Journal of Chromatography, 230 (1982) 319—325
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1255

SEPARATION OF NANOGRAM QUANTITIES OF HYDROXY METABOLITES OF VITAMIN D_3 IN PLASMA BY THIN-LAYER CHROMATOGRAPHY ON SILICA GEL

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(First received December 30th, 1981; revised manuscript received February 5th, 1982)

SUMMARY

Thin-layer chromatography (TLC) on silica gel coated HPTLC plates, using chloroform—ethanol—water as mobile phase, is highly effective in the quantitative separation of biologically active metabolites of vitamin D. The combination of TLC and competitive protein-binding assay results in a rapid, sensitive and reproducible method for the analysis of nanogram quantities of metabolites of vitamin D_3 (25-hydroxycholecalciferol, 24,25-dihydroxycholecalciferol and 25,26-dihydroxycholecalciferol) in plasma samples.

INTRODUCTION

It is now well established that the actions of the D vitamins on intestine and bone are mediated through their hydroxylated metabolites [1]. At the present time there are many modifications of the assays for hydroxy metabolites of vitamin D [2—5]. All of them involve, prior to the application of a proper quantitative method, preparative chromatography using Sephadex LH-20 [6], Lipidex 5000 [7] and/or, recently, silica Sep-Pak [8,9] and high-performance liquid chromatography for the finest separation of the metabolites of vitamin D in serum (reviewed in refs. 2, 10 and 11). The use of these chromatographic steps results in very good separation of vitamin D metabolites but, on the other hand, it requires apparatus with specialized accessories for separation of larger series for routine analysis which may not be available in laboratories specializing in the methods of saturation analysis.

Extending our previous work on the description of thin-layer chromatographic (TLC) separation of metabolites of vitamin D [12], we present a detailed study of the determination of nanogram quantities of hydroxy

0378-4347/82/0000—0000/$02.75

metabolites of vitamin D_3 [25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3]* in plasma after their chromatographic separation on thin layers of silica gel. We have tried to simplify this separation step. The elution of the separated metabolite from silica gel [12] was omitted, and for final determination diluted osteomalacic plasma as binding protein [13] was applied directly to scraped silica-gel areas.

EXPERIMENTAL

Materials

All solvents (Lachema, Brno, Czechoslovakia) were of analytical grade and were used without further purification.

Crystallized 25-OH-D_3 (a gift from Philips Duphar, Weesp, The Netherlands), 24,25-$(OH)_2$-D_3, 25,26-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 (a gift from Dr. M. Uskokovič, Hoffmann-La Roche, Nutley, NJ, U.S.A.) and D_3 (purchased from E. Merck, Darmstadt, G.F.R.) were dissolved in 96% distilled ethanol to a concentration of 0.24 μM and stored at −20°C.

25-Hydroxy[26(27)-methyl-^{3}H]cholecalciferol (0.43 T Bq/mmol) was purchased from the Radiochemical Centre, Amersham, Great Britain. Ethanolic solution of 8.6 μM was stored at −20°C. Prior to use in the assay, ^{3}H-labelled 25-OH-D_3 was purified by TLC on silica gel in ethyl acetate—*n*-hexane (1:6, v/v).

The following pre-coated plates (for nano-TLC) were tested: silica gel 60 without fluorescent indicator, silica gel 60 F_{254} with fluorescent indicator (both manufactured by E. Merck), and silica gel for HPTLC without fluorescent indicator from Whatman (Maidstone, Great Britain).

Chloroform—ethanol—water (183:16:1, v/v) was used as the mobile phase for the development. The plates were stored at a relative humidity of about 45%.

The charcoal—dextran suspension was prepared by mixing equal volumes of 0.05% (w) dextran (Koch-Light Labs., Colnbrook, Great Britain) and 0.5% (w) suspension of charcoal Norit A (Serva, Heidelberg, G.F.R.) in 0.05 *M* phosphate buffer, pH 7.2.

Heparinized plasma from a patient suffering from osteomalacia who had not been treated with vitamin D previously, was diluted 1:1500 (v/v) with 0.05 *M* phosphate buffer, pH 7.2, and used as binding protein for competitive protein-binding assay (CPBA).

Plasma samples for analysis were collected from healthy men aged 40—50 years.

Radioactivity in 0.5-ml aqueous aliquots was counted in 10 ml of Bray's scintillation fluid on a Model 2425 Packard Tricarb liquid scintillation spectrometer with counting efficiency of about 50%.

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*Abbreviations: D_3 = cholecalciferol, vitamin D_3; 25-OH-D_3 = 25-hydroxycholecalciferol; 1,25-$(OH)_2$-D_3 = 1α,25-dihydroxycholecalciferol; 24,25-$(OH)_2$-D_3 = 24,25-dihydroxycholecalciferol; 25,26-$(OH)_2$-D_3 = 25,26-dihydroxycholecalciferol.

Sample preparation

The plasma samples were analysed by a modification of our method previously described [13]. Three volumes of ammonium sulphate (66% saturation) were used to precipitate carrier proteins for metabolites of vitamin D in 1-ml plasma samples. Following centrifugation the precipitate was dissolved in 1 ml of water. Precipitation was repeated three times. After the addition of 1 ml of methanol the metabolites were extracted with 3 ml of toluene. An aliquot of 1 ml of toluene was evaporated and redissolved in 20 μl of ethanol and applied to the silica-gel thin layer.

Thin-layer chromatography

Prior to use the plate was developed in the mobile phase used for a development and dried gently. The sample (2—20 μl) was then applied to the plate. After developing in the solvent mixture microgram quantities of the separated compounds were visualized either as yellow—red spots by spraying the plate with concentrated sulphuric acid [14] or as violet spots on a fluorescent background under a UV source at 254 nm. For smaller quantities, 0.2—4.0 ng, the sample must be run alongside a larger standard sample and the spot position determined with the aid of a template. The spot is then marked out, scraped off and the compound is, after wetting with 50 μl of water, diluted with 20 μl of 96% ethanol [for analysis of 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3], or with 20 μl of the developing solvent (for analysis of 25-OH-D_3). After thoroughly mixing for 20 min the mixture is prepared for CPBA.

Competitive protein-binding assay

The CPBA was performed essentially as we have described elsewhere [13,15]. Ethanol (20 μl) was added to all assay tubes. Incubation was started by the addition of 1 ml of a cold (−4°C) incubation solution. This solution contained, per 1 ml of a solution of binding protein, 20 μl of ethanolic solution of ^{3}H-labelled 25-OH-D_3 of about 22,000 cpm. After the addition of 0.25 ml of dextran-coated charcoal, phase separation was achieved by centrifugation at 1000 *g*. Aliquots of the supernatant (1 ml) were solubilized in Bray's scintillation solution and monitored for radioactivity.

RESULTS AND DISCUSSION

Separation efficiencies of silica-gel-coated HPTLC plates of different origin for the separation of vitamin D metabolites are listed in Table I. Using high-performance silica-gel plates a much higher separation efficiency was achieved than with the other types of TLC on silica gel [12]. Naturally occurring vitamin D metabolites of biological importance, listed in Table I, are distinctly separated by TLC in nanogram quantities.

From studying the binding properties of the metabolites of vitamin D we have found that 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 are equipotent in displacing ^{3}H-labelled 25-OH-D_3 from the plasma binding protein (diluted plasma from a patient with non-cured osteomalacia). Neither D_3 (within the concentration range 0—100 ng) nor 1,25-$(OH)_2$-D_3 (within the concentration range 0—100 pg) affects the competitive protein-binding reaction.

TABLE I

R_F VALUES OF METABOLITES OF VITAMIN D AFTER TLC ON SILICA-GEL LAYERS IN CHLOROFORM—ETHANOL—WATER (183:16:1)

HPTLC silica-gel layers	R_F values				
	D_3	25-OH-D_3	24,25-$(OH)_2$-D_3	25,26-$(OH)_2$-D_3	1,25-$(OH)_2$-D_3
Kieselgel with indicator* (Merck)	0.69	0.56	0.41	0.30	0.20
Kieselgel without indicator* (Merck)	0.65	0.51	0.38	0.28	0.22
Silica gel without indicator* (Whatman)	0.70	0.56	0.48	0.39	0.32

*Fluorescent indicator at 254 nm.

Using the above CPBA method for final quantitative determination of metabolites of vitamin D, we have checked for evidence of separation of nanogram quantities of 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 by TLC. The characteristics of the CPBA evaluated by establishing standard curves of these metabolites were used in this assay [15]. Determinations of 1,25-$(OH)_2$-D_3 and D_3 — after their separation on TLC — by CPBA using a specific binding protein from the intestine of chicken are under investigation. Our attempts to simplify the procedure of determination of metabolites after their separation on thin layers of silica gel [12] by omitting the elution step are illustrated in Fig. 1 and Table II.

TABLE II

PERCENTAGE RECOVERY AND ACCURACY OF ADDED AMOUNTS OF 25-OH-D_3, 24,25-$(OH)_2$-D_3 AND 25,26-$(OH)_2$-D_3 THROUGH THE PROCEDURE OF TLC + CPBA

Compound	Amount applied to TLC* (ng)	Amount found after TLC + CPBA** (ng)	No. of replicates (n)	Recovery (%)	C.V.*** (%)
25-OH-D_3 24,25-$(OH)_2$-D_3 25,26-$(OH)_2$-D_3	0	0.00 ± 0.01	10	100.01	2.57
25-OH-D_3	0.40	0.38 ± 0.03	5	95.00	7.50
24,25-$(OH)_2$-D_3	0.40	0.39 ± 0.01	6	98.59	1.68
25,26-$(OH)_2$-D_3	0.40	0.39 ± 0.01	5	96.50	3.59
25-OH-D_3	1.00	0.99 ± 0.01	6	99.30	1.72
24,25-$(OH)_2$-D_3	1.00	1.00 ± 0.07	4	100.50	6.57
25,26-$(OH)_2$-D_3	1.00	1.03 ± 0.05	4	103.10	4.67

*On silica gel (Merck, without indicator).
**Mean ± S.D.
***Coefficient of variation.

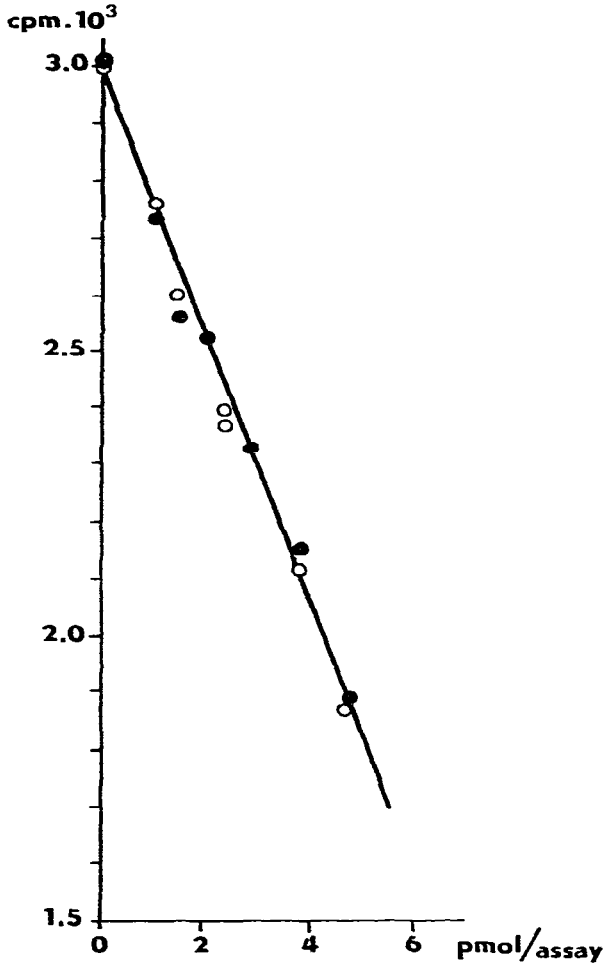

Fig. 1. Competitive protein-binding displacement of 25,26-$(OH)_2$-D_3, within the concentration range 0.0—3.0 ng, without or after TLC on silica gel (Whatman). (●) CPBA, (○) TLC + CPBA in the presence of silica gel.

Standard curves of 25,26-$(OH)_2$-D_3 constructed from the results of CPBA of 25,26-$(OH)_2$-D_3 (without TLC) and the same CPBA (after TLC) in the presence of silica gel resulted in practically the same values. This means that the precision of the above procedure does not decrease the usefulness of TLC; it is the same as for CPBA itself.

Recoveries of added standards of 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 to thin layers of silica gel (Merck) after TLC and following CPBA, summarized in Table II, confirm that the presence of silica gel did not interfere with the CPBA of the above metabolites.

Results of the analysis of the metabolites of vitamin D in 1 ml of plasma after TLC and following CPBA are listed in Table III. The accuracy of the procedure was calculated from the values of recoveries of the added amounts of 25-(OH)-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3 to the extract of the

TABLE III

ACCURACY OF TLC (SILICA GEL, MERCK) OF PLASMA SAMPLES EVALUATED BY RECOVERY OF ADDED 25-OH-D_3, 24,25-$(OH)_2$-D_3 AND 25,26-$(OH)_2$-D_3 TO PLASMA SAMPLES

Sample	Amount added (ng)			Amount recovered* (ng)					
	25-OH-D_3	24,25-$(OH)_2$-D_3	25,26-$(OH)_2$-D_3	25-OH-D_3	C.V.** (%)	24,25-$(OH)_2$-D_3	C.V. (%)	25,26-$(OH)_2$-D_3	C.V. (%)
Plasma A	0.00	0.00	0.00	3.28 ± 0.18	5.49	0.65 ± 0.04	6.91	0.28 ± 0.01	5.71
Plasma A'	0.00	0.40	0.40	3.28 ± 0.18	5.49	1.03 ± 0.07	7.20	0.66 ± 0.03	5.62
Plasma B	0.00	0.00	0.00	3.82 ± 0.29	7.61	1.02 ± 0.06	6.13	0.21 ± 0.01	6.66
Plasma B'	0.00	1.00	0.30	3.82 ± 0.29	7.61	2.00 ± 0.11	5.62	0.52 ± 0.03	6.42
Plasma C***	0.00	0.00	0.00	2.41 ± 0.09	3.72	0.44 ± 0.04	9.10	0.02 ± 0.00	4.00
Plasma C'	0.60	0.60	0.60	2.95 ± 0.18	6.09	1.13 ± 0.09	7.98	0.70 ± 0.04	5.78

*Mean ± S.D. ($n = 5$).
**Coefficient of variation.
***Plasma C was analysed using TLC on silica gel from Whatman.

plasma sample prior to TLC. Added amounts correspond to those of naturally occurring endogenous vitamin D metabolites in plasma. The criteria (accuracy and precision) showed that TLC can be used for reliable quantitative separation and further determination of metabolites of vitamin D occurring in plasma in nanogram quantities. There are no differences in quantitative parameters using either silica-gel plates from Merck or from Whatman.

TLC on silica gel provides a convenient and fast procedure for the quantitative separation of metabolites of vitamin D in plasma available for routine analysis. The major advantage of using silica-gel-coated HPTLC plates in comparison to the currently used column chromatography on Sephadex LH-20, even when silica Sep-Pak cartridges are used for prepurification [8,9], is not only saving of time in column packing but complete quantitative separation of all biologically active metabolites of vitamin D including 1,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3. Both techniques are comparable in cost, convenience, reproducibility and sample recovery. As can be seen from our results, TLC is highly effective for the quantitative separation of nanogram quantities of biologically active metabolites of vitamin D in plasma capable of replacing column chromatography on Sephadex LH-20 with subsequent high-performance liquid chromatography.

ACKNOWLEDGEMENTS

We are grateful to Dr. M. Uskokovič for samples of vitamin D_3 metabolites, and to Whatman Ltd. for providing us with HPTLC precoated silica-gel plates.

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