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Application of the standard addition approach for the quantification of urinary benzene

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Abstract

Urinary benzene is used as biomarker of exposure to evaluate the uptake of this solvent both in non-occupationally exposed population and in benzene-exposed workers. The quantitative determination of benzene in urine is carried out in a three steps procedure: urine collection, sample analysis by head space/solid phase microextraction/gas chromatography/mass spectrometry and analyte quantification. The adopted quantification method influences the initial step, hence the whole procedure. Two quantification approaches were compared as regards precision and accuracy: the calibration curves and the standard addition method. Even if calibration curves obtained by using urine samples from different subjects were always linear, their slopes and intercepts showed noteworthy variations, attributable to the influence of the biological matrix on benzene recovery. The standard addition method showed to be more suitable for compensating matrix effects, and a three-point standard addition protocol was used to quantify benzene in urine samples of 11 benzene-exposed workers (smokers and non-smokers). Urine from occupationally exposed workers was collected before and after work-shift. Besides urinary benzene, the applicability of the method was verified by measuring the urinary concentration of the *S*-phenylmercapturic acid, a specific benzene metabolite, generally adopted as biomarker in biological monitoring procedures. A similar trend of concentration levels of both analytes measured in urine samples collected before work-shift with respect to the after work-shift ones was found, showing the actual applicability of the standard addition method for biological monitoring purposes.

Keywords: Urinary benzene; Standard addition approach; Occupational exposure

1. Introduction

Benzene is an important chemical used world wide for plastic, as chemical intermediate and solvent. Moreover, it is a constituent of engine emissions and combustion, a component of tobacco smoke and gasoline.

Hence it has acquired great relevance as ubiquitous pollutant of the outdoor and indoor human environment [1–3]. Owing to its low boiling-point and its high lipophilicity benzene is rapidly absorbed *via* inhalation or dermal contact. Data from epidemiological studies evidence its toxicity to humans; benzene is associated with the development of acute non-lymphocytic leukemia [4–6], aplastic anemia [7], chromosomal aberrations [8–11] and a progressive degeneration of the bone marrow [12]. The American Conference of Governmental Industrial Hygienists (ACGIH) classifies benzene in group A1 (carcinogen to human) and defines a threshold limit value-time weighted average (TLV-TWA) of 0.5 ppm [13].

Due to the toxicological properties of benzene, there is an interest in the development of specific analytical procedures

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to measure the exposure to this aromatic hydrocarbon in biological fluids. Among the exposure biomarkers, two benzene metabolites have been currently adopted by ACGIH: the *S*-phenylmercapturic acid (S-PMA) and the *trans,trans*-muconic acid. The first one, representing about 1% of the absorbed dose of benzene [14], is a specific metabolite, and it is considered a useful biomarker also for the measurement of low levels of benzene exposure; nevertheless it needs high levels of sensitivity, requiring sophisticated instrumentation and expertise. The second one derives not only from the metabolism of benzene but also from the sorbic acid (a widely used food preservative), hence the presence of *trans,trans*-muconic acid in urine is not depending exclusively on exposure to benzene. Currently, the measurement of the unmetabolized benzene excreted in urine, which represents about 1% of the absorbed quantity [15], has been proposed as useful biomarker and it is used for biological monitoring purposes [16–19].

The determination of urinary benzene is often performed by head space/solid phase microextraction (HS/SPME) followed by gas chromatography/mass spectrometry (GC/MS) [17,18,20–22].

The HS technique results particularly useful because of the high volatility of benzene, involving an easy transition of benzene contained in the urine matrix to the vapour.

SPME is a solvent-free extraction technique that combines sampling, pre-concentration and the direct transfer of the analytes into a gas chromatography system [23]. The extraction step is based on the partitioning of volatile compounds between a fiber coated with a stationary phase and the gaseous phase above the sample. With SPME, the amount of analyte removed by the fiber, is proportional to the concentration of the compound in the sample.

The method combines the advantages of HS/SPME with the high chromatographic resolution of capillary GC and high specificity and sensitivity afforded with mass spectrometric detection.

Even if the analytical method used for the detection of benzene is sufficiently sensitive and specific, the quantitative determination of the analyte is noteworthy influenced by the initial step of the whole procedure, i.e. by the modality of urine collection, sample preparation and sample storage. In fact, factors such as environmental pollution and the quality of lab materials that have to be used could involve considerable quantification errors, as well as sample freezing and thawing could have a not negligible effect on benzene recovery from biological matrix. That is why special care must be taken during the sample preparation in order to have accurate and reproducible results.

The decision of which analytical procedure and which quantification approach to choose, particularly when using SPME, depends on the sample matrix, on its complexity, and on the extraction method being used [24]. As for benzene, the standard addition approach is essential to obtain accurate measurements by minimizing the influence of pollution, samples handling and matrix effects that would otherwise contribute to quantification bias. In fact, with this method, each urine sample is divided into three aliquots: one is the "unknown" sample and the others are spiked with known amounts of the analyte and then used for calibration. So that the calibration step is carried out by using the same urine specimen that has to be analyzed, hence any interference due to matrix complexity (proportional systematic errors) is taken into account during the analyte quantification.

Here, quantitation using the standard addition approach was compared with the calibration curve one. The assay was applied to the analysis of urine samples from 11 smoker and non-smoker workers occupationally exposed to benzene, in samples collected before and after work-shift. In the same urine samples, in order to evaluate the obtained results with respect to a commonly used biomarker proposed by ACGIH, also S-PMA levels were determined. S-PMA concentrations were measured by liquid chromatography/negative electrospray ionization/tandem mass spectrometry ionization with selected reaction monitoring (LC/ESI-NI/MS^2), following a procedure previously set up in our laboratory [25].

2. Experimental

2.1. Materials

Benzene was purchased from Merck (Darmstadt, Germany), deuterated benzene was from Carlo Erba (Milan, Italy). 10 ml vials and silicone/Teflon lined (0.1 mm thick coating) septa, "superior standard", were purchased from Carlo Erba (Milan, Italy). SPME fiber (fused-silica fiber 10 mm long, coated with an 85 μm film thick layer of polydimethylsiloxane/carboxen) and fiber holder were from Supelco (Bellafonte, PA, USA). GC/MS analyses were carried out by using a gas chromatograph HRGC MEGA Series II (Fisons Instruments, Milan, Italy), interfaced with a single quadrupole mass detector QMD 1000, equipped with an Electron Ionization source (CE Instruments, Milan, Italy). The gas chromatograph was equipped with a split/splitless injector (0.75 mm i.d. inlet liner for SPME) from Supelco (Bellafonte, PA, USA) and a ZB-50 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) from Phenomenex (Torrance, CA, USA).

2.2. Benzene spiked urine sample preparation

Urine from non-smoker, non-occupationally exposed to benzene volunteers were collected and 4 ml aliquots were put in 10 ml vials containing 1 g of NaCl previously dried (200 °C, 1 h). The amount of benzene eventually present in the fluid was eliminated by bubbling a stream of nitrogen for 5 min, then vials were sealed with silicone/Teflon lined septa and each sample was added with 61.0 μg/l aqueous solution of deuterated benzene, in order to have a constant urinary internal standard concentration of 0.76 μg/l.

The samples were also added with different volumes of a 11.6 μg/l aqueous solution of benzene, in order to have five different benzene concentrations (0.086, 0.17, 0.29, 1.16 and 2.30 μg/l) to be used for the construction of calibration curves, and three different benzene concentrations (0.145, 0.58, 2.23 μg/l) to be used for the evaluation of variation coefficients and inaccuracy of the standard addition method. One aliquot was not added with benzene and it was subsequently used to verify that benzene was undetectable by using the HS/SPME/GC/MS analysis described below. All samples were stored at −4 °C. Vials were left at room temperature until completely thawed and then analyzed.

The whole procedure was repeated three times by using different urine from different subjects.

2.3. Real urine sample collection and preparation

Urinary samples were obtained from smokers and non-smokers benzene occupationally exposed workers of a gasoline storage.

Spot urine specimens were collected, directly from the donors, in 100 ml sterile containers. For every donor, three aliquots (4 ml of urine each one) were immediately transferred into 10 ml vials, containing 1 g of NaCl and previously purged by a stream of nitrogen and crimped shut. The specimens were taken to the laboratory and added with a solution of deuterated benzene in order to have a constant urinary internal standard concentration of 0.76 μg/l.

Standard addition samples were prepared as follow: a "zero" point was prepared just as described above; the first standard addition sample was spiked with 100 μl of a benzene solution, 11.6 μg/l, in order to have a concentration of 0.29 μg/l; the second standard addition sample was spiked with 800 μl of the same solution in order to have a concentration of 2.30 μg/l of benzene.

Then samples were frozen before analysis.

2.4. HS/SPME/GC/MS analysis

The sample was heated at 55 °C and kept at this temperature for 30 min to allow the volatile compounds to reach the equilibrium between gaseous and aqueous solutions. Then the SPME device was inserted into the vial, the fiber was pushed out from the holder and exposed directly on the head space above the sample for 15 min. At the end of the sampling time the fiber was pulled in the stainless steel needle and immediately inserted into the GC injector. After insertion, the SPME fiber was pushed out of the needle and thermally desorbed at 260 °C.

The GC oven temperature was kept at 40 °C for 2 min, then the temperature was increased to 70 °C at 6 °C/min. Helium (purity: 99.5%) was used as the carrier at 1 ml/min constant flow. The MS detector (source temperature, 170 °C) was operating in the selected ion monitoring mode. The acquired masses were *m/z* 51, 77 and 78 for benzene, 82 and 84 for deuterated benzene.

3. Results and discussion

3.1. Contamination of the laboratory atmosphere

Benzene is a ubiquitous pollutant; moreover, it can be used as solvent during industrial processing in the production of septa. That is why, factors such as the environmental pollution of laboratories of analysis and the quality of lab materials that have to be used, were taken into account when the quantitative determination of benzene was carried out.

The HS/SPME/GC/MS analysis of vials sealed with common septa, and containing just environmental air, without any fluid inside, showed a chromatographic peak (retention time 2.8 min) corresponding to detectable amount, and sometimes remarkable amount, of benzene. The presence of the molecule in the sample is attributable either to the environmental pollution of the air or to the release of the analyte from the septa used during the analysis. In order to investigate the source of pollution, two vials were differently prepared. The first was sealed with treated septa previously heated at 200 °C for 1 h, dried and then covered with aluminium sheet before sealing the vial; and the second vial was purged by a stream of nitrogen and then sealed with untreated septa. In both cases the analysis showed benzene was still present and the interference was eliminated only when vials both purified with nitrogen and sealed with treated septa were analyzed.

The adoption of a different type of septa, covered with a silicone/Teflon film (superior standard quality) did not show any benzene release from untreated septa, hence this kind of septa seems particularly suitable for the determination of aromatic solvents. Nevertheless, different batches of the same product can have different levels of contamination, that is why even when using septa specific for solvents, it is advisable to test each new lot before analysis.

The pollution of environmental air involves not only particular care during vial preparation but also the desorption of benzene from the SPME fiber before using. In fact, when a fiber pulled in the stainless steel needle was left at room temperature in contact with air, the GC/MS analysis showed appreciable levels of benzene that can only derive from benzene present in the air of the analysis laboratory. That is why the fiber has to be kept at 260 °C before being used for analysis.

3.2. Urine sample storage

Urine samples are usually frozen for storage before analysis. We investigate the influence of freezing and thawing on the release of benzene from the biological matrix to the gaseous phase.

Two urine samples from the same subject were spiked with the same amount of benzene and of deuterated benzene; then they were differently treated. The first one was immediately analyzed, the second one was frozen, thawed and then analyzed. The analytical responses (ratio between chromatographic peaks areas of benzene/deuterated benzene)

were similar but the signal to noise ratio was about thirty times better for the frozen sample.

This fact could be ascribed to the corpuscular material present in urine sample that forms a precipitate after defrosting. The corpuscle may absorb interference, leading to a decrement of background noise.

This result demonstrates that the release and the recovery of benzene from the biological matrix are noteworthy influenced by modalities of storage and of sample preparation before analysis. That is why, in order to compare results from different laboratories, it should be desirable to follow the same analytical procedure, from urine collection to sample analysis. In any case it is indispensable to add the internal standard soon after the urine collection; in this way it undergoes the same treatment of the analyte that has to be quantified. So that the analytical response, being a relative ratio of areas and not only an absolute area, does not care about different sample handling.

3.3. Quantification approaches: linearity and reproducibility

Our initial attempts, to quantitatively measure urinary benzene, were based on using samples containing undetectable levels of benzene as a starting material for spiking to generate calibration curves. Benzene contained into specimen (from non-smokers volunteers) was sent away by a nitrogen stream, then known amounts of benzene and deuterated benzene were added and samples were analyzed by HS/SPME/GC/MS.

Calibration curves were constructed by reporting the nominal benzene concentration in spiked calibration samples on the *x*-axis and on the *y*-axis the peaks areas ratio between benzene and the internal standard. At first consideration these samples yielded calibration curves with excellent linearity. However, when urinary samples from different donors, with same benzene amount spiked, were analyzed, differences in the relative response factors suggested that matrix had not negligible effects. This suggested that a more thorough investigation was needed. Five-point calibration curves were established using urinary samples from three different donors. The obtained equations of calibration curves were: $y = 2.3244x + 1.6383$; $y = 2.2359x + 0.7011$ and $y = 1.6245x + 0.4096$ and they showed good linearity with coefficients of determination (R^2) of 0.998, 0.996, 0.998 respectively. Nevertheless, the slopes and/or the intercepts of the calibration curves obtained from these three different urine samples varied considerably.

The intra-assay repeatability (i.e. the repeatability calculated by using urine from just one donor) was expressed as the percent coefficient of variation (intra-assay CV%) and it was estimated through repeated analysis of urine samples (three for each concentration) spiked with benzene, with urinary concentrations of 0.086, 0.17, 0.29, 1.16 and 2.32 μg/l. The obtained results are reported in Table 1. The inter-assay repeatability (inter-assay CV%) was estimated by using the same benzene concentration levels reported above but on three different urine matrices, from three different donors, for each concentration (Table 1). The results were compared and showed a good repeatability for the samples coming from the same urine unlike those coming from different urine.

Table 1
Comparison between quantification approach: calibration curve and standard addition method

Calibration curve: intra- and inter-assay repeatability

Nominal [benzene] (μg/l)	Intra-assay precision (CV%)	Inter-assay precision (CV%)
0.086	3.3	32.2
0.17	3.6	46.5
0.29	4.0	51.0
1.16	1.8	28.9
2.32	1.0	25.4

Standard addition method: inaccuracy and precision

Nominal [Benzene] (μg/l)	CV%	% Inacc.	Calculated [benzene] ± SD (μg/l)
0.145	2.0	2.1	0.148 ± 0.003
0.580	2.5	3.4	0.600 ± 0.015
2.230	0.1	0.9	2.340 ± 0.002

This fact confirms the necessity of relying upon an analytical method independent from interference due to the biological matrix.

Hence, the traditional calibration curve strategy was abandoned in favour of the standard addition approach that compensated for differences in the urinary matrix.

This technique is suitably used when a blank matrix is not available. It makes use of the addition of known concentrations of the analyte of interest to multiple aliquots of the sample, and of another aliquot, called “zero-point”, that is not spiked. Then samples are analyzed and detector responses versus the amount spiked for each analysis is plotted. A straight line is drawn and the value of the *x* intercept represents the amount of the analyte in the unknown sample [26].

In the case here reported, two aliquots of urinary samples are added with two known concentrations of benzene, while

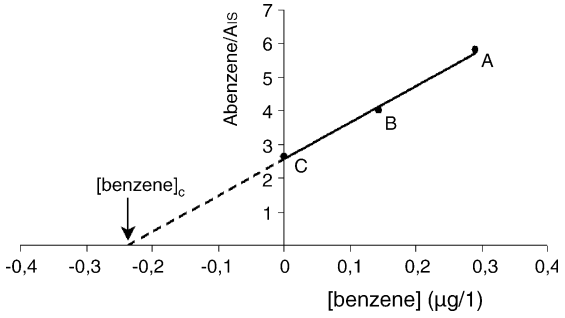

Fig. 1. Quantification by standard addition approach. IS = internal standard; A and B = urine samples spiked with 2.30 and 0.29 μg/l of benzene, respectively; C = “zero-point”. Equation of straight line $y = 10.863x + 2.5748$, $R^2 = 0.9981$. “Zero-point” benzene concentration ($[benzene]_C$) is obtained as follows $y = 0$: $x = -0.237$ and $[benzene]_C = 0.237$ μg/l.

the third one is not spiked. Samples are analysed and the peak areas ratio between benzene and the internal standard is worked out and considered as the detector response, as showed in Fig. 1.

To determine the percent inaccuracy (% Inacc.) and precision (CV%) of the standard addition quantification approach, urine samples were bubbled by a nitrogen stream (in order to eliminate the benzene eventually present), spiked with three known amount of benzene and each sample was treated as unknown. Namely, each sample was quantified by using the other two. The measurements were repeated three times in three different days, with three different urines.

The obtained results, reported in Table 1, showed that the method could be applicable to the quantification of benzene in real unknown samples from subjects occupationally exposed to benzene.

3.4. Standard addition method: urine real samples analysis

Urinary determinations of benzene were not performed in order to provide data for a biological monitoring but only to verify the feasibility of the proposed method in real sample analysis.

Urine from 11 occupationally benzene-exposed subjects, working in a gasoline storage was collected before and after the work-shift. Information regarding smoking habits and specific tasks performed by each examined worker were

Table 2
Urine samples from benzene occupationally exposed workers

Sample	Cigarettes per day	[Benzene] μg/g creatinine	[S-PMA] μg/g creatinine
1[a]			
Before the work-shift	0	0.009	3.8
After the work-shift		0.031	7.1
2[a]			
Before the work-shift	0	0.019	8.0
After the work-shift		0.011	10.1
3[b]			
Before the work-shift	0	0.098	10.0
After the work-shift		0.256	14.8
4[b]			
Before the work-shift	0	0.171	29.8
After the work-shift		0.019	24.0
5[c]			
Before the work-shift	0	0.202	10.6
After the work-shift		≫3	176.5
6[c]			
Before the work-shift	0	0.028	9.3
After the work-shift		0.331	43.8
7[c]			
Before the work-shift	0	0.084	2.8
After the work-shift		0.208	15.5
8[c]			
Before the work-shift	0	0.130	4.3
After the work-shift		1.293	19.8
9[c]			
Before the work-shift	0	0.054	3.8
After the work-shift		0.203	11.6
10[c]			
Before the work-shift	15	1.244	14.2
After the work-shift		0.088	21.2
11[d]			
Before the work-shift	20	0.073	11.4
After the work-shift		>3	25.2

Benzene and S-PMA urinary concentrations. Theoretic benzene exposure levels based on worker specific tasks.

[a] Very low.
[b] Low.
[c] Medium.
[d] High.

recorded and each urine sample was divided into five aliquots; three were used for benzene quantification by the standard addition approach, the other two were used for the determination of S-PMA and creatinine levels, respectively.

Information were recorded because urinary benzene levels depend upon smoking habits, and because a good knowledge of specific tasks was necessary for having a theoretic scale about exposure levels to be expected, in order to understand if the benzene concentrations found in urine could be related to hypothetical exposure levels. S-PMA urinary concentration was measured because S-PMA is considered a useful biomarker for the measurement of low levels of benzene exposure, that is why the obtained benzene concentrations were compared with *S*-phenylmercapturic acid levels determined in the same urine samples. Creatinine levels were measured to normalize S-PMA concentrations as usually reported in literature. Urine was collected before and after work-shift so to verify if the benzene and the S-PMA excretion follow a similar trend.

No substantial differences were found by expressing benzene concentration either in μg of benzene per urine liter or with respect to creatinine, so that both benzene and S-PMA concentrations were corrected for the creatinine level. Results are schematized in Table 2.

The majority of subjects showed a proportional increase of both analytes in urine collected after the work-shift, except for the smoker subjects 10 and 11. For the subject 10, there is an increment of S-PMA concentration and a decrement of urinary benzene; for subject 11, both analytes concentrations increase in after work-shift urine, but the increment is not proportional because of the high benzene concentration. In these samples the results obtained for benzene and S-PMA are dissonant owing to their smoking habits and to the different excretion time of analytes, in fact, urine collected soon after smoking reveals high amount of benzene, while S-PMA takes some hours to be excreted.

From the recorded information about specific tasks, the following theoretic scale of exposure levels was established: very low (administrative employee, samples 1 and 2), low (person in charge of installation and employed to the storehouse, samples 3 and 4), medium (installation employee, samples 5–10), high (pumps installation employee, sample 11).

The increment of benzene and S-PMA concentration levels found in urine collected before and after work-shift reflects expected exposure levels. Subjects 1 and 2 did not show remarkable increment and the analytes levels are similar to those of the general population non-occupationally exposed to benzene. Subjects 3, 6–9 showed a benzene concentration increase reflecting the expected exposure levels. Subject 4 represents an exception because both benzene and S-PMA concentrations decrease in after work-shift urine. This fact is attributable to exposure factors (environmental pollution, vehicle traffic, passive smoking) other than occupational ones. Samples 11 and 5 showed high benzene concentrations levels in after work-shift urine. The first one, as above discussed, is a smoker subject but he also is the worker more exposed to benzene, hence the high levels found can depend on both factors. For the second one, we found very high levels of both benzene and its metabolite suggesting a remarkable occupational exposure. Besides, while in the majority of samples, the level of S-PMA returns in limits provided for ACGIH (25 μg/g creatinine) [13], in this case, we found a concentration value seven times higher, hence, this case would need further investigation.

4. Conclusions

In recent years urinary benzene is being adopted by more and more authors as exposure biomarker; we verified the actual feasibility of this biomarker, on condition that interference due to environmental pollution and sample collection and storage are taken into account. Besides, given that many quantification errors may depend upon the complexity of the urinary matrix, the standard addition method turns out to be the most suitable quantification approach. The obtained results suggest that urinary benzene could be used as biomarker of occupational exposure because there is an appreciable difference of its concentration in urine collected before and after work-shift. Nevertheless, the collection and the analysis of two urine samples for every subject that have to be investigated is both time consuming and economically disadvantageous. Therefore, when the biological monitoring is periodically repeated, the analysis of before work-shift samples could be limited only to the first time the biological monitoring is carried out, so that background levels can be established for each subject under study. In any case, such analyses have to be repeated every time a meaningful variation in the life style of the subjects occurs and/or for smoking subjects. Otherwise, even the analysis of only after work-shift urine can reflect the occupational exposure level and it can be used for a general screening of samples for biological monitoring purposes in the evaluation of occupational exposure to benzene.

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