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Complete separation of urinary metabolites of xylene in HPLC/DAD using β-cyclodextrin: Application for biological monitoring☆

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ABSTRACT

To determine the biomarkers of exposure to xylene, urinary 2-, 3- and 4-methyl-hippuric acids, a new HPLC/DAD analytical method has been developed, which uses β-cyclodextrin as an additive for elution; its complexing abilities are exploited to achieve complete chromatographic separation of the three isomers. The mobile phase was a 3% aqueous solution of β-cyclodextrin, pH 3, and methanol, 80:20, in isocratic conditions, with a flow rate of 1 mL/min. To optimize quantitative analysis three wavelengths were employed for detection: λ = 198 nm, λ = 200 nm, and λ = 202 nm. SPE was applied for the extraction from urine samples of analytes. Validation parameters show recoveries always above 82%; LOD was set at 1 μg/mL with an LOQ of 3 μg/mL. The linear dynamic range (from 4 to 100 μg/mL) showed excellent correspondence. This method is rapid and inexpensive and can be applied to several samples simultaneously using a manifold for SPE extraction. The analytes were separated completely and could be fully quantified. The method was used for the analysis of urine samples from 54 workers exposed to xylene in hospital laboratories and showed a good applicability while allowing quantification even at low doses.

1. Introduction

Xylene isomers are widely used as industrial solvents and occupational exposure to them has been extensively studied and is widely controlled. A frequently applied method of estimating exposure is by determining the urinary levels of o-, m- and p-methyl-hippuric acids (MHA). Xylenes are metabolized by the oxidation of one of the methyl groups, followed by conjugation to glycine, leading to the excretion of more than 95% of the absorbed dose as MHA. Excretion is rapid, peaking at around 8 h post-exposure, with a total elimination of 80–90% within 24 h [1,2].

These compounds have proved useful as biomarkers of occupational exposure as there are close correlations with environmental concentrations [3–5]. To obtain a biologically useful indicator it was suggested that the urinary concentration should be corrected for creatinine [6,7] and this is now used as the basis for establishing the Biological Exposure Index (BEI) [8] by the American Conference of Governmental Industrial Hygienists (ACGIH).

Various methods have been published for the quantitative determination of MHA. Gas chromatography [9–12] is specific, with excellent precision and accuracy, but the sample has to be derivatized, e.g. using diazomethane [9,10], trimethyl-silyl derivatives [13], or methanol in hydrochloric acid [14], resulting in a longer sample work-up procedure and hazardous analytes as well as by products.

Thus liquid chromatography (LC) is an alternative, and has been widely applied, giving highly satisfactory results even for exposure to multiple solvents (toluene, xylenes, and styrene) [15–21]. The US National Institute for Occupational Safety and Health (NIOSH) proposes it as the reference method (NIOSH 8301) for biological monitoring of workers occupationally exposed to styrene [22].

Published LC methods have the disadvantage that it does not allow the separation of the meta- and para-MHA isomers. This separation is in fact problematic and some researchers suggest adding tetra n-butylammonium bromide [4], tetrahydrofurane [21] or β-cyclodextrin to the mobile phase [20]. The latter compound offers advantages in terms of cost, separation capacity and ease of use. Cyclodextrins are molecules with a fairly rigid conical structure, with apolar hydrophobic characteristics inside the cavity and hydrophilic properties outside; β-cyclodextrin in particular easily forms inclusion complexes with a wide range of compounds, but especially with the aromatic ring [23,24].

The present study set out to finalize an HPLC analysis procedure for determining the three MHA separately, as a means of quantifying occupational exposure to xylenes. The method, described here, was then used for biological monitoring of workers in the research and diagnosis laboratories of a large hospital in Rome (Italy).

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The use of biomonitoring for occupational exposure assessment has the advantage of providing a more comprehensive data than environmental monitoring, since it takes into account the potential dermal route of absorption, indeed xylene has got a skin notation by ACGIH so that some high MHA concentrations might be explained by dermal absorption.

2. Materials and methods

2.1. Chemicals and instrumentation

MHA standards were purchased from Sigma–Aldrich (St. Louis, MO, USA); all other reagents were analytical-grade and were purchased as follows: β-cyclodextrin from Sigma–Aldrich (St. Louis, MO, USA); methanol and HPLC-grade water from Carlo Erba Reagenti (Milan, Italy); glacial acetic acid from Merck (Darmstadt, Germany).

Urine samples for quality control and calibration curves were obtained from volunteers not exposed to xylenes. Test samples were taken from laboratory staff in a hospital at the end of a short work shift, at 2 p.m., and at the end of a longer shift, at 7 p.m., at the beginning and end of the week.

We used a Shimadzu HPLC system (Kyoto, Japan), comprising two model LC-10AD VP pumps, an SIL-10AD VP autosampler, a CTO-10AS VP column oven, an SPD-M10A VP diode array detector, with an SCL-10A VP control system. Shimadzu LC-Solution software was used for graphic visualization and data acquisition. The HPLC was equipped with an Alltima C18 column, 150 mm × 4.6 mm, packed with 5-μm particles, and a C8 precolumn, 7.5 mm × 4.6 mm, also packed with 5-μm particles, purchased from Alltech-Italia (Milan, Italy).

Quality control standards and test samples were collected in urine containers and promptly transferred to polypropylene test-tubes, then frozen at −20 °C until analysis. A 1-mL sample was used to extract the analytes through SPE C18 packed columns (100 mg, 1 mL), purchased together with a vacuum extraction manifold from Alltech-Italia (Milan, Italy).

2.2. Methods

The SPE columns were conditioned before use with a sequence of 2 mL water then 2 mL methanol, then 1 mL of the test sample was applied. The column was brought to dryness under vacuum for 3 min and the sample was extracted using a mixture of H_2O/CH_3OH 50:50 (v/v) in two successive 1-mL portions. The eluate was collected in glass tubes and then transferred to autosampler vials, and 20 μL was injected into the HPLC column.

MHA standard solutions were prepared by dissolving 5 mg of each compound, accurately weighed, in 50 mL of a mixture of H_2O/CH_3OH (50:50, v/v), to give a 100 μg/mL solution. Quality control samples were prepared by adding mixtures of the MHA at different concentrations to volunteers' urine samples (to nominal concentrations of 4, 8, 15, and 25 μg/mL). Samples for the calibration curve in urine were prepared at the nominal concentrations of 4, 8, 15, 25, 50 and 100 μg/mL with volunteers' urine.

The mobile phase consisted of an aqueous phase of 3% β-cyclodextrin acidified to pH 3 with acetic acid and methanol as the organic phase. For separation we used 80% aqueous phase and 20% organic phase, v/v, in isocratic conditions, at a flow rate of 1 mL/min. The column temperature was kept thermostatically at 40 °C. The elution was followed over the whole UV spectrum (190–350 nm) for qualitative analysis, and at wavelengths of 198 nm for 2-methyl hippuric acid (2-MHA), 200 nm for 3-methyl hippuric acid (3-MHA), and 202 nm for 4-methyl hippuric acid (4-MHA) for quantitative analysis. Despite the small difference in wavelength, there was an approximately 4% increase in the signal for 3-MHA and 7% for 4-MHA, which we deemed worthy of consideration. Retention times were 11.0 min for 2-MHA, 21.0 min for 3-MHA, and 19.7 min for 4-MHA.

To confirm the normal distribution of data we first of all used the Shapiro Wilk test on the results of quality control samples' analysis, the final score, $|z|$, was 0.112, substantially lower than the tabulated $z_{\alpha/2}$ (1.96) for this test, with 99% significance, in order to accept the hypothesis of Gaussian distribution. To complete the preliminary analysis we used Dixon's test to assess the significance of each finding: the parameters R_{min} and R_{max} were considerably lower than the tabulated T values, so this second hypothesis could be accepted too.

3. Results and discussion

3.1. Validation parameters

To achieve the best possible peak resolution for 3- and 4-MHA we ran tests with different concentrations of β-cyclodextrin to optimize the mobile phase. The selectivity factor (α) for 3-MHA/4-MHA calculated at various percentages of β-cyclodextrin gave: 0.49 at 1.5%, 0.84 at 2.8% and 1.22 at 3%. The standard deviation for α was lower than ±0.01. We decided to use a β-cyclodextrin concentration of 3% because α was larger than 1 under these conditions. The separation of isomers was sufficient for a separate quantification of each MHA.

We also calculated the resolution (R_s) with different percentages of β-cyclodextrin in the mobile phase: at 0% the chromatographic peaks for 3- and 4-MHA did not separate; R_s was 0.62 ± 0.02 at 1.5%, 0.94 ± 0.03 at 2.8%, and 1.39 ± 0.02 at 3%.

Figs. 1–3 show the representative chromatograms.

To plot the calibration curve in urine we read off the peaks for the three isomers, at the different wavelengths, all the curves showed excellent linearity in a concentration range of 4–100 μg/mL; the coefficient of determination (R^2) was 0.959 ± 0.002 for 2-MHA, 0.999 ± 0.001 for 3-MHA, and 0.982 ± 0.001 for 4-MHA.

The comparison of the calibration curve in urine with the curve obtained from standard solutions at the same nominal concentrations showed the absence of a significant matrix effect since the values of the slopes and intercepts were comparable (CV% 0.01–1.8).

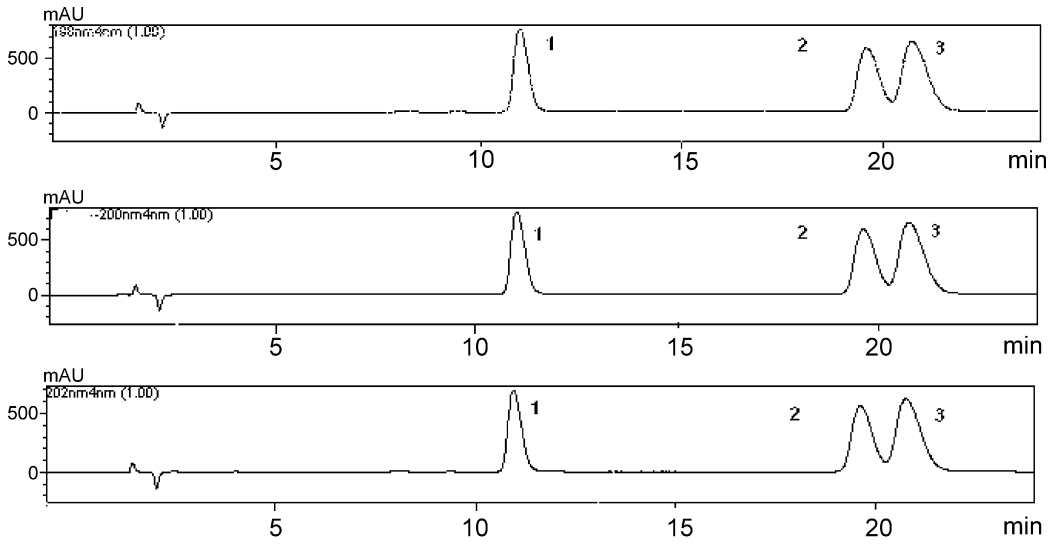
Recoveries of the MHA from volunteers' urine spiked with the analytes were examined at four concentrations (4, 8, 15 and 25 μg/mL), each run in triplicate, and we compared the peak areas for the extracted sample and the standard solutions. Recovery data are shown in Table 1.

The limit of detection (LOD) was set at 1 μg/mL for all three analytes (signal-to-noise ratio 3) and the limit of quantification (LOQ), determined on four replications, at 3 μg/mL, i.e. the lowest concentration with an intra-day coefficient of variation (C.V.) lower than 10%.

Intra- and inter-day precision and accuracy were calculated by measuring urinary MHA levels in four quality control samples: each sample was analyzed in quadruplicate for intra-day information while, for inter-day 1, the same sample was analyzed in four different days. Table 1 sets out the results.

To check the accuracy of the method the calculated mean concentration was expressed as a percentage of the real concentration added; precision was expressed as the coefficient of variation, calculating the standard deviation as a percentage of the mean concentration.

Sample stability was in line with published data; urinary MHA stored at −18 °C are stable for at least 6 months [25].



peak area:

	198nm	200nm	202nm
2-MHA	3119539	2929993	2474278
3-MHA	4209290	4326593	4000715
4-MHA	2928125	3238981	3340123

Fig. 1. Chromatograms of a mixture of standards at the nominal concentration of 10 μg/mL: (1) 2-MHA, (2) 4-MHA, and (3) 3-MHA.

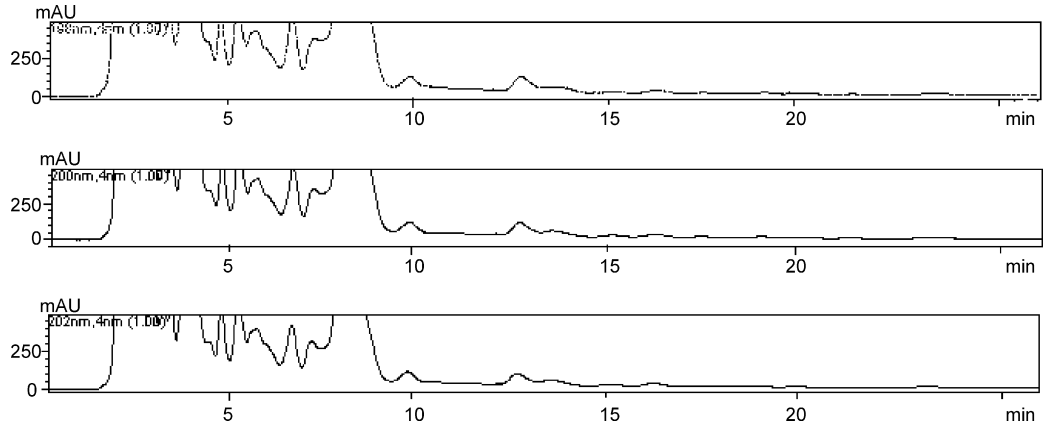


Fig. 2. Chromatograms of a urine sample of a non-exposed subject (blank).

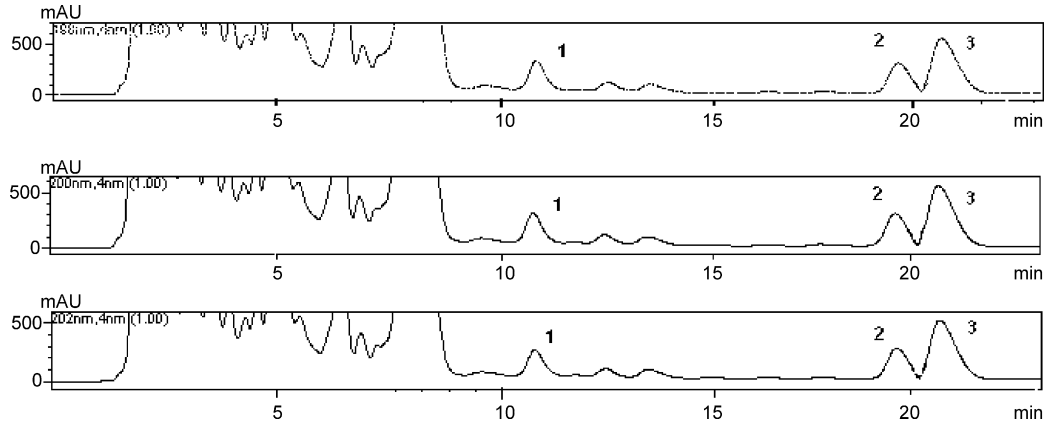


Fig. 3. Chromatogram of a urine sample of a laboratory worker exposed to xylene: (1) 2-MHA, (2) 4-MHA, and (3) 3-MHA.

Table 1
Validation parameters: intra-day and interday accuracy and precision; average recovery data.

	Intra-day		Inter-day		Recovery (%)
	Accuracy %	Precision (CV%)	Accuracy %	Precision (CV%)	
2-MHA					
4 μg/mL	114.0	9	115.0	12	109
8 μg/mL	106.0	5	108.0	9	106
15 μg/mL	95.0	5	100.0	8	99
25 μg/mL	99.5	7	99.6	7	99
3-MHA					
4 μg/mL	98.0	9	101.7	11	97
8 μg/mL	94.0	8	90.5	12	94
15 μg/mL	101.0	10	100.8	10	102
25 μg/mL	101.0	5	95.1	7	98
4-MHA					
4 μg/mL	92.0	4	88.9	7	99
8 μg/mL	89.0	5	87.8	11	89
15 μg/mL	95.6	7	93.8	8	99
25 μg/mL	109.0	9	95.5	13	102

Table 2
Results of urinary MHA in exposed workers and average value of environmental monitoring.

Environmental monitoring	Σ xylene (μg/m³)	Σ MHA (μg/g creatinine)	Range					Percentile	
	Average		*n*	Average	Min	Max	Median	5th	95th
PL	2754.70	PL	10	0.25	0.000	1.011	0.109	0.023	0.789
MOL	421.55	MOL	7	0.05	0.000	0.073	0.054	0.008	0.071
MCBL	184.61	MCBL	10	0.04	0.000	0.071	0.039	0.000	0.073
VPL	166.93	VPL	24	0.06	0.000	0.274	0.037	0.000	0.181
AIL	84.38	AIL	3	0.04	0.000	0.092	0.042	0.004	0.087

3.2. Application for biomonitoring of workers

To assess the application of the analytical method we recruited 54 workers occupationally exposed to xylene in six research and diagnosis laboratories in a large hospital in Rome (Italy). These were the Pathology Laboratory (PL), the Molecular Oncology Laboratory (MOL), the Molecular and Cellular Biology laboratory (MCBL), the Vascular Pathology Laboratory (VPL) and the Allergology and Immunology Laboratory (AIL). There were 15 men and 39 women, mean age 37 years; 30 were biologists, 7 chemists, 3 doctors and 14 laboratory technicians.

As the exposure levels obviously differed in the various laboratories, we first administered a questionnaire to find out which chemicals each person handled, how frequently, and in what volumes/amounts. We also enquired about the methods and instrumentation employed. We then surveyed the premises to establish where the various chemicals were used, and the standard procedures for using them. We did environmental checks for xylenes, alcohols and formaldehyde in the premises, and none of the concentrations exceeded the exposure limits for either 8 h or 15 min: xylenes 221 mg/m³ (8 h), 442 mg/m³ (15 min); methanol 260 mg/m³ (8 h); formaldehyde 0.37 mg/m³ (15 min).

Urine samples were obtained from each worker at the end of shifts at the beginning and end of the week. For a correct interpretation of data we also determined creatinine in the urine, so as to relate the final data to these levels, and to permit a comparison with the biological exposure limits proposed by the ACGIH (sum of the three MHA = 1.5 g/g creatinine).

The urinary determinations confirmed the environmental findings (Table 2), with values three orders of magnitude below the BEI, but we were nevertheless able to quantify personal exposure. In the pathology laboratory, which uses far more xylene than other laboratories, staffs were exposed to much higher levels, with a peak (3PL) one order of magnitude higher. Fig. 4 shows these results.

For comparison with levels of MHA in the general population only few data is available in literature. In particular only one published article shows results for a larger a numerous group of subjects (281) [18]. Since these data were collected in a Chinese population a comparison with our results might be complicated by

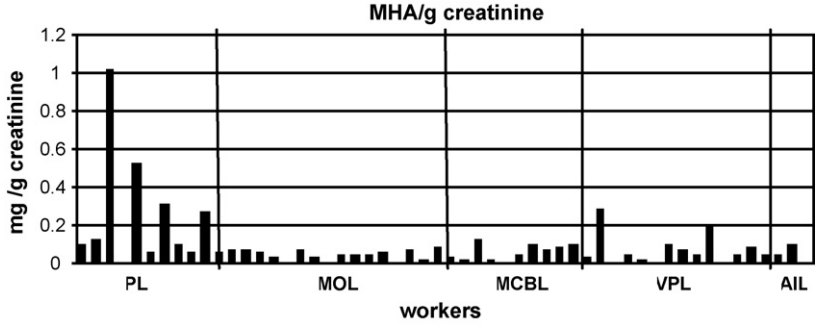


Fig. 4. Results of urinary MHA analysis in workers' samples of different laboratories.

the fact that exposure is closely linked with lifestyle, life habitat and life environment.

4. Conclusions

This method proved useful for biomonitoring purposes, the β-cyclodextrin concentration selected achieved complete resolution of the chromatographic peaks for all three isomers, and the analytical procedure is simple and easy to use, with a manifold for solid-phase extraction that can process up to 30 urine samples simultaneously. Quantification at the three wavelengths gave optimal detection, thereby optimizing the analytical conditions proposed by others [20] obtained a lower detection limit for the acid 3MHA half, and a wider linear dynamic range for all three isomers, even at lower concentrations (4–100 μg/mL) against 43–106 μg/mL for 2-MHA, 11–27 μg/mL for 4-MHA and 21–53 μg/mL for 3-MHA furthermore the calibration curve of this method is applicable to all kind of urine samples, with no matrix effects, and that means the possibility of a higher throughput of samples. Analysis times and costs are comparable with those reported elsewhere, and validation parameters confirmed the stability of the response and its intra- and inter-day accuracy; at the various concentrations tested recovery of the analytes from the matrix was sufficient.

The low LOD and LOQ allow for biological monitoring of exposed workers and the detection of low levels of MHA several orders of magnitude lower than the current BEI value of the ACGIH. This enabled us to characterize even "low-dose" exposure, a situation of particular interest in various work settings.

Application of the method "on site" showed that pathology laboratory workers were more definitely exposed to xylenes than staffs in the other laboratories, as were to be expected considering the amounts required for processing anatomical specimens.

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