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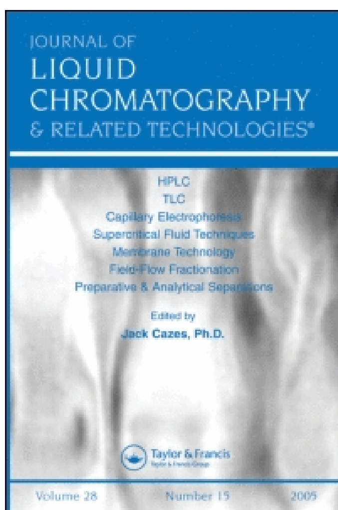

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SPECIFIC HPLC METHOD FOR THE SEPARATION OF VERAPAMIL AND FOUR MAJOR METABOLITES AFTER ORAL DOSING

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ABSTRACT

We have developed a high performance liquid chromatographic (HPLC) method which resolves verapamil, norverapamil, D620, D617 and what we believe to be another verapamil metabolite which has been previously unreported. An alkyl-phenyl column is used with a mobile phase of 0.005% sulfuric acid in methanol. The extraction recoveries of verapamil, norverapamil and the internal standard (imipramine) from plasma ranged between 98% and 104%. The day-to-day, and within-day coefficients of variations for verapamil and norverapamil at plasma concentrations of 7.3 and 233 ng/ml ranged between 1.7 and 6.1%. The limit of sensitivity was slightly less than 1 ng for both verapamil and norverapamil. Chromatograms of extracts of serum and urine obtained from five normal subjects who took single oral verapamil doses, indicated the presence of verapamil, norverapamil, and two other known metabolites. Chromatograms of serum extracts also indicated an additional peak which is probably another verapamil metabolite.

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INTRODUCTION

Verapamil is a calcium channel antagonist with antiarrhythmic, antianginal and antihypertensive properties (1). Verapamil undergoes extensive hepatic extraction; only 3-4% of the drug's total clearance is renal, and 12 metabolites have been identified in the urine of human subjects following single 80-mg oral doses (2). The major metabolite, norverapamil, is the only one that has been shown to possess some pharmacologic activity (3). Numerous high performance liquid chromatographic (HPLC) and gas-liquid chromatographic (GLC) assays for quantitation of verapamil and verapamil and norverapamil have been reported in the literature (4-30). A few HPLC methods report the separation of the two major N-dealkylated metabolites, D620 and D617 from verapamil and norverapamil (19-22). An additional assay reports the separation of seven metabolites from verapamil, but does not report data for the major metabolite, norverapamil (23). In this report, we describe an HPLC method which separates verapamil, norverapamil, D620, D617 and what we believe to be a fourth verapamil metabolite after oral administration. This method offers several advantages over existing HPLC assays; (i) the specificity of all peaks (including the internal standard) in relationship to the other verapamil metabolites has been demonstrated after oral dosing; and (ii) the method is superior to other published methods in either, the recovery and sensitivity of the method for verapamil and norverapamil or the

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simplicity and cost of mobile phase preparation. We also report the application of this method to a study of verapamil pharmacokinetics in five subjects who have taken single oral verapamil doses.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a pump (Model 2010, Varian Associates, Inc., Palo Alto, California, U.S.A.), an injector with a 50 ul sample loop (Model 7125, Rheodyne Inc., Cotati, California, U.S.A.) a fluorescence detector (Model 980, Spectro Inc., Ramsey, New Jersey, U.S.A.) and a linear chart recorder (Omniscribe, Houston Instruments, Austin Texas, U.S.A.). An alkyl-phenyl column (150 mm x 4.5 mm I.D., 5um particle diameter, IBM Instruments Inc., Danbury, Connecticut, U.S.A.) was used for separation of compounds. Equipment for extractions included 20-ml glass culture tubes with PTFE-lined screw caps and disposable 13 x 100 mm borosilicate culture tubes (American Scientific Products, McGaw Park, Illinois, U.S.A.).

Chemicals and Reagents

Hydrochloride salts of verapamil and eight of its metabolites (norverapamil, D620, D617, PR-21, PR-22, PR-23, PR-24, and PR-25) were provided by Knoll Pharmaceuticals, Ludwigshafen, G.F.R.. The internal standard, imipramine HCl, was obtained

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from Sigma Chemical Co., St. Louis, Missouri, U.S.A.. The concentrated sulfuric acid and anhydrous diethyl ether were A.C.S. reagent grade from J.T. Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.. Methyl alcohol (Mallincrodt, Paris, Kentucky, U.S.A.) was HPLC grade. The sodium hydroxide solution was prepared using double distilled water and A.C.S. reagent grade sodium hydroxide from Fisher Scientific (Fair Lawn, New Jersey, U.S.A.). High purity dry grade nitrogen was obtained from Union Carbide, Danbury, Connecticut, U.S.A.. Out-dated blood-bank plasma used for the preparation of drug standards was obtained from the Red Cross, Columbus, Ohio, U.S.A..

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Drug Standards

A reference mixture containing verapamil and eight metabolites (0.93 ug/ml of each) and imipramine (45 ug/ml) was prepared in water. A stock plasma standard containing verapamil and norverapamil (0.93 ug/ml of each) was prepared by spiking plasma with appropriate volumes of aqueous verapamil and norverapamil stock solutions (186 ug/ml). A urine stock solution was prepared in the same way, except urine was diluted 1:100 with distilled water before spiking. Working plasma and urine standards were prepared by serial dilution of stock plasma and urine standards to final concentrations of 3.5 and 465 ng/ml. Standards were stored in polypropylene tubes at -20 ^{o}C until used, and freshly prepared every three months. A working inter-

nal standard solution was prepared as 8.9 ug/ml of imipramine in water, and stored protected from light at 8 ^{o}C between uses.

Extraction Procedure

One milliliter of plasma, serum or diluted urine (1:100 with water) was pipetted into extraction tubes along with 50 ul (445 ng) of imipramine and 400 ul of 5N NaOH. Following the addition of 5 ml anhydrous diethyl ether, the mixture was agitated for 10 min on a reciprocating shaker and centrifuged for 10 min. The aqueous phase was frozen in a bath of dry ice and acetone, and the ether was decanted into disposable glass culture tubes and evaporated to dryness under a mild stream of filtered air in a 45 ^{o}C water bath. The residue was reconstituted with 50 ul of methyl alcohol, vortexed, and the entire volume injected onto the column.

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Chromatography and Calibration

The mobile phase was prepared by adding 50 ul of concentrated sulfuric acid to one liter of methyl alcohol, and degassed using sonication under a vacuum. The detector excitation wavelength was 209 nm and no emission cut-off filter was used. The initial operating range for detection was 0.02 relative fluorescence units and was adjusted as needed for increasing verapamil and norverapamil concentrations. A slow stream of dry nitrogen was run into the monochromator through the purge nipple

on a continuous basis during instrument use. The mobile phase flow was set at 1.0 ml/min; the chart speed was 0.25 cm/min.

Standard curves were constructed by plotting peak height ratios of drug to internal standard against known concentrations of drug standards. Non-weighted linear regression analysis was used for determination of unknown concentrations.

Analytical Variables

The extraction recoveries of verapamil, norverapamil and imipramine from plasma were determined by comparing the peak heights of extracted samples to those of unextracted standards. Extraction recoveries of verapamil and norverapamil were determined at concentrations of 46.5 and 465 ng/ml, respectively. The recovery of the internal standard from plasma was tested at the concentration used in samples, 445 ng/ml. Extraction recoveries of the seven verapamil metabolites (excluding norverapamil) from the aqueous reference mixture were also determined.

Linear ranges for detection were determined by injecting known amounts of verapamil and norverapamil and measuring the resulting peak heights. Linear regression analysis of peak height versus amount was performed on the three lowest values. The equation for this line was then used to calculate the next highest amount from its measured peak height. If the calculated amount deviated less than 5% from the known amount, it was included for regression analysis and the process was repeated.

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This was continued until the calculated amount deviated more than 5% from the known amount. This injected amount was considered the upper end of the linear range (31). The limit of sensitivity (lower limit of linear range) was calculated as the amount of verapamil or norverapamil that corresponds to a detector response equal to twice the baseline noise (32).

Precision was evaluated by determination of coefficients of variation (C.V.) for verapamil and norverapamil at plasma concentrations of 7.3 and 233 ng/ml. Within-day precision was evaluated by replicate analyses of ten samples on the same day; between-day precision was evaluated by analyses of ten samples on separate days.

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Application

Concentrations of verapamil and norverapamil were measured in the serum and urine obtained from five healthy volunteers at various times following the administration of single oral 80 or 120-mg verapamil hydrochloride doses (Isoptin^{R}, Knoll Pharmaceuticals, Whippany, New Jersey, U.S.A.). Chromatograms were also examined for the presence of other metabolites. Areas under the serum concentration versus time curves from zero to infinity were determined for subjects who received 80-mg doses.

RESULTS

The separation of verapamil, eight of its metabolites, and imipramine is shown in the chromatogram of an unextracted

reference mixture (Fig. 1a). The capacity factors (k') for the reference mixture components are presented in Table 1. Complete separation of all ten components was not obtained. Metabolites D620 and PR-22 had the same capacity factors (represented by peak 1, Fig. 1a) as did metabolites PR-23 and PR-24 (peak 6). Total chromatography time was between 16 and 17 min.

No interferences with verapamil, norverapamil or imipramine were evident in chromatograms of pre-dose serum as shown in Figure 1b.

Fig. 2 shows chromatograms of extracted serum (Fig. 2a) and urine (Fig. 2b) samples obtained 1 hr after the administration of 120-mg oral verapamil hydrochloride dose to a volunteer subject. Peaks 1, 3, 5, 5a, 7 and 8 represent D620, norverapamil, D617, what we believe to be an unidentified metabolite, verapamil and the internal standard, respectively. The measured serum concentrations of verapamil and norverapamil were 84.8 and 81.3 ng/ml, respectively.

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TABLE 1. Capacity Factors

D620	3.1
PR-22	3.1
PR-25	3.4
Norverapamil	3.7
PR-21	4.0
D617	4.3
PR-23	5.0
PR-24	5.0
Verapamil	6.4
Imipramine	8.3

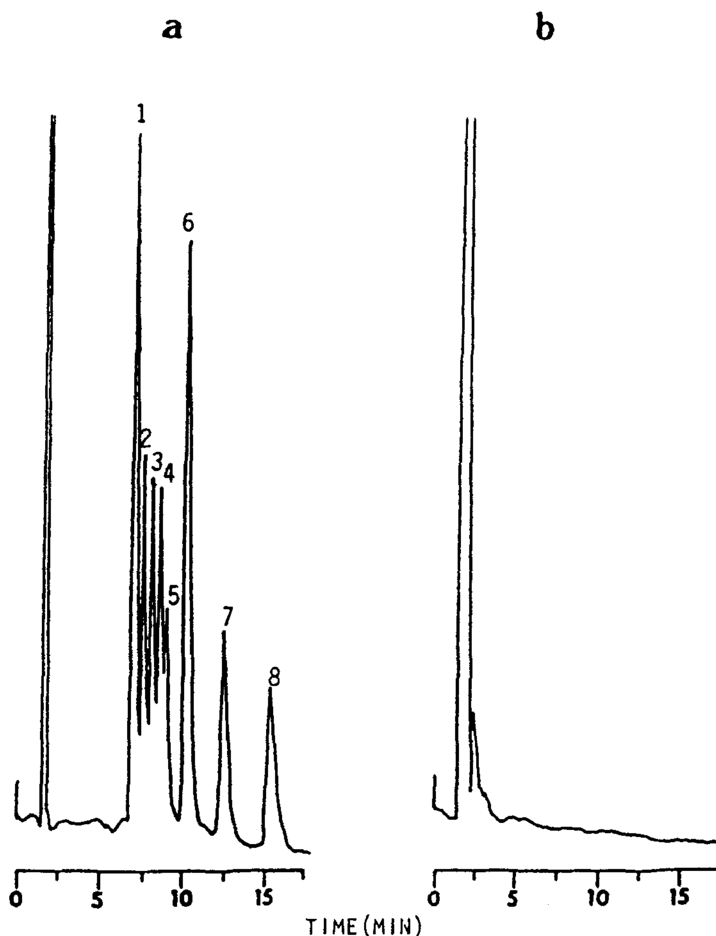

FIGURE 1a: Chromatogram of an unextracted aqueous reference mixture of verapamil, eight metabolites, and imipramine (internal standard). Detection range is 0.04 relative fluorescence units. Peak 1=D620; Peak 2=PR21; Peak 3=Norverapamil; Peak 4=PR-25; Peak 5=D617; Peak 6=PR-23; Peak 7=Verapamil; Peak 8=Imipramine (internal standard). FIGURE 1b: Chromatogram of drug-free pre-dose serum.

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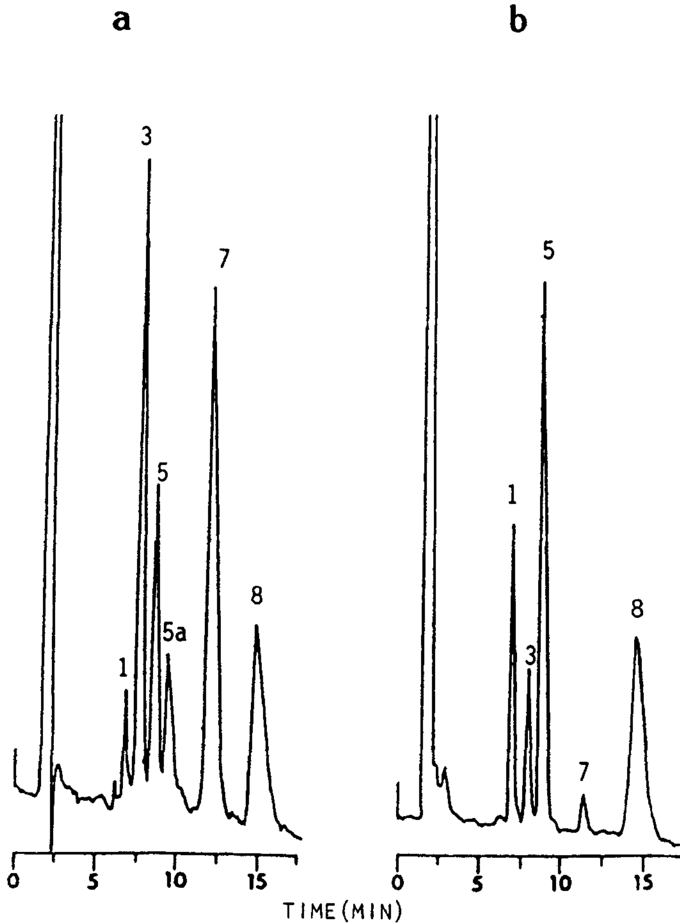

FIGURE 2. Chromatograms of serum (a) and urine (b) extracts from samples taken 1 h after a single 120-mg oral verapamil hydrochloride dose to a volunteer subject. Detection range is 0.04 relative fluorescence units. Peak 1=D620; Peak 3=Norverapamil; Peak 5=D617; Peak 5a=Unknown metabolite; Peak 7=Verapamil; Peak 8=Imipramine (internal standard).

Verapamil, norverapamil, and imipramine were completely recovered from plasma, and recovery was independent of drug concentration over the range studied (Table 2.).

Extraction recoveries of several other metabolites from an aqueous reference mixture were less than complete, as summarized in Table 3.

The recoveries of metabolites PR-22 and PR-25 were found to be higher when water was not alkalinized. This neutral extraction method has been previously reported by Kuwada et al. (23). Re-analysis of subject sera and urines using the neutral

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TABLE 2. Mean (C.V.) Extraction Recoveries

	Plasma Drug Concentration	
	46.5 ng/ml	465 ng/ml
Verapamil	100% (5.0)	104% (1.0)
Norverapamil	98% (3.8)	100% (3.6)
Internal Standard		101% (3.8)

TABLE 3. Verapamil Metabolite Extraction Recoveries from Water

D620	70%
D617	80%
PR-21	0%
PR-22	30%
PR-23	100%
PR-24	100%
PR-25	0%

extraction procedure did not reveal additional peaks on the chromatograms, thereby confirming that metabolites other than those shown in Fig. 2 were either absent or undetectable. This was also tested and confirmed on samples obtained from a subject who had taken oral verapamil chronically.

Although the standard curves for verapamil and norverapamil were apparently linear, the use of linear regression analysis for the entire range of standard values resulted in large prediction errors at the low concentration values. Two standard curves were therefore used: a low range (3.6 to 29 ng/ml) and a high range (29 to 465 ng/ml). This resulted in less than 5% deviation of calculated from known values for all except the 3.6 ng/ml standard. Average percent deviation values for this standard was less than 10% for verapamil and less than 15% for norverapamil. No significant systematic time-dependent changes were noted over the 3-month period that each set of standards was used.

The upper limits of the linear ranges for verapamil and norverapamil were 580 ng and 665 ng, respectively. The limits of sensitivity were slightly less than 1 ng for both compounds. The precision data for verapamil and norverapamil concentration measurements are presented in Table 4.

Mean (SD) peak plasma concentrations of verapamil and norverapamil following the administration of an 80-mg verapamil hydrochloride dose in five subjects were 58.5 (29.5) ng/ml and

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TABLE 4. Coefficients of Variation

	Within Day		Between Day	
	7.3 ng/ml	233 ng/ml	7.3 ng/ml	233 ng/ml
Verapamil	2.8%	2.3%	4.7%	1.7%
Norverapamil	4.4%	4.4%	5.7%	6.1%

42.8 (11.2) ng/ml, respectively. Concentrations of verapamil measured 16 h after the dose ranged from non-detectable to 5.1 ng/ml, while concentrations of norverapamil 24 h after the dose ranged from non-detectable to 5.8 ng/ml. The mean (SD) areas under the verapamil and norverapamil concentration-time curves were 217 (106) and 429 (111) (ng/ml)h, respectively.

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DISCUSSION

We have shown our method to be specific for verapamil, norverapamil, D620, D617 and the internal standard with respect to all other verapamil metabolites after oral dosing. We also report the separation of what we believe to be another verapamil metabolite, which has been previously unreported. Our method is unique in both of these aspects. In addition, the precision and sensitivity for quantitation of verapamil and norverapamil is equal to or superior to other published methods (4-30). Our method was sensitive enough to quantitate verapamil and norverapamil serum concentrations obtained as long as 16 and 24 h,

respectively, after a single 80-mg dose in most subjects. Peak serum concentrations, areas under the curves, oral clearances and half life values for verapamil in our subjects were similar to those reported by Eichelbaum et al. (33) who used a sensitive and specific mass fragmentographic method (34) and studied a similar subject population. We therefore conclude that our method is reliable and is useful for single or multiple-dose pharmacokinetic studies of verapamil.

Although not all the verapamil metabolites were resolved from one another, three major metabolites (D620, D617 and norverapamil), and an unidentified metabolite (peak 5a) were separated. We cannot state with certainty that peak 5a is a metabolite of verapamil, but offer the following in support of this (i) peak 5a was present in the chromatograms of extracts of serum obtained from all subjects following verapamil administration; (ii) it was absent in the chromatograms of extracts of plasma obtained from all subjects just prior to drug administration; (iii) its time course was similar to that of other metabolites.

The internal standard that was initially tested for our method D517, (a verapamil analogue) interfered with D617 and peak 5a; consequently another internal standard was selected. This emphasizes the need to test for potential interferences by metabolites, especially for orally administered drugs which are extensively metabolized. Even though the structure of im-

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ipramine differs from that of verapamil, it performed extremely well as an internal standard under the conditions of our method.

The range for verapamil and norverapamil concentrations used for plasma and urine standards in this study encompasses the "therapeutic range" for verapamil (100-500 ng/ml) and falls within our defined linear range for detection. It is advised that any sample whose concentration measurement falls above this range be appropriately diluted and the measurement repeated.

The results of our study suggest either that the metabolic pathways responsible for the production of metabolites PR-21, PR-22, PR-23, PR-24 and PR-25 are negligible, or that these metabolites are substrates for further, rapid metabolism. This is because the chromatograms of basic and neutral extracts of plasma and urine obtained from normal subjects who volunteered to receive verapamil orally contained no peaks corresponding to these metabolites. Although metabolites PR-22 and D620 co-elute, peak 1 observed in serum and urine extracts probably represent only D620. This is because PR-22 is a minor metabolite (1), and its extraction recovery is only 30% as compared to 80% recovery for D620. The presence of D617, D620 and norverapamil in the serum and urine of these subjects suggests that dealkylation and demethylation are major pathways of verapamil metabolism in man, and are consistent with data reported by others (19-22).

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The method presented here offers several advantages over previously published HPLC methods for verapamil and metabolites. Of the verapamil assays published (4-30) only four are able to completely separate verapamil, norverapamil, D620 and D617 (19-22). Our method is similar in this respect, however, we report a fifth compound which appears to be another verapamil metabolite. Further, our method offers either better recovery and sensitivity or less expensive and simpler mobile phase preparation.

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