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Review

Determination of benzene and its metabolites: Application in biological monitoring of environmental and occupational exposure to benzene

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Abstract

Methods for the biological monitoring of benzene and its metabolites in exhaled air, blood and urine are reviewed. Analysis of benzene in breath can be carried out by using an exhaled-air collection tube and direct analysis by GC or GC–MS; however, this technique is less reliable when compared to analysis using blood or urine. For the determination of non-metabolized benzene in blood and urine, GC head-space analysis is recommended. Phenol, the major metabolite of benzene can be monitored by either HPLC or GC methods. However, urinary phenol has proved to be a poor biomarker for low-level benzene exposure. Recent studies have shown that *trans,trans*-muconic acid, a minor metabolite of benzene can be determined using HPLC with UV detection. This biomarker can be used for detection of low-level benzene exposure. Urinary S-phenylmercapturic acid is another sensitive biomarker for benzene, but it can be detected only by GC–MS. Hydroquinone, catechol and 1,2,4-benzenetriol can be measured using HPLC with either ultraviolet or fluorimetric detection. Nevertheless, their use for low-level assessment requires further studies. Eventually, for the assessment of health risks caused by benzene, biological-exposure reference values need to be established before they can be widely used in a field setting.

Contents

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List of abbreviations

BEI	Biological exposure indices
BQ	Benzoquinone
BT	1,2,4-Benzenetriol
CAT	Catechol
CRE	creatinine
DNA	Deoxyribonucleic acid
ECD	Electrochemical detection
FID	Flame-ionization detection
FD	Fluorimetric detection
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrophotometry
Hb	Haemoglobin
HPLC	High-performance liquid chromatography
HQ	Hydroquinone
MSD	Mass-selective detector
PID	Photoionization detection
RP	Reversed-phase
SPC	S-Phenylcysteine
SPE	Solid-phase extraction
SPMA	S-Phenylmercapturic acid
TLV	Threshold limit value
ttMA	*trans,trans*-Muconic acid
TWA	Time-weighted average
UV	Ultraviolet

1. Introduction

Benzene has been classified as a group I carcinogen by the International Agency for Cancer Research [1]. It is an important component in gasoline, a constituent of engine emissions, and tobacco smoke [2]. Benzene is also widely used in chemical, paint and dye industries. The evidence for the association between exposure and leukaemogenic effect has been adequately reviewed [3,4]. However, much controversy still continues on what level of exposure to benzene constitutes an acceptable risk [5].

The European Community Benzene Directive calls for an action level of 1 ppm benzene and a limit value of 5 ppm time-weighted average [6]. With new evidences on the risk of benzene associated with neoplasia, the American Conference of Government Industrial Hygienists (ACGIH) has recently proposed to lower the threshold limit value (TLV) for 8-h exposure from 1 ppm to 0.1 ppm [7]. Thus, any future studies on biological monitoring must consider the need for identifying biomarkers that are sensitive and reliable at these low-level exposure concentrations. Analytical methods for biomarkers to be adopted for the monitoring of benzene exposure should also be validated. Without reliable methods it is impossible to discern the dose–response relationship between exposure and effect.

The growing concern on benzene exposure and its effect on health has called for more studies to be conducted to identify appropriate biomarkers and assess the health risk. Biological monitoring, which may be used in conjunction with personal monitoring to evaluate the exposure information more accurately0, would provide more precise information for risk assessment. The aims of this article are of two-fold: (1) To review the methods commonly used for determination of benzene and its metabolites in

exhaled air, blood and urine, and (2) to evaluate the use of these biomarkers in environmental and occupational exposure.

2. Biomarkers for benzene exposure

Biological markers or biomarkers, are broadly defined as indicators of cellular or biochemical components or processes, or functions that are measurable in biological system or samples [8]. For environmental health research, the main interest in biological markers is to identify the early stages of changes and to understand the basic mechanisms of exposure and response.

A biological marker should be a measurable quantity that is initiated by a chemical and which results in pathological or functional changes. The prerequisite of a biomarker however, must be validated by establishing the existence of a relationship between environmental exposure and the biological response. Two characteristics determine the validity of a marker: sensitivity and specificity. It is desirable for a marker to be as specific and sensitive as possible. A specific biomarker is one that originates from the exposure to a particular toxic substance. A sensitive biomarker should be present even if the exposure level is low.

Although the analysis of hydrocarbon in exhaled breath has been postulated as a method of biological monitoring for volatile organic solvent exposure, its use has been limited. By measuring exhaled breath after exposure it is theoretically possible to estimate the exposure to benzene. This technique is however complex. One of the major drawbacks is that the results can vary considerably depending on the type of sampling technique and time of sampling [9]. Several other factors may also affect breath analysis, e.g. the solubility of the hydrocarbon compound, metabolic clearance, vital lung capacity and physical workload [10].

On the other hand, because of its invasive nature, collection of blood samples for benzene determination is not always appreciated by the workers. Therefore, traditionally the most common method used for biological monitoring of hydrocarbon exposure is based on measuring the urinary metabolites. For benzene biomonitoring, urinary phenol is most widely used. However, due to the high background of phenol caused by its presence in many foodstuffs and arising from metabolism of aromatic amino acids, measurement of urinary phenol has been noted to be unreliable especially for low levels of benzene exposure [11]. The low sensitivity and specificity of this approach calls for an urgent need to identify and evaluate more reliable biological markers for benzene exposure, so that prevention measures can be taken.

In addition, attempts have been made to estimate the uptake of benzene in order to evaluate the biological response in workers exposed to benzene. These tests have included cytogenetic studies and haematological tests. Unfortunately, most of these effect markers or genetic markers are poor predictors and thus less useful for risk assessment.

3. Metabolism of benzene

The identification, validation, and use of biomarkers in biomedicine depend fundamentally on the understanding of the metabolic processes of the toxic substance. It is a prerequisite for evaluation of the specificity of a biomarker.

The metabolism of benzene has been well studied [3,4] and the postulated pathways are shown in Fig. 1. Absorption of benzene occurs mainly through inhalation of vapours and secondarily through skin contact. Srbova et al. [12] exposed human volunteers to 47–110 ppm benzene vapour for 2 h and found that ca. 50% of the inhaled benzene was absorbed. A fraction of the absorbed benzene is excreted unchanged in the exhaled air. Several reports showed that in man, the fraction eliminated in the exhaled air varies between 10 and 50%, depending on the metabolic activities [13–15]. The remaining fraction is metabolised and the metabolic pathways are rather complex (Fig. 1). The absorbed benzene is first transformed by microsomal oxidase to benzene oxide. This compound is a very reactive intermediate that either binds directly to

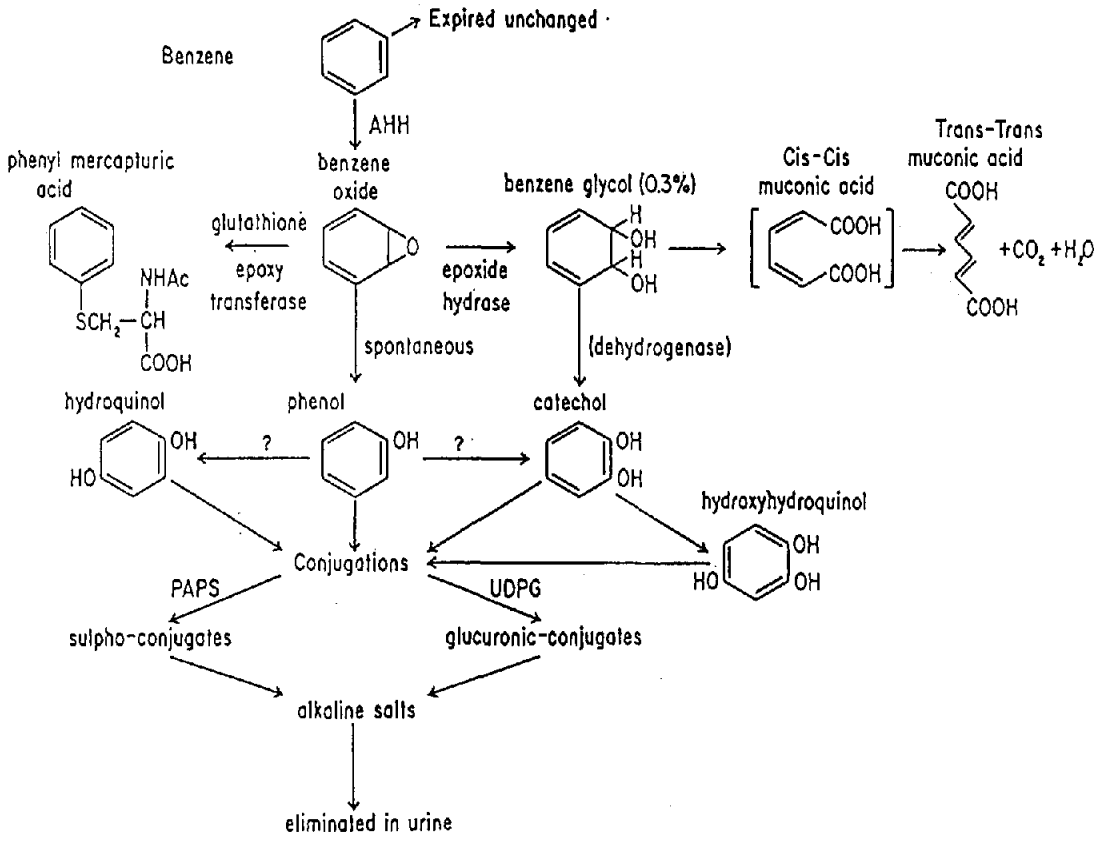

Fig. 1. Pathways of benzene metabolism and elimination.

cellular constituents (e.g. DNA or proteins), or is further transformed to other benzene derivatives [16]. Benzene epoxide is suspected of being responsible for the myelotoxic action of benzene [17].

Benzene epoxide may be transformed non-enzymatically to phenol, which is then conjugated with glucuronic acid or sulphate. The glucurono and sulphoconjugates of phenol are then excreted in the urine [18]. Phenol (free or conjugated) constitutes the main urinary metabolite of benzene.

The epoxide may also react with glutathione, the product formed being S1 glutathione. The subsequent action of a glutathionase in the presence of a glutamine acceptor, a peptidase and acetyl CoA acetyltransferase, results in the formation of premercapturic acid, i.e. S1 acetyl-L-cysteine premercapturic acid, which is subsequently excreted as S-phenylmercapturic acid (S-PMA) in the urine. Several animal studies have demonstrated that this metabolite is excreted in a dose–response relationship to benzene exposure.

trans-1,2-Dihydroxybenzene is formed by the action of the enzyme epoxidehydrase, and is quickly transformed to catechol (CAT). Very slight amounts of hydroquinol (hydroquinone, HQ) and 1,2,4-benzenetriol (1,2,4-trihydroxybenzene, BT) have also been identified in urine [19]. After a ring scission, benzene glycol is transformed to *trans,trans*-muconaldehyde and subsequently to *trans,trans*-muconic acid (ttMA), which is excreted in urine.

Based on various laboratory and field studies, an average of 12.1% (3.8–27.8%) of the total absorbed benzene is eliminated by the pulmonary route through exhaled air. Urinary excretion

accounts for 33% of the dose, mainly in the form of conjugated phenols, averaging 23.5% of the absorbed benzene [5]. Other phenolic metabolites are CAT (2.2%) and HQ (4.8%). Muconic acid accounts for 1.3% and S-PMA for 0.5%. A very small proportion of unchanged benzene (0.1 to 0.2%) is excreted in the urine. Based on field evaluation, Sherwood [20] has estimated that ca. 28% of absorbed benzene is biotransformed into phenol.

The formation of SPMA, sulphate and glucuronides is generally considered to be a detoxification pathway leading to excretion of benzene metabolites via the kidney, whereas all other pathways lead to potentially toxic metabolites [5].

It has been demonstrated that benzene metabolism occurs not only in the liver, but also in other tissues, e.g. the bone marrow [21]. This observation may have some bearing on benzene toxicity. Excretion of the metabolites is usually completed within 24–48 h after a single exposure which represents a biological half-life of less than 12 h [22].

4. Analytical methods for the determination of non-metabolised benzene

4.1. Benzene in breath

Evaluation of environmental and occupational exposure to aromatic volatile organic solvents based on the determination of the unchanged parental compounds has been of growing importance in recent years. The determination of non-metabolised compounds is generally achieved by gas chromatographic (GC) methods. Exhaled air analysis has been developed to measure non-metabolised benzene and applied for the assessment of occupational exposure. The usefulness of this biological method was primarily investigated by Sherwood [23]. Elimination curves for benzene in exhaled air demonstrated a distinct three-phase elimination process: a very rapidly falling rate during the first one to two hours after exposure, a less rapid fall over the next few hours and then a steady decline to natural background levels over a period of as much as 70 h.

Two methods have been developed for sampling exhaled air: breath sampling tubes, and a breath sampling respirator [22]. The sampling tube has the advantage of instantaneous collection, complete absence of chemical pretreatment prior to gas chromatography and ready collection of duplicate samples. It provides a more consistent measure of elimination, as all exhaled breath over the period is sampled and it reduces the risk of interference from ambient benzene vapour.

For routine monitoring, Sherwood [23] has proposed taking samples at the end of the work shift and analysing them promptly. Any follow-up samples needed can then be taken before the next shift commences, which will allow a better estimation of the integrated exposure during the preceding day.

Earlier studies have shown that the detection limit was rather high when using packed-column GC [24]. After extraction of benzene from "Sorsil" silica gel, the sample was injected onto a GC with a polyethylene glycol 400 column and detected by flame-ionization detection (FID). Sensitivity was in the range of 100 ppb in breath (319 $\mu g/m^3$), but this was improved to ca. 20 ppb (64 $\mu g/m^3$) by using a gas sampling tube with PTFE connections [23,24].

When carrying out exhaled-breath monitoring, quantitative assessment is best made immediately after exposure as it provides the most specific technique. The present BEI as recommended by ACGIH is 80 ppb for mixed-exhaled air and 120 ppb for end-exhaled air [25].

Perbellini et al. [26] conducted a study on 34 chemical workers exposed to an average benzene concentration of 0.35 ppm (1.12 $\mu g/l$). The authors used a 5% phenylmethylsilicone capillary column and a MSD for benzene in alveolar air quantification. Benzene concentration was found to be higher in smokers than in non-smokers. Although alveolar benzene showed a highly significant correlation with blood benzene concentration, the alveolar benzene concentration was not associated with environmental benzene exposure.

Studies on non-occupational exposure using breath sampling and GC–MS have shown that as low as 0.02 ppb (0.06 $\mu g/m^3$) can be detected [27]. Linear extrapolation indicates that occupational exposure to 10 ppm of benzene over an 8-h exposure period would produce 0.12 ppm in breath [27].

However, the proposed GC–MS method is not a practicable approach for routine monitoring as analysis takes ca. 1.5 h per sample. Furthermore, assessment of occupational exposure at these levels is confounded by the presence of benzene in the breath of tobacco smokers, reported as ranging from 2–60 ppb [28,29]. Studies in California showed that concentrations in the exhaled air of 5% of the citizens exceeded 2 ppb, and a peak non-occupational concentration of 300 ppb (958.2 $\mu g/m^3$) was determined [30].

For occupational exposure in the coke oven industry, Drummond et al. [31] have shown that low levels of benzene exposure were best measured in the exhaled breath at the end of the shift. Breath benzene measurements were made directly using a respiratory GC–MS. For benzene measurement the selected ion m/z 78 was used for monitoring with a detection limit of 12.2 ppb (0.5 nmol/l). With this sensitive method exposure to low concentrations of benzene, less than 0.1 ppm, could be detected. However, this method is sophisticated and costly which may restrict its use for routine monitoring. These authors also showed that determination of the benzene concentration in breath collected before the next shift is non-specific in the case of smokers [31].

Pekari et al. [32] recently reported the use of GC equipped with a FID detector for the determination of benzene in exhaled air. The separation was carried out with a HP-1 capillary column and a detection limit of 5 ppb could be achieved. Studies of the benzene metabolism in experimental subjects suggested that benzene is eliminated from the exhaled air rather rapidly. Nevertheless, with the use of their technique the concentration of benzene could still be detected in a sample taken the morning after a 4-h exposure to 1.7 ppm of benzene.

Table 1 summarises some of the methods recently used for benzene measurement and their detection limits. Determination of benzene in exhaled air is certainly a valuable method to confirm exposure to benzene. It is highly sensitive and more specific than metabolite determination. It may, however, be relevant to stress that benzene is present in very high concentrations in cigarette smoke (47–64 ppm) [33,34]. Therefore, although determination of benzene in exhaled air is specific, its detection does not necessarily imply exposure to chemicals containing benzene.

Table 1
Methods for determination of non-metabolised benzene in exhaled air, blood and urine

Specimen	Method	Sensitivity	Advantages	Disadvantages	Ref.
Exhaled breath	GC-FID	20 ppb	Specific and simple	Not widely used	[23]
	GC–MS	0.02 ppb		Required immediate	[26]
	GC–MS	12.2 ppb		analysis, reliability varied	[31]
Benzene in blood	GC-FID	100 $\mu g/l$	Specific and sensitive	Invasive	[36]
	GC-FID	20 $\mu g/l$			[37]
	GC–MS	0.04 $\mu g/l$			[27]
	GC-FID	0.4 $\mu g/l$			[42]
	GC-FID	0.5 $\mu g/l$			[39]
	GC-PID	005 $\mu g/l$			[43]
Urinary benzene	GC-FID	50 ng/l	Specific and sensitive	Not widely used	[44]
	GC-PID	40 ng/l			[43]

4.2. Benzene in blood

The measurement of benzene in blood has been proposed as a biological monitoring method [28]. Although a few procedures have recently been developed, the use of this approach has rarely been studied as a method of evaluating environmental or occupational exposure.

Direct injection of blood into a packed Chromosorb W column followed by FID was described by Szadkowski et al. [35]. However, this method resulted in serious contamination of the column. Although the method was specific, the detection limit was poor and the background values were high [6].

Methods using a clean-up procedure are more specific. Snyder et al. [13] reported an extraction method for blood benzene using toluene. Methylene chloride was used as the internal standard. Separation was achieved on a packed Chromosorb W AW DCMS column with 10% UC-W using FID. The recovery was ca. 79%.

An improved extraction procedure with recovery rates of over 95% was reported by Jirka and Bourne [36]. Hemolysed blood was extracted with purified toluene, and benzene was separated on a Supelco SP-2100 packed column followed by FID. A detection limit of 100 μg/l of blood was achieved.

Both direct methods and extraction methods suffer from poor sensitivity and selectivity. The detection limits are too high to cope with decreasing occupational threshold values and for environmental exposure levels in the low ppm range. They are thus not recommended for routine use.

As exposure levels decrease, the determination of benzene requires increasingly sophisticated methods. These methods must not only be sufficiently sensitive and specific to determine low levels of benzene exposure, they must also be able to detect natural background levels. In this respect, head-space analysis offers a useful method for the separation of volatile benzene from other biological constituents. When interference from the biological matrix is eliminated, the head-space technique could achieve detection limits at the ng/l level.

In 1971 Sato [37] used a syringe-equilibration method for the determination of benzene and toluene in blood. After establishing equilibrium at 37°C for 1 h, a 1-ml volume of the overlying air was submitted to GC analysis. The detection limit was 20 μg/l for benzene. This procedure was however very tedious and thus not suitable for field studies. By equilibrating a blood sample in an airtight vial and injection with a gastight syringe onto a column packed with Chromosorb GAW DMCS with 4% phenylsilicone oil, a similar detection limit was achieved by Angerer [38].

Brugnone et al. [39] reported a detection limit of 0.5 μg/l achieved on a column packed with Apiezon L on Supelcoport and a FID. The results to showed good correlations between blood benzene concentration and average exposure concentration during the shift. Even though the data showed wide variation, exposure to an average of 0.3 ppm benzene during a workshift appeared to produce a blood benzene concentration of ca. 579 ng/l 16 h later. Good correlation was also found between blood and alveolar benzene concentrations in exposed workers: 313 ppb (1 μg/l), equivalent to ca. 37.6 ppb (0.12 μg/l) in alveolar air. This fits well with studies of Sato and Fujiwara [40] and Sherwood and Carter [24] on human volunteers.

Drummond et al. [31] analysed blood benzene using a semiautomated head-space technique with a 10% Carbowax 1500 on Chromosorb W column. Toluene was used as an internal standard and benzene was detected using FID. The detection limit was 0.05 μmol/l (39 μg/l). Unfortunately, the concentrations were only just above the detection limit for workers exposed to benzene concentrations of around 1 ppm at the workplace. The authors concluded that determination of benzene in blood was less reliable than breath analysis for low-level exposure.

Significant improvement in the specificity of the head-space technique was achieved with the use of capillary columns. Better peak shapes and baseline led to improved sensitivity. Angerer [41] reported the use of a fused-silica dimethylpolysilocane capillary column for the separation of benzene from various volatile hy-

drocarbons. The detection limit of this method was 3 μg/l for benzene, using FID.

The simultaneous determination of benzene and toluene on methylsilicone or phenylmethylsilicone capillary columns with PID was reported by Pekari et al. [42]. As PID is sensitive to double bonds and aromatic rings, interference from the biological matrix was reduced; thus a detection limit of 0.4 μg/l (5 nmol/l) was achieved for benzene in blood. By using dynamic head-space analysis with a purge and trap procedure, Perbellini et al. [26] have shown that the detection limit can be further lowered by a factor of at least 10, when the method is combined with MSD. With a silica capillary column with crosslinked 5% phenylmethylsilicone oil as the stationary phase and a quadrupole mass detector for benzene quantification, they reported benzene levels in blood for environmental and occupational exposure ranging from 1–377 μg/l.

Using a silicone-gum capillary column and a PID, Kok and Ong [43] have recently shown a detection limit of 0.64 nmol/l (0.05 μg/l) (Fig. 2). With this low detection limit the method proved suitable for the determination of benzene in blood for non-occupational exposure studies.

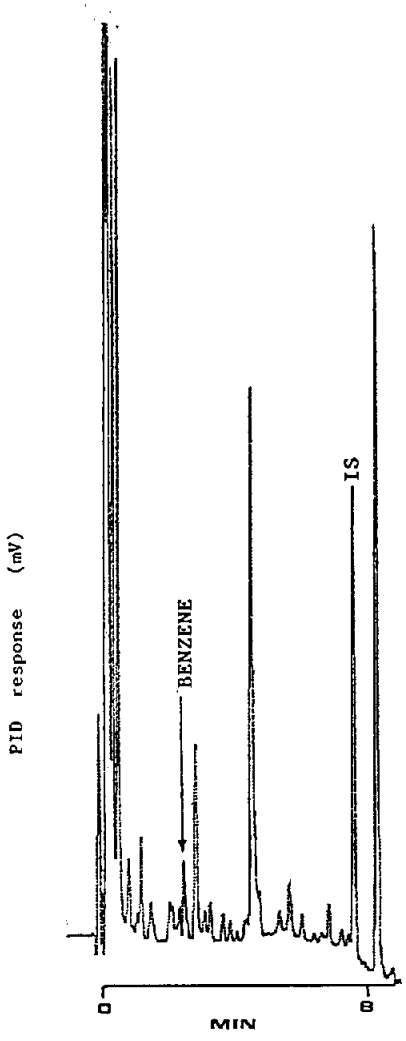

Fig. 2. Head-space chromatogram of a blood sample collected at the end of shift from a petroleum worker. A temperature controlled incubator was used to incubate the sample vial at 60°C. The column used was a crosslinked methylsilicone capillary column (30 m × 0.53 mm I.D.). The oven temperature was programmed as follows: 50°C for 2.5 min, increase at 10°C/min to 150°C and hold for 1 min. The injector and detector temperatures were set at 120°C and 180°C, respectively. PID was used for detection.

4.3. Benzene in urine

Although several studies have been conducted on the determination of benzene in blood, the quantification of benzene in urine is less investigated. Ghittori et al. [44] have recently reported the use of a thermal desorption technique and FID for low-level urinary benzene determination. Benzene was first stripped from the urine and concentrated on a Carbotrape tube by means of a suction pump. This was followed by passing filtered air through the GC. The benzene detection limit of this method was 50 ng/l (6.4 nmol/l) and the average recovery was ca. 82%. A significant correlation ($r = 0.60$) was observed between air benzene concentrations and benzene concentrations in urine for occupational exposure to benzene concentration of 2–4.5 μg/m^3. This method managed to eliminate interferences from the biological matrix, but the procedure was rather time consuming. Kok and Ong reported the determination of benzene in urine by head-space analysis and PID [43]. A gas chromatograph equipped with a crosslinked methylsilicone-gum capillary column was used for separation and, as PID is highly sensitive to

aromatic rings, a detection limit of 0.51 nmol/l urine (40 ng/l) was achieved (Table 1).

The method was validated with urine and blood samples collected from 25 non-smokers and 50 smokers. A significant correlation (r = 0.61) was found between benzene in blood and benzene in urine. The authors suggested that determination of benzene in urine could replace the determination of benzene in blood for biological monitoring, as the method is non-invasive and the relatively simple.

One of the major drawbacks of the determination of non-metabolised benzene in biological fluids for low-level occupational exposure was that their is usually interference from exposure to tobacco smoke. Caution should be exercised when interpreting the data on occupational exposure.

Table 2 summarises some of the values of non-metabolised benzene in exhaled air, blood and urine of non-exposed populations. The results clearly indicate that the average benzene concentrations in the blood or urine of cigarette smokers are generally 3–5 times higher than the concentrations in non-smokers.

5. Determination of benzene metabolites

The toxicity of benzene is generally thought to be mediated by its metabolites. The metabolites usually have a longer half-life in the body than unchanged benzene, thus a time-weighted assessment of exposure could be estimated more accurately. The main disadvantage of this approach is that some of the biomarkers are diagnostically unspecific.

5.1. Ratio between inorganic and organic sulphates in urine

The ratio between inorganic and total sulphates in urine is normally more than 85%. Exposure to benzene produces a decrease in this

Table 2
Reference values of non-metabolised benzene in exhaled air, blood and urine of non-exposed persons

Biomarker	Average concentration (ng/l)	Range	Smoking habit[a]	Ref.
Exhaled breath	9	1– 77	NS	
	40	2– 171	S	[26]
Blood	218 ± 96	112– 455	NS	
	547 ± 195	287– 947	S	[87]
	127 ± 54	49– 191	NS	
	584 ± 300	109–1136	S	[26]
	130 ± 96	10–1067	NS/S	[88]
	332 ± 320			
	176 ± 62	80– 300	NS	
	211 ± 85	130– 430	S	[89]
	110 ± 53	49– 219	NS	
	328 ± 165	81– 600	S	[43]
Urine	116 ± 73	52– 344	NS	
	404 ± 269	110–1450	S	[43]
	139 ± 42	–	NS	
	943 ± 550	–	S	[44]

[a] NS = non-smokers, S = smokers.

ratio, since some metabolites of benzene are eliminated as sulpho-conjugates [45].

A significant decrease in the ratio can only be observed when benzene exposure exceeds 40 ppm. When using this approach the sensitivity of the test is too low to use as a biomarker for exposures in the order of 10 ppm. The specificity of this test [45] is also limited since numerous hydroxylated organic chemicals are also excreted in urine as sulphoconjugates. Thus, this test can no longer be recommended for the evaluation of benzene exposure.

5.2. Urinary phenol

The primary metabolite of benzene is phenol. Phenol and its oxidation product are excreted as glucuronide and sulphate conjugates in urine.

Measurement of phenol in urine was the most commonly used technique for biological monitoring of benzene exposure. The analysis of phenol in urine has evolved from the classical spectrophotometric method to the more sensitive capillary column GC and RP-HPLC methods. The early methods failed to distinguish between phenol and cresols, and hence lacked specificity. The introduction of capillary GC as a routine tool enabled a more specific measurement of phenol, which is normally present in urine in a much smaller concentration than the methyl phenols, such as *o*-and *m*-cresols.

The most commonly used columns for determination of urinary phenol by HPLC are C_{18} or C_8 combined with UV detection at 205 or 275 nm. The mobile phases used are mixtures containing more polar solvents such as methanol and acetonitrile; the detection limits are in the range of 1–2 mg/l urine [46–52].

It has been reported that urine without prior treatment could be analysed for phenol on a Nucleosil-5 C_{18} column using methanol–H_2O–acetic acid (50:50:0.2, v/v), containing 0.05 *M* PIC reagent (tetra-*n*-butylammonium bromide) in the mobile phase. Detection was carried out at 254 nm [46]. However, owing to the large concentration of impurities in the urine, the life of the analytical columns tends to be shortened considerably (unpublished observations, Ong and Lee).

Since phenol is normally excreted as glucuronide or sulphate conjugates, its determination requires pretreatment of the sample prior to analysis. These two compounds could be hydrolysed either enzymatically [47,48] or by acid [49–52]. The released phenol can then be subjected to solvent extraction [53] prior to chromatographic measurement.

Solvent extraction after enzymatic hydrolysis was reported by Brega et al. [48]. Urine was extracted with methylene chloride and analysed on a C_{18} column with methanol–water–orthophosphoric acid (30:70:0.1, v/v) as the mobile phase with detection at 210 nm.

Although enzymatic hydrolysis is found to be quite reliable, this approach is hampered by the long time required for complete hydrolysis. This method is also susceptible to the interference by *o*-phenylphenol, which has a retention time close to that of phenol [53]. A more recently developed method has shown that concentrated hydrochloric acid was able to hydrolyse the conjugated glucuronide and sulphate for the subsequent analysis of phenol [53]. It seems that the most essential aspect of urinary phenol determination is to ensure that hydrolysis of the conjugated phenol is complete before extraction (Fig. 3).

Eadsforth and Coveney [51] extracted acidified urine with dibutyl ether and separated the phenol on a Spherisorb-NH_2 silica gel column. The mobile phase used was *n*-hexane–propanol (98:2, v/v) and phenol was detected at 265 nm.

Although phenol is preferably determined by HPLC methods, GC methods for the determination of phenol in urine have also been developed. The preparation of urine samples and the extraction of phenol is similar to the procedures used in the HPLC methods. The acidified and hydrolysed urine was subjected to solvent extraction [53,54] or steam distillation [49]. In the past, separation was achieved using packed Universal "A" or "B" support with 2% polyethyleneglycol columns and FID was used for detection [23]. The reported detection limits ranged from 0.1 mg/l to 2 mg/l [54–56].

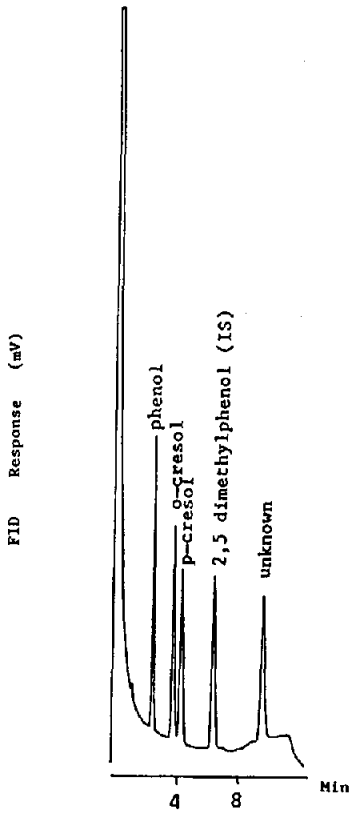

Fig. 3. Capillary chromatogram of a urine sample collected at the end of the workshift from a worker exposed to about 2 ppm of benzene. Separation was accomplished with a methylsilicone-gum capillary column (10 m × 0.63 mm I.D.). The oven temperature was 60°C for 2 min, increased by 10°/min to 70°C for 0.5 min, followed by 80°C for 2 min. Detector: FID.

Field and experimental studies have been initiated by Sherwood [23] and Sherwood and Carter [24] to correlate urinary phenol concentration with environmental exposure. Immediately after exposure, 25 ppm in air would give a phenol concentration of 100 mg/l which decreases to 50 mg/l 16 h after exposure. Thus the extrapolated urinary phenol concentration was 50 mg/l. The latter has been estimated to correspond to the current BEI of 50 mg/g creatinine [55].

To date, capillary columns are much more commonly used for urinary phenol determination. With the achieved improvement in resolution, the specificity of the detection has also been increased. It has been possible to quantitate phenol and various alkyl phenols (cresols) in a single run using capillary columns coated with methylsilicone [53] or SE-54 [54]; detection has usually been carried out by FID (Table 3). Detection limits of less than 0.2 mg/l have been reported [57].

A summary of values reported in the literature for the phenol concentration in urine for persons who were not occupationally exposed to benzene is shown in Table 4. It can be seen that for non-occupationally exposed persons, the phenol concentration in urine usually does not exceed 20 mg/l. Nevertheless, in some cases the values may reach 30 mg/l. This is because phenol is also a physiological product of the metabolism of various aromatic amino acids and certain pharmaceuticals, such as pheny silicate and many food stuffs [58]. The normal background range of the urinary phenol excretion in non-occupationally exposed persons is between 0 and 20 mg/g creatinine [50]. The current American Conference of Governmental Industrial Hygienists' (ACGIH) occupational exposure limit for benzene in air is 1 ppm with an intended reduction to 0.1 ppm [7]. Monitoring of exposure to benzene in air at a concentration in this range is thus limited by the inadequate specificity of the phenol determination. Furthermore, dermal application of phenol containing preparations, exposure to phenol itself and ingestion of salicylate containing drugs increase the urinary phenol concentration [56]. Thus in today's context, urinary phenol is not a useful biomarker for risk assessment of benzene exposure.

5.3. Urinary *trans,trans*-muconic acid

Several new biomarkers for monitoring exposure to low benzene concentrations have been developed during the last few years.

Both human and animal studies indicated that *trans,trans*-muconic acid (ttMA), a metabolite of *trans,trans*-mucoaldehyde through the oxidation of benzene oxide, could be suitable as a biomarker for human exposure to benzene [59].

Gad-El Karim et al. [60] reported an HPLC method for the determination of ttMA in urine

Table 3
Methods for the determination of benzene metabolites in urine

Benzene metabolite	Method	Sensitivity (mg/l)	Advantages	Disadvantages	Ref.
Urinary sulphate ratio	Spectrophotometry	Not given	Unknown	Not validated	[45]
Urinary phenol	GC-FID	1 mg/l	Useful for high level exposure	High background level	[87]
	GC-FID	0.1–1 mg/l			[46–52]
Urinary	HPLC-UV	0.1 μg/l	Low background level	Purification of urine samples is essential	[61]
	HPLC-UV	0.05 μg/l			[62]
	HPLC-UV	0.025 μg/l			[63]
	GC–MS	0.1 μg/l			[59]
Urinary S-PMA	HPLC-UV	6 μg/l	Highly specific	Tedious, require sophisticated technique	[65]
	GC–MS	1 μg/l			[66]
	GC–MS	1–5 μg/l			
Urinary CAT	HPLC-UV	0.5 mg/l	Highly sensitive	High background level	[71]
	HPLC-UV	7 mg/l			[72]
	HPLC-FL	0.2 mg/l			[73]
Urinary HQ	HPLC-UV	1.0 mg/l	Very sensitive	Research method	[71]
	HPLC-FL	0.03 mg/l			[73]
Urinary BT	HPLC-UV	0.5 mg/l	Sensitive	Unproven	[74]

Table 4
Reference values of urinary metabolites of benzene of non-exposed persons

Metabolite	Average concentration (mg/l)	Range	Smoking habit	Ref.
Phenol	7.5	2–18		[54]
	4.6	0.6–12.8		[49]
	3.4	0.5–17.3		[52]
	11.3/24 h			[47]
ttMA	0.16	0.1–0.5	NS	[62]
	260	6–870	NS	
	310	17–3830	S	[63]
SPMA	0.004	–	–	[67]
HQ	580	420	NS	
	1180	920	S	[73]
CAT	1054	1610	NS	
	4750	3830	S	[73]

samples by using an RP column. However, the recoveries were poor. An improved analytical method was developed by Inoue et al. [61]. Urinary ttMA was analysed using a Spherisorb ODS 5 column and detected with a UV detector at 265 nm. The mobile phase used was methanol–0.1% acetic acid (1:9, v/v). In field application, they established that the natural background of the metabolite should be less than 0.1 mg/l. The authors estimated that about 2% of benzene inhaled is excreted into the urine as ttMA. Using a similar approach, Ducos et al. [62] were able to achieve a detection limit of 50 μg/l, when a clean-up procedure using SPE extraction cartridges was applied to urinary samples prior to HPLC analysis (Table 4). Separation was carried out on a LiChrosorb C_{18} column with detection at 259 nm. The mobile phase used was methanol–1% aqueous acetic acid (10:90, v/v). A human volunteer study showed that ttMA has half-life of ca. 12 h, which is similar to that of phenol. However, field data on workers are insufficient to confirm the reliability of the method at low levels of exposure, as too few measurements were made at low levels of exposure. Nevertheless it appears that this technique should be able to assess exposure in the order of 1 ppm.

Bechtold et al. [59] also studied ttMA elimination in rats and in a small group of exposed workers using a GC–MS technique. A 25-m Ultra-1 fused-silica capillary column was used for separation and detection was by a mass-selective detector. Unfortunately their exposure assessment is weak. Although there was a significant correlation between urinary phenol and ttMA, the limited data (14 exposed and 8 controls) only confirmed that workers exposed at 4.4 ppm showed significantly higher levels of the metabolite than non-exposed subjects.

A recent study by Lee et al. [63] has shown that purification of urine samples by ion-exchange chromatography eliminates the matrix interferences and results in better resolution and sensitivity (Fig. 4). The mobile phase used was acetic acid–methanol–sodium acetate (1:10:89, v/v) with a pH of 3.2. The ttMA was detected at 265 nm and a Partisphere ODS cartridge column

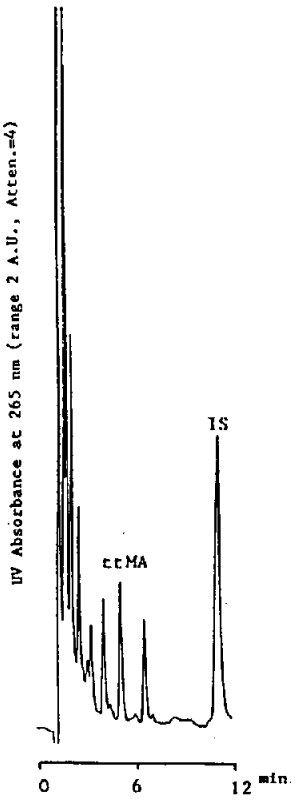

Fig. 4. HPLC chromatogram of a processed urine sample collected from a subject exposed to about 1.3 ppm of benzene. Column: Partisphere 5 ODS cartridge, 5 μm. UV detection at 265 nm. The mobile phase was acetic acid–methanol–sodium acetate (5 mmol/l) (1:10:89, v/v). Reproduced from Ref. [63] with permission.

was used. The initial flow-rate was 10 ml/min and it was increased to 1.5 ml/min after 6 min. Vanillic acid was used as internal standard and a detection limit of 125 pg (0.05 mg/l) of ttMA could be achieved. The method was validated with urine samples collected from non-occupationally exposed subjects and from refinery workers exposed to benzene concentration around 1 ppm. A close correlation (Fig. 5) was observed between the increased urinary ttMA concentrations (after vs. before exposure) and the environmental benzene concentrations (r = 0.81). The results also showed that for non-occupationally exposed subjects the urinary ttMA concentrations in smokers are significantly higher than in non-smokers.

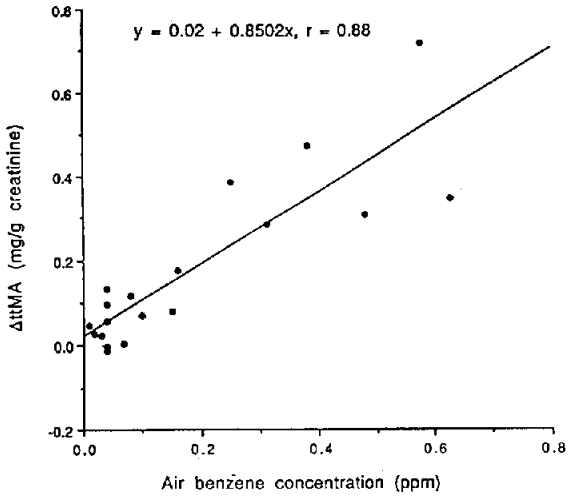

Fig. 5. Relationship between benzene exposure and ttMA excretion. ΔttMA: difference between end-shift and pre-shift urinary concentrations of ttMA. Reproduced from Ref. [63] with permission.

The stability of ttMA in acid-treated urine allows sample storage, however using this approach a sample cleaning procedure is essential, especially for smoker's urine, which is known to contain more organic metabolites that cause matrix interference.

In a similar study, Lauwerys et al. [64] have recently shown that ttMA is a reliable biomarker for occupational exposure to low levels of benzene. The mean postshift ttMA concentrations corresponding to an 8-h TWA exposure to 0.5 and 0.8 ppm benzene were 0.8 and 1.4 mg/g creatinine, respectively (Table 5).

Since the method used to determine urinary ttMA levels is highly sensitive and has the ability to detect background environmental exposure in the general population (Table 5), the use of this biomarker may be promising for risk assessment of exposure to low levels of benzene.

Table 5
Biomarkers for workplace exposure to benzene

Biomarker	Exposed level (ppm)	Estimated value	Ref.
Exhaled breath	1.12 μg/l	70 ng/l[a]	[26]
Blood	0.3	1.6 μg/l	
	1.7	7.8 μg/l	[26]
Urine	0.46	3.3 μg/l	
	0.41	5.0 μg/l	[44]
Phenol	25	50 mg/l	[54]
	10	50 mg/g cre.	[25]
ttMA	10	14.31 mg/g cre.	[61]
	0.5	0.445 mg/g cre.[b]	[63]
	0.5	0.8 mg/g cre.	[64]
	0.8	1.4 mg/g cre.	[64]
SPMA	0.07–1.1	0.025–0.071 mg/g cre.	[67]
	1	0.046 mg/g cre.	[68]
CAT	10	17.3 mg/g cre.	[71]
HQ	10	59.4 mg/g cre.	[71]
BT	10	2.26 mg/g cre.	[74]

[a] 16 h after exposure.
[b] Differences between after exposure and before exposure; cre. = creatine.

5.4. Urinary S-phenylmercapturic acid

SPMA (S-phenylmercapturic acid or S-phenyl-N-acetylcystine), a minor metabolite of benzene resulting from conjugation with glutathione, has been found to be specific for benzene exposure. This metabolite is physiologically excreted only in small amounts.

Jongeneelen et al. [65] were the first to describe a gradient HPLC method for SPMA. They used a C_{18} column and water–methanol–phosphate buffer (80:10:10, v/v) and water–methanol (10:90, v/v) as the mobile phase and UV detection at 256 nm. Unfortunately, the physiological background level of SPMA could not be detected with this method, as the detection limit (6 μg/l urine) was too high for low-level evaluation.

Mueller et al. [66] showed that this metabolite is a more specific indicator for benzene exposure than phenol. They used a modified amino acid analysis following ethyl acetate extraction and acid hydrolysis. The highest value reported was 140 μg/l in 7 workers exposed to 5–7 ppm of benzene, close to the detection limit of about 100 μg/l. This method was further developed by Stommel et al. [67] using both amino acid analysis and GC–MS. The column used was a 25-m PB-2Q (WGA). A detection limit of 50 nmol (about 1 μg/l) SPMA/l of urine was reported. Workers exposed to 0.005–0.15 ppm showed an increased SPMA excretion during the shift from 12.0 to 48.5 μg/g creatinine, while a second group of workers exposed to 0.07–1.13 ppm showed a change from 25.1 to 70.9 μg/g creatinine [67] (Table 5). For non-exposed subjects a normal value of 4 μg/l urine could be determined.

Using the method of Stommel et al. with some changes [67], Van Sittert et al. [68] recently showed that a detection limit of 1–5 μg SPMA/l urine could be achieved. The column used was a 60-m fused-silica column coated with DB-1. Quantification of SPMA was carried out using GC–MS and monitoring the ion m/z 194. The authors also validated the use of this biomarker for low concentrations of benzene in 12 separate investigations in three types of industrial settings. A strong correlation was found between 8-h exposure to airborne benzene (0.3 ppm and higher) and urinary SPMA concentrations in end-of-shift samples. It was calculated that an 8-h exposure to 1 ppm benzene would correspond to an average SPMA concentration of 46 μg/g creatinine (Table 5). The authors concluded that with the sensitivity of ca. 1–5 μg/g creatinine exposure to benzene levels of 0.3 ppm and higher can be measured.

It appears that GC–MS is required for a sufficiently sensitive determination of SPMA. Although the method is promising, it is not yet effective enough for SPMA to be used as a biomarker for routine monitoring.

5.5. Urinary catechol and hydroquinone

As early as 1953, Parke and Williams [69] identified catechol (CAT) and quinol (hydroquinone, HQ), in addition to phenol, in the urine of laboratory animals given [^{14}C]benzene. However until recently very few reports on the analysis of CAT in biological fluids have been published, probably due to technical difficulties. A method for the separation of phenolic metabolites of benzene using RP-HPLC was described by Greenlee et al. in 1981 [70]. HPLC separation of CAT, HQ and phenol was carried out under linear gradient elution from 10 to 90% of aqueous methanol containing 200 μl/l of formic acid. To prevent auto-oxidation of various phenolic metabolites a constant stream of nitrogen was blown across the liquid phase during the separation. Detection of phenolic metabolites was performed at 260 nm. This method however suffers from serious biological matrix interferences and thus is not commonly adopted for routine use. Furthermore, as the method requires a nitrogen atmosphere, application to validate the use of CAT and HQ in environmental or occupational studies has not been attempted.

Inoue et al. [71] reported the separation of urinary CAT and HQ in urine of workers ex-

posed to benzene. Hydrolysed urine after carbon disulphide–diethyl ether extraction was analysed using a H-3056 column with detection at 280 nm. The mobile phase was acetonitrile–acetic acid–water (15:1.5:83.5, v/v). The detection limit was 0.5 mg/l for CAT and 1.0 mg/l for HQ. The method was validated with a field study comparing CAT excretion in 152 benzene exposed workers and 131 non-occupationally exposed subjects. The authors found a linear relationship between occupational exposure, as determined by personal sampling, and concentrations of CAT and HQ in urine. It was estimated that ca. 2% and 10% of the inhaled benzene would be excreted in urine as CAT and HQ, respectively. However, as the exposure levels were rather high, benzene concentrations below 10 ppm were not assessed. The geometric mean values for non-exposed subjects were 13.32 and 5.43 mg/g creatinine for CAT and HQ, respectively. Correlation indicates that exposure to a concentration of 10 ppm produces 17.3 mg CAT/g creatinine and 59.4 mg HQ/g creatinine in urine (Table 5).

Schad et al. [72] reported a method for the simultaneous determination of benzene metabolites by SPE and HPLC. An anion-exchange Bond Elut extraction cartridge filled with Sax sorbent was used to pretreat the urine samples before diethyl ether extraction. Analysis was carried out using a Nucleosil C_{18} column and detection was performed at 270 nm. The eluent was a solution of 5 mmol/l sodium phosphate (pH 3.4) containing 30% (v/v) of methanol. The detection limits of CAT and HQ were 7 and 60 mg/l, respectively. The method was validated in female mice dosed with 300 ppm of benzene for one week. This method is tedious and lacks sensitivity and has not been validated for either occupational or environmental exposure.

Lee et al. [73] recently developed a HPLC method using variable wavelength fluorimetric detection for the simultaneous determination of urinary CAT, HQ and phenol (Fig. 6). Acid hydrolysed urine samples were saturated with sodium sulphate and extracted with diethyl ether before HPLC analysis. The two buffers used for gradient elution were (a) 10 m*M* sodium acetate containing 0.5% (v/v) acetic acid, and (b) the same buffer but additionally containing 20% (v/v) acetonitrile. The metabolites were separated with a Partisphere-5 ODS cartridge column. The lowest detection limits when using variable wavelength fluorimetric detection were 0.03, 0.2 and 0.3 mg/l of urine for HQ, CAT and phenol, respectively. The concentrations of HQ and CAT in non-exposed subjects were 0.56 and 2.12 mg/g creatinine, respectively. These findings confirm the earlier results of Inoue et al. [71] that the urinary CAT concentration was generally higher than that of HQ.

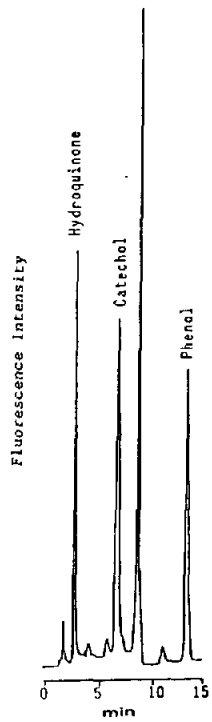

Fig. 6. Simultaneous determination of HQ, CAT and phenol in a processed urine using HPLC with a variable-wavelength fluorimetric detector. The two buffers used for gradient elution were (a) 10 m*M* sodium acetate containing 0.5% (v/v) acetic acid and (b) the same buffer but additionally containing 20% (v/v) acetonitrile. Hydroquinone: ex = 304 nm, em = 338 nm; catechol: ex = 284, em = 313 nm. Chromatogram is presented with integrator attenuation programme as follows: at 0 min, 32 mV; 4 min, 64 mV; 9 min, 512 mV. Reproduced from Ref. [73] with permission.

5.6. Other metabolites in urine

1,2,4-Benzenetriol or hydroxyquinol (BT), was identified as a minor urinary metabolite after administration of benzene to rabbits, and catechol has been assumed to be a precursor of this compound. Greenlee et al. [70] proposed a HPLC method for determination of BT under nitrogen atmosphere. Inoue et al. [74] subsequently developed an aerobic HPLC method for detection of this metabolite in the urine of workers exposed to benzene. Urine specimens were hydrolysed with hydrochloric acid and 2% pyrogallol in methanol. Separation was achieved with a Spherisorb ODS 5 column. The mobile phase was methanol–water–acetic acid (20:971:9, v/v) and the eluate was detected at 290 nm. The detection limit under aerobic condition was 0.5 mg/l. The results showed that ca. 0.5% of the absorbed benzene was converted to BT and excreted in the urine at the end of the workshift. According to the authors [74] urinary analysis of BT has an advantage over other methods since the BT level is zero in non-occupationally exposed subjects. It was estimated that about 2.26 mg/g creatinine would have been excreted at the end of the workshift after exposure to 10 ppm of benzene (Table 5). One of the serious drawbacks of this method was that only benzene exposure above 10 ppm could be assessed. Furthermore, excretion of BT is significantly suppressed by co-exposure to toluene. Coexistence of toluene and benzene is common in many workplaces.

Dehnen [75] reported on N-acetylcystine and thiophenol in urine by using HPLC, including also measurements after experimental inhalation of benzene. The author showed that these two compounds could be determined after alkaline hydrolysis of S-phenyl-N-acetylcystine. So far, no study has been conducted to validate this method for low-level benzene monitoring. Considerable development will be required since the proposed method is too elaborate for routine monitoring.

N-7-Phenylguanine formation has been suggested by Norpoth et al. [76] as a biomarker since it is a critical reaction during the carcinogenic process of benzene; however, exposure studies were limited to the rats. As the tests were carried out on rats treated with relatively high concentrations of benzene (up to 400 mg/kg), the authors suggested that an increased sensitivity of the analytical methods is essential if exposures below 5 ppm are to be assessed. The method still lacks adequate sensitivity for low-level benzene monitoring and considerable work would be needed before this procedure could be considered a routine monitoring tool for risk assessment.

6. Protein and DNA adducts of benzene

Benzene is a known human carcinogen. The identification and assessment of its genetic markers is important for disease prevention. Because biological monitoring in environmental or occupational health practice can not performed on samples from target organs such as bone marrow, attempts have been made to use nucleated blood cells and blood protein (hemoglobin, albumin) as surrogates for the target tissue. The common characteristic of chemical carcinogens is their covalent binding to DNA or protein molecules and this is thought to be a key step in the initiation of cancer. The stability of these adducts could allow monitoring long after exposure and also investigation of damage not detectable by common analytical methods.

The binding of benzene metabolites to DNA was first observed by Lutz and Schlatter [77]. They estimated that one in every 10^6 nucleotides of hepatic DNA was bound by a benzene metabolite. Snyder et al. [78] showed that benzene metabolites bound covalently to mitochondrial DNA in vitro. The adducts were characterised by UV, fluorescence, GC–MS and nuclear magnetic resonance spectroscopy. Sun et al. [79] demonstrated that one of the minor metabolites of benzene, benzoquinone, formed adducts with Hb and accumulated linearly in animals exposed to benzene.

Norpoth et al. [76] reported excretion of the

benzene adduct N-7 phenylguanine in the urine of rats after benzene exposure. The adduct was further investigated using HPLC and GC–MS. It was suggested that this adduct could be used as a biomarker for benzene exposure. However, the same group of investigators was unable to detect this metabolite in rats dosed with benzene and analysed using refined HPLC or a sensitive immunological assay (ELISA) [80]. With GC–MS trace amounts of this compound were found in concentrated rat urine samples. The authors assumed that hydroxylated phenylguanine has a highly reactive intermediate OH group which might be lost because of the high temperatures during GC–MS measurements.

The carcinogenicity of benzene has been considered to be in part mediated by its chemically reactive phenolic metabolites, such as benzoquinone (BQ), which is formed from the intermediary metabolites phenol and hydroquinone (HQ). Reddy et al. [81] have shown that phenol, HQ and BQ produce adducts in vitro, but the corresponding adducts were not detected in vivo, even measured with nuclease P_1 ^{32}P postlabelling methods capable of detecting 1 adduct in 10^{9-10} DNA bases.

Using a similar technique, Levay and Bodell [82] showed that hydroquinone (HQ) was 7–9 times more effective than CAT and BT in inducing DNA adduct formation in HL-60 cells. The authors also observed a significant synergistic effect of benzene metabolites in vitro. A recent report of Bodell et al. [83] also showed that human bone marrow could convert HQ to *p*-benzoquinone (*p*BQ) and caused DNA adduct formation.

The formation of adducts of benzene with Hb might also be used as an alternative biomarker for benzene exposure [84]. Radiolabelled benzene has been given to rats and the adducts were characterised by HPLC. Two major adducts which cochromatographed with S-2,5-dihydroxyphenyl cystine (HQ cystine) and S-phenylcysteine (SPC) have been subsequently identified by GC–MS. These two adducts accounted for 60–75% of the total radioactivity associated with rat globin.

SPC, however, could not be detected in the globins of humans occupationally exposed to benzene at concentrations of up to 28 ppm [85]. Using another approach, Bechtold et al. [85] examined the binding of benzene to cysteine groups of a different blood protein, albumin. The isolated albumin was analysed for SPC by isotope dilution GC–MS. An Ultra 1 capillary column was used together with electron-impact ionization and selected-ion monitoring for SPC of m/z 236 ions. Levels of SPC were measured in 12 workers exposed to benzene at concentrations ranging from 0 to 23 ppm. The results showed a linear increase of SPC with benzene exposure. Although the findings suggest that SPC in serum albumin may prove useful as a biomarker for benzene exposure, the extraction and analytical processes are tedious and require sophisticated instrumentation. Its use for routine biological monitoring is thus limited.

In short, many of the biomarkers for genetic risks as described here are currently in the validation stage. It is not clear which markers would reflect most accurately the exposure as well as the biological effects. The current application of these assays in the workplace or in general population studies is still preliminary.

7. Evaluation of various biomarkers

There is a growing interest in the use of biological markers to study the health effects of exposure to environmental toxicants in environmental and occupational medicine. Epidemiology uses biomarkers as indicators of exposure, internal dose or health effect; toxicology uses markers to help determine underlying mechanisms of diseases, develop better estimates of dose–response relationships, and improve the technical bases for assessing risks at low levels of exposure.

The ideal biomarker for benzene exposure should be specific, available for analysis with non-invasive techniques, detectable in trace concentration, inexpensive to detect and most importantly quantitatively related to the degree of

exposure. Very rarely a biomarker will satisfy all these qualities. Nevertheless, they provide valuable information that may improve our ability to determine the extent of environmentally induced diseases.

Among the methods which have been proposed for evaluating exposure to benzene, two approaches appear to have some practical application: (1) determination of non-metabolised benzene in urine, and (2) measurement of the concentration of urinary ttMA.

For risk assessment of low-level environmental exposure, the determination of the unchanged compounds is of greater value as they tend to be more specific [86]. The measurement of non-metabolised benzene in exhaled breath is a very specific and sensitive method to confirm exposure to benzene. However, the data obtained are not adequate to correlate the benzene concentration in breath with integrated exposure. It must also be kept in mind that since benzene is present in cigarette smoke, its detection in exhaled breath does not necessarily imply occupational exposure to benzene. The other major drawback for breath analysis is that the collected specimen has a short half-life, analysis has to be performed immediately after collection or at least on the same day. In addition, breath analysis could be influenced by a number of biological parameters as well as exposure conditions. Deep respiration caused by heavy work could result in significant absorption of hydrocarbons as compared with normal breathing associated with a low physical workload [10]. Unless more reliable techniques become available, breath analysis does not appear to be useful as a biomarker for low-level benzene exposure.

Measurement of benzene in urine is usually preferred since the procedure is non-invasive and is thus suitable for routine monitoring. Although only few studies have been conducted on urinary benzene determination, good correlations between urine benzene concentrations and environmental exposure were found [42–44], making the determination of benzene in urine a suitable approach for biological monitoring. For laboratory analysis of benzene in urine, head-space GC using capillary columns is the obvious choice in the case of both environmental and occupational exposure.

The determination of phenol, the major metabolite of benzene, can be carried out by either capillary GC or HPLC using isocratic elution. However, because of the low specificity of urinary phenol and the decreasing concentrations occurring in environmental exposure, it is expected that determination of urinary phenol will become less significant for risk assessment.

The minor metabolites of benzene, such as ttMA and SPMA, have been demonstrated to be more specific and sensitive than the main metabolite for the estimation of exposure [63,64,68]. SPMA determination requires GC–MS analysis for adequate sensitivity and thus is not an efficient method to be used in routine monitoring. On the other hand, recent field investigations showed that ttMA determination is highly sensitive and can detect background environmental exposure in a general population [63,64]. The use of this biomarker is promising in risk assessment of low benzene exposure. RP-HPLC separation with UV detection is the method preferred by most analytical laboratories for determination of ttMA (Table 5).

Analytical methods for the determination of phenolic metabolites such as CA, HQ and BT have been developed recently. These metabolites are usually determined preferably by HPLC using fluorimetric detection because of its specificity and lower detection limits. However, considerable work will be required to validate their usefulness for the monitoring of low-level benzene exposure.

It should be noted that the biological half-life of benzene metabolites is usually short (less than 12 h); therefore the time of sampling of biological material in relation with the time of exposure is very important. When biological monitoring involves sampling and analysis of non-metabolised benzene or its metabolites, the collection methods and the means of expressing the results should be standardised. For most methods mentioned above, the best time for

sample collection appears to be at the end of the workshift.

Measurements of DNA and protein adducts of benzene may provide a better basis for the estimation of the carcinogenic risk of benzene, although the predictability of these biomarkers in terms of adverse health effects in occupationally or environmentally exposed persons is very difficult to validate. Furthermore, many of the molecular biomarkers are currently under validation. One can only state that, at the present stage, the levels of the carcinogen-adducts are only a measure of exposure rather than of health effects. Although HPLC has been used for the detection of benzene–Hb or benzene–protein adducts, they are determined preferably by ^{32}P post-labelling techniques [81].

Recently there has been much discussion on what level of exposure to benzene constitutes an acceptable risk. If an occupational or environmental exposure limit has to be considered, both the normal values in a non-exposed population and the most appropriate analytical techniques have to be established first. Selection of biological exposure indices should take into account the specificity and sensitivity of the analytical technique, as well as its usage for individual or group exposure. One of the major confounding factors in the risk assessment of benzene is that the exposure is ubiquitous. A reference value for a non-exposed population would have to be defined before a biomarker can be widely used in a field setting. In order to advise on standard settings, the scientific community would also have to decide on what level of exposure to benzene constitutes an acceptable risk. More collaborative efforts from different disciplines are obviously needed to achieve these objectives.

8. Conclusions

Benzene is a proven human carcinogen and therefore constitutes a relevant health problem. During the last decade, numerous methods have been developed for the determination of benzene and its metabolites in exhaled breath, blood and urine. Nevertheless, many of these methods have not been validated, especially those related to low-level environmental exposure.

The ideal biomarker for benzene exposure should be specific, available for analysis with non-invasive techniques, sensitive to trace concentration, inexpensive and, most importantly quantitatively related to exposure concentrations. Very few biomarkers available for measurement of benzene exposure fulfil these criteria.

There are primarily two approaches for the biological monitoring of persons occupationally or environmentally exposed to benzene:

(1) Determination of the parent compound in breath, blood or urine.

(2) Measurement of various metabolites in the urine.

Owing to dietary intake or biological matrix interferences, the measurement of metabolites tends to be less specific than the determination of non-metabolised benzene, this being especially true for the main metabolite, phenol.

The determination of benzene in urine using GC head-space analysis appears to be simple and specific. As an analytical method, the determination is specific enough to trace exposure down to relatively low concentrations.

Recent methods developed using RP-HPLC for the determination of ttMA appear to present an alternative approach for biological monitoring of low-level benzene exposure. With a detection limit of about 25 μg/l environmental exposure to less than 1 ppm of benzene could be detected. Several field studies have also shown good correlations between benzene exposure and ttMA concentration in urine.

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