STEROIDS

CCXV. THE QUANTITATIVE ANALYSIS OF STEROIDS BY THIN-LAYER CHROMATOGRAPHY*

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(Received May 8th, 1962)

The development of thin-layer chromatography (TLC) by KIRCHNER *et al.* in 1951[2] and 1952[3,4] was not fully appreciated until its extension by STAHL[5] in Europe beginning in 1953. Since then TLC has spread to every field involving separations and its usefulness is attested to by the literature (for reviews see ref. 6). Often, the resolution by TLC surpasses that of paper partition chromatography and in some cases it is as good as gas chromatography[7,8]. The first application of TLC to the separation of steroids[9] was rapidly followed by many others (see ref. 10) showing that this method is applicable to many different types of steroids.

The excellent resolution and rapid development times with TLC offers many advantages for quantitative applications. Methods of quantification reported in the literature have included quantitative analysis by gas chromatography after separation by TLC[11], densitometry of sprayed plates[12] and comparison techniques[13]. In some of his original work, KIRCHNER[2,14] detected U.V. absorbing steroids by adding an inorganic phosphor[15] to the coating. Under ultra violet irradiation, U.V. absorbing compounds stand out as dark spots against a bright background. The spots were then eluted and quantitative measurements made with a spectrophotometer. GÄNSHIRT AND MORIANZ[16] also used this technique for the separation and quantitative analysis of methyl and propyl *p*-hydroxybenzoates with excellent results.

The quantitative analysis of non-U.V. absorbing steroids has usually presented a more difficult problem. They must first be detected in a non-destructive way so that they may be extracted and determined quantitatively by colorimetric or physical methods. In general, non-U.V. absorbing steroid zones are detected by colorimetric reactions. Of the several general reagents used for detecting steroids, iodine vapor[17] appears to be the mildest. It was found during our investigations that detection of steroids by iodine vapor does not affect them markedly for quantitative analysis by other methods afterwards. The effect seems to be one of adsorption since the iodine spots generally disappear shortly after the plates have been removed from the iodine vapor. The use of a phosphor in the thin layer did not affect their detection with iodine vapor.

These techniques have been adapted to the quantitative analysis of steroids by TLC on a semi micro scale. The procedures, and our results with this method are described.

* This work was supported by National Institutes of Health contract No. SA-43-PH-2448. For Part CCXIV see ref. 1.

EXPERIMENTAL

Preparation of coating material

The phosphor used in this work was GS-115 green emission phosphor of U.S. Radium Corp. With 100 mg of phosphor/30 g of coating material (silica gel G or aluminum oxide G with $CaSO_4$ binder)* the green fluorescence was adequate when viewed under a lamp peaking at 254 mμ. The contrast is seen best in a U.V. viewing box (Chromato-Vue) although a hand lamp will serve. In order to remove solvent extractable interferences, the 30 g of adsorbent with 100 mg of phosphor added was extracted three times with 75 ml of boiling methanol (redistilled) with stirring. Fines were effectively removed by filtering with suction through a coarse porosity fritted Pyrex glass filter funnel. After washing and filtering three times, the powder was dried in an oven before it was applied to the plates in the usual manner.

In extracting steroids from zones, erratic or high results were obtained if the coating material was not extracted before preparation of the plates. Tests with methanol and ethanol extraction showed that the lowest blanks were obtained after extraction with methanol. In all quantitative determinations the extracted steroids were read against a blank.

Steroid extraction from zones

After solvent development, the plate is viewed under U.V. light and the U.V. absorbing zones are marked with an ample margin around the zone. The powder in the zone is extracted from the plate by means of the zone extractor shown in Fig. 1. The

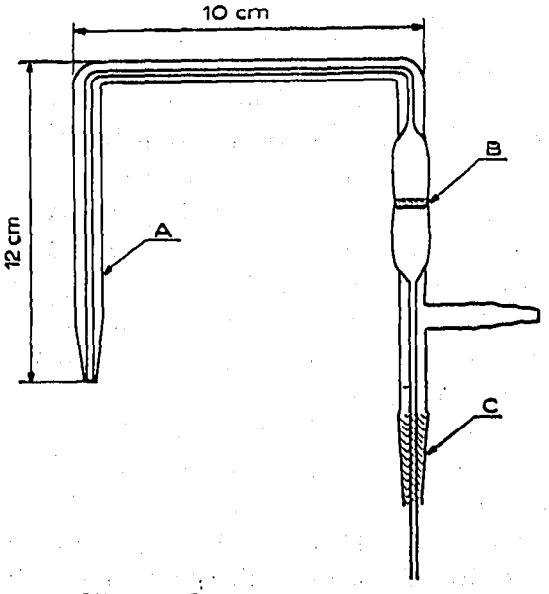

Fig. 1. Zone extractor.

T/S 10/30 joint can fit 5, 10, 25 or 50 ml Kimble or Pyrex volumetric flasks. Throughout this work, 10 ml volumetric flasks were used. With a volumetric flask attached, suction is applied at the hose connection and the steroid zone can be sucked off the

* Obtained from Brinkmann Instruments Inc., 115 Cutter Mill Road, Great Neck, N.Y. (U.S.A.)

plate by rubbing it loose with the inlet tube, A. The powder is carried by the rushing air and stops on top of medium porosity disc, B. After the powder in the zone has been completely removed it is extracted *in situ*. With the vacuum still applied, ethanol is sucked up through A and the steroid is extracted directly into the volumetric flask. The zone extractor can be cleaned by reverse flushing with water and methanol. Occasionally it is necessary to clean the extractor with concentrated sulfuric acid.

After the U.V. absorbing zones have been removed the plate is inserted into a clear jar containing iodine crystals. In a few seconds brown spots appear wherever there are non-U.V. absorbing steroids. The plate is removed immediately and the zones marked quickly. After the spots disappear, the zones are extracted as above. Any trace of adsorbed iodine left is probably removed under the conditions (air and partial vacuum) of zone removal.

Extraction of the steroid from the adsorbent was complete with the first ml of ethanol when quantities of 50 μg of progesterone were used. Using quantities of 1–8 ml of ethanol, recoveries of 94–100% were obtained with an average recovery of 96.4% from silica gel and 97.8% from alumina (Table I). In general practice 8 ml of ethanol was used for extraction.

TABLE I

% RECOVERY OF 50 μg PROGESTERONE FROM ALUMINA AND SILICA GEL

Solvent vol. (ml)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*Average*
Alumina	99.8	98.1	94.4	96	98.1	96	100	100	97.8
Silica gel	96.3	94.1	94.8	96	96	99.8	96	98.1	96.4

Application of steroids

Since the quantities of steroid which are used are small there would be an appreciable error in weighing the amounts applied. Furthermore, the coating on the plate is too fragile to stand repeated applications at the same spot such as is done in paper chromatography in order to quantitatively transfer a weighed sample. In this work the solution was applied to the starting line from a 100 μl Kirk type transfer micro pipet. The use of the 100 μl pipet led to much greater reproducibility than could be obtained with smaller micro pipets or the microliter syringes commonly used in gas chromatography.

The concentration of the steroid solution was such that the steroid was applied to the plate in one application from a 100 μl pipet. In any given experiment the solutions were applied with the same micro pipet to eliminate errors due to differences between pipets. For strongly U.V. absorbing steroids such as Δ^4-3-ketones, 100 μg of steroid or 1 mg/ml concentrations were used. For weak U.V. absorbers such as estrogens, 300 μg of steroid were applied. In order to reduce the errors in weighing and dilution, the appropriate amount of sample was weighed in a 5 or 10 ml volumetric flask and dissolved in distilled chloroform. Other solvents did not prove as suitable as chloroform for sample application. The more polar solvents such as methanol tended to leave the steroid as a ring rather than a spot. It was found convenient to warm the plate on an electric hot plate at about 50° while applying the solution in order to increase the rate of evaporation, thus speeding up sample application and keeping the spot small.

RESULTS

The results of multiple determinations are shown in Table II. In these experiments pure testosterone and 4-chloro-17α-hydroxyprogesterone standards were used. The spots containing approximately 100 μg each of steroid were placed on a silica gel plate without development and extracted with 8 ml of ethanol into 10 ml volumetric flasks which were then made to the mark. The exact amount of steroid applied to the plate with the 100 μl pipet was determined by placing the same volume in a 10 ml volumetric flask (in triplicate), making to volume and determining the absorbance in the ultraviolet. The results listed for testosterone and 4-chloro-17α-hydroxyprogesterone thus represent the percentage recovery from the plate. In these cases the $E^{1\%}_{1\,cm}$ calculated for the standard was taken as 100%. The percentage in any case was calculated as follows:

$$\frac{E^{1\%}_{1\,cm}\ \text{problem}}{E^{1\%}_{1\,cm}\ \text{reference}} \times 100 = \% \qquad (1)$$

TABLE II

STATISTICAL ANALYSIS OF REPETITIVE DETERMINATIONS

Steroid	*Results*	$\bar{X}$	*d*	*s*
Testosterone (std.)	95.6, 94.0, 95.6, 94.0, 95.6	94.96	0.77	± 0.87
4-Chloro-17α-hydroxy progesterone (std.)	96.1, 94.8, 95.1, 94.8, 95.9, 96.1	95.47	0.58	± 0.63

$\bar{X}$ = mean.

d = average deviation = $\frac{\Sigma X_i}{n}$ where X_i is deviation of individual values from the mean $\bar{X}$ and n is the number of determinations.

s = standard deviation = $\sqrt{\frac{\Sigma(X_i)^2}{n-1}}$.

The analysis of different steroids of different degrees of purity are listed in Table III. In each case the determination was carried out in triplicate and compared against standards run under the same conditions. The results obtained by TLC are compared with those obtained by other methods. Many analyses were made by more than one operator and are designated by the letters A, B or C. All these analyses were carried out on silica gel G with phosphor. The results listed for 6α-fluoro-16α-hydroxy-dihydrocortisone-16,17-acetonide-21-acetate are for two different lots as indicated by the brackets.

In addition to the analysis of impure lots of steroids, a synthetic blend of testosterone and isotestosterone was separated and determined quantitatively (Table IV). In this experiment a mixture of 130.8 μg testosterone and 80 μg isotestosterone was run on aluminum oxide G with phosphor for a distance of 15 cm using benzene–ether (1:1). The determination was performed in triplicate against a standard run at the same time.

TABLE III

ANALYSIS OF IMPURE STEROIDS

	Steroid	Solvent system*	% Purity found	
			TLC	Other**
1	11-Desoxycortisone	C:E 3:2	95.5 (A) 95.6 (B)	97.4 (p)
2	19-Nor-progesterone	B:E 1:1	93	95.5 (p)
3	4-Chloro-17α-hydroxyprogesterone	C:E 3:1	95	96 (p)
4	3-Methyl ether of 17α-ethynylestradiol	B:E 9:1	97.5 (B) 98.6 (C)	97 (g)
5	6α-Fluoro-16α-hydroxydihydrocortisone-16,17-acetonide-21-acetate	C:E 3:2	92.9 (A) 92.2 (B) 95.2 (C)	95.8 (p)
			82.8 (A) 82.0 (C)	83.5 (p) 81.5 (cp)

* C = chloroform; E = ethyl acetate; B = benzene.

** p = paper partition chromatography; cp = column partition; g = gas–liquid chromatography.

TABLE IV

SEPARATION AND ANALYSIS OF TESTOSTERONE AND ISOTESTOSTERONE

Steroid	R_F	% Found	Average	% (Theory)
Testosterone	0.37	62.3, 63.0, 63.2	62.8	62.1
Isotestosterone	0.47	39.1, 39.1, 38.8	39.0	37.9

TABLE V

DETECTION OF PURE STEROIDS WITH IODINE VAPORS

Steroid (standards)	$E^{1\%}_{1cm}$				% Recovery after iodine	Thin layer*
	U.V.		Iodine			
	Duplicate	Average	Duplicate	Average		
Testosterone acetate	507 509	508	482 491	487	96	S
	493 493	493	477 483	480	97	A
3-Methyl ether of 17α-ethynylestradiol	63.2 64.4	63.8	62.2 60.8	61.5	96	S
	63.7 64.3	64.0	60.8 60.8	60.8	95	A
Cortisone	403 413	408	421 431	426	104	S
	400 395	398	390 385	388	98	A
Corticosterone	409 409	409	410 430	420	103	S
	416 426	421	416 408	412	98	A
6-Dehydro-testosterone acetate	759 771	765	750 769	760	99.3	S
	768 750	759	750 750	750	98.8	A

* S = Silica gel G with phosphor. A = Aluminum oxide G (neutral) with phosphor.

As mentioned in the introduction, the analysis of non-U.V. absorbing steroids was carried out by first detecting the zone with iodine vapors. In order to establish that this method of detection did not interfere with the subsequent colorimetric reaction for quantification, the effect was first studied on U.V. absorbing steroids containing in aggregate the most common steroid groups or groupings.

The experiments were performed in duplicate both on silica gel G and aluminum oxide G (neutral). The procedure followed was to place four equal spots of the steroid on a plate containing phosphor. Two of the spots were detected and marked under U.V. light and determined as described above. After these were removed, the plate was placed in a clear jar with iodine vapors and the steroid spots marked and extracted in the normal manner. The values obtained with the steroids detected by U.V. light were taken as 100% and the values obtained after iodine vapor detection were related to this. The results are given in Table V.

This method of detecting steroids was then applied to the separation and quantification of a synthetic mixture of androsterone and isoandrosterone. The method of quantification chosen was the Zimmermann reaction. For each steroid a calibration curve was prepared as follows: duplicate samples of 10, 50 and 100 μg were placed in 10 ml volumetric flasks and the solvent evaporated. To each flask was added 0.2 ml of a 2% ethanol solution of *m*-dinitrobenzene and 0.2 ml of an ethanolic 2.5 *N* potassium hydroxide solution. The flasks were stoppered, shaken and left in the dark at 25° for one hour. Immediately before being read against a blank on a Beckman DK-2, each flask was made to the mark with ethanol (the ethanol used was 96% spectroscopic grade).

After the steroid mixture had been separated and detected, each zone was eluted with 2 ml of ethanol into a 10 ml volumetric flask. This was evaporated on a steam bath under a stream of nitrogen and treated as above. Table VI lists the results.

TABLE VI

ANALYSIS OF ANDROSTERONE AND ISOANDROSTERONE IN MIXTURE

System: neutral alumina; 15 cm; hexane–ethyl acetate (1 : 1)

Steroid	R_F	*Found (μg)*	*Average*	*Theory*
Isoandrosterone	0.95	48, 53.5, 56.5	52.7	51.5
Androsterone	0.76	48.5, 51.5, 52.5	50.8	52

Analysis of an impure lot of isoandrosterone by this method gave 92% purity while analysis by gas chromatography gave 89%.

DISCUSSION

As mentioned earlier the use of thin-layer chromatography has distinct advantages over paper partition chromatography. Not the least of these is the fact that there is less diffusion in TLC than in partition chromatography. This results in smaller more compact zones with less tendency to streak and tail. Thus greater resolution is possible and more reproducible quantitative results are obtained. Due to variations in layer

thickness and activity as well as variables in solvent composition, the R_F values in TLC are not as reproducible as in paper partition chromatography. However, this is not a problem when analyzing steroids for purity since obviously the biggest zone is the steroid whose purity is being determined. Quantitative analysis of steroids by gas–liquid chromatography has the disadvantage that all steroids do not give the same molar response[18]. Unless the impurities are known, and calibration curves prepared, this may lead to considerable error when using the method of internal normalization. This problem does not arise in quantitative analysis by TLC.

The limitless variations in solvent composition that can be used in TLC allows one to make experiments with high concentrations of the impure steroid so that even minor impurities may be resolved and detected by iodine once the best solvent mixture is found. It also permits one to observe whether any impurities are travelling close to the major component. Allowances can then be made for this when marking the major zones after detection, so that these impurities are not included in the zone being extracted. Again, the application of samples as small spots aids in this respect since resolution is enhanced. The formation of small spots is aided by warming the plate to about 50° while the 100 μl of solution is applied. The flow rate of solution onto the thin layer must also be controlled to keep the spot small.

All the above procedures for quantitative analysis can probably be scaled down by using microliter syringes and smaller volumes and sample weights but with probable attendant increases in error and decreases in reproducibility. If it is desired to detect much lower quantities of U.V. absorbing steroids it would be advisable to increase the phosphor content in the layer so that the contrast becomes greater under U.V. light.

The detection of steroids by iodine vapor was investigated on compounds containing in aggregate the following functional groups or groupings: aromatic ring A; methyl ether of phenolic ring A; tertiary hydroxyl; primary hydroxyl; ketone; Δ^4-3-ketone; $\Delta^{4,6}$-3-ketone; 17α,21-diol-20-ketone; 21-ol-20-ketone; ethynyl; and ester (Tables V and VI). Although in some cases there is a discrepancy of as much as 4 or 5% after detection by iodine as compared with U.V. detection, the net results indicate that there is very little reaction of iodine with the U.V. chromophore, *i.e.* the Δ^4-3-ketone, $\Delta^{4,6}$-3-ketone or aromatic ring A groups. The addition of iodine to double bonds is known to be very slow even in solution and furthermore is easily reversible. Under the conditions of detections used here, there should be little or no reaction since it is in the dry state where there is likely to be less reaction than in solution. To determine if any reaction occurred when testosterone acetate was detected by this method, about 0.5 mg was placed on a silica gel plate and exposed to iodine vapor. When the spot became visible the plate was removed and the color of iodine allowed to disappear. Another equal amount of testosterone acetate was then placed beside it and the chromatogram developed with chloroform. No new spots were visible in iodine vapor after solvent development indicating that no new products had been formed by this method of detection. The same procedure was followed for each steroid in Table V using solvent systems which had been shown to resolve the largest number of impurities. The only steroid which gave a new compound was cortisone, although the amount was very small compared to the total.

The functional grouping in cortisone which might be sensitive to iodine vapor is the α-ketolic side chain. It has been reported that cortisone gives a blue color when

detected by an iodine solution[19] indicating that there may be some reaction taking place. However, when cortisone is detected by iodine vapor in TLC the appearance of the spots is the same as for any other steroid at the concentrations used in this method. Only when high concentrations were used (500 $\mu g/cm^2$) was a blue color visible. In order to check that there is no appreciable reaction with the α-ketolic group when detected by iodine vapor, cortisone was tested with blue tetrazolium reagent. For this experiment six equal spots of pure cortisone were placed on a silica gel G plate containing phosphor. Three were detected by U.V. light, extracted and the blue tetrazolium reaction run. The other three spots were detected first by iodine vapor, then treated in exactly the same manner as the previous three spots. The absorbances were measured on a DK-2. The absorbances obtained with the cortisone detected by U.V. gave values of 0.318, 0.333 and 0.365 for an average value of 0.339. The cortisone detected by iodine vapor gave values of 0.311, 0.335 and 0.354 for an average value of 0.333. The difference is less than 2% indicating that there is little if any reaction of iodine vapor with the α-ketolic group of cortisone.

The foregoing indicates that the detection of steroids in TLC by iodine vapor is primarily an adsorption phenomenon and there is very little if any chemical reaction with most functional groups usually present in steroids. Detection by this method does not interfere with subsequent colorimetric reactions.

SUMMARY

A method has been developed for the quantitative analysis of U.V. and non-U.V. absorbing steroids utilizing thin-layer chromatography. U.V. absorbing steroids are detected by means of a phosphor in the thin layer, removed and determined quantitatively in a spectrophotometer. Non-U.V. absorbing steroids are detected by iodine vapor, then removed before being determined quantitatively by colorimetric reactions.

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