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The detection and identification of synthetic steroids in horse urine

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A scheme for the detection of synthetic steroids which could be used in the "doping" of race-horses is described. The method involves extraction from urine into ethyl acetate : ether (1 : 1) followed by initial two-dimensional thin-layer chromatography using (1) ethyl acetate (2) methylene chloride : dioxan : water (100 : 50 : 50). Elution of the ultraviolet absorbing spots for extinction measurements is followed by further one way thin-layer chromatography as free alcohols in amyl acetate : acetone (1 : 1). Further chromatography of the steroid alcohols in chloroform : ether : water (80 : 20 : 0·5) on formamide-impregnated plates and chromatography of the acetates in ether helps to identify the unknown steroid. Additional identification is made by colour reactions with (1) a tetrazolium reagent (2) vanillin : perchloric acid.

ANTI-INFLAMMATORY steroids are now widely used both in veterinary and human medicine. Their possible use to influence the performance of racehorses caused us to investigate methods for their detection. We report a method for the detection of the steroids most likely to be used in this way. The method enables a separation to be made between any of these synthetic steroids and the naturally occurring steroids in horse urine. For the purpose of identifying the presence of "dope", we developed a method which would detect unchanged drug because (i) this was easier than looking for conjugated forms of drug which would need to be hydrolysed; (ii) it made the diagnosis of "doping" more certain in cases where metabolites might be the same as those from endogenous steroids, e.g. metabolites from prednisolone and hydrocortisone; (iii) detailed metabolism studies were not necessary (although work on this aspect has already commenced). For speed, we considered a method based on thin-layer chromatography would be preferable to any based on paper chromatography. From our attempts to obtain samples, we found that the only accessible anti-inflammatory steroids were: betamethasone, dexamethasone, fludrocortisone, 6α-methylhydrocortisone, 6α-methylprednisolone, prednisolone, prednisone and triamcinolone. These were used as the alcohols since their 21-esters would be hydrolysed to the parent alcohols in the body (Voigt, 1959; Melby & St. Cyr, 1961). These steroids, and hydrocortisone and cortisone, and their separation, form the subject of this paper.

Experimental

MATERIALS

Triamcinolone, dexamethasone and betamethasone were gifts from Dr. R. W. H. Edwards. Further samples of these steroids together with fludrocortisone, 6α-methylhydrocortisone and 6α-methylprednisolone and the corresponding acetates were gifts from Mr. J. S. Wragg (Boots Pure Drug Co. Ltd.). Prednisolone, cortisone, hydrocortisone, and the

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corresponding acetates, and prednisone were obtained from Koch-Light Laboratories. Other acetates were prepared by the method of Edwards (1960).

METHODS

A Unicam SP.800 recording spectrophotometer was used for ultraviolet and visible absorption measurements.

The ethyl acetate was redistilled before use; other solvents and chemicals were of analytical grade where this was available (with the exception of the ether for chromatography which was solvent ether B.P.)

Thin-layer chromatography was on silica gel containing a fluorescent additive (Kieselgel GF.254-Merck) and of layer thickness 0·25 mm. The plates were prepared by slurrying 50 g silica gel, particle size 5-25 μ, with 100 ml distilled water, spread, dried in air at room temperature for 15 min, and dried and activated at 110° for 1 hr in an oven with internal fan.

Formamide-impregnated plates were prepared by ascending development of the plates with 20% formamide in acetone, followed by drying for 30 min in air at room temperature before use. A Hanovia 'Chromatolite' was used for ultraviolet (254 mμ) examination of the plate after chromatography. Reducing steroids were detected by spraying with a solution of 0·05% w/v 2,5-diphenyl-3-(4-styrylphenyl) tetrazolium chloride and 8% w/v sodium hydroxide in methanol. This was made immediately before use by mixing equal volumes of 0·1% tetrazolium solution and 16% sodium hydroxide solution, the strength used by Clifford, Wilkinson & Wragg (1964) to prepare their spray reagent containing tetrazolium blue.

Steroids generally were detected by spraying with vanillin-perchloric acid reagent. This reagent is made immediately before use by mixing equal volumes of 10% w/v vanillin in glacial acetic acid and 72% perchloric acid. Colours due to the steroids appear after heating at 90° for 10 min. This is essentially an application of the method of Few (1965).

URINE FRACTIONATION

All solvent extracts were taken to dryness under reduced pressure (water-pump) in a rotary film evaporator keeping the temperature below 40°. When these residues were submitted to chromatography, they were transferred to the plate (completely) using sodium sulphate-dried ether as solvent.

Urine (100 ml) was extracted with ethyl acetate:ether (1:1, 100 ml) by mechanical rolling in a bottle at one 1 rev./sec for 15 min. The solvent was *completely* separated, washed without delay with 1/10th volume ice-cold 0·1 N sodium hydroxide and then with 1/10th volume 0·1 N hydrochloric acid. The solvent layer was filtered and after thorough drying (sodium sulphate), evaporated to dryness and submitted to two dimensional thin-layer chromatography. The plates were first run in ethyl acetate and then in the system of Hall (1964) prepared by mixing methylene chloride:dioxan:water (100:50:50) and using the lower layer. Spots were located by ultraviolet examination.

Substances in the areas of absorption corresponding to any abnormally present steroid were eluted with 1 × 2 ml ethanol by shaking mechanically for 10 min. The ultraviolet absorption curve was then determined on the ethanolic solution after centrifuging. The Δ^4-3 ketones give a maximum at approximately 240 mμ (see Table 1). The remaining area of the plate was sprayed with the tetrazolium reagent followed by the vanillin: perchloric acid reagent. This detects any other non-ultraviolet absorbing steroids which may be present and also indicates whether they are reducing. This technique is useful in revealing the presence of certain metabolites of these drugs. After ultraviolet examination the ethanolic extracts were taken to dryness at room temperature, and chromatographed on silica gel using amyl acetate: acetone (1 : 1) as solvent. The steroids were located by ultraviolet light and their presence confirmed by spraying with the tetrazolium reagent, and then overspraying with the vanillin reagent (see Table 1).

Further confirmation of the presence of any of these steroids was obtained by submitting a urine extract (from 500 ml urine) to the two dimensional chromatography described and eluting the spots as before with ethanol. Approximately half of each eluate was taken to dryness, dissolved in 1 ml pyridine and 1 drop acetic anhydride added (Edwards, 1960). After leaving overnight at room temperature, the solution was taken to dryness under vacuum (water-pump), dissolved in ether and the acetates submitted to thin-layer chromatography using ether as solvent. The spots were located by ultraviolet light and then sprayed with the tetrazolium reagent to indicate the presence of the reducing side-chain.

The remaining ethanolic eluates were spotted on formamide impregnated plates and run in chloroform : ether : water (80 : 20 : 0·5) (Clifford & others, 1964) and the steroids were located as above.

Results and discussion

Rf values, determined on standard solutions and on steroids in urine extracts, are summarised in Table 1. At least five assessments were made of each Rf value, and these were usually obtained from different plates and in most instances on different days. In no case did the standard deviation of the results exceed 0·06. When identifying an unknown it is, of course, essential to run standards on the same thin-layer plate. For the best results, all solvent systems should be freshly prepared every 2 days and allowed to equilibrate for at least 1 hr before use.

The Rf values obtained from urine extracts do not necessarily correspond with those from pure standards. In the ethyl acetate system, separation of standards of cortisone or hydrocortisone and prednisolone or prednisone is not great, but in urine extracts the separation is marked. Triamcinolone in urine extracts gives a much increased Rf value compared with standard solutions. The Rf values of pure steroids and steroids in urine extracts are given in the Table for the systems used for the original separation, i.e. ethyl acetate and methylene chloride : dioxan : water. In the event of an abnormal steroid being detected, the Rf value for identification is listed in Table 1 under urine extract.

TABLE 1. Rf VALUES OF STEROIDS IN DIFFERENT SOLVENT SYSTEMS (on Silica gel plates)

Steroid	Ethyl acetate		Methylene chloride: dioxan: water		Chloroform: ether: water on formamide plates	Amyl acetate: acetone	λ_{max}*	Colour with vanillin-perchloric acid sprayed over the tetrazolium reagent	Rf of steroid acetate in ether
	Standard solutions	Steroids in urine extracts	Standard soln.	Steroids in urine extracts					
Betamethasone	0·47	0·40	0·48	0·45	0·33	0·69	240	Grey	0·44
Hydrocortisone	0·35	0·46	0·45	0·47	0·38	0·62	241	Purple/Brown	0·42
Cortisone	0·36	0·45	0·62	0·60	0·77	0·66	238	Red/Brown	0·34
Dexamethasone	0·47	0·41	0·45	0·45	0·34	0·69	242	Grey/Purple	0·45
Fludrocortisone	0·49	0·45	0·46	0·46	0·29	0·71	244	Purple/Brown	0·55
6α-Methylhydrocortisone	0·40	0·37	0·48	0·47	0·57	0·64	241	Red/Brown	0·42
6α-Methylprednisolone	0·38	0·35	0·41	0·41	0·45	0·66	243	Red/Brown	0·37
Prednisolone	0·30	0·27	0·38	0·38	0·25	0·61	243	Green/Grey	0·40
Prednisone	0·30	0·32	0·55	0·53	0·60	0·64	238	Grey-Purple	0·29
Triamcinolone	0·23	0·29	0·24	0·36	0·04	0·56	243	Light Brown	0·22

* Determined for ethanol solutions containing 20 μg/ml of steroid

The ethyl acetate conditions are critical and considerable variations in Rf values can occur if either the extract added to the plate is wet, or impurities, including water, are present in the ethyl acetate. Also all traces of ethyl acetate must be removed before the second run is commenced. The position of the original spot and the solvent used to transfer the steroid to the plate can also alter the Rf values. This is particularly true of triamcinolone. The papers by Quesenberry & Ungar (1964), Dallas (1965) and Clifford & others (1964) are of interest with reference to these points.

In the separation of the acetates using ether as solvent, the tank should be prepared 24 hr before use. The grade of ether can be critical and we have found that solvent ether B.P. (Boots Pure Drug Co. Ltd.), 'aged' for 24 hr as above, gave the greatest separations. The values quoted in Table 1 have been determined in this way.

The scheme has been applied to urines containing added steroids and it has been possible to detect and identify any of the steroids concerned at levels of 2 μg/ml in original urine. Although detection of 1 μg/ml is possible on the 2-dimensional chromatogram at this level there is usually insufficient material to allow the ultraviolet detection after chromatography in the amyl acetate:acetone system. The losses involved in manipulations and elutions at this level appear to be relatively large. The volume of urine available for extraction then becomes the limiting factor to the certain diagnosis of "doping".

We have found no materials present normally in horse urine which run within the same area and give an ultraviolet maximum within the same region as the steroids under discussion. There is a frequently ocurring "normal" spot in horse urine which does show up on the initial two-dimensional chromatogram in the area of these synthetic steroids, but usually only faintly, and the ultraviolet extinction of its eluate is virtually zero between 235 and 260 mμ. (Weak maxima are observed at approximately 230 and approximately 285 mμ; the identity of this spot is unknown). The initial two-dimensional chromatography and ultraviolet absorption measurement together serve as a quick and useful screen for the detection of foreign steroids in horse urine.

Where difficulty is experienced in obtaining a satisfactory ultraviolet absorption curve the acetate may be eluted, after chromatography in ether, and used for ultraviolet measurement.

For complete differentiation of dexamethasone from betamethasone, the acetate is submitted to chromatography on alumina as suggested by Hall (1964). The Rf of betamethasone acetate is 0·45 and that of dexamethasone acetate 0·59.

We feel that the scheme gives a quick and sound identification of any of these foreign steroids. The evidence of identification can be summarised as follows:

1. A neutral material which, on thin-layer chromatography, shows as an ultraviolet-absorbing spot on the original two-dimensional chromatogram in a region normally free from ultraviolet absorbing material.
2. Rf values in agreement with those found in four systems: (i) ethyl

acetate; (ii) methylene chloride: dioxan: water; (iii) amyl acetate: acetone and (iv) in a partition system formamide-chloroform: ether: water for the parent steroid.

3. An Rf value for the steroid acetate in ether.

4. An ultraviolet maximum (in ethanol) between 235–245 mμ (see Table 1), the reaction with the tetrazolium reagent and the colour produced with the vanillin-perchloric acid reagent.

Any substance conforming is an 'abnormal' in horse urine belonging to the *neutral* group of drugs. This 'abnormal' would contain in the same molecule a group giving absorption in the ultraviolet (of which a Δ^4-3-one structure is one possibility), a fairly easily esterifiable group (presumably a primary or unhindered secondary alcohol), a reducing structure capable of reducing the tetrazolium reagent in the *cold* (of which an α-ketol structure is the most likely), a grouping reacting with vanillin-perchloric acid (see Few, 1965) and agreeing in Rf value, in 5 systems, with a standard steroid.

In experiments on the metabolism of prednisolone and prednisone which we hope to publish shortly, we found that the administration of either gave a mixture of both in the urine. Therefore as a further confirmation of prednisolone (or prednisone) administration, we expect to identify by our procedures both the 11-hydroxy- and the 11-oxo-compound in the urine.

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