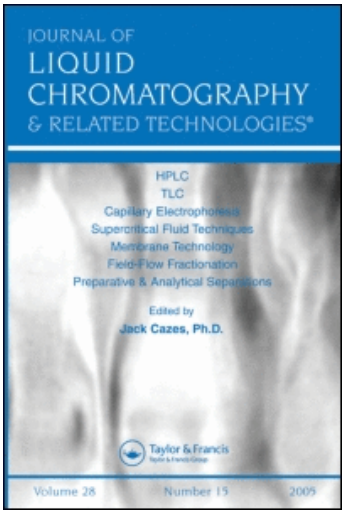

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NORMAL PHASE HPLC WITH EC DETECTION: 1,25-DIHYDROXY VITAMIN D_3 AND 25-HYDROXY-16-ENE-23-YNE VITAMIN D_3

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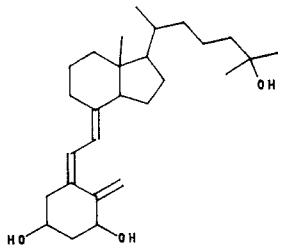
ABSTRACT

An analytical method for the separation and detection of 1,25-dihydroxy vitamin D_3 and 25-hydroxy-16-ene-23-yne vitamin D_3 by normal phase HPLC with electrochemical detector is reported. A post column electrolyte technique was adopted in order to apply electrochemical detection in a normal phase chromatographic system. Warfarin was used as an internal standard. The response and concentration was linear over the 10^{-3} mg/ml-10^{-5} mg/ml concentration range for both compounds. The sensitivity is improved 1000 fold by the use of amperometric detection instead of UV detection. The detection limit was approximately 3 pg/ml. The amperometric detector can detect some of the degradation products of 25-hydroxy-16-ene-23-yne vitamin D_3 which can not be detected by UV.

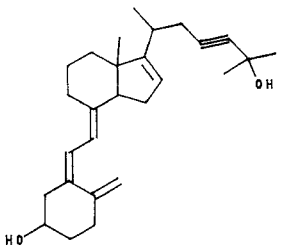
INTRODUCTION

The most important activated metabolite of vitamin D_3 is 1,25-dihydroxy vitamin D_3. It has been shown that this metabolite has a significant effect in maturation, differentiation and proliferation of immune system (1). In order to reduce the side effects such as intestinal calcium absorption (ICA) and bone calcium metabolization (BCM), an analog of 1,25-dihydroxy vitamin D_3 (25-hydroxy-16-ene-23-yne vitamin D_3) has been synthesized (2) (Figure 1).

The analysis methods hitherto reported for 1,25-dihydroxy vitamin D_3 are mainly bioassay techniques (3,4). Disadvantages of these assays include relatively long analysis time and variability of protein binding. Although there are several investigations with GC-MS (5) and LC-MS (6), they are still not suitable as routine



Calcitriol (Ro. 21-5535)



25-Hydroxy-16-ene-23-yne-Vit D_3

(Ro. 24-2090)

Figure 1 Chemical Structures.

clinical assay techniques. No analysis method has been reported for the synthetic analog, 25-hydroxy-16-ene-23-yne vitamin D_3. Our report offers a separation and detection method for 1,25-dihydroxy vitamin D_3 and its analog 25-hydroxy-16-ene-23-yne vitamin D_3 by normal phase HPLC with both electrochemical and UV detectors. Post column electrolyte was used in the amperometry experiment in order to combine the normal phase HPLC with electrochemical detection (7).

EXPERIMENTAL

Instrumentation

The liquid chromatography system consisted of two HPLC reciprocating piston pumps, both were Model 6000A (Waters Chromatography Division, Millipore Corp., Milford, MA.). Pump B assembled with a Sapphire Pump Head Retrofit Kit (Swip Precision, Verona, WI); the injector was a manual 50 μl loop Model 7125 Rheodyne, (Cotati, CA.); the column was a 5 μm Spherisorb silica analytical column (Universal Scientific, Norcross, GA.), 25cm x 4.6mm i.d. slurry packed in 90/10:MeOH/H_2O; a 100 psi back-pressure regulator (Upchurch, Oak Harbor, WA) was used to maintain the stability of the system pressure. A three electrode

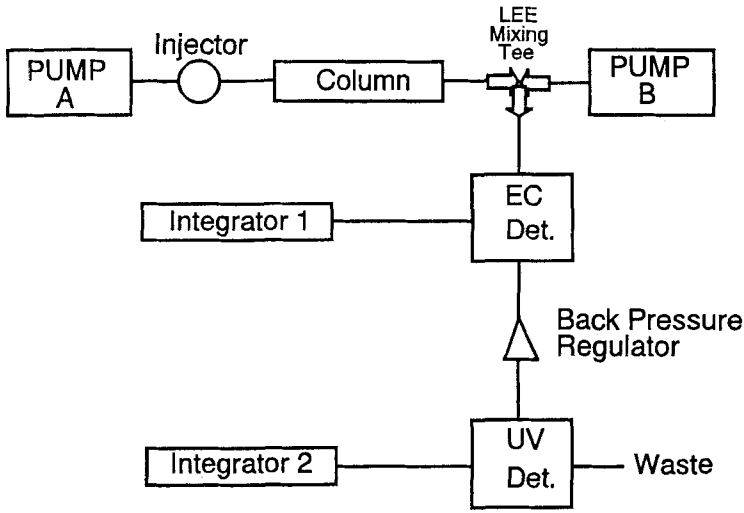


Figure 2 Apparatus schematic diagram.

amperometric detector (EC) was used with the BAS potentiostat. The system consisted of a 3mm glassy carbon working electrode, an Ag/AgCl reference electrode filled with 3.0 M NaCl and the stainless steel body as the auxiliary electrode. Ultraviolet Detector (UV) was a Beckman Model 160 equipped with a 254 nm filter (Beckman, Berkley, CA). Integrator models 3390A and 3392A (Hewlett Packard, Palo Alto, CA) were used for UV detector and EC detectors respectively. A schematic diagram of the apparatus is shown in Figure 2.

Reagents and Chemicals

1,25$(OH)_2$ vitD_3 (Ro. 21-5535) and 25(OH)-16-ene-23-yne vitD_3 (Ro. 24-2090) were kindly supplied by Hoffmann-La Roche, Nutley, N.J. Both compounds were stored under argon at -5°c. The organic solvent hexane (hex), methylene chloride ($MeCl_2$), tetrahydrofuran (THF) Iso-propanol (IpOH) and Methanol (MeOH) were HPLC grade (J. T. Baker Chemical Co., Phillipsburg, NJ). The THF was stored under nitrogen. Nitrogen (99.95%) and argon (99.999%) were obtained from Selox (Chattanooga, TN). The (α-acetonylbenzyl)-4-hydroxycoumarin (warfarin) was obtained from Sigma Chemical Company. Electrochemical grade tetrabutylammonium
hexafluorophosphate (TBAHFP) (99.0%) was obtained from Fluka Chemical Company (Ronkonkoma, NJ). Actinic volumetric flasks were used in the preparation of solutions.

Post-column electrolyte concentration

The concentration of 0.1 M TBAHFP in $MeCl_2$ was used as a stock solution. The salt concentration of TBAHFP was tried in the range of 0 to 0.1 M. The

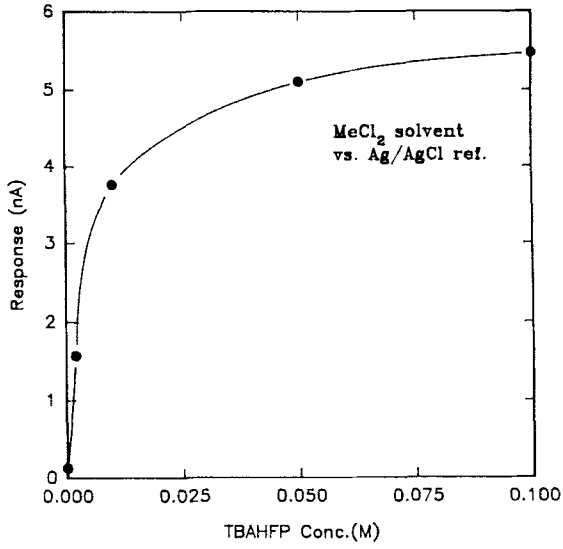


Figure 3 Response of amperometric detector vs. TBAHFP in $MeCl_2$.

voltage applied was 1.12 V. The sensitivity control of the amperometric detector was set at 10 nAfs, integrator attenuation was 8.

Hydrodynamic voltammetry

Ro. 21-5535 and Ro. 24-2090 solutions (0.5 μg/ml) were prepared separately by dilution with the mobile phase. The potential applied was 0.8 v to 1.5 v at 50 mv intervals. The attenuation of the integrator was set at 10, the sensitivity of the EC detector was 5 nAfs. When the response was over 5 nAfs, the full scale response was attenuated to a higher value to decrease the sensitivity. During area calculation, the value obtained from the integrator is multiplied by the sensitivity value shown on the potentiostat to give the corrected area. The area value was converted to nA by multiplying the transfer factor: (1 count x 0.125 μV-sec/12V) x full scale (nA).

Chromatographic mobile phase

The mobile phase was prepared by mixing 700 ml of $MeCl_2$, 200 ml of hex, 100 ml of THF and 60 ml of IpOH. The solution was passed through a 0.45 μm filter and sonicated for 10 min. The concentration of post column electrolyte solution was 0.05 M in $MeCl_2$. The flow rate was 1.5 ml/min for both pump A and pump B.

Photodegradation and air degradation

A solution of Ro. 24-2090 was prepared by dissolving 0.012 mg/ml of the compound into the mobile phase. Solutions of the same concentration were

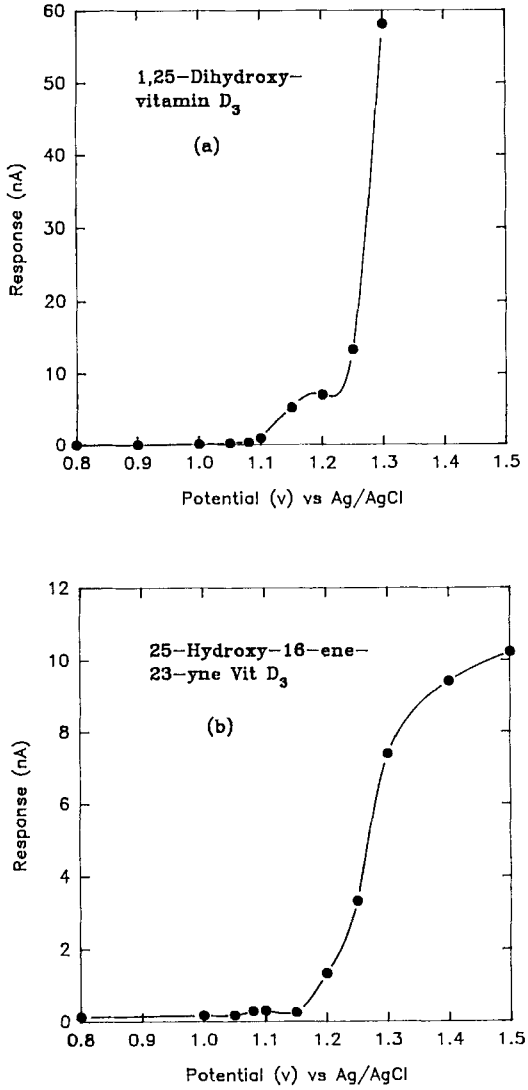


Figure 4 Hydrodynamic voltammograms (vs. Ag/AgCl) of 4 ng/ml 1,25$(OH)_2$vitamin D_3 (a) and 25(OH)-16-ene-23-yne vitamin D (b), TBAHFP=0.0025 M.

Table 1 Linearity and Detection Limits of Ro. 21-5535 and Ro. 24-2090. Mean of 3 values.

	Ro. 21-5535	Ro. 24-2090
Linear Range (EC)	$4.8x10^{-3}$ - $4.8x10^{-5}$ mg/ml	$6.5x10^{-3}$ - $6.5x10^{-5}$ mg/ml
Linear Range (UV)	$4.8x10^{-2}$ - $4.8x10^{-4}$ mg/ml	$6.5x10^{-2}$ - $6.5x10^{-4}$ mg/ml
Detection Limit (EC)	0.13 pg	0.14 pg
Detection Limit (UV)	2.4 ng	2.8 ng
Signal/Noise Ratio	3/1	3/1

exposed to four different conditions: 1. exposed to incandescent light and maintained in an argon atmosphere, 2. exposed to air and protected from light, 3. exposed to the incandescent light and air, 4. protected from light and maintained in an argon atmosphere. These solutions were chromatographed after 8h, 36h and 72h of treatment and detected by UV and EC detectors simultaneously. The chromatograms were compared with the chromatogram of freshly prepared Ro. 24-2090 solution.

RESULTS AND DISCUSSION

The plot of the signal vs. salt concentration (Figure 3) shows that the curve is essentially flat when the salt concentration is greater than 0.1 M, therefore this concentration of TBAHFP solution was adopted as the post column electrolyte solution. When the salt concentration was above 0.05 M, the system produced a large amount of bubbles, which interfere with the detector. This might be caused by the limited solubility of TBAHFP in mobile phase. The hydrodynamic voltammograms of Ro. 21-5535 and Ro. 24-2090 are shown in Figure 4.

The attenuation of the integrator was set to the highest (10), and the range of the detector was set as low as possible (1 nA) in the detection limit test. The detection limit concentration which would have a signal/noise ratio of 3 was obtained by the following calculation: (estimated conc.) = 3 x (diluted conc.)/$(\text{signal/noise})_{\text{exptl}}$ (Table 1).

Both analytes are not soluble in water, so normal phase chromatography has an obvious advantage in the separation of these two compounds, but insolubility of inorganic salts in normal phase solvents precludes the use of electrochemical detection, which needs an electrolyte to conduct current. The organic salt solution was used as post column electrolyte. Since the salt did not go through the column, it would not interfere with the separation. Also the normal phase solvent causes less baseline noise than an aqueous solution when the potential was raised to 1.3 v.

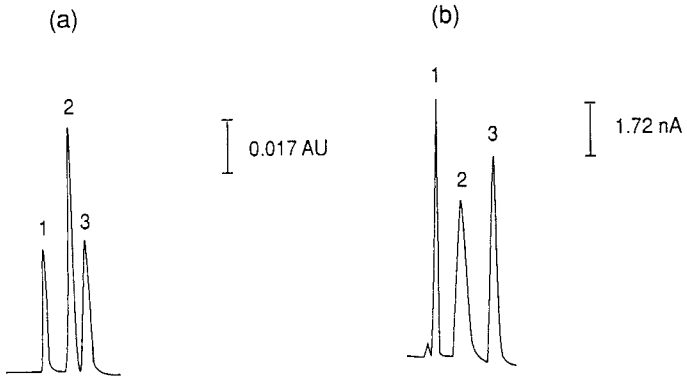


Figure 5 Chromatograms. (a) Ro. 21-5535 (3,1mg\100ml), Ro. 24-2090 (1,1mg\100ml) and warfarin (internal standard) (2,3mg/100ml) in 20/68/10/2:hex/$MeCl_2$/THF/MeOH solvent by UV detection at 254 nm, flow rate=1.2ml/min. (b) Ro. 21-5535 (3,1.5μg/1ml), Ro. 24-2090 (1,1.5μg/ml) and warfarin (2,20μg/1ml in 20/70/10/6:hex/$MeCl_2$/THF/IpOH, flow rate=1.5 ml/min with EC detection at 1.3 v.

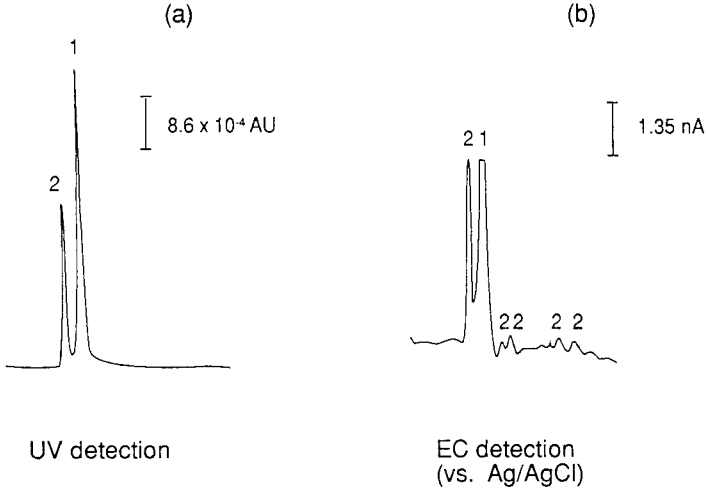


Figure 6 Chromatograms of Ro. 24-2090 (1,40μg/ml) and Degradation Products (2), after 36 hrs exposure to light and air. (a) UV detector, 254nm, sensitivity=0.05Aufs. (b) EC detector, sensitivity=5nAfs. Mobile phase: 20/70/10/6=hex/$MeCl_2$/THF/IpOH.

When a mixture of 68/20/10/2 methylene chloride/hexane /THF/methanol was used as the mobile phase, the capacity factor of Ro. 24-2090 was better and the broadening of the warfarin peak was reduced (Figure 5). However, methanol is immiscible with hexane, and when this mobile phase is used in the EC cell, it caused an increase in baseline noise because of poor mixing. A 70/20/10/3 methylene 2hloride/hexane/THF/iso-propanol was used as mobile phase for EC detection.

The comparison of the chromatograms in the degradation test showed that the compound 25-hydroxy-16-ene-23-yne vitamin D_3 is more sensitive to the ultraviolet light than air and that the amperometric detector could detect some degradation compounds that the UV detector could not detect (Figure 6).

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