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# Note

## Thin-layer chromatography on silica-coated aluminium sheet as an adjunct to radioimmunoassay of steroids

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Sephadex LF-20 [1–5] and Celite [6–9] microcolumns are now widely used for the separation of steroids. However, the preparation and packing of such columns is time-consuming, and good separation of steroids sometimes requires large volumes of solvents (*e.g.* Sephadex LH-20 columns). Another disadvantage of column chromatography is that the polarity of the solvents has to be changed frequently (Celite columns), and small changes in flow-rates greatly influence the elution profile.

With the availability of silica-precoated aluminium sheets, the rapid separation of some steroids and their elution from cut-out pieces has been reported [10], but to our knowledge this approach has not been investigated with the idea of maximum recovery, at nanogram levels, of glucocorticosteroids and sex steroids.

## EXPERIMENTAL

### *Reagents and solvents*

All reagents and solvents (analytical grade) were purchased from Merck (Darmstadt, Germany), and chromatoplates (Alugram SIL G, 0.25 mm, 20 × 20 cm) with fluorescence indicator from Macherey and Nagel (Düren, Germany). Tritiated steroids were supplied by NEN Chemicals (Dreieich, Germany). The specific activities (Ci/mmol) of steroids were as follows: 103 (progesterone); 44 (17-hydroxyprogresterone); 102 (deoxycorticosterone); 100 (cortisol), 102 (corticosterone); 103 (testosterone). Antisera for steroid assays were generously supplied by Professor Dr. F. Bidlingmaier (Department of Clinical Biochemistry, University of Bonn, Bonn, Germany).

### *Chromatography, elution and radioimmunoassay of steroids*

To test whether steroids can be quantitatively eluted from the silica matrix, chromatoplates were cut into 1 × 1 cm pieces, and trace amounts of tritiated

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steroids (50–100 pg, dissolved in 20 μl ethanol) were applied to them. After ethanol had been evaporated, pieces were placed into plastic scintillation vials containing 2.0 ml of methanol. The samples were gently shaken and 5, 30 and 60 min later, 0.1-ml aliquots were withdrawn for scintillation counting to calculate the recovery. To determine whether standard curves of the six radioimmunoassays are influenced by the solvent and/or chromatoplate impurities, 0.5-ml aliquots of a steroid-free human plasma pool were placed onto kieselguhr minicolumns (Extrelut, Merck) as previously described [11]. Half of the samples were extracted with dichloromethane (glucocorticosteroids), the other half with diethyl ether. Extracts of plasma were redissolved in 100 μl of ethanol, and 20-μl aliquots were applied to TLC plates. They were scored to give twelve 1.5-cm-wide lanes. Steroids (1 μg/10 μl ethanol) were spotted on the outer lanes as markers, and the inner lanes were used for plasma extracts. After development twice in chloroform–methanol–water (188:12:1, v/v/v), the steroid markers were located under the UV lamp. The centres of the spots were marked, and a band 0.5 cm on either side of the calculated mid-point of the extracts was cut out for each steroid. The pieces were placed into scintillation vials and steroids were eluted with 2.0 ml of methanol. After 60 min, pieces were removed and vials were allowed to stand at room temperature for 14 h. Methanol extracts were taken up in 1.0 ml of phosphate buffer (0.5 *M*, pH 7.3) with 0.1% bovine serum albumin (Serva, Heidelberg, Germany). To normal standards (triplicates), 100-μl aliquots of dichloromethane or diethyl ether extracts ("standards without TLC extract") or 100-μl aliquots of methanol extracts from chromatoplates ("standards with TLC extracts") were added. After addition of 0.5 ml of diluted antiserum and 0.1 ml of tracer, assay tubes were incubated at 4°C. After 12–14 h. 0.5 ml of dextran-coated charcoal were added and tubes were centrifuged at 4°C for 15 min. Then the supernatant was decanted into scintillation vials, mixed with 3 ml of liquid scintillation cocktail and counted for radioactivity. Further details of the assay systems, including dilutions and cross- reactions of the antisera, are given elsewhere [5,11–13].

*Applicability to biological samples*

In order to test the applicability of the separation/elution method to the analysis of biological samples, urine samples from adult male guinea-pigs were placed on kieselguhr minicolumns [11] and steroids were extracted either with dichloromethane or diethyl ether. After evaporation of the organic phase, the residues were dissolved in 100 μl of ethanol and 20-μl aliquots were applied to TLC plates. After development in chloroform–ethanol–water (188:12:1, v/v/v), steroid markers were located under the UV lamp, and corresponding bands of glucocorticosteroids and sex hormones located and eluted with 2.0 ml of methanol. Methanol extracts were taken up in 2.0 ml of phosphate buffer, and after appropriate dilution, steroid concentrations were determined by radioimmunoassay.

RESULTS AND DISCUSSION

The mobilities of the six steroids tested are shown in Table I. Solvent fronts travelled 14 cm in 60 min. The $R_F$ values listed are the average of six replicate determinations. The maximum variability of placements was 0.07 $R_F$ units. Mobilities of steroids, when applied as mixtures, were in good agreement with those of single compounds and no differences in spot size or shape were observed after development.

Table II shows that all steroids were eluted within 15 min and that longer elution times did not significantly increase recoveries (15 min, 92 ± 5%; 60 min, 93 ± 4%; $n = 7$) or precision (coefficient of variation: 15 min, 4.8%, 60 min, 4.8%). In contrast to the work of Murphy [2], who stated that "TLC techniques produce substances which subsequently denature the assay proteins, causing er-

TABLE I

$R_F$ VALUES OF GLUCOCORTICOSTEROIDS AND SEX STEROIDS CHROMATOGRAPHED ON ALUGRAM SIL G SHEETS

| Steroid | $R_F$ values (mean ± S.D., $n = 6$) | |
|---|---|---|
| | First run | Second run |
| Progesterone | 1.00 ± 0.01 | 1.00 ± 0.01 |
| 17-Hydroxyprogesterone | 0.76 ± 0.06 | 0.90 ± 0.04 |
| 11-Deoxycorticosterone | 0.81 ± 0.03 | 0.90 ± 0.05 |
| Cortisol | 0.24 ± 0.05 | 0.43 ± 0.06 |
| Corticosterone | 0.45 ± 0.07 | 0.71 ± 0.05 |
| Testosterone | 0.69 ± 0.06 | 0.85 ± 0.04 |

TABLE II

RECOVERY OF TRITIATED STEROIDS FROM ALUGRAM SIL G SHEETS AFTER DIFFERENT ELUTION TIMES

| Steroid | Recovery (mean ± S.D., $n = 5$) (%) | | |
|---|---|---|---|
| | 15 min | 30 min | 60 min |
| Progesterone | 97 ± 2 | 96 ± 3 | 98 ± 3 |
| 17-Hydroxyprogesterone | 87 ± 5 | 91 ± 6 | 93 ± 6 |
| 11-Deoxycorticosterone | 90 ± 6 | 91 ± 5 | 90 ± 5 |
| Cortisol | 86 ± 5 | 85 ± 4 | 88 ± 6 |
| Corticosterone | 96 ± 3 | 94 ± 3 | 98 ± 5 |
| Testosterone | 96 ± 2 | 95 ± 3 | 95 ± 2 |

ratic blank values" (p. 21), Table III illustrates that blanks are negligible if Alugram SIL G chromatoplates are used.

In summary, the TLC technique described here provides a simple method for the separation and elution of glucocorticosteroids and sex steroids prior to analysis by radioimmunoassay. Distinct advantages include good resolution, high reproducibility, low costs of solvents and absence of finely dispersed silica particles, which are known to increase blank values [2,14] and to decrease recoveries [15,16]. Furthermore, multiple simultaneous sample processing enables us to separate and elute a large number of samples ($n$ = 50) containing physiological levels of glucocorticosteroids and sex steroids in plasma or urine in a single after-

TABLE III

STANDARD CURVES OF GLUCOCORTICOSTEROIDS AND SEX STEROIDS WITHOUT OR WITH TLC EXTRACT

For further information, see Experimental.

| Steroid | Tracer binding (mean ± S.D., $n$ = 3) (cpm) | | | | | |
|---|---|---|---|---|---|---|
| | 0 ng | 0.1 ng | 0.2 ng | 0.5 ng | 1.0 ng | 2.0 ng |
| Progesterone | 7114 | 5685 | 4772 | 3009 | 2017 | 1392 |
| without TLC extract | ±233 | ±85 | ±90 | ±60 | ±56 | ±15 |
| Progesterone | 7189 | 5801 | 4799 | 3121 | 2189 | 1387 |
| with TLC extract | ±188 | ±78 | ±101 | ±36 | ±22 | ±34 |
| 17-Hydroxyprogesterone | 7499 | 2987 | 1951 | 1120 | 860 | 691 |
| without TLC extract | ±51 | ±187 | ±46 | ±13 | ±13 | ±17 |
| 17-Hydroxyprogesterone | 7502 | 3002 | 1946 | 1099 | 862 | 668 |
| with TLC extract | ±72 | ±103 | ±35 | ±18 | ±16 | ±12 |
| 11-Deoxycorticosterone | 11470 | 8602 | 6249 | 3117 | 1939 | 1408 |
| without TLC extract | ±108 | ±238 | ±187 | ±49 | ±54 | ±28 |
| 11-Deoxycorticosterone | 11504 | 8597 | 6266 | 3001 | 1878 | 1452 |
| with TLC extract | ±123 | ±144 | ±138 | ±67 | ±63 | ±32 |
| Cortisol | 11461 | 8585 | 6252 | 3134 | 1932 | 1492 |
| without TLC extract | ±122 | ±450 | ±172 | ±31 | ±37 | ±18 |
| Cortisol | 11448 | 8570 | 6199 | 3160 | 1899 | 1492 |
| with TLC extract | ±101 | ±411 | ±168 | ±25 | ±49 | ±45 |
| Corticosterone | 11003 | 8256 | 5898 | 2873 | 1645 | 1406 |
| without TLC extract | ±164 | ±142 | ±140 | ±36 | ±36 | ±23 |
| Corticosterone | 11086 | 8301 | 5920 | 2923 | 1668 | 1428 |
| with TLC extract | ±187 | ±131 | ±162 | ±42 | ±41 | ±14 |
| Testosterone | 12390 | 4468 | 2768 | 1554 | 1075 | 858 |
| without TLC extract | ±280 | ±112 | ±77 | ±56 | ±27 | ±58 |
| Testosterone | 12411 | 4650 | 2962 | 1604 | 1102 | 847 |
| with TLC extract | ±301 | ±108 | ±61 | ±61 | ±31 | ±61 |

TABLE IV

URINARY EXCRETION OF GLUCORTICOSTEROIDS AND SEX STEROIDS IN SALINE- AND (1–24)ACTH-TREATED MALE GUINEA-PIGS

Animals ($n$ = 4/group) were injected with either 0.2 ml of saline or 20 I.U. of (1–24) ACTH (Synacthen depot), and urine samples were collected over 24 h. For further details, see Experimental. Values are mean ± S.D.

| Steroid | Excretion ($\mu$g per day) | |
|---|---|---|
| | Saline | (1–24)ACTH |
| Progesterone | 0.08 ± 0.02 | 0.12 ± 0.03 |
| 17-Hydroxyprogesterone | Not detectable | |
| 11-Deoxycorticosterone | Not detectable | |
| Cortisol | 93 ± 36 | 412 ± 54 |
| Corticosterone | 0.27 ± 0.14 | 1.51 ± 0.75 |
| Testosterone | 0.05 ± 0.03 | 0.15 ± 0.14 |

noon. Data for glucocorticosteroid excretion in both normal and (1–24)ACTH-treated guinea-pigs (Table IV, Fig. 1) are in agreement with those of earlier investigations, demonstrating that cortisol is the major corticosteroid excreted by the guinea-pig [17–19]. Hence, the method presented in this work should prove to

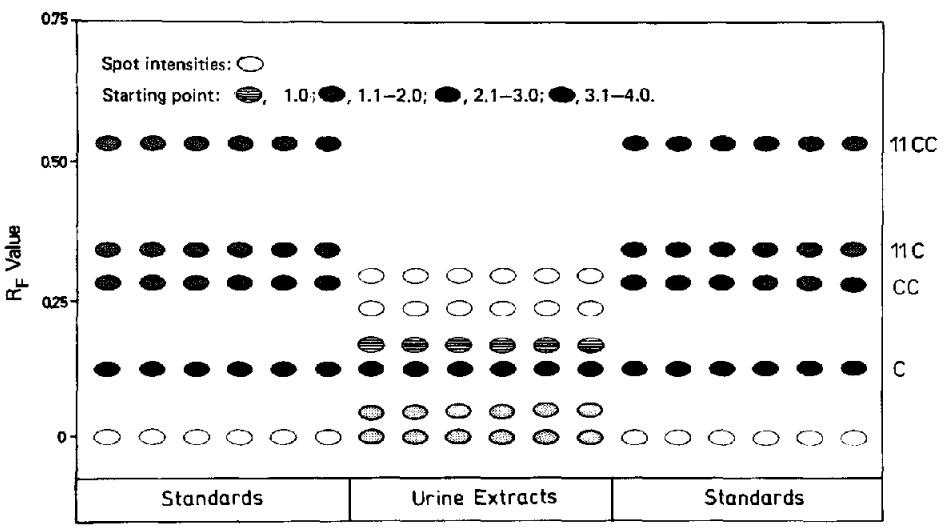


Fig. 1. Separation of glucocorticosteroids on Alugram SIL G. Urine samples from (1–24)ACTH-treated guinea-pigs were extracted, and 10-$\mu$l aliquots were spotted onto TLC plates. They were developed in chloroform–ethanol–water (188:12:1, v/v/v) and spots were detected under UV. Abbreviations: C = cortisol; CC = corticosterone; 11C = 11-deoxycortisol; 11CC = 11-deoxycorticosterone.

be a simple but accurate alternative to other separation techniques, *e.g.* chromatography on Sephadex LH-20 or Celite minicolumns.

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