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ASSESSMENT OF A TWO-STEP HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY USING DUAL-WAVELENGTH ULTRAVIOLET MONITORING FOR 25-HYDROXYERGOCALCIFEROL AND 25-HYDROXYCHOLECALCIFEROL IN HUMAN SERUM OR PLASMA

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SUMMARY

The technique of dual-wavelength monitoring was used to verify the purity of high-performance liquid chromatographic (HPLC) peaks quantified as 25-hydroxyergocalciferol and 25-hydroxycholecalciferol. The data obtained show the need for a second HPLC step prior to quantitation. Potential inaccuracy arising from inadvertent collection of radio-labelled decomposition products was assessed. Between-day coefficients of variation were 7.3, 5.0 and 3.6%, respectively for 11.3 (n = 12), 17.1 (n = 14), and 32.9 (n = 8) ng/ml of 25-hydroxycholecalciferol. For 25-hydroxyergocalciferol, these values were 6.4 and 3.8% for 11.4 (n = 12) and 20.1 (n = 8) ng/ml concentrations, respectively. Comparison of total 25-hydroxycalciferol with a competitive protein binding assay was made. The comparison produced a correlation coefficient (r) of 0.94 and a relationship of $y = 1.03x + 3.3$. Four of the samples contained more than 10 ng/ml of 25-hydroxyergocalciferol and the results are consistent with the reported 100% cross-reactivity of the competitive binding protein method for 25-hydroxyergocalciferol and 25-hydroxycholecalciferol A simple regeneration procedure is also described which enables Sep-Pak C_{18} cartridges to be reused up to eighteen times. Samples may be stored at —18°C for upto several months before assay and either serum or plasma may be used.

INTRODUCTION

The vitamin D status of a given subject is usually assessed by the plasma concentration of 25-hydroxycalciferol [25(OH)D] [1]. This value is effectively the sum of the concentrations of 25-hydroxycholecalciferol [25(OH)D3] and 25-hydroxyergocalciferol [25(OH)D2]. Paterson [2] has also highlighted the

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need for this assay in clinical management of patients being administered milligram doses of vitamin D.

Recent reports [3—5] of inter-laboratory comparisons suggest that problems currently exist in the accuracy of many assays for 25(OH)D. Accuracy of a given method is usually assessed by spiking known amounts of the compound of interest into a blank matrix, the same as that used in the assay. This matrix is also often extracted and assayed as per samples to determine if any co-eluting peaks are presented in the chromatgram. When dealing with an endogenous compound such as 25(OH)D3, no suitable blank matrix is readily available. It has been suggested that charcoal-treated plasma is a suitable matrix for this purpose. However, as charcoal treatment removes a wide range of compounds non-specifically, there is a high probability that any co-eluting compounds would also be removed from the plasma.

We have therefore found it necessary to assess the accuracy of this method by indirect means. This has been achieved by comparison of assay results with those obtained using a theoretically distinct assay for the same samples. Competitive protein binding (CPB) has been used as this comparative assay. Assessment of other potential sources of inaccuracy in the high-performance liquid chromatographic (HPLC) assay has also been undertaken. For example, purity of the chromatographic peaks was assessed by dual-wavelength monitoring, as was the potential inclusion of radiolabelled decomposition products in the recovery determination.

EXPERIMENTAL

Apparatus

HPLC was carried out using an LKB Model 2150 pump (LKB, Bromma Sweden). The detector was an LKB Uvicord S, fitted with an HPLC flow cell and 254 nm lamp and filter. The detection was carried out at 0.0025 AUFS. A Tracor Model 970A variable-wavelength detector (Tracor Instruments, Austin, TX, U.S.A.), set at 285 nm and 0.01 AUFS was also used in series with the LKB detector. Chromatograms were recorded on an Analytical Instruments dual-pen recorder (Analytical Instruments, Strathpine, Australia). All solvents were of HPLC grade (Waters Assoc., Milford, MA, U.S.A.). De-ionised water was obtained from a Milli R015 system, coupled with a Super-Q water system (Millipore, Bedford, MA, U.S.A.).

All glassware used in sample preparation was silanised by overnight soaking in 10% dimethylchlorosilane in toluene.

Sample extraction

Samples were stored at −18°C until the assay. A 2-ml volume of plasma or serum was added to 15-ml culture tubes with PTFE-lined screw caps. A 10-μl aliquot of ethanol containing approximately 10 000 disintegrations per min (dpm) of 80—120 Ci/mmol 25(OH)[23,24*n*-^{3}H]D3 (Amersham International, Amersham, U.K.) was also added. This radiolabelled tracer had been previously purified by using the same chromatographic procedure as will be described for samples.

Three 10-μl volumes of tracer were also pipetted directly into respective

scintillation vials for determination of percent recovery. Methanol (8 ml) was added to each tube containing a sample. After capping, the tubes were mixed thoroughly and centrifuged at 1500 *g* for 15 min to remove the precipitated protein.

A Sep-Pak C_{18} cartridge (Waters Assoc.) was rinsed with 10 ml of methanol and 10 ml of de-ionised water and then fitted to a 50-ml glass syringe. De-ionised water (3 ml) was added to the syringe followed by the sample supernatant. After rinsing the sample tube with a further 3 ml of de-ionised water, these washings were also added to the syringe. After mixing, the plunger was re-inserted and the syringe contents applied to the Sep-Pak. The sample was rinsed with 10 ml of distilled water, followed by 5 ml of air to remove excess water. Finally, the sample was eluted from the Sep-Pak with 6 ml of methanol which was collected in a 12-ml tapered base centrifuge tube. After addition of 2–3 ml of dichloromethane, each sample was evaporated to dryness at 40°C using high purity dry nitrogen. Between samples, the Sep-Pak cartridge was regenerated by reverse flushing with 10—15 ml of methanol, followed by 10—15 ml of de-ionised water. Batches of eighteen samples were extracted using a single cartridge.

Standards

Appropriate volumes of a working standard of 25(OH)D3 in ethanol were added to each of five 12-ml centrifuge tubes to produce a range of approximately 8 to 80 ng per tube. A 10-μl aliquot of tracer was also added to each standard to determine recoveries in the HPLC eluate after chromatography. To each tube 2—3 ml of dichloromethane were added to enable rinsing and mixing of the contents. The solvent was then evaporated at 40°C using nitrogen.

Chromatography

Extracted samples were redisssolved in 100 μl of mobile phase and centrifuged for 10 min at 1500 *g* to remove insoluble particulate matter. A maximum amount of the sample was then injected into a 100-μl HPLC loop. The eluate fraction which contained both 25(OH)D2 and 25(OH)D3 was collected into a further 12-ml centrifuge tube and evaporated under nitrogen at room temperature.

Chromatographic conditions used in the first HPLC step included the use of two Brownlee Labs. S160 5-μm columns (25 cm × 4.6 mm) connected in series and preceded by a Brownlee Labs. pre-column (Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase was isopropanol—hexane (9:91) with a flow-rate of 0.90 ml/min.

For the second HPLC step, a single Brownlee Labs. Spherisorb-ODS 5-μm column (25 cm × 4.6 mm) was used. The mobile phase was methanol—water (88:12) at a flow-rate of 1.0 ml/min. Samples were treated similarly to the first step, with the appropriate mobile phase being used. The fraction containing 25(OH)D3 was collected to determine radiolabel recovery.

Calculations

From the standards chromatographed, a standard curve of peak height versus nanograms of 25(OH)D3 injected, was constructed. From this curve, the

nanograms of 25(OH)D2 and 25(OH)D3 injected for each sample were determined. These values were then corrected for recoveries and volume of sample to give ng/ml in the original serum or plasma.

Competitive protein binding

This assay was performed as reported by Mason and Posen [6]. Columns of Sephadex LH-20 (Pharmacia (South Seas), North Ryde, Australia) were used for preliminary purification. Rat serum was used as the source of binding protein during quantitation.

Scintillation counting

Tritiated 25(OH)D3 was measured using a Packard Tri-Carb Model 2650 liquid scintillation spectrometer with ACS scintillation fluid (Amersham, Arlington Heights, IL, U.S.A.). Quench corrections were automatically applied using an efficiency curve generated from quench standards (Packard, Downers Grove, IL, U.S.A.).

Re-use of Sep-Pak cartridges

Thirteen batches of samples (eighteen samples per batch) were chosen at random and the overall recoveries from samples 1, 6, 12 and 18 were analysed by one-way analysis of variance for differences in recoveries at each sample number.

Radiochemical purity of fractions collected for recovery determinations

Eluate fractions of 0.3 ml each were collected across the chromatographic band in which 25(OH)D3 elutes during the second HPLC step. This was performed for nine consecutive samples.

Comparison of HPLC and CPB

Plasma samples were obtained from ten haemodialysis patients, ten patients being treated with continuous ambulatory peritoneal dialysis, nine samples submitted for clinical purposes, ten samples from geriatric patients and eleven samples from normal subjects. Each sample was subdivided into two aliquots and stored until analysed by either HPLC or CPB.

Comparison of plasma and serum

Both heparinised plasma and serum were collected from each of thirteen normal subjects. All samples were analysed by HPLC for 25(OH)D2 and 25(OH)D3.

Storage of samples

Heparinised plasma was obtained from each of eleven normal subjects and subdivided into two aliquots prior to storage at −18°C. One aliquot for each subject was analysed by HPLC within fourteen days. The other aliquots remained in storage for eleven months before being assayed.

RESULTS AND DISCUSSION

Retention behaviour of 25(OH)D2 and 25(OH)D3 is in general agreement

with that reported by others [7—10], given the differences in chromatographic conditions. Peak heights per ng injected and peak height ratios for 25(OH)D2 and 25(OH)D3 are similar. This is expected due to the presence of the same chromophore in these molecules and similar molecular weights.

The between-day coefficient of variation obtained using this method is reported in Table I and indicates quite acceptable reproducibility.

Data showing the overall recoveries, and the recoveries during re-use of Sep-Pak cartridges, are presented in Table II. As there was no statistically significant reduction in the overall recovery for up to eighteen samples per Sep-Pak cartridge, it is concluded that up to eighteen samples can be extracted per cartridge when using the regeneration procedure described. This is in agreement with the findings of Cannell et al. [11] who were able to re-use Sep-Pak cartridges up to ten times [12]. The build-up of material at the Sep-Pak inlet has effectively been overcome by reverse flushing of the cartridge. The relatively low overall recovery is not unexpected for the number of transfers. In view of the coefficient of variation for this assay (see Table I), these recoveries are acceptable. The lower limit of quantitation for the HPLC assay has been arbitrarily set at a chromatographic signal-to-noise ratio of 5:1. This represents 3.0 ng/ml of 25(OH)D2 or 25(OH)D3.

TABLE I

BETWEEN-DAY COEFFICIENT OF VARIATION (C.V.) FOR THE HPLC ASSAY

Units of means are ng/ml.

		Sample		
		1	2	3
25(OH)D3	n	12	14	8
	$\bar{x}$	11.3	17.1	32.9
	C.V. (%)	7.3	5.0	3.6
25(OH)D2	n	12	14	8
	$\bar{x}$	11.4	<3.0	20.1
	C.V. (%)	6.4	—	3.8

TABLE II

RE-USABILITY OF SEP-PAK CARTRIDGES AND RECOVERIES USING THE HPLC ASSAY

The table shows the mean recoveries obtained for samples 1, 6, 12 and 18 from thirteen batches of samples. One-way analysis of variance shows no statistically significant differences for the recoveries between samples 1, 6, 12 and 18 (F = 0.63). This lack of difference suggests Sep-Pak cartridges do not deteriorate after re-use for up to eighteen samples.

	Sample			
	1	6	12	18
n	13	13	13	13
$\bar{x}$	0.51	0.50	0.50	0.49
S.D.	0.04	0.04	0.04	0.06

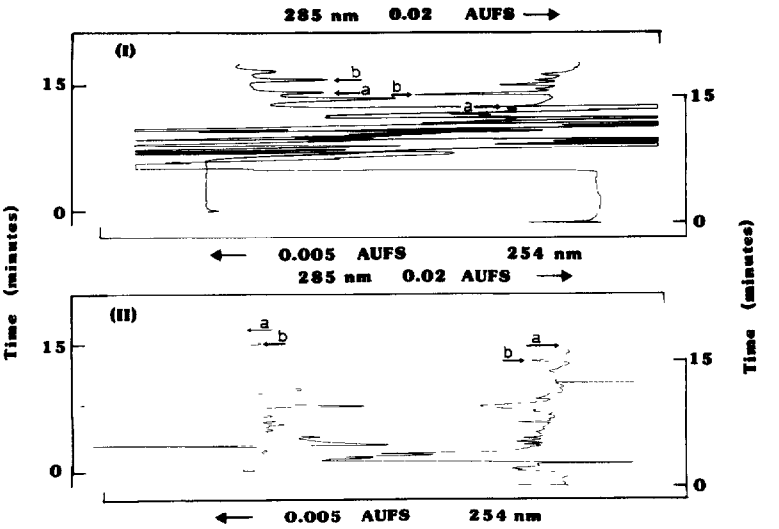

Fig. 1. Chromatograms obtained for samples during HPLC step 1 (I) and step 2 (II). Despite apparent separation of 25(OH)D3 after step 1, dual-wavelength monitoring shows the presence of an underlying peak (see Table III). The 25(OH)D3 peak in (II) represents 16 ng of material. a = Retention time of 25(OH)D2; b = retention time of 25(OH)D3.

Fig. 1 shows the chromatograms obtained at 254 nm and 285 nm for both the first and second HPLC steps. The chromatogram for step one suggests adequate separation to allow quantitation. Dual-wavelength monitoring, however, shows the presence of an underlying peak for both 25(OH)D2 and 25(OH)D3 which is not detectable by simple visual evaluation (see Table III) An acceptable range of peak height ratios was defined as being within two standard deviations (S.D.) of the mean ratio obtained for pure standards in

TABLE III

PEAK HEIGHT RATIOS (285 nm/254 nm) FROM DUAL-WAVELENGTH MONITORING

Standards and samples were assessed. The standards shown were run on the second HPLC step only. All 25(OH)D3 data were obtained in a single batch and all 25(OH)D2 data were normalised to a ratio of 0.66 for the 25(OH)D3 standards of the same batch. To allow for instrumental variations, acceptable limits for the ratio were set at $\bar{x} \pm 2$S.D. for standards (i.e 0.58—0.74) Medians are quoted as step 1 data are not normally distributed.

	25(OH)D3			25(OH)D2	
	Step 1	Step 2 (After step 1)	Step 2 (Standards)	Step 1	Step 2
n (Total)	16	18	5	12	12
Median	0 71	0.675	0.65	0.62	0.665
Range	0.61—1.00*	0.61—0.73	0.63—0.72	0.49—1.69	0.61—0.72
n (Outside limits)	6	0	0	4	0

*This range is underestimated as two samples went off scale and could not be included in the data.

the same batch. Samples having ratios outside these limits are considered as containing excessive impurities. On this basis, six of the sixteen 25(OH)D3 samples and four of the twelve 25(OH)D2 samples were unacceptable after the first HPLC step, while all samples were acceptable after the second HPLC step. Owing to day-to-day variation in detector performance and manual selection of the 285 nm wavelength, the peak height ratios reported in Table III vary. For this same reason, the 25(OH)D2 data were normalised as they were not obtained in a single batch.

Early studies by Chen et al. [13] highlight the instability of vitamin D compounds, especially in the presence of water. Inadvertent collection of decomposition products, resulting from the extraction, could therefore produce erroneously high recovery values. This would produce inaccurate results.

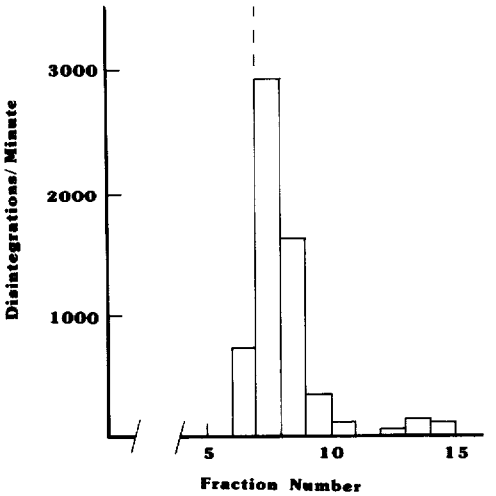

Fig. 2. A typical histogram of radiolabel in fractions obtained from HPLC step 2 (single sample). Fractions 12—14 present possible errors in recoveries if inadvertently collected with the 25(OH)D3.

Fig. 2 is a histogram of the radiolabelled 25(OH)D3 in fractions collected from one sample. Fractions 12—14 inclusive represent a potential interference in the recovery determination if collected with the tritiated 25(OH)D3 for recovery determination. This peak is undoubtedly a decomposition product produced during the extraction procedure as no other radiolabel has been added to the system. Fractions were collected in a similar manner from a total of nine samples. From these data, the percent error in recoveries was calculated. The mean error was 2.4% and the range 0.6—5.1%. Although this is not a large error, exclusion of this decomposition product from the recovery fraction will contribute to the accuracy of results. No radiolabel was found in fractions other than those mentioned above. Interference from any late eluting decomposition products (i.e., from previous samples) is therefore unlikely.

The comparison of results obtained from the HPLC assay with CPB, is presented as Fig. 3. These data show agreement between the two methods for a wide range of sample concentrations and subjects types. This agreement between two methods of quite distinct theoretical basis supports the accuracy of the results obtained.

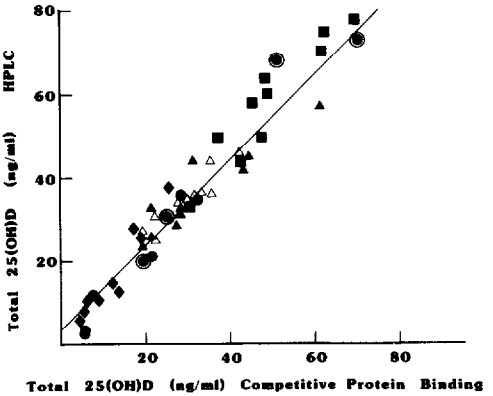

Fig. 3. Comparison of total 25(OH)D as determined by HPLC and CPB on each of fifty samples. Regression analysis of the data yielded a correlation coefficient (r) of 0.94 and the relationship $y = 1.03x + 3.3$. The four samples encircled contained measurable amounts of 25(OH)D2 (i.e. > 3.0 ng/ml). From lowest to highest total 25(OH)D they contained 11, 17, 68 and 64 ng/ml of 25(OH)D2, respectively. ♦ = Geriatrics; ● = clinical; ▲ = continuous ambulatory peritoneal dialysis; ■ = haemodialysis; △ = normals; ○ = 25(OH)D2 present.

The agreement between HPLC and CPB methods, for samples containing measurable 25(OH)D2 concentrations, is consistent with 100% cross-reactivity between 25(OH)D2 and 25(OH)D3 in the CPB assay. This is in agreement with the report of Jones et al. [14].

It has been argued that chromatographic separation of 25(OH)D2 and 25(OH)D3 invalidates the use of tritiated 25(OH)D3 to estimate the recovery of 25(OH)D2 [15]. A widespread practice in HPLC is to use chromatographically distinct analogues as internal standards. This depends on consistent relative recoveries of internal standard and the compound of interest. In view of this practice, it must be assumed that any error in the recovery of 25(OH)D2 as assessed by tritiated 25(OH)D3 will be systematic. Assuming the reported 100% cross-reactivity, the agreement with the CPB assay (for samples containing measurable 25(OH)D2 concentrations), suggests that no systematic error of clinical relevance has occurred in the assay presented here.

Of the samples analysed to compare serum and plasma, none contained measurable 25(OH)D2 concentrations. The paired two-tailed Student t test showed no statistically significant difference between results obtained for serum or plasma. The mean 25(OH)D3 ± S.D. for the serum and plasma samples were 31.7 ± 7.5 ng/ml and 30.5 ± 6.1 ng/ml, respectively.

A paired two-tailed t test comparing the two storage periods shows a statistically significant difference in the 25(OH)D concentrations between the storage times ($p < 0.05$). This is in contrast to a previous report [16] which suggests there is no change during storage. This contradiction may be due to either the longer storage time used in the present study or it may be a reflection of an improved coefficient of variation for the present method (see Table I). The respective means for the fourteen-day and eleven-month treatments were 26.8 and 24.3 ng/ml, respectively. These relatively minor differences suggest that storage times up to several months are acceptable. However, long term storage is not advised in view of the statistically significant difference shown.

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