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Note

Quantification of gallopamil and norgallopamil in human plasma by high-performance liquid chromatography with fluorescence detection

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Gallopamil, 2-methyl-3-cyano-3-(3,4,5-trimethoxyphenyl)-7-methylaza-9-(3,4-dimethoxyphenyl)nonane hydrochloride, is a calcium channel blocking drug, which was recently introduced for treatment of coronary heart diseases, such as angina pectoris. In humans, the drug is metabolized to several metabolites (Fig. 1), the most important of which is reported to be N-desmethylgallopamil (norgallopamil) [1]. This paper describes the first liquid chromatographic procedure for quantification of gallopamil and norgallopamil, which is sensitive down to subnanogram concentrations and selective to metabolites and biogenic interferences.

EXPERIMENTAL

Materials and reagents

Gallopamil, N-desmethylgallopamil, norverapamil and five other minor metabolites of gallopamil were kindly supplied by Dr. Brode, Knoll (Ludwigshafen, F.R.G.) and received as hydrochloride salts with an analytical purity of more than 99.5% by thin-layer chromatography. All other solvents and reagents were of either analytical or certified HPLC grade (E. Merck, Darmstadt, F.R.G.).

Chromatographic system and conditions

The modular liquid chromatograph consisted of a high-performance liquid chromatography (HPLC) pump (Model 1000, Merck-Hitachi), an autosampler (Model 116, Gilson-Abimed), a fluorescence detector (Model FS 970, Kratos) operated at an excitation wavelength of 230 nm with an emission filter (cut-off 340 nm), and an integrator (Model C-RlB, Shimadzu). The analytical column

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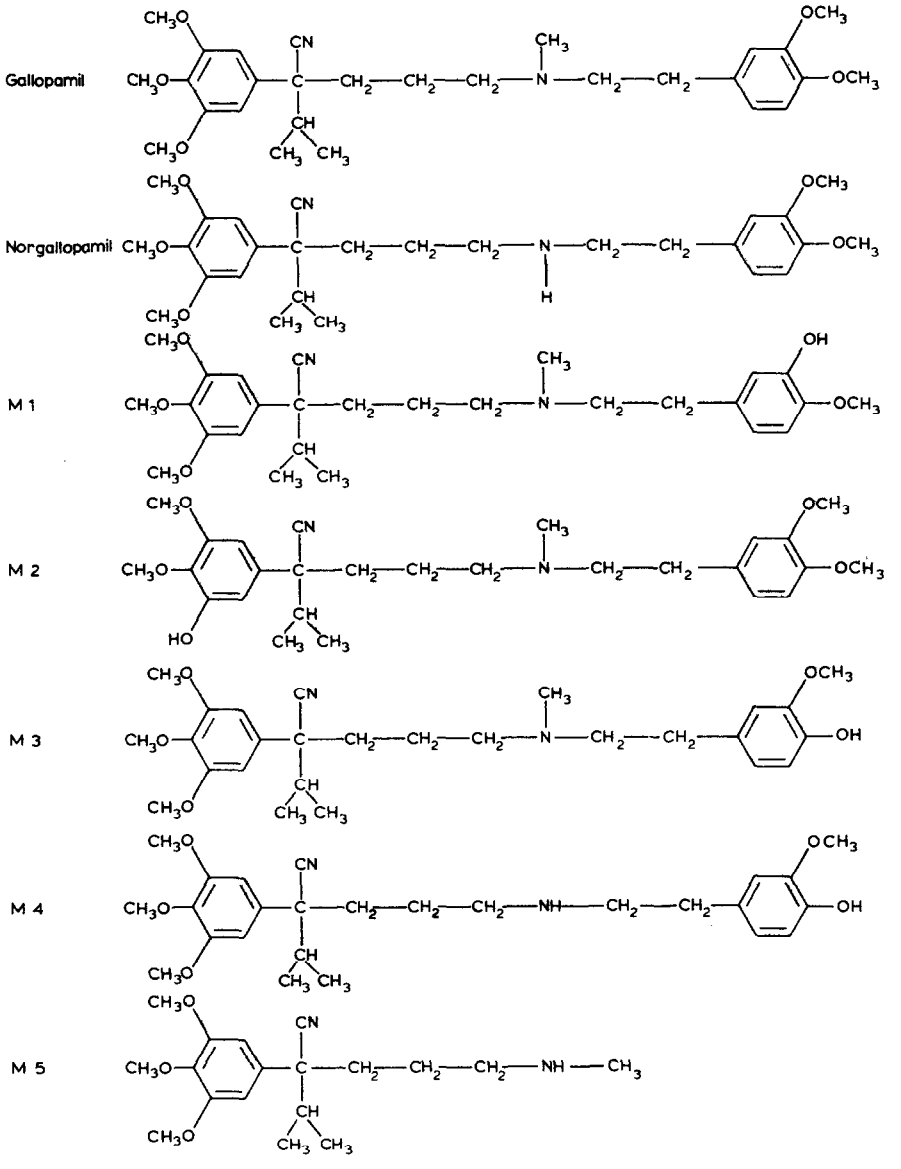

Fig. 1. Chemical structures of gallopamil and its metabolites.

(25 cm×4.0 mm I.D., packed with Shandon Hypersil ODS, particle size 5 μm) was thermostatted to a constant temperature of 30°C. The mobile phase consisted of 600 ml of acetonitrile, 400 ml of water and 4.0 ml of concentrated perchloric acid. The flow-rate was maintained at 1.0 ml/min, to create a typical backpressure of 6.0 MPa. Under the chosen conditions N-desmethylgallopamil, gallopamil and internal standard norverapamil were eluted at 12.5, 14.2 and 10.8 min, respectively. The fluorescence detector had a typical multiplier voltage adjust of −1200 V and a current sensitivity of 0.2 μA full scale. The integrator deter-

mined the peak-area ratios of the analytes and internal standard.

Solution preparation

Gallopamil- and N-desmethylgallopamil-spike solutions for use in plasma assay were prepared by dilution of the methanolic 100 μg/ml stock solutions with water to concentrations of 10.0 and 1.00 μg/ml. The standard spiking solutions were kept in the refrigerator for short periods (4°C) and in the freezer (−20°C) for long-term storage.

An internal standard spiking solution was prepared by diluting the methanolic 100 μg/ml stock solution with water to a concentration of 1.00 μg/ml. The internal standard spiking solution was kept in the refrigerator for storage. The stock and spiked solutions for quality-control (QC) samples were prepared and stored independently.

Spiked plasma samples

Standard spiking solutions of gallopamil and N-desmethylgallopamil and drug-free human plasma from healthy volunteers were added to tubes to give analyte concentrations of 0.20 and 0.02, 0.50 and 0.05, 1.00 and 0.10, 2.50 and 0.25, 5.00 and 0.50, 10.0 and 1.00 and 20.0 and 2.00 ng of gallopamil and norgallopamil per ml plasma, respectively. After mixing, the samples were frozen immediately and stored in the freezer until analysis. QC samples were prepared just before extraction in drug-free plasma.

Extraction method for plasma

Volumes of 1000 μl of plasma, 100 μl of 0.1 *M* sodium hydroxide, 10 μl of internal standard spiking solution and 6.00 ml of diethyl ether were pipetted into a conical glass tube. The tube was capped, shaken in the overhead shaker for 20 min and centrifuged for 20 min at 4000 *g*. Then 5.0 ml of the organic phase were transferred into another tube filled with 250 μl of 0.1 *M* hydrochloric acid. The tube was capped, shaken in the overhead shaker for 20 min and centrifuged for 20 min at 4000 *g*. The diethyl ether phase was then removed and the aqueous phase transferred to the microinserts of the sampler. Aliquots of 100 μl were injected automatically onto the column.

Concentration calculation

The printout of the integrator (peak-area ratio) for the calibration samples was used to establish a calibration curve with concentration-weighted linear regression. With this calibration curve the unknown sample concentrations were calculated. The calibration was valid from the lower limit of quantitation (LLQ) to the upper limit of quantitation (ULQ). Values below LLQ were not quantitated and given as "smaller than" in the printouts. Blanks, i.e. "zero concentrations", were not included in the calibration.

Prevalidation procedure

Two calibration ranges were investigated, 0.20–20.0 ng/ml for gallopamil and 0.02–2.00 ng/ml for norgallopamil. The procedure was evaluated in terms of sen-

sitivity, linearity of response, inaccuracy, imprecision, selectivity and recovery. For this purpose seven calibration curves in five different sequences were measured. The sensitivity of the method was evaluated by analysing spiked samples at the presumed LLQ. The linearity of response was investigated using spiked plasma standards carried through the method in seven different sequences. The inaccuracy of the method was assessed by statistical evaluation of the mean value of four QC concentrations and their nominal value. The imprecision of the method was assessed by statistical evaluation of the percentage standard deviation at those concentrations. Within-day and between day imprecision was also evaluated from these data.

In one sequence, plasma samples from patients dosed with gallopamil were processed to get an indication of real plasma levels. The selectivity was tested by processing the metabolites M1–M5, caffeine, nicotine, theophylline and propranolol through the method to evaluate their retention times.

Recovery from plasma

The recovery was evaluated by comparison of the peak areas of extracted concentrations with the peak areas of directly injected amounts with respect to the volumes handled during extraction. The following formula was used:

$$\text{Recovery}(\%) = \frac{P_{aa}}{P_{ae}} \cdot \frac{V_{oi}}{V_{oo}} \cdot \frac{V_{di}}{V_{in}} \cdot 100$$

in which P_{aa} = peak area of the directly injected amount, P_{ae} = peak area of the extracted concentration, V_{oi} = volume of extraction solvent added, V_{oo} = volume of extraction solvent transferred, V_{di} = volume dissolved for injection, and V_{in} = volume injected. The recovery was evaluated at 2.5 and 0.25, 10.0 and 1.00 and 20.0 and 2.00 ng of gallopamil and norgallopamil per ml plasma, respectively, in triplicate.

RESULTS AND DISCUSSION

Gallopamil and norgallopamil both have an intense native fluorescence, which is emitted after excitation at 276 and 230 nm. The emission ranges from 300 to 390 nm, with a peak wavelength of 340 nm. Excitation at 230 nm in combination with a 340-nm cut-off filter allows very selective and sensitive detection with a detection limit of ca. 0.10 ng/ml and an LLQ of 0.20 ng/ml for both analytes. The signal-to-noise ratio at the LLQ was more than 20; instrumental noise had no influence on quantitation. Fig. 2 shows the chromatograms of a plasma blank, a 0.20 and 0.02 ng/ml spike, a 2.50 and 0.25 ng/ml spike, a 20.0 and 2.00 ng/ml spike and a sample taken from a subject 1 h after oral administration of one 50-mg tablet of gallopamil hydrochloride.

The method is not interfered with by caffeine, nicotine, theophylline or propranolol. As shown in Table I, the method is also selective for the metabolites, which were eluted before the internal standard and the analytes.

Statistical evaluation of imprecision and inaccuracy data showed the LLQ for gallopamil to be 0.20 ng/ml, and for norgallopamil to be 0.25 ng/ml. Lower con-

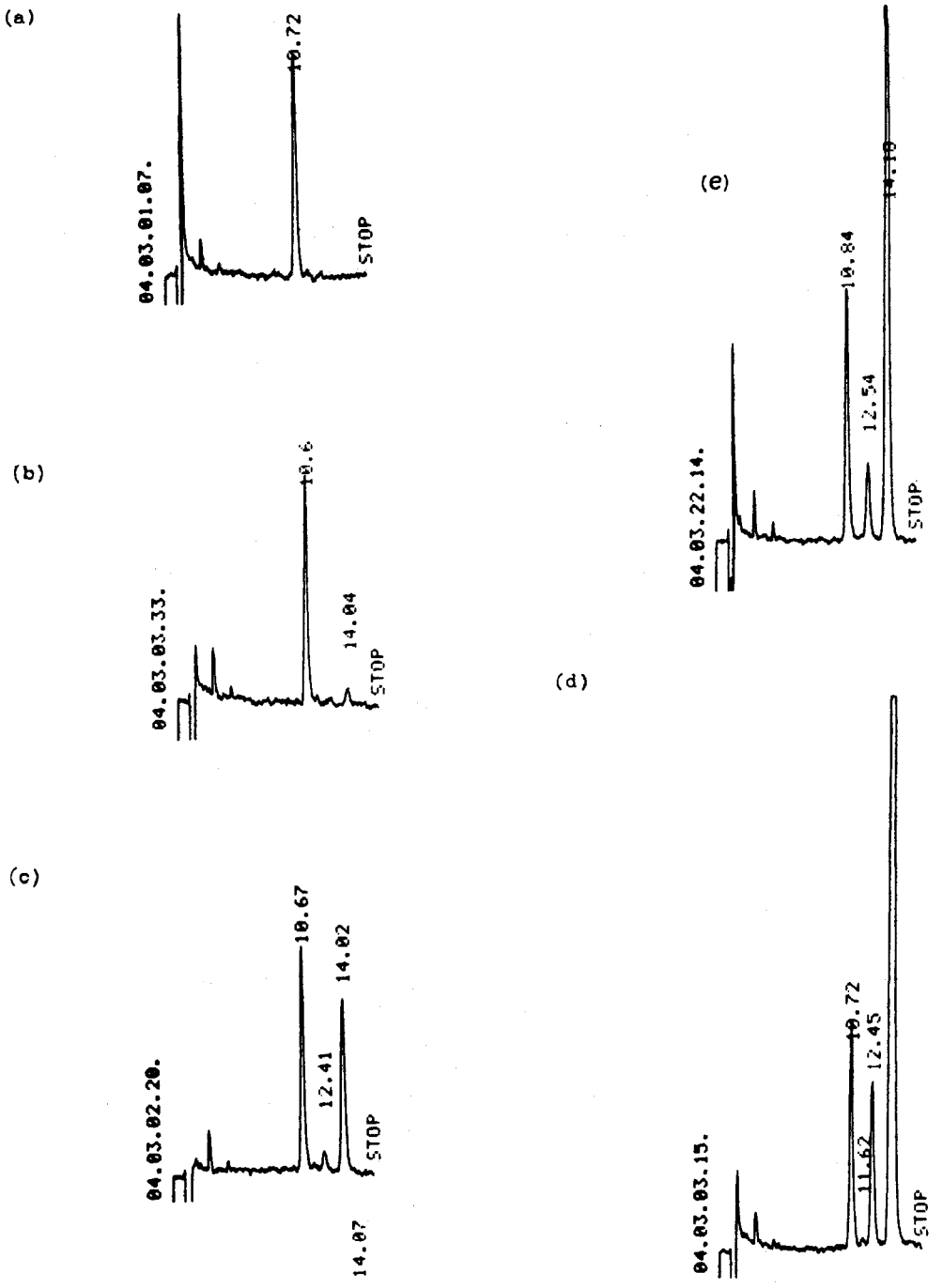

Fig. 2. Chromatograms of spiked and real plasma samples: (a) Blank plasma; (b) 0.20 ng of gallopamil per ml of plasma; (c) 0.25 ng of norgallopamil and 2.5 ng of gallopamil per ml of plasma; (d) 2.00 ng of norgallopamil and 20.0 ng of gallopamil per ml of plasma; (e) real plasma sample drawn from a healthy volunteer 1 h after oral administration of one 50-mg tablet of gallopamil hydrochloride; the peak at 10.8 min is internal standard norverapamil, that at 12.5 min is 0.95 ng/ml norgallopamil and that at 14.1 min is 13.7 ng/ml gallopamil.

TABLE I

INTERFERENCE STUDIES

Compound	Concentration in plasma (ng/ml)	Retention time (min)
Gallopamil		14.2
Norgallopamil		12.5
Norverapamil		10.5
Caffeine	1000	No peak
Nicotine	1000	No peak
Theophylline	2500	No peak
Propranolol	100	4.7
M5	100	4.8
M3	100	8.6
M2	100	8.0
M1	100	8.5
M4	100	7.4

centrations can be detected qualitatively with an imprecision of more than 20%.

The calibration curves for gallopamil were linear from 0.20 to 20.0 ng/ml, with typical correlation coefficients greater than 0.999. For norgallopamil they were linear from 0.25 to 2.0 ng/ml with typical correlation coefficients greater than 0.995. The calibration curves of both analytes had constant slopes and intercepts near the origin.

For gallopamil, the imprecision was 9.51% at the LLQ and 1.99% at the ULQ.

TABLE II

STATISTICS OF CALIBRATION CURVES 1-7 FOR GALLOPAMIL IN PLASMA

	Found concentration (ng/ml)			r^2	Found QC concentration (ng/ml)		
	Spiked with 0.20 ng/ml	Spiked with 2.50 ng/ml	Spiked with 20.0 ng/ml		Spiked with 0.20 ng/ml	Spiked with 2.50 ng/ml	Spiked with 20.0 ng/ml
	0.21	2.37	20.1	0.9999	0.22	2.54	19.1
	0.20	2.53	20.0	0.9999	0.20	2.46	19.8
	0.16	2.51	19.8	0.9999	0.17	2.54	19.7
	0.19	2.28	20.2	0.9997	0.14	2.49	19.5
	0.19	2.53	20.6	0.9997	–	2.73	20.7
	0.18	2.63	21.02	0.9940	0.25	2.67	19.9
	0.21	2.49	20.27	0.9996	0.24	2.78	19.5
Mean	0.19	2.48	20.3	0.9991	0.20	2.60	19.7
S.D.	0.02	0.12	0.40		0.04	0.13	0.50
Inaccuracy (%)	−4.1	−0.9	1.44		0.62	4.00	−1.3
Imprecision (%)	9.51	4.67	1.99		21.1	4.83	2.51

TABLE III

STATISTICS OF CALIBRATION CURVES 1-7 FOR NORGALLOPAMIL IN PLASMA

	Found concentration (ng/ml)			r^2	Found QC concentration (ng/ml)	
	Spiked with 0.25 ng/ml	Spiked with 0.50 ng/ml	Spiked with 2.00 ng/ml		Spiked with 0.25 ng/ml	Spiked with 2.00 ng/ml
	0.22	0.55	1.90	0.9920	0.26	1.83
	0.22	0.58	1.96	0.9960	0.27	1.82
	0.23	0.53	1.95	0.9989	0.30	1.97
	0.27	0.45	2.01	0.9989	0.26	1.87
	0.22	0.59	1.97	0.9960	–	1.90
	0.26	0.52	2.11	0.9956	0.26	1.95
	0.26	0.49	2.05	0.9988	0.22	1.93
Mean	0.24	0.51	1.99	0.9963	0.26	1.90
S.D.	0.25	0.51	0.69		0.04	0.58
Inaccuracy (%)	−4.3	5.74	−0.40		4.66	−5.20
Imprecision (%)	10.3	9.67	3.49		14.9	3.07

The inaccuracy was −4.1% at the LLQ and 1.44% at the ULQ. The respective QC data for imprecision ranged from 21.1% (low QC) to 2.51% (high QC). The respective QC data for inaccuracy ranged from 0.62% (low QC) to −1.3% (high QC). Table II shows the statistics of seven calibration curves and the corresponding QCs.

For norgallopamil, the imprecision was 10.3% at the LLQ and 3.49% at the ULQ. The inaccuracy was −4.3% at the LLQ and −0.4% at the ULQ. The respective QC data for imprecision were 17.5% (low QC) and 3.07% (high QC). The respective QC data for inaccuracy were 7.83% (low QC) and −5.2% (high QC). Table III shows the statistics of seven calibration curves and the corresponding QCs.

The recovery from plasma for gallopamil at 2.5 ng/ml was 101%, at 10.0 ng/ml 99% and at 20.0 ng/ml 99%. The recovery from plasma for norgallopamil at 0.25 ng/ml was 104%, at 1.00 ng/ml 98% and at 2.00 ng/ml 101%. The recovery from plasma therefore was quantitative for both analytes.

CONCLUSION

The data presented demonstrate the ability of our method to quantify gallopamil and norgallopamil in human plasma selectively with subnanogram sensitivity. The extraction procedure is simple and rapid with quantitative analytical recovery. Using automated HPLC about 60 samples can be processed daily.

REFERENCE

1 M. Kaltenbach and R. Hopf (Editors), Gallopamil, Springer, Berlin, 1983.