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Vitamin D determination using high-performance liquid chromatography with internal standard–redox mode electrochemical detection and its application to medical nutritional products☆

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ABSTRACT

The selectivity and sensitivity of high-performance liquid chromatographic analysis for the determination of vitamin D_3 and D_2 content in medical nutritional products were improved with the aid of electrochemical detection in internal standard–oxidation/reduction mode. The relative standard deviation at the 13-nmol level for the analysis of vitamin D in products was 3.6% (n = 5). Recovery rates of added vitamin D_3 were 97.5 ± 3.0% (mean ± S.D.). It is concluded that this method is much more selective and accurate for detection of vitamin D at the nanomolar level than ultraviolet detection methods.

INTRODUCTION

It is well established that vitamin D plays an important role in preventing infantile rickets and adult osteomalacia. The daily requirement of vitamin D in adult humans has been estimated to be in the range 200 to 400 I.U. [1]. In addition, the recommended dietary allowance has been evaluated to be 400 I.U. [2], where one international unit (I.U.) of vitamin D is defined as the activity of 25 ng of *cis*-vitamin D_3 (cholecalciferol). However, a continuous vitamin D intake as low as 50–120 μg per day is known to be toxic [3,4]. Therefore, the contents of vitamin D derivatives in fortified milk and infant formulas should be carefully controlled: they are adjusted to about 400 and 350–500 I.U./l [5], respectively. Thus, estimation of the content of vitamin D in medical nutritional products is very important.

The determination of vitamin D contents in foods has been extensively elaborated by several methods [6,7]. High-performance liquid chromatography (HPLC), for example, has been well established to be useful for evaluating the vitamin D contents of infant formulas [8–11] and milk [5,12–21]. Generally, the usual HPLC methods for vitamin D determination require pretreatments of crude materials. Such HPLC methods involve UV absorbance detection, which is not selective for detecting vitamin D.

Agarwal [21] developed a method in which vitamins D_2 and D_3 are converted into isotachysterols with antimony trichloride and the isotachysterols quantitated using HPLC with UV detection at an absorption maximum of 301 nm. This method has

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the advantage that the other components present in the sample do not give any adsorption at this wavelength. The only disadvantage of this method is that it cannot separately determine vitamins D_2 and D_3.

Recently, we found that dual-electrode detection in the electrochemical reduction/reoxidation mode [22] is a selective and sensitive method of detecting hydrophobic vitamins. Vitamin K contained in human milk, for instance, was accurately evaluated by the HPLC method, as reported previously [23].

In this report, we discuss an HPLC–internal standard–redox mode electrochemical detection (ED) method and apply it to the determination of vitamin D in medical nutritional products.

EXPERIMENTAL

Chemicals and materials

Crystalline vitamin D_3 [Japanese Pharmacopoeia (JP) reference standard cholecalciferol] was used as the standard. Crystalline vitamin D_2 (JP reference standard ergocalciferol) was also used as an internal standard. HPLC-grade *n*-hexane, ethanol, isopropanol and acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium perchlorate and 60% perchloric acid were purchased from Nakarai Chemicals (Kyoto, Japan). Other reagents were of analytical grade. The test sample was Ensure · liquid. The commercial medical nutritional product, a nutritionally complete liquid-formula diet, was obtained from Meiji Milk Products (Gunma, Japan). It is claimed that the product is fortified with vitamin D_3 at the level of 1.25 μg per can (0.5 μg per 100 ml). The test samples were prepared by adding vitamin D_3 to the nutritional product so as to increase the vitamin D_3 level by about 30%.

Sample preparation

In a 300-ml amber boiling flask, the product sample (40 ml) was mixed with 50 ml of 0.6% ethanolic pyrogallol solution and with 6 g of solid potassium hydroxide. Then, the mixture was saponified by refluxing for 30 min in a boiling water bath. The saponified mixture was transferred to a separatory funnel, and the boiling flask was rinsed with two 25-ml portions of water and with 25 ml of diethyl ether successively. The solution was shaken vigorously in the separatory funnel. The aqueous layer was removed and the extraction procedure was repeated twice with 35 ml of diethyl ether. The diethyl ether layers were combined with those from the first extraction. The diethyl ether extracts were washed with 30-ml portions of water until there was no longer any red colour due to phenolphthalein in the aqueous layer. [When the product contains vitamin D_2, the diethyl ether solution is exactly divided into two parts; the working standard solution (0.25 μg/ml vitamin D_2) is added one of the diethyl ether solutions.] Then a 1-ml volume of the working standard solution (0.25 μg/ml vitamin D_2) was added as an internal standard [24,25] to the extracted ether solution and the solution evaporated in a flask under reduced pressure at 40°C. Then, a 5-ml volume of ethanol was added and re-evaporated to remove any trace of water in the sample. The concentrated extract was diluted with *n*-hexane and transferred to a 10-ml amber sample vial, and again evaporated to dryness under a nitrogen atmosphere. A 0.3-ml volume of *n*-hexane was added to the sample vial and the solution was used for HPLC analysis.

A 100-μl aliquot was injected onto a semipreparative HPLC column. The fraction of D_3 with D_2 was collected and evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 100 μl of methanol. A 10-μl aliquot was injected onto an HPLC column for quantitative analysis. The working standard solutions in methanol were prepared for calibration. Concentrations of the standard solutions of vitamin D_2 and D_3 were 0.25, 0.50, 0.75 and 1.0 μg/ml.

Semipreparative high-performance liquid chromatography

Generally, preliminary clean-up of the HPLC system in the analysis of vitamin D has employed the reversed-phase mode [26]. However, the highly concentrated residue obtained in saponification is often insoluble in the eluting solvents used for reversed-phase HPLC. Therefore, semipreparative HPLC in normal-phase mode was employed in the first stage.

The semipreparative HPLC system consisted of a Hitachi (Tokyo, Japan) Model L-6000 pump, a Rheodyne (Berkeley, CA, USA) Model 7125 syringe injection valve and a Hitachi Model 638-41 variable-

wavelength UV detector at 265 nm for vitamin D_2 and D_3. A column (Nucleosil 50-5, 5 μm particle size, 25 cm × 4.6 mm I.D.) produced by Macherey-Nagel (Düren, Germany) was used for semipreparation. The eluent was a mixture of *n*-hexane–isopropanol (99.5:0.5) and elution rate was maintained at 4 ml/min.

Quantitative high-performance liquid chromatography

The phase system was optimized in terms of the selectivity between vitamins D_2 and D_3 and co-extracted sample constituents. The quantitative HPLC system consisted of a Hitachi Model L-6200 pump and a Rheodyne Model 7125 sample valve. An ESA 5100A Coulochem system (Bedford, MA, USA) was used for ED of the eluting compounds. The 5100A system was composed of a Model 5011 dual analytical cell and a Model 5020 guard cell. The former dual cell was located in series at the end of the HPLC column; the potentials applied to detectors 1 and 2 in the dual cell were adjusted to +0.65 V and −0.20 V, respectively. The latter guard cell was located between the pump and the sample injector; the potential applied to the guard cell was adjusted to +0.65 V.

An H_2/H^+ (NHE) electrode was used as a reference in our reaction system. All chromatograms from detector 2 were recorded. In our HPLC system, a UV detector as a monitor was also set between the HPLC column and the dual cell. A column (Hitachi Gel 3056 reversed-phase column, 5 μm particle size, 25 cm × 4.0 mm I.D.) for quantitative analysis was purchased from Hitachi. The eluent was acetonitrile–methanol −50% perchloric acid (970:30:1.2) containing 0.057 *M* sodium perchlorate. The mobile phase was run at a flow-rate of 1.2 ml/min. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

HPLC for semipreparation

Typical chromatograms of a standard solution of vitamin D_2 (2.5 μg/ml) and D_2 (2.5 μg/ml) and an extracted sample are shown in Fig. 1. Vitamins D_3 and D_2 in the sample were eluted at about 6.2 min. It is clear from Fig. 1A that vitamin D_3 is not separated from vitamin D_2, *i.e.*, a single peak is observed at 6.2 min. Therefore, vitamins D_3 and D_2 in the extracted sample should be eluted between 6 and 6.5 min. However, the peak of vitamins D_3 and D_2 in the extracted sample could not be identified on the chromatogram, as shown in Fig. 1B, because of peaks due to contaminants and the relativity low concentration of vitamins in the sample.

Optimization of electrochemical detection

The mean half-peak oxidation potential of vitamin D_3 and D_2 in water containing 25% methanol was estimated to be about 0.95 ± 0.02 V *vs.* Ag/Ag^+ [27]. We found that the oxidation potential of vitamin D_3 and D_2 in acetonitrile–methanol–50% perchloric acid (970:30:1.2) containing 0.057 *M* sodium perchlorate agreed with the above value within the experimental error. The ESA electrochemical detector used here was supposed to either oxidize or reduce 99% of each compound in the sample, depending on the positive or negative potential applied [28,29]. To obtain the optimum potential applied to our HPLC detection system, the

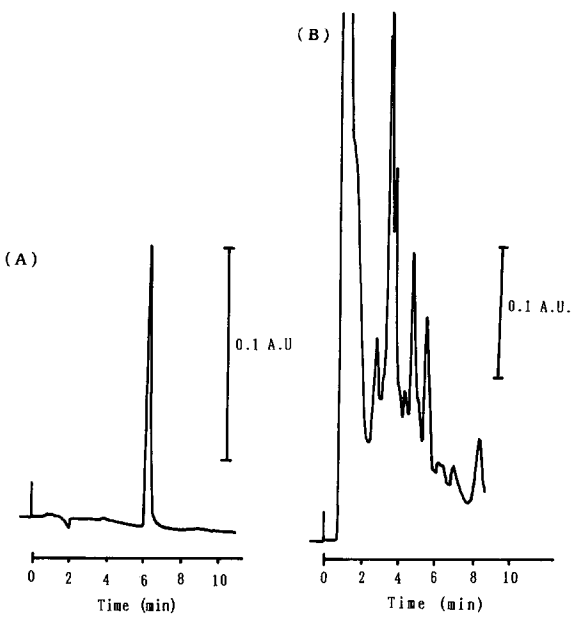

Fig. 1. Profiles of the semipreparative HPLC of the mixture of standard vitamins D_3 and D_2 and the unsaponifiable sample from a medical nutritional product as described in the Experimental section. (A) Chromatogram of an authentic mixture of vitamins D_3 and D_2 in solution (each 2.5 μg/ml); (B) chromatogram of the unsaponifiable sample.

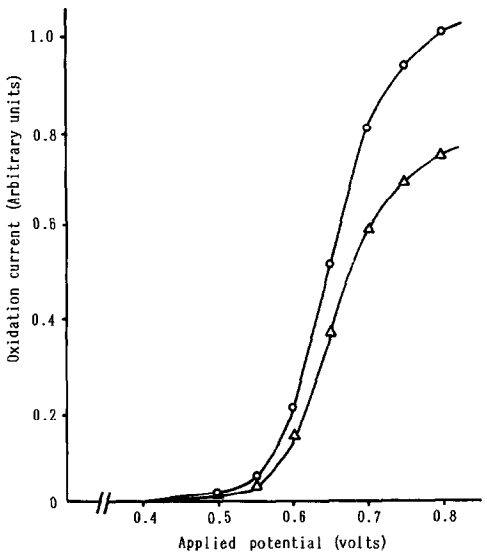

Fig. 2. Hydrodynamic voltammograms of vitamins D_3 and D_2. Hydrodynamic voltammetry was carried out for standard solutions containing vitamins D_3 and D_2: ○ = 1.0 μg/ml cholecalciferol (vitamin D_3); △ = 1.0 μg/ml ergocalciferol (vitamin D_2). The applied potential was variable at the downstream electrode, whereas it was fixed at zero at the upstream electrode.

hydrodynamic voltammetry was carried out for standard solutions containing vitamins D_3 and D_2 (Fig. 2). Fig. 2 shows the hydrodynamic voltammograms of vitamins D_3 and D_2, plots of oxidation currents against the applied potentials to cell 2 (in detector 2) with zero potential to cell 1 (in detector 1). It is apparent from Fig. 2 that vitamins D_3 and D_2 are converted to their oxidized forms by electrochemical oxidation, and that the higher applied potential gives a higher oxidation current for the standard solution. The half-wave potentials for both vitamins are nearly identical, *ca.* +0.65 V. On the basis of the observed noise level, the detection limit for vitamins in the standard solution was estimated to be about 8 pg when an applied potential of +0.65 V was used. Although the sensitivity by this detection mode was much higher than that with the UV method, the chromatograms of the peak fraction from the preparative HPLC were complicated because of strong background due to contaminants. Also, an applied potential higher than about 0.7 V gave rise to greater noise. Another drawback to the electrochemical oxidation method as described above is the problem of recovery of vitamin D. Wilske and Hulthe [30] tried to detect of 25-hydroxyvitamin D_3 in human serum by HPLC–electrochemical oxidation methods. This achieved only a low extraction efficiency (53%) of vitamin D_3.

We have found that the vitamin D was converted to an unidentified oxidated form at the upstream electrode (cell 1) and that it was reconverted to the reduced form at the downstream electrode (cell 2). Keeping a constant potential of +0.65 V at the upstream electrode, the current of the downstream electrode was measured as a function of the downstream electrode potential in the range 0 V to −0.5 V. Fig. 3 shows the plots of reduction current as a function of applied potential. The reduction current is linearly changed as the applied potential is increased from 0 to −0.3 V. A larger current resulted in a more negative potential, but background noise was increased. Thus, the optimum potentials for the oxidation and reduction were +0.65 V and −0.20 V, respectively.

Fig. 4A shows a typical chomatogram of a medical nutritional product obtained under the ED conditions described above. It is remarkable that well resolved peaks of vitamins D_2 and D_3 are observed and that the peaks are also separated from the majority of unsaponifiable compounds. As a result, vitamins D_2 and D_3 can be accurately quantitated by this method. Fig. 4B is a chromatogram of the same sample without vitamin D_2 as an

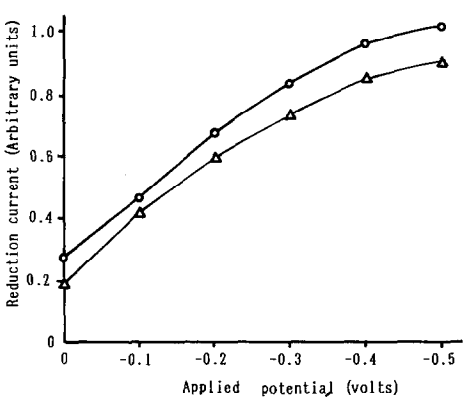

Fig. 3. Hydrodynamic voltammograms of vitamins D_3 and D_2. Hydrodynamic voltammetry was carried out for standard solutions containing vitamins D_3 and D_2: ○ = 1.0 μg/ml vitamin D_3; △ = 1.0 μg/ml vitamin D_2. The applied potential was variable at the downstream electrode, whereas it was fixed at +0.65 V at the upstream electrode.

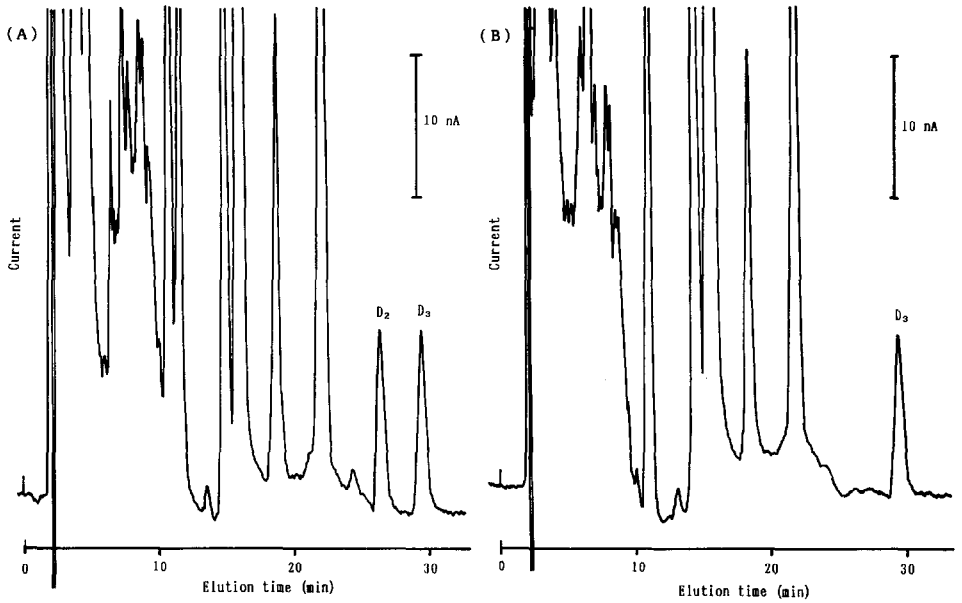

Fig. 4. Typical chromatograms of the extract from a medical nutritional product recorded by ED in the oxidation (+0.65 V)/reduction (−0.20 V) mode. (A) With addition of vitamin D_2 as an internal standard (0.625 μg per 100-ml sample); (B) without addition of vitamin D_2. Peaks D_2 and D_3 were assigned to vitamins D_2 and D_3, respectively.

internal standard. In the absence of vitamin D_2, only one peak due to vitamin D_3 is clearly observed in range 29–30 min. In comparison with the chromatograms observed in the presence and absence of vitamin D_2, the background at peak position is almost at the baseline level.

In conclusion, ED in the redox mode has many advantages over UV detection in terms of both selectivity in the region of elution of vitamins D_2 and D_3 and sensitivity. This method is able to detect both vitamins D_2 and D_3 because the vitamin D_2 and D_3 peaks obtained by UV detection are broad peaks compared with the ED peaks. Therefore, it appears that the UV peaks for vitamins D_2 and D_3 contain co-eluting substances.

Linearity, recovery and precision

Calibration curves for vitamins D_3 and D_2 were obtained based on ED in redox mode. They were found to be linear over the range 0.05–1.0 μg/ml. The peak observed for vitamin D_3 solution at a concentration as low as 50 ng/ml was reproducible and 5 mm in height. Assuming that the detection limit is twice the noise level of the baseline, the limit was estimated to be about 200 pg based on the observed peak height. The amount of vitamin D_3 in a given sample is calculated using internal standard of vitamin D_2.

The relative response factor (R_{rf}) of D_3 is:

$$\frac{\text{Peak height of } D_2}{\text{Concentration of } D_2} \times \frac{\text{concentration of } D_3}{\text{peak height of } D_3}$$

The amount of vitamin D_3 in the product sample (μg per 100 ml) is:

$$\frac{\text{Peak height of } D_3}{\text{Peak height of } D_2} \times \frac{100}{40} \times$$

$$\times \text{ added } D_2 \text{ standard } (\mu g) \times R_{rf} \times F_c$$

where F_c is the conversion factor [31], which was assumed to be 1.25 for pre-vitamin D [32,33].

The precision of our method may be evaluated by its reproducibility in the analysis of medical nutritional products. The results are shown in Table I. When the same sample was analysed five times on different days, a mean value of 5.78 and an S.D. of ±0.21 ng/ml were obtained, meaning that the relative standard deviation from the mean value was 3.6%. Recoveries of added vitamin D_3 were 97.5 ± 3.0% (mean ± S.D.).

TABLE I

REPRODUCIBILITY AND RECOVERY RATE OF VITAMIN D_3 ANALYSIS IN A MEDICAL NUTRITIONAL PRODUCT

Test	Before adding D_3 (μg/100 ml)	After adding 1.5 ng/ml D_3 (μg/100 ml)	Recovery rate	
			μg/100 ml	%
1	0.588	0.736	0.0148	98.7
2	0.585	0.725	0.0140	93.3
3	0.549	0.696	0.0147	98.0
4	0.564	0.708	0.0144	96.0
5	0.602	0.754	0.0152	101.3
Mean	0.578	0.724	0.0146	97.5
S.D.	0.021	0.023	0.0045	3.0

In conclusion, dual ED of vitamin D in the oxidation/reduction mode with an internal standard appears to be much more selective and accurate than UV detection at the nanomolar level for the measurement of vitamin D in medical-nutritional products. Its selectivity can be dramatically improved by converting vitamin D to its oxidized form at the upstream electrode in a dual-electrode system before detecting reduced current at the downstream electrode. The method is applicable to the pharmacokinetic study of vitamin D as well as the analysis of vitamin D in other medical nutritional products, fortified milks and infant formulas.

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