THIN-LAYER PARTITION CHROMATOGRAPHY FOR URINARY METABOLITES OF CORTISOL

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In 1962 VAEDTKE AND GAJEWSKA[1] described a pure partition thin-layer system for steroid separation. They obtained very good results for pharmaceutical preparations with a wide range of polarity, from sterol esters to prednisolone.

In this work VAEDTKE's method has been used in a slightly modified form to separate urinary metabolites of cortisol. By using Zaffaroni type systems on TLC plates coated with a practically inactive support the authors have obtained very good results. Various solvent systems were tried using steroid reference standards. Ethylene glycol–dichloromethane and ethylene glycol–chloroform proved to be the most suitable and when applied to urinary extracts of PORTER–SILBER chromogens have given a very good separation of THF, ATHF, THE, F and E* in one chromatographic run.

EXPERIMENTAL

Five 20 × 20 cm or ten 10 × 20 cm glass plates were covered with a slurry prepared by mixing 35 g of Kieselguhr G Merck and 60 ml of water. To spread the slurry on the plates, standard Shandon TLC equipment was used. The thickness of the layer obtained was 0.25 mm. The plates were then dried in an oven for 2 h at 120°. The dried plates were kept in a desiccator. The layer of kieselguhr was removed from each side of the plate so as to provide a margin 1 cm wide.

Solvent systems were prepared as in paper chromatography, *i.e.*, the solvents were shaken in a separatory funnel and allowed to stand till separation of the phases occurred.

After coating, the plates were impregnated with the stationary phase either by means of spraying or by soaking as in ascending chromatography. For spraying the stationary phase alone was used. In the case when soaking was used for impregnation 5 %, 10 %, 20 % and 30 % v/v solutions of the stationary phase in acetone (respectively), were used. This method of impregnation determined the amount of the stationary phase on the plate. The impregnation was stopped when the front of the

* Abbreviations and trivial names used refer to the following chemical substances: THF = tetrahydrocortisol = 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one; ATHF = allo-tetrahydrocortisol = 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one; THE = tetrahydrocortisone = 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione; F = cortisol = 11β,17α,21-trihydroxy-Δ^4-pregnene-3,20-dione; E = cortisone = 17α,21-dihydroxy-Δ^4-pregnene-3,11,20-trione; BT = blue tetrazolium; TTC = triphenyltetrazolium chloride.

solvent had reached the upper edge of the layer on the plate. The plates were then allowed to stand in air at room temperature until the acetone had evaporated. Immediately afterwards the material to be chromatographed was applied to the starting lines (4 cm from the lower edge of the plate and at distances of 1.5 cm from each other) by means of a microlitre syringe and the plate was placed in a chamber containing the mobile phase. The chromatogram was developed by the ascending technique. Chromatography was carried out until the solvent front had reached the upper edge of the kieselguhr layer. After drying at room temperature or in an oven (but not exceeding 40°) the colour reaction with alkaline BT or TTC spray solution[2] was performed.

Standard solutions were prepared by dissolving pure substances in chloroform–ethanol (1:1) mixture. F, E, THF and THE were used in the experiments. ATHF was used only in some instances because our supply of this substance was very small.

For the separation of urinary metabolites of cortisol, the solvent system was chosen on the basis of the work with standard substances.

The preparation of urinary extracts was carried out according to the PORTER–SILBER method for 17-hydroxy-corticosteroids in urine[3]. Crude extracts or when necessary after additional silica gel column purification[4], were dissolved in a 0.2–0.3 ml chloroform–ethanol mixture (1:1) and applied to the starting lines.

RESULTS

The following solvent systems were tested: formamide–chloroform; formamide–dichloromethane; ethylene glycol–chloroform; ethylene glycol–dichloromethane; ethylene glycol–ethylene chloride. For most of the solvent systems four concentra-

TABLE I

R_F VALUES OF SOME STEROIDS ON PARTITION THIN-LAYER CHROMATOGRAPHY: INFLUENCE OF THE AMOUNT OF STATIONARY PHASE

Abbreviations: F = formamide; EtG = ethylene glycol. Subscripts to the stationary phase symbols express percentage of concentration (v/v) of stationary phase in acetone solution used for impregnation.

Solvent system	*E*	*F*	*THE*	*ATHF*	*THF*
$F_5/CHCl_3$	0.86	0.62	0.62	—	0.33
$F_{10}/CHCl_3$	0.47	0.21	0.21	—	0.09
$F_{20}/CHCl_3$	0.43	0.15	0.15	—	0.06
$F_{30}/CHCl_3$	0.41	0.14	0.14	—	0.04
F_5/CH_2Cl_2	0.93	0.72	0.72	—	0.49
F_{10}/CH_2Cl_2	0.71	0.42	0.42	—	0.21
F_{20}/CH_2Cl_2	0.59	0.27	0.27	—	0.13
F_{30}/CH_2Cl_2	0.55	0.24	0.24	—	0.12
$EtG_5/CHCl_3$	0.90	0.66	0.54	—	0.27
$EtG_{10}/CHCl_3$	0.84	0.48	0.33	—	0.14
$EtG_{30}/CHCl_3$	0.70	0.30	0.18	—	0.08
EtG_5/CH_2Cl_2	0.92	0.81	0.71	—	0.43
EtG_{10}/CH_2Cl_2	0.90	0.68	0.55	0.32	0.26
EtG_{20}/CH_2Cl_2	0.57	0.24	0.16	—	0.06
$EtG_{10}/C_2H_4Cl_2$	0.60	0.23	0.14	—	0.05
$EtG_{20}/C_2H_4Cl_2$	0.53	0.17	0.11	—	0.04

tions (mentioned above) of the stationary phase were tried. Solvent systems and R_F values are given in Table I.

As expected, the more stationary phase on the plate, the more compact were the spots, the lower the R_F values and the differences between them. Very small amounts of stationary phase on the plate such as in a case of impregnation with a 5 % solution of stationary phase in acetone, gave diffused spots with a tendency to tailing. R_F values were high and even for the same standard steroid substances chromatographed on the same plate, have differed considerably.

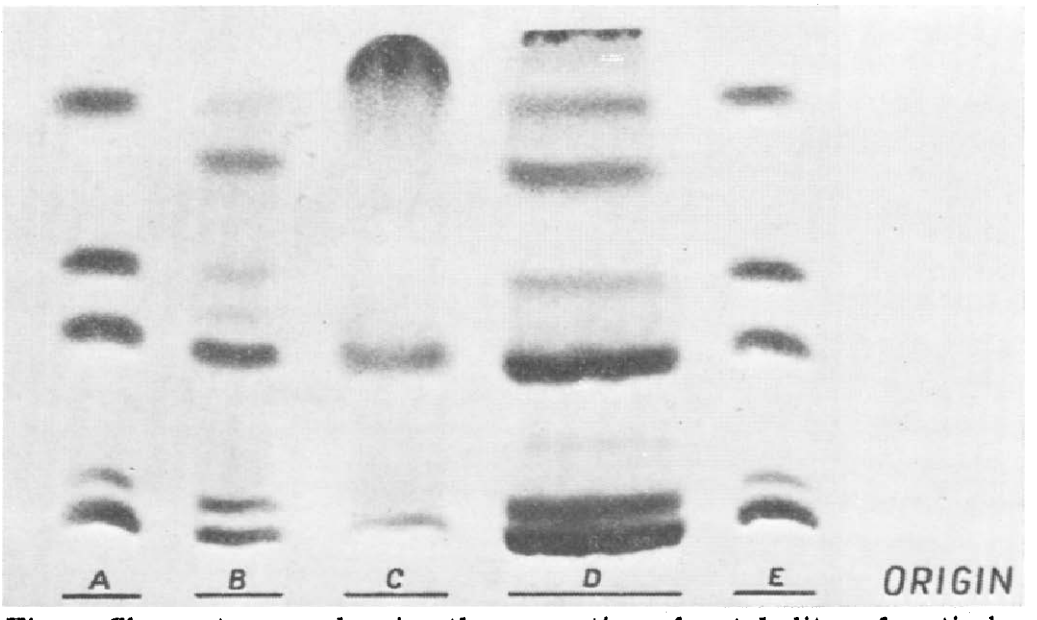

Fig. 1. Chromatogram showing the separation of metabolites of cortisol on a TLC plate. A and E represent the mixture of reference standards. C is the urinary extract before ACTH stimulation. B and D are urinary extracts after ACTH stimulation. Extract B however contains equal concentration of PORTER–SILBER chromogens as extract C and shows the effect of ACTH on ratio of particular metabolites. Extract D was prepared from the same volume of urine as extract C and therefore shows both qualitative and quantitative changes due to ACTH stimulation.

Among the systems being tested the most suitable appeared to be ethylene glycol–dichloromethane (10 % v/v ethylene glycol in acetone used for impregnation). It was possible to separate THF, ATHF, F, THE and E in one chromatographic run. Hence this solvent system was chosen for the separation of urinary metabolites of cortisol. Results of separation of urinary extracts were as good as for standard substances.

Among the urine samples analysed in our clinic were those taken under basal conditions, as well as those during dynamic studies (stimulation with ACTH, metopyrone test, suppression with dexamethasone, urine samples from patients under the influence of different kinds of stress, etc.). In Fig. 1 a chromatogram of a urinary extract after ACTH stimulation is presented. 25 mg of ACTH Prolongatum was given intramuscularly every 12 h for three days (see Fig. 1 and legend).

DISCUSSION

Full separation of urinary metabolites of cortisol by means of paper chromatography in BUSH or ZAFFARONI type systems is a time-consuming procedure and needs

at least two chromatographic steps, namely preliminary separation in a formamide–chloroform system, followed by the second step in propylene glycol–toluene (to separate THF from ATHF), and in the BUSH C system[5] (to separate F from THE) needs about 5 days[6]. Using the thin-layer partition technique described above we have been able to separate THF, ATHF, THE, F and E in one chromatographic step which takes about one hour only.

The purity of the separation and the distances between the steroid spots on the chromatogram seem to allow one to quantitate the steroids after having eluted them from the sorbent. This is the subject of work in preparation.

SUMMARY

A thin-layer partition method for the separation of urinary metabolites of cortisol is described. From amongst the solvent systems tested the most suitable appeared to be ethylene glycol–dichloromethane. Using this system the authors have obtained very good separation of tetrahydrocortisol, *allo*-tetrahydrocortisol, tetrahydrocortisone, cortisol and cortisone in one chromatographic step which takes only about one hour.

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