Results and Discussion

No attempts were made to determine quantitatively the water concentration under the described conditions. The calculation of the individual amine concentrations was made assuming the total area under the amine peaks as 100 %.

The results are presented in Table I.

It will be seen that the average error does not exceed ± 2.5 % although in some cases maximum errors of about 10 % were recorded.

Application

This method was applied to the analysis of propylamine in a complex mixture containing in addition to acrylonitrile, propionitrile, adiponitrile and potassium chloride, in acid solution. Butylamine was added as a marker.

On neutralization with NaOH, two phases appeared. The mixture was vigorously stirred with a magnetic stirrer and samples were withdrawn for chromatographic analysis during the stirring.

The determination of propylamine by the present method gave reproducible results, within the limits of ± 2.5 %.

The nitriles were determined on a separate column, as described earlier[1].

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Color detection of bile acids using thin-layer chromatography

There is common agreement that the usefulness of R_F values in thin-layer chromatography (TLC) is limited. To solve this difficulty relative mobility values are often suggested. However, use of these values, as well as of R_F, is at times unsatisfactory because of the problems of concave solvent fronts and/or incomplete separation of some acids having similar mobilities[1,2].

Recently KRITCHEVSKY, MARTAK AND ROTHBLAT[3] have demonstrated the usefulness of color detection in bile acid identification.

The present study expands on the use of color detection with particular stress

on advantages of specific detecting reagents in resolving the two problems stated above.

Materials

Solvent systems for TLC. Preliminary investigations, using several solvent systems, disclosed that two systems consistently gave satisfactory results in TLC of bile acids:

Solvent I, used for free acids: ethyl acetate (freshly distilled)–glacial acetic acid (96:4, v/v).

Solvent II, used for conjugated acids: amyl acetate–glacial acetic acid–*n*-propanol–water (40:30:20:10, v/v).

Detecting reagents for bile acids. The following detecting reagents should be freshly prepared in an ice bath:

A = 15 ml concentrated sulfuric acid in 85 ml of anhydrous *n*-butanol.

B = 20 g antimony trichloride (anhydrous) dissolved in 50 ml anhydrous *n*-butanol and mixed with 10 ml concentrated sulfuric acid and 20 ml glacial acetic acid.

C = 5 ml concentrated sulfuric acid in 95 ml of acetic anhydride[4].

D = 2 g ferric chloride dissolved in 83 ml anhydrous *n*-butanol and mixed with 15 ml concentrated sulfuric acid.

Methods

A suspension of Silica Gel G* was prepared by adding 30 g of dry gel to 90 cc of distilled water. Acidification of the gel to pH 3.0 with acetic acid improved the sharpness of bile acid spots, especially those of hyodeoxycholic acid (HDCA) and deoxycholic acid (DCA). Glass plates, 200 × 200 mm and/or 25 × 75 mm, were prepared by soaking in a chromic–sulfuric acid mixture. The acidic gel suspension was applied to the plates at a thickness of 75–100 μ, according to the method of STAHL[5]. They were next air dried at room temperature for about 5 min and then activated at 100° for 2 h. When not in use, the plates were stored in a desiccator over silica gel.

The bile acids were made up in concentrations of 20 μg/μl in *n*-butanol. Five to twenty micrograms of each acid was sufficient for color detection. In order to prevent a concave solvent front—a condition particularly common with some of the highly mobile solvent systems—linear grooves, on each side of the bile acid origin, were made with a small hypodermic needle.

Rectangular chromatographic chambers, 29 × 28 × 10 cm, were lined with Whatman No. 3 filter paper and saturated with appropriate solvents. The chromatoplates were allowed to develop in the chambers at room temperature from 1 to 3 h, depending on whether free acids were being separated with Solvent I or conjugated acids with Solvent II. In either case, the chromatoplates were removed from the chambers just before the solvent front reached the top of the plate, were air dried for several minutes, and then thoroughly dried at 110° for a minimum of 3 h. After cooling, the plates were sprayed with freshly prepared detecting reagents, air dried for 15 min and placed in an air-circulating oven at 110°. Since color development is dependent upon careful control of heat exposure, the exposure time should not exceed 25–30 min for conjugated acids, or 45–50 min for free acids. During the development

* Available from E. Merck, A. G., Darmstadt, Germany.

of the spots, the background remains white. It is advisable to record the pertinent color information as soon as the plates have cooled, preferably within an hour or two, although some of the free bile acids, particularly deoxycholic and 3α,12α-dihydroxy-7-ketocholanic acids, retain their initial color for several days after spraying if the chromatoplates are covered with clear glass plates.

Discussion

Color reactions of the free and conjugated bile acids are shown in Table I. In those instances where two colors are noted the first color predominates. The use of color charts for standardization would seem in order, but it is sugggested that each investigator, when feasible, prepare his own color standards. It is to be noted that the bile acids having a hydroxyl at 12 carbon position gave a yellow color, except when detecting reagent D (containing ferric chloride) was used. The conjugated acids gave colors similar to their free-acid counterparts. Current studies in this laboratory, using biological materials, indicate that color detection reagents facilitate bile acid identification.

TABLE I

COLOR REACTIONS OF FREE AND CONJUGATED BILE ACIDS

*Free bile acids, solvent system I**	*Detecting reagents*			
	A	*B*	*C*	*D*
Cholic	Y	Y-Gr	Y	Gr-Bk
Hyocholic	Gy	Br	Br	Gr
Hyodeoxycholic	Bk	Bk	Gy-Gr	Bk-Bl
Chenodeoxycholic	Gy-Gr	Gr-Y	Gy-Gr	P-Bk
Ursodeoxycholic	Gy-Gr	Gr-Y	Gy-Gr	P-Bk
Deoxycholic	Y	Y	Y-Br	Br
Apocholic	Y	Y-Gr	Y-Br	P-Bk
Lithocholic	P	Pk-P	P	P-Bk
3α,7α-Dihydroxy-12-ketocholanic	Y	Pk	Y	Y-Gr
3α,12α-Dihydroxy-7α-ketocholanic	Ys	Ys	Ys	Ys
Dehydrocholic	Pk	Pk	Pk	O-Br
Dehydrodeoxycholic	Pk	O	Pk	O-Br
*Conjugated bile acids, solvent system II**	*Detecting reagents*			
	A	*B*	*C*	*D*
Taurocholic	Y-Gr	Y	Y	Gr-Bk
Taurohyodeoxycholic	Bk	Br	Br	Bk-Bl
Taurodeoxycholic	Y-Gr	Ys	Ys	Br
Glycocholic	Y	Y	Y	Gr-Bk
Glycohyodeoxycholic	Bk-Bl	Br	Br	Bk-Bl
Glycodeoxycholic	Y-Gr	Ys	Ys	Br
*Sterol, solvent system I**	*Detecting reagents*			
	A	*B*	*C*	*D*
Cholesterol	P	P	P	P-Bk

Color code: Bl = blue; Bk = black; Br = brown; Gr = Green; Gy = Grey; O = Orange; P = Purple; Pk = Pink; Y = Yellow; Ys = Sun Yellow.

* For composition of solvent systems and detecting reagents, see text: "Materials".

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Separation of equol from oestrogens by thin-layer chromatography

Equol may be present in hen's urine in relatively large amounts[1,2]. It can be separated from steroid oestrogens by thin-layer chromatography (TLC). The procedure involves (i) separation by TLC of the phenols into (a) oestrone, (b) oestradiol-17β plus equol and (c) oestriol; (ii) methylation of (b); and (iii) subsequent TLC of the methyl ethers of (b), which affords excellent separation of these two compounds.

A mixture of oestrone, oestradiol-17β, oestriol and equol was chromatographed on silica gel G (Merck) in benzene–methanol (85:15)[3]. The two terminal strips of the chromatoplates were sprayed with 1 % (w/v) *p*-nitrobenzenediazonium fluoborate in acetic acid–water (1:1 v/v) while the middle section was protected by a plastic plate. The respective R_F values were 0.63, 0.41, 0.24 and 0.41. The blank area corresponding to the oestradiol-17β plus equol spot was removed, eluted with ethanol and methylated[4]. The methyl ethers were chromatographed in benzene–methanol (95:5) and the spots detected by spraying with 2 % (v/v) sulphuric acid in aqueous ethanol[3]. Methylation renders equol less "polar" than oestradiol in consequence of the formation of the dimethyl ether[5], which moves far ahead of the 3-methyl ether of oestradiol-17β in this solvent system (R_F: 0.78 and 0.37 respectively).

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