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RAPID ENZYMATIC DETERMINATION OF 3-OXO-BILE ACIDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method for the rapid quantification of 3-oxo-5β-cholan-24-oic acids has been developed. The acids are separated on silica gel G and located using a water spray or iodine vapor. Each oxo acid is eluted from the gel and reduced with sodium borohydride. The resulting α- and β-hydroxy acids are then oxidized in a reaction catalyzed by 3-hydroxysteroid dehydrogenase during which NAD is reduced to NADH. The absorbance of the reaction mixture is determined at 340 nm and is directly proportional to the amount of 3-oxo acid originally present on the thin-layer plate.

INTRODUCTION

The 3-oxo-5β-cholan-24-oic acids (3-oxo bile acids) are a group of substances which (1) are products of bile acid metabolism in the gastrointestinal tract and are found in feces and the portal blood[1], (2) are intermediates in the organic synthesis of bile acids, and (3) are sometimes used as choleretics, *viz.* dehydrocholic acid. In the past, the only practical approach to quantification of small amounts of these substances was gas–liquid chromatography[2]. This method, although accurate, is time-consuming and involves the preparation of derivatives prior to assay. We have developed a rapid new method which can be applied to large numbers of samples. The principal of the method is: 3-oxo-5β-cholan-24-oic acids, separated by thin-layer chromatography (TLC), are reduced to 3α- and 3β-hydroxy-5β-cholan-24-oic acids using sodium borohydride. The hydroxy bile acids are then oxidized to 3-oxo acids in a reaction catalyzed by a mixture of 3α- and 3β-hydroxysteroid dehydrogenases[3,4]. β-Nicotinamide–adenine dinucleotide (NAD) accepts hydrogen and is reduced to NADH, while hydrazine traps 3-oxo acids and forces the reaction to completion. The amount of NADH formed is directly proportional, on a one to one molar basis, to the amount of 3-oxo-5β-cholan-24-oic acids originally present. The concentration of NADH is determined by reading the absorbance of the reaction mixture at 340 nm.

MATERIALS

All of the chemicals used were reagent grade and all solvents were distilled prior to use. 3-Oxo-5β-cholan-24-oic, 3,6-dioxo-5β-cholan-24-oic, 3,7,12-trioxo-5β-cholan-24-oic, 3,12-dioxo-5β-cholan-24-oic, 3α,7α,12α-trihydroxy-5β-cholan-24-oic, 3α,7α-dihydroxy-5β-cholan-24-oic, and 3α,12α-dihydroxy-5β-cholan-24-oic acids were purchased from Steraloids (Wilton, N.H., U.S.A.). All were checked for purity by TLC and purified when necessary using preparative TLC (see below). 7α-Hydroxy-3-oxo-5β-cholan-24-oic, 6α-hydroxy-3-oxo-5β-cholan-24-oic, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic, and 12α-hydroxy-3-oxo-5β-cholan-24-oic acids were synthesized as outlined below. NAD, sodium borohydride and 3-hydroxysteroid dehydrogenase (3HSD; desiccated *Pseudomonas testosteroni* cells) were all purchased from Sigma (St. Louis, Mo., U.S.A.). Hydrazine hydrate (64% in water) was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.) and silica gel G from E. Merck, (Darmstadt, G.F.R.).

Pyrophosphate buffer (PPB), pH 10, 0.1 *M*, is prepared by dissolving 44.61 g of sodium pyrophosphate decahydrate in 900 ml of distilled water. The pH of the solution is adjusted to 10 with 0.1 *M* hydrochloric acid. The buffer is then diluted to 1 l with water. NAD solution is made by dissolving 45 mg of NAD in 100 ml of distilled water. Hydrazine hydrate solution is made by diluting 12.5 g of commercial hydrazine hydrate (64% in water) to 90 ml with PPB. The pH of the resulting solution is adjusted to 10 with 6 *M* hydrochloric acid and the final volume adjusted to 100 ml with PPB. 3HSD solution is prepared by homogenizing 100 mg of dried *Pseudomonas testosteroni* cells together with 10 ml of PPB for 5 min in an ice bath. The content of the homogenizer is centrifuged at 50,000 *g* for 20 min. The supernatant solution containing 3HSD is decanted and stored in an ice-bath until used. Alternately, dried extracts of *Pseudomonas testosteroni* cells are available (Sigma) and are simply dissolved in PPB prior to use.

METHODS

Synthesis of 3-oxo-5β-cholan-24-oic acids

One gram of the 3α-hydroxy-5β-cholan-24-oic acid corresponding to the desired 3-oxo-5β-cholan-24-oic acid dissolved in 50 ml of PPB, 2.5 g of NAD dissolved in 100 ml of PPB and 5 g of hydrazine hydrate dissolved in 40 ml of PPB are placed in a liter erlenmeyer flask. Crude desiccated *Pseudomonas testosteroni* cells (700 mg) are homogenized in 70 ml of PPB. The resulting suspension is centrifuged for 45 min at 20,000 *g*. The supernatant containing 3HSD is then added to the flask, 140 ml of PPB are added and the contents of the flask mixed. The flask and its contents are then incubated with shaking at 37° in a water-bath. Small aliquots of the solution are removed at 15-min intervals and their absorbance determined at 340 nm to check for NADH which results from the oxidation of the 3α-hydroxyl group of the bile acid. When there is no further increase in the absorbance, the flask is removed from the incubator and the pH of its content adjusted to 1 using 12 *M* hydrochloric acid. The solution is then transferred to a separatory funnel and extracted four times with two volumes of diethyl ether. The extract which contains the hydrazone of the 3-oxo-5β-cholan-24-oic acid is evaporated to dryness. The hydrazone is suspended in

100 ml of 2 *M* hydrochloric acid and refluxed for 30 min. The suspension is cooled, transferred to a separatory funnel and extracted four times with three volumes of ethyl ether. The ether extract containing the 3-oxo-5β-cholan-24-oic acid is evaporated to dryness. The crude acid is dissolved in chloroform–methanol (2:1, v/v) and purified via preparative TLC using plates coated with silica gel G and developed with ethyl acetate–isooctane–acetic acid (10:10:2, v/v/v). The final products were chromatographically pure; overall yields averaged from 50 to 60%.

Thin-layer chromatography

Preparation of plates. Silica gel G soft-coated plates (200 μm) were used in all quantitative and qualitative studies. To prepare them, 30 g of silica gel G are mixed with 70 ml of distilled water acidified with five drops of glacial acetic acid. The mixture is stirred until it just begins to thicken (about 8 min) and is then rapidly spread on 20 × 20 cm glass plates. After spreading, the plates are allowed to air-dry and are then stored in desiccators over silica gel. Prior to use, the plates are channeled so that each plate has 12 to 14 separate channels. The plates are then activated in a drying oven at 100° for 1 h.

R_F and relative mobility determinations. A 2-μl portion (10 μg) of each of the bile acid solutions (methanolic) is applied to the individual channels of the plate and the methanol is allowed to evaporate. The plates are developed in equilibrated (4 h) filter paper-lined glass tanks using ethyl acetate–isooctane–acetic acid (10:10:2). When development is complete, the plates are removed from the tanks and air-dried. The plates are then lightly sprayed with 0.05% of pyrene dissolved in hexane[5]. The bile acids are visualized using short-wave ultraviolet radiation. The positions of the centers of the spots are marked and the necessary measurements made.

Quantitative determination of 3-oxo-5β-cholan-24-oic acids. Aliquots (1–6 μl; 5–120 μg) of methanolic bile acid solutions are applied to the individual channels of TLC plates. The methanol is allowed to evaporate and the plates are developed in equilibrated (4 h) filter paper-lined glass tanks using ethyl acetate–isooctane–acetic acid (10:10:2). When developed, the plates are removed from the tanks and allowed to dry in a filtered stream of air at room temperature. The dry plates are sprayed with water[6] and the positions of the acids outlined with a sharp stylus. The plates are then allowed to dry thoroughly and the silica gel G in the marked areas is transferred to centrifuge tubes by scraping the gel from the plates with the squared end of a stainless-steel spatula. Silica gel from a blank plate developed and dried in the same way is transferred to centrifuge tubes for use in Blanks and Standards. Two ml of chloroform–methanol (2:1) are added to each centrifuge tube. The gel is suspended in the solvent by swirling the tubes with a vortex mixer; they are then warmed and swirled again. The tubes are now centrifuged for 5 min at 800 *g*. The supernatant fluid is quantitatively transferred to 10 × 100 mm screw-capped or glass-stoppered test tubes. The extraction is repeated three times. When extraction is complete, the fluid in the tubes is evaporated to dryness using a stream of air or nitrogen and a hot water-bath. The content of each tube, except the standards, is then dissolved in 0.5 ml of PPB. A 0.5-ml portion of a standard solution containing 90 μg of 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid per 0.5 ml is added to the blank tubes to be used as standards. A 0.2-ml portion of sodium borohydride solution (18 mg per 10 ml of PPB) is next added to each tube. The tubes are allowed to stand for

1 h at room temperature to effect complete reduction of 3-oxo-5β-cholan-24-oic acids to 3-hydroxy-5β-cholan-24-oic acids. A 0.2-ml portion of 12 *M* hydrochloric acid is now added to each tube and the tubes are placed in a hot water-bath to destroy excess sodium borohydride. After 10–15 min, 0.4 ml of 6 *M* sodium hydroxide is added to each tube to neutralize hydrochloric acid. It is well to test the pH of the first few tubes after adding alkali to make sure that they are slightly basic. If not, a small adjustment of the volume of alkali may be necessary. Exact pH adjustment is unnecessary at this point since PPB is added at a later stage in the procedure. Next, 1 ml of NAD solution, 1.2 ml of hydrazine hydrate solution and 0.3 ml of 3HSD solution are added. The tubes are thoroughly mixed and placed in a water-bath maintained at 37° for 30 min. Exact timing of the samples is unnecessary since the reaction is essentially complete in 30 min and after that time the absorbance of the solutions does not change appreciably for over 1 h. After incubation the absorbance of the solutions is determined at 340 nm.

RESULTS AND DISCUSSION

The μ*M* absorbance figures in the right hand columns of Table I show two things. First, in the range from 20 to 60 μg, absorbance is directly proportional to concentration, *i.e.*, Beer's law is followed. Second, since the standard deviations of the μ*M* absorbance values are small, the method has precision. The range of the method is greater than the figures in Table I imply since the relationship between mass and absorbance is linear from 5 μg to at least 120 μg.

A number of solvent systems were investigated to find a mixture which would

TABLE I

THIN-LAYER CHROMATOGRAPHY AND QUANTITATIVE DETERMINATION OF 3-OXO-5β-CHOLAN-24-OIC ACIDS

Solvent system: isooctane–ethyl acetate–acetic acid (10:10:2); TLC data are the average of four determinations.

-5β-Cholan-24-oic acid	R_F	*Relative mobility**	*μM Absorbance** ± S.D.*		
			μg 3-oxo-5β-cholan-24-oic acid applied to plate		
			20	*40*	*60*
3-Oxo-	0.64	9.25	1.46 ± 0.02	1.47 ± 0.01	1.44 ± 0.03
3,6-Dioxo-	0.48	6.92	1.51 ± 0.04	1.41 ± 0.03	1.43 ± 0.02
3,12-Dioxo-	0.44	6.41	1.44 ± 0.03	1.42 ± 0.01	1.38 ± 0.02
3,7,12-Trioxo-	0.24	3.50	1.43 ± 0.02	1.43 ± 0.05	1.46 ± 0.01
6α-Hydroxy-3-oxo-	0.32	4.58	1.38 ± 0.04	1.40 ± 0.02	1.43 ± 0.03
7α-Hydroxy-3-oxo-	0.42	6.00	1.43 ± 0.03	1.42 ± 0.05	1.47 ± 0.07
12α-Hydroxy-3-oxo-	0.39	5.58	1.47 ± 0.02	1.42 ± 0.05	1.44 ± 0.06
7α, 12α-Dihydroxy-3-oxo-	0.16	2.33	1.44 ± 0.04	1.45 ± 0.05	1.38 ± 0.03
3α,7α-Dihydroxy-	0.24	3.64	1.48 ± 0.05	1.40 ± 0.01	1.43 ± 0.04
3α,12α-dihydroxy-	0.29	4.25	1.40 ± 0.02	1.44 ± 0.03	1.39 ± 0.02
3α,7α,12α-Trihydroxy-	0.07	1.00	1.43 ± 0.04	1.44 ± 0.02	1.42 ± 0.01

* With respect to 3α, 7α,12α-trihydroxy-5β-cholan-24-oic acid.

** The absorbance at 340 nm (1-cm path) of the reaction products of 1 μ*M* of acid, 3HSD, NAD and hydrazine hydrate in a 3.8 ml total volume.

successfully separate the 3-oxo acids. Ethyl acetate–isooctane–acetic acid (10:10:2) was the most satisfactory. The R_F and relative mobility data in Table I show that this solvent system effectively separates the acids. Very small samples of some of the acids, however, must be applied to the plates to achieve complete separation because of similarities in some R_F values.

Several types of substances were tested before a detection reagent was found that was compatible in the quantitative procedure. Iodine vapor was tried and was capable of detecting from 5 to 20 μg of oxo acid. Iodine could be removed from the plates by allowing them to stand in a stream of air at room temperature. Although it would appear that iodine would be satisfactory for locating the acids prior to quantification, we have encountered cases (fecal extracts) where it is very difficult or impossible to remove the last traces of this substance. A spray reagent containing 0.05% pyrene dissolved in hexane can be used to detect very small amounts of the oxo acids (<0.5 μg) under ultraviolet radiation[5]. Unfortunately, pyrene interferes during quantification. Although it is possible to remove pyrene from the plates by redeveloping with ethyl ether–light petroleum (b.p. 36–54°) (2:3, v/v) prior to quantification of bile acids, this cannot be done in the case of oxo acids since many of them migrate to some extent during redevelopment. A water spray[6] appears to be a satisfactory detecting agent since it is easy and completely removable. Although it is not as sensitive as pyrene, it is capable of detecting from 1 to 2 μg of the oxo acids which is somewhat below the practical limits of the quantitative procedure. Care must be taken to observe the plates during drying since various oxo acids show up most clearly at different stages of this process.

It might appear that the method could be simplified by carrying out borohydride reduction and quantification with 3HSD without prior removal of silica gel. In fact, it has been shown[7] that it is unnecessary to elute bile acids from silica gel during their quantification with 3HSD. We therefore attempted this modification of the procedure. The results were entirely unsatisfactory with losses ranging up to 55%. Interestingly, if silica gel was added to a solution containing 3-oxo acids and reduction and quantification effected, recoveries were satisfactory. This probably means that borohydride reduction does not take place if the acids are absorbed on silica gel. 3HSD, on the other hand, is capable of catalyzing the oxidation of absorbed bile acids.

The reduction of the 3-oxo group is very rapid in the presence of sodium borohydride: 0.3 mg of borohydride is able to reduce 60 μg of oxo acid in less than 30 min; 6- and 7-oxo groups are also easily reduced. 12-Oxo groups are slowly reduced and as much as 24 h are required to effect complete reduction of the 12-oxo group of 3,7,12-trioxo-5β-cholan-24-oic acid.

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