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# Some aspects of the use of thin-layer chromatography in a limit test for related foreign steroids

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To attain the maximum separation of the steroid impurities in pharmaceutically important synthetic corticosteroids the running of two or more chromatograms using different solvent systems is recommended. Using adsorption chromatography, solvent systems of the type 1,2-dichloroethane : methanol : water, 95 : 5 : 0·2, give the most satisfactory general separations. Some steroids can best be separated by partition chromatography, using formamide as the stationary phase and chloroform : ether : water, 80 : 20 : 0·5, or cyclohexane : tetrachloroethane : water, 50 : 50 : 0·1, as the mobile phase. A spray reagent of 0·05% tetrazolium blue and 8% sodium hydroxide in methanol is recommended. The amounts of foreign related steroids can be limited by running standard amounts of impurities alongside the steroid under test and comparing the colour intensities of the impurities in the steroid with those of the standards.

CORTISONE acetate and a number of related synthetic steroids are assayed in the British Pharmacopoeia 1963 by measuring the colour of the formazan produced by the reducing action of the steroids on triphenyltetrazolium. In addition, to limit the presence of impurities that are tetrazolium-reactive, a paper chromatographic procedure is included in which the impurities are separated and then reacted with a triphenyltetrazolium reagent.

The well-known advantages of thin-layer chromatography over paper chromatography have led us to investigate this newer technique as an alternative means of testing for tetrazolium-reacting impurities.

## Experimental and results

### MATERIALS AND APPARATUS

*Adsorbents.* Kieselgel G and Kieselgel GF 254 (Merck). With Kieselgel GF 254 the chromatograms were examined in ultra-violet light at 254 m$\mu$.

The steroid solutions were applied to the thin layers by micro-capillary (Micro-cap) pipettes. The reproducibility of the delivery of ten 5$\mu$l pipettes was checked by weighing the amount of water delivered. For any given pipette, no delivery deviated by more than 2% from the mean and the means ranged from 4·7–5·0 $\mu$l.

The steroids examined are those listed in Table 1.

Analytical grade solvents were used, purified where necessary (Bush, 1961). 2-Methoxyethyl acetate was purified by the procedure 19·054 of the Official Methods of Analysis of the A.O.A.C. (1960).

### GENERAL PROCEDURE

Shake the adsorbent (25 g) for 1 min with water (50 ml) and spread the slurry at a thickness of 0·25 mm over twenty 5 × 20-cm or five

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TABLE 1. CHROMATOGRAPHY IN VARIOUS SOLVENT SYSTEMS

| Steroid | Number of hydroxyl groups | Number of carbonyl groups | Running distances (hydrocortisone acetate = 1·00) Solvent system A | Solvent system B | Solvent system C | Running distances (hydrocortisone = 1·00) Solvent system D | Running distances (hydrocortisone acetate = 1·00) Solvent system E |
|---|---|---|---|---|---|---|---|
| Deoxycortone acetate | 0 | 2 | 2·44 | 2·51 | 1·42 | 3·0 | 2·0 |
| Reichstein's Compound S 21-acetate | 1 | 2 | 1·62 | 1·90 | 1·36 | 3·0 | 2·0 |
| Deoxycortone | 1 | 2 | 1·76 | 1·96 | 0·98 | 3·0 | 2·0 |
| Cortisone acetate | 1 | 3 | 1·25 | 1·45 | 1·02 | 2·9 | 1·72 |
| Prednisone acetate | 1 | 3 | 1·16 | 1·24 | 0·89 | 2·8 | 1·54 |
| Dexamethasone acetate | 2 | 2 | 1·11 | 1·14 | 1·18 | 2·7 | 1·05 |
| Fludrocortisone acetate | 2 | 2 | 1·03 | 1·14 | 1·16 | 2·6 | 0·62 |
| 6-α-Methylhydrocortisone 21-acetate | 2 | 2 | 1·00 | 1·10 | 1·08 | 2·8 | 1·42 |
| Hydrocortisone acetate | 2 | 2 | 1·00 | 1·00 | 1·00 | 2·7 | 1·00 |
| Methylprednisolone acetate | 2 | 2 | 0·92 | 0·90 | 1·00 | 2·7 | 1·19 |
| Reichstein's Compound S | 2 | 2 | 0·84 | 1·02 | 0·86 | 2·7 | 1·27 |
| Presnisolone acetate | 2 | 2 | 0·78 | 0·82 | 0·92 | 2·5 | 0·72 |
| Triamcinolone acetonide | 2 | 2 | 0·59 | 0·73 | 0·74 | 2·6 | 0·99 |
| Cortisone | 2 | 3 | 0·51 | 0·63 | 0·53 | 1·92 | 0·24 |
| Prednisone | 2 | 3 | 0·43 | 0·51 | 0·44 | 1·70 | 0·16 |
| 6-α-Methylhydrocortisone | 3 | 2 | 0·27 | 0·31 | 0·50 | 1·55 | 0·13 |
| Dexamethasone | 3 | 2 | 0·27 | 0·35 | 0·68 | 0·91 | 0·04 |
| Hydrocortisone | 3 | 2 | 0·24 | 0·29 | 0·45 | 1·00 | 0·06 |
| Fludrocortisone | 3 | 2 | 0·24 | 0·33 | 0·63 | 0·70 | 0·01 |
| Betamethasone | 3 | 2 | 0·22 | 0·35 | 0·69 | 0·91 | 0·04 |
| Methylprednisolone | 3 | 2 | 0·22 | 0·24 | 0·45 | 1·24 | 0·09 |
| Presnisolone | 3 | 2 | 0·19 | 0·22 | 0·39 | 0·72 | 0·04 |
| 16-α-Hydroxyfludrocortisone | 4 | 2 | 0·14 | 0·18 | 0·31 | 0·19 | 0·01 |
| Triamcinolone | 4 | 2 | 0·08 | 0·14 | 0·27 | 0·04 | 0 |

On chromatograms run 15 cm, the approximate distances in cm travelled by hydrocortsone acetate in solvent systems A, B, C and E are 3·7, 5·1, 6·2 and 6·8 and by hydrocortisone in solvent system D is 4·7.
Solvent systems: A—1,2-Dichloroethane : methanol : water—95 : 5 : 0·2.
B—1,2-Dichloroethane : 2-methoxyethyl acetate : water—80 : 20 : 1.
C—Cyclohexane : ethyl acetate : water—25 : 75 : 1.
D—Stationary phase: Chromatoplate run in 20% v/v solution of formamide in acetone. Mobile phase: Chloroform : ether : water—80 : 20 : 0·5.
E—Stationary phase: Chromatoplate run in 25% v/v solution of formamide in acetone. Mobile phase: Cyclohexane : tetrachloroethane : water—50 : 50 : 0·1.

20 × 20-cm glass plates. Activate the resulting thin layers at 105–110° for 1 hr after drying at room temperature for 30 min. Remove a thin border of silica gel from the sides of the chromatoplate to reduce "edge-effects." Store the chromatoplates, unless used immediately, in desiccators over anhydrous silica gel, for a maximum of four days.

Introduce the solvent mixture into the tank, line the sides of the tanks with Whatman No. 1 filter-paper, saturated with solvent and allow to equilibrate for 1–2 hr.

Spot a suitable quantity of the steroid, usually 5–10 μg in 2–5 μl of chloroform, 2 cm from the lower edge of the chromatoplate. Allow the running solvent to ascend the chromatogram until 15 cm past the spotting line. Remove the solvent by drying for 10 min in a stream of cold air from a hair drier.

## SOLVENT SYSTEMS

(a) *Adsorption chromatography.* The running distances of the steroids, relative to the movement of hydrocortisone acetate, in three useful solvent systems on Kieselgel GF 254 are listed in Table 1 (systems A, B and C). System A is similar to those used by Bennett & Heftmann (1962). They were selected from 1500 systems examined.

(*a*) *Partition chromatography.* An examination of 400 partition systems was made using formamide on silica gel as the stationary phase. The formamide was applied by running the activated chromatoplate in a solution of formamide in acetone. The steroid solutions were spotted and the chromatograms run in the mobile phase. The running distances of the steroids, relative to that of hydrocortisone or of hydrocortisone acetate, in two partition systems are listed in Table 1 (systems D and E).

### TETRAZOLIUM BLUE REAGENT

A spray reagent of 0·05% tetrazolium blue and 8% sodium hydroxide in methanol reacts with the steroids on the chromatogram to give blue spots on a white background. The reagent is in two parts. Part A, 0·1% w/v tetrazolium blue in methanol. Part B, 16% w/v sodium hydroxide in methanol. Equal parts of A and B are mixed immediately before use. Over-spraying with the reagent must be avoided, otherwise the chromatogram will disintegrate. 1–1·5 ml is suitable for a 5 × 20 cm chromatogram.

The tetrazolium blue reagents suggested by Matis, Adamec & Galvánek (1962) and Nishikaze & Staudinger (1962) and the 2,5-diphenyl-3-(4-styryl-phenyl)tetrazolium reagent suggested by Stevens (1964) were examined but they were found to be appreciably less sensitive than the proposed reagent. Other tetrazolium salts were examined but were also less sensitive.

Cortisone, hydrocortisone, prednisolone, prednisone, prednisolone acetate, hydrocortisone acetate, cortisone acetate, prednisone acetate, dexamethasone and betamethasone all gave approximately the same response to the tetrazolium blue reagent. The approximate limit of detection of each was about 0·03 $\mu$g when each was run 3 cm in the same solvent system.

### FACTORS AFFECTING THE RESPONSE TO THE TETRAZOLIUM BLUE REAGENT

(*a*) *Removal of excess solvent from the chromatogram.* The use of heat to remove excess solvent from the chromatogram results in a loss of sensitivity when the steroids are subsequently sprayed with the reagent. The limit of detection of prednisolone after chromatography with 1,2-dichloroethane: acetone (2:1), was 0·02 $\mu$g when the chromatogram was dried under a stream of cold air from a hair-drier for 10 min but 0·03 $\mu$g when dried at 100° for 10 min.

(*b*) *Application of steroid solution to the chromatoplate.* The area occupied by the steroid when applied to the chromatoplate initially, determines the area it occupies after chromatography and hence its apparent response to the reagent. This area is dependent upon the solvent used to dissolve the steroid, the way in which the solution is applied and the concentration of the solution.

5 $\mu$g of prednisolone, dissolved in chloroform, chloroform:methanol (97·5 + 2·5) and dioxane and spotted in one 5 $\mu$l application gave spots at the point of application of 2·9, 3·5 and 9·0 mm diameter, respectively.

5 $\mu$g of prednisolone dissolved in chloroform, when applied in two 2·5 $\mu$l amounts and in one 5 $\mu$l amount, gave spots of 2·6 and 2·9 mm diameter, respectively. 5-$\mu$l applications of 2·5 and 10 $\mu$g amounts of prednisolone dissolved in chloroform gave spots of 2·5 and 3·0 mm diameter, respectively.

(*c*) *Running distance.* The distance moved by a steroid on the chromatogram affects its response to the reagent. The approximate limit of detection of hydrocortisone acetate, run 1, 2, 3 and 5 cm, was 0·01, 0·02, 0·03 and 0·04 $\mu$g, respectively.

(*d*) *Amount of steroid and concentration of impurity.* Various amounts of cortisone acetate, prednisone acetate and prednisolone acetate were added to solutions of "pure" hydrocortisone acetate and 5-$\mu$l applications were chromatographed in the solvent system A. The response of the added impurities is indicated in Table 2.

TABLE 2. DETECTION OF IMPURITIES AT DIFFERENT CONCENTRATIONS

| Amount of hydrocortisone acetate applied in one 5-$\mu$l application $\mu$g | Added steroid | Response of added steroids at concentrations of | | | | | Running distance of the added steroid cm |
|---|---|---|---|---|---|---|---|
| | | 0·5% | 1·0% | 2·0% | 3·0% | 4·0% | |
| 5 | Cortisone acetate | − | ? | + | ++ | +++ | 4·8 |
| 5 | Prednisone acetate | − | ? | + | ++ | +++ | 4·2 |
| 5 | Prednisolone acetate | ? | + | + | ++ | +++ | 2·7 |
| 10 | Cortisone acetate | ? | + | ++ | +++ | +++ | 5·1 |
| 10 | Prednisone acetate | ? | + | ++ | +++ | +++ | 4·5 |
| 10 | Prednisolone acetate | + | ++ | +++ | +++ | +++ | 2·8 |
| 15 | Cortisone acetate | + | ++ | +++ | +++ | +++ | 5·1 |
| 15 | Prednisone acetate | + | ++ | +++ | +++ | +++ | 4·5 |
| 15 | Prednisolone acetate | ++ | ++ | +++ | +++ | +++ | 2·7 |

Symbols: − Not detectable. ? Very faint spot. + Faint spot. ++ Easily detectable. +++ Very easily detectable.

## Discussion

Using adsorption chromatography, solvent systems such as ethylene dichloride (or chloroform)/methanol (or dioxane or 2-methoxyethyl acetate) separate the steroids according to the number of hydroxyl and carbonyl groups each possesses (Table 1, solvent systems A and B). Steroids that differ only in their less polar groups are less easily separated by this type of solvent system. Prednisolone and hydrocortisone are just separated from each other, but fludrocortisone and dexamethasone are not separated from hydrocortisone nor is methylprednisolone separated from prednisolone.

The solvent system of cyclohexane: ethyl acetate, though giving a less effective separation of the steroids according to their polarity, does separate fludrocortisone and dexamethasone from hydrocortisone.

Up to 2% water added to the solvent system often results in a considerable reduction of the tailing to which the more polar steroids are prone. An optimum separation of steroids within a certain range of polarity

is obtained by adjusting the proportions of the solvents. Thus 1,2-dichloroethane : methanol : water, 95 : 5 : 0·2 is suitable for separating the acetates listed in Table 1, while the alcohols are better separated when the solvents are in the proportions 92 : 8 : 0·5.

Compared with adsorption chromatography, the disadvantages of partition chromatography are that the spot sizes tend to be larger, especially those of the less polar steroids, and that it is a more time-consuming procedure. The advantages of partition chromatography are that the spread of the steroids over the chromatogram is improved and that some separations are achieved that are not possible by adsorption chromatography. Thus a better separation is obtained between hydrocortisone and prednisolone and a good separation is obtained between prednisolone and methylprednisolone.

The apparent response of a steroid on a chromatogram to the reagent depends on the nature and volume of the solvent in which the steroid is applied to the chromatoplate, the amount of steroid applied and the distance moved by the steroid. In developing a test it is necessary to standardise these conditions or to compensate for any that cannot be readily controlled.

A test in which a sample is deemed satisfactory when the impurities are not visible suffers from two disadvantages: (1) It is often difficult to decide whether or not an impurity is just visible. (2) Any variations in test conditions that affect the distance the impurities travel on the chromatogram will affect their limits of detection.

These disadvantages can be overcome by applying standard impurities to each chromatoplate and comparing the intensities of the sample and standard impurity spots. The standard impurities should be easily visible, but not too intensely coloured or comparison becomes difficult.

Assuming a limit of not more than 3% of any tetrazolium-reactive impurity, hydrocortisone for example, could be examined using the following adsorption systems and standards:

(i) Apply 5 $\mu$l of a 0·10% chloroform solution of the sample and 5 $\mu$l of a chloroform solution containing 0·10% of pure hydrocortisone and 0·003% each of prednisolone, prednisone and prednisolone acetate. Run the chromatogram with the solvent system 1,2-dichloroethane : methanol : water, 92 : 8 : 0·5.

(ii) Apply 5 $\mu$l of a 0·10% chloroform solution of the sample and 5 $\mu$l of a chloroform solution containing 0·10% of pure hydrocortisone and 0·003% each of cortisone, hydrocortisone acetate and cortisone acetate. Run the chromatogram with the solvent system 1,2-dichloroethane : methanol : water, 95 : 5 : 0·2.

(iii) Apply 5 $\mu$l of a 0·10% chloroform solution of the sample and 5 $\mu$l of a chloroform solution containing 0·10% of pure hydrocortisone and 0·003% of fludrocortisone. Run the chromatogram with the solvent system cyclohexane : ethyl acetate : water, 25 : 75 : 1.

After the spraying with the tetrazolium blue reagent, for chromatograms (i) and (ii) compare the intensity of any impurity from the sample

lying within the region of the chromatogram between the highest and lowest standard impurity spots with that of the nearest standard impurity spot; for chromatogram (iii) compare with the standard impurity spots the intensity of any impurity from the sample at a similar running distance.

## References

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