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Development and validation of two chromatographic methods for the quantification of E-6087 and one of its metabolites, E-6132, in rat plasma

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Abstract

E-6087 is a nonsteroidal anti-inflammatory compound under development that selectively inhibits cyclooxygenase-2. In vitro studies have shown that one of its metabolites, E-6132, also inhibits this enzyme. Due to chromatographic reasons, two reverse phase HPLC methods were developed and validated in order to elucidate which compound is responsible for the pharmacological activity in vivo. Chromatographic separation of E-6087 was achieved using acetonitrile–phosphate buffer (pH 2.5; 25 mM) (60:40, v/v) as mobile phase and two 4.6 × 150 mm × 5 μm Inertsil ODS-2 columns. For E-6132, two Inertsil ODS-3 columns and 52% of acetonitrile were used instead. Internal standards and fluorescence detection differed between both methods. The same on-line solid-phase extraction method was used. Mean retention times for E-6087 and E-6132 were 15.2 ($\pm$1.3) and 36.1 ($\pm$0.6) min, respectively. The methods were selective and linear over the concentration range of 10–500 ng ml^{-1} ($r^2 > 0.996$) for E-6087 and 5–200 ng ml^{-1} ($r^2 > 0.997$) for E-6132. The limits of quantitation were 10 ng ml^{-1} (E-6087) and 5 ng ml^{-1} (E-6132) with a precision and accuracy $< 16\%$ (E-6087) and $< 11\%$ (E-6132). Mean recoveries from plasma were 43.2–61.9% (E-6087) and 60.4–65.2% (E-6132). For both compounds, both inter-assay and intra-assay precision and accuracy were within acceptable limits ($< 15\%$). As an example of the suitability of these methods, the results from a pharmacokinetic study are reported. After single oral administration of 5 mg kg^{-1} of E-6087 to rats, plasma concentrations of E-6087 at peak time were higher than those of E-6132, suggesting that activity is mainly due to E-6087.

Keywords: Anti-inflammatory agents; E-6087; Validation; Prospekt; HPLC; On-line solid-phase extraction

1. Introduction

E-6087 is a nonsteroidal anti-inflammatory agent under development that has shown in animal models anti-inflammatory, analgesic and antipyretic activities. The mechanism of action of

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E-6087 is based upon an inhibition of prostaglandin synthesis, via inhibition of cyclooxygenase-2 (COX-2). Unlike conventional nonsteroidal compounds, at therapeutic concentrations in animals, E-6087 does not inhibit cyclooxygenase-1 (COX-1), limiting the secondary effects associated to the inhibition of this enzyme [1].

Preliminary studies on in vitro metabolism have shown that E-6087 is metabolised, among other metabolites, to E-6132. This metabolite is formed by oxidation of the pyrazoline ring of E-6087 (Fig. 2) and also inhibits COX-2.

The aim of the present study was to develop and validate an analytical method to quantify E-6087 and E-6132 (metabolite) in rat plasma. Given that E-6132 also exhibits pharmacological activity, the second aim of this study was to elucidate which compound is responsible for the activity in vivo. Hence, as an example of the suitability of the two developed methods, the results of a pharmacokinetic study in which a single dose of E-6087 was orally administrated to rats are reported.

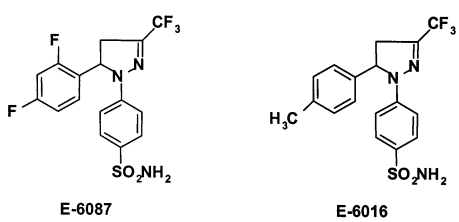


Fig. 1. Chemical structures of E-6087 and internal standard (E-6016).

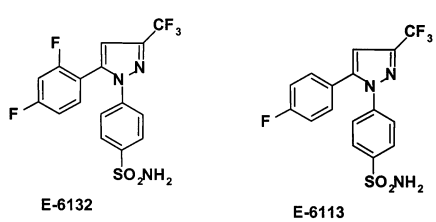


Fig. 2. Chemical structures of E-6132 and internal standard (E-6113).

2. Experimental

2.1. Chemical and reagents

E-6087, (±)4-[5-(2,4-difluorophenyl)-4,5-dihydro-3-trifluoromethyl-1H-pyrazol-1-yl] benzenesulfonamide, and the internal standard (E-6016), 4-[4,5-dihydro-5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide, were synthesised and provided by the Department of Synthesis of Laboratorios Dr Esteve (Barcelona, Spain). E-6087 metabolite, namely E-6132, 4-[5-(2,4-difluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide and internal standard (E-6113), 4-[5-(4-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide, were also synthesised and provided by the above Department. Potassium dihydrogen phosphate was obtained from Panreac (Barcelona, Spain) and 85% phosphoric acid was supplied by Carlo Erba (Milano, Italy). Acetonitrile and Methanol were of high-performance liquid cromatography (HPLC) grade and were purchased from Scharlau (Barcelona, Spain). Demineralized water was purified in a Milli-Q filtration system (Millipore Corporation, Bedford, MA) to obtain water HPLC grade. Drug-free plasma was obtained in the Department of Pharmacokinetics and Metabolism, Laboratorios Dr Esteve (Barcelona, Spain) from rats by cardiac puncture and subsequent blood centrifugation. Once separated, plasma was stored at −80°C until assay.

2.2. Equipment

The cromatographic system consisted of a Hewlett Packard model HP-1050 quaternary pump and a Hewlett Packard model HP1046A fluorescence detector (Waldbronn, Germany). For E-6132, a Shimadzu model RF-10XL fluorescence detector (Kyoto, Japan) was used. Chromatograms were acquired by Access*Chrom software supplied by Perkin Elmer (Cupertino CA) and kept as data processing files.

The automated sample handling system consisted of a Prospekt (Progammable On-Line Phase Extraction Technique), a refrigerated autosampler

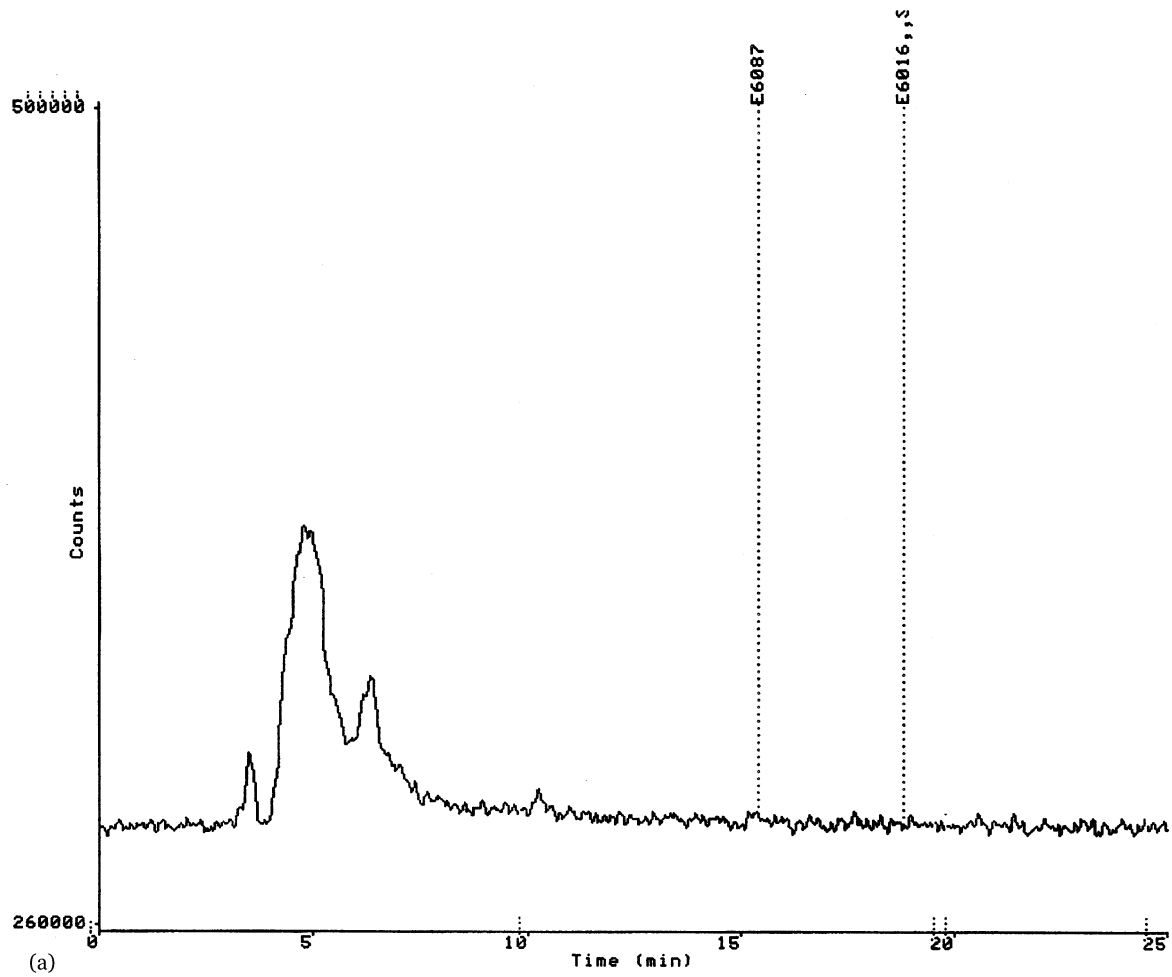


Fig. 3. Chromatograms of an extracted drug-free plasma (a) and 100 ng ml^{-1} calibration standard of E-6087 (b).

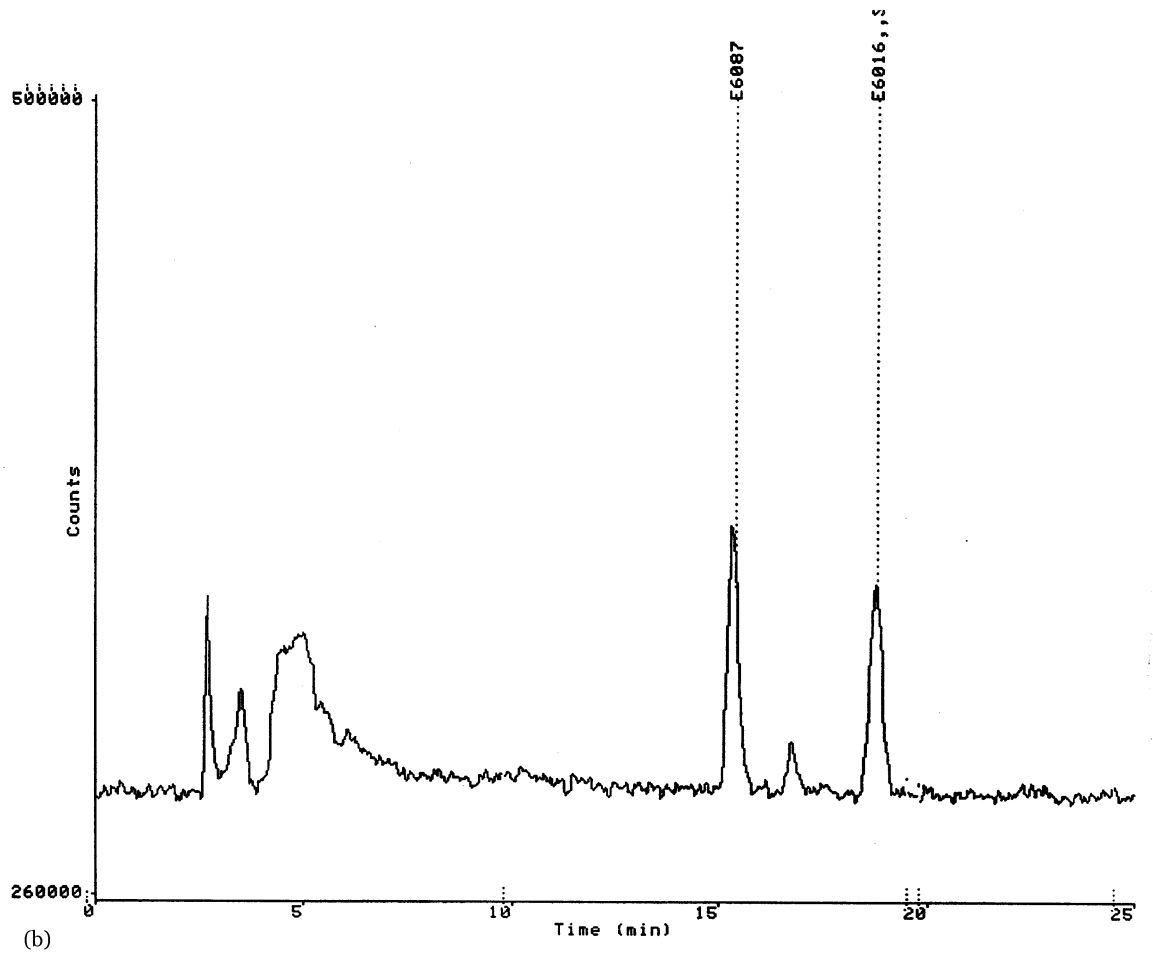
(Triathlon) and a solvent delivery unit; all provided by Spark Holland (Emmen, The Netherlands).

2.3. Chromatographic conditions

Two methods were developed with chromatographic conditions dependent on the compound being analysed; that is, E-6087 or metabolite.

Chromatographic separations of E-6087 or E-6132 and their corresponding internal standards were achieved by reverse phase high-performance liquid chromatography (HPLC). The columns used were supplied by GL-Sciences (Tokyo, Japan) and differed between compounds. To determine E-6087, two Inertsil ODS-2 columns (150 × 4.6 mm, 5 μm particle size) were used whereas for E-6132 two Inertsil ODS-3 columns (150 × 4.6 mm, 5 μm particle size) were used instead. For both determinations, the two columns were connected in series and protected by using a Tracer ODS cartridge (Kromasil; 10 × 3 mm i.d.; 5 μm particle size) obtained from Tecnokroma (Barcelona, Spain). This cartridge was changed daily.

To determine concentrations of either E-6087 or E-6132 in rat plasma, plasma was eluted iso-



(b)

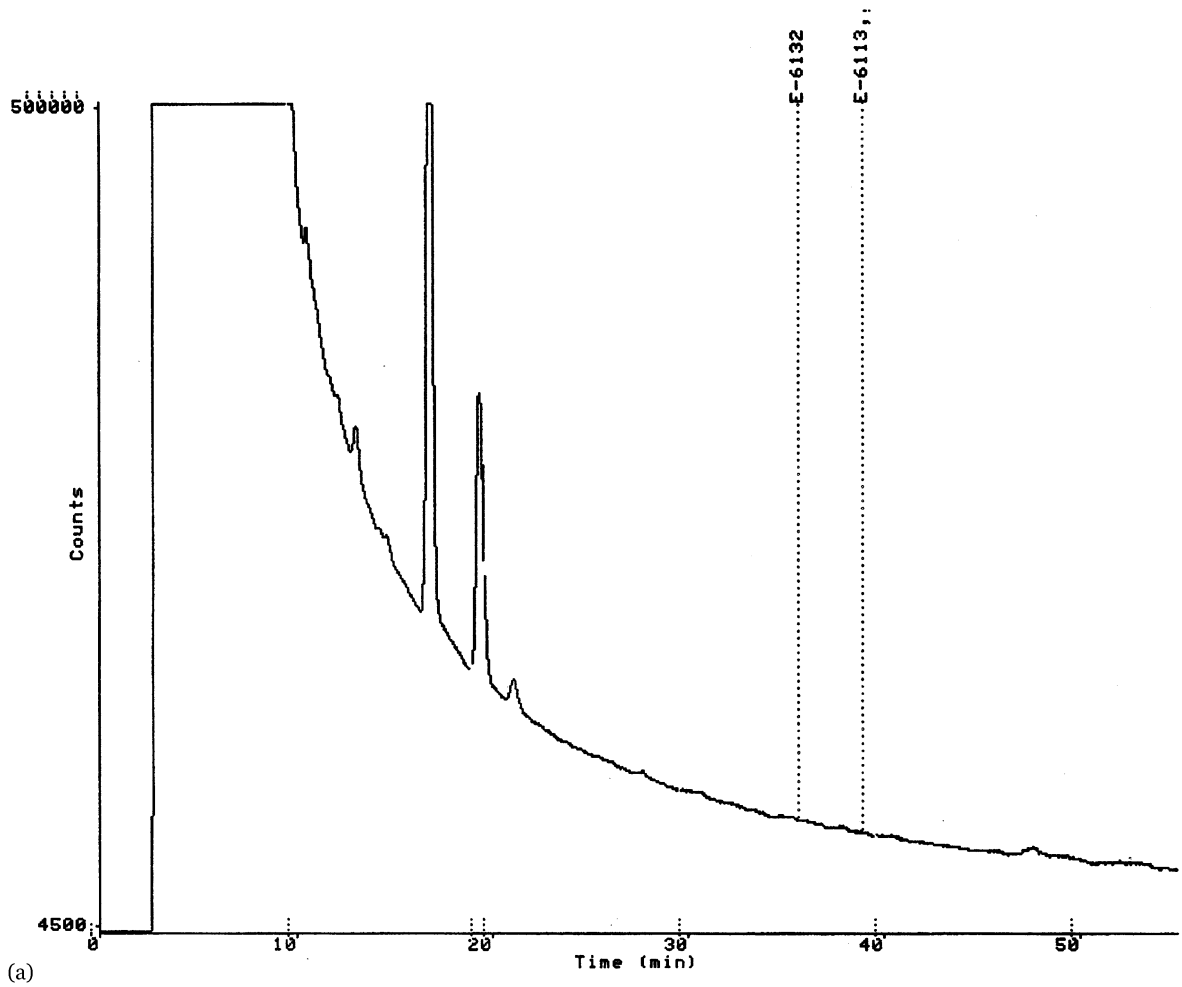
Fig. 3. (*Continued*)

cratically with a mobile phase consisting of acetonitrile and potasium dehydrogen phosphate (pH 2.5; 25 mM) at a flow rate of 0.7 ml min^{-1}. The acetonitrile content was dependent on the compound being analysed. To determine E-6087 a mixture of acetonitrile–phosphate buffer of 60:40 (v/v) was used whereas for E-6132 a lower percentage of acetonitrile was employed (52%). Both mobile phases were degassed before and during the chromatographic process. Prior to use, degassification was done by filtration under vacuum through a 0.2 μm Millipore membrane and during the analysis by using helium. Injection volume was 100 μl. Samples in the autosampler were kept at 20°C.

Both compounds were detected by fluorescence, this detection being done at wavelengths dependent on the compound. E-6087 detection was done at 320 nm excitation and 425 nm emission wavelengths whereas E-6132 was detected at excitation and emission wavelengths of 282 and 355 nm, respectively.

2.4. Preparation of stock and working standard solutions

Stock solutions of related compounds, containing 100 μg ml^{-1} of E-6087 or E-6132, were prepared in acetonitrile-water (30:70, v/v) and methanol, respectively. E-6087 concentrations in



(a)

Fig. 4. Chromatograms of an extracted drug-free plasma (a) and 100 ng ml^{-1} calibration standard of E-6231 (b).

the working solutions were 5, 4, 1, 0.8, 0.4, 0.2 and 0.1 μg ml^{-1}. For E-6132, a smaller range of concentrations was used, namely 2, 1.5, 1, 0.8, 0.4, 0.2, 0.1 and 0.05 μg ml^{-1}. All working solutions were made by further dilution with water of the corresponding stock solution.

The internal standards used for determining E-6087 and E-6132 were E-6016 (Fig. 1) and E-6113 (Fig. 2), respectively. The stock solutions of the internal standards (100 μg ml^{-1}) were prepared as described above. By diluting with water, standard solutions of 1 μg ml^{-1} of E-6016 and 10 μg ml^{-1} of E-6113 were obtained.

2.5. Preparation of plasma standards, samples and quality control samples

Drug-free rat plasma used to prepare plasma standards and samples was thawed at room temperature, vortexed and centrifugated at $2000 \times g$ for 10 min prior to use.

Calibration and plasma standards were prepared by spiking 180 μl aliquots of blank rat plasma with 20 μl of the internal standard working solution, and 20 μl of the E-6087 or E-6132 solution. Vials were then vortexed and placed in the autosampler. Concentration ranges of 10–500 ng ml^{-1} (E-6087) and 5–200 ng ml^{-1} (E-6231) were used.

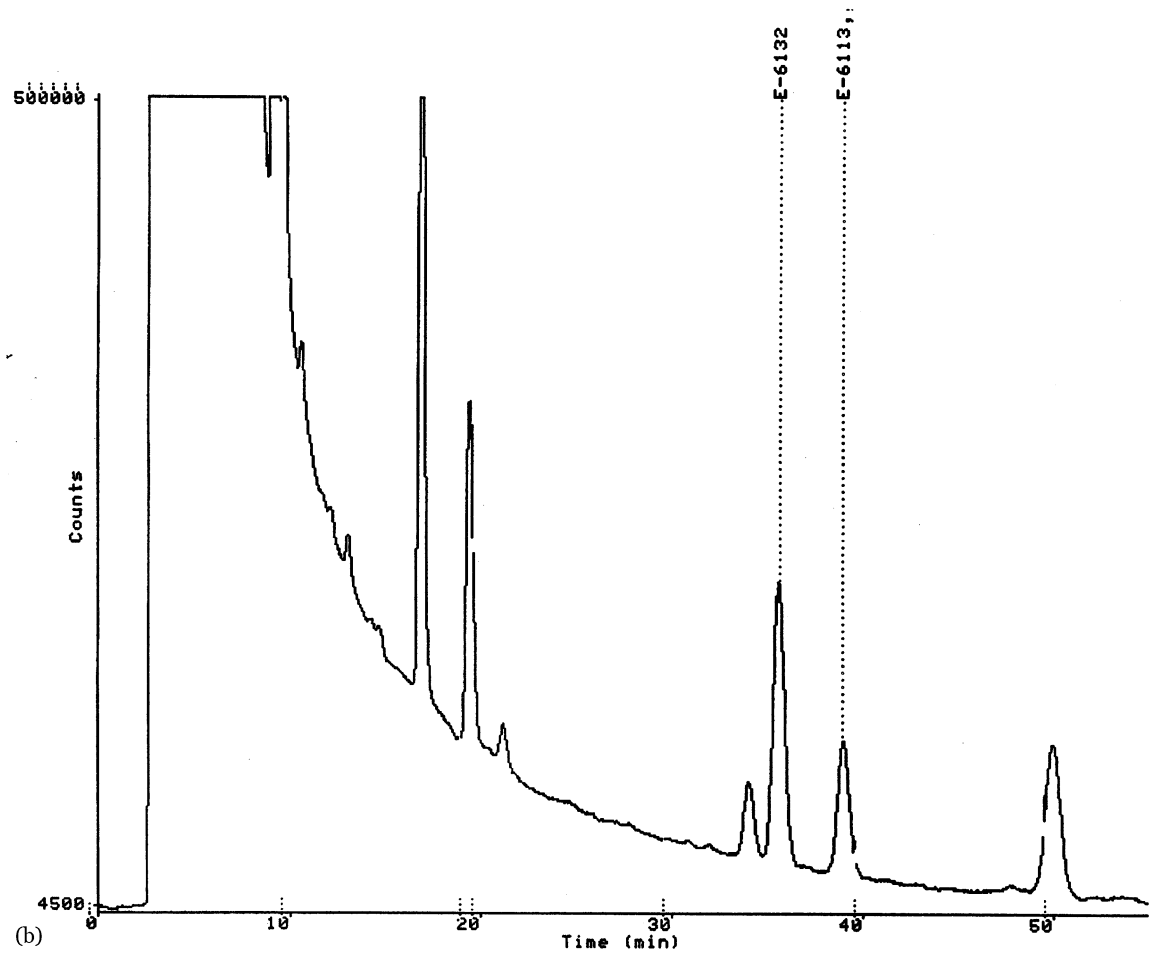


Fig. 4. (*Continued*)

In the pharmacokinetic study, 200 μl of plasma samples were spiked with internal standard (20 μl). Quality control samples were prepared by spiking drug-free rat plasma with the appropriate working solution of compound and internal standard. The same procedure described above was followed with all these samples.

2.6. Extraction method

Compounds were extracted from rat plasma using an on-line solid-phase extraction system (Prospekt). The same extraction method was followed for E-6087 and metabolite. Extraction was made on disposable C18 cartridges (10 × 2 mm

Table 1
Calibration curves for E-6087 and E-6132, and confidence intervals for N [a]

	r^2	y-intercept	Slope	N
E-6087	0.996 ± 0.003	0.013 ± 0.007	0.814 ± 0.148	0.952 ± 0.218
E-6132	0.997 ± 0.003	-0.003 ± 0.016	17.962 ± 1.685	1.094 ± 0.152

[a] Results expressed as mean ± SD ($n = 6$–7).

Table 2
Back-calculated concentrations (mean ± SD) of calibration standards of E-6087 ($n = 6$) and E-6132 ($n = 7$)

Concentration (ng ml^{-1})			RSD (%)		RE (%)	
Nominal	found					
	E-6087	E-6132	E-6087	E-6132	E-6087	E-6132
5		5.1 ± 0.5		9.7		8.0
10	9.7 ± 0.8	10.2 ± 0.9	8.2	9.3	7.1	8.2
20	19.0 ± 1.3	20.4 ± 2.4	6.9	11.7	6.6	9.8
40	40.6 ± 2.5	38.0 ± 2.6	6.2	6.9	5.2	5.6
80		79.2 ± 3.2		4.0		2.8
100	104.7 ± 4.8	99.1 ± 3.8	4.5	3.8	4.7	3.0
150		145.7 ± 2.4		1.7		3.4
200	208.2 ± 13.0	206.8 ± 6.2	6.3	3.0	6.3	4.0
400	393.4 ± 16.8		4.3		4.0	
500	494.3 ± 25.7		5.2		3.9	

Table 3
Precision and accuracy of the two analytical methods developed for the determination of E-6087 and E-6132 in rat plasma ($n = 7$–8)

Concentration (ng ml^{-1})	Mean concentration found (ng ml^{-1})		Precision (% RSD)		Accuracy (% RE)		Total error (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
E-6087								
10	9.2 ± 0.5	9.1 ± 0.9	3.6	16.4	7.9	10.8	11.5	27.2
100	109.4 ± 3.8	96.5 ± 8.3	3.5	14.5	9.4	5.2	12.9	19.7
400	372.1 ± 18.6	376.4 ± 20.0	5.6	12.6	6.7	5.8	12.3	18.4
E-6132								
5	4.7 ± 0.4	4.9 ± 0.6	6.7	9.0	7.9	10.6	14.6	19.6
10	10.0 ± 0.6	9.8 ± 0.9	4.6	10.7	4.1	6.4	8.7	17.1
80	76.8 ± 3.8	82.6 ± 4.4	5.0	7.3	4.4	3.6	9.4	10.9
150	157.2 ± 13.9	150.7 ± 13.1	6.9	9.9	8.0	6.5	14.9	16.4

Table 4
Mean recoveries (± SD) of E-6087 and E-6132, and their internal standards from spiked rat plasma ($n = 7$–8)

Concentration	Recovery ± SD (%)			
(ng ml^{-1})	E-6087	I.S. (E-6016)	E-6132	I.S. (E-6113)
5			65.2 ± 5.2	
10	43.2 ± 7.0		64.1 ± 4.0	
80			60.4 ± 5.9	
100	61.9 ± 3.0	49.4 ± 7.0		
150			64.0 ± 6.5	
400	61.6 ± 1.9			
1000				71.1 ± 5.2

i.d.) supplied by Spark Holland (Emmen, The Netherlands). The extraction procedure was as follows: after activation of the cartridge with methanol (1.5 ml min^{-1}, 1 min) and water (1.5 ml min^{-1}, 2 min), the plasma sample was loaded, washed with water (first at 0.5 ml min^{-1} for 1 min and later at 1.0 ml min^{-1} for 1 min) and subsequently eluted with the mobile phase (0.7 ml min^{-1}, 2 min).

2.7. Assay validation

To validate the analytical techniques developed to quantify E-6087 and the metabolite (E-6132) in rat plasma, the following parameters were determined: selectivity, linearity and range, inter- and intra-assay precision and accuracy, limit of quantitation, recovery, stability and ruggedness [2,3].

2.8. Application of the methods

The suitability of the two methods developed was assessed by analysing rat plasma samples from a pharmacokinetic study. In this study, a single oral dose of E-6087 (5 mg kg^{-1}) was administered by gastric gavage (10 ml/kg) in a 5% gum arabic suspension to male and female Wistar rats (250 ± 50 g; Harlan España). Blood samples were collected by cardiac puncture at pre-determined times up to 96 h. Blood from three animals at each time was drawn. After centrifugation, plasma was separated and stored at -80°C until assay of E-6087 and E-6132.

All animals were fasted for 15–24 h prior to E-6087 administration. Food was provided 6 h post-dose but water was allowed ad libitum.

3. Results and discussion

3.1. Chromatography

E-6087 and its metabolite (E-6132) could not be determined in the same run under the chromatographic conditions of E-6087. When using these conditions, i.e. mobile phase, chromatographic columns and fluorescence wavelengths, E-6132 could not be detected due to the different fluorescence spectrums of both compounds. Keeping the mobile phase composition and columns used for E-6087, interferences from plasma at the retention time of E-6132 were observed when the fluorescence wavelengths of E-6132 were used. These results implied the different fluorescence spectrum and presence of interferences from plasma when using the fluorescence wavelengths of E-6132 implied that an analytical method different from that for E-6087 had to be developed to determine E-6132.

Chromatograms of drug-free plasma and plasma spiked with E-6087 are shown in Fig. 3. Chromatograms obtained for E-6132 using the second method developed are displayed in Fig. 4. The use of different wavelengths depending on the compound being analysed would account for the differences found in the chromatograms (Figs. 3

Table 5
Mean relative errors of plasma quality control samples (%RE) at three concentrations of E-6087 from two pharmacokinetic studies done in rats

Concentration (ng ml^{-1})			RE (%)	
Nominal	Found			
	3.5 Months[a]	5.5 Months[b]	3.5 Months[a]	5.5 Months[b]
20	20.2 ± 1.6	21.0 ± 1.4	6.9	7.3
100	105.8 ± 7.1	104.9 ± 6.8	7.9	7.5
400	422.5 ± 28.2	412.0 ± 51.6	7.6	8.5

[a] $n = 44$–56.
[b] $n = 44$–63.

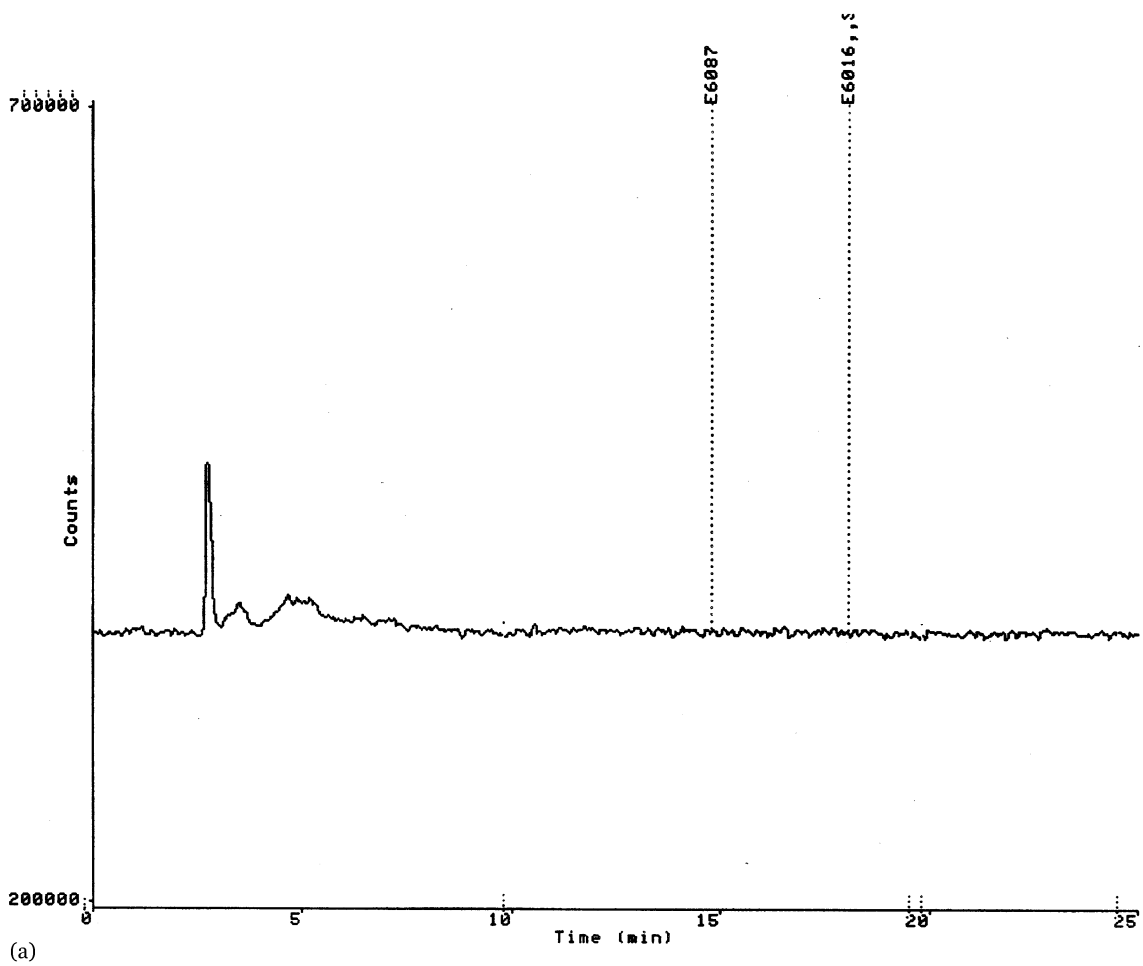


Fig. 5. Chromatograms of a pre-dose: (a) and 3 h post-dose; (b) plasma samples from a male rat after administration of a single oral dose of 5 mg kg^{-1} of E-6087. Columns: Inertsil ODS-2 (150 × 4.6 mm × 5 μm); injection volume: 100 μl; mobile phase: Acetonitrile-potasium dehydrogen phosphate (25 mM, pH 2.5) (60:40; v/v); flow rate: 0.7 ml min^{-1}; fluorescence detection at wavelengths of 320 nm (excitation) and 425 nm (emission).

and 4). Good separation for each pair of compounds: E-6087 and E-6016 (internal standard), and E-6132 and E-6113 (internal standard) was achieved. The retention times for E-6087 and internal standard were 15.5 ($\pm$ 1.3; $n = 146$) min and 18.6 ($\pm$ 1.6; $n = 146$) min respectively. E-6132 and internal standard eluted at longer times: 36.1 ($\pm$ 0.6; $n = 183$) min for E-6132 and 39.5 ($\pm$ 0.6; $n = 183$) min for internal standard. Run times set for the determination of E-6087 and E-6132 were 25 and 55 min, respectively.

The good operating conditions of the equipment and suitability of the methods were assessed at the beginning of each batch analysis. Tailing factor, resolution and system reproducibility were calculated in four replicates of quality controls prepared in mobile phase. Quality controls of 100 ng ml^{-1} of E-6087 and 80 ng ml^{-1} of E-6132 were prepared. The concentrations of E-6087 calculated, expressed as percentage of the nominal value, were 100.0 ($\pm$ 2.7; $n = 22$)%. For E-6132, a percentage of 98.5 ($\pm$ 5.3; $n = 20$)% was obtained.

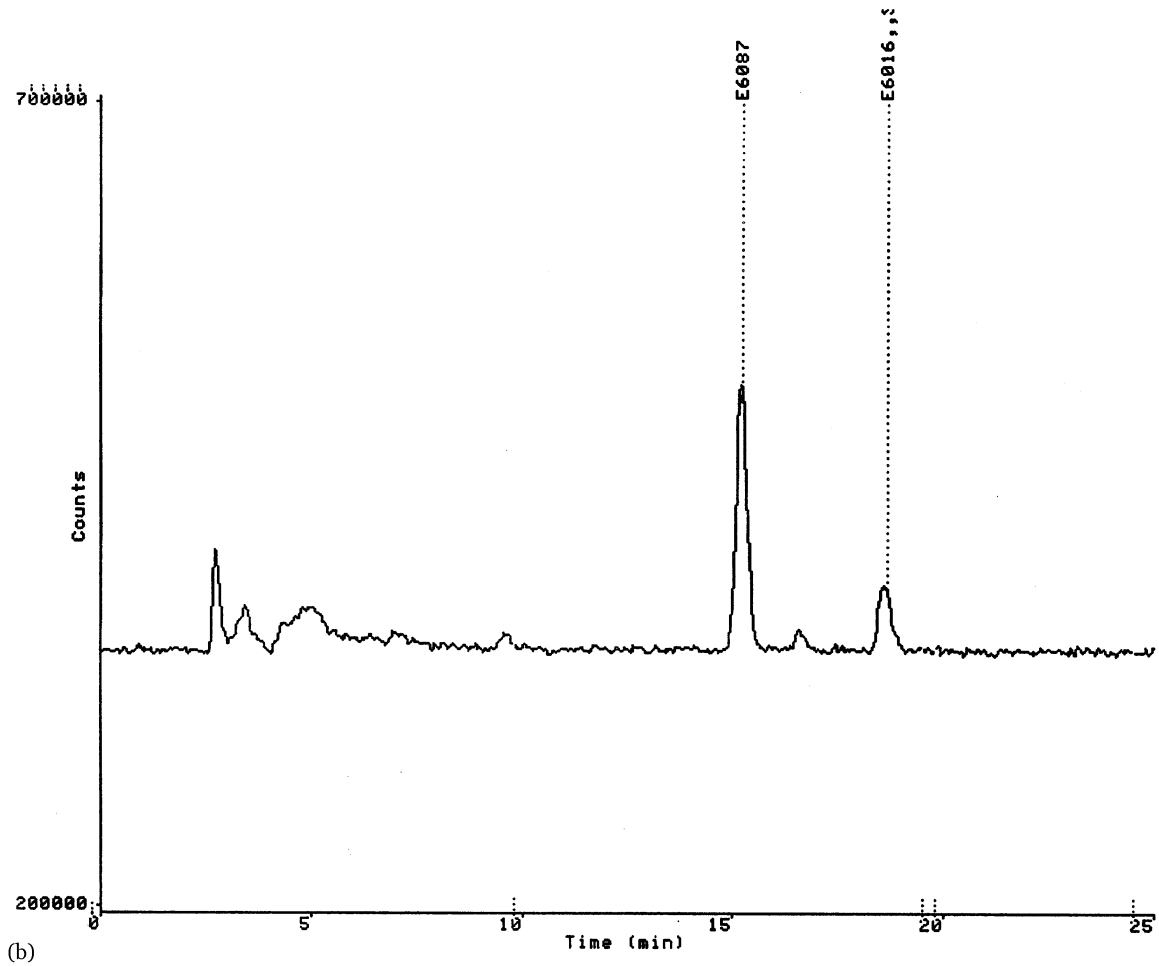


Fig. 5. (*Continued*)

These data indicate that both systems were suitable to perform the analysis.

3.2. Selectivity

The selectivity of the two methods developed was assessed by analysing drug-free plasma from 6–7 different sources. For both assayed methods, no apparent rat plasma components eluted at the retention times of the compounds under study (Fig. 3A and Fig. 4A). The absence of interferences indicates that both methods have adequate selectivity [3,4].

3.3. Linearity and range

Calibration curves for E-6087 and E-6132 were constructed from the compound-to-standard internal peak area ratio and the corresponding concentration ratio. The best-fit line was determined by weighted least-squares linear regression of calibration data to an equation of the type $y = mx + b$, where y is the peak area ratio, x is the concentration ratio, m the slope and b the y-intercept of the calibration curve. The weighting factor chosen was the inverse of the concentration.

Using this equation, linearity of peak area ratios of E-6087 over the range of 10 and 500 ng

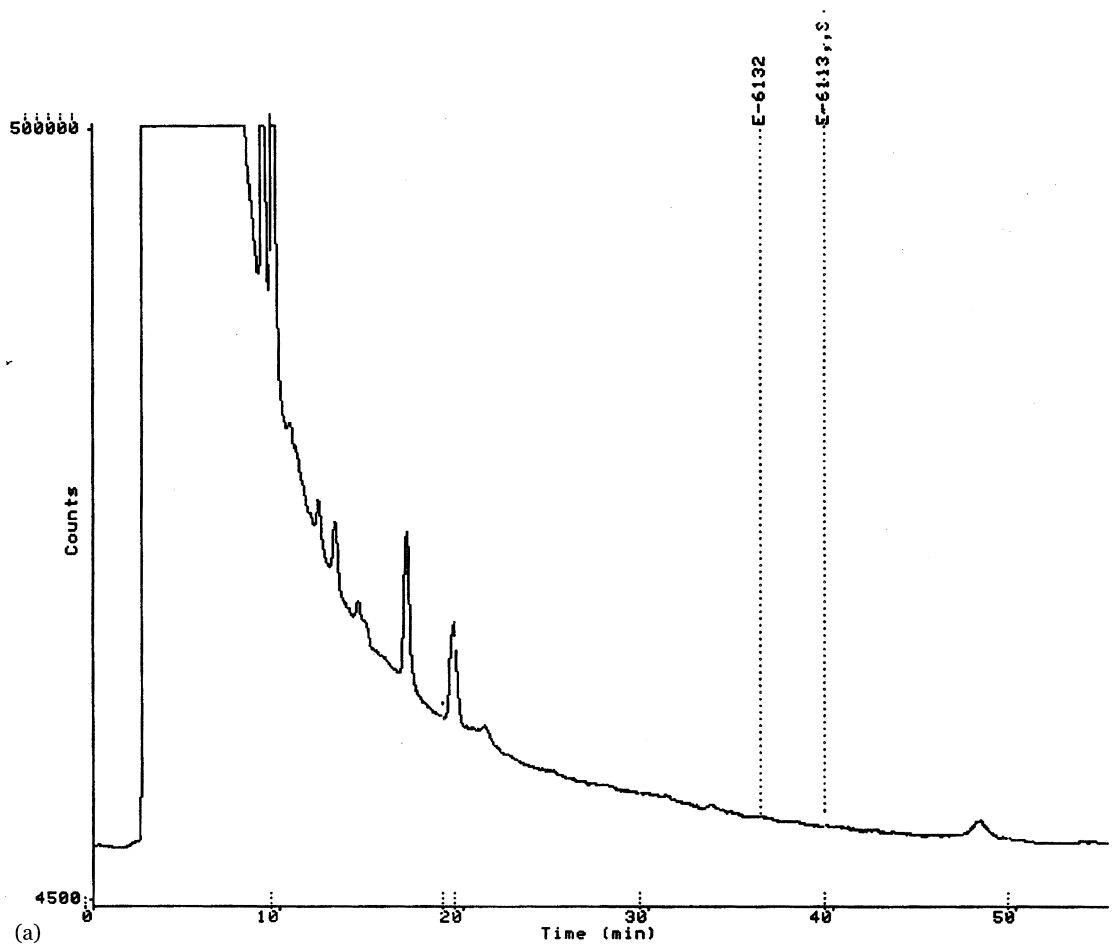


Fig. 6. Chromatograms of a pre-dose: (a) and 3 h post-dose; (b) representative plasma samples from a male rat after administration of a single oral dose of 5 mg/kg of E-6087. Columns: Inertsil ODS-3 (150 × 4.6 mm × 5 μm); injection volume: 100 μl; mobile phase: Acetonitrile-potasium dehydrogen phosphate (25 mM, pH 2.5) (52:48; v/v); flow rate: 0.7 ml min^{-1}; fluorescence detection at wavelengths of 282 nm (excitation) and 355 nm (emission).

ml^{-1} concentrations was studied. For E-6132 the linear concentration range was 5–200 ng ml^{-1}.

Individual calibration curves for E-6087 from 6 different days were pooled giving a mean curve with a slope of 0.814 ($\pm$ 0.148) and coefficient of determination (r^2) of 0.996 ($\pm$ 0.003). The mean curve obtained for E-6132 from 7 days had a slope of 17.962 ($\pm$ 1.685) and a coefficient of determination of 0.997 ($\pm$ 0.003) (Table 1). Values obtained for r^2 (>0.99) indicates a good fitting of data to the linear model [4].

Besides the coefficient of determination, the linearity of the calibration curves over the concentration range studied was also demonstrated by fitting the calibration data to the equation $y = mx^N + b$, and establishing that the coefficient N was not significantly different from the unity. The latter was checked by calculating the 95% confidence intervals for this coefficient using the Student's t distribution. The confidence intervals were given by $N \pm \mathrm{SE} * t_{\alpha/2,\mathrm{df}}$, where SE is the standard error of the coefficient N and $t_{\alpha/2,\mathrm{df}}$ is the Student's

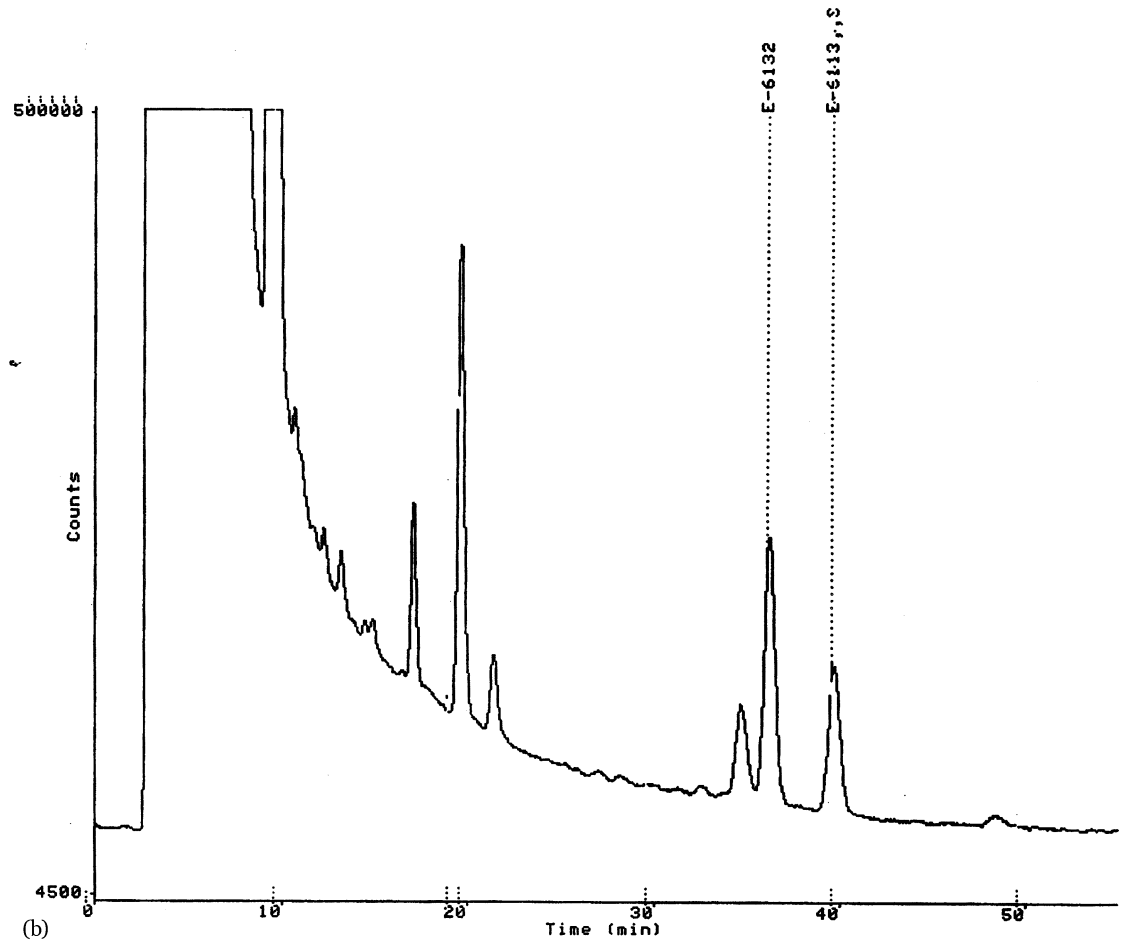


Fig. 6. (*Continued*)

t distribution with $n-3$ degrees of freedom for the one-tailed probability level of 95% ($\alpha/2 = 0.025$).

The mean values of the confidence intervals so calculated are summarised in Table 1. Mean confidence intervals for E-6087 (1.170, 0.734) and E-6132 (1.246, 0.942) included the unity, suggesting that both methods are linear over the concentration ranges studied.

Linearity was also assessed by calculating the concentrations of calibration standards from individual peak area ratios using the calibration curve parameters. The mean deviation from nominal values of these back-calculated concentrations, expressed as percent relative error (% RE), ranged between 3.9 and 7.1% for E-6087 and between 2.8 and 9.8% for E-6132 (Table 2). The deviations found can be taken as acceptable as they are lower than 15% for concentrations different from the limit of quantitation ($< 20\%$) [3].

Collectively, these data suggest that the two analytical methods proposed are linear over the concentration ranges of 10–500 ng ml^{-1} for E-6087 and 5–200 ng ml^{-1} for E-6132.

3.4. Precision and accuracy

Inter- and intra-assay precision and accuracy were assessed at E-6087 concentrations of 10, 100 and 400 ng ml^{-1} and E-6132 concentrations of 5,

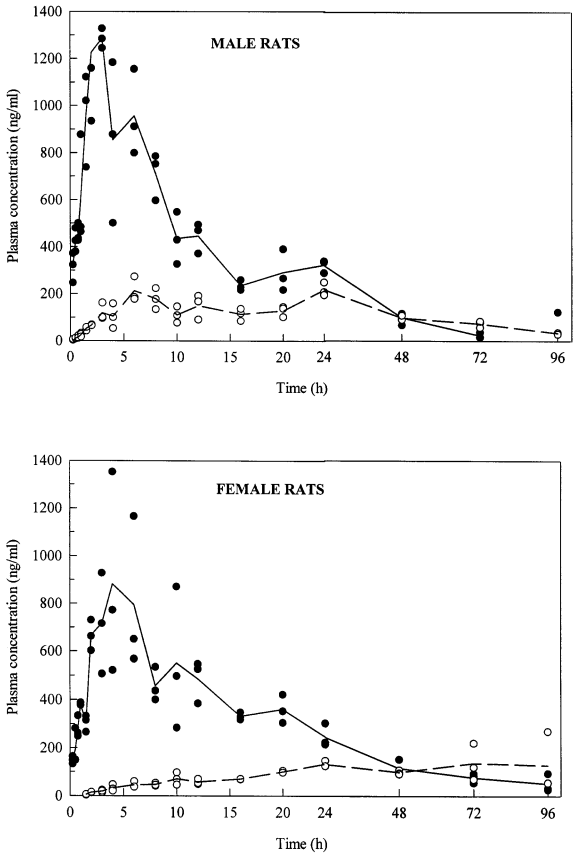


Fig. 7. Plasma concentration–time profiles of E-6087 (●) and E-6132 (○) obtained in female (a) and male (b) rats after single dose administration of 5 mg/kg of E-6087. Lines represent the mean concentrations of E-6087 (—) and E-6132 (...).

10, 80 and 150 ng ml^{-1}. Both parameters were determined by analysing 7–8 replicates per concentration in the same day and in 6–7 different days. The influence of the calibration curve in the determination of these parameters was minimised by using plasma standards different from the calibration standards [5,6]. The precision and accuracy associated to each of the analytical methods are shown in Table 3.

Assay precision was expressed as the percentage of the relative standard deviation (% RSD) of the peak area ratios measured for each concentration. For E-6087, intra-assay precision had values lower than 5.6% whereas for inter-assay precision higher values were found (16.4% for 10 ng ml^{-1} and 12.6–14.5% for the remaining concentrations). The precision of the analytical method developed to determine E-6132 showed values of intra- and inter-assay precision ranging between 4.6 and 10.7%.

Predicted concentrations for each compound were compared with the corresponding nominal values to calculate the assay accuracy, which was expressed as percent relative error (% RE). The values of the intra-assay accuracy for E-6087 ranged from 6.7 to 9.4%. Lower values were found for E-6132 ($< 8.0\%$). Inter-assay accuracy of both methods was $< 11.0\%$.

Accuracy and precision of the two methods had values within the acceptable limits in bioanalysis, taken to be less than 15% at all concentrations except the limit of quantitation where is less than 20% [3]. Accordingly, all these data indicate that both methods are precise and accurate.

Another parameter recently mentioned in the AAPS Workshop on Bioanalytical Methods Validation held in Washington D.C. (2000) is the total error, which is expressed as percentage and it is given by the sum of assay precision and accuracy. Both inter- and intra-assay total error had values than met the acceptance criteria ($< 30\%$ at the limit of quantitation and $< 20\%$ at concentrations other than this one).

3.5. Limit of quantitation (LOQ)

The limit of quantitation was defined as the concentration of the lowest standard on the calibration curve that can be measured with acceptable accuracy and precision. Based on the accordance with these criteria, the limits of quantitation determined in this study were 10 ng ml^{-1} for E-6087 and 5 ng ml^{-1} for E-6132 (Table 3).

3.6. Recovery

The recovery of E-6087 and E-6132 from rat plasma following the extraction method proposed in this study was determined at the same concentrations used to calculate the assay precision and accuracy. As for the internal standards, recovery was only determined at the working concentration of 100 ng ml^{-1} for E-6016 (internal standard of E-6087) and 1 μg ml^{-1} for E-6113 (internal stan-

dard of E-6132). Recovery at each concentration was calculated by comparing the peak area of extracted plasma samples with that of the corresponding nonextracted standard solution. Different solid phase extraction methods as well as type of cartridges (C2, C8, C18, CN) were investigated. Among the cartridges tested those with C18 filling proved to extract the compounds from plasma more efficiently.

Mean recovery of E-6087, expressed as percentage, was approximately 62% at all concentrations assayed but the lowest one, which had a recovery of 43.2%; no satisfactory explanation could be found. For E-6132 mean recoveries were 60.4% at the concentration of 80 ng ml^{-1} and 64.1–65.2% at the remaining concentrations. Recoveries of the internal standards were greater than 49% (Table 4). No relationship between the recovery of the compounds under study and their concentrations was found. Variability in compound recoveries, expressed as coefficient of variation, for either of the compounds was low at all the concentrations ($< 10\%$), implying that the extent of E-6087 and E-6132 recovery is reproducible. According to the Guidance on 'Bioanalytical methods validation for human studies' given by FDA (1998), recovery values of 50–60% can be accepted if the recovery is reproducible, as found in this study.

3.7. Stability

After 7 days at 4°C, the chromatographic responses of the stock solutions, expressed as percentage of initial response were $103.6 \pm 12.6\%$ for E-6087 and $97.0 \pm 2.3\%$ for E-6016. All these values were greater than 95% of the initial values [7], indicating that these compounds are stable in stock solutions kept at 4°C for at least this time period.

Stability of E-6087 in rat plasma after storage al -80°C during the time required to complete the analysis of plasma samples from a pharmacokinetic study, was also evaluated. At the beginning of the study, quality control samples were prepared in rat plasma at low, medium and high concentrations of the linear range of E-6087 and stored at -80°C. At the beginning and end of the analysis, E-6087 concentrations in these quality controls were determined and compared with the nominal values. Results were expressed as percentage. On starting the analysis, the mean percentage of E-6087 recovered was $101.1 \pm 16.5\%$ ($n = 12$) and after analysis completion $94.8 \pm 10.7\%$ ($n = 12$). No statistical differences between these percentages were found ($p > 0.09$). A one-way ANOVA using a rank test was applied. This finding suggests that E-6087 remained stable in rat plasma samples stored at -80°C during the time frame of the sample analysis.

The stability of E-6087 during sample preparation and analysis was ensured by preparing and analysing in each batch quality controls at low, medium at high E-6087 concentrations. A mean relative error of 103.8 (± 8.0; $n = 159$)% was obtained in 93% of the quality controls included in all batches analysed. When the study was aimed at analysing E-6132, plasma quality controls of E-6132 were also prepared and included in each batch for analysis. The mean relative error obtained for all quality controls was $98.5(\pm 8.1)\%$. Meeting of the acceptance criterion ($> 15\%$) indicates that both E-6087 and E-6132 were stable in rat plasma during sample processing and analysis.

3.8. Ruggedness

The ruggedness of the method developed to determine E-6087 was assessed by analysing plasma quality controls from two studies which lasted 3.5 and 2 months and had 1 month of time lapse between them. During the analysis time, different analytical columns and reagents were used. Mean relative error values of the quality controls included in these studies were $< 8.5\%$ at all concentrations assayed (Table 5), implying that the method is reproducible [4].

3.9. Application of the analytical methods

Fig. 5A is a chromatogram of a typical plasma sample taken from a male rat just before 5 mg kg^{-1} of E-6087 were orally administered. A chromatogram of a representative plasma sample taken 3 h after E-6087 administration is shown in Fig. 5B. These chromatograms indicate the suc-

cessful application of the method developed to assay E-6087. Good suitability of the method proposed to assay E-6132 in the same samples can also be drawn from the observation of the chromatograms obtained before and after E-6087 administration (Fig. 6).

Concentration–time profiles of E-6087 and E-6132 in male and female rats (Fig. 7) are an example of the potential utility of the developed methods in pharmacokinetic studies. The comparison of profiles for E-6087 and E-6132 shows that at early times, when pharmacological activity is evaluated, there is a higher percentage of E-6087. This suggests that in vivo, activity is mainly due to the unchanged compound (E-6087).

4. Conclusions

Two liquid chromatographic methods have been validated to determine E-6087 or its metabolite, E-6132, in rat plasma. Two different methods had to be developed because there were chromatographic problems to determine E-6132 under the chromatographic conditions of E-6087. Validation results of both methods showed that both of them were suitable to quantitate in rat plasma E-6087 or E-6132, depending on the method, at concentration ranges of 5–200 ng ml^{-1} for E-6132 and 10–500 ng ml^{-1} for E-6087. The application of both methods in a pharmacokinetic study in rats indicated that pharmacological activity in vivo is mainly due to E-6087.

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