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CHROMATOGRAPHIC DETERMINATION OF TOLUENE AND ITS METABOLITES IN URINE FOR TOLUENE EXPOSURE – A REVIEW

(Penentuan Kromatografi Toluena dan Metaboliknya di dalam Urin bagi Pendedahan Toluena – Satu Ulasan)

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Abstract

The determinations of toluene and their metabolites in biological samples such as urine and blood allow the estimation of the degree of exposure to this chemical. Chromatographic methods and preliminary methods are now universally employed for this purpose. Preliminary color test methods are well established for qualitative determination of toluene and its metabolites. Mobile test kits using color test methods are a vast tool for screening urine samples but chromatographic methods are still needed for confirmation and quantitative analysis. Gas chromatography (GC) methods are well-adapted for the determination of toluene metabolite in urine, but these methods often require several pretreatment steps. Meanwhile, high performance liquid chromatography (HPLC) is becoming a powerful tool for the accurate and easy determination of toluene metabolites considering its decisive advantages for routine monitoring. Furthermore, recent development in HPLC could widen the usefulness of this method to solve the most complex analytical problems that could be encountered during the measurement.

Keywords: toluene, toluene metabolites, preliminary test, gas chromatography, high performance liquid chromatography

Abstrak

Penentuan toluena dan metabolitnya dalam sampel biologi seperti urin dan darah membolehkan anggaran tahap pendedahan terhadap bahan kimia ini. Kaedah kromatografi dan kaedah awalan merupakan kaedah yang sering digunakan untuk tujuan ini pada masa kini. Kaedah awalan menggunakan ujian warna telah dibangunkan untuk penentuan kualitatif toluena dan metabolit. Kit ujian mudah alih menggunakan ujian warna meluas digunakan bagi pemeriksaan sampel urin tetapi kaedah kromatografi masih diperlukan untuk pengesahan analisis kuantitatif. Kaedah kromatografi gas adalah kaedah yang kerap digunakan bagi menentukan metabolit toluena dalam urin namun kaedah ini sering memerlukan beberapa langkah rawatan awal. Sementara itu, kromatografi cecair berprestasi tinggi (HPLC) menjadi alat yang berkuasa untuk penentuan tepat dan mudah metabolit toluena memandangkan kelebihannya yang jelas untuk pemantauan rutin. Tambahan pula, perkembangan terkini dalam HPLC boleh memperluaskan kegunaan kaedah ini bagi menyelesaikan masalah analisis rumit yang boleh dihadapi semasa pengukuran.

Kata kunci: toluena, metabolit toluena, ujian awalan, kromatografi gas, kromatografi cecair berprestasi tinggi

Introduction

Volatile organic compounds (VOCs) are used as a component in a variety of domestic or industrial consumer products. This statement illustrates that humans always use and are frequently in contact with VOCs. Consumer products such as cleaning products, paints or thinner, glues, lighter, aerosols, cosmetics, fuels and others contain VOCs. Despite these, prolonged exposure to VOCs may lead to poisoning and sudden death [1, 2].

One of the major current issues among adolescents is misuse of the consumer products. In most of the cases, organic compounds contained in the consumer products are inhaled by them using different methods such as huffing, bagging and sniffing. Such conducts will deliver the desired effect such as feeling of pleasure, disinhibition, euphoria and hallucination [3, 4]. Unfortunately, the most difficult facet of the inhalant problem to confront is the fact that inhalants are everywhere.

Even though, there are many VOCs in consumer products, the concern is largely on toluene. This is because toluene has been the most widely used organic solvent in the industries since the second half of the last century [5-7].Such exposure to high concentrations of toluene has been reported by Watson [8]. Toluene biotransformation consists of several steps [9] involving side-chain oxidation where toluene is metabolized into benzoic acid and further conjugated with glycine to form hippuric acid [10]. Meanwhile, small amounts of absorbed toluene are oxidized at the aromatic ring and excreted in urine as *o*- and *p*-cresol.

This review aims to present a summary of the toluene exposure pertaining to toluene metabolites and its potential biomarker, preliminary tests for toluene metabolites, sample pretreatment and determination of toluene metabolites using various instrumentations. From this review, researchers in this area are expected to gain a good idea of analytical procedures, analytical techniques and analytical instrumentations involved. It is our hope that this review will facilitate researchers to plan their own research on toluene exposure and to develop fast method and new separation technique using green technology, electrochemical sensors with mobile instrumentation and other related techniques. The discussion begins with a general topic about toluene exposure and its effects on humans. The metabolism of toluene in human body from its inhalation until excretion in urine is then described. The selection of potential biomarkers and indicators of exposure to toluene are also described in a considerable detail.

Toluene exposure effects and analysis

The preliminary test has become a vast tool for on-site screening of urine sample as many drugs and other poisons give characteristic colors with appropriate reagents if present in sufficient amounts and in the absence of interfering compounds. A few of these tests are only for practical purposes but usually compounds containing similar functional groups will also react and thus interferences from other poisons, metabolites or contaminants are to be expected. Color tests for toluene metabolites have been described including for hippuric acid [11] and *o*-cresol [12].

Methods for toluene analysis and the identification of a reliable indicator in toluene exposure have been described by many researchers. High Performance Liquid Chromatography (HPLC) represents a valuable tool for the routine monitoring of workers exposed to toluene. The determination of urinary metabolites of toluene using HPLC has been extensively studied including those focusing on hippuric acid [13-17], cresol [18] and S-benzyl mercapturic acid [19-20]. For the urinary determination of solvent metabolites, especially for monitoring purposes, HPLC is becoming a method of choice. This method offers decisive advantages in this area as it permits the simultaneous determination of several metabolites whether it is run in isocratic mode or by gradient analysis. This method provides a complete metabolic profile, detection of isomer compound, it is easily automated, and does not require extraction, derivatization steps or pre-treatment of samples.

Determination of toluene metabolites in complex mixtures such as biological fluids is well-known and established. The separation of toluene metabolites from biological fluid can easily be achieved by gas chromatography (GC) with headspace method [21, 22]. However, gas chromatography-mass spectrometry (GC-MS) appears very suitable for the identification of toluene and their metabolites [23-27]. Unfortunately, not all target compounds are amenable to GC due to their poor thermal stability or volatility. Polar compounds are an example of analytes that need the pretreatment of sample using derivatization processes and these together rendered GC less widely-employed compared to HPLC in the analysis of toluene and its metabolites.

Metabolism and potential biomarker of toluene: Historical background

Hippuric acid has been a historical biomarker for toluene exposure but the validity of hippuric acid as an exposure marker is questionable as non-exposed subjects (background levels) were found to contain higher levels of hippuric acid compared to exposed subjects [28-31]. The value of the hippuric acid as a biomarker of occupational toluene exposure was further challenged by the presence of benzoate in some preservatives [32, 33] as benzoate is metabolized and excreted as a hippuric acid in urine [32, 34, 35].

Several researchers have considered a few other possible biomarkers for toluene exposure namely *o*-cresol [27, 36, 37], benzylmercapturic acid [19, 20, 38] and toluylmercapturic acid [39]. Although hippuric acid has attracted considerable attention as an indicator of exposure to toluene, the fact that it can deliver false results of toluene exposure has prompted a number of researchers to suggest *o*-cresol be used as a biomarker, especially for the determination of low-level exposures to toluene. However, it was found that urinary *o*-cresol demonstrated false results too when it involved smoking and drinking counterpart [40] where it can reduce the amount of this chemical particularly at low levels of toluene exposure.

More recently, researchers found a new specific biological marker for toluene metabolites namely S-benzylmercapturic acid (SBMA) [19, 20, 41]. SBMA was found to be a more specific biomarker compared to hippuric acid and *o*-cresol. It was found that SBMA was dependent only on airborne toluene and not effected by food preservative, smoking and drinking habit even at low level of exposure to toluene [42].

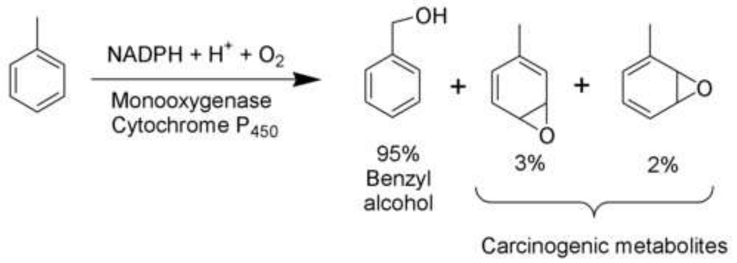
Toluene metabolism

According to Park et al. [43], toluene is the major solvent with the most documentation of abuse, possibly because of its relative low risk of sudden death and the ease of detection in blood. When many aromatic compounds such as toluene and benzoic acid are taken internally, they are converted to hippuric acid by reaction with the amino acid glycine [43]. In another paper, Anderson et al. [44] explained in detail that the principal metabolite of toluene is benzoic acid (approximately 80% of dose) which is conjugated with the glycine to form hippuric acid and directly excreted in urine (half-life of 2-3 hours).

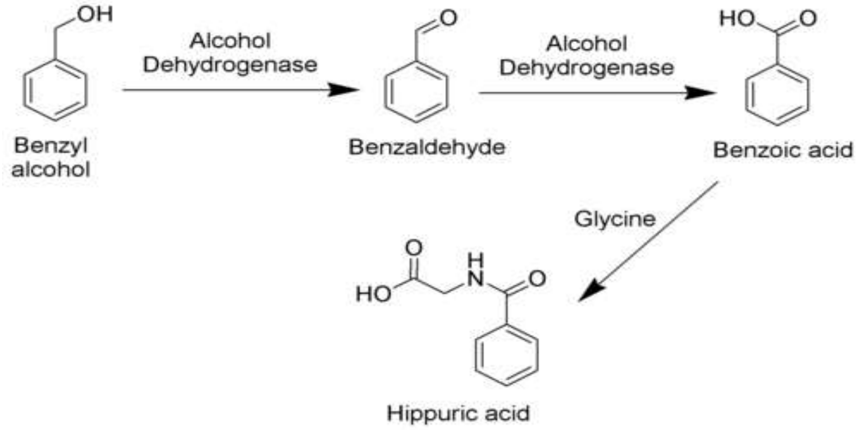
Toluene can be absorbed into the blood stream from the lung and the gastrointestinal tract and through the skin and mucosa. In late 1980s, Ameno et al. described that the brain and liver serve as reservoir for toluene [45]. A few years later, Yamazaki et al., in their work explained that more than 80% of absorbed toluene is metabolized by mix-oxidase enzyme system to benzoic acid and hippuric acid in the liver and kidney before excretion into the urine. Meanwhile, some absorbed toluene (0.4 – 1.1 %) is hydroxylated and excreted as a mixture of *ortho*-, *para*-, and *meta*-cresol. A schematic diagram for toluene metabolism in humans is shown in Scheme 1 [46].

Later on, Zuppi et al. [47] stated that the hippuric acid is biosynthesized from glycine, benzoic acid and CoA by enzymes located in the mitochondrial matrix of liver and kidney cells. This metabolic pathway provides an alternative flux of nitrogen from the usual urea precursors (ammonia, alanine and glutamate) to glycine and it is utilized for the detoxification of aromatic compounds such as toluene. Toluene is very hazardous to our health, with the potential of inflicting considerable pain and suffering until death to the sniffer.

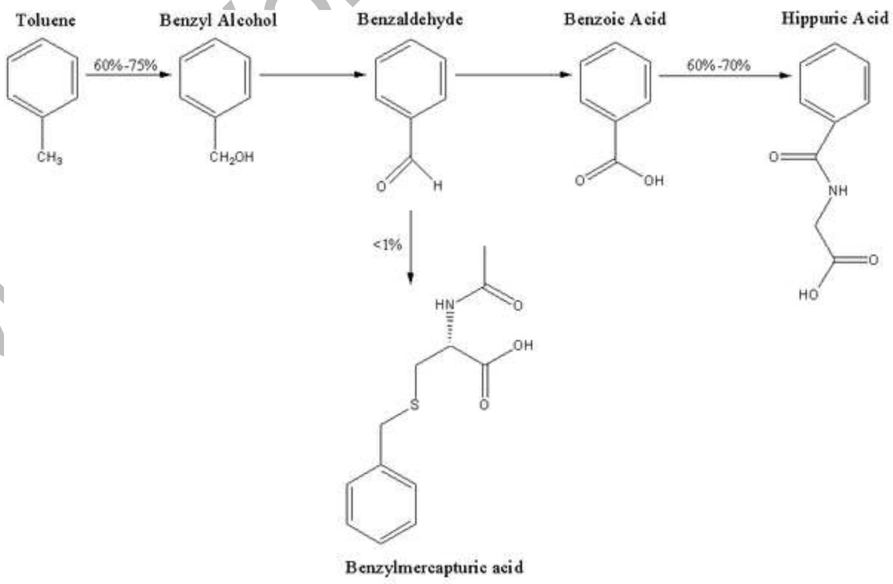
Following inhalation or oral exposure to toluene, approximately 60 – 75 % of absorbed toluene is metabolized to benzoic acid. The initial step involves side chain oxidation to benzyl alcohol by cytochrome P450 enzymes (Scheme 2). Benzyl alcohol is further oxidized to benzoic acid by alcohol dehydrogenase and aldehyde dehydrogenase. Benzoic acid is subsequently conjugated with glycine to form hippuric acid. Benzoic acid may also be conjugated with glucuronic acid to form benzoyl glucuronide in the urine. Less than 1% of absorbed toluene undergoes ring hydroxylation to form *o*-, and *p*-cresol, which are excreted in the urine as glucuronide or sulphate conjugates as reported by Amorim et al. [48].



Scheme 1. First step of toluene metabolism [46]



Scheme 2. Second step of toluene metabolism [46]



Scheme 3. Complete toluene metabolism in urine [47]

According to the World Health Organization (WHO), following inhalation, toluene is predominantly excreted in the urine as the metabolite, hippuric acid. Approximately 7 – 20 % of absorbed toluene is eliminated in air unchanged. Following a single acute exposure, toluene and its metabolites are almost completely eliminated within 24 hours [49].

Scheme 3 illustrates a full steps of toluene metabolism in urine. Approximately 60 to 75 % toluene is converted to benzyl alcohol before further oxidized to benzaldehyde. Less than 1 % benzaldehyde is converted to benzyl mercapturic acid while the rest is converted to benzoic acid. About 60 to 70 % hippuric acid is produced from conjugating benzoic acid with glycine.

Analytical methods: Sample collection and storage

It is a good practice that biological specimens should be stored at 4 ºC before transported to the laboratory. Exceptions to this include hair and nail, which are stable at room temperature and filter paper with adsorbed dried blood, which is a convenient way of storing and transporting blood samples for specified analyses whenever refrigerated transport and storage is not feasible [50].

Each specimen bottle should be securely sealed to prevent leakage and individually packaged in a separate plastic bag. Particular attention should be given to the packaging of the samples to be transported by post or courier in order to comply with current health and safety regulations. Sample volumes or amounts smaller than those indicated in Table 1 are often sufficient to complete the analyses required. However submission of very small sample may result in reduced sensitivity and scope of the analyses undertaken but nevertheless such samples should always be forwarded to the laboratory. Any residual specimen should be kept at -20 ºC or below until investigation of the incident has been concluded.

Table 1. Sample requirements for general analytical toxicology

Sample	Notes
Whole Blood	10 mL (lithium heparin or EDTA tube - use fluoride/oxalate/citrate if ethanol is suspected; plastic tube if paraquat is suspected; glass or plastic tube with minimal headspace if carbon monoxide or other volatile is suspected)
Plasma/Serum	5 mL (send whole blood if volatiles, metals and some other compounds are suspected)
Urine	20 - 50 mL (plain bottle, sodium fluoride)
Gastric contents	25 - 50 mL (plain bottle, no preservative)
Other samples	Vitreous humor (maximum available collect separately from both eyes), bile (2 mL) or liver (about 5g) can be a substitute for urine postmortem work. Other tissues (brain, liver, kidney, lung, and subcutaneous fat – 5g) may also be valuable, especially if organic solvents or other volatile poison is suspected.

Preliminary Test

Color tests are useful in minimizing the use of equipment and expertise required. However, the reagent has to be easily available and stable. Sensitivity is limited and the tests are usually applicable only to urine. It is possible to extract the metabolite and apply the color test to the residue, though this is rarely done for biological fluids. False negatives are a risk even when a test is used for its intended purposes in appropriate samples. Positive results with poison, metabolite or others serve to indicate the need for quantitative measurement. Urine samples are commonly used for preliminary test. This is because of urine is easy to collect and it can be useful to provide qualitative information about exposure to a particular substance (Table 2).

Researches in toluene exposure are extensive whether they involve quantitative or qualitative analyses. One of the most varied tools for qualitative analysis is a color test method. Many years ago, only a few of researchers employed color test method to determine toluene metabolites [51]. Among the metabolites of toluene, hippuric acid is a traditional biomarker for the biological monitoring of toluene exposure but both *o*-cresol and hippuric acid are used by the endogenous and dietary sources [52].

Table 2. Color test methods for determination of toluene metabolites in urine

Sample Pre-treatment	**Procedure**[a]	**Analysis Method**	**Color Changes**	**Detection Limit**	**Ref.**
Extracted three times with methanol	Detection of hippuric acid based upon azlactone formation resulting from the reaction of an aldehyde with hippuric acid.Hippuric acid was converted to a deep orange-colored azlactone, 2-phenyl 4- (p-dimethylamino)benzal 5-oxazolone, by treatment of hippuric acid with acetic anhydride and p-dimethylamino-benzaldehyde.	Colorimetry method. λ_{max} = 410 nm	Yellow to deep orange color.	N.R	[52]
Acid extraction of urine sample	0.5 mL hippuric acid of pyridine (Pyr) solution mixed to 0.2 mL of benzenesulphonyl chloride (BSC). The solution was allowed to stand >20 min. It is then diluted to 5 mL with chloroform and recorded at 380 nm.	Colorimetry method. λ_{max} = 420 (red color) and 360 nm (orange color).	Yellow to orange red color within 40 min.	0.135 mg/mL	[53]
No extraction	0.5 mL of urine and 0.5 mL of Pyr were mixed and added with 0.2 mL of BSC and mixed for 5 s with a vibration mixer. The colored solution was allowed to stand for 30 min at room temperature, diluted to 5 mL with ethanol and mixed. The solution was centrifuged for 5 min to remove a little turbidity. The absorbance was determined at 410 nm in ethanol.	Colorimetry method. λ_{max} = 410 nm	Yellow to deep red color within 20 min.	0.20 mg/mL	[54]
No extraction	Urine sample (0.1 or 0.2 mL) was mixed with 0.25 mL Pyr, and subsequently with 0.1 mL BSC. Then, 0.125, 0.25, 0.5. 1.0 or 1.5 mL ultra-pure water was added.	Colorimetry method. λ_{max} = 410 nm	Yellow to deep red	0.125 mg/ml	[55]
No extraction	0.1 mL of urine added with 0.25 mL Pyr and 0.1 mL BSC and 1.0 mL distilled water.	Spectrophotomety UV at λ_{max} = 417 nm	Yellow to deep red within 1 min.	0.125 mg/ml	[11]

[a] Pyr, Pyridine; BSC, Benzenesulphonyl Chloride. N.R; Not reported.

Color test methods for hippuric acid have been developed since 1950s and further investigated by researchers in early 1980s. The founders of color reaction for hippuric acid, Gaffney et al. employed the formation of azlactone resulting from the reaction of an aldehyde with hippuric acid [51]. In this reaction, hippuric acid was converted to a deep orange colored azlactone, 2-phenyl-4-(p-dimethylamino) benzal-5-oxazolone by treatment of hippuric acid with acetic anhydride and p-dimethylaminobenzaldehyde. Urine sample was extracted three times with methanol for sample pre-treatment. The absorption was recorded with colorimetry method at λ_{max} = 410 nm.

A few years later, Umberger and Fiorese found a new method using pyridine and benzenesulfonyl chloride to develop deep red color in the presence of hippuric acid. Urine sample was extracted using ethyl acetate before recording using colorimetric method at λ_{max} = 420 nm (red) and 360 nm (orange) with detection limit of 0.135 mg/mL [53]. An investigation using colorimetric method was also reported by Tomokuni and Ogata [54]. The procedure employed 0.5 mL of urine, 0.5 mL of pyridine and 0.2 mL of BSC that were mixed for about 5 s with a vibration mixer and the colored solution was allowed to stand for 30 min at room temperature and then diluted with 5 mL of ethanol. Then the solution was centrifuged for 5 min to remove a little turbidity and the absorbance was determined at 410 nm in ethanol. This spectrophotometer method was simple but the yellow coloration obtained was indistinguishable from the yellowish color of urine because of the ethanol dilution.

In 2005, Yoshida et al. [55] modified the color test method that was established by Umberger and Fiorese, [53]. It involves a new color test method where the addition of pyridine and benzenesulfonyl chloride with existing distilled water develops a deep red color in the presence of hippuric acid. The colorimetric method for urine hippuric acid investigated in this study is based on the color reaction (reddening) of the specimen, which can be judged more clearly than the method of Tomokuni and Ogata [54] which involves a change to yellowish color. This method required only several minutes and used only commercially-available reagents without any pre-treatment. This work demonstrated the color changed but they it did not illustrate the color changes with different concentrations of hippuric acid. Besides that, the work was only focused on samples of people exposed to toluene and did not provide the equation and reaction of the color test method [55].

More recently, Yacob and Zinalibdin, developed a simple mobile test kit for screening and detecting glue sniffers based on the color test method [11]. The results obtained using the kit is are similar to those using UV–Vis result but it technique is faster with the results obtained within only 5 min and is also comparable to the dip strip urine drug testing kits. Thus, this kit can be a fast and in situ tool to help the National Anti-Drug agency for in the screening and detecting glue abusers among school students and teenagers [11].

Sample pre-treatment: Hippuric acid sample pre-treatment prior to HPLC

Liquid-liquid extraction (LLE) has been used for sample pretreatment of toluene metabolites. Poggi et al. illustrated the extraction procedure was performed on acidified urine, after addition of 4-hydroxybenzoic acid as internal standard using a butylchloride/isopropanol mixture and 0.5 mL of the organic layer was dried under nitrogen flow. The residue obtained was dissolved in 0.1 mL water/acetonitrile and 5 µL was injected into a HPLC [15]. Matsui et al. in his research used ethyl acetate for partial clean-up of biological samples [56]. Bevine et al. conducted a sample pretreatment which is made alkaline and extracted stepwise with methylene chloride and ethyl acetate and the addition of internal standard (3-methoxysalicylic acid) in plasma samples [57]. Meanwhile, several works have demonstrated sample pretreatments that do not require extraction step but effected only by dilution with distilled water followed by centrifugation [14, 58, 59].

Liquid-liquid-liquid microextraction (LLLME) method was developed for the determination of hippuric acid in urine and serum sample [60]. The analyte was extracted from an acidic aqueous sample solution (pH 3) through a thin layer of organic solvent membrane and back-extracted to a basic acceptor drop (pH 11) suspended on the tip of a 10-µL HPLC syringe in organic layer. Green technology was adapted in this method that offers the advantages of being simple, sensitive and inexpensive.

A simple molecularly imprinted polymer coated with modifed polysulfone membrane was introduced by Moein et al. [61]. The membrane was utilized for online extraction of hippuric acid in biological matrices. This method provided fast, sensitive, selective and robust sample preparation method for hippuric acid in biological fluids.

Hippuric acid sample pre-treatment and derivatization procedure prior to gas chromatography

Methods for the determination of hippuric acid based on GC usually require a derivatization procedure in the sample pretreatment step. A number of researchers have established the derivatization procedure for the analysis of toluene metabolites. Some researchers used diazomethane but it is an explosive, carcinogenic and highly toxic reagent [62-64].Other researchers used expensive substances as reagents to obtain methylsilyl derivatives which require frequent cleaning of the GC detector [65, 66] and these derivatives are moisture-sensitive. In 1991, De Carvalho et al. [23] proposed the derivatization of hippuric acid using methanol in acid medium (HCl), a low-cost and low toxicity reagent. About ten years later, Kongtip et al. modified the method using a GC equipped with a DB-1 capillary column and a flame ionization detector [25]. The method successfully separated and eluted methyl esters of hippuric acid, *o*-, *m*- and *p*-methylhippuric acid within 11 min.

Extraction step is often the first step for the detection of toluene metabolites using GC. Before this century, many researcher used LLE as extraction step where the sample was acidified with acid and then the analyte was extracted using ethyl acetate [23, 25, 62-64]. The organic layer was collected for the derivatization step.

A novel approach of the extraction of hippuric acid using solid phase extraction [67] has been introduced. In the approach, an online automatic method for extraction of hippuric acid from urine sample using µ-solid phase extraction with polypyrrole adsorbent system was established [68]. However, post derivatization of hippuric acid by conversion to its methyl ester with methanol in hydrochloric acid was still needed for monitoring the toluene metabolites using GC.

Cresol pre-treatment prior to gas chromatography

The urinary cresol is present in urine sample usually as sulphate or glucuronic acid conjugates. For this reason, hydrolysis and neutralization steps are required to determine the urinary cresol. Truchon et al. in 1996 described a sensitive and reproducible gas chromatographic procedure for the determination of urinary *o*-cresol as follows. Acid hydrolysis was the first step encountered to produce unconjugated *o*-cresol. After extraction with methylene chloride at pH 2, the organic layer was concentrated by evaporation and the sample was analyzed by GC-flame ionization detection [27].

Solid phase microextraction (SPME) was established for determination of urinary *o*-cresol. Acid-hydrolyzed step is still required for the sample and a CW/DVB fiber was directly immersed in the sample solution for 20 min under magnetic stirring for the extraction. This SPME device was then introduced into chromatography injector port where the analyte is thermally desorbed from the fiber [26].

Analysis by High Performance Liquid Chromatography

Numerous sample pretreatment and HPLC methods have been proposed for the separation of toluene metabolites in biological samples, some of which are listed in Table 3. Several researchers have worked on the determination of hippuric acid in urine and blood samples. A few of them studied on the determination of hippuric acid in urine and blood for exposure of toluene, xylene and other chemicals for occupational and safety health. Ogata and his co-workers [59] determined hippuric acid as indicator of toluene exposure by HPLC. They used mixed solution of methanol-water-acetic acid (20: 80: 0.2) as the mobile phase and UV detection at 254 nm and without solvent extraction and pretreatment. Their results showed a low detection limit of 0.2 µg and analysis time of < 30 min.

HPLC method for simultaneous quantitative determination of the urinary metabolites of toluene, m-xylene and styrene (hippuric acid, *m*- methyl hippuric acid, phenylglyoxylic acid, mandelic acid) was described by Poggi et al. [15]. In the method, the residue obtained was dissolved in 0.1 mL of water/acetonitrile and 5 µL of the solution was injected into a HPLC system for analysis. Absorbance measurement was performed at 225 nm using UV detector. All metabolites were clearly separated within 12 min [15].

The direct determination of hippuric acid (HA) and *o*-, *m*- and *p*-methylhippuric acids (MHA) in the urine, metabolites of toluene and *o*-, *m*- and *p*-xylenes by HPLC have been described and established. A mixed solution of methanol-water-acetic acid (80: 20: 0.2) containing tetra-*n*-butylammonium bromide (0.2 % w/v) as mobile phase was used. Concentrations of HA and MHAs were estimated from their peak height at a wave length of 225 nm. Urine can be analyzed directly without solvent extraction or pretreatment to obtain complete separation of HA and *o*-, *m*- and *p*-MHAs. Authentic samples of glycine conjugates, HA and *o*-, *m*- and *p*-MHAs, in water and urine were well separated from each other and eluted within 25 min [69].

An automated HPLC method for the direct determination of urinary concentrations of hippuric acid (HA), and *o*-, *m*- and *p*-methyl hippuric acids (MH As), metabolites of toluene and *o*-, *m*- and *p*-xylenes, and of urinary phenyl glyoxylic acid (PGA) and mandelic acid (MA), metabolites of styrene or ethylbenzene have been described. Methanol was added to urine, the mixture was centrifuged and the supernatant was injected into HPLC A stainless-steel column packed with octadecyl silanized silicate was used and the mobile phase was a mixture solution of 5 mM potassium phosphate monobasic-acetonitrile (90:10) The method is simple and specific. Urine can be analyzed without solvent extraction. Analysis can be performed satisfactorily within 45 min for samples containing HA, MHAs, PGA and MA, and within 15 min for those containing HA, PGA and MA [70].

Matsui et al. [56] used HPLC for the determination of hippuric acid in urine sample. The separation was carried out on a reversed-phase column using 20 % methanol in 0.01 M potassium phosphate containing 0.5 % acetic acid as the mobile phase. The column effluent was monitored using UV detection at 254 nm. Hipuric acid was separated from other normal urine constituents in less than 10 min.

Kubota et al. [58] described the monitoring of hippuric acid and benzoic acid simultaneously in human biological fluids and considered it to be clinically important. They developed a simple and accurate HPLC method for the simultaneously determination of hippuric acid and benzoic acid in human urine and plasma. This research required no extraction step. Aliquots of urine and plasma were added to a solution of internal standard (*o*-chlorobenzoic acid) in acetonitrile and directly injected into a HPLC system using an acidic (pH 2.7) eluent and UV detection at 235 nm.

A method has been developed for the isocratic HPLC analysis of hippuric acid in human blood plasma. After the addition of internal standard (3-methoxysalicylic acid), plasma samples were made alkaline and extracted stepwise using methylene chloride and ethyl acetate. The detection limit was 50 pmol of hippuric acid per mL plasma. This work was conducted on samples from human occupationally exposed to organic solvent as described by Bevine et al. [57].

A simple HPLC procedure was described as a method for simultaneous determination of the six metabolites in urine namely hippuric acid , *o*-, *m*- and *p*-methylhippuric acid, phenylglyoxylic acid and mandelic acid. Urine analysis was selected in the study because of its non-invasive nature in sampling with minimal burden on exposed workers. A mobile phase of methanol-acetic acid-water (200: 8: 792) by volume was allowed to flow at a rate of 0.85 mL/min and monitored at a wavelength of 257 nm [13].

Yang [14] described a simple method for the simultaneous determination of creatine, uric acid, creatinine and hippuric acid in urine using HPLC. Chromatography was performed with a 0.02 M KH_2PO_4 as a mobile phase and UV detection at 220 nm without any extraction method. This method was applied for biomedical application to analyze urine samples from normal subjects and some patients. A HPLC method for determination of hippuric acid in urine was described by Yoshida et al. [55]. The mobile phase was 30% methanol containing 0.1% acetic acid and UV detection at 225 nm. No extraction method was used in this work. Urine samples were centrifuged (12 000 rpm, 10 min) and 5 µL supernatant was injected for analysis. This method was applied to the analysis of glue sniffer's urine [55].

A simple method for the simultaneous determination of hippuric acid and benzoic acid in urine using reversed-phase HPLC was described by Yacob and Zinalibdin [71]. Chromatography was performed with a mobile phase of mixed solution methanol-water-acetic acid (20: 80: 0.2) and UV detection at 254 nm in < 10 min. The detection limit of this method was 0.01 mg/L for hippuric acid and 0.06 mg/L for benzoic acid. This method was applied to the analysis of urine samples from the suspected of toluene abuser or glue sniffer among secondary school students. Meanwhile, Lee et al. [18] developed a simple and rapid HPLC-UV method for the simultaneous determination of eight urinary metabolites which is hippuric acid, mandelic acid, *o*-, *m*- and *p*-methylhippuric acid and *o*-, *m*- and *p*-cresols. All eight metabolites were successfully resolved within 5 min in 10 % aqueous ethanol containing 0.3 % acetic acid and 1.6 % β-cyclodextrin, using a gradient flow rate 1.0 - 5.0 mL/min [18].

Table 3. Conditions for sample pretreatment and HPLC separation of toluene metabolites in biological samples

Sample Pre-treatment[a]	**HPLC Column**	**Detection Limit[b]**	**Mobile Phase[c]**	**Detection wavelength**	**Target Analyte[d]**	**Ref.**
Acidified urine sample and extracted with ethyl acetate by shaking. Centrifuge for 5 min at 1000 rpm.	Bondapak C18 (30 cm × 4 mm) Flow rate : 0.70 mL/min Time : 11 min	N.R	20 % MeOH in 0.01M K_2PO_4 containing 0.5 % v/v HOAC.	254 nm	HA	[56]
Acidified urine, add 4-hydroxybenzoic acid using butylchloride/IPA and dried with N_2. Residue dissolve in 0.1 mL water /acetonitrile.	HC ODS Sil-X Flow rate: 1.0 mL/min Time: 12 min	MA: 25µg/mL HA: 7.5µg/mL m-mHA: 10µg/mL PHGA: 1µg/mL	Water: ACN: HOAC 95: 5: 0.02	225 nm	MA, HA, m-mHA, PHGA.	[15]
1ml MeOH mix with 1 mL urine and centrifuge at 2000 rpm for 5 min.	Silica gel Flow rate: 1.2 mL/min. Time: 25 min	N.R	MeOH-water-HOAC 80: 20: 0.2 With tetra-*n*-butyl ammonium bromide 0.2 % w/v	225 nm	HA, o-mHA, m-mHA	[69]
Urine dilute with mobile phase and centrifuge at 2000 rpm for 5 min.	Octadecyl-silanized silica gel (0.4 mm × 150 mm) Flow rate: 0.7 mL/min Time: 20 min	MA: 5ng/mL HA: 2ng/mL o-mHA: 4 ng/mL m-mHA : 4ng/mL PHGA: 1 ng/mL	0 mM KH_2PO_4 (pH3.3) containing 3 mM 1-decane-sulfonate/CH_3CN (85:15)	225 nm	HA, o-mHA, MA, Creatinine	[70]
100µl sample mix with 200µl contain ISTD	Yanapak ODSA (250 mm × 4.6 mm) Flow rate: 1.0 mL/min Time: 10 min	BA & HA: 1µg/mL	CAN-water-HOAC 35:63: 2	235 nm	HA & BA	58]

Table 3 (cont'd). Conditions for sample pretreatment and HPLC separation of toluene metabolites in biological samples

Sample Pre-treatment[a]	**HPLC Column**	**Detection Limit**[b]	**Mobile Phase**[c]	**Detection wavelength**	**Target Analyte**[d]	**Ref.**
Urine mix with MeOH and centrifuge 1600 rpm at 60 min	Intersil ODS-2 (4.6 mm × 250 mm) Flow rate: 0.85 mL/min Time: 20 min	HA: 0.5 mg/L o-mHA: 2.0mg/L m-mHA: 0.8 mg/L p-mHA: 0.2 mg/L PHGA: 0.1 mg/L MA: 3 mg/L	MeOH-HOAC-water 200: 8: 792	257 nm	MA, HA, o-mHA, p-mHA, m-mHA, PHGA.	[13]
N.R	Novapak- C18 (3.9 mm × 150 mm) Flow rate: 0.5 mL/min Time: 10 min		0.02 mol/L KH2PO4	220 nm	Creatine, Uric acid, Creatinine, HA	[14]
2 mL of urine centrifuge at 12000 rpm for 10 min.	Devolosil C18 (15 cm × 4.6 mm) Flow rate: 0.70 mL/min	HA: 0.1 ng/mL	MeOH-water-HOAC 20: 80: 0.2	254 nm	HA	[55]
Acidified urine and extracted with diethyl eter/methanol (9:1)	Novapak-C18 (3.9 mm × 150 mm) Flow rate: 1.0 mL/min Time: 8 min	HA: 0.01 mg/L BA: 0.06 mg/L	MeOH-water-HOAC 20: 80: 0.2	254 nm	HA & BA	[71]

Table 3 (cont'd). Conditions for sample pretreatment and HPLC separation of toluene metabolites in biological samples

Sample Pre-treatment[a]	**HPLC Column**	**Detection Limit[b]**	**Mobile Phase[c]**	**Detection wavelength**	**Target Analyte[d]**	**Ref.**
Vortex sample and ISTD. Dillute with 20 fold deionized water and centrifuge for 5 min.	Onyx Monolith RP-18 (4.6 mm × 100 mm) Flow rate gradient: 1.0-5.0 mL/min Time: 6 min	HA: 2μg/mL MA: 0.8 μg/mL o,m,p-mHA: 0.4 μg/mL o, m, p-cresol: 0.8 μg/mL	EtOH-HOAC-1.6% β-cyclodextrin 10 : 0.3 :90	225 nm	HA, MA, o,m,p-mHA, o, m, p-cresol	[18]

[a] IPA, Isopropanol; N_2, Nitrogen; MeOH, Methanol; ISTD, Internal Standard.

[b] NR, not recorded; MA, mandalic acid; HA, Hippuric acid; BA, Benzoic acid; o-mHA, o-methylhippuric acid; p-mHA, p-methylhippuric acid; m-mHA, m-methylhippuric acid, PHGA, phenylglyoxylic acid; o, ortho; p, para; m, meta.

[c] MeOH, methanol; K_2PO_4, Potassium phosphate; HOAC, acetic acid; ACN, acetonitrile; KH_2PO_4, Potassium dihydrogen phosphate; EtOH, ethanol.

[d] MA, mandalic acid; HA, Hippuric acid; BA, Benzoic acid; o-mHA, o-methylhippuric acid; p-mHA, p-methylhippuric acid; m-mHA, m-methylhippuric acid, PHGA, phenylglyoxylic acid; o, ortho; p, para; m, meta.

Analysis of VOCs by gas chromatography

Gas chromatography (GC) offers some advantages for determination of volatile substances such as toluene due to its easy procedure. Selected examples of conditions for sample pretreatment and GC separation of toluene metabolite in biological sample are listed in Table 4. Head space method can achieve good separation of volatile solvents from the biological matrix [22]. In addition, the determination of toluene and its metabolites was not a problem as the GC peaks were well separated [21].

De Carvalho in his work [23] described the determination of hippuric acid by GC-flame ionization detection. This research proposed the derivatization of hippuric acid using methanol in acid medium which is a low cost reagent with a low level of toxicity. After the extraction procedure, hippuric acid and internal standard were converted to the methyl esters using methanol in acid medium. All methyl ester were detected in < 45 min [23]. Besides that, a sensitive and reproducible GC procedure for the quantitative determination of urinary o-cresol was also described [27]. The first step involves acid hydrolysis (2 N HCl, 100 ºC, 10 min) which yields unconjugated *o*-cresol. After extraction (methylene chloride, pH 2), the organic layer is concentrated by evaporation and samples are analyzed by GC-flame ionization detection with a DB-5 column. The detection limit of the method is 0.36 µmol/L [27].

Determination of hippuric acid and *o*-, *m*- and *p*-methylhippuric acid in urine was described by Kongtip et al. [25]. These metabolites were extracted using ethyl acetate and then derivatized it to methyl ester derivatives and quantitated using a gas chromatography flame ionization detector and DB-1 capillary column. The derivative of hippuric acid and *o*-, *m*- and *p*-methylhippuric acid were well separated within 11 min [25].

Simultaneous determination of hippuric acid, *o*-, *m*- and *p*-methylhippuric acids, mandelic acid and phenyl glyoxylic acid was developed and described by Ohashi et al. and it proved to be a fast and sensitive technique [72]. These metabolites were converted to methyl esters derivatives using methanol in hydrochloric acid and then quantitated by gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring using a DB-1 capillary column. The target analytes were quantitatively analyzed and resolved within 19 min with low detection limit.

Table 4. Conditions for sample pretreatment and GC separation of toluene metabolites in biological sample

Sample Pre-treatment[a]	**GC Procedure**[b]	**Target Analyte**[c]	**Derivatization Reagent**[d]	**GC Temperature**[e]	**Detection Limit**[f]	**Ref.**
Urine was acidified with 0.5 N HCl and extracted with ethyl acetate 20 min in horizontal shaker. Sample was centrifuged at 4000 rpm for 4 min and organic phase was collected.	SE-30 in Chromosorb W-Silanized glass column using GC-FID	PGHA, OA, *o*-mHA, *p*-mHA, BA, SA, MA, *m*-cresol	MeOH in HCl medium.	Carrier N_2 : 40 mL/min- TPGC Oven : 200 ºC Injector: 240 ºC Detector: 240 ºC	N.R	[23]
Hydrolyzed (2N HCl, 100ºC, 10 min) and extracted with methylene chloride, pH 2. Organic layer evaporated and collected	DB 5-column using GC-FID	o-cresol	Hydrolyzed using HCl and incubated in oven at 100 ºC for 10 min.	Carrier N_2 : 40 mL/min TPGC 30 ºC for 12 min and then increased 2ºC/min to 93 ºC	o-cresol: 0.36 µmol/L	[27]
1 mL of urine mix with 1 mL distilled water and acidified with 0.5M HCl then extracted with 3 mL ethyl acetate. Sample was evaporated to dryness and the residue reconstituted with derivatizing reagent and left in oven at 60ºC for 45 min. Solution was extracted with 1 mL chloroform and add 2 mL distilled water.	DB 1-column using GC-FID	HA, *o*,*m*,*p*-mHA	MeOH in HCl medium.	Carrier He: 10 mL/min Isothermal programmed Oven : 200 ºC Injector: 250 ºC Detector:250 ºC	HA: 0.05 mg/mL o,m,p-HA: 0.015 mg/mL	[25]
Acidified and extracted with diethyl ether. Sample was centrifuged at 2500 rpm for 10 min and dry incubated at 70ºC for 15 min. Next derivatize step was take part.	DB1-column using GC-MS. MA-mz:107 PHGA-mz: 105 HA-mz: 105 *o*,*p*,*m*-mHA-mz: 119	HA, o,m,p-mHA, MA, PHGA .	MeOH in HCl medium.	Carrier He: 1.8 mL/min TPGC 150 ºC for 3 min at 10 ºC/min and then increased 250 ºC for 7 min at 10º C/min Injector: 260 ºC Detector:260 ºC	HA, o,m,p-mHA, MA, PHGA : 8-27 pg/L	[72]

Table 4 (cont'd). Conditions for sample pretreatment and GC separation of toluene metabolites in biological sample

Sample Pre-treatment[a]	**GC Procedure**[b]	**Target Analyte**[c]	**Derivatization Reagent**[d]	**GC Temperature**[e]	**Detection Limit**[f]	**Ref.**
Acidified using HCl and kept in oven at 100ºC for 1 h. Hydrolyzed and adjusted pH 7 with NaOH & Na$_2$SO$_4$. Immersed with CW/DVB for 20 min under magnetic stirrer.	ZB-35 column using GC-FID	o-cresol	Hydrolyzed using HCl and kept in oven at 100ºC for 1 hour.	Carrier N$_2$: 8.0 mL/min Isothermal GC Oven: 190 ºC Injector: 210 ºC Detector:220 ºC	o-cresol: 0.1 mg/L	[26]
DI-SPME directly immersed in 10 mL vial for 20 min at 60ºC and under agitation for 20 min.	SLB-5 MS using GC-MS	FA, HA, m-mHA, MA, PGHA, SA trans muconic acid	Terimethyloxonium tetrafluoroborate	Carrier He:1.2 mL/min TPGC 50 ºC (1min); increased at 10 ºC/min to 300 ºC (3 min).	FA: 0.9µg/mL, SA: 8.1 µg/mL, t,t-MA: 0.9 µg/mL.	[24]
Centrifuge urine at 4500 rpm for 10 min. µ-SPE system condition with 2.0 mL MeOH and 3.0 mL of Britton-Robinson buffer (3.0 $\times 10^{-3}$mol/L, pH2). 0.1 mL aliquot sample dilute with Britton Robinson buffer at 10 mL volumetric flask. Preconditioned catridge 20 mg polypyrrole and washed with Britton Robinson buffer.	CP Sil 8 CB using GC-FID	HA in urine and water	MeOH in HCl medium.	Carrier He:10 psi. TPGC. 80 ºC (1 min) at 20 ºC/min to 140 ºC hold for 1 min at 20ºC/min then increased 280ºC (10 min). Injector: 280ºC Detector:280ºC	HA: Urine: 16.5 ng/mL Water: 12.1 ng/mL	[68]

[a] HCl, Hydrochloric acid; NaOH, Sodium hydroxide; Na$_2$SO$_4$, Sodium sulfate, CW/DVB, Carbowax-divinylbenzene; Direct immersion-solid phase microextraction, DI-SPME; MeOH, Methanol.

[b] GC-FID, Gas chromatography-flame ionization detector; GC-MS, Gas chromatograph-mass spectrometry; MA, Mandelic acid; PHGA, Phenyglyoxylic acid; HA, Hippuric acid; o-, ortho; m-, meta; p-, para; mHA, methylhippuric acid.

[c] HA, Hippuric acid; o-, ortho; m-, meta; p-, para; mHA, methylhippuric acid; MA, Mandelic acid; PHGA, Phenyglyoxylic acid; OA, Oxalic acid; SA, Stearic acid; FA, Furoic acid.

[d] MeOH, Methanol; HCl, Hydrochloric acid.

[e] N$_2$, Nitrogen; He, Helium; TPGC, temperature programmed GC.

[f] N.R, Not reported; HA, Hippuric acid; o-, ortho; m-, meta; p-, para; mHA, methylhippuric acid; MA, Mandelic acid; PHGA, Phenyglyoxylic acid; FA, Furoic acid; SA, Stearic acid; t-tMA, trans-trans muconic acid.

De Paiva et al. [26] developed a method for the determination of urinary *ortho*-cresol using solid-phase microextraction (SPME), followed by capillary GC-flame ionization detection (GC/FID). After optimization of the SPME variables and validation of the method, it was applied to analysis of *o*-cresol in urine collected from 27 workers exposed to solvents in automotive repair shops. The maximum extraction efficiency was obtained using a carbowax-divinylbenzene (CW/DVB) fiber (70 µm), immersed 20 min in acid hydrolyzed urine added with 3 g

Na_2SO_4 at pH 7.0 and under magnetic stirring. The SPME-GC/FID method –proved to be fast, simple and can be applied to the occupational toluene biomonitoring.

A method for the determination of the organic acids directly in the urine employing derivatization with trimethyloxonium tetrafluoroborate as a methylating agent and sequential extraction by head space and direct immersion-solid phase microextraction is reported. Furoic acid, hippuric acid, methylhippuric acid, mandelic acid, phenylglyoxylic acid and *trans*-muconic acid contained in urine and determined by GC-ion trap-mass spectrometry/tandem mass spectrometry analysis within 20 min [24].

Meanwhile, Ahmadi et al. [68] described a rapid, simple and high sensitive method for extraction of hippuric acid from human urine samples by using an automated micro solid phase extraction system (µ-SPE). However in order to increase sensitivity of gas chromatography with flame ionization detector, a post derivatization procedure was developed. In this work, a polypyrrole was synthesized by chemical oxidation of the pyrrole monomer in non-aqueous solution and applied as an excellent and efficient sorbent for µ-SPE. This method was successfully used to analyze trace amounts of HA in human urine samples without any interference from coexisting.

Conclusion

Biological monitoring of toluene exposure is a growing field of interest in toxicological and occupational laboratories. Chemical and biomedical analysts should be aware and have a broad knowledge to choose the most appropriate analytical method because it can remain a major problem. They are no ideal method *per se* but the best method selected should be able to deliver good results and compromise between several conflicting requirements.

Preliminary test is one of the indicators for screening purposes. It could be used on site for qualitative analysis with fast results using the mobile kit. The color test has proved a powerful method for preliminary test since early this decade. However, this method is not sensitive for low concentrations of toluene exposure and it can give a false results. Thus, quantitative instrumental analysis is still required for enforcement and research purposes such as using GC-MS and HPLC. Indeed, in these situations, cost, length, complexity and workload problems generated by the analytical method are not necessarily major concerns. In contrast, for the routine monitoring of toluene exposure and forensic cases in industrial environment and enforcement agency, the cost per determination, simplicity and automation will be important considerations.

HPLC appears to be the primary choice for the determination of toluene metabolites. The apparatus is relatively economical, automatable and sufficiently versatile to be used for other determination in related areas such as drug monitoring or clinical toxicology that are currently of interest in the same laboratory. As previously indicated, the analyst now has a choice between several methods, well adapted to routine and simultaneous determination of urinary metabolites of some common volatile solvents that require biological monitoring. Moreover, HPLC is also becoming an attractive tool in the growing field of more elaborate approaches with new urinary markers such as specific conjugates or isomers.

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