ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1497–1502

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

A novel high-performance liquid chromatographic assay for vitamin D metabolites using a coulometric electrochemical detector[1]

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Received 2 September 1996; accepted 16 October 1996

Abstract

A new, highly sensitive HPLC assay method using an electrochemical detector (ECD) for multiple assay of vitamin D metabolites is reported. The assay involves extracting lipids from plasma with methylene chloride and methanol, purification on Zorbax SIL column with 5.5% (v/v) iso-propanol in hexane and quantification by HPLC-ECD. A coulometric system, composed of the dual electrode analytical cell and a guard cell, was used for ECD of the eluting compounds. The potentials applied to detectors 1 and 2 in a dual electrode analytical cell were adjusted to +0.20 V and +0.60 V, respectively. This method is sensitive to 20 pg of 25-hydroxyvitamin D_3 [25(OH)D_3] and of 24*R*,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]. Calibration curves gave linearity from 20–1000 pg for 25(OH)D_3 and 24,25$(OH)_2D_3$. The detection limit was approximately 50 pg ml^{-1} for 25(OH)D_3 and 24,25$(OH)_2D_3$ in plasma. This sensitivity combined with an overall recovery of 25(OH)D_3 (81.5 ± 2.6%, mean ± S.E.) allows the measurement of trace amount of 25(OH)D_3 with only 20 μl of plasma. Intra- and interassay RSD values were 5.3 and 9.7% for 25(OH)D_3 and 6.3 and 9.7% for 24,25$(OH)_2D_3$, respectively. Plasma levels of 25(OH)D_3 and 24,25$(OH)_2D_3$ in normal adults were 15.9 ± 2.8 ng ml^{-1} ($n = 10$) and 1.4 ± 0.5 ng ml^{-1} ($n = 10$), respectively. This method allows the determination of 25(OH)D_2 and 25(OH)D_3 for evaluating their nutritional and clinical status. From these results, it is concluded that the proposed HPLC-ECD assay system is useful for the determination of vitamin D metabolites in biological fluids as a highly sensitive physicochemical method. © 1997 Elsevier Science B.V.

Keywords: 25(OH)D; 24,25$(OH)_2$D; HPLC; Electrochemical detector; Plasma

1. Introduction

Vitamin D is metabolized to 25-hydroxyvitamin D [25(OH)D] in the liver and subsequently to 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D] or

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[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August, 1996, Osaka, Japan.

PII S0731-7085(96)02005-5

24*R*,25-dihydroxyvitamin D [24,25(OH)$_2$D] in the kidney [1,2]. 1,25(OH)$_2$D, which is known to be an active form of vitamin D, promotes calcium absorption in the small intestine and increase the resorption of calcium from bone. It is well recognized that serum levels of 25(OH)D reflects the nutritional status of vitamin D in mammals [3,4]. Although 24,25(OH)$_2$D was considered as an inactive form of vitamin D, several recent reports have demonstrated its physiological activity [5]. The analytical methods reported for vitamin D metabolites mainly depend on bioassay techniques. The disadvantage of methods of saturation analysis, especially the competitive protein binding assay (CPBA), is the inability to achieve physiocochemical proof of the identity of the compound being determined. Methods using HPLC-UV and GC-MS are accurate since the substrate can be detected by the physiocochemical methods but the sensitivity is not enough for measuring most of the vitamin D metabolites in plasma. In the present study, a new, highly sensitive HPLC assay method has been investigated using an electrochemical detector (ECD) for multiple assay of vitamin D metabolites.

2. Materials and methods

2.1. Apparatus

The HPLC-UV system comprised a Shimadzu LC 6A pump, a Shimadzu SPD-6A ultraviolet detector and a Gilson Model 201 fraction collector. The HPLC-ECD system comprised a Shimadzu LC 9A pump and a ESA model 5100A electrochemical detector; (the analytical cell was Model 5011 and the guard cell was Model 5020). The dual analytical cell was located at the outlet of HPLC column and the guard cell was located between the pump and the sample injector. In this HPLC-ECD system, a UV detector was also placed between the HPLC column and the dual analytical cell as a monitor.

2.2. Reagents

Commercially available crystalline 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ (Solvay Duphar, The Netherlands) were used. 25(OH)D$_2$ was obtained by an in vivo method as reported previously [6]; 24*R*,25(OH)$_2$D$_2$ was chemically synthesized in the laboratory [7]. Organic solvents of analytical grades were distilled before use.

2.3. Plasma sample

Plasma samples were obtained from apparently healthy individuals ($n = 10$), patients with liver cirrhosis ($n = 7$), leukemia ($n = 7$) and osteoporosis ($n = 5$). Plasma samples obtained from healthy volunteers with supplementation of multivitamin preparation for 4 weeks were used for the separate assay of the metabolites of vitamin D$_2$ and D$_3$ ($n = 7$).

2.4. Determination of 25(OH)D and 24,25(OH)$_2$D in plasma

Exactly 100 μl of plasma sample was placed in a glass tube and diluted with distilled water to 500 μl. The diluted plasma sample was extracted with methanol and methylene chloride [8]. The lipid was dissolved in 200 μl of 5.5% (v/v) of iso-propanol in hexane and exactly 180 μl of the solution was subjected to preparative HPLC-UV. Conditions of preparative HPLC-UV were: column, Zorbax SIL (4.6 × 250 mm); mobile phase, 5.5 % (v/v) iso-propanol in hexane; flow-rate, 1.5 ml min^{-1}; retention time of 25(OH)D$_2$, 7.05 min (952 drops); 25(OH)D$_3$, 8.05 min (1073 drops); collection as 25(OH)D fr., 860–1300 drops; 24,25(OH)$_2$D fr., 2100–2780 drops. Both the 25(OH)D fraction and the 24,25(OH)$_2$D fraction obtained as above were dried under reduced pressure. The residue was dissolved in 200 μl of 5% (v/v) methanol in acetonitrile. Exactly 50 μl of the 25(OH)D fraction was subjected to analytical HPLC-ECD. In the case of 24,25(OH)D, exactly 180 μl of sample solution was subjected to analytical HPLC-ECD. Conditions of analytical HPLC-ECD were: column, Nucleosil 5C$_{18}$ (7.5 × 300 mm); mobile phase; 5% (v/v) methanol in acetonitrile with 0.025 M $HClO_4$; flow-rate: 1.2 ml min^{-1}; voltage; guard cell, +0.65 V, analytical cell 1, +0.20 V, analytical cell 2, +0.60 V; retention time; 25(OH)D$_2$, 25.20 min, 25(OH)D$_3$, 23.40

min, $24,25(OH)_2D_2$, 18.69 min, $24,25(OH)_2D_3$, 18.48 min. The peak heights corresponding to $25(OH)D_3$ and $24,25(OH)_2D_3$ on the chromatogram obtained from analytical HPLC-ECD were estimated. The concentration of $25(OH)D_3$ or $24,25(OH)_2D_3$ ng ml^{-1} was calculated using the following formula:

Concentration of $25(OH)D_3$ or $24,25(OH)_2D_3$ ng ml^{-1}

$$= S \times \frac{Psa}{Pst} \times \frac{200}{V_1} \times \frac{200}{180} \times \frac{1}{V}$$

where S is the quantity of $25(OH)D_3$ or $24,25(OH)_2D_3$ in the respective standard solution (ng), Psa is the peak height of $25(OH)D_3$ or $24,25(OH)_2D_3$ on the HPLC chromatogram obtained from a sample, Pst is the peak height on the HPLC-ECD chromatogram obtained from the respective standard solution, V_1 is injection volume (μl) for HPLC-ECD, 180 is injection volume (μl) for HPLC-UV and V is volume of a sample (μl) taken for assay.

3. Results

3.1. Optimization of electrochemical detection of vitamin D compounds

The influence of the applied potential and concentration of the supporting electrolyte was studied. In order to determine the optimum potential for the detection of vitamin D compounds, their current-voltage curve was obtained by injecting fixed amounts of standard solution of vitamin D compound and verifying the applied potentials of analytical cell 2 in 100 mV steps (Fig. 1). The curve describes the behaviour of $25(OH)D_3$ to oxidation potenials and the mean half-wave potential of $25(OH)D_3$ was nearly +0.60 V. Applied potentials greater than +0.70 V gave larger signals accompanied by greater noise. Therefore the optimum potential for $25(OH)D_3$ was set at +0.60 V for oxidation. The potential, +0.60 V, was also chosen for the detection of $24,25(OH)_2D_3$ since a current-voltage curve of $24,25(OH)_2D_3$ showed a similar pattern to $25(OH)D_3$. The potential applied to analytical cell 1 was adjusted to +0.20 V in order to eliminate expected other substances derived from plasma samples but not to affect the sensitivity for vitamin D metabolites. The applied potential of the guard cell was set to +0.65 V, which is 0.05 volt greater than the potential of analytical cell 2, according to the usual protocol. The influence of concentration of $HClO_4$ in the mobile phase was studied in the range 0.01–0.1 M. The peak height reached a constant value for concentrations > 0.025 M. Hence, an $HClO_4$ concentration of 0.025 M was chosen for the proposed method.

3.2. Extraction and purification of 25(OH)D and 24,25(OH)$_2$D from plasma

Chromatograms of analytical HPLC-ECD for 25(OH)D are shown in Fig. 2a. Under these HPLC conditions, $25(OH)D_2$ and $25(OH)D_3$ were successfully separated. Satisfactory results with good separation were obtained in human plasma after administration of multivitamin preparation. Calibration curves of $25(OH)D_2$ and $25(OH)D_3$ showed a linearity from 20–1000 pg under these conditions (Fig. 2b). Chromatograms of analytical HPLC-ECD for $24,25(OH)_2D_3$ are shown in Fig. 3a. $24,25(OH)_2D_3$ was separated from other sub-

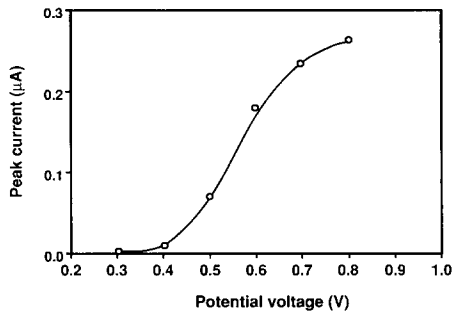

Fig. 1. Current-Voltage curve of $25(OH)D_3$. To produce a current-voltage curve, a constant weight was injected while the applied potential was varied. Each 5 ng/50 μl of standard solution of $25(OH)D_3$ was subjected to HPLC-ECD. The applied potential was varied from +0.30 V to +0.80 V at the analytical cell 2 electrode, whereas it was fixed at +0.20 V at the analytical cell 1 electrode. HPLC-ECD conditions were: column, Nucleosil $5C_{18}$ (7.5 × 300 mm); mobile phase, 5% (v/v) methanol in acetonitrile with 0.025 M $HClO_4$; flow-rate, 1.2 ml min^{-1}.

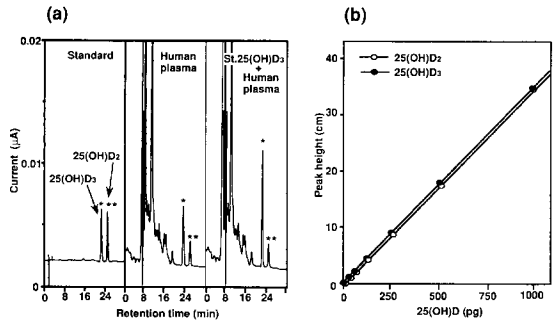

Fig. 2. (a) Chromatograms of HPLC-ECD for 25(OH)D. HPLC-ECD conditions were: column, Nucleosil $5C_{18}$ (7.5 × 300 mm); mobile phase, 5% (v/v) methanol in acetonitrile with 0.025 M $HClO_4$; flow-rate, 1.2 ml min^{-1}. (b) Calibration curves of $25(OH)D_2$ and $25(OH)D_3$ obtained from HPLC-ECD.

stances in plasma but was not successfully separated from $24,25(OH)_2D_2$ (retention time of $24,25(OH)_2D_2$, 18.69 min; 24,25(OH) $2D_3$, 18.48 min). A calibration curve of $24,25(OH)_2D_3$ gave a linearity between 20–1000 pg under these conditions (Fig. 3-b).

3.3. Sensitivity, precision and accuracy

This method is sensitive to each 20 pg of $25(OH)D_3$ and $24,25(OH)_2D_3$ (Table 1). The lower detection limit was approximately 250 pg ml^{-1} for $25(OH)D_3$ and $24,25(OH)_2D_3$ in plasma.

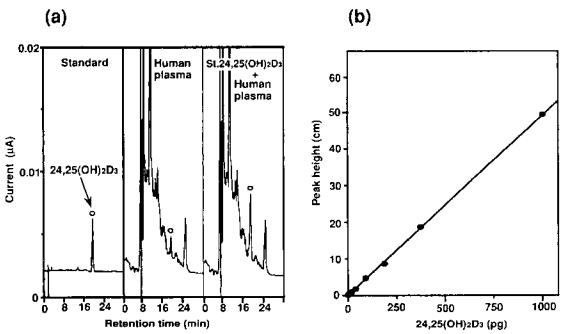

Fig. 3. (a) Chromatograms of HPLC-ECD for $24,25(OH)_2D$. HPLC-ECD conditions were: column, Nucleosil $5C_{18}$ (7.5 × 300 mm); mobile phase, 5% (v/v) methanol in acetonitrile with 0.025 M $HClO_4$; flow-rate, 1.2 ml min^{-1}. (b) Calibration curve of $24,25(OH)_2D_3$ obtained from HPLC-ECD.

Table 1
Summary of assay method of HPLC-ECD for vitamin D metabolites in plasma

	$25(OH)D_3$	$24,25(OH)_2D_3$
Detection limit (pg)	20	20
Dillution test: plasma volume (μl)	0–200	0–200
Recovery *n*	10	10
Mean ± S.E. (%)	98.7 ± 2.2	87.2 ± 5.4
RSD (%)	9.2	13.7
Intra-assay *n*	10	10
Mean ± S.E. ng ml^{-1}	30.5 ± 1.6	1.2 ± 0.1
RSD (%)	5.3	6.3
Inter-assay *n*	10	10
Mean ± S.E. ng ml^{-1}	29.0 ± 1.6	1.3 ± 0.1
RSD (%)	9.7	9.7

This sensitivity combined with an overall recovery of $25(OH)D_3$ (81.5 ± 2.6%, mean ± S.E., $n = 5$) allowed the measurement of $25(OH)D_3$ with only 20 μl of plasma. Intra- and interassay/RSD values were 5.3 and 9.7% for $25(OH)D_3$ and 6.3 and 9.7% for $24,25(OH)_2D_3$, respectively. Satisfactory results with high sensitivity and good reproducibility were obtained by the proposed method.

3.4. Concentration of $25(OH)D_3$ and $24,25(OH)_2D_3$ in healthy subjects

This method was applied to the plasma samples obtained from 10 healthy subjects aged in their twenties (Fig. 4). Plasma levels of $25(OH)D_3$ and $24,25(OH)_2D_3$ in healthy subjects were 15.9 ± 2.8 (mean ± S.E.) ng ml^{-1} and 1.4 ± 0.5 ng ml^{-1}, respectively. The respective values were close to those values reported previously [9,10] and the result suggested that the determination of 25(OH)D and $24,25(OH)_2D$ using HPLC-ECD was satisfactory.

3.5. Nutritional and clinical applications of the proposed method

To investigate the possibility of this method for a clinical study, plasma samples obtained from patients with liver cirrhosis, leukemia or osteoporosis were assayed (Fig. 5). Plasma levels of $25(OH)D_3$ showed a seasonal variation. The

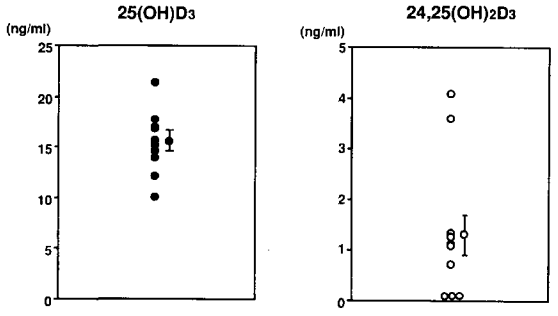

Fig. 4. Plasma levels of $25(OH)D_3$ and $24,25(OH)_2D_3$ in healthy subjects. These values were expressed as the mean ± S.E. of 10 samples.

plasma levels of $25(OH)D_3$ in patients with liver cirrhosis, leukemia and osteoporosis were significantly lower than that of healthy subjects in the summer ($P < 0.001$). Next, this method was applied to the plasma samples of subjects receiving a multivitamin preparation containing 200 IU (5 μg) of vitamin D_2 for 4 weeks to investigate the simultaneous determination of $25(OH)D_2$ and $25(OH)D_3$ (Fig. 6). No $25(OH)D_2$ was observed before supplementation of the multivitamin preparation; the levels were increased 4 weeks after the daily supplementation (3.5 ± 1.7 ng ml^{-1}) and were decreased 1 week after withdrawal of the supplementation (2.3 ± 1.1 ng ml^{-1}). On the other hand, the levels of $25(OH)D_3$ did not change during the administration of the multivitamin preparation.

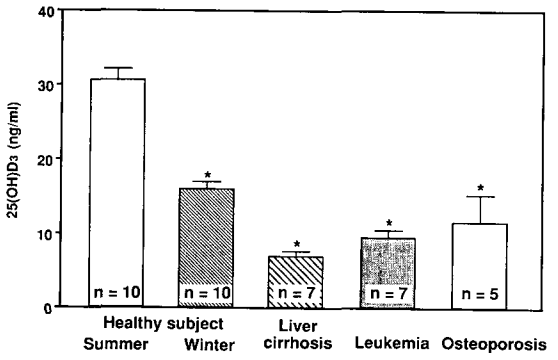

Fig. 5. Plasma levels of $25(OH)D_3$ in various diseases. These values were expressed as the mean ± S.E. *: Significantly different from the value of the normal subject in summer. Statistical analysis was performed by Student's t-test ($P < 0.001$).

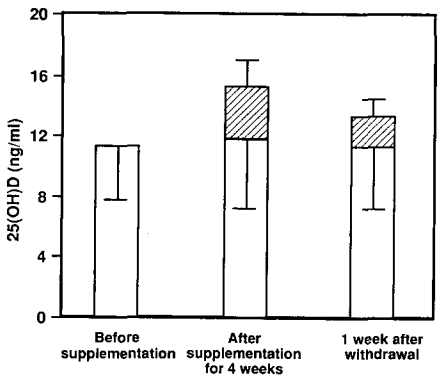

Fig. 6. Changes of plasma levels of 25(OH)D after the supplementation of the multiviatmin preparation. Multivitamin preparation containing 200 IU (5 μg) of vitamin D_2 was supplemented to healthy volunteers for 4 weeks. Plasma was obtained before the supplementation, 4 weeks after the daily supplementation and 1 week after withdrawal of the supplementation. These values were expressed as the mean ± S.E. of 7 samples. ▨ $25(OH)D_2$; □ $25(OH)D_3$.

4. Discussion

Numerous assays for quantification of 25(OH)D and $24,25(OH)_2D$ in plasma or serum have been reported in the last decade, using biological procedures such as CPBA or RIA [11]. These biological methods have shown great advantages in sensitivity; however they do not provide any proof of the identity of the compounds being measured. Although a physiocochemical method for measuring vitamin D metabolites, i.e. GC-MS [12], is very credible, it has not been successful in a routine assay for biological fluids because of the lower sensitivity than that of CPBA. Recently, dual-electrode detection in the electrochemical reduction/reoxidation mode has been reported as a selective method of detecting fat-soluble vitamins in nutritional products [13,14]. However, these methods employed amperometric detection; therefore, the sensitivity is not enough for detection of vitamin D metabolites in plasma. In the present study, the determination of vitamin D metabolites in plasma has been investigated using HPLC-ECD with coulometric detection.

In the coulometric detection system, the dual electrode analytical cell contains two porous

graphite in-line working electrodes. The potential of analytical cell 2 (+ 0.60 V) is the most important factor to obtain greater sensitivity. It is possible to eliminate other substances derived from samples possessing lower potential for oxidation than vitamin D metabolites by adjusting the potential of analytical cell 1 to + 0.20 V. This gave a good advantage for the selectivity of vitamin D metabolites in this system. The guard cell is useful for eliminating background noise resulting from the electolysis of other substances that may occur in solvents. The result that these potentials of analytical cell 1 and 2 were not changed by the vitamin D metabolites suggested that the reaction occurred at the same moiety in the vitamin D molecule. In addition, vitamin D compounds possessing a conjugated triene, e.g. tachysterol, pre-vitamin D_3, and conjugated diene, e.g. lumisterol, pro-vitamin D_3, were detected by the HPLC-ECD system. (data not shown). When the eluent from HPLC-ECD was subjected to HPLC-UV and GC-MS, it was confirmed that the structure was not changed. From these results taken together, it is postulated that the conjugated diene or conjugated triene structure in the vitamin D molecule may be responsible for HPLC-ECD detection.

As shown in Fig. 2 and Fig. 3, and Table 1, satisfactory results with good separation and reproducibility were obtained. Precision and accuracy of coulometric HPLC-ECD obtained above were similar to those data previously reported [9,10]. This method is also useful for the evaluation of clinical and nutritional status since simultaneous measurements are made of exogenous 25(OH)D_2 and endogenous 25(OH)D_3 in plasma (Fig. 5 and 6).

In summary, the proposed method has several advantages: high sensitivity; small samples; a non-radiosotope method; multiple assay of 25(OH)D and 24,25$(OH)_2$D. It is concluded that a sensitive method for the separate determination of 25(OH)D and 24,25$(OH)_2$D in plasma has been established. The proposed methods are useful as a routine assay for 25(OH)D and 24,25$(OH)_2$D in plasma.

Acknowledgements

The authors wish to thank Drs Hirotoshi Morii, Kiyoshi Nakatsuka of Osaka City University, Medical School, Dr Hakumei Oh of Chiba University, Department of Medicine, Drs Makoto Mino and Hiroshi Tamai of Osaka Medical School, Department of Pediatrics for providing the plasma samples from patients. We also appreciate Miss Takako Doi and Kyoko Fujimoto of Kobe Pharmaceutical University for their excellent technical assistance.

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