sampatrilat down to 0.20 ng ml^{-1} in plasma and 5 ng ml^{-1} in urine. Throughput is such that all samples from a small clinical trial (generating in the order of 600 samples) can be analysed within one week, and samples from a large, multiple-dose crossover study (more than 2000 samples) can be analysed within one month. We have used this DELFIA method to analyse samples from all clinical trials on the compound following the first two studies, which were analysed using the HPLC-APCI-MS-MS assay [1]. The assay has also been successfully established in the laboratories of a contract research organisation. Fig. 6

shows a typical plasma concentration-time plot for a volunteer given 2 mg of sampatrilat intravenously. The throughput of the DELFIA was such that in this dose-escalation i.v. study, all samples up to 24 h from all subjects dosed in one period were analysed and the results reported within two days of receipt of samples. This compares extremely well with assays using HPLC and, in particular, with the derivatisation HPLC-APCI-MS-MS assay described previously for sampatrilat [1]. This latter involved two solid-phase extractions and a derivatisation step, with three evaporation stages; it took 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. The derivatisation APCI.

MS-MS assay, being selective and sensitive, was used to cross-validate the immunoassay which showed no positive or negative bias with respect to the selective physical method.

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