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Determination of the aza alkyl lysophospholipid 3-methoxy-2-N,N-methyloctadecylaminopropyloxyphosphorylcholine in rat plasma by liquid chromatography–particle beam–mass spectrometry

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ABSTRACT

A sensitive and specific method for the determination of the aza alkyl lysophospholipid (AALP) 3-methoxy-2-N,N-methyloctadecylaminopropyloxyphosphorylcholine (I) in rat plasma is described. The target molecule was analyzed by high-performance liquid chromatography (HPLC)–mass spectrometry (MS) after one single liquid–liquid extraction with chloroform–methanol (2:1, v/v). 1,2-Didecanoyl-*sn*-glycero-3-phosphocholine was used as internal standard. HPLC was carried out using a polymeric reversed-phase column; the coupling to the mass spectrometer was a particle beam (PB) interface, and the ionization method was electron impact (EI). This simple and rugged method permits the measurement of I in rat plasma in the range of 25 ng/ml–5 μg/ml with good accuracy and precision and is used in pharmacokinetic studies.

INTRODUCTION

Platelet activating factor (PAF), a family of ether-linked phospholipids [1], has been reported to have a broad spectrum of biological activities [2–4]. Among the numerous structural analogs of PAF that have been described [5–7], the group of alkyl lysophospholipids (ALP) shows an interesting cytostatic activity, ET-18-OCH_3 being one of the most active compounds of this series [8,9]. Recently, a new series of ALP analogs has been reported, the most significant characteristic of which is the presence of an amino group. These compounds have been named aza alkyl lysophospholipids (AALP) and they show immunomodulatory [10] and cytotoxic activities [11,12]. A representative molecule of this series is the 3-methoxy-2-N,N-methyloctadecylaminopropyloxyphosphorylcholine (I) the structure of which is shown in Fig. 1.

In order to gain more insight into the mechanism of action of AALP, precise analytical methods are necessary. High-performance liquid chromatography (HPLC) is useful in the analysis of endogenous phospholipids but the absence of a good chromophoric group in these molecules forces one to work in the low region of the UV spectrum, resulting in low sensitivity [13–15]. Attempts to increase the sensitivity have been made by derivatization and using UV [16] and fluorescence detection [17] or phosphorous analysis [18]. MS coupled to HPLC with a thermospray (TSP) interface has been used in the qualitative and quantitative analysis of phospholipids with good sensitivity and selectivity [19–21].

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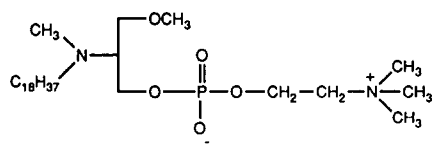
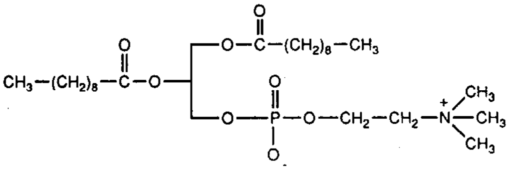


3-methoxy-2-N,N-methyloctadecylaminopropyloxyphosphorylcholine (I)

$C_{28}H_{61}N_2O_5P$ MW= 536



1,2-didecanoyl-sn-glycero-3-phosphocholine (Internal Standard)

$C_{28}H_{56}NO_8P$ MW= 565

Fig. 1. Structures of the compounds used in this study.

The analysis of exogenous alkyl lysophospholipids in biological fluids is not a straightforward process. To the previously mentioned problem of lack of sensitivity must be added the occurrence of interfering peaks coming from endogenous phospholipids of the matrix that usually show similar chemical and chromatographic properties as the target compound.

Alkyl lysophospholipids have been determined in biological fluids by HPLC using phosphorous analysis [22] or light scattering detection [23], by high-performance thin-layer chromatography (HPTLC) with densitometric quantitation [24] and by gas chromatography (GC) of their trimethylsilyl derivatives [25].

In this paper we describe a method to measure the level of I in rat plasma by HPLC–MS after a one single liquid–liquid extraction. The coupling between the chromatograph and the mass spectrometer has been achieved by a particle beam (PB) interface that allows one to use the most common volatile HPLC solvents. This simple and rugged method permits the measurement of concentrations in the range 25 ng/ml–5 μg/ml of rat plasma with good accuracy and precision.

EXPERIMENTAL

Reagents

HPLC-grade methanol was from SDS (Peypin, France). HPLC-grade 2-propanol was from Aldrich (Steinheim, Germany). Water was purified by a Milli Q system from Millipore (Bedford, MA, USA) until a resistivity of 18 MΩ cm was achieved. Ammonium acetate was from Aldrich, A.C.S. reagent grade. Ammonia solution p.a. 25% was from Merck (Darmstadt, Germany). Compound I was from Institute Henri Beaufour (Paris, France), corporative name BN52205. 1,2-Didecanoyl-*sn*-glycero-3-phosphocholine (synthetic: approximately 99%) was from Sigma (St. Louis, MO, USA).

Preparation of calibration curves

A standard stock solution (500 μg/ml) was prepared by dissolving 5.0 mg of I in 10 ml of methanol–water (1:1, v/v). Serial dilutions were carried out to achieve the suitable range of calibration standard solutions of 100, 50, 20, 10, 5, 2, 1 and 0.5 μg/ml. Internal standard stock solution (100 μg/ml) of 1,2-didecanoyl-*sn*-glycero-3-phosphocholine was prepared by dissolving 1.0 mg of this compound in 10 ml of methanol–water (1:1, v/v) and the working internal standard solution (50 μg/ml) was prepared by a 1:2 (v/v) dilution with methanol–water (1:1, v/v).

Two series of four calibration curves were prepared as follows. Eight aliquots of 1.0 ml of untreated rat plasma were transferred into 10-ml glass centrifuge tubes fitted with PTFE lined screw caps and spiked with 50 μl of internal standard solution (effective concentration 2.5 μg/ml) and 50 μl of each standard solution. Effective concentrations of I in plasma samples were 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05 and 0.025 μg/ml. One plasma blank with only 100 μl of methanol–water (1:1, v/v) and another with 50 μl of internal standard and 50 μl methanol–water (1:1, v/v) were also prepared for each calibration curve.

Sample preparation

The plasma samples to which the suitable standard solutions were added were extracted with 5 ml of chloroform–methanol (2:1, v/v) during 30 min using a tube shaker. The tubes were then centrifuged at 2000 *g* for 10 min and the lower organic layer was removed and transferred into 6-ml conical glass tubes. After evaporation of the solvent under a stream of nitrogen, the samples were resuspended in 200 μl of the mobile phase [methanol–2-propanol–0.1 *M* ammonium acetate pH 9.0 buffer (7:1:2, v/v/v)], centrifuged at 2000 *g* for 10 min and placed in low-volume vials for automatic injection.

Preparation of recovery test samples

To reproduce exactly the same LC–MS conditions for all plasma samples, reference samples were analyzed in the presence of plasma extracts. Nine blank plasma samples were extracted following the general procedure without addition of the standard solutions. Aliquots of 50 μl of working internal standard solution (effective concentration 2.5 μg/ml) and 50 μl of standard solutions of effective concentrations 5, 0.5 and 0.025 μg/ml were added in triplicate before the evaporation step. Triplicate calibration samples with the spiked concentrations of 5, 0.5 and 0.025 μg/ml were prepared following the general procedure; however, the internal standard solution was added before drying the samples under nitrogen.

HPLC-particle beam–MS

The LC–MS system used for this work was a Hewlett-Packard (Palo Alto, CA, USA) HP-59980B PB interface coupled to a HP-5989A quadrupole MS working in the electron impact ionization (EI) mode that was also linked to a HP-5890 series II GC. Data were processed by HP-59944C MS ChemSystem software running on an Apollo Series 400 networked data processing workstation. The PB nebulizer was operated at 370 kPa helium inlet pressure as was determined by a standard optimization procedure. The temperature of the PB desolvation chamber was set at 55°C and the momentum separator second stage pressure was 16–27 Pa. The ion source temperature was 300°C, the MS analyzer was held at 100°C and the manifold pressure was around $7 \cdot 10^{-3}$ Pa. The MS electron multiplier was run 200 V above the tuning values. All data were obtained in EI mode at 70 eV. Selected-ion monitoring (SIM) of the ions of *m/z* 283 and 296 at the time ranges of 10–17 min and 17–24 min respectively were carried out with a dwell time of 2 s. When necessary a complete mass spectrum was obtained in the scan mode, the range being 50–600 a.m.u. Tuning was carried out manually over *m/z* 314 and *m/z* 326 of the PFTA (perfluorotributylamine).

HPLC was carried out on a Hewlett-Packard HP-1090 liquid chromatograph fitted with an automatic injector. A Spark Holland (AJ, Emmen, Netherlands) Must HP 6 multiport stream switch allowed the flow automatically to be sent from the column to the MS or to waste. All LC parts were controlled by HP-79988A operating software running on a HP-9000/300 computer. The column was an Asahipak ODP 50 from Asahi Chemical Industry (Yakoh, Japan), 5 μm packing, 15 cm × 4.6 mm I.D. A 2-μm pore screen filter from Supelco (Bellefonte, CA, USA) was placed ahead of the column. Elution was carried out by a mixture of methanol–2-propanol–0.1 *M* ammonium acetate pH 9.0 buffer following the program shown in Table I. This program also controlled the valve switching the flow between the waste (W) and the MS positions. The flow-rate was 0.5 ml/min and the injection volume was 75 μl. The total run time was 45 min.

RESULTS

Mass spectra

Fig. 2 shows the electron impact mass spectra of the internal standard and I obtained from a spiked plasma sample. A comparison with the mass spectra obtained from methanol is also shown to demonstrate the integrity of these products in rat plasma.

The PB–EI mass spectrum of 1,2-didecanoyl-*sn*-glycero-3-phosphocholine did not show the molecular-ion peak. Some of the most abundant peaks of the spectrum were assigned as follows:

m/z 155	$[CO-C_9H_{19}]^+$
m/z 171	$[O-CO-C_9H_{19}]^+$
m/z 382	$[M-((HO)_2PO-O-CH_2-CH_2-N(CH_3)_3)]^+$
m/z 283	Ion II, that could be obtained by cyclization of ion I with a loss of a neutral fragment (Fig. 3).

In the PB–EI mass spectrum of I, the molecular-ion peak was also absent. This molecule showed few intense peaks in the high mass region, the most intense being *m/z* 296 whose structure could be interpreted as ion III (Fig. 3).

TABLE I

GRADIENT PROGRAM FOR THE LC–MS ANALYSIS OF COMPOUND I

Solvent A: methanol. Solvent B: 2-propanol. Solvent C: 0.1 *M* ammonium acetate pH 9.0.

Time (min)	Valve position	Solvent A (%)	Solvent B (%)	Solvent C (%)
0.01	Waste	70	10	20
10.00	Waste	70	10	20
10.01	Waste	85	10	5
11.50	Mass spectrometer	85	10	5
20.00	Mass spectrometer	85	10	5
21.00	Mass spectrometer	90	10	0
24.00	Waste	90	10	0
30.00	Waste	90	10	0
31.00	Waste	70	10	20
40.00	Mass spectrometer	70	10	20

Positive and negative chemical ionization using both methane and ammonia were also investigated and did not show any additional advantage since extensive fragmentation occurred.

Specificity

The mass chromatograms shown in Fig. 4 indicate that no interfering peaks appear at the retention times of the compounds studied. The mean retention time for sixteen runs was 12.9 min (C.V. 0.8%) for the internal standard and 20.2 min (C.V. 1.4%) for I.

Linearity

The measured response ratio of I was calculated as the quotient between its peak area and the peak area of the internal standard. An upper range between 0.5 μg/ml and 5 μg/ml and a lower range between 0.025 μg/ml and 0.5 μg/ml were defined. Weighted least squares (weighting factor = concentration^{-2}) fitted the effective concentration *vs.* response ratio to equation: C(concentration) = Slope × response ratio ± intercept. Quantitation was found to be linear in the two concentration ranges studied. In all cases the correlation coefficient was between 0.986 and 0.999 for the lower range and between 0.993 and 1.000 for the upper range.

Sensitivity

The limit of quantitation was established as 25 ng I/ml of plasma with an accuracy of 6% and with a precision of 3% for eight spiked samples.

Accuracy and precision

Accuracy of the analysis was estimated as the percent differences (error) between the mean values and the true or known concentrations. Precision was estimated as the relative standard deviation (coefficient of variation) of the measured concentrations of replicate samples.

Intra-day values were calculated for two series of four replicates and inter-day data were obtained when the calculation was carried out over these two series considered as one series of eight replicates. Table II shows the accuracy and precision data.

Extraction recovery

The extraction recovery at 0.025, 0.5 and 5 μg/ml were established from the response ratio of triplicate spiked plasma samples relative to reference samples of identical effective concentrations. Mean extraction recoveries at concentrations of 0.025, 0.5 and 5.0 μg/ml were 103%, 85% and 98% respectively. The mean recovery for these three concentrations was 96% (C.V. 10%).

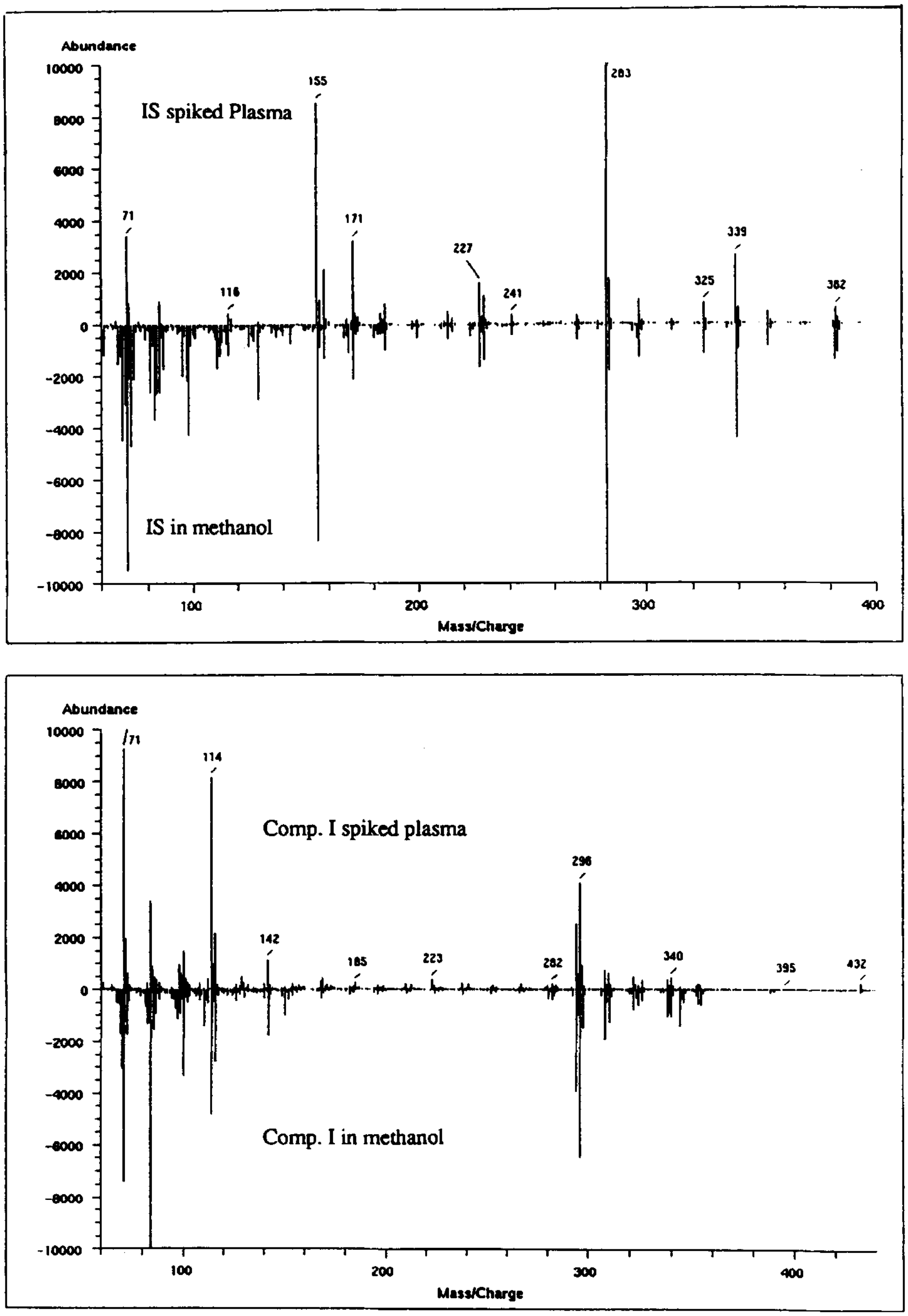


Fig. 2. Comparison between the electron impact mass spectra of the internal standard and I from a spiked plasma sample and a methanol solution.

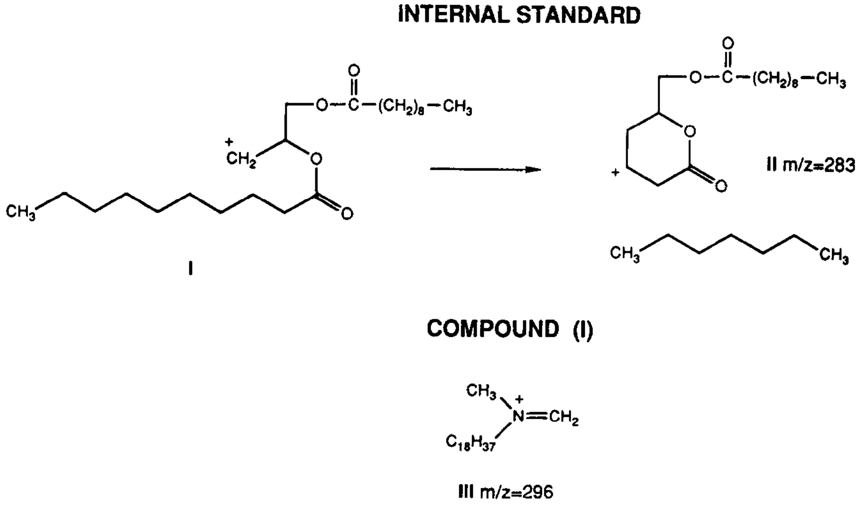


Fig. 3. Proposed structures for the ions of the internal standard and I used in the selection-ion monitoring detection method.

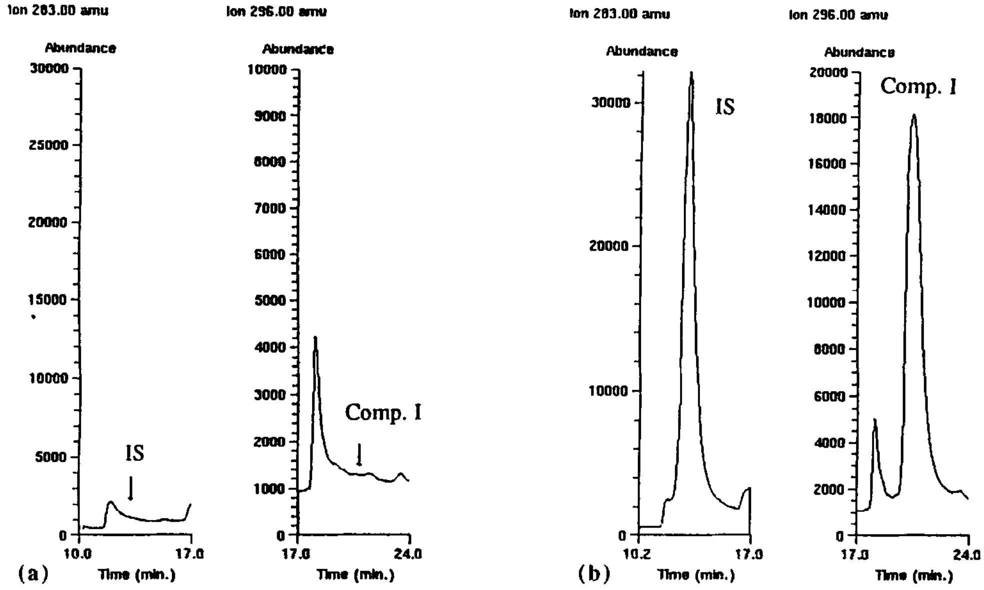


Fig. 4. (a) Representative mass chromatogram of a blank rat plasma sample extract before intravenous administration. (b) Representative mass chromatogram of a rat plasma sample extract after intravenous administration of I at a dose of 10 mg/kg.

DISCUSSION

One of the most serious problems encountered in the measuring of I in rat plasma arose from the difficulty in achieving good HPLC conditions. Although phospholipids can be chromatographed using octadecyl silica (ODS C_{18}) columns [14,15,19,21], in our case this kind of stationary phase did not allow the elution of the target compound whatever combination of PB-compatible eluents were tested (methanol, 2-propanol, acetonitrile, ammonium acetate buffers and 0.05% trifluoroacetic acid solutions). The presence of the octadecyl chain in both the stationary phase and I probably produced a strong interaction between the molecule and the bonded phase that the eluent could not overcome. The exhaustive endcapping of the free

TABLE II

ACCURACY AND PRECISION DATA FOR THE LC–MS ANALYSIS OF COMPOUND I

Day	Parameter	Low concentration curve					High concentration curve			
		0.025 μg/ml	0.050 μg/ml	0.10 μg/ml	0.25 μg/ml	0.50 μg/ml	0.50 μg/ml	1.00 μg/ml	2.50 μg/ml	5.00 μg/ml
1	Mean ($n = 4$) (μg/ml)	0.027	0.045	0.089	0.26	0.53	0.52	0.92	2.44	5.25
	Error (%)	7.0	−10.0	−11.5	2.4	6.7	3.8	−7.6	−2.4	5.1
	C.V. (%)	1.9	4.1	5.7	8.5	9.8	0.7	2.1	3.1	1.9
2	Mean ($n = 4$) (μg/ml)	0.026	0.047	0.097	0.25	0.52	0.51	0.97	2.49	5.06
	Error (%)	5.0	−7.0	−3.3	−0.9	4.6	1.3	−2.5	−0.5	1.2
	C.V. (%)	3.7	6.7	6.1	4.4	5.7	2.9	7.2	3.2	1.0
Overall	Mean ($n = 8$) (μg/ml)	0.027	0.046	0.093	0.25	0.53	0.51	0.95	2.46	5.16
	Error (%)	6.0	−8.5	−7.4	0.8	5.7	2.5	−5.0	−1.5	3.1
	C.V. (%)	2.9	5.5	7.3	6.6	7.5	2.3	5.8	3.1	2.5

C.V. = Coefficient of variation.

silanol groups of this type of columns render them even more apolar, thereby enhancing the interaction. The use of octyl silica columns (a less hydrophobic phase) and elution with mixtures of methanol, 2-propanol and pH 7.5 ammonium acetate buffers allowed us to obtain peaks, but they were so broad that they were not useful for quantitation purposes, due to the low sensitivity obtained under these conditions. Asahipak ODP C_{18} is a phase with spherical porous particles that have octadecyl groups bonded to a polar polymeric bead [26]. The higher overall polarity of this stationary phase as compared with the endcapped silica ODS columns decreased the high affinity for the target molecule, giving narrower peaks and better sensitivity.

As mentioned before, isolation of I and the internal standard from plasma was performed only with liquid–liquid extraction with chloroform–methanol (2:1, v/v). This is a simple and quick method but it also removes all the endogenous lipids from the plasma. A cleaning step with a high concentration of methanol in the presence of 2-propanol in the HPLC program preserved column performance during many injections, but due to the high concentration of lipid material eluting from the column, the skimmers of the PB interface become clogged after only a single injection. One attempt to solve this problem was to introduce a purification step for the total lipid extract using prepacked silica columns bonded with different phases. This method did not give good extraction recoveries and furthermore would add an additional step to the sample preparation. The use of an automatic switching valve to send the HPLC flow to the MS only when the desired compounds are eluting from the column permits one to run series of 60–80 plasma samples extracts while keeping the interface working properly. To prevent the skimmers from clogging during a new series of samples they were cleaned after each batch.

During the validation procedure it was found that the responses of both I and the internal standard were significantly higher when they were injected from a plasma extract than from a methanol solution. This effect has been reported as "carrier effect" and is defined as the appearance of increased ion abundances for coeluting compounds [27]. The described mechanism involves coeluting compounds "carrying" analyte particles through the PB momentum separator resulting in an increase of transfer efficiency. For this reason, reference samples were prepared with plasma extracts to include this effect in the recovery calculations. This phenomenon may produce a deviation from linearity if the composition of the endogenous lipids that coelute with the analytes is not constant; in our case, however, good linearity was observed. Some factors that are beneficial for the preservation of linearity may be the use of an internal standard, the use of two ranges of concentrations in the calibration curves and the use of a mobile phase additive such as ammonium acetate [27,28].

CONCLUSIONS

The coupling of high-performance liquid chromatography with mass spectrometry in the electron impact ionization mode using a particle beam interface permits measurement of I with good accuracy and precision in the range of concentrations between 25 ng/ml and 5 μg/ml from a rat plasma sample volume of 1 ml. The limit of quantitation of this method was established as 25 ng/ml with an accuracy of 6% and with a precision of 3% for eight spiked samples. The high selectivity of mass spectrometric detection allowed us to reduce the purification step to a single liquid–liquid extraction of the total lipids. This method has been used with good reproducibility in the analysis of more than 500 samples over a period of four months during pharmacokinetics studies in the rat.

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