Journal of Chromatography, 222 (1981) 507—511
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 743

Note

Simultaneous determination of verapamil and its seven metabolites by high-performance liquid chromatography

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(Received August 7th, 1980)

Verapamil is an effective antiarginal and antiarrhythmic agent which exerts its effect through inhibition of membrane transport of calcium [1]. Pharmacokinetic studies have shown that verapamil undergoes extensive biotransformation in men and animals, and several N-dealkylated and O-demethylated metabolites have been identified [2,3]. Thus, it seems to be desirable to measure verapamil and its N-dealkylated and O-demethylated metabolites in biological fluids in order to evaluate the pharmacodynamics and disposition kinetics of verapamil.

Recently, Harapat and Kates [4] reported a simple and sensitive high-performance liquid chromatographic (HPLC) method for simultaneous determination of verapamil and norverapamil (N-demethylated metabolite) in plasma. This method is found to be adequate for the determination of verapamil and norverapamil, but it does not allow measurement of the other N-dealkylated metabolites or, moreover, the O-demethylated metabolites.

This paper describes a simple, rapid and selective HPLC method for the simultaneous determination of verapamil and its seven major metabolites (Fig. 1) in plasma. The procedure involves a simple extraction, paired-ion chromatography with reversed-phase column and fluorescence detection. The method can detect 2.5 ng/ml of each component in plasma, which is sufficiently sensitive for pharmacokinetic studies in human subjects.

EXPERIMENTAL

Materials

Verapamil, its seven metabolites (PR21, PR22, PR23, PR24, PR25, D617, D620) and internal standard (D600) were kindly supplied from Knoll (Ludwigs-

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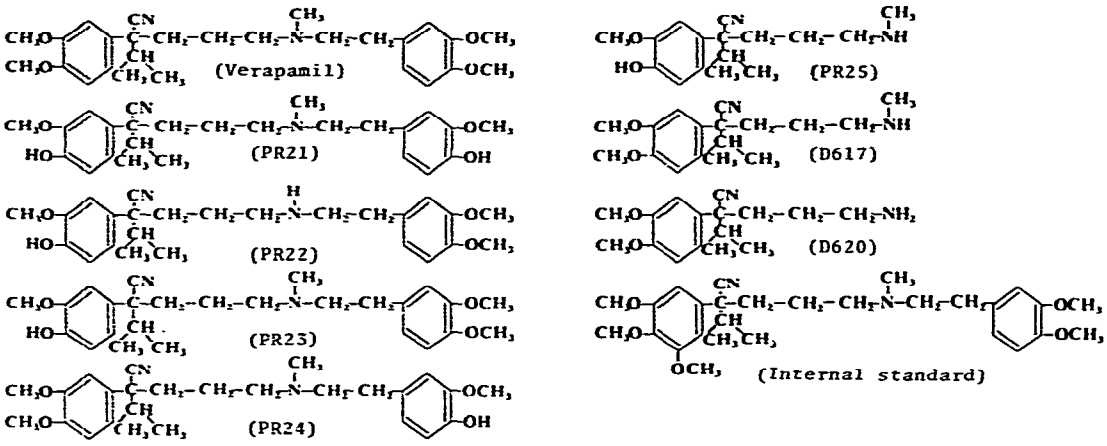

Fig. 1. Chemical structures of verapamil, its seven metabolites and the internal standard.

hafen, G.F.R.). The HPLC-grade acetonitrile and distilled water used for a mobile phase preparation were obtained from Wako Pure Chemical (Tokyo, Japan). The paired-ion reagent, 1-heptanesulfonic acid sodium salt, was obtained from Tokyo Kasei Kogyo (Tokyo, Japan).

Instrumentation

Chromatography was performed on a component system consisting of a Model 100A pump (Altex, Berkeley, CA, U.S.A.), Model 7120 injector (Rheodyne, Berkeley, CA, U.S.A.), Model FS-970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.) and Model U-225M dual-pen recorder (Nihon Denshi Kogyo, Tokyo, Japan).

Chromatographic separation was carried out on a 20 cm × 4.6 mm I.D. Nucleosil $5C_{18}$ column (Macherey, Nagel & Co., Düren, G.F.R.).

Extraction procedure

Samples (1 ml) of fresh human plasma spiked with known amounts of verapamil and seven metabolites were transferred to PTFE-lined screw-cap tubes, in which 25 μl (25 ng) of an aqueous solution of internal standard and 1 ml of distilled water were added. The mixture was extracted with 5 ml of diethyl ether for 20 min on a reciprocating shaker. After centrifugation (1000 *g*, 5 min), the aqueous layer was frozen in a dry-ice—acetone bath and the ether layer was decanted into a clean tube. A second extraction was performed in the same way. The combined ether layers were evaporated to dryness under a stream of nitrogen. The residue was reconstituted by vortex mixing in 200 μl of HPLC mobile phase and injected into the column.

Chromatographic conditions

The mobile phase consisted of acetonitrile and aqueous buffer solution. The buffer solution was prepared by dissolving 0.71 g of sodium hydrogen phosphate, 300 μl of phosphoric acid and 2 g of 1-heptanesulfonic acid sodium salt in 1 liter of distilled water. The solution was filtered through a Type HA

membrane filter (Millipore, Bedford, MA, U.S.A.) in vacuo. The mobile phase was then prepared by mixing buffer solution and acetonitrile (1:1, v/v) and the pH adjusted to 4.60 with 0.1 *M* phosphoric acid solution. Chromatographic analysis using the above solvent was carried out at a flow-rate of 1.5 ml/min.

The column temperature was maintained at 30°C by a regulated water-jacket. The excitation monochrometer of a fluorescence detector was fixed at 203 nm and the emission radiation was passed through a 320-nm cut-off filter. A detector range setting of 1.0 μA was used together with a recorder setting of 2 and 10 mV output.

RESULTS

Simultaneous separation of verapamil and its seven metabolites by gas chromatography was difficult because it was not possible to resolve verapamil and its O-demethylated metabolites. Reversed-phase and normal-phase chromatography provided incomplete separations. However, using paired-ion chromatography with a reversed-phase column, separation of verapamil and its seven metabolites in biological samples could be accomplished. Separation of each compound with maximum resolution from each other and from co-extracted contaminants was obtained using a mobile phase of acetonitrile—0.01 *M* phosphoric acid—sodium hydrogen phosphate buffer containing 0.2% 1-heptanesulfonic acid sodium salt (1:1, pH 4.60).

Fig. 2 shows the chromatograms of an extract of 1 ml of blank plasma (A), an extract of 1 ml of plasma sample spiked with verapamil, seven metabolites and internal standard (B), and a mixture of verapamil, seven metabolites and internal standard (C). As can be seen, the nine compounds were separated and eluted within 12 min. The retention times of the nine compounds are as follows: PR25 3.5 min; D620 4.0 min; D617 4.9 min; PR21 5.5 min; PR22 6.7 min; PR23 7.4 min; PR24 7.7 min; verapamil 10.7 min; internal standard

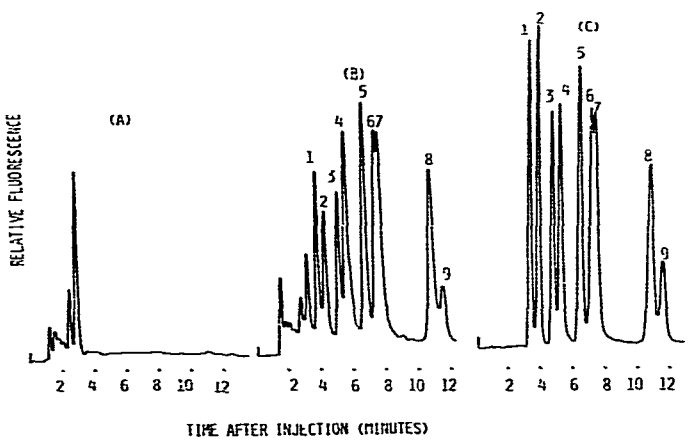

Fig. 2. Chromatograms of (A) an extract of 1 ml of blank plasma, (B) an extract of a 1-ml plasma sample spiked with 25 ng of internal standard and 31.25 ng each of verapamil and seven metabolites, and (C) a mixture of 25 ng of internal standard and 31.25 ng each of verapamil and seven metabolites. Peaks: 1 = PR25, 2 = D620, 3 = D617, 4 = PR21, 5 = PR22, 6 = PR23, 7 = PR24, 8 = verapamil, 9 = internal standard.

11.5 min. The chromatogram of blank plasma demonstrated no interfering peaks under these conditions. PR23 (peak 6) and PR24 (peak 7) were not completely separated from one another (resolution factor 0.60), but quantitation of these compounds was possible by measuring peak heights [5].

Fig. 3 shows the calibration curves which were obtained by analyzing plasma samples spiked with verapamil and seven metabolites in the concentration range 2.5—125 ng/ml. Excellent linearity was achieved for each compound, with the extrapolated plots passing through the origin (correlation coefficient 0.99). The minimum detectable concentration was 2.5 ng/ml of plasma for each compound. The coefficients of variation for the normalized peak height ratios were less than 9.0% for each compound.

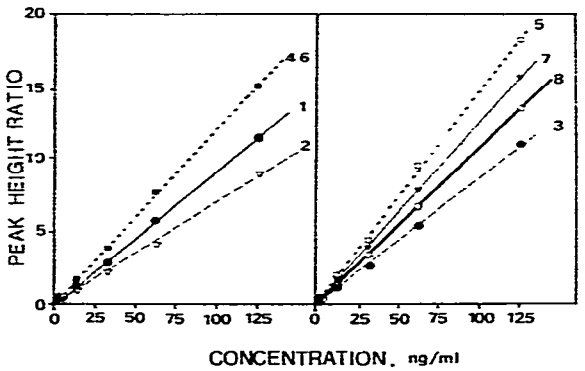

Fig. 3. Calibration curves of verapamil and seven metabolites. 1 = PR25, 2 = D620, 3 = D617, 4 = PR21, 5 = PR22, 6 = PR23, 7 = PR24, 8 = verapamil.

Table I shows the recoveries of each compound over the concentration range stated. The recoveries were estimated by comparing the peak heights of chromatograms obtained from extracted and directly injected samples of each compound. The coefficient of variation for each compound was found to be less than 8.3%. During extraction all compounds were observed to be stable.

TABLE I

RECOVERY OF VERAPAMIL AND ITS SEVEN METABOLITES FROM PLASMA

Values given are percentage recoveries representing the mean ± S.D. of three determinations.

Compound	Concentration in plasma (ng/ml)				
	2.5	12.5	31.25	62.5	125.0
PR25	63 ± 2	63 ± 2	67 ± 4	61 ± 3	58 ± 2
D620	45 ± 3	48 ± 3	55 ± 8	46 ± 5	46 ± 1
D617	55 ± 1	69 ± 8	66 ± 5	71 ± 1	70 ± 2
PR21	80 ± 9	93 ± 8	95 ± 4	97 ± 5	96 ± 2
PR22	80 ± 1	89 ± 5	90 ± 5	94 ± 1	94 ± 3
PR23	87 ± 6	89 ± 4	97 ± 3	99 ± 1	96 ± 4
PR24	93 ± 2	92 ± 4	96 ± 4	99 ± 6	97 ± 4
Verapamil	90 ± 7	90 ± 6	87 ± 4	99 ± 1	98 ± 3

DISCUSSION

An HPLC method for the simultaneous determination of verapamil and norverapamil in plasma has been reported by Harapat and Kates [4]. Their method involves extraction with diethyl ether at basic pH and reversed-phase chromatography using a mobile phase of acetonitrile—0.004 *N* sulfonic acid (pH 2.4). However, this method does not allow one to measure O-demethylated and N-dealkylated metabolites except for norverapamil, since extraction at the basic pH used resulted in a reduction in the extraction efficiency of the O-demethylated metabolites. When a strongly acidic solution is used as mobile phase, the N-dealkylated metabolites are not retained by the reversed-phase column due to the fact that these compounds are ionized in the mobile phase solvent.

In this study, improvement of these disadvantages could be made by changing the extraction procedure and chromatographic conditions. This method has advantages that include simple and specific determination of verapamil metabolites but do not involve special and time-consuming procedures. In addition, the method can detect up to a level of 2.5 ng of verapamil and its seven metabolites in 1 ml of plasma, which is sufficiently sensitive for pharmacokinetic studies.

ACKNOWLEDGEMENTS

We are grateful to Knoll AG for the generous gift of verapamil and its metabolites. We also thank Dr. T. Fujita, Director, Section of Drug Metabolism and Biopharmaceutics, for discussion of the manuscript.

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