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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF VERAPAMIL AND NORVERAPAMIL IN BLOOD PLASMA OR SERUM

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SUMMARY

A simple high-performance liquid chromatographic method for the simultaneous measurement of plasma verapamil and norverapamil concentrations has been developed. The sample (100 μl) is vortex-mixed for 30 sec with 4 *M* sodium hydroxide solution, pH 13 (50 μl), internal standard solution (aqueous 5,6-benzoquinoline, 0.20 mg/l) (50 μl) and methyl *tert.*-butyl ether (200 μl). After centrifugation at 9950 × *g* for 2 min, a portion (100 μl) of the resulting extract is analysed on a microparticulate (5 μm) silica column using a methanolic solution of potassium bromide (3.0 m*M*) and perchloric acid (0.37 m*M*) as the mobile phase, and the column effluent is monitored by fluorescence detection using an excitation wavelength of 203 nm. A specimen, together with a quality control sample, can be analysed, in duplicate, within 30 min. The limit of accurate measurement of the assay is 2 μg/l, and no potential sources of interference have been identified. The method has advantages of speed, small sample requirement and complete resolution of the three major metabolites of verapamil.

INTRODUCTION

Verapamil (DL-2-(3,4-dimethoxyphenyl)-2-isopropyl-5-(N-methyl, N-β(3,4-dimethoxyphenyl)ethylamino)valeronitrile; Fig. 1) has anti-anginal, anti-hypertensive and anti-arrhythmic properties[1]. This compound is extensively metabolised by both N-demethylation and N-dealkylation (Fig. 1), and the N-demethylated metabolite (norverapamil) is pharmacologically active and can accumulate to plasma concentrations equal to or greater than those of verapamil itself[2,3].

Previously published techniques for the measurement of plasma verapamil concentrations have either measured metabolites together with the parent com-

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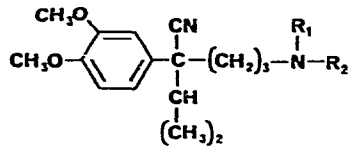


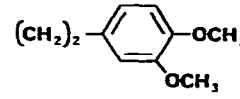
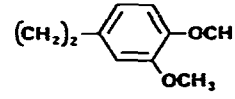
	R_1	R_2
VERAPAMIL (D365)	CH_3	
NORVERAPAMIL (D591)	H	
D617	CH_3	H
D620	H	H

Fig. 1. Structural formulae of verapamil, norverapamil and two other metabolites.

pound[4] or required relatively large sample volumes, long extract preparation times or the formation of derivatives prior to chromatographic analysis[5–9]. The method presented here is based on the direct high-performance liquid chromatographic (HPLC) analysis of an extract of a relatively small sample volume, and permits the simultaneous measurement of verapamil and norverapamil in the presence of the two remaining principal metabolites (Fig. 1).

EXPERIMENTAL

Materials and reagents

Verapamil and norverapamil hydrochlorides and the additional metabolites D617 and D620 (Fig. 1) were obtained from Abbott Labs. (Queenborough, Great Britain). The internal standard, 5,6-benzoquinoline, was obtained from Aldrich (Gillingham, Great Britain) and was used as a 0.20 mg/l solution in glass-distilled water. (This latter solution was prepared by dilution from a 1.00 g/l solution of 5,6-benzoquinoline in water–methanol, 80:20.) Methanol and methyl *tert.*-butyl ether were HPLC grade (Rathburn, Walkerburn, Great Britain), and perchloric acid (70%), sodium hydroxide and potassium bromide were all analytical-reagent grade (BDH, Poole, Great Britain). Sodium hydroxide was used as a 4 *M* solution in glass-distilled water.

High-performance liquid chromatography

The solvent delivery system was a constant-flow reciprocating pump (Applied

Chromatography Systems, Model 750/04) and sample injection was performed using a Rheodyne Model 7125 syringe-loading valve fitted with a 100-μl sample loop. Stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet port of the valve to the analytical column, a stainless-steel tube 125 × 5 mm I.D. packed with Spherisorb 5 silica (Hichrom, Woodley, Great Britain), which was used at ambient temperature (normally 22°C). The column effluent was monitored using a Schoeffel Model FS 970 fluorescence detector, with an excitation wavelength of 203 nm, no emission filter and a time constant of 0.5 sec. The mobile phase was a solution of potassium bromide (3 mM) and perchloric acid (0.37 mM, equivalent to 0.004%, v/v) in methanol, and was helium-degassed before use. The flow-rate was 2.0 ml/min, maintained by a pressure of approximately 60 bar.

The chromatography on this system of a methanolic solution containing verapamil and the three metabolites under study, together with the internal standard, is illustrated in Fig. 2. The retention times, measured relative to the internal standard, of verapamil, the metabolites under study and some additional compounds are given in Table I.

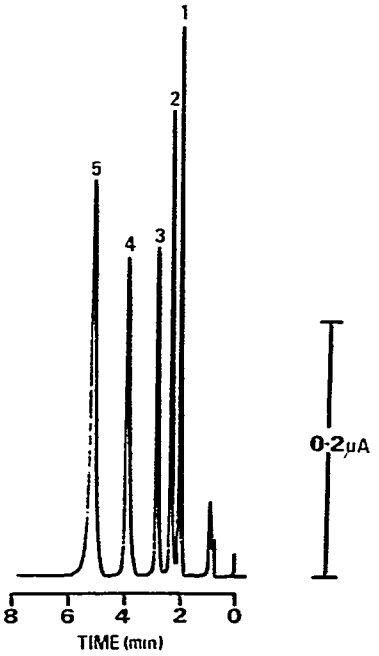


Fig. 2. Chromatogram obtained on analysis of a methanolic solution of D620 (1), norverapamil (2), D617 (3), verapamil (4) (all 0.10 mg/l) and 5,6-benzoquinoline (5) (0.20 mg/l); 100 μl injection. For chromatographic conditions, see text.

Sample preparation

Plasma or serum (100 μl) was pipetted into a small (Dreyer) test-tube (Poulton, Selfe and Lee, Wickford, Great Britain). Internal standard solution (50 μl), sodium hydroxide solution (50 μl) and methyl *tert.*-butyl ether (200 μl) were added using Hamilton repeating mechanisms fitted with Hamilton gas-tight luer-fitting glass sy-

TABLE I

RETENTION TIMES RELATIVE TO 5,6-BENZOQUINOLINE OF VERAPAMIL, NORVERAPAMIL AND SOME OTHER COMPOUNDS

See text for chromatographic conditions.

Compound	*Relative retention time*
Desalkylflurazepam	0.30
Mexiletine	0.31
Ajmaline	0.32
Nitrazepam	0.32
D620	0.37
Propranolol	0.42
4-Hydroxypropranolol	0.42
Nortriptyline	0.43
Norverapamil	0.45
D617	0.55
Doxepin	0.59
Desipramine	0.62
Amitriptyline	0.65
Dothiepin	0.69
Trimipramine	0.69
Atenolol	0.73
Verapamil	0.75
Orphenadrine	0.78
Imipramine	0.81
Chlorpromazine	0.83
Flurazepam	0.60–0.68*
Mianserin	0.85
Prajmalium	0.86
5,6-Benzoquinoline	1.00
Quinidine	4.60
Quinine	4.73

* Tailing peak: retention times measured at 100 mg/l and 1.00 g/l.

ringes and stainless steel needles. The contents of the tube were vortex-mixed for 30 sec and centrifuged at 9950 *g* for 2 min in an Eppendorf centrifuge 5412 (Anderman, East Molesey, Great Britain). Subsequently, a portion (approximately 110 μl) of the extract was taken and used to fill the sample loop of the injection valve.

Duplicate sample analyses were performed, and the mean result taken.

Instrument calibration

Standard solutions containing both verapamil and norverapamil at concentrations equivalent to 100, 200, 300, 500, 750 and 1000 μg/l of analyte free-base were prepared in heparinised human plasma by serial dilution of solutions of the appropriate hydrochlorides in methanol at concentrations equivalent to 1.00 g/l free-base. These standards were stable for at least 3 months if stored in small aliquots at −20°C in the absence of light. On analysis of these solutions, the ratio of the peak height of the analyte to the peak height of the internal standard, when plotted against analyte concentration, was linear and passed through the origin of the graph in each case

(Fig. 3). The calibration gradients (peak height ratio/analyte concentration) normally obtained were 0.0108 and 0.0126 l/μg for verapamil and norverapamil, respectively.

A further set of solutions prepared in heparinised human plasma containing verapamil and norverapamil at concentrations of 5, 10, 20 and 50 μg/l were available for use when indicated. The calibration obtained on analysis of these solutions was again linear and passed through the origin of the graph in each case. The calibration gradients obtained were the same as those quoted above.

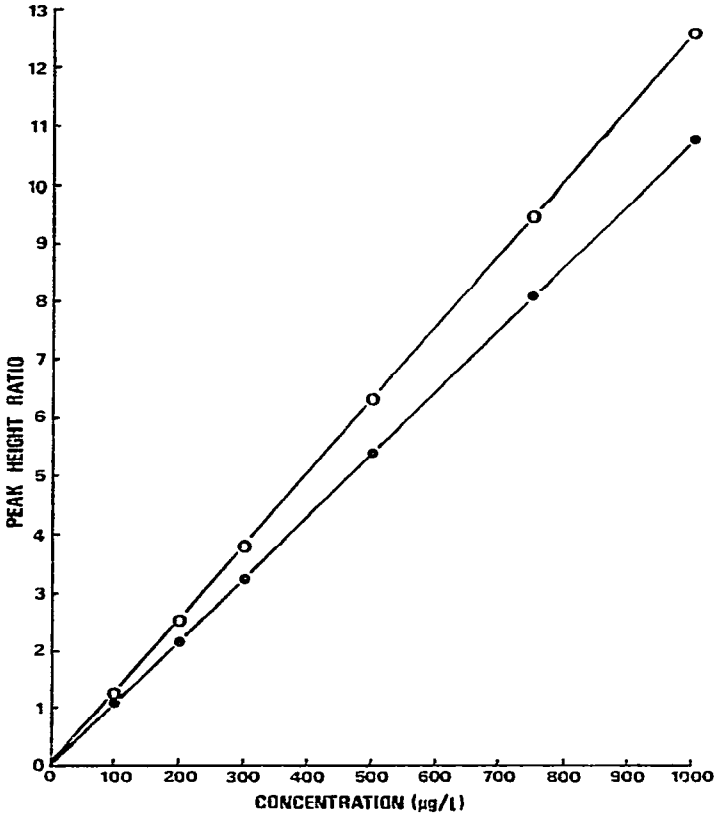


Fig. 3. Calibration graph of peak height ratio of verapamil (●) and norverapamil (○) against analyte concentration.

RESULTS AND DISCUSSION

Choice of chromatographic conditions

The use of inorganic anions such as bromide or perchlorate to promote the elution of basic and neutral drugs from silica columns has been described previously[10,11]. In this case, the use of perchlorate alone at a concentration of 1.85 m*M* (0.02%, v/v, perchloric acid) in methanol did not achieve complete resolution of metabolite D620 (Fig. 1) from norverapamil, even if a 250 × 5 mm I.D. column packed with Spherisorb 5 silica was used. On the other hand, the use of

bromide as counter-ion at a concentration of 3.0 m*M* gave complete resolution of verapamil and the metabolites under study, but did not promote the elution of the internal standard selected on the basis of the work with perchlorate, 5,6-benzoquinoline. However, the combination of both bromide and perchlorate used not only maintained the separation of verapamil and the metabolites studied but also promoted the elution of the internal standard at a satisfactory retention time (Fig. 2).

The system was found to be both stable and reproducible, and during routine use the relative retention times of the compounds under study (Table I) varied by ± 0.01, although the absolute retention times did vary up to 15%. Any deterioration in resolution or peak shape which did occur, however, was normally attributable to contamination of the chromatographic system with water or other compounds, or accumulation of insoluble material at the top of the column or in the microbore tubing from the injection port. Flushing with diethyl ether (approximately 50 ml) was often successful in removing soluble contaminants from the column, while simple cleaning and/or repacking of the top 2–3 mm of the column using a slurry of Spherisorb 5 silica in methanol was usually effective in prolonging the life of the column.

Selectivity

No endogenous sources of interference have been observed. The chromatogram obtained on analysis of a specimen of drug-free human plasma is illustrated in Fig. 4, and the chromatogram obtained from a plasma specimen from a patient treated chronically with verapamil is given in Fig. 5. Analyses of these and other specimens performed without the addition of 5,6-benzoquinoline have not revealed the presence of interfering compounds.

Compounds which were extracted under the conditions of the assay were studied further as potential sources of interference (Table I). Mexiletine, propranolol, orphenadrine, quinidine and quinine were detectable if present at concentrations similar to those attained during normal therapy, *i.e.* 5 mg/l or less, but were at least partially resolved from the compounds of interest on the chromatographic system (Table I). Other cardio-active drugs studied (amiodarone, disopyramide, lignocaine, lorcainide, metoprolol, procainamide, oxprenolol and tocainide) were not detected on this system. The relative responses of the remaining compounds studied (Table I) were generally much lower than those of verapamil and its metabolites, and so serious interference is unlikely to occur except following overdosage.

Detection conditions

The use of an emission cut-off filter was not thought to be necessary in view of the high selectivity shown by the system in normal use. This had the advantage of giving enhanced sensitivity (increased signal to noise ratio), and directly facilitated the measurement of very low verapamil concentrations. The lowest-attainable detector time-constant (0.5 sec) was used in view of the very rapid elution of the compounds of interest (Fig. 2) and also to minimise problems such as that reported with propranolol[12] where analyte peak area was reported to be inversely proportional to eluent flow-rates up to 1.4 ml/min using a time-constant of 6 sec.

Recovery studies

Standard solutions containing verapamil and norverapamil at concentrations

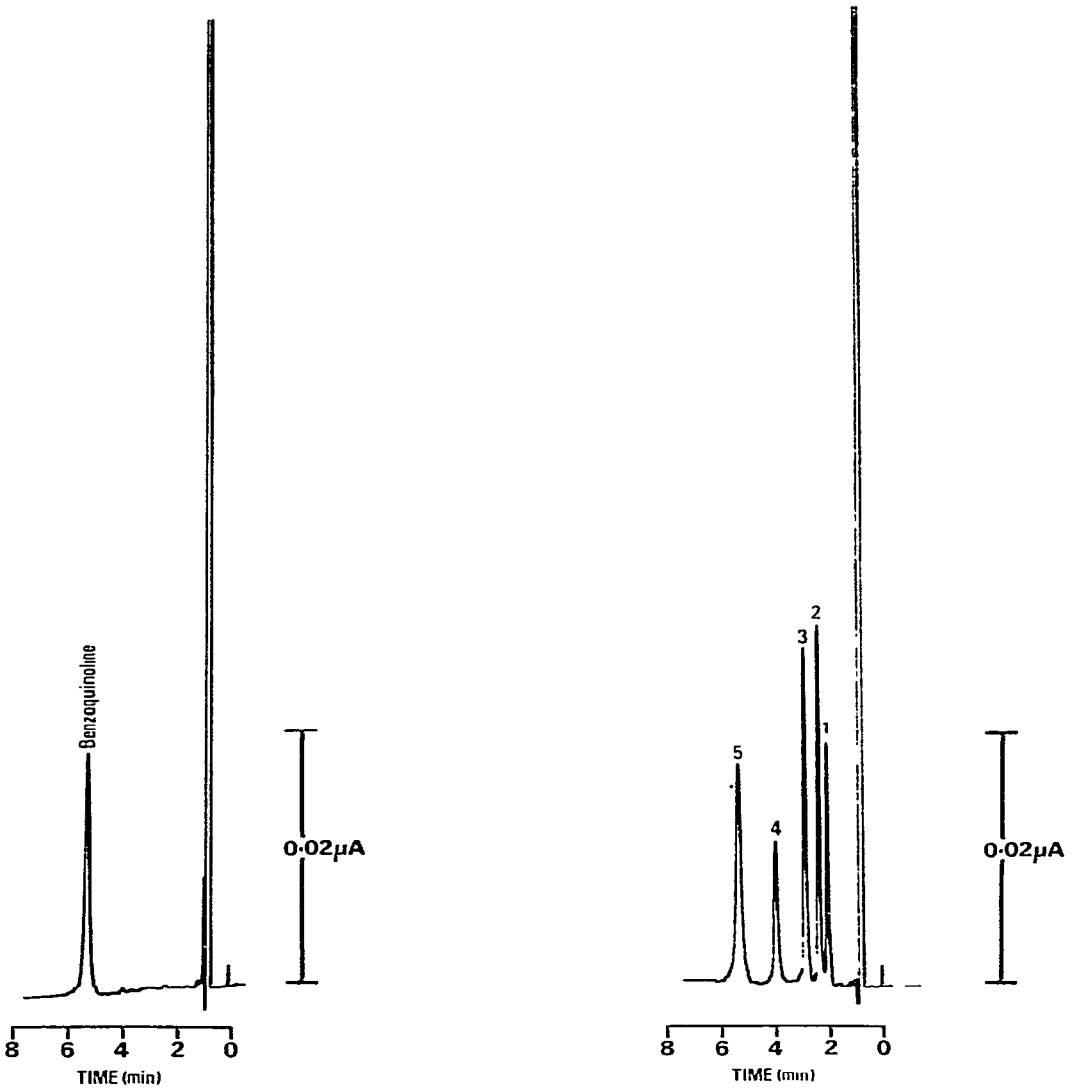


Fig. 4. Chromatogram obtained on analysis of a specimen of drug-free human plasma; 100 μl injection. The initial 5,6-benzoquinoline concentration was 0.20 mg/l.

Fig. 5. Chromatogram obtained on analysis of a plasma specimen obtained from a patient treated chronically with verapamil (3 × 120 mg/day); 100-μl injection. The initial 5,6-benzoquinoline (5) concentration was 0.20 mg/l, and the plasma verapamil (4) and norverapamil (2) concentrations were found to be 60 and 135 μg/l, respectively. (1 and 3 were D620 and D617, respectively; *cf.* Fig. 1.)

of 25, 50 and 100 μg/l were prepared in methanol by dilution from the 1.00 g/l stock methanolic solutions. The recovery of analyte from the corresponding plasma standards, *i.e.* 50, 100 and 200 μg/l, was calculated by comparison of the peak heights of verapamil and norverapamil obtained on analysis of 100 μl portions of the methanolic standards to those obtained from freshly-prepared sample extracts, and the

TABLE II

RECOVERY OF VERAPAMIL AND NORVERAPAMIL FROM HEPARINISED HUMAN PLASMA

$n = 10$ at each concentration.

Concentration (μg/l)	*Recovery (%; mean ± S.D.)*	
	Verapamil	*Norverapamil*
50	97.7 ± 2.9	94.5 ± 3.2
100	96.6 ± 3.4	88.7 ± 3.8
200	94.3 ± 3.0	89.4 ± 3.6

results are presented in Table II. The recovery of 5,6-benzoquinoline was only approximately 60% under the conditions of the assay.

Reproducibility

The intra-assay coefficients of variation (C.V.) measured from replicate analyses ($n = 10$) of standard solutions prepared in heparinised human plasma containing verapamil and norverapamil at concentrations of 5, 20, 50, 100 and 200 μg/l are presented in Table III. The inter-assay C.V. at 100 μg/l for verapamil was 2.98% and for norverapamil was 2.80% ($n = 10$ in both cases).

TABLE III

INTRA-ASSAY REPRODUCIBILITY OF THE ASSAY

$n = 10$ at each concentration.

Concentration (μg/l)	*Coefficient of variation (%)*	
	Verapamil	*Norverapamil*
5	4.4	4.8
20	3.1	4.0
50	2.9	3.0
100	1.3	2.8
200	1.2	2.2

Limit of sensitivity

The limit of accurate measurement of the assay was 2 μg/l using a 100 μl sample; the intra-assay C.V. at this concentration was 6.2% ($n = 10$). The use of larger sample sizes (*e.g.* 250 μl) could enable accurate measurements to be made down to concentrations of the order of 1 μg/l, should this be indicated.

CONCLUSIONS

The method described here has been used for the measurement of the plasma concentrations of both verapamil and norverapamil attained during therapy, and also

for the measurement of verapamil following single intravenous doses. Only 200 μl of specimen are required for a duplicate analysis, which can be completed, together with the analysis of a quality control specimen, within 30 min. No potential sources of interference have been identified, and it should prove possible to measure additional metabolites of verapamil if this was indicated.

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