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THE QUANTITATIVE THIN LAYER CHROMATOGRAPHY OF CORTICOSTEROIDS

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SUMMARY

Apparatus and methods for the quantitative thin layer chromatography of corticosteroids down to the 0.001 μg level are described. The stability of cortisol throughout the procedure has been investigated and found to be satisfactory. Some sources of error are discussed and methods of minimising these are described.

The large number of papers describing the application of thin layer chromatography to problems of steroid analysis is striking evidence of the importance of this technique. However the development of quantitative techniques of thin layer chromatography in the steroid field has been relatively slow, particularly in the case of the corticosteroids. Of the reports that do fall in this category most are concerned with analytical problems where the quantity of steroid is relatively large (10 μg or more)[1–7].

We wished to use thin layer chromatography in the final purification of corticosteroids extracted from blood plasma, urine and sweat, prior to quantitation by acid induced fluorescence. This combination of thin layer chromatography and fluorimetry had previously been applied to the purification of cortisol extracted from blood plasma[8] and urine[9] by other workers. Poor recovery of both labelled and inactive steroids lead us to investigate the quantitative thin layer chromatography of corticosteroids and it is the results of this investigation that are reported in this paper.

The apparatus and manipulative techniques were perfected before the present detailed study of the problems involved in the quantitative thin layer chromatography of corticosteroids at the sub-microgram level.

The results of experiments in which we chromatographed sub μg quantities of cortisol and corticosterone to which tracer amounts of the radioactive steroids were added, suggested to us that there were two main problems:

(a) low absolute recoveries of steroid after thin layer chromatography and

(b) the inhibition of the fluorescent reaction of the eluted steroids by interfering substances from the gel, together with a high fluorescent blank.

We found that most commercially available silica gels exhibited (b) to a lesser

or greater extent, even after exhaustive washing with methanol, but one gel, Machery, Nagel HR_{254} was found to be satisfactory without purification. The detailed study of (a) reported in this paper and ways of overcoming this problem are based on the use of this silica gel.

IDLER, KIMBALL AND TRUSCOTT[10] have reported quantitative (98%) recoveries of steroid from thin layer chromatograms but we have found this only down to the 2 μg level and that below this there tends to be excessive losses of steroid.

These workers have also shown that there may be destruction of steroid during elution from silica gel depending on the brand of gel and the elution technique used. Evidence will be given to show that with our choice of gel and procedure there is little or no destruction of sub μg quantities of cortisol.

MATERIALS AND METHODS

Materials

Ethanol. Burroughs RR grade.

Other solvents were of analytical reagent grade where available. Ethyl acetate, ethoxyethyl acetate, *n*-butyl ether and isopropanol were redistilled freshly daily.

Silica gel. Macherey, Nagel-HR/UV_{254}.

Sulphuric acid. British Drug Houses "Analar".

Ethanol–sulphuric acid fluorescence reagent; 25:75 ethanol–sulphuric acid was used for the fluorimetry of the cortisol and 35:65 ethanol–sulphuric acid for the corticosterone.

Radioactive steroids. These were obtained from the Radiochemical Centre, Amersham, England. They were purified by paper chromatography in the BUSH system C[12] or in the EBERLEIN AND BONGIOVANNI E_2B system[13], eluted with methanol and stored in methanol–benzene (1 + 4) at −20°.

Apparatus

The apparatus used for spotting the samples on to the plates is shown in Fig. 1. Nine samples can be spotted on to each of two plates simultaneously. The centres of the spots are 2 cm apart. The pipettes are made from glass capillary tubing 1 mm bore by 7 mm O.D. The ends were ground to a taper and fire polished. The chromatograms are held rigidly on aluminium plates which can be raised and lowered by means of laboratory jacks. The teats used are of the heavy rubber type (Esco (Rubber) Ltd., London). Ordinary latex teats are not satisfactory.

Fig. 2 shows the apparatus used for scraping the gel off the chromatograms into tubes for elution. It is designed to take off a 1.5 cm band off a chromatogram. Wider bands can be taken off in two sections but it is not possible to remove narrower bands.

All glass moulded chromatography tanks either 30 × 22 × 30 cm or 30 × 10 × 22 cm provided with ground glass lids were used. They were lined with blotting paper and the plates were supported on glass rods so that they did not touch the bottom of the tank. In this way the bottom of the tank may be covered with solvent during equilibration without initiating development of the chromatograms. The tanks were protected from drafts.

U.V. absorbing steroids were located using the "Chromatolite" short wave U.V. lamp.

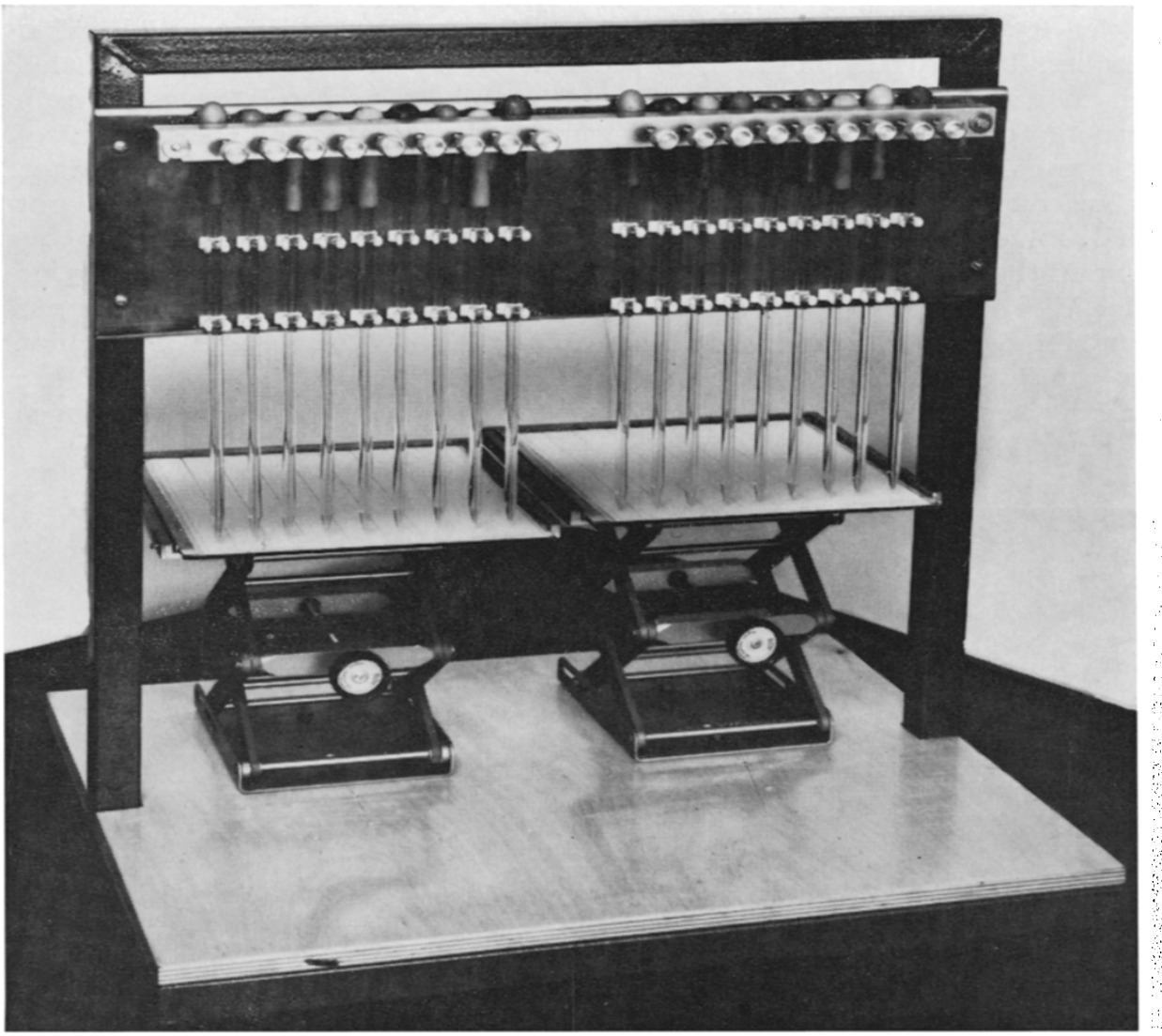

Fig. 1. Apparatus for spotting samples onto thin layer chromatograms.

METHODS

Thin layer chromatography

The plates were coated to a thickness of 0.25 mm with silica gel using an "Unoplan" leveller and adjustable spreader (Shandon Ltd., London). For a batch of five 20 × 20 cm plates a slurry was prepared using 30 g silica gel and 60 ml distilled water which were vigorously shaken together for 1 min.

After coating the plates were left in the leveller for 30 min and were then dried in a stream of warm air (30°) for 60 min. The prepared plates were stored in a cabinet over silica gel desiccant; although the plates were usually used within 48 h they can be kept for at least a week without deterioration. Before use the plates were scored to give 9 lanes each 2 cm wide. When subliminal ($< 1\ \mu g$) quantities of steroids were being run, the outer two lanes were used for standards (2–5 μg) and when the eluates were to be assayed fluorimetrically the centre lane was used as a blank ("gel blank").

Samples for chromatography were evaporated to dryness in 15 ml conical centrifuge tubes. The dry residue was dissolved in about 0.1 ml ethyl acetate–methanol (19:1). This solution was taken up into the spotting pipettes; the plate was put into position, brought up to within 2–3 mm of the tips of the pipettes and the contents of the pipettes were deposited dropwise on to the plates about 2 cm from the end. Each drop was allowed to dry before the next was applied. The tubes were rinsed with about 0.05 ml of the solvent mixture which was similarly applied to the plate.

To obtain the origin as a sharp line the plates were developed to a height of 3 cm with ethanol. When dry the plates were placed in the thin layer chromatography tanks to equilibrate for 45 min at an ambient temperature of 20–25°. At the end of this time sufficient solvent was added to cover the bottom of the plates to a depth of 1 cm. When the solvent front had reached within 0.5 cm of the top of the plates, they were removed from the tank and dried in air in a horizontal position.

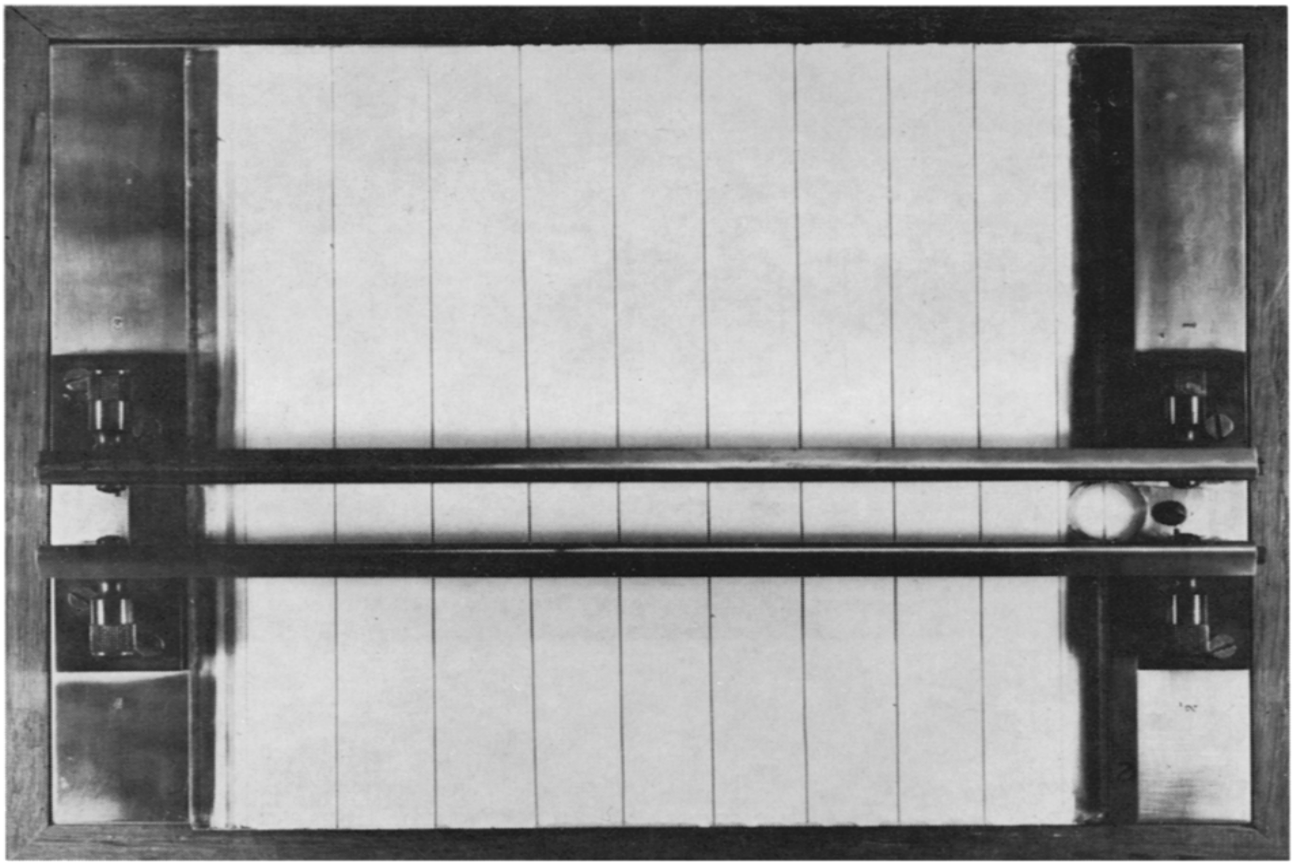

Fig. 2. Apparatus for scraping silica gel off thin layer chromatograms into test tubes. One edge of the chromatographic plate overlaps a funnel which is fixed into the base plate of the apparatus. Successive bands of gel are scraped down the funnel into tubes for elution.

When dry the developed plates were examined under the U.V. lamp and the position of the standards was marked. The plates were placed in the scraping apparatus (Fig. 2) and the appropriate areas of the gel were scraped off into 10 ml glass stoppered tubes. 5 ml methanol was added to each tube and, when tightly stoppered, the contents were mixed vigorously for 10 sec on a vortex mixer and then gently for 5 min on the

"Rolamix". The silica gel was centrifuged down (1500 r.p.m., 1 min) and aliquots of the supernatant were taken for fluorimetry and/or liquid scintillation counting. These aliquots were evaporated to dryness in test tubes or glass bottles respectively by placing them in the vacuum desiccator over silica gel for several hours—commonly overnight.

The following solvent systems have been used in this work:

1. Ethyl acetate
2. Isopropanol–*n*-butyl ether (3:7)
3. Toluene–ethyl acetate–ethanol (60:35:5)
4. Toluene–*β*-ethoxyethyl acetate (2:3)

Paper chromatography

The following solvent systems were used:

A. Toluene–isooctane–methanol–water (150:50:160:40) (DE COURCY)[11]
B. Toluene–ethyl acetate–methanol–water (9:1:5:5) (System C, BUSH)[12]
C. Isooctane–*tert.*-butanol–water (10:5:9) (System E_2B, EBERLEIN AND BONGIOVANNI)[13].

Whatman No. 2 paper was used for systems A and B and No. 1 for system C. Chromatograms were run using 23 cm wide sheets divided into 2 cm wide strips; the tanks were equilibrated overnight and developed with the ambient temperature in the range 20–25°.

Fluorimetry

A Locarte LFM 5 fluorimeter equipped with a zinc lamp was used; the primary filter was LF 11 (this isolates the 468-472-481 lines from the zinc lamp) and the secondary was LF 7 which transmits above 510 mμ. The dry steroid residues were dissolved in 1.5 ml of the appropriate ethanol–sulphuric acid reagent; fluorescence measurements were taken after 20 min in the case of cortisol and after 40 min in the case of corticosterone.

Liquid scintillation counting

A Packard Tri-Carb model 3314 liquid scintillation spectrometer was used. Samples were dissolved in 5 ml of the scintillant solution. The instrument was operated on a pre-set time which was chosen so that at least 5,000 counts would be recorded in the majority of samples. In those samples where the recovery of radioactivity was low this total was not always achieved. The counting efficiency was monitored using the Radium-226 external standard which has been previously calibrated against standard ^3H and ^{14}C toluene. As the counting efficiencies were extremely constant, all the results have been expressed in counts per minute (c.p.m.). When ^3H and ^{14}C were counted simultaneously the count rate for ^3H was obtained by the screening method of OKITA, KABARA, RICHARDSON AND LEROY[14].

RESULTS

The R_F values of some corticosteroids in the solvent systems that we have found most useful are listed in Table I.

The efficiency of our elution technique was studied as follows: 10 μl volumes of

TABLE I

THE R_F VALUES OF SOME CORTICOSTEROIDS

	Solvent system			
	1	*2*	*3*	*4*
Aldosterone	0.14	0.20	0.10	0.27
Cortisol	0.31	0.41	0.18	0.42
Cortisone	0.34	0.36	0.23	0.51
Corticosterone	0.30	0.36	0.23	0.51
Dehydrocorticosterone	0.31	0.24	0.23	0.53
Cortexolone	0.44	0.45	0.31	0.63
Progesterone	0.57	0.56	0.56	0.93
17α-Hydroxyprogesterone	0.51	0.56	0.45	0.75
Prednisone	0.31	0.37	0.19	0.39
Prednisolone	0.28	0.44	0.12	0.34
Tetrahydrocortisone	0.24	0.47	0.12	0.24
Tetrahydrocortisol	0.27	0.46	0.13	0.24
allo-Tetrahydrocortisol		0.46	0.14	
Cortisol acetate	0.53	0.57	0.35	0.67

ethyl acetate containing $4 \cdot 10^4$ c.p.m. ^{3}H-cortisol (0.013 μg) were deposited onto prepared plates using an "Agla" micrometer syringe. Standard areas (2.0 × 1.5 cm) were scrapped off and eluted with 5 ml solvent. The results are summarised in Table II.

In Table III is listed the recovery of standard quantities of ^{3}H and of ^{14}C steroids to which various amounts of inactive carrier steroid have been added; and in Table IV the recovery of mixtures of ^{14}C-cortisol and ^{3}H-aldosterone are presented. In the latter experiment there was negligible cross contamination of the two steroids.

From Table III it will be seen that for a given steroid and solvent system the recovery of the steroid is often related to the weight of steroid chromatographed. In order to improve the recovery of nanogram quantities of ^{3}H-cortisol we have investigated the possibility of using a "foreign" steroid as the carrier. Some results of this approach were summarised in Table V. While cortexolone and progesterone, which have much greater mobility than cortisol, are without significant effect, prednisolone, which moves very closely to cortisol, is equally as effective as cortisol.

To demonstrate that the material eluted from the silica gel does not influence the fluorimetric assay of the eluted steroids various quantities of ^{14}C-cortisol and of ^{3}H-corticosterone were chromatographed and the recovery determined by both fluorimetry and by liquid scintillation counting. It will be seen from Table VI that both end points give essentially the same result.

TABLE II

ELUTION OF ^{3}H-CORTISOL FROM THIN LAYERS OF SILICA GEL

Solvent	*n*	*% Recovery (range)*
Ethyl acetate	6	80 (76–83)
Acetone	5	90 (86–93)
Methanol	6	95 (90–98)
Chloroform	3	36 (34–39)

TABLE III

THE RECOVERY OF SOME PURE STEROIDS MEASURED BY LIQUID SCINTILLATION COUNTING

Steroid	*Quantity* (μg)*	*Solvent system*	*n*	*% Recovery (range)*
^{3}H-Corticosterone	0.001	4	7	77 (66–87)
	0.001	3	10	71 (56–81)
	0.5	3	3	84 (78–88)
^{14}C-Cortisone	0.01	3	12	57 (37–90)
	0.01	1	6	26 (20–37)
	0.5	1	6	45 (36–53)
	2.0	1	6	60 (56–67)
	0.01	2	5	46 (32–57)
	0.5	2	6	53 (44–59)
	2.0	2	6	76 (73–79)
^{3}H-Cortisol	0.001	1	16	44 (19–66)
	0.20	1	4	70 (63–74)
	0.50	1	4	77 (70–84)
	1.0	1	3	84 (75–91)
	2.0	1	4	90 (83–96)

* Approximately 3,000 c.p.m. were used per sample.

When working with very small quantities of steroids the possibility exists that chromatography or even evaporation of a solution to dryness may lead to chemical changes[4,5]. To determine if, and to what extent, this happens we have carried out a dilution analysis of carrier free ^{3}H-cortisol that had been chromatographed in system 1. Three aliquots of the eluate were taken for liquid scintillation counting; to three more aliquots were added 100 μg of carrier cortisol and three further aliquots were evaporated to dryness in the vacuum desiccator during 18 h prior to the addition of 100 μg of carrier cortisol. These latter six aliquots were chromatographed on paper first in the BUSH C system and then in the E_2B system. The recovery of carrier cortisol was measured by spectrophotometry at 245 mμ, and that of tritium by scintillation counting. After correcting for losses of the carrier cortisol we obtained recoveries of 101, 102 and 97% for the first three aliquots and 98, 96 and 103% for the second three aliquots; from this data we conclude that at least in this case there was no destruction of the cortisol either during the chromatography or the evaporation of the eluate to dryness.

TABLE IV

SEPARATION OF MIXTURES OF ^{3}H-ALDOSTERONE AND ^{14}C-CORTISOL

Solvent system	*% Recovery*	
	Aldosterone	*Cortisol*
1	62	69
1	67	71
4	65	79
4	65	75
2 (n = 3)	56 (54–58)	69 (65–73)

Quantities: Cortisol = 0.1 μg. Aldosterone = 0.001 μg.

TABLE V

THE INFLUENCE OF THE ADDITION OF CARRIER STEROIDS (2 μg) ON THE RECOVERY OF ^{3}H-CORTISOL*

Carrier steroid	*n*	*% Recovery (range)*
Nil	8	33 (19–49)
Cortisol	6	69 (63–72)
Prednisolone	4	72 (69–75)
Progesterone	5	36 (26–49)
Cortexolone	5	38 (25–61)

* 3,000 c.p.m. (0.001 μg) used per test. Solvent system = ethyl acetate.

In another experiment we attempted to determine whether the chromatography could cause tritium to be lost from ^{3}H-cortisol. Aliquots of a mixture of ^{3}H and of ^{14}C-cortisol were chromatographed in systems 1 and 2. In each case the recovery of ^{14}C was very slightly higher than that of tritium. When the recovery of ^{14}C was given an arbitary value of 100% then the recovery of tritium was 96% (range 93–99%). Dilution analysis of the ^{3}H-cortisol used gave values of 104, 98 and 100% so this shortfall in the recovery of tritium is not likely to be due to radiochemical impurity in the starting material. Whether this apparent loss of tritium is really due to its removal from the steroid molecule or to isotope fractionation cannot be decided with certainty from the present data.

We have investigated the distribution of tritium along chromatograms of ^{3}H-cortisol by scraping off consecutive 1.5 cm bands and counting aliquots of the eluates for tritium. These results are shown in Fig. 3. The cutting of the fractions was slightly different from normal as the U.V.-absorbing band was in the middle of the peak fraction whereas we usually set the front blade of the scraper only about 2 mm

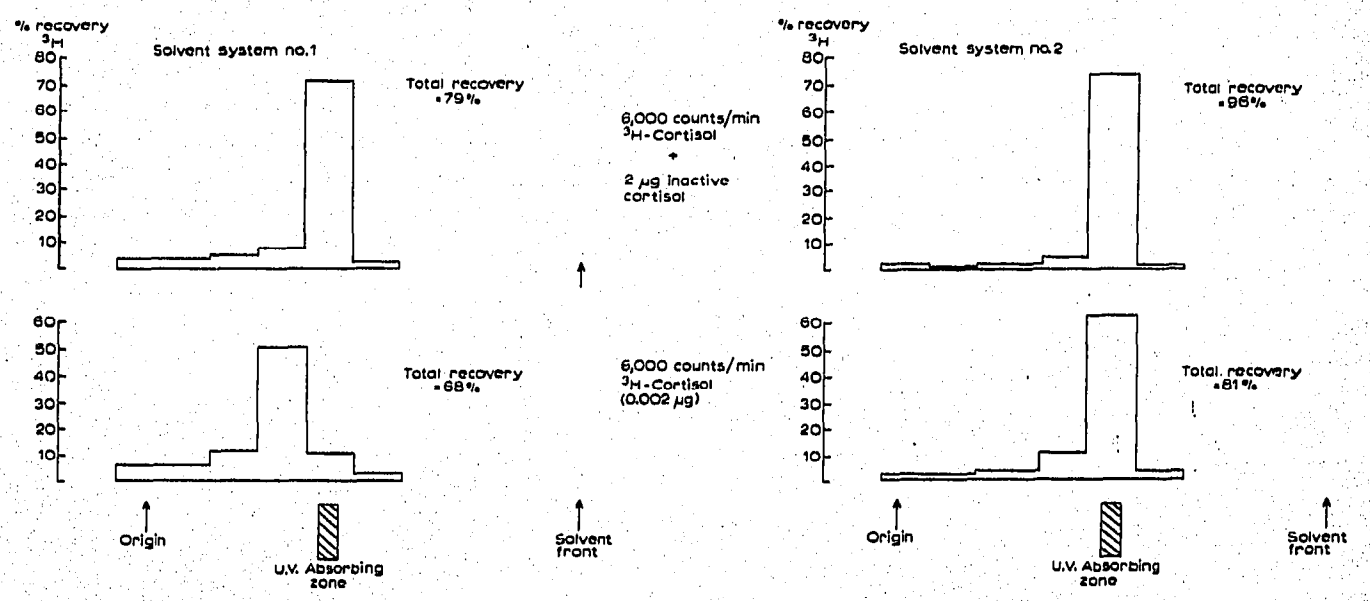

Fig. 3. The influence of the addition of 2 μg cortisol on the chromatography of 0.002 μg ^{3}H-cortisol.

TABLE VI

RECOVERY OF CORTISOL AND CORTICOSTERONE AFTER TLC. COMPARISON OF RESULTS OBTAINED BY FLUORIMETRY AND BY LIQUID SCINTILLATION COUNTING

Steroid	*Isotope*	*Solvent system*	*Weight (μg)*	*n*	*% Recovery (range)*	
					Fluorimetry	*Scintillation*
Corticosterone	^{3}H	3	0.05	6	83 (76–110)	83 (67–102)
	^{3}H	3	0.1	6	83 (77– 91)	77 (74– 91)
	^{3}H	3	0.15	6	77 (74– 80)	80 (73– 88)
	^{3}H	3	0.2	6	74 (64– 77)	76 (64– 81)
Cortisol	^{14}C	1	0.25	6	73 (53–102)	73 (56– 85)
	^{14}C	1	1.0	7	76 (57– 88)	76 (55– 86)

ahead of the front edge of the standard. It can be seen that when 0.002 μg ^{3}H-cortisol is chromatographed in system 1 it moves more slowly than the 2 μg standard thus accounting for the poor and variable recovery one obtains with this combination. On the contrary the distribution of tritium is very similar between 0.002 and 2.0 μg ^{3}H-cortisol when system 2 is used. In both solvent systems the total recovery of tritium is rather lower in the absence of the carrier steroid. This could be accounted for by irreversible binding of a small quantity of the steroid by the silica gel.

DISCUSSION

The two major problems that we have encountered in the quantitative thin layer chromatography of sub-microgram quantities of corticosteroids have been poor overall recovery of steroids as measured by liquid scintillation counting, and inhibition of the acid induced fluorescence of the eluted steroids. As we have shown in this paper both of these difficulties can be overcome by the use of appropriate solvents and silica gel. The root cause of the first effect is well illustrated in Fig. 3 where it will be seen that in one case elution of the gel in parallel with the standards results in missing the major part of the ^{3}H-cortisol sample.

The nature of the substances that can be eluted from some grades of silica gel which inhibit the fluorescence of the corticosteroids is unknown to us. However, we have found that addition to the ethanol–sulphuric acid reagent of the salts of copper, (ferric) iron, mercury and silver and of most common oxidising agents results in a decrease in or complete absence of fluorescence from standard quantities of cortisol.

In order to obtain a reasonably quantitative transfer of the steroids onto the plates it is necessary to use relatively large volumes of solvent. Because these volumes of solvent have to be evaporated down onto one spot it is essential that the layers of gel have good mechanical strength. When we activated our plates for 30–60 min at 110° their strength was inadequate but by omitting this step we get consistently good layers. We have found very little difference in the mobility of the steroids whether the layers are activated or not.

We have found ethyl acetate–methanol (19:1) very satisfactory as the solvent for transferring the steroids onto the plates. With care less than 3% of the sample is left in the tubes from which the transfer is made. The use of water-miscible solvents,

in these volumes, tends to make the gel lift up from the glass plate, while it is difficult to control the flow of the heavy chlorinated hydrocarbons. In order to hasten the application of the samples to the plates relatively large (1 cm diameter) spots were applied and these were then compressed into a thin line by the preliminary development in ethanol. The final developed chromatograms show the steroids as bars rather than spots.

The choice of solvent system is mainly governed by two considerations. Firstly, it must achieve the degree of resolution necessary for the problem in hand; secondly, the quantity of steroid being chromatographed must be recoverable in reasonable yield. If the first consideration suggests a system in which the recovery is unsatisfactory then this may be improved by the addition of a foreign carrier. Obviously the problem becomes very much simpler if the quantity of steroid being chromatographed is sufficient to give visible spots under the U.V.-lamp. When analysing biological fluids we use paper chromatography for the preliminary separations; it is fortuitous that some separations that are difficult on paper can be readily achieved by TLC (such as cortexolone and corticosterone).

When thin layer chromatography is used in routine analytical work there is some advantage in using a single component solvent as variations in composition cannot occur and better reproducibility should result. It is for this reason that we have experimented with ethyl acetate (system 1) despite it being unsatisfactory at very low levels.

The choice of the elution technique is governed by the need to handle large numbers of samples. Methanol has been used as the eluant as such residue as it leaves on evaporation neither fluoresces with the ethanol–sulphuric acid reagent nor does it inhibit the fluorescence of cortisol or corticosterone. When trying other types of gel we tried diluting the methanol with less polar solvents in the hope that the elution of interfering material would be diminished, but any advantages obtained were offset by corresponding solvent residue problems, or by difficulties in the removal of the solvents. This technique is much simpler than others that have been described[9,15,16] and no less effective.

The apparatus and technique described in this paper were designed for routine use. One worker, using eight plates (20 × 20 cm) can chromatograph 48 samples a day if two standards and a gel blank have to be included on each plate. If all nine channels on a plate are available for analysing samples then this rate increases to 72 samples a day.

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