# QUANTITATIVE DETERMINATION OF BILE ACIDS BY DIRECT DENSITOMETRY OF THIN-LAYER CHROMATOGRAMS*

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A method for the quantitative determination of bile acids in biological samples, using densitometry, was developed. The results by this method were compared with those obtained by the spectrophotometric method of GÄNSHIRT *et al.*[1]. The instrument used was a Photovolt Recording Transmission Densitometer equipped with an automatic recording integrator and a thin-layer chromatoplate stage, which allowed for the automatic scanning of thin-layer chromatography (TLC) plates. Curves were determined for several bile acids, both free and conjugated, to establish if a linear relationship exists between the quantity of acid in the spot and the area of the density peak, as determined by the automatic integrator.

## EXPERIMENTAL

### *Preparation of the plates*

Thin-layer plates, 150 $\mu$ thick, were prepared according to STAHL[2], using Silica Gel G. The applicator used, a Unoplan Leveller**, has a special leveling device which insures uniformity in the heights of the surfaces of the several plates. The plates were activated at 110° for 2 h, and stored over silica-gel desiccant until used.

### *Preparation of the standards*

Standard solutions were prepared of several concentrations of the following acids: cholic (CA), chenodesoxycholic (CDCA), desoxycholic (DCA), lithocholic (LCA), hyocholic (HCA), hyodesoxycholic (HDCA), glycocholic (GCA), glycodesoxycholic (GDCA), glycohyodesoxycholic (GHDCA), and taurodesoxycholic (TDCA). CA and CDCA were purified by TLC. The impurities were separated by developing in the appropriate solvent system, locating by spraying with water[1], and eluting the spot with ethanol. Approximately 50 mg of pure acid was obtained from each chromatogram by this method.

### *Preparation of biological samples*

Female Sprague-Dawley rats were sacrificed, their small intestines removed and lyophylized. The dried samples were ground with sand and placed in extraction thimbles. Fecal steroids were removed by continuous extraction with ethanol for

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** Manufactured by Colab Laboratories Inc., Chicago Heights, Illinois.

48 h, using Bailey-Walker extractors. The samples were then hydrolyzed with 7 *N* NaOH, by autoclaving at 15 lb pressure for 3 h. Extraction in liquid–liquid extractors with petroleum ether for 2 h removed $\alpha$ and $\beta$ sterols. After acidification with $H_2SO_4$, fatty acids were removed by extraction with petroleum ether and extraction continued for 6 h with ethyl ether in liquid–liquid extractors. This final ether extract was evaporated to dryness, and the residue taken up in ethanol and chromatogrammed.

## DENSITOMETRY

For each bile acid, a TLC plate, marked off in channels 8–10 mm in width to prevent the spots from spreading, was spotted with varying quantities of the acid, using 1 $\mu$l disposable pipets*, and developed in the appropriate solvent system. A system of trimethyl pentane–ethyl acetate–acetic acid (10:10:2, v/v) was used for the DCA, LCA, CDCA and HDCA standards, and the determination of CDCA in biological samples; for the CA and HCA standards and the spectrophotometric determination of CA in biological samples, the system was ethyl acetate–acetic acid (96:4, v/v), and for the conjugated bile acids, an amyl acetate–acetic acid–propanol–water (20:15:10:5, v/v) system was employed.

A variety of solvent systems were used to effect maximum separation and ease of scanning. For example, the trimethyl pentane system, used for the CDCA determination in the biological samples, separates desoxycholic acid from chenodesoxycholic acid[3], but does not move cholic acid far enough from the origin to permit a quantitative determination of the spot. On the other hand, the ethyl acetate–acetic acid solvent permits a CA determination but does not separate CDCA and DCA.

The plates were sprayed with a 20% solution of phosphomolybdic acid in ethanol, and heated at 110° for 15 min. After positioning under the photometer to obtain a maximum density reading for each spot, the plate was held in place by means of clamps attached to the stage, and the recorder zeroed in a clear region just above the spot. The areas of the peaks were then computed by the automatic integrator. An L-5 response setting was used for most determinations, including the biological samples, and a D-2 response when a range of 0.5 $\mu$g to 4.0 $\mu$g was required.

The same procedure was followed for both standard and biological samples, except that each biological sample was done in duplicate with an internal standard of 0.5 $\mu$g of CA or CDCA added to one of the samples by spotting 1 $\mu$l of a 0.5 $\mu$g/$\mu$l solution of the acid to be determined along with the sample. The ratio between the integrator units of the peaks of the standard and the biological sample was used to determine the amount of bile acid present in each sample spot, according to the formula:

$$\text{Bile acid (mg)} = \frac{\text{(mg of standard) (peak area of sample)}}{\text{peak area with internal standard} - \text{peak area without standard}}$$

In order to insure accuracy and reproducibility in this method, the TLC plates must be absolutely uniform. Furthermore, the spraying procedure must be standardized by using the same volume of spray for each plate, spraying evenly and thoroughly, and heating at 110° for no longer than 15 min. HARA *et al.*[4] have also investigated the quantitative aspects of the preparation of the plate and conditions for coloring.

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## SPECTROPHOTOMETRY

The densitometry results were checked by the spectrophotometric method of GÄNSHIRT *et al.*[1], using the same biological samples evaporated to a smaller volume, spotted again in quantities of 5–10 $\mu$l, and developed. A set of standards of known concentration was also spotted on each plate. The plate was sprayed with water to locate the desired bile acid spot, which was then marked. After drying, the spot was scraped off into a test tube to which 3.5 cc of 65 % $H_2SO_4$ was added. The mixture was heated at 60° for exactly 1 h, and cooled under running water. The absorbance of the standards and biological samples was read at a wave length of 385 m$\mu$ on a Beckman DU Spectrophotometer. A curve of the amount of standard *vs.* absorbance was plotted, and the quantity of bile acid read from this graph.

## RESULTS AND DISCUSSION

When plots were made of the bile acid concentrations against the logarithmical densitometric units, a linear relationship was obtained in all cases, provided the density of the spot was within the range (0.25 $\mu$g to 4.0 $\mu$g) of the densitometer (Figs. 1 and 2). Standard curves had to be prepared for each bile acid since the slopes

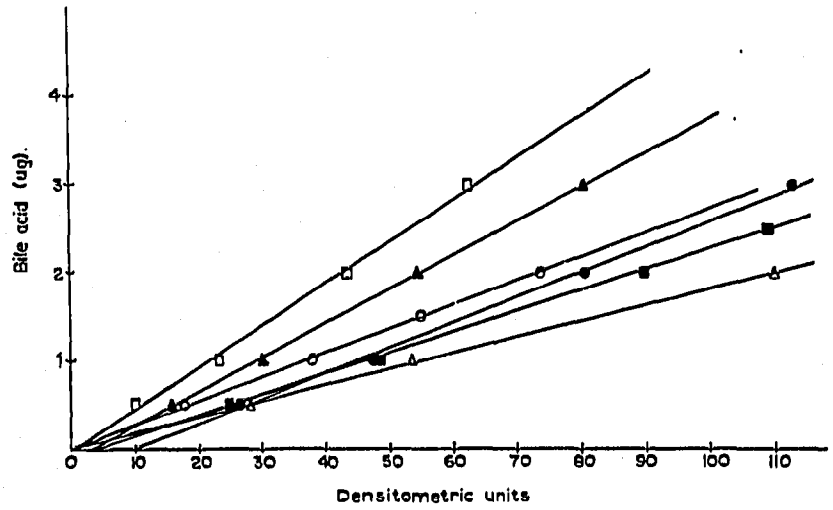


Fig. 1. Densitometry of free bile acids. Peak area in densitometric units plotted against concentration of the acid. ● = cholic, ○ = chenodesoxycholic, ▲ = hyodesoxycholic, △ = hyocholic, ■ = lithocholic and □ = desoxycholic acid.

of the several curves varied somewhat. Therefore, it was found advisable to use an internal standard of the anticipated size of the biological sample rather than make a standard curve for each determination. The variation of the slopes may be attributed to several factors such as the intensity of stray or reflected light[5], the amount of background color caused by the detection spray (which can be minimized by the use of filters), and the purity of the standards (Fig. 3).

A comparison of the results of the CA and CDCA determinations by the two methods is given in Table I. The agreement between the two sets of values is good considering the great difference in the conversion factors used to calculate the final

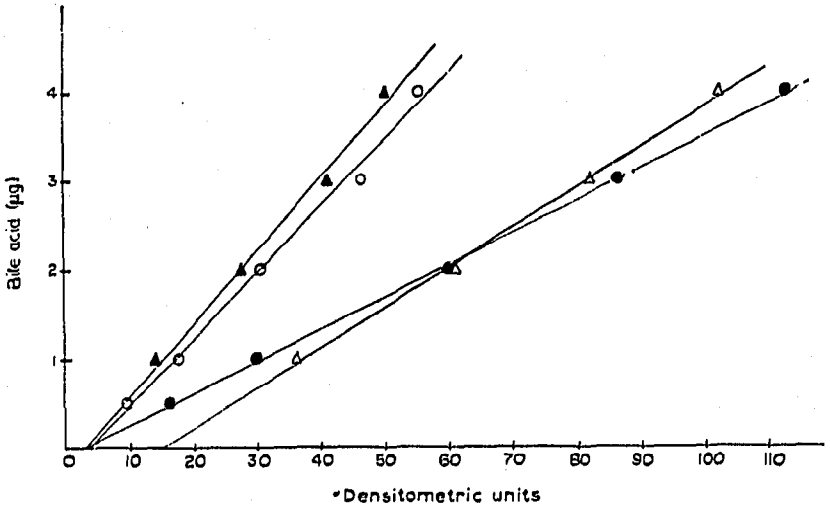


Fig. 2. Densitometry of conjugated bile acids. Peak area in densitometric units plotted against concentration of the acid. ● = glycocholic, ○ = glycohyodesoxycholic, ▲ = taurodesoxycholic, and △ = glycodesoxycholic acid.

result. In the densitometric method, approximately 0.5 µg must be determined quantitatively on the plate, and be converted to about 20 mg, employing an aliquot dilution factor of approximately 28, while the spectrophotometric method needs a dilution factor of only 3. In this way any error in the actual determination of the acid by densitometry is magnified 9 times more than an error in the spectrophotometric value. (However, densitometry of 0.5 µg of either CA or CDCA in biological samples resulted in an average standard deviation of 0.030 µg when the same sample was determined repeatedly on different channels of the same plate, and of 0.033 µg when the sample was determined on several different plates.)

Total time for the densitometric analysis of biological samples (from final extract to results) is about 4 h. Since several samples may be run simultaneously, and there is no removal of the spots or other extraneous procedure, this method has

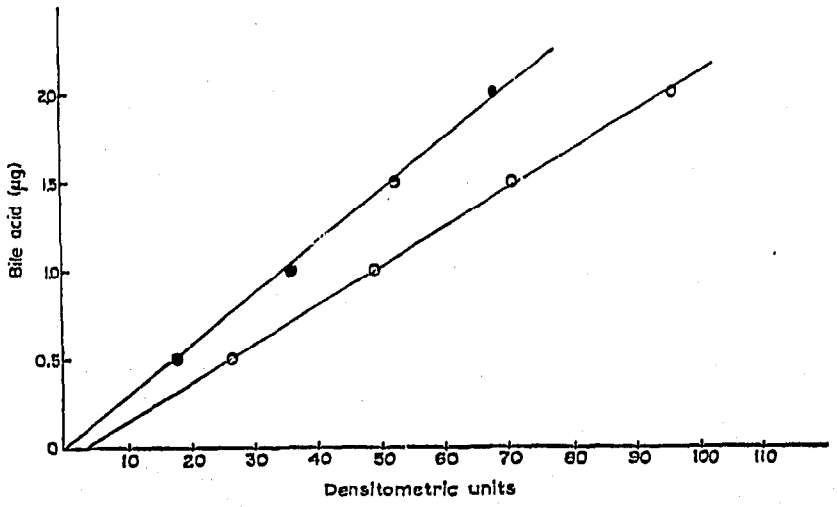


Fig. 3. Effect of purification on the slope of a standard densitometry curve. ○ = impure chenodesoxycholic acid, ● = chenodesoxycholic acid purified by TLC.

TABLE I

COMPARISON OF BILE ACID DETERMINATION BY DENSITOMETRY AND SPECTROPHOTOMETRY
Each value represents the average of 3 or 4 determinations.

| *Sample No.* | *Cholic acid (mg)* | | *Chenodesoxycholic acid (mg)* | |
|---|---|---|---|---|
| | *Densitometer* | *Spectrophotometer* | *Densitometer* | *Spectrophotometer* |
| 1 | 19.4 ± 1.8 | 23.0 ± 3.5 | 2.00 ± 0.12 | 2.66 ± 0.28 |
| 2 | 20.2 ± 1.7 | 21.3 ± 2.9 | 2.56 ± 0.18 | 3.54 ± 0.17 |
| 3 | 15.7 ± 0.8 | 18.9 ± 1.8 | 2.69 ± 0.57 | 3.57 ± 0.59 |
| 4 | 21.7 ± 1.0 | 21.3 ± 2.6 | 2.66 ± 0.36 | 3.38 ± 0.21 |
| 5 | 24.6 ± 3.7 | 20.3 ± 1.6 | 5.06 ± 1.50 | 4.67 ± 0.36 |
| 6 | 17.8 ± 1.7 | 20.6 ± 2.0 | 4.84 ± 0.05 | 4.48 ± 0.09 |

distinct advantages in comparison with others currently employed for bile acid analysis.

A preliminary investigation by HARA and coworkers[6] indicated the feasibility of using thin-layer densitometry for the quantitative determination of bile acids. They designed a densitometer which permitted scanning of a chromatoplate in two directions, and with this instrument determined standard curves for taurocholic and glychocholic acids. They did not, however, apply their method to free bile acids or to the quantitative determination of bile acids in biological samples.

SUMMARY

A method was developed for quantitative analysis of bile acids by chromatography and densitometry. Within the range of experimental error, the results of cholic and chenodesoxycholic acid determinations on biological samples agreed with those obtained by the spectrophotometric method of GÄNSHIRT[1]. Reproducibility was influenced by the uniformity of the plate-coating and of the spraying, by width of channels, and by spot size. Since several samples may be run simultaneously and it is unnecessary to locate and remove them from the plates for spectrophotometric analysis, this method has advantages over methods currently employed for bile acid analysis.

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