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Extração em fase sólida molecularmente impressa de ácido hipúrico e ácido metil-hipúrico em amostras de urina seguido de análise por cromatografia líquida de alta eficiência

Molecularly imprinted solid phase extraction of hippuric and methyl hippuric acids from urine samples followed by high performance liquid chromatography analysis

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Resumo

Tolueno e xileno são solventes amplamente usados e podem causar vários efeitos indesejáveis no sistema nervoso central quando em altas concentrações no ambiente. A monitorização biológica destas substâncias tem sido frequentemente realizada analisando os seus metabólitos na urina como o ácidos hipúrico (AH) e 3-metil hipúrico (3-AMH), respectivamente para tolueno e xileno. Assim, métodos de preparo de amostras seletivos e sensíveis para a extração destes metabólitos em amostras de urina são desejados. Desta forma, este trabalho descreve uma nova alternativa para a análise simultânea de AH e 3-AMH a partir de amostras de urina, utilizando extração em fase sólida molecularmente impressa e cromatografia líquida de alta eficiência. A faixa linear, limite de quantificação e coeficiente de correlação foram 0,5-8,0 mg mL^{-1}, 0,5 mg mL^{-1} e >0,992 para ambos os analitos, respectivamente. Além disso, o método é confiável, preciso e exato na faixa linear e pode ser eficientemente aplicado em análises de rotina de AH e 3-AMH em amostras de urina de trabalhadores expostos ao tolueno e xileno em seu ambiente de trabalho.

Palavras-chave: ácido hipúrico, ácido metil-hipúrico, cromatografia líquida de alta eficiência, polímero de impressão molecular, tolueno, xileno.

Abstract

Toluene and xylene are widely used solvents and can cause several undesirable effects in the central nervous system when in high concentrations. The biological monitoring of these substances has often been performed analyzing their urine metabolites as hippuric (HA) and 3-methyl hippuric (3-MHA) acids, respectively for toluene and xylene. Thus, selective and sensible sample preparation methods for the extraction of these metabolites from urine samples are welcome. Therefore, this paper describes a new alternative for the simultaneous analysis of HA and 3-MHA from human urine samples using molecularly imprinted solid phase extraction and high performance liquid chromatography. The linear range, limit of quantification and correlation coefficient were from 0.5 to 8.0 mg mL^{-1}, 0.5 mg mL^{-1} and >0.992 for both analytes, respectively. Moreover, the method is reliable, precise and accurate in the linear range and it can be efficiently applied in routine analysis of HA and 3-MHA in urine samples from workers exposed to toluene and xylene in their occupational environment.

Keywords: high performance liquid chromatography, hippuric acid, methyl hippuric acid, molecularly imprinted solid phase extraction, toluene, xylene.

1. Introduction

Toluene and xylene are organic solvents widely used in industries in the production of resins, paints, paint thinners, glues, fuels, among others. In high environmental concentrations, these compounds can be easily absorbed during breathing, causing several health problems mainly in the central nervous system[1]. The biological monitoring of these solvents has often been accessed by the determination of their metabolites in urine samples, for example, the hippuric (HA) and 3-methyl hippuric acids (3-MHA) for toluene and *para*-xylene, respectively[2]. The biological exposure index for HA and 3-MHA in Brazil are 2.5 and 1.5 g per gram of creatinine[3].

The HA and 3-MHA quantifications frequently depend on a previous sample preparation procedure based on either solvent[1] or solid phase (SPE) extractions[4] to eliminate undesirable interferents present in urine, as for example, proteins, salts, lipids and organic compounds. In this way, selective, sensible, simple and fast extraction procedures are welcome instead of the conventional techniques, as those based on molecular imprinting for example[5]. Molecular imprinted polymers (MIPs) are alternative materials that have been highlighted in literature due to their high selectivity when compared with other sorbents. These polymers consist of a rigid three-dimensional network synthesized around a template molecule, which is usually the analyte itself[6]. After the synthesis, the material is washed to remove the mold and the polymer acquires binding sites able to re-bind the template[6]. The sorbents commonly used in SPE present retention mechanism based on hydrophobic interactions, with the disadvantage of co-eluting interfering species. In MIPs, these non-specific interactions are minimized, prevailing specific bonds between the polymer and the template molecule or other molecules similar to the template[7].

Several studies have been published in recent years demonstrating the usefulness of the MIPs in the extraction of several compounds from different matrices as biological samples[8-11], food[12,13], water[14,15], etc. However, molecularly imprinted solid phase extractions of HA and 3-MHA have not been reported so far, to the best of our knowledge. So, in the present paper we evaluate the efficiency of a new MIP for selective extraction of HA and 3-MHA from human urine samples, followed by high performance liquid chromatography analysis with UV detector.

2. Experimental

2.1. Instrumentation

A water bath from B. Braun Biotech International (Melsungen, Germany) was used for the polymers synthesis. The pH was adjusted with a NT PH2 pH-meter from Nova Técnica (São Paulo, Brazil) equipped with a combined glass electrode. A spectrophotometer UV-Vis Biomate 5 (Thermo Electron Corporation, Rochester, USA) operating at 228 nm was used during the washing and adsorption experiments. All the chromatographic analyses were performed in a HPLC Shimadzu model LC-10AV (Kyoto, Japan) equipped with a LC-10ATvp pump, a CTO-10ASvp column oven, a SIL-10AF automatic injector, a SPD-10Avp UV detector and a Supelcosil™ LC-18 reverse-phase column (150 × 4.6 mm, 5 µm particle size) from Supelco® (Bellefonte, PA, USA) protected by a similar guard-column (10 x 4.0 mm). A Visiprep DL vacuum manifold from Supelco® (Bellefonte, USA) was employed in the SPE.

2.2. Reagents and solutions

Ultrapure water was obtained from a Milli-Q Plus gradient water system (Millipore, São Paulo, Brazil). 3-MHA, 4-vinylpyridine, ethylene glycol dimethacrylate, 2,2'-azobisisobutyronitrile (all from Sigma-Aldrich®, Steinheim, Germany) and HPLC grade acetonitrile (Vetec, Rio de Janeiro, Brazil) were used in the synthesis as template, functional monomer, crosslinker, initiator and solvent, respectively. Ethyl acetate, methanol, acetone and hexane (all of HPLC grade) used during the extraction optimization were

purchased from Honeywell (Muskegon, USA). The sodium hydroxide and nitric acid were purchased from Labsynth (Diadema, Brazil) and Dinâmica (Diadema, Brazil), respectively. Standards solutions of 50 mg mL^{-1} HA and 3-MHA (Sigma-Aldrich, Louis, USA) were prepared in methanol and stored in amber flasks at -4ºC.

2.3. MIP synthesis

The MIP synthesis was performed as described by Vieira et al.[9]. 2.0 mmol of 3-MHA (template), 8.0 mmol of 4-vinylpyridine (functional monomer), 40.0 mmol of ethylene glycol-dimethacrylate (crosslinking reagent), 1.5 mmol of 2,2'-azobisisobutyronitrile (initiator) and 11 mL of acetonitrile were added into a 100 mL glass flask, and the mixture was purged with nitrogen during 20 min to eliminate oxygen[16]. The flask was sealed and maintained in a water bath during 24 h at 60°C. After this, it was opened and the polymer was ground in a mortar. A steel graduated sieve was used to select the particles <150 µm in size. Finally, 50 mg of the MIP was packed into a SPE cartridge and submitted to consecutives washing cycles with 3 mL of methanol to eliminate the template and residues from the synthesis. The polymer was considered clean when no spectrophotometric signal (at 228 nm) was observed in the eluates from washing cycles. The non-imprinted polymer (NIP) was synthesized by the same way, except for template addition step.

2.4. Samples

The urine samples handling was approved for the Ethics Committee of the Federal University of Alfenas (no: 23087.001825/2009-81). All samples were collected in an appropriate flask and filtered before the analysis. The HA and 3-MHA standard solutions were prepared by fortifying a blank urine samples (from individuals non-exposed to toluene and xylene) with specific concentrations.

2.5. Molecularly imprinted solid phase extraction

A solid phase extraction (SPE) cartridge contained 50 mg of MIP were conditioning with 3.0 mL of ethyl acetate and 5.0 mL of water at 1 mL min^{-1}. Next, 1.0 mL of standard/sample was percolated through the cartridge and the HA and 3-MHA were retained in the MIP. Finally, 1.0 mL of mobile phase (792:200:8-v/v/v water:methanol:acetic acid) was employed to elute the analytes. 20 µL of the eluate were injected in the HPLC system.

2.6. Chromatographic conditions

The guard and analytical columns were maintained at 40ºC and the UV detector was set at 228 nm. The 792:200:8 (v/v/v) water:methanol:acetic acid was used as isocratic mobile phase at 1.3 mL min^{-1} flow rate, and the injection volume was 20 µL.

2.7. Validation

The method validation was carried out according to the Guidance for analytical methods validation of Brazilian Health Surveillance Agency (ANVISA) and the FDA's Guidance for Industry for Bioanalytical Method Validation[17,18].

Calibration curves (linear regression analysis) were constructed by plotting peak areas of the HA and 3-MHA (subtracted from the blank) vs. the original concentrations.

Intra-assay precision and accuracy were determined by six replicates assay with blank urine spiked with three concentrations. The assays were realized at the same day. Inter-assay precision and inter-assay accuracy were evaluated by analyzing three values of concentration at three different days.

Robustness test was performed evaluating the method susceptibility to the changes on the experimental conditions. Younden test consists in a factorial combination of two variables from seven parameters that result in eight assays. The results of assays are statistically analyzed to estimate the influence of the chosen parameters in the analytical method[19,20].

3. Results and discussion

The spectrophotometric signal of each fraction of methanol (3.0 mL) used in washing procedure was gradually decreasing until its total absence after about 20 cycles. So, 20 cycles were considered enough to clean completely the MIP.

All the optimization studies were carried out by using a blank urine sample (from individuals non-exposed to toluene and xylene) fortified with 0.5 mg mL^{-1} of HA and 3-MHA. The initial conditions were: i) conditioning with 5 mL of 0.5 mol L^{-1} nitric acid aqueous solution and 10 mL of water, ii) extraction of 2 mL of standard/sample and iii) elution with 1 mL of methanol. All the solutions were flowed through a cartridge with 100 mg of MIP at 1 mL min^{-1}.

Initially, methanol and mobile phase (792:200:8 - v/v/v water:methanol:acetic acid) were evaluated as eluent and no differences were observed in terms of analytical signal. On the other hand, when the methanol was used, the obtained chromatogram presented more peaks of concomitants attesting that this solvent was less selective as eluent in comparison with the mobile phase. Therefore, mobile phase was selected as the working condition.

The washing step has been often used to eliminate interferents bonded to the MIP by unspecific interactions without to elute the own analytes[21]. In this way, several washing solvents were appraised and the results are presented in Figure 1. As can be seen, the analytes were almost completely eluted during the washing when polar solvents (methanol, water and acetonitrile) were used. Additionally, the best results in terms of recoveries were found when no washing step was used. Moreover, the chromatographic profiles obtained after extraction using hexane as washing solution were very similar with those obtained without washing steps, and no interferents peaks were observed in the retention times of the analytes. Thus, the SPE procedure was optimized without any washing step.

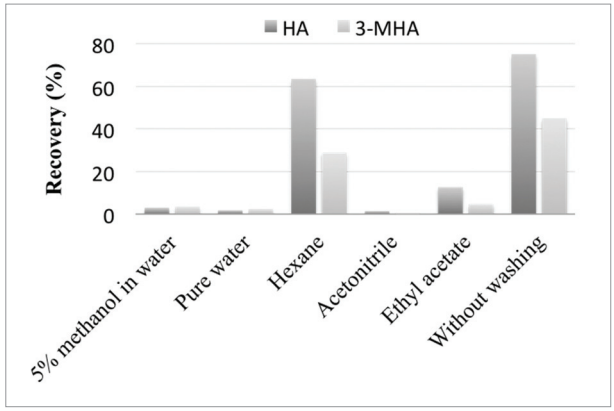

Figure 1. Effect of the washing solutions in the extraction recovery.

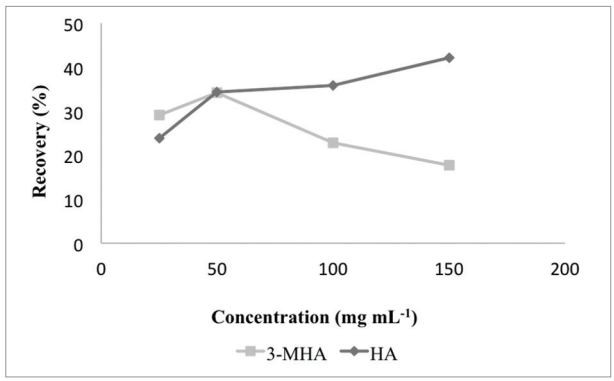

Figure 2. Relation between the recovery rates of hippuric Acid (HA) and 3-Methyl hippuric acid (3-MHA) and the amount of MIP used into the cartridge.

The sample pH was studied from 3.0 to 10.0 (without buffer), and the best extraction efficiency was obtained for pH 3.0. Therefore, hydroxyl groups from the analytes are in their uncharged form, whereas the amine group from the 4-vinylpyridine is positively charged, resulting in an ion-dipole interaction.

The mass of MIP into the cartridge was tested from 50 mg to 150 mg, and 50 mg was selected as working condition because it presented good adsorption efficiency and a low resistance to the flow. Additionally, for masses > 50 mg, an increase in the HA retention was observed, probably due to its chemical similarity with the 3-MHA, as well as lower molecular weight. As it can be seen in Figure 2, recovery rates of HA and 3-MHA were equal by using 50 mg of MIP in the cartridge.

The final condition of extraction was defined by A) conditioning with 3 mL of ethyl acetate; B) conditioning with 5 mL of ultra pure water; C) sample extraction; D) elution with 2 mL of water:methanol:acetic acid (792:200:8. v/v). Figure 3 shows a typical chromatogram of a urine sample fortified with 3 mg mL^{-1} of HA and 3-MHA, and extracted by these conditions before the HPLC analysis. As it can be seen, there are not interferent peaks in the chromatogram, attesting the selectivity of the molecularly imprinted solid phase extraction.

NIP is a polymer synthesized on the same conditions at MIP, using the same reagents e following the same procedure. However the template is not added at the synthesis mixture and the selective cavities are not formed. Due to absence of the selective cavities, the efficiency of extraction using NIP cartridges were 46.05% to HA and 20.09% to 3-MHA, whereas for MIP the values were 60.53% to HA and 58.63% to 3-MHA. The lower adsorption capacity of the NIP can be explained by the absence of selective biding sites.

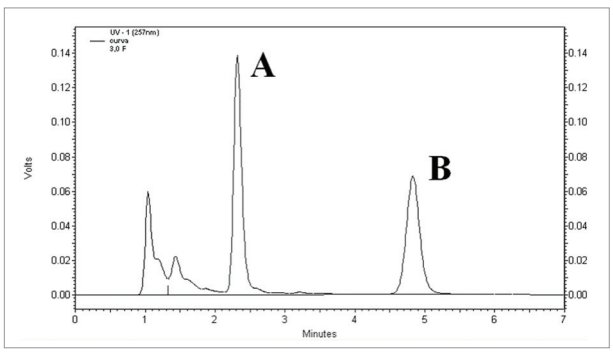

Figure 3. Chromatogram of extraction of HA (A) and 3-MHA (B) from urine by SPE using MIP as extraction phase.

The limit of quantification was defined as 0.5 mg mL^{-1} to HA and 3-MHA. Calibration curves were established from 0.5 mg mL^{-1} to 6.0 mg mL^{-1} for both analytes. These ranges include the biological exposure index values that are 1.5 and 2.5 g g^{-1} of creatinine to HA and 3-MHA, respectively[3], adopting the creatinine excretion of about 1.5 g L^{-1}. Equations and determination coefficients were y = 236064x+24718 and 0.9966 to HA and y = 177220x-46872 and 0.9921 to 3-MHA.

Precision and accuracy values of both analytes are presented in Table 1. The results of precision and accuracy were expressed as a percentage of the relative standard deviation and relative error, respectively, and the obtained results attested that the method is precise and accurate.

Robustness was evaluated by Youden test as described previously. Table 2 shows the analyzed parameters, factorial combination and equations used to calculate the influence of each condition. Signal (+) was attributed to the value correspondent to nominal

Table 1. Accuracy and precision of HA and 3-MHA calculated as relative error and RSD values.

		Concentration ($mg.mL^{-1}$)	0.5	3.0	6.0
Hippuric Acid	Accuracy RE[1] (%)	Intra-assay (n=5)	-2.00	10.30	-13.00
		Inter-assay (n=3)	-6.00	8.70	-0.50
	Precision RSD[2] (%)	Intra-assay (n=5)	8.41	8.18	4.94
		Inter-assay (n=3)	8.07	2.57	1.70
3-metthyl hippuric Acid	Accuracy RE (%)	Intra-assay (n=5)	9.10	6.70	11.00
		Inter-assay (n=3)	9.40	-0.30	8.00
	Precision RSD (%)	Intra-assay (n=5)	7.97	7.22	8.29
		Inter-assay (n=3)	0.85	9.48	3.15

[1]Relative error, [2]relative standard deviation.

Table 2. Analytical parameters, Youden test factorial combination and equations used to evaluated robustness.

Parameters	Nominal (+)	Variation (-)	Factorial Combination								Formula for variation effect
			1	2	3	4	5	6	7	8	
Methanol in mobile phase	200mL	204mL	+	+	+	+	-	-	-	-	(a+b+c+d)/4 – (e+f+g+h)/4
Column temperature	40°C	35°C	+	+	-	-	+	+	-	-	(a+b+e+f)/4 – (c+d+g+h)/4
Sample volume	1,0mL	1,1mL	+	-	+	-	+	-	+	-	(a+c+e+g)/4 – (b+d+f+h)/4
Room temperature	20ºC	no control	+	+	-	-	-	-	+	+	(a+b+g+h)/4 – (c+d+e+f)/4
Elution volume	2,0mL	1,8mL	+	-	+	-	-	+	-	+	(a+c+f+h)/4 – (b+d+e+g)/4
Urine pH	pH 3,0	pH 3,2	+	-	-	+	+	-	-	+	(a+d+e+h)/4 – (b+c+f+g)/4
Ethyl acetate volume	3,0mL	3,2mL	+	-	-	+	-	+		-	(a+d+f+g)/4 – (b+c+e+h)/4
Results			**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	

Table 3. Effects on concentration results caused by changing conditions.

Parameters	Concentration HA (%)	Concentration 3-MHA (%)
Volume de methanol in mobile phase	16.10	13.59
Column temperature	-0.29	6.58
Sample volume	-9.53	-4.17
Room temperature	6.39	3.41
Elution volume	-18.66	-9.60
Urine pH	3.63	4.05
Ethyl acetate volume	2.18	7.96

conditions and a signal (-) was attributed to value corresponding to the variation. The influence of each parameter is showed in Table 3. The more influential parameter was the elution volume, showing that more caution is required to this parameter when performing the extraction. Nevertheless the method can be considered robust because the variation values are statistically acceptable.

The method was used to analyze HA and 3-MHA in different samples from healthy volunteers from our laboratory, and the obtained concentrations were lower than the biological exposure indexes for HA and 3-MHA.

4. Conclusions

In this paper, a fast, simple and practical method to simultaneous analyzes HA and 3-MHAfrom urine samples was developed and validated. The molecular imprinted polymer showed to be a very useful tool to extract these analytes from urine. Precision, accuracy, selectivity, linearity and robustness were satisfactory.

Therefore this analytical method can be employed to routine analysis of HA and 3-MHA for biological monitoring of occupationally exposed workers to organic toluene and xylene.

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