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RAPID AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ERCALCIDIOL AND CALCIDIOL (25-HYDROXYVITAMINS D_2 AND D_3) USING *trans*-CALCIDIOL AS AN ULTRAVIOLET-ABSORBING INTERNAL STANDARD

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SUMMARY

A system is described using high-performance liquid chromatography to separate and quantify, by spectrophotometry in a simple one-stage procedure, ercalcidiol (25-hydroxyvitamin D_2) and calcidiol (25-hydroxyvitamin D_3). The novel feature of the method is the employment of an ultraviolet-absorbing internal standard to monitor recovery. This has the advantage of permitting total automation of the quantification by eliminating the need for radioactivity counting. The method gives results that compare well with those obtained in other systems and has particular application in clinical studies where rapid separate determination of ercalcidiol and calcidiol is required.

INTRODUCTION

The assay of ercalcidiol and calcidiol (25-hydroxyvitamins D_2 and D_3, respectively*) is of considerable clinical importance because these hepatic metabolites represent the major circulating pool of the vitamins and their concentration is used as an index of the state of vitamin D nutrition [2]. Two types of vitamin D may be found in the body, calciol (vitamin D_3) the endogenous form, synthesised from 7-dehydrocholesterol in the skin by the action of UV light and ercalciol (vitamin D_2) formed exogenously by irradiation of the plant sterol ergosterol; the latter is used for supplementation of food stuffs and for pharmaceutical preparations in many parts of the world including the U.K. Separate measurement of the 25-hydroxylated derivatives of ercalciol and calciol can therefore be valuable

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*The names used for vitamin D metabolites in this paper are the trivial names recommended by the IUPAC-IUB Commission on the nomenclature of vitamin D [1].

in assessing the response to treatment, or compliance with it as ercalcidiol, and distinguishing this from the concentration of calcidiol which largely reflects exposure to sunlight.

Many methods for the assay of calcidiol have been described using radioimmunoassay (RIA), protein binding assay (PBA) or high-performance liquid chromatography (HPLC), and have been reviewed by Seamark et al. [3]. Methods based on RIA or PBA cannot distinguish between ercalcidiol and calcidiol unless these compounds have first been separated by HPLC, thus introducing a further step into the assay. Several methods have been reported for ercalcidiol and calcidiol assay by quantifying UV absorbance on HPLC but in all these the recovery has been assessed by addition of [³H]-calcidiol, requiring a sample to be counted before a final result is obtained [4–9]. Consequently, even where automated HPLC equipment has been used, a correction needs to be made before the result is available [9].

This paper describes the development of a method that eliminates the need for a radioactive standard by substituting an internal UV-absorbing standard thus enabling both the quantification and the recovery determination to be performed in the same single HPLC step. The integrator can then complete all necessary calculations and deliver a final result.

EXPERIMENTAL

Vitamin D metabolites

Ercalcidiol and calcidiol were the generous gifts respectively of Upjohn (Kalamazoo, MI, U.S.A.) and Roussel (Paris, France). Tritium-labelled calcidiol, 25-hydroxy-[26,(27)-methyl-³H]cholecalciferol (22.3 Ci/mmol) was obtained from Amersham International (Little Chalfont, U.K.).

Solvents

All solvents were HPLC grade supplied by Rathburn (Walkerburn, U.K.).

Instrumentation

HPLC was performed using a liquid chromatograph consisting of a Model 6000A solvent delivery system, WISP 710B automatic sample injector and Model 440 UV detector controlled by a Model 730 data module, all equipment from Waters Assoc. (Harrow, U.K.). Fractions were collected using a programmable Superac fraction collector from LKB (South Croyden, U.K.). Samples were applied to a stainless-steel Zorbax-Sil column (25 cm × 4.6 mm I.D., 10 μm particle size) fitted with a guard column packed with H.C. Pellosil (Whatman, Clifton, NJ, U.S.A.) and eluted with mixtures of methanol, propan-2-ol and hexane.

Preparation of the internal standard (5,6-trans-calcidiol)

The method used was based on the procedure of Lawson and Bell [10]. Calcidiol (0.75 mg) was dissolved in 20 ml hexane, 0.1 ml of a solution of iodine in hexane (0.1%, w/v) was added and the mixture left in diffuse daylight for 1 h. The solution was then washed with 0.1 M sodium thiosulphate and evaporated

to dryness. The residue was dissolved in hexane–methanol–propan-2-ol(96:2:2, v/v/v) and a sample was injected on to a Zorbax-Sil column developed with hexane–methanol–propane-2-ol(96:2:2, v/v/v) to follow the course of the reaction. The main part of the solution was then taken up in hexane–propan-2-ol(98:2, v/v) and applied to a Zorbax-Sil column developed with the same solvent to prepare the *trans* isomer product separate from the *cis*-calcidiol starting material.

UV spectrophotometry was used to confirm the identity of the two peaks; the *cis* isomer has minimum absorbance at 228 and maximum at 265 nm whereas the *trans* form shows minimum at 232 and maximum at 274 nm. The latter absorbance was used to determine the mass of the *trans* isomer assuming a molar absorptivity of 23 600.

Preparation of isotacalcidiol (25-hydroxyisotachysterol$_3$)

This isomer of calcidiol was prepared by the method of Seamark et al. [11] in which the isomerisation is effected by hydrogen chloride gas. The product was harvested by HPLC on a Zorbax-Sil column developed with hexane–propan-2-ol (96:4) and identified and quantified by UV spectrophotometry using the characteristic absorbance maximum at 290 nm, molar absorptivity 41 800.

Extraction of samples for assay

A volume of 2.0 ml serum or plasma was normally used for extractions; smaller volumes were made up to 2.0 ml with 0.9% saline solution. Recovery standard was added in 50 μl ethanol and left to equilibrate for 30 min with occasional vortex-mixing. A volume of 2.0 ml acetonitrile was added and vortexed for 30 s. The resulting homogenate was centrifuged (1000 g, 3 min) and the supernatant loaded directly on to a pre-packed reversed-phase silica cartridge (C_{18} Sep-Pak, Waters Assoc.), which had been pre-treated as follows. Methanol (2 ml) was passed through the Sep-Pak (1 drop/s emerging from the cartridge) using a 5-ml glass syringe, 5 ml distilled water were applied in the same manner; the Sep-Pak was then ready to receive the sample dissolved in acetonitrile–water (50:50, v/v). After loading, the cartridge was washed with 3 ml methanol–water (70:30, v/v), this wash was discarded. The fraction eluted with 3 ml acetonitrile was retained. The solvent was evaporated by a stream of nitrogen to leave a residue that was resuspended in 200 μl of hexane–propan-2-ol (98:2) for analysis by HPLC.

HPLC for quantification of calcidiol

Samples were injected on to a Zorbax-Sil column eluted with hexane–propan-2-ol at 2.0 ml/min. This system was capable of resolving ercalcidiol and calcidiol as shown by the UV absorbance at 254 nm (0.005 a.u.f.s.) of unlabelled compound when passed through the detector or by collecting the appropriate fractions and counting them in a liquid scintillation counter when ^{3}H-labelled preparations were used.

Standard curves were constructed by injecting a known volume (200 μl) of appropriate dilutions of a standard solution of ercalcidiol or calcidiol which had been calibrated by UV absorbance at the maximum extinction, 265 nm. Quan-

tities from 1–100 ng were injected: over this range the response (peak area) was proportional to the mass.

PBA for calcidiol

This method [12] was used to check the results of the new method described in the paper and employs rat kidney cytosol as the source of binding protein.

HPLC assay for calcidiol using radioactive internal standard

This method was also used to check results of the new method and has already been described [13].

Statistical analysis

Results are expressed as mean ± standard deviation of the mean; differences were examined by Student's t test for unpaired samples. Correlation was examined by the Pearson r test.

RESULTS

Preparation of 5,6-trans-calcidiol

Separation of the cis and trans isomers of calcidiol was achieved by HPLC. The course of the reaction was followed by peaks being collected at 8 and 9 min, respectively, which were then identified by UV spectrophotometry (Fig. 1). Quantification of the trans peak gave a final yield of 0.20 mg (27%).

Preparation of isotacalcidiol

Isotacalcidiol was separated from calcidiol by HPLC using conditions as in Fig. 1; the peak had the characteristic UV-absorbance spectrum and showed a 69% yield from 20 μg starting material.

Choice of internal standard

Three UV-absorbing compounds were assessed as possible internal standards 5,6-trans calcidiol, isotacalcidiol and 24-hydroxycalcidiol (24,25-dihydroxyvitamin D_3). Isotacalcidiol was found to be unsuitable as it was not recovered to the same extent as calcidiol (53 and 67%, respectively); in addition when added to serum samples a peak of UV-absorbing activity was seen to run close to the internal standard in several HPLC systems. 24-Hydroxycalcidiol was rejected because of low recovery relative to calcidiol giving inflated results for serum calcidiol assay when compared to the same samples assayed using radioactive calcidiol as internal standards.

trans-Calcidiol was assessed for recovery as follows. Twelve 2-ml aliquots of a serum pool were supplemented with 85 ng ercalcidiol, 87 ng calcidiol and 100 ng trans-calcidiol; a further twelve aliquots were supplemented only with trans-calcidiol. Samples were extracted and chromatographed as described in Experimental. Subtraction of the peak heights for ercalcidiol and calcidiol in the unsupplemented samples from those in the supplemented samples enabled recovery of these compounds to be calculated. Recoveries for ercalcidiol, calcidiol and
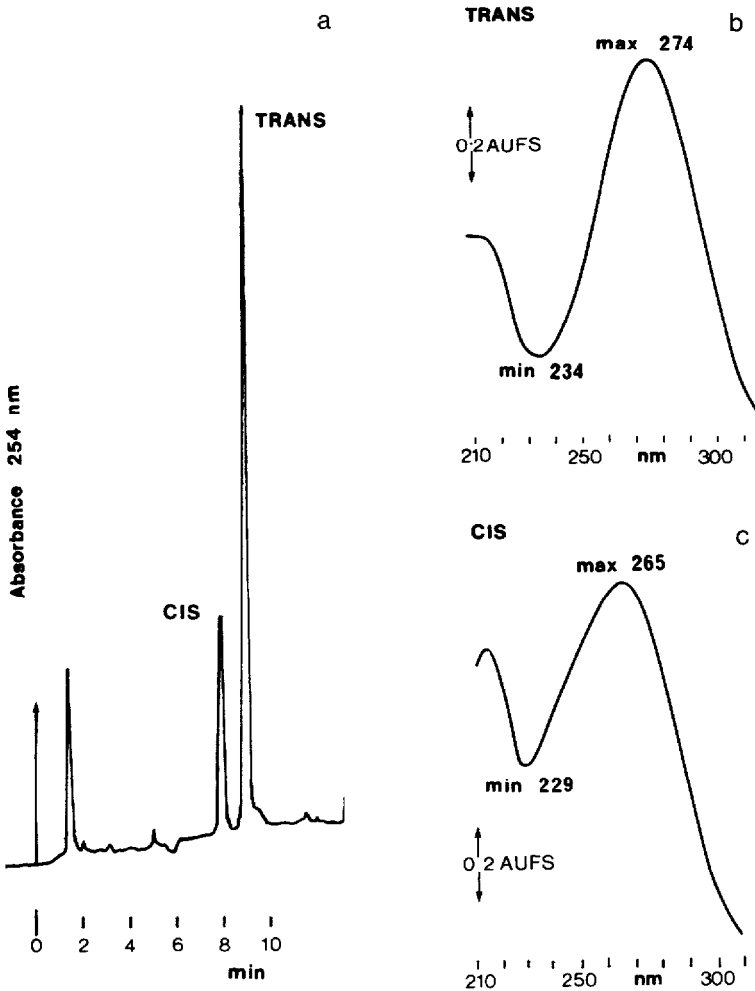
Fig. 1. Use of HPLC to monitor conversion of 5,6-*cis*-calcidiol to 5,6-*trans*-calcidiol; samples were applied to a Zorbax-Sil column eluted with hexane–methanol–propan-2-ol (96:2:2) at 2.0 ml/min. (a) Absorbance at 254 nm (0.5 a.u.f.s.) was used to detect the peaks corresponding to the two isomers which were collected at 8 and 9 min, and identified by their UV absorbance spectra. (b,c) UV absorbance spectra for the separated 5,6-*trans* (b) and 5,6-*cis* (c) isomers of calcidiol.

trans-calcidiol were 69.4 ± 1.4, 70.1 ± 1.4 and $71.2 \pm 1.3\%$ (mean $\pm$ S.D.), respectively; none of the differences between these results was significant.

The UV absorbance of *trans*-calcidiol at 254 nm was found to bear a linear relation to the mass injected over the range 1–200 ng.

No UV-absorbing peaks were observed in serum that ran in the region of *trans*-calcidiol; on this basis and because of the satisfactory recovery characteristics, this compound was considered suitable for use as an internal standard.

Method for calcidiol assay using trans-calcidiol as internal standard

The procedure adopted for the *trans*-calcidiol internal standard assay (TCISA) is summarised in Fig. 2. Extraction and HPLC were performed as described in

CALIBRATION   ANALYSIS

Calibration standard   Serum sample (2 ml)

            Internal standard ——┐
            50 - 100 ng   ┆
                            Extraction
                            vial
Ercalcidiol 50 ng ——┐
Calcidiol   50 ng ——┐   Incubate 20°C, 30 min

Autosampler vial ◄——   Add 2 ml acetonitrile
                            mix vigorously 30 s
                            then centrifuge

                    Pellet   Supernatant
                    (discard)
                                  Sep-pak
                                  (reversed phase)

        HPLC   Dry and transfer to
    CALIBRATION INJECTION   autosampler vial
Integrator formulates response
  factors from std quantities
                                  HPLC
                            ANALYSIS INJECTION
                            Integrator completes
                                calculation using
                                analysis data and
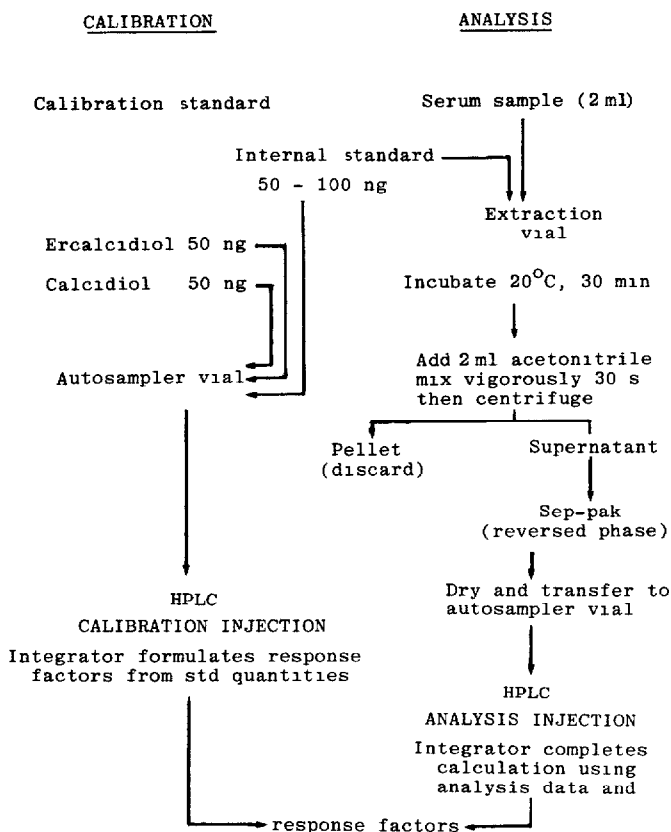   ——► response factors ◄——┘

Fig. 2. Flow diagram of the method developed for the assay of ercalcidiol and calcidiol using 5,6-*trans*-calcidiol as an internal UV-absorbing standard (TCISA).

Experimental. Results were obtained using the data module as follows. The same quantity of internal standard (which need not be known precisely but which must be within the linear response range) that was added to the samples was also added to a calibration standard containing known quantities of ercalcidiol and calcidiol. The integrator calculated response factors which were used to quantify and assess recovery in serum samples giving a final result as ng/ml.

Examples of chromatograms for standards and serum samples are displayed in Fig. 3 showing well separated peaks with no interference in the areas of interest even when sera from a vitamin D-treated patient or one on anticonvulsant therapy were assayed.

Comparison with established methods

Protein binding assay. Twenty seven serum samples were assayed using both the TCISA method described above to obtain values for calcidiol and ercalcidiol which were then summed and the PBA which measures the two forms together. The results (Fig. 4a) show a close correlation between the two methods ($r = 0.98$, $p < 0.001$) and an equivalence in value ($y = 1.00x + 0.75$).
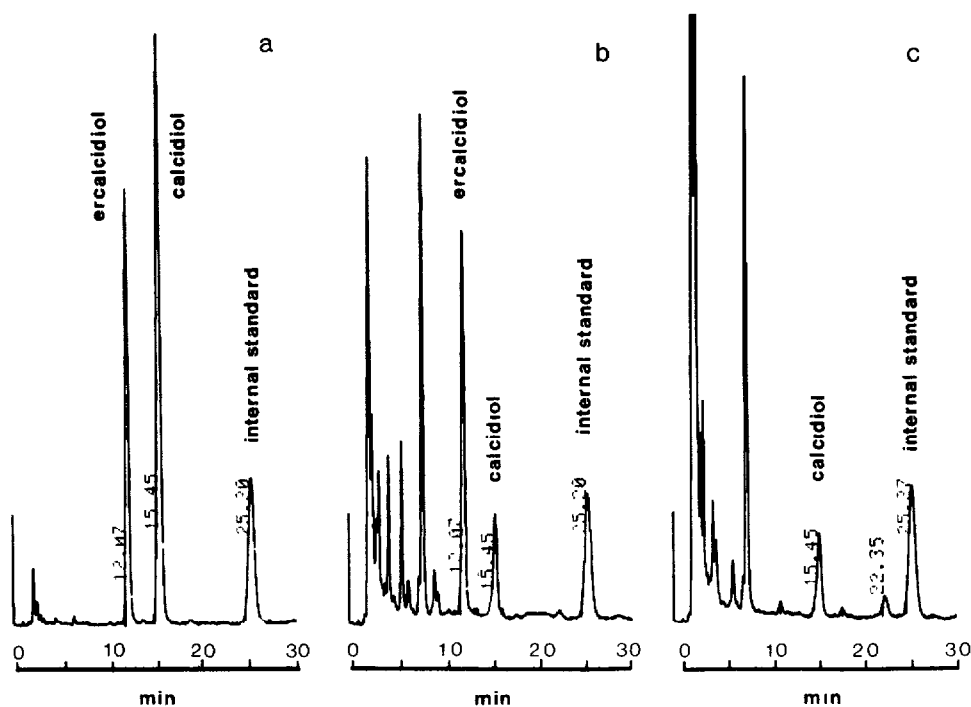
Fig. 3. Sample chromatograms showing elution positions of ercalcidiol, calcidiol and *trans*-calcidiol (internal standard). HPLC was on a Zorbax-Sil column developed with hexane–propan-2-ol (98:2) at 2.0 ml/min. Detection was by UV absorbance at 254 nm (0.005 a.u.f.s.). (a) Standards; (b) serum sample from patient treated with ercalciol showing large peak for ercalcidiol; (c) serum sample from patient on anti-convulsant therapy showing absence of interfering material.

HPLC assay with internal radioactive standard. Twenty four serum samples containing different amounts of ercalcidiol (present in only thirteen of the samples) and calcidiol were extracted in duplicate using either *trans*-calcidiol or radioactive calcidiol as internal standard. The results in Fig. 4b and 4c show close correlations and equivalence in the two methods; $r = 0.99, p < 0.001, y = 1.02x + 0.76$ for ercalcidiol; $r = 0.99, p < 0.001, y = 1.05x - 0.04$ for calcidiol.

Accuracy and precision of the method

Accuracy and precision were determined by supplementing serum pools with known amounts of ercalcidiol and calcidiol and measuring the increase in the assayed peaks. Serum supplemented with 42.5 ng/ml ercalcidiol and 43.5 ng/ml calcidiol showed mean increases of 41.6 ± 0.5 and 42.9 ± 0.7 ng/ml, respectively (mean $\pm$ S.D., $n = 10$) giving recoveries of 97.9 and 98.6%. Intra-assay coefficients of variation (C.V.) at this level were 3.5 and 5.6%, respectively. A pool of serum measured at 13.4 ng/ml calcidiol by PBA assayed at 13.3 ± 0.2 ng/ml (mean $\pm$ S.D., intra-assay C.V. 3.0%) using TCISA. To assess accuracy and precision at lower concentrations a further serum pool was supplemented with 2.5, 5.0 and 10.0 ng/ml of either ercalcidiol or calcidiol and assayed by TCISA in

triplicate on two occasions together with the unsupplemented pool. The results are shown in Table I; in each case the amount assayed corresponded closely with the amount added. Regression analysis showed close correlations; for ercalcidiol $r=0.99$, $y=1.01x-0.22$ and for calcidiol $r=1.00$, $y=1.04x-0.13$. The small intercepts and closeness to unity of the slopes indicate a high degree of accuracy. Precision at lower calcidiol levels was measured by supplementing a serum pool assayed by PBA at 2.6 ng/ml with 5 ng/ml calcidiol. The level detected by TCISA was 7.9 ± 0.2 ng/ml (mean $\pm$ S.D., $n=14$, intra-assay C.V. 7.3%).

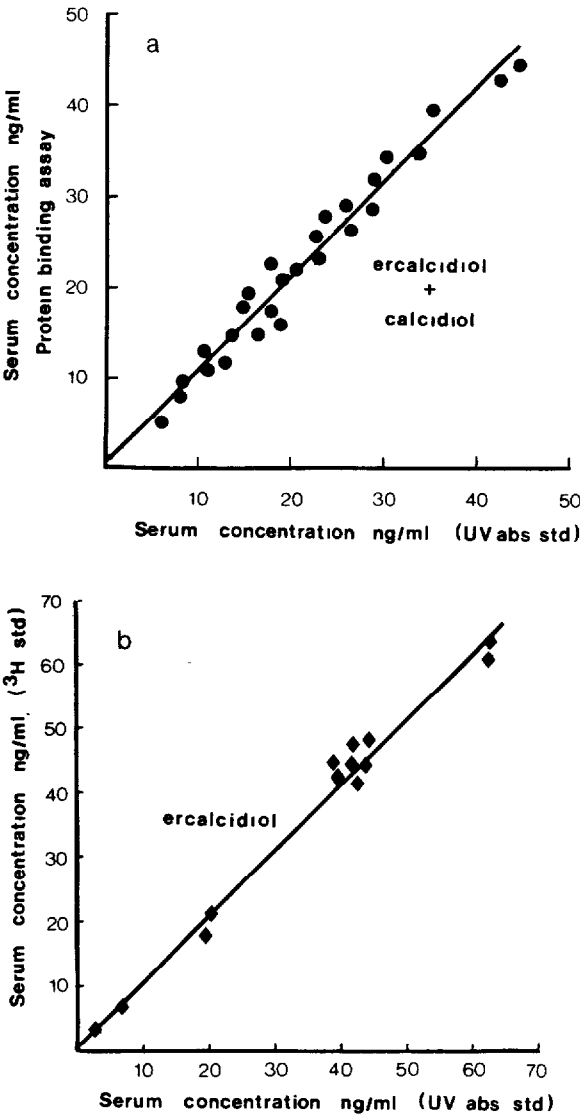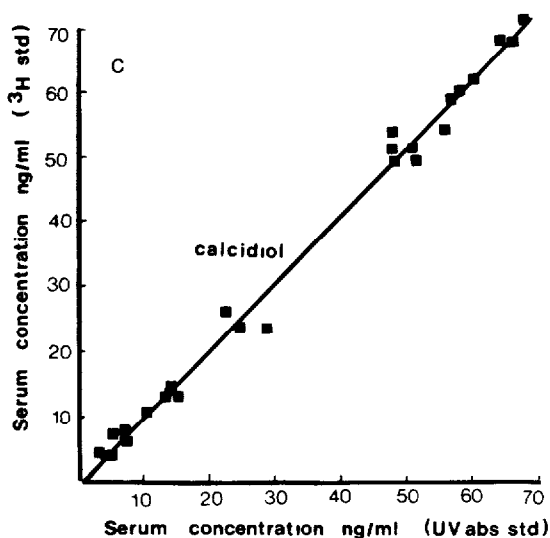Inter-assay variation was monitored by collecting results for the same control

Fig. 4.

Fig. 4(a) Comparison of measurements for the sum of ercalcidiol and calcidiol in 27 serum samples using protein-binding assay or the UV-absorbing internal standard HPLC assay. Correlation coefficient $r = 0.98$, $p < 0.001$, $y = 1.00x + 0.75$. (b) Comparison of ercalcidiol concentrations in 13 serum samples using UV-absorbing HPLC assays with either [^{3}H]calcidiol or 5,6-*trans*-calcidiol (UV-absorbing standard) as internal standards to monitor recovery. Correlation coefficient $r = 0.99$, $p < 0.001$, $y = 1.02x + 0.76$. (c) Comparison of calcidiol concentrations in 24 serum samples, details as in b. Correlation coefficient $r = 0.99$, $p < 0.001$, $y = 1.05x - 0.04$.

sample run on fourteen assays over a three-month period. The value was 21.6 ± 0.5 ng/ml (mean ± S.D.) with a C.V. of 8.7%.

The limit of detection was 2 ng, or 1 ng/ml if a 2-ml sample was used. Saline samples have consistently given undetectable results.

Clinical applications of the assay

The TCISA has been in use for three years in this laboratory and over 3000 samples have been assayed. Subjects investigated have included patients with chronic renal and liver disease and patients with a high intake of drugs such as

TABLE I

RECOVERY OF ERCALCIDIOL AND CALCIDIOL ADDED TO SERUM

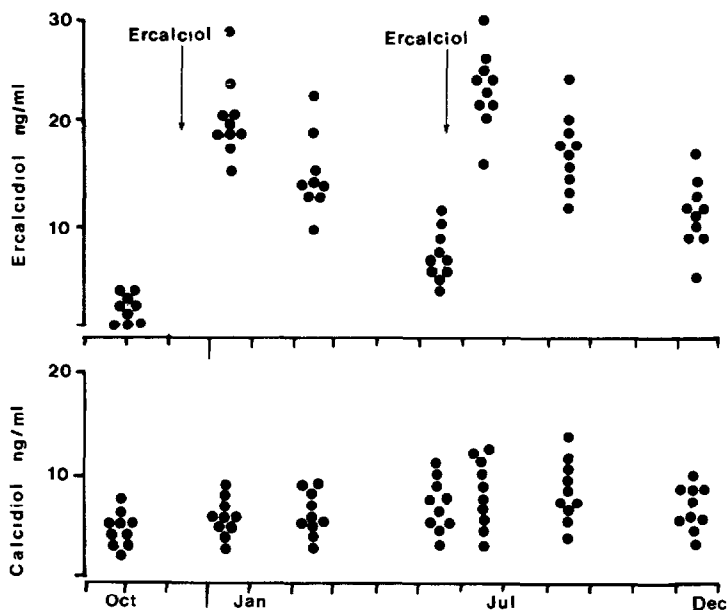| Compound	Added (ng/ml)	Found (mean ± S.D., $n = 6$) (ng/ml)
Ercalcidiol	2.5	1.86 ± 0.48
	5.0	5.30 ± 0.63
	10.0	9.58 ± 0.36
Calcidiol	2.5	2.32 ± 0.32
	5.0	5.33 ± 0.63
	10.0	10.19 ± 0.63

Fig. 5. Serum concentrations of ercalcidiol and calcidiol over a fourteen-month period in a group of ten elderly patients treated with ercalciol in December and June showing an increase in ercalcidiol following treatment. Results were obtained using the *trans*-calcidiol internal standard assay.

anti-convulsants. In no case has material been observed to co-elute with the internal standard peak (see Fig. 3). It has been noticed, however, that the standard peak is diminished, with the concomitant appearance of a new peak of lower polarity when left in contact with serum extract for more than 24 h even at $-20°C$. Pure standard does not deteriorate under these conditions. It is important, therefore, that extraction and HPLC separation be performed on the same day. Final results for up to 48 samples can be obtained in about 24 h, depending on the HPLC analysis time used.

The assay has proved particularly useful for the analysis of a large number of samples from a study on vitamin D supplementation in the elderly. Results showing the state of ercalciol and calciol nutrition before treatment have been published [14] as has a preliminary account of treatment in a subgroup of the population studied [15]. The TCISA has permitted the changes in concentration of ercalcidiol and calcidiol to be monitored following treatment with ercalciol (Fig. 5).

DISCUSSION

The development of an assay for calcidiol and ercalcidiol using 5,6-*trans*-calcidiol as internal standard has meant that the full potential of automated HPLC equipment can be realised. Component retrieval, necessary to assess recovery when a radioactive standard is used, is eliminated, and a final result can be produced by the integrator. As far as we are aware, this approach has not been reported in any published procedure for calcidiol assay.

Various compounds were assessed for use as an internal standard but only *trans*-calcidiol was found to have the appropriate extraction characteristics in the system used; however, it is possible that other compounds might have proved suitable if the extraction procedure had been modified. Prolonged use of the TCISA in this laboratory has not revealed any problems due to interfering peaks in the position of the internal standard, even in patients on high drug intakes and in people on a variety of dietary regimes. This is in contrast to the trial of isotacalcidiol as internal standard where interference was observed. It is possible that by appropriate manipulation of solvent systems this interference might have been eliminated, since conversion to isotacalcidiol has formed the basis of a published method for calcidiol assay [16]; in addition, however, recovery was relatively low with this compound.

A particular advantage of the TCISA is its speed and simplicity; only one HPLC analysis is required for both the quantification and recovery determination. This is in contrast to some other published methods where a second HPLC run is required [4,5,7], usually because the assay of calcidiol is part of a more detailed investigation involving measurement of other vitamin D metabolites in the same sample. In clinical use, however, calcidiol (together with ercalcidiol) is the assay most frequently demanded. The present method addresses this demand and is thereby simplified, the combination of a reversed-phase extraction procedure with normal-phase HPLC and a relatively long analysis time provides samples sufficiently clean that uncontaminated peaks are encountered for all the compounds of interest.

The accuracy and precision of the TCISA have been well tested and results are virtually identical with those obtained by other methods, as evidenced by the high correlation coefficients and mathematical equivalence when results are compared. The TCISA is less sensitive than the PBA, requiring a 2-ml volume to give detectable results in vitamin D-deficient serum or plasma. This is not likely to be a problem, except in paediatric samples, and smaller volumes can be used when the level of calcidiol is not expected to be very low. The advantage of the TCISA over the PBA is the ability to measure both ercalcidiol and calcidiol; this is not possible with the PBA unless the extract has been previously fractionated by HPLC.

The ability to measure ercalcidiol and calcidiol accurately and quickly has applications in both routine clinical use and in large-scale research surveys. Although the main use of the TCISA is to measure 25-hydroxylated vitamin D compounds per se, a further application of this rapid assay is to screen for the presence of ercalcidiol in samples where measurement of the 1,25-dihydroxylated derivatives by RIA is required. Most antisera used to assay calcitriol (1,25-dihydroxyvitamin D_3) do not cross-react equally with ercalcitriol (1,25-dihydroxyvitamin D_2). If the precursor ercalcidiol is first shown to be present then steps can be taken to collect ercalcitriol separately at the HPLC stage and to assay it appropriately, thus avoiding underestimation of this metabolite.

Commercial supplies of ercalcidiol for use as standard are no longer available; the metabolite can, however, be isolated from the plasma of patients or animals given large doses of ercalciol. To conserve limited stocks of ercalcidiol standard

it is possible, after some experience of running this compound on HPLC and knowing its retention time relative to calcidiol, to enter a calculated value for ercalcidiol at the calibration stage. The accuracy of this prediction can be tested by running, as the first sample, a control serum known to contain ercalcidiol.

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