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Determination of 2-butoxyethanol and butoxyacetic acid in rat and human blood by gas chromatography–mass spectrometry

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Abstract

A sensitive and selective gas chromatographic–negative-ion chemical ionization mass spectrometric method was developed to simultaneously quantitate 2-butoxyethanol (BE) and butoxyacetic acid (BAA) in rat and human blood at low ng/g levels as pentafluorobenzoyl and pentafluorobenzyl derivatives, respectively. Analysis of ^{13}C-labeled analogs of BE and BAA were found to improve the limits of quantitation to below 2 ng/g. Deuterium-labeled BE and BAA were used as internal standards. Calibration curves were generally linear over three orders of magnitude, with limits of quantitation of 16–18 ng/g for both BE and BAA, and 1.5 and 0.4 ng/g for [$^{13}C_2$]BE and [$^{13}C_2$]BAA, respectively, in human blood. Linearity in rat blood was similar, with limits of quantitation of 22 ng/g for BE and 5 ng/g for BAA. This method was developed for the support of mammalian metabolism studies and human biomonitoring studies involving exposure to BE or [$^{13}C_2$]BE.

1. Introduction

Butoxyethanol (BE) is a key ingredient in water- or solvent-based coatings and industrial and consumer cleaning products [1]. Mammalian in-vivo and in-vitro studies, including rats and humans, have been carried out to perform risk assessments for human BE exposure. Toxicological findings in animal studies have been decreased body weight gains, hemolysis, liver and kidney toxicity and skin irritation [2]. The hemolytic effects of BE are the most sensitive indicators of toxicity in several species of laboratory animals and have, therefore, received the most attention for human risk assessment. It has been well documented, in both in-vitro and in-vivo studies that the major metabolite of BE, butoxyacetic acid (BAA), is primarily responsible for the hemolysis of red blood cells [3–5]. Human erythrocytes have been shown to be much less susceptible to butoxyacetic acid-induced hemolysis compared to rats [6,7]. The analysis of BE and BAA in blood is a critical component in developing and validating a physiologically-based pharmacokinetic model that describes the uptake, metabolism and disposition of BE and BAA in humans. This methodology can also be used as an alternative to the current practice of measuring urinary BAA as the procedure of monitoring human exposure to BE.

Analytical methods previously published for the analysis of BE and BAA have been limited

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by their sensitivity, selectivity and/or extensive sample preparation [8–13]. These methods also lack the ability to measure both BE and BAA in a single method. Much of this work has been done using gas chromatography (GC) with either flame ionization detection (FID) or electron capture detection (ECD), which are both limited by low selectivity for the analytes of interest after forming halogenated derivatives, such as the pentafluorobenzyl derivatives. The presence of matrix interferences was the major limitation to quantitation at low levels. As many of these methods were developed for the analysis of urine samples, they have used sample sizes which are greater than that possible for in-vivo sampling of sequential rat blood samples and have had limits of quantitation above those necessary for low-dose studies. Initial efforts in the development of this method have been previously described [14]. This work describes a selective and sensitive technique for simultaneously analyzing BE and BAA in rat or human blood which involves relatively little sample preparation, as well as the use of deuterated internal standards and stable isotope-labeled analytes.

2. Experimental

2.1. Preparation of standards/samples in human blood

Control human blood was drawn from four volunteers and mixed with sodium heparin to prevent clotting. Using proper biohazard handling precautions, control blood was pooled to prepare standards, except for the preparation of blanks, which were used to determine the variability in endogenous BAA. Aliquots of heparinized control human blood (250 mg) were combined with 0.75 ml of an ion-pairing reagent (0.2 *M* NaOH, Fisher Scientific, Chicago, IL, USA; 0.1 *M* tetrabutylammonium hydrogen sulfate, 97%, Aldrich Chemical Co., Milwaukee, WI, USA) in a 4-ml glass vial and 60–70 ng of the deuterated internal standards, $CD_3(CD_2)_2CH_2O(CH_2)_2OH$, D_7-BE, and $CD_3(CD_2)_2CH_2OCH_2CO_2H$, D_7-BAA [15] contained in 5 μl of deionized water. The standards were then fortified with known quantities of BE and BAA or [$^{13}C_2$]BE and [$^{13}C_2$]BAA [15] contained in 1–50 μl aqueous stock solutions. After mixing, an extractive alkylation was performed on BAA by adding 30 μl of pentafluorobenzyl bromide (PFBBr, 99 + %, Aldrich Chemical Co.) and 2 ml of methylene chloride, and vortex-mixing the mixture for 20 min at 45°C on an Evapotec vortex-evaporator (Haake-Buchler Instruments, Saddle Brook, NJ, USA). The layers were separated by centrifugation (1278 *g* for 8 min; IEC Centra-8, International Equipment Company, Needham Heights, MA, USA). The methylene chloride layer was transferred to a 2-ml GC autosampler vial. The BE was derivatized with the addition of 20 μl of pentafluorobenzoyl chloride (PFBCl, 99%, Aldrich Chemical Co.) and heating for 15 min at 70°C.

2.2. Preparation of standards in rat blood

Heparinized control rat blood was obtained from male Fischer 344 rats (Charles Rivers Breeding Laboratory, Kingston, NY, USA) which were sacrificed via cardiac puncture while anesthetized with carbon dioxide. Aliquots of control blood (100 mg) were combined with 0.3 ml of the ion-pairing reagent previously described, 10–90 ng of the deuterated internal standards contained in 5–10 μl of deionized water, and 2–40 μl of aqueous stock solutions containing BE and BAA. After mixing, 20 μl of PFBBr and 1.5 ml of methylene chloride were added and the standards were vortex-mixed for 20 min at 45°C. The layers were separated by centrifugation (1460 *g* for 7 min) and the methylene chloride layer transferred to a 2-ml GC autosampler vial where 15 μl of PFBCl was added. Standards were heated at 70°C for 15 min.

2.3. Preparation of samples for stability determination

Aliquots of heparinized human blood (250 mg) and rat blood (100 mg) were fortified with known levels of $^{13}C_2$-labeled BE and BAA and

rapidly frozen by placing the vials on dry ice. The samples were stored for 30 days at −80°C and −20°C for the rat and human blood, respectively. To determine the analyte levels in the samples after 30 days, the samples were prepared using the procedures described above. Matrix standards were prepared using freshly collected control blood from each species.

2.4. Preparation of standards and samples in rat urine for the investigation of conjugate stability

In early metabolism studies, pooled urine samples from rats dosed with BE (10 and 125 mg/kg, gavage) and control rat urine fortified with known amounts of BE and BAA were prepared using the same procedure as described for the preparation of rat blood samples with the following exceptions: 150-mg aliquots were used; 2-methylhexanoic acid and 3-methoxy-1-butanol (Aldrich Chemical Co.) were used as internal standards and were contained in the methylene chloride; the final methylene chloride solution was evaporated to dryness and reconstituted in 0.6 ml of acetone.

For the analysis of total urinary BE and BAA, conjugates were hydrolyzed by first reacting with β-glucuronidase (20 units, type VII-A, Sigma, St. Louis, MO, USA) for 22 h at 37°C followed by the 10% v/v addition of concentrated HCl (36.5–38.0%, Fisher Scientific) and heating at 70–80°C for 22 h. Urine samples were neutralized with 5 *M* NaOH and prepared as described previously.

2.5. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were performed using a HP5890 Series II gas chromatograph coupled to either a HP5989X mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) or a Finnigan TSQ-700 (Finnigan MAT, San Jose, CA, USA). Samples were introduced by a 0.3-min splitless injection of 2 μl from a HP7673B autosampler onto a fused-silica DB-5MS capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness; J and W Scientific, Folsom, CA, USA) heated initially at 100°C with a 0.3-min hold to 185°C at 15°C/min and then to 300°C at 30°C/min with a 0.5-min hold. Helium carrier gas was used at a column head pressure of 0.7 bar (measured at an oven temperature of 100°C). The injection port and transfer line were maintained at 250°C and 300°C, respectively. The source was maintained at 200°C. The mass spectrometer was operated in the negative chemical ionization (NCI) mode using methane as the reagent gas at a source pressure of 1.3 mbar. Quantitation was performed with the instrument operated in the selective-ion monitoring mode, with dwell times of 40–60 ms for each ion monitored. Ions 312, 314 and 319 were monitored, corresponding to $[M]^-$ for $[^{12}C]$-, $[^{13}C_2]$- and the $[D_7]$BE, respectively. Similarly, ions 131, 133, and 138, corresponding to $[M - CH_2C_6F_5]^-$ were monitored for $[^{12}C]$-, $[^{13}C_2]$- and the $[D_7]$BAA, respectively. For the analysis of selected rat urine samples, ion 298 was monitored for 3-methyl-1-butanol and 129 for 2-methoxyhexanoic acid, which corresponded to $[M]^-$ and $[M - CH_2C_6F_5]^-$, respectively.

3. Results and discussion

3.1. Chromatography

The GC–MS method used in this analysis was found to be sufficiently sensitive and selective for the determination of BE and BAA in rat and human blood. Selected-ion monitoring was performed for the parent $[M^-]$ ion of the BE isotopomers and the $[M - CH_2C_6F_5]^-$ fragment ion of the BAA isotopomers, as their respective derivatives (Fig. 1). GC–NCI-MS chromatograms of control and fortified human and rat blood, as well as blood from humans exposed to $[^{13}C_2]$BE [16], are presented in Figs. 2–6. An interference was observed for BE which contributed the equivalent of 19 ng BE/g for the rat blood standards (Fig. 5) and 10 ng BE/g for the human blood standards (Fig. 2). The interference was observed in solvent standards at levels similar to those observed in the blood standards, indicating that the source was the reagents used.

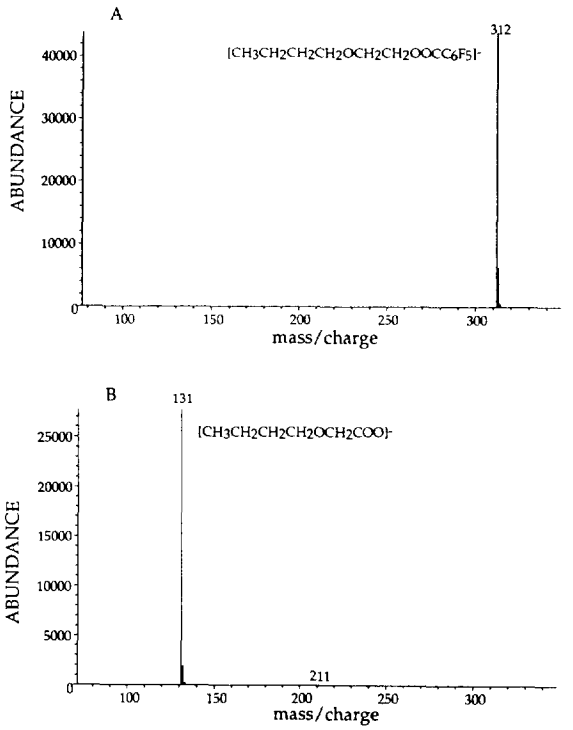

Fig. 1. NCI-mass spectra. (A) standard solution of BE as pentafluorobenzoyl derivative (0.2 mg/ml); (B) standard solution of BAA as pentafluorobenzyl derivative (0.2 mg/ml).

As a result, the magnitude of the interference was consistent, which allowed for accurate correction of the BE analyte response. Efforts to identify which reagent(s) was the source of this background were unsuccessful. The relative magnitude of the background interference was observed to increase dramatically if the standard extract was concentrated after derivatization, so this step was not performed. No interferences were observed for the other analytes or internal standards.

The blood matrix did not negatively impact the analysis of BE and BAA. No difference was observed for the BE and BAA peak shapes between standards prepared with and without blood. Peak response, though, was found to be impacted by the presence of blood. Absolute peak areas were found to be five-fold greater for standards prepared with blood. As a result of the deuterated internal standards, the analyte:internal standard ratio was not affected by this change in absolute response. The use of the internal standard as described in this method normalized not only the instrument response, but also the sample preparation (e.g. derivatization and extraction efficiencies), both of which are potential causes of the observed response differences between solvent and matrix standards.

3.2. Quantitative analysis

Triplicate standards prepared in control whole rat blood demonstrated excellent linearity by GC–NCI-MS over ranges of 22–29 000 ng/g BE and 5–27 000 ng/g BAA. Using a least-squares plot of the data for each analyte (concentration vs. peak-area ratio), the correlation (r) was 0.99983 and 0.99933 for BE and BAA, respectively. The limit of quantitation for BE was limited by the background interference, not the response of the instrument. No background BAA was observed in the control rat blood. Based on prior metabolism studies in rats [17,18] or guinea pigs [19,20] in which blood concentrations of BAA and BE were investigated, the quantitation range of this method goes well below the reported concentrations. This expansion of the analytical range allows for analysis of samples with lower concentrations resulting from lower dose concentrations, different routes of exposure, blood collection further past the time of dosing, etc.

Human blood standards also demonstrated excellent linearity and the use of the $^{13}C_2$-labeled analytes allowed for a reduction in the limit of quantitation. The linear range of BE was 16–1104 ng/g ($r = 0.99993$) while [$^{13}C_2$]BE was linear over a range of 1.5–745 ng/g. The difference in linear range was due to the interference for unlabeled BE. Endogenous BAA was observed which limited the linear range of [^{12}C]BAA. In the four volunteers sampled, the background BAA ranged from 2.5 to 12 ng/g. The linear range of BAA was determined to be 18–1150 ng/g ($r = 0.99967$). For the [$^{13}C_2$]BAA, in which no interference was observed, the linear

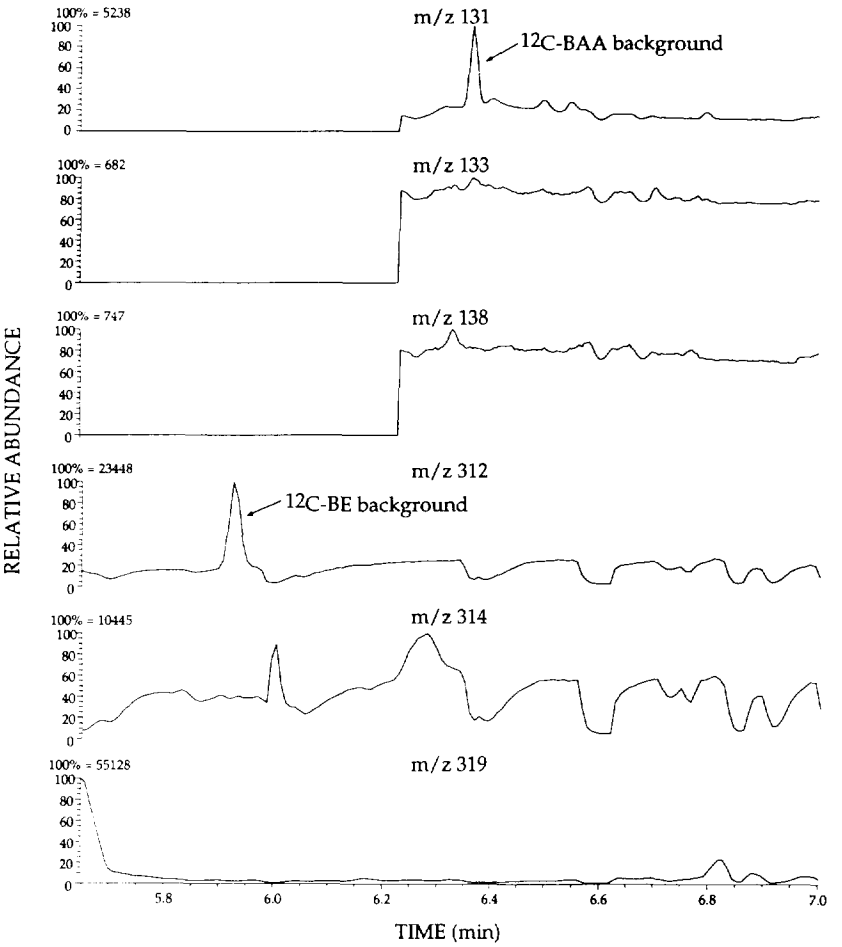

Fig. 2. Selected-ion chromatograms (NCI) of a derivatized control human blood extract.

range was 0.4–638 ng/g. These data demonstrate the advantage of using stable isotope labeled analytes for test materials in controlled experiments. One such experiment in which human volunteers were dermally exposed to a [$^{13}C_2$]BE vapor [16] demonstrates the utility of this method.

The precision of the method was determined by preparing triplicate standards in both human and rat blood. The relative recovery (mean ± S.D.) for triplicate standards of each compound is presented in Tables 1–3. For all analytes, the coefficient of variation was generally less than 10% for concentrations above 5 ng/g and did not exceed 20% at any concentration.

Inter-day precision of this method was investigated using fortified samples and standards prepared with human blood. Sets of matrix standards and blood samples fortified with [$^{13}C_2$]BE and [$^{13}C_2$]BAA were prepared and analyzed on several different days (represented by "*n*" in Table 4). Single samples were prepared each day within the concentration ranges. The mean relative recovery of the fortified samples compared to matrix standards of the same day was determined along with the relative standard deviation of this recovery across all analyses in a given concentration range (Table 4). The relative standard deviations across all concentration levels for [$^{13}C_2$]BE and [$^{13}C_2$]BAA were 5.5–

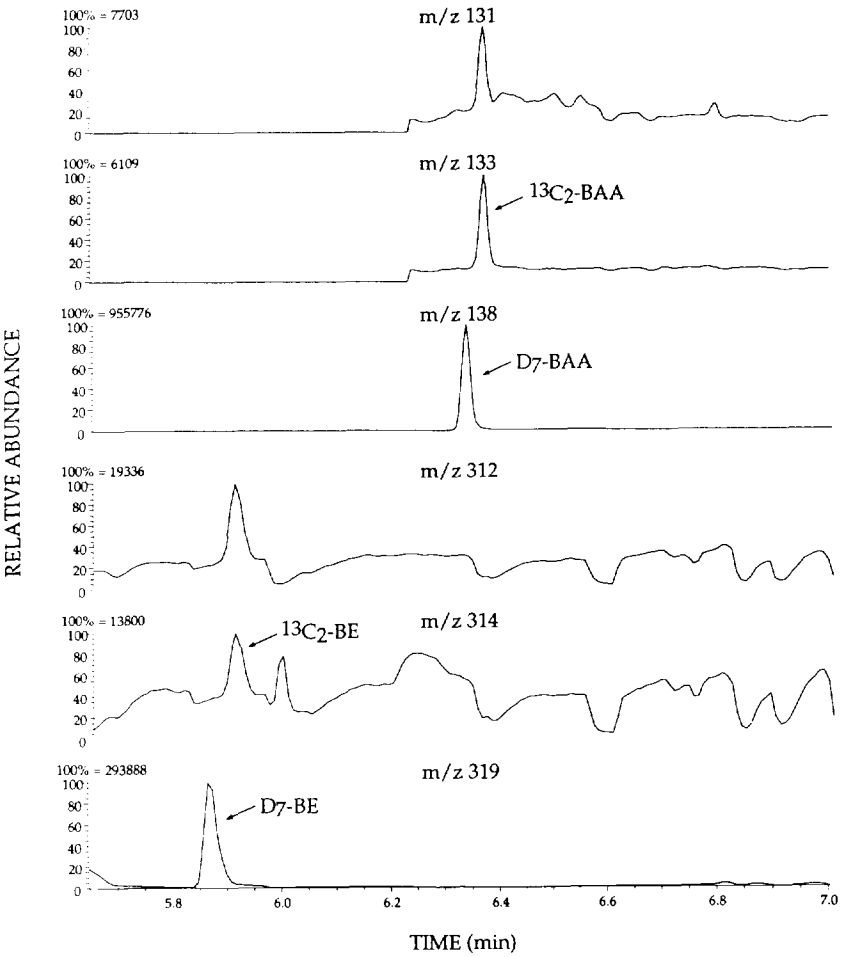

Fig. 3. Selected-ion chromatograms (NCI) of fortified human blood containing [$^{13}C_2$]BAA (1.6 ng/g), [D_7]BAA (480 ng/g), [$^{13}C_2$]BE (12 ng/g) and [D_7]BE (460 ng/g), as pentafluorobenzyl and pentafluorobenzoyl derivatives, respectively.

9.5% and 7.3–13.5%, respectively, with 5–7 determinations at each level.

3.3. Analysis of rat urine to investigate the stability of conjugates during sample preparation

Glucuronide and sulfate conjugates of BE have been identified as urinary metabolites of BE in previous rat metabolism studies [21]. The GC–MS method described in this paper was developed to measure only nonconjugated BE and BAA present in rat blood. To examine the stability of any phase II conjugates of these two analytes during the sample preparation for GC–MS quantitation, urinary metabolites obtained from metabolism studies using [^{14}C]BE were analyzed [18]. Urine samples, hydrolyzed with enzyme and acid, were analyzed via HPLC as well as with the current GC–MS quantitative method. Analysis by both GC–MS and HPLC (radiochemical detection) found that the BE levels were ten-fold higher in the hydrolyzed samples compared to those in unhydrolyzed urine. These data indicate that the sample preparation for the GC–MS analysis of BE in blood, described in this paper, would not be expected to hydrolyze any conjugates of BE present in this matrix.

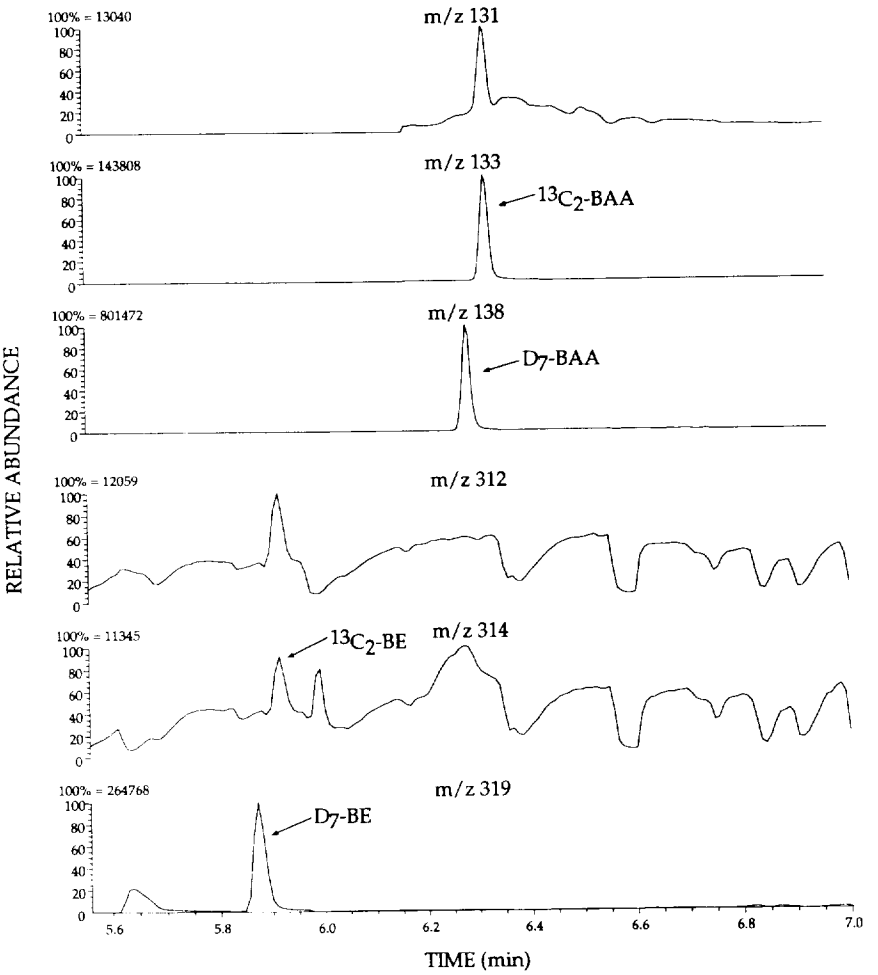

Fig. 4. Selected-ion chromatograms (NCI) of human blood containing 59 ng [$^{13}C_2$]BAA/g and 11 ng [$^{13}C_2$]BE/g from volunteers exposed dermally to a 50 ppm vapor of [$^{13}C_2$]BE for 2 h (internal standards fortified at 480 ng [D_7]BAA/g and 460 ng [D_7]BE/g), as pentafluorobenzyl and pentafluorobenzoyl derivatives, respectively.

No conjugates of BAA were observed in the prior rat metabolism studies, so similar data for BAA is not available. However, Rettenmeier et al. [22] have reported a glutamine conjugate of BAA present in urine from humans exposed to BE. Future studies with BAA in humans, using the GC–MS method described in this paper, should provide data on the stability of that conjugate during sample preparation and derivatization.

Methylene chloride extracts of blood containing derivatized BE and BAA were found to be quite stable. Measurement of analyte:internal standard ratios showed no significant change over a period of three weeks (data not shown).

3.4. Storage stability

The stability of blood samples stored at −80°C (rat) and −20°C (human) was investigated since it is probable that blood samples will not be analyzed immediately following collection. The recovery of ^{13}C-labeled analytes was determined from fortified rat and human control blood stored for 32 days ($n = 3$ at all levels). Human blood fortified at 57 and 570 ng BE/g had

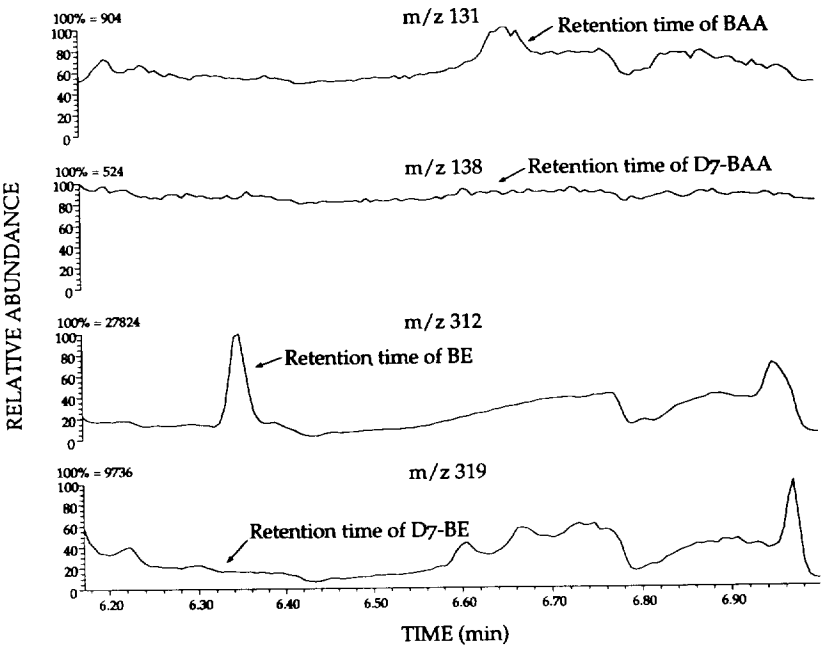

Fig. 5. Selected-ion chromatograms (NCI) of a derivatized control rat blood extract.

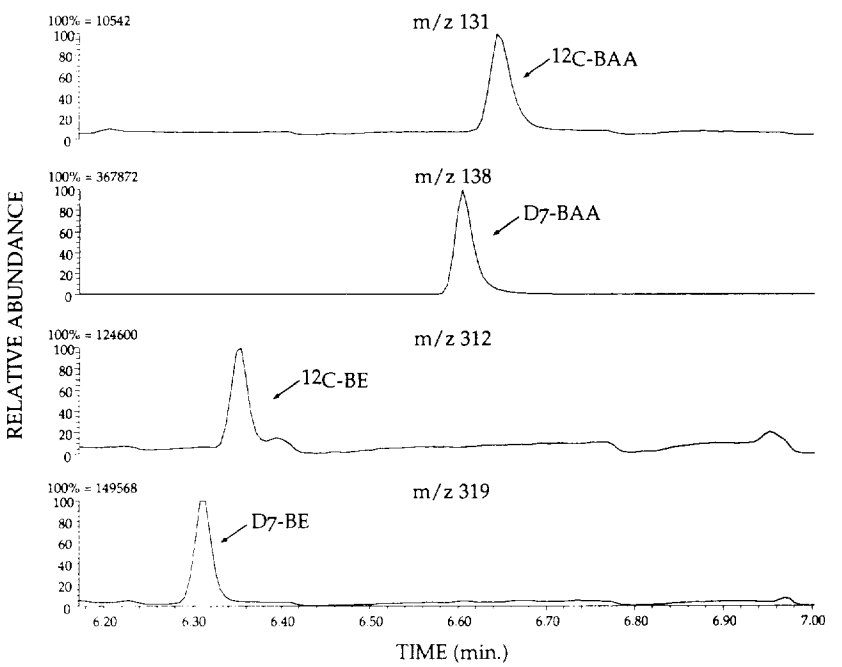

Fig. 6. Selected-ion chromatograms (NCI) of control rat blood fortified with BAA (32 ng/g), [D_7]BAA (900 ng/g), BE (30 ng/g) and [D_7]BE (900 ng/g), as pentafluorobenzyl and pentafluorobenzoyl derivatives, respectively.

Table 1
Precision of BE and BAA in rat blood ($n = 3$)

BE			BAA		
Concentration (ng/g)	Relative recovery (%)	C.V (%)	Concentration (ng/g)	Relative recovery (%)	C.V. (%)
21	105	9	6	93	19
30	104	3	12	105	16
104	90	6	22	109	8
345	97	3	32	107	10
1036	106	3	108	100	8
2761	103	1	360	100	6
Mean	101	6	1079	97	6
			2876	97	3
			Mean	101	6

Table 2
Precision of unlabelled BE and BAA in human blood ($n = 3$)

BE			BA		
Concentration (ng/g)	Relative recovery (%)	C.V. (%)	Concentration (ng/g)	Relative recovery (%)	C.V. (%)
17	97	7	17	104	7
28	102	5	29	99	1
83	104	5	86	98	3
276	100	3	288	97	3
1104	98	1	1150	104	3
Mean	100	3	Mean	100	3

Table 3
Precision of [$^{13}C_2$]BE and [$^{13}C_2$]BAA in human blood ($n = 3$)

[$^{13}C_2$]BE			[$^{13}C_2$]BAA		
Concentration (ng/g)	Relative recovery (%)	C.V. (%)	Concentration (ng/g)	Relative recovery (%)	C.V. (%)
1.5	101	10	0.4	97	12
4	87	14	1.3	97	12
10	105	2	3	103	1
22	106	4	9	102	6
74	103	4	19	106	4
248	101	3	64	101	4
745	96	3	213	98	1
Mean	100	6	638	97	4
			Mean	100	3

Table 4
Inter-day precision in the determination of [$^{13}C_2$]BE and [$^{13}C_2$]BAA from fortified human blood

[$^{13}C_2$]BE				[$^{13}C_2$]BAA			
Concentration (ng/g blood)	Recovery (%)		n	Concentration (ng/g blood)	Recovery (%)		n
	Mean	R.S.D.			Mean	R.S.D.	
4–6	92.3	5.5	6	3–5	102.6	13.5	7
10–15	97.7	7.2	5	9–13	107.8	8.9	5
38–75	101.8	9.5	6	40–64	103.0	10.7	5
91–143	103.3	6.6	7	100–168	102.4	8.0	7
226–287	103.4	7.0	7	213–299	102.1	7.3	6

recoveries of 85 ± 2% and 96 ± 6%, respectively. Human blood fortified with BAA at levels of 49 and 490 ng/g had recoveries of 91 ± 1% and 91 ± 4%, respectively. The recovery from control rat blood fortified at 62 ng BE/g and 53 ng BAA/g was 100 ± 8% and 102 ± 8%, respectively. No interferences with either ^{13}C-labeled analyte were observed in the control blood stored along with the fortified samples.

4. Conclusions

A sensitive and selective GC–MS method has been developed for the determination of BE and BAA in rat and human blood. The sample preparation overcomes two major limitations of previously published methods by being relatively simple and allowing for the simultaneous quantitation of both analytes. BAA is extractively alkylated with pentafluorobenzyl bromide into methylene chloride. BE is also extracted into the methylene chloride and subsequently derivatized with pentafluorobenzoyl chloride to yield the pentafluorobenzyl and pentafluorobenzoyl derivatives of BAA and BE, respectively.

Deuterated internal standards were synthesized and used for quantitation. The limits of quantitation of unlabeled BE and BAA (17 ng/g in human blood; 22 and 5 ng/g, respectively, in rat blood) were significantly lower than those of previously reported methods. The limits of quantitation for ^{13}C-labeled BE and BAA were 1.5 and 0.4 ng/g, respectively, demonstrating that a ^{13}C-labeled test material would yield a lower quantitation limit for BE and its metabolites relative to an unlabeled test material.

Acknowledgement

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