PAPER CHROMATOGRAPHY OF TRITERPENOID AND STEROID ACIDS

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(Received September 7th, 1961)

Many solvent systems have been reported which are suitable for the chromatography of bile acids on paper[1–4]. These acids and their ammonium salts are relatively water soluble, and the systems used are all of the BUSH type[5,6]. For those steroid acids which are extremely insoluble in water, such as eburicoic acid and other triterpenoid acids[7–10], a ZAFFARONI type[6,11,12] system will obviously be more advantageous[6,13]. In the present paper, we wish to report two ZAFFARONI type systems suitable for the chromatography of the triterpenoid acids on paper.

A BUSH type system resembling those reported for the sulfate and glucuronide conjugates of steroids[14–16] also was found satisfactory for these triterpenoid acids, and will be described. BUSH type systems with a relatively polar solvent as the mobile phase can be adapted to the chromatography of the more polar steroid acids such as the etianic acids and the bile acids. A description of a system of this type which we found to be especially satisfactory is also included in this communication.

PROCEDURE

System I (Zaffaroni type)

Ethylene glycol, *n*-butyl ether and piperidine in the volume ratio of 10:20:1 are shaken together. The lower glycol phase is withdrawn and diluted with 1.5 volumes of methanol. A Whatman No. 1 paper strip is drawn through this mixture and allowed to air dry for 15 min to evaporate off the methanol. After spotting the samples, the strip is equilibrated with the upper ether phase for one hour in the chromatographic jar. A descending chromatogram is then developed with the ether phase as the mobile solvent at room temperature (22–28°).

System II (Zaffaroni type)

n-Amyl acetate is used in place of *n*-butyl ether in the above system.

System III (Bush type)

n-Hexane, *tert.*-butanol and 4 *N* NH_4OH in the volume ratio of 10:3:10 are shaken together. The Whatman No. 1 paper strip bearing the sample spots is thoroughly equilibrated with the lower aqueous phase for at least 2 h at 37°. A descending chromatograph is then developed with the upper hexane phase. Equilibration overnight produces the most satisfactory result.

System IV (Bush type)

The procedure of System III is followed, using a mixture of equal volumes of chloroform, methanol and 1 *N* NH_4OH. Equilibration for one hour at room temperature gives entirely satisfactory results.

Detection methods

The following detection methods are applicable to ZAFFARONI type papergrams which have been dried at 100° in an oven equipped with an air blowing system, or to BUSH type papergrams which have been air dried at room temperature.

Steroids with an α,β-unsaturated ketone structure are readily detected with HAINES' u.v.-scanner[17]. The phosphomolybdic acid reagent[18–21]—10 % (w/v) in 95 % ethanol has been found very effective for detecting steroids in general. Not only the 3-hydroxy-steroids, but those with a 3-acetoxy group, a Δ^4-3-keto structure as well as some with only a 3-keto group are detectable. It is true, however, that 3-hydroxy-steroids respond especially strongly. It is essential to apply the reagent heavily by either dipping or spraying and to heat the wet papergrams immediately after the application of the reagent at 100° for 5 min.

The iodine vapor method[22] is an effective alternative of the phosphomolybdic acid test.

Demonstration of acidic nature

To show that a spot is an acid, naturally a very weak acid, a modified borate–phenol red spray reagent as originally devised by BRADFIELD AND FLOOD[23] and HOCKENHULL[24] for polyols was found applicable. To exclude the detection of *vic*-glycols, a Tris-buffer was used in place of the borate. The reagent consists of a mixture of 9 ml of methanol, 1 ml of a 0.1% phenol red solution in 95% ethanol and 0.4 ml of an aqueous 0.1 *M* tris-(hydroxymethyl)-aminomethane solution. The color of the mixture is adjusted to decidedly red by dropwise addition of 0.1 *N* NaOH. On spraying, the acids appear as bright yellow spots on a pink background. The incorporation of a small amount of buffer in the reagent is essential so that the paper background remains basic to phenol red. Indicators with end points at lower pH values proved useless for detecting very weak acids.

This spray reagent is not recommended for the detection of acid spots on a papergram because the sensitivity is relatively low. A papergram spot of approximately 15 γ of eburicoic acid on an area of 1 cm^2 is required to show a decisive positive test. It is therefore rather recommended for demonstrating the acid nature of a compound after its presence in sufficient quantity as a papergram spot is assured.

Small jar paper chromatography

For the solvent systems described here as well as for other neutral systems of both ZAFFARONI and BUSH types, the small jar chromatography as recommended by UNDERWOOD AND ROCKLAND[25, 33], was shown to work as satisfactorily as the usual large jars, *e.g.* 8 1/2-in. (diameter) × 20-in. In our laboratory, 4 1/2-in. × 3 1/2-in. × 9-in. jars made by cutting off the top of diphtheria toxin bottles (Corning Cat. No. 590) have been conveniently used.

RESULTS

The R_F values of some of the steroid acids used in the present study and the recommended detection methods are presented in Table I. The triterpenoid acids (sample Nos. 1, 3, 4, 5, 6) were obtained from Dr. HALSALL of the Oxford University. The bile acids (sample Nos. 7, 11, 12) were commercially available materials. The 3-keto-

TABLE I
PAPER CHROMATOGRAPHIC SEPARATION OF STEROID ACIDS

Sample No.	*Acids*	*R_F values in solvent system**				*Recommended detection method*
		I	*II*	*III*	*IV*	
1	Acetyl eburicoic acid	0.77	0.95	0.93	0.95	PMA
2	3-Keto-$\Delta^{8,24(28)}$-ergostadien-21-oic acid	0.42	0.85	0.92	0.95	PMA
3	Eburicoic acid	0.23	0.66	0.73	0.93	PMA
4	Damarenolic acid	0.22	0.66	—**	—	PMA
5	Oleanolic acid	0.20	0.60	0.67	—	PMA
6	Polyporenic acid-A	0.00	0.14	0.30	0.20	PMA
7	Dehydrocholic acid	0.00	0.00	0.06	0.35	DNPH
8	3-Keto-Δ^4-etienic acid	0.00	0.00		0.47	UV
9	17α-Hydroxy-3-keto-Δ^4-etienic acid	0.00	0.00		0.39	UV
10	11β,17α-Dihydroxy-3-keto-Δ^4-etienic acid	0.00	0.00		0.17	UV
11	Desoxycholic acid	0.00	0.00	0.15***	0.20	PMA
12	Cholic acid	0.00	0.00		0.07	PMA

* *Solvent systems*: I = Ethylene glycol–piperidine/*n*-butyl ether–piperidine. II = Ethylene glycol–piperidine/*n*-amyl acetate–piperidine. III = *n*-Hexane–butanol–4 *N* NH_4OH (10:3:10). IV = Chloroform–methanol–1 *N* NH_4OH (1:1:1).

PMA = Phosphomolybdic acid reagent, UV = u.v.-scanning; DNPH = Dinitrophenylhydrazine reagent[30,32].

** Spot appeared as a smear.

*** With tailing.

$\Delta^{8,24(28)}$-ergostadien-21-oic acid (sample No. 2) was the chromic acid oxidation product of eburicoic acid. It was prepared by using a microchemical technique similar to those used by ZAFFARONI[26], BUSH[27] and BERLINER[28] and their co-workers. A reagent of chromic acid in acetone was used[29] and the compound was not isolated in pure form. The details of the procedure are being published elsewhere[30]. The etienic acids, sample Nos. 8, 9 and 10, were prepared by oxidizing cortexone, cortexolone and cortisol respectively with periodate, using a similar microchemical technique as follows:

To 0.1 ml of a methanolic solution containing 100 γ of each of these three neutral corticoids, 0.1 *M* of a saturated aqueous KIO_4 solution is added. The test tube is left to stand at room temperature. After 4 h or longer, 0.5 ml of water, 0.4–0.5 g $(NH_4)_2SO_4$ and 2.5 ml of chloroform are added. The test tube, tightly closed with a polyethylene stopper, is shaken vigorously for one minute and then centrifuged. The upper aqueous layer is removed with a capillary medicine dropper and the chloroform extract is transferred into a clean and dry test tube. After removing droplets of aqueous phase by shaking with a small piece of filter paper, the chloroform extract is evaporated to dryness by keeping the test tube in a 40–50° water bath while blowing a gentle current of air onto the surface of the liquid. The residue is taken up in 0.1 ml of a 1:1 methanol–chloroform mixture. This solution is used for application to the paper.

For samples 2, 8, 9 and 10, the major spot is recognized as representing the compound listed in Table I.

DISCUSSION

The R_F values given in Table I are in full agreement with the polarity as demanded by the structure of the acids tested[6,31]. These four systems are obviously adequate for handling steroid acids of a wide range of polarity.

In a system of ethylene glycol *vs.* *n*-butyl ether or other similar solvent pairs without the addition of piperidine, eburicoic acid moves with a satisfactory R_F value, but it moves either as a smear or as two spots; one of them remaining at the origin. The addition of piperidine renders both phases efficient solvents. The system can then function well even with acids which are highly insoluble in either of the two neutral solvents, such as the acetyl eburicoic acid.

By using other mobile phases, *e.g.* benzene, carbon tetrachloride, chloroform, etc., satisfactory papergrams can also be obtained. The mobility will naturally vary according to the polarity of the mobile phase. Formamide can be used in place of ethylene glycol as the stationary phase with little change in mobility.

System IV can be easily adapted to the chromatography of still more polar acids by simply increasing the proportion of methanol. Thus, in a system of chloroform–methanol–1 *N* NH_4OH (2:3:2), cholic acid moves with $R_F = 0.46$. Such a system would be satisfactory for steroid acids more polar than those listed in Table I.

SUMMARY

Four systems are described for the chromatography of steroid acids of a wide range of polarity. These systems are based on the principles involved in ZAFFARONI and BUSH type systems for chromatographing neutral steroids on paper.

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