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Determination of the anti-platelet-activating factor BN-50727 and its metabolites in human plasma by high-performance liquid chromatography–solid-phase extraction

J. Pruñonosa*, L. Parera, C. Peraire, F. Pla, O. Lavergne, R. Obach

Pharmacokinetic Department, S.A. Lasa Laboratorios, Crta. Laureà Miró 395, 08980 Sant Felíu de Llobregat, Barcelona, Spain

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Abstract

A sensitive and selective HPLC–solid-phase extraction procedure was developed for the determination of platelet-activating factor antagonist BN-50727 and its metabolites in human plasma. The procedure consisted of an automated solid-phase extraction of the drug and metabolites on disposable propylcarboxylic acid cartridges, followed by on-line chromatographic separation. The method was linear from 3.75 to 2400 ng/ml and the limit of quantitation for BN-50727 in plasma samples was 3.75 ng/ml. The within-run precision of the method, expressed as relative standard deviation, ranged from 2.1 to 8.1%. The accuracy, expressed as relative error, ranged from −3.5 to 4.0%. For the main metabolite, the O-demethylated BN-50727 product, the method was linear from 7.5 to 2400 ng/ml and the limit of quantitation in plasma was 7.5 ng/ml. The within-run precision ranged from 2.1 to 11.0% and the accuracy from −5.3 to 1.1%. This paper describes the validation of the analytical methodology for the determination of BN-50727 in human plasma and also of its metabolites. The method has been used to follow the time course of BN-50727 and its metabolites in human plasma after administration of single and multiple doses.

1. Introduction

BN-50727 (9-(4-methoxyphenylcarbamoyl)-6-(2-chlorophenyl)-1-methyl-4,7,8,10-tetrahydro-pyrido[4′,3′-4,5]thieno[3,2-*f*]-1,2,4-triazolo[4,3-*a*]-1,4-diazepine) is a synthetic derivative with potent platelet-activating factor (PAF) antagonizing properties [1,2]. This compound (Fig. 1, compound III) is currently under investigation for its anti-allergic, anti-ischemic and anti-inflammatory activities.

The structure of compound III is closely related with that from another anti-PAF, the BN-50730 (Fig. 1). This compound is metabolized to the corresponding O-demethylated product and also to compound III [3] after a desulphurization reaction.

Preliminary metabolic studies revealed that compound III is primarily metabolized to 9-(4-hydroxyphenylcarbamoyl)-6-(2-chlorophenyl)-1-methyl-4, 7, 8, 10-tetrahydro-pyrido[4′, 3′-4, 5]thieno[3, 2-*f*]-1, 2, 4-triazolo[4, 3-*a*]-1, 4-diazepine, the O-demethylated BN-50727 product (Fig. 1, compound II) in dogs, monkeys, rats and humans [4]. The compound 6-(2-chorophenyl)-1-methyl-4, 7, 8, 10-tetrahydro-

* Corresponding author.

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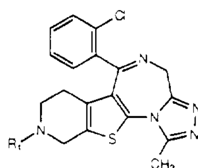

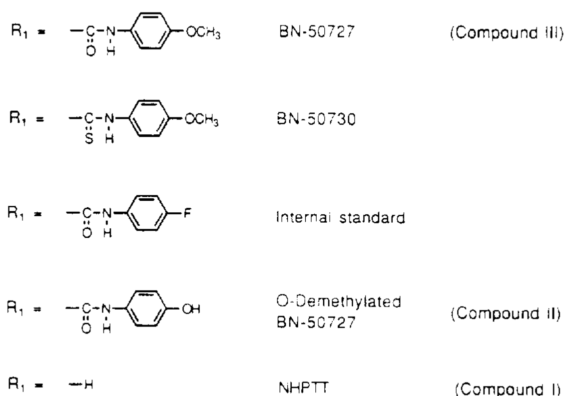

Fig. 1. Structures of BN-50727 (compound III), its metabolites and the internal standard.

pyrido[4′,3′-4,5]thieno[3,2-*f*]-1,2,4-triazolo[4,3-*a*]-1,4-diazepine (NHPTT, Fig. 1, compound I) was also detected in the urine of rats after administration of compound III, indicating another possible metabolite of the parent compound.

The development of this potentially new and original drug, compound III, has made it necessary to optimize the analytical methodology for the determination of the parent drug as well as its known metabolites. Recently, a very sensitive method has been published for the determination of compound III in human plasma and urine by liquid chromatography in combination with negative ion chemical ionization mass spectrometry [5]. The present paper describes a selective and sensitive HPLC technique using a solid-phase extraction (SPE) method for the determination of the compounds I, II and III. The methodology provides enough sensitivity to thoroughly follow the time course in human plasma after compound III administration, at doses of 2.5, 10, 20, 40, 80 and 160 mg. Although the method has been mainly used for human plasma, it has also been applied, with minor modifications, to follow the time course of compound III and metabolites in dog, monkey, rabbit and rat plasma.

2. Experimental

2.1. Chemicals and reagents

Compounds I and III were supplied by Expansia (Aramon, France). Compound II and 9-(4-fluorophenylcarbamoyl)-6-(2-chorophenyl)-1-methyl-4, 7, 8, 10-tetrahydro-pyrido[4′, 3′-4, 5]thieno[3, 2-*f*]-1, 2, 4-triazolo[4, 3-*a*]-1, 4-diazepine, used as internal standard (Fig. 1), were synthesized by treating compound III with BBr_3, and compound I with 4-fluorophenylisocyanate, respectively. Acetonitrile (for HPLC) and acetic acid were obtained from Panreac (Barcelona, Spain). Triton X-100 (for gas chromatography), ammonium hydroxide and sodium chloride were supplied by Merck (Darmstadt, Germany). All reagents were analytical-reagent grade if not otherwise stated. Sodium heparinate (161.4 USP heparin units/mg) was obtained from Kraeber (Hamburg, Germany). Water was purified through a Milli-Q system (18 MΩ cm resistivity).

2.2. Plasma preparation

Heparinized blood (0.75 mg sodium heparinate per ml of blood) was obtained from healthy donors by venepuncture. Blood was centrifuged at 2000 *g* (4°C) for 10 min using Merck's "separating agent for the preparation of erythrocyte-free plasma", according to the instructions supplied by the manufacturer. The plasma thus obtained was collected with a pipette and stored frozen at −22°C in polypropylene tubes.

2.3. Apparatus

Chromatographic separations were performed using a Waters HPLC equipment (Millipore-Waters, Milford, MA, USA) consisting of an M-600 pump, an M-486 UV detector and an M-845 data and chromatography control station using Waters

Expert Ease Chromatography Software (V. 3.0). Sample injections were made with a Prospekt (Programmable On-Line Solid-Phase Extraction Technique) system (Spark Holland, Emmen, Netherlands). The complete system contains three modules: the main Prospekt solid-phase extraction controller unit, the Marathon autosampler and the solvent delivery unit. It is a fully automated on-line sample clean-up and injection system. Samples are automatically loaded on a disposable cartridge, purged with the appropriate solvent(s) for clean-up and are subsequently eluted to the analytical HPLC system.

2.4. Solid-phase extraction

SPE of the samples were made on disposable CBA (propylcarboxylic acid) ion-exchange cartridges (10×3 mm I.D., Analytichem). The cartridges were initially treated with methanol (2 ml), water (2 ml) and ammonium acetate, 20 m*M*, pH 7.0 (2 ml); all steps at a flow-rate of 2 ml/min. Plasma samples (500 μl) prepared as described below were automatically loaded onto the cartridges which were purged with ammonium acetate, 20 m*M*, pH 7.0 (0.5 ml/min during 1 min) and a mixture of acetonitrile–20 m*M* ammonium acetate, pH 7.0, 5:95 (1 ml/min during 2 min) for cleaning-up. Finally, the cartridges were inserted on line with the chromatographic system by means of a switching valve in order to elute them using the appropriate solvent gradient programme.

2.5. Chromatography

Chromatographic separations were made on a Nova-Pak C_{18} (4 μm particle diameter) Radial Pak cartridge (Waters, 10×0.8 cm I.D.), inserted in a Radial Compression Module, RCM 8×10. A Newguard RP-18 precolumn (Applied Biosystems, 15×3.2 mm, 7 μm) was also used. Elution of the compounds was carried out in the gradient mode. A concave gradient programme (curve 8) from 28 to 60% solvent A in 19 min was applied (solvent A = acetonitrile; solvent B = ammonium acetate, 20 m*M*, pH 7.0). After each injection, a column-washing programme was used consisting of 2 min 100% solvent A and 4 min conditioning to starting conditions. Separations were performed at a flow-rate of 2.0 ml/min. UV detection was set at a wavelength of 250 nm.

2.6. Validation protocol

The assay parameters determined as part of the validation process were: accuracy, precision, sensitivity, specificity, linearity, recovery and acceptance criteria for the routine analysis. For this purpose, replicates of calibration curves and samples analyzed as unknowns, all at eight different concentration levels, were prepared on five different days. Pooled plasma from six different healthy human donors was used in this study. The plasma of each donor was checked before the study in order to verify the chromatographic profiles of the different samples from the control matrix.

Within-batch (intra-assay) validation
The within-batch validation of the assay was carried out by analyzing, as unknowns, 6-fold replicate samples prepared in control matrix at each of the calibration sample concentrations. These spiked samples were analyzed against a duplicate calibration line. The results provided the data used to calculate the within-batch accuracy, precision and linearity of the assay. The batch was bigger in size than those of real samples predicted to be analyzed when the assay was carried out routinely. Precision was expressed as relative standard deviation and accuracy as relative error, both in percentages.

Between-batch (inter-assay) validation
The between-batch validation was carried out on four different days. All the batches included were similar in size to the batches of real samples predicted to be analyzed when the assay was carried out routinely. The batches comprised the following:

(a) A duplicate set of calibration standards. The data from these were used to construct the calibration line against which all the remaining samples were measured.

(b) A duplicate set of spiked samples at each of the calibration standard concentrations made up in the control matrix. Comparison of the results for these samples over four days gave the between-batch accuracy and precision data.

(c) Duplicates of stock solutions containing the analytes at concentrations similar to the spiked plasma sample. These stock solutions (reference solutions) were injected directly and were made up in 100 m*M* sodium phosphate buffer, pH 7.4. Comparison of the analytical results for these stock solutions with those for the spiked samples gave the recovery of the assay procedure throughout the calibration range. The absolute recoveries were established by comparing the corresponding peak heights (for compound I) and peak areas (for compounds II and III) for spiked plasma samples after the extraction procedure with those of the reference solutions.

(d) Duplicate quality controls (QC) at three concentrations. The QC samples were made up in bulk and were used to monitor the performance of the assay when used routinely. These samples were prepared by spiking blank plasma from healthy donors with known amounts of the standards to be analyzed. The data from the QC samples provide information used to establish the acceptance criteria for the routine analysis.

2.7. Standard and sample preparations

Calibration standards were prepared for compounds I, III and the internal standard from stock solutions of 750 μg/ml in acetonitrile, and for compound II from a stock solution of 375 μg/ml in methanol. The stock solutions were protected from light by storing them in amber glass sealed vials. These solutions were stored at 4°C for no more than a week. The working calibration standards were prepared daily by accurate and appropriate dilutions of the stock solutions in an acetonitrile–water mixture (1:9).

Plasma calibration curves were performed using plasma from healthy donors. For this purpose, 0.5-ml aliquots of blank plasma were spiked with 150 μl of 0.9% sodium chloride solution, 25 μl of standard mixture solution containing appropriate concentrations of the compounds I, II and III, 25 μl of internal standard solution and 50 μl of a 20% Triton X-100 solution. The final concentrations were 3.75, 7.5, 15, 75, 150, 600, 1200 and 2400 ng/ml plasma for the compounds I, II and III, and 300 ng/ml plasma for the internal standard.

Plasma samples to be analyzed (pharmacokinetic studies) were prepared in the same manner by substituting the addition of the standard mixture solution with the same volume of acetonitrile–water mixture (1:9) solution.

The samples, prepared as described above, were introduced in the autosampler (8°C) for direct injection of 500 μl onto the disposable cartridges.

2.8. Quantitation

The peak height ratios of compound I/internal standard, peak area ratios of compound II/internal standard and compound III/internal standard were plotted versus known concentrations of the compounds I, II and III, respectively. Peak height was used for compound I because it gave a more precise result than peak area due to the broader and tailed shape of this peak. Data were fitted by weighted least-squares linear regression to obtain the corresponding calibration curves, the weighting factor being the reciprocal of the square concentrations. A weighted least-squares linear regression was used because of the clear improvement in the precision and accuracy obtained for the back-calculated values, mainly at low concentration levels. Data were processed with Waters Expert Ease Chromatography Software (V. 3.0).

3. Results and discussion

3.1. Chromatographic separation

The chromatographic profiles obtained from plasma blanks corresponding to six different healthy plasma donors were similar. A representative chromatogram is shown in Fig. 2A. A chromatogram corresponding to a plasma sample spiked with 150 ng/ml plasma of the compounds

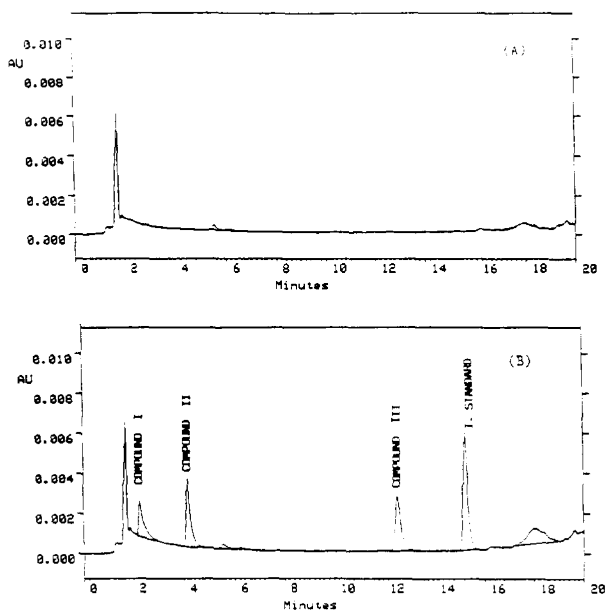

Fig. 2. Chromatograms of compounds I, II and III in human plasma. (A) Plasma from a healthy donor (plasma blank). (B) Plasma from a healthy donor spiked with 150 ng/ml of the compounds I, II and III, and 300 ng/ml of the internal standard.

I, II and III and 300 ng/ml plasma of the internal standard is shown in Fig. 2B. A comparison of the chromatograms of the blank plasma and the spiked plasma shows the absence of interfering peaks at the expected retention times of the compounds of interest.

3.2. Precision and accuracy

The results obtained for 6-fold replicate samples prepared in control matrix and analyzed against a duplicate calibration line provided the within-run (intra-assay) precision and accuracy data. These results are shown in Table 1. The precision ranged from 2.9 to 13.0%, 2.1 to 11.0% and 2.1 to 8.1% for compounds I, II and III, respectively. The within-run (intra-assay) accuracy ranged from −10.3 to 8.0%, −5.3 to 1.1% and −3.5 to 4.0% for compounds I, II and III, respectively.

The results obtained for 2-fold replicate samples prepared in control matrix and analyzed on four different days plotted against duplicate calibration lines provided the between-run (inter-assay) precision and accuracy data. These results are shown in Table 2. The precision, expressed as relative standard deviation in percentages, ranged from 8.1 to 18.9%, 3.0 to 14.3% and 2.6 to 18.7% for compounds I, II and III, respectively. The between-run (inter-assay) accuracy, expressed as relative error (%), ranged from −8.0 to 10.1%, −5.1 to 10.7 and −4.5 to 8.0% for compounds I, II and III, respectively.

The concentration ranges summarized in Tables 1 and 2, after analysis of the linearity described below, were 7.5 to 1200 ng/ml for compound I, 7.5 to 2400 ng/ml for compound II and 3.75 to 2400 ng/ml for compound III.

3.3. Specificity and limit of quantitation

The blank plasma chromatograms did not show interfering peaks at the expected retention times of the compounds I, II, III and the internal standard. Individual coinjections of blank plasma spiked with these compounds confirmed peak identification. Coinjection of blank plasma spiked with all the compounds demonstrated that they did not affect each other in the elution pattern.

The limit of quantitation (LOQ) was defined as the concentration of the compound that produces a peak height or area equal to the mean blank plus three standard deviations, at least, with acceptable accuracy and precision (20%). This was fixed at 7.5 ng/ml plasma for compounds I and II and 3.75 ng/ml for compound III.

3.4. Linearity

The linearity for compounds I, II and III was checked in the 3.75 to 2400 ng/ml plasma concentration range. Response ratio of data versus effective concentration was fitted by a weighted least-squares linear regression to the equation:

$$\textit{Response ratio} = \textit{slope} \cdot C(\textit{concentration}) \pm \textit{intercept}$$

Table 1
Means, standard deviations (S.D.), relative standard deviations (R.S.D.) and relative errors (R.E.) derived from within-run accuracy and precision studies for the determination of compound III and its metabolites (compounds I and II) in human plasma ($n = 6$)

Effective concentration (ng/ml)	Compound I			Compound II			Compound III		
	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)
3.75	–	–	–	–	–	–	3.68 ± 0.20	5.4	−1.9
7.5	8.1 ± 0.74	9.1	8.0	7.1 ± 0.78	11.0	−5.3	7.8 ± 0.60	7.7	4.0
15	14.7 ± 1.22	8.3	−2.0	14.3 ± 1.04	7.3	−4.7	15.2 ± 1.23	8.1	1.3
75	67.3 ± 8.77	13.0	−10.3	72.4 ± 4.62	6.4	−3.5	73.0 ± 2.57	3.5	−2.7
150	139.0 ± 14.84	10.7	−7.3	145.9 ± 3.49	2.4	2.7	146.3 ± 3.93	2.7	−2.5
600	562.0 ± 52.51	9.3	−6.3	594.7 ± 24.58	4.1	−0.9	579.3 ± 14.75	2.6	−3.5
1200	1078.4 ± 30.78	2.9	−10.1	1205.4 ± 25.32	2.1	0.5	1189.5 ± 25.28	2.1	−0.9
2400	–	–	–	2426.3 ± 68.44	2.8	1.1	2391.7 ± 70.69	3.0	−0.3

Table 2
Means, standard deviations (S.D.), relative standard deviations (R.S.D.) and relative errors (R.E.) derived from between-day accuracy and precision studies for the determination of compound III and its metabolites (compounds I and II) in human plasma ($n = 6$)

Effective concentration (ng/ml)	Compound I			Compound II			Compound III		
	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)
3.75	–	–	–	–	–	–	3.96 ± 0.74	18.7	5.6
7.5	7.4 ± 1.40	18.9	−1.3	8.3 ± 1.19	14.3	10.7	8.1 ± 1.34	16.5	8.0
15	13.8 ± 1.51	10.9	−8.0	15.0 ± 1.22	8.1	0.0	15.2 ± 1.14	7.5	1.3
75	70.3 ± 6.46	9.2	−6.3	71.2 ± 2.86	4.0	−5.1	72.0 ± 2.89	4.0	−4.0
150	147.2 ± 11.95	8.1	−1.9	143.7 ± 7.84	5.5	−4.2	143.2 ± 5.33	3.7	−4.5
600	610.3 ± 85.69	14.0	1.7	600.1 ± 30.73	5.1	0.0	592.0 ± 21.08	3.6	−1.3
1200	1320.6 ± 122.31	9.3	10.1	1210.8 ± 73.98	6.1	0.9	1216.7 ± 51.42	4.2	1.4
2400	–	–	–	2540.7 ± 75.56	3.0	5.9	2544.2 ± 66.30	2.6	6.0

The weighting factor used was $1/C^2$. The analysis of linearity was made by comparison of the normalized response ratios (ratio of peak height or peak area between the corresponding compound and the internal standard, divided by the concentration of the compound) as obtained at different concentration levels. A concentration range was considered linear when the analysis of variance of the normalized response ratios could warrant constancy in the concentration range studied.

For compound I, peaks could not be detected at the 3.75 ng/ml concentration level and some of the studied replicates at 2400 ng/ml were clearly out of the linear response. For compound II, peaks at 3.75 ng/ml could not be detected with acceptable precision. Thus linearity was thoroughly analyzed in the concentration ranges: 7.5 to 1200 ng/ml for compound I, 7.5 to 2400 ng/ml for compound II and 3.75 to 2400 ng/ml for compound III.

The application of one-way analysis of variance to the normalized response ratios did not show significant differences ($p > 0.05$) for each compound with respect to the different selected concentration levels studied. The regression coefficients corresponding to the calibration lines involved during the validation study, expressed as mean ± S.D., were 0.9974 ± 0.00151 for compound I, 0.9984 ± 0.00216 for compound II and 0.9977 ± 0.00161 for compound III ($n = 5$). The accuracy and precision summary of back-calculated values from the calibration curves are shown in Table 3. The linear ranges were fixed between 7.5 and 1200 ng/ml for compound I, between 7.5 and 2400 ng/ml for compound II and between 3.75 and 2400 ng/ml for compound III.

3.5. Recovery

The recovery of the analytical method was studied the at concentration levels 7.5, 15, 75, 150, 600 and 1200 ng/ml for compound I, at 7.5, 15, 75, 150, 600, 1200 and 2400 ng/ml for compound II, and at 3.75, 7.5, 15, 75, 150, 600, 1200 and 2400 ng/ml for compound III. The results obtained for the absolute recovery of the analytical method for the determination of these compounds in human plasma are summarized in Table 4. The application of one-way analysis of variance to the mean recovery values did not show significant differences ($p > 0.05$) among the different concentration levels. Therefore, the recovery remained constant in the concentration ranges studied, being ca. 38, 59 and 58% for compounds I, II and III, respectively.

Recovery for the internal standard, studied only at the working concentration, was 60.8 ± 4.7% (data not shown), a value very close to those obtained for compounds II and III.

3.6. Acceptance criteria for the routine analysis

The quality, and therefore acceptability, of the routine analytical results was monitored for each analysis sequence to demonstrate whether a batch of analytical data is valid. For this purpose, six quality control samples (duplicates at three concentration levels) were included in each analysis sequence. The QC samples were distributed homogeneously in the carrousel of the autosampler and one of them was always fixed at the end of the daily run.

The acceptance criteria for the QC samples permitted a maximum bias of 15% for accuracy and precision, except for the LOQ where the maximum deviation allowed was 20%. For a batch of analytical data to be valid, four of the six QC samples had to meet these criteria, with at least one at each concentration.

The results obtained for quality control samples included during the between-batch validation (four days) are summarized in Table 5. The accuracy of the quality controls obtained by comparing the mean measured concentrations with the effective ones, and expressed as the mean percentage error values were less than 10.7% for compound I, 13.3% for compound II and 2.9% for compound III. The precision of quality control samples, expressed as variation coefficients, did not exceed 22.4%, 24.0% and 12.5% for the compounds I, II and III, respectively.

The QC sample values of Table 5 were analyzed by regression analysis by plotting the

Table 3
Accuracy and precision summary of back-calculated values from calibration curves ($n = 5$)

Effective concentration (ng/ml)	Compound I			Compound II			Compound III		
	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)	Found (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)
3.75		–	–	–	–	–	3.76 ± 0.21	5.6	0.3
7.5	7.7 ± 0.11	1.4	2.7	7.6 ± 0.13	1.7	1.3	7.5 ± 0.87	11.6	0.0
15	14.5 ± 0.38	2.6	3.3	14.8 ± 0.52	3.5	−1.3	14.8 ± 0.56	3.8	1.3
75	73.9 ± 3.91	5.3	−1.5	73.0 ± 4.59	6.3	−2.7	73.9 ± 2.51	3.4	−1.5
150	147.6 ± 8.10	5.5	−1.6	146.3 ± 3.21	2.2	−2.5	144.7 ± 3.70	2.6	−3.5
600	596.9 ± 34.61	5.8	−0.5	589.0 ± 19.62	3.3	−1.8	588.3 ± 15.68	2.7	−2.0
1200	1261.4 ± 112.3	8.9	5.1	1256.5 ± 65.18	5.2	4.7	1258.2 ± 59.59	4.7	4.9
2400		–	–	2456.9 ± 133.6	5.4	2.4	2463.1 ± 106.82	4.3	2.6

Table 4
Absolute recovery of the analytical method for the determination of compound III and its metabolites (compounds II and III) in human plasma ($n = 8$)

Concentration (ng/ml)	Recovery (mean ± S.D.) (%)		
	Compound I	Compound II	Compound III
3.75	–	–	60.6 ± 6.11
7.5	42.9 ± 5.06	62.6 ± 15.56	55.7 ± 15.89
15	39.0 ± 5.19	59.0 ± 9.07	56.0 ± 4.63
75	36.8 ± 4.55	54.5 ± 6.72	56.7 ± 5.25
150	37.7 ± 8.09	57.0 ± 3.12	58.5 ± 3.30
600	36.4 ± 4.78	59.2 ± 6.43	59.6 ± 6.89
1200	36.2 ± 4.19	59.0 ± 4.90	59.6 ± 3.93
2400	–	59.0 ± 4.17	60.4 ± 2.98

measured concentrations versus the theoretical ones. The slope and the intercept were statistically compared to the theoretical values (1 and 0, respectively), using Student's t-test. The intercept values (± S.D.) for compounds I, II and III were -0.8180 ± 5.63167, 1.3109 ± 29.00356 and 1.6943 ± 26.23762, respectively. The corresponding slope values (± S.D.) were 1.0057 ± 0.05488, 1.0170 ± 0.02089 and 1.0096 ± 0.01890, respectively. The intercept values were not different from 0 at the 95% probability level, with p values of 0.887, 0.964 and 0.949 for respectively compounds I, II and III. The slope values did not differ from the theoretical value 1, with p values of 0.919, 0.424 and 0.617, respectively. Therefore, it can be concluded that no significant bias indicating a systematic error was observed.

The methodology described here has been used for pharmacokinetic studies in humans, and provided enough sensitivity to follow thoroughly the time course of compound III in plasma after administrations of 2.5, 10, 20, 40, 80 and 160 mg. After the administration of compound III in humans, the main metabolite in plasma was

Table 5
Accuracy and precision summary of quality control sample values for compounds I, II and III obtained during the validation of the analytical method

Effective concentration (ng/ml)	Measured concentration (ng/ml)					C.V. (%)	R.E. (%)
	Day 1	Day 2	Day 3	Day 4	Mean ± S.D.		
Compound I							
7.5	6.8–6.9	6.4–3.8	8.8–6.2	6.6–8.3	6.7 ± 1.50	22.4	−10.7
150	149.5–160.3	131.4–161.7	154.0–180.8	112.6	150.0 ± 22.18	14.8	0.0
Compound II							
7.5	9.2–6.5	6.1–4.8	8.2–4.9	6.6–5.4	6.5 ± 1.56	24.0	−13.3
150	149.5–164.2	142.8–162.3	144.9–164.8	126.9–196.6	156.5 ± 20.76	13.3	4.3
2400	2508.2–2655.4	2541.4–2500.0	2569.6–2495.9	2123.6–2142.3	2442.1 ± 197.68	8.1	1.7
Compound III							
7.5	8.1–8.1	8.1–5.7	7.4–7.5	7.8–8.4	7.6 ± 0.85	11.2	1.3
150	145.9–159.3	141.9–155.4	145.5–159.5	131.8–196.0	154.4 ± 19.27	12.5	2.9
2400	2523.2–2583.8	2509.1–2466.9	2527.8–2473.2	2263.2–2049.4	2424.6 ± 178.82	7.4	1.0

Table 6
Accuracy and precision summary of quality control sample values for the compounds II and III obtained during different pharmacokinetic studies

Theoretical concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	R.S.D. (%)	R.E. (%)	*n*
Compound III				
3.75	3.60 ± 0.47	13.1	−4.0	102
7.5	7.2 ± 0.74	10.3	−4.0	108
150	146.5 ± 6.22	4.2	−2.3	110
1200	1222.1 ± 52.82	4.3	1.8	111
Compound II				
7.5	7.6 ± 0.94	12.4	1.3	109
150	148.3 ± 9.24	6.2	−1.1	110
1200	1231.1 ± 76.98	6.3	2.6	111

compound II and no presence of compound I was observed. Table 6 summarizes the accuracy and precision of the values of quality control samples for compounds II and III obtained in different pharmacokinetic studies, indicating acceptable values for the analytical technique.

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