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TERNARY SOLVENT MIXTURES FOR IMPROVED RESOLUTION OF HYDROXYLATED METABOLITES OF VITAMIN D_2 AND VITAMIN D_3 DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

This paper reports the development of three new ternary solvent mixtures for the liquid-chromatographic separation of metabolites of vitamin D on microparticulate silica. All solvent systems offer reduced peak tailing and improved resolution of vitamin D compounds, particularly of 24(*R*),25-$(OH)_2D_3$, when compared to the commonly used hexane—isopropanol mixture. The new mixtures can be substituted for hexane—isopropanol systems presently used for preparative liquid-chromatographic steps prior to radioimmunoassay or competitive protein-binding assay of 24,25-$(OH)_2D$ and 1,25-$(OH)_2D$ in human plasma. Hexane—isopropanol—methanol (87:10:3) mixtures are recommended where the lipid content of samples is high, whereas hexane—ethanol—chloroform (80:10:10) promises to be a useful mixture for differentiating vitamin D_3 metabolites from their vitamin D_2 analogs. A combination of the two solvent systems permits the separate assay of both 24(*R*),25-$(OH)_2D_3$ and 24(*R*),25-$(OH)_2D_2$ as well as 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$.

INTRODUCTION

High-performance liquid chromatography (HPLC) on microparticulate silica columns is becoming increasingly popular for the separation of metabolites of vitamin D during clinical assay [1, 2]. Though other solvent systems have been suggested [3, 4], a mixture of isopropanol and hexane (ca. 10:90) is the eluting solvent most frequently used [5], since it permits the partial resolution of most of the known metabolites of vitamins D_2 and D_3. Since the initial demonstration of the resolving power of this solvent system four years ago [5], we have

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applied HPLC using isopropanol—hexane to a number of problems including ultraviolet (254 nm) assay of 25-OH-D_2, 25-OH-D_3 and vitamin D in human serum [6], the separation of 24(R,S)$(OH)_2D_2$ stereoisomers [7] and the identification of products of 25-OH-D_3 in an isolated kidney perfusion system [8]. In the majority of cases, particularly where proportions of isopropanol are kept low, this solvent system provides adequate resolution. However, in some applications where higher solvent strengths are required and proportions of isopropanol must be raised above 10%, severe tailing occurs. This can also occur when anhydrous solvents are used and no attempt is made to equilibrate the solvent with water [9]. In applications where vitamin D metabolites are prepared for competitive protein assay [1, 2] or radioimmunoassay [10, 11], we perceived the need to develop a solvent system able to reduce tailing of one peak into another and to provide better resolution of vitamin D_3 metabolites from their vitamin D_2 analogs. In this paper we present three new alternative solvent mixtures and compare them to the standard isopropanol—hexane solvent system.

EXPERIMENTAL

Solvents

All solvents except ethanol were from Burdick & Jackson Labs., Muskegon, MI, U.S.A., "distilled-in-glass" spectroscopic grade. Ethanol was technical grade supplied by Consolidated Alcohols, Toronto, Canada.

Vitamin D metabolites

Crystalline 25-OH-D_2 and 25-OH-D_3 were generous gifts from Drs. J.A. Campbell, Jack Hinman and John Babcock of Upjohn, Kalamazoo, MI, U.S.A. Crystalline 24(R),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were kind gifts of Dr. M. Uskokovic of Hoffmann LaRoche, Nutley, NJ, U.S.A. 24(R),25-$(OH)_2D_2$ and 1,25-$(OH)_2D_2$ were synthesized respectively from stigmasterol by a chemical route [12] and 1,25-(OH)-D_2 by a biosynthetic method [13]. The latter product, 1,25-$(OH)_2D_2$, was purified by Sephadex LH-20 chromatography [14] and HPLC [15] prior to identification using mass spectrometry [16].

Concentrations of solutions of vitamin D metabolites were measured by a Model SP 1800 spectrophotometer (Pye-Unicam, Cambridge, Great Britain) assuming ϵ at 265 nm is 18 300.

High-performance liquid chromatography

The chromatograph used in these studies was a Model LC 204 fitted with a Model 6000A pumping system, U6K injection valve and a Model 440 ultraviolet fixed-wavelength (254 nm) detector (all from Waters Assoc., Milford, MA, U.S.A.). Stainless-steel columns (25 cm × 6.2 mm I.D.) prepacked with 6 μm diameter microparticulate silica (Zorbax-SIL) used in most of the experiments were purchased from Dupont Instruments, Wilmington, DE, U.S.A. For certain experiments indicated in the text, 25 cm × 4.6 mm I.D. columns of Zorbax-SIL (also from Dupont) or LiChrosorb SI-100, 10 μm (Brownlee Labs., Karlsruhe, G.F.R.) were used for the separation. A Sigma-10 chromatography Data System (Perkin-Elmer, Norwalk, CT, U.S.A.) was used to plot and

integrate the chromatograms.

Resolution, R_S, was calculated using the equation

$$R_S = \frac{1}{4}\left[\frac{\alpha - 1}{\alpha}\right]\sqrt{\bar{N}}\left[\frac{K'}{K' + 1}\right]$$

where $\bar{N}$ is the average number of theoretical plates based upon two peaks of the chromatogram and is calculated using the peak widths measured at the baseline (not from the peak width of a triangle drawn under the peak).

RESULTS

In extreme cases, chromatography of the principal metabolites of vitamin D_3 (25-OH-D_3, 24(R),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$) on microparticulate silica (in this case Zorbax-SIL, 25 cm × 4.6 mm) with the solvent system hexane—isopropanol (85:15) can produce severe tailing effects (Fig. 1).

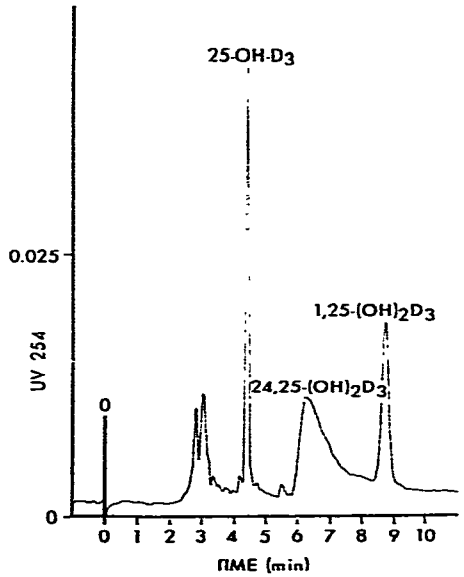

Fig. 1. Severe tailing during liquid chromatography of a mixture of synthetic metabolites of vitamin D_3. Chromatographic conditions: Zorbax-SIL, 25 cm × 4.6 mm; hexane—isopropanol (85:15); flow-rate 1.5 ml/min; 900 p.s.i.

Substitution of a new LiChrosorb SI-100 column (25 cm × 4.6 mm) for the Zorbax-SIL column in this situation produced no change in the degree of tailing under these particular conditions. It seems unlikely, therefore, that the tailing can be attributed to column voids but is solvent- or adsorbant-related. The possibility that 24,25-$(OH)_2D_3$ was contaminated with an impurity on the tail end of the peak was investigated by collecting subfractions of the broad peak and re-injecting them under identical conditions. The subfractions all gave a single peak which retained a tail and had a retention time identical to that of the parent peak. Extracolumn mixing, eddying and other chromatograph-related problems were ruled out when improvements were observed with

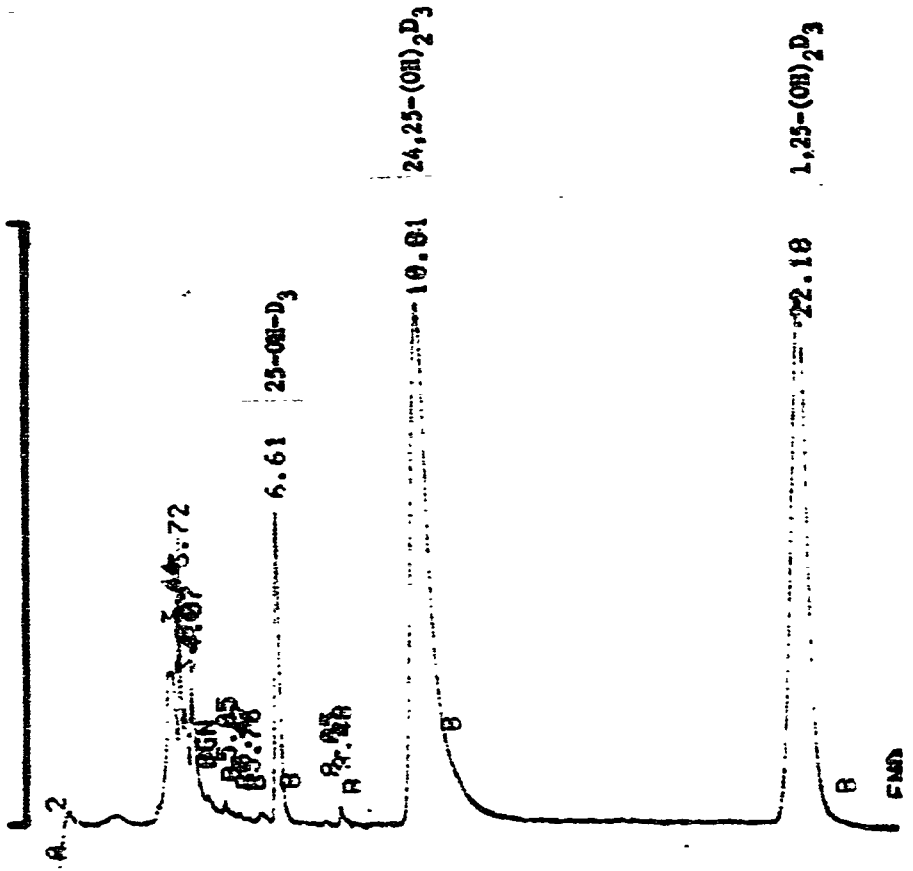

Fig. 2. Typical separation of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ on microparticulate silica using hexane—isopropanol (85:15) as solvent. Chromatographic conditions: Zorbax-SIL, 25 cm × 6.2 mm; flow-rate 1.5 ml/min; 600 p.s.i. Numbers above peaks are retention times in minutes. Bar on abscissa represents ultraviolet (254 nm) response of 0.006 absorbance units.

modified solvent mixtures. In fact, as we can see from a more typical run shown in Fig. 2 (Zorbax-SIL, 25 cm × 6.2 mm), other batches of the solvent hexane—isopropanol (85:15) produced less extensive tailing of the peaks. Nevertheless, as illustrated in Figs. 1 and 2, each metabolite was affected to a different degree by the tailing effect. 25-OH-D_3 and 1,25-$(OH)_2D_3$ were, at worst, only slightly broadened by hexane—isopropanol (85:15) whereas the other dihydroxylated metabolite of vitamin D_3, 24(*R*),25-$(OH)_2D_3$, was noticeably asymmetrical.

When ternary solvent systems based upon hexane—isopropanol—methanol (87:10:3), hexane—ethanol—chloroform (80:10:10), or hexane—methanol—methylene chloride (80:10:10) were used with the same 25 cm × 6.2 mm Zorbax-SIL column on the same day, significant reductions in tailing occurred and resolution of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ was greatly improved (Figs. 3—5 and Table I). This improvement was in the face of no change in flow-rate, column, primary solvent or approximate solvent strength.

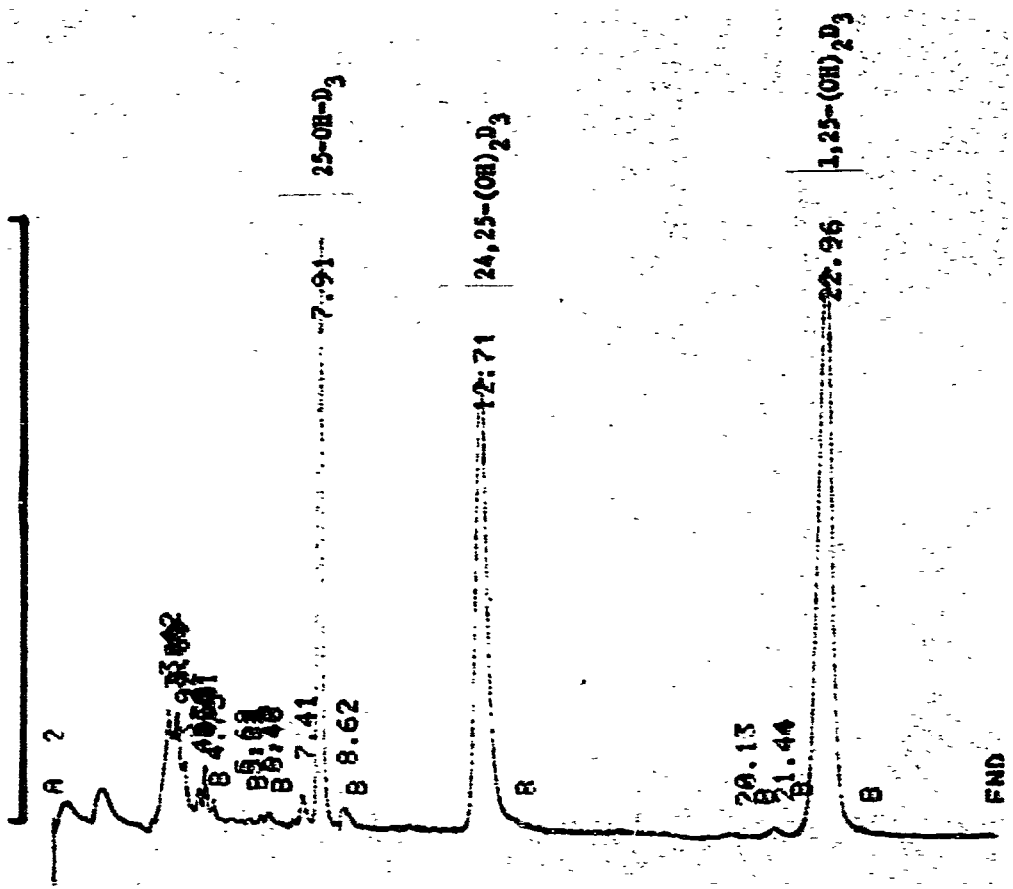

Fig. 3. Separation of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ on Zorbax-SIL using hexane—isopropanol—methanol (87:10:3) as solvent. Column and other chromatographic conditions as described in Fig. 2.

There was not only a marked reduction in the tailing of the 24(*R*),25-$(OH)_2D_3$ peak but also a sharpening of the 1,25-$(OH)_2D_3$ peak.

When we examined the separation of 1,25-$(OH)_2D_3$ from its vitamin D_2 analog, 1,25-$(OH)_2D_2$, using these three systems, we also observed improvement over the traditional hexane—isopropanol (85:15) mixture (Fig. 6). Whereas hexane—isopropanol (85:15) (Fig. 6A) gave only marginal separation of 1,25-$(OH)_2D_3$ (22.35 min) and 1,25-$(OH)_2D_2$ (20.89 min), this was slightly improved using hexane—isopropanol—methanol (87:10:3) (Fig. 6B) [1,25-$(OH)_2D_3$ (22.35 min); 1,25-$(OH)_2D_2$ (20.71 min)], and baseline resolved using hexane—ethanol—chloroform (80:10:10) (Fig. 6C) [1,25-$(OH)_2D_3$ (20.76 min); 1,25-$(OH)_2D_2$ (18.54 min)] or hexane—methanol—methylene chloride (80:10:10) (Fig. 5) [1,25-$(OH)_2D_3$ (13.0 min); 1,25-$(OH)_2D_2$ (11.8 min)].

Both solvent systems containing chlorinated hydrocarbons also provided the best separation of 24(*R*),25-$(OH)_2D_3$ from its vitamin D_2 analog, 24(*R*),25-$(OH)_2D_2$. Hexane—ethanol—chloroform (80:10:10) (not illustrated) gave retention times of 10.19 and 11.20 min for 24(*R*),25-$(OH)_2D_2$ and 24(*R*),25-$(OH)_2D_3$, respectively. Hexane—methanol—methylene chloride (80:10:10)

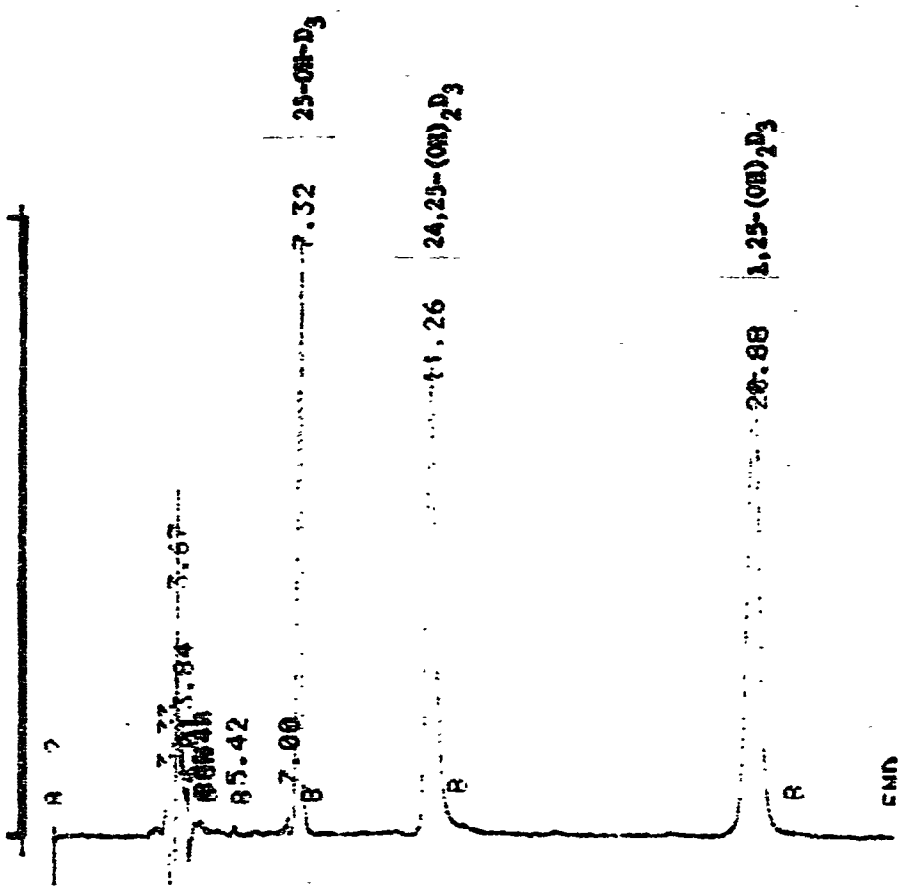

Fig. 4. Separation of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ on Zorbax-SIL using hexane—ethanol—chloroform (80:10:10) as solvent. Column and other chromatographic conditions as described in Fig. 2.

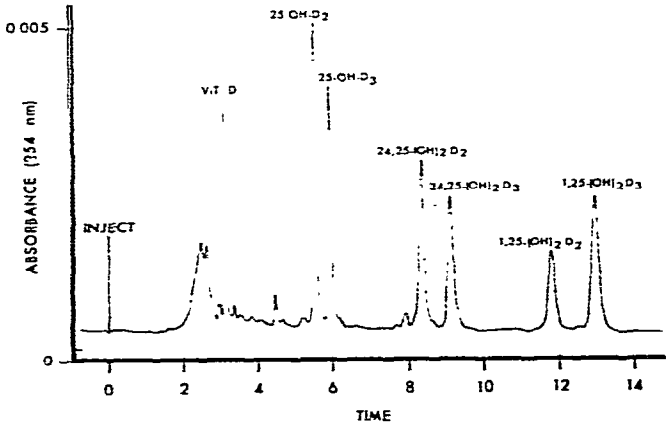

Fig. 5. Separation of the metabolites of vitamin D_2 and vitamin D_3 on Zorbax-SIL using hexane—methanol—methylene chloride (80:10:10) as solvent. Chromatographic conditions: Zorbax-SIL, 25 cm × 6.2 mm; flow-rate 2 ml/min; 900 p.s.i.

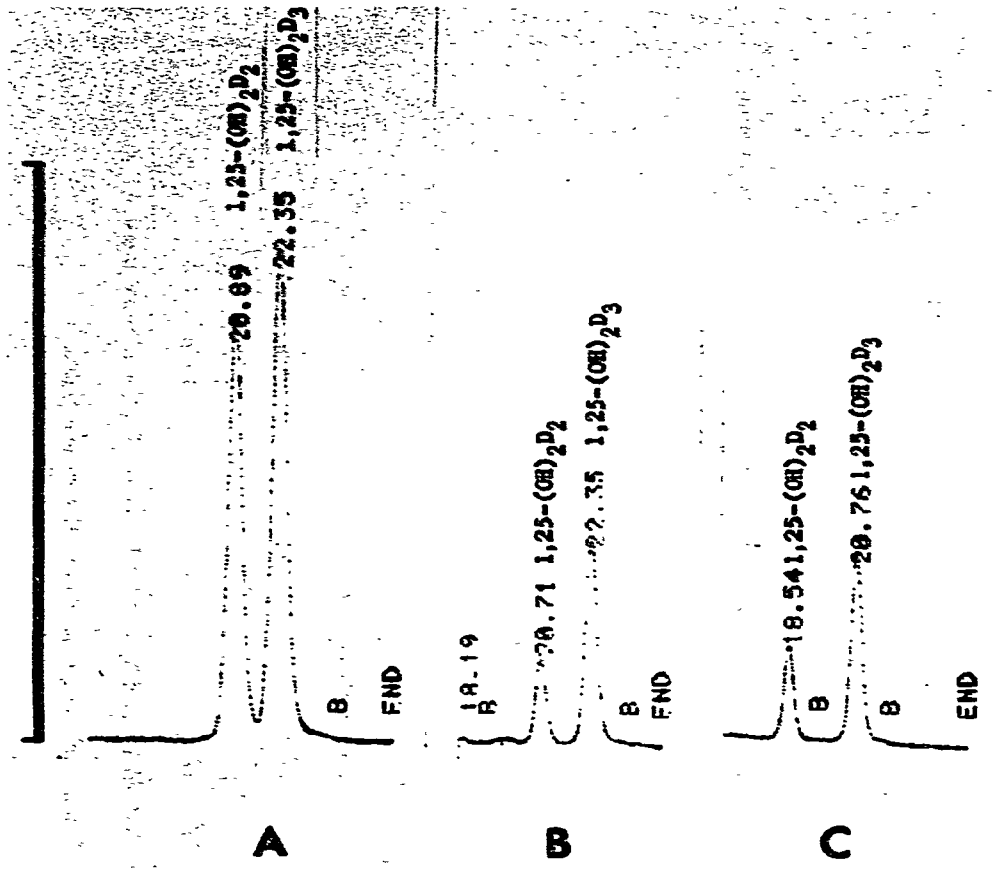

Fig. 6. Separation of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ and Zorbax-SIL using (A) hexane—isopropanol (85:15), (B) hexane—isopropanol—methanol (87:10:3), and (C) hexane—ethanol—chloroform (80:10:10) as solvent. Chromatographic conditions as in Fig. 2. Only the relevant region of each chromatogram is reproduced in the figure.

(Fig. 5) gave retention times of 8.6 and 9.1 min for these peaks with the same order of elution. The solvent system hexane—isopropanol (90:10) only partially separates 24(*R*),25-$(OH)_2D_2$ and 24(*R*),25-$(OH)_2D_3$ [5].

Tables I and II summarise the chromatographic data contained in Fig. 1—6 and provide resolution and theoretical plate counts for each of the solvent systems used. Though, as can be seen, the three new ternary solvent systems provided improved resolution of hydroxylated vitamin D_3 metabolites from each other and from their vitamin D_2 analogs, one major disadvantage was observed. The solubility of blood lipid in the solvents hexane—ethanol—chloroform (80:10:10) and hexane—methanol—methylene chloride (80:10:10) was much lower than for hexane—isopropanol—methanol (87:10:3) or hexane—isopropanol (85:15). This led to the formation of a two-phase system in the sample container whenever excessive lipid was dissolved in hexane—ethanol—chloroform (80:10:10) or in hexane—methanol—methylene chloride (80:10:10). If the sample was injected into the chromatograph in this form, peaks were invariably doublet in nature. The problem was not apparent with the new ternary system hexane—isopropanol—methanol (87:10:3).

TABLE I

CHROMATOGRAPHIC DATA FROM FIGS. 1–5

Fig. No.	Solvent system	K' 25-OH-D_3	K' 24,25-$(OH)_2D_3$	K' 1,25-$(OH)_2D_3$	$\overline{N}$*	R_s**
1	Hexane–isopropanol (85:15)***	0.48	1.21	2.03	707	1.81
2	Hexane–isopropanol (85:15)§	0.84	2.00	5.16	1061	4.17
3	Hexane–isopropanol–methanol (87:10:3)§	1.20	2.53	5.38	1764	4.69
4	Hexane–ethanol–chloroform (80:10:10)§	1.03	2.13	4.80	2010	5.16
5	Hexane–methanol–methylene chloride (80:10:10)§	1.20	2.39	3.81	2808	3.92

*$\overline{N}$, average number of theoretical plates based upon 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ peaks. Peak width measured at baseline.
**Resolution of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ based upon the average number of theoretical plates, $\overline{N}$.
***Zorbax-SIL, 25 cm × 4.6 mm, 6 μm silica.
§ Zorbax-SIL, 25 cm × 6.2 mm, 6 μm silica.

TABLE II

CHROMATOGRAPHIC DATA FROM FIGS. 5 AND 6

Fig. No.	Solvent system	K' 1,25-$(OH)_2D_2$	K' 1,25-$(OH)_2D_3$	$\overline{N}$*	R_s**
6A	Hexane–isopropanol (85:15)§	4.80	5.21	2196	0.77
6B	Hexane–isopropanol–methanol (80:10:3)§	4.75	5.21	4059	1.17
6C	Hexane–ethanol–chloroform (80:10:10)§	4.15	4.77	3745	1.64
5	Hexane–methanol–methylene chloride (80:10:10)§	3.37	3.81	3538	1.37

*$\overline{N}$, average number of theoretical plates based upon 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ peaks. Peak width measured at baseline.
**Resolution of 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ based upon the average number of theoretical plates, $\overline{N}$.
§ Zorbax-SIL, 25 cm × 6.2 mm, 6 μm silica.

DISCUSSION

This paper reports three new ternary solvent mixtures for HPLC of the metabolites of vitamin D_3 and their vitamin D_2 analogs on microparticulate silica. These ternary solvent mixtures offer improved resolution and reduced peak tailing when compared to the hexane–isopropanol systems presently used for the separation of metabolites of vitamin D [5, 11, 17, 18]. These systems, particularly the solvent hexane–isopropanol–methanol (87:10:3), are well-suited for the preparation of purified fractions containing 24,25-$(OH)_2D$ and 1,25-$(OH)_2D$ during the assay of these compounds in human blood. Present methodology [10, 11] for the radioimmunoassay of 1,25-$(OH)_2D_3$ involves extensive purification of 1,25-$(OH)_2D_3$ fractions because of the almost equal affinity of antibodies for 1,25-$(OH)_2D_3$ and 24(*R*),25-$(OH)_2D_3$, and a 50-fold higher concentration of the 24(*R*) compound over the 1-hydroxylated compound in human plasma. Use of the new hexane–isopropanol–methanol (87:10:3) system ensures minimal tailing of the 24(*R*),25-$(OH)_2D_3$ peak into the 1,25-$(OH)_2D_3$ region of the chromatogram, thereby minimizing the possibility of 24(*R*),25-$(OH)_2D_3$ contamination of the 1,25-$(OH)_2D_3$ fraction. As antibodies with greater specificity for 1,25-$(OH)_2D_3$ become available, this improved resolution should lessen the need for pre-purification of plasma extracts prior to HPLC.

Peak tailing is a common problem in adsorption chromatography and is believed to be due to heterogeneity of the chromatographic surface due in turn to lack of total hydration of the active groups of the column [19]. It is not clear why the side-chain-dihydroxylated metabolite 24(*R*),25-$(OH)_2D_3$ is more severely affected than its A ring-dihydroxylated positional isomer, 1,25-$(OH)_2D_3$, but it is possibly related to the vicinal nature of the hydroxyl functions in the former compound and the interaction of these groups with the absorptive surface of the silica. Interestingly, the difference in the degree of tailing cannot be explained simply by an increase in the polarity of the molecule, since 1,25-$(OH)_2D_3$ is more strongly retained than 24(*R*),25-$(OH)_2D_3$ yet does not tail so severely. Peak tailing has been overcome in other situations by saturation of the eluting solvent with water [20], a modification not desirable here due to the labile nature of the solutes. Furthermore, because of the need to use any chromatographic development as a purification tool in the assay of 1,25-$(OH)_2D_3$, we avoided the use of agents, such as water, that would lengthen the time taken to evaporate solvent prior to radioimmunoassay. Thus, we used small percentages of methanol or ethanol [20] as a substitute for water and were able to significantly reduce tailing without the problems of using water referred to above. It is presumed that the addition of alcohol works by providing a more homogeneous hydration of the absorptive surface, thereby producing a more symmetrical peak.

Certain of the new solvent systems, particularly those containing a halogenated hydrocarbon (e.g., hexane–ethanol–chloroform, 80:10:10), offer improved resolution of the vitamin D_3 metabolites from their vitamin D_2 analogs. The baseline separation of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ or of 24(*R*),25-$(OH)_2D_3$ and 24(*R*),25-$(OH)_2D_2$ permits the separate assay of these metabolites in plasma extracts. However, in view of the poor solubility of plasma lipids in hexane–ethanol–chloroform (80:10:10), we suggest that this

is best achieved by rechromatography of fractions obtained after initial chromatography using silica and hexane—isopropanol—methanol (87:10:3). Development of separate assays for vitamin D_2 and vitamin D_3 analogs should open up the possibility of testing the validity of assays which purport to measure total 1,25-$(OH)_2$D and total 24(R),25-$(OH)_2$D in patients receiving large doses of vitamin D_2 for treatment of various mineral disturbances [21, 22]. Assays which claim to measure total 24,25-$(OH)_2$D or 1,25-$(OH)_2$D are under suspicion because of their differential sensitivity to vitamin D_2 analogs [23] or because of interference by 25,26-$(OH)_2D_2$ [24, 25], or calcidiol 26-23 lactone [26]. Methods described in this paper may help us to test these assays more rigorously and perhaps offer improvements.

The use of hexane—ethanol—chloroform in conjunction with (but after) hexane—isopropanol—methanol overcomes the only disadvantage noted for the halogenated solvent mixtures (that they provide poor lipid solubility) by minimizing the lipid content of the samples to be injected. Alternatively, the solvent mixtures hexane—ethanol—chloroform and hexane—methanol—methylene chloride may find their usefulness in the separation of closely similar compounds (e.g., isomers) and where exploitation of the solvent selectivity factor, α, is required. The importance of the factor α in the resolution equation (described in Experimental) is often overlooked since most separations are based upon increasing the theoretical plate count of the column or changing the nature of the chromatographic surface.

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