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Note

Detection of bile salts with Komarowsky's reagent and group specific dehydrogenases

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A number of spray reagents for the detection of bile salts and neutral steroids on thin-layer chromatography (TLC) are in common use. Some of the more widely used reagents include various sulfuric acid solutions[1], molybophosphoric acid[1] antimony(III) chloride[2] and anisaldehyde-sulfuric acid solution[3]. Kellogg[4] has documented the use of 8-hydroxy-1(3,6-pyrene)trisulfonic acid for bile salt and neutral steroid detection. More recently, Goswami and Frey[5] have introduced a novel copper–ammonium molybdate–sulphuric acid reagent to detect a variety of bile salts, lecithin and cholesterol on TLC plates.

Komarowsky's reagent (*p*-hydroxybenzaldehyde–sulfuric acid solution) has been used to detect a limited number of keto-steroids and sapogenins[1,6]. It is the purpose of this communication to describe the general usefulness of this reagent in the detection of hydroxy- and keto- bile salts and the application of group specific dehydrogenases against eluates from sprayed thin-layer plates.

MATERIALS

Preparation of Komarowsky's reagent was modified from Stevens[6]: 5 ml of concentrated sulfuric acid were added to 100 ml of a 2% solution of *p*-hydroxybenzaldehyde (Baker, Phillipsburg, N.J., U.S.A.) in methanol. All bile salts and neutral steroids were dissolved (5 mg/ml) in methanol–water (4:1). TLC plates (20 × 20 cm) were made as described earlier[7].

Solvent systems consisted of chloroform–methanol–acetic acid: (A), 40:4:2; (B), 40:2:1; (C), 40:1:1.

3α-Hydroxysteroid dehydrogenase (3α-HSDH) was purchased from Worthington (Freehold, N.J., U.S.A.) enzymes; 7α-hydroxysteroid dehydrogenase (7α-HSDH) was made as previously described[7].

METHODS

Standard steroids (5–20 μl) were spotted approximately 2 cm from the edge of a TLC-plate (pre-washed by leaving in a tank containing chloroform–methanol–water (85:35:5) and pre-activated at 100° for 1 h). Prior to spotting, plates were divided

into 10 lanes and the appropriate solvent system allowed to equilibrate in a glass tank for 2 h. On completion of spotting, the plates were dried by blowing cold air onto the surface and quickly placed in a solvent tank. Plates were developed for 2 h, dried (in a fumehood), sprayed liberally with Komarowsky's reagent and left at 80° for 5–8 min. Alternately, plates were gently warmed by use of a heat gun (1–2 min). Colors and R_F values were noted, when the plate was cooled (approximately 15 min).

The colors were allowed to partially fade (1 or 2 days); individual spots corresponding to selected compounds were scraped from the plate and the scrapings were placed in pasteur pipettes, pre-packed with cotton-wool (approximately 1 cm high). Scrapings were eluted directly into a cuvette with 0.5 ml methanol (or methanol–diethyl ether, 50:50, for the more non-polar compounds; *e.g.* di- or tri-keto bile salts). The solvent was blown down to dryness and 3.0 ml reaction mixture, consisting of 0.17 *M* glycine–NaOH buffer pH 9.5, $0.8 \cdot 10^{-3}$ *M* NAD, was added. A baseline was obtained by reading the cuvette at 340 nm in a Beckman DBGT spectrophotometer and recorder at 0.1 in./min. A 50-μl volume of purified 3α-HSDH (2 mg/ml) or 7α-HSDH (4 mg/ml) were added and the change in absorbance at 340 nm observed.

RESULTS AND DISCUSSION

Results are summarized in Table I. The reagent reacted with both hydroxylated and ketonic bile salts and gave a wide range of colors. It appears to be able to differentiate isomers such as 3α,7α-dihydroxy- and 3α-,12α-dihydroxy-cholanoates, and 3,7 diketo- and 3,12 diketo-cholanoates which are not generally differentiated by their R_F values. It can be noted however that there was no color difference between a free bile salt and the methylester or glycine or taurine conjugates (not shown) or between 5α-H bile salts and 5β-H bile salts. Thus the color formed with *p*-hydroxybenzaldehyde appears to be dependent on the number and positions of the hydroxyl- and keto-substituents. The range and type of compounds detected by this reagent is far greater than the original paper suggests[6]. All compounds detected under visible light, fluoresced when viewed under the long wave or short wave ultraviolet light although the former gave a more intense fluorescence.

The spectrum of colors seen with *p*-hydroxybenzaldehyde is similar (but not identical) to that seen with anisaldehyde, which has been extensively investigated by Kritchevsky *et al.*[3] and Lisboa[8]. Similar to their finding, the limit of sensitivity was found to be approximately 1 μg of steroid in day light. The use of a long wave UV-lamp will detect less than 0.5 μg cholic acid not visible in day light. Colors were stable for a period of approximately 6 h although some fading of the more "delicate" shades was evident after about 2 h. A plate which had been allowed to fade nearly completely (over a weekend) could be re-stored by simply re-heating at 80° although the restored color was not necessarily identical to the original.

The solvent systems A, B, and C in our hands gave excellent results. Behaviour of most bile salts in solvent B was somewhat similar to their behaviour in solvent S_1 of Eneroth[9] but with less diffusion and improved separations (Benzene–dioxane–acetic acid, 75:20:2). An increase or decrease in the chloroform–methanol ratio (solvents C and A respectively) permits the separation of less polar or more polar compounds without essentially changing the order on the TLC plate. However,

TABLE I

THIN-LAYER CHROMATOGRAPHY OF TRI- DI- AND MONO-FUNCTIONAL BILE SALTS AND COLORS WITH KOMAROWSKY'S REAGENT

Cholanoate	*Source**	*Color*	*Solvent system*	R_F**
3α,7α,12α-Trihydroxy-5β-	b	deep purple	A	0.21
			B	0.06
3α,7α,12α-Trihydroxy-5β-methyl	a	deep purple	A	0.39
			B	0.12
3α,7α,12α-Trihydroxy-5α-methyl	d	deep purple	A	0.28
			B	0.11
7α,12α-Dihydroxy-3-keto-5β-methyl	a	rose wine	A	0.70
			B	0.42
3α,12α-Dihydroxy-7-keto-5β-	a	khaki green	A	0.40
			B	0.15
3α,12α-Dihydroxy-7-keto-5β-methyl	a	khaki green	A	0.65
			B	0.37
3α,7α-Dihydroxy-12-keto-5β-	a	green-gray	A	0.40
			B	0.15
3α,7α-Dihydroxy-12-keto-5β-methyl	a	green-gray	A	0.65
			B	0.37
3α-Hydroxy-7,12-diketo-5β-	c	lime	B	0.37
7α-Hydroxy-3,12-diketo-5β-	e	bright red	B	0.39
12α-Hydroxy-3,7-diketo-5β-	e	brown	B	0.39
3,7,12-Triketo-5β-	a	orange-crimson	B	0.52
			C	0.36
3α,7α-Dihydroxy-5β-	b	violet	B	0.25
3α,7α-Dihydroxy-5β-methyl	a	violet	B	0.49
3α,7α-Dihydroxy-5α-methyl	d	violet	B	0.41
3α,7β-Dihydroxy-5β-	b	blue-purple	B	0.32
3α-Hydroxy-7-keto-5β-	a	violet-red	B	0.51
			C	0.44
7α-Hydroxy-3-keto-5β-	a	oxblood	C	0.48
3,7-Diketo-5β-	a	rust-yellow	B	0.57
			C	0.48
3α,12α-Dihydroxy-5β-	b	mauve-gray	B	0.25
3α,12α-Dihydroxy-5β-methyl	a	mauve-gray	B	0.49
3α-Hydroxy-12-keto-5β-	a	green	B	0.40
			C	0.27
12α-Hydroxy-3-keto-5β-	a	mahogany	C	0.42
3,12-Diketo-5β-	c	brick red	C	0.47
3α,6α-Dihydroxy-5β-	b	medium blue	B	0.22
3α,12α-Dihydroxy-5β-	a	blood red	B	0.45
7α-Hydroxy-5β-	c	navyblue-purple	B	0.66
			C	0.48
3-Keto-5β-	e	brick red	B	0.74

* (a) Steraloids (Wilton, N.H., U.S.A.); (b) Calbiochem. (Los Angeles, Calif., U.S.A.); (c) Applied Science Labs. (State College, Pa., U.S.A.); (d) kindly donated by Dr. W. H. Elliot (St. Louis University, Mo., U.S.A.); (e) Synthesized enzymatically (refs. 7 and 13).

** Average of two determinations.

similar to those results of Eneroth[9], certain isomeric pairs were not separated (*e.g.* 3α, 7α- and 3α,12α-dihydroxy-5β-cholanoates).

Low concentrations of *p*-hydroxybenzaldehyde (10^{-5} *M*) in the cuvette did not

inhibit either *P. testosteroni* 3α-HSDH or *E. coli* 7α-HSDH, however higher concentrations absorbed excessively at 340 nm and thereby interfered. Sulfuric acid in correspondingly small amounts was readily buffered in the glycine–NaOH reaction system. Thin-layer eluates of 3α,7α,12α-trihydroxy-5β-cholanoate, 3α,7α-dihydroxy-12-keto-5β-cholanoate and 3α,12α-dihydroxy-7-keto-5β-cholanoate from a sprayed plate were tested as substrates. In each case, the presence of the appropriate OH-group, a measurable evolution of NADH occurred on addition of 3α-HSDH or 7α-HSDH or 7α-HSDH. Approximately 1.5 μg of bile salt was required for enzymatic detection. The color complex when eluted into a cuvette, caused a somewhat variable increase in background, but did not interfere in the enzymatic reactions. Although enzymatic reactions could be observed to go to "completion", no attempt was made to obtain quantitative results.

Because of the fairly mild conditions required for detection of bile salts and sterols with Komarowsky's reagent[6], this spray reagent is essentially non-destructive; its use with appropriate solvent systems enables the investigator to obtain rapid preliminary structural evidence on the basis of 3 criteria: mobility, color and reactivity with enzymes of established position and stereospecificity. Preliminary evidence suggests that this reagent can also be used with two independent sources of 12α-HSDH and can serve as a valuable aid in the identification of microbial degradation products[10].

It must be emphasized that the last criterion depends strongly on substrate specificity pattern of the enzyme involved. For example, Haslewood[11] has demonstrated that sulfation of the 3 position of cholate (or side chain elongation) will abolish reactivity of 7α-HSDH at the 7α-OH position. Similarly we have demonstrated[10] that oxidation of the 3α-OH group to the ketone will drastically reduce reactivity of the molecule with 7α-HSDH. Additionally, since the visualization of bile salts is more sensitive than the enzymatic detection, insufficient amounts may be present in the eluate for the latter. Thus a positive enzyme reaction with an unknown compound may be used for evidence that a given OH-group is present but a negative test in the absence of other evidence may not permit an investigator to claim the absence of this OH-group in the molecule. The importance of the investigation of substrate specificity patterns of recently discovered enzymes[11–13] cannot be understated.

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