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Note

Simultaneous quantitation of verapamil, norverapamil, and N-dealkylated metabolites in human plasma following oral administration

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Verapamil, 5-[(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (I), a slow channel inhibitor, undergoes extensive hepatic metabolism after an oral dose with some of its metabolites reaching concentrations in plasma equal to or greater than those of verapamil [1]. Major metabolites include the N-demethylated compound, norverapamil (III), and two N-dealkylated compounds, 5-methylamino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (IV) and 5-amino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (V), shown in Fig. 1. Norverapamil in particular has been reported to have a coronary vasodilator potency of 20% compared to that of verapamil [2].

Six other methods, all with various limitations, have been published addressing the separation of verapamil from its metabolites and their quantitation by high-performance liquid chromatography (HPLC). The original procedure of Harapat and Kates [3] was suitable for the separation of two N-dealkylated metabolites but was inadequate for the quantitation of norverapamil. Jaouni et al. [4] modified this procedure and acetylated norverapamil to prevent its extraction and interference with verapamil. Harapat and Kates [5] also modified their original procedure and thereby were able to measure norverapamil but not the N-dealkylated metabolites. Cole et al. [6] quantitated verapamil, norverapamil and the N-dealkylated metabolites but required potassium bromide, which is corrosive to stainless-steel solvent delivery systems, as a component of the mobile phase. Piotrovski et al. [7] separated the same four compounds but only quantitated verapamil. Kuwada et al. [8] have reported the separation and quantitation of verapamil, five O-demethylated metabolites and the two N-dealkylated metabolites, but did not report the separation of norverapamil.

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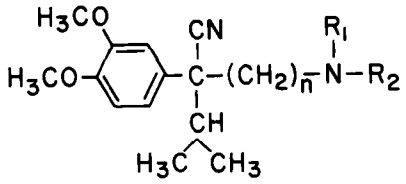


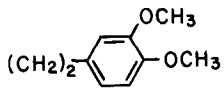
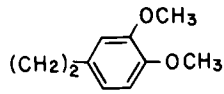
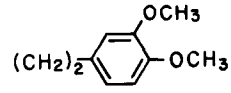
	n	R_1	R_2
I	3	CH_3	
II	2	CH_3	
III	3	H	
IV	3	CH_3	H
V	3	H	H

Fig. 1. Structural formulae of verapamil (I), internal standard (II), norverapamil (III), and two N-dealkylated metabolites (IV and V).

This laboratory has previously reported a rapid, simple, and sensitive method for the extraction of verapamil and its internal standard (II) from plasma with the subsequent quantitative determination of verapamil by HPLC separation and fluorescence detection [9]. The present study confirms the suitability of this method for the simultaneous separation and quantitative determination of verapamil as well as norverapamil, and the N-dealkylated metabolites IV and V, in human plasma following oral administration.

EXPERIMENTAL

Verapamil hydrochloride (I) was obtained from Knoll Pharmaceutical (Whippany, NJ, U.S.A.). Its internal standard, 4-[(3,4-dimethoxyphenylethyl)-methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylbutyronitrile (II), norverapamil (III), and IV and V, were obtained as the hydrochloride salts from Knoll (Ludwigshafen, F.R.G.). Glass-distilled heptane (Mallinckrodt, St. Louis, MO, U.S.A.) and HPLC-grade acetonitrile (MCB, Cincinnati, OH, U.S.A.) were used. Sodium hydroxide (2 *M*), sulfuric acid (0.1 *M*), and 0.1 *M* phosphate buffer, pH 3.0 were prepared with deionized water.

Other reagents and instruments, as well as the extraction, separation, and detection procedures were identical to our previous report [9]. A liquid chromatograph (Model 33, Altex, Berkeley, CA, U.S.A.) was used with a

fluorescence detector (Model 970, Altex). The column (15 cm × 4.6 mm I.D., particle size 5 μm) (Ultrasphere ODS, Altex) was a C_{18} reversed-phase. The only exception was a change in the proportions of the mobile phase to acetonitrile—0.1 *M* potassium phosphate, pH 3.0 (34:66). The solvent was degassed prior to use by applying a vacuum. The flow-rate was set at 1 ml/min. The detector settings were 203 nm for excitation with a 320-nm emission filter.

To 0.5 ml of plasma were added 50 ng of internal standard, II, 0.2 ml of 2 *M* sodium hydroxide, and 3 ml of heptane. The mixture was shaken mechanically for 15 min and then centrifuged. The organic layer was transferred to a 5-ml conical tube, and 50 μl of 0.1 *M* sulfuric acid were added. The contents were mixed in a vortex mixer for 1 min and centrifuged, and a 10-μl sample was injected into the chromatograph.

RESULTS AND DISCUSSION

An extracted blank plasma sample is shown in Fig. 2A. In Fig. 2B a representative chromatogram is shown of a 0.5-ml plasma sample spiked to the following concentration: I = 94 ng/ml; internal standard, II = 85 ng/ml; III = 92 ng/ml; IV = 75 ng/ml; V = 90 ng/ml. The retention times for I, II, III, IV, and V are 14.8, 12.2, 13.3, 3.9, and 3.3 min, respectively. The concentrations of I, III, IV, and V, as determined from the chromatogram were 95, 95, 73 and 84 ng/ml respectively.

Fig. 3 shows the calibration curves obtained from spiked plasma samples containing verapamil, III, IV and V in concentrations up to 400 ng/ml. Samples that had lower verapamil concentrations also had lower concentrations of the metabolites. Peak height ratios were plotted as a function of the concentration of each compound and were linear within this range ($r \geqslant 0.99$). The

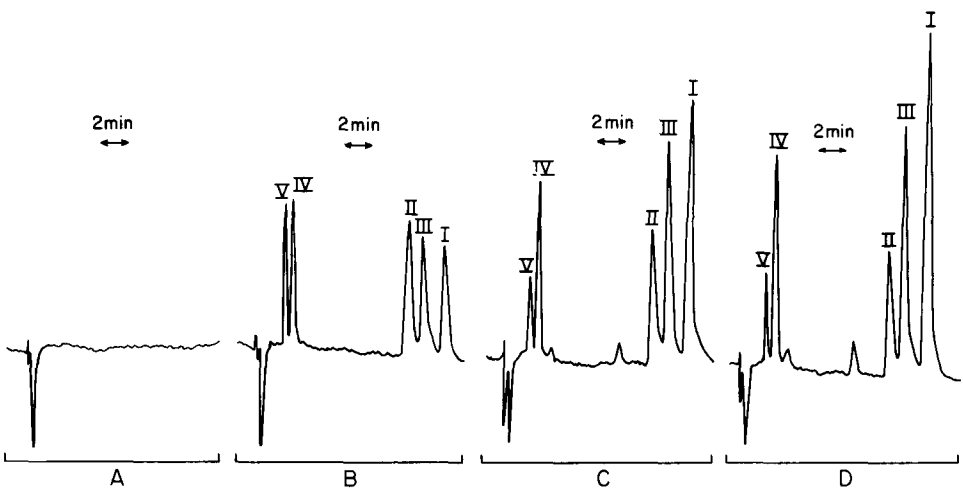


Fig. 2. Chromatograms of the extracts of 0.5-ml plasma samples. A = blank plasma; B = spiked plasma; C = patient plasma after four doses of oral verapamil, 120 mg every 6 h; D = plasma from the same patient as C, after one month of such oral verapamil therapy. Peaks: I = verapamil; II = internal standard; III = norverapamil; IV and V = N-dealkylated metabolites. Concentrations of the compounds are given in the text.

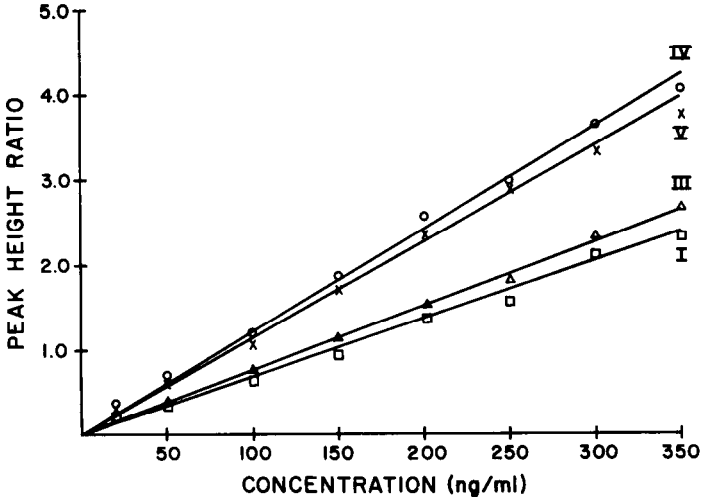


Fig. 3. Calibration curves of verapamil (I, □), norverapamil (III, △), and N-dealkylated metabolites (IV, ○ and V, ×).

coefficients of variation for the normalized peak height ratios of compounds I, III, IV and V over a concentration range of 10—500 ng/ml, assayed on seven different days over a two-week period, were 4.9%, 4.1%, 6.8%, and 6.9%, respectively. To evaluate intra-assay reproducibility, repeated analyses were performed of spiked plasma samples (n = 8) containing 94 ng/ml verapamil, 92 ng/ml III, 75 ng/ml IV, and 90 ng/ml V, to which was added 85 ng/ml internal standard, with coefficients of variation of 3.6%, 4.0%, 4.4% and 5.7%, respectively.

The recoveries of the four compounds were determined by comparing the peak heights of chromatograms obtained from extracted and directly injected samples of each compound at concentrations of 40, 120, and 350 ng/ml. The results are given in Table I, showing the consistency of compound recovery over the concentration range of interest. The minimum detectable concentration for the simultaneous determination of the five compounds was approx. 4 ng/ml of plasma, by requiring a signal-to-noise ratio of greater than 5:1.

Chromatograms of extracted plasma samples from a 46-year-old male patient receiving 120 mg of verapamil orally, every 6 h, for the treatment of

TABLE I

RECOVERY OF VERAPAMIL, INTERNAL STANDARD, AND METABOLITES FROM PLASMA

Concentration in plasma (ng/ml)	n	Percent recovery* (mean ± S.E.)				
		I	II	III	IV	V
40	4	63 ± 1	68 ± 1	65 ± 3	66 ± 1	51 ± 1
120	5	61 ± 3	64 ± 3	62 ± 4	65 ± 2	48 ± 2
350	4	65 ± 2	67 ± 2	67 ± 3	66 ± 2	48 ± 2

*I = verapamil; II = internal standard; III = norverapamil; IV and V = N-dealkylated metabolites.

paroxysmal atrial tachycardia refractory to other modes of therapy are shown in Fig. 2C and D. Fig. 2C is the chromatogram of plasma drawn after the first four doses of oral verapamil therapy. The concentrations of verapamil, III, IV and V after four doses (Fig. 2C) are 220, 175, 76, and 43 ng/ml, and in the one-month sample (Fig. 2D) are 320, 217, 100, and 56 ng/ml, respectively. The patient was taking no other medication of record to explain the peak noted between IV and II in Fig. 2C and D with a retention time of 9.5 min, which may represent a minor metabolite; however, it does not interfere whatsoever with the interpretation of peaks I through V.

The detailed evaluation of verapamil pharmacokinetics with regard to metabolism and disposition after oral administration depends upon a sufficiently sensitive and selective analytical method for the determination not only of verapamil, but of the major metabolites found in significant concentrations in the plasma of patients undergoing oral therapy. The contribution of active metabolites of antiarrhythmic agents to unanticipated drug efficacy or toxicity, as well as their accumulation with abnormalities of renal or hepatic function, has been recently reviewed [10]. Impaired hepatic function has been demonstrated to prolong the elimination phase after intravenous verapamil administration [11], but even in patients with normal hepatic and renal function, the elimination of both verapamil and norverapamil has been found to be prolonged during chronic oral therapy [12]. Measurements of plasma concentrations of verapamil and metabolites during oral therapy, therefore, may help decrease the frequency of adverse drug effects and improve the likelihood of successful therapy.

Toward these ends, the present method has been satisfactorily used to quantitatively determine verapamil as well as three major metabolites in human plasma. Following bolus intravenous verapamil administration, when no metabolites are anticipated, plasma samples can be rapidly analyzed [9]. For samples following long-term or oral administration, it is important to increase the aqueous fraction of the mobile phase during chromatography to provide for adequate separation of verapamil from norverapamil, with the concomitant determination of IV and V. With this modification the total retention time is still only 15 min. The suitability of this method for the assay of verapamil plus the three metabolites in multiple samples daily has the potential to significantly contribute to the understanding of interpatient pharmacokinetic differences and to aid the individualization of dosage and scheduling during oral therapy with verapamil.

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