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Methodological issues in the biological monitoring of urinary benzene and S-phenylmercapturic acid at low exposure levels☆

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ABSTRACT

Biological monitoring of low level exposure to pollutants is a very challenging analytical activity, and the quality of results is difficult to assess, especially when a certified reference material is unavailable. The aim of this work was to evaluate the reliability of the assays used to measure urinary benzene (Benz-U) and S-phenylmercapturic acid (SPMA), by applying an internal quality control protocol. Urine spot samples from 705 subjects who were either members of the general urban population, gasoline station attendants, or refinery plant workers were assayed for Benz-U and SPMA, using GC/MS and LC/MS/MS, with quantification limits of 15 ng/L and 0.10 μg/L. The median Benz-U concentration was 263 ng/L (60–2789 ng/L, 5th–95th percentile), and the median SPMA concentration was 0.19 μg/L (<0.1–2.5 μg/L, 5th–95th percentile). Linearity of both assays was good, but a less-than-proportional response was found for SPMA concentrations below 1 μg/L. Between-run precision and accuracy for Benz-U concentration determination were assessed using quality controls at 120 ng/L and 1000 ng/L and were 10.3% and 4.8%, and 104.8% and 98.9%, respectively; while the precision and accuracy for SPMA concentration determination at 0.3 μg/L, 2.5 μg/L, and 20 μg/L were 40.3%, 6.2%, and 6.2%, and 48.3%, 96.3%, and 98.8%, respectively. Precision, estimated using duplicates of unknown samples, was 13.4% for Benz-U and 26.5% for SPMA analyses. Control charts for the means of the slope of the linear calibration curve of Benz-U showed good stability of the means over a five-year period. For SPMA, a two-laboratory comparison revealed acceptable agreement between ln-transformed data pairs, with a slope of the linear regression of 0.863 (confidence interval 0.774–0.952), null intercept, and a Pearson's *r* value of 0.844. Reliable results were obtained for Benz-U analyses over the entire concentration range, and for high and medium SPMA levels. However, the determination of SPMA concentrations at levels close to the limit of quantification was less reliable.

1. Introduction

Benzene is one of the chemicals more widely spread into the environment and a ubiquitous pollutant of indoor and outdoor air, with auto vehicular traffic and cigarette smoking as its major sources. Benzene is a known carcinogen to humans [1], and evidence indicates that benzene causes hematotoxicity at exposure levels below 1 ppm [2].

For the biological monitoring of low levels of benzene, both urinary benzene (Benz-U) and urinary S-phenyl mercapturic acid (SPMA) have been indicated as the markers of choice [3–5]. Both are specific and well correlated with external exposure, and offer the advantage, over blood benzene, of not requiring an invasive blood drawing, which may be considered a relevant advantage for studies involving the general population.

From an analytical chemistry point of view, biological monitoring is a very challenging activity, requiring the determination of tiny concentrations of toxic compounds or their metabolites in complex biological fluids, typically blood and urine, that are often available in unique and small samples.

Nowadays, sophisticated analytical instrumentation is used in biological monitoring, with mass spectrometry as the technique of choice for specific and sensitive detection of analytes, coupled with different techniques, such as gas chromatography, liquid chromatography, and inductively coupled plasma source. A critical issue of these analytical techniques is the quality of results. In fact, it is known from basic analytical chemistry that imprecision becomes greater as the concentration to be determined becomes smaller (the Horwitz trumpet, as reported in [6]). Moreover, for several toxins

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of interests, and especially for the newest ones, certified reference material is lacking, and/or is available at concentrations not suitable for monitoring low exposures, as those observed in the general population. To face the issue of quality controls, specific proficiency testing schemes or external quality assessment are organized by some institutions such as the German Society for Occupational and Environmental Medicine, the Danish National Institute of Occupational Health, and the Centre de Toxicologie du Québec, Canada [7]. In addition, not all chemicals of interest are available as certified material. For example, there is wide coverage of metal analytes at a range of concentrations, but the possibilities are much more limited for organic compounds.

To cope with the issue of analytical quality in the case of chemicals for which no certified materials and no proficiency testing are available, there is a need for designing intra-laboratory protocols, which assess the precision and accuracy of data and check the performance of the assay with time.

The aim of this work was to evaluate the reliability of Benz-U and S-SPMA concentration determinations using gas chromatography–mass spectrometry (GC/MS) and liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS), in the presence of isotopically labeled internal standards. An internal quality control protocol was set up which included the use of multiple calibration curves, quality controls at different concentrations, independently prepared duplicates of unknown samples, use of Shewhart charts for the means and the ranges of the slope of the calibration curve, and a two-laboratory comparison.

2. Methods

2.1. Study samples and pre-analytical phase

Study samples consisted of 705 urine spot samples collected from subjects exposed to benzene in living or working environments. One group of subjects belonged to the general urban population living and working in the Milan area (269 samples), the second group consisted of gasoline station attendants (267 samples), and the third group consisted of petrochemical workers (169 samples).

Samples were collected in different studies, each of which was authorized by the appropriate ethics committee. All subjects were informed about the aim of the study and gave their written informed consent.

Urine spot samples were collected in plastic tubes. For Benz-U analysis, a 7 ml aliquot was promptly poured into a pre-evacuated and thermally-cleaned 8 ml glass vial, closed with a rubber lid with a PTFE lining, and crimped with an aluminum seal [8]. For SPMA analysis, a 5 ml aliquot was simply poured into a plastic tube. All samples were coded, and delivered to the laboratory at room temperature where they were stored at −20 °C until analysis.

All samples were analysed in the laboratory of Occupational and Environmental Toxicology, Milan, in 2007–2008. A subgroup of 149 samples from petrochemical workers, from whom duplicates were available, was also analysed in 2008 for SPMA in the laboratory of Industrial Toxicology, Parma, to perform a two-laboratory comparison.

2.2. Urinary benzene

The determination of Benz-U concentration was performed by headspace solid-phase microextraction (SPME) followed by GC/MS analysis in the presence of benzene-d_6 (Sigma–Aldrich, St. Louis, MO, USA) as the internal standard, according to a published method [9], with some modifications. Briefly, a 0.6 ml urine aliquot was poured into a 2 ml crimped top vial (National Scientific, Rockwood, TN, USA) containing 300 mg of NaCl. A volume of 0.5 μl of the internal standard solution of benzene-d_6 in methanol (IS_{Benz} at 475 μg/L) was added, and the vial was immediately sealed with a magnetic crimp cap with a silicone-PTFE septum (Gerstel, Mülheim an der Ruhr, Germany). The urine headspace was sampled for 5 min by the SPME technique using a polymethyldisiloxane (PDMS) 100 μm fiber (Supelco, Sigma–Aldrich, St. Louis, MO, USA) and thermally desorbed for 3 min by inserting the fiber into the chromatographic injection port. Sampling was operated at room temperature by a Gerstel MPS2 autosampler (Gerstel, Mülheim an der Ruhr, Germany) equipped with the SPME device. Analyte separation was performed by GC (Agilent 6890 Plus, Agilent Technologies Inc., Santa Clara, CA, USA) using a DB1 column (60 m length, 0.25 mm internal diameter, 1 μm film thickness; J&W Scientific Inc., Folsom, CA, USA). Quantification was done using an Agilent 5975 mass spectrometric detector (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an inert EI source (electron energy 70 eV). The split/splitless injector, operating in the splitless mode, was equipped with an inlet liner for SPME (0.75 mm internal diameter, Supelco, Sigma–Aldrich, St. Louis, MO, USA). The GC analysis was performed under the following conditions: helium carrier gas at a constant flow rate of 1 ml/min; injector temperature at 250 °C, gas chromatograph oven temperature programmed from 40 °C (3 min initial hold) to 70 °C at 3 °C/min, hold 1 min, and then to 250 °C at 20 °C/min (final temperature 5 min hold). The retention times of benzene and benzene-d_6 were 13.46 min and 13.34 min, respectively. The MS detection was performed under the following conditions: transfer line temperature at 280 °C; ion source temperature at 300 °C, single ion monitoring mode, registering the ions *m/z* 78 for benzene and 84 for benzene-d_6. The quantification limit was 15 ng/L.

2.3. Urinary SPMA (Milan laboratory)

The determination of urinary SPMA concentration was based on solid phase extraction (SPE) followed by LC/MS/MS analysis in the presence of SPMA-d_2 as the internal standard (CDN Isotopes Inc., Pointe-Claire, Quebec, Canada), following a previously published procedure with some modifications [10]. To a 2 ml urine sample was added 20 μl of internal standard solution containing SPMA-d_2 in methanol (IS_{SPMA} at 1.2 mg/L), and 1 ml of this solution was loaded onto a 3 ml Hypersep-SAX SPE tube (Thermo Fisher Scientific Inc., Waltham, MA, USA). The tube was rinsed with 2 ml of water, 2 ml of 2 mM phosphate buffer pH 6, 2 ml of 1% aqueous acetic acid, and finally 1 ml of 15% aqueous acetic acid. SPMA was eluted with 0.5 ml of 15% aqueous acetic acid. An aliquot of 10 μl of the eluate was analysed by high performance liquid chromatography (HPLC, Surveyor, Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a Betasil C18 column (150 mm length, 2.1 mm internal diameter, 5 μm particle size, Thermo Fisher Scientific Inc., Waltham, MA, USA) kept at room temperature. For the elution, an isocratic mixture of 0.5% aqueous acetic acid and methanol (1:1) at a flow rate of 0.25 ml/min was used. The HPLC instrument was interfaced with a triple quadrupole mass spectrometric detector (TSQ Quantum Access, Thermo Fisher Scientific Inc., Waltham, MA, USA). The retention times of SPMA and SPMA-d_2 were 4.55 min and 4.54 min, respectively. Analytes were ionized in negative-ion ESI, by applying a voltage of −5000 V, and keeping the temperature of the ion transfer tube at 290 °C. Quantification was based on selected reaction monitoring (SRM) following the transitions *m/z* 238 → 109 for SPMA and *m/z* 240 → 109 for SPMA-d_2 (collision energy 12 eV). The quantification limit was 0.1 μg/L, as evaluated from the sample giving a signal equal to five times the concentration, which corresponded to the standard deviation of the signal in the blank.

2.4. Urinary SPMA (Parma laboratory)

SPMA concentration was determined in untreated urine samples by isotopic dilution LC/MS/MS using a PE-Sciex API 365 triple quadrupole mass spectrometer (Applied Biosystems, Thornhill, Canada) equipped with a Turboionspray interface (TIS) for pneumatically assisted electrospray. SPMA-d_5, used as the internal standard, was purified from urine of rats treated with benzene-d_6, and purified by SPE and HPLC, as previously described [10]. Before analyses, urine samples were centrifuged at $3000 \times g$ for 10 min, SPMA-d_5 was added to the samples, and the samples were acidified with 0.1 M formic acid. A volume of 20 μl was then injected onto a Supelcosil LC-18-DB column (75 mm length, 3.0 mm internal diameter, 3 μm particle size; Supelco, Sigma–Aldrich, St. Louis, MO, USA). Elution was achieved at a flow rate of 0.50 ml/min by running a linear gradient starting from 2% (hold 1.5 min) to 80% methanol (in 6.5 min, and then holding for 1 min) in 20 mM aqueous formic acid. The retention times of SPMA and SPMA-d_5 were 9.08 min and 9.06 min, respectively. Analytes were ionized in negative-ion ESI, by applying a TIS voltage of −4000 V and keeping the ion source at 350 °C. Detection was performed in SRM mode following the transitions characteristic of the analyte and internal standard, m/z 238 → 109 for SPMA, and m/z 243 → 114 for SPMA-d_5 (collision energy 12 eV). The limit of detection, calculated as the signal to noise ratio greater than 3, was 0.1 μg/L.

2.5. Quality control procedures

2.5.1. Calibration and quality control solutions

Benz-U. To set the calibration curve, standard solutions of benzene in methanol at concentrations of 12,000, 8700, 6000, 3000, 1500, 600, 300, 150, 75, 37.5, and 12.5 μg/L were prepared. Quality control solutions containing benzene in methanol at concentrations of 144 μg/L and 1200 μg/L were independently prepared.

SPMA. To prepare the calibration curve and the quality controls, calibration standard solutions of SPMA in methanol/0.5% aqueous acetic acid (5:95) at concentrations of 2.5 mg/L, 0.25 mg/L, and 0.05 mg/L were prepared.

All the solutions, kept at −20 °C in glass vials, were stable up to six months.

2.5.2. Calibration curve and analytical sequence

For both Benz-U and SPMA analyses, matrix-added standard calibration curves and quality controls were prepared at the time of sample preparation. The appropriate calibration or quality control solution was added to blank urine, which was obtained from non-smoking donors.

Benz-U. Calibration solutions were obtained by adding 0.5 μl of each benzene standard solution and 0.5 μl of IS_{Benz} to 0.6 ml of urine. Two different calibration curves were prepared depending on the expected level of Benz-U in samples; for expected low exposures the curve included the final added benzene concentrations: 2500, 1250, 500, 250, 125, 60, 30, 10, and 0 ng/L; for expected high exposures the curve included the concentrations: 10000, 5000, 2500, 1250, 500, 250, 125, 60, and 0 ng/L. Two replicates for each calibration level were prepared. Low level quality control and high level quality control (L-$QC_{Benz\text{-}U}$ and H-$QC_{Benz\text{-}U}$) samples were obtained by adding 0.5 μL of each quality control solution and 0.5 μL of IS_{Benz} to blank urine at the final added benzene concentrations: 120 ng/L and 1000 ng/L.

SPMA. Calibration solutions were obtained by adding suitable volumes of SPMA standard solutions and 20 μl of IS_{SPMA} to 2 ml of urine. Also, in this case two different calibration curves were prepared; for low exposures the curve included the final added SPMA concentrations: 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.25, 0.12, and 0 μg/L; for high exposures the curve included the concentrations 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0 μg/L. Two replicates for each calibration level were prepared. Low level quality control, medium level quality control, and high level quality control (L-QC_{SPMA}, M-QC_{SPMA}, and H-QC_{SPMA}) samples were similarly prepared at the final added SPMA concentrations: 0.3 μg/L, 2.5 μg/L, and 20 μg/L; two quality controls were introduced in each sequence, based on the expected level of SPMA in the samples.

For the determination of both Benz-U and SPMA concentrations, between 20 and 60 samples were prepared in the same batch; about 7–10% of samples were prepared as independent duplicates; a suitable number of quality controls was also prepared so that each level was run about every 10–15 samples. The typical analytical sequence was set beginning with a calibration curve, followed by the unknown samples and duplicates, in which the quality controls were inserted at regular intervals, and ending with a second calibration curve

2.5.3. Shewhart charts for means and ranges

For Benz-U analyses, Shewhart charts for means and ranges of the slope of the linear regression were prepared using data from 20 consecutive analytical sequences, each of which yielded a slope (as mean value of two calibration curves), and a mobile range R, that were used to calculate the target mean value for the slope μ_0 and the mean mobile range $\overline{R}$. These values were used to allocate the target mean line, the upper and lower warning lines, and the upper and lower action lines on the y-axis [11,12].

2.6. Statistical analysis

A value corresponding to half of the quantification limit was assigned to measurements below the quantification limit. Due to the highly positively skewed distribution of both Benz-U and SPMA, median and percentiles were used to describe the data

Least squares linear regression analysis was applied to estimate the slope (m) and the intercept (q) of the function $y = mx + q$, where y is the ratio between the chromatographic peak area of the analyte *vs*. the chromatographic peak area of the internal standard subtracted by the analogous ratio of blank urine, and x is the concentration of the analyte in the calibration solution. In cases in which a less-than-proportional response was observed (i.e. for SPMA ≤ 1 μg/L), a quadratic curve was used to fit the experimental data.

Precision of the assay, expressed as percent coefficient of variation (%CV), was estimated using two different approaches. The first approach was based on quality controls, and allowed the evaluation of both within- and between-run precision at different concentrations; it was calculated as the ratio between the standard deviation and the mean of the repeated measurements [13].

The second approach was based on the use of data of the duplicates of unknown samples, and precision was estimated according to the equation:

$$\%CV = \sqrt{(e^{S_w} - 1)} \cdot 100$$

where S_w is the within-subject variability calculated using two-way ANOVA on the ln-transformed data [14].

Accuracy was determined by analysis of the quality controls and was expressed as a percent ratio between the concentration estimated from the calibration curve subtracted by the signal of blank urine, and the spiked concentration [13].

For the statistical evaluation of the two-laboratory comparison, SPMA data were ln-transformed to fit normal distribution and to reduce the orders of magnitude of the values to be compared; on these data the t-test for paired samples and linear regression analyses were applied [6]. A two-sided p-value of 0.05 was considered significant.

Table 1
Summary of results for Benz-U and SPMA in the study samples.

	N samples	LOQ	*N* samples >LOQ (%)	5°	25°	50°	75°	90°	95°	99°
Benz-U (ng/L)	705	15	703 (99.7%)	60	114	263	782	1919	2789	6530
SPMA (μg/L)	705	0.10	440 (64%)	<0.10	<0.10	0.19	0.71	1.51	2.50	6.32

The statistical analyses were carried out using Excel for Windows (Microsoft Corporation, Seattle, WA, USA) and SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

The Benz-U and SPMA concentration analyses results of the 705 samples are summarized in Table 1. The distribution of Benz-U and SPMA was positively skewed, with median levels of 263 ng/L and 0.19 μg/L, indicating a low exposure to benzene. Notably, Benz-U was detected in almost all samples, while the distribution of SPMA was left-truncated. In fact, as much as 36% of the samples had SPMA below the quantification limit and few samples showed relatively high levels of either urinary marker. The 99th percentile of distribution was 6530 ng/L for Benz-U and 6.32 μg/L for SPMA.

The BEN-U concentrations found in the present study were in good agreement with those previously reported in Italian subjects working as gasoline station attendants, traffic policemen, and controls without occupational exposure to benzene [3]. The levels of SPMA found were also in good agreement with those obtained using similar analytical conditions in nonoccupationally exposed subjects, which were 0.12 μg/L in non-smokers and 1.31 μg/L in smokers (overall range <0.05–3.33 μg/L) [15], and below 0.5 μg/L in non-smokers and 0.7–9.5 μg/L in smokers [16]. Conversely, the SPMA concentrations reported in the present study were much lower than those reported in the above cited investigation [3], where median concentrations ranging from 4.1 μg/L to 13.7 μg/L were reported. This difference is attributable to, at least, two reasons: the first is that in the previous study samples were acidified with hydrochloric acid as a preserving agent, with consequent hydrolysis of premercapturic acid to SPMA, which therefore increased the concentration of this analyte [17,18]; the second is that in the previous study the analyses were done using an immunoassay kit.

3.1. Linearity of calibration curves and Shewhart control charts

For Benz-U concentrations, calibration curves were linear throughout the investigated range of concentrations, with determination coefficients (R^2) typically greater than 0.99.

Fig. 1 shows the Shewhart charts for the means (a) and the ranges (b) of the slope of the calibration curve. Slopes (as a mean value of two calibration curves) and ranges obtained in forty-two analytical sequences, resulting from analytical activity spread over about five years, were introduced into the charts. The charts show that slopes were mostly within the warning lines, three slopes were between the warning lines and the action lines, and one slope was above the action line. The chart for ranges shows two sequences with *R* outside of the upper warning line, and four exceeding the upper action line. The application of Westgard's rules indicated that an out-of-control process was in effect in situations in which the action line was exceeded [19]. Thus, the related analytical work was discarded. This occurred in four out of forty-two analytical sequences, with a failure rate of approximately 10%. Considering the long period of time covered by these Shewhart charts, the discontinuous use of the assay, the fact that it was a research survey rather than part of a daily routine, and different operators were involved in the assay execution, the overall result is considered satisfactory.

For SPMA concentrations, the calibration curves also were linear in the investigated range of concentrations, with determination coefficients (R^2) typically greater than 0.99. However, a less-than-proportional response was observed for concentrations less than 1 μg/L. This may be due to difficulties in the desolvatation of small molecules in the ESI source of the mass spectrometer, with consequent loss of the analyte, which instead of entering the spectrometer was discarded with the solvent by the vacuum system. For these data, the best fit was obtained using a quadratic curve, for which again determination coefficients (R^2) were typically greater than 0.99

Due to the limited number of analytical sequences available for SPMA concentration determination, as the assay was only recently introduced in our laboratory, the construction of Shewhart charts was not feasible. With the data available, however, the mean and standard deviation of the slope of the linear regression were calculated to be 0.0837 ± 0.0115, with a %CV of 13.7% (n = 15).

3.2. Precision and accuracy

Results of within- and between-run precision for both the Benz-U and SPMA concentration determinations were estimated using the quality controls and are presented in Tables 2 and 3.

Good within-run precision was obtained for Benz-U analyses, in line with the US Food and Drug Administration (FDA) requirements for the validation of bioanalytical methods [13], with a CV less than 20% for the H-$QC_{Benz\text{-}U}$ samples, and for the majority of the L-$QC_{Benz\text{-}U}$ samples. Accuracies were also generally good for the H-$QC_{Benz\text{-}U}$ samples, while more problematic for the L-$QC_{Benz\text{-}U}$ samples, with five cases out of the twelve exceeding the range of the spiked concentration ±20%. In one case a gross error was probably the cause of the 42% accuracy determination, and this value was excluded from further evaluations. The other four cases were probably due to the fact that the QC samples were prepared by adding benzene to blank urine that already contained a low level of benzene. Since accuracy is evaluated by the difference between the spiked amount of benzene and the amount intrinsically present, it is largely impacted in cases in which these levels are comparable, as in the low QC samples. To overcome this problem in future

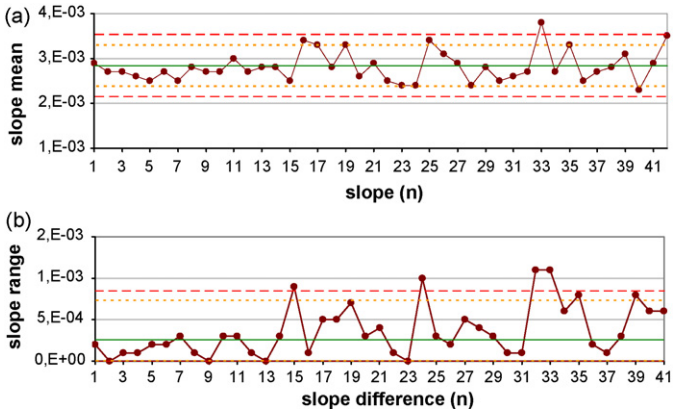

Fig. 1. Shewhart charts for the means (a) and the ranges (b) of the slope of the calibration curve for Benz-U concentrations. The solid line represents the target value μ_0; the dotted lines represent the upper and lower warning limits; and the slashed lines represent the upper and lower action limits.

Table 2
Benz-U: within- and between-run precision and accuracy, assessed using low and high level quality controls.

Sequence ID	QC (*n*)	Within-run precision, %CV		Accuracy%	
		L-QC$_{Benz-U}$ (120 ng/l)	H-QC$_{Benz-U}$ (1000 ng/l)	L-QC$_{Benz-U}$ (120 ng/l)	H-QC$_{Benz-U}$ (1000 ng/l)
1	4	6.5	1.1	114.5	107.1
2	3	4.7	3.3	100.9	94.4
3	2	7.8	14.5	42.1[a]	96.8
4	2	1.5	0.4	100.6	85.4
5	2	11.1	2.1	127.7	88.6
6	3	3.0	1.8	90.9	102.2
7	5	10.3	2.4	100.5	99.1
8	4	6.4	3.1	88.0	94.8
9	3	16.1	3.3	123.7	101.2
10	3	9.8	10.7	89.1	128.5
11	3	25.5	8.5	139.4	101.1
12	3	20.7	6.8	77.0	87.9
Between-run precision, %CV		10.3	4.8	104.8	98.9
SD		7.2	4.4	19.3	11.3

[a] Value not included in the calculation of the mean and SD.

experiments, a pre-tested pool of urine samples containing very low levels of benzene will be used.

Similarly, good within-run precision and accuracy were obtained for the SPMA analyses, using both M-QC$_{SPMA}$ and H-QC$_{SPMA}$ samples. Conversely, at 0.3 μg/L L-QC$_{SPMA}$, both precision and accuracy were very low. Again, as for the Benz-U analysis, the addition of SPMA to urine containing similar intrinsec amounts of analyte toghether with the already mentioned less-than-proportional response below 1 μg/L may explain these results.

Table 4 shows the Benz-U and SPMA precision results that were calculated using the duplicates of unknown samples. Considering all of the data, the Benz-U concentration precision was 13.4%. Given the fact that the median concentration of duplicate samples was 304 ng/L, this result is compatible with that obtained for quality controls at 120 ng/L. The SPMA concentration precision was 26.5%. As the median concentration of duplicate samples was 0.15 μg/L, this precision is better compared to that obtained using the L-QC$_{SPMA}$, for which a precision of 40.3% was found. This discrepancy may be explained by considering that as much as 64 data pairs for duplicates of unknown samples were used in this evaluation, while only 14 repeats were used in the experiment with the L-QC$_{SPMA}$.

Due to the fact that duplicates were randomly chosen among the unknown samples, and since it is known that analyte concentration in the sample plays a role in determining assay precision, the effect of sample concentration on precision was evaluated by arbitrarily dividing the duplicates into two groups: low and high concentration. For Benz-U ≤ 120 ng/L, the CV was 9.0%, which was unexpectedly lower than the CV of 14.8% found for Benz-U > 120 ng/L. For SPMA ≤ 1 μg/L, the CV was 27.6%, which was reasonably greater than the CV of 16.5% found for SPMA > 1 μg/L.

Although this method of using duplicates to evaluate assay precision is not indicated in the FDA requirements for bioanalytical method validation [13], it seems to yield a better evaluation compared to the use of quality controls since precision is evaluated on real samples. When precision is determined from *ad hoc* prepared samples, additional systematic and/or random error can more easily be introduced.

An overall evaluation of the precision and accuracy data show that, with few exceptions, the assay for Benz-U is reliably working over the entire range of investigated concentrations, whereas the assay for SPMA yields acceptable results only at medium and high levels, while the determination appears to be less reliable close to the quantification limit.

Previously described analytical procedures for SPMA concentration determination using LC/MS/MS have shown much better analytical performance at concentrations comparable to L-QC$_{SPMA}$. Particularly, a CV of 10.4% for intra-day precision and 12.2% for inter-day precision, and accuracy in the range of 88–125% were reported at 0.43 μg/L of SPMA by Schettgen et al. [15], and within-batch precision of 4.6% and accuracy of about 108% were reported

Table 3
SPMA: within- and between-run precision and accuracy, assessed using low, medium and high level quality controls.

Sequence ID	*N* QC at each level	Within-run precision, %CV			Accuracy %		
		L-QC$_{SPMA}$ (0.3 μg/L)	M-QC$_{SPMA}$ (2.5 μg/L)	H-QC$_{SPMA}$ (20 μg/L)	L-QC$_{SPMA}$ (0.3 μg/L)	M-QC$_{SPMA}$ (2.5 μg/L)	H-QC$_{SPMA}$ (20 μg/L)
1	6	–	7.0	4.5	–	68	96
2	5	–	3.6	3.1	–	96	92
3	4	–	18.8	19.4	–	107	108
4	3	–	3.7	6.2	–	94	84
5	3	–	2.3	2.0	–	119	124
6	3	–	4.5	5.2	–	103	120
7	3	–	12.4	8.1	–	90	81
8	3	–	3.7	6.4	–	99	103
9	3	–	9.0	7.6	–	98	89
10	2	–	3.8	2.0	–	86	89
11	4	–	5.9	6.6	–	88	98
12	3	–	2.8	3.1	–	75	101
13	3	41.0	5.1	-	73	98	–
14	6	30.3	3.9	-	36	107	–
15	5	49.5	6.8	-	36	117	–
Between-run precision, %CV		40.3	6.2	6.2	48.3	96.3	98.8
SD		9.6	4.4	4.7	21.4	13.9	13.4

Table 4
Precision of Benz-U and SPMA, as %CV calculated using duplicates of unknown samples, according to the equation $CV\% = \sqrt{(e^{Sw} - 1)} \cdot 100$.

Analyte	Number of duplicates	Median	5–95°	Precision, %CV
Benz-U (ng/l)				
All data	48	304	69–4937	13.4
≤120 ng/l	13	91	63–118	9.0
>120 ng/l	35	492	125–4955	14.8
SPMA (μg/l)				
All data	64	0.15	<0.10–2.21	26.5
≤1 μg/l	56	0.12	<0.10–0.78	27.6
>1 μg/l	8	2.14	1.05–3.87	16.5

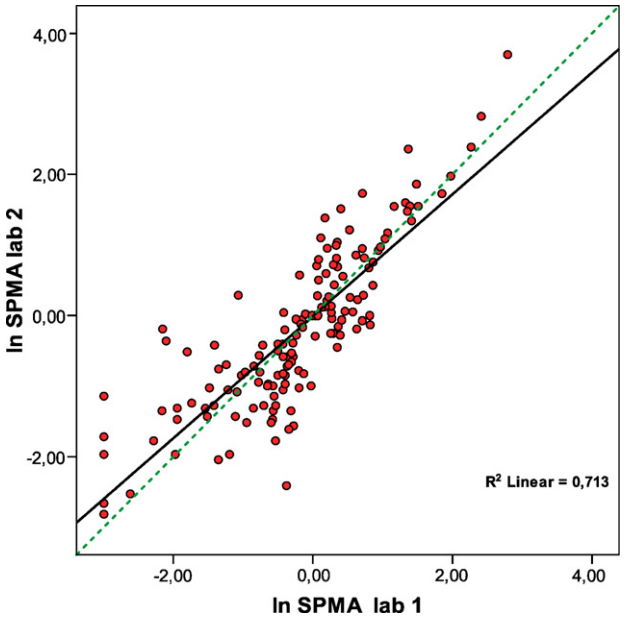

Fig. 2. Two-laboratory comparison for SPMA concentration analysis: scatter plot between ln-transformed data pairs. The solid line represents the linear regression between data; and the slashed line represents the reference line with a slope of 1 and a null intercept.

by Li et al. [16]. These better results may be due to higher linearity in the response of the instruments used, i.e. a Sciex API 3000 in the previous assays, and a TSQ Quantum Access in the present procedure. However, it should be underlined that better performances are generally obtained in method development, when instrument conditions and assay variability can be strictly controlled, as compared to real working conditions when increased numbers of real samples are run, which increases the probability of soiling the instrument and critically affecting its performance.

3.3. Two-laboratory comparison for SPMA analysis

A subset of 149 samples was analysed for SPMA for the two-laboratory collaborative trial. The Milan laboratory determined the median SPMA concentration to be 0.82 μg/L (<0.10–4.26 μg/L, 5th–95th percentile), while the Parma laboratory determined the median SPMA concentration to be 0.76 μg/L (0.14–5.63 μg/L, 5th–95th percentile). Transformation of the data by taking the natural logarithms provided normally distributed variables (Kolmogorov–Smirnov normality test). Data from the two laboratories were initially compared using a *t*-test for paired samples, which showed no statistically significant difference between the means of the ln-transformed variables. A second evaluation was performed using a scatter plot between the ln-transformed data pairs, as shown in Fig. 2. The solid line represents the linear regression between the SPMA data from laboratory 1, taken as an independent variable, and the data from laboratory 2, taken as a dependent variable. Data were correlated with a Pearson's coefficient of 0.844. The linear regression equation was:

$$\ln \text{SPMA}_{\text{lab2}} = 0.863 \cdot \ln \text{SPMA}_{\text{lab1}} - 0.010$$

The 95% confidence interval (CI) of the slope was 0.774–0.952, which was significantly different from 1; the CI of the intercept was −0.113 to 0.093, which was not significantly different from zero. The linear regression indicates a difference of about 14% between the data produced by the two laboratories. Since this difference was the same order of magnitude as the assay's inaccuracy, the results of the trial were considered acceptable. This is especially true, considering that the two laboratories used different LC/MS/MS equipment (a new generation *vs*. an old fashioned mass spectrometer), applied different sample preparation procedures (SPE sample clean up *vs*. direct injection), and used internal standards with different isotope-labelling, e.g. commercially available SPMA-d_2 for lab 1 and biosynthetically obtained SPMA-d_5 for lab 2. Lab 2 performed analysis on an API365 mass spectrometer, which was not equipped with a collision cell designed to avoid cross-talking phenomena occurring when different SRM events lead to the same product ion(s) from different precursor ions. Therefore, SPMA-d_5 was chosen as the internal standard by lab 2 to avoid cross-talking phenomena between the analyte and the internal standard. Whereas SPMA-d_2 is labelled on the cysteine residue, which is released as a neutral moiety upon fragmentation leading to a light product ion (at *m/z* 109, the same as unlabeled SPMA), SPMA-d_5 is labelled on the aromatic ring, which keeps the negative charge leading to a heavier product ion (at *m/z* 114).

The value of *r* of 0.844 and the spread of points around the fitting line indicates that, at least for some samples, a relevant and systematic divergence existed between the two laboratories, especially at lower and higher values, while a better agreement was observed in the central part of the distribution.

Since neither of the two laboratories involved in the collaborative trial could be considered as a reference, it was arbitrarily chosen to set the results of lab 1 on the *x*-axis and results of lab 2 on the *y*-axis. However, the line of regression of *y* on *x* was calculated on the assumption that the errors in the *x*-values were negligible—all errors were assumed to occur in the *y*-direction. This assumption is evidently not justified when the regression line is used for comparison purposes; it is certain that random errors will occur in both analytical methods, i.e. in both the *x* and *y* directions [6]. To check the consequence of this arbitrary assignment of variables, the dependent and independent variables were switched, obtaining a new regression line with a different slope (0.826, CI: 0.714–0.911), and intercept (−0.063, CI: −0.164 to 0.037), $r = 0.844$. Given the fact that considerations similar to those reported above can be used to comment on this result, the agreement between the SPMA assays of the two-laboratory comparison was determined to be good. Moreover, this result assures the comparability of the data coming from the studies already carried out independently by the two laboratories involved in the comparison.

4. Conclusions

The present study assessed the quality of the proposed analytical procedures for the determination of Benz-U and SPMA concentrations; however, the evaluation of the intrinsic characteristics of these biomarkers was beyond the aim of this paper. To our knowledge, this is the first detailed example of quality controls applied to a research protocol for determination of the analytical reliability of biomarkers suitable to assess environmental exposure to low levels of benzene. The results of this study show that the described assay for Benz-U reliably worked over the range of concentrations investigated, with acceptable and constant performances during long time periods. In addition, the assay for SPMA yielded good results at medium and high concentrations, but less reliable results were obtained at concentrations below 1 μg/L. Considering that the use of these assays was discontinuous and limited to research surveys, the overall results were satisfactory.

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