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DETERMINATION OF 1,25-DIHYDROXYVITAMIN D_3 IN PLASMA USING THIN-LAYER CHROMATOGRAPHY AND MODIFIED COMPETITIVE PROTEIN BINDING ASSAY

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SUMMARY

A simplified assay for calcitriol in plasma samples, using high-performance thin-layer chromatography (HPTLC) followed by competitive protein binding assay (CPBA), is described. For quantitative separation of femtomol concentrations of calcitriol from other metabolites of vitamin D, HPTLC can be used instead of column chromatography on Sephadex LH-20 and HPLC on silica gel. Since the CPBA with duodenal cytosol from chickens as a binding protein and [^{3}H]1,25-dihydroxycholecalciferol as radioligand is highly sensitive, detection limit 10 fmol per assay, the volume of plasma sample can be reduced to 1 ml.

INTRODUCTION

Calcitriol (1,25-dihydroxyvitamin D_3)[1] has been shown[2,3] to be the major hormonal form of vitamin D. Since the physiological concentration of calcitriol is very low, fmol per ml of plasma, it is very difficult to determine this hormone in biological materials.

The competitive protein binding assay (CPBA) techniques have been very successful in measuring calcitriol[4].

Unfortunately, all these techniques require this steroid to be separated from closely related vitamin D metabolites. A frequently used method of separation employs column chromatography on Sephadex LH-20 and high-performance liquid chromatography (HPLC) on silica gel[5,6].

For the clinical measurement of calcitriol it is necessary to devise a method which is not only accurate and sensitive but also simple and fast, does not require specialized apparatus and is suitable for routine applications.

We have previously[6] separated metabolites of vitamin D by high-performance thin-layer chromatography (HPTLC) on silica gel: picomol quantities of 25-hydroxyvitamin D_3 (25OHD_3), 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] and 25,26-dihydroxyvitamin D_3 [25,26$(OH)_2D_3$] occurring in plasma samples. In the present paper we report our studies on the TLC separation of fmol quantities of calcitriol from other metabolites of vitamin D in plasma samples. A modified CPBA technique with

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intestinal cytosol receptor from chickens as binding protein and tritium-labelled 1,25-dihydroxycholecalciferol was used to quantify femtomol amounts of calcitriol[8].

EXPERIMENTAL

Materials

All solvents were of analytical grade and were distilled before use.

Crystalline standards of vitamin D_3 metabolites, $25OHD_3$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$, including calcitriol [$1,25(OH)_2D_3$], were kindly provided by Dr. M. Uskokovič of Hoffmann-La Roche (Nutley, NJ, U.S.A.). 1,25-Dihydroxy[23,24(n)-$^3H_2$]vitamin D_3, 3.00 TBq/mmol, was obtained from the Radiochemical Centre (Amersham, U.K.). Prior to use it was purified by TLC on silica gel in ethyl acetate–*n*-hexane (1:6, v/v).

Silica gel 60 HPTLC plates, without fluorescent indicator, were purchased from E. Merck (Darmstadt, F.R.G.). Chloroform–ethanol–water (183:16:1, v/v) was used for the development.

Phosphate buffer (50 mmol/l, pH = 7.4) from Lachema (Brno, Czechoslovakia), containing 1 mmol/l of dithiothreitol (Serva, Heidelberg, F.R.G.) and 50 mmol/l of KCl (Lachema) was used throughout the CPBA procedure.

The suspension of Charcoal, Norit A (0.5%, w/w; Serva), was mixed with an equal volume of dextran (0.05%, w/w; Koch-Light, Colnbrook, U.K.).

Binding protein in phosphate buffer was prepared from the intestine of normal 40-days-old chickens, kept on a normal diet, not enriched with vitamin D_3, according to a modification of the method of Jongen *et al.*[9].

Plasma samples for analysis were collected from healthy individuals aged 30–50 years.

Radioactivity was measured in Bray's scintillation solution using a Packard Tricarb 2425 liquid scintillation spectrometer with counting efficiency of about 29%.

Methods

The treatment of plasma samples prior to determination of calcitriol is summarized in Table I. Briefly, plasma sample, containing metabolites of vitamin D bound to proteins, was 72% saturated with ammonium sulphate[10]. The precipitate which formed was solubilized in water and after extraction with methanol and toluene, the metabolites of vitamin D were separated chromatographically on a thin layer of silica gel.

As fmol quantities of calcitriol in plasma samples cannot be detected by the method used, standards of vitamin D_3 metabolites, $25OHD_3$, $24,25(OH)_2D_3$, $25,26(OH)_2D_3$ and $1,25(OH)_2D_3$, in μmol amounts were chromatographed simultaneously with plasma samples. A calibration curve was constructed by subjecting standards of calcitriol, within the concentration range 0.0–1.0 pmol, to the same chromatographic system. After development with chloroform–ethanol–water, the R_F values of metabolites of vitamin D were determined by spraying μmol quantities of the above-mentioned metabolites on a strip of silica gel with concentrated sulphuric acid[11].

The area of silica gel, corresponding to the R_F value of calcitriol, in fmol quantities, of the chromatographed samples was scraped off and after adding water

TABLE I

FLOW CHART FOR $1,25(OH)_2D_3$ ASSAY IN PLASMA

(I) Extraction
Plasma (1.0 ml)
Water (1.0 ml)
Ammonium sulphate 72% saturation (5.0 ml)
Centrifugation (2000 *g*, 20 min, 4°C)
Precipitate
Water (2.0 ml)
Methanol (2.0 ml, 30 min, mix)
Toluene (6.0 ml, 30 min, mix)
Centrifugation (1000 *g*, 10 min, 4°C)
Aliquot of toluene (2.5 ml)

(II) HPTLC
Plasma sample (aliquot of toluene), metabolites*
(0.2 μmol), $1,25(OH)_2D_3$ (0.0–1.0 pmol)
Evaporation under nitrogen
Ethanol (60 μl)
HPTLC
Development (chloroform–ethanol–water, 183:16:1, v/v)
Spray with sulphuric acid (only metabolites in μmol)

(III) CPBA
Scraped silica gel zones (1 × 1 cm^2), corresponding to R_F values
of $1,25(OH)_2D_3$
Water (50 μl)
$[^3H]1,25(OH)_2D_3$ (20 μl, 20,000 dpm)
Cytosol (0.6 mg per 750 μl)
Incubation (2 h, 4°C)
Dextran-coated charcoal (200 μl)
Centrifugation (1000 *g*, 10 min, 22°C)
Supernatant (500 μl)
Scintillation solution (10.0 ml)

* $25OHD_3$ + $24,25(OH)_2D_3$ + $25,26(OH)_2D_3$ + $1,25(OH)_2D_3$.

a mixture was prepared for CPBA[8]. Intestinal cytosol from chickens was used as a binding protein and tritium-labelled 1,25-dihydroxycholecalciferol as a tracer. The mixture was left in a refrigerator for 2 h, then the incubation was stopped by addition of a suspension of dextran-coated charcoal. After centrifugation an aliquot of the supernatant was solubilized in Bray's solution and monitored for radioactivity.

RESULTS AND DISCUSSION

As we have reported previously[6,7], endogenous metabolites of vitamin D_3 are well separated by TLC on silica gel.

To determine whether the separation is quantitative even for femtomol concentrations, a CPBA was used to quantify chromatographed standards of calcitriol within the concentration range 0.0–1.0 pmol. A calibration curve constructed from the TLC–CPBA data (●) is compared with the corresponding data for CPBA alone (◆) in Fig. 1. The results show that TLC is an useful method for quantitative separation of femtomol amounts of calcitrol. The similarity of the two sets of data

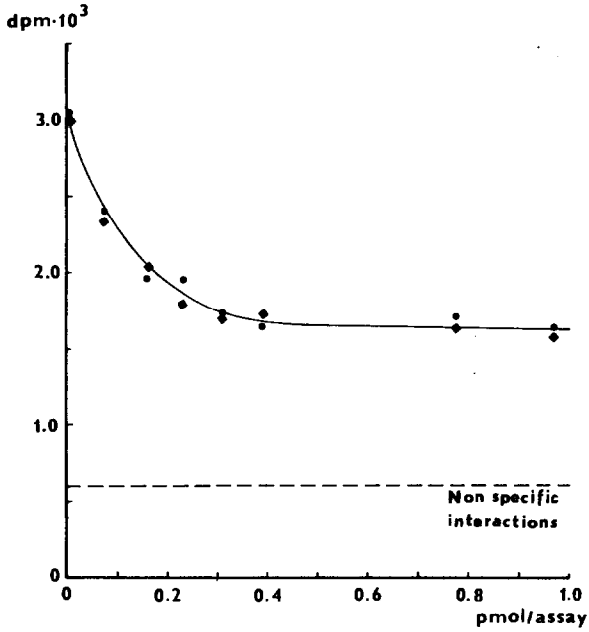

Fig. 1. CPBA displacement curve of calcitriol, within the concentration range 0.0–1.0 pmol per assay, with TLC (●) and without TLC (◆).

indicates that TLC does not influence the sensitivity (detection limit *ca.* 10 fmol per assay) and precision of the CPBA. The technique employed for transferring the metabolite from the thin layer to the CPBA, *i.e.*, without elution from the silica gel, resulted in almost 100% recovery of the chromatographed sample. Thus, the presence of silica gel does not interfere with the CPBA.

The recovery of calcitriol in the analysis of 1 ml of plasma expressed as a percentage of the added labelled 1,25-dihydroxycholecalciferol is shown in Table II. Calcitriol is bound predominantly to the albumin fraction of plasma proteins. This fraction is precipitated from a solution 67–80% saturated with ammonium sulphate. However, it is very difficult quantitatively to separate the precipitated fraction from solutions at >75% saturation. Therefore we chose 72% saturation, which enables a recovery of 73% with respect to the recovery of [^{3}H]1,25(OH)$_2$D$_3$ added in all

TABLE II

PERCENTAGE RECOVERY OF [^{3}H]1,25(OH)$_2$D$_3$ IN THE OVERALL PROCEDURE FOR DETERMINATION OF 1,25(OH)$_2$D$_3$

Procedure	*Recovery*		*No. of replicates, n*
	%	*S.D.*	
Precipitation	73.0	7.8	9
Extraction	99.6	5.5	8
HPTLC	98.9	6.2	9

TABLE III

ACCURACY [% RECOVERY OF ADDED $1,25(OH)_2D_3$] AND PRECISION (INTRA-ASSAY VARIATION COEFFICIENT) OF TLC-CPBA OF $1,25(OH)_2D_3$ IN PLASMA

Sample	*Amount added (fmol)*	*Amount recovered*			*C.V.** (%)
		*fmol***	S.D.	%	
Plasma A	0.0	24.0	0.5	–	2.1
	76.9	92.0	10.5	91.2	11.4
	230.8	250.0	19.5	98.1	7.8
Plasma B	0.0	22.1	0.5	–	2.3
	76.9	92.5	12.0	93.4	12.9
	230.8	280.4	29.5	110.9	10.5
	307.7	334.1	21.0	101.3	6.3
Plasma C	0.0	148.0	24.0	–	8.4
Plasma D	0.0	20.4	2.5	–	8.8
Plasma E	0.0	61.3	9.8	–	9.5

* Coefficient of variation.
** Mean ($n = 3$).

plasma samples. Recoveries of the labelled standard after extraction with toluene and after TLC on silica gel were nearly 100%.

The ability of TLC quantitatively to separate calcitriol from the other metabolites present in plasma was tested by determining the recovery of added non-radioactive standard calcitriol to the chromatographed plasma sample. Several samples of plasma were studied; each was chromatographed without and with addition of a standard of calcitriol. The added amounts corresponded to those occurring naturally in 1 ml of plasma. The results expressed in terms of the accuracy and precision are summarized in Table III. The values are the means from three analyses of the same sample and the standard error of the means. The accuracy of the TLC–CPBA is expressed as the percentage recovery of added standard. The precision of the method is characterized by the intra-assay coefficient of variation for replicate analysis of the same plasma sample. The results are in good agreement with those calculated from column chromatography on Sephadex and HPLC on silica gel[5,12,13].

In conclusion, HPTLC is a highly effective method for quantitative separation of metabolites of vitamin D_3, occurring at femtomol concentrations in plasma samples. Together with the CPBA, it can be used instead of HPLC to determine metabolites of vitamin D in biochemical laboratories do not possess specially designed chromatographic apparatus. Since the CPBA is highly sensitive, allowing the determination of 10 fmol, the analysed volume of plasma can be reduced to 1 ml.

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