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Note

Separation of functional hydroxymetabolites of vitamin D_3 by thin-layer chromatography

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The examination of hydroxycholecalciferols, hormonally active functional metabolites of vitamin D_3, has recently received considerable attention in clinical investigations because of the effect of vitamin D on the regulation of mineral metabolism in man. For this reason, there is a general interest in the development of the methodology for studying hydroxycholecalciferols in body fluids. Radioligand assays for these hormones have been developed using either cytoplasmic receptor protein, antibodies raised against hydroxycholecalciferols or 25-hydroxycholecalciferol specific plasmatic transport protein as the binding protein. However, the specificity of these binding proteins is not high enough to discriminate between the individual mono- and dihydroxycholecalciferols which differ in their biological activities. Thus, a chromatographic separation prior to radioligand assay is required[1] for exact measurements. For the separation of nanogram quantities of D_3, 1-OH-D_3, 25-OH-D_3, 1,25-$(OH)_2$-D_3, 24,25-$(OH)_2$-D_3 and 25,26-$(OH)_2$-D_3* a simple column chromatography on Sephadex LH 20 or silicic acid was not fully satisfactory[2–5], and in most recent methods high-performance liquid chromatography (HPLC) was used for the separation of individual mono- and dihydroxycholecalciferols[6–17], in combination with prior purification on a Sephadex column. The use of HPLC results in very good separation. On the other hand, this additional step complicates the processing of larger series of routine analyses of hydroxycholecalciferols and it requires apparatus which may not be available in laboratories specialising in radioimmunoassays.

As a part of our study on hydroxycholecalciferol action in disorders of calciophosphate metabolism, we have developed a thin-layer chromatographic (TLC) system allowing the separation of mono- and dihydroxycholecalciferols which is a prerequisite for the radioligand assay of the individual compounds.

MATERIALS AND METHODS

[26,27-^{3}H]-25-OH-D_3 and [1α,2α(n)-$^3H_2$]-D_3, obtained from the Radiochemical Centre, Amersham, Great Britain, had a specific activity of 436 GBq/mmol and 455

* Abbreviations: D_3 = cholecalciferol, vitamin D_3; 1-OH-D_3 = 1α-hydroxycholecalciferol; 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.

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GBq/mmol, respectively. Ethanolic solutions of 8.6 μM [^{3}H]25-OH-D_3 and 4.05 μM [^{3}H]D_3 for checking recovery were stored at −20°C. The purity of the radioactive material was checked prior to TLC on silica gel in ethylacetate–*n*-hexane (1:6, v/v).

Non-radioactive 24,25-$(OH)_2$-D_3, 25,26-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 were kindly donated by Dr. M. Uskokovic (Hoffman-La Roche, Nutley, NJ, U.S.A.). 25-OH-D_3 was a gift from Philips-Duphar (Weesp, The Netherlands). Vitamin D_3 was purchased from E. Merck (Darmstadt, G.F.R.). All crystalline cholecalciferols were dissolved in 96% ethanol to a concentration within the range 0.40–0.55 mM and were kept at 4°C.

Radioactivity was counted in a liquid scintillation counter (Packard Tri-Carb 2425) using 10 ml of Brayś solution per vial. Quenching was estimated for each sample by an external standard method; the counting efficiency was *ca.* 46–50%.

Aluminium foils coated with Kieselgel 60 F_{254} (E. Merck), aluminium foils coated with silica gel Silufol UV 254 (Kavalier, Sázava, Czechoslovakia) and Celluloid foils coated with silica gel 6061 without fluorescent indicator (Eastman-Kodak, Rochester, NY, U.S.A.) were used without previous activation. The foils were stored at a relative humidity of 50%.

All TLC runs for qualitative separations were carried out by applying 0.7–1.0 μg of the sample in 2–10 μl of ethanol to the plate with a Hamilton syringe. The plate was allowed to dry, placed in a rectangular glass tank and developed by the ascending technique at room temperature (21 ± 2°C). Chloroform–ethanol–water (183:16:1, v/v/v) was used for the development. The time required for a run was *ca.* 20 min. The spots of the authentic cholecalciferols were visualized under a UV source at 254 nm, or by spraying the foil with a solution of anisaldehyde in sulphuric acid (2%, w/v) mixed with glacial acid (1:10, v/v) and warming at 40°C. In biological samples the contents of the hydroxymetabolites of vitamin D_3 are at the nanogram level which is below a limit of sensitivity of detection by both UV and the anisaldehyde reagent. The areas of compounds under investigation were therefore localized with corresponding metabolites used as markers, then scraped off and extracted five times with 0.1 ml 96% ethanol. The pooled extracts were separated from traces of silica gel by centrifugation at 3500 *g* at room temperature and the eluates were used for the radioactivity counting.

For the estimation of recovery, [^{3}H]D_3 (3.3 kBq, 4.68 ng) and [^{3}H]25-OH-D_3 (16.9 kBq, 0.34 ng) were cochromatographed with the samples.

RESULTS AND DISCUSSION

The separation of biologically active metabolites of vitamin D_3 by TLC in the system chloroform–ethanol–water was very satisfactory and allowed further determination of biologically active hydroxycholecalciferols by radioligand methods. The spots of the compounds were circular, well defined, without tailing and were about 3 mm in diameter. The optimal separation was achieved on Kieselgel 60 F 254 foils as shown in Table I.

The influence of the chromatographic purification step on the quantitation of metabolites of vitamin D_3 was checked by recovery of labelled vitamin D_3 and 25-OH-D_3 after chromatography and elution. The recovery of the markers in five replicate determinations was 97.8 ± 2.9% for vitamin D_3 and 98.7 ± 1.5% for 25-OH-

D_3, respectively, when only the effect of elution was verified. The effect of the complete chromatographic procedure on the accuracy and precision of the determination is shown in Table II. The amounts of vitamin D_3 and 25-OH-D_3 used in the experiments correspond to those found in biological samples.

TABLE I
R_F VALUES OF VITAMIN D_3 AND ITS FUNCTIONAL HYDROXYMETABOLITES ON SILICA GEL LAYERS

Compound	*Kieselgel (Merck)*	*Silufol (Kavalier)*	*Silica gel (Eastman)*
D_3	0.69	0.56	0.65
1-OH-D_3	0.35	0.39	0.40
25-OH-D_3	0.56	0.43	0.57
1,25-$(OH)_2$-D_3	0.20	0.15	0.33
24,25-$(OH)_2$-D_3	0.41	0.26	0.51
25,26-$(OH)_2$-D_3	0.30	0.17	0.35

TABLE II
RECOVERY OF LABELLED VITAMIN D_3 AND 25-HYDROXYCHOLECALCIFEROL AFTER CHROMATOGRAPHY ON THIN LAYERS OF KIESELGEL (MERCK)

Compound	*Amount applied (ng)*	*Amount found* (ng)*	*Coefficient of variation (%)*
D_3	4.68	4.44 ± 0.33	5.85
25-OH-D_3	0.34	0.33 ± 0.01	3.03

* Mean ± S.D. (n = 5).

The reliability of the method confirms that TLC is a suitable alternative method for separation of individual functional metabolites of vitamin D_3. It can serve as a purification step for various types of radioligand assays of these compounds. The sensitivity, accuracy and precision of the overall method are more dependent on the radioassay than the TLC. The sensitivity is practically uneffected by the TLC step because of the low experimental losses. TLC is a simple method which does not require specially designed chromatographic apparatus and which enables routine determination of hydroxycholecalciferols in large series more conveniently than HPLC or by use of Sephadex column.

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REFERENCES

1 R. K. Skinner and M. R. Wills, *Clin. Chim. Acta*, 80 (1977) 543.
2 M. F. Holick and H. F. DeLuca, *J. Lipid Res.*, 12 (1971) 460.
3 J. G. Haddad, C. Min, J. Walgate and T. J. Hahn, *J. Clin. Endocrinol. Metab.*, 43 (1976) 712.
4 C. M. Taylor, S. E. Hughes and P. Silva, *Biochem. Biophys. Res. Commun.*, 70 (1976) 1243.

5 M. R. Haussler and H. Rasmussen, *J. Biol. Chem.*, 247 (1972) 2320.
6 E. W. Matthews, P. G. H. Byfield, K. W. Colston, I. M. A. Evans, L. S. Galante and I. McIntyre, *FEBS Lett.*, 48 (1974) 122.
7 G. Jones and H. F. DeLuca, *J. Lipid Res.*, 16 (1975) 448.
8 N. Ikekawa and N. Koizumi, *J. Chromatogr.*, 119 (1976) 227.
9 K. T. Koshy and A. L. VanDerSlick, *Anal. Biochem.*, 74 (1976) 282.
10 P. W. Lambert, B. J. Syverson, C. D. Arnaud and T. C. Spelberg, *J. Steroid Biochem.*, 8 (1977) 929.
11 J. A. Eisman, R. M. Shepard and H. F. DeLuca, *Anal. Biochem.*, 90 (1977) 298.
12 K. T. Koshy and A. L. VanDerSlick, *J. Agr. Food Chem.*, 25 (1977) 1246.
13 K. T. Koshy and A. L. VanDerSlick, *Anal. Biochem.*, 85 (1978) 283.
14 T. J. Gilbertson and R. P. Styrd, *Clin. Chem.*, 23 (1977) 1700.
15 P. C. Shaefer, *J. Lab. Clin. Med.*, 91 (1978) 104.
16 G. Jones, *Clin. Chem.*, 24 (1978) 287..
17 A. D. Care and J. L. H. O'Riordan, *Clin. Endocrinol. (Oxford)*, 11 (1979) 225.