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Note

Separation of the hydroxylated metabolites of vitamin D_3 by high-performance thin-layer chromatography

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Procedures for the analysis of the mono- and dihydroxylated metabolites of vitamin D_3 by conventional thin-layer chromatography (TLC) have been described[1,2]. However, no studies have been published on the analysis of these compounds by high-performance TLC (HPTLC). This technique is faster and requires less solvent than conventional thin-layer chromatography. Typically, in vitamin D research biological samples are first partially purified using column chromatography such as Sephadex LH-20[3] or conventional TLC and are then analyzed by high-performance liquid chromatography (HPLC)[4–6]. For routine analysis HPTLC may be superior to HPLC in rapidity of analysis because of its capability to separate many samples at a time[7]. We have developed a solvent system for separating the mono- and dihydroxylated metabolites of vitamin D_3 by HPTLC and have tested two published conventional TLC solvent systems[1] for their ability to separate vitamin D_3 metabolites by HPTLC.

EXPERIMENTAL

The di- and trihydroxylated metabolites of vitamin D_3 were donated by Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ, U.S.A.). Vitamin D_3 was purchased from Sigma (St. Louis, MO, U.S.A.) and 25-hydroxyvitamin D_3 ($25OHD_3$) was a gift from UpJohn (Kalamazoo, MI, U.S.A.). All metabolites were stored at $-20°C$.

Silica gel HPTLC-HLF and GHLF plates (Analtech, Newark, DE, U.S.A.) were used without previous activation. The solvent systems were dichloromethane–isopropanol (90:10), hexane–isopropanol (85:15)[1], and chloroform–ethyl acetate (50:50)[1]. All solvents were HPLC grade.

Standard solutions (1–1.3 mg/ml) were obtained by dissolving the metabolites in hexane–isopropanol or in ethanol. A 0.5–1.0-μl volume of each standard was deposited on the HPTLC plates and 1–2 μl on the conventional TLC plates. The TLC plates were developed by the ascending technique in a 20 × 20 cm rectangular glass tank. A 10 × 10 cm twin-trough chamber (Applied Analytical Industries, Wilmington, NC, U.S.A.) was used to develop the HPTLC plates so that only 20 ml were used for development compared with 200 ml in conventional TLC. Migration distances were 7 cm for the HPTLC plates and 14 cm for the conventional TLC plates.

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TABLE I

SEPARATION OF THE HYDROXYLATED METABOLITES OF VITAMIN D_3 BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

Compound	R_F *values*		
	Dichloromethane–isopropanol (90:10)	*Chloroform–ethyl acetate (50:50)*	*Hexane–isopropanol (85:15)*
$1,24,25(OH)_3D_3$	0.21	0.05	0.14
$1,25(OH)_2D_2$	0.37	0.19	0.26
$25,26(OH)_2D_3$	0.44	0.29	0.35
$24,25(OH)_2D_3$	0.61	0.44	0.46
$23,25(OH)_2D_3$	0.64	0.51	0.51
$25OHD_3$	0.73	0.71	0.59
Vitamin D_3	0.89	0.87	0.66

Developing times were 11–12 min for the HPTLC plates and 35–45 min for the conventional TLC plates. The spots were visualized under a UV source at 254 nm. All manipulations were carried out at room temperature.

RESULTS AND DISCUSSION

The dichloromethane–isopropanol system developed by us adequately separates vitamin D_3 from its monohydroxylated and dihydroxylated metabolites. Among the dihydroxylated metabolites the single region of possible overlap is between 23,25-dihydroxyvitamin D_3 [$23,25(OH)_2D_3$] and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] (Table I). The two conventional systems tested here were also effective in separating the metabolites. Again the compounds less effectively separated were $23,25(OH)_2D_3$ and $24,25(OH)_2D_3$. The system of choice would depend on the metab-

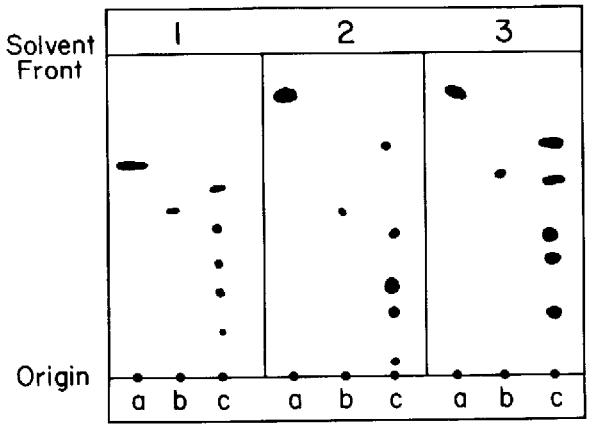

Fig. 1. Pattern of migration of vitamin D_3 metabolites on HPTLC. Panel 1, hexane–isopropanol (85:15); panel 2, chloroform–ethyl acetate (50:50); panel 3, dichloromethane–isopropanol (90:10). Row a, vitamin D_3; b, $23,25(OH)_2D_3$; c, in order of increasing R_F values $1,24,25(OH)_3D_3$, $1,25(OH)_2D_3$, 25,26-dihydroxyvitamin D_3 [$25,26(OH)_2D_3$], $24,25(OH)_2D_3$ and $25OHD_3$.

olite of interest. For example, the dichloromethane–isopropanol system is preferable for the isolation of 1,24,25-trihydroxyvitamin D_3 [1,24,25$(OH)_3D_3$] in a biological sample because of the greater distance of 1,24,25$(OH)_3D_3$ from contaminating lipids at the origin in that system (Fig. 1). The chloroform–ethyl acetate system, although ineffective in separating 1,24,25$(OH)_3D_3$ from contaminating lipids at the origin, is the most effective solvent system in separating 25OHD_3 from 24,25$(OH)_2D_3$. The hexane–isopropanol system provides the least distance between 25OHD_3 and vitamin D_3, but the greatest distance between vitamin D_3 and contaminating lipids at the front. Samples spotted in ethanol tended to spread, as seen by the increased diameter of the vitamin D_3 spot on all plates and the five standards spotted on the plate developed with dichloromethane–isopropanol (Fig. 1).

The R_F values of vitamin D_3 and its metabolites were determined by conventional TLC for comparison (Table II). The effectiveness of separation by conventional TLC was very similar to that by HPTLC. For example, R_F 25OHD_3/R_F 1,25-dihydroxyvitamin D_3 [1,25$(OH_2)D_3$] on HPTLC plates developed with our new system (dichloromethane–isopropanol) was 2.0 compared with 2.1 on conventional plates. The R_F values of vitamin D_3 and its metabolites were also determined by conventional TLC using the two published systems since R_F values in the literature were listed only for 25OHD_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$. Our R_F values for these three metabolites are different from those previously published[1], probably because the TLC plates were not identical.

The separation of the vitamin D_3 metabolites by HPTLC is rapid and, given the capacity to analyze several samples on a 10 × 10 cm plate, can be a time-saving alternative to HPLC for routine analysis of partially purified samples. The metabolite of interest can be quantitatively extracted[2] from the plate for further use. The choice between TLC and HPTLC would depend on the material to be purified. TLC is definitely the choice for whole lipid extracts of tissue homogenates because of its greater capacity (microgram amounts of material as opposed to nanograms in HPTLC), but HPTLC can be used as a second purification step for the extracted region of interest. These three solvent systems are very effective when used sequentially in the purification of metabolites from a biological sample because of their

TABLE II

SEPARATION OF THE HYDROXYLATED METABOLITES OF VITAMIN D_3 BY CONVENTIONAL THIN-LAYER CHROMATOGRAPHY

Compound	R_F *values*		
	Dichloromethane–isopropanol (90:10)	*Chloroform–ethyl acetate (50:50)*	*Hexane–isopropanol (85:15)*
1,24,25$(OH)_3D_3$	0.17	0.05	0.12
1,25$(OH)_2D_3$	0.29	0.14	0.22
25,26$(OH)_2D_3$	0.35	0.19	0.28
24,25$(OH)_2D_3$	0.49	0.33	0.40
23,25$(OH)_2D_3$	0.53	0.36	0.46
25OHD_3	0.62	0.55	0.55
Vitamin D_3	0.76	0.71	0.69

varying ability to remove different types of interfering lipids from the desired metabolite.

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