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Achieving greater selectivity for the analysis of o-, m-, p-methylhippuric acids in workers' urine by ultra performance liquid chromatography coupled with tandem mass spectrometry

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ABSTRACT

A selective method analyzing separately o-, m- and p-methylhippuric acid isomers in workers' urine samples has been developed using ultra performance liquid chromatography coupled with tandem mass spectrometry. Chromatographic separation has been optimized to resolve the three isomers at baseline. Combined with this optimal separation, the mass spectrometer allowed rapid switching from MRM scan to full scan and product ion scan within the chromatographic peak. This feature allowed the retention of analyte chemical structure information for the three methylhippuric acid isomers in parallel with the simultaneous acquisition of quantitative data. Such an approach is unequaled for the reliability of the data generated and it can be applied to each isomer separately. The method was adjusted to a dynamic range between 0.2 mM and 8.12 mM for o-methylhippuric acid and p-methylhippuric acid, and between 0.41 mM and 16.23 mM for m-methylhippuric acid in order to cover the biological exposure index. A negligible matrix effect was observed with the conditions used. Also, intra-day and inter-day precisions were both <6% for all the concentration levels tested and the accuracy was evaluated at 97 ± 4%. The inclusion of simultaneous full scan acquisitions did not prevent the robustness of the quantitative data. The method applied to the determination of inter-laboratory proficient test samples led to results in the tolerated range. Moreover, urine samples from workers were robustly quantified and the MHA levels were all below the biological exposure index reference value.

1. Introduction

Xylene is commercially available as 3 different isomers: ortho-xylene, meta-xylene and para-xylene. These xylene isomers are widely used as solvent, in paints and coatings, and as a blend in gasoline [1]. It is recognized that workers can be occupationally exposed to the three forms of xylene [2–5]. The three isomers are harmful for workers exposed at significant levels. Following an unintended absorption in the body, the three xylene isomers undergo metabolic cascades leading to the formation of different metabolites by oxidation, glucuronidation and glycine conjugation [1,6]. Glycine conjugation produces o-, m-, p-methylhippuric acids (MHAs) as the metabolites as shown in Table 1. MHAs are recognized by the American Conference of Governmental Industrial Hygienists (ACGIH) as biomarkers of exposure for monitoring occupational xylene exposure [7]. The o-MHA, m-MHA and p-MHA detected in urine at the end of the work shift must be added to perform biological monitoring. The recommended Biological Exposure Index (BEI) based on total MHA is set at 0.89 mmol/mmol creatinine to prevent workers' illness. Due to high similarities between the isomers, the selective analysis of each isomer in urine represents a challenge.

Different analytical methods have been developed over the years to analyze MHAs in urine. An initial technique involved gas chromatography coupled with mass spectrometry (GC–MS) [8,9]. With this technique, MHAs must be extracted, followed by derivatization prior to injection into the GC–MS. Such an approach is labor intensive due to the multiple steps required for the preparation of the samples. Another approach relied on high performance liquid chromatography (HPLC) coupled with an ultraviolet (UV) detector [10]. This technique offers some advantages compared to GC–MS methods because no derivatization is needed, but it still requires sample extractions prior to injection into the HPLC. Subsequently, approaches based on HPLC coupled with tandem mass spectrometry (MS/MS) [11,12] were developed. These approaches were either incomplete in terms of the MHA isomers analyzed, or required a long sample preparation procedure by solid phase extraction (SPE).

Recently, new approaches to analyze solvent metabolites have been developed using ultra performance liquid chromatography (UPLC) coupled with MS/MS [13–15]. The approaches focused

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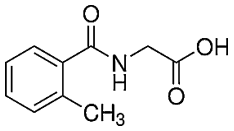
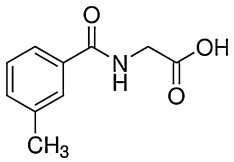
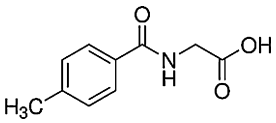
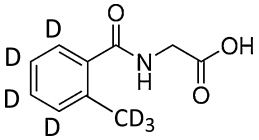
Table 1
Compound structures and MRM parameters used.

Compounds	Structure	Cone (V)	Collision energy (V)	MRM transition (*m*/*z*)
o-MHA		28	12 18	192 > 148 (quant.) 192 > 91 (conf.)
m-MHA				
p-MHA				
d7-o-MHA (ISTD)		30	12	199 > 155

either on small scale studies where specific solvent metabolites such as mandelic acid, phenylglyoxylic acid and trans, trans-muconic acid were analyzed, or on a larger scale study where a group of volatile organic compound (VOC) metabolites was simultaneously analyzed. The small scale studies showed valuable strategies, such as the acquisition of analyte chemical structure information at the same time as quantitative data, an improved sample preparation procedure, and an improved chromatographic run. However, these approaches are too specific and did not report the analysis of MHAs in urine. The large scale study showed good efficiency analyzing 28 VOC metabolites simultaneously, but the approach is lacking in terms of specificity for the analysis of MHAs where the three isomers are not chromatographically separated and limited analyte chemical structure information is acquired. A method achieving greater selectivity for MHA analysis by UPLC–MS/MS is currently needed. The objective of this paper is to describe an optimized strategy for the selective measurement of the three MHA isomers by UPLC–MS/MS in parallel with the simultaneous acquisition of structural information about each isomer.

2. Experimental

2.1. Chemicals

o-MHA, m-MHA and p-MHA (98% purity) were obtained from Sigma–Aldrich (Milwaukee, USA) and were used without any further purification. The internal standard (ISTD) d7-o-MHA (99.4% atom D) was purchased from C/D/N Isotopes (Pointe-Claire, Canada). Methanol (MeOH), acetonitrile (ACN), water (H_2O) and formic acid (FA), all LC–MS grade, were obtained from Fisher Scientific (Canada). Clincheck® clinical diagnostic reference material for metabolites in urine was provided by Recipe [16] (Munich, Germany). Inter-laboratory proficient test urine samples were provided by the German External Quality Assessment Scheme (G-EQUAS) [17].

2.2. Instruments and analytical conditions

The UPLC–MS/MS system consisted of a Waters Acquity UPLC coupled with a Waters Xevo TQ triple quadrupole mass spectrometer (Beverly, MA, USA) equipped with an electrospray source. The analytical column used was an Acquity UPLC BEH C8 1.7 μm, 2.1 mm × 150 mm from Waters (Santry, Ireland). The software used to operate the system and analyze the data was Masslynx. The peak integration was done using the automatic feature for integrating the peak area. Manual adjustments were done on integrations not covering the entire peak. The calibration curve regression was linear fit with a weighting factor of 1/*x*.

The mobile phase was composed of MeOH + 0.1% FA (eluent A), and water + 0.1% FA (eluent B). UPLC separation was achieved using an isocratic program of 15% eluent A for 7.5 min. The eluent A composition was then ramped to 55% for 2.5 min to clean the column, followed by a 1.0-min equilibration period at 15% eluent A. The flow rate was 0.45 mL/min and the column was kept at 60 °C. The flow was diverted from the mass spectrometer to the waste containers in periods extending from 0–3.0 min and 7.5–10.0 min. The injection volume was 10 μL using the partial loop with needle overfill feature. The samples were kept at 15 °C in the auto sampler. The Xevo TQ was operated in negative mode, the capillary voltage was set at −2.8 kV, the source temperature at 150 °C, the desolvation temperature at 500 °C, the desolvation flow at 1000 L/h, the collision gas flow at 0.15 mL/min, and the data were acquired in multiple reaction monitoring (MRM) mode with the product ion confirmation scan and full scan MS options activated. The parallel product ion confirmation scan was set at daughter scan with an activation threshold of 20, a reset threshold level of 50%, a mass above parent of 10, a minimum mass of 50, a scan speed of 5000 amu/s, a duration of 0.4 s, and the data were acquired in continuum mode. The parallel full scan was conducted in continuum mode between 50 and 650 *m*/*z* with a scan time of 0.3 s. The MRM transition and conditions used for MHAs and the ISTD are listed in Table 1.

2.3. Subject

Urine was randomly obtained from individuals. For occupationally exposed individuals, urine samples of 20 mL or more had to be collected at the end of their work shift. The urine samples for analysis were kept at 4 °C for less than 30 days. No stability issues were observed at 4 °C during this period.