Journal of Chromatography B, 738 (2000) 137–147

JOURNAL OF CHROMATOGRAPHY B

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Rapid and highly sensitive method for the determination of verapamil, [$^2H_7$]verapamil and metabolites in biological fluids by liquid chromatography–mass spectrometry

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Received 25 August 1999; received in revised form 22 October 1999; accepted 28 October 1999

Abstract

A rapid and highly sensitive method for the determination of verapamil [2,8-bis-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile] and [$^2H_7$]verapamil and their primary metabolites D-617 [2-(3,4-dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile], D-703 [2-(4-hydroxy-3-methoxyphenyl)-8-(3,4-dimethoxy-phenyl)-6-methyl-2-isopropyl-6-azaoctanitrile], D-702 [2-(3,4-dimethoxy-phenyl)-8-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile], norverapamil [2,8-bis-(3,4-dimethoxyphenyl)-2-isopropyl-6-azaoctanitrile] and secondary metabolites D-620 [2-(3,4-dimethoxyphenyl)-5-amino-2-isopropylvaleronitrile], D-717 [2-(4-hydroxy-3-methoxyphenyl)-5-amino-2-isopropylvaleronitrile], and D-715 [2-(4-hydroxy-3-methoxyphenyl)-8-(3,4-dimethoxy-phenyl)-2-isopropyl-6-azaoctanitrile] has been developed using high-performance liquid chromatography–electrospray mass spectrometry. D-832, the gallopamil analogue of D-617 and [$^2H_3$]norverapamil were used as internal standards. The analytes were extracted automatically from plasma and intestinal perfusate using end-capped CN- and C_2 solid-phase extraction cartridges. Separation of the eight analytes was achieved on a LUNA C8 analytical column (150×2 mm I.D., 5 μm particle size) with 5 m*M* ammonium acetate–acetonitrile as the mobile phase run with a gradient from 70:30 to 40:60 and run times of 15 min. With the mass spectrometer operated in the selected-ion monitoring mode, the limits of quantification in plasma and intestinal perfusate were 1 pmol/ml for D-620, D-617, D-702, D-703, norverapamil, verapamil, and [$^2H_7$]verapamil and 2.5 pmol/ml for D-717 and D-715 using a sample size of 1 ml plasma and intestinal perfusate. The method described was successfully applied to the determination of verapamil, [$^2H_7$]verapamil and their metabolites in human plasma and intestinal fluid in pharmacokinetic studies. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Verapamil

1. Introduction

The calcium channel-blocker verapamil undergoes extensive presystemic elimination after oral administration which results in a variable and low bioavailabilty [1]. Multiple members of the cytochrome P450 superfamily (e.g. CYP3A and CYP2C) catalyse the formation of verapamil metabolites [2,3]. It is increasingly recognised that these enzymes are not only expressed in the liver but also in the intestine [4] and that the latter can be an important site of verapamil metabolism [5].

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PII: S0378-4347(99)00508-3

In order to investigate the extent of intestinal presystemic extraction of a drug absolute bioavailability derived from AUCs (area under the plasma concentration-time curve) for AUC after oral and i.v. dosing has to be determined. The stable labelled isotope technique is an elegant method to determine intestinal and hepatic drug metabolism because the simultaneous administration of the unlabelled drug orally and deuterium labelled drug intravenously is carried out in one experiment, thus excluding day-to-day variability in drug disposition and increasing the power of the study [6–8]. The use of mass spectrometric discrimination of unlabelled and deuterated drug is a prerequisite in applying this technique.

Intestinal perfusion catheters have been used to study drug absorption [9–11] in humans. In combination with stable labelled isotope technique and simultaneous i.v. and oral administration, in addition to absolute bioavailability and gut wall metabolism can be studied. These approaches require the measurement of parent drug and metabolites which can be determined by HPLC. In addition in order to differentiate between isotopic species, MS is required.

We therefore have developed a rapid and highly sensitive LC–MS method which enables the quantification of verapamil, $[^2H_7]$verapamil and seven unlabelled and $[^2H_7]$metabolites (Fig. 1) in human plasma and intestinal perfusate after simultaneous oral verapamil and intravenous $[^2H_7]$verapamil administration. Assay times were kept below 15 min to reduce full. The achieved limits of quantification of 1 pmol/ml for D-620, D-617, D-703, D-702, norverapamil and $^1H/^2H_7$verapamil and 2.5 pmol/ml for D-717 and D-715 are an improvement over current published HPLC methods. Salama et al. [12] describes a fluorimetric method with limits of quantification of 2.5 ng/ml (5.5 pmol/ml) while Garcia et

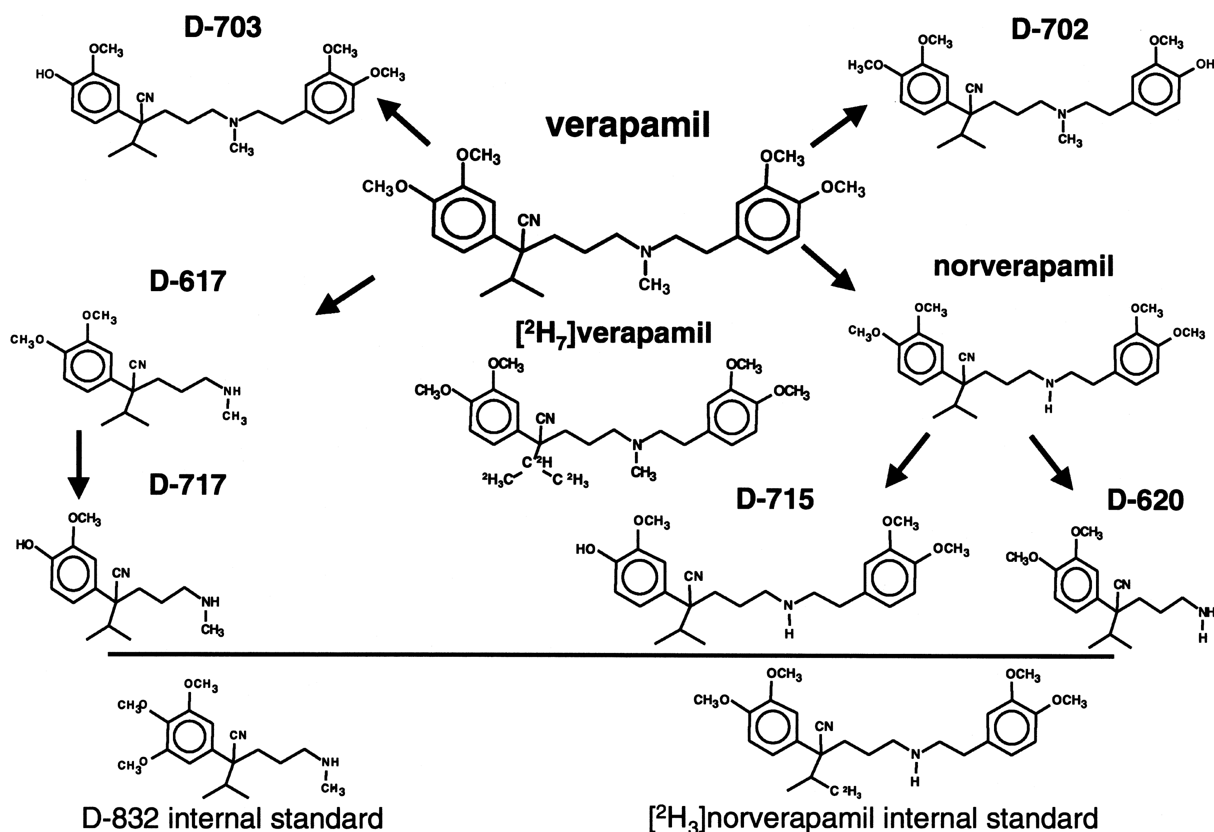

Fig. 1. Structure formulas of verapamil and metabolites.

al. [13] achieved 25 ng/ml (55 pmol/ml) for norverapamil and verapamil. Furthermore, the sensitivity of this LC–MS method for verapamil and norverapamil is comparable to GC–MS assays [14,15] but in contrast to GC–MS, the determination of more polar metabolites, which require derivatization for GC, is easier to achieve with adequate sensitivity.

2. Experimental

2.1. Materials

Solvents used were of HPLC quality; chemicals were of analytical grade. Verapamil–HCl, [$^2H_7$]verapamil–HCl, D-620–HCl, D-617–HCl, D-715–HCl, D-717–HCl, D-702–HCl, D-703–HCl, norverapamil–HCl [$^2H_3$]norverapamil-oxalate, and D-832–HCl (Fig. 1) were obtained from Knoll AG (Ludwigshafen, Germany). Isoamyl alcohol was obtained from Sigma (Deisenhofen, Germany). Solid-phase extraction (SPE) cartridges, CN endcapped (50 mg) and C_2 endcapped (100 mg) were supplied by Isolute (Bad Homburg, Germany).

2.2. Preparation of standard solutions

Stock standard solutions (1 mg/ml) of verapamil–HCl, [$^2H_7$]verapamil–HCl, D-620–HCl, D-617–HCl, D-715–HCl, D-717–HCl, D-702–HCl, D-703–HCl, norverapamil–HCl, [$^2H_3$]norverapamil-oxalate, and D-832–HCl were prepared in 2-propanol. Working standard solutions were prepared from the stock solutions in distilled water with (1% v/v) isoamyl alcohol. All standard solutions were kept below −20°C.

2.3. Sample preparation

Samples (1 ml of plasma or 0.5 ml of intestinal perfusate+0.5 ml potassium phosphate buffer, 0.1 *M*, pH 7.4) were spiked with 25 μl of internal standard mix (2 pmol/μl D-832 and [$^2H_3$]norverapamil). Sample work-up was performed automatically on an ASPEC XL system (Gilson Abimed, Langenfeld, Germany) using CN (EC) 50 mg columns for plasma and C_2 (EC) 100 mg columns for intestinal perfusate. Each column was first conditioned with 1 ml of methanol and 1 ml of potassium phosphate buffer (0.1 *M*, pH 7.4) at a flow-rate of 6 ml/min. The sample was loaded on the column at a flow-rate of 0.5 ml/min, then washed with 1 ml of potassium phosphate buffer (0.1 *M*, pH 7.4) at a flow-rate of 3 ml/min, and dried by applying 2 ml of air. The analytes were eluted with 1 ml of methanol containing 0.25% (v/v) triethylamine and 1% (v/v) isoamyl alcohol at a flow-rate of 1 ml/min. The eluate was evaporated to dryness under a stream of nitrogen and redissolved in 150 μl of mobile phase. Volumes of 40 μl were injected onto the column.

For recovery experiments the peak areas obtained with SPE from serum and intestinal perfusate samples were compared to peak areas obtained from the same amounts directly injected into the LC–MS system without extraction.

2.4. HPLC conditions

A HP1100 LC-system (Hewlett Packard, Waldbronn, Germany) equipped with a binary pump was used. Chromatographic separation was performed on a LUNA C8 analytical column (150×2 mm I.D., 5 μm particle size; Phenomenex, Hösbach, Germany) in combination with an Eclipse XDB-C8 (12.5×2.1 mm I.D., 5 μm particle size) guard column (Hewlett Packard). The mobile phases were: (A) 5 m*M* ammonium acetate buffer, pH 4.2 and (B) acetonitrile. Gradient runs were programmed as follows: 30% B at a flow-rate of 0.25 ml/min for 9 min, decrease to 20% B in 0.1 min, 20% B for 1.9 min, increase B to 50% and flow-rate to 0.5 ml/min in 1.5 min, 50% B at a flow-rate of 0.5 ml/min for 2 min, then re-equilibration with 30% B at a flow-rate of 0.25 ml/min for 4 min, until the next sample was injected. A column switching valve was used that switched the liquid flow on the detector from 2.5 to 14.5 min after injection.

2.5. Mass spectrometry

A HP 1100 single quadrupole mass spectrometer (Hewlett Packard) equipped with an electrospray source was used. Electrospray parameters were: spray voltage, 3.5 kV; gas temperature 350°C; sheath gas pressure 50 p.s.i.; auxiliary gas 11 ml/min. Positive ionisation with selected ion monitoring

mode (SIM) was used for all analytes, the multiplier gain was set on one. The following ions were detected: m/z 277 for D-717 and D-620, m/z 284 for [$^2H_7$]D-717 and [$^2H_7$]D-620, m/z 291 for D-617, m/z 298 for [$^2H_7$]D-617 and m/z 321 for D-832 for the first 6.5 min after injection. From 6.5 to 9 min m/z 427 was used for D-715, m/z 434 for [$^2H_7$]D-715, m/z 441 for D-703, D-702 and norverapamil, m/z 448 for [$^2H_7$]D-702, [$^2H_7$]D-702 and [$^2H_7$]norverapamil. After 9 min m/z 441 was used for D-703, D-702 and norverapamil, m/z 448 for [$^2H_7$]D-703, [$^2H_7$]D-702 and [$^2H_7$]norverapamil, m/z 455 for verapamil, m/z 462 for [$^2H_7$]verapamil and m/z 444 for [$^2H_3$]norverapamil.

2.6. Standardisation

Calibration samples were prepared by adding increasing amounts of D-717, D-620, D-617, D-715, D-703, D-702, norverapamil, verapamil, and [$^2H_7$]verapamil to control plasma and intestinal perfusate. Standard curves were evaluated by weighted ($1/x$) linear regression based on internal standard calibration and were obtained by plotting peak-area ratios against the amount of the substance. D-717, D-620, and D-617 were standardised against D-832, the gallopamil analogue of D-617. D-715, D-703, D-702, norverapamil, verapamil and [$^2H_7$]verapamil were standardised against [$^2H_3$]norverapamil. For standardisation of [$^2H_7$]metabolites the calibration curves of the corresponding unlabelled analogue were used. All standardisation was performed with HP Chemstation (98) software (Hewlett Packard).

2.7. Assay validation

To determine assay variability, quality control samples were prepared by adding known amounts of metabolites, verapamil and [$^2H_7$]verapamil to 30 ml of drug free plasma and intestinal perfusate, which were divided into aliquots and stored at −20°C. Quality control samples were always extracted and analysed together with samples of corresponding matrices.

In addition, 86 plasma samples which were previously assayed for verapamil with liquid–liquid extraction [16] following GC–MS analysis [15] served for inter-assay validation.

3. Results and discussion

3.1. Solid phase extraction and HPLC separation

Sample extraction was performed automatically by a modification of the method developed by Hubert et al. [17]. Different cartridges were tested for plasma and intestinal fluid, and the best results with respect to recovery and accuracy were obtained with 50 mg CN (EC) cartridges for plasma and 100 mg C_2 (EC) cartridges for perfusate (Table 1). Addition of 1% (v/v) isoamyl alcohol to the eluting methanol prevents verapamil, a very lipophilic compound from adsorption to polypropylene (e.g. cartridge surface) and glass surfaces. Without the use of 1% (v/v) isoamyl alcohol, the linear range drops from 1 to 500 pmol/ml down to 10–500 pmol/ml as do the recovery rates from solid-phase extraction. Recovery

Table 1
Recoveries from different SPE cartridges (n=4) using 1 ml of plasma and 0.5 ml of intestinal fluid (IF), respectively, spiked with 50 pmol/ml D-717, D-620, D-617, D-715, D-703, D-702, norverapamil, verapamil and [$^2H_7$]verapamil

Cartridge	Recovery [%] mean±SD								
	D-717	D-620	D-617	D-715	D-703	D-702	Norverapamil	Verapamil	[$^2H_7$] Verapamil
CN (EC) plasma	64.2±8.0	74.0±7.7	71.4±6.9	79.1±5.9	77.5±5.0	76.8±5.1	88.2±6.3	81.9±4.2	83.1±4.1
C_2 (EC) plasma	67.3±11.8	68.7±11.2	68.7±6.7	63.5±11.9	66.5±9.2	70.4±5.1	61.9±15.6	70.7±13.7	71.7±14.3
CN (EC) IF	65.8±2.0	88.7±3.0	77.3±2.5	47.0±3.2	51.1±4.2	50.6±2.6	56.0±3.1	47.4±3.4	49.7±4.0
C_2 (EC) IF	68.6±3.8	88.2±3.9	75.3±2.1	54.9±2.6	58.6±1.5	60.5±2.0	64.6±2.9	57.4±0.5	58.7±0.4
Phenyl (EC) IF	61.2±2.0	79.0±2.5	6.20±2.1	36.0±2.4	33.1±4.1	33.5±3.4	30.1±4.4	20.2±4.2	20.6±3.8

rates of verapamil and norverapamil are equivalent to published liquid–liquid extraction procedures [14,18]. The same extraction procedure optimised for intestinal fluid samples could be used for urine with a restriction in sample volume of 100 μl to avoid an overload of the cartridge. Bile cannot be automatically extracted with either cartridges because of excessive impurities in the eluate. Liquid–liquid extractions [14,18] can be used when analysing bile (data not shown).

Verapamil and its metabolites are usually separated by ion-pair reversed-phase HPLC. For LC–MS ion-pair additives and non-volatile buffers should be avoided. With regard to short run times, height equivalent of a theoretical plate (HETP) and peak asymmetry (A_s) a Luna RP8 column (150×2 mm I.D., 5 μm particle size) and an ammonium acetate buffer (5 m*M* pH 4.2)–acetonitrile gradient was used. Baseline separation for verapamil and all its metabolites was achieved with retention times of 3.2 min for D-717, 3.9 min for D-620, 4.3 min for D-617, 4.9 min for D-832, 7.5 min for D-715, 8.2 min for D-703, 8.9 min for D-702, 12.1 min for norverapamil and [$^2H_3$]norverapamil and 12.9 min for verapamil and [$^2H_7$]verapamil. The gradient program employed was optimised for the separation of norverapamil and verapamil increasing peak height. The signal reduction of [$^2H_3$]norverapamil and [$^2H_7$]verapamil caused by ion suppression due to increasing amounts of norverapamil and verapamil within the linear range of 1–500 pmol/ml was not observed. Ion suppression phenomena appeared at concentrations above 1000 pmol/ml. The gradient program also prevented column contamination with late-eluting compounds. With this wash step and regularly changing the precolumn after 100 samples, the HPLC column could be used for more than 1500 samples. In blank plasma and intestinal perfusate no interfering peaks could be detected (Fig. 2). Chromatograms of a perfusate sample spiked with [$^2H_7$]verapamil, verapamil and metabolites and of a plasma sample after treatment with verapamil and [$^2H_7$]verapamil are shown in Figs. 3 and 4.

3.2. Mass spectrometry

With the electrospray ionisation (ESI) the protonated molecular ions (MH^+) are observed as base peaks and were used for determination of all compounds in the single ion monitoring mode (SIM). Fragmentation was below an abundance of 15% of MH^+ peaks. For D-620 fragmentation to m/z 260 occurred, which did not interfere with the other ions monitored. Mass spectrometric parameters (capillary voltage, gas temperature, gas flows) were adjusted to get a maximum signal for the verapamil MH^+ ion.

3.3. Validation

The method has good linearity over the entire range measured: 1–500 pmol/ml for D-620, D-617, D-703, D-702, norverapamil, verapamil and [$^2H_7$]verapamil and 2.5–500 pmol/ml for D-717 and D-715. The assay precision and accuracy and limit of quantification were determined by repeatedly analysing aliquots of plasma and intestinal fluid samples spiked with known amounts of analytes. The intra-assay variability and LOQ of plasma are given in Table 2. Precision ranges from −11.7 to 4.4% and the accuracy is better than 7%. At the LOQ the variation was less than 7% with a bias of less than 14% for all analytes determined. The day-to-day variation in intestinal fluid as shown in Table 3 was less than 10% except for verapamil and D-715 which was less than 12% over a period of 4 weeks.

Comparison of verapamil determination in 86 plasma samples which were previously assayed with liquid–liquid extraction [16] following GC–MS analysis [15] with verapamil data obtained with the described method served as an inter-assay validation and revealed a correlation of r=0.995 (Fig. 5).

3.4. Assay application

The sensitivity achieved for D-717, D-620, D-617, D-715, D-703, D-702 is better than with previously published methods [13,14,18]. Fig. 6 shows a representative concentration-time curve in intestinal fluid of verapamil and its metabolites norverapamil, D-617 and D-702 after an 80 mg oral dose of verapamil. As the verapamil metabolites are generated by different cytochrome P450 isoforms (e.g. D-617, norverapamil by CYP3A; D-703, D-702 by CYP 2C) [2,19] and those isoforms exhibit highly variable expression in liver and intestine [20,21], C_{max} values vary over a great concentration range. C_{max} of D-617

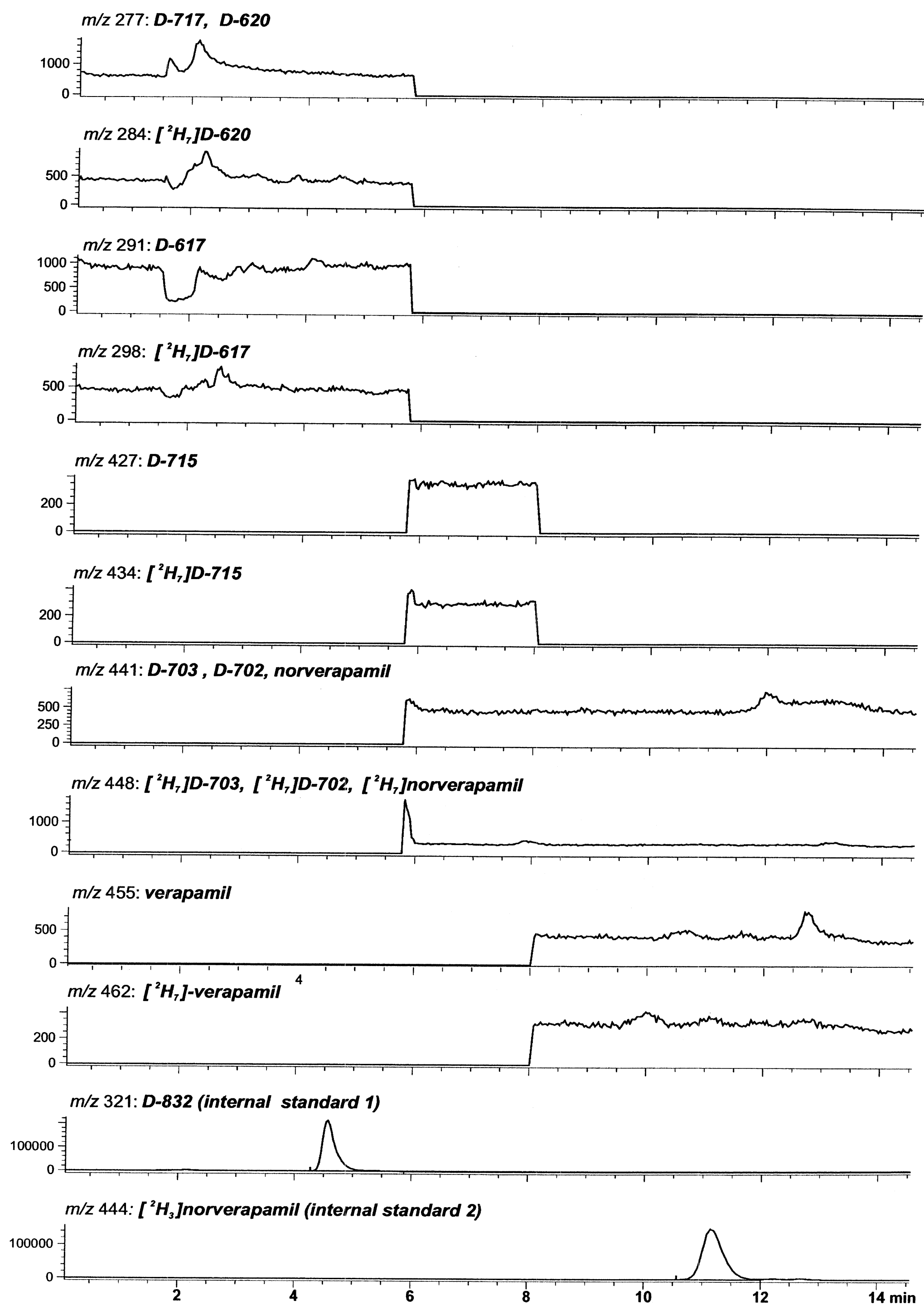

Fig. 2. Mass chromatograms of an extract of 0.5 ml intestinal fluid spiked with internal standards (100 pmol/ml) D-832, 100 pmol/ml [$^{2}H_{3}$]norverapamil.

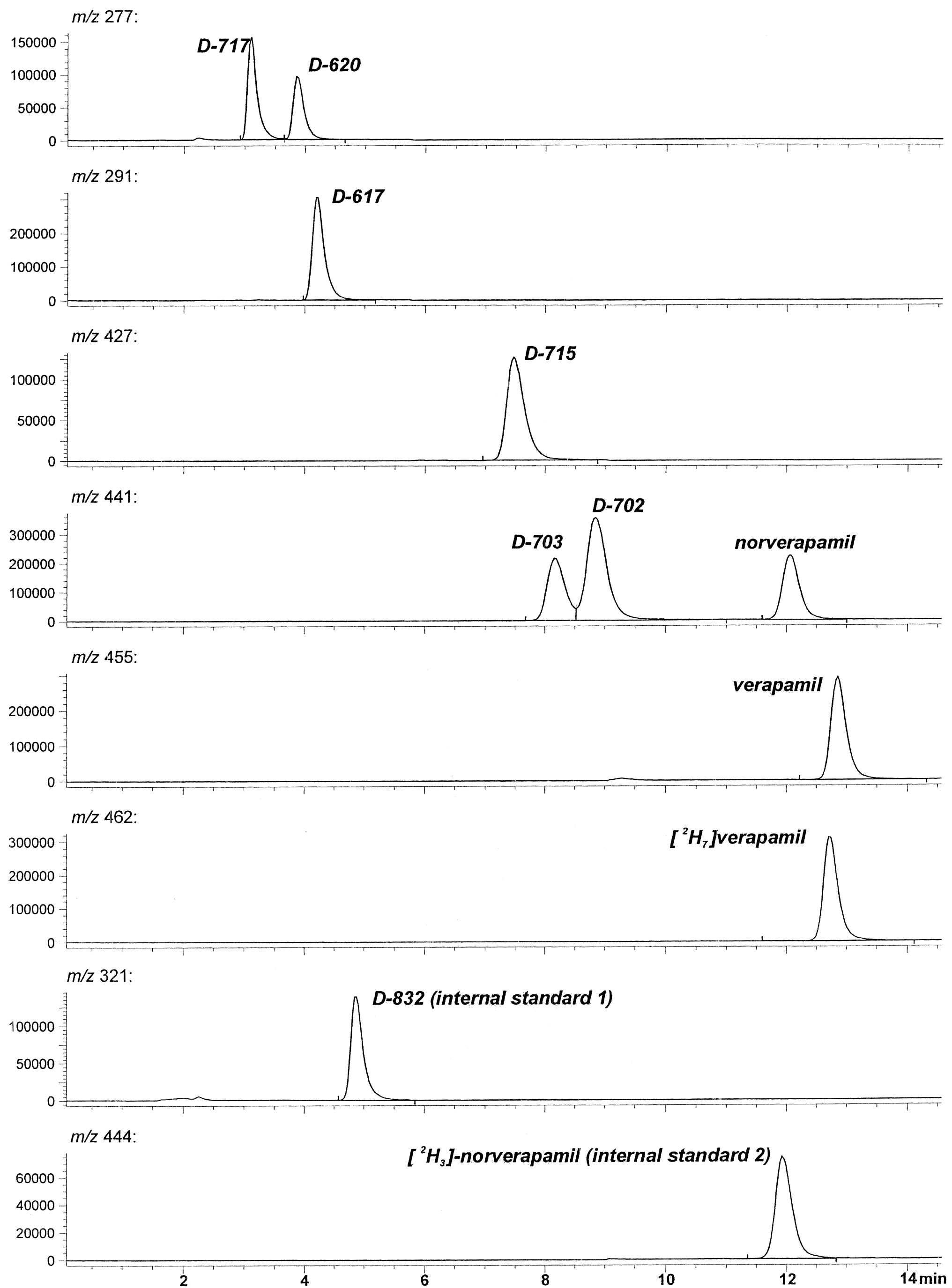

Fig. 3. Mass chromatograms of an extract of 0.5 ml intestinal fluid spiked with 500 pmol/ml of D-717, D-620, D-617, D-715, D-703, D-702, norverapamil, verapamil, [$^{2}H_{7}$]verapamil and 100 pmol/ml of D-832 and [$^{2}H_{3}$]norverapmil.

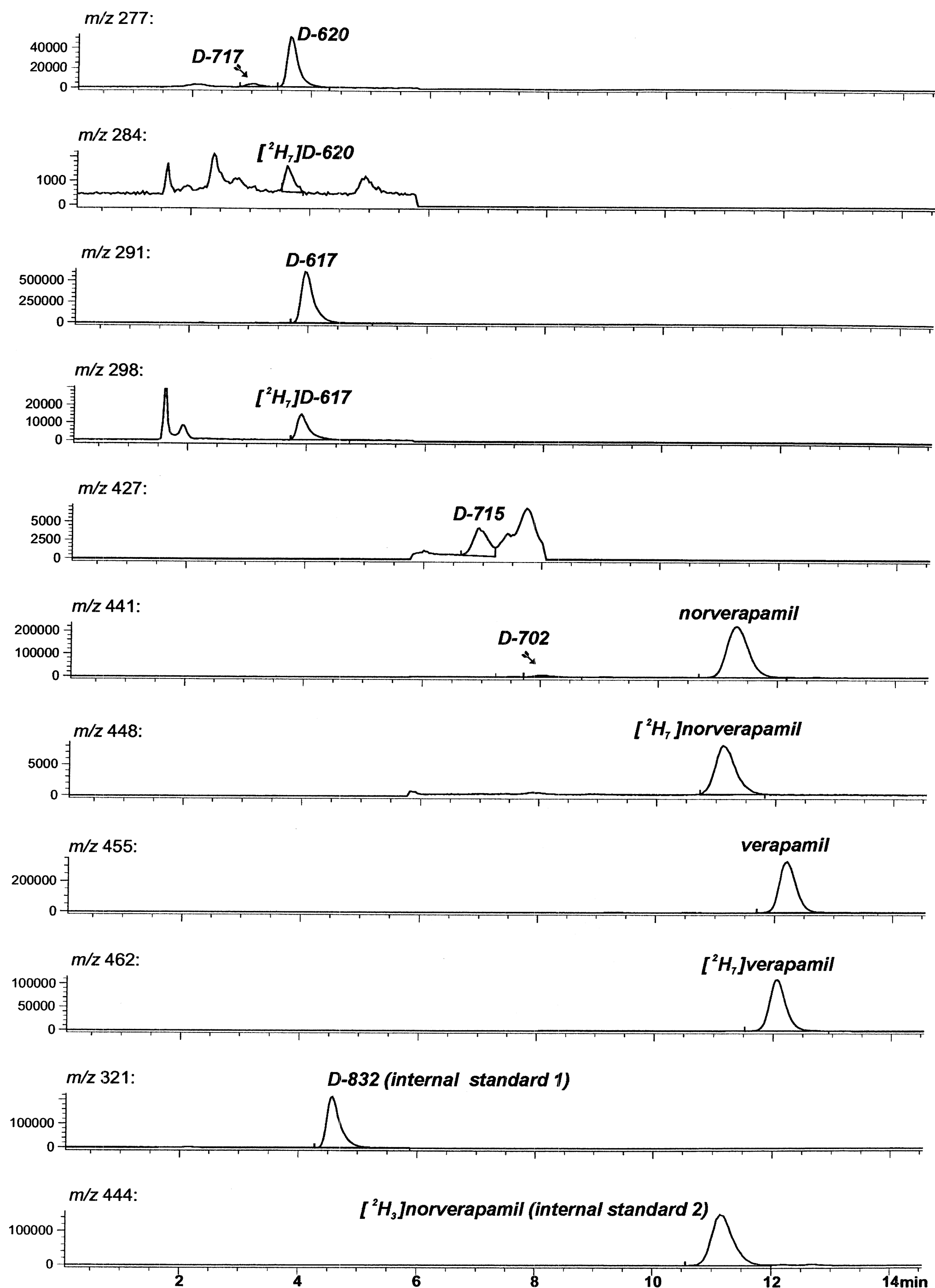

Fig. 4. Mass chromatograms of an extract of 1 ml plasma after treatment with verapamil and [$^2H_7$]verapamil. Concentrations found [pmol/ml]:1.3 D-717, 27.8 D-620, 1.03 [$^2H_7$]D-620, 122.4 D-617, 2.6 [$^2H_7$]D-617, 1.5 D-715, 4.4 D-702, 132.4 norverapamil, 5.4 [$^2H_7$]norverapamil, 120.8 verapamil, and 30.6 [$^2H_7$]verapamil.

Table 2
Intra-assay precision and accuracy for the determination of verapamil, [$^2H_7$]verapamil and metabolites in plasma

	Concentration added (pmol/ml)	*n*	Concentration found (pmol/ml)	Bias (%)	C.V. (%)
D-717	2.5	4	2.27	−9.3	7.1
	5	4	5.0	0.3	2.5
	50	4	51.3	2.5	2.5
	250	4	260.9	4.4	2.0
D-620	1	4	1.03	2.7	6.1
	5	4	4.6	−8.7	1.4
	50	4	47.7	−4.6	2.2
	250	4	253.8	1.5	2.0
D-617	1	4	0.91	−8.5	2.6
	5	4	5.0	−0.4	1.0
	50	4	51.5	3.1	3.8
	250	4	259.6	3.9	2.2
D-715	2.5	4	2.4	−3.9	3.1
	5	4	4.6	−7.8	1.8
	50	4	51.9	3.8	6.8
	250	4	254.1	1.6	2.9
D-703	1	4	0.93	−6.5	1.4
	5	4	4.7	−5.7	0.9
	50	4	51.0	1.9	7.1
	250	4	251.5	0.6	1.3
D-702	1	4	1.03	3.4	1.5
	5	4	4.9	−1.2	1.5
	50	4	49.7	−0.6	2.3
	250	4	260.7	4.3	2.1
Noverapamil	1	4	1.11	10.6	3.8
	5	4	4.4	−11.7	1.6
	50	4	49.1	−1.9	2.5
	250	4	257.4	3.0	3.0
Verapamil	1	4	1.13	13.4	3.5
	5	4	4.8	−4.9	2.7
	50	4	50.0	0.1	1.9
	250	4	253.5	1.4	2.4
[$^2H_7$]Verapamil	1	4	1.12	12.2	3.7
	5	4	4.7	−6.5	1.9
	50	4	49.5	−1.0	6.8
	250	4	256.2	2.5	3.1

in perfusate (8.75 nmol/ml) is 16-fold higher than C_{max} of D-702 (0.54 nmol/ml) which is shown in Fig. 6. In addition samples from pharmacokinetic studies using stable isotope-labelling techniques often contain the two differently labelled compounds and their metabolites at greatly different concen-

Table 3
Inter-assay precision and accuracy for the determination of verapamil, $[^2H_7]$verapamil and metabolites in intestinal fluid

	Concentration added (pmol/ml)	*n*	Concentration found (pmol/ml)	Bias (%)	C.V. (%)
D-717	5	20	5.4	8.7	6.0
	50	17	53.2	6.3	3.7
	250	10	238.9	−4.5	7.7
D-620	5	20	5.0	0.4	7.3
	50	17	51.2	2.4	5.9
	250	10	247.7	−0.9	6.8
D-617	5	20	5.1	1.2	2.5
	50	17	53.9	7.8	2.8
	250	10	255.2	2.1	3.0
D-715	5	20	5.1	1.6	8.2
	50	17	56.7	13.4	10.9
	250	10	256.9	2.8	12.1
D-703	5	20	4.8	−3.6	6.9
	50	17	52.0	4.0	7.1
	250	10	261.7	4.7	9.7
D-702	5	20	4.9	−2.9	6.1
	50	17	54.3	8.6	9.3
	250	10	255.1	2.0	9.0
Noverapamil	5	20	4.6	−7.1	6.4
	50	17	49.2	−1.6	4.1
	250	10	260.7	4.3	2.4
Verapamil	5	20	4.7	−6.2	10.5
	50	17	50.1	0.2	3.8
	250	10	258.1	3.2	2.8
$[^2H_7]$Verapamil	5	20	4.7	−6.5	6.9
	50	17	50.6	1.2	3.0

trations due to the different doses applied orally and intravenously, e.g. Fig. 7 shows plasma concentration time curves of D-617 and $[^2H_7]$D-617 after oral administration of 80 mg verapamil and 5 mg $[^2H_7]$verapamil intravenously. Both metabolites D-617 and $[^2H_7]$D-617 can be determined up to 26 h after verapamil administration.

The assay presented here represents a robust method to determine verapamil and $[^2H_7]$verapamil and their metabolites over a broad concentration range with high precision and accuracy in human plasma and intestinal fluid.

Acknowledgements

This work was supported by grants from the Robert Bosch Foundation (Stuttgart, Germany) and the Bundesministerium für Bildung, Wissenschaft,

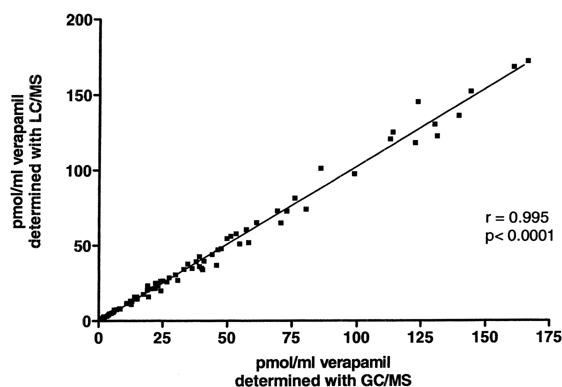

Fig. 5. Inter-assay validation of 86 plasma samples determined with solid-phase extraction – LC–MS and liquid–liquid extraction – GC–MS.

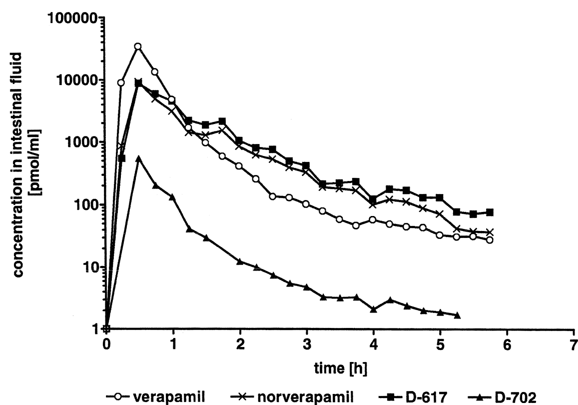

Fig. 6. Typical intestinal fluid concentration-time curves for verapamil and its metabolites norverapamil, D-617 and D-702 after an oral dose of 80 mg verapamil solution.

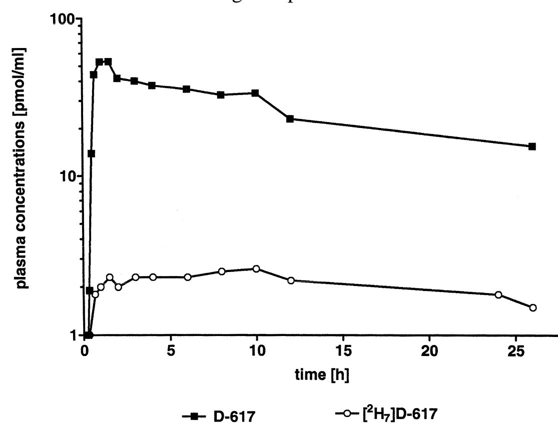

Fig. 7. Typical plasma concentration-time curves of D-617 and $[^2H_7]$D-617 after an oral dose of 80 mg verapamil and a simultaneous i.v. dose of 5 mg $[^2H_7]$verapamil.

Forschung und Technologie (BMBF grant BEO 0311782).

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