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Note

Determination of gallopamil in serum by gas chromatography–mass spectrometry

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Gallopamil, the methoxy derivative of verapamil (Fig. 1), is a calcium antagonist that has been demonstrated to be effective in the treatment of angina [1]. The pharmacological profile of gallopamil is similar to that of verapamil. However, gallopamil is three to five times more potent than verapamil as assessed by the negative inotropic effects produced in isolated heart muscle preparations from several species and the relaxation of smooth muscle in rat aortic strips [2]. To date little information on the pharmacokinetics and pharmacodynamics of gallopamil is available in the literature [3]. As a result of extensive first-pass metabolism, very low serum concentrations of gallopamil are observed [1]. It has been reported that the half-life of gallopamil is longer following oral than intravenous administration [3]. This observation is in all probability due to the limit of detection of the analytical techniques used, which

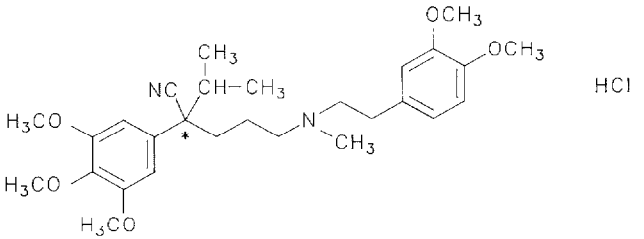

Fig. 1. Structure of gallopamil hydrochloride. The chiral carbon is indicated with an asterisk.

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can measure gallopamil concentrations in serum for only 5 h following intravenous administration or 6–8 h after oral dosing. In order to characterize the disposition of gallopamil fully, an extremely sensitive analytical technique, able to measure concentrations for up to 24 h after administration, which are as low as 0.1 ng/ml, is required.

The high-performance liquid chromatographic (HPLC) [4,5] and gas chromatographic (GC) techniques [6] recently published have a limit of sensitivity in the order of 1 ng/ml. The HPLC method of Nieder and Jaeger [7] employs fluorescence detection and has a limit of sensitivity of 0.2 ng/ml. However, to ensure selectivity, extensive sample clean-up is required. A number of methods [5–7] also assay the N-desmethyl metabolite norgallopamil. As less than 5% of the dose is eliminated as norgallopamil, which has only 10% of the activity of gallopamil [8], quantification of this metabolite in addition to gallopamil is unlikely to provide any further clinically significant information.

This paper reports a sensitive, specific and rapid gas chromatographic–mass spectrometric (GC–MS) method for the quantification of gallopamil in serum, which enables serum concentrations to be determined for up to 25 h following a single oral dose of 25–50 mg. The selectivity of MS allows for the extraction procedure to be rapid and simple. Up to twenty samples, representing a detailed characterization of the serum concentration–time curve of gallopamil, can be assayed daily. Detection by MS means that the method is suitable for investigating gallopamil disposition and metabolism using stable isotope labelling. The technique has been applied to a study of the disposition of the enantiomers of gallopamil administered as the pseudoracemate, a 1:1 mixture of unlabelled *R*-gallopamil and *S*-gallopamil labelled with two deuterium atoms at a metabolically inert position.

EXPERIMENTAL

Standards, reagent and glassware

R-(+)-Gallopamil hydrochloride (MW 521.1), *S*-(−)-[$^2H_2$]gallopamil hydrochloride (MW 523.1) and *R*/*S*-[$^2H_7$]gallopamil hydrochloride monohydrate (MW 546.1) were synthesized and generously supplied by Knoll (Ludwigshafen, F.R.G). The two hydrogen atoms at C-5 of *S*-gallopamil are replaced by deuterium atoms, and for the racemic internal standard the seven hydrogen atoms of the isopropyl group are replaced with deuterium atoms. The optical and isotopic purity of each compound was greater than 98%. Stock solutions were prepared in 0.01 *M* hydrochloric acid and dilutions for pipetting the standard curves were prepared in 20 μM verapamil hydrochloride. All gallopamil solutions were stored in the dark at 4°C. Diethyl ether (nanograde) was obtained from Promochem (Wesel, F.R.G.). Hexane (J.T. Baker, Phillipsburg, NJ, U.S.A.) was distilled prior to use. Acetonitrile was obtained from

Rathburn (Walkerburn, U.K.). All other reagents were of analytical grade and obtained from Merck (Darmstadt, F.R.G). Distilled water was used throughout. All glassware was washed with Extran MA03 (Merck) and rinsed with distilled water and acetone prior to air-drying. The tapered glass evaporation tubes were deactivated by treatment with 10% dichlorodimethylsilane (Merck) in toluene.

Instrumentation and chromatographic conditions

Gallopamil was analysed by GC–MS using a 5890A gas chromatograph (Hewlett-Packard, Palo Alto, U.S.A.) directly interfaced with a 5790B mass-selective detector (Hewlett-Packard) which operated in electron-impact selected-ion monitoring (SIM) mode at an electron energy of 70 eV and an emission current of 220 μA. A 1.2 m $\times$ 0.32 mm I.D. retention gap and a fused-silica capillary column (13.5 m $\times$ 0.2 mm I.D.) coated with OV-1 at a film thickness of 0.33 μm (HP-1, Hewlett-Packard) were used. Splitless injection at a temperature of 300 °C and a hot-needle injection technique were selected. The carrier gas was helium at a flow-rate of 1 ml/min, leading to a column head-pressure of 100 kPa. The initial oven temperature was 80 °C, which resulted in cold-trapping of the sample applied to the column. This oven temperature was maintained for 0.8 min and was then increased by 35 °C/min to 300 °C. The detector transfer line was maintained at 300 °C. *R*- and *S*-[$^2H_2$]gallopamil eluted with retention times of 11.06 and 11.05 min, respectively, and the internal standard *R*/*S*-[$^2H_7$]gallopamil eluted at 11.0 min. Initial mass spectra of gallopamil and the stable labelled analogues were performed in electron ionization mode. The base peaks (M – 151) resulted from cleavage of the C–C bond β to the substituted amine nitrogen atom. The ions monitored were *m/z* 333, 335 and 340 for *R*-gallopamil, *S*-[$^2H_2$]gallopamil and *R*/*S*-[$^2H_7$]gallopamil, respectively. Each *m/z* can be focused upon with an accuracy of 0.1 *m/z*, and the exact masses monitored (dwell time 100 ms, frequency 2.6 Hz) were selected daily after the initial autotune procedure by injecting a test solution containing all three gallopamil species. The chromatograms were analysed using a 9000 series 300 Chemstation® (Hewlett-Packard) and macros written to assist in automatization of the integration procedure.

Serum extraction

To 1 ml of serum in a glass extraction tube fitted with a PTFE-lined cap were added 5 ng internal standard, *R*/*S*-[$^2H_7$]gallopamil hydrochloride (20 μl of a 250 ng/ml solution) and 125 μl of 2 *M* sodium hydroxide. After shaking briefly using a vortex mixer, 5 ml of the organic extracting solvent (diethyl ether–hexane, 50:50, v/v) was added. The tubes were mixed for 10 min on a head-to-tail mixer and then centrifuged for 10 min at 1500 *g*. The organic phase was transferred to tapered evaporation tubes and evaporated to dryness under

a gentle stream of nitrogen at 40°C. The residue was reconstituted in 30 μl of acetonitrile, and 0.8–1.8 μl was injected into the GC–MS system.

Standardization, accuracy and reproducibility

Standard curves were prepared on each day that analyses were performed, using drug-free heparinized plasma obtained from healthy volunteers. The standards spanned three order of magnitude, i.e. 0.05, 0.1, 0.25, 0.5, 2, 20 and 50 ng/ml, and were prepared using appropriate volumes of gallopamil standard solutions. The standard curves were evaluated by linear regression analysis, and gallopamil concentrations in serum samples were calculated from the mean of the slope parameter generated for each individual standard concentration. To ensure that concentrations determined on different days could be directly compared, quality control sera were prepared by adding known amounts of *R*- and *S*-[$^2H_2$]gallopamil hydrochloride to 30 ml of drug-free serum, which was divided into 1.2-ml aliquots and stored at −20°C. Quality control samples were assayed on each day that analyses were performed. The within-day precision of the method was assessed by extracting and analysing up to nine identical samples in one day. The between-day precision was determined by measuring each day aliquots of up to five different quality control sera and comparing the results obtained for each single assay on the consecutive analytical runs. The between-day variability was also evaluated by reassaying each day a random sample from the previous analytical run and comparing the results obtained from the two analyses. The accuracy over the entire concentration range measured was assessed by adding various amounts of gallopamil standard solutions to drug-free plasma and measuring the concentration present.

RESULTS AND DISCUSSION

The method described for the determination of gallopamil in serum by GC–MS has excellent sensitivity. The lowest standard routinely assayed was 0.05 ng/ml, and concentrations as low as 0.02 ng/ml could be assayed with a signal-to-noise ratio of 5:1. The excellent sensitivity was primarily due to the SIM detection technique chosen and the favourable fragmentation pattern of gallopamil. The base peak monitored represents 32% of the total ions formed. The excellent sensitivity is also a result of the high recovery of the single-step extraction procedure. As determined by comparing the response of extracted standards with standard solutions injected directly into the GC–MS system, the recovery of the extraction technique was 92%. The sensitivity of the technique is also enhanced by using a deuterated analogue of gallopamil as the internal standard, which will behave as a carrier of other gallopamil species when present at low concentration.

The specificity of the MS technique enables gallopamil to be measured after

a single extraction with hexane–diethyl ether (50:50). The methods for the analysis of gallopamil in serum published to date require multi-step back-extractions [4–7], which prolong substantially the analysis time required for each sample. Other organic solvents, such as diethyl ether, hexane, dichloromethane and ethyl acetate, were evaluated for their suitability as a gallopamil extraction solvent. The serum extracts obtained, however, were not as clean at the ions monitored, and/or the overall recovery was diminished. Typical chromatograms of serum extracts from a healthy volunteer before and after the administration of 50 mg of pseudoracemic gallopamil hydrochloride are shown in Fig. 2. No interfering peaks at the three masses monitored have been identified in the sera of healthy volunteers to date. The main metabolites of gallopamil do not interfere with the analysis of the parent drug as the compounds are not extracted at the high pH selected, the species chromatograph at retention times, or fragment to masses that are not monitored, or they require derivatization prior to GC.

The retention times of the gallopamil species labelled with deuterium are, as anticipated, shorter than that of the unlabelled compound. The chromatographic run time is 13 min. The total run time is, however, longer (18 min) owing to the temperature programme selected and execution of an automated integration procedure developed specifically for this assay with the Chemstation software.

During development of the analytical technique, loss of gallopamil at low concentrations (less than 1 ng/ml), presumably due to adsorption to the glassware, was observed. All evaporation tubes were therefore silylated prior to use. In addition all dilutions of gallopamil standard solutions were prepared in 20 μM verapamil hydrochloride. This structural analogue of gallopamil presumably saturates the remaining active sites on the glassware and consequently even low concentrations of gallopamil produce the GC–MS response expected relative to high concentrations. Ruggirello et al. [6] have also reported that gallopamil is lost to glassware during evaporation of the organic extraction solvent. These authors have added isopropyl alcohol to the silylated evaporation tubes to prevent adsorption. The verapamil present in the gallopamil solutions elutes at a shorter retention time (10.5 min) than gallopamil and therefore does not interfere with the gallopamil analysis in any way. Indeed, owing to the structural similarity of gallopamil and verapamil and the excellent chromatographic and MS characteristics of verapamil, this compound would also be a suitable internal standard for the quantification of gallopamil if the deuterated analogues were not available.

The method described has excellent linearity over the entire range of concentrations assayed. A typical standard curve for *R*-gallopamil is $y = 0.177x + 0.001$ ($r = 0.9997$) and for *S*-[$^2H_2$]-gallopamil is $y = 0.187x - 0.010$ ($r = 0.9999$). To prevent overloading of the electron multiplier at high concentrations, smaller sample volumes were injected and the electron-multiplier volt-

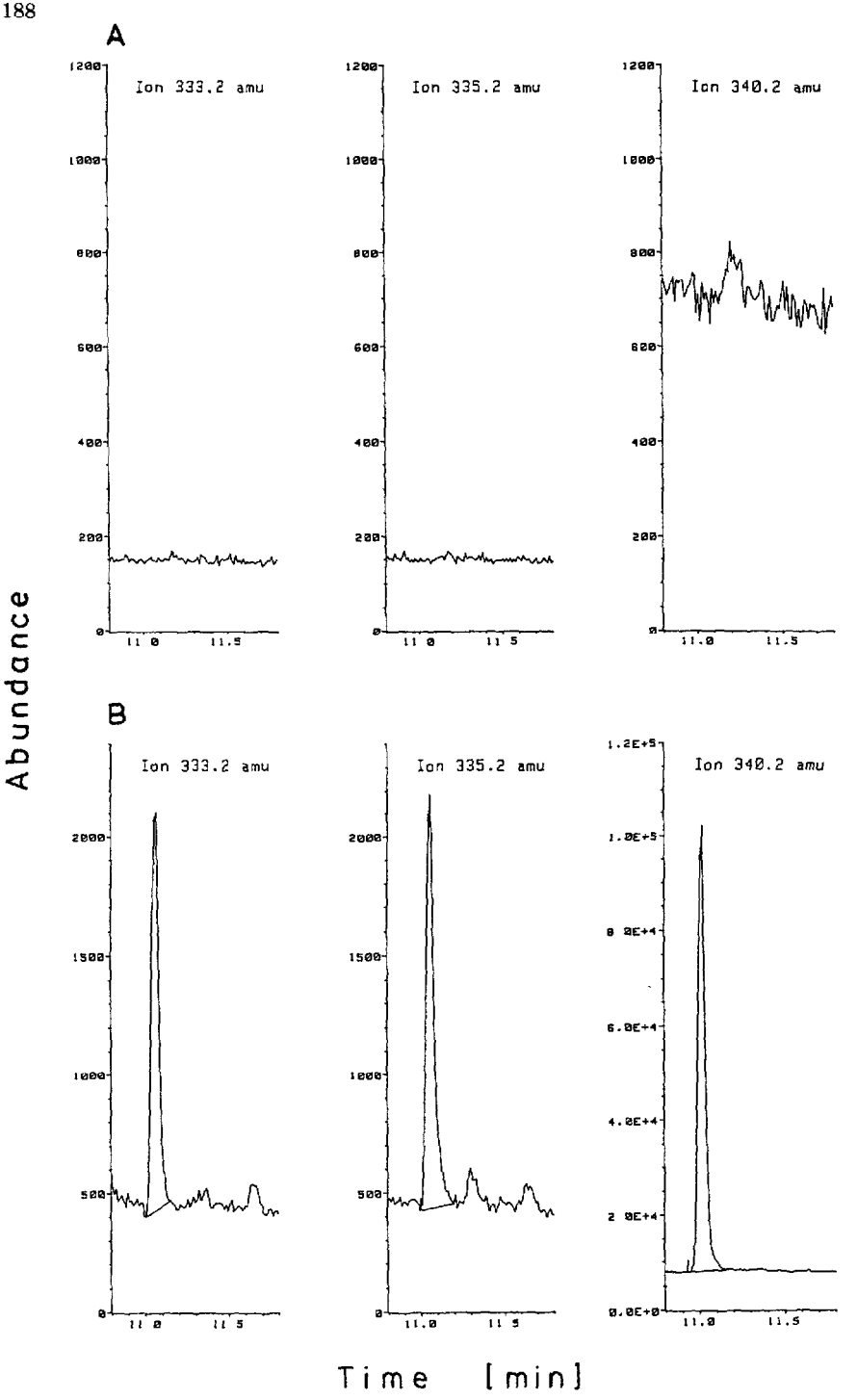

Fig. 2. Chromatograms of extracts from (A) drug-free serum and (B) serum from a healthy volunteer 25 h after administration of 50 mg of pseudoracemic gallopamil hydrochloride containing 0.089 ng/ml *R*-gallopamil hydrochloride (ion 333.2 *m/z*), 0.099 ng/ml *S*-[$^2H_2$]gallopamil hydrochloride (ion 335.2 *m/z*) and 5 ng/ml internal standard, [$^2H_7$]gallopamil hydrochloride (ion 340.2 *m/z*).

TABLE I

ACCURACY OF THE DETERMINATION OF *R*-GALLOPAMIL HYDROCHLORIDE AND *S*-[$^2H_2$]GALLOPAMIL HYDROCHLORIDE IN SERUM BY GC–MS.

The concentrations added to drug-free serum and the concentrations measured by GC–MS are given. The ratio of the assayed to the added concentration is shown as a percentage. The relative concentrations of *R*- to *S*-gallopamil present in the sample (*R*/*S* ratio) and the ratios calculated from the concentrations measured are given. The ratio of the relative concentration of the gallopamil enantiomers assayed to that added is expressed as a percentage.

R-Gallopamil hydrochloride			*S*-[$^2H_2$]Gallopamil hydrochloride			*R*/*S* ratio		
Concentration added (ng/ml)	Concentration assayed (ng/ml)	Assayed/added (%)	Concentration added (ng/ml)	Concentration assayed (ng/ml)	Assayed/added (%)	Added	Assayed	Assayed/added (%)
8.00	7.82	97.8	8.00	7.74	96.8	1.00	1.01	101
4.00	4.03	100.8	2.00	2.04	102.0	2.00	1.98	99
0.00	N.D.[a]		0.40	0.39	97.5			
0.80	0.81	101.3	0.70	0.68	97.1	1.14	1.19	104.4
0.30	0.32	106 7	0.25	0.26	104.0	1.20	1.23	102.5
0.20	0.21	105.0	0.30	0.30	100.0	0.67	0.70	104.5
0.90	0.91	101.1	0.90	0.89	98.9	1.00	1.02	102
45.00	40.54	90.1	25.00	23.42	93.7	1.80	1.73	96.1
30.00	29.54	98.5	40.00	40.69	101.7	0.75	0.73	97.3
0.40	0.48	120 0	0.00	N.D.				
20.00	19.03	95.2	24.00	23.87	99.5	0.83	0.80	96.4

[a]N.D. = not detected.

age was progressively reduced. The accuracy of the technique is demonstrated in Table I. The is an excellent correlation between the concentration of both *R*- and *S*-[$^2H_2$]gallopamil added and the actual concentration determined ($y = 1.0x + 0.003$, $r = 0.999$). The relative concentrations of *R*- and *S*-gallopamil determined therefore also agree well with the relative concentrations added. The ratio of the concentrations of the gallopamil enantiomers can therefore be determined accurately.

The concentrations of *R*- and *S*-gallopamil measured in the quality control sera assayed daily are also in good agreement with the expected concentrations present (Table II). The within-day and between-day precisions of the determination of both *R*- and *S*-[$^2H_2$]gallopamil are shown in Table II. Over the entire concentration range studied, the within-day precision for both gallopamil species is less than 10%. The results of the analyses of single aliquots of five different quality control sera, which were assayed on 47 consecutive days on which analyses were performed, are given as the between-day precision. At concentrations of both gallopamil species greater than 0.5 ng/ml the day-to-day variability of the assay is less than 10%. At the lowest concentration assessed (0.1 ng/ml) the day-to-day variation in the measurement of both *R*- and *S*-gallopamil is acceptable (12.3 and 14.4%, respectively). The good accuracy and precision of the method described are reflected in the excellent

TABLE II

WITHIN-DAY AND BETWEEN-DAY PRECISION

Values are mean ± S.D. of the measurement of gallopamil in serum by GC–MS. The accuracy of the method can be assessed from the difference between the concentration added and the concentration assayed.

R-Gallopamil hydrochloride				*S*-[$^2H_2$]Gallopamil hydrochloride			
Concentration added (ng/ml)	*n*	Concentration assayed (ng/ml)	C.V. (%)	Concentration added (ng/ml)	*n*	Concentration assayed (ng/ml)	C.V. (%)
Within-day							
0.10	9	0.10 ±0.005	4.8	0.1	9	0.09 ±0.007	7.9
0.25	7	0.25 ±0.01	4.7	0.25	7	0.24 ±0.01	6.2
10	8	11.0 ±0.2	1.8	10	8	10.5 ±0.2	1.6
Between-day							
0 10	47	0.097 ± 0.012	12.3	0.10	44	0.097 ± 0.014	14.4
0.60	27	0.62 ±0.05	7.5	0.80	27	0.79 ±0.07	9.3
0.90	20	0.93 ±0.09	9.9	1.20	20	1.23 ±0.12	9.6
22.5	21	22.5 ±2.0	9.1	17.5	21	18.0 ±1.9	10.6
57.9	9	54.9 ±4.5	8.1	47.8	9	47.4 ±2.0	4.2

TABLE III

SERUM CONCENTRATIONS OF *R*-GALLOPAMIL HYDROCHLORIDE

Values are determined during two consecutive analytical runs on samples from healthy volunteers administered unlabelled gallopamil hydrochloride (25 mg).

Sample	Concentration (ng/ml)	
	Assay 1	Assay 2
1	9.22	7.86
2	0.48	0.66
3	1.70	1.59
4	1.31	1.47
5	5.49	6.17
6	10.45	10.42
7	20.90	19.31
8	15.45	13.12
9	56.44	57.64
10	12.60	12.40
11	2.80	3.29
12	14.99	16.99

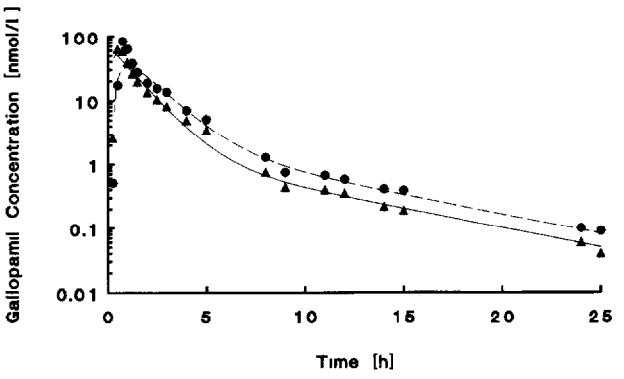

Fig. 3. Typical serum concentration–time profile of (▲) *R*- and (●) *S*-[$^2H_2$]gallopamil hydrochloride in a healthy male volunteer administed 50 mg of pseudoracemic (*R*- and *S*-[$^2H_2$]) gallopamil hydrochloride. The biexponential relationships fitted to the serum concentration–time data generated using non-linear least-squares regression analysis are also shown.

agreement between gallopamil concentrations determined during two consecutive analytical runs using serum samples obtained from healthy subjects administered 25 mg of unlabelled gallopamil hydrochloride (Table III). The correlation observed between the results of the two assays is $y=1.01x-0.22$ ($r=0.998$).

The technique described has been used to determine the concentration of gallopamil in serum samples obtained from young healthy volunteers administered a 50-mg dose of pseudoracemic (25 mg *R* and 25 mg *S*-[$^2H_2$] gallopamil hydrochloride. A typical serum concentration–time curve is shown in Fig. 3. The half-life of gallopamil determined after oral administration using this method is 4–8 h, which is longer than the previously reported values of 4–5 h [3], 2.5 h [5] and 1.9 h [6]. These studies utilized HPLC and GC analytical techniques, respectively, which have a limit of sensitivity in the order of 1 ng/ml. Using these methods gallopamil serum concentrations can only be measured for 6–8 h following oral administration. The excellent sensitivity of the method described here enables concentrations as low as 0.1 ng/ml, which are present 24–25 h after a single oral dose of 25 mg, to be measured with a high degree of accuracy and precision. The terminal half-life of gallopamil can therefore be determined accurately, and the pharmacokinetics of gallopamil following oral administration can be characterized reliably. Preliminary experiments indicate that without major modifications the technique is also suitable for the quantification of gallopamil in whole blood and urine.

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