ELSEVIER

Journal of Chromatography B, 676 (1996) 77–85

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of the calcium antagonist SIM6080 and its N- and O-demethylated metabolites in plasma, urine and tissues by high-performance liquid chromatography

Pierfrancesco Castelnovo

Medicinal Chemistry Analytical Laboratory, Zambon Group, Bresso (Milan), Italy

First received 13 July 1995; revised manuscript received 25 September 1995; accepted 25 September 1995

Abstract

A sensitive and versatile high-performance liquid chromatographic assay for the determination of the calcium antagonist SIM6080 and its four N- and O-demethylated metabolites in plasma, urine and tissues has been developed and validated. A two-step extraction procedure is employed followed by reversed-phase liquid chromatographic analysis using ultraviolet detection. An isomer of SIM6080 was used as the internal standard. The analysis of spiked plasma, urine and tissues demonstrated the accuracy and precision of the assay with quantitation limits of 5 ng/ml (plasma and urine) or 100 ng/g (tissues). This assay has been used for urinary recovery and tissue distribution studies, as well as for toxicokinetic protocols.

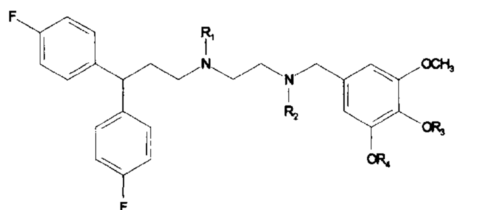
Keywords: SIM6080; Calcium antagonist

1. Introduction

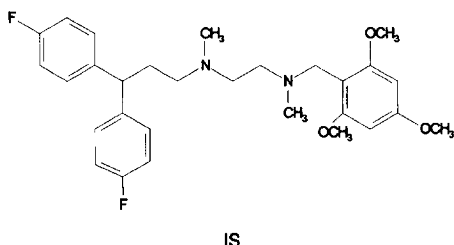
SIM6080 (I) (Fig. 1) is a calcium antagonist that belongs to a class of compounds structurally related to the phenylalkylamines [1]. The drug combines transmembrane and intracellular calcium antagonistic properties as well as anti-atherosclerotic effects [2,3]. A sensitive high-resolution gas chromatographic (HRGC) assay was developed in our laboratory and extensively employed to investigate the pharmacokinetic profile of the drug in laboratory animals [4]. The first investigations on the metabolism of I revealed the presence of two N-demethylated and at least one of the two possible O-demethylated metabolites. Therefore a method able to determine the demethylated metabolites in addition to the parent drug was required in order to investigate the urinary excretion and the tissue distribution as well as for toxicokinetic studies.

The first efforts were towards a modification of the existing HRGC assay. Since both the N- and the O-demethylated metabolites have an active hydrogen in their structure, they must be derivatized in order to be analyzed by gas chromatography. Among the derivatization reactions studied acylation with MBTFA as derivatizing reagent and N-methyl imidazole as catalyst [5] was fast and reproducible and the trifluoroacetyl derivatives were successfully separated, but partial loss of the analytes during the additional evaporation step required to remove reagent and

0378-4347/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(95)00414-9



	R1	R2	R3	R4
I	CH3	CH3	CH3	CH3
M-I	H	CH3	CH3	CH3
M-II	CH3	H	CH3	CH3
M-III	CH3	CH3	CH3	H
M-IV	CH3	CH3	H	CH3



IS

Fig. 1. Structure of I (SIM6080), the N- and O-demethylated metabolites and the internal standard (I.S.).

catalyst occurred, thus reducing sensitivity. Since we were not able to eliminate such loss and taking into account that the addition of the derivatization reaction to the three-step purification procedure would have made the entire sample preparation too lengthy, we decided to develop a new assay based on HPLC which would have required no derivatization of the metabolites and possibly allowed a simpler sample preparation.

This paper reports an assay for the quantification of I and its four N- and O-demethylated metabolites (Fig. 1) in plasma and urine and in a variety of tissues such as heart, kidney, lung, liver, brain, ileum, aorta, suprarenal gland, muscles and fat. The drug and its metabolites were isolated by a two-step extraction procedure and analyzed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Tissues were homogenated in water prior to the extraction. These features give the method the selectivity and the versatility required to determine five closely related substances in a wide variety of biological matrixes. Due to the availability of an automated sample injector up to 48 samples can be assayed daily by a single operator.

2. Experimental

2.1. Materials

N_1,N_2-Dimethyl-N_1-[3,3-di-(4-fluorophenyl)-propyl]-N_2-(3,4,5-trimethoxybenzyl)ethylenediamine dihydrochloride (SIM6080, I), its N-demethylated (M-I and M-II) and O-demethylated (M-III and M-IV) metabolites and the internal standard (I.S.), an isomer of I (Fig. 1), were synthesized by the Medicinal Chemistry Dept. of Zambon Group. Heptane (Resy-analyzed reagent) was purchased from Baker (Deventer, Netherlands). Analytical-grade organic solvents and chemicals were purchased from Carlo Erba (Milan, Italy). Sulfatase (type H-2 from *Helix pomatia*) crude solution was from Sigma (St. Louis, MO, USA). Purified water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). The disposable pyrex glass culture tubes with PTFE-lined screw-cap (16 × 100 mm) and pipettes (5 ml) were from Corning (Corning, NY, USA). Automatic micropipettes were from Brand (Wertheim-Main, Germany). The sonicator was a Model W-380 from Heat System-Ultrasonics (Farmingdale, NY, USA) equipped with a 2.4-mm microtip.

2.2. Equipment and chromatographic conditions

The liquid chromatographic system consisted of a Series 3 pump, a ISS-101 autosampler, an LC-90 UV detector, all from Perkin-Elmer (Monza, Italy). The chromatograms were acquired using a SP-4270 integrator linked to a Chromstation/2 system, all from Spectra-Physics (San Jose, CA, USA).

The following reversed-phase HPLC columns

were tested during the method development: Supelcosil LC-18 5 μm 150×4.6 mm I.D., Supelcosil LC-18-DB 3 μm 75×4.6 mm I.D. and Suplex pKb-100 5 μm 150×4.6 mm I.D. all from Supelchem (Milan, Italy), Partisphere C_{18} 5 μm 125×4.6 mm I.D. from Whatman (Clifton, NY, USA), Lichrosorb RP 18 7 μm, Hypersil APS-2 5 μm, Spherisorb ODS-2 5 μm, Nucleosil 100 5 μm, Chromspher C_8 5 μm and Chromspher C_{18} 5 μm all 100×3.0 mm I.D. from Chrompack (Middelburg, Netherlands).

The final choice was the Nucleosil 100 column. The mobile phase was 0.05 *M* sodium perchlorate pH 3.0–acetonitrile (63:37) at a flow-rate of 0.6 ml/min with a counter-pressure of 7.5 MPa. The column was used at room temperature. The detector wavelength was 210 nm and 20 μl were injected.

2.3. Preparation of stock and working solutions

Stock solutions (1 mg/ml) of I, of the demethylated metabolites and of the I.S. were prepared in methanol and proved to be stable for at least one month when stored at −20°C.

The working solution containing I (1 μg/ml) and the metabolites (2 μg/ml) and the working solution of the I.S. (1 μg/ml) were prepared daily by dilution of the corresponding stock solutions with acetonitrile–0.01 *M* HCl (25:75, v/v).

2.4. Extraction procedures

2.4.1. Extraction from plasma

The frozen plasma (−20°C) was thawed at room temperature and a 1-ml aliquot was pipetted into a disposable glass culture tube. Internal standard (100 ng = 100 μl of the working solution), 1 *M* carbonate buffer pH 9.0 (125 μl) and 5 ml of the organic extracting solvent (heptane–*n*-pentanol, 95:5, v/v) were added to each tube. The plasma sample was extracted for 20 min on a horizontal shaker and then centrifugated for 1 min at 1200 *g*. The organic phase was transferred to another disposable tube, 0.05 *M* sulphuric acid (100 μl) was added and the tube was vortex-mixed for 30 s. After centrifugation (1 min at 1200 *g*) the organic phase was aspirated off and the aqueous phase transferred to the autosampler vial.

2.4.2. Enzyme hydrolysis and extraction from urine

The frozen urine was thawed at room temperature and a 0.1–0.5 ml aliquot was pipetted into a disposable glass culture tube. Sodium acetate buffer (0.5 *M*) pH 5.0 (100 μl), sulfatase crude solution (100 μl) and 200 μl of a EDTA–sodium metabisulfite solution (5 mg/ml each) were added. The mixture was incubated at 37°C for 16 h and then extracted as described above for plasma. The only modification was the use of 1 *M* carbonate buffer pH 10.0 to correct the pH before extraction.

2.4.3. Homogenation and extraction from tissues

A 100-mg amount of tissue was transferred to a polypropylene 16×100 mm tube and homogenized by sonication in 2.0 ml of water keeping the tube in an ice bath. Sonication time was in the range 0.5–5 min. Aorta was placed in a mortar, immersed in liquid nitrogen and pulverized before sonication. An 0.1–1.0 ml aliquot of homogenate was transferred into a disposable glass culture tube and then extracted as described above for plasma.

2.5. Calibration

Standard curves were prepared using 1 ml of drug-free heparinized plasma, urine or the appropriate homogenate depending on the samples under investigation. The plasma and urine standards spanned one order of magnitude (25, 50, 75, 100, 150 and 200 ng/ml for I and 12.5, 25, 37.5, 50 and 100 ng/ml for each metabolite), while tissue homogenate standards were 50, 100 and 200 ng/ml for I and 25, 50 and 100 ng/ml for the metabolites. All standards were prepared by adding appropriate volumes of the working solution containing I and the metabolite. The calibration curves were obtained from the least squares linear regression of the peak-area ratio of the analyte to the internal standard against analyte concentration.

3. Results and discussion

3.1. Choice of the chromatographic system

This soon turned out to be the most challenging task in the development of the assay since we were faced with the problem of separating four closely related substances such as the N- and O-demethylated metabolites. In addition both I and the metabolites exhibit strong base behaviour due to the presence of one or more tertiary nitrogen atoms in their molecular structure. Therefore a highly efficient LC separation system was needed with small and narrowly distributed particles as well as an effective surface deactivation. In fact basic drugs are often difficult to handle because of the strong ionic interaction of the charged solutes with free silanol groups of the packing and it is well known that LC columns from different vendors exhibit a quite different behaviour towards basic compounds [6,7]. We tested the chromatographic behavior of I and its demethylated metabolites on a variety of phases available in our laboratories: Supelcosil LC-18 and LC-18-DB, Suplex pKb-100, Hypersil ODS, Spherisorb ODS-2, Chromspher C_8 and C_{18}, Lichrosorb RP18, Partisphere C_{18} and Nucleosil C_{18} using phosphate buffer pH 4.5, phosphate buffer with the addition of an amine (triethylamine) or of an ion-pairing reagent (sodium heptansulphonate), and perchlorate buffer as mobile phase. In most instances the resolution was not satisfactory because of broad and asymmetrical peaks even though in some cases the addition of a modifier brought about an improvement in the chromatographic performance.

Only Nucleosil C_{18} and Suplex pKb-100 gave very good peak shapes especially with mobile phase containing sodium perchlorate which at the same time acts as buffering system and ion-pairing reagent. Nucleosil C_{18} was preferred over Suplex pKb-100 because of the much shorter equilibration time and the much lower price. It turned out to be a very rugged chromatographic system since the column could withstand thousands of injections before being replaced, provided a regular change (twice a week) of the precolumn was scheduled.

3.2. Extraction

The prechromatographic sample purification should ideally afford high recoveries of the analytes and the elimination of compounds that would otherwise interfere in the chromatogram. HPLC generally requires a less stringent purification procedure than HRGC in order to eliminate endogenous material such as proteins and lipids that could rapidly decrease the efficiency of the chromatographic system. We then tried to simplify the three-step extraction procedure previously developed for the HRGC assay [4] but we found that if only the first step [extraction with heptane–*n*-pentanol (95:5) at strongly alkaline pH] was used two drawbacks occurred: O-demethylated metabolites were extracted with low recoveries and clean blank chromatograms were difficult to obtain. Lowering the pH to 9, using carbonate buffer instead of sodium hydroxide to correct plasma pH before extraction, allowed good recoveries of the O-demethylated metabolites while back-extraction in 0.01 *M* sulphuric acid eliminated interferences from the biological matrix.

A new internal standard, which is essential for obtaining a high precision in the assay, was also required. In fact, being less lipophilic than I, the defluorinated analogue previously used for the HRGC assay [4] eluted just before I preventing its detection when present in low amounts. The structural isomer I.S. (Fig. 1), which eluted after I, was then chosen as internal standard.

3.3. Selectivity and sensitivity

Chromatograms of drug-free plasma, plasma spiked with known concentration of I and of the N- and O-demethylated metabolites and plasma from a healthy volunteer receiving a 120-mg oral dose of I taken 2 h after administration are shown in Fig. 2. They show both the sensitivity of the assay, which gives a quantitation limit of 5 ng/ml and a detection limit ($S/N = 3$) of 1 ng/

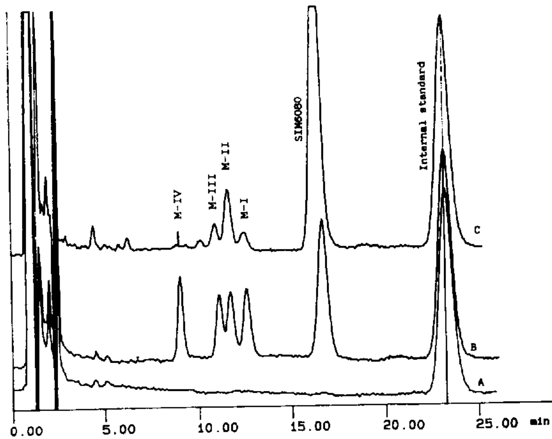


Fig. 2. Chromatograms of extracts from (A) drug-free plasma sample spiked with internal standard (200 ng/ml), (B) plasma sample spiked with I (SIM6080), (50 ng/ml), N- and O-demethylated metabolites (25 ng/ml each) and internal standard (200 ng/ml) and (C) plasma sample from a healthy volunteer 2 h after a 120-mg oral dose of I. Concentrations are: I (SIM6080), 141 ng/ml; M-I, 5.6 ng/ml; M-II, 18 ng/ml; M-III, 27 ng/ml and M-IV, 0.65 ng/ml.

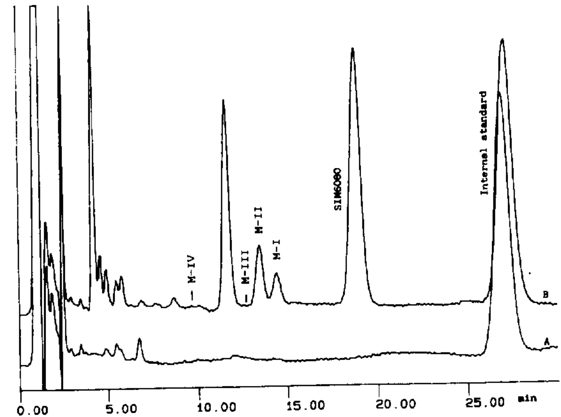


Fig. 4. Chromatograms of extracts from (A) drug-free kidney homogenate spiked with internal standard (4000 ng/ml) and (B) kidney homogenate from a rabbit receiving a multiple 3 mg/kg subcutaneous dose of I and sacrificed 2 h after administration on day 50. Concentrations are: I (SIM6080), 7.0 ng/g; M-I, 0.46 ng/g and M-II, 1.3 ng/g. The O-demethylated metabolites M-III and M-IV are below the detection limit.

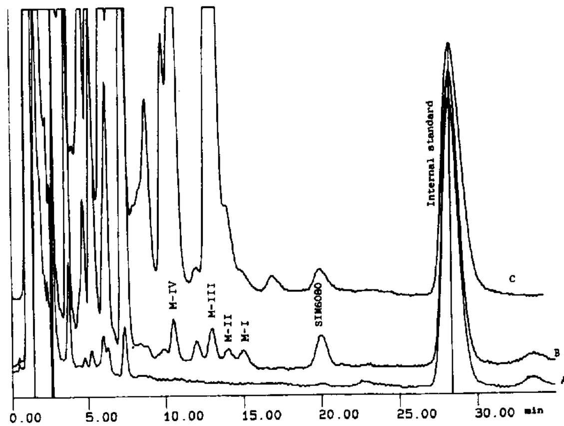


Fig. 3. Chromatograms of extracts from (A) drug-free urine sample spiked with internal standard (200 ng/ml), (B) urine sample from healthy volunteer receiving a multiple 60-mg oral dose of I collected for 6 h after administration on day 11 and (C) same as (B) after enzymatic hydrolysis. Concentrations are: (B) I (SIM6080), 14 ng/ml; M-I, 6 ng/ml; M-II, 7 ng/ml; M-III, 14 ng/ml and M-IV, 12 ng/ml, (C) I (SIM6080), 12 ng/ml; M-III, 532 ng/ml and M-IV, 203 ng/ml.

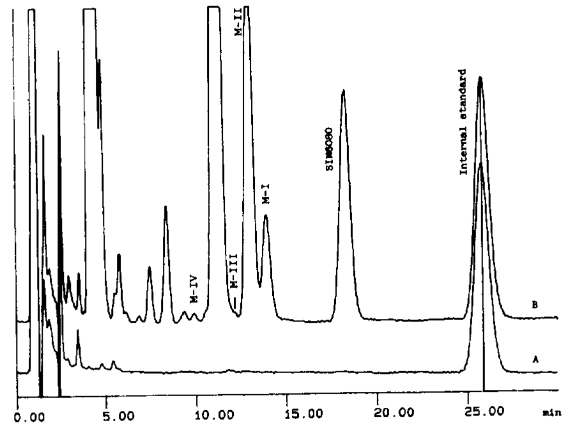


Fig. 5. Chromatograms of extracts from (A) drug-free liver homogenate spiked with internal standard (4000 ng/g) and (B) liver homogenate from a rabbit receiving a multiple 3 mg/g subcutaneous dose of I and sacrificed 2 h after administration on day 50. Concentrations are: I (SIM6080), 2.8 μg/g; M-I, 0.78 μg/g; M-II, 4.1 μg/g and M-IV, 0.6 μg/g.

ml, and the selectivity as no interfering peaks are seen at the retention times of I and metabolites.

The same results in terms of sensitivity and selectivity were obtained for the analysis of urine samples. Fig. 3 shows chromatograms of drug-free urine, urine from a healthy volunteer receiving a multiple 60-mg dose of I collected for 6 h after administration on day 11 and the same urine sample after enzymic hydrolysis. As can be seen, I and both N- and O-demethylated metabolites were present in trace amounts in the non-hydrolyzed sample while a dramatic increase in the concentration of the two O-demethylated metabolites occurs after hydrolysis indicating that O-demethylation followed by conjugation is one of the main metabolic routes.

Chromatograms with no interfering peaks were also obtained for the homogenates of the tissue analyzed (kidney, lung, liver, brain, heart, aorta, suprarenal gland, ileum muscle and fat from rabbits and rats). Figs. 4 and 5 shows chromatograms of drug-free homogenates and homogenates taken from rabbits which were given I at the subcutaneous dose of 3 mg/kg for 50 days killed 2 h after the last administration. Quantitation limit was 100 ng/g of tissue for all the analytes while detection limit ($S/N = 3$) was 20 ng/g.

3.4. Calibration

The analyte concentration of the standard solutions, when plotted against the ratio of the peak area of each analyte to the peak area of internal standard, showed a linear response in the concentration range tested and passed through the origin in each case. The correlation coefficients were >0.997 for all the analytes.

Similar results were also obtained for urine and tissues. Due to the high specificity of the assay, which gives blank chromatograms with no interfering peaks, the parameters for the calibration graphs in all the tissues were quite similar as shown in Table 1.

3.5. Validation

The within-day accuracy and precision of the method were assessed by extracting and analyzing plasma and urine samples spiked with I and the N- and O-demethylated metabolites at five different concentrations four times on one day.

Table 1
Parameters of the calibration graphs (concentration vs. peak-area ratio in tissue homogenates) for SIM6080 and N-demethylated (M-I and M-II) and O-demethylated (M-III and M-IV) metabolites

	Parameter	Aorta	Ventricle	Kidney	Liver	Lung	Ileum	Muscle	Fat	Brain	Supra-renal gland
I	Slope	173.9	151.5	170.9	159.5	156.9	162.9	143.9	141.8	151.6	153.6
	Intercept	3.8	−1.9	−6.3	−1.5	−0.4	0.3	−4.3	3.4	−0.7	−2.1
	r	0.9991	0.9997	0.9968	0.9998	0.9999	0.9999	0.9986	0.9992	0.9999	0.9994
M-I	Slope	130.1	134.1	166.9	156.4	156.3	152.2	139.9	154.4	150.0	154.1
	Intercept	1.7	−0.6	2.9	−0.5	−0.1	0.6	−1.8	0.5	0.1	−1.3
	r	0.9992	0.9998	0.9974	0.9999	0.9999	0.9998	0.9990	0.9999	0.9999	0.9998
M-II	Slope	152.8	158.1	196.6	186.3	185.8	185.1	161.6	177.7	179.8	178.6
	Intercept	2.7	−1.2	−2.6	−0.2	0.3	0.7	−1.8	0.8	0.3	−0.9
	r	0.9981	0.9998	0.9979	0.9999	0.9999	0.9998	0.9990	0.9998	0.9999	0.9997
M-III	Slope	229.8	207.9	190.5	220.5	210.0	203.9	205.6	231.4	229.1	235.6
	Intercept	0.2	−0.2	2.3	1.2	2.3	1.2	2.8	−0.2	4.3	3.1
	r	0.9981	0.9992	0.9969	0.9999	0.9997	0.9995	0.9930	0.9999	0.9999	0.9991
M-IV	Slope	225.3	214.4	195.6	221.3	217.3	200.1	199.3	235.6	229.3	231.5
	Intercept	0.8	−1.2	−2.3	0.2	0.1	1.7	2.9	−0.3	−0.4	0.1
	r	0.9991	0.9995	0.9981	0.9999	0.9998	0.9999	0.9990	0.9999	0.9999	0.9996

The concentrations were 50, 100 and 200 ng/ml for SIM6080 and 25, 50 and 100 ng/ml for the demethylated metabolites. The table shows slope, y-intercept and correlation coefficient (r) from the least squares regression analysis of each compound.

Table 2
Within-day precision and accuracy ($n = 5$) of the determination of I and demethylated metabolites in spiked plasma by HPLC–UV analysis

	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Accuracy (%)
I	25	24.3 ± 1.5	6.2	−2.8
	50	52.3 ± 3.0	5.7	+4.6
	75	77.4 ± 3.4	6.6	+3.2
	100	102.5 ± 3.4	3.3	+2.5
	200	198.5 ± 1.5	0.8	+0.8
M-I	12.5	13.4 ± 1.2	9.0	+7.2
	25.0	27.0 ± 2.3	8.5	+8.0
	37.5	34.3 ± 2.1	6.1	−8.5
	50.0	50.9 ± 1.7	3.3	+1.8
	100.0	100.0 ± 1.1	1.1	+0.0
M-II	12.5	14.2 ± 1.5	10.6	+13.6
	25.0	24.7 ± 1.3	5.3	−1.2
	37.5	34.8 ± 2.9	8.3	−7.2
	50.0	49.8 ± 1.2	2.4	−0.4
	100.0	100.9 ± 0.8	0.8	+0.9
M-III	12.5	12.0 ± 1.3	10.8	−4.0
	25.0	22.4 ± 0.9	4.0	−10.4
	37.5	38.9 ± 1.7	4.4	+3.7
	50.0	49.8 ± 0.6	1.2	−0.4
	100.0	98.5 ± 0.8	0.8	−1.5
M-IV	12.5	11.8 ± 0.5	4.2	−5.6
	25.0	25.5 ± 1.1	4.6	+0.4
	37.5	35.4 ± 0.8	2.3	−5.6
	50.0	51.4 ± 1.5	2.9	+2.8
	100.0	100.4 ± 0.9	0.9	+0.4

Table 3
Within-day precision and accuracy ($n = 5$) of the determination of I and demethylated metabolites in spiked urine by HPLC–UV analysis

	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Accuracy (%)
I	25	23.8 ± 2.3	9.7	−4.8
	50	48.8 ± 4.2	8.6	−2.4
	75	75.4 ± 4.2	5.6	+0.5
	100	103.4 ± 2.7	2.6	+3.5
	200	200.9 ± 1.9	0.9	+0.4
M-I	12.5	11.6 ± 0.9	7.8	−7.2
	25.0	27.7 ± 1.9	6.9	+10.8
	37.5	36.3 ± 3.4	9.4	−3.2
	50.0	48.9 ± 1.7	2.5	−2.2
	100.0	101.1 ± 0.7	0.7	+1.1
M-II	12.5	12.0 ± 0.9	7.5	−4.0
	25.0	25.3 ± 2.2	8.7	+1.2
	37.5	34.7 ± 1.8	5.2	−7.5
	50.0	50.3 ± 0.9	1.8	+0.6
	100.0	99.1 ± 2.4	2.4	−0.9
M-III	12.5	11.5 ± 1.5	13.0	−8.0
	25.0	23.3 ± 1.7	7.3	−6.8
	37.5	39.2 ± 2.4	6.1	+4.5
	50.0	47.5 ± 2.2	4.6	−5.0
	100.0	99.5 ± 1.7	1.7	−0.5
M-IV	12.5	11.3 ± 0.8	7.1	−9.6
	25.0	26.2 ± 1.1	4.2	+4.8
	37.5	37.2 ± 1.2	3.2	−0.5
	50.0	50.2 ± 3.1	6.2	+0.4
	100.0	99.4 ± 0.4	0.4	−0.9

Both the accuracy and the coefficient of variation were generally below 10%, with only few exceptions. The results are summarized in Tables 2 and 3.

The day-to-day accuracy and precision were determined by extracting and analyzing plasma and urine samples spiked with I and metabolites at five different concentrations on five consecutive days. All samples were evaluated using the calibration curves obtained on day 1. The results are shown in Tables 4 and 5 and show the good accuracy and precision of the method in the concentration range tested.

4. Conclusions

The HPLC method described allows the determination of I and of its N- and O-demethylated metabolites in a variety of biological matrixes such as plasma, urine and tissue. It offers about the same sensitivity as a previously HRGC assay developed for I but with a simpler sample preparation procedure, a more rugged chromatographic system and the ability of analyzing also the demethylated metabolites with no need of derivatization. This method has been extensively used to study the urine recovery after single-dose administration and the tissue distribution after long-term administration in laboratory animals, to support toxicity study in rats and dogs, as well as for in vitro studies on the metabolism of I with cultured cells and hepatic microsomes and to determine the uptake of I in perfused isolated organ during in vitro pharmacological experiments.

Table 4
Day-to-day precision and accuracy of the determination of I and demethylated metabolites in spiked plasma by HPLC–UV analysis

	Concentration added (ng/ml)	Concentration found (ng/ml)					Mean ± S.D.	C.V. (%)	Accuracy (%)
		Day 1	Day 2	Day 3	Day 4	Day 5			
I	25	23.8	24.3	22.5	24.8	23.3	23.8 ± 0.9	3.9	−5.0
	50	46.7	61.7	52.1	52.3	48.8	52.3 ± 5.7	11.0	+4.6
	75	69.7	75.5	77.8	83.3	73.9	76.1 ± 5.2	6.8	+1.5
	100	96.3	100.1	105.5	108.7	102.1	102.5 ± 4.8	4.7	+2.5
	200	195.1	202.3	201.5	206.2	198.3	200.7 ± 4.2	2.1	+0.3
M-I	12.5	14.7	12.2	11.9	11.3	13.9	12.8 ± 1.4	11.2	+2.4
	25.0	28.1	25.5	26.3	26.1	26.3	26.5 ± 1.0	3.7	+5.8
	37.5	35.1	37.3	36.1	34.8	35.9	35.8 ± 1.0	2.7	−4.4
	50.0	49.5	50.9	50.6	51.4	48.7	50.2 ± 1.1	2.2	+0.4
	100.0	98.6	99.6	100.0	100.5	100.7	99.9 ± 0.8	0.8	−0.1
M-II	12.5	12.1	12.9	14.9	13.8	14.5	13.6 ± 1.1	8.4	+9.1
	25.0	24.7	26.6	24.8	24.1	25.3	25.1 ± 0.7	3.7	+0.4
	37.5	35.1	34.8	34.5	37.2	38.1	35.9 ± 1.6	4.5	−4.2
	50.0	49.8	49.0	49.1	48.9	53.1	50.0 ± 1.8	3.6	+0.0
	100.0	100.9	98.3	98.9	99.9	99.1	99.4 ± 1.0	1.0	+0.6
M-III	12.5	11.0	12.6	11.1	11.1	11.9	11.5 ± 0.7	6.0	−7.7
	25.0	22.4	24.5	22.3	22.9	24.1	23.2 ± 1.0	4.3	−7.0
	37.5	35.1	42.2	35.9	34.5	36.9	36.9 ± 3.1	8.4	−1.5
	50.0	49.8	47.5	47.9	47.1	48.2	48.1 ± 1.0	2.2	−3.8
	100.0	98.5	98.7	96.9	98.9	101.4	98.9 ± 1.6	1.6	−1.1
M-IV	12.5	12.2	12.5	11.8	13.1	11.2	12.2 ± 0.7	5.9	−2.7
	25.0	24.0	26.3	25.1	25.9	25.1	25.3 ± 0.9	3.5	+1.1
	37.5	41.0	37.1	37.5	38.1	36.9	38.1 ± 1.7	4.4	+1.7
	50.0	48.9	51.4	51.1	51.1	50.9	50.7 ± 1.0	2.0	+1.4
	100.0	100.4	100.4	101.2	101.9	100.1	100.8 ± 0.7	0.7	+0.8

Table 5
Day-to-day precision and accuracy of the determination of I and demethylated metabolites in spiked urine by HPLC–UV analysis

	Concentration added (ng/ml)	Concentration found (ng/ml)					Mean ± S.D.	C.V. (%)	Accuracy (%)
		Day 1	Day 2	Day 3	Day 4	Day 5			
I	25	25.4	22.2	23.1	23.9	24.1	23.7 ± 1.2	5.0	−5.0
	50	48.2	45.3	48.2	49.5	48.8	48.0 ± 1.6	3.3	−4.0
	75	77.8	71.3	78.3	80.3	75.9	76.7 ± 3.4	4.4	+2.3
	100	102.3	102.4	98.7	102.4	100.9	101.3 ± 1.6	1.6	+1.3
	200	198.7	202.4	195.1	195.6	198.4	198.0 ± 2.9	1.5	−1.0
M-I	12.5	12.9	11.8	15.3	12.3	13.0	13.1 ± 1.3	10.3	+4.5
	25.0	23.1	23.9	27.3	25.0	23.1	24.5 ± 1.8	7.2	−2.1
	37.5	37.3	38.1	40.2	38.6	35.0	37.5 ± 1.5	4.0	+0.1
	50.0	52.3	50.9	48.3	51.7	52.3	51.6 ± 0.8	1.5	+3.1
	100.0	99.7	100.9	103.7	100.5	100.9	100.5 ± 0.5	0.5	+0.5
M-II	12.5	12.0	13.8	14.1	12.1	14.9	13.4 ± 1.3	9.6	7.0
	25.0	28.3	25.1	27.3	27.9	26.9	27.1 ± 1.2	4.6	8.4
	37.5	40.2	37.5	40.2	40.9	38.8	39.5 ± 1.4	3.4	5.4
	50.0	48.1	47.7	48.3	50.7	48.7	48.7 ± 1.2	2.4	−2.6
	100.0	101.7	100.3	103.7	103.3	100.0	101.8 ± 1.7	1.7	+1.8
M-III	12.5	10.4	11.7	10.9	10.3	11.1	10.9 ± 0.6	5.2	−13.0
	25.0	24.5	22.3	22.1	20.9	23.0	22.6 ± 1.3	5.9	−9.8
	37.5	35.5	31.2	33.3	35.0	35.1	34.0 ± 1.8	5.3	−9.3
	50.0	50.3	57.1	50.3	51.1	50.9	48.1 ± 1.7	5.6	+3.9
	100.0	98.7	99.9	99.7	97.6	97.1	98.6 ± 1.2	1.3	−1.4
M-IV	12.5	12.0	11.5	11.8	14.1	12.0	12.3 ± 1.0	8.5	−1.6
	25.0	24.5	27.3	25.1	24.9	25.3	25.4 ± 1.1	4.3	+1.6
	37.5	40.0	38.3	39.5	37.4	37.9	38.6 ± 1.7	2.8	+2.9
	50.0	49.9	54.7	51.1	56.8	49.9	52.4 ± 2.9	5.6	+4.8
	100.0	101.4	100.2	100.2	99.0	99.5	100.4 ± 0.9	0.9	+0.4

References

[1] C. Casagrande, F. Santangelo, M.L. Calabi, C. Saini, R. Ferrini, G. Miragoli, L. Merlo, N. Giglioli and C. Semeraro, Actual. Chim. Ther., 15 (1988) 119.
[2] R. Ferrini, N. Giglioli, C. Semeraro and C. Casagrande, Pharmacol. Res. Commun., 20 (Suppl. II) (1988) 151.
[3] F. Bernini, M. Fantoni, A. Corsini and R. Fumagalli, Pharmacol. Res., 22 (1990) 35.
[4] P. Castelnovo, J. Chromatogr. B, 652 (1994) 240.
[5] M. Ervik, K. Hoffmann and K. Kylberg-Hansenn, Biomed. Mass Spectrom., 8 (1981) 322.
[6] J.W. Dolan, LC·GC, 7 (1989) 476.
[7] R.J.M. Vervoort, F.A. Maris and H. Hidrinks, J. Chromatogr., 623 (1992) 207.