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Note

Thin-layer chromatographic separation of keto derivatives of free bile acids

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Chavez and Krone [1] reported that the 3-keto derivatives of cholic and lithocholic acids can be separated by thin-layer chromatography (TLC) on pre-coated silica-gel plates using Petcoff's developing solution (hexane—methyl-ethylketone—glacial acetic acid, 56:36:8, v/v). Reported here is the verification of this observation and in addition the separation of the 3-keto derivatives of deoxycholic and chenodeoxycholic acids.

METHODS

Experimental conditions were identical to those previously reported [1]. The 3-keto derivatives of cholic, chenodeoxycholic, deoxycholic and lithocholic acids (Supelco, Bellefonte, Pa., U.S.A.) were prepared by exposure of these bile acids separately to the 3-α-hydroxy steroid dehydrogenase (STDHP; Worthington Biochemicals, Freebold, N.J., U.S.A.) enzymatic mixture under the conditions described by Iwata and Yamasaki [2] and MacDonald [3]. The bile acids were allowed to react for 15 min and then acidified with 5 *N* hydrochloric acid. Then the bile acids and their derivatives were extracted with diethyl ether, the ether phase washed three times with distilled water, exposed to anhydrous sodium sulfate and then filtered. The ether phase was evaporated under a gentle stream of air in a warm water bath. The remaining material was re-dissolved in methanol and subjected to TLC (plates, EM 5763 from EM Labs., Elmesford, N.Y., U.S.A.) in Petcoff's solution along with the parent free bile acids. Under these conditions each 3-keto derivative appears as a major band though additional minor bands are seen. All bands were detected with a fine water spray [4] and the major bands were marked, scraped and eluted with ether, filtered and then evaporated to dryness. All the eluted material was

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re-dissolved in methanol and re-chromatographed. This procedure was performed twice with each band to assure purity. The presence of 3-keto derivatives was confirmed by phenylhydrazone formation when reacted with 2,4 dinitrophenol hydrazine, keto absorption in infra-red spectroscopy and failure to produce reduced diphospho, ‐ridine nucleotide (DPNH) upon re-exposure to the 3-α-hydroxy steroid dehydrogenase enzymatic mixture. In addition, standards of 3-keto derivatives of cholic and lithocholic acid obtained commercially (Steraloids, Wilton, N.H., U.S.A.) chromatographed at the position identical with those prepared as mentioned.

The 3-keto derivatives of chenodeoxycholic and deoxycholic acids when chromatographed with Petcoff's solution migrated at the same R_F. Separation of these two keto derivatives was accomplished by using a second developing solution of 1% glacial acetic acid in diethyl ether. A sample of human duodenal fluid was deconjugated according to Nair and co-workers [5—7] and chromatographed with the two solutions.

RESULTS

Fig. 1 shows the separation of the four free bile acids found in human bile and their respective 3-keto derivatives achieved with Petcoff's solution. Fig. 2* is a chromatogram of deconjugated bile acids from intestinal fluid along with appropriate standards developed in Petcoff's solution. Although the 3-keto

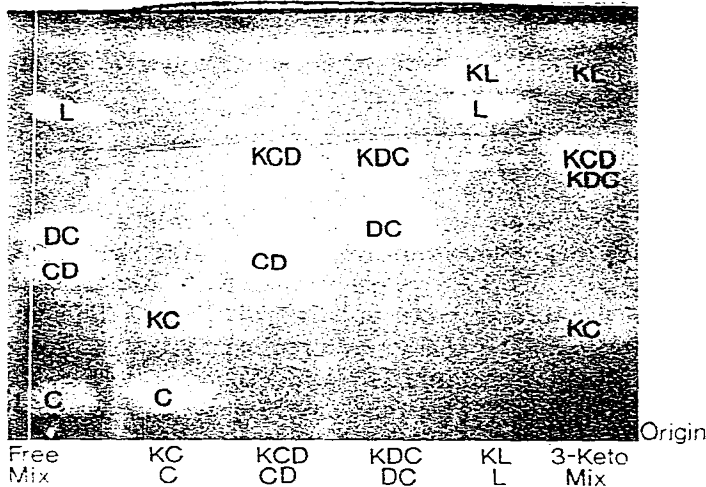

Fig. 1. Separation of four free bile acids and their respective 3-keto derivatives in Petcoff's solution. From left to right: Free mix, top to bottom, lithocholic acid (L), deoxycholic acid (DC), chenodeoxycholic acid (CD), and cholic acid (C); 3-keto cholic acid (KC) and cholic acid; 3-keto chenodeoxycholic acid (KCD) and chenodeoxycholic acid; 3-keto deoxycholic acid (KDC) and deoxycholic acid; 3-keto lithocholic acid (KL) and lithocholic acid and 3-keto bile acid mix, top to bottom, 3-keto lithocholic, 3-keto deoxycholic with 3-keto chenodeoxycholic and 3-keto cholic acids.

*Figs. 2 and 3 are both composite photographs of separate TLC plates to illustrate more clearly the TLC separation of the compounds.

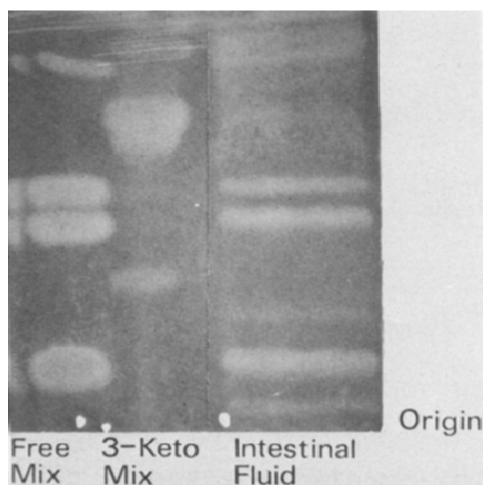

Fig. 2. Separation of four free bile acids, 3-keto derivatives of these four bile acids and hydrolyzed intestinal fluids in Petcoff's solution. From left to right: Free mix and 3-keto derivatives same as Fig. 1 and hydrolyzed intestinal fluid. Minor bands are unknown compounds.

derivatives of cholic and lithocholic acid separate well from each other and their parent compounds, the 3-keto derivatives of chenodeoxycholic and deoxycholic acids migrate as one band.

Figs. 3* and 4 show the separation of these compounds in 1% glacial acetic acid in ether. Although this developing solvent clearly separates the 3-keto derivatives of cholic, chenodeoxycholic, deoxycholic and lithocholic acids from each other, it fails to separate chenodeoxycholic and deoxycholic acids. The trailing seen on Figs. 2 and 4 in the intestinal fluid sample can, in actual prac-

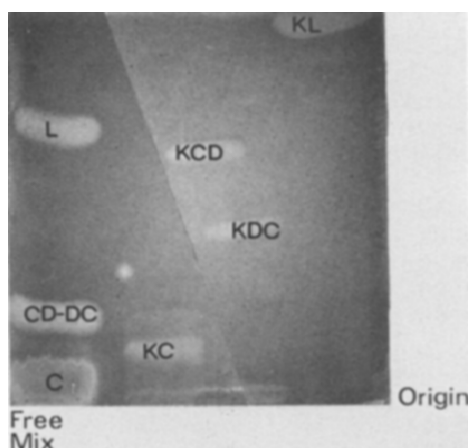

Fig. 3. Separation of four free bile acids and the 3-keto derivatives of the four free bile acids in 1% glacial acetic acid in diethyl ether. Free bile acids mix, from top to bottom: Lithocholic acid (L), chenodeoxycholic acid (CD) with deoxycholic acid (DC); and cholic acid (C); 3-keto derivatives, from left to right: KC, KCD, KDC and KL.

*See footnote on p. 72.

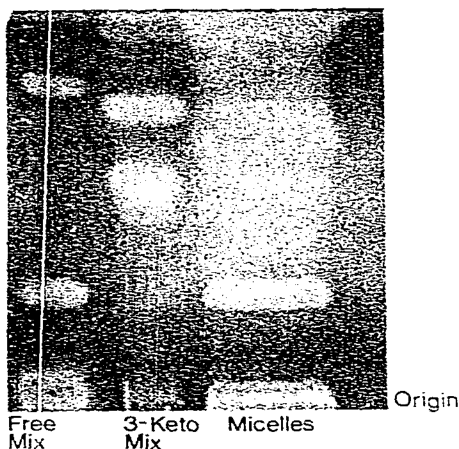

Fig. 4. Separation of four free bile acids, 3-keto derivatives and hydrolyzed intestinal fluid in 1% glacial acetic acid in diethyl ether. Free mix and 3-keto mix same as Fig. 3 (3-keto cholic acid is not seen because of very low concentration). Hydrolyzed intestinal fluid same as Fig. 2.

tice, be avoided by applying it as a longer streak to the plate. Obviously, the intestinal fluid contains only traces of the keto bile acids.

Table I shows the approximate R_F values for the compounds in the solvent systems reported here. Standards should always be included in the chromatograph as these values vary somewhat with each run.

TABLE I

R_F VALUES FOR FREE BILE ACIDS AND THEIR 3-KETO DERIVATIVES.

Solvent 1 = Hexane—methylethylketone—glacial acetic acid (56:36:8, v/v).
Solvent 2 = Glacial acetic acid—diethyl ether (1:99, v/v).

	Solvent 1 (Petcoff's Solution)	Solvent 2 R_F 1.00 = 14.0 cm
Free bile acids		
Cholic acid	0.11	0.04
Chenodeoxycholic acid	0.46	0.20
Deoxycholic acid	0.52	0.20
Lithocholic acid	0.77	0.68
3-Keto derivatives of:		
Cholic acid	0.31	0.11
Chenodeoxycholic acid	0.68	0.61
Deoxycholic acid	0.68	0.41
Lithocholic acid	0.99	0.96

DISCUSSION

Depending on the individual investigator's interest, one may use one or both developing systems to separate the four basic human bile acids and their respective 3-keto derivatives. If quantitation is desired the plates must be prewashed with the developing solution, otherwise a faint yellow band will separate along or near the lithocholic acid band. This band will be eluted with ether along with lithocholic acid and turns into a deeper yellow color at pH 9.5, increasing the spectrometric quantitative readings of the DPNH. This may be the same interfering compound discussed by Sandberg, et al. [8] and explains the unaccountably high recovery of lithocholic acid reported by Bruusgaard [9]. Quantification of the 3-keto compounds of cholic and chenodeoxycholic acids might be achieved by using the enzymatic mixture of 7-α-hydroxy steroid dehydrogenase as suggested by Hazelwood et al. [10] and MacDonald et al. [11]. The derivatives of deoxycholic might be determined by using the 12-α-hydroxy steroid dehydrogenase enzyme if it becomes available. In certain situations the preliminary separation by TLC may make the quantification by gas—liquid chromatography easier and more accurate.

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