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Short communication

A respiration–metabolism chamber system and a GC–MS method developed for studying exhalation of perfluorobutane in rats after intravenous injection of the ultrasound contrast agent Sonazoid™

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Abstract

Sonazoid™ is a new contrast agent for ultrasound imaging comprising an aqueous suspension of lipid-stabilised perfluorobutane (PFB) gas microbubbles. A respiration–metabolism chamber system was developed to collect exhaled air following intravenous administration of Sonazoid™ to rats. Analysis of PFB in the exhaled rat air was performed using a modified version of an earlier published method for blood samples, i.e. an automatic headspace gas chromatographic mass spectrometric (GC–MS) method using electron impact ionisation. The calibration standards were PFB diluted in air (2.5–1800 pg/ml). Perfluoropentane (PFP) was used as an internal standard and the MS detector was set to single ion monitoring of the base fragment ions of PFB (*m*/*z* 69 and 119) and PFP (*m*/*z* 69). The calibration curve, made by plotting the peak area ratios of PFB (*m*/*z* 69) to PFP (*m*/*z* 69) against the theoretical concentration of PFB, was fitted to a linear equation with weighting $1/y^2$ and found to be reproducible. The lower limit of quantification (LLOQ) was 2.5 pg PFB/ml. The between-day variation of the method was below 2.6% relative standard deviation (R.S.D.) and the within-day variation of the method was below 6.4% R.S.D. The accuracy of the method was evaluated and showed a relative error less than 5.2%. PFB was found to be stable for 14 days when stored in Tedlar sample bags at room temperature. An even lower detection limit may be obtained by using the more time-consuming process of solid-phase micro extraction; thus, by concentrating PFB on carboxen–PDMS fibres an LLOQ of 0.5 pg PFB/ml was obtained. When five rats were given an i.v. bolus injection of Sonazoid™ at a dose of 8 μl microbubbles/kg a mean recovery of 96% (range, 81–110%) was found during 24 h; more than 50% was exhaled during the first 30 min after injection.

Keywords: GC–MS; Headspace; Perfluorobutane; Exhalation; Ultrasound; Contrast agent

1. Introduction

Sonazoid™ is an ultrasound contrast agent under development for detection of focal lesions in liver [1]. The agent may also be used for left ventricular border enhancement, myocardial perfusion mapping and several vascular applications, such as characterisation of the vascularity of tumours [2,3]. The contrast agent is a lipid-stabilised dispersion of perfluorobutane (PFB) gas microbubbles with a median volume diameter of approximately 3 μm [4]. The product is supplied as a freeze-dried powder, which is reconstituted with water before injection. The clinical dose of Sonazoid™ for liver imaging is 0.12 μl microbubbles/kg body weight.

Investigations of the biodistribution of PFB in rat tissues after injection of Sonazoid™ showed that the highest amount of PFB was found in liver at the first sampling time at 5 min after injection. At this time point the recovery in liver was approximately 50% and the total recovery from blood, liver, spleen, kidney, fatty tissue, muscle, heart, lung and brain was

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approximately 70% of the injected dose. At 24 h after dosing the total recovery of PFB from these tissues was approximately 2% of the injected dose (unpublished data). In a study using electron microscopic analysis of perfused fixed rat livers, intact gas microbubbles were observed exclusively within the Kupffer cells, i.e. in the macrophages located in the liver sinusoids, but not within parenchymal, stellate or endothelial cells [5].

As no metabolic system for degradation of PFB is known and no metabolites of PFB have been observed during the preclinical testing of this agent it has been assumed that PFB is rather rapidly excreted via the lungs. To study the elimination of PFB in exhaled air following intravenous administration of Sonazoid™ to rats, we developed a gas collection system and modified an earlier developed automatic headspace capillary GC–MS method [6] in order to quantify PFB in exhaled rat air. This system was applied in a study where five rats were given Sonazoid™ at a dose of 8 μl microbubbles/kg and the amount of PFB determined in exhaled air up to 24 h after intravenous injection. As no differences were observed in biodistribution data or toxicokinetic data when rats were injected 0.8 or 8.0 μl microbubbles/kg (unpublished data), we decided to use the highest dose for the exhalation study in order to obtain as high recovery data as possible.

2. Experimental

2.1. Material

Sonazoid™ was produced by GE Healthcare Bio-Sciences, Norway. Perfluoro-*n*-butane (PFB, C_4F_{10}) was purchased from F2 Chemicals Ltd., England, and perfluoro-*n*-pentane (PFP, C_5F_{12}) from Fluorochem, England. Saline (NaCl) was from Kabi Pharmacia AB, Sweden, and sterile water from Braun, Germany. Helium (99.9999%) and medical air were from AGA Gas AS, Norway.

2.2. Instrumentation

2.2.1. Respiration–metabolism chamber system

The respiration–metabolism chamber system is shown in Fig. 1. A medical air tank with a two-stage cylinder regulator (AGA Gas AS, Norway) provided a stable airflow of 250 ml/min that was regulated by an adjustable stainless steel flow regulator (No. B-SS6MM, Swagelok, USA) and monitored by a flow meter (model GFM-171, Aalborg, USA). The flow at the system outlet was in addition controlled manually by a bubble flow meter (Supelco, USA). The metabolism chamber was a 1650 cm^3 glass cage (Harvard, USA) with a specially constructed lid and glass tubes connected with low-pressure fittings (Legris, USA). The different parts of the system were coupled together with 6 mm polyurethane tubing (Legris, USA) and fittings from Legris and Swagelok. A three-way valve (No. 0452-06-13, Legris, USA) directed

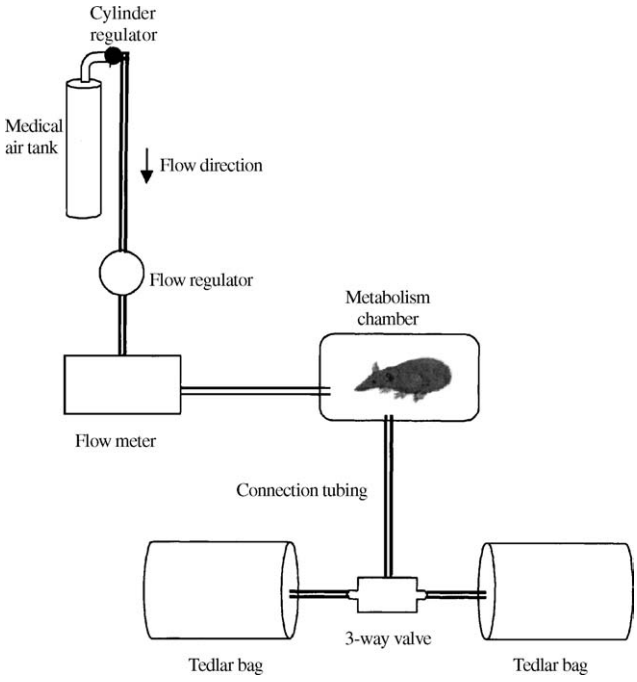

Fig. 1. The gas collection system used to collect expired air from rats.

the flow into 10–100-l Tedlar sample bags with dual stainless steel fittings (SKC, USA).

2.2.2. Headspace GC–MS

The automatic headspace sampler was an HS40XL coupled to an Autosystem gas chromatograph connected with a TurboMass mass spectrometer detector (all from Perkin-Elmer, USA). The column used for the chromatographic analysis was a Chrompack CP-PoraBOND Q, 25 m × 0.25 mm i.d. and 8 μm film thickness (Varian, USA). The data sampling and handling were performed by using TurboMass ver. 4.1.1 (Perkin-Elmer, USA). The conditions used for the headspace sampler, GC and MS are shown in Table 1.

2.2.3. SPME

The SPME fibre was 75 μm carboxen–PDMS (polydimethylsiloxane) for manual holder from Supelco, USA. New fibres were conditioned at 300 °C for at least 60 min prior to use. Extraction time was 25 min at room temperature and desorption time 1 min at 275 °C. The split-flow was 30 ml/min, with the split-valve closed 1 min before and opened 1.1 min after injection. The other GC–MS settings were as described above for analysis of the headspace samples.

2.3. Standard and sample preparation

All standards and QC samples were prepared at room temperature (approximately 22 °C). The PFB standards were prepared by filling an "empty" 10 ml headspace vial (Chromacol Ltd., England) with PFB gas. The amount of PFB in

Table 1
Conditions for the headspace sampler, gas chromatograph and mass spectrometer

Headspace sampler		Gas chromatograph		MS conditions	
HS mode	Constant	Carrier gas	Helium (1.0 ml/min)	Interface temperature	250 °C
Sample shaker	Off	Vacuum compensation	On	Source temperature	220 °C
Oven temperature	60 °C	Split-flow	5 ml/min	Ionisation mode	Electron impact (El)
Needle temperature	85 °C	Injector temperature	225 °C	Ion monitoring	SIM of *m/z* 69 and 119
Transfer temperature	105 °C			Inter channel delay	0.02 s
GC cycle time	25 min	*Oven temperature programme*		Dwell time	0.1 s
Thermostating time	10 min	Initial temperature	40 °C for 0.25 min	Span	0.2 Da
Pressurise time	3 min	Ramp 1	45 °C/min	Start time	2.0 min
Injection time	0.2 min	Final temperature	230 °C for 4 min	End time	4.5 min
Withdrawal time	0.5 min			Electron energy	70 eV
Vial venting	Off				

the vial was calculated from the increased weight of the vial corrected with a factor of 1.138 for displacement of air from the vial. This factor is estimated from densities of 9.72 and 1.18 mg/ml for PFB and air, respectively. PFB was then transferred with a gas-tight syringe (Vici, USA) to a 1-l Tedlar sample bag (SKC, USA) containing 800 ml of air. Additional stock mixtures were made by transferring aliquots from this mixture to another 1-l Tedlar sample bag containing 800 ml of air. From the stock mixtures, the appropriate amount of PFB was transferred to 50-ml Tedlar mini bags (SKC, USA) containing 40 ml of air and added internal standard, PFP. After equilibration for about 1 h, 15 ml of each sample was transferred with a gas-tight syringe (SGE, USA) to vented and capped 22.4 ml headspace vials (Perkin-Elmer, USA) containing 14 ml of saline. The target concentrations of PFB in the calibration standards were 2.5, 5, 15, 60, 240, 900 and 1800 pg/ml. The quality control (QC) samples used to validate the method were prepared by transferring the appropriate amount of PFB from the stock mixtures to 3-l Tedlar sample bags containing 2000 ml of air; 40 ml aliquots from the Tedlar sample bags were then transferred to 50-ml Tedlar mini bags with a 50 ml gas-tight syringe (SGE, USA) and treated similarly to the standards. The QC samples were made fresh from the same standard stock mixtures for each analytical sequence (the standard stock mixtures were stable for at least one month). The concentrations of the QC samples were 7.45 pg/ml (QCL), 91.8 pg/ml (QCM) and 1376 pg/ml (QCH). The exhaled rat air samples were prepared by transferring 40-ml aliquots from the 10–100-l Tedlar sample bags to 50-ml Tedlar mini bags with a 50 ml gas-tight syringe (SGE, USA). The samples were then treated similarly to the standards. PFP was prepared by transferring 5 μl cold liquid PFP (2–8 °C) to a capped 10 ml headspace vial and then diluting the PFP gas in a 1-l Tedlar bag containing 800 ml of air.

The standard and sample preparations were similar for the SPME–GC–MS method, but without the use of headspace vials. The SPME fibre was placed directly into the 50-ml Tedlar mini bags, and the concentrations of the standards were approximately 0.5–50 pg PFB/ml air and 25 pg PFP/ml air. The concentrations in the QC samples were approximately 1.5, 7.5 and 37.5 pg PFB/ml air, respectively. Lyophilised Sonazoid™ was reconstituted in 2 ml sterile water to give a PFB concentration of approximately 80 μg/ml.

2.4. Validations parameters

The standards were analysed in six separate analytical sequences on six different days. The precision and accuracy of the method were evaluated by analysing the three QC samples in triplicate on six different days. After analysis the following were calculated: the mean, the pooled within-series standard deviation of the mean ($S.D._w(p)$), the between-series standard deviation ($S.D._b$) and the relative error (R.E.) from theoretical value. The standard deviations were determined from the mean square values of an ANOVA single factor analysis. The QC samples prepared on the first day were used to evaluate the stability of PFB, and the QC samples in triplicate were analysed after 2, 7, 14, 21 and 28 days of storage at room temperature.

2.5. Animals

Male Sprague–Dawley rats, 6–7 weeks old and weighing between 155 and 175 g at study start were obtained from Harlan, The Netherlands. Rats were housed three per cage and given free access to water and Rat & Mouse No. 1 Maintenance Diet (Special Diet Service, Northwich, UK). The rats were individually accustomed to being in the metabolism chambers for approximately 0.5–1 h during an acclimatising period of at least five days before study start. A total of five rats were used in the study.

2.6. Validation of the respiration–metabolism chamber system

The respiration–metabolism chamber system was set up as described, and a rat was placed in the chamber. Using gas-tight syringes, PFB at three levels (14.7, 180 and 2700 ng) and three parallels per level, were injected into the chamber through an injection port. Airflow through the system was 250 ml/min, but the airflow was stopped for 1 min during injection of the standards. Air was then collected in 10-l Tedlar sample bags for 8 min and analysed with the headspace

GC–MS method. The recovery was calculated by using the same standards spiked directly into Tedlar sample bags containing 2 l of air as reference standards.

2.7. Collection of exhaled air

The chamber system was set up as described. The respiration–metabolism chamber was disconnected from the gas collection system and the gas inlet and outlet were properly sealed. Enough food and drinking water for 24 h consumption were placed in the chamber. The rat was anaesthetised (propofol, 10 mg/kg) and placed in the chamber. After a bolus injection of Sonazoid™ (approximately 8 μl microbubbles/kg) into the lateral tail vein, the lid of the chamber was immediately tightened and the chamber was connected to the airflow. Expired air was sampled in Tedlar sample bags of different volumes for several time intervals up to 24 h after injection and analysed with the headspace GC–MS method within two days. The exact amount of PFB in the injected Sonazoid™ solution was determined by withdrawing 100 μl aliquots prior to injection and analysed with a headspace GC–MS method similar to the one described.

3. Results and discussion

3.1. Validation of the headspace GC–MS method

The fragment ions at *m/z* 69 and 119 were the main peaks in the PFB spectrum, whereas the fragment ion at *m/z* 69 was the main peak in the PFP spectrum. The MS was, therefore, set to monitor these fragments ions for PFB and PFP; the fragment ion at *m/z* 119 was, however, only used for confirmation of the identity of the PFB peak. There were no endogenous peaks interfering with either PFB or PFP in blank air using these settings (Fig. 2).

Based on initial experiments (data not presented), 2.5 pg PFB/ml was chosen as the lowest standard in the calibration curve. The validation showed that the precision (R.S.D.) and accuracy (R.E.) at this concentration were 6.0 and 11.1%, respectively, with a signal-to-noise ratio of 19 (five parallels, one day). This concentration was, therefore, accepted as the analytical LLOQ of the method. The calibration standards were analysed as described in Section 2 and a calibration curve was made by plotting the peak area ratio of PFB (*m/z* 69) to PFP (*m/z* 69) against the theoretical amount of PFB. Both linear and non-linear regression analysis were tested and the calibration curve was found to be linear throughout the concentration range. Weighting (by $1/y^2$) was, however, essential to give the best fit of the lowest calibration standards to the calibration curve. The regression parameters of the standard curve are given in Table 2. The goodness of fit of the calibration points to the calibration curve showed deviations less than 6.5% from the theoretical amounts of PFB (Table 3). The within-day and between-day variation were found to be below 6.4% $R.S.D._w(p)$ and 2.6 $R.S.D._b$, respectively (Table 4).

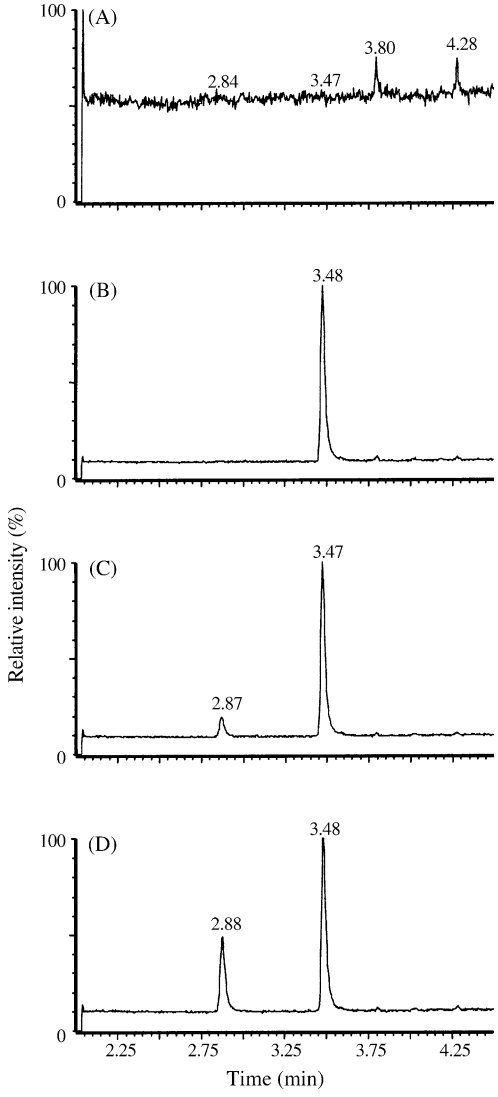

Fig. 2. Chromatograms using detection at *m/z* 69. (A) Blank air; (B) blank air added 110 pg PFP; (C) blank air added 14 pg PFB and 110 pg PFP; (D) exhaled air sample added 110 pg PFP.

The accuracy of the method ranged from −0.2 to 5.2% R.E. (Table 4). PFB in air was stable for 14 days when stored in Tedlar bags at room temperature (data not shown).

3.2. Validation of the SPME–GC–MS method

Validation of the SPME–GC–MS method showed a linear calibration range from approximately 0.5–50 pg PFB/ml.

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

Regression coefficient	Mean ± S.D. ($n = 6$)
a	0.000153 ± 0.00237
b	0.00754 ± 0.000810
r^2	0.998 ± 0.000598

The standards were analysed in six analytical series (2.5, 5, 15, 60, 240, 900 and 1800 pg PFB/ml).

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

Target concentration (pg PFB/ml)	Mean recovery ± S.D. (relative error (%) of theoretical value)
2.5	−2.0 ± 1.5
5	5.1 ± 3.9
15	−0.3 ± 2.0
60	−0.9 ± 1.6
240	5.1 ± 1.5
900	−6.5 ± 1.1
1800	2.2 ± 0.7

Table 4
The precision and accuracy of the method were estimated from three control samples analysed in triplicate on six different days

	Mean (pg PFB/ml)	$S.D._w(p)$	$S.D._b$	$R.S.D._w(p)$	$R.S.D._b$	Accuracy
Control 1[a]	7.84	0.50	0.17	6.4	2.1	5.2
Control 2[b]	91.6	1.54	2.35	1.7	2.6	−0.2
Control 3[c]	1416	24.3	26.2	1.7	1.9	2.9

The following were calculated; the mean of the within-series means, the pooled within-series standard deviation of the daily mean ($S.D._w(p)$), the between-run standard deviation ($S.D._b$) and accuracy as relative error from the theoretical value.

[a] Theoretical concentration: 7.45 pg PFB/ml.

[b] Theoretical concentration: 91.8 pg PFB/ml.

[c] Theoretical concentration: 1376 pg PFB/ml.

Precision and accuracy were determined for three different PFB concentrations (in triplicate on three different days) to be below 7.6% R.S.D. and within ±2.1% R.E., respectively, except at LLOQ (five parallels on one day) where precision and accuracy were 11.1% R.S.D. and 10.2% R.E., respectively, with a signal-to-noise ratio of 12. This method was not used for analysis of study samples as it is more time-consuming than the method not including SPME.

3.3. Recovery of PFB from the respiration–metabolism chamber system

Recovery of PFB injected into the respiration–metabolism chamber system was 92.6 ± 5.4, 95.8 ± 9.6 and 92.5 ± 6.6% (mean ± S.D., $n = 3$) for the low, medium and high concentrations of PFB, respectively. Thus, close to 100% recovery was obtained for all three concentration levels. It is not known if these slightly less than 100% recoveries are due to analytical uncertainty or to some minor loss of PFB due to absorption to the surface of the glass chamber, tubing and valves. However, the data showed that the respiration–metabolism chamber system was suitable for quantitative collection of exhaled air from rats following injection of Sonazoid™.

3.4. Recovery of PFB after intravenous injection of Sonazoid™ in rats

The accumulated recovery of PFB from each animal is shown in Fig. 3. The amount of PFB recovered during the 24 h collection period was in the range 80.5–110.4% of the injected dose for the five rats with a mean value of 96.4%. More than 50% of the recovered PFB was exhaled during the first 30 min post injection. The individual variations observed in the present study are most likely due to uncertainty in the determination of the amount of PFB in injected Sonazoid™ in addition to random errors in the measurements. It is less likely that this variation is due to different exhalation rates from the five animals, as the accumulated recovery curves are quite parallel (Fig. 3).

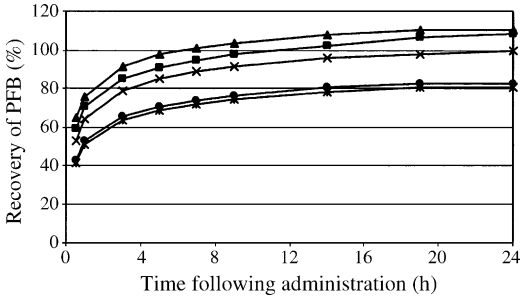

Fig. 3. Accumulated amounts of PFB in exhaled rat air as percent of injected dose for each of the animals.

These exhalation results correspond well with results from rat pharmacokinetic studies done with Sonazoid™ where, 5 min after injection, a total of approximately 70% of the injected dose was recovered in blood, liver, spleen, kidney, fatty tissue, muscle, heart, lung and brain, whereas only approximately 2% was recovered from these tissues 24 h after injection (our unpublished data). It should be noted that these recovery data are liable to considerable uncertainty due to summing up the recovery from nine different tissues, and several of these tissues were assumed to constitute a fixed percent of each animal's total weight.

To our knowledge the combination of biodistribution and exhalation data has not been published for any other perfluorocarbon-based compound developed for ultrasound imaging. There is, however, one report describing blood and exhalation kinetics of perfluoropropane after injection of Optison™ in mongrel dogs [7] and one report describing exhalation kinetics of the same agent in humans [8]. Moreover, there is one report describing blood and exhalation kinetics of perfluoropentane after injection of EchoGen™ in humans [9]. Optison™ consists of perfluoropropane gas encapsulated in albumin microspheres, whereas EchoGen™ consists of a 2% (w/v) suspension of perfluoropentane, which results in a gaseous dispersion by means of a hypobaric activation technique and elevation to body temperature on injection (boiling point of perfluoropentane is approximately 28 °C). In the Optison™ studies, nearly 100% of the perfluoropropane was exhaled within 6 min in humans and 10 min in dogs. In the EchoGen™ study, perfluoropentane was eliminated from blood with a half-life of 1.8–2.5 min and nearly 100% was exhaled 2 h after injection in humans. Thus, it appears that the perfluorocarbon component of these agents

is more rapidly exhaled than the PFB from Sonazoid™. All these perfluorocarbons seem to be exhaled without formation of any metabolites.

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