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PAPER

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Quantitation of drugs *via* molecularly imprinted polymer solid phase extraction and electrospray ionization mass spectrometry: benzodiazepines in human plasma

Eduardo Costa Figueiredo,[*ab] Regina Sparrapan,[b] Gustavo Braga Sanvido,[b] Mariane Gonçalves Santos,[a] Marco Aurélio Zezzi Arruda[cd] and Marcos Nogueira Eberlin[b]

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The association of solid phase extraction with molecularly imprinted polymers (MIP) and electrospray ionization mass spectrometry (ESI-MS) is applied to the direct extraction and quantitation of benzodiazepines in human plasma. The target analytes are sequestered by MIP and directly analyzed by ESI-MS. Due to the MIP highly selective extraction, ionic suppression during ESI is minimized; hence no separation is necessary prior to ESI-MS, which greatly increases analytical speed. Benzodiazepines (medazepam, nitrazepam, diazepam, chlordiazepoxide, clonazepam and midazolam) in human plasma were chosen as a proof-of-principle case of drug analyses by MIP-ESI-MS in a complex matrix. MIP-ESI-MS displayed good figures of merits for medazepam, nitrazepam, diazepam, chlordiazepoxide and midazolam, with analytical calibration curves ranging from 10 to 250 μg L^{-1} ($r > 0.98$) with limit of quantification <10 μg L^{-1} and acceptable within-day and between-day precision and accuracy.

Introduction

Mass spectrometry (MS), due to its high selectivity, sensitivity and speed, is currently the gold standard technique for multi-target analysis in complex matrices. For precise quantitation, however, a separation step[1,2] is often required prior to MS analysis in order to avoid ion suppression effects during analyte ionization. Ion suppression is a common matrix effect in spray based ionization techniques such as electrospray ionization[3] that causes other components present in the same droplet to hamper or preclude the ionization of the target analyte.[4] Coupling mass spectrometry with a pre-separation technique permits proper separation and individual ionization (no competition) from the ESI droplets and hence more precise quantitation. Selective extraction also minimizes sample complexity, and consequently ion suppression,[5,6] and solid phase extraction (SPE) has been widely used for sample clean up.[7]

[a]*Laboratory of Toxicants and Drugs Analysis, Faculty of Pharmaceutical Sciences, Federal University of Alfenas—Unifal-MG, 700 Gabriel Monteiro da Silva street, 37130-000, Alfenas, MG, Brazil. E-mail: eduardocfig@yahoo.com.br; Fax: +55 19 3299 1063; Tel: +55 35 3299 1342*
[b]*ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas—Unicamp, 13083-970 Campinas, SP, Brazil*
[c]*Group of Spectrometry, Sample Preparation and Mechanization—GEPAM, Institute of Chemistry, University of Campinas—Unicamp, 13083-970 Campinas, SP, Brazil*
[d]*National Institute of Science and Technology for Bioanalytics, Institute of Chemistry, University of Campinas—Unicamp, PO Box 6154, 13083-970 Campinas, SP, Brazil*

Molecularly imprinted polymers (MIP) are becoming increasingly more popular for selective extraction from complex matrices. MIP, first proposed by Wulff and Sarhan,[8] are synthesized for a specific target analyte (template)[9] being therefore capable of selectively binding *via* its molded cavities to the target molecule.[10] MIP have been used as sensors,[11] stationary phases for HPLC[12] and capillary electrochromatography[13] and for SPE.[14,15] Recently, MIP have been coupled to MS such as in liquid chromatography mass spectrometry,[16] ion mobility mass spectrometry[17] and ambient ionization mass spectrometry.[7]

Screening and detection are greatly simplified and/or improved by such approaches but for robust quantitation, pre-separation[16] or post-separation (*via* ion mobility)[17] has been used and seems to be an indispensable step.

MIP extracts may allow, however, direct ESI-MS analyses without pre-separation, since a few molecules of the same class (or eventually a single molecule) should predominate in such extracts. Ion suppression during ESI should therefore be greatly minimized or eliminated. In this study, we tested the application of MIP-ESI-MS to quantitate target drugs in complex mixtures, and used benzodiazepines in human plasma as a proof-of-principle case.

Experimental

Instrumentation, reagents and solutions

MS analysis was performed using a LCMS 2010 monoquadrupole mass spectrometer from Shimadzu® Corporation (Kyoto, Japan) equipped with an ESI source.

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The solutions were prepared with analytical grade chemicals and deionized water (>18.2 MΩ cm) obtained from a Milli-Q water purification system (Millipore, Bedford, USA). For the MIP synthesis, diazepam was used as the template, methacrylic acid as the functional monomer, ethylene glycol dimethacrylate as the crosslinking reagent and 2,2′-azobisisobutyronitrile as the initiator (all from Sigma-Aldrich, Steinheim, Germany). HPLC grade chloroform (Merck, Darmstadt, Germany) was used as solvent.

Stock solutions of medazepam, nitrazepam, diazepam, chlordiazepoxide, clonazepam and midazolam (all from Sigma-Aldrich, Steinheim, Germany) were prepared at 7.0 mg L^{-1} in HPLC grade methanol (J. T. Baker, Phillipsburg, USA). An aqueous solution of HNO_3 (Merck, Darmstadt, Germany) at pH 1.0 was used as the washing solution.

MIP synthesis

The diazepam-imprinted polymer was prepared according to Ariffin *et al.*:[18] 0.8 mmol of diazepam, 4.6 mmol of methacrylic acid, 23 mmol of ethylene glycol dimethacrylate and 0.51 mmol of 2,2′-azobisisobutyronitrile were added to a 30 mL glass flask. The solution was placed in an ice bath and purged with nitrogen for 5 min. The flask was sealed and a water-bath was used to keep the temperature at 60 °C for 24 h. After polymerization, the polymer monolith obtained was mechanically ground and the particle size was selected using a steel sieve (>100 μm). The template was extracted from the MIP using 10 fractions (10 mL each) of methanol : acetic acid solution (9 : 1, v/v). The polymer particles were then dried and maintained at room temperature. Six benzodiazepines (Fig. 1) were selected as a proof-of-principle class of drug analytes due to their wide use in conventional therapy, which routinely requires quantitation in human plasma.

Sample preparation

Human plasma samples were submitted to liquid–liquid extraction (for proteins elimination) using ethyl acetate as solvent: 3 mL of plasma were spiked with 50 μL of benzodiazepine solution (from 0.6 to 40 mg L^{-1}) and 3 mL of 0.1 mol L^{-1} NaOH; 5 mL of ethyl acetate were then added to the test tube which was vortexed for 1 min and centrifuged at 1000*g* (15 min). The organic phase was evaporated under a nitrogen atmosphere. The residue was then dissolved in 0.5 mL of a 0.01 mol L^{-1} phosphate buffer, pH 7.0, and then analyzed by MIP-ESI-MS.

All plasma samples were collected after the approval of the Ethical Committee of the Medical Science Faculty of the University of Campinas (CAAE: 0411.0.146.000-07).

MIP-ESI-MS system

The MIP-ESI-MS system comprises a syringe pump (SP), a MIP column and a single quadrupole mass spectrometer equipped with an ESI source (Fig. 2). Each analytical cycle was executed by pumping through the MIP column: 500 μL of H_2O, 500 μL of sample, 500 μL of washing solution (HNO_3, 0.1 mol L^{-1}) and

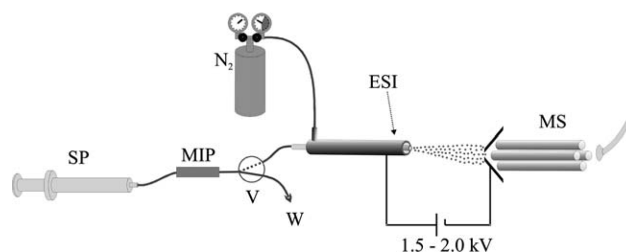

Fig. 2 MIP-ESI-MS system used to sequester and quantitate benzodiazepines directly from plasma samples. SP: syringe pump, V: three-way valve, MS: mass spectrometer, W: waste, ESI: electrospray ionization.

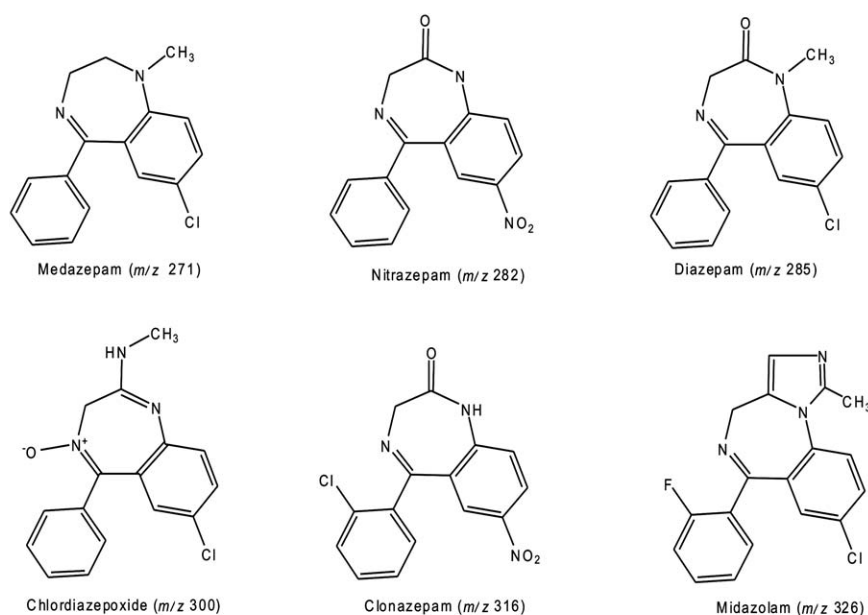

Fig. 1 Chemical structures and *m*/*z* values of the protonated molecules for the six benzodiazepine drugs evaluated in this study.

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then 500 μL of H_2O at 1 mL min^{-1}. The analytes were presumably retained in the MIP and interferences were eluted by the wash solution. During washing, the flow originating from the column was driven to the waste. Soon after, the valve (V) was switched and the column effluent was then directed to the ESI source of the mass spectrometer. To promote analyte elution, 500 μL of HNO_3 : methanol (1 : 99, v/v) was percolated through the MIP column at 100 μL min^{-1}. ESI mass spectra were obtained in the positive ion mode and the drugs were detected as their protonated molecules. System variables were optimized using a mixed standard solution prepared in aqueous phosphate buffer containing 200 μg L^{-1} of each benzodiazepine (medazepam, nitrazepam, diazepam, chlordiazepoxide, clonazepam and midazolam). ESI-mass spectra were summed and averaged for each peak obtained during the elution step.

Results and discussions

MIP-ESI-MS optimization

Fig. 3 shows the total ion current (TIC) plot for duplicate injections of the MIP extract from plasmas spiked with the pool of benzodiazepines at 200 μg L^{-1}. The inset shows the *m/z* region of the ESI mass spectrum in which the six drugs are detected *via* their protonated molecules. For TIC monitoring, the protonated molecules of the six drugs (Fig. 1) were used.

Initially, the amount of MIP used to pack the column was varied to verify the best performance and 20 mg was found to be ideal. Packing the columns with more than 20 mg of MIP increases too much the pressure of the system, hampering sample percolation. Less than 20 mg of MIP decreases sensitivity; for 15, 10 and 5 mg of MIP, TIC intensity decreased by *ca.* 27, 38 and 55%, respectively.

The eluent composition is important to guarantee the efficient elution of the analytes from the MIP column as well as for reconditioning the polymer for the next cycle. To evaluate this effect, 1, 5 and 10% (v/v) HNO_3 in methanol were tested as eluent. The results showed that there were no significant changes in terms of sensitivity (less than 7%) for the three concentrations tested. Therefore, 1% (v/v) HNO_3 in methanol was selected as the eluent solution, preserving the selective sites of the MIP. It is important to point out that the nitric acid was used for six months and no corrosion was observed in the instrument.

Binding between the sorbent and the analyte in SPE is pH dependent, which influences the ionization (which occurs by protonation) of the analyte and protonation of the sorbent binding site.[15] Therefore, a phosphate buffer at 0.01 mol L^{-1} was used, since this buffer has been routinely used for pH stabilization of biological samples.[7,15] The pH of the sample solution was varied from 6.0 to 8.0 and the best result for medazepam, nitrazepam, diazepam, chlordiazepoxide and midazolam, in terms of sensitivity, was obtained at pH 7.0. Clonazepam showed little changes in response as a function of pH. Coincidentally, this molecule is inefficiently retained in the diazepam–MIP. A possible explanation for this low adsorption is the *o*-chloride substitution that may hinder proper fitting in the MIP cavities.

During the extraction step, other molecules than the target analytes can bind to the MIP either in the specific binding site (molecules similar to the template) or binding can also occur with much less selectivity to the polymer surface (dissimilar molecules).[19,20] This problem can be circumvented using acidic or alkaline washing solutions at high enough concentrations to eliminate these concomitants but the washing solution should be also selective and not remove the target analytes. In this work, an aqueous HNO_3 solution was used to wash the MIP and its pH was evaluated for optimal concomitant removal. The pH was varied from 1.0 to 4.0, and the best result was obtained for pH 1.0. For pH < 1.0, a considerable decrease in sensitivity was observed for all analytes.

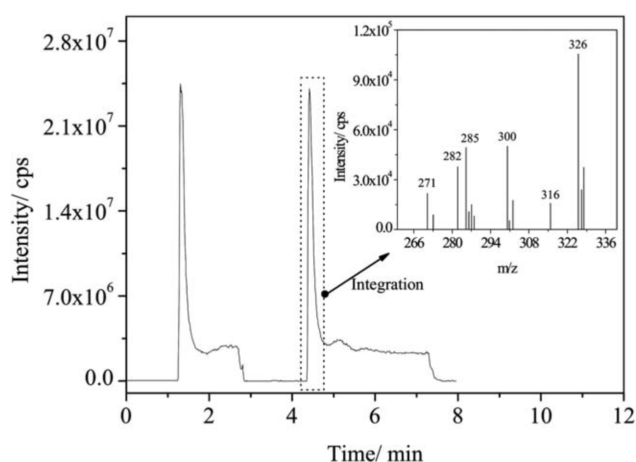

Fig. 3 Analytical signals obtained *via* ESI-MS analysis after sequential injection (in duplicate) of a human plasma sample spiked with 200 μg L^{-1} of each benzodiazepine drug. The inset shows a full ESI mass spectrum in which the six drugs are detected *via* their protonated molecules: medazepam (*m/z* 271), nitrazepam (*m/z* 282), diazepam (*m/z* 285), chlordiazepoxide (*m/z* 300), clonazepam (*m/z* 316) and midazolam (*m/z* 326).

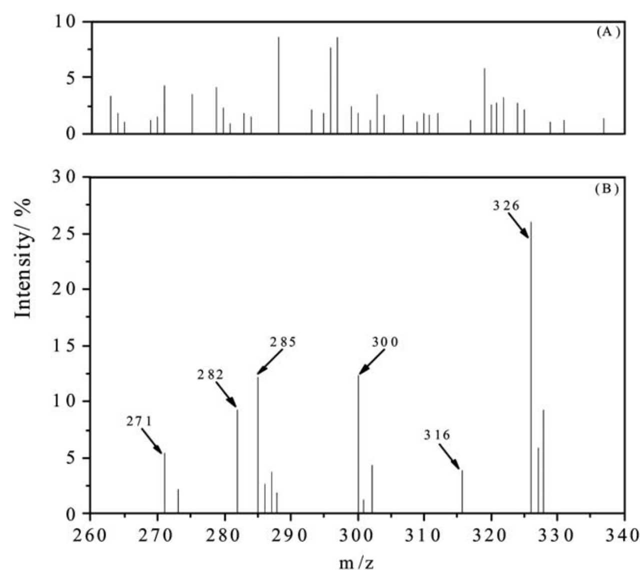

Fig. 4 ESI mass spectra of a human plasma sample spiked with the pool of six benzodiazepines at 100 μg L^{-1} and analyzed either after (A) liquid–liquid extraction or (B) liquid–liquid extraction followed by MIP extraction.

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Table 1 Linear equations, correlation coefficients (r) and ranges ($n = 3$) obtained for the determination of six benzodiazepines in plasma by MIP-ESI-MS

Benzodiazepines	Linear equation	r	Range/μg L^{-1}
Medazepam	$y = 230x + 2927$	0.980	10–250
Nitrazepam	$y = 283x + 7442$	0.986	10–250
Diazepam	$y = 494x + 3914$	0.994	10–250
Chlordiazepoxide	$y = 451x + 3164$	0.984	10–250
Clonazepam	$y = 63x + 11\,390$	0.745	10–250
Midazolam	$y = 1297x - 7602$	0.977	10–250

Selectivity study

MIP selectivity was checked using a human plasma sample fortified with all six benzodiazepines (150 μg L^{-1}). For comparison, ESI-MS analysis was performed after: (a) simple liquid–liquid extraction or (b) liquid–liquid extraction followed by MIP extraction. For liquid–liquid extraction only (Fig. 4A), ions of the target drugs were not observed, perhaps due to great sample complexity and therefore considerable ion suppression during ESI. But for liquid–liquid plus MIP extraction, however, abundant benzodiazepine ions were observed (Fig. 4B) likely due to much reduced ion suppression. This result confirms that selective MIP extraction is essential to eliminate concomitants present in high concentrations in the plasma sample as well as in the liquid–liquid extract, avoiding substantial ion suppression during ESI.

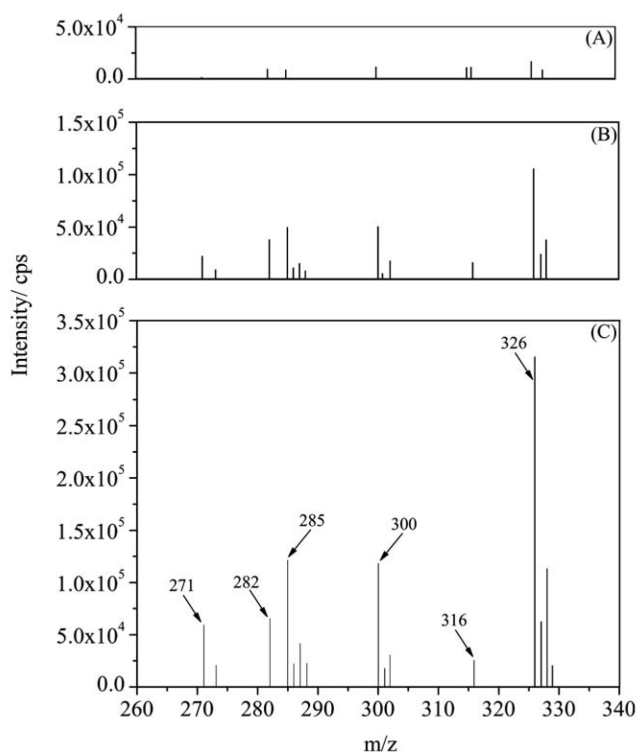

Fig. 5 MIP-ESI mass spectra of a plasma sample spiked with the pool of the six benzodiazepines at (A) 10 μg L^{-1}, (B) 100 μg L^{-1} or (C) 200 μg L^{-1}. Note the detection of all six drugs as their protonated molecules: medazepam (*m/z* 271), nitrazepam (*m/z* 282), diazepam (*m/z* 285), chlordiazepoxide (*m/z* 300), clonazepam (*m/z* 316) and midazolam (*m/z* 326), and the rather clean mass spectra obtained from complex plasma matrices.

Table 2 Speed of analysis and LOQ of different methods applied for diazepam determination

Technique	Sample preparation	Speed of analysis/h^{-1}	LOQ/ μg L^{-1}	Reference
HPLC-MS	SPE[a]	2	6.8	22
CE-UV	LLE[b]	3	20	23
CG-MS	SPME[c]	3.5	10	24
HPLC-UV	SPE	4.8	2.5	25
MIP-ESI-MS	MISPE	12	10	This paper

[a] Solid phase extraction. [b] Liquid–liquid extraction. [c] Solid phase microextraction.

Figures of merit

The analytical curves, which were prepared using a pool of human plasma, presented linear ranges from 10 to 250 μg L^{-1} for medazepam, nitrazepam, diazepam, chlordiazepoxide and midazolam (Table 1). Higher concentrations resulted in linearity deviation, probably due to MIP saturation. Fig. 5 shows ESI-MS for three plasma samples spiked with 10, 100 and 200 μg L^{-1} of the six benzodiazepines. Note the rather clean mass spectra obtained from complex plasma matrices and the proper detection of all six drugs of the pool. Poor linearity was observed for clonazepam, probably due to the low retention capacity of the MIP to sequester this molecule as described in MIP-ESI-MS optimization. The limit of quantitation (LOQ), calculated as ten times the noise level of the blank, was *ca.* 10 μg L^{-1} for medazepam, nitrazepam, diazepam, chlordiazepoxide and midazolam. The speed of analysis was calculated as 12 samples per hour (MIP extraction plus ESI-MS).

Precision and accuracy (intra-day and inter-day) were measured as the relative standard deviation (RSD) and relative error (E), respectively, using a human plasma sample spiked with 150 μg L^{-1} of each benzodiazepine. The RSD and E values (both for $n = 5$) ranged from 9 to 18% and −7 to 18%, respectively, in agreement with FDA (Food and Drug Administration) guidelines.[21] The same MIP column was used during all optimization and validation steps and non-significant differences (<10%) were observed in the analytical signal after *ca.* 70 cycles.

Conclusions

Short filters packed with properly designed MIP can be advantageously used for fast sample clean-up and to sequester specific classes of target molecules from complex biological matrices during drug analysis by direct infusion ESI-MS. Ion suppression is greatly minimized (or nearly eliminated) and quite equal ionization efficiencies are attained due to selective extraction of target molecules of the same class. These features permit proper quantitation in short times (*ca.* 5 min or less by analysis) with no prior separation steps. The MIP-ESI-MS method, as exemplified here for medazepam, nitrazepam, diazepam, chlordiazepoxide and midazolam in human plasma, presents good figures of merits in terms of precision, accuracy, LOQ, and speed of analysis (Table 2). The MIP-ESI-MS coupling seems therefore promising for high throughput analyses of target molecules in complex matrices and applicable in several areas such as clinical and

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toxicological monitoring and diagnoses and pharmaceutical, environmental toxicological, biochemical and food technologies.

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