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Selective pretreatment and determination of phenazopyridine using an imprinted polymer-electrospray ionization ion mobility spectrometry system

B. Rezaei*, M.T. Jafari, O. Rahmanian

Department of Chemistry, Isfahan University of Technology, Isfahan 84156-83111 Islamic Republic of Iran

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ABSTRACT

In this research, selective separation and determination of phenazopyridine (PAP) is demonstrated using molecular imprinted polymer (MIP) coupled with electrospray ionization ion mobility spectrometry (ESI-IMS). In the non-covalent approach, selective MIP produced using PAP and methacrylic acid (MAA) as a template molecule and monomer, respectively. The created polymer is utilized as a media for solid-phase extraction (SPE), revealing selective binding properties for the analyte from pharmaceutical and serum samples. A coupled MIP-IMS makes it possible to quantitize PAP in the range of 1–100 ng mL^{-1} and with a 0.2 ng mL^{-1} detection limit. Furthermore, the MIP selectivity is evaluated by application of some substances with analogous and different molecular structures to that of PAP. This method is successfully applied for the determination of PAP in pharmaceutical and serum samples.

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1. Introduction

Phenazopyridine (PAP) [2,6-diamino-3-(phenylazo)pyridine], has been used in the treatment of urinary tract infections since 1930s and is utilized to alleviate the pain in conditions such as cystitis and urethritis. Overdose using of the PAP may cause some side effects such as, jaundice, shortness of breath, skin rash, blue or purple coloring in skin, swelling of face, fever and confusion [1]. Therefore, in clinical treatment, it is necessary to develop a rapid and sensitive method with simple sample preparation and determination steps for PAP analysis. Some techniques exist for the determination of PAP in biological fluids, including polarography [2], adsorptive stripping voltammetry [3], high-performance liquid chromatography (HPLC) [4,5], gas chromatography–mass spectrometry (GC–MS) [6,7] and liquid chromatography–mass spectrometry (LC–MS) [8]. However these methods have not sufficient sensitivity owing to chromatographic interferences, need to use large biological fluid volumes and also expensive instruments and toxic solvents.

Molecular imprinting is a method for making artificial receptor sites in a polymer. MIPs are typically prepared by polymerizing a combination of a target molecule (template), functional monomers and an excess of cross-linker. Binding sites with molecular recognition properties are formed after removing template molecules from the polymer, leaving behind cavities complementary in size and shape to the template for the subsequent rebinding process.

Nowadays, MIP has been utilized in a diversity of applications, because of its molecular identification ability, such as drugs [9], small analytes [10], peptides [11] and proteins [12–15]. In contrast with some of other sorbents, the construction time for MIPs is short, building is easy and the substance is steady under various circumstances [16,17]. MIP solid phase extraction can clean up the sample before analysis in the complex matrices such as plasma serum.

Ion mobility spectrometry (IMS) has been developed as a sensitive analytical tool for identifying organic compounds based upon the mobility of gas phase ions in a weak electric field. IMS equipment is robust and easy to miniaturize for field operation and offers a very rapid, susceptible and inexpensive tool for the capable analysis and characterization of various chemical compounds [18]. This method acts a significant role in useful applications such as industrial and environmental screening and detection of drugs and explosives [19]. Furthermore, IMS is not only a simple detector; it can also be employed to separate molecule ions based on their mobility. This ion mobility depends on mass, charge state, and shape of the ion. The capability of IMS lets us identify sample molecules with the same masses, in addition to identical functional groups, such as isomeric compounds [18]. Many detectors of liquid chromatography (LC) do not supply other analyte information beyond the retention times and can only analyze ions with definite properties. The coupling of LC with IMS would give a selective and susceptible detector not including the expensive and extensive requirements of mass spectrometry [20]. O'Donnell et al. [21] have recently published a review on pharmaceutical applications of IMS.

Quite recently [22,23], we have introduced IMS as a powerful detection technique for molecular imprinted polymer (MIP) separation. Based on these studies, IMS is extremely compatible with

* Corresponding author. Tel.: +98 3113912351; fax: +98 3113912350.
E-mail addresses: rezaei@cc.iut.ac.ir, rezaeimeister@gmail.com (B. Rezaei).

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MIP separation methods, and the MIP-IMS system has great capability for providing highly selective analysis of trace chemical and biochemical compounds. In fact, the chemically similar compounds which are not separated by MIP have an additional chance for separation in IMS.

In this study, the application and facility of ESI-IMS is demonstrated as a sensitive detection system after MIP technique for selective extraction and pre-concentration of PAP from complex matrices such as pharmaceutical and serum samples.

2. Experimental

2.1. Chemicals

Phenazopyridine, hydrochlorothiazide, dibucaine were supplied from Sigma–Aldrich. MAA, ethylene glycol dimethacrylate (EDMA), 2,2′-azobis(2-isobutyronitrile) (AIBN), dimethylformamide (DMF), HPLC-grade methanol and acetic acid were purchased from Merck.

2.2. Ion mobility spectrometry

The ESI-IMS used for this study was designed and constructed at Isfahan University of Technology and has been described previously [24]. In short, the major parts of the instrument are: the IMS cell, the electrospray needle, two high voltage power supplies, a pulse generator, an analogue to digital converter and a computer. In this research, the IMS cell of the system was built from 16 aluminum discs, which were separated from each other by thin PTFE rings. The IMS cell length was divided into three sections, the electrospray region (3 cm), the desolvation region (4 cm), and the drift region (11 cm in length). The drift and desolvation rings have the inner diameter of 4 and 2.1 cm, respectively. The aluminum rings are joined by sequences of resistors to form the electric field ramp. The electrospray needle (P/N 7768-01, Hamilton, Reno, NV, USA) inserted into a Teflon tube to eliminate the corona discharge problem and it is fixed at one end of the cell. The Bradbury–Nielsen shutter grid is prepared of two series of parallel wires biased to a potential, creating an orthogonal field relative to the drift field, to block ion passage to the drift tube. The grid potential is eliminated for a short time by the pulse generator, to give an ion pulse to the drift region. Generally, this period of time was selected 200 μs. To get the nearly complete desolvation, preheated nitrogen gas was utilized with flow rates of 500 and 900 mL min^{-1} for drift and desolvation gas, respectively. The default Faraday plate detector configuration comprised of a 21-mm-diameter stainless steel plate positioned ∼1.0 mm behind the aperture grid. The high-speed A/D module (12-bit dynamic range) was employed to determine the spectrometer output and to alter the analogue signal to a digital one. All mobility data were gathered by data acquisition software and each IMS spectrum was the average of 50 individual spectra. Table 1 shows the used conditions under which the IMS spectra were obtained.

2.3. Preparation of the PAP imprinted polymer

The monomer MAA (3.9 mmol), PAP print molecule (0.8 mmol) and 8.5 mL of DMF were placed in an 18 mL glass sample vial. Then, cross-linker EDMA (19.5 mmol) and the reaction initiator AIBN (0.3 mmol) were added. After being purged with nitrogen for 5 min, the bottle was sealed under nitrogen and then left to polymerize in an oil bath at 60 °C for 24 h. The tube was smashed and the bulk polymer was ground into fine particles using a mortar and pestle. The product, after drying in overnight, was possessed a rigid structure. A non-imprinted polymer (NIP) containing no template was also prepared using the same procedure.

Table 1
Typical operating circumstances throughout the experimental runs.

Operating parameters	Setting
Needle voltage	11.40 kV
Target electrode voltage	9.00 kV
Liquid flow rate	6 μL min^{-1}
Drift field	600 V cm^{-1}
Desolvation field	600 V cm^{-1}
Drift gas flow (N_2)	500 mL min^{-1}
Desolvation gas flow (N_2)	900 mL min^{-1}
Drift tube length	11 cm
Shutter grid pulse	0.2 ms
Number of IMS averages	50
Scan time	20 ms
Number of points per ion mobility spectrum	500

2.4. Extraction of the PAP from MIP

The soxhlet apparatus was used to remove the template from the polymer matrix. The polymer was extracted for 48 h using a mixture of methanol-acetic acid (9:1 v/v), resulting at a rate that caused a filling and eventual emptying of the extraction chamber every 45 min with 150 mL of solvent mixture. Quantification of the removed PAP from the polymer was done by using IMS instrument. Soxhlet extraction was not carried out on the non-imprinted polymer particles.

2.5. Preparation of column

A steel sieves was used to select particles with the sizes between ∼150 and 200 μm. This was distributed into a stainless steel column (as SPE cartridge) measuring 5 cm in length and 0.4 cm i.d. until the column bed was packed. A 10 μm frit was employed at the end of column fitting to make sure no loss of polymer particles and column was capped. Methanol (2 mL) was run through the column to guarantee uniform particle packing. The column was capped and joined to a 5.0 mL gastight syringe (Hamilton, Reno, NV) via a PTFE tube. Sample solution was carried into the MIP-SPE by a programmable syringe pump (New Era Pump System Inc. made in USA). The preparation of a NIP column was achieved in a similar manner.

2.6. Pharmaceutical and serum sample analysis

The pharmaceutical samples were chosen from PAP tablet (Shahre Daru Laboratories Co.). Three PAP tablets were precisely weighed in order to get the average weight of each tablet. Then, the tablets were lightly powdered via a mortar. An equivalent quantity of the powder including a known amount of the active material was weighed and sonicated for 15 min. This was filtered into a volumetric flask and completed to the mark with methanol to make a sample solution of PAP.

A 0.5-mL human serum sample (taken from the health center of Isfahan University of Technology) was spiked with PAP to give a working concentration of 0.50, 1.0 and 1.5 μg mL^{-1}. This sample was placed into a glass vial including 4.0 mL of methanol, vortexed for 10 s, and centrifuged at 4000 rpm for 20 min. A 1.0-mL aliquot of the methanol layer was placed into another glass vial and 3.0 mL methanol was added and centrifuged again at 4000 rpm for 10 min. One milliliter of the supernatant was diluted to 10 mL, and 2.0 mL of the aliquot was introduced onto the anti-PAP column. After the solution had surpassed through the MIP column, it was washed by 2.0 mL of methanol:water (1:9). This solution washed out unwanted materials, which were chemically close to the ana-

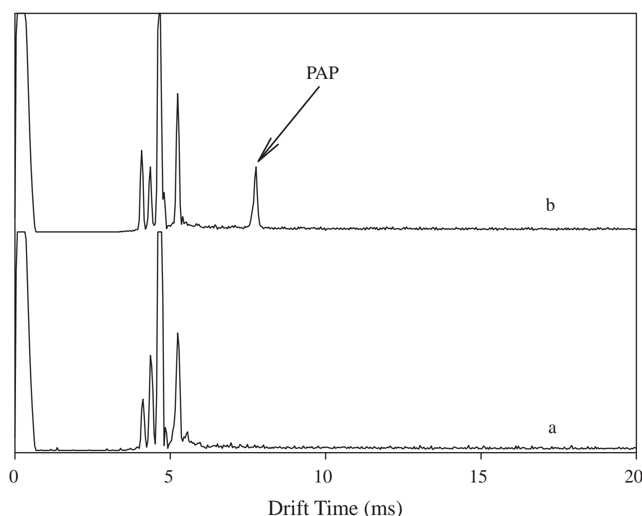

Fig. 1. Ion mobility spectrum of (a) methanol–H_2O (9:1) solvent as a background and (b) PAP solution, 1.0 μg mL^{-1}.

lyte or the analyte molecules adsorbed to nonspecific binding point of MIP. Finally, the analyte was eluted with 2 mL of methanol. The flow rate was 0.12 mL min^{-1} in this step. Then, this solution was directly injected into the electrospray needle of the IMS.

3. Results and discussion

3.1. Ion mobility spectrum

The ion mobility spectrum of PAP in comparison with background spectrum is illustrated in Fig. 1. These spectra were taken in the optimized IMS circumstances given in Table 1. The reduced mobility value of PAP ions was measured to be 1.46 cm^2 V^{-1} s^{-1}. So there is not any report on reduced mobility for the result ions originating from PAP in the literatures, the chemical formula of the ions could not be distinguished.

3.2. Calibration curve and analytical parameters ESI-IMS

In this work, a series of standards in the range 0.05–5.0 μg mL^{-1} of PAP was prepared in methanol: water (90:10) and used to determine the analytical parameters of the instrument. When the standard solution was sprayed, the PAP peak was appeared after a short time. The area of this ion peak was calculated and considered as the response of ESI-IMS for each concentration of the compound. Ten ion mobility spectra were acquired to get the averaged data points. The response of ESI-IMS was schemed against the concentration of this compound (Fig. 2) and calibration curve equation was build up by least-squares method. The linear dynamic range for the PAP determination was 0.05–5.0 μg mL^{-1} that is 2 orders of magnitude which are common for ESI and for most IMS systems. The detection limit was 0.01 μg mL^{-1}, and the relative standard deviation (R.S.D. %) for four replicate measurements of 1.0 μg mL^{-1} of PAP was 3.3%.

3.3. Effect of variables on MIP application

3.3.1. Effect of solvent ratio and extraction time

It is very important to mention that in order to obtain the best extraction time and solvent ratio (methanol–acetic acid) for the extraction of PAP from the polymer matrices, different solvent ratio (9:1, 7:3 and 5:5) and extraction times (3–17 h), were evaluated by IMS instrument. Fig. 3 demonstrated that the solvent ratio of 9:1 is the better solvent ratio for removing PAP from the polymer

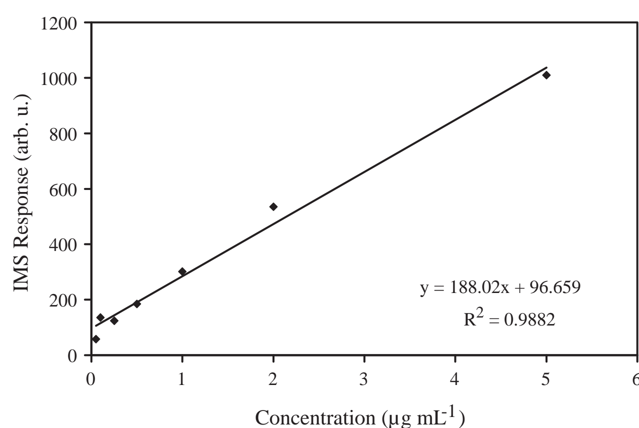

Fig. 2. Scheme of IMS response against the concentration of PAP, the scheme confirms the linear dynamic range of 0.05–5.0 μg mL^{-1}.

with extraction percentage of about 90% until 17 h. After that, in the longer time, IMS responses were varied with very low slope which showed a little amount of PAP was extracted. Therefore, because of bleeding PAP from MIP, the extraction time of 48 h was more favorable for complete extraction of PAP. The solvent ratio of 9:1 and extraction time of 48 h were selected as optimum conditions for further studied.

3.3.2. Effect of pH

The effect of pH on the performance of MIP was studied in the pH range of 2.0–8.0 (Fig. 4). The results show that, the PAP recovery was increased to pH 7.0. The optimal pH should be applied to better embed of analyte in the polymer cavities. Because of make convenient configuration of PAP, the pH of sample loading should be adjusted. In these circumstances suitable hydrogen bonding with polymer was attained. Therefore, pH 7.0 was chosen as the suitable pH for further studies.

3.3.3. Effect of flow rate

The influence of analyte retention time was estimated by transmitting 2.0 mL of PAP solution (3.0 μg mL^{-1}) at the pH 7.0 in different flow rates. The selective binding of PAP to the imprinted polymer had to be optimized in order to evade of reduced sensitivity of the MIP sorbent. In this system, the selectivity can be improved by ease of access to the binding sites within the porous polymer particles. As can be seen (Fig. 5) the best flow rate was attained to be 0.12 mL min^{-1}. In higher flow rates, the analyte does not have sufficient residence time for effective interactions with

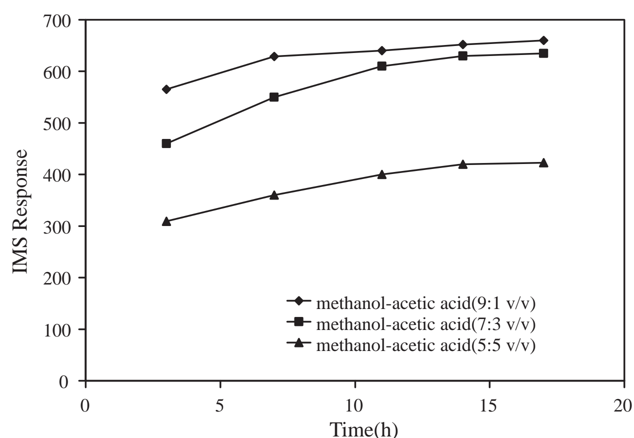

Fig. 3. Optimization of solvent ratio and time extraction. Condition: volume of extraction solvent, 150 mL.

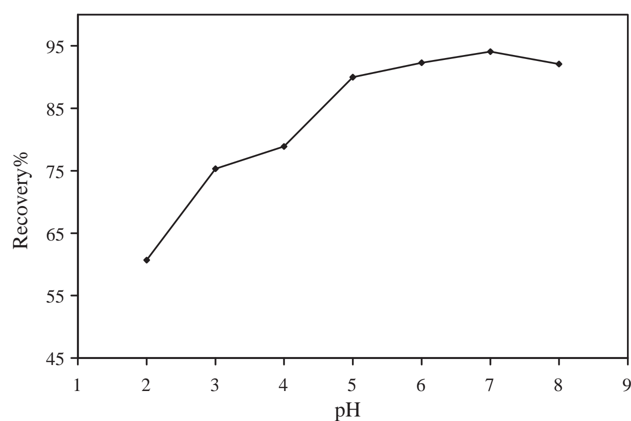

Fig. 4. Effect of analyte pH on the percentage recovery. Conditions: amount of column packing material, 50 mg; buffer solutions volume, 9.0 mL; PAP concentration, 1.0 μg mL^{-1}; flow rate, 0.12 mL min^{-1}; solution volume passed through the column, 2.0 mL; elution volume, 2.0 mL.

sorbent and in lower flow rates, the template molecule reaches to equilibrium with MIP and have enough time to diffusion from MIP cavities to solution.

3.3.4. Retention capacity and enrichment factor of MIP

A significant step in any MIP-based procedure is the determination of retention capacity because it permits to achieve high extraction efficiency. Various concentration of PAP in the range 50.0–400.0 μg mL^{-1} at the same volume (2.0 mL) was passed through the column for measuring retention capacity. The retention capacity (mg adsorbed PAP/g of sorbent) was obtained to be 12.0 mg g^{-1}.

The enrichment factor as an important parameter on preconcentration step was determined by passing 100.0 mL of PAP solution with the concentration of 0.10 μg mL^{-1} through the MIP column. After analyte elution with 2.0 mL methanol, IMS analysis was carried out and the results show that the enrichment factor is 50. Therefore the detection limit of ESI-IMS could be improved to 0.2 ng mL^{-1} when the MIP is coupled to the system.

3.3.5. Evaluate the selectivity

In order to assess the selectivity, two other drugs such as hydrochlorothiazide and dibucaine were selected. The motive for the election of these drugs would be that, they have many like functional groups which are able to bind with MIP, but with different conformations. It is essential to declare that in usual SPE methods the separation is based on size, charge and functional groups and it is independent of the molecular shape and conformation. Therefore, influence of these species on the determination of 1.0 μg mL^{-1} of PAP was investigated. The concentration of bothersome species is selected one hundredfold of PAP. Washing, elution and other processes for sample preparation were carried out according to those for PAP which were mentioned previously. Ion mobility spectra of the respective solutions were obtained with no significant hydrochlorothiazide and dibucaine ion peaks, revealing that the MIP polymer did not adsorb a significant amount of selected interference material (Fig. 6). The results represented that the affinity and specificity of MIPs for PAP is more than other examined drugs. This fact can be featured to the high selectivity of prepared MIP. In this figure, the ion peak originated by dibucaine is not observed because of its week adsorption on MIP sorbent and low intensity in the IMS. The major problem correlated with SPE columns packed with ordinary stationary phases is low selectivity of the retention mechanism. For more investigation of MIP selectivity, the NIP sorbent has been provided. A PAP standard solution (1.0 μg mL^{-1}) was passed through the NIP column and the obtained recovery was compared with that of MIP, indicating lower than 10% non-specific adsorption.

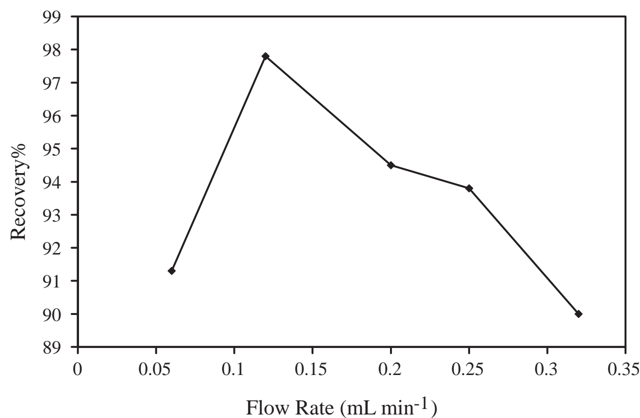

Fig. 5. Effect of flow rate on the percentage recovery. Conditions: amount of column packing material, 50 mg; buffer solutions volume, 9.0 mL; PAP concentration, 50.0 μg mL^{-1}; flow rate, 0.12 mL min^{-1}; solution volume passed through the column, 2.0 mL; elution volume, 2.0 mL.

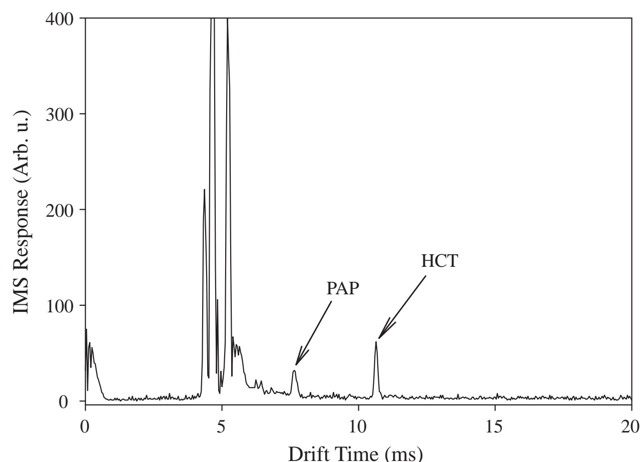

Fig. 6. Evaluate the selectivity. The MIP polymer did not adsorb a significant amount of selected interference material.

3.3.6. Response characteristics

In Table 2, figures of merits of the proposed method are compared with some reported techniques [5–8,25–28]. It is quite obvious from the results; proposed method has a better detection limit and linear dynamic range. This method is simple and does not need expensive equipments, whereas the other reported

Table 2
Comparison of some methods for determination of PAP with proposed method.

Method	Linear dynamic range (μg mL^{-1})	Detection limit (ng mL^{-1})	Reference
HPLC	2–20	10	[5]
GC–MS	0.01–1.0	0.3	[6]
GC–MS	0.005–0.5	0.3	[7]
LC–MS	0.0001–0.150	–	[8]
Derivative spectrophotometry	2–18	–	[25]
HPLC–UV	0.05–10	10	[26]
Amperometric	1–30	–	[27]
Spectrophotometric	1–5	–	[28]
Proposed method	0.001–0.1	0.2	–

Table 3
Results of PAP determination in real sample analysis ($n = 3$).

Sample	PAP added ($\mu g\, mL^{-1}$)	Average of PAP found ($\mu g\, mL^{-1}$)	Recovery (%)
Serum sample	–	Not detected	–
	0.5	0.55 ± 0.07	110
	1.0	0.91 ± 0.06	91
	1.5	1.55 ± 0.04	103
Tablet	–	0.42 ± 0.03	–
	1.0	1.34 ± 0.11	92
	1.5	1.85 ± 0.04	95

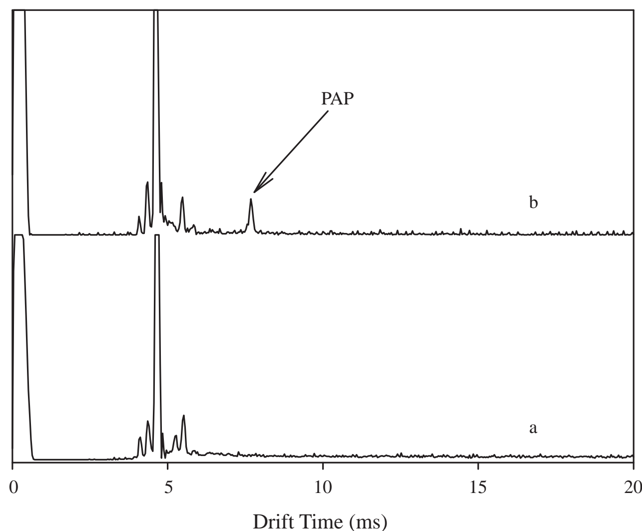

Fig. 7. Ion mobility spectrum of (a) serum sample and (b) spiked serum sample with $1.5\, \mu g\, mL^{-1}$ of PAP.

methods such as HPLC and GC or LC–MS need expensive instruments, time consuming, hazardous solvents, and complicated tools.

3.3.7. Real sample analysis

The recommended method has been applied successfully for the extraction and determination of PAP in the pharmaceutical and serum samples. The analysis was performed on human serum and PAP tablet by using the spiked samples. The results are summarized in Table 3 (each analysis was done three times) and good recoveries in all samples were achieved. In order to remove the interference of real sample constituents, the column was washed with washing solution (methanol 10%) after loading real samples on it and then it was eluted by elution solvent to measure PAP content (the same as described procedure). In this work, the applied method not exhibited interference in the ion mobility spectrum as it can be seen in Fig. 7. Furthermore, it is clearly observed that the ion mobility spectrum achieved from serum sample have low background and so low detection limit. Therefore, MIP has performed very clean in extraction of PAP from complicated sample such as human serum.

4. Conclusions

In view of the require for a selective and sensitive method to determine PAP in complex matrix samples, in this work a imprinted polymer was synthesized and applied as sorbent in SPE to achieve a selective PAP with high recoveries. The coupling of synthesized polymer with IMS as detection system was successfully applied for high sensitive determination of PAP. There are not found bothersome peaks with application of IMS. ESI-IMS has many attractive characteristics to be incorporated with MIP separations. The application of MIP-IMS system in pharmaceutical and serum samples was successfully demonstrated. Furthermore, because extraction selectivity is notably improved, a lower background is observed, allowing us to get lower detection limits.

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