Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 919–925, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00

A semi-automated high-performance liquid chromatographic system for the determination of 25-hydroxyvitamin D in human plasma: elimination of interference by barbiturates and use of photodiode array detection*

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Abstract: A semi-automated high-performance liquid chromatographic (HPLC) method for the measurement of 25-hydroxyvitamins D_2 and D_3 is described. Plasma was extracted using acetonitrile and a Bond-Elut C_{18} cartridge system, eluted with methanol and fractionated on Sep-Pak SIL. After formation of isotachysterol isomers straight-phase HPLC was carried out monitoring the mobile phase with a photodiode array detection system. Two internal standards have been used, namely [^{3}H]25-hydroxy-vitamin D_3 and 25-hydroxydihydrotachysterol$_3$ both of which are shown to give satisfactory results. The use of the extraction system described eliminates interference from barbiturates which had previously been reported to interfere. Photodiode array detection allows confirmation of the identity of the analytes.

Keywords: *25-Hydroxyvitamin D; plasma; HPLC; photodiode array detection; barbiturates.*

Introduction

25-Hydroxyvitamin D_3 ($25OHD_3$) is the metabolite of vitamin D_3 which circulates in greatest concentration in human plasma. It is also the substrate for the renal 1α-hydroxylase enzyme which catalyses conversion to 1,25-dihydroxyvitamin D_3, the active calcium homeostatic hormone. Measurements of the plasma concentration of $25OHD_3$ are widely used as a means of assessing vitamin D status in patients with suspected osteomalacia. Methods for the measurement of $25OHD_3$ have recently been reviewed [1] and usually use either saturation analysis or high-performance liquid chromatography (HPLC). The metabolite formed from *in vivo* generated vitamin D_3 is $25OHD_3$ but in addition $25OHD_2$ is formed from exogenous vitamin D_2 which is sometimes added to

*Presented at the "International Symposium on Pharmaceutical and Biomedical Analysis", September 1987, Barcelona, Spain.

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foodstuffs. Saturation analysis does not usually distinguish between the two metabolites, whereas HPLC can be used to measure both metabolites independently. Conversion to isotachysterol isomers prior to HPLC improves sensitivity by two-fold [2]. It is usual to monitor the HPLC effluent by means of UV detectors at fixed wavelength, usually around 254 nm, which may not distinguish between 25OHD and interfering compounds present in the plasma extract. One such case of interference has been reported in a patient taking therapeutic levels of amylobarbitone [3]. This paper describes the development of a semi-automated HPLC system for the determination of 25-hydroxy-vitamin D in human plasma using an automated HPLC system attached to a photodiode array detector, which allows peaks to be examined for their UV spectra. Vitamin D metabolites are extracted from plasma, prior to HPLC, using a solid phase C18 cartridge system [2].

Experimental

Materials and methods used have previously been described [2], with the exception that HPLC was carried out using a programmable 590 pump, a 712 WISP automated sample injection system, and a model 990 photodiode array detector (all supplied by Millipore Waters UK Ltd., Harrow). Short HPLC columns (Microsorb short-one, 100 × 4.6 mm, 3 μm silica, Rainin Instrument Co., Ltd., Woburn, MA, USA) were obtained from Anachem UK Ltd. Unless otherwise specified reagents were all of analytical grade and were used without further purification. HPLC solvents were generally obtained from Rathburn Chemicals Ltd., Walkerburn, Scotland, UK, and were used as supplied after degassing in a sonic bath. Bond-Elut C18 (300 mg) (Analytchem International, Harbor City, CA, USA) packed into small syringes (3 ml) was obtained from Jones Chromatography Ltd. (Hengoed, Glamorgan, Wales, UK). These cartridges after receipt and prior to use were washed sequentially with 4 ml hexane, 4 ml methanol and 4 ml of water. Isotachysterol isomers were formed by passing dry HCl gas into a small glass vial (1.3 cm diameter × 4 cm height, 1 dram, FBG-Trident Ltd., Temple Cloud, Bristol, UK) containing the seco-steroids plus 200 ng vitamin D_2 dissolved in 0.1 ml of chloroform.

The method used for plasma extraction was based on that described previously [2] and is summarised here: after addition of internal standard, plasma (2 ml) was extracted with acetonitrile, centrifuged and the supernatant diluted with 1 ml phosphate buffer (0.1 M, pH = 10.5) and passed through a small Bond-Elut C18 cartridge as previously described. After washing with water (3 ml) and methanol–water (60:40, v/v, 3 ml), 25OHD was eluted with 3 ml of methanol. The methanol extract was further fractionated using a Sep-Pak SIL cartridge and isopropanol–hexane solvent mixtures as previously described [4] (after sample application in 2 × 100 μl 1% isopropanol–hexane, washing with 10 ml 1% isopropanol–hexane the 25-hydroxyvitamin D is eluted with 10 ml 3% isopropanol–hexane). Isotachysterol isomers (25-OHITS) were formed and subjected to straight-phase HPLC using a short 3 μm SIL column, eluting with a ternary solvent system, methanol–isopropanol–hexane (1:3:96, v/v/v, 1.5 ml min^{-1}) and monitoring the effluent with a Waters 990 photodiode array assembly. The system was modified to enable a semi-automatic HPLC analysis to be carried out by addition of an automated sample injector (712 WISP system) and a programmable pump. In addition to the radiolabelled internal standard ($[^3H]$-$25OHD_3$) previously used, a second internal standard, 25-hydroxydihydrotachysterol ($25OHDHT_3$), was investigated since the use of

[^{3}H]25OHD$_3$ required modification after the UV detector to allow for the collection of the 25OHITS$_3$ fraction for liquid scintillation counting. The complete system is illustrated in Fig. 1. The programmable 590 pump can be programmed to switch valve 1 to direct solvent flow to the fraction collector at the beginning of a peak and switch off at the end of the peak. At the end of the peak, the fraction collector was moved to the next collection tube. This requires consistency of retention times which was achieved by enclosing the HPLC column in a water jacket which was kept at 25°C.

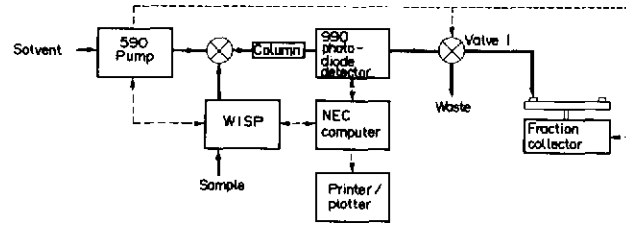

Figure 1
Diagrammatic representation of the automated HPLC system. Interrupted lines (----) indicate information flow, and continuous lines (——) indicate solvent per sample flow.

The effluent was continuously monitored by the diode array assembly which had been modified to include a narrow slit on the incident light to improve resolution to 1.4 nm, and this enables clear resolution of the three maxima of the absorption peaks of isotachysterol and dihydrotachysterol which are separated by 10 nm. The advantage of the use of radiolabelled 25OHD$_3$ as an internal standard was that it monitored the efficiency of isotachysterol formation using the acidic reagent, but the disadvantage was that it required liquid scintillation counting prior to final calculation. In an attempt to overcome this latter difficulty 25OHDHT$_3$ was used as an internal standard. Although 25OHDHT$_3$ has a UV spectrum which is similar in shape to that of the isotachysterol isomers, the three maxima occur at different wavelengths. Figure 2 illustrates the spectra of these two types of compound. Using the ternary HPLC solvent system already described, 25OHITS$_2$, 25OHITS$_3$ and 25OHDHT$_3$ were all resolved. By monitoring at different wavelengths (i.e. 253 and 301 nm) any interference which arises from inadequate resolution of the isotachysterol isomers from 25OHDHT$_3$ could be eliminated. Peak area ratios were used for quantitation (the ratio peak area of 25OHITS: counts min^{-1}[^{3}H]25OHITS$_3$ being used when [^{3}H]25OHD$_3$ was used as an internal standard, and the ratio peak area of 25OHITS:peak area 25OHDHT$_3$ when 25OHDHT$_3$ was used as an internal standard). Graphs relating peak area ratio (y axis) to mass of 25OHD (x axis) were straight lines (using [^{3}H]25OHD$_3$, $y = 12.9x - 1.15$, correlation coefficient: 0.9999; when using 25OHDHT$_3$, $y = 0.56x + 1.89$, correlation coefficient: 0.9880) and there was no difference between the lines obtained for 25OHD$_2$ and 25OHD$_3$.

Results

The use of the photodiode array assembly enabled the peaks of interest to be examined for homogeneity and identified as isotachysterol derivatives by their UV spectra. A typical 3-D trace obtained from a plasma sample to which 25OHD$_2$ had been added, together with 25OHDHT$_3$ as an internal standard, is illustrated in Fig. 3. The

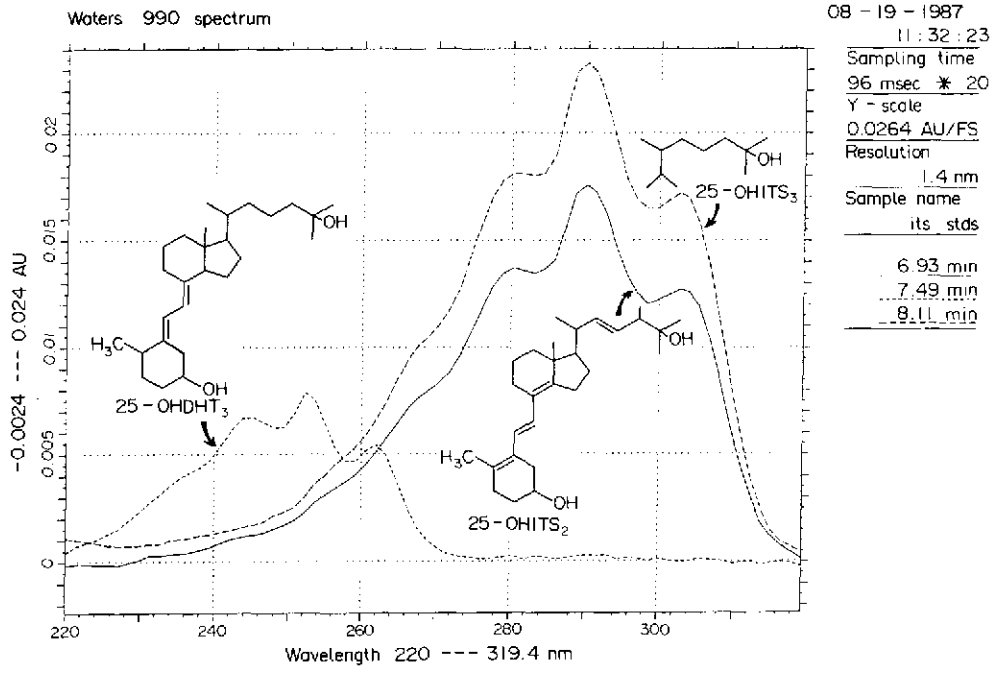

Figure 2
UV spectra of 25OHDHT$_3$, 25OHITS$_2$, and 25OHITS$_3$. The retention times of these compounds are given at the bottom of the right hand panel and indicate that the three peaks are completely resolved in the HPLC system.

change in UV spectra and retention times when 25OHD$_3$ is converted to 25OHITS$_3$ are illustrated by Fig. 4.

Amylobarbitone in therapeutic doses has been reported to interfere in an HPLC assay for 25OHD [3]. The retention behaviour of a number of barbiturates in the ternary solvent system was examined, some of the values obtained are given in Table 1. To examine the possibility of such interference, three barbiturates (hexo-, amylo-, and pheno-barbitone) representing the range of polarity in the straight phase SIL system were added to plasma in $\mu g\ ml^{-1}$ concentrations. The various fractions from the Bond-Elut C18 cartridge were then examined for the presence of these barbiturates, as was the final extract prior to HPLC. Table 2 summarises the results of these experiments.

Discussion

A simple semi-automated HPLC system has been developed for the analysis of 25OHD$_2$ and 25OHD$_3$ in human plasma. This system uses either [^{3}H]25OHD$_3$ or 25OHDHT$_3$ as an internal standard. Quantitation can be carried out using a diode array detector monitoring at a number of different wavelengths. The interpolation of a specific chemical reaction (formation of isotachysterol) which produces compounds with characteristic spectra, adds an extra degree of specificity to this assay as well as improving the sensitivity, since isotachysterols have a molar extinction coefficient which is roughly twice that of the unisomerised vitamin D metabolites. The specificity of peaks with the correct retention times can be examined by inspection of their UV spectra which

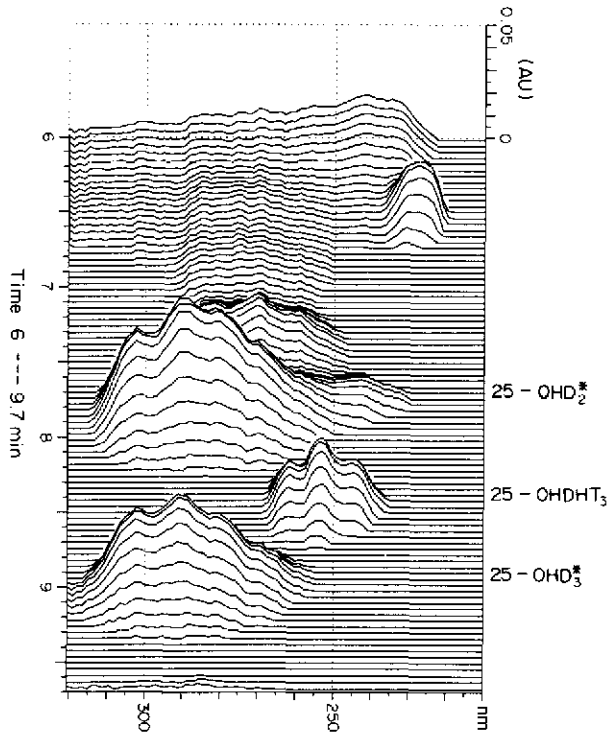

Figure 3
Three dimensional UV spectra obtained by HPLC of an extract of plasma to which 25OHD$_2$ had been added, using 25OHDHT$_3$ as an internal standard. Hexobarbitone (5 μg ml^{-1}) had also been added to the plasma prior to extraction. The positions of the parent compounds are indicated on the right hand side (* indicates that these compounds are actually present as their isotachysterol isomers).

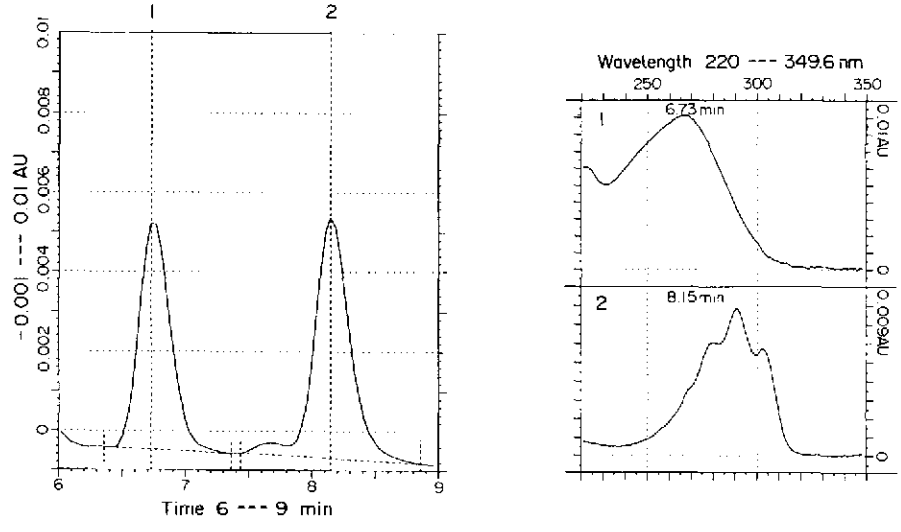

Figure 4
HPLC of 25OHD$_3$ (1) and 25OHITS$_3$ (2) illustrating the increase in polarity in a straight-phase HPLC system and change in UV spectrum which occur on isomerisation of a vitamin D metabolite to its isotachysterol isomer.

Table 1
Retention times of various barbiturates in the straight-phase HPLC system used for the separation of $25OHD_2$ and $25OHD_3$ and their isotachysterol isomers

Compound	Retention time
$25OHD_2$	7.58
$25OHITS_2$	8.81
$25OHD_3$	8.51
$25OHITS_3$	9.97
Hexobarbitone	5.53
Amylobarbitone	6.62
Pentobarbitone	7.10
Butobarbitone	8.44
Cyclobarbitone	8.55
Barbitone	9.48
Phenobarbitone	9.76

Retention times of the barbiturates examined were not reproducible using the HPLC system described in the text. On one occasion the relative retention times of pento- and amylo-barbitones were reversed.

Table 2
Recoveries of barbiturates added to plasma at various stages of extraction

Fraction	% Recovery of added hexobarbitone	amylobarbitone	phenobarbitone*
A. Application	98.8	87.8	77.2
B. Water wash	trace	3.8	4.7
C. MeOH:water wash	—	—	—
D. Methanol	—	—	—

Barbiturates were added to plasma at a concentration of 5 $\mu g\ ml^{-1}$. Plasma samples (2 ml) were extracted as described in the text. Fraction A was that part of the acetonitrile extract which passed through the Bond-Elut cartridge on application, Fraction B was the 3 ml water wash, Fraction C was the 3 ml methanol:water wash, and Fraction D was the 3 ml methanol eluent. Fractions were evaporated to dryness, or in cases where large quantities of water were present, acidified and the barbiturates extracted into organic solvent. HPLC was carried out on all fractions and the amount of each barbiturate present was measured.

*Low recoveries of phenobarbitone were obtained in all experiments and it is suggested that this may be due to the absorption of this barbiturate, and to a lesser extent amylobarbitone, to the protein pellet produced when acetonitrile is added to plasma. The percentage recoveries recorded are the mean of two experiments.

will give the characteristic UV maxima at 280, 291 and 301 nm of the isotachysterol isomers. It is shown that by using the Bond-Elut C18 solid phase extraction procedure, interference by barbiturates at therapeutic concentrations is eliminated since these compounds are largely not retained by the reverse-phase Bond-Elut column and the small quantity which is retained is washed off in the first water wash. While amylobarbitone could be separated on Bond-Elut C_{18} from the vitamin D metabolites in low concentrations, if present in high concentrations it might well interfere, and other barbiturates had retention times close to that of the 25-hydroxylated isotachysterol isomers. However the UV spectra of barbiturates is distinctly different from that of isotachysterols. Figure 5 shows the 3-D UV spectrum of amylobarbitone. The use of the

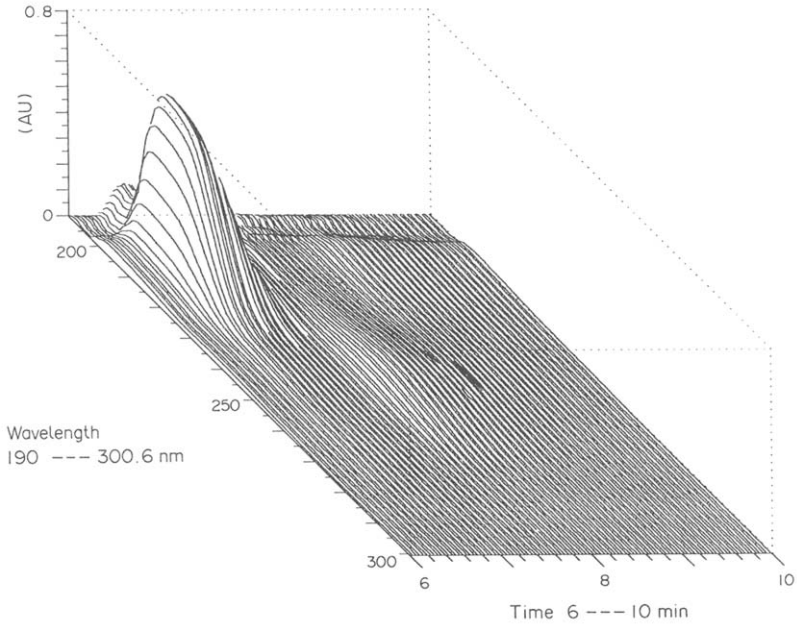

Figure 5
Three dimensional ultraviolet spectrum obtained by HPLC of amylobarbitone.

photodiode detector should therefore give an indication of possible interference. The extraction method used in the present procedure involves a reversed-phase extraction step on the Bond-Elut C18 cartridge and it might be expected that barbiturates would be removed at this stage, particularly in view of the use of alkaline phosphate buffer. No trace of the three barbiturates studied could not be detected in the HPLC extract and appeared to be removed during the initial stages of the solid-phase extraction procedure. Recoveries, reproducibilities and sensitivity of this assay are the same as that previously described [2].

Acknowledgements We should like to thank Millipore (UK) Ltd. for financial assistance during the course of this investigation.

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[*Received for review 23 September 1987; revised manuscript received 15 January 1988*]