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Note

Measurement of verapamil in human plasma by reversed-phase high-performance liquid chromatography using a short octyl column

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Verapamil (Fig. 1) is a potent inhibitor of calcium flux across the cell membrane which makes it an active cardiovascular agent with antianginal, antihypertensive and antiarrhythmic properties [1,2]. After oral administration, verapamil is almost completely absorbed from the gastrointestinal tract. The parent drug is highly protein-bound and is extensively metabolized by the liver [3]. Although verapamil is highly absorbed when administered orally, its bioavailability is relatively low due to rapid first-pass metabolism.

Several gas chromatographic (GG) methods to determine verapamil in human biological samples have been described in the literature [4–7]. High-performance liquid chromatography (HPLC) has also been extensively used to

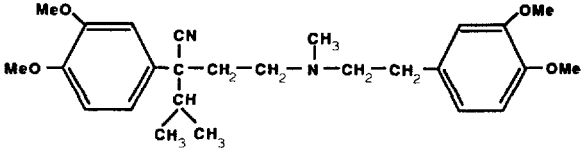

Fig. 1. Structure of verapamil.

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analyze verapamil and its major metabolites in human plasma, whole blood and/or urine [8–15]. Most of the methods suffer severe limitations in the procedure of pre-chromatographic isolation of verapamil from human biological samples. The use of a lengthy and complex extraction procedure is common for most of the methods as described in the literature.

In this paper, verapamil was isolated from human plasma in a single-step procedure. The isolated parent drug was analyzed by reversed-phase HPLC using a 80 mm×2.0 mm semi-microbore slurry packed column. This method is simple, rapid and has adequate sensitivity to conduct routine analysis even in the presence of few commonly prescribed drugs.

EXPERIMENTAL

Equipment

The HPLC system used was a Perkin-Elmer Series Bio-410 solvent delivery pump (Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with a Rheodyne 7275 sample injector with a 100-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). A slurry packer (Micromeritics, Norcross, GA, U.S.A.) was used to pack the 80 mm×2.0 mm analytical column with 5-μm octyl (C_8) particles. The UV–visible variable-wavelength HPLC detector (Model 783) was purchased from ABI Analytical (Ramsey, NJ, U.S.A.). The chromatograms were recorded on a Houston Instrument D5000 strip chart recorder (Houston Instrument, Austin, TX, U.S.A.). Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.). A 5.0-ml gas-tight syringe was used to filter the plasma samples of verapamil (Hamilton, Reno, NV, U.S.A.). A 0.45-μm Nylon-66 membrane filter-tip was used for the syringe (Rainin Instrument, Woburn, MA, U.S.A.). A Model 2200 Branson sonicator was used to degas the mobile phase (Branson Cleaning Equipment, Shelton, CT, U.S.A.). The samples were centrifuged by an IEC centrifuge, Model HN (Damon, IEC Division, Needham Heights, MA, U.S.A.).

Materials

Methanol, acetonitrile, isopropanol and *n*-propanol (all HPLC grade) were purchased from EM Science (Cherry Hill, NJ, U.S.A.). Magnesium sulfate, zinc sulfate and potassium dihydrogenphosphate were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). The 5-μm octyl (C_8) stationary phase (Spherisorb) was purchased from Alltech Assoc. (Deerfield, IL, U.S.A.). The borosilicate glass culture tubes and the disposable glass pipettes were purchased from Curtin Matheson Scientific (Elk Grove, IL, U.S.A.). The aged, pooled plasma was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI, U.S.A.). Verapamil was obtained from Knoll Pharmaceutical (Whippany, NJ, U.S.A.) and was used as received.

Preparation of stock solutions

A stock solution of verapamil was prepared by dissolving 10 mg of verapamil in 10 ml of acetonitrile. This solution was diluted appropriately to prepare the standard solutions of verapamil in plasma and water which were used to construct the calibration curves. A standard solution of verapamil (100 ng/ml) was injected into the HPLC system to determine the retention time under the chromatographic conditions used in this experiment.

Chromatographic conditions

The analytical column (80 mm × 2.0 mm), was slurry packed with 5-μm C_8 particles using an HPLC column slurry packer. The procedure of slurry packing has been described elsewhere [16]. The mobile phase consisted of 60% acetonitrile and 40% (v/v) 0.01 *M* potassium dihydrogenphosphate (dissolved in deionized water). The pH of the mobile phase mixture was adjusted to 7.1 with dilute sodium hydroxide (0.10 *M*). The flow-rate of the mobile phase was 0.80 ml/min. Verapamil was monitored by a UV–visible detector at 220 nm and 0.10 to 0.002 absorbance unit full scale (a.u.f.s.).

Purification of $MgSO_4$, $ZnSO_4$ and cleaning of borosilicate culture tubes

Purification of $MgSO_4$, $ZnSO_4$ and cleaning of borosilicate culture tubes was conducted by using a procedure described elsewhere [17].

Isolation of verapamil from plasma prior to chromatography

Verapamil was isolated from plasma by adding 250 μl of acetonitrile, 20 mg of $MgSO_4$ and 10 mg of $ZnSO_4$ in 1.0 ml of plasma solution. The effectiveness of an organic solvent to precipitate endogenous substances from human plasma was also investigated using ethanol, methanol, isopropyl alcohol, acetonitrile and *n*-propanol.

The frozen plasma ($-10\,^{\circ}C$) was thawed at room temperature and mixed, and a 1.0-ml aliquot was pipetted into a clean borosilicate culture test tube. An aliquot of standard verapamil solution was added to the plasma and vortex-mixed for 1 min. A 250-μl aliquot of acetonitrile and approximately 20 mg of $ZnSO_4$ and 10 mg of $MgSO_4$ were added to the plasma–drug mixture. This solution was vortex-mixed for 3 min and centrifuged for 6 min at 2000 *g*. The supernatant was transferred into a fresh borosilicate test tube with a disposable glass pasteur pipette. This solution was finally filtered through a 0.45-μm Nylon 66 membrane using a 5.0-ml gas-tight syringe. A 100-μl aliquot of the filtered solution was injected into the HPLC system.

Construction of the calibration curve

The stock solution of verapamil in acetonitrile was stored at $-25\,^{\circ}C$ and was found to be stable for at least three months. Six standard solutions of verapamil in plasma and water were prepared by adding enough stock solution (or

diluted stock solution) to obtain concentrations between 25 and 150 ng/ml. The prepared water and plasma solutions of verapamil were treated as described above. Under the experimental conditions, the calibration curves were linear from at least 20 ng/ml to 500 ng/ml.

RESULTS AND DISCUSSION

Fig. 2 is a typical chromatogram of human pooled plasma containing no verapamil. Fig. 3 is a chromatogram of plasma containing verapamil. From Fig. 2 it is clear that the retention time region of verapamil is free of interfering peaks from plasma endogenous substances. The verapamil quantitated in Fig. 3 was 110 ng/ml.

A linear calibration curve method was used instead of an internal standard method to quantitate verapamil in plasma samples. The calibration curves in plasma and water were straight lines ($r=0.998$) and essentially passed through the origin. The slope of the calibration curve of verapamil in plasma was approximately 25% lower than verapamil's calibration curve in water. This result indicates that 25% verapamil has been lost during sample preparation by coprecipitation with the plasma proteins. Therefore, one must use a calibration curve of verapamil obtained from standard solutions of verapamil in human plasma to determine the drug in unknown samples. The sensitivity of the

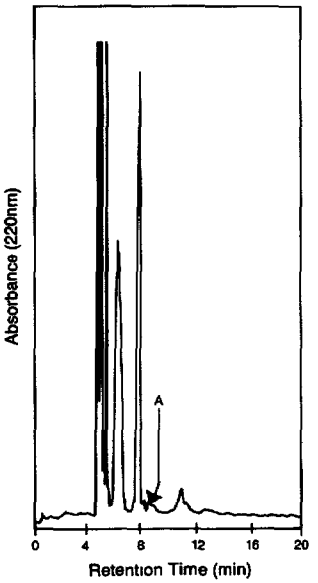

Fig. 2. Chromatogram of human pooled plasma with no verapamil. The retention time of verapamil is indicated by A. The detector was set at 220 nm and 0.005 a.u.f.s.

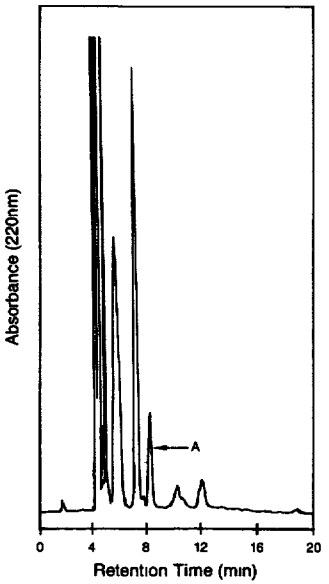

Fig. 3. Chromatogram of human plasma for verapamil. Peak A = verapamil. The detector was set at 220 nm and 0.005 a.u.f.s. The verapamil concentration was 110 ng/ml.

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ASSAY FOR WITHIN-DAY ANALYSIS

Actual concentration (ng/ml)	Concentration determined[a] (mean ± S.D., $n=4$) (ng/ml)	Accuracy (%)	R.S.D. (%)
40	42 ± 2	95.0	4.7
60	59 ± 3	98.3	5.1
80	82 ± 2	97.5	2.4
100	103 ± 3	97.1	2.9
150	146 ± 4	97.3	2.7

[a]Samples were prepared as described in the Experimental section.

method was adequate for typical sample concentrations (of patients) even after the loss of 25% of the drug during sample preparation.

The reproducibility and accuracy of the method were determined by multiple analyses of plasma samples spiked with an aliquot of standard verapamil solution to yield concentrations between 40 and 150 ng/ml. Table I shows the results for within-day analysis and Table II shows the results for between-day analysis. The relative standard deviation (R.S.D.) varied from 2.4 to 5.1% for within-day analysis and 2.5 to 5.1% for between-day analysis. The accuracy was greater than 95% for both within-day and between-day analysis. High ac-

TABLE II

REPRODUCIBILITY AND ACCURACY OF THE ASSAY FOR BETWEEN-DAYS ANALYSIS

Actual concentration (ng/ml)	Concentration determined[a] (mean ± S.D., $n=4$) (ng/ml)	Accuracy (%)	R.S.D. (%)
40	39 ± 2	97.5	5.1
60	62 ± 3	96.6	4.8
80	79 ± 2	98.8	2.5
100	103 ± 3	97.0	2.9
150	154 ± 4	97.3	2.6

[a]Samples were prepared in the Experimental section. Samples were analyzed every other day for seven days.

curacy and reproducibility for the assay indicates that the use of an internal standard in this method was not necessary.

Investigation of different organic solvents, sulfate salts of zinc, cadmium and magnesium and cupric chloride were investigated to obtain the one which precipitates the maximum amount of plasma proteins without precipitating verapamil. The 2:1 ratios of zinc and magnesium sulfate (with acetonitrile) used in this experiment was optimum for pre-chromatographic isolation of verapamil from plasma endogenous substances.

The cleaning of borosilicate culture tubes and purification of $ZnSO_4$ and $MgSO_4$ are critical steps of this experiment in order to achieve an interference-free verapamil peak. The addition of acetonitrile in the plasma sample caused precipitation of plasma proteins. $ZnSO_4$ and $MgSO_4$ caused an additional precipitation of plasma endogenous substances that were not affected by acetonitrile. If any of the above steps are omitted from the sample clean-up procedure, large peaks of plasma constituent elute with identical retention times as that of verapamil. The mobile phase ratios used in this experiment were optimum for the analytical column with respect to sensitivity and resolution of verapamil.

The HPLC method described in this report can be used for routine analysis of verapamil in plasma. The limit of quantification of the method is about 30 ng/ml for a 100-μl injection volume with an R.S.D. of 7.0% or less. Interference of norverapamil and some common prescription and non-prescription drugs was also tested and was found negative.

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