Journal of Chromatography, 428 (1988) 61–69
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4170

ASSAY OF ISRADIPINE AND OF ITS MAJOR METABOLITES IN BIOLOGICAL FLUIDS BY CAPILLARY GAS CHROMATOGRAPHY AND CHEMICAL IONIZATION MASS SPECTROMETRY

CHRISTIAN JEAN and ROBERT LAPLANCHE*

Biopharmaceutical Department, Sandoz Ltd , 4002 Basle (Switzerland)

(First received December 23rd, 1987; revised manuscript received February 15th, 1988)

SUMMARY

A method is described for the determination of isradipine, a dihydropyridine calcium antagonist, and five of its metabolites in plasma and urine. The neutral compounds were extracted in toluene and analysed in a wide-bore silica capillary column. The acidic compounds were extracted in two steps, then esterified with diazomethane and assayed separately using the same column. Detection was performed by negative-ion mass spectrometry with chemical ionization. The limit of detection of isradipine was 0.04 ng/ml when the compound was determined alone and 0.7 ng/ml when its oxidized metabolite was determined simultaneously. The limits of detection of the metabolites in plasma ranged from 0.15 to 2 ng/ml. The method was successfully used in conventional pharmacokinetic studies and in a multicentre study of population pharmacokinetics.

INTRODUCTION

Isradipine [PN 200-110, Lomir®, Dynacirc®, isopropylmethyl 4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridine dicarboxylate] is a new potent calcium channel blocking agent [1–5]. Although its major metabolites [6] are much less potent than the parent drug [7], their determination is of interest in the investigation of possible accumulation in cases of hepatic or renal diseases or for the detection of non-compliers in population pharmacokinetics [8].

Like other dihydropyridine derivatives, isradipine can be determined by gas chromatography (GC) with electron-capture detection [9–12]. However, because of the low dosage used and extensive metabolism of the drug, a more sensitive and specific mass spectrometric (MS) detection method was developed [13–15]. The present methodology includes a three-step extraction of the compounds and a methylation of the acidic metabolites prior to GC–MS analysis with chemical ionization and negative-ion detection.

0378-4347/88/$03.50

EXPERIMENTAL

Materials

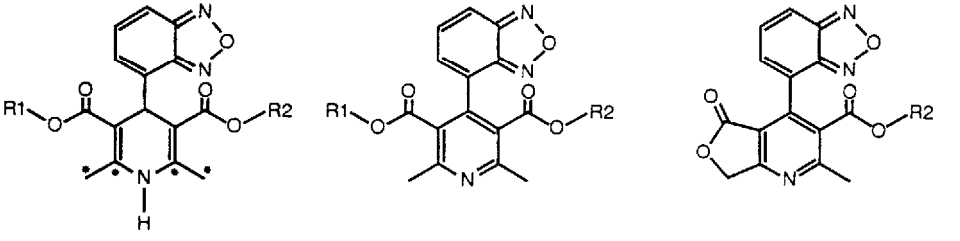
The following compounds were synthesized at Sandoz (Basle, Switzerland) (see Fig. 1): isradipine (0), its pyridinic metabolite 203-831 (1), the hydrolysed products 204-144 (2), 202-787 (3), 204-145 (11) and 204-775 (14), the lactone 921-013 (16), and the two compounds used as internal standards, [$^{13}C_4$]-PN 200-110 (IS1) and 202-479 (IS2).

Sodium hydroxide (1 *M*) and 1 *M* and 2 *M* hydrochloric acid were prepared with Titrisol® from Merck (Darmstadt, F.R.G.). The pH 3 buffer was an undiluted Titrisol ampoule. Methanol, ethanol, toluene, 2-propanol and diethyl ether from Merck were all of analytical grade (Uvasol®). Diethyl ether (2 l) was shaken with 200 ml of a mixture of equal volumes of 15% ferrous sulphate solution and 1.5% sulphuric acid to remove peroxides. Water was purified by ion exchange on a Milli-Q® filter system (Millipore, Kloten, Switzerland). Diazomethane in diethyl ether solution (2%, w/w) was prepared according to DeBoer and Backer [16].

Human plasma was prepared by centrifugation of outdated heparinized blood from a blood bank (Cantonal Hospital, Basle, Switzerland) and stored at −20 °C.

Standards

Stock solutions were made by dissolving 5 mg of each compound in 50 ml of methanol. Solution A, containing 200 ng/ml IS1, was prepared by diluting the stock solution of labelled isradipine in water. Solution B, containing IS2, was



Base A, dihydropyridines — Base B pyridines* — Base C Lactone

Compound	Base	R1	R2	Number
0	A	CH_3	$CH_2(CH_3)_2$	200-110
1	B	CH_3	$CH_2(CH_3)_2$	203-831
2	A	CH_3	H	204-144
3	A	H	$CH_2(CH_3)_2$	202-787
11	B	CH_3	H	204-145
14	B	H	$CH_2(CH_3)_2$	204-775
16	C	–	$CH_2(CH_3)_2$	921-013
IS2	A	C_3H_7	C_3H_7	202-479

*Position of Carbon 13 in labelled PN 200-110

Fig. 1. Structures of isradipine and of its major metabolites.

prepared by diluting the stock solution of 202-479 in toluene. The concentration of B was typically 200 ng/ml for plasma samples and 1 μg/ml for urine samples.

Two calibration standards, S1 and S2, were prepared by diluting the stock solutions of isradipine and its metabolites in plasma or urine so that the concentrations of all compounds were ten times higher in S2 than in S1. For S1 the following concentrations were used for the compounds 0, 1, 2, 3, 11, 14 and 16, respectively: 2, 5, 4, 1, 8, 2 and 13 ng/ml in plasma and 1, 2, 200, 100, 50, 25 and 200 ng/ml in urine.

Two quality control samples, Q1 and Q2, of different concentrations for determination as unknowns were prepared in the same way as the calibration standards, but independently, by persons not taking part in the analytical work.

Calibration standards and quality controls were provided for a whole study, distributed in 4.5-ml portions and stored at −20°C, as were the unknown samples. One portion was analysed in each series of determinations.

Washed Pyrex glass tubes of two sizes were used for sample preparation: 125 mm×16 mm I.D. (15 ml) and 125 mm×20 mm I.D. (20 ml), both with conical bottoms. For automatic injection, 0.3-ml disposable conical polypropylene vials with aluminium crimp caps were purchased from Weidmann Plastic (Romanshorn, Switzerland).

Instrumentation

The solvents and solutions were dispensed from Oxford® pipettors or dispensors (Kontron, Zurich, Switzerland), whereas biological material and extracts were handled with MLA® pipettes (Ismatec, Zurich, Switzerland). Extraction was performed on a horizontal shaker (Buhler, Tubingen, F.R.G.) at 150 cycles/min, and centrifugation with a Multex centrifuge from MSE (Zivy, Basle, Switzerland) using an universal angle-head rotor at 400 *g*. Mixing was performed with a standard Vortex mixer and evaporation with a Buchler Vortex evaporator (Kontron).

A Hewlett-Packard (Palo Alto, CA, U.S.A.) HP 5982 gas chromatograph–mass spectrometer, modified for the detection of negative ions [17], was equipped with an HP 7671 autosampler for injection and an HP 5934 data system for monitoring the analysis and processing the chromatograms. The chromatographic column was a 10 m×0.53 mm I.D. fused-silica open column coated with 2.65 μm of a cross-linked methylsilicone gum, obtained from Hewlett-Packard. Methane was used as carrier gas for chromatography and as reactant gas for ionization. The methane flow-rate was adjusted to 10 ml/min at the outlet end of the column (atmospheric pressure). During the analysis the total effluent was directed into the ion source. The working temperatures were 350°C for both the injector and the GC–MS transfer line and 200°C in the ion source.

The column temperature was 230°C for the analysis of extract A. For extract B, it was held at 230°C for 2 min and then increased to 270°C at 32°C/min. If only the unchanged drug was determined, the oven was heated isothermally at 260°C. The mass spectrometer was operated under chemical ionization conditions with an electron energy of 250 eV. The voltage applied to the detector shelf was 3000 V, and the detection gain was 10 for extract A and 6 for extract B. The

analysis was monitored in selected-ion mode after optimization of the source and mass filter parameters (gain, offset, blades, draw-out, repeller, ion-focus, electron-focus, emission current, entrance lens) on the mass peak of isradipine (*m/z* 311). The selected masses were *m/z* 311 for isradipine and for the methylated derivatives of 2 and 3, 369 for 1 and the methylated derivative of 14, 341 for the derivative of 11 and 353 for the lactone 16. The internal standards IS1 and IS2 were detected on *m/z* 315 and 339, respectively. The resolution at 10% valley was ca. 0.6 dalton.

Procedures

Biological samples and standards, stored at −20 °C, were thawed in a water-bath (37 °C) and equilibrated for 5 min at room temperature. To 1 or 2 ml of plasma or urine in a 15-ml extraction tube were added 50 µl of the solution A of IS1, 0.5 ml of 1 *M* sodium hydroxide and, after mixing, 5 ml of toluene. The tubes were stoppered and shaken mechanically for 20 min, opened and centrifuged for 10 min. The organic phase was transferred to a 15-ml tube, and the solvent was evaporated to dryness in vacuo at a temperature not exceeding 70 °C (extract A).

To the aqueous phase from the first extraction were added 0.5 ml of 1 *M* hydrochloric acid, 0.5 ml of pH 3 buffer and 5 ml of toluene–2-propanol (90:10). The tubes were stoppered, shaken mechanically for 20 min and centrifuged for 10 min. A 4.5-ml aliquot of the organic phase was transferred to a 20 ml-glass tube T, and 0.1 ml of the diazomethane solution was added. To the aqueous layer remaining from the second extraction were added 0.5 ml of 2 *M* hydrochloric acid and 5 ml of diethyl ether. The tubes were stoppered and shaken for 20 min. They were centrifuged for 10 min using plastic stoppers to avoid evaporation. A 4-ml aliquot of the ether phase was transferred to the same tube T, to which were added 0.5 ml of methanol, 0.1 ml of solution B of IS2 and 0.2 ml of the diazomethane solution. The mixture was vortexed for 5 s and allowed to stand for 5 min. It was washed successively with 1 *M* sodium hydroxide and 1 *M* hydrochloric acid in the following manner: 2 ml of the reagent were pipetted into the tube, which was stoppered and shaken for 5 min, opened and centrifuged for 5 min. The sodium hydroxide layer was drawn off with a Pasteur pipette and hydrochloric acid was added. An 8-ml aliquot of the organic phase was transferred to a 15-ml tube, and the solvent was evaporated to dryness in vacuo at a temperature not exceeding 70 °C (extract B).

Extracts A and B were taken up in 50 µl of toluene, transferred to a vial and analysed separately; 2-µl samples were injected.

Unknown concentrations were calculated by interpolation between two calibration standards, assuming a linear relationship between the signal and the concentration of the compounds. Premedication samples were used as a calibration standard of zero concentration. The signal of a compound was the area ratio of the compound peak to the peak of the internal standard IS1 (extract A) or IS2 (extract B). For the unchanged isradipine, the signal Y had to be corrected for the mutual interferences with IS1: $Y=(A-A^*R^*)/(A^*-AR)$, with A = peak area at m/z 311, A^* = peak area at m/z 315, $R=A^*/A$ for isradipine alone and $R^*=A/A^*$ for IS1 alone.

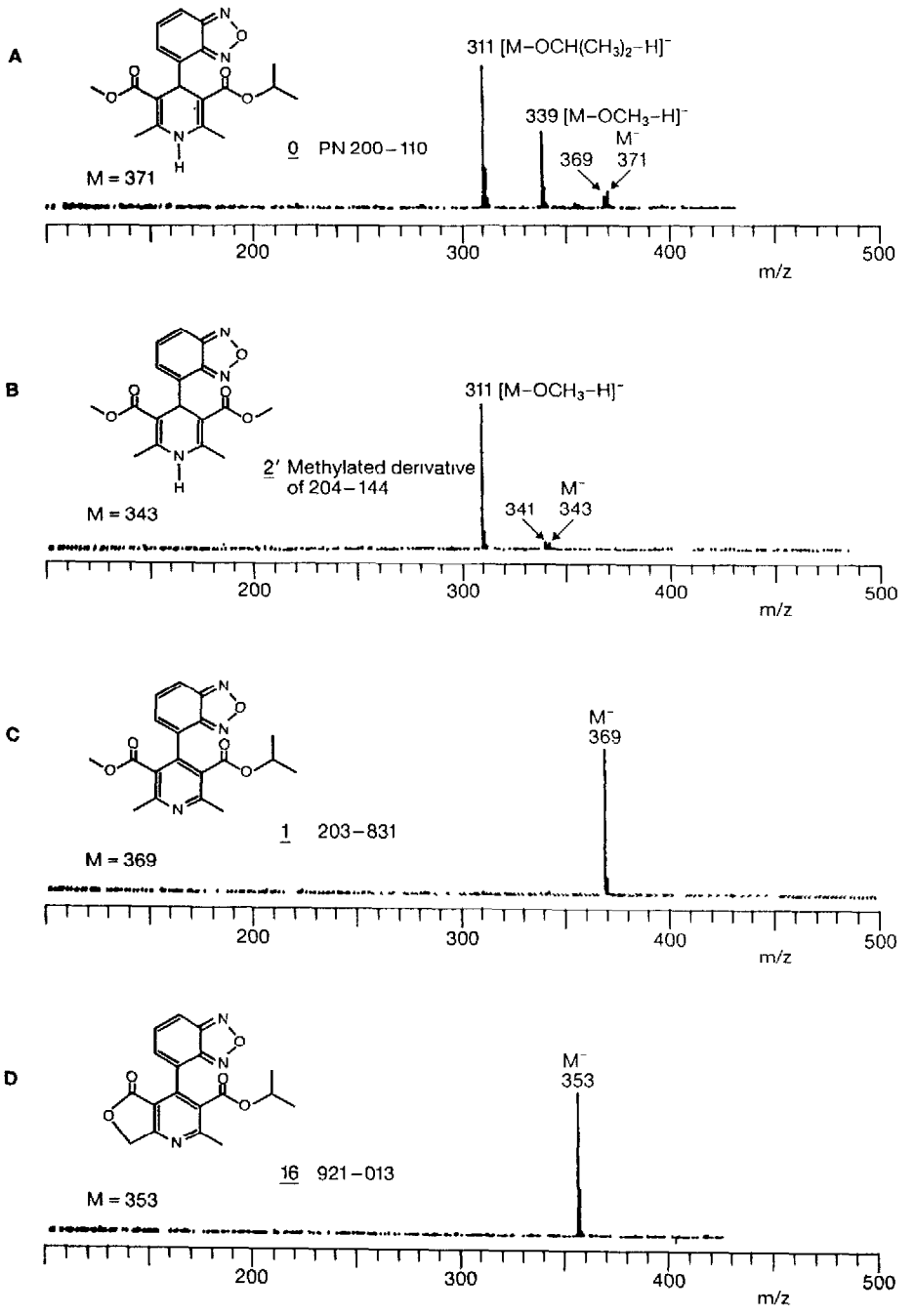


Fig. 2. Negative-ion spectra using chemical ionization with methane: (A) and (B) dihydropyridine structures; (C) a pyridine structure; (D) the lactone.

RESULTS AND DISCUSSION

Chromatography

The ions chosen for the detection in selected-ion mode were the base peaks of the negative-ion spectra of the compounds (Fig. 2). For the pyridines, they corresponded to the molecular ion M^-. For the dihydropyridines, they were fragment ions arising from α-cleavage of the carbonyl group with hydrogen abstraction: $[M-(OCH_3+H)]^-$ and $[M-(OC_3H_7+H)]^-$. Where competition occurred, the latter fragment was preponderant. This fragmentation did not appear in the spectrum of dihydropyridines which, like nifedipine, contain a nitrophenyl group [14].

The retention times (Fig. 3) were 1.3 min for 1 and 2.5 min for 0 (extract A), 1.1, 1.3, 2.2, 2.4 min for the derivatives of 11, 14, 2 and 3, respectively, 2.35 min for 16 and 3.0 min for IS2 (extract B). The interference of compound 16 at mass 311 was not negligible for the determination of the derivative of 3. As compound 3 appeared as a minor metabolite relative to 11, 14 and 16, its determination was not further investigated.

As, in spite of repeated washings, extract B was rather dirty, the glass inlet of the injector port was changed each day in order to avoid peak broadening. It was thus possible to analyse automatically ca. 35 clinical samples per day, the two analytical runs being achieved within 8 h.

Method validation

Isradipine was stable in plasma for two months at −20 °C and for 24 h at ca. 22 °C. New determinations with freshly prepared calibration standards did not

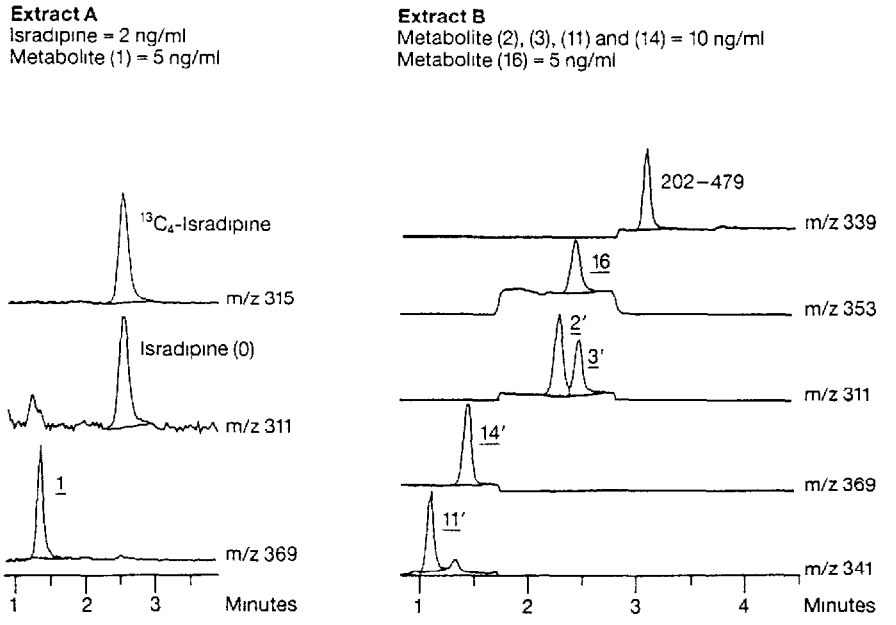


Fig. 3. Specimen chromatograms (selected-ion monitoring).

TABLE I

METHOD VALIDATION FOR ISRADIPINE AND FIVE OF ITS METABOLITES IN PLASMA

Control sample	Compound	n*	Concentration (ng/ml)		Found/ expected (%)	Coefficient of variation (%)	
			Expected	Found		Within assays**	Between assays***
A	0	7	11.3	10.6	94	4.4	6.2
	1	6	29.3	31.8	109	7.6	8.5
	2	5	27.9	30.0	108	9.6	10.2
	11	5	47.0	49.9	106	9.8	13.3
	14	5	15.7	15.9	101	9.7	1.3
	16	5	115.9	132.0	114	8.0	8.6
B	0	6	2.5	2.3	92	5.3	1.8
	1	6	3.8	4.2	111	7.5	7.6
	2	6	9.2	8.8	96	21.9	6.8
	11	6	10.2	9.3	91	25.5	19.4
	14	6	7.6	5.9	78	11.5	10.6
	16	6	32.8	36.8	112	6.6	9.4

*Number of duplicate determinations over a period of ca one month.
**Mean coefficient of variation of duplicate determinations (quadratic mean).
***Coefficient of variation of n means.

TABLE II

METHOD VALIDATION FOR ISRADIPINE DETERMINED ALONE IN PLASMA (TWO STUDIES)

Control sample	n'/n''*	Concentration (ng/ml)		Found/expected (%)	Coefficient of variation (%)	
		Expected	Found		Within assays**	Between assays***
C3	9/0	0.00	0 07	–	–	92.0
C1	11/0	0.04	0.12	300	–	35.0
A	3/7	0.66	0 70	106	4.8	10.3
B	3/7	1.87	1.73	93	5.8	3.8
C	3/7	2.00	1.93	97	3.7	3.7
C4	12/0	3.09	2.82	91	–	5.4
C2	10/0	3.10	2.79	90	–	4.8
D	3/7	4 00	3 82	96	7.3	6.0
C5	11/0	10.00	9.99	100	–	4.2

*Number of single/duplicate determinations.
**Mean coefficient of variation in duplicate determinations (quadratic mean).
***Coefficient of variation of n single or duplicate determinations.

reveal significant changes in the concentrations of metabolites in plasma and urine controls after 1.5 months storage at $-20\,^{\circ}C$.

The extraction of the compounds was investigated with spiked plasma samples, which were compared with standard solutions added to blank plasma extracts.

The recovery was 100% for 1 (10 ng/ml) and 16 (100 ng/ml), 95% for 0 (10 ng/ml) and 90% for the acidic metabolites 2, 3, 11 and 14 (100 ng/ml each).

The calibration curves in plasma were linear in the investigated ranges up to 10 ng/ml for 0 and 1 (mean of range 3.1 and 3.6, respectively), up to 50 ng/ml (mean 13.4) for 16 and up to 100 ng/ml (mean 28) for the others. The deviation from the regression at zero concentration corresponded to 0.14, 0.25, 0.15, −1.3, −1.6 and −0.74 ng/ml of 0, 1, 2, 11, 14 and 16, respectively. Because of this deviation, calibration standards of two concentrations were prepared in plasma and urine.

Accuracy, precision and reproducibility were investigated with the control samples determined along with unknown plasma samples over a period of ca. one month of analytical work. They are documented in Table I for the metabolites and in Table II for the determination of unchanged isradipine alone. The values in urine, not reported here, are very similar to the plasma values.

The limit of detection (L) of each compound was calculated for duplicates using the mean S.D. observed for three determinations of three different concentrations around the expected value of L. It was ca. 0.7 ng/ml for 0, 2 for 1, 0.15 for 2, 0.25 for 11, 0.4 for 14 and 0.5 for 16. When isradipine was determined alone, L was ca. 0.04 ng/ml for double determinations on the same day. The limit of quantitation is dependent on the concentrations of the standards chosen for calibration and on the range of measurements. In the example of Table II, it was ca. 0.15 ng/ml, owing to a difference of 0.07 ng/ml between the measured and the expected values in the lower concentration range of the control samples. That systematic error was attributed to the presence of an interfering compound in the outdated blood bank plasma used in the preparation of the control samples.

Assay of clinical samples

The method was sufficiently sensitive to allow the plasma and urine concentrations to be followed up to 24 h after administration of therapeutic doses of the

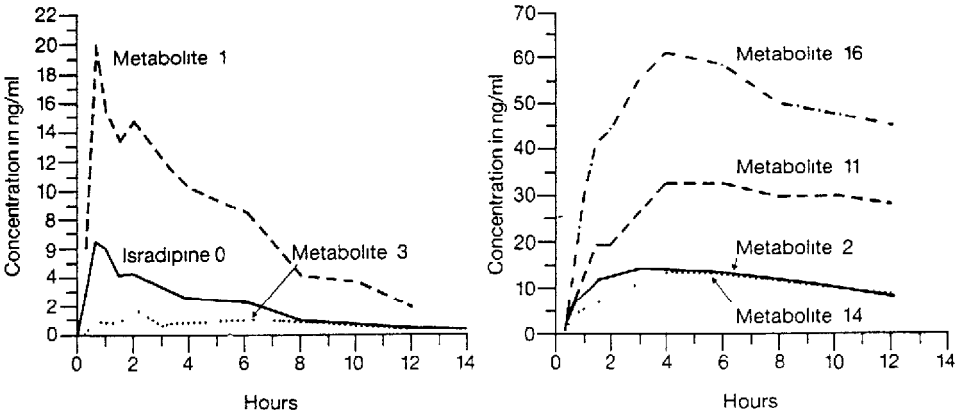


Fig. 4. Plasma levels of isradipine and metabolites following administration of a single dose of 5 mg of isradipine (means of eight subjects).

drug (1.25–10 mg two times a day). Fig. 4 shows the mean plasma levels obtained with eight healthy volunteers after a single oral dose of 5 mg of isradipine.

The assay was successfully applied to clinical studies involving a total of ca. 15 000 samples. Unchanged disradipine was determined in order to compare various formulations of the drug and to assess its absolute bioavailability. In addition, the metabolites were assayed in conventional pharmacokinetic studies in patients suffering from renal or hepatic insufficiency, and in a multicentre study of population pharmacokinetics, which aimed to detect atypical and non-complying patients.

ACKNOWLEDGEMENTS

We are grateful to H. Andres, A. Vogel and R. Voges for the synthesis of the reference compounds and to P. Ryser for his assistance in sample analysis.

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