J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 17*T*–23*T* Received April 28, 1964

The detection and identification of other 17,21-dihydroxy-20- oxosteroids in corticosteroids

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A method is described for the separation of corticosteroids by paper chromatography. It is more sensitive than the present official test and will control the presence of related foreign steroids to a uniform degree and in a reproducible manner. Details of the application of this test to many pharmaceutically important steroids are given and the relationship of molecular structure to mobility in the solvent systems used is discussed.

THE paper chromatography of steroids has been extensively reviewed by Bush (1961). Much of this work has been concerned with the isolation, identification and estimation of steroids as an aid to clinical studies. Our interest, on the other hand, was to define a test to limit the proportion of related foreign steroids in corticosteroids of pharmaceutical significance. With many such substances, now available, each capable of manufacture by a variety of syntheses, the problem of devising a test of general applicability is complex. To be effective as an official criterion of purity, such a test should control all possible related compounds to approximately the same extent, and it should be uniformly applicable in all laboratories. Tests designed to fulfil this need, and based on the solvent systems used by Zaffaroni, Burton & Keutmann (1950) and Burton, Zaffaroni & Keutmann (1951a, b) have been described (British Pharmacopoeia 1963; United States Pharmacopeia XVI). These systems have proved particularly effective because they have a sufficiently high capacity to allow an adequate sample to be chromatographed so that minor constituents may be recognised and estimated; no doubt, however, other systems might also be applicable. Considerable work in conjunction with the establishment of a collection of authentic specimens of various corticosteroids has shown that the published tests are not uniformly sensitive for all 17,21-dihydroxy-20-oxosteroids since diffusion of a substance as it moves down the paper necessarily results in a decrease of sensitivity. The extent to which this diffusion can affect the conclusions to be drawn from the test has been determined and a standardised procedure has been developed that will enable impurities to be equally detected, irrespective of their Rf values.

Experimental and results

The sensitivity of various means of detection, in relation to distance moved, was studied as follows: Papers (Whatman No. 1) were dipped in a 40% v/v solution of formamide in methanol, and blotted. Chromatograms were developed as described in the British Pharmacopoeia 1963 page 1075 for Related Foreign Steroids, various loadings of cortisone acetate were used and the chromatograms were run (with mobile phase B)

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for different lengths of time. After removal from the tank, the papers were dried in a current of air for 5 min then heated at 105° for 40 min.

The minimum quantities of cortisone acetate detectable under different conditions are given in Table 1.

TABLE 1. DETECTABILITY OF CORTISONE ACETATE WITH VARIOUS REAGENTS IN RELATION TO DISTANCE MOVED

Distance of spot from starting line (cm)	Method of detection used				
	254 mμ U.V. light	TPTZ	TPTZ fluorescence after heat*	BT	DPST
	Amount visible (μg)				
4	0·25	4 (?)	0·5	0·25	0·25
6·5	0·5	8	1	0·25	0·25
8	0·5	8	0·5	0·5	0·25
13	1	8	0·5	0·5	0·5
15	2	8	1	1	0·5
22	2	12	2	1	0·5
30	2	12	1	2	1

TPTZ. Alkaline triphenyltetrazolium chloride solution. B.P. 1963, page 1076.

BT. Blue tetrazolium solution. A 0·1% w/v solution of blue tetrazolium in 2N sodium hydroxide.

DPST. A 0·1% solution of diphenylstyrylphenyltetrazolium chloride prepared as described in the recommended procedure.

* No fluorescence is obtained with $\Delta^{1,4}$ corticosteroids.

A second series of experiments explored the sensitivity of diphenylstyrylphenyltetrazolium chloride (Brooks & others, 1958); this is the preferred means of detection because at the concentration specified, it has good sensitivity and gives a violet spot on a pale yellow background.

Solutions were prepared containing equal amounts of six steroids having different Rf values. These solutions were then chromatographed by the official method (using mobile phase B), but using diphenylstyrylphenyltetrazolium chloride (DPST) as the detecting reagent. Loadings equivalent to 0·125, 0·25, 0·5, 1, 2 and 4 μg of each steroid were used: the distance moved and the minimum quantity detected were then recorded for each steroid (Table 2).

TABLE 2. DETECTABILITY OF CORTICOSTEROIDS* WITH DIPHENYLSTYRYLPHENYLTETRAZOLIUM CHLORIDE IN RELATION TO DISTANCE MOVED

Solution 1	Distance moved (cm)	Minimum quantity detected (μg)
Dexamethasone	2·3	0·125
Methylprednisolone	3·6	0·125
Prednisone	5·9	0·25
Fludrocortisone acetate	17	0·5
Dexamethasone acetate	22·5	0·5
Cortisone acetate	30·2	1·0
Solution 2		
Prednisolone	2·1	0·125
Hydrocortisone	3·3	0·125
Prednisolone acetate	18·5	0·5
Hydrocortisone acetate	22·1	0·5
Prednisone acetate	28·0	0·5
Prednisolone trimethylacetate	33·4	1·0

* When each of these steroids is spotted on prepared Whatman No. 1 paper, the minimum quantity detectable with DPST reagent is the same for all, viz. 0·08 μg.

The results in Tables 1 and 2 show that a 4- to 8-fold difference in detectability is to be expected according to the Rf value of the steroid, and that triphenyltetrazolium chloride is insufficiently sensitive for use in a limit test. The precise loss in sensitivity depends on the degree to which the spots diffuse during chromatography, but we believe that the results quoted are typical for the standardised procedure detailed below.

The tables also indicate the minimum amount of a corticosteroid that can be detected at any given distance from the starting line.

The test described below has been designed to limit related foreign steroids to about 3%, a figure at present judged to be an acceptable practical limit. By increasing the loading or decreasing the distance that the solvent front moves, however, it is possible to make the test more stringent. In the standardised procedure, strict adherence to the details is necessary if inter-laboratory agreement is to be achieved. For example, there are many valid ways in which paper may be impregnated with formamide, each giving rise to different amounts of stationary phase. Such differences can lead to considerable variation in the time required for the mobile phase to move a given distance: in our experience this affects the degree of diffusion of the spot.

Apparatus. A rectangular glass tank sufficiently tall to enable a descending chromatogram about 45 cm in length to be prepared.

Reagents. Mobile phase A. A saturated solution of formamide in chloroform.

Mobile phase B. A saturated solution of formamide in a mixture of equal volumes of benzene and chloroform.

Formamide. Reagent grade material (vacuum distillation is unnecessary).

Diphenylstyrylphenyltetrazolium chloride solution (DPST solution). Add one volume of a 0·1% w/v solution of 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium hydrochloride in ethanol (95%) to nine volumes of 2N sodium hydroxide just before use. This reagent is 66 times weaker than the reagent of Brooks & others (1958).

Other reagents were of analytical reagent quality.

METHOD

Saturate the tank by placing some of the specified mobile phase in the bottom *and also in the solvent trough,** close the lid and allow to stand (24 hr) at 26° $\pm$ 1°. Impregnate suitable sheets of Whatman filter paper No. 1 by dipping in a 40% v/v solution of formamide in methanol and blotting twice between sheets of clean filter paper. 100 ml of the formamide solution, which should be prepared immediately before use, is sufficient to treat four papers 20 cm $\times$ 52 cm. With the minimum of delay, apply separately to the paper in 5 μl of acetone, the quantities specified in Table 3 of (*a*) the substance being examined, (*b*) an authentic

* With a tall tank this ensures that both the top and the bottom of the tank is saturated.

specimen of the substance and (*c*) cortisone acetate, limiting the spot diameters to a maximum of 4 mm. Transfer the prepared papers to the tank as soon as possible, having first emptied and dried the solvent trough. Allow the closed tank to equilibrate for an hour and a half,

TABLE 3. LOADINGS TO BE APPLIED IN THE RECOMMENDED METHOD

Mobile phase	Distance of solvent front from starting line (cm)	Authentic specimen loading (μg)	Substance under examination loading (μg)	Cortisone acetate loading (μg)
A	35±2	25	25 50	1·0
B	22±2	25	25 —	0·5

introduce the specified mobile phase into the solvent trough and let the solvent front travel the distance from the starting line specified in Table 3. Remove the paper from the tank, allow to dry at room temperature for 5 min, heat at 105° for 40 min and develop by rapid passage through a shallow layer of DPST solution. Blot between sheets of filter paper and lay on a clean sheet of filter paper for examination. Secondary spots develop more slowly than the primary spot and also fade more quickly. The chromatograms should be examined in a subdued light for 2 min immediately after development. *The cortisone acetate spot must always be visible; if not the test should be rejected.*

The substance being examined and the authentic specimen each yield, equidistant from the starting line, a violet spot. If no secondary spots are visible when using mobile phase B, the substance passes the test. With mobile phase A, if no secondary spots are visible above 22 cm from the starting line on the 25 μg loading, or below 22 cm from the starting line on the 50 μg loading, the substance passes the test.

Where the substance does not pass the test, the following procedure is adopted. Measure the distance that any secondary spot has travelled from the starting line and repeat the procedure described above, using the loadings of the substance being examined as specified in Table 4 and the same loading of cortisone acetate as in the previous test. The larger of the two loadings may show a secondary spot but the smaller loading should not. As before, the cortisone acetate spot must be visible.

TABLE 4. LOADINGS TO BE APPLIED IN THE RECOMMENDED METHOD WHEN A SECOND CHROMATOGRAM IS NECESSARY

Distance of secondary spot from starting line (cm)	Larger loading (μg)	Smaller loading (μg)
4	4	2
8	8	5
12	12	7
16	16	10
20	20	12
24	24	14
28	30	18
32	38	23
36	50	30

With mobile phase B, certain impurities may move such a short distance that it is not possible to suggest suitable loadings. Where any impurity has moved less than 4 cm, the test should be repeated using mobile phase A.

The recommended mobile phase for individual corticosteroids, together with Rf values, are listed in Table 5. With each substance, materials from as many manufacturing sources as possible were examined, together with W.H.O. Authentic Chemical Substances and U.S.P. Reference Preparations, where available.

TABLE 5. RECOMMENDED MOBILE PHASES AND RF VALUES FOR THE MORE FREQUENTLY ENCOUNTERED CORTICOSTEROIDS

Mobile phase A		Mobile phase B	
	Rf		Rf
Betamethasone	0·16	Betamethasone acetate	0·44
Cortisone	0·62	Betamethasone 17-valerate	0·86
Dexamethasone	0·21	Betamethasone 21-valerate	0·89
Hydrocortisone	0·26	*Cortisone acetate	0·80
6-Methylhydrocortisone	0·36	Dexamethasone acetate	0·60
Methylprednisolone	0·27	Fludrocortisone acetate	0·45
Prednisolone	0·15	Fluocinolone acetonide	0·40
Prednisone	0·55	Hydrocortisone acetate	0·59
		Prednisolone acetate	0·49
		Prednisolone trimethylacetate	0·89
		Prednisone acetate	0·74
		Triamcinolone acetonide	0·52

* As a test of reproducibility, seven samples of cortisone acetate from different sources have been examined repeatedly over a period of several months. Values recorded for the Rf ranged between 0·76 and 0·80.

Hydrocortisone sodium and hydrogen succinates, betamethasone sodium phosphate and the hydrogen and sodium phosphates of prednisolone remain on the starting line with both mobile phases; with these substances the test is only of value in detecting unesterified material. Triamcinolone remains on the starting line with both mobile phases and in this case the test may be used to detect impurities having no hydroxyl group at position 16. Deoxycortone acetate and deoxycortone trimethylacetate move with the solvent front in both mobile phases and the test can be applied to detect impurities substituted with a hydroxyl or oxo group at position 11.

The proposed test depends on the assumption that all the corticosteroids under consideration (and impurities having the 17,21-dihydroxy-20-oxo-side-chain) respond with equal sensitivity to the reagent (see footnote to Table 2). This assumption is also supported by the similar responses that many corticosteroids show to 2,3,5-triphenyltetrazolium chloride in the assays of the British Pharmacopoeia. With substances esterified with a large group at position 21 (hydrocortisone sodium succinate and prednisolone trimethylacetate for example) the response to DPST is delayed a little probably owing to the time required for hydrolysis of the ester. The inclusion of a small loading of cortisone acetate with each test ensures that the overall sensitivity of that test is satisfactory and thus that any conclusions drawn are valid.

Discussion

The test is designed to limit the presence of any one foreign steroid in a particular corticosteroid to not more than about 3%. However if two or more impurities are each present in amounts just below this limit, the sample would often pass the test despite the aggregate of impurities exceeding the desired limit. The presence of more than one impurity in

significant quantity however, is usually detected in a preliminary chromatogram. It is then often possible to gain further information by chromatographing with suitably larger loadings or for shorter distances.

Information about the nature of unknown impurities can be gained by comparing the Rf values with those of corticosteroids of known structure. Such correlations have been extensively discussed by Bush (1961). In the test described above, using mobile phase B the following relationships between molecular structure and mobility have been noted. Acetylation of the 21-hydroxyl group brings about an increase in Rf value of approximately 0·5 whilst esterification with trimethylacetic acid (pivalic acid) increases the value still more. In a 21-acetate, replacement of the 11-hydroxyl group by an 11-oxo group causes an increase of between 0·2 and 0·25; similar replacement in a free 21-alcohol produces an increase of about 0·1. In the absence of hydroxyl or oxo substituents at position 11 and of hydroxyl substitution at position 17 (as in deoxycortone acetate and deoxycortone trimethylacetate), there is a marked increase in mobility. A double bond in the 1,2 position in addition to that in the 3,4 position causes a decrease of between 0·05 and 0·1, both in the free 21-alcohols and the corresponding esters. The presence of a non-polar substituent such as a methyl group (usually in position 6 or 16) enhances mobility so that 6-methylhydrocortisone and 6-methylprednisolone have Rf values approximately 0·05 greater than the unsubstituted compounds. Polar groups, on the other hand, reduce mobility and 9-fluorohydrocortisone acetate has an Rf value about 0·14 less than that of hydrocortisone acetate. In dexamethasone acetate, which has the 9-fluoro- substituent and also a methyl group at position 16, the opposing effects of polar and non-polar groups eliminate their respective influences so that this substance is comparable to hydrocortisone acetate in mobility. Very strongly polar substitution prevents movement in mobile phase B and substances such as hydrocortisone hydrogen (or sodium) succinate, prednisolone acid phosphate and triamcinolone (which has fluoro-substitution at position 9 and hydroxyl substitution at position 16) remain at the point of application. For triamcinolone acetonide, where a 16-hydroxyl group participates in acetonide formation, considerable mobility is restored (Rf, 0·52) whilst in fluocinolone acetonide, which differs only in having a second fluoro-group at position 6, the Rf value is reduced by 0·12. This reduction is similar to that caused by the introduction of a single fluoro- group into hydrocortisone acetate.

With the slower moving corticosteroids these differences may be accentuated by running in mobile phase A, but similar relationships exist. Dexamethasone and betamethasone, which differ only in the orientation of the methyl group at position 16, can be differentiated in this system when allowed to run side by side on the same paper. The Rf value for dexamethasone (16α-methyl) is 0·21 and that for betamethasone (16β-methyl), 0·16. From these observations the substituents present in unexpected contaminants can often be deduced and related to the synthetic process used.

Acknowledgements. We thank members of the Hormones Committee of the British Pharmacopoeia for helpful comments.

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The paper was presented by MR. JOHNSON