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Matrix interferences in the analysis of benzene in urine

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Abstract

The analysis of benzene in urine of the general population or of exposed workers can be performed with different methods using the 'purge and trap' or 'solid-phase microextraction' techniques in combination with gas chromatographic analysis and photoionisation or mass spectrometric detection. The published results, however, are deeply conflicting. Differences in sample preparation by different research groups and our own preliminary observations prompted us to investigate pre-analytical and analytical factors potentially capable of modifying the urinary benzene quantification results. Benzene concentrations were measured in 20 urine samples in relation to different conditioning conditions (at 24, 40 and 80°C) and at basic or acid pH. Urinary protein concentrations were measured in the same samples. Urine heating at 80°C yields benzene concentrations on average five times higher than at 24°C. On acidification of urine, the benzene released increases up to 28-fold in comparison to that obtained at uncorrected 'physiological' pH. Despite a widely scattered data distribution, a statistically significant linear correlation was found between 'heat-released' and 'acid-labile' benzene values. There was no correlation between total urinary proteins present in 'physiological' concentrations (between 12 and 110 mg/l) and the different kinds of benzene in urine. Our results could perhaps be explained if it is supposed that part of the benzene in urine is absorbed onto sediment, or bound to specific proteins, or derived from parent molecules and is released with pH modification or heat administration. Our observations may also help to explain why the urinary benzene concentrations reported by different investigators vary considerably even when environmental levels are comparable.

Keywords: Benzene

1. Introduction

Benzene is a carcinogenic aromatic hydrocarbon [1,2] widely used as a basic component for chemical synthesis and as an organic solvent in many industrial applications; it is also added to gasoline as an antiknocking agent. Benzene has become a ubiquitous pollutant of urban air, mainly related to vehicle exhaust fumes [3]. Smoking is a further source of exposure to benzene, because the substance is released during the burning of tobacco [4]. Individual occupational or non-occupational exposure to benzene can be measured by individual samplers (though this is a very expensive method) or by biological monitoring. The concentration of benzene in blood can be used as a good parameter of the

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'body burden' of the solvent, absorbed during work or from ubiquitous pollution [5]. The urinary concentration of *trans,trans*-muconic or phenylmercapturic acids, which are two specific metabolites of benzene, can also be used in the biological monitoring of very low-level exposure to benzene [6,7]. Recent research suggests that urinary benzene is also a reliable biological index of exposure, but comparison of data reported in the literature shows wide differences between the urinary concentrations found in relation to similar exposure to benzene [8–10]. Urinanalysis of benzene has been performed using different versions of the 'head-space' technique: 'dynamic head space' [8,11,12] and solid-phase microextraction both combined with gas chromatography with mass spectrometry [13] or photoionisation [14]. During the pre-analytical phase, urine samples were added with different salts and conditioned at room temperature [8,11,13] or at 60°C [9,14]. Bearing in mind these operating conditions and our own unpublished experience, in this paper we report the effects of a number of analytical or physiological factors such as acidic or alkaline pH, temperature of conditioning and sampling, and concentration of proteins, capable of substantially influencing the measurement of benzene in urine.

2. Material and methods

2.1. Chemicals

Benzene, hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), zinc sulphate and anhydrous sodium sulphate were obtained from C. Erba (Milan). All products were of analytical grade.

2.2. Project lay-out

To study the effect of the urine incubation temperature, the significance of urinary pH (acid or basic) and the influence of protein precipitation on benzene concentration, 20 urine samples were used. These biological samples were collected from healthy subjects non-occupationally exposed to benzene. Ten of them were non-smokers and ten smokers (from five to 20 cigarettes a day). After collection, each urine sample was immediately subdivided into six 5-ml fractions which were placed in 70-ml sealed head-space vials containing NaCl (500 mg). Three of the vials were used to analyse the urinary benzene concentration after 1 h of conditioning (and shaking) at 25, 40 and 80°C, respectively. Two other urine samples placed in vials were added with concentrated HCl (40 μl) and NaOH (100 μl of a 4 *M* solution) to obtain urinary pH values of 2–3 and 9–10. The individual pH of each urine sample was measured after benzene analysis. The urine of the sixth vial was deproteinised by addition of 100 μl of 40% (w/v) zinc sulphate aqueous solution together with NaOH (100 μl of a 4 *M* solution). All acid or alkaline samples were analysed after room temperature conditioning.

Other fractions of the same 20 urine samples were used to measure their total protein concentrations, specific gravity, glucose, bilirubin and sediment: all urinalysis results were normal.

A benzene solution in water (500 ng/l) was used to perform a parallel experiment to that with urine. Vials containing 5 ml of the benzene solution were prepared and treated in the same way as the urine samples: four of them were analysed at room temperature, four at 40°C, and four at 80°C; another four were acidified, four alkalinised and four added with zinc sulphate. All urine and water samples were maintained at 4°C pending testing, which was done within 5 days. After these tests, we studied the influence of pH modification on benzene determination in detail in another ten urine samples (five from non-smokers and five from smokers): each of the ten urine samples was subdivided into five aliquots of 5 ml and placed in head-space vials containing 0, 20, 40 or 200 μl of concentrated HCl or 100 μl NaOH 4 *M*, respectively. The individual pH of each urine sample was measured after benzene analysis; depending on the volume of acidic or alkaline modifier added, five different pH values were obtained for each urine specimen: lower than 2, between 2 and 4, between 4 and 5, between 5 and 8 and higher than 8. To compare the five benzene concentrations obtained from each urine sample a weight of 100% was attributed to the highest benzene concentration among the five values; weights of the other results were calculated as percentages of the latter.

Each experimental series of samples, analysed at different temperature or pH, was added to a concomitant calibration curve, prepared with urine samples (obtained from non-smokers) and treated in the same way as the real samples (Fig. 1). The benzene concentration of each urine sample was calculated by multiplying the angular coefficient of the specific calibration curve by the abundance of the benzene peak (adjusted to the internal standard abundance) found in the individual urine samples.

2.3. Analytical methods

The urinary concentration of benzene was measured using a purge and trap technique previously described [11,12] and briefly summarised here: 5 ml of urine were placed in a 70-ml sealed head-space vial. Originally, 5 ng of tetrachloromethane were added as internal standard, and later 1,1,1-trichloroethane was substituted for this solvent. Each sample was shaken for 60 min at the programmed temperature (24, 40 or 80°C); then the vial was connected up to a six-way valve system attached to the carrier gas flow of the gas chromatograph–mass spectrometer (GC–MS). Volatile compounds in the vial head space were trapped in a 'U'-shaped tube containing 300 mg of Tenax maintained at −30°C during the sampling (3 min); the Tenax was then heated to 300°C within 2.5 s and the desorbed products were driven into the GC column. A Hewlett-Packard 5890 gas chromatograph equipped with silica capillary HP Ultra 2 column, 50 m long, I.D. 0.2 mm, film thickness 0.33 μm was used for benzene quantification The initial column temperature was held constant for 5 min and then programmed from 35 to 120°C at 15°C/min. The carried gas was purified helium delivered at a flow-rate of 0.8 ml/min. A Hewlett-Packard 5970B mass selective detector (quadrupole) was used for benzene identification and quantification (m/z 78 and 52 ions); 1,1,1-trichloroethane was monitored by m/z 97 ion.

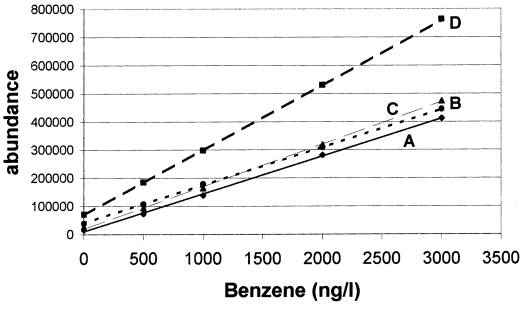

Fig. 1. Calibration curves obtained from four series of vials prepared with the same urine sample. Each series was added with 0, 500, 1000, 2000 and 3000 ng/l of benzene. One series was also acidified. Samples analysed at 24°C. Line A: $y=133x+12173$; $r=0.9997$. Acidified samples analysed at 24°C. Line B: $y=134x+40856$; $r=0.9999$. Samples analysed at 40°C. Line C: $y=150x+20769$; $r=0.9996$. Samples analysed at 80°C. Line D: $y=231x+69280$; $r=0.9999$.

2.4. Data analysis and statistics

The usual descriptive statistical tests were calculated. As the distributions of the individual groupings of data were not normal, but skewed, the Wilcoxon's non-parametric test (Z), the Kruskal–Wallis test (H) and the Spearman test (rank correlation coefficient) were used. The linear regression was studied when the Spearman test was statistically significant. A test was considered statistically significant when $P<0.05$ (software: Statgraphics®).

3. Results

The tests performed with the water solution of benzene (500 ng/l) yielded similar results in the various different test conditions: acidification, alkalinisation, no treatment, adding zinc sulphate, conditioning at 25, 40 or 80°C. The variation coefficients within the individual series of samples ranged from 2 to 8.1%. The variation coefficient obtained from all the differently treated samples was 6.3%, thus confirming the good precision of the analytical method.

In Fig. 1 the calibration curves corresponding to the different groups of urine samples are reported. Samples analysed at 24°C, without any treatment or acidified, had the same angular coefficient. Samples analysed at 40 and 80°C had increasing slopes related to the higher air/urine partition coefficient of benzene than at 24°C. The intercept of the regression line obtained from untreated samples is the lowest; increasing values were found at 40 and 80°C. The intercept of the acidified samples is higher than that

found in untreated samples and in samples analysed at 40°C.

Fig. 2 shows the GC/MS analyses of the same urine sample without any treatment and after acidification. The urine acidification 'increases' the benzene concentration about 4-fold in view of the fact that the abundance of the internal standard is similar.

Table 1 reports all data obtained from the 20 urine

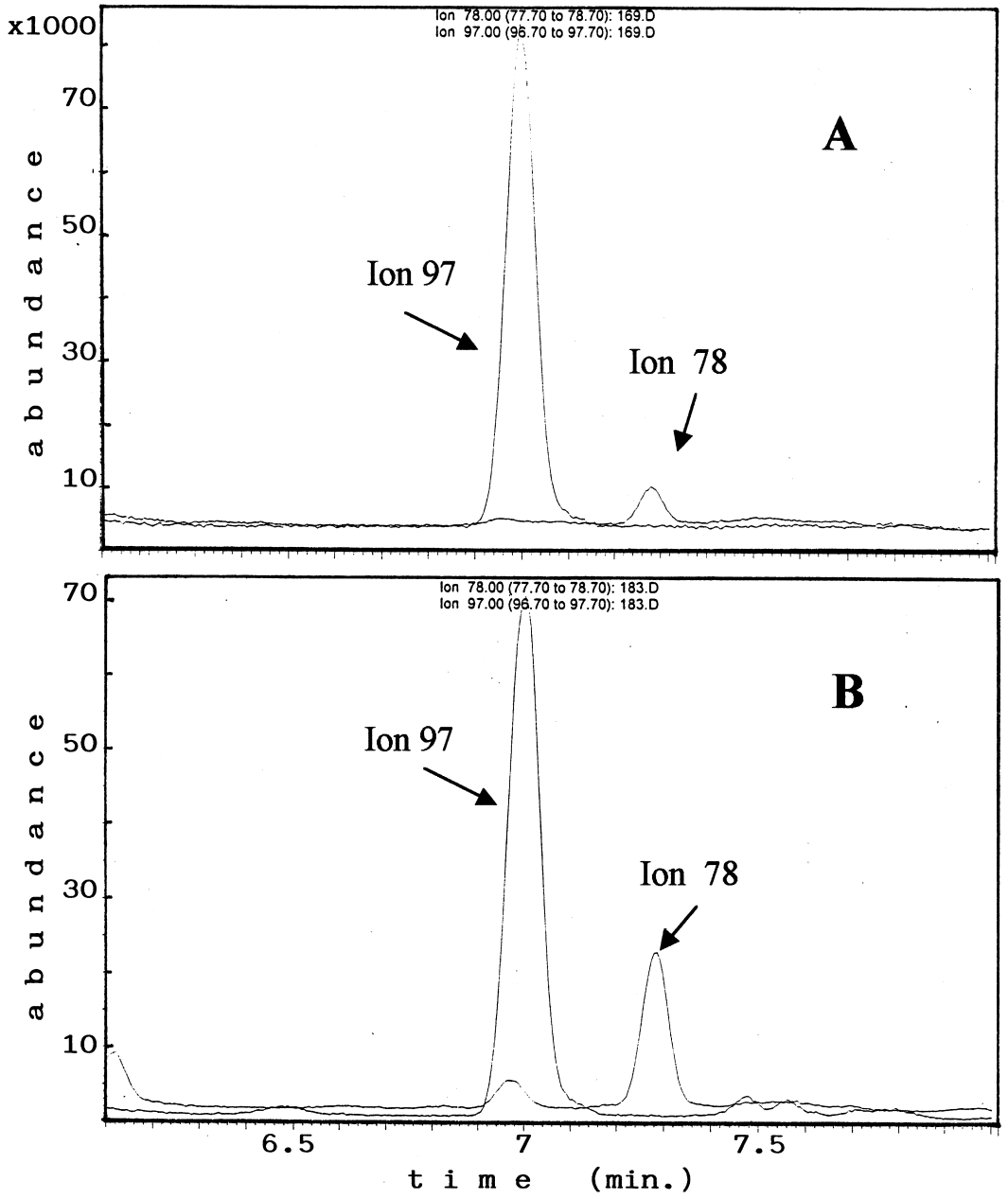

Fig. 2. GC–MS analysis of benzene at 24°C of the same urine sample without any treatment (A) and acidified (B). The internal standard (1,1,1-trichloroethane=ion 97) has a retention time of 7 min; benzene has a retention time of 7.3 min (ion 78). In the acidified sample the peak abundance of benzene is about 4-fold higher than in the untreated urine.

Table 1
Benzene concentrations (ng/l) in the 20 urine samples analysed in different conditions, associated with their main statistical parameters[a]

No.	At 24°C	At 40°C	At 80°C	Acidified	Alkalinised
1	39	164	489	902	37
2	41	48	204	141	44
3	55	71	350	227	50
4	59	75	175	145	59
5	60	136	341	1727	48
6	102	115	127	377	105
7	104	236	719	1815	113
8	107	101	254	1129	103
9	108	140	563	369	102
10	541	406	1808	2040	536
11	45	59	338	640	57
12	68	79	211	1013	58
13	90	116	399	368	120
14	159	237	1474	1751	199
15	182	210	1670	935	138
16	237	273	2652	1699	288
17	239	197	708	1831	369
18	268	225	1618	1885	195
19	402	406	2756	1673	412
20	1051	981	3981	2238	1029
Mean	198	214	1042	1145	203
S.D.	240	208	1074	717	239
Median	105	152	526	1071	109
Geom. mean	126	160	635	847	128
Minimum	39	48	127	141	37
Maximum	1051	981	3981	2238	1029

[a] Geom. mean, geometrical mean; S.D., standard deviation.

samples analysed after the different treatments together with the main descriptive statistical tests. The first ten groups of results were obtained from urine of non-smokers, and the other ten from smokers.

The temperature at which the samples were conditioned before and during sampling appeared to play an important role in determining the amount of benzene released from the biological matrix. Increasing the temperature resulted in a significant increase in benzene values (Table 1). Results obtained at 40°C were about 20% higher than the corresponding values at 24°C, while at 80°C results as high as five times those obtained at 24°C were observed ($H=22$; $P<0.001$).

The regression lines between benzene values obtained at 24 vs. 40°C and 24 vs. 80°C are shown in Fig. 3. The slopes suggest comparable results at 24 and 40°C, while urine samples heated at 80°C show an approximately 4-fold mean increase. The ratio of the results obtained at room temperature to those at 80°C appeared to be unrelated to the smoker or non-smoker status of the subjects studied.

The influence of urinary pH modification on benzene concentration is summarised in Table 1 and

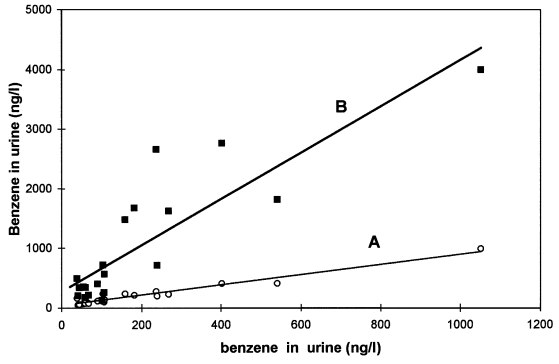

Fig. 3. Relationship between benzene concentrations in urine analysed at 40°C (y) and 24°C (x) (○) (line A: $y=0.844x+47$; $n=20$; $r=0.9723$; $P<0.001$) or at 80°C (y) and 24°C (x) (■) (line B: $y=3.88x+274$; $n=20$; $r=0.8663$; $P<0.001$).

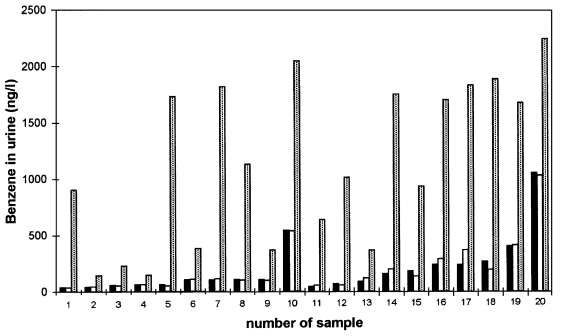

Fig. 4. Benzene concentrations in urine samples with different pH: alkalinised pH (black column), physiological pH (white column), acid pH (grey column). The first ten samples refer to non-smokers, the others to smokers.

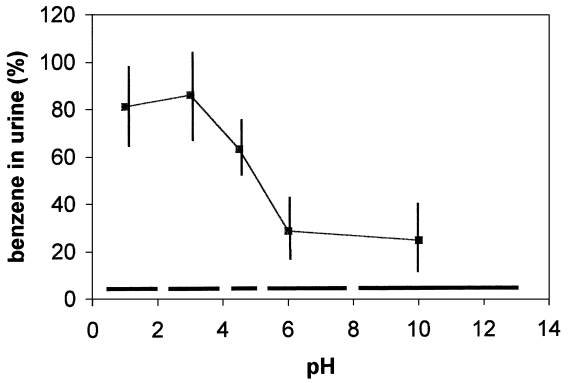

Fig. 5. Relationship between benzene concentrations in urine and pH. Black dots and vertical bars denote mean values and their standard deviations, respectively.

illustrated in Fig. 4: for each individual sample, three different benzene values, obtained by analysing urine at pH≤3, at physiological pH and at pH≥9, respectively are reported. On urine acidification a variable amount of benzene is fortuitously released from different samples. The increase in benzene concentration obtained on acidification in comparison with the results obtained at physiological pH is statistically significant ($Z=27$; $P<0.001$). The ratio of the results obtained on acidification to those at physiological pH appeared to be independent of the smoker or non-smoker status of the subjects studied: 13 samples (eight from smokers) showed 2- to 10-fold increased values, and seven samples (two from smokers) showed 10- to 28-fold increases over basal values.

Benzene in alkalinised urine shows no significant differences as compared to benzene concentrations obtained at 24°C.

The effect of urine acidification on benzene concentrations was also confirmed when we studied in detail the influence of pH modification on urinary benzene (see Section 2.2, for a description of sample preparation). As can be seen in Fig. 5, on acidification (pH≤4) peak benzene concentration values were obtained, while, at pH higher than 5, only 20–25% of the highest values could be detected.

When paired values obtained by treatment of individual urine at 80°C (uncorrected pH) (y) or at pH≤4 (at 24°C) (x) were plotted, a significant correlation between the two series of results was evidenced (Fig. 6).

'Physiological' total protein values ranging from 12 to 110 mg/l (mean 61 mg/l) were observed in all urine samples studied. No correlation could be found between protein and benzene concentrations in urine analysed in different conditions.

Benzene concentrations after protein precipitation by adding zinc sulphate and NaOH to urine were not statistically different from those obtained in unmodified urine.

4. Discussion

For several years now organic solvents have been measured in biological media using the 'head-space technique', which is easy to perform and yields accurate and precise results. We were unable to find

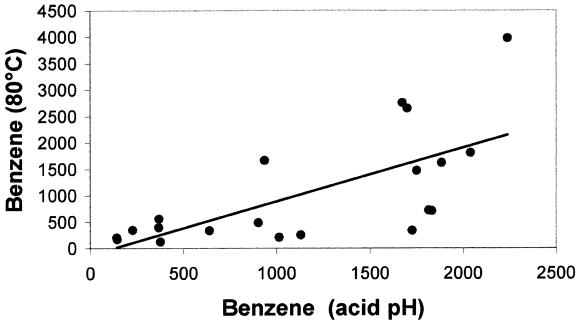

Fig. 6. Correlation between benzene concentrations (ng/l) in heated (80°C) (y) and in acidified urine (x): $y=1.01x-121$; $n=20$; $r=0.6785$; $P<0.01$

any evidence in the literature to support the hypothesis that changing the pre-analytical factors induces such marked differences in results. Our data demonstrate that the urinary concentration of benzene can differ by as much as 30-fold if the urine sample is heated or acidified.

The urinary benzene concentration measured at 24°C can be considered 'free benzene'. A different amount of benzene in urine can be released by heating the sample to 40 or 80°C. The 'heat-released' benzene is on average five times higher than 'free benzene' (Table 1 and Fig. 3). Urine acidification also releases remarkable amounts of benzene ('acid-labile benzene') which are related to the 'heat-released' ones (Fig. 6). but with a widely scattered data distribution. Free and 'acid-labile' concentrations for carbon disulfide in blood were previously found [15].

We were unable to find any significant correlation between total urinary protein concentration and 'free', or 'heat-released' or acid-labile' benzene in urine. This lack of correlation does not contrast with the hypothesis that benzene may be bound by a specific protein, as recently found for acrylonitrile [16]. The latter has a high affinity for a parvalbumin weighing about 10 000 Da and its measurement in urine requires lengthy hydrolysis at 90°C.

We have no rational explanation for our results, but a number of hypotheses are possible. As already suggested, it may be that part of the benzene in urine is bound to specific proteins and released by temperature or acid pH. Another hypothesis suggests the possibility that part of the benzene may derive from 'physiological' compounds present in urine such as benzaldehyde or methoxybenzene when the temperature or pH of urine changes. A third possibility is related to the urinary sediment which can adsorb different molecules and releases them in specific temperature or acidity conditions. These hypotheses, of course, are not mutually exclusive.

Urinary benzene concentrations of non-occupationally exposed people have rarely been studied. Ghittori et al. [8] found a benzene concentration of 131 ng/l (geometrical mean G.M.) in 20 non-smokers as against 790 ng/l (G.M.) in heavy smokers. Their analysis was performed at 24°C. Comparable results were obtained by Kok and Ong [14] with 99 ng/l (G.M.) in non-smokers and 258 ng/l (G.M) in smokers; their measurements were performed at 60°C for 30 min. Our concentrations obtained at 24°C are slightly lower than those of the other researchers, but those obtained at 80°C or with acid urine are much higher both in non-smokers and in smokers (Tables 1 and 2). The results of Kok and Ong [14] obtained at 60°C are at variance with our data.

On the other hand, even more at variance are the results of Ong et al. [9,10] and Ghittori et al. [8] in workers occupationally exposed to benzene. Both groups of researchers found a statistically significant linear correlation between environmental and urinary benzene concentrations, but at the same occupational exposure of 1 ppm there is approximately a 5-fold difference in results between these two studies. We believe that differences in analytical methods mainly account for the divergent results.

There is a certain body of evidence to support the hypothesis that the urinary excretion of solvents is mainly related to their blood/urine partition coefficient [17], but our present results and those of other

Table 2
Main benzene concentration statistical parameter values in urine samples analysed at 24 or 80°C or in acidified urine (see Table 1) after splitting samples into non-smokers and smokers (data in ng/l)[a]

	At 24°C		At 80°C		In acid urine	
	N.S.	SM	N.S.	SM	N.S.	SM
Mean	122	274	503	1581	887	1403
S.D.	150	293	495	1242	747	616
Median	81	209	345	1546	639	1683
Geom. mean	85	186	371	1088	579	1240
Minimum	39	45	127	211	141	368
Maximum	541	1051	1808	3981	2040	2238

[a] N.S., non-smokers; SM, smokers.

studies [18,19] suggest that the mechanism of urinary excretion of organic solvents is subject to more complicated and unknown rules. In dogs exposed to benzene, the concentration of the solvent in urine was much higher than in blood [18]. Brugnone et al. [19] reported similar mean concentrations of benzene in blood and urine in non-occupationally exposed people. Kok and Ong [14] found comparable concentrations of benzene in blood and urine in non-smokers, while, in smokers, the urinary concentrations of benzene were higher than in blood. These findings might suggest that the benzene concentrations in blood are equal to or lower than that in urine: this is neither true nor possible. At 37°C the 'in vitro' blood/air partition coefficient of benzene is close to 8 [20], while the water/air, like the urine/air partition coefficient is close to 3. On this basis, blood concentrations of benzene should always be 2–3-fold higher than in urine.

In conclusion, the results presented here suggest that more studies are needed if we are to understand the mechanisms of excretion of organic solvents in urine; moreover, comparison of urinary concentrations of benzene (and perhaps of other organic solvents as well), obtained by different research groups will be possible, only if the analytical conditions yield comparable results.

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