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Multidimensional on-line sample preparation of verapamil and its metabolites by a molecularly imprinted polymer coupled to liquid chromatography–mass spectrometry

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Abstract

A new molecularly imprinted polymer (MIP) material was synthesized selective for verapamil and utilized for on-line metabolic screening of this common calcium antagonist in biological samples. Since some metabolites of verapamil have also shown pharmacological properties, a selective and sensitive sample preparation approach that provides a metabolic profile in biologically relevant samples is important. The MIP material was coupled on-line to a restricted access material (RAM) precolumn. The multidimensional nature of this set-up removed large matrix interferents such as proteins from the sample, while the selectivity of the MIP enabled further cleanup of the smaller analytes. The selectivity and extraction efficiency of the MIP for verapamil and its metabolites was evaluated in various biological matrices, such as cell cultures and urine. The experimental set-up with the developed method enabled the direct injection of biological samples for the selective isolation, preconcentration, identification and analysis of verapamil and its phase I metabolites by LC–MS^n. This multidimensional approach provided much qualitative information about the metabolic profile of verapamil in various biological matrices. An analytical method was developed for the quantification of verapamil and gallopamil in urine, plasma and cell culture. Acceptable linearity ($R^2 = 0.9996$, 0.9982 and 0.9762) with an average injection repeatability ($n = 3$) of 10, 25 and 15% R.S.D. was determined for urine, plasma and cell culture, respectively. This is the first application of the procedure for the selective metabolic screening of verapamil in biological samples.

Keywords: Verapamil; Molecularly imprinted polymers

1. Introduction

Verapamil hydrochloride is a well-known calcium channel blocker with antianginal, antihypertensive and antiarrythmic properties [1]. The activity of verapamil is complicated since its metabolism leads to pharmacological inactivation, while the *N*-demethylated metabolite (norverapamil) has shown some pharmacologically activity (about 20% of verapamil). As a result, the patient requires frequent dosing of the drug but risks accumulation of the metabolites in the body at concentrations equal or greater than those of the parent drug. Therefore, the development of bioanalytical sample preparation and analytical methods, especially suited to the demands of verapamil pharmacokinetic studies, are important.

Traditional approaches such as liquid–liquid extraction [2], or protein precipitation [3] have been employed but can present automation difficulties. The more common approach of using silica based solid-phase extraction (SPE) [4] materials such as C18 or C8, is also limited by awkward solvent wetting requirements, poor extraction efficiency for polar drugs, undesirable interaction between residual silanols and basic analytes, and time and labour consumption [5]. More recently, a new class of SPE materials, known as restricted access materials (RAM) have been developed for online and direct injection of biological samples [6–11]. They possess a biocompatible surface and a pore size that restricts proteins from entering the interior extraction phase based on size. Simultaneous to this size exclusion process, an extraction phase located on the inner pore surface

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is responsible for isolation of the low molecular weight compounds.

Although, automation with RAM columns is fairly straightforward, the selectivity is still limited. A very pronounced difference in selectivity toward the target analyte can be achieved with a molecularly imprinted polymer (MIP) material. MIPs are extensively cross-linked polymers containing synthetic cavities or recognition sites with a predetermined selectivity [12–14]. As a result of the chemical and physical robustness of the MIP [15], in combination with the polymer's selectivity, this material has proven to be a good adsorbent for molecularly imprinted solid-phase extraction (MISPE) applications [16,17]. Coupling of RAM and MIP precolumns has been accomplished in a six-SPE procedure (size selective sample separation and solvent switch) and has been applied for routine drugs and environmental contaminants [18–20]. The procedure consists of initially fractionating the macromolecular matrix components from the low molecular weight analytes with a RAM precolumn. Back flushing the RAM precolumn with an organic solvent, such as acetonitrile, enables transfer of the RAM extract in a solvent that is suitable for the selective binding of the analytes by the MIP precolumn. The target analytes are then transferred to the analytical column for separation and eventual detection. Preparation of a RAM–MIP material has been accomplished by Haginaka et al. [21,22]. Although this single precolumn configuration is simpler, the loading solvent is restricted to aqueous buffers to prevent the precipitation of proteins and this aqueous environment can minimize the MIP's selectivity.

The objective of this work was to prepare a novel MIP material to improve the extraction selectivity available for verapamil metabolism studies and to further evaluate the application of the six-SPE. Coupling of this sample preparation method to LC–MS^n, enabled the selective isolation of verapamil and structurally similar metabolites from complex biological samples providing metabolic pathway elucidation.

2. Experimental

2.1. Chemicals

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA) and 2,2′-azobis (2-isobutyronitrile) (AIBN) were purchased from Merck (Darmstadt, Germany). The MAA was purified by vacuum distillation prior to use [23]. All solvents were HPLC grade from Caledon (Georgetown, Ont., Canada). The structures of verapamil and various metabolites are shown in Fig. 1. The standard

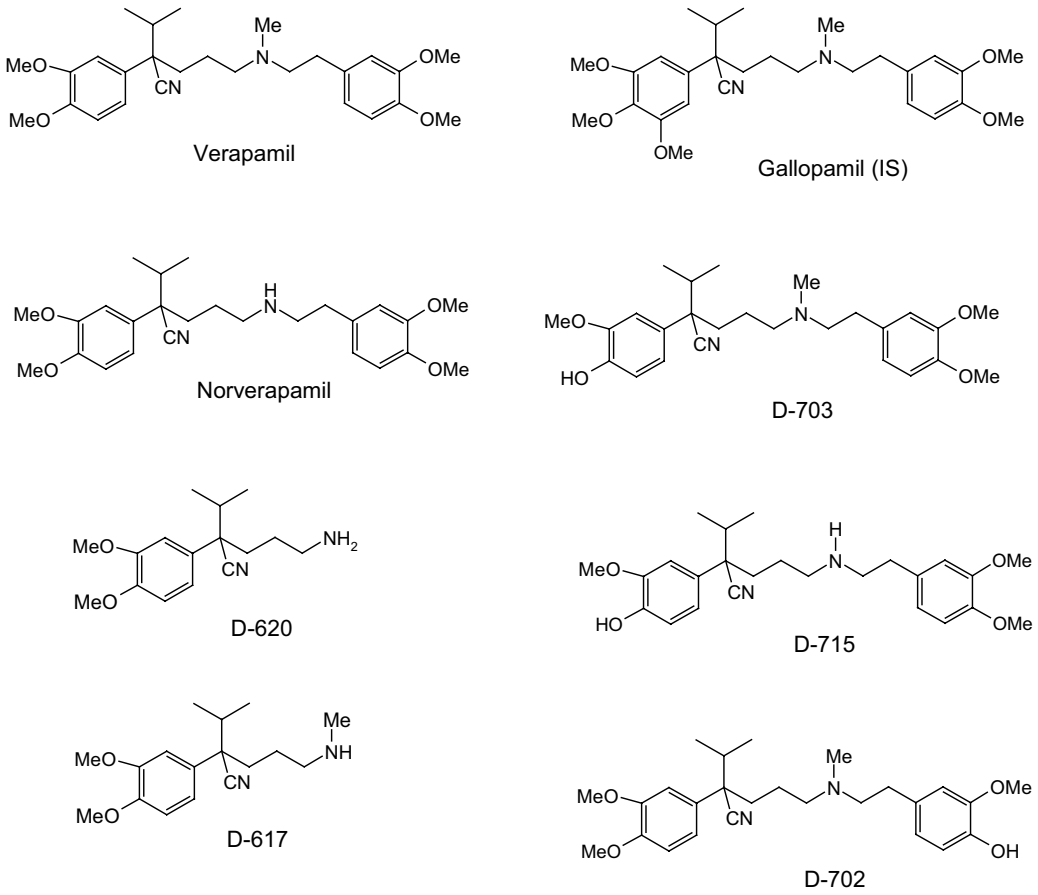


Fig. 1. Structure of verapamil and various analytes.

norverapamil (5-*N*(3,4-dimethoxyphenethyl)amino-2-(3′,4′-dimethoxyphenyl)-2-isopropyl-valeronitrile) was purchased from Research Biochemicals International (Natick, MA, USA) and verapamil (5-*N*(3,4-dimethoxyphenethyl)methyl-amino-2-(3′,4′-dimethoxyphenyl)-2-isopropyl-valeronitrile) and gallopamil (5-*N*(3,4,5-dimethoxyphenethyl) methyl-amino-2-(3′,4′-dimethoxyphenyl)-2-isopropyl-valeronitrile) were from Sigma (Lot56H0925) (Steinheim, Germany) (purity unknown). Deionized water, from a Millipore Milli-Q water system (Eschborn, Germany), was used for all experiments.

2.2. Molecularly imprinted polymer

The monomer MAA (242 mg), verapamil print molecule (107 mg) and 25 ml of chloroform were placed in a 25 ml glass sample vial. The cross-linker EDMA (2.59 g) and the reaction initiator AIBN (57 mg) were then added. The mixture was degassed under vacuum in a sonicating water bath while being purged with nitrogen for a period of 5 min. While maintaining a flow of nitrogen, the reaction flask was removed from the sonicating bath, sealed and placed inside a 60 °C water bath to begin the reaction. Under these conditions, the reaction was continued for 24 h. The product polymer, after drying in air overnight, was white and possessed a rigid structure. It was ground into fine particles using a mortar and pestle. A control polymer was also prepared when polymerization was carried out in the absence of verapamil.

2.3. Extraction of verapamil from the MIP

Removal of the verapamil print molecule from the MIP particles, yielding a blank polymer, was accomplished through a Soxhlet extraction. A sample of the verapamil-imprinted polymer was placed inside the cellulose extraction thimble. The extraction solvent (150 ml) was a mixture of methanol and acetic acid (9:1). Heat was applied to the flask containing the solvent, at a rate that caused a filling and eventual emptying of the extraction chamber every 45 min. The extraction was continued for 24 h.

2.4. Batch binding assay

One hundred milligrams each of the verapamil and blank polymer particles were placed in separate glass vials containing 2 ml of a 10 μg/ml verapamil standard solution prepared in acetonitrile. Each suspension was magnetically stirred for 24 h and then passed through a 0.45 μm filter. The concentration of verapamil in the filtrate was analyzed by HPLC and compared to the original standard concentration.

2.5. Preparation of columns

The verapamil polymer particles were sieved and the >50 μm size fraction was used. A small volume of methanol was added to the polymer particles to produce a slurry and the finest particles were removed by sedimentation of the slurry several times. This was dispensed into a stainless steel column measuring 40 mm in length and 4 mm i.d. until the column bed was fully packed. A 2 μm frit was used in the end column fitting to ensure no loss of the polymer particles. Ten milliliters of methanol were run through the column to ensure uniform particle packing. The column was capped and set up in a HPLC system. The preparation of a blank polymer column was achieved in a similar manner.

2.6. Instrument and analytical conditions

The experimental set-up is shown in Fig. 2. Pump system 1 consisted of two Waters 490 pumps (Milford, MA, US), pump 2 was Shimadzu 9A (Columbia, MD, US) and pump 3 was integrated in a Merck-Hitachi OSP2 unit (Darmstadt, Germany). The restricted access precolumn (RAM) was a LiChroCART® 25-4mm filled with LiChrospher® RP-8 ADS (25 μm) and purchased from Merck KGaA (Darmstadt, Germany). The Lab Pro switching valve was received from Rheodyne (Cotati, US). The HPLC mobile phase (flow 0.6 ml/min) was split at a ratio of 1:4 via an Accurate-Splitter (LC Packings, San Francisco) before entering the Esquire ion trap mass spectrometer from Bruker-Daltonik (Bremen, Germany). HPLC–MS analyses were done on the same HPLC coupled to the atmospheric pressure (AP) ion source of the MS operated under positive ion electrospray (ESI) conditions in the full scan, MS^2 and in some instances in the MS^3 mode. The nebulizer pressure was set to 40 psi and the dry gas temperature to 350 °C, while +3 kV were applied to the nebulizing capillary. Full mass spectra were acquired by scanning the mass range of *m*/*z* 100–500. CID spectra were obtained from the protonated molecules $[M + H]^+$. HPLC analysis was carried out with an isocratic elution of 50:50 (v/v) ammonium acetate buffer (0.01 M, pH 3.0):acetonitrile. The chromatographic column was a LiChrospher® RP-SelectB (12.5 cm × 4.0 mm i.d.; 5.0 μm particle size) from Merck KGaA (Darmstadt, Germany). A LiChrospher® RPSelectB (1.0 cm × 4.0 mm i.d.; 5.0 μm particle size) guard column from Merck KGaA (Darmstadt, Germany) was installed at the inlet of the chromatographic column.

The samples were loaded onto the RAM precolumn using a mobile phase composition of 95:5 (v/v) ammonium acetate solution (0.01 M, pH = 6):acetonitrile. After 10 min, valve 1 (V1) was switched and the previously extracted analytes were desorbed from the RAM precolumn using a mobile phase of 100% acetonitrile (pump 2, flow rate = 0.6 ml/min). The transfer time of the analytes to the MIP precolumn was 5 min. The analytes were desorbed and transferred to the analytical column for chromatographic separation by switching of valve 2 (V2) and using a mobile phase of 50:50 (v/v) acetonitrile/ammonium acetate (0.01 M, pH = 3) at a flow rate of 0.6 ml/min.

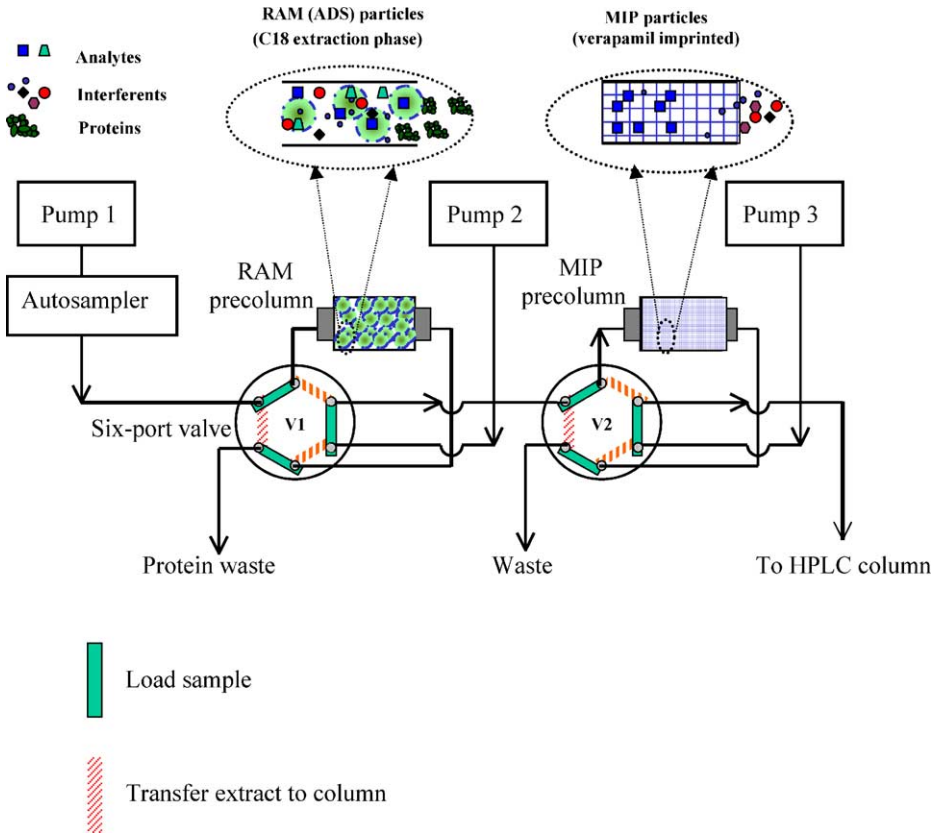


Fig. 2. Experimental configuration of six-SPE.

2.7. Sample preparation

2.7.1. Cell cultures

All animal procedures described in this report were approved by the local authorities. The hepatocytes were isolated from a male "Sprague–Dawley" rat as described in Hansen et al. [24]. A 30 ml pool was generated from the cell culture media and 800 μl were used for this study. Incubations were performed with verapamil and D_6-verapamil in a 5 μM concentration.

2.7.2. Urine

For estimating the linearity and reproducibility, the matrices were spiked with five different amounts of verapamil and gallopamil to reach a final concentration of 25, 50, 100, 250, and 500 ng/ml. A 50 μl aliquot was injected into the LC–MS^n system. For sample analysis, urine from a healthy volunteer was used. A total of 80 mg verapamil hydrochloride was given as a single dose to a healthy male volunteer and urine was collected for 10 h.

2.7.3. Plasma

Verapamil was injected into three male Sprague–Dawley rats (250–300 g bodyweight) via the tail vein (dosage: 0.5 mg/kg bodyweight). The animals were anesthesized after 120 min and then 50 international units of Heparin were given i.p. Animals were humanely killed. The blood was collected and centrifuged (4000 rpm) for 15 min at 4 °C and the resulting plasma supernatant was stored at −20 °C.

2.7.4. Recovery

The recovery of the spiked human urine, plasma, and cell culture media was investigated with standard concentrations of 50, 100, 200 and 500 ng/ml. The recovery was calculated by comparing the MS-peak area of the spiked sample to a direct injection of a 100 ng/ml standard solution.

3. Results and discussions

3.1. Characterization of pre- and post-polymerization imprinting system

A critical step in the synthesis of a molecularly imprinted polymer material is the formation of a strong complex between the print molecule and the chosen monomer in the pre-polymerization mixture. Several spectroscopic techniques, such as NMR [25,26], IR [27] or UV [28,29] have been developed to quantify this interaction. The interaction between verapamil and methacrylic acid was evaluated by a UV spectroscopic approach, as previously outlined by Mullett et al. [28]. In summary, a 0.1 mM standard solution of verapamil (prepared in chloroform) was titrated with consecutive 1 μl injections of MAA (1.25 M), yielding a fi-

nal MAA concentration of 0.1, 0.5, 0.5 and 10 mM, prior to recording the UV spectrum. An increase in the absorbance at $\lambda = 290$ nm was observed with the increased addition of MAA, indicating the formation of a complex between verapamil and MAA. Unfortunately, the background absorbance spectrum of MAA slightly overlapped at this wavelength making quantification of this monomer-template interaction difficult. Regardless, the pre-arrangement or interaction of verapamil and MAA in the pre-polymerization mixture was confirmed.

After synthesis of the polymer, a batch-binding assay was performed to determine the binding efficiency and functionality of the MIP particles. The MIP and non-imprinted (blank) polymers (control MIP) were evaluated in a verapamil standard solution prepared in acetonitrile. This solvent was chosen since it will be used as the transfer solvent for the analytes from the RAM precolumn to the MIP precolumn. As shown in Table 1, a large decrease in the amount of verapamil detected (as indicated by the HPLC peak area), relative to the blank polymer, was observed with the imprinted particles. The MIP material was successful at binding of verapamil with a greater efficiency than the blank material, indicating the success of the imprinting procedure. In order to calculate the magnitude of the imprinting effect, a control experiment, consisting of stirring the verapamil MIP particles in a solution of pure acetonitrile, was performed to determine the presence of any template bleed. The possibility of the initial print molecule bleeding from the polymer has become widely recognized [30] and will negatively impact the calculation of the imprinting effect. As summarized in Table 1, verapamil was detected in the acetonitrile after stirring with the MIP particles and confirmed the presence of template bleed. This value was used to adjust the binding efficiency of the imprinted material and a binding enhancement of 50% was calculated for the newly prepared verapamil MIP. The MIP particles were then packed into a column for validation as a selective on-line adsorbent.

3.2. Working principle of coupled columns in six-SPE

The working principle of the six-SPE procedure has been previously described [19]. The first step was the direct sample injection onto the RAM precolumn where the alkyl-diol-silica (ADS) packing material possessed two different chemical surfaces. Hydrophilic electroneutral diol groups were bound to the external surface of the spherical particles, protecting the sorbent from contamination by proteins, while the inner surface of the porous particles contained a C8 alkyl hydrophobic bonded phase that was responsible for simultaneous extraction of the target compounds. Only smaller molecules (<15,000 g/mol) can penetrate the pores for absorption, while larger molecules such as proteins, eluate out the end of the column to waste. It is important to ensure the sample loading mobile phase was limited to <15% of organic phase to prevent sample proteins from precipitating and to ensure a high recovery of the analytes on the hydrophobic extraction phase [31].

Transfer of the extracted analytes from the RAM to the MIP precolumn, for a second dimension of cleanup, was possible by switching valve (V1) at the appropriate time. The transfer time was determined by a return of the signal to a baseline value after the direct injection of the biological sample or transfer of the extracted analytes.

Back flushing the RAM precolumn with 100% acetonitrile resulted in the quantitative elution of the analytes and allowed their transfer onto the MIP precolumn. The presence of an organic solvent was important to ensure complete elution of the analytes from the hydrophobic RAM column, but more importantly, also provided a solvent environment for enhancing the recognition of target analytes on the MIP material. As shown by the batch binding results above and previous MIP studies [32], the presence of aprotic solvents, such as acetonitrile, are important for recognition of the target analytes. The successful extraction of the target analytes by the MIP precolumn under the switched solvent conditions was confirmed by the absence of any significant analyte breakthrough.

Once the transfer of the sample extract to the MIP precolumn was complete, the second valve (V2) was switched and a higher polarity and protic mobile phase was back flushed though the MIP precolumn for elution and transfer of the analytes to the analytical column. A low pH (pH = 3) was used for the mobile phase to ensure quantitative removal of the target compounds from the MIP. Since the pK_a-values of the analytes are between 9 and 10 [33], the positive charge on each compound will result in faster elution from the neutral charge of the polymer coating.

Table 1
Summary of verapamil batch binding results

Polymer	HPLC peak area ($\times 10^8$)	R.S.D. (%) ($N = 3$)	Imprinting effect[a] (%)
MIP	2.98	2	50
Control MIP	0.85	3	
Blank polymer	4.31	1	

[a] Imprinting effect calculated as: [(control HPLC peak area (blank polymer) − adjusted MIP HPLC peak area)/control HPLC peak area (blank polymer)] × 100%; where adjusted MIP = MIP − control MIP.

3.3. Biological sample analysis

3.3.1. Qualitative analysis

3.3.1.1. Investigation of selectivity. The coupling of the two sample preparation precolumns with an LC–MS^n system allowed the rapid and selective isolation, preconcentration, separation and identification of drug metabolites. The commercially available standards, verapamil and gallopamil, were selected to initially evaluate the selectivity of the MIP precolumn, relative to a blank MIP material. A % imprint factor was calculated for both compounds in the various biological matrices. As summarized in Table 2, the imprinted material was able to extract more of the target compounds

Table 2
Matrix dependence on imprinting factor

Matrix	Imprinting factor[a] (%)	
	Verapamil (absolute recovery)	Gallopamil
Urine	50 (80)	80
Plasma	20 (46)	10
Cell culture	10 (31)	10

[a] Imprinting factor calculated as: [(mass extracted by MIP − mass extracted by blank polymer)/mass extracted by blank polymer] × 100%.

in all matrices. However, the effect of imprinting was most predominant in the urine matrix. The higher protein concentration of the plasma or cell culture matrix likely contributed to this reduced recovery as the analyte may complex with these interferents. In addition, the presence of other matrix interferences, such as small hydrophobic molecules in the plasma or cell culture media, could have reduced the binding interaction between MIP and analyte.

In addition to verapamil and gallopamil, five metabolites could be isolated from urine, plasma and cell culture samples with the MIP precolumn, as illustrated by the low breakthrough values listed in Table 3. Most interestingly, the highest amount of breakthrough was recorded for the verapamil print molecule. However, this value may have been influenced by the presence of template bleed, resulting in a higher than expected amount of analyte breaking and washing through the column. Alternatively, a structural analogue such as gallopamil could have been used to imprint the polymer. However, the polymer was imprinted with verapamil since the main aspect of this work was the successful isolation of drug metabolites by the MIP and previous studies have used gallopamil for quantification of verapamil and metabolites in various matrices, allowing better comparison with previous results. The evaluated metabolites were structurally close to the initial verapamil print molecule and could be extracted by the MIP precolumn, further indicating very high extraction selectivity for this class of compounds.

All the extracted compounds were phase I metabolites. Normally in urine, most of the metabolites are excreted as glucuronide conjugates (phase II metabolites), with the D617 compound appearing as the phase I as the major metabolite. As these conjugates are known to be cleaved under the acidic conditions of the mobile phase (pH = 6), only phase I metabolites could be trapped on the MIP material. However, experiments under neutral conditions have shown, that the glucuronide conjugates were not trapped on the MIP material but were detected in the breakthrough. Therefore, modification of the metabolite with a glucuronic acid's moiety produced a significant change in the size and shape of the compound to prevent its recognition by the MIP material.

Table 3
Percentage recovery of verapamil and metabolites from urine samples using MIP precolumn

Compound	Breakthrough	Wash	Eluate
D620	0	0	100
D617	0	13	87
D703	2	1	97
D702	2	1	97
Norverapamil	3	16	81
Verapamil	9	25	65

See Fig. 1, for structures of compounds; 20 μl of urine sample injected. Wash solvent = 100% acetonitrile; elution solvent = 95:5 ammonia acetate (0.01 M, pH = 6):acetonitrile (v/v); verapamil and gallopamil concentration ~200 ng/ml; content of breakthrough, wash and elute for norverapamil and D620 (only available standard metabolites) was set as 100% to estimate the recovery for the other metabolites.

3.3.2. Structure elucidation of verapamil metabolites by HPLC–MS^n

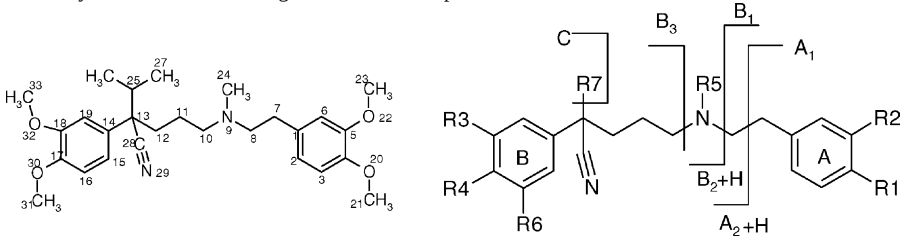
The coupling of these two sample preparation methods with an HPLC–MS^n system allowed the rapid identification of drug metabolites. Six phase I metabolites (glucuronides) were observed in the matrices, i.e. rat hepatocytes, plasma and human urine, using the above described two experimental approaches.

Comparing the metabolite's MS fragmentation pattern with that of verapamil, the structures were elucidated. The MS fragmentations of gallopamil, verapamil and its metabolites are summarized in Table 4. Only significant ions (relative intensity: >5%) are discussed. The collision-induced dissociation (CID) of the protonated molecules of all metabolites can be described by the general fragmentation scheme shown in Table 4. The fragmentation leads to structure-specific ions formed by bond cleavages in the aliphatic moiety of the molecule (with and without hydrogen transfer). This fragmentation predominantly leads to thermochemically stable products, i.e. even-electron ions and neutral molecules (see also Table 4) with the exception of the loss of the isopropyl group observed with several metabolites, which leads to an odd electron fragment ion and a neutral radical. The fragments in general allow an unambiguous identification of the metabolites, except for the metabolites formed by *O*-demethylation (D-715, D-703 and D 702) where the exact site of the demethylation remains uncertain and can only be elucidated by additional NMR experiments, reported elsewhere [39]. The MS spectra summarized in Table 4 have been previously discussed in detail [39,40] and will not be repeated here. The confirmed structures of all metabolites are shown in Fig. 1.

3.3.3. Quantitative analysis

The developed six-SPE LC–MS method for verapamil and several metabolites was validated using plasma, urine and cell culture medium. The additional effect of the MIP precolumn on the elimination of interference compounds is shown Fig. 3. Sample chromatograms of human urine were recorded using six-SPE and conventional RAM sample preparation procedures. Comparison of these two methods, as illustrated by the total ion chromatograms in Fig. 3A and B, show a significant reduction in the amount of matrix with the six-SPE method. The elimination of these

Table 4
Summary of LC–MS^n data-fragmentation of verapamil and metabolites



Compound	Retention time (min)	Functional group						Phase I	α-Cleavage		N–C cleavage				Relative abundance		
		R1	R2	R3	R4	R5	R6	$[M+H]^+$	A_1	A_2+H	B_1	B_2+H	B_3	C	Hepato-cytes[a,b]	Plasma[a,b]	Urine[a,b]
D-620	7.6	–	–	OCH_3	OCH_3	H	–	277	–	–	–	–	260	234	–	2	10
D-617	8.5	–	–	OCH_3	OCH_3	CH_3	–	291	–	–	–	–	260	248	20	20	85
D-715	12.4	OCH_3	OCH_3	OCH_3	OH	H	–	427	151	275	165	–	–	384	–	–	2
D-703	13.1	OCH_3	OCH_3	OCH_3	OH	CH_3	–	441	–	289	165	–	–	246	10[c]	28[c]	35[c]
D-702	13.1	OCH_3	OH	OCH_3	OCH_3	CH_3	–	441	–	303	151	291	260	248	10[c]	28[c]	35[c]
Norverapamil[d]	16.5	OCH_3	OCH_3	OCH_3	OCH_3	H	–	441	151	289	165	–	–	398	30	40	50
Verapamil	17.4	OCH_3	OCH_3	OCH_3	OCH_3	CH_3	–	455	–	303	165	–	–	–	100	100	100
Gallopamil (IS)	19.8	OCH_3	OCH_3	OCH_3	OCH_3	CH_3	OCH_3	485	–	333	165	–	–	–	–	10	10

[a] Metabolites were semiquantified comparing their peak ratio in the MS-mode to verapamil, data are shown in %.
[b] Peak ratios after 2 h exposure.
[c] Sum of D703 and D702 is shown.
[d] A further fragment is observed at *m/z* 246 (see text).

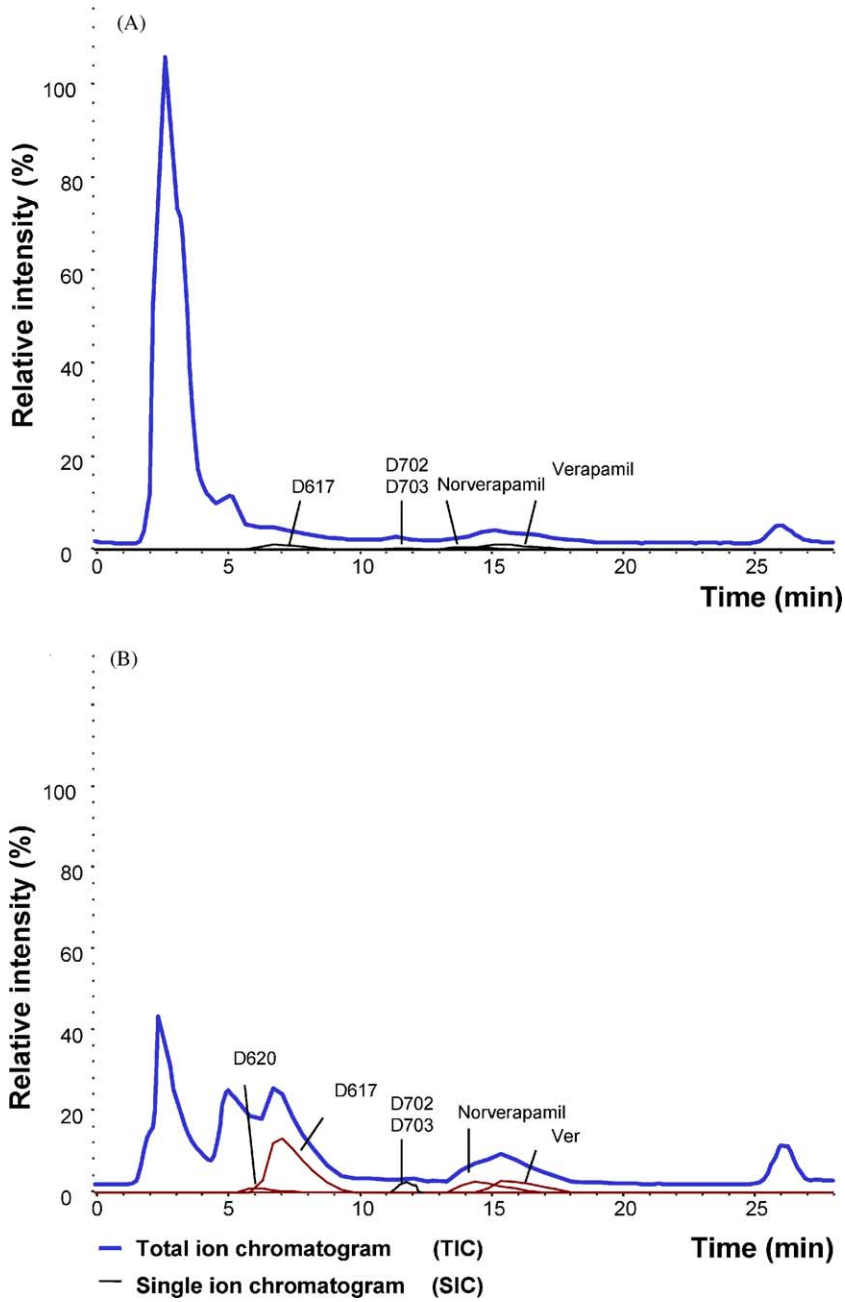


Fig. 3. Comparison of urine chromatograms for SPE (A) and six-SPE approach (B). SPE configuration utilized same configuration as Fig. 2, but without MIP column.

interferents is important to minimize matrix effects, since an analyte's response by LC–MS can be negatively affected by matrix components in biological samples [34]. The ability to provide cleaner sample extracts is important to enhance the overall performance of any developed method. Shorter chromatographic runs times, improved column lifetimes and less fouling of the mass spectrometer, with better signal to noise ratios, are possible with cleaner extracts [34].

Various analytical figures of merits were evaluated for the developed method in all matrices. The linearity of the standard calibration curve for verapamil was evaluated over the range of 25–500 ng/ml in urine, plasma and cell culture and produced average linear regression coefficients (R^2-value) of 0.9996, 0.9982 and 0.9762, respectively. However, only the precision of verapamil in human urine was sufficient enough for quantitation. For example, the average precision for verapamil and gallopamil was calculated with five repeated injections of spiked urine, plasma and cell culture media and was determined to be 10, 25 and 15% R.S.D. The intra assay precision were also evaluated for injections of urine, plasma and cell culture media spiked with 50 ng/ml verapamil and gallopamil and calculated to be 12, 25 and 16% R.S.D. Sim-

ilar results of 12, 26 and 16% R.S.D. were achieved for the day-to-day reproducibility of these samples.

As previously mentioned, one disadvantage of MIP materials is the appearance of template bleed. This is especially critical when determining trace amounts of the analyte. Often, this drawback can be avoided by imprinting with a structural analogue of the analyte, which is chromatographically separated from the analyte prior to quantification [35]. Unfortunately, a standard compound with a structure similar to verapamil was unavailable, with the exception of gallopamil, which was used as an internal standard. To overcome this difficulty, cell culture incubations were performed with D_6-labeled verapamil. When the sample extracts were quantified by mass spectrometry, the presence of verapamil from template bleed could be distinguished from the extracted verapamil in the cell culture, based on a mass difference in the mass spectrum. This approach was used to determine the amount of template bleed and method's limit of detection. The results show that template bleed were not very high, consistently less than 10% of the initial template remaining in the polymer. The limit of detection (LOD) for D_6-verapamil in cell culture media at a concentration, where the signal/noise ratio was equal to 3, was calculated to be 5 ng/ml.

The multidimensional nature of this approach may appear slightly complicated, however, the elimination of matrix components are crucial especial for MS detection, as the lack of selectivity in sample preparation can result in ion suppression caused by excess sample matrix components [36]. In addition, the online and direct nature of the approach minimized the amount of sample manipulation, which can also help eliminate interference and matrix effects [37]. The whole system can be fully automated with a total analysis time of 35 min including sample preparation, selective fractionation, chromatographic separation and detection. A single ADS and MIP precolumn were utilized for all the experiments, with more than 300 direct injections of biological samples, with no significant changes in the extraction performance, confirming the robustness of the system.

4. Conclusions

The successful coupling of a restricted access material, to a verapamil molecularly imprinted polymer precolumn allowed the direct injection of various biological fluids and was applied to verapamil drug metabolism studies. The multifunctional nature of this Six SPE setup provided an on-line and automated method for the selective extraction and detection of verapamil and several metabolites. The complete elimination of matrix components and some sample interferents from the target compounds was accomplished, providing improved isolation and mass spectrometric identification of metabolites in biological fluids. In comparison to conventional SPE or RAM column switching approaches, an improvement in the extraction selectivity of verapamil was achieved. Sample preparation was minimal with a total analysis time of 35 min, which is an important consideration to achieve higher sample throughput.

The straightforward coupling of the precolumns to LC–MS^n, provided much qualitative and quantitative information about the metabolic profile of verapamil in various biological matrices. The MIP material showed high extraction selectivity for this class of compounds and was suitable for drug metabolism studies with phase I metabolites, where trace metabolites could be isolated. Difficulties with quantification resulting from template bleed were overcome with the use of a verapamil isotope. Lastly, the unique properties of the MIP material may also extend the application of this approach to the isolation and investigation of enantiomers in biological samples, which plays an important role in the pharmacological effect of verapamil [38].

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