ELUTION OF STEROIDS AFTER THIN-LAYER CHROMATOGRAPHY*

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(Received August 27th, 1966)

Since the standardization of thin-layer chromatography (TLC) by STAHL[1-4], this method has found wide application for steroid separation during the last few years. Eluting steroids from the coating material of the thin-layer plate has, however, been a problem: the recovery from such elution is irregular and sometimes quite low. Moreover, techniques using fluorimetry or the detector devices of the gas chromatograph for end point analysis require steroids in a high state of purity. This is not always achieved by current methods of steroid elution from thin-layer plates.

The purpose of this communication is to present a method for steroid elution from silica gel after thin-layer chromatography.

MATERIALS

Solvents

Methanol: anhydrous, analytical reagent (AR Mallinckrodt) distilled off 2,4-dinitrophenylhydrazine.

Benzene: nanograde (AR Mallinckrodt).

Ethyl acetate: analytical reagent (AR Mallinckrodt), first fractionally distilled, then refluxed 4 h with acetic anhydride (100 ml/l) and concentrated sulfuric acid (5 ml/l), and finally redistilled through a fractionating column.

Hexane: analytical reagent (AR Mallinckrodt), fractionally distilled; washed in sequence with concentrated sulfuric acid (3 × 100 ml/l), water (3 × 180 ml/l), 1.3% (w/v) $KMnO_4$ in 4 *N* H_2SO_4 (3 × 100 ml/l), then with water (150 ml/l) to neutrality, dried over Na_2SO_4 and fractionally distilled.

Toluene: analytical reagent (AR Mallinckrodt).

Silica gel: Silica Gel G (Merck, according to Stahl for thin-layer chromatography), was washed 3 times with boiling water and 3 times with boiling methanol and dried overnight at 100°.

Phosphor: (DuPont Luminescent chemical, Index 609) was washed 3 times with boiling methanol and dried overnight at 100°.

* This work was supported in part by a U.S. Public Health Training Grant No. TO1 CA 5000.
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Cotton: regular cotton was washed 6 times with methanol and dried overnight at 100°.

Syringes: 3 cc capacity (Travenol Laboratories, Inc.).

Needles: Yale B-D 25 gauge.

2,5-Diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), scintillation grade (Packard Instrument Co., Inc.) were used without further purification.

Radioactive steroids (^{3}H and ^{14}C): New England Nuclear Corporation.

METHODS

Gas chromatography

A standard Barber-Colman Model 10 instrument equipped with an Aerograph electron capture detector (Wilkens Instrument & Research, Inc.) and a Model 214a pulse generator (Hewlett-Packard Co.) was used as previously described by our laboratory[5].

A glass column 2 ft. long and 0.4 cm in diameter was packed with XE 60 (1 %) on 80–100 Gas-Chrom Q (Applied Science Laboratories).

Column temperature 204°, detector temperature 220°, flash heater temperature 230°.

Thin-layer chromatography

The plates were prepared with a Desaga spreader to give layers of 0.2 cm thickness. Fifteen grams silica gel mixed with phosphor (50 mg DuPont 602) and 45 ml water were used to prepare 5 plates (20 × 20 cm). The plates were kept at 100° during at least 1 h before use. Chromatograms were developed in an ascending fashion.

Detection of steroids

Radioactive ^{14}C steroids were localized on the thin-layer plates by a specially built radioactive plate scanner; nonradioactive steroid standards were visualized by a Haines' fluorescent scanner or by iodine vapor (estrogens and their derivatives).

Assay of radioactivity

^{3}H and ^{14}C were measured using a Packard Tri-Carb liquid scintillation spectrophotometer. Samples were dried in 20 ml glass vials and dissolved in 10 ml of scintillation fluid (4 g PPO and 40 mg POPOP in 1 l toluene).

All evaporations were done in nitrogen at +40°.

PRACTICAL PROCEDURE

This method of elution is based on the principle of column chromatography. The area of steroid spotted on the thin-layer plate was scraped off with a spatula and transferred into a 3 ml syringe equipped with a 25 gauge needle. A small amount of cotton was placed in the bottom of the syringe. This column was then washed and the steroids eluted with an appropriate solvent (Table I). To prevent the dispersion of silica gel, the solvent was run carefully down the wall of the syringe.

TABLE I

ELUTION OF STEROIDS* FROM SILICA GEL

	Wash fraction			*Elution fraction*			
	Hexane–toluene (v/v)	*Hexane–benzene (v/v)*	*% Steroid eluted by this solvent*	*Methanol–toluene (v/v)*	*Methanol–benzene (v/v)*	*Ethyl acetate–benzene (v/v)*	*% Steroid eluted by this solvent*
Estrone	Hexane only		0.16			EtOAc only	89.61
17β-Estradiol	4:1		0.13	1:19			95.37
Testosterone	1:9		1.29	1:49			93.66
Androstenedione		2:3	0.86		1:49		92.81
DHEA		1:1	0.85		1:49		94.23
Progesterone	3:2		0.29	3:97			91.30
F_K	Toluene only		0.17	1:9			97.32
B_K	Toluene only		0.04	1:49			93.47
DOC	1:9		0.41	1:49			94.28
DOCA	3:2		0.01	3:97			90.60
E_1-OCH_3		Hexane only	0.29		1:19		90.12
E_2-OCH_3		Hexane only	3.2		1:9		98.25
20β-Hydroxy-Δ^4-pregnen-3-one	1:9		0.75	1:49			92.45
E_2-OCH_3-ClAc		Hexane only	2.3		Benzene only		87.7
20β-Hydroxy-Δ^4-pregnen-3-one-ClAc	3:2		0.6	1:49			95.2
Testosterone-ClAc		4:1	0.01			1:9	87.9

* For trivial names, see p. 171.

RESULTS AND DISCUSSION

The study was carried out on several steroids and their derivatives. For each compound, 6 ml of a suitable solvent combination was used as a wash-fraction on the column and the steroid was then eluted by 6 ml of an appropriate solvent. In order to obtain high recoveries, it is advisable to get rid of the wash-fraction trapped in the cotton at the bottom of the column. For this purpose, steroid elution was done in the following way: 0.5 ml of the eluting solvent was applied to the column twice followed by the application of 5 ml of this solvent. These fractions were combined. Table I shows the losses due to washing of this column and the recovery of steroid following elution by different solvent combinations. The results are means from five experiments for each steroid studied.

In some instances, silica gel passed through the column when the steroid was eluted. In such samples, adequate assay by the technique of gas-phase chromatography with electron capture detector was not possible. Silica gel could, however, be removed from the organic solvent by stirring with 0.5 ml of water, centrifuging for 10 min at 1500 r.p.m. and removing the organic solvent layer containing the steroid sample.

In order to compare compound purity by the current method with the more common method of eluting steroids from silica gel, the following experiment was

carried out: testicular venous blood was obtained from a dog[6] and its concentration of plasma testosterone assayed by the method of BROWNIE *et al.*[5]. The isolated testosterone 17-monochloroacetate was divided into four equal parts and chromatographed separately in the solvent system benzene–ethyl acetate 4:1 on thin-layer plates coated with silica gel. Two samples of testosterone chloroacetate were scraped off the thin-layer plate and the steroid contained in the coating material was extracted by 2 ml benzene × 3 after the addition of 0.5 ml water to the silica gel. The combined benzene was evaporated to dryness. The two other samples of testosterone chloroacetate were scraped off the thin-layer plate and the silica gel transferred to a 3 ml syringe. The silica gel was first washed with 6 ml of hexane–benzene (20:80, v/v) and the testosterone chloroacetate contained in the silica gel eluted with 6 ml ethyl acetate–benzene (10:90, v/v). This fraction was washed with 0.5 ml of water and then evaporated to dryness. All residues were dissolved in toluene and subjected to gas-phase chromatography. Representative gas chromatography tracings of these samples are presented in Fig. 1.

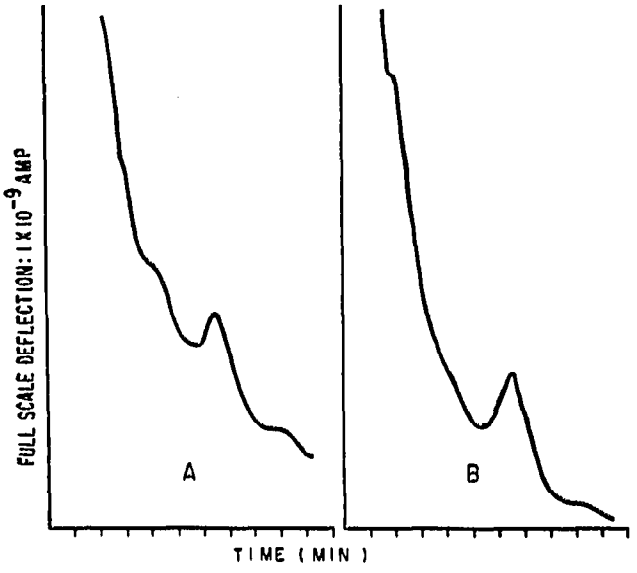

Fig. 1. Gas-phase tracings of testosterone 17-monochloroacetate after processing testicular dog plasma by the method of BROWNIE *et al.*[5]. (A) Steroid removed from silica gel by extraction. (B) Steroid removed from silica gel by column chromatography.

It can be seen from these tracings that a thinner front and a more distinct peak of plasma testosterone were obtained when the hormone contained in the silica gel was removed by column chromatography. If high purity is not required, all the steroids listed in Table I can be eluted by our column technique with 6 ml of methanol without preliminary washing. Such elution will, however, result in considerable removal of silica gel with the steroid, thus it is advisable to use other solvents for steroid elution when the column has not been exposed to a wash fraction. For instance, ethyl acetate may be used for testosterone, progesterone, estrone, DHEA, DOCA, 20β-hydroxy-Δ^4-pregnen-3-one, E_1-OCH_3, Δ^4-androstenedione elution, or benzene for E_1-OCH_3 elution. In such experiments the steroid recovery is as high as shown in Table I.

CONCLUSION

A method has been presented for the elution of steroids from Silica Gel G after thin-layer chromatography. One of the advantages of this technique is a high recovery[7]. The compound purity achieved by this procedure is of value for techniques of steroid quantification like gas-phase chromatography with electron capture detection.

TRIVIAL NAMES

The following trivial names have been used in this paper:
Estrone: $\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one ;
17β-Estradiol: $\Delta^{1,3,5(10)}$-estratriene-3,17β-diol;
Testosterone: Δ^{4}-androsten-17β-ol-3-one;
Androstenedione: Δ^{4}-androstene-3,17-dione;
DHEA: Δ^{5}-androsten-3β-ol-17-one;
Progesterone: Δ^{4}-pregnene-3,20-dione;
F_K: Δ^{4}-pregnene-11β,17α,21-triol-3,20-dione;
B_K: Δ^{4}-pregnene-11β,21-diol-3,20-dione;
DOC: Δ^{4}-pregnen-21-ol-3,20-dione;
DOCA: Δ^{4}-pregnen-21-ol-3,20-dione acetate;
E_1-OCH_3: $\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one methyl ether;
E_2-OCH_3: $\Delta^{1,3,5(10)}$-estratriene-3,17-diol 3-methyl ether;
E_2-OCH_3-ClAc: $\Delta^{1,3,5(10)}$-estratriene-3,17-diol 3-methyl ether 17-monochloroacetate;
20β-Hydroxy-Δ^{4}-pregnen-3-one-ClAc: Δ^{4}-pregnen-20β-ol-3-one monochloroacetate;
Testosterone-ClAc: Δ^{4}-androsten-17-ol-3-one monochloroacetate.

SUMMARY

A method of steroid elution from Silica Gel G after thin-layer chromatography is presented. The method is based on the principle of column chromatography. This technique gives adequate and reproducible recoveries and a high state of compound purity.

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