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Assay method for the perfluorooctyl bromide (perflubron) in rat blood by gas chromatography–mass spectrometry

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Abstract

This paper describes a GC–MS method (SIM mode) for the analysis of perfluorooctyl bromide (perflubron, **I**) in rat blood. The chromatographic separation was performed by injection in the split mode using a CP-select 624 CB capillary column. Following destruction of the emulsion by addition of ethanol, the analytical procedure involves a liquid–liquid extraction with 1,1,2-trichlorotrifluoroethane. The bis(*F*-butyl)ethene **(II)** was used as internal standard. Observed retention times were 3.22 min for **I** and 2.32 min for **II**. Two calibration curves were used; linear detection responses were obtained for concentrations ranging from 0.009 to 0.9 mg/ml and from 0.9 to 13.5 mg/ml. The extraction efficiency averaged 50% for **I** and 93% for **II**. Precision ranged from 0.7 to 14%, and accuracy was between 91 and 109%. The limit of quantification was 9 μg/ml. The method validation results indicate that the performance characteristics of the method fulfilled the requirements for assay method for use in pharmacokinetic studies.

Keywords: Perfluorooctyl bromide; Perflubron

1. Introduction

Since several years, our group of research works on methods allowing the detection of misuse by recombinant human erythropoietin (rHuEpo) [1–5]. Indeed, this drug is widely used by athletes to increase oxygen transport and aerobic power in the hope to improve their endurance capacity and recovery during training and competition [6–8]. The main action of rHuEpo is to stimulate the proliferation and the differentiation of erythroid progenitor cells in bone marrow and their evolution into mature erythrocytes [9,10]. Beside rHuEpo, perfluorocarbon (PFC) emulsions have been recently used to increase oxygen transport [11]. Indeed, perfluorocarbons are passive gas transporters. This is in contrast to hemoglobin, which actively binds oxygen to the iron in the heme moiety [12]. Gas transport by PFCs is based on enhanced solubility. This enhanced solubility is caused by the physics of the molecules involved making them less polar. The uptake and release of oxygen (nitrogen or carbon dioxide) from

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a PFC essentially depend on the partial pressure of the gas [11]. Because there is no specific interaction between oxygen molecules and PFCs, by contrast to the strong chemical bonding that prevail between oxygen and an iron atom in hemoglobin, most of the PFCs can dissolve and completely release 50 to 60 vol% of oxygen [13], as compared to only 21% for the whole blood with normal haemoglobin and haematocrit. PFCs exchange gases more rapidly and more completely than do red blood cells because they load and unload gases by simple diffusion. Perfluorooctyl bromide (perflubron) based emulsions are presently investigated under clinical trials as oxygen carriers (temporary blood substitutes) during surgery [14]. Neat perfluorooctyl bromide is also tested in clinics to treat the respiratory distress syndrome during liquid ventilation [12,15]. Due to their exceptional O_2-carrying capacity, there is a risk for PFCs to be diverted from their initial therapeutic applications and employed for prohibited uses. These compounds are indeed in instance to be officially included on the International Olympic Committee (IOC) list of products considered to be illegal drugs.

Today, few methods are available to quantify PFCs in biological samples [16–19]. A gas chromatography method with a flame ionization detector has been proposed by Yamanouchi et al. to quantify perfluorochemicals in organs and body fluids [16]. In a recent paper, Henry et al. [18] compare fluorocrit measurements to those obtained by gas chromatography analysis using an electron capture detector. A method to quantify perflubron in tissues using a cylindrical CT phantom has been also described [19]. On the other hand, a sensitive gas chromatography-electron-capture negative ion chemical ionisation mass spectrometry method is proposed for the detection of a range of perfluorocarbon tracers in atmosphere [20]. However, these published methods did not report assay validation.

The objective of this work was to validate a gas chromatography-mass spectrometry method to quantify perflubron (**I**) in rat blood. The bis(*F*-butyl)ethene (F-44E) was used as internal standard. This method was validated according to validation procedures, parameters and acceptance criteria [21–23] based on USP XXIII guidelines [21] and recommendations of Shah et al. [22]. This method has enhanced precision due to the high selectivity of gas chromatography coupled with mass spectrometry. It was used to assay samples from a preclinical study in rats.

2. Experimental

2.1. Materials and reagents

Perfluorooctyl bromide (perflubron, **I**, molecular mass 499) (Fig. 1), a perfluorochemical-based oxygen carrier, was provided by Atochem (Pierre Benite, France). The perflubron emulsion was prepared and characterized at the Charles Sadron Institute (UPR 22, Strasbourg, France) by high-pressure homogenization (5000 p.s.i.) (Microfluidizer® M-110 T, Microfluidics Corp., Newton, MA, USA). It is a 90% weight-by-volume emulsion. The emulsion is prepared under thorough exclusion of oxygen in a clean room. After preparation, the emulsion is sterilized in an autoclave at 121°C for 15 min. The average particle size was 0.18 ± 0.02 μm, as measured by photosedimentation (Horiba Capa 700, Horiba Ltd., Kyoto, Japan). The composition of perflubron emulsion and physical constants are listed in Table 1. The emulsion is emulsified by egg yolk phospholipids

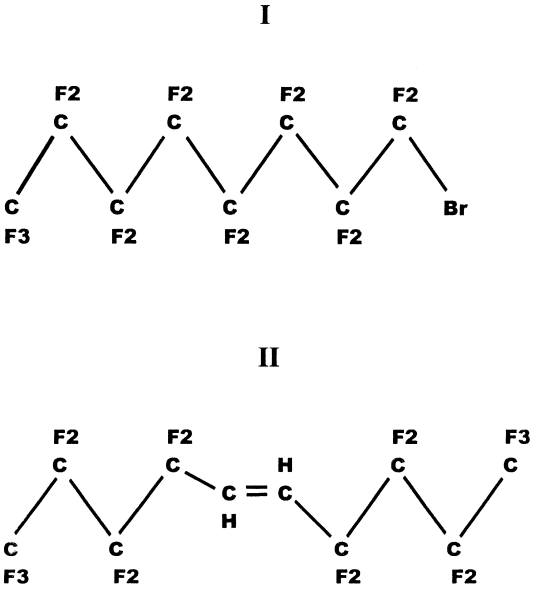

Fig. 1. Molecular formulae of **I** (perfluorooctylbromide) and **II** (bis(*F*-butyl)ethene).

Table 1
Characteristics of perflubron (**I**) and internal standard (**II**)

	Physico-chemical properties	
	Perflubron	Internal standard
Chemical formulae	$C_8F_{17}Br$	$C_{10}F_{18}H_2$
Boiling point (°C)	141	132
Density (g/ml)	1.93	1.667
Vapor pressure (Torr, at 37°C)	14.0	12.8
	Perflubron emulsion	
Composition	Perfluorooctylbromide	90% w/v
	Egg yolk phospholipids	4.5% w/v
	Perfluorohexyldecane	2.8% w/v
	Sodium dihydrogen phosphate, H_2O	0.052% w/v
	Disodium hydrogen phosphate, 7 H_2O	0.355% w/v
	D α-tocopherol	0.002% w/v
	Sodium chloride	0.25% w/v
	Water QSP	

and is efficiently stabilized by a semifluorinated alkane $C_6F_{13}C_{10}H_{21}$ (perfluorohexyldecane) [24]. The average particle size was 0.2±0.02 μm, after 1 year, when stored at 40°C. It did not change significantly when the emulsion was stored at room temperature. The bis(*F*-butyl)ethene (**II**, molecular mass 464), internal standard was a gift from Atochem.

The 1,1,2-trichlorotrifluoroethane (FC113) was obtained from Fluka (Buchs, Switzerland). Absolute ethanol of High Purity Solvent (HPLC grade) was purchased from Carlo Erba (Val de Reuil, France). The 9‰ sodium chloride was purchased from Aguettant (Lyon, France).

Fourteen dilutions of the perflubron emulsion ranging from 0.36 to 540 mg/ml were prepared in a room thermostated at 10°C in 9‰ sodium chloride; they were used to prepare calibration curves and quality control (QC) samples.

An internal standard solution containing 0.5 ml/l of FC113 was prepared and kept at 4°C.

2.2. Equipment and chromatographic conditions

An HP 5989A GC–MS system (Hewlett-Packard, Palo Alto, CA, USA) was used. The chromatographic separation was performed by injection in the split mode (split: 40 ml/min) of 1 μl of the extract in a CP-select 624 CB capillary column (length: 30 m; I.D. 0.32 mm; film thickness: 1.8 μm) (Chrompack, Middelburg, The Netherlands). Helium pressure was 40 kPa. The injector temperature was 200°C and the initial oven temperature was 40°C. This temperature was maintained for 1 min, the temperature was then programmed as follows: 11°C/min up to 65°C. The transfer line temperature was set to 200°C.

Analysis was performed by electronic impact ionisation. The ion source temperature was set to 200°C. The electron energy was 70 eV. PFC was analyzed in the SIM mode. The ions *m/z* 131 $(C_3F_5)^+$ and *m/z* 419 $(C_{18}F_{17})^+$ were selected to monitor **I** due to their abundance and to their specificity (Fig. 2). The ion *m/z* 295 $(C_7H_2F_{11})^+$ was selected for the internal standard; another ion (*m/z* 126) was also recorded for the selectivity.

2.3. Analytical procedure

2.3.1. Calibration curves and quality control samples

All preparations were performed at 10°C. Quantitation was based on the internal standard method. Twenty five microliters of each dilution of the perflubron emulsion were used to spike drug free rat blood sample (1 ml) in order to obtain calibration standards at the concentrations of 0.009, 0.045, 0.09, 0.225, 0.45, 0.90, 2.25, 4.5, 9 and 13.5 mg/ml.

QC samples were prepared at the concentrations of

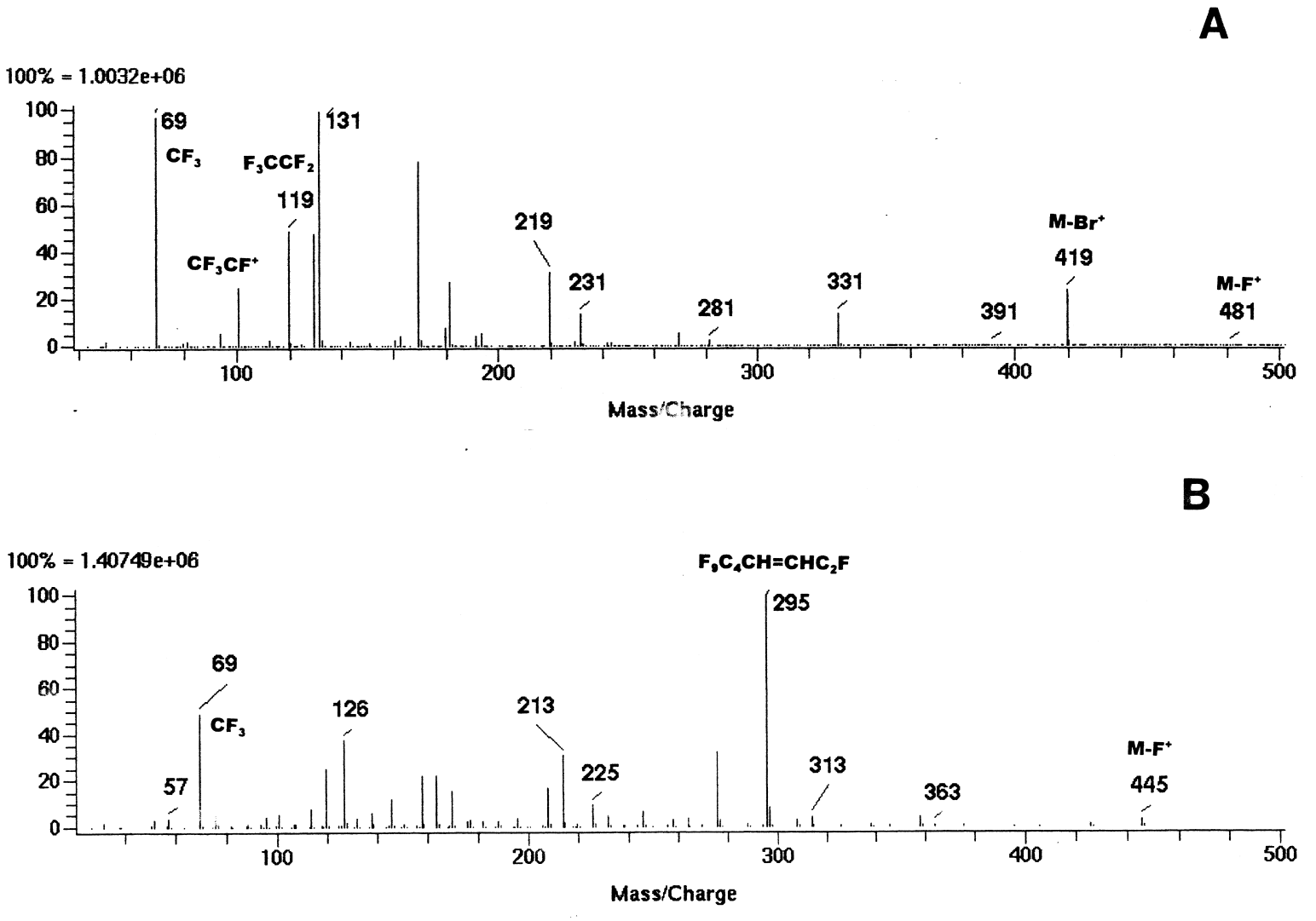

Fig. 2. Mass spectra (SCAN mode) for the perfluorooctylbromide (**I**) (A) and bis (*F*-butyl)ethene used as internal standard (**II**) (B).

0.027, 1.35, 0.405 and 11.25 mg/ml in drug free rat blood.

In a 5 ml screw capped glass centrifuge tube, 100 μl of each blood sample (calibration standards or QC samples) were stored at −20°C for at least 24 h before extraction in order to destroy the emulsion.

The calibration curve and four QC samples were run with every set of ten unknown samples.

2.3.2. Extraction procedure

As rapid losses of perflubron due to evaporation occurred at room temperature, all experiments were performed at 10°C. Prior to their analyses, samples were brought to 10°C and 1 ml of FC113 (4°C) containing the internal standard was added; the tubes were immediately sealed. The mixture was vortex-mixed for 60 s, then 100 μl of ethanol (4°C) was added and the tubes were immediately sealed. The content was mixed during 15 min in an ultrasonic bath maintained at 4°C then vortex-mixed for 120 s. The tubes were centrifuged (4°C) at 1500 *g* for 5 min and thawed at −30°C for at least 30 min. Just before analysis by GC–MS, the ethanolic phase was withdrawn and the organic phase put into another 5-ml glass tube, 1 μl of this solution was injected into the chromatograph.

Each determination was performed in triplicate; in the interval of two injections, extracts were stored at −30°C.

2.4. Data analysis

From recorded peak areas, the ratios of the drug to internal standard were calculated. The peak area ratios were plotted against theoretical concentrations.

Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data (formula: $y = a + bx$; where $x =$ concentration and $y =$ peak area ratio). Two different calibrations curves were constructed, the first one at the low concentrations (0.009 to 0.9 mg/ml) and the second one at high concentrations (0.9 to 13.5 mg/ml).

The quality of fit was evaluated by comparing back-calculated concentrations to the nominal ones. The resulting slopes and intercepts were used to obtain concentration values for that day's quality control samples and unknown samples.

The linearity of the method was confirmed using the classical statistical tests; i.e., comparison of intercepts with zero and correlation coefficients with 1. Moreover, the Kolmogorov-Smirnov test was used to compare the distribution of the residuals (difference between nominal and back-calculated concentrations) to the expected one ($N(0,1)$, i.e., normal distribution and centered around zero).

2.5. Precision and accuracy

Inter-day and intra-day reproducibilities of the assay were assessed by performing replicate analyses of QC samples in blood against a calibration curve. The procedure was repeated on different days on the same spiked standards to determine inter-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate the same day.

The accuracy was evaluated as [mean found concentration/theoretical concentration]×100. Precision was given by the coefficients of variation.

2.6. Extraction efficiency

The extraction efficiency (recovery) was determined three times at four concentration levels for **I** (0.009, 0.045, 0.9, and 13.5 mg/ml) and at the concentration used during the assay for the internal standard. The peak areas obtained after extraction were compared to the ones obtained using standard solutions prepared at the same concentrations and injected in the GC–MS system.

2.7. Determination of the limit of quantitation (LOQ)

The LOQ estimated on spiked samples was defined as the lowest drug concentration which can be determined with a precision ≤20% and an accuracy between 100±20% on a day-to-day basis [21–23].

2.8. Specificity

To evaluate the specificity of the method, three different drug free rat blood pools were tested. The retention times of endogenous compounds in the matrix were compared with that **I** and **II**.

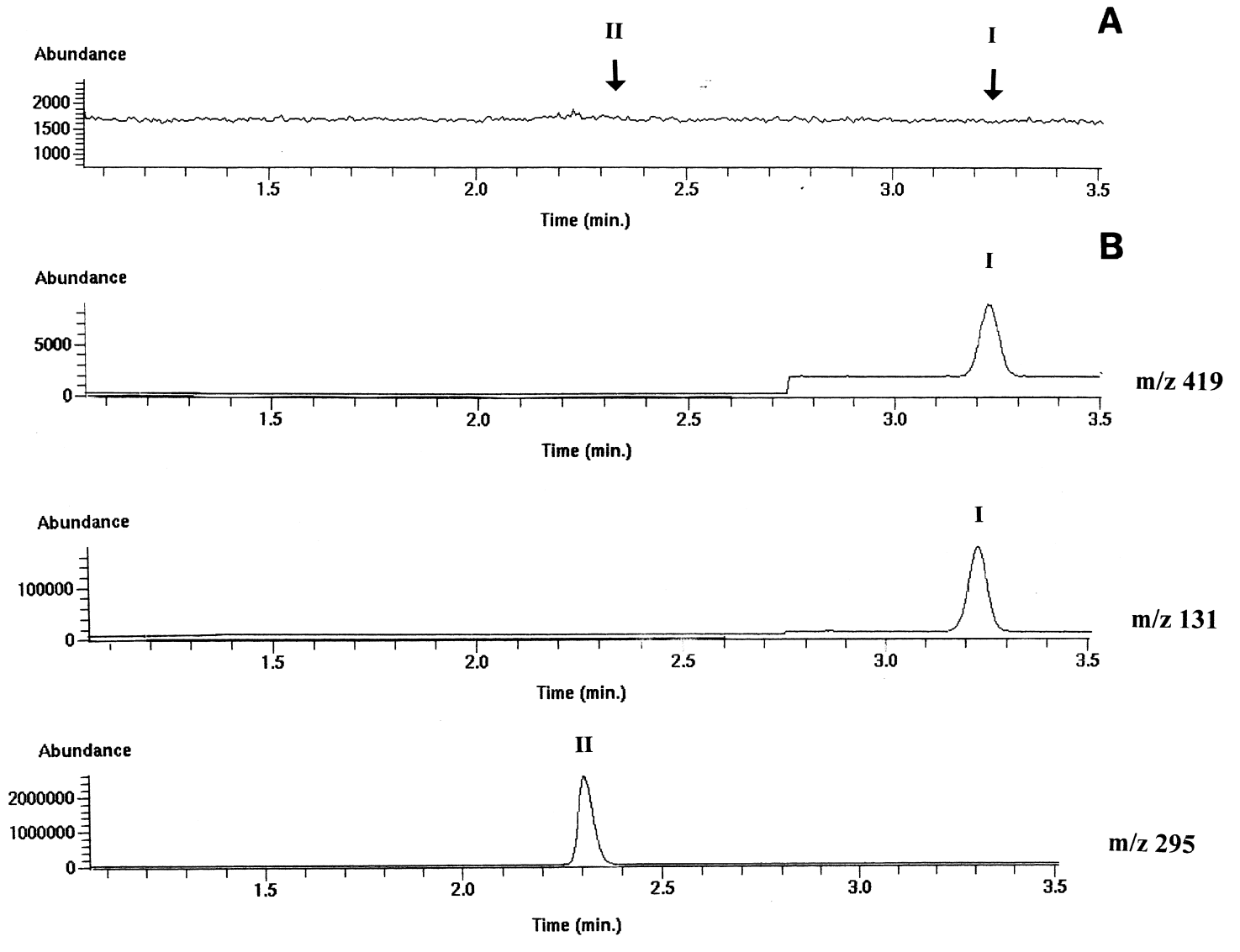

Fig. 3. Typical chromatograms (SIM mode) of drug free rat blood (*m/z* 419, 131, and 295) (A); and of a blood sample collected one day after administration of perflubron in rat (concentration: 0.056 mg/ml) (B). Peak **II** is the internal standard, peak **I** is perflubron. For chromatographic conditions see Sections 2.2 and 2.3.

2.9. Pharmacokinetic study in rat

The animals used were Wistar strain male rats weighing 250–280 g. Animals, previously anesthetized with diethylether, received 1.8 g of perflubron per body weight in the emulsion form from the penis vein. Blood samples (100 μl) were collected from the retro-orbitary sinus in glass tubes coated with sodium heparinate, at the following time: 2, 4, 6, 24 and 72 h, and 10 and 25 days after drug administration.

The determination of **I** excreted through expiration was also carried out. Rats having received the perflubron emulsion were placed in a glass hermetic vessel (9 litres capacity) for 30 min at several intervals after drug administration (1.5, 4, 7, 24, 48 and 72 h, and 4, 7, 8, 9, 10, 11, 14, 16, 17, 21, 22, 25, 35, 39 and 50 days). The air (1 ml) was inspired through a septum using a gas syringe; 5 min before air collection, the atmosphere was homogenized using a ventilator. Concentration of **I** was determined using a calibration curve done in the same conditions by evaporating (37°C) varying proportions of **I** in the hermetic vessel; each standard curve contains 5 data points.

A pharmacokinetic analysis of the blood concentration versus time and of the excretion rate of the perflubron through expiration versus time (rate plot) was undertaken for each rat using the pk-fit software [25].

3. Results

3.1. Retention times and specificity

The extraction procedure and the chromatographic conditions allow to separate **I** and **II**. Observed retention times were 3.22 min for **I** and 2.32 min for **II**. Chromatogram derived from a blood sample of a treated animal is shown in Fig. 3.

Chromatographic analysis of drug free blood samples confirmed that there were no endogenous peaks that coeluted with the respective analytes (Fig. 3A).

3.2. Calibration curves

For calibration curves prepared on different days ($n=12$), linear relationships were obtained between the ratio of the area of ions m/z 419 and m/z 131 of **I** to ion m/z 295 of **II**, and the concentration of **I** [$y=0.0762$ (±0.0161)$x+0.0001$ (±0.0003), $r=0.999$ (C.V.$=0.17\%$) at low concentrations; and $y=0.115$ (±0.035)$x-0.0489$ (±0.0348), $r=0.997$ (CV$=0.44\%$) at high concentrations for ion m/z 419; and $y=1.10$ (±0.268)$x+0.0041$ (±0.0059), $r=0.998$ (C.V.$=0.22\%$) at low concentrations; and $y=1.57$ (±0.269)$x-0.586$ (±0.852), $r=0.997$ (CV$=0.56\%$) at high concentrations for ion m/z 131].

Table 2 reports back-calculated concentrations of **I**

Table 2
Inter-day precision and accuracy from calibration curves ($n=12$)[a]

Theoretical concentration (mg/ml)	m/z 419			m/z 131		
	Back-calculated concentration (mg/ml)	C.V. (%)	Recovery (%)	Back-calculated concentration (mg/ml)	C.V. (%)	Recovery (%)
0.009	0.008	5.1	91.2	0.009	13.5	104.7
0.045	0.048	8.5	106.4	0.045	6.62	101.1
0.090	0.092	3.5	102.2	0.093	5.64	103.0
0.225	0.224	6.0	99.7	0.221	6.54	98.2
0.450	0.442	4.3	98.3	0.450	3.41	99.9
0.900	0.904	0.82	100.5	0.901	0.71	100.2
2.25	2.22	5.8	98.8	2.25	8.07	99.9
4.50	4.47	3.0	99.3	4.48	4.20	99.6
9.00	9.02	4.5	100.2	9.01	4.35	100.1
13.5	13.5	1.9	100.0	13.5	1.81	100.0

[a] n = Number of replicates.

from the corresponding calibration lines. A linear regression of the back-calculated concentrations vs. the nominal ones provided a unit slope and an intercept equal to 0 (Student *t*-test).

The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The distribution of the residuals (difference between nominal and back-calculated concentrations) shows random variations, the number of positive and negative values being approximately equal. Moreover, they were normally distributed and centered around zero (Kolmogorov-Smirnov test).

3.3. Extraction efficiency, precision and accuracy

Extraction efficiency of **I** was 50±3.2% ($n=18$) over the range studied. For the internal standard it averaged 93±8.0% ($n=16$).

For concentrations of calibration standards the precision around the mean value ranged from 0.7 to 13.5% (Table 2). Inter-day and intra-day precision and accuracy of the method were assessed by analyzing QC samples prepared in rat blood at different concentrations, in replicate, on the same day and on different days. The results are presented in Table 3.

3.4. Limit of quantitation

The limit of quantitation was 9 μg/ml. At this level, the analytical error was less than 14%.

3.5. Pharmacokinetic study

Fig. 4A shows a blood concentration vs. time profile. The elimination half-life was 6.9 days and the total clearance 0.17 ml/min. The major route of elimination of perflubron from the body was via expiration. There was no measurable perflubron eliminated in the urine at any times of the kinetic. Fig. 4B shows the variations with time of the excretion rate by expiration. Data were consistent with a three-compartment model. The apparent elimination half-lives of the three observed phases were 0.41, 1.95 and 15.1 days, respectively. In mean, 93% was recovered in the expiratory air.

4. Discussion and conclusion

In this manuscript we described a GC–MS method to quantify a PFC, perfluorooctyl bromide (perflubron), in blood and through expiration. Compared to electron-capture detection, mass spectrometry allows the identification of the drug and therefore provides a

Table 3
Assessment of the accuracy and precision of the method[a]

Theoretical concentration (mg/ml)	*m/z* 419			*m/z* 131		
	Experimental concentration (mg/ml)	C.V. (%)	Recovery (%)	Experimental concentration (mg/ml)	C.V. (%)	Recovery (%)
Within-day ($n=6$)						
0.027	0.0263	11.1	97.4	0.0293	10.0	108.5
0.405	0.379	9.6	93.5	0.389	7.7	96.0
1.35	1.31	3.0	97.3	1.23	4.2	90.9
11.25	11.3	4.6	100.6	11.9	4.9	105.8
Between-day ($n=14$)						
0.027	0.0273	11.1	101.2	0.027	13.9	100.0
0.405	0.398	10.4	98.3	0.403	8.7	99.4
1.35	1.35	8.2	100.3	1.25	10.9	92.7
11.25	11.4	2.83	101.4	11.0	7.9	97.7

[a] n = Number of replicates.

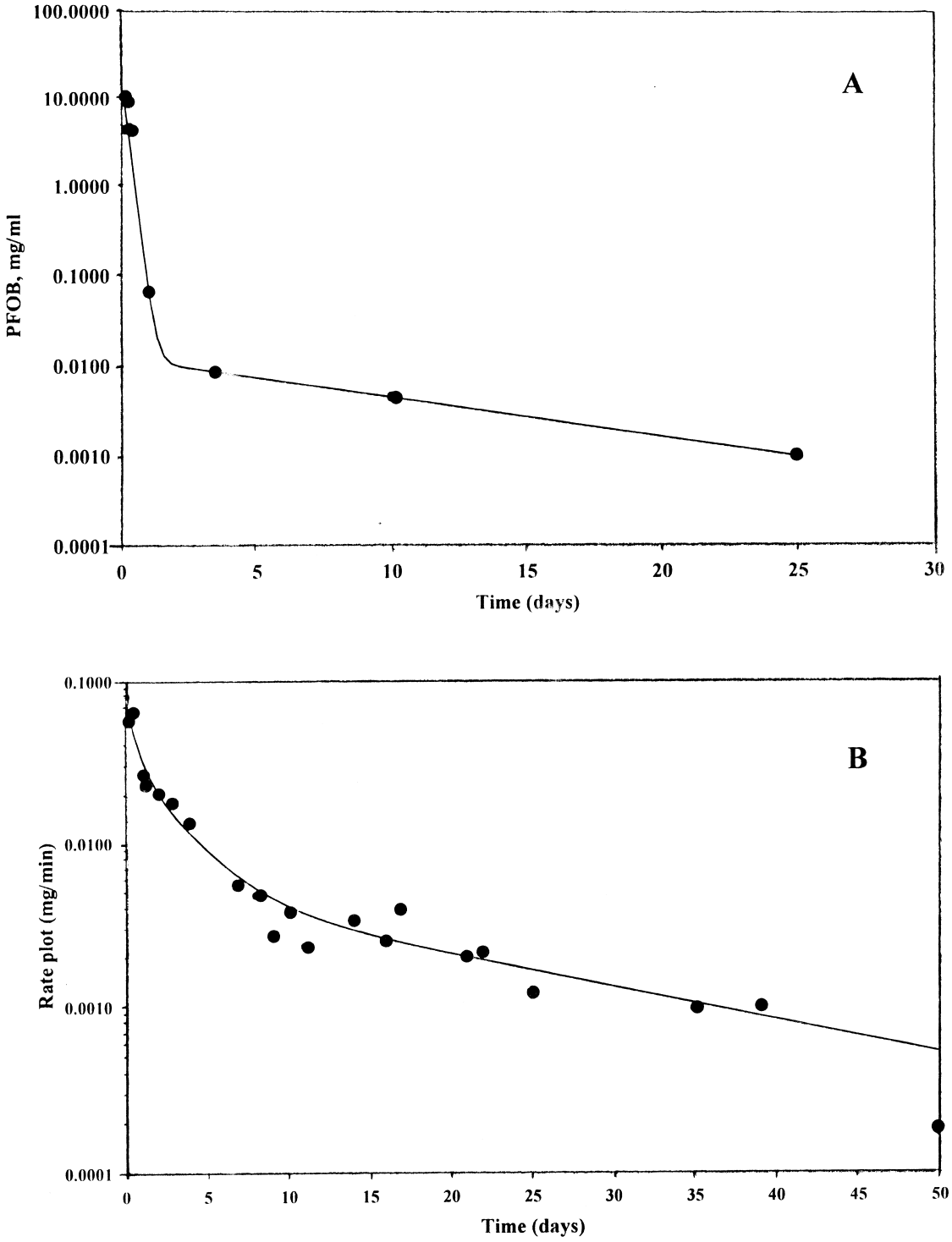

Fig. 4. Kinetics of perflubron in blood (**A**) and through expiration (**B**) after i.v. administration of perflubron emulsion (2 ml/kg) in rat.

very selective method for identification and quantification of PFCs in doping control. As opposed to previously published methods, the present method was validated according to the Washington conference [22]. Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and from the results of quality control samples. The present method validation results indicate that the performance characteristics of the method fulfilled the requirements for a sufficiently accurate and precise assay method to carry out pharmacokinetic studies. This method was used to determine the pharmacokinetic profile of perflubron in rats.

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