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Quantitative analysis of dipeptidyl peptidase inhibitor P32/98 and its main metabolite in rat, dog, mouse, monkey, human plasma and human urine using liquid chromatography–tandem mass spectrometry: application to pharmacokinetic evaluation

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Abstract

A sensitive, specific and robust assay was developed for the simultaneous determination of the oral antidiabetic drug candidate P32/98 and its main metabolite P57/99 in different biological fluids using LC–MS/MS in the atmospheric pressure chemical ionization (APCI) positive mode. Both analytes were isolated from the biological matrices by solid phase extraction using a strong cation exchanger. This assay was successfully cross-validated for rat, dog, mouse, monkey, human plasma and human urine. The pre-study validation results, as well as the in-study quality control (QC) data obtained, demonstrate the feasibility of the assay for pharmacokinetic evaluation of the compounds in different species and confirm the robustness of the assay for routine use.

Keywords: Pharmacokinetics; Dipeptidyl peptidases; DP IV-inhibitor

1. Introduction

Type 2 diabetes is a metabolic disease regarded as an increasing worldwide epidemic [1,2]. The therapeutic potential of dipeptidyl peptidase IV (DP IV)-inhibitors in the treatment of type 2 diabetes has been the focus of recent pharmaceutical research.

In the blood circulation DP IV inactivates the insulinotropic gut hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) by removing dipeptides from their N-termini. It was shown that stabilization of post-prandially released GLP-1 and GIP after oral application of DP IV-inhibitors led to an improved glucose tolerance in vivo [3,4]. This new therapeutic concept has the chance to revolutionize diabetes therapy if chronic application of DP IV-inhibitors will prove to be safe [5,6]. Hence, the detailed investigation of the pharmacokinetics of appropriate drug candidates in preclinical and early clinical development is of particular importance.

Di-[(2*S*,3*S*)-2-amino-3-methyl-pentanoic--1,3-thiazolidide] fumarate (P32/98) was shown to be a potent

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competitive inhibitor of the enzyme DP IV. For pharmacokinetic evaluation of this new drug candidate during toxicological and clinical studies there was a need for a robust bioanalytical assay. Our aim within this project was to develop a sensitive and specific assay for simultaneous determination of the parent drug and its major metabolite (P57/99) in biological fluids from different species. Furthermore the established assay was validated by determining specificity, linearity, limit of quantification, intra- and inter-run precision and accuracy as well as recovery and stability in rat, dog, mouse, monkey, human plasma and human urine according to international standard guidelines [7].

2. Experimental

2.1. Chemicals

P32/98 (di-[(2*S*,3*S*)-2-amino-3-methyl-pentanoic-1,3-thiazolidide] fumarate) was synthesized by Heumann Pharma (Nürnberg, Germany); the metabolite P57/99 (di-[(2*S*,3*S*)-2-amino-3-methyl-pentanoic-1,3-thiazolidine-*S*-oxide] fumarate) and the internal standard P36/98 (di-[2-amino-3-methyl-butanoic-1,3-thiazolidide] fumarate) were synthesized by the Department of Medicinal Chemistry at Probiodrug (Halle, Germany) according to standard methods as described in [8]. The chemical structures of the active compounds are shown in Fig. 1. High purity solvents were purchased from Baker (Atlantic Labo, France) and Merck (Darmstadt, Germany), and were used without further purification. All other chemicals were commercially available and of analytical grade. Drug free EDTA animal plasma was obtained from LPT GmbH (Laboratory of Pharmacology and Toxicology, Hamburg, Germany). Human EDTA plasma from non-infectious subjects was supplied by Biomedia (Boussens, France).

2.2. Preparation of calibration standards and quality control samples

Stock solutions of P32/98 and P57/99 were prepared by dissolving the pure reference compounds in water to achieve final concentrations of 1 mg/ml (human plasma and urine) and 10 mg/ml (for animal plasma), respectively. Intermediate solutions, containing both analytes, were prepared by mixing aliquots of the respective stock solutions (final concentrations of P32/98 and P57/99: 1 and 4 μg/ml for human studies and 100 μg/ml for animal studies). Working standard solutions were obtained by appropriate dilutions of the intermediate solutions in water and adjusted to the calibration range of each species. Calibration standards were prepared while mixing blank matrix (50, 100 and 20 μl, respectively) and additionally 50 μl of the respective working solution with 900 μl buffer an 50 μl internal standard working solution (see Section 2.3 for sample preparation procedure). For human plasma analysis the concentrations of calibration standards were 1, 2, 5, 20, 50, 100, 200, 400 and 500 ng/ml. Calibration standards for human urine were prepared at concentrations of 10, 20, 100, 500, 2000, 5000, 8000 and 10,000 ng/ml whereas for animal plasma the concentrations were 10, 20, 50, 250, 500, 2000, 4000 and 5000 ng/ml.

Working solution of internal standard was prepared by appropriate dilution of the stock solution (1 mg/ml) in water to achieve a final concentration of 50 ng/ml (human plasma) and 500 ng/ml (human urine and animal plasma), respectively.

Standard curves were performed in parallel to each batch of samples. The calibration curves were obtained by plotting the chromatographic peak height ratios of analyte and internal standard versus known concentrations of P32/98 and P57/99. A $1/x^2$ weighted linear regression analysis was employed to determine the

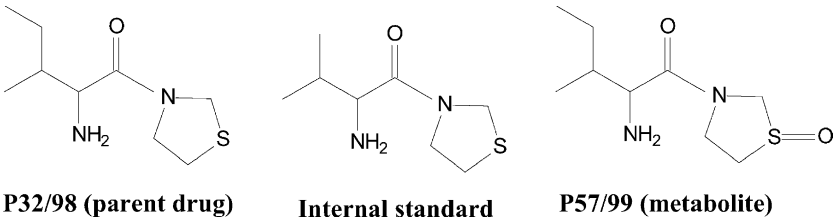

Fig. 1. Chemical structures of P32/98, P57/99 and internal standard.

slope and intercept. These parameters were applied to calculate the unknown concentrations of P32/98 and P57/99 in the quality control (QC) and study samples.

QC samples were obtained using a second set of working standard solutions. For analysis of human plasma samples the final concentrations of QC samples were 2, 50 and 500 ng/ml while for human urine 20, 2000 and 10,000 ng/ml. QC samples of animal plasma were prepared at concentrations of 20, 250 and 4000 ng/ml. A further set of animal QC samples at 5 and 100 μg/ml was prepared to check the possible influence of dilution of high dose samples. These QC samples were diluted (1:20) with blank plasma before analysis.

2.3. Analytical procedure

2.3.1. Sample preparation

Fifty microliters of animal plasma samples (100 and 20 μl of human plasma and human urine, respectively) were mixed with 900 μl of 60 mM citrate buffer solution (pH 6, Merck) and 50 μl internal standard working solution. After briefly stirring using a vortex mixer, the tubes were centrifuged for 10 min at 3500 rpm. The mixture was applied onto the top of a 1 ml cartridge containing 100 mg of a strong cation exchanger (Bond Elute PRS, Varian) previously conditioned with 1 ml of methanol and 1 ml of water. The cartridges were washed successively with 1 ml of buffer (pH 6) and 1 ml acetonitrile and afterwards placed over 10 ml disposable glass tubes and the analytes were eluted under atmospheric pressure using 3 × 1 ml of a methanol/ammonia solution (95/5; v/v). The solvent was evaporated to dryness under a gentle stream of nitrogen at 45 °C using an evaporator (TurboVap, Zymark). The dry residue was dissolved with 200 μl of water and a 20 μl aliquot was injected into the LC–MS/MS system (see below).

2.3.2. LC–MS/MS analysis

Liquid chromatography was performed using a HP1100 system from Agilent. The analytical column (50 mm × 4 mm) was packed with Lichrosphere RP Select B stationary phase (5 μm). Mobile phase was a mixture of water/acetonitrile (70:30; v/v) containing formic acid (0.02%; v/v). Mobile phase flow was kept isocratic at a flow rate of 0.4 ml/min. The HPLC was connected to a API 3000 MS/MS system (Applied Biosystems) operating in the atmospheric pressure chemical ionization (APCI) positive mode. Source temperature was 400 °C. Nitrogen was used as both nebulizer (70 psi) and collision gas (collision energy: 25 eV). Air was applied as auxiliary gas (50 psi). Corona discharge current was set to 3 μA. The MS/MS system was focused in the Multiple Reaction Monitoring (MRM) mode to monitor the following ion transitions: 203.2 → 86.1 for P32/98, 219.2 → 86.1 for P57/99 and 189.2 → 72.1 for the internal standard. Declustering potential was set between 21 and 26 V. Data were collected using a MAC OS2 operating system (Applied Biosystems). Final data processing was performed by using the software CORVETTE developed and validated by PAREXEL. An internal standard method was used for quantification. All calculations were based on peak height ratios.

3. Results and discussion

3.1. LC–MS/MS method development

The aim of the present study was to develop and validate a robust method for determination of P32/98 and its major metabolite in plasma and urine using LC–MS/MS. A structural analogue was synthesized as internal standard.

A simple solid phase extraction procedure using propylsulfonic acid (PRS) as a strong cation exchanger for selective interaction with the amino group of the dipeptide derivatives was found to be a suitable technique for sample preparation resulting in high recoveries of the compounds of interest as well as good chromatography due to clean extracts. In the analytical conditions described above, retention times were approximately 2.3 min for P57/99, 3.2 min for the internal standard and 4.0 min for P32/98 (see Figs. 2–4).

Comparative tests were performed by using two ionization techniques in the positive mode: electrospray (ESI) and APCI. Preliminary results have shown for all tested matrices that the ion suppression phenomenon was negligible when using APCI. Moreover, signals were more sensitive and reproducible compared to the ones obtained with the ESI device. Thus, APCI has been chosen as a robust and reliable ionization technique as also mentioned in [9]. MS/MS conditions

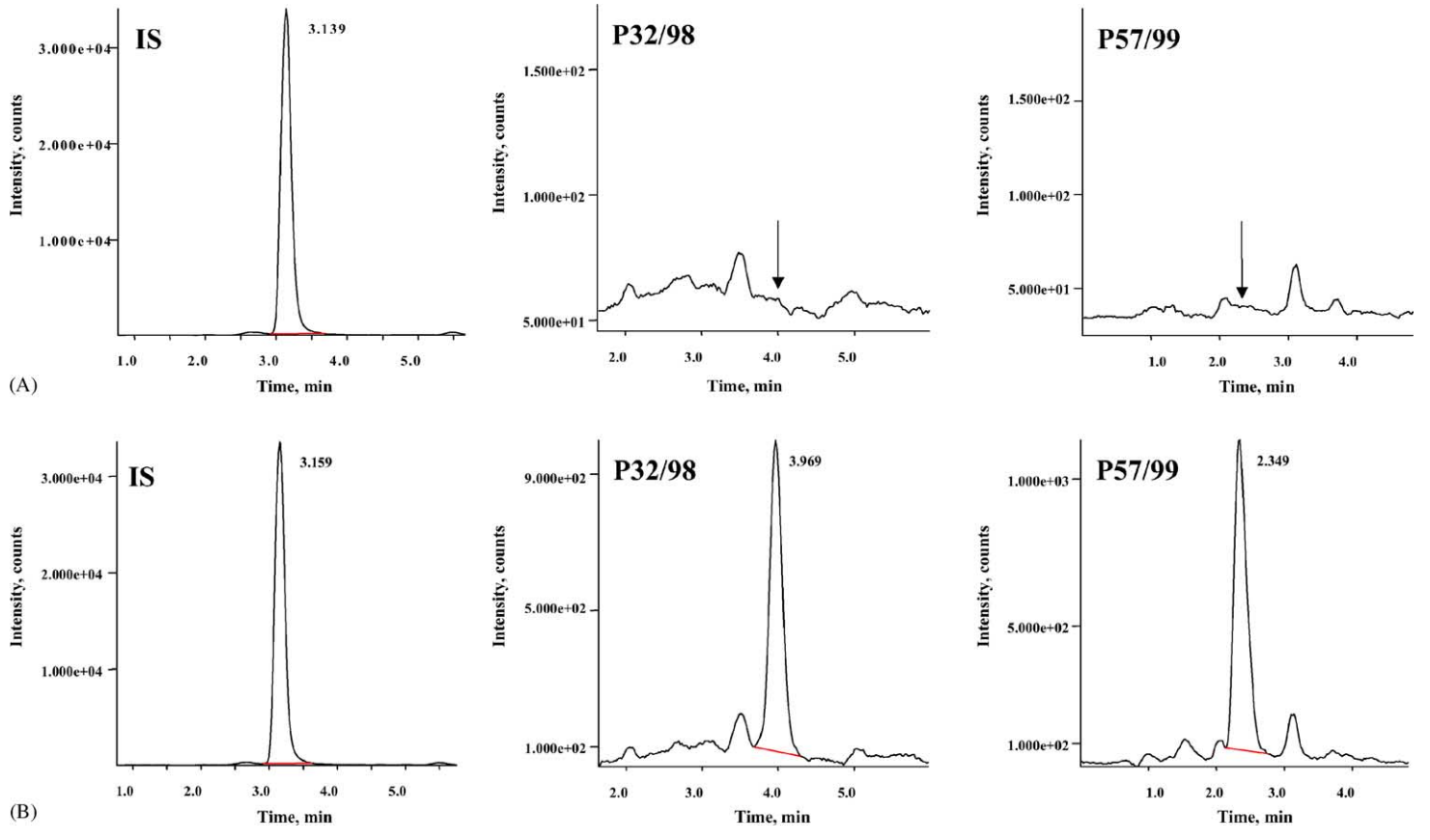

Fig. 2. Representative chromatograms of human plasma extracts obtained from a blank sample (A) and a plasma sample spiked with P32/98 and P57/99 at the LLOQ of 1 ng/ml (B).

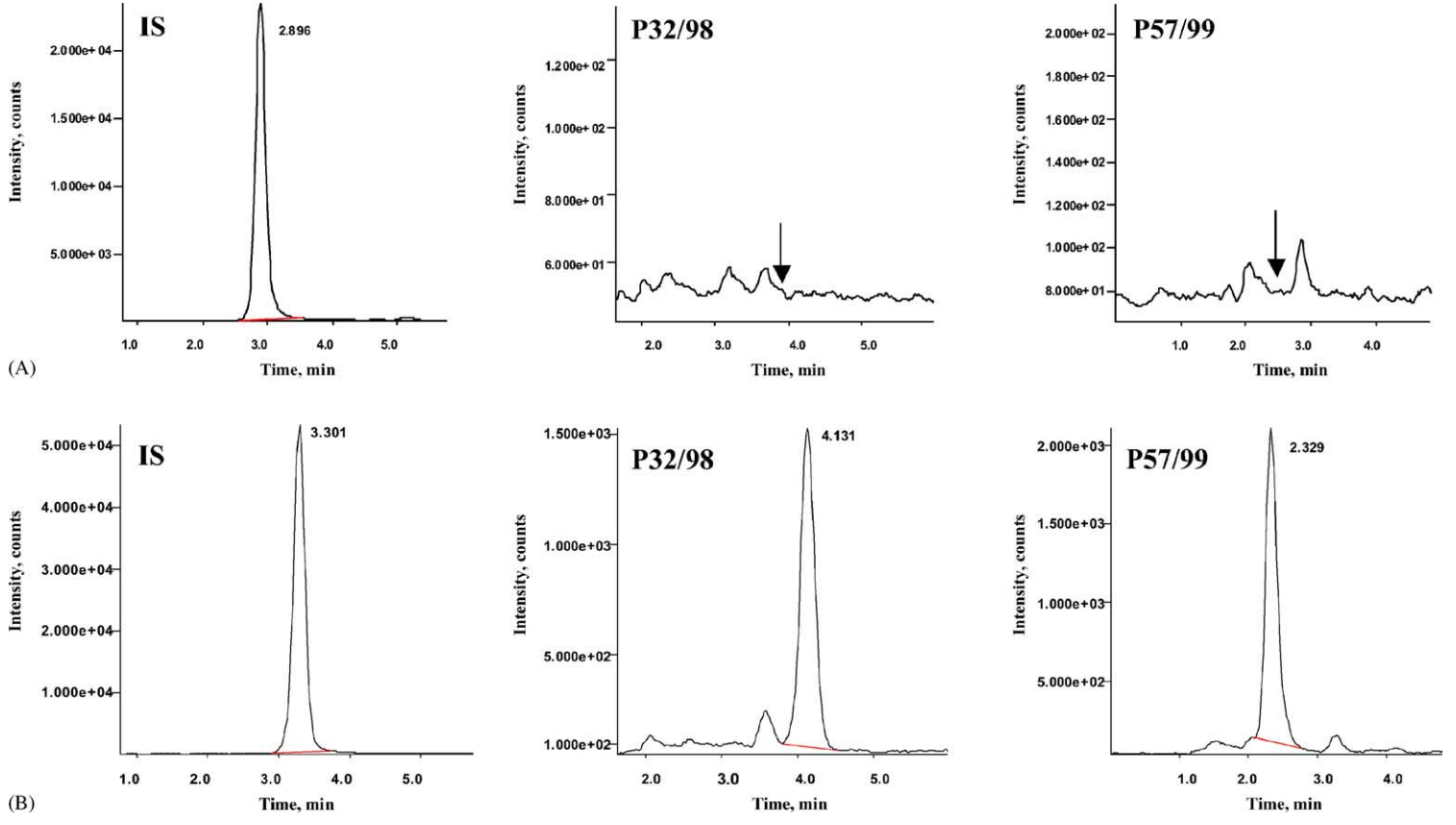

Fig. 3. Representative chromatograms of human urine extracts obtained from a blank sample (A) and a plasma sample spiked with P32/98 and P57/99 at the LLOQ of 10 ng/ml (B).

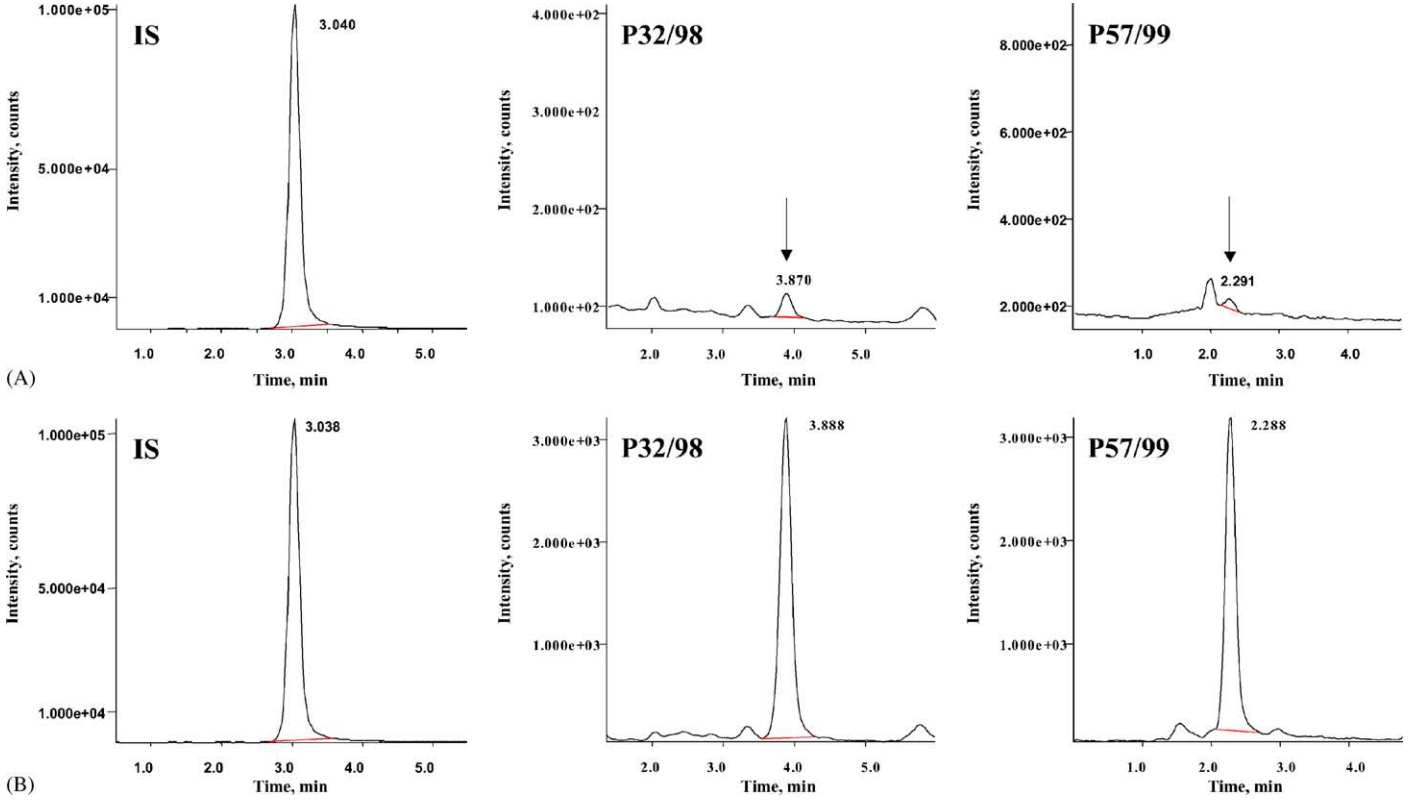

Fig. 4. Representative chromatograms of rat sample extracts obtained from a blank sample (A) and a plasma sample spiked with P32/98 and P57/99 at the LLOQ of 10 ng/ml (B).

were optimized by tuning the instrument to obtain maximum response and stable product ions. A dwell time of 400 ms per mass range led to a minimum of at least 35 data points for the measurement of P32/98 and P57/99. Typical mass chromatograms obtained for human, rat plasma and human urine samples spiked with P32/98 and P57/99 are presented in Figs. 2(B), 3(B) and 4(B).

3.2. Pre-study validation

3.2.1. Instrument qualification

A pure reference standard solution (50 ng/ml) of each compound was injected 100 times (comparable to

Table 1
Mean recovery for extraction of parent drug and metabolite from plasma and urine from different species ($n = 6$)

Species	Matrix	Mean recovery (%)	
		Parent drug	Metabolite
Rat	Plasma	87	98
Dog	Plasma	85	100
Mouse	Plasma	90	95
Monkey	Plasma	90	95
Human	Plasma	70	95
Human	Urine	88	100

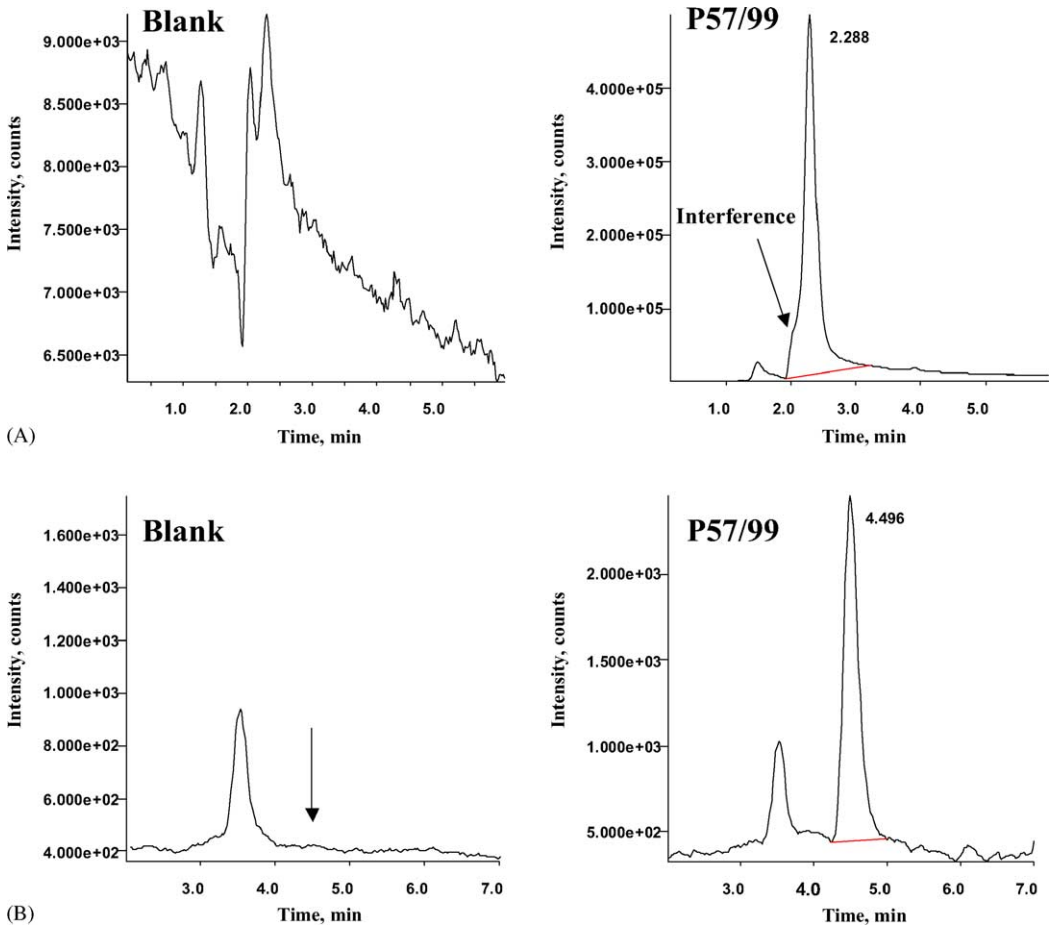

Fig. 5. Representative chromatograms for the determination of the metabolite P57/99 in monkey plasma extracts obtained from a blank sample and a plasma sample spiked with P57/99 at the LLOQ of 10 ng/ml using a short (50 mm × 4 mm) column (A) and a long (125 mm × 4 mm) column (B).

Table 2
Pre-study validation data: Inter-run repeatability for parent drug and metabolite at the LLOQ (1 ng/ml for human plasma and 10 ng/ml for animal plasma and human urine) obtained by replicate QC sample analysis in different species

Species	Matrix	Parent drug		Metabolite	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Rat	Plasma	3.37	4.73	3.26	4.71
Dog	Plasma	3.25	4.54	3.76	3.14
Mouse	Plasma	4.47	4.03	4.31	6.68
Monkey	Plasma	5.11	−4.64	7.32	−0.84
Human	Plasma	3.40	−2.61	4.79	−3.24
Human	Urine	3.38	1.08	4.67	−1.85

Precision expressed as R.S.D. and accuracy expressed as bias (n = 6).

a batch size) in order to evaluate the LC–MS/MS system precision. The relative standard deviation (R.S.D.) was calculated to be 1.02% for P32/98 and 1.52% for P57/99, thus demonstrating that the system was suitable for accurate analysis of P32/98 and P57/99.

3.2.2. Specificity

Six different batches of drug free control plasma were investigated from each species except mice (only two different mouse plasma batches available) in order to assess interferences from endogenous compounds. Typical mass chromatograms obtained from blank human, rat plasma and human urine samples are shown in Figs. 2(A), 3(A) and 4(A). No chromatographic interferences were detected in the batches of control plasma from human, rat and dog plasma and human urine. For mouse and monkey plasma samples an interference at the retention time of the metabolite was observed in blank samples (see an example for monkey in Fig. 5(A)). Furthermore we observed a small drop in baseline due to ion suppression (see blank sample in Fig. 5(A)). Therefore, for the analysis of those matrices a longer column (125 mm × 4 mm) was used to avoid both problems due to a shift in retention times (see Fig. 5(B)).

3.2.3. Recovery

The recovery was determined by comparing the signal obtained for each compound in a control plasma sample that had been spiked with known concentration of P32/98, P57/99 and internal standard before the sample preparation procedure with one that had been spiked after the extraction procedure. The results provided evidence that there was no major loss during sample processing. The recovery was evaluated at three concentration levels, assessed in all matrices and ranged from 70 to 90% and 95 to 100% for P32/98 and P57/99, respectively, as summarized in Table 1.

3.2.4. Linearity

Calibration curves obtained by plotting the peak height ratios of the ion transitions versus nominal concentrations of P32/98 and P57/99 resulted in straight

Table 3
In-study QC data: inter-run precision and accuracy calculated from replicate human plasma QC sample analysis during three different clinical studies

Nominal concentration (ng/ml)	Study no.	QC no.[a]	Parent drug		Metabolite	
			Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
500	1	20	2.45	3.79	4.84	−2.68
	2	8	2.67	0.81	2.56	1.74
	3	4	0.41	−6.34	2.76	−7.54
50	1	20	2.77	3.28	4.11	−1.18
	2	8	1.16	3.72	1.80	4.03
	3	4	3.75	−4.04	2.40	−8.03
2	1	20	3.56	5.10	4.48	0.48
	2	8	3.36	6.43	4.59	5.86
	3	4	6.22	−5.50	3.74	−5.50

Precision expressed as R.S.D. and accuracy expressed as bias.
[a] Total number of QC samples analyzed (two QC samples per concentration level and batch).

Table 4
In-study QC data: inter-run precision and accuracy calculated from replicate human urine QC sample analysis during a clinical study (20 QC samples per concentration level)

Nominal concentration (ng/ml)	Parent drug		Metabolite	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
8000	4.46	−1.04	4.43	−1.40
2000	3.36	1.21	3.17	2.16
20	3.71	0.79	4.13	−0.96

lines over the concentration range. No bias and R.S.D. beyond 10% was observed.

3.2.5. Lower limit of quantitation (LLOQ)

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the analyte that can be measured with acceptable precision (±15%) and bias (<15%). A LLOQ of 1 ng/ml was specified for P32/98 and the metabolite in human plasma. For all other matrices the LLOQ was set to 10 ng/ml.

3.2.6. Precision and accuracy of the assay

To determine precision and accuracy of the assay the R.S.D. and the bias of the intra- and inter-assay variations were calculated from four runs of replicate sample analysis at four concentration levels. Inter-run precision at LLOQ was ranging from 3.25 to 5.11% for the parent drug and from 3.26 to 7.32% for the metabolite. Inter-run accuracy was calculated between −4.64 and 4.73% for parent drug and −3.24 and 6.68% for the metabolite (see Table 2).

3.2.7. Stability

Quality control samples were kept frozen during at least 24 h at −20 °C and then analyzed after one and three freeze-thaw cycles. Another set of quality control samples were kept at room temperature for 24 h.

Table 5
In-study QC data: inter-run precision and accuracy calculated from replicate animal plasma QC sample analysis during toxicological studies in different species

Nominal concentration (ng/ml)	Species	QC no.[a]	Parent drug		Metabolite	
			Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
100,000[b]	Rat	14	3.20	−3.95	6.79	−3.47
	Dog	8	3.43	−2.03	4.21	−4.27
	Mouse	4	1.90	−9.28	1.56	−7.01
	Monkey	14	3.78	1.23	4.46	1.19
5000[b]	Rat	14	6.39	−0.79	2.68	0.93
	Dog	8	3.32	2.39	5.39	1.60
	Mouse	4	6.69	−5.00	4.34	−3.79
	Monkey	14	2.76	−1.92	3.75	−1.67
4000	Rat	14	2.88	0.20	5.06	−0.02
	Dog	8	3.23	−1.93	5.54	−0.46
	Mouse	4	0.67	1.22	0.80	1.60
	Monkey	14	8.51	1.46	8.02	2.16
250	Rat	14	5.55	−1.34	4.06	2.61
	Dog	8	5.00	3.67	4.85	4.42
	Mouse	4	1.71	−1.22	1.75	−1.47
	Monkey	14	5.42	1.82	5.55	2.63
20	Rat	14	3.73	2.66	7.53	0.67
	Dog	8	4.42	3.66	4.34	2.71
	Mouse	4	2.28	4.76	1.59	3.28
	Monkey	14	5.62	−4.84	6.10	−6.80

Precision expressed as R.S.D. and accuracy expressed as bias.
[a] Total number of QC samples analyzed (two QC samples per concentration level and batch).
[b] For analysis diluted 1:20.

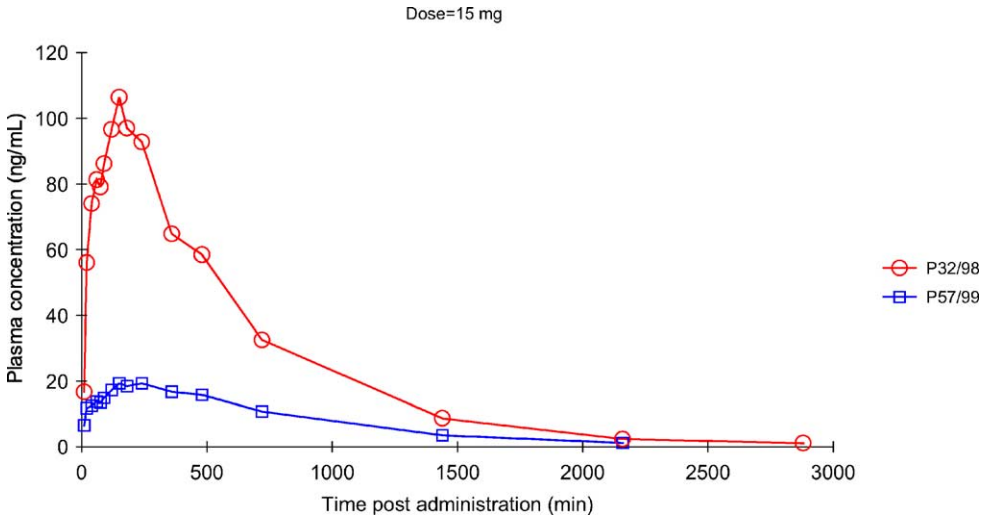

Fig. 6. Pharmacokinetic profile of P32/98 and P57/99 after oral administration of 15 mg P32/98 tablets to healthy human subjects ($n = 9$).

The results of this stability tests demonstrated that freeze-thaw cycles and storage at room temperature for 24 h had no consequence on the stability of P32/98 and its metabolite in the different plasma and urine samples.

Stability of both analytes was also given in plasma extracts kept at room temperature for at least 48 h.

3.3. In-study validation

The results of the analysis of quality control samples during routine use are presented in Tables 3–5. Table 3 shows the precision and the accuracy calculated from human plasma QC samples analyzed during three clinical studies while Table 4 presents human urine QC data derived from a clinical study. Table 5 shows QC data obtained from sample analysis during different toxicological studies in animal species. The data revealed high precision and accuracy as indicated by low R.S.D. and bias and confirm the reliability of the developed assay during routine use.

3.4. Pharmacokinetic evaluation

The validated assay was successfully applied to determine concentration levels of parent drug and metabolite in animal plasma samples obtained from toxicological studies as well as human plasma and urine samples obtained from clinical Phase I and II studies [10,11].

Fig. 6 shows a representative pharmacokinetic profile of the parent drug and the metabolite after oral administration of 15 mg P32/98 to healthy human subjects ($n = 9$) during a clinical study. Mean plasma concentrations are plotted versus post-dose sampling time. The pharmacokinetic parameters area under curve (AUC), maximal concentration (C_{max}), time to maximal concentration (T_{max}) and the elimination half-life ($t_{1/2}$) were derived from the calculated plasma concentrations (see Table 6).

Table 6
Pharmacokinetic parameters calculated from determination of parent drug and metabolite plasma levels following single oral administration of 15 mg P32/98 tablets to human subjects (mean ± S.D.; $n = 9$)

Parameter[a]	Parent drug	Metabolite
C_{max} (ng/ml)	126.9 ± 40.8	22.10 ± 5.76
T_{max} (h)	2.2 ± 1.2	2.5 ± 1.3
$AUC_{0\rightarrow t}$ (ng h/ml)	1118.5 ± 210.2	283.0 ± 56.4
$AUC_{0\rightarrow \infty}$ (ng h/ml)	1135.0 ± 212.0	306.0 ± 51.1
$t_{1/2}$ (h)	6.3 ± 0.7	7.2 ± 0.6

[a] C_{max}: peak plasma concentration; T_{max}: time of C_{max}; AUC: area under curve; $t_{1/2}$: elimination half-life.

4. Conclusion

The objective of the study was to develop and validate a method for the determination of P32/98 and its metabolite (P57/99) in plasma and urine samples from different species with sufficient sensitivity, specificity and reliability for routine use. The sample clean up procedure using cation exchanger for extraction led to similar recoveries from all matrices. When LC–MS/MS in the APCI positive mode is used, the validated LLOQ of the analytical method is equal to 1 ng/ml for human plasma and 10 ng/ml for rat, dog, mouse, monkey plasma and human urine, respectively. The R.S.D. and bias calculated from the different repeatability assays demonstrate that, even at the limit of quantification, the precision and accuracy of the method is suitable for routine analysis of these compounds which was in turn confirmed by in-study quality control data derived from sample analysis during toxicological studies in different species as well as clinical trials.

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