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Note

High-performance liquid chromatographic measurement of verapamil in plasma using a diol column

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Verapamil is a calcium antagonist drug used in the treatment of cardiac arrhythmias, angina and hypertension [1]. Several high-performance liquid chromatographic (HPLC) methods for assay of verapamil in plasma, using C_8 or C_{18} reversed-phase columns and fluorimetric detection, have been reported [2—7]. However, in some of these methods, norverapamil, a major metabolite of verapamil with some pharmacological activity, has not been considered [2, 3, 7]. This metabolite has been reported to accumulate in man during repeated dosing with verapamil, and plasma levels of norverapamil comparable to those of the parent drug have been observed [8, 9]. Other published HPLC procedures [4—6] have described incomplete chromatographic resolution of verapamil and norverapamil and this problem has been addressed by introducing an acetylation step into the sample preparation [4, 5].

The use of fluorimetric detection limits the choice of compounds that can be used as internal standards to those with fluorescence spectra similar to those of verapamil. Analogues of verapamil such as D600 and D517, which do not form part of the metabolic pathway for the drug, have been used but complete chromatographic separation of these compounds from verapamil or norverapamil using reversed-phase columns has not yet been achieved in a run time acceptable for routine analysis [4—6]. Satisfactory resolution, however, has been obtained through the use of silica [10] or cation-exchange columns [11].

The present study describes an HPLC assay of verapamil in plasma using a

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diol column and fluorimetric detection. The method is both sensitive and selective in that complete chromatographic resolution of verapamil, norverapamil and internal standard is readily obtainable.

MATERIALS AND METHODS

Chemicals and solutions

Verapamil {α-isopropyl-α-[(N-methyl-N-homoveratryl)-γ-aminopropyl]-3,4-dimethoxyphenylacetonitrile}, the N-desmethyl metabolite, norverapamil, and the internal standard α-isopropyl-α-[(N-methyl-N-homoveratryl)-β-aminoethyl]-3,4-dimethoxyphenylacetonitrile (D517, I.S.) were obtained as hydrochloride salts from Knoll. Stock solutions of each compound were prepared in water at a concentration of 5.0 μg/ml base (Ajax Chemicals, Melbourne, Australia). All other chemicals were of analytical quality. Water was distilled. Glassware was silanized using Aqua Sil (Pierce, IL, U.S.A.).

Instrumentation

A Constametric IIG high-pressure pump (Labortory Data Control, Riviera Beach, FL, U.S.A.), a Model 7120 sample injection valve with a 20-μl loop (Rheodyne, Berkeley, CA, U.S.A.) and a Model MPF 3L fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) fitted with a 20-μl flow cell formed the basis of the liquid chromatograph. Sample fluorescence was monitored using an excitation wavelength of 280 nm and an emission wavelength of 314 nm. A slit width of 7 nm was used for both excitation and emission.

Chromatography was performed on a 250 mm × 4 mm, 5 μm particle size LiChrosorb Diol column (E. Merck, Darmstadt, F.R.G.) at ambient temperature. The mobile phase consisted of methanol–acetonitrile–0.001 mol/l aqueous diammonium hydrogen phosphate (70:10:30). The pH of the phosphate buffer was adjusted to 6.6 with 0.2 mol/l orthophosphoric acid prior to mixing of the mobile phase. The flow-rate was constant at 1.0 ml/min. Column back-pressure was approximately 17 MPa.

Sample preparation

To 1-ml plasma samples in PTFE-lined screw-cap glass tubes were added 30 μl (150 ng) of the internal standard stock solution, 200 μl of saturated aqueous sodium carbonate (pH 11.5) and 3 ml of 20% *tert.*-amylalcohol in hexane. After vortex-mixing for 3 min and centrifugation for 5 min at 1500 *g*, the organic layer was transferred to a clean glass tube, acidified with 200 μl of 1 mol/l hydrochloric acid and vortex-mixed for 2 min. After centrifugation, the organic layer was discarded. The aqueous phase was made alkaline by addition of 200 μl of saturated aqueous sodium carbonate (pH 11.5) and extracted with 2 ml of 20% *tert.*-amylalcohol in hexane as described above. The organic layer was separated by centrifugation, transferred to a silanized tapered tube and evaporated to dryness at room temperature under a gentle stream of nitrogen. The extract was reconstituted with 50 μl of the mobile phase of which 20 μl were injected into the chromatograph.

The assay was calibrated by addition of verapamil and the internal standard

to pooled blood bank plasma. Calibration curves were established for concentrations of verapamil up to 400 ng/ml, and for concentrations of norverapamil up to 200 ng/ml. Peak height ratios were used in quantitation of the assay.

RESULTS AND DISCUSSION

Typical chromatograms of extracted plasma specimens are shown in Fig. 1. The retention times for verapamil, norverapamil and the internal standard (I.S.) were 6.5, 9.0 and 3.6 min, respectively. Chromatograms of blank plasma extracts were free from any interfering peaks and no endogenous substances with prolonged retention were detected. Chromatograms of plasma extracts from patients who had been taking verapamil consistently contained an additional substance which eluted immediately after norverapamil (Fig. 1c). This was assumed to be one of the minor metabolites of verapamil. Monitoring of the chromatography up to 15 min did not reveal any additional peaks.

Calibration curves for verapamil passed through the origin and were linear up to 400 ng/ml, the maximum concentration used. The limit of sensitivity for the assay was approximately 1 ng/ml. The recovery of verapamil from plasma, following extraction, was assessed by comparison of the peak heights from plasma extracts with those obtained from standard solutions of the drug in water. At a concentration of 40 ng/ml verapamil, mean recovery was 78.5% (S.D. 5.3; $n = 4$). The intra-assay coefficient of variation for plasma extracts at a concentration of 40 ng/ml was 2.3% ($n = 7$). For plasma concentrations of 20 ng/ml and 80 ng/ml, the respective inter-assay coefficients of variation, assessed over a period of four weeks, were 2.1% ($n = 7$) and 4.3% ($n = 7$).

For norverapamil, mean recovery from plasma at a concentration of 20

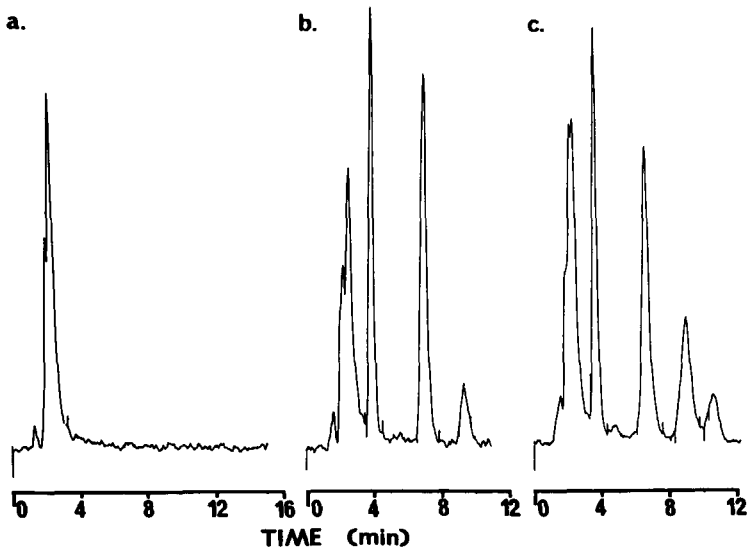

Fig. 1. Chromatograms of human plasma extracts: (a) blank plasma, (b) plasma containing 150 ng/ml I.S., 200 ng/ml verapamil and 100 ng/ml norverapamil, (c) plasma from a patient taking verapamil 80 mg t.d.s. Retention times of I.S., verapamil and norverapamil were 3.6 min, 6.5 min and 9.0 min, respectively.

ng/ml was 54.5% (S.D. 9.7; $n = 4$). The minimum detectable concentration was 3 ng/ml. The calibration curve was linear up to 200 ng/ml, the maximum concentration used, and at a concentration of 40 ng/ml, the intra-assay coefficient of variation was 2.3% ($n = 7$).

The assay has been evaluated using plasma samples from patients receiving verapamil for treatment of heart disease. Despite concurrent medication with a number of drugs including prazosin, theophylline, frusemide, chlorothiazide, disopyramide, propranolol, quinidine, hydrallazine and procainamide, no interference with the chromatographic measurement of verapamil or norverapamil has been detected. However, the potassium-sparing diuretic, amiloride, elutes in 11.0 min and may be detected after the elution of norverapamil.

The use of a diol column has resulted in improved separation of verapamil, norverapamil and I.S. in comparison to earlier methods which employed alkyl-chain reversed-phase columns [2—7]. Using the diol column, norverapamil elutes after verapamil, probably as a result of a greater degree of hydrogen bonding of the more polar norverapamil with —OH groups of the diol-bonded stationary phase. Chromatography on the diol columns has proven stable and reproducible. Unlike silica columns, the diol column undergoes rapid equilibration and is not susceptible to deterioration in performance through contamination of the chromatographic system with water. As a result, the method represents a reliable alternative to normal-phase chromatography of verapamil [10], and is of sufficient sensitivity and selectivity to be used in pharmacokinetic studies.

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