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SOLID PHASE EXTRACTION SYSTEM FOR VITAMIN D AND ITS MAJOR METABOLITES IN HUMAN PLASMA

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SUMMARY

A new procedure using C_{18} and silica cartridges for the extraction and subsequent separation of vitamin D and its major metabolites from plasma has been developed and compared to a conventional extraction procedure with respect to lipophilic material extracted as evaluated by high-performance liquid chromatographic profiles. The C_{18} cartridges were efficient in extracting all compounds tested while subsequent chromatography of the extract on silica cartridges was effective in resolving vitamin D and its metabolites based on increasing polarity. High-performance liquid chromatographic profiles of each silica cartridge fraction clearly demonstrated that the newly conceived solid phase extraction was superior to conventional extraction methods with respect to cleanliness of sample fractions. This difference in lipophilic load between the new and conventional extraction systems was most apparent in the vitamin D and 25-hydroxyvitamin D containing fractions. The new extraction system can be used when total extraction and subsequent analysis of vitamin D and its major metabolites is desired.

INTRODUCTION

Analysis of vitamin D and its metabolites in plasma has become quite prominent during the last decade [1—6]. The majority of these analyses are based on competitive protein binding analysis (CPBA) following organic extraction of the sample and subsequent chromatographic separation. The extraction procedures used in these analyses are based on conventional methods such as that developed by Bligh and Dyer [7]. These conventional extraction procedures are often difficult for a number of reasons, including large volume requirements, emulsion formation and erratic recoveries of the desired compounds. This type of extraction method, when applied to vitamin

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D analysis, has resulted in procedures that require a plethora of chromatographic steps prior to final quantitation [1- 6]. The alternative is to avoid the necessary steps with this type of extraction and risk obtaining artificially elevated compound levels due to lipid interference in the final CPBA [8].

Solid phase extraction systems have been developed for the more polar metabolites of vitamin D including 25-hydroxyvitamin D (25-OH-D), 24,25-dihydroxyvitamin D (24,25-$(OH)_2$-D) and 1,25-dihydroxyvitamin D (1,25-$(OH)_2$-D) [9—11]. However, these systems have been totally inefficient in removing the parent compound, vitamin D, from plasma. One exception to this was the work of Dabek et al. [12] which used C_{18} cartridges to quantitatively extract and separate vitamin D compounds from plasma using mixtures of methanol—water. It was our purpose to develop a solid phase extraction system that would efficiently and effectively extract all vitamin D compounds from a plasma sample along with a subsequent system that would give adequate resolution on the important vitamin D compounds. Herein, we describe such a system and demonstrate its superiority to a conventional extraction system.

EXPERIMENTAL

Materials

[1,2-^{3}H]Vitamin D_3 (12.3 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). [25,26-^{3}H]-25-OH-D_3 (90 Ci/mmol), [26,27-^{3}H]-25-OH-D_2 (90 Ci/mmol), [26,27-^{3}H]-24,25-$(OH)_2$-D_3 (90 Ci/mmol), [26,27-^{3}H]-1,25-$(OH)_2$-D_2 (90 Ci/mmol) and [26,27-^{3}H]-1,25-$(OH)_2$-D_3 (90 Ci/mmol) were obtained from Drs. R.L. Horst, T.A. Reinhardt (Ames, IA, U.S.A.) and J.L. Napoli (Dallas, TX, U.S.A.). Crystalline 25-OH-D_3, 24(*R*),25-$(OH)_2$-D_3; 1,25-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_2 were generous gifts of Dr. Milan Uskokovic of Hoffmann-LaRoche (Nutley, NJ, U.S.A.). 25-OH-D_2 and 24,25-$(OH)_2$-D_2 were obtained from Drs. Horst and Reinhardt. Vitamin D_2 and vitamin D_3 were purchased from Sigma (St. Louis, MO, U.S.A.). Silica Bond-Elut cartridges and C_{18} Bond-Elut cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.). A Sep-Pak cartridge rack was purchased from Waters Assoc. (Milford, MA, U.S.A.). HPLC grade methanol, methylene choride, chloroform, hexane, isopropanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Procedures

Plasma (0.5 ml) was incubated with tracer quantities (1000 cpm) of radiolabelled vitamin D_3, 25-OH-D_3, 25-OH-D_2, 24,25-$(OH)_2$-D_3, 1,25-$(OH)_2$-D_2 and 1,25-$(OH)_2$-D_3 for 30 min at 20°C. Plasma samples were extracted by two methods with the first being performed using methanol- methylene chloride [6]. The second extraction method is new and was performed in the following manner. An equal volume of saturated ammonium sulfate solution was added to a plasma sample and vortex-mixed for 1 min followed by the addition of two plasma volumes of absolute ethanol and 1 min additional vortex mixing. The sample was then centrifuged at 1000 *g* for 10 min at 20°C. The supernatant was removed and placed in a 13 × 100 mm disposable glass tube followed by the addition of two plasma volumes of 0.4 *M* dipotassium

phosphate (pH 10.5), mixing for 1 min and further addition of two plasma volumes of acetonitrile. This solution was vortex-mixed for 1 min, centrifuged at 1000 *g* for 10 min at 20°C, the superantant removed and subsequently subjected to solid phase extraction using a Bond-Elut C_{18} silica cartridge.

The Bond-Elut C_{18} cartridges were prepared before use by successively washing each cartridge with 2 ml hexane, 3 ml isopropanol, 3 ml methanol and 5 ml distilled water. The sample was applied to the cartridge under 100 mmHg vacuum using the Waters Sep-Pak cartridge rack. The cartridge was then washed successively with 5 ml distilled water, 2.5 ml methanol—water (70:30) and finally 5 ml methanol, which eluted vitamin D and its metabolites. The methanol was dried under nitrogen at 40°C and the residue redissolved in 0.30 ml methylene chloride for subsequent chromatography using a silica Bond-Elut cartridge.

The silica Bond-Elut cartridges were prepared before use by successively washing each cartridge with 3 ml isopropanol, 4 ml hexane and finally 4 ml methylene chloride. The samples were applied to the cartridge in two 0.30-ml washes of methylene chloride under a 50-mmHg vacuum using the following solvent sequence: 2 ml of 0.2% isopropanol in methylene chloride (discard); 8.5 ml of 0.2% isopropanol in methylene chloride (vitamin D); 3.5 ml of 2% isopropanol in hexane (discard); 6 ml of 5% isopropanol in hexane (25-OH-D); 9 ml of 8% isopropanol in hexane (24,25-$(OH)_2$-D) and 6 ml of 15% isopropanol in hexane (1,25-$(OH)_2$-D). The fractions collected were dried under nitrogen in preparation for HPLC.

HPLC was performed on a Beckman Model 344 high-performance liquid chromatograph. Normal-phase HPLC was performed using a 0.4 × 25 cm Zorbax-Sil column packed with 5 μm silica (DuPont, Wilmington, DE, U.S.A.), whereas reversed-phase HPLC was carried out using a 0.4 × 25 cm Vydac ODS column packed with 5 μm C_{18}-bonded silica (Separations Group, Hisperia, CA, U.S.A.). The solvent mixtures used to separate vitamin D and its metabolites have been described in detail elsewhere [13] although a brief description will follow. Normal-phase chromatography of vitamin D was achieved using hexane—methylene chloride—isopropanol (49.5:49.5:0.5) at a flow-rate of 2 ml/min. Reversed-phase chromatography of vitamins D_2 and D_3 was achieved using acetonitrile—chloroform (95:5) at a flow-rate of 1.75 ml/min. 25-OH-D_2 and 25-OH-D_3 were resolved using a hexane—isopropanol (97:3) mixture at a flow-rate of 2 ml/min. 24,25-$(OH)_2$-D_2 and 24,25-$(OH)_2$-D_3 were separated using a quaternary solvent system composed of hexane—methylene chloride—isopropanol–methanol (80:16:3.5:0.5) at a flow-rate of 2 ml/min. A similar quaternary system in the proportions of (79.5:14:5.5:1) was utilized to separate 1,25-$(OH)_2$-D_2 and 1,25-$(OH)_2$-D_3, again at a flow-rate of 2 ml/min. All fractions were dried under nitrogen and subjected to liquid scintillation counting in order to determine the final recoveries of the radioactive tracers throughout the procedure.

RESULTS

The elution profiles of [^{3}H]-D_3, [^{3}H]-25-OH-D_3, [^{3}H]-24,25-$(OH)_2$-D_3 and [^{3}H]-1,25-$(OH)_2$-D_3 from the silica Bond-Elut cartridge are displayed in Fig. 1.

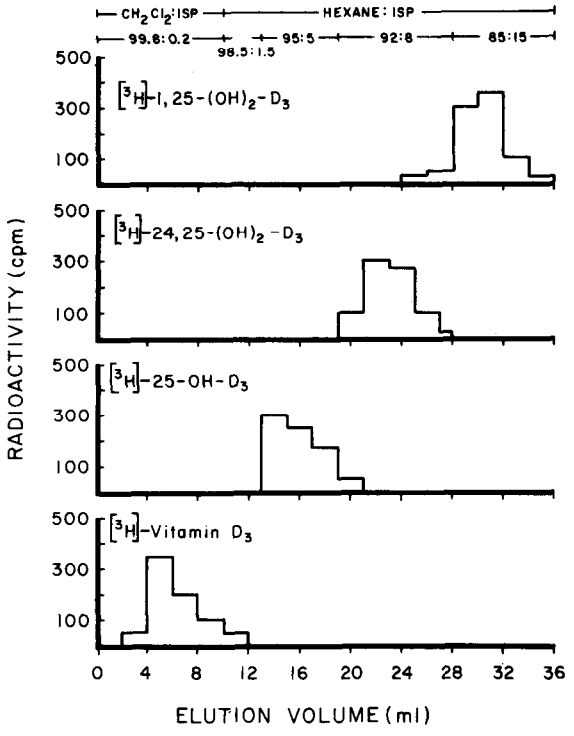
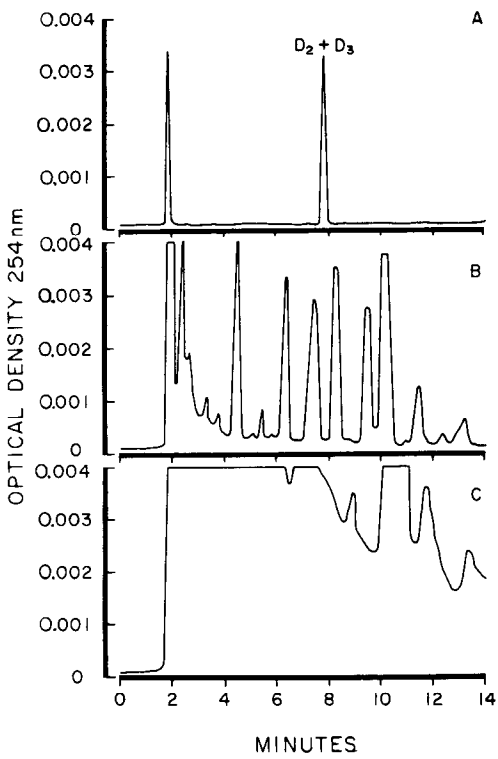


Fig. 1. Elution profiles of radioactive vitamin D_3 and its metabolites chromatographed on a silica Bond-Elut cartridge. Each bar depicts the amount of radioactivity in 2 ml of eluent during batch elution with various mixtures of isopropanol in methylene chloride or hexane.

The batch elution technique used was very efficient in resolving the vitamin D metabolites tested. Slight amounts of carry-over were observed between some of the compounds but this never exceeded 10%. Although not displayed, the corresponding elution of vitamin D_2 compounds were nearly identical to their vitamin D_3 counterpart on this Bond-Elut system.

The amount of extraneous lipid contained in the various Bond-Elut silica fractions was compared between the present extraction system and that performed using methanol—methylene chloride [6]. These results can be observed in Figs. 2 and 3. Fig. 2 illustrates the normal-phase HPLC separation of the vitamin D fraction from the Bond-Elut cartridge. Fig. 2A shows the elution profile of standard vitamins D_2 and D_3. Fig. 2B and C depict the ultraviolet profile of the new solid phase extraction system and the methanol—methylene chloride system [6], respectively. The ultraviolet HPLC profile comparison of 25-OH-D is similarly displayed in Fig. 3.

Final recoveries of tracer compounds, expressed as percent ± S.D., through the entire extraction and purification procedure (HPLC) were as follows: vitamin D_3, 45.8 ± 4.3 (reversed phase), 51.4 ± 4.9 (normal phase); 25-OH-D_2, 60.3 ± 3.5; 25-OH-D_3, 55.9 ± 8.5; 24,25-$(OH)_2$-D_3, 43.4 ± 3.2; 1,25-$(OH)_2$-D_2, 43.0 ± 2.9; and 1,25-$(OH)_2$-D_3, 42.5 ± 4.2. All recovery data were based on ten observations except vitamin D which was five observations per system. These recoveries equaled or exceeded those obtained with a conventional extraction system [13].



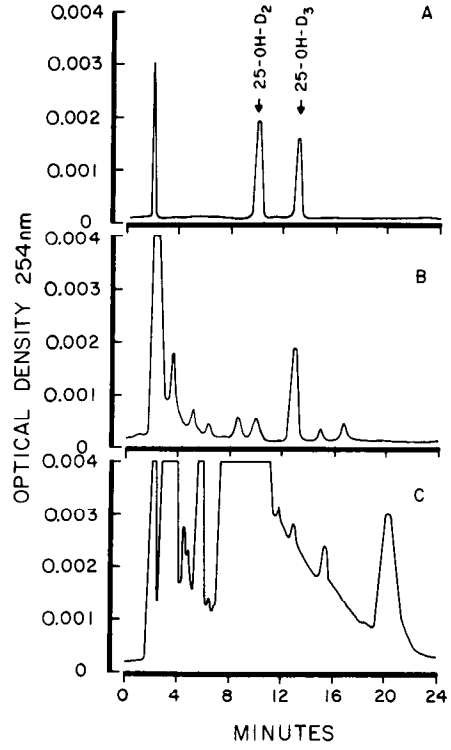


Fig. 2. HPLC profiles of standard vitamins D_2, D_3 and vitamin D containing preparative chromatographic fractions from the Bond-Elut cartridge. Column calibration was achieved on a Zorbax-Sil column by injecting 10 ng of both vitamins D_2, D_3 and monitoring optical density at 254 nm (A). The column was developed in and eluted with hexane—methylene chloride—isopropanol (49.5:49.5:0.5) at a flow-rate of 2.0 ml/min. Plasma samples (0.5 ml) were first extracted using either the newly described method (B) or methylene chloride—methanol procedure [6] (C), processed through the silica Bond-Elut cartridge and applied to HPLC. Optical density of the extracts was monitored at 254 nm during elution.

Fig. 3. HPLC profiles of standard 25-OH-D_2, 25-OH-D_3 and 25-OH-D containing preparative chromatographic fractions from the Bond-Elut cartridge. Column calibration was achieved on a Zorbax-Sil column by injecting 10 ng of both 25-OH-D_2 and 25-OH-D_3 and monitoring optical density at 254 nm (A). The column was developed in and eluted with hexane—isopropanol (97:3) at a flow-rate of 2.0 ml/min. Plasma samples (0.5 ml) were first extracted using either the newly described method (B) or methylene chloride—methanol procedure [6] (C), processed through the silica Bond-Elut cartridge and applied to HPLC. Optical density of the extracts was monitored at 254 nm during elution.

DISCUSSION

C_{18} reversed-phase cartridges have been successfully used for the extraction of 25-OH-D, 24,25-$(OH)_2$-D and 1,25-$(OH)_2$-D from plasma [9—11]. However, these methods have proven to be ineffective in removing the parent compound, vitamin D, from plasma. The present method was universally efficient in removing all major vitamin D compounds from plasma samples. The methods of Rhodes et al. [11], Fraher et al. [9] and Turnbull et al. [10] all use acetonitrile as an extraction solvent for the removal of vitamin D compounds from

plasma. Herein lies the problem that vitamin D itself has limited solubility in this organic medium and thus is not extracted from plasma by its use. We have overcome this problem by using a combination of ammonium sulfate and ethanol which removes all vitamin D compounds with near equal efficiency. We have also included an additional step by adding basic dipotassium phosphate to the ethanol extract. The purpose of this step is important in that it transforms charged lipids, such as free fatty acids, into their respective potassium soaps for easy elution from the C_{18} cartridge under an aqueous eluent. Vitamin D and its metabolites were eluted from the C_{18} cartridge with methanol. Previous procedures have used acetonitrile to accomplish this elution although in our hands we found acetonitrile relatively ineffective in eluting the parent compound. Recently, Dabek et al. [12] described a combined solid phase extraction and separation system of vitamin D compounds from plasma using C_{18} cartridges. While this method removed all desired compounds from plasma the resolution of the dihydroxylated vitamin D compounds from one another was not satisfactory. This procedure also involved large amounts of methanol—water mixtures which would be difficult to evaporate in order to recover the desired vitamin D metabolite.

The extracted vitamin D compounds were further separated using a silica Bond-Elut chromatography cartridge eluted with mixtures of methylene chloride—isopropanol and hexane—isopropanol. Similar methods have been published using silica cartridges for resolving vitamin D and its metabolites into their respective polar fractions. Adams et al. [14] published a method of separation based on hexane–ethyl acetate elution. This method was effective in separating vitamin D from 25-OH-D but was totally ineffective in separating 24,25-$(OH)_2$-D from 1,25-$(OH)_2$-D. Similar systems have since been published, however, these systems, which use Waters silica Sep-Pak cartridges, still provide inadequate resolution between 24,25-$(OH)_2$-D and 1,25-$(OH)_2$-D [15, 16]. The present system offers excellent resolution for the separation of vitamin D, 25-OH-D, 24,25-$(OH)_2$-D and 1,25-$(OH)_2$-D using Bond-Elut silica columns (Fig. 1).

A comparison of the present extraction system and a conventional extraction [6] with respect to lipophilic content clearly demonstrated the superiority of the new system. The vitamin D fraction was substantially cleaner with the present method as compared to the conventional procedure when separated by normal-phase HPLC (Fig. 2B versus C). A comparable improvement in sample cleanliness was observed in the vitamin D fraction when the fraction was separated by reversed-phase HPLC (not shown). This reduced lipid load diminishes the likelihood of lipophilic interference inherent in the CPBA of vitamin D [3, 4, 17] as well as decreased HPLC run times between samples. Similar dramatic differences between the two extraction methods were observed from the HPLC profile for 25-OH-D (Fig. 3) and the dihydroxylated vitamin D metabolites (not shown). While the conventional extraction [6] contains much contaminating lipid the new method is very clean actually allowing direct ultraviolet quantitation of 25-OH-D_2 and 25-OH-D_3. This type of direct ultraviolet quantitation of 25-OH-D has been reported using previous solid phase extraction methods [9—11]. While the lipophilic load differences between methods for the dihydroxylated metabolites are not as striking as that

of vitamin D and 25-OH-D it is still apparent that the new procedure provides cleaner extracts. This decreased lipid load has also allowed us to quantitate 1,25-$(OH)_2$-D by protein binding assay [15] following silica Bond-Elut chromatography, something not possible when using the classic extraction procedure.

The newly described system provides a rapid and superior alternative to total vitamin D extraction from plasma. As a result we have incorporated this method into our overall assay procedure [13] resulting in a substantial increase in efficiency due both to increased sample handling capacity and decreased HPLC run time as well as CPBA reliability.

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