ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 487–494

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Determination of perfluorobutane in rat blood by automatic headspace capillary gas chromatography and selected ion monitoring mass spectrometry

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Received 1 August 2000; accepted 20 August 2000

Abstract

A new contrast agent (Sonazoid™; NC100100) for ultrasound imaging has been developed. It is an aqueous suspension of lipid stabilised perfluorobutane (PFB) gas microbubbles. An automatic headspace capillary gas-chromatographic mass spectrometric method using electron impact ionisation was developed for analysis of Sonazoid™ PFB in rat blood. The calibration standards were gaseous PFB dissolved in ethanol in the range of 0.5–5000 ng PFB. Fluorotrichloromethane (CFC 11) was used as an internal standard of the method and the MS detector was set to single ion monitoring of the base fragment ions of PFB (m/z 69 and 119) and CFC 11 (m/z 101). The calibration graph, made by plotting the peak area ratios of PFB (m/z 69) to CFC 11(m/z 101) against the amount of PFB, was fitted to a second-order polynomial equation with weighting $1/y^2$ and found to be reproducible. The limit of quantification of the method was set to 0.4 ng PFB. The between-day variation of the method was below 9.2% relative standard deviation (RSD) and the within-day variation of the method was below 7.6% RSD. The accuracy of the method, as compared to Coulter counter, was estimated by determination of PFB in samples where Sonazoid™ was added to saline and found to range from 91.5% to 105.2%. PFB, added as Sonazoid™, was found to be stable for at least 7 months in rat blood samples when stored at −20°C. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Rat blood; Headspace gas chromatography–mass spectrometry; Perfluorobutane; Validation; Ultrasound imaging; Microbubbles

1. Introduction

Sonazoid™ (NC100100) is a new ultrasound contrast agent under development for indications such as left ventricular border enhancement, myocardial perfusion mapping, detection of focal

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PII: S0731-7085(00)00432-5

lesions in the liver and several vascular applications such as characterisation of the vascularity of tumours [1]. The contrast agent is a lipid-stabilised suspension of perfluorobutane (PFB) gas microbubbles with a median volume diameter of 2.4–3.5 μm.

The perfluorocarbons have structures analogous to the familiar hydrocarbons but possess very different chemical and physical properties. Perfluorocarbons are normally much less reactive than hydrocarbons towards all chemical reagents except alkali metals. Temperatures approaching 1000°C are required to decompose perfluoroethane and perfluoropropane, but even though perfluorocarbons of higher molecular weights are thermally less stable, most perfluorocarbons are stable below 300°C. Completely fluorinated alkanes are essentially non-toxic [2]. Perfluorocarbon liquids have thus been used in vitreoretinal surgery as an instrument for manipulating intraocular tissues [3,4] and selected perfluorocarbons have been used as tracers for monitoring the movement of the atmosphere [5–7] and in reservoir studies [8].

Gas chromatography (GC) has been the preferred analytical technique for the determination of perfluorocarbons. One of the requirements for separating volatile compounds is the use of a stationary phase that provides sufficient retention for the compounds to be measured. With the introduction of chemically bonded phases on capillary columns, selectivity could be combined with a high theoretical plate number. Adsorption materials such as Al_2O_3 and porous polymer types of materials in porous layer open tubular (PLOT) capillary columns have been found to be effective for analyses of several fluorocarbons [9,10]. Perfluorocarbon compounds have been analysed with alumina PLOT [11], fused-silica SE-30 [12], fused-silica SE-54 [13] and fused-silica DB-Petro 100 [14] capillary columns. The perfluorocarbons have been detected with high sensitivity by electron-capture detection [12,14–16] or electron-capture negative ion chemical ionisation mass spectrometry (MS) [13]. GC–MS with electron impact (EI) ionisation has been extensively used for determination of aerosol propellant fluorocarbons in biological tissues [17–21].

To determine the pharmacokinetic properties of the new contrast agent, Sonazoid™, in animals, we developed an automatic headspace sampler capillary GC–EI–MS method for specific determination of PFB in rat blood. The method was validated and the validation results are presented in this paper.

2. Experimental

2.1. Materials

PFB, C_4F_{10}, was purchased from 3M Speciality Chemicals Division, USA, or from F2 Chemicals Ltd., England. Fluorotrichloromethane (CFC 11) $>99\%$ was from Sigma-Aldrich, England. Perfluoropropane, perfluoropentane and perfluorohexane were from Fluorochem, England. Sonazoid™ was from Nycomed Imaging AS, Norway. Ethanol (96%) was spiritus fortis and 2-propanol was analytical grade. Sodium chloride (NaCl), 9 mg/ml, was purchased from Kabi Pharmacia AB, Sweden. Heparinised blood was collected from rats.

2.2. Instrumentation

The automatic headspace sampler was a Dani (Milan, Italy) HSS 3950 equipped with a constant heating time accessory and a 1 ml sample loop. The headspace injector was coupled to a Fisons 8000 gas chromatograph connected with a Fisons MD 800 mass spectrometer. The column used for the chromatographic analysis was a Chrompack CP-PoraPLOT Q, 25 m × 0.25 mm i.d. and 8 μm film thickness (Chrompack, The Netherlands).

The bath temperature, equilibration time, sampling time and injection time of the automatic headspace sampler was optimised for determination of PFB in blood. The optimisation was carried out during the development of the method using a factorial experimental design (2^4), and showed that two of the factors, the bath temperature and the injection time, had significant effects on the PFB response (data not shown). Based on these experiments, the optimal settings for the headspace sampler were found and are shown in

Table 1. For clarity, the conditions of the GC and MS instruments are also shown in Table 1.

2.3. Preparation of samples and standards

The PFB standards were prepared by filling an empty 10 ml headspace vial (Chromacol Ltd., England) with gaseous PFB. The amount of gaseous PFB in the vial was calculated from the increased weight of the vial. Gaseous PFB was then transferred with a gas-tight syringe to a capped headspace vial (4 ml, actual volume 4.8 ml; Kimble, Mexico) containing 4.5 ml of ethanol and mixed. Additional stock solutions of PFB were subsequently prepared by serial dilution to capped headspace vials containing 4.5 ml of ethanol. From the stock solutions, the appropriate amount of PFB was transferred to 10 ml capped headspace vials containing 1.0 ml of blood and 6.0 ml of saline. Gas-tight syringes were used for all standard preparations. The target amount of PFB in the calibration standards was 0.5, 2.0, 5.0, 25, 150, 750 and 5000 ng. Since the calibration standards were prepared fresh for every analytical sequence, the amount of PFB in the standards varied slightly from day to day since the initial weight of gaseous PFB varied. The variation was, however, by not more than approximately 10%. Initially, the standards were dissolved in 2-propanol but this was changed to ethanol, because, eventually, a large solvent peak with the same retention time as the internal standard at m/z 101 started to appear in the chromatograms. Changing the solvent of the PFB standards had no significant effect on the calibration (not shown).

Any possible effect of diluting gaseous PFB in ethanol on the accuracy of the calibration standards was also examined. Two sets of standards were prepared from stock solution of gaseous PFB either diluted in air or ethanol. There was no significant difference between the two sets of standards (not shown).

CFC 11 was used as internal standard of the method and was also prepared fresh for every analytical sequence. CFC 11 was first diluted in ethanol and, prior to analysis, approximately 46 ng was added to all standards and samples.

Three levels of control samples were prepared from gaseous PFB in the same manner as the

Table 1
Conditions for the headspace sampler, GC and MS

Headspace sampler		**Gas chromatograph**	
Bath temperature	60°C	Injector temperature	240°C
Valve/loop temperature	85°C	Split ratio	Splitless
Carrier gas pressure	1.0 bar	*Oven temperature programme*	
Equilibration time	30 min	Initial temperature	50°C for 2 min
Injection parameters		Rate	20°C/min
Probe down	0 s	Final temperature	230°C
Pressurise on/off	0 s	Final time	5 min
Vent on/off	1/11 s	Interface temperature	250°C
Inject on/off	12/47 s	Carrier gas	Helium ($\approx$ 10 psi)
Probe up	48 s		
		MS conditions	
		Ionisation mode	Positive electron impact
		Ion monitoring	SIM of m/z 69, 101 and 119
		Inter channel delay	0.02 s
		Dwell time	0.02 s
		Span	0 Da
		Start time	4 min
		End time	16 min
		Electron energy	70 eV
		Source temperature	220°C

standards. The appropriate amount of gaseous PFB was added to capped headspace vials containing 7.0 ml of saline and stored at −20°C. The amount of PFB in the control samples were 5.1, 128 and 2200 ng.

The rat blood samples were prepared by transferring 1.0 ml heparinised blood to 10 ml headspace vials containing 6.0 ml of saline and capped. The vials were stored at −20°C until they were analysed.

Prior to analysis, all standards and samples were sonicated for 5 min and vortex mixed for 10 s.

2.4. Drug product

Drug product samples were prepared by reconstitution of Sonazoid™ dry powder in sterile water and dilution in sucrose (92 mg/ml). After carefully mixing for 1 min, 10 μl of the reconstituted drug product was transferred to headspace vials containing 7.0 ml of saline and capped immediately. The headspace vials were stored at −20°C until they were analysed. The amount of PFB in these samples was determined from gaseous PFB standards prepared as described in Section 2.3, but added to capped headspace vials containing 7.0 ml of saline.

2.5. Coulter counter

The microbubble concentration and size distribution of all samples were determined by Coulter counting with a Coulter Multisizer Mrk II (Coulter Electronics Ltd., Luton, England) fitted with a 50 μm aperture with a measuring range of 1.00–31.1 μm. Analysis was performed with a 64 logarithmically spaced size channels. A suitable sample volume was diluted in 200 ml double filtered Isoton II (Coulter Electronics Ltd., Luton, England) at ambient temperature and stirred for four minutes prior to analysis. From the detected microbubble volume concentration (V_m, μl microbubbles/ml), the concentration of PFB (C_{PFB}, ng/ml) was calculated assuming that the entire microbubble volume contained pure PFB at ambient pressure and temperature. PFB concentration was calculated as:

$$C_{PFB} = V_m \times \rho_{PFB}$$

where ρ_{PFB} is the density of PFB at ambient pressure and temperature (9.8 mg/ml).

2.6. Sample analysis and validation parameters

The samples were analysed in sequences together with standards, control samples and blank samples. The standards were positioned at the beginning of the sequence while the samples and control samples were randomly placed in the sequence.

The standard curve was evaluated from seven calibration graphs analysed on seven different days. The precision of the method was evaluated by analysing the three control samples (5.1, 128 and 2200 ng PFB) in triplicate on three different days. After analysis, the following was calculated: the mean of the within-series means, the standard error of the mean (SDx) and the pooled within-series standard deviation of the daily mean ($SD_w(p)$). The standard deviations were determined from the mean square values of an ANOVA single factor calculation of the results where $SD_w(p)^2$ is within group mean square and $n\mathrm{SD}x^2$ is between group mean square (where n is the number of replicates per analysis).

The accuracy of the method for measuring PFB in reconstituted Sonazoid™ was evaluated by determining PFB in Sonazoid™ with GC–MS and comparing this with the volume concentration of the microbubbles estimated by Coulter counter. Five different concentrations of reconstituted Sonazoid™ solutions were prepared and 10 μl aliquots were added to headspace vials containing 7.0 ml of saline and analysed with PFB standards in saline. The same Sonazoid™ solutions were subsequently analysed by Coulter counter.

2.7. Data handling

Finnigan MassLab data sampling system, ver. 1.4, was used for sampling and integration of the chromatograms. GraphPad PRISM, ver. 2.0, was used for preparation of calibration curves and estimation of PFB amount. Microsoft Excel, ver. 5.0, was used for statistical calculations.

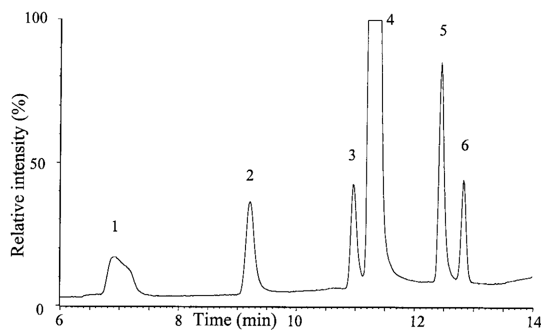

Fig. 1. Typical chromatogram of a mixture of perfluorocarbons (≈ 1 μg of each) analysed with the MS in the full scanning mode: (1) perfluoropropane, (2) PFB, (3) perfluoropentane, (4) ethanol, (5) perfluorohexane and (6) CFC 11.

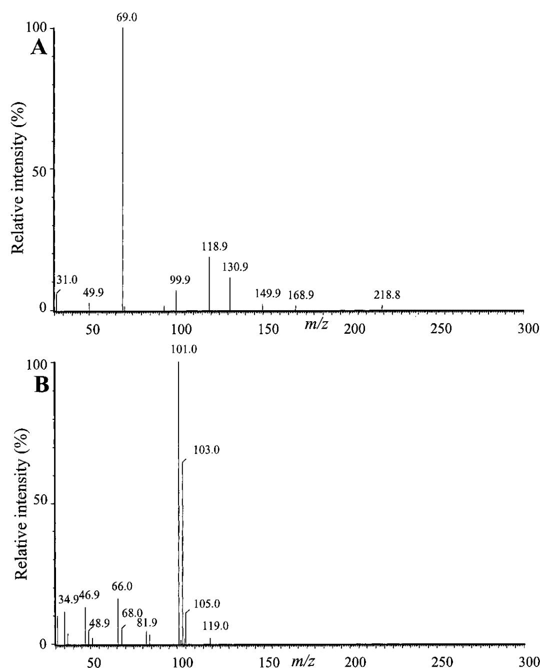

Fig. 2. Full scan spectrum of (A) PFB and (B) CFC 11.

3. Results and discussion

The main purpose of this study was to develop a method for the determination of PFB in rat blood after i.v. injection of the ultrasound contrast agent Sonazoid™. Since PFB is a highly volatile substance, headspace injection using an automatic headspace sampler coupled with GC–MS was the method of choice. Fig. 1 shows that PFB was chromatographically separated from some of the closely related perfluorocarbons. CFC 11 was chosen as the internal standard of the method and Fig. 2 shows the EI + spectrum of PFB and CFC 11. As shown in Fig. 2, the fragment ions at m/z 69 and 119 were the base peaks in the spectrum of PFB and the fragment ion at m/z 101 was the base peak in the spectrum of CFC 11. The MS was therefore set to monitoring these fragment ions of PFB and CFC 11; the fragment ion at m/z 119 was, however, only used for confirmation of PFB peak identity. Fig. 3 (A) and (C) shows that, when the MS was set to m/z 69 and 101, there were no endogenous peaks interfering with neither PFB nor CFC 11 in rat blood.

According to published recommendations, the limit of quantification (LOQ) of a method can be set to a specific concentration provided that the repeatability of analysing at this concentration is below 20% relative standard deviation (RSD) of the mean [22]. Based on the prevalidation work, the lowest standard at approximately 0.5 ng was chosen as the LOQ of the method. Since the calibration standards were made fresh for every analytical sequence, the PFB amount in the lowest calibration standard varied slightly from sequence to sequence. Standards containing gaseous PFB at 0.4 ng were therefore prepared and the precision of analysing this standard, expressed as the RSD of the mean of the peak area ratio of PFB to CFC 11, was found to be 2.8% RSD (n = 6). Femtogram detection of perfluorocarbons with electron-capture negative ion chemical ionisation MS detection has previously been published [13]. But since our LOQ was approximately 0.01% of the highest standard we felt that for practical reasons the sensitivity of our method was adequate.

The calibration standards were analysed as described in Section 2 and a calibration curve was made by plotting the peak area ratio of PFB to CFC 11 against the theoretical amount of PFB. The curvature was estimated from the following equation: $y = a + bx^m$ and the parameter m was found to be $m = 1.08 \pm 0.07$ (mean ± SD, n = 7). The calculated m value indicated that the

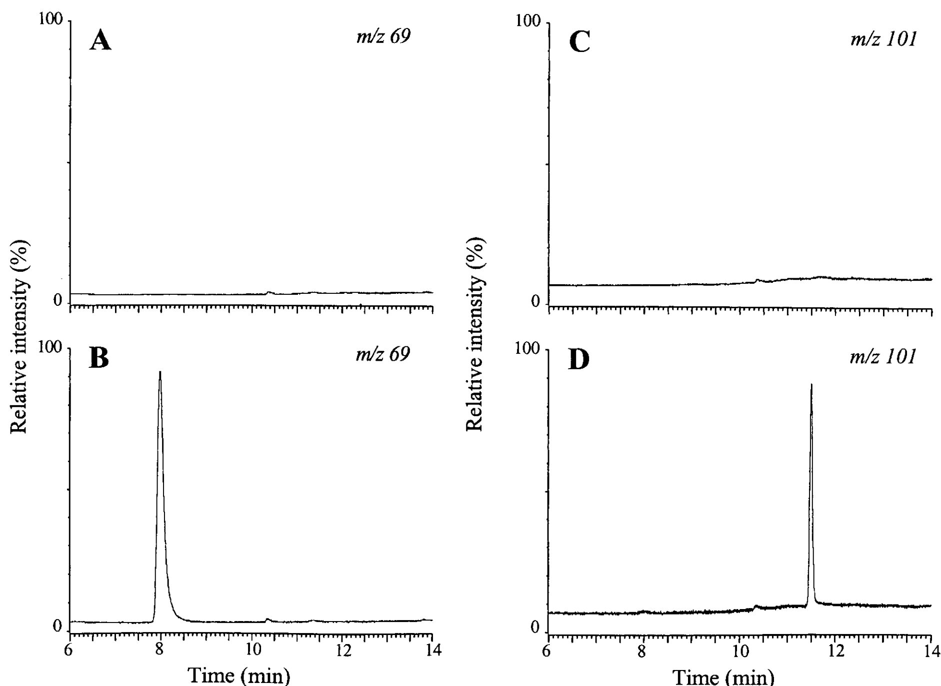

Fig. 3. Chromatograms of (A) blank rat blood using detection at m/z 69, (B) rat blood added 128 ng PFB using detection at m/z 69 with the same relative intensity as in (A), (C) blank rat blood using detection at m/z 101 and (D) rat blood added 46 ng CFC11 using detection at m/z 101 with the same relative intensity as in (C).

calibration curve deviated slightly from linearity. It was therefore decided to fit the curve to a second-order polynomial equation $y = a + bx + cx^2$. In addition, to get the best fit of the lowest standards to the calibration curve, it was weighted $1/y^2$. The estimated parameters of the calibration curve from seven series of analysis are listed in Table 2. The goodness of fit of the calibration points to the calibration curve was also estimated and the largest deviation from the theoretical amount of PFB was 12.7% (Table 3).

For the preparation of each standard, 1.0 ml of rat blood was used. In order to examine whether blood could be omitted in the standards, they were prepared by adding PFB to headspace vials containing either 7.0 ml of saline or 1.0 ml rat blood added to 6.0 ml saline. The amount of PFB in the control samples was then estimated from the two sets of standards and a significant difference ($P < 0.05$) was found using a two-tailed paired Students t-test when the results were compared. Fig. 4 shows that there was a slight overestimation of the PFB amount when the standards were prepared with only saline.

Initially, the control samples were prepared by adding aliquots of Sonazoid™ to headspace vials but since this product consists of floating microbubbles, it is not a homogenous solution. Preparation of large amounts of control samples from Sonazoid™ yielded, therefore, samples with

Table 2
The estimated regression parameters of the standard curve fitted to the equation; $y = a + bx + cx^2$ with weighting $1/y^2$ [a]

Regression coefficient	Mean ± SD ($n = 7$)
a	0.0047 ± 0.0113
b	0.064 ± 0.025
c	$3.1 \times 10^{-6} \pm 3.5 \times 10^{-6}$
r^2	0.99959 ± 0.00042

[a] The standards were analysed in seven series with two parallels for each standard.

Table 3
The goodness of fit of the calibration points to the calibration curve was estimated from seven analytical series

Target concentration (ng PFB)	Mean recovery ± SD (% of theoretical value)
0.5	102.5 ± 5.0
2	99.2 ± 7.6
5	90.9 ± 5.3
25	103.4 ± 2.4
150	112.7 ± 7.8
750	100.1 ± 7.9
5000	99.9 ± 1.9

unequal amounts of PFB. The precision of the method was therefore evaluated with control samples prepared from gaseous PFB (Section 2). The within- and between-day variation of the method was found to be below 7.6% RSD_w (p) and 9.2% RSDx, respectively (Table 4). This shows that the daily precision of analysing the samples was acceptable and that the calibration curve (and thus the quantification) was reproducible.

The accuracy of the method was evaluated with samples prepared from Sonazoid™ and the amount of Sonazoid™ PFB determined by GC–MS was compared to the volume concentration of the microbubbles estimated by Coulter counter as described in Section 2. Assuming 100% purity of PFB, the microbubble concentration can be converted to μg PFB by using the molar gas volume of PFB at 1 atm and 22°C i.e. 24.25 l/mol. The molecular weight of PFB is 238 g/mol, giving the following relationship between PFB mass and volume: 9.8 g/l. Table 5 shows that the accuracy of the method ranged from 91.5% to 105.2% when compared to Coulter counter. When plotting the amount of PFB determined by GC–MS to the amount determined by Coulter counter, the slope of the linear regression curve was estimated to 0.96 ± 0.06 and the y-intercept was estimated to 0.3 ± 4.8. Evaluated by comparison to the volume concentration determined by Coulter counter, the method was linear in the investigated range of approximately 80–1000 ng PFB, with a slope and intercept equal to one and zero, within the 95% confidence interval.

The control samples were stable for at least 7 months when stored at −20°C (not shown) and no difference was found between the stability of control samples with Sonazoid™ PFB and gaseous PFB (not shown).

In conclusion, the method developed was found to be precise and accurate for the determination of Sonazoid™ PFB in rat blood. Using headspace

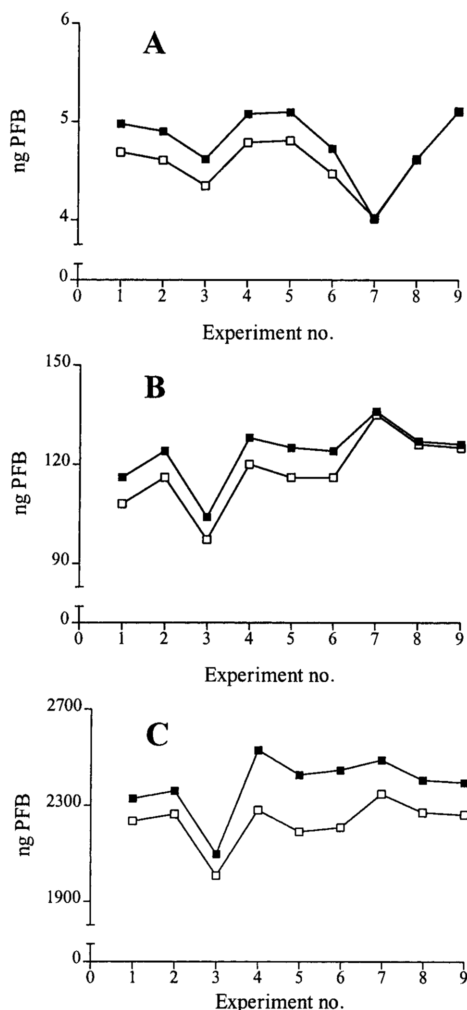

Fig. 4. The amount of PFB in control samples was determined from two different sets of standards in each experiment. The control samples were either determined from standards prepared by adding gaseous PFB to headspace vials containing 7.0 ml of saline (■) or 1.0 ml of rat blood and 6.0 ml of saline (□). Control sample containing (A) 5.1 ng of PFB, (B) 128 ng of PFB and (C) 2200 ng of PFB.

Table 4
The precision of the method was estimated from three control samples analysed in triplicate on three different days[a]

	Mean (ng PFB)	SDx	SD_w (p)	RSDx (%)	RSD_w (p) (%)	Recovery (%)
Control 1[b]	4.61	0.073	0.349	1.6	7.6	90.4
Control 2[c]	118	10.8	6.45	9.2	5.5	92.2
Control 3[d]	2228	62.4	90.1	2.8	4.0	101.3

[a] The following were calculated; the mean of the within-series means, the standard error (SDx) of the mean and the pooled within-series standard deviation of the daily mean ($SD_w(p)$).
[b] 5.1 ng of PFB (theoretical concentration).
[c] 128 ng of PFB (theoretical concentration).
[d] 2200 ng of PFB (theoretical concentration).

Table 5
The accuracy of the method as compared to Coulter counter[a]

Coulter counter Mean ± SD [b] (ng PFB)	GC–MS Mean ± SD [c] (ng PFB)	Recovery GC–MS/Coulter (%)
81.3 ± 12.5	74.4 ± 5.5 ($n=6$)	91.5
326 ± 8.3	343 ± 19 ($n=3$)	105.2
750 ± 1.4	703 ± 28 ($n=6$)	93.7
951 ± 28	920 ± 175 ($n=19$)	96.7
995 ± 6.9	946 ± 28 ($n=6$)	95.1

[a] The results are given as ng PFB in 10 μl reconstituted Sonazoid™ solution.
[b] Two sample replicates.
[c] Sample replicates indicated in parenthesis.

sampling very little sample preparation was necessary and the method have now been used for analysing several hundred rat blood samples for evaluation of the pharmacokinetics of this new ultrasound contrast agent. These results will be published separately.

Acknowledgements

We thank Per Christian Sontum for the work with obtaining the Coulter data.

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