CHROM. 3440

THIN-LAYER CHROMATOGRAPHY OF VITAMIN D AND RELATED STEROLS*

GERARD PONCHON** AND FRANCIS X. FELLERS***
Department of Pediatrics, Harvard Medical School and Children's Hospital Medical Center, Boston, Mass. 02115 (U.S.A.)

(First received October 17th, 1967; modified February 8th, 1968)

SUMMARY

Thin-layer chromatography (TLC) on Silica Gel G or on Alumina GF is a simple and useful tool for the investigation of vitamin D and related sterols. The use of Alumina GF layers and chloroform or petroleum ether (b.p. 37°–59°)–benzene (50:50, v/v) as solvents yields the best separation and sensitivity. It allows specific detection of samples on the basis of their U.V. absorbance or fluorescence characteristics, followed by staining with antimony trichloride or other spray reagents, and finally charring with 75% sulfuric acid. Vitamin D can also be quantitatively recovered after TLC by elution of the layer with methanol.

Metabolic studies of vitamin D necessitates the use of very precise separation methods for various sterols. Such procedures must respect the physico-chemical structure of isolated compounds. It is well-known that vitamin D and related substances are highly sensitive to oxidation and to photochemical transformations[1]. Various chromatographic techniques have been proposed: paper chromatography[2], column chromatography[3–5,12–14], thin-layer chromatography[10–20], and gas–liquid chromatography (GLC)[6–10]. The former gives poor resolution in spite of long development times. Good separation is achieved on columns, but minute amounts of sterols are not readily detectable by chemical means. GLC allows very fine resolution, but induces thermal rearrangement of certain sterols. Compared to those methods, thin-layer chromatography has the advantage of being easy, rapid, and inexpensive. It allows sensitive and specific detection of developed sterols which can be eluted for further characterizations.

In this paper results obtained on silica gel and alumina layers with various solvent systems are studied. Sterols are not only detected in submicrogram amounts on the chromatoplates, but can also be quantitatively recovered for further characterization.

* This study was supported in part by National Institutes of Health, Division of Arthritis and Metabolic Diseases, Roi AM 09278-01 and 02.

** Research Fellow of the Harvard Medical School and the Children's Hospital Medical Center. C.R.B. Graduate Fellow of the Belgian American Educational Foundation.
Present address: Department of Biochemistry, University of Wisconsin, Madison, Wisc. 53706.
*** Assistant Professor of Pediatrics, Harvard Medical School. Senior Associate in Medicine at the Children's Hospital Medical Center.

MATERIALS

The Desaga-Brinkmann "applicator", with a fixed exit slot of 275 μ high, is used to coat the 10 × 20 cm glass plates.

An "application box" Desaga-Briukmann Model DB is convenient for spotting the samples under nitrogen simultaneously on two plates.

Chromatoplates are developed in rectangular glass "developing tanks" 9.7 × 27.2 × 30 cm (d × h × w).

A layer suction apparatus consists of a fine stainless steel tube (1 mm internal

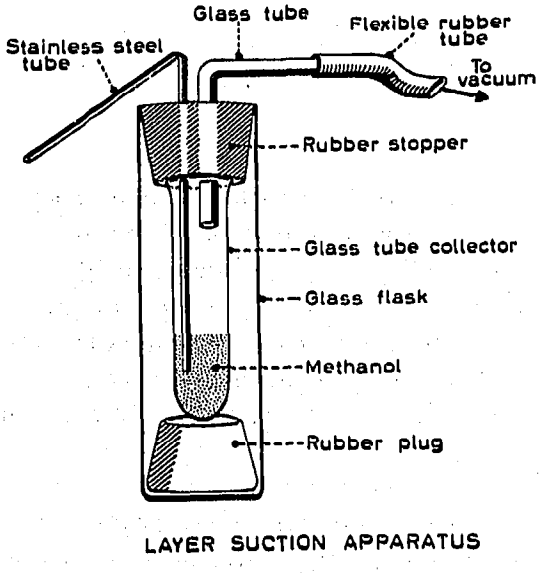

Fig. 1. Layer suction apparatus.

diameter) connected to vacuum, through an intermediate glass collector filled with methanol (Fig. 1). This apparatus was suggested by Dr. N. GOLD*.

Adsorbents: Silica Gel G Merck No. 7731, average particle size 5–25 μ, $CaSO_4$ binder, neutral pH.

Aluminum Oxide GF Merck No. 1092, average particle size 40 μ, $CaSO_4$ binder; manganese activated zinc silicate as fluorescing indicator (peak 2540 Å).

Color reagents: 50% antimony trichloride in glacial acetic acid; 20% phosphotungstic acid in absolute ethanol; 75% sulfuric acid; phosphomolybdic acid–Brinkmann spray reagent No. 531.

METHOD

Preparation of the chromatoplates

A slurry is made of 30 g adsorbent per 60 ml distilled water, and applied in a uniform layer of about 250 μ thickness.

Silica, as well as alumina gels, are activated at 140° in the oven for at least 12 h. The dry layer is scraped off along the edges of the plate and separation lines are made to form 9 mm "lanes" parallel to the length of the plate. The plates are then activated

* N. GOLD, Ph.D., Harvard Medical School and the Children's Hospital Medical School.

again for 2 h at 140°. If not immediately used after cooling, they may be stored on racks in a vacuum dessicator. When the period of storage is longer than four days, the plates are reactivated in a similar way.

Spotting of the samples

The samples are dissolved in petroleum ether (b.p. 37°–59°) at a concentration of 5 μg/μl. This solvent is chosen because the very low polarity and high volatility will allow the greatest concentration of the material to be adsorbed onto the layer. When the substances do not dissolve in petroleum ether, carbon tetrachloride or chloroform may be used, although not as effectively.

Five μl aliquots (25 μg) are delivered by calibrated 5 μl Drummond microcaps. Two or three successive 5 μl aliquots can be spotted without difficulty, except when carbon tetrachloride or chloroform are used as solvent. In those cases special care must be taken to avoid spreading of the samples. Five μl of dye mixture are spotted on both edge-lanes as indicators of development.

Generally, compounds to be analysed are run on the central lanes while known sterol standards are chromatographed on the peripheral lanes as "markers". The plates are spotted in a special application box under a stream of nitrogen. Red light is used during the entire procedure which must not last longer than 10 min for two plates.

Development

The chromatographic jars are filled with the solvent mixture to a depth of 2 cm 24 h before use. Local saturation is obtained with filter paper sheets covering the walls of the tank and dipping in the solvent.

Immediately after spotting, the plates are allowed to develop at room temperature (20°). Development is stopped before the front reaches the upper limit of the layer giving runs of approximately 12 cm. However, a method where the solvent front moves up to an "end line" at 15 cm from the starting line can be used. Development time is approximately 1 h.

The following adsorbents and solvent systems were studied:

I. Silica Gel G: *n*-hexane–acetone (95:5, v/v)
II. Silica Gel G: *n*-hexane–acetone (90:10, v/v)
III. Silica Gel G: chloroform
IV. Aluminum Oxide GF: chloroform
V. Aluminum Oxide GF: chloroform–methanol (99:1, v/v)
VI. Aluminum Oxide GF: chloroform–methanol (95:5, v/v)
VII. Aluminum Oxide GF: petroleum ether (b.p. 37°–59°)–benzene (90:10, v/v)
VIII. Aluminum Oxide GF: petroleum ether–benzene (80:20, v/v)
IX. Aluminum Oxide GF: petroleum ether–benzene (50:50, v/v)
X. Aluminum Oxide GF: *n*-hexane–chloroform (95:5, v/v)
XI. Aluminum Oxide GF: *n*-hexane–chloroform (90:10, v/v)
XII. Aluminum Oxide GF: *n*-hexane–chloroform (75:25, v/v)
XIII. Aluminum Oxide GF: *n*-hexane–chloroform (50:50, v/v)
XIV. Aluminum Oxide GF: *n*-hexane–diethyl ether (80:20, v/v)

Detection

After chromatography the plates are allowed to dry for 2 min in the darkness.

First examination is done under U.V. light with a long-wave (366 mμ) and a short-wave (255 mμ) lamp. The absorbing and fluorescing spots are recorded within 20 min after drying.

The chromatoplates are then sprayed with one of the color reagents mentioned above and dried in the oven at 80° for 5 min. The localization and colors of the spots are again recorded under regular light at that time (initial color). Fluorescence of the colored spots under long-wave U.V. light is examined after prolonged heating at 110° for 20 min, or after a delay of one day at room temperature.

Colors and fluorescence of sterols are also recorded following charring with 75% H_2SO_4 at greater than 150° on a hot plate.

Records

In each case the R_F of the sterol is measured from the point of maximum intensity, not necessarily the actual center of the spots. The R_s values are calculated with reference to cholesterol, or to the dye azobenzene when cholesterol remains on the starting line with solvents of very low polarity.

Permanent records of the stained chromatoplates are made by contact prints (black and white) on Kodak Velox F or Azo F, with regular light. It is also possible to obtain black and white pictures of the fluorescence under 366 mμ U.V. light or of absorbance under 255 mμ U.V. light before staining by the use of Panchromatic (red fluorescence) or Orthochromatic "Royal Ortho-Eastman Kodak" (yellow-green fluorescence) films with a Wratten AA filter. Exposure time is at least 2 min with a U.V. lamp (0.1–0.2 A) at a distance of 20 cm. Color prints of stained plates or of fluorescence under 366 mμ U.V. light are taken on Kodak Ektachrome. With the U.V. lamp at a distance of 20 cm, exposure time for color prints is a matter of trial and error, around 5 min.

Extraction of sterols from the layer

The dye mixture and the sterol standard "markers" are visualized on the "edge-lanes", while the center of the plate is covered with a thick sheet of paper or opaque plastic. Location of "unknown" sterols on central lanes is determined by reference to the edge "markers" and the corresponding area of layer is sucked off in the apparatus described above*. The adsorbent is collected in a glass tube containing 3 ml of methanol. The stainless steel tube is then rinsed with an additional 2 ml of methanol. The 5 ml suspension is vigorously shaken for one minute, and centrifuged in the same glass tube at 3,000 r.p.m. for 15 min. The volume of packed adsorbent at the bottom of the tube is less than 0.1 ml per spot removed. Thus, the solvent methanol contains the sterol eluted in a volume of 5.0 ml, neglecting the small volume of adsorbent. Four milliliters of this supernatant are easily pipetted for subsequent analysis.

Quantitative determination and analysis of purity of vitamin D

Methanol solutions of calciferol standards or extracts from TLC are analysed for U.V. absorption using a Beckman DU spectrophotometer equipped with 1 cm rectangular cuvettes of standard silica. Quantitative determination is done at the maximum of 264 mμ for ergo- and chole-calciferol. Absorption curves are recorded

* This process must be done very rapidly after development, while the chromatoplate is still impregnated with solvent.

from 220 mμ up to 320 mμ. Blanks are pure methanol for calciferol standards. When the sterol is recovered from a chromatoplate, the blank consists of a methanolic extract of an equivalent area of adsorbent taken off from a lane free of sterol.

RESULTS

Separation and mobility of sterols

Tables I and II summarize the R_F and R_s values of various sterols and dye mixtures chromatographed on activated Silica Gel G and Alumina GF, using the solvent systems described in the method*.

In TLC the R_F values are not very reproducible from one plate to another. On the other hand, good reproducibility is noted on runs of the same samples on a given plate.

From the series of solvents used, the best separation between cholesterol, ergo- and pre-ergo-calciferol, was reached with the system IV, pure chloroform. On the other

TABLE I

R_F AND R_s VALUES ON SILICA GEL G

Samples	*Solvent systems*					
	*I***		*II*		*III*	
	R_F	R_s	R_F	R_s	R_F	R_s
Cholesterol	0.03 ± 0.004 (5)*	1.0	0.09 ± 0.003 (7)	1.0	0.11 ± 0.01 (7)	1.0
Ergocalciferol	0.07 ± 0.010 (5)	2.0	0.16 ± 0.004 (7)	1.8	0.22 ± 0.01 (6)	1.9
Pre-ergocalciferol	0.10 ± 0.010 (5)	2.9	0.20 ± 0.006 (7)	2.2	0.27 ± 0.02 (6)	2.4

* Values = mean ± S.E. Brackets = number of plates.
** Roman numerals indicate the solvent systems, as described under METHODS.

hand, the esters of cholesterol are better separated with a system of lower polarity, such as IX—petroleum ether (37°–59°)–benzene (50:50, v/v).

Various chromatographic conditions were tested in order to determine the most accurate procedure. The following observations are based upon repeated chromatographic trials.

Compared to room dried (20°) plates, the activation at 140° results in a 25% increase of the R_F values of the sterols as well as of the aromatic dyes on Silica Gel G layers. This increase does not seem to be influenced by the solvent used. Little effect is observed on Alumina GF where the activation seems to decrease the mobility of the sterols and dye mixture by about 12%.

Presaturation of activated plates is performed by placing the plates in the chromatographic tanks as for development and allowing the solvent to migrate freely through the layer for 6 h. The samples are immediately spotted thereafter and de-

* The R_s given may differ slightly from the value expected on the basis of the mean R_F when an isolated determination of the "standard" cholesterol is made without concommittant chromatography of the sterol considered.

TABLE II

R_F AND R_s VALUES ON ALUMINA GF

Samples		*IV*[a]	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*	*XIII*	*XIV*
Cholesterol	R_F	0.30 (3)[b]	0.67 (1)	0.75 (1)	0.00 (2)	0.00 (1)	0.01 (2)	0.01 (2)	0.02 (1)	0.05 (1)	0.18 (2)	0.01 (1)
	R_s	1.00	1.00	1.00			1.00	1.00	1.00	1.00	1.00	1.00
β-Sitosterol	R_F	0.30 (3)	0.68 (1)	0.74 (1)	0.00 (1)	0.00 (1)	0.01 (1)		0.01 (1)	0.05 (1)	0.15 (1)	0.01 (1)
	R_s	1.01	1.01	1.00			1.00		0.81	1.04	1.00	1.00
Stigmasterol	R_F	0.30 (3)	0.68 (1)	0.74 (1)	0.00 (1)	0.00 (1)	0.01 (1)	0.01 (1)		0.04 (1)	0.16 (1)	0.01 (1)
	R_s	1.01	1.01	1.00			1.00	1.00		0.96	1.00	1.00
Lanosterol	R_F		0.77 (1)	0.77 (1)	0.003 (1)	0.01 (1)	0.04 (2)	0.03 (2)	0.05 (1)	0.21 (1)	0.42 (2)	0.05 (1)
	R_s		1.14	1.03	0.01*	0.02*	3.72	6.32	3.25	4.65	2.35	5.00
Ergocalciferol	R_F	0.46 (2)	0.74 (1)	0.84 (1)	0.01 (3)	0.02 (1)	0.05 (2)	0.04 (2)	0.05 (1)	0.15 (1)	0.33 (2)	0.05 (1)
	R_s	1.58	1.10	1.12	0.02*	0.04*	4.54	9.19	3.06	3.25	1.85	5.20
Pre-ergocalciferol	R_F	0.55 (2)	0.78 (1)				0.06 (2)	0.04 (1)	0.07 (1)	0.27 (1)	0.49 (1)	0.10 (1)
	R_s	1.90	1.16				4.66	3.77	4.69	5.93	3.27	10.0
Cholesterol acetate	R_F	0.70 (3)	0.83 (1)	0.82 (1)	0.14 (2)	0.22 (1)	0.51 (2)	0.45 (2)	0.52 (1)	0.73 (1)	0.81 (2)	0.52 (1)
	R_s	2.36	1.23	1.10	0.28*	0.37*	42.4	96.1	32.4	15.8	4.58	52.3
Cholesterol benzoate	R_F	0.70 (3)	0.84 (1)	0.83 (1)	0.15 (2)	0.28 (1)	0.64 (2)	0.56 (2)	0.64 (1)	0.77 (1)	0.84 (2)	0.62 (1)
	R_s	2.37	1.24	1.11	0.30*	0.48*	53.7	120.0	40.2	16.8	9.36	61.7
Stigmasterol acetate	R_F	0.71 (3)	0.82 (1)	0.83 (1)	0.11 (1)	0.22 (1)	0.48 (1)	0.44 (1)	0.54 (1)	0.75 (1)	0.74 (1)	0.54 (1)
	R_s	2.40	1.22	1.11	0.24*	0.38*	43.5	39.6	33.7	16.4	4.97	53.7
Cholestanol acetate	R_F		0.82 (1)	0.83 (1)	0.18 (1)			0.49 (1)			0.90 (1)	
	R_s		1.22	1.11	0.33*			164.0			4.27	

[a] Roman numerals indicate the solvent systems, as described under METHOD.

[b] Numbers in brackets refer to number of plates. An asterisk indicates that the R_s is referred to azobenzene, otherwise to cholesterol.

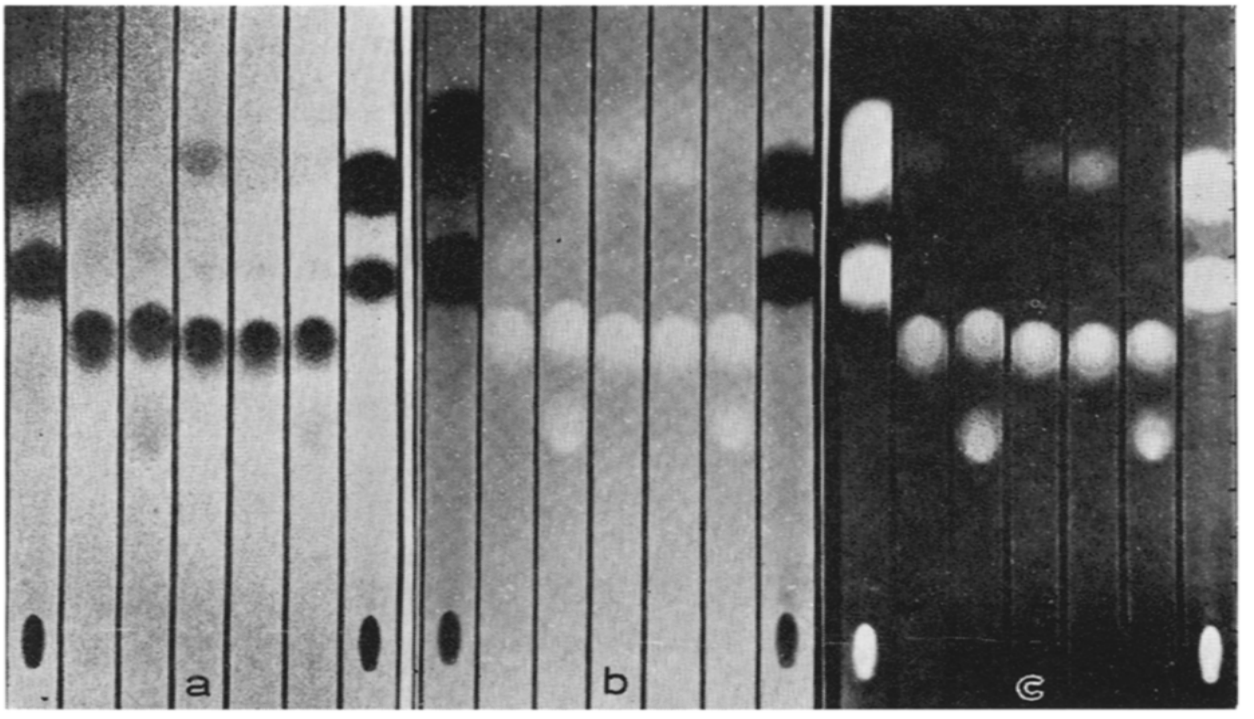

Fig. 2. Reproductions of thin-layer plates. TLC on activated Alumina GF, solvent chloroform. The same plate is examined by U.V. absorption, fluorescence, and antimony trichloride staining. Samples chromatographed from left to right: dye mixture, ergocalciferol and stigmasterol acetate, β-sitosterol and ergocalciferol, ergocalciferol and cholesterol benzoate, ergocalciferol and cholesterol acetate, cholesterol and ergocalciferol, dye mixture. (a) Natural U.V. absorption with 255 mμ U.V. light, 15 min after chromatography. (b) Natural fluorescence under 366 mμ U.V. light, 15 min after chromatography. (c) Antimony trichloride stained plate, one day after chromatography. (a and b are orthochromatic Royal Ortho Eastman Kodak reproductions; c is a contact print with Kodak Velox F.)

veloped in the same solvent, as usual. Such a procedure only slightly modifies the R_F values of sterols and dye mixture.

By allowing the solvent to migrate up to the "end" line at 15 cm from the starting point for 1 hour, the R_F values are increased by about 20%.

Multiple successive developments of the same chromatoplate were tried on Silica Gel G layer with *n*-hexane–acetone (95:5, v/v) as solvent. It appeared that the effect on R_F depends on the mobility of the compound; the most rapid are gradually decelerated, while the slowest appear to be accelerated.

Detection of sterols on developed chromatoplates

The use of fluorescing layers like Alumina GF lowers the detection limits with the 255 mμ U.V. light. Absorbing sterols appear as dark spots quenching on a bright green fluorescing back-ground (Fig. 2, plate a). In order of decreasing intensity of absorption, the following are noted successively: ergo- and chole-calciferol, previtamin D, ergosterol, cholesterol benzoate, and lanosterol. Under the experimental conditions, other sterols examined fail to absorb U.V. light, or at least are not visible. Chole- and ergo-calciferol can be visualized in amounts smaller than 1.0 μg (see Table III).

Long-wave (366 mμ) U.V. light brings about fluorescence of all but three of the

TABLE III

DETECTION CHARACTERISTICS OF STEROLS AND RELATED COMPOUNDS ON ALUMINA GF

Key: Sensitivity (+++) less than 0.5 μg; (++) 0.5–1.0 μg; (+) 1.0–5.0 μg; (±) 5.0–25.0 μg; (?) questionable; (o) no detection. Colors: bl = blue; br = brown; gr = gray; gre = green; or = orange; pur = purple; re = red; vio = violet. Colors exhibited after staining with spray reagents are recorded under regular light. Natural absorbance and fluorescence are seen under U.V. light of respectively 255 and 366 mμ wave length, immediately after drying of developed plates. Fluorescence after $SbCl_3$ spray or when charring with concentrated H_2SO_4 is detected under U.V. light at 366 mμ.

	Natural absorbance	*Natural fluorescence*	*Phosphotungstic acid*	*$SbCl_3$ initial colors*	*$SbCl_3$ fluorescence*	*Phosphomolybdic acid*	*H_2SO_4 initial colors*	*H_2SO_4 fluorescence under charring*
Ergo- and chole-calciferol	++	o	br	re-br	++	bl	gr-vio	+++
Pre-ergocalciferol	+	o	br	re-br	++	bl	gr-vio	+++
Ergosterol	+	o	vio-br	gr-vio	++	bl		
Cholesterol	o	+	or-br	pur-bl-gre	++	bl	gr-vio	+++
β-Sitosterol	o	+	or-br	pur-bl-gre	++	bl	gr-vio	+++
Stigmasterol	o	+	or-br	pur-bl-gre	++	bl	gr-vio	+++
Lanosterol	±	+		or-br	++	bl		
3β-Cholestanol	o	?		o	o	o		±
Cholic acid	o	+		o	?	bl	gre	+++
Cholesterol acetate	o	+	br	pur-bl-gre	++	bl	gr-vio	+++
Cholesterol benzoate	+	+	br	pur-bl-gre	++	bl		
Stigmasterol acetate	o	+	br	pur-bl-gre	++	bl		
Δ^3-Cholesten-3-ol 3-acetate	o	+		o	?	?		

substances analysed. They appear as white or yellowish spots on a dark violet background with alumina as well as Silica Gel G (Fig. 2, plate b). The sensitivity of the latter procedure is not as good as the short-wave U.V. absorption. The detection limit for cholesterol is of the order of 5 μg. The only compounds which absorb under these conditions are ergo- and chole-calciferol and their previtamins (Table III).

It must be noted that dark absorbing spots tend to fade. As far as ergo- and chole-calciferol and the previtamins are concerned, fluorescence under 366 mμ U.V. light develops progressively at room temperature from the periphery to the center of the spots. This reflects the degradation of the sterols on the dry plate. Therefore, detection of absorbing sterols must be done by 20 min after development.

After U.V. examination of the chromatoplates, color reagents provide an additional method of detection.

With phosphotungstic acid slight differences in color exist between the sterols examined (Table III), but after one day all the spots become uniformly brown.

Antimony trichloride brings about more intense and various colors (Table III). Among the substances tested Δ^3-cholesten-3-ol 3-acetate, 3-β-cholestanol and cholic acid do not show colored spots. The most intense and rapid colorations are observed with the vitamins; these become apparent immediately after spraying and before drying (at concentrations of 25 μg/spot). If heating of the sprayed plates is prolonged

for 20 min at 110°, the colored spots tend to fade and become uniformly brown or gray brown. Long-wave U.V. light then shows fluorescence of various colors with all recognized spots. The same result is obtained when the colored plates are allowed to stand for one day at room temperature. Fig. 2, plate c shows a contact print of a chromatoplate stained with antimony trichloride.

Phosphomolybdic acid is a sensitive reagent and gives dark blue spots on a pale yellow background. Only 3 β-cholestanol is not revealed by this procedure (Table III). Detection limits with the 3 color reagents tested above are of the order of 0.5–1.0 μg of ergocalciferol submitted to complete chromatographic procedure.

The use of 75% sulfuric acid as a charring agent gives interesting results. Immediately after spraying and drying at 80°, the substances tested appear as colored spots and the sensitivity is similar to that of the aforementioned reagents. During charring on an electric hot plate, these spots become progressively fluorescent when examined under long-wave U.V. light. At a higher temperature, over 150°, fluorescence becomes brighter and maintains the same color as the initial spots. At such a temperature it is necessary to use special heat resistant glass plates to avoid breakage.

By this charring technique 3 β-cholestanol can be detected. The detection limits for the vitamin D are lowered below 0.5 μg. The observed fluorescences disappear when the plate is allowed to cool, and reappear on heating again even 24 h later. The charring technique can be used with very similar results after the plates have been stained with another reagent, such as phosphotungstic acid or antimony trichloride.

Permanent records of excellent quality are obtained either by contact prints of the stained chromatoplates (Fig. 2, plate c), or by photographs of the fluorescing or absorbing spots under U.V. light (Fig. 2, plates a and b).

Quantitation of chromatographed sterols

Known amounts of ergocalciferol are chromatographed on Alumina GF and then extracted from the layers and submitted to U.V. spectophotometry for quantitative estimation. Chromatography is carried out on activated plates. Spotting was done under red light and in a stream of nitrogen in one series of experiments and

TABLE IV

ERGOCALCIFEROL RECOVERY FROM TLC ON ALUMINA GF (SOLVENT CHLOROFORM)

	Under red light and stream of N_2 (Standard = 28.5 μg)		*Under red light without stream of N_2 (Standard = 37.5 μg)*	
	μg	*% standard*	*μg*	*% standard*
	27.5	96	34.25	91
	26.5	93	36.0	96
	26.5	93	36.0	96
	28.5	100	34.25	91
	28.5	100	33.0	88
	27.0	95	36.0	96
	29.5	104	36.0	96
	29.5	104	32.5	87
Mean ± S.E.	27.9 ± 0.5	98 ± 1.6	34.8 ± 0.5	93 ± 1.4

without nitrogen in the other. In both series, eight spots of ergocalciferol are chromatographed on the same plate with pure chloroform as the solvent. As seen on Table IV, recoveries are 98% ± 2 (mean ± S.E.) when spotting under nitrogen and 93% ± 1 when spotting without a nitrogen stream. U.V. spectrophotometry does not show appreciable change in absorption curves between the standards and the chromatographed ergocalciferol; the maximum remains at 264 mμ.

DISCUSSION

Silica gel has been generally accepted for TLC of sterols and vitamin D in either esterified or free forms[10–25,27–29]. In the present experiments, the R_F values of vitamin D and sterols on Silica Gel G are about one half of those cited by NORMAN AND DELUCA[12], using the same solvent system. This is probably due to differences in the adsorbent, which is free of binder in their method. Surprisingly, activation leads to a definite increase in the R_F value of about 25% (lowers the adsorptive strength). It is well known that the adsorptive strength of silicic acid is critically dependent on its water content, the less hydrated, the more adsorptive, up to a maximum which corresponds to a minimum hydration or structural water of 3–4%. The prepared plates must be more hydrated than that, since that structural water can only be removed by heating to about 1,100°. Since the free water can be evaporated below 200° one would expect greater adsorptivity with activated silica gel plates. NORMAN AND DELUCA left plates at room temperature for more than 45 minutes after activation and showed decreasing adsorptivity[12]. In the present studies, the $CaSO_4$ as binder, might alter the characteristic properties of the activated silicic acid. Among the different solvent systems, pure chloroform gave the best results. Two other systems of lower polarity gave very low R_F values and, consequently, poor separation. Cholesterol, which is highly concentrated in many biological materials, could overlap the vitamin D area. In addition, reproducibility of R_F data with *n*-hexane–acetone (95:5, v/v) is very poor as a result of inadequate separation.

A search for better eluants seemed less practical than looking for another adsorbent which should bind sterols less strongly. Theoretically, solutes of low polarity are better analysed using both solvent and adsorbents of low polarity. The best separations between closely related sterols and vitamin D have been realized on "attenuated" adsorbents such as a mixture of Silica Gel G and kieselguhr[25], or on "reversed-phase partitioning systems"[26,27]. It must be noted that these chromatographic studies were carried out on ester derivatives of sterols. Esterification of sterols markedly diminishes their polarity by masking the hydroxyl group, and allows better separations based upon the structural features of the solute molecule, such as number and location of double bonds and length of the lateral chain. Separation between vitamins D_2 and D_3 has been reported on Silica Gel G impregnated with paraffin with a reversed-phase system[16].

The use of silver nitrate-impregnated silicic acid allowed separation of different esters of cholesterol[22,27], as well as of vitamin D[23], on the basis of the chain length and the degree of unsaturation of the fatty acid moiety. DITULLIO *et al.*[24] used the same system for the separation of free sterols, with respect to their number and configuration of double bonds. However, no attempt was made to recover the sterols separated by such a system.

The addition of bromine to the moving phase of a chromatographic system can improve the separation of some sterols on the basis of their respective number of double bonds. However, this system cannot be applied in the case of vitamin D or other sterols having conjugated double bonds or lacking the $\Delta 5$ double bond, which are destroyed by the bromine[27]. Alumina appeared adequate for analysing mixtures of free as well as esterified sterols present in biological materials. Data obtained with alumina can add significantly to that of silica gel. Mechanisms of adsorption are different.

Eleven different solvent systems were tried. Among those the most useful are pure chloroform and petroleum ether (37°–59°)–benzene (50:50, v/v). The former is of general use, while the latter is particularly suited for separation of esters on the basis of their acidic portion.

The commercial grades of alumina are already activated, but may be deactivated during storage of the opened container. Further activation does not seem to have much effect on Alumina GF (Merck), as it is commercially available, but this might vary according to the solvent used. It may be advantageous to maintain a constant moisture content of the layer. Presaturation of the activated alumina gel layers with the corresponding solvent does not significantly change the R_F values, which indicates that saturation of the layer is quickly reached when placed in the chromatographic jar.

The procedure described by BENNETT AND HEFTMANN[28] which allows the front to reach an "end-line" over which the flow ceases, secures a more uniform front than the usual technique. The R_F values of sterols increase definitely and separation is at least as satisfactory. No control is needed during the development.

Multiple successive developments do not improve the separation, since the slowest compounds tend to migrate faster, and the fastest to move more slowly. In addition, this procedure enchances the risk of destruction of the chromatographed sterols. It is well known that the R_F values of TLC are not very reproducible from one plate to another, and this is confirmed with Silica Gel G as well as with Alumina GF. It emphasizes the need to run adequate standards on each chromatoplate. However, a sample chromatographed in duplicate on the same plate gives very close R_F values and is without any "edge-effect". Owing to such "intra plate" R_F reproducibility, spots on central lanes can be located and identified by reference to the standards developed on the edge lanes.

The best separations are achieved with small amounts of spotted material of the order of 5 μg or less. Since large variations in the amounts of samples can produce some change in the R_F values, standards should be spotted in approximately the same quantities as the material under examination.

Different methods of detection were evaluated for sensitivity, specificity and stability, as well as the ability to recover the sterol following recognition. Brief examination of less than 1 minute under U.V. light (emission peak at 255 mμ and 366 mμ) does not seem to significantly affect the sterols. Natural absorbance of sterols under 255 mμ U.V. light is a highly sensitive and specific way of detection. The use of a fluorescent layer, such as Alumina GF facilitates the detection. Only substances having at least two conjugated double bonds show absorbance. Intensity of U.V. absorption is a function of the number, and probably also of the position of the conjugated double bonds. The following compounds are listed in decreasing order of absorption intensity:

(1) Ergo- and chole-calciferol: three conjugated double bonds at C5, 7, and 10.

(2) Pre-ergocalciferol: three conjugated double bonds at C5(10), 6, and 8.
(3) Ergosterol: two conjugated double bonds at C5 and 7.
(4) Cholesterol benzoate: three conjugated double bonds on the aromatic ring.

U.V. absorption is a most useful means of detection of vitamin D, as well as of their pre- and provitamins. It is suitable for biological analysis, since the detection limit is below 1.0 μg for both ergo- and chole-calciferol. Natural fluorescence of sterols under 366 mμ U.V. light is far less sensitive and specific. Most sterols fluoresce under such conditions, except the vitamin D, previtamins and provitamins. In this way it is useful in distinguishing these compounds.

On the other hand, color reagents do not allow the recovery of the stained compounds. Further application of another reagent is impossible, except for the charring method which shows similar features before and after phosphotungstic acid or antimony trichloride staining. Among the reagents tested, antimony trichloride gives the highest specificity, owing to the wide range of colors characteristic of each sterol. It provides an additional means of detection by the fluorescence exhibited by the sterols examined under 366 mμ U.V. light after prolonged heating at 110°. The best sensitivity is obtained by charring with 75% H_2SO_4. According to BOLLIGER *et al.*[16], vitamin D_2 and D_3 could be distinguished from each other using concentrated sulfuric acid, as brown and green colored spots respectively. However, such differences could not be recognized in the present studies with the small amounts of sterols chromatographed, less than 25 μg.

By combining different procedures, sensitive detection and good characterization can be achieved. Thus, very good results are obtained by successive U.V. light examination for absorbance and fluorescence, antimony trichloride for initial colors and fluorescence, and eventually H_2SO_4 charring.

When permanent records are needed, black and white contact prints give very fine and inexpensive reproduction. The sensitivity of the print allows detection of the faintest spots detectable on the original chromatoplate.

TLC on Alumina GF does not seem to alter vitamin D to any significant extent, provided that the samples are chromatographed immediately after quick spotting under nitrogen and red light, and that the spots are removed just after localization while the plates are still impregnated with solvent. Elution from the layer with pure methanol yields a better than 95% recovery of unaltered vitamin D, as shown by the unchanged U.V. absorption curves. This compares favorably to the destruction of significant quantities of steroids[29] and of vitamin D on silica gel[16]. When developed chromatoplates are allowed to stand on the bench for several hours, progressive destruction of vitamin and previtamin D occurs which results in a decrease of U.V. absorptivity and the appearance of a peripheral ring of fluorescent material under 366 mμ U.V. light. The occurrence of previtamin D does not reflect an artefact of the TLC procedure, but results from the reversible thermal transformation of vitamin D which occurs during storage of the standard solution[16,30–32].

REFERENCES

1 P. S. CHEN, A. R. TEREPKA, K. LANE AND A. MARCH, *Anal. Biochem.*, 10 (1965) 421.
2 E. KODICEK AND D. R. ASHBY, *Biochem. J.*, 57 (1954) xii.
3 J. GREEN, *Biochem. J.*, 49 (1951) 45.
4 P. S. CHEN, A. R. TEREPKA AND N. REMSEN, *Anal. Chem.*, 35 (1963) 2030.
5 S. W. JONES, J. B. WILKIE AND D. A. LIBBY, *J. Assoc. Offic. Agr. Chemists*, 48 (1965) 1212.

6 H. ZIFFER, W. J. VANDENHEUVEL, E. O. HAAHTI AND E. C. HORNING, *J. Am. Chem. Soc.*, 82 (1960) 6411.
7 P. P. NAIR, C. BUCANA, S. DELEON AND D. A. TURNER, *Anal. Chem.*, 37 (1965) 631.
8 T. K. MURRAY, K. C. DAY AND E. KODICEK, *Biochem. J.*, 98 (1966) 293.
9 L. V. AVIOLI AND S. W. LEE, *Anal. Biochem.*, 16 (1966) 193.
10 J. R. CLAUDE, *J. Chromatog.*, 23 (1966) 267.
11 H. JANECKE AND I. MAAS-GOEBELS, *Z. Anal. Chem.*, 178 (1960) 161.
12 A. W. NORMAN AND H. F. DELUCA, *Anal. Chem.*, 35 (1963) 1247.
13 A. W. NORMAN, AND H. F. DELUCA, *Biochemistry*, 2 (1963) 1160.
14 P. F. NEVILLE AND H. F. DELUCA, *Biochemistry*, 5 (1966) 2201.
15 R. STROHECKER AND H. M. HENNING, *Vitamin Assay—Tested Methods*, Verlag Chemie, 1966.
16 H. R. BOLLIGER, in E. STAHL (Editor), *Thin-layer Chromatography*, Academic Press, 1965, p. 223.
17 H. R. BOLLIGER AND A. KÖNIG, *Z. Anal. Chem.*, 214 (1965) 1.
18 D. SCHACTER, J. D. FINKELSTEIN AND S. KOWARSKI, *J. Clin. Invest.*, 43 (1964) 787.
19 L. T. HEAYSMAN AND E. R. SAWYER, *Analyst.*, 89 (1964) 529.
20 P. S. CHEN, *Anal. Chem.*, 37 (1965) 301.
21 C. K. PAREKH AND R. H. WASSERMAN, *J. Chromatog.*, 17 (1965) 261.
22 L. J. MORRIS, *J. Lipid Res.*, 4 (1963) 357.
23 J. PASALIS AND N. H. BELL, *J. Chromatog.*, 20 (1965) 407.
24 N. W. DITULLIO, C. S. JACOBS AND W. L. HOLMES, *J. Chromatog.*, 20 (1965) 354.
25 R. D. BENNETT AND E. HEFTMANN, *J. Chromatog.*, 9 (1962) 359.
26 J. W. COPIUS-PEEREBOOM AND H. W. BEEKES, *J. Chromatog.*, 9 (1962) 316.
27 J. W. COPIUS-PEEREBOOM AND H. W. BEEKES, *J. Chromatog.*, 17 (1965) 99.
28 R. D. BENNETT AND E. HEFTMANN, *J. Chromatog.*, 9 (1962) 348.
29 D. R. IDLER, N. R. KIMBALL AND B. TRUSCOTT, *Steroids*, 8 (1966) 865.
30 L. VELLUZ, G. AMIARD AND A. PETIT, *Bull. Soc. Chim. France*, (1949) 501.
31 L. VELLUZ, G. AMIARD AND B. GOFFINET, *Bull. Soc. Chim. France*, (1955) 1341.
32 P. S. CHEN AND K. LANE, *Arch. Biochem. Biophys.*, 112 (1965) 70.