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Quantitative determination of the 1,3-butadiene urinary metabolite 1,2-dihydroxybutyl mercapturic acid by high-performance liquid chromatography/tandem mass spectrometry using polynomial calibration curves☆

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ABSTRACT

1,3-Butadiene is used in the production of synthetic rubber and is also a widespread environmental pollutant, produced by car exhaust, heating and cigarette smoke. According to IARC it is probably carcinogenic to humans. A method was developed and validated for the quantification in human urine of 1,2-dihydroxybutyl mercapturic acid, a butadiene metabolite for which the American Conference of Governmental Hygienists suggests a biological exposure index of 2500 μg/L. Solid phase extraction was used for analyte extraction and HPLC–MS/MS for detection. The calibration range from 20 to 2500 μg/L required the use of polynomial calibration curves, and the performance of the analytical method was tested according to an international validation guideline. Accuracy was never less than 85%, precision always higher than 15% and the LOD 3.6 μg/L. The method was applied to 33 non-smokers, non-occupationally exposed to butadiene, and gave urinary concentrations between 16 and 599 μg/L.

1. Introduction

1,3-Butadiene (BD) is a flammable, colorless, reactive gas with a pungent, aromatic, gasoline-like odor. It is a major industrial chemical used in the manufacture of synthetic elastomers (rubbers and latexes) and for producing raw materials for nylon. Occupational exposure to BD arises during its production, its use as a chemical feedstock in the manufacture of other chemicals, the use of these other chemicals, and in a wide variety of miscellaneous processes involving petroleum refining, secondary lead smelting and wastewater treatment.

BD is also an ubiquitous environmental pollutant whose major source is traffic; other non-occupational sources of BD exposure include inhalation of cigarette smoke [1,2] combustion products of fossil fuel [3], and products of incomplete combustion during forest fires.

In mainstream cigarette smoke, BD levels ranged from 16 to 75 μg/cigarette and in sidestream smoke from 205 to 361 μg/cigarette [1]. Environmental tobacco smoke (ETS)-related concentrations of this compound ranged from 3 to 19 μg/m^3 [1,4,5]. A study conducted in six European cities found median BD levels of 1.2 and 1.37 μg/m^3 respectively outside and inside homes [7].

Long-term exposure of humans has been associated with a wide variety of toxic responses, including lymphohematopoietic cancers [8–13]. Several national organizations and agencies such as IARC, ACGIH, EPA, NIOSH and DFG have classified BD as a carcinogen or probable carcinogen to humans and have established occupational exposure limits [4,6,14–17].

Inhalation is the main route of exposure: inhaled BD is partly eliminated unmetabolized in exhaled air or in urine and the rest is metabolized through cytochrome P450-catalyzed oxidation processes to highly reactive epoxides (butadiene monoepoxide and 1,2:3,4-diepoxybutane). The epoxides can be hydrolyzed to the corresponding hydroxy-metabolites and conjugation with glutathione leads to mercapturic acids which are excreted in urine [2,18,19]. The major BD-derived mercapturic acids are *R,S*-1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene (monohydroxybutenyl-mercapturic acid) and *R,S*-1,2-dihydroxy-

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4-(*N*-acetylcysteinyl)-butane [dihydroxy-butyl-mercapturic acid (DHBMA)] [20,21].

Recently ACGIH has proposed DHBMA in urine as a biomarker to assess the exposure to BD and has established a biological exposure index (BEI) of 2.5 mg/L with the notations Semi-quantitative "Sq" (the substance is an indicator of exposure to the chemical, but the quantitative interpretation of the measurement is ambiguous) and Background "B" (the substance may be present in biological fluids from subjects who have not been occupationally exposed). Assays for DHBMA in urine of workers occupationally exposed to BD have employed GC–MS or GC–MS/MS but lacked sensitivity to measure low exposures [22,23]. Some HPLC–MS/MS methods have now been developed with high sensitivity in a concentration range up to 1 mg/L of DHBMA in urine [24–26].

The aim of this study was to develop and validate a simple, reproducible and accurate method for the quantification of DHBMA in human urine for assessing occupational and environmental exposure to BD in the range to the BEI of 2.5 mg/L.

2. Experimental

2.1. Chemicals and supplies

The analytical reference standard of DHBMA and its deuterium-labeled isotope DHBMA-d_7 (used as internal standard) were purchased from Spectra 2000 (Rome, Italy). Glacial acetic acid (100%; Merck, Darmstadt, Germany) was used for preparing the mobile phase and for the solid phase extraction (SPE), with purified water from a Milli-Q Plus system (Millipore, Milford, MA, USA). Methanol for HPLC/MS and SPE was supplied by J.T. Baker (Deventer, Holland). OASIS HLB cartridges (6 mL, 0.2 g) for SPE and the SPE vacuum manifold were supplied by Waters (Milford, MA, USA). Anotop 10 LC syringe filter devices (0.2 μm pore size, 10 mm diameter) were purchased from Whatman Inc. (Maidstone, UK). A Phenomenex Synergy 4U Fusion RP C-18 column (150 mm × 4.6 mm i.d., 80A) was supplied by Chemtek Analytica s.r.l. (Bologna, Italy). Control human urine samples for standard calibration curves and quality control (QC) samples were obtained from healthy, non-smoking volunteers.

2.2. Standard solutions

One milligram of DHBMA was dissolved in 10 mL of methanol to obtain a standard solution of 100 mg/L (A). One further dilution was prepared at the concentration of 10 mg/L (B).

A solution containing 100 mg/L of internal standard was obtained from 1 mg of DHBMA-d_7 dissolved in methanol (solution C). By mixing suitable volumes of A, B and C, nine calibration standards in methanol were prepared containing DHBMA concentrations of 20, 50, 100, 250, 500, 1000, 1500, 2000 and 2500 μg/L, and 500 μg/L of internal standard. Solutions A, B and C were stored in the dark at 4 °C for subsequent use.

2.3. Urine standards

Urine calibration standards were prepared by adding DHBMA and internal standard to 1 mL of urine from healthy, non-smoking donors, and adjusted to pH 2 with 6 M HCl, to reach the theoretical concentrations of 0, 20, 50, 100, 250, 500, 1000, 1500, 2000 and 2500 μg/L of DHBMA and 500 μg/L of DHBMA-d_7. Urine standards were then purified on SPE cartridges using a modification of the procedure described in Ref. [25] as described below.

The SPE cartridge was conditioned with 3 mL methanol, then with 3 mL 0.1% acetic acid. The urine standard was loaded onto the cartridge which was washed with 1 mL 0.1% acetic acid, then eluted with 2 mL methanol. The eluate was filtered on a 0.2 μm syringe filter, and 10 μL were injected into the HPLC–MS/MS system. Each standard was tested in duplicate.

Since DHBMA can be found in the urine of non-smokers non-occupationally exposed to butadiene, true blank urine samples do not exist. Therefore we call the samples from non-smoking, non-occupationally exposed subjects, without added DHBMA, our "blanks". The DHBMA concentration in urine standards is the sum of the theoretical concentration plus the amount already present in the "blank" urine.

2.4. Matrix effect

To determine the matrix effect of different urines on the instrumental response following the procedure suggested by Matuszewski et al. [27], nine blank urine samples were submitted to the SPE procedure and spiked after elution in order to reach the same final concentrations as the nine urine calibration standards described above. These samples were analyzed by HPLC–MS/MS in duplicate. This procedure was repeated with five separate urines from different donors.

2.5. Calibration curves

Five independent sets of calibration curves, in methanol and in urine, and five sets of the corresponding matrix standards were analyzed on five different days, three of them not consecutive. Each pair of urine and matrix calibration curves was prepared from the urine of a different donor. Samples were analyzed in duplicate and the average was used.

2.6. Quality control samples (QC)

Five replicates of six standards in urine spiked with concentrations of 20, 50, 100, 500, 1000 and 2000 μg/L of DHBMA, independent of the calibration standards, and containing 500 μg/L of deuterated internal standard were prepared (5 mL each) and 1 mL was tested the same day in order to assess intra-day precision (for a total of 30 samples); the remainder was divided into 1-mL aliquots, stored at −80 °C and tested on four subsequent days in order to assess the inter-day precision.

2.7. HPLC–MS/MS conditions

Samples were analyzed on a Series 200 LC quaternary pump (PerkinElmer, Norwalk, CT, USA) using a 150 mm × 4.6 mm, 80A Phenomenex Synergy 4U Fusion RP C-18 analytical column, maintained at 70 °C. Elution followed the scheme reported in Table 1, using methanol (mobile phase A) and 0.1% acetic acid v/v in water (phase B), flow rate 1 mL/min. In these conditions, the retention time of DHBMA and of the internal standard is 5 ± 0.5 min (when working with the column at 70 °C, the retention time can vary slightly due to the solvent temperature and column aging. This variability does not affect the peak area). Total run time was 9 min.

The ion source of the AB/MDS Sciex API 4000 triple quadrupole mass spectrometer can accept a mobile phase flow rate up to 1 mL/min so the HPLC eluate was fed entirely into the Turbo Ion

Table 1
HPLC elution scheme.

Step	Time (min)	% A phase	% B phase
Isocratic	1	10	90
Linear gradient	3	70	30
Isocratic	3	70	30
Linear gradient	2	10	90
Total run time	9		

Table 2
Triple quadrupole MS/MS detector experimental conditions.

API 4000 acquisition parameters	
Source type	Turbo spray
Source temperature	400 (°C)
Scan type	MRM
IS (ion spray)	−4500 (arbitrary unit)
CEM (continuous electron multiplier)	2000 (V)
Dwell time	200 (ms)
CUR (curtain gas)	20.00 (psig)
GS1 (gas 1)	16 (psig)
GS2 (gas 2)	0 (psig)
CAD (collisionally activated dissociation)	4.00 (arbitrary unit)
EP (collision cell entrance potential)	−10.00 (ΔV)
CXP (collision cell exit potential)	−8.00 (ΔV)
CE (collision energy value)	−20 (ΔV)
DP (cluster-breaking orifice voltage)	−34.5 (ΔV)

Spray (TIS) probe. The source temperature was set at 400 °C. Detection was in the negative ion, multiple reaction monitoring (MRM) mode, and parameters were optimized for the analytes by the automated "Infusion Quantitative Optimization" procedure and subsequently refined by flow injection analysis (FIA) using the pure standards; values are reported in Table 2. The following ion pairs (precursor → product) were monitored: m/z 250 → m/z 121 for DHBMA and m/z 257 → m/z 128 for the deuterated internal standard. Fig. 1 shows the chemical structures of DHBMA (parent ion, m/z 250) and the product ion selected (m/z 121). Version 1.4 of the Analyst® software was employed for instrument control and data acquisition.

2.8. Analyses of urine samples

The urine of 33 non-smoking volunteers, non-occupationally exposed to BD, were collected and tested with this method for their DHBMA concentration. The urine samples were stored at −20 °C until analysis (maximum 2 weeks).

3. Results and discussion

3.1. Data processing and calibration curves

The peak areas generated by HPLC/MS–MS analysis of the samples were integrated by the 1.4 Analyst® software. The calibration range selected is from 20 to 2500 μg/L of added DHBMA. The lowest calibration level is chosen on the basis of the lowest value found in controls [24], and the highest point is the ACGIH BEI.

Best fitting of the curve was done using the statistical software R (free on line). Although a simple linear regression is sufficient in some cases, a higher order polynomial law should be introduced to get better results. In this case the linearity range is from 0 to 500 μg/L, but on account of saturation phenomena, a second order polynomial regression curve is needed for a wider range. The International Conference of Harmonization (ICH) guidelines for method validation [28] mentioned that for some analytical procedures which do not show linearity the analytical response should be described by an appropriate function of the concentration of an analyte sample. Picò et al. [29] report that the MS/MS detector has a low dynamic range, and that second and even third order equations

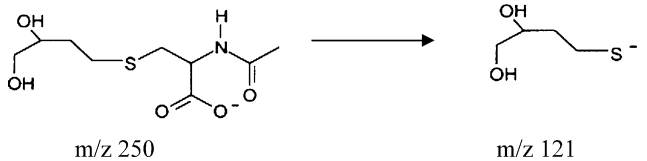

Fig. 1. Chemical structure of 1,2-dihydroxybutyl mercapturic acid (DHBMA) (parent ion, m/z 250) and of the ion fragment selected for monitoring (m/z 121).

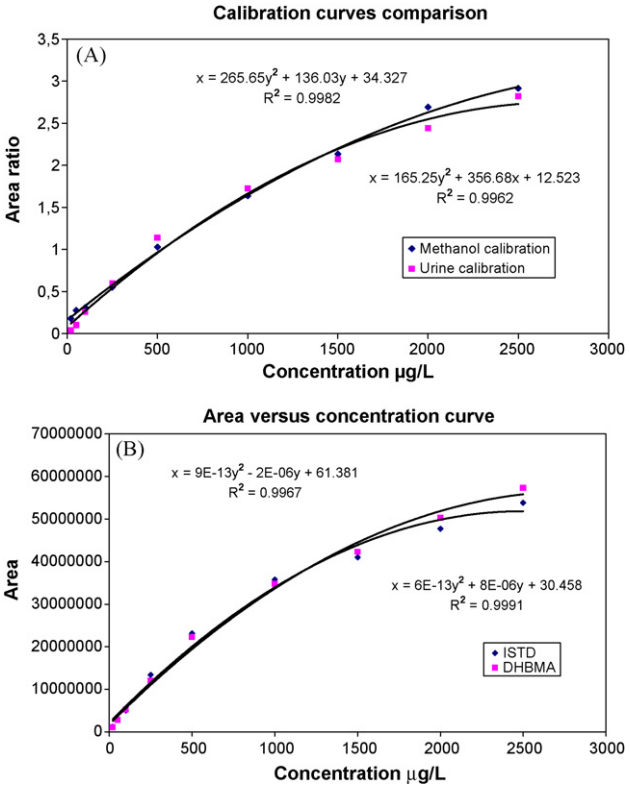

Fig. 2. Typical calibration curves in methanol and in urine (DHBMA/ISTD area ratio versus concentration) (A) and polynomial regression curves for instrumental response (area) versus concentration of DHBMA and internal standard (B).

may be used to express the detector response to concentration relationships when wide calibration ranges are needed. This solution has also been applied in other studies [30].

To distinguish a simple model (i.e. linear correlation) from a more complicated one (i.e. second or third order polynomial) two different procedures were used: (1) the Akaike information criterion (AIC) [31] which adds to the log-likelihood function a penalty increasing with the number of parameters of the fitted model: the smaller the AIC function, the better the fit; (2) an F-test in which the F ratio quantifies the relationship between the relative increase in sum-of-squares and the relative increase in degrees of freedom:

$$F = \frac{(SS1 - SS2)/SS2}{(DF1 - DF2)/DF2} \quad (1)$$

where SS1 and SS2 are the sum-of-squares respectively for a simple model and a more complicated one, and DF1 and DF2 are the degrees of freedom of the two models. If the p value (probability of the null hypothesis) for the F variable of the test is low, one can conclude that the more complicated model 2 is significantly better than the simpler model 1.

The calibration curves were generated using polynomial regression analysis according to the equation $y = ax^2 + bx + c$, where x is the ratio of the DHBMA peak to that of the internal standard, and y is the DHBMA concentration for each calibration standard, in methanol and in urine. Typical calibration curve concentration to area ratios in methanol and in urine are reported in Fig. 2A. The AIC was used to discriminate between the quadratic polynomial law and the simpler linear model and the more complex third order polynomial law. The second order polynomial equation resulted in the curve with the lowest AIC function value. The test based on the F ratio, defined in Eq. (1), confirmed the AIC results. Five different calibration curves in methanol were obtained; the data fitting a second order polynomial law always yielded determination coefficients greater than 0.993.

Table 3
Validation report of the proposed HPLC/MS/MS method.

Calibration range	(20 + blank)–(2500 + blank) μg/L	
Calibration curve	Second order polynomial	
Detection limit (LOD)	3.6 μg/L	
Quantitation limit (LOQ)	12.2 μg/L	
	Mean accuracy	Precision
Inter-assay–low spike (20 μg/L)	96.1%	12.1%
Inter-assay–high spike (2000 μg/L)	111.3%	9.1%
Intra-assay–low spike (20 μg/L)	88.2%	4.8%
Intra-assay–high spike (2000 μg/L)	94.8%	3.6%

For the calibration curves in urine the results were very similar to those in methanol. In some cases a simple linear law yielded the prediction of negative concentrations corresponding to very small DHBMA/ISTD area ratios. Like in methanol, five curves were obtained in urine with determination coefficients always better than 0.993. Again, polynomial laws of different orders were compared by means of the AIC and the F ratio-based test.

Fig. 2B presents the calibration curves in methanol in the range 20–2500 μg/L, both for DHBMA and for the internal standard DHBMA-d_7, in order to verify that the relationship between the concentration and the relative areas followed a polynomial equation for both analytes.

In analytical methods where calibration curves are generated using linear regression analysis, the area of the "blank" urine has to be subtracted from the areas of the calibration samples. This is not applicable to polynomial regression curves as areas are not additive. Therefore the concentration of the "blank" was evaluated on the polynomial calibration curve in methanol and added to the theoretical concentration of urine calibration samples in order to generate the polynomial calibration curve in urine matrix.

3.2. Method validation

The analytical method was validated according to the ICH [28] and results are summarized in Table 3. In addition, the matrix effect and the uncertainty of the measurement were determined.

3.2.1. Matrix effect and SPE yield

The matrix effect was evaluated for DHBMA and for the deuterated internal standard in five separate urines from five donors. The relative analyte response is the proportion (expressed as a percentage) between the DHBMA peak areas of the matrix standards and for the same concentrations of standards dissolved in methanol: we call it the "matrix effect yield". In these samples DHBMA was not subjected to SPE and values were between 39 and 116%, confirming that the MS/MS response varies significantly between urine donors.

SPE yield was calculated by comparing the peak areas produced by analysis of the urine calibration standards (spiked before SPE) to those of the matrix standards (prepared with the same urine but spiked after SPE) and was more than 78% over five independent experiments. We called "total yield" the product of matrix effect yield and SPE yield, which equals the ratio between the peak areas of the urine standards and those of the same concentrations of standards dissolved in methanol. Table 4 reports the complete results. (The values are the average of five results for each urine, and the last row shows the means of the five experiments.)

The best method of compensating for the matrix effect in quantitative analysis is to use an internal standard, preferably a stable isotope-labeled compound that mimics the analyte's behavior in terms of retention time, fragmentation and ionization, and will therefore give a very similar instrumental response. The matrix effect, if not compensated, can affect the accuracy and precision of the quantitation results.

3.2.2. Accuracy and precision

The inter-day accuracy and precision were determined from analysis of six independent QC samples at concentrations of 20, 50, 100, 500, 1000 and 2000 μg/L of added DHBMA tested over the 5 days of the validation study. The accuracy was determined by calculating the ratio between the concentrations found in the QC samples (from the regression curve) and the theoretical values calculated as the sum of the nominal concentration plus the blank concentration (see Section 3.1 on data processing). The results range from 93 to 111%. Precision is expressed as the relative standard deviation (R.S.D.) of the values found over the mean for each concentration and was 12.4%.

Table 4
Total yield and its components: solid phase extraction (SPE) yield and matrix effect accuracy both for DHBMA and internal standard.

Urine donor	DHBMA in blank urine (μg/L)	Creatinine (g/L)	SPE recovery DHBMA (%)	SPE recovery DHBMA-d_7 (%)	Matrix effect accuracy DHBMA (%)	Matrix effect accuracy DHBMA-d_7 (%)	Total recovery DHBMA (%)	Total recovery DHBMA-d_7 (%)
1	17.5	1.2	87.1	91.9	116.0	93.9	102.3	86.6
2	79.9	1.2	102.7	94.0	49.6	39.3	48.4	37.0
3	47.4	0.7	78.7	86.9	107.6	85.1	84.2	73.8
4	259.8	0.9	102.5	116.9	46.1	66.7	46.4	77.6
5	282.4	1.8	84.3	78.4	38.7	61.0	32.3	47.0
Mean			91.1	93.6	71.6	69.2	62.7	64.4
Standard deviation			11.0	14.3	37.0	21.4	29.3	21.3
R.S.D.			12.0	15.3	51.7	30.9	46.7	33.0

Table 5
Inter- and intra-assay accuracy and precision on urine quality controls.

	Added DHBMA (μg/L)					
	20	50	100	500	1000	2000
Mean accuracy (%)[a]	96.1	98.7	98.8	102.8	92.9	111.3
Standard deviation	11.6	5.8	11.9	12.8	11.4	10.1
Interday precision R.S.D. (%)	12.1	5.9	12.1	12.4	12.2	9.1
Mean accuracy (%)[b]	88.2	102.2	104.5	109.3	109.1	94.8
Standard deviation	4.2	3.6	2.3	4.9	1.0	3.4
Intra-day precision R.S.D. (%)	4.8	3.5	2.2	4.5	0.9	3.6

[a] Value is the average of % accuracy of five replicates tested in different days using different calibration curves.
[b] Value is the average of % accuracy of five replicates tested in the same day using the same calibration curve.

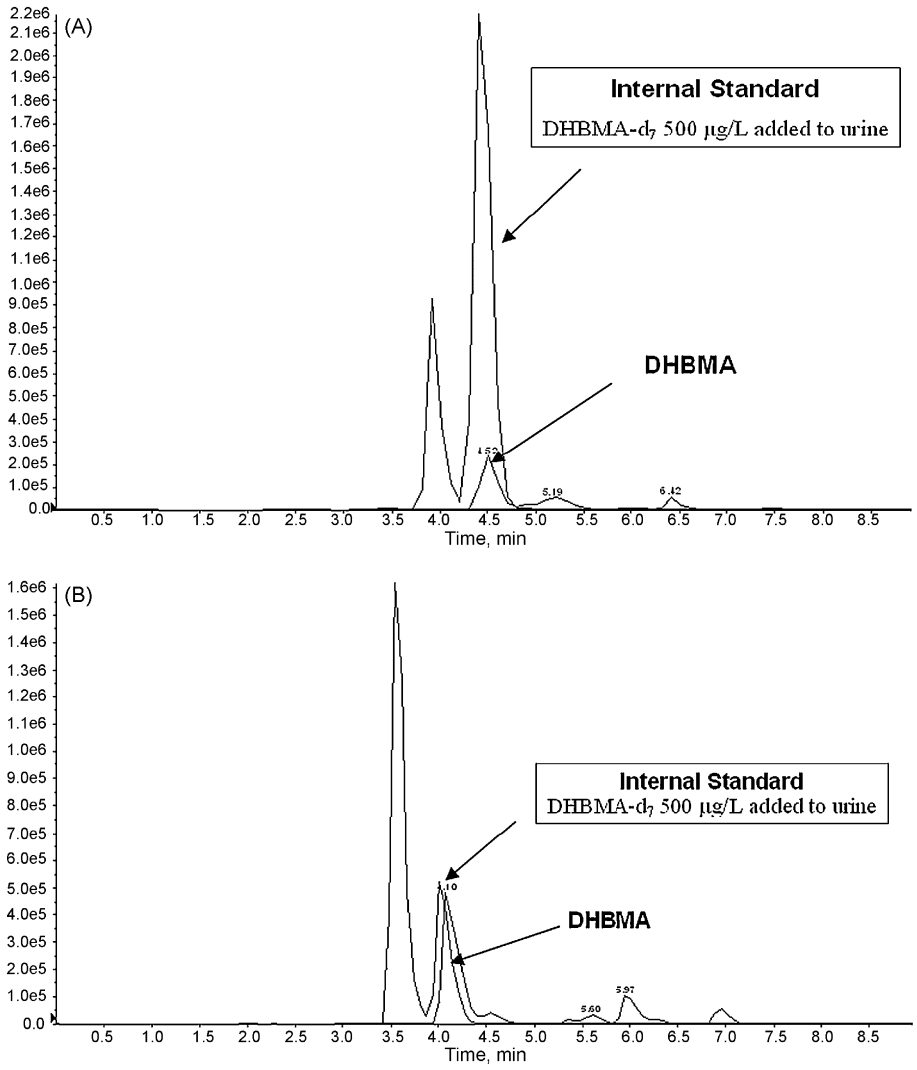

Fig. 3. Chromatogram of the urine sample of a non-smoking volunteer, containing 47 μg/L of DHBMA (A) and chromatogram of the urine sample of another volunteer, containing 599 μg/L of DHBMA (B).

The intra-day accuracy and precision were calculated by testing five independent replicates (five separate SPE columns) of the six QC samples (30 samples) on the same day. The accuracy, determined by comparing the means of the concentrations found in the QC samples with the theoretical values, ranged from 88 to 109% with the R.S.D. less than 5%. Table 5 summarizes the results.

3.2.3. Limits of detection and quantification

In order to determine the limits of detection and quantification (LOD and LOQ) we applied the approach based on the standard deviation according to ICH [28]. The analytical background was measured by analyzing 10 urine samples not spiked with the analyte, considering the area at a retention time very close to the analyte. The response is the ratio between the background and internal standard areas; three and ten times the standard deviation and the polynomial regression were used to calculate the LOD and LOQ, which were 3.6 and 12.2 μg/L. This sensitivity was considered adequate because the urinary levels in the general population reported in the literature [25] are well above 12 μg/L.

3.2.4. Uncertainty of measurement

The uncertainty of measurement was evaluated according to EURACHEM/CITAC guidelines [32]. Two independent sources of uncertainty were recognized: the component associated with the precision $u(p)$ and the component associated with the calibration curve $u(cal)$.

The contribution to uncertainty due to the precision was calculated from the R.S.D.s of the six QCs measured on different days. The *F*-test showed they were not significantly different, so they were combined to obtain the $u(p)$. The two components were combined according to the following equation, obtaining the relative combined uncertainty for the measurement of the DHBMA concentration:

$$\grave{u}(c) = \sqrt{(\grave{u}(p))^2 + (\grave{u}(cal))^2}$$

The relative expanded uncertainty was calculated using a coverage factor of 1.96 at a confidence level of 95%. The relative uncertainty in precision was 0.110 and that of the calibration curve at the concentration of 2000 μg/L, close to the BEI, was 0.012. The relative combined uncertainty was 0.11, and the relative expanded uncertainty was 0.22 (or 22%).

3.3. Application of the method

The analytical method was employed for the quantitative determination of DHBMA in the urine of 33 non-smoking subjects, non-occupationally exposed to BD. The mean value (166 μg/L,

range 16–599 μg/L) was lower than reported by other authors [21,24,25,33]. The chromatograms of two urine samples, one containing 47 μg/L and the other 599 μg/L of DHBMA and 500 μg/L of isotope-labeled internal standard are reported in Fig. 3A and B.

4. Conclusions

The analytical method presented is useful for the quantitative determination of DHBMA in human urine for biological monitoring of BD exposure. The isotopic dilution method, which is strongly recommended for quantitative HPLC–MS/MS determination, using a commercially available deuterium-labeled isotope of the analyte, rendering the results independent of the relative instrumental response and ion suppression or ion enhancement effects due to the urine matrix.

The wide calibration range selected, from 20 to 2500 μg/L, requires the use of polynomial regression curves, and the optimal performances of the analytical method were verified using nine calibration points and six independent QC samples. A calibration range with an upper end lower than 2500 μg/L, for which linear regression can be used, would require the dilution and re-analysis of the samples with higher concentrations in order to avoid the risk of obtaining unreliable DHBMA values. This is particularly important in the assessment of occupational exposure as the values near to or higher than the BEI indicate a risk of adverse health effects.

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