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Verapamil drug metabolism studies by automated in-tube solid phase microextraction

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Abstract

Verapamil is a common calcium antagonist described with antianginal, antihypertensive and antiarrythmic properties. The metabolites of verapamil have also shown pharmacological properties and therefore sample preparation and analysis techniques capable of metabolic screening for verapamil are important. In-tube SPME is a relatively new method integrating sample extraction, concentration and introduction into one single step without the use of organic solvents. The capability of in-tube SPME in bioanalysis has been reviewed but there has been no application described in the field of drug metabolism. Since automation and interfacing of in-tube SPME coupled to liquid chromatography-mass spectrometry (LC-MS) is possible, we confirm in this study that it is a powerful method to monitor the main metabolites of verapamil in various biological matrices like plasma, urine and cell culture media. Further, we show that it could also be used in routine pharmacokinetics measurements. An in-tube SPME LC-MS method was developed to extract and analyze the metabolic profile of verapamil from biological matrices. The detection limit for verapamil, gallopamil, norverapamil and PR22 were 52, 53, 65 and 83 ng/ml (UV detection) and 5, 6, 6 and 8 ng/ml (MS detection), respectively. The precision of the method was calculated in various biological matrices and the average % R.S.D. ($N = 5$) for verapamil, gallopamil, norverapamil and PR22 was 3.9, 3.7, 3.8 and 4.3% (MS detection), respectively. The linear dynamic range was determined to be 100–800 ng/ml (UV detection) with a total sample preparation and analysis time of 34 min.

Keywords: In-tube SPME; Verapamil; Drug metabolism; Pharmacokinetics; Analysis

1. Introduction

Verapamil hydrochloride [DL-2-(3,4-dimethoxyphenyl)-2-isopropyl-5-(*N*-methyl-*N*-β-(3,4-dimethoxyphenyl-9-ethylamino)valeronitril, a synthetic papaverin derivate, is a well known calcium

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channel blocker with antianginal, antihypertensive and antiarrythmic properties [1]. Verapamil is commercially used as a racemic mixture of equals amounts of two optical isomers, (+)-(R)-verapamil and (−)-(S)-verapamil. The optical isomers differ considerably in their pharmacological potency with (−)-(S)-verapamil being 20 times more potent than the (+)-(R)-verapamil concerning negative dromotropic effects on atrioventricular (AV) conduction. Moreover, the metabolism and plasma protein binding of the S enantiomer is 6 times as high as from the R-enantiomer. Verapamil suffers from extensively first pass metabolism [2]. This results in low drug bioavailability and considerable variability of therapeutic plasma levels. Metabolism of verapamil in general leads to pharmacological inactivation and thus patients require frequent dosage of this particular drug. This compound is extensively metabolized by *N*-demethylation, *O*-demethylation and *N*-dealkylation. The *N*-demethylated metabolite (norverapamil) is also pharmacologically active (about 20% of verapamil). Moreover, it can accumulate in the body at concentrations equal or greater than those of the parent drug. Because of the different pharmacological properties of this important compound, an effective sample preparation and sensitive analytical method is required to monitor verapamil and its metabolites in different biological matrices like plasma, urine and cell culture media.

A majority of methods for the determination of verapamil and its main metabolites in biological samples has been reported using high performance liquid chromatography [3–5] and HPLC-MS [6,7]. For the analysis of low levels of metabolites and the parent compound in biological samples, sample treatments such as extraction, preconcentration and clean-up steps are often required to improve the sensitivity and selectivity. Traditional procedures such as solvent extraction [8], solid phase extraction [9] and other techniques are time consuming and labor intensive, require large volumes of sample and solvent and need additional instrumentation to automate. Solid phase microextraction (SPME), which has obtained widespread acceptance in many areas, can overcome these problems by integrating sample extraction, concentration and introduction into one single step without the use of organic solvents [10]. This method uses a small amount of extraction phase coated on a convenient silica fiber and is more rapid and less expensive than the traditional methods. However, it is often restricted to headspace extractions in biological samples to prevent fouling of the fiber from protein adsorption, limiting the analysis to small volatile drugs [11]. In addition, SPME fiber automation with HPLC is presently difficult.

In tube solid phase microextraction (in tube SPME) is a relatively new microextraction and preconcentration technique, which can easily be coupled online to HPLC for automated analysis of less volatile and polar compounds like drug metabolites [12,13]. In-tube SPME utilizes an open tubular capillary (with an extraction coating on its inner surface) as an SPME device enabling simple automation of the extraction process. In this article we present an in-tube SPME method coupled to an LC-ESI-MS system for the determination of verapamil metabolites in different biological matrices like urine, plasma and cell culture media from in vitro assays. To the author's best knowledge, this is the first application of in-tube SPME in drug metabolism studies.

2. Experimental

2.1. Chemicals and reagents

Pyrrole (98%) (Aldrich, ON, Canada) was distilled prior to use. Ferric perchlorate ($Fe(ClO_4)_3 \cdot 6H_2O$) and perchloric acid (70%) were used as received (BDH, Toronto, ON, Canada). Ammonia solution (25%) was purchased from Sigma (Steinheim, Germany), acetonitrile and methanol from Malinckrodt-Baker (Deventer, Holland), ammonium acetate from Merck (Darmstadt, Germany) and acetic acid from Fluka (Buchs, Switzerland). The standard norverapamil (5-*N* (3, 4-dimethoxyphenethyl) amino-2-(3′,4′-dimethoxyphenyl)-2-isopropyl-valeronitrile) was purchased from Research Biochemicals (Natick, MA) and verapamil (5-*N* (3,4-dimethoxyphenethyl) methylamino-2-(3′,4′-

dimethoxyphenyl)-2-isopropyl-valeronitrile) and gallopamil (5-*N* (3,4,5-dimethoxyphenethyl) methylamino-2-(3′,4′-dimethoxyphenyl)-2-isopropyl-valeronitrile) was from Sigma (Lot56H0925) (Steinheim, Germany). D-715 was a kindly gift of Professor Dr W.L. Nelson (University of Washington, Seattle, US). Deionized water, from a Millipore Milli-Q water system (Eschborn, Germany) was used for all experiments.

2.2. Instrument and analytical conditions

All experiments were carried out on an Agilent Technologies (Waldbronn, Germany) 1100 series HPLC coupled with an autosampler and variable wavelength detector. The HPLC-MS analyses were done on the same HPLC coupled to an Esquire ion trap mass spectrometer from Bruker–Daltonik (Bremen, Germany) 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 300 °C, while +3 kV were applied to the nebulizing capillary. Full mass spectra were acquired by scanning the mass range of m/z 100–700. CID spectra were obtained from the protonated molecules $[M+H]^+$. HPLC analysis was carried out with an isocratic elution of 1:1 ammonium acetate buffer (0.01 M, pH 6.0):acetonitrile. The chromatographic column was a LiChrospher® RP-Select B (15.0 cm × 4.0 mm i.d.; 5.0 μm particle size) from Merck KGaA (Darmstadt, Germany). A LiChrospher® RPSelectB (4.0 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.

For all analyses (plasma, urine, and cell culture media) 800 μl of the sample matrix were filled into one 2-ml HPLC vial. A total of 150 μl of a 1% ammonia solution and 50 μl of the internal standard (gallopamil) were also added. Extraction was done with a 3 times coated capillary with 40 draw/eject cycles at an extraction speed of 100 μl/min.

2.3. Sample preparation

2.3.1. 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. [14]. A 30-ml pool was generated from the cell culture media and 800 μl were used for this study.

2.3.2. Urine

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. A total of 1 ml of this urine was diluted 1:10 with water.

2.3.3. Plasma for in-tube and pharmacokinetic studies

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 frozen at −20 °C.

For pharmacokinetic studies at specified time periods of 10, 20, 40, 60, 120, 240 min after the injection, blood was collected from the animal, heparinized and centrifuged [15].

The proteins were removed from the plasma samples as follows: 6 ml of methanol was added to 1 ml plasma and mixed by vortex. The sample was then centrifuged for 10 min at 3000 RPM. The supernatant was evaporated until all MeOH was removed. A total of 800 μl of the solution were used for drug metabolism studies.

2.4. 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 UV-peak areas of the spiked samples with the direct injection of standard solutions of equal concentration at pH 11.

2.5. Preparation of PPY coated capillary for in-tube SPME

A polypyrrole (PPY) film was coated on the inner surface of a fused silica capillary (60 cm long, 0.25 mm i.d.) by a chemical polymerization method as described previously [16]. In brief summary, the capillary was first cleaned with acetone and then dried with N_2 before treatment. The PPY inner surface coating was prepared by passing first the monomer solution (pyrrole in isopropanol, 50% v/v) through the capillary with the aid of nitrogen gas to form a thin layer of monomer on the capillary inner surface, and then allowing oxidant solution (0.2 M ferric perchlorate in 0.4 M perchloric acid) to flow through the capillary in the same way as for the monomer. The polymer was formed by oxidative reactions when the oxidant reagent reached the monomer coating in the capillary. During polymerization, the color of the capillary gradually changed from yellow to black, indicating the formation of PPY on the inner wall of the capillary. The coating thickness could be controlled by repeated the oxidative reaction several times. Finally, the PPY coated capillary was washed with methanol for 2 min, dried with nitrogen and coupled to the autosampler as described in the next section. For comparison purposes, a commercially available extraction capillary, Omegawax 250 (0.25 μm film thickness, 0.25 mm i.d.) from Supelco (Bellefonte, PA), was also evaluated.

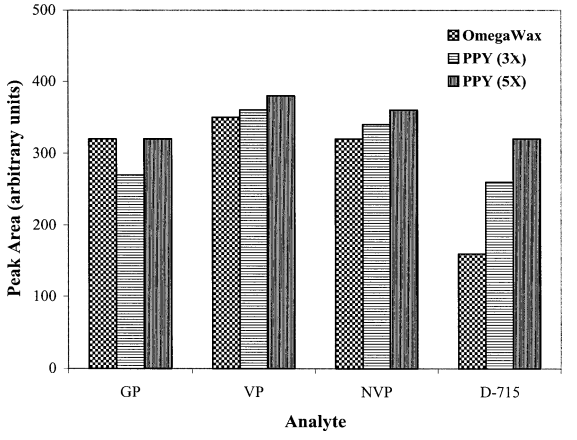


Fig. 1. Comparison of three capillaries for exraction efficacy of verapamil and metabolites. In-tube SPME conditions: sample concentration = 2.5 μg/ml; sample draw/eject volume = 30 μl; # draw/eject cycles = 40; draw/eject speed = 50 μl/min; HPLC conditions outlined in Section 2.

2.6. In-tube solid phase microextraction

The in-tube SPME configuration has been previously reported in the literature [17,18] In summary, the 60 cm capillary column was connected between the injection needle and the injection loop of the autosampler. Under control of the autosampler, the analytes were extracted from the sample by repeatedly aspirating (drawing) and dispensing (ejecting) the sample through the capillary. Desorption of the extracted analyte was then possible by redirecting the mobile phase through the capillary column by switching the six port injection valve from the load to inject position for transport to the analytical column.

Several experimental parameters, such as the extraction coating, sample pH, number of draw/eject cycles and speed of the draw/eject cycle were evaluated as part of the optimization for the developed method.

3. Results and discussions

3.1. Optimization of in-tube SPME conditions

3.1.1. Selection of extraction capillary

In metabolism studies, sample preparation is a crucial task since metabolites often appear at very low concentrations present in complex matrices containing potential interferents such as proteins, salts and other small organic molecules of less interest. The challenge of the analytical chemist is therefore to extract the highest possible amount of analytes, preferably with a rapid, simple and automated method.

In-tube SPME uses an extraction capillary, connected between the injection needle and the sample loop of a commercial autosampler. Extraction of analytes from the sample was possible using the autosampler to simply draw and eject a specified volume of the sample through the extraction capillary. Application of in-tube SPME for the

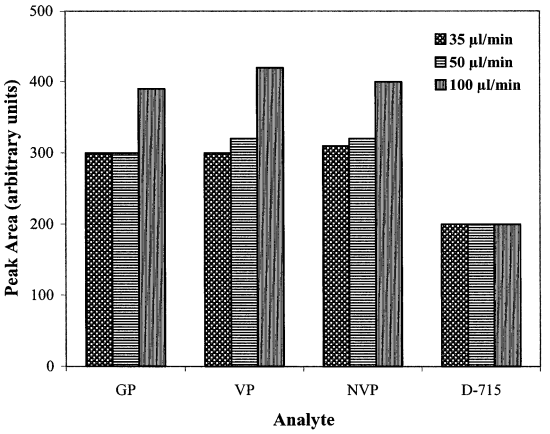


Fig. 2. Effect of draw/eject rate on the amount of verapamil and several metabolites extracted. In-tube SPME conditions: sample concentration = 2.0 μg/ml; other conditions outlined in Fig. 1.

metabolism study of verapamil was evaluated using several capillaries with different extraction phases coated on the inside wall of the capillary. The extraction coatings were tested for their ability to simultaneously extract verapamil and its metabolites (see Fig. 6 for structures). The evaluated extraction phases were selected on their ability to extract polar drug compounds as previously confirmed [16,18]. As illustrated in Fig. 1, the amount of analyte extracted varied among the commercially available (Omega Wax GC column) and two custom made capillaries (polypyrrole coated 3 and 5 times). In most cases, the polypyrrole capillaries had the highest extraction efficiency for verapamil, gallopamil and the two metabolites norverapamil and D-715. In particular the PPY is better suited than Omega Wax for the extraction of the most polar metabolites, such as D-715 (a secondary amine). The multifunctional properties of the PPY film, such as permeability (porous structure) and the ability to form intermolecular interactions between the coating and analytes based on acid–base, π–π, dipole–dipole, and hydrophobic interactions, hydrogen bonding and ion exchange, all enhance the extraction efficiency for this class of analytes. For example, since polypyrrole contains a conjugated π structure, it is expected that to extract aromatics through π–π and hydrophobic interactions. In addition, the presence of a hydrogen-bonding donor in the polymer should enhance the extraction of compounds that contain a hydrogen-bonding acceptor. More recently, these theoretical expectations have been confirmed by different analytical methods such as chromatographic [19], electrochemical [20] and surface methods [21].

A significant increase in the coating thickness with the 5 × coated capillary, as previously confirmed with scanning electron microscopy (SEM) [16], enabled higher binding capacities and further improvements in the amount of analyte extracted.

3.1.2. Speed dependence

The extraction speed (sample draw/eject speed) will effect the time (t_e) required to reach the equilibrium value of extracted analyte as shown in Eq. (1) [22]:

$$t_e = \frac{L\left(1 + K_{fs}\frac{V_f}{V_v}\right)}{\mu} \quad (1)$$

where L is the length of the capillary, K_{fs} is the sample/coating distribution constant, V_f is the volume of the extraction phase, V_v is the void volume of the capillary and μ is the linear velocity of the sample traveling through the capillary. Higher draw/eject speeds will reduce the time required to reach the equilibrium value of extraction, where maximum sensitivity is achieved. In other words, before equilibrium is reached increasing the draw/eject rate will result in more analyte being extracted over shorter time periods, therefore reducing the required number of draw/eject cycles and the total analysis time. As seen in Fig. 2, this effect was confirmed as increasing the extraction rate resulted in a larger amount of analytes being extracted. An exception to this trend was compound D-715, where the extraction rate did not appear to affect the amount extracted. Although not investigated, our hypothesis was that at the lower rates, this analyte may have already reached its equilibrium value and further increases in the extraction speed would therefore have no effect. The effect between 35 and 50 μl/min was relatively small and may have been the result of poor mass diffusion in the capillary at these low extraction rates. Regardless, the faster

extraction speed has the additional advantage of reducing the total sample preparation time and the value of 100 μl/min (the maximum possible by the autosampler) was used for all subsequent analysis.

3.1.3. Extraction time profiles at various sample pH

An important part of the in-tube SPME method development was to determine an extraction time profile at various sample pH. The extraction time profile can indicate the number of draw/eject steps (extraction cycles) required to reach equilibrium. The available standard compounds were subjected to an increasing number of extraction cycles, prior to desorption of the extracted analytes by the mobile phase. As seen in Fig. 3, the amount of analytes extracted (corresponding to the peak area) continued to increase over the range of 0–40 draw/eject cycles for all pH values. However, at higher numbers of extraction cycles (> 20), a proportional increase in the amount extracted was not observed. The plateau for these profiles represents the approach of equilibrium extraction. For a given initial concentration, the amount of analyte extracted at equilibrium represents the maximum amount and is determined by the magnitude of the distribution constant between the extraction coating and the analyte. To ensure maximum detection sensitivity,

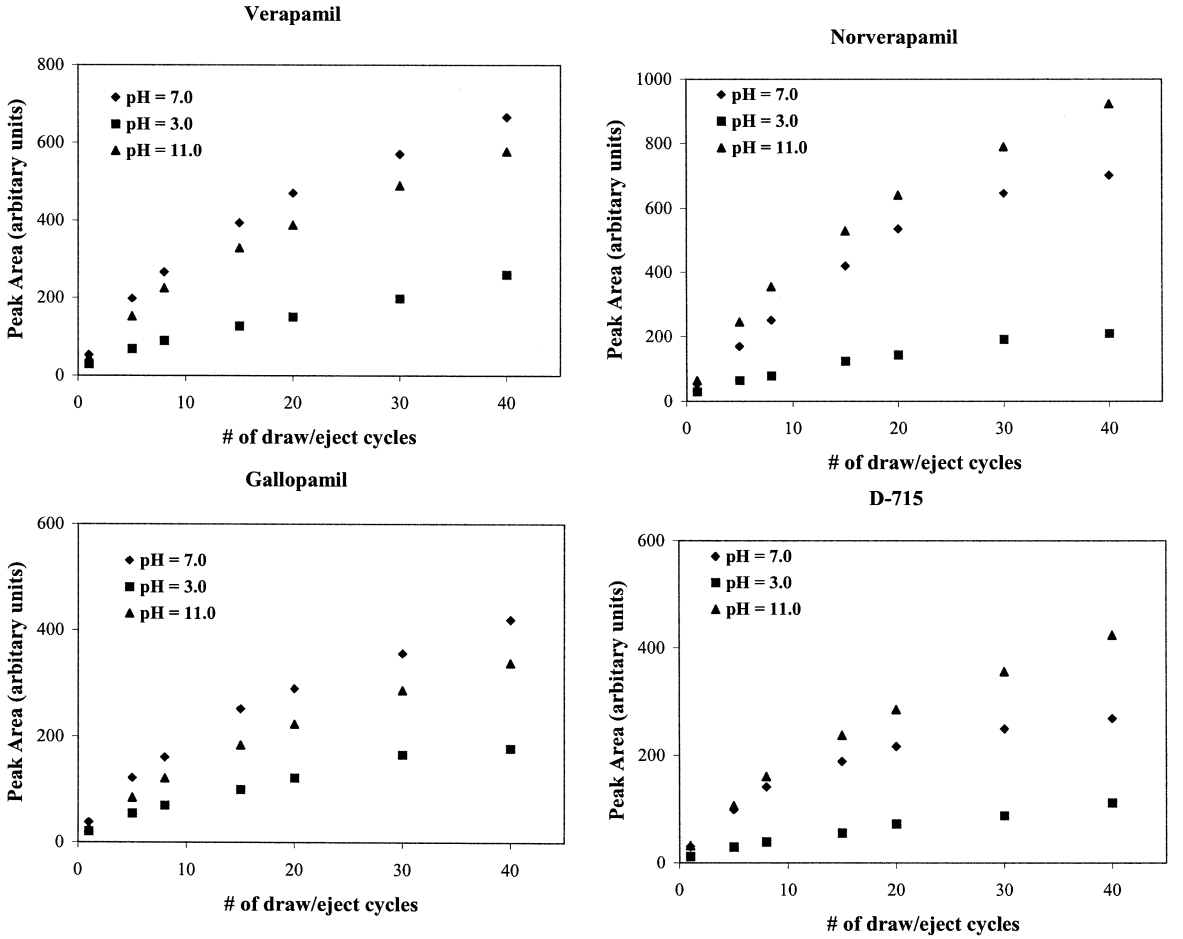


Fig. 3. Extraction time profile of verapamil and several metabolites at various sample pH. In-tube SPME conditions: sample concentration = 2.0 μg/ml; other conditions outlined in Fig. 1.

Table 1
pK_a values for NNK and several metabolites

Compound	pK_{a_1}*	pK_{a_2}
Verapamil	9.04	
Norverapamil	9.87	
Gallopamil	9.01	
D-617	10.35	
D-620	9.84	
D-702	10.32	9.80
D-703	9.15	9.0
D-715	9.88	9.09
D-717	10.35	9.09

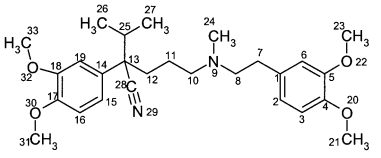
* ACD/pK_a calculates accurate acid–base ionization constants (pK_a values) for 25 °C and zero ionic strength in aqueous solutions.

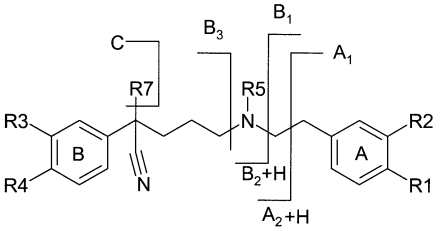
all future sample extractions were performed at 40 draw/eject cycles.

The magnitude of the distribution constant can be influenced by the pH and therefore previous studies have shown the pH of the sample was an important parameter for compounds possessing a pH-dependant dissociable group [23]. As summarized in Table 1, the actual or predicted values of pK_a for verapamil and several metabolites were obtained using the ACD/I-Lab Web service [24]. The pK_{a_1} value for all compounds was in the range of 9–10. Therefore, the available standards were prepared in solutions of pH 11, 7 and 3 equilibrated with acetic acid and ammonia solution to fully validate the effect of extraction pH.

As shown in Fig. 3, the extraction efficiency of PPY for norverapamil and D-715 increased significantly when the sample pH increased from 3 to 10, while the best extraction efficiency for verapamil and gallopamil were obtained at pH 7. These results can be better understood when considering the acid base equilibrium of the analytes.

Table 2
Structure and fragmentation scheme of verapamil and metabolites





		Functional Group					Phase I	Phase II	α-Cleavage		N-C-Cleavage				Concentration (ng/ml)		
Compound	Retention Time (min)	R1	R2	R3	R4	R5	$[M+H]^+$	$[M+H]^+$	A_1	A_2	B_1	B_2	B_3	C	Urine (human)	Plasma (rat)	Hepatocytes (rat)
D-620	6.6	-	-	OCH_3	OCH_3	H	277	-	-	-	-	-	260	234	260	2	-
D-617	8.2	-	-	OCH_3	OCH_3	CH_3	291	-	-	-	-	-	260	248	4200	47	22.5
D-715	4.8	OCH_3	OCH_3	OCH_3	OH	H	427	603	-	275	165	-	260	384	[1]	-	-
D-703	11.2-13.5	OCH_3	OCH_3	OCH_3	OH	CH_3	441	617	-	289	165	-	-	246	[1]	55	16
D-702	11.2-13.5	OCH_3	OH	OCH_3	OCH_3	CH_3	441	617	-	303	151	291	260	248	[1]	55	16
Norverapamil	14.2	OCH_3	OCH_3	OCH_3	OCH_3	H	441	-	151	289	165		-	398	140	84	26
Verapamil	15.9	OCH_3	OCH_3	OCH_3	OCH_3	CH_3	455	-	-	303	165	-	-	-	1030	470	79
Gallopamil (IS)	17.1	OCH_3	OCH_3	OCH_3	OCH_3	H	485	-	-	333	165	-	-	-	-	-	-

[1] Metabolites have been detected as glucuronides.

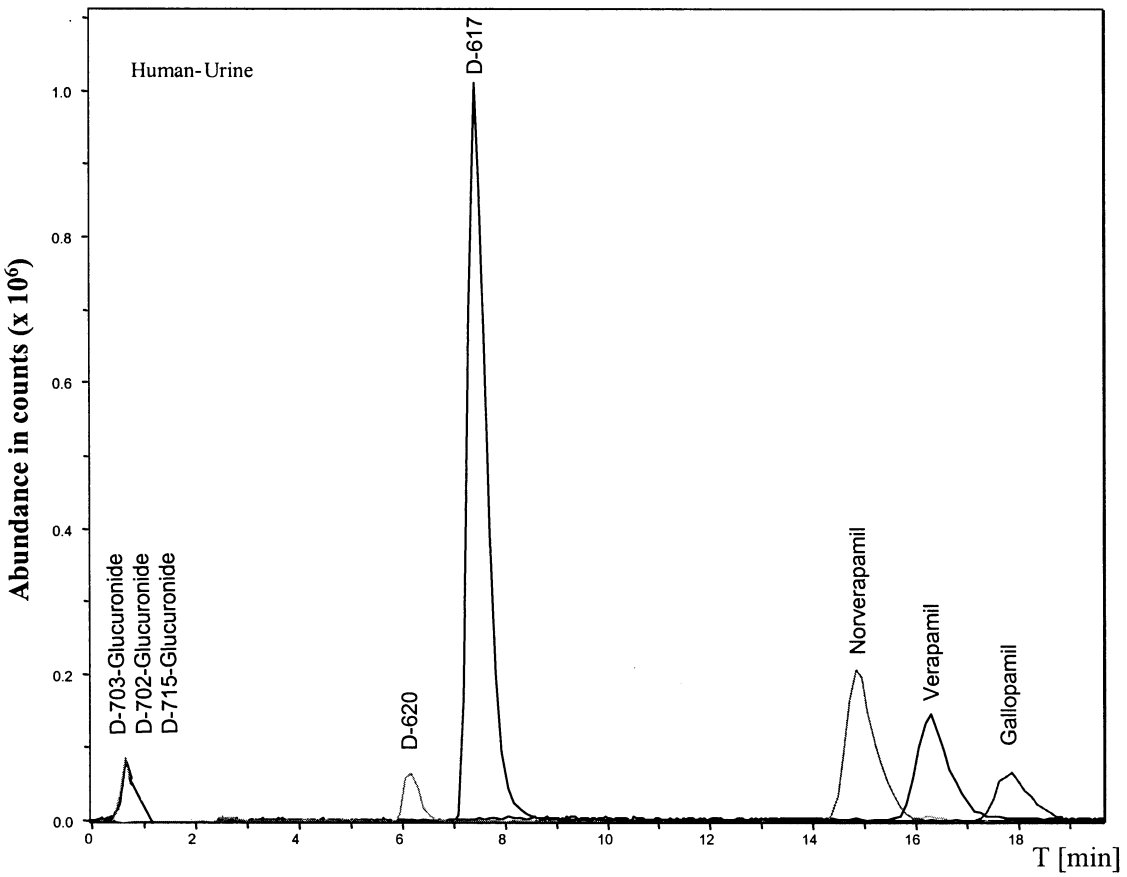


Fig. 4. Typical HPLC chromatogram of verapamil and metabolites extracted from cell culture by in-tube SPME with PPY capillary. Conditions outlined in Fig. 1.

Table 3
% R.S.D. summary for verapamil and available metabolite standards in various biological matrices

Matrix	% R.S.D.			
	Verapamil	Gallopamil	Norverapamil	D-702
Urine	4.5 ± 0.2	3.7 ± 0.2	3.7 ± 0.3	3.2 ± 0.6
Plasma	3.3 ± 0.2	3.7 ± 0.4	3.7 ± 0.3	6.5 ± 0.5
Cell culture	4.0 ± 0.3	3.7 ± 0.3	4.0 ± 0.5	3.2 ± 0.3

The pK_a-values for verapamil and gallopamil are between 9 and 10. Because the PPY coating is positively charged in its oxidized form (a weak acid) and the analytes also had positive charges at low pH solutions (the acid form), the electrostatic repulsion between the coating and analytes led to the lower extraction efficiency in the low pH sample solutions. With an increase in the pH of the sample solutions, the positive charges on the analytes were reduced, the base forms were increased, and the attractive inter-molecular interactions between analytes and PPY became

dominant, therefore the extraction efficiency increased. The pK_as for verapamil and gallopamil are around 9 while the pK_as of the metabolites norverapamil and D-702 are around 10. It is obvious that these metabolites are extracted better at a pH value around 11, when in their free base form and not protonated.

Table 4
% Recovery of verapamil and selected metabolites from various biological matrices

Analyte	Urine	Plasma	Cell-culture media
Verapamil	95 ± 5	89 ± 8	87 ± 6
Gallopamil	98 ± 6	91 ± 9	90 ± 7
Norverapamil	89 ± 6	87 ± 5	90 ± 4
D-715	87 ± 4	82 ± 7	93 ± 6

Error calculated from % R.S.D. ($N = 3$).

Table 5
Summary of in-tube SPME HPLC detection limits for UV and MS detectors

Detector	Detection limit[a] (ng/ml)			
	Verapamil	Gallopamil	Norverapamil	PR22
UV	52	53	65	83
MS	5	6	6	8

[a] Detection limit calculated at S/N = 3.

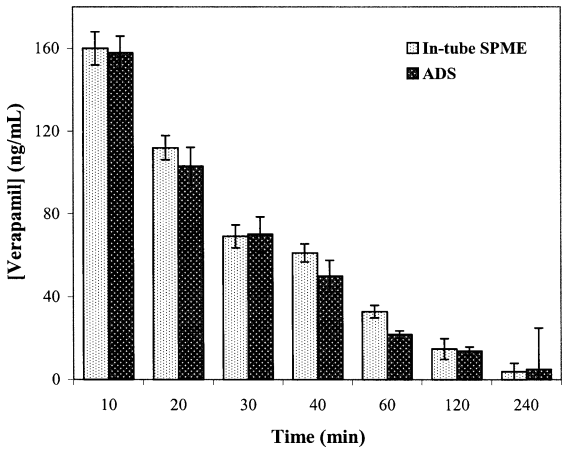


Fig. 5. In-tube SPME LC-MS pharmacokinetic plot for verapamil after intravenous injection.

As we want the highest extraction efficiency for the metabolites in drug metabolism studies, all experiments were performed with a sample pH 11.

3.2. Qualitative analysis of cultured hepatocytes, plasma and urine

The developed in-tube SPME method was applied to the analysis of verapamil and its metabolites in various matrices. Mass spectrometry was used to analyze and identify the extracted analytes. As shown in Table 2, the in-tube SPME coupled to LC-MS was an appropriate method to monitor the major metabolites of verapamil in various matrices like cultured hepatocytes, plasma or urine.

After extraction and separation (see Fig. 4), the structures of the metabolites were elucidated by comparing their MS-fragmentation pattern (see Table 2) with that of verapamil. Verapamil and gallopamil were available as a standard compounds and were identified by their chromatographic retention time.

Our study demonstrates the utility and effectiveness of electrospray ionization and collision induced dissociation mass spectrometry in the identification and structural elucidation of novel metabolites of verapamil. Mass spectrometry is the first choice in the search of new metabolites as the concentration of biotransformation products are too low to obtain informative ^{1}H NMR-data. The collision-induced dissociation of the protonated molecules of all six metabolites can be described by a general fragmentation scheme as shown in Table 2. 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 2) with the exception of the isopropyl group loss as observed with several metabolites, which leads to an odd electron fragment ion and a neutral radical. This surprising loss of the isopropyl radical may be due to influence of the cyano group at the same C-atom. However, the competing (thermodynamically more favorable) loss of an isopropene molecule is

often also observed. The observed neutral losses are particularily valuable for the identification of structural features. Thus, loss of 176 amu (corresponding to glucuronic acid–H_2O) is characteristic for a glucuronide conjugate (see Fig. 8), loss of methylamine from the protonated molecule or a primary fragment proves the presence (or collision induced formation) of a secondary amine. The relative abundance of the fragment ions of the parent compound and the six metabolites depends on the substitution pattern. For instance, oxydation of the phenylalkylamine moiety (norverapamil and D-702) leads to N–C-cleavage with charge retention on the nitrogen, oxydation in the remaining part of the molecule to a corresponding cleavage with charge migration (D-617, D-703) to the phenylalkyl moiety.

3.2.1. Metabolism of verapamil

In urine, dealkylverapamil (D-617) was the major metabolite observed, with D-620 and norverapamil also being expressed in significant amounts. The metabolites D-715 and D-702 were detected as glucuronide conjugates. The response of these glucuronides in positive ESI was much lower than the corresponding unconjugated metabolites, so the glucuronides could only be detected in trace amounts, and possibly indicated why the other glucuronides were not been detected. Previous studies have also shown that in human urine, the metabolite D-617 is preferably formed and that most metabolites are excreted as conjugates [25,26]. Although the only glucuronide described in literature is the norverapamil glucuronide, which was isolated from rat bile [27], the glucuronides D702, D-703 and D-715 have been described for the first time directly by us.

In plasma, the concentrations of the metabolites were much lower with norverapamil being detected as the major metabolite. Besides the *O*-demethylverapamilisomers (D-702 and D-703), the dealkylverapamil-metabolites (D-715 and D-620) have also been detected. The results from the rat plasma are similar to published results [28]. Here also norverapamil is formed as the major metabolite. The metabolic profile obtained from the rat plasma was similar to the results of the incubations with rat hepatocytes.

3.3. Quantitative method validation

The developed in-tube SPME HPLC method was validated using plasma, urine and cell culture medium. Fig. 4 represents a typical chromatogram for a spiked urine sample. The in-tube SPME approach was able to isolate verapamil and several metabolites providing a simple and automated procedure for obtaining the metabolic profile from a biological sample.

Various analytical figures of merits were evaluated for the developed method in all matrices. For example, the average precision was calculated with five repeated injections of the available standards spiked into each matrix and is summarized in Table 3. The recovery of the spiked biological samples was calculated by comparison to the standard solutions and has been shown in Table 4. In all matrices, good recovery was observed, however on average the plasma exhibited the lowest values. The higher protein concentration in this matrix likely contributed to this reduced recovery as the analyte may complex with these interferents.

The linearity of the standard calibration curve of verapamil, gallopamil, norverapamil and compound D-715 was evaluated over the range of 100–800 ng/ml and produced an average linear regression coefficient (R^2 value) of 0.9992, 0.9991, 0.9990 and 0.990, respectively. The limit of detection (LOD) for each available compound was determined in all available biological matrices at a concentration where the signal/noise ratio was equal to 3 and these values, for both the UV and mass spectrometer detectors, have been included in Table 5.

One routine application is the pharmacokinetics of a drug and the accuracy of the in-tube SPME method was compared against an established method. In Fig. 5, the pharmacokinetics of verapamil after intravenous application is shown and compared to the results obtained with an ADS-column switching device [29]. As shown in Fig. 5, both methods produce very similar results and the clearance of the drug followed the same trend as a previously published pharmacokinetic plot [30], confirming the suitability of the developed in-tube SPME method for these type of studies. The

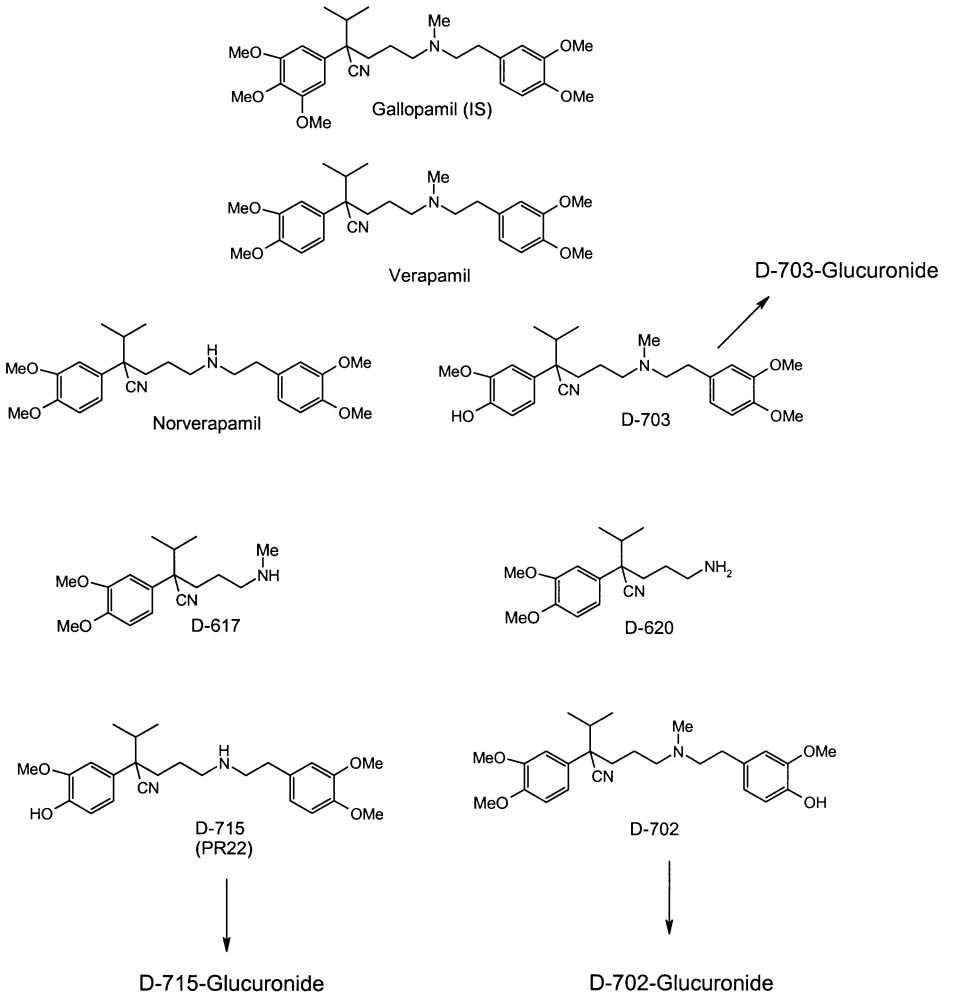


Fig. 6. Structures of verapamil, gallopamil, and identified metabolites.

standard deviation (calculated from both the standard deviation of the verapamil injection and the gallopamil injection) was slightly better for the in-tube SPME method. In general, the standard deviation of the ion trap mass spectrometer is higher than that obtained with quadrupoles. In both methods, a range of 1–12% R.S.D was observed, however most sample concentrations were determined at a % R.S.D. of less than 6%. As expected, the higher % R.S.D. values was observed at the lowest concentration level, where the S/N was decreased and approaching the detection limit.

In this work, a single PPY coated capillary was used for all the experiments, and no significant changes in the extraction performance were observed using different matrices like urine, plasma, and cell culture media, confirming the robustness of the method. In comparison to other sample preparations methods for verapamil and its metabolites [31,32], the developed in-tube SPME approach was fully automated, used a simple apparatus (single autosampler) and minimized the solvent and time requirements (Fig. 7).

4. Conclusions

The developed in-tube SPME method was sensitive, simple and reproducible for the determination of verapamil and metabolites in several biological matrices. The easy automation also provided an advantage over many existing sample preparation techniques used for metabolism studies. The effect of several experimental parameters, such as extraction rate and sample pH, were discussed and optimized for the method. In addition, the multifunctional nature of the PPY film was shown to be a useful extraction phase for the isolation of the target analytes, and when coupled to LC-MS, much qualitative and quantitative information about the metabolic profile of verapamil was acquired.

In summary, in-tube SPME was shown to be an appropriate method for drug metabolism studies, routine analysis or pharmacokinetics as the parent compound and main metabolites could be monitored in various matrices of interest.

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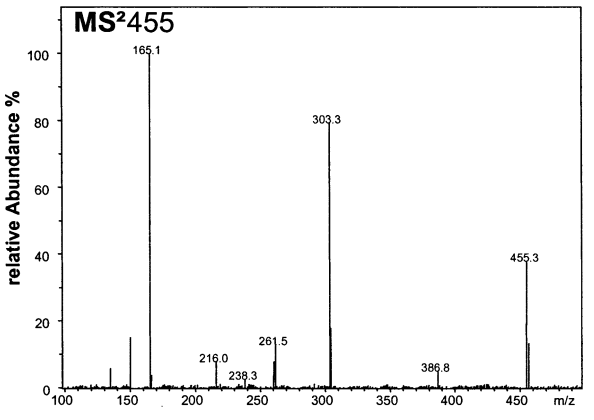

Fig. 7. MS/MS spectra of verapamil.

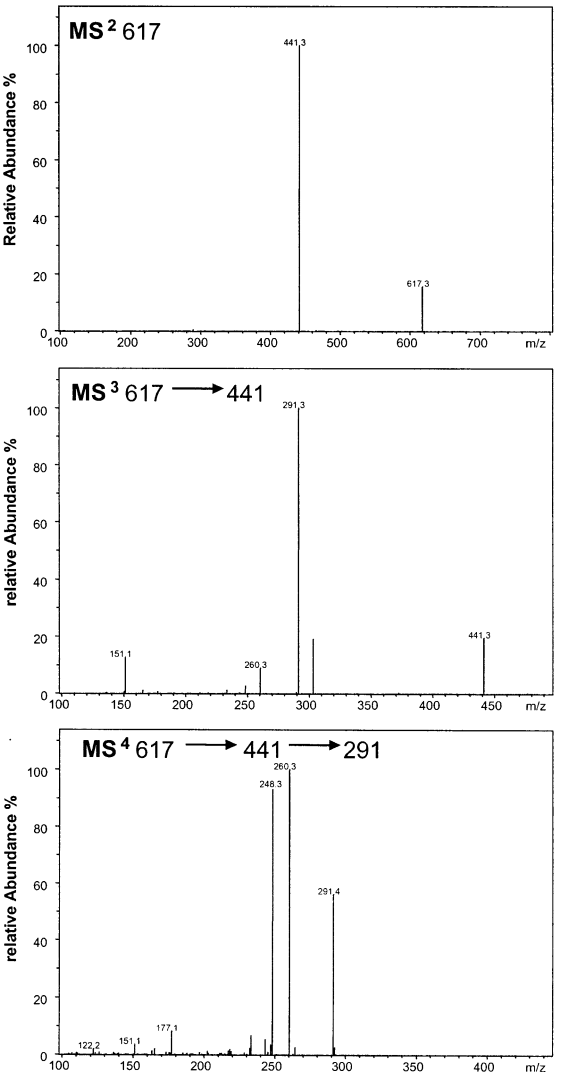

Fig. 8. MS^n-spectra of the D702-glucuronide.

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