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HIGH-PERFORMANCE LIQUID-CHROMATOGRAPHIC SEPARATION OF INDIVIDUAL BILE ACIDS: FREE, GLYCINE AND TAURINE CONJUGATED BILE ACIDS

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High-performance liquid-chromatographic separation of bile acids, free and conjugated with taurine and glycine, is described. The analysis of free and glycine conjugated bile acids is based on the esterification of the carboxylic group of bile acids with 1-p-nitrobenzyl-3-p-tolyltriazene. On the other hand, taurine conjugated bile acids are separated and detected by an ultraviolet detector (210 nm), directly.

Despite the improvement of gas-liquid chromatographic analysis for the estimation of free bile acids[1),2)], it is unable to separate individual conjugated bile acids by this method. Moreover, the preparation of sample for gas-liquid chromatography is complicated, and this procedure has the difficulties in the accuracy. On the other hand, the separation of individual bile acids, free and conjugated, by thin layer chromatography was reported on the biological samples[3)]. However, there were the difficulties about the quantitative analysis on the sample of low content of bile acids like serum. From necessity of data on conjugated as well as free bile acids in clinical medicine, we have developed a method using high-performance liquid chromatography.

As we have found that p-nitrobenzyl esters of glycine conjugated and free bile acids possess characteristic ultraviolet absorption spectra, an attempt to application of this character for the analysis of these bile acids was done. On the other hand, taurine conjugated bile acids were separated by high-performance liquid chromatography, and detected by UV detector.

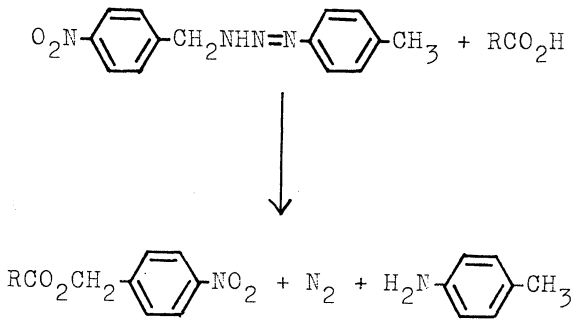
Materials and Methods

The bile acids were obtained from Sigma, St. Louis. Mo., Calbiochem, San Diego, Calif. and Katayama Kagaku Kogyo, Japan. 1-p-Nitrobenzyl-3-p-tolyltriazene was obtained from Regis Chemical Co.

A Varian 4200 liquid chromatograph and a Jasco 350 liquid chromatograph were used throughout this work. These instruments were fitted with UV (254 nm) detector and a gradient elution accessory, and with UV (210 nm) detector, respectively. MicroPak-NH_2 column, which was commercially available from Varian instrument division, was used for the analysis of p-nitrobenzyl esters of bile acids. The analysis for taurine conjugated bile acids was done by the use of μ bondapak/C_{18} column prepared by Waters Associates. Sample injection was done from a 25 μl syringe, with the column flow stopped. On the analysis of free and glycine conjugated bile acids, the condition was as follows; column: MicroPak-NH_2 (25 cm x 1/8" i.d.), mobile phase: A- iso-octane/dichloromethane= 1, B- iso-octane/dichloromethane= 1/9, gradient slope: 0.5 %/min, flow rate: 60 ml/hr, detector: UV (254 nm). Moreover, on the analysis of taurine conjugated bile acids, the condition was as follows; column: μ Bondapak/C_{18} (1' x 1/4"i.d.), mobile phase: methanol/0.01 M KH_2PO_4= 3 (V/V), flow rate: 60 ml/hr, detector: UV (210 nm).

Procedure

In order to apply this method to the analysis of biological samples, individual bile acids were prepared by the extraction with XAD-2 column[4], followed by the recovery as free carboxylic acids through CM-cellulose column. The resulting bile acids were esterified with a threefold excess of 1-p-nitrobenzyl-3-p-tolyltriazene in ethanol as illustrated in the following equation[5].



Results and Discussion

The separation of p-nitrobenzyl esters of the individual bile acids including unconjugated and glycine conjugated bile acids was investigated by using high-performance liquid chromatography. Fig. 1 shows the result. Each peak is equivalent with approximately 4 μg of bile acids. The identification of each peak was done according to the addition of p-nitrobenzyl ester of each bile acid which was synthesized, respectively. Especially, the structure of p-nitrobenzyl ester of cholic acid was established by the analysis of IR, NMR, and Mass spectra. Separation between deoxy-

cholic acid and chenodeoxycholic acid was not recognized. It is supposed that other peaks except the peaks of p-nitrobenzyl esters of bile acids result from the excess reagent and degradation products of the reagent such as p-toluidine etc. in the esterification of bile acids.

The analysis of a small amount of cholic acid was attempted. Two μg of cholic acid was esterified with 6 μg of 1-p-nitrobenzyl-3-p-tolyltriazene in a sealed tube at 80° for 30 minutes. After the reaction, the total product concetrated under reduced pressure was injected into liquid chromatograph. Consequently, one μg of cholic acid could be detected on chromatogram.

Fig. 2 shows analysis of taurine conjugated bile acids (each 5 μg).

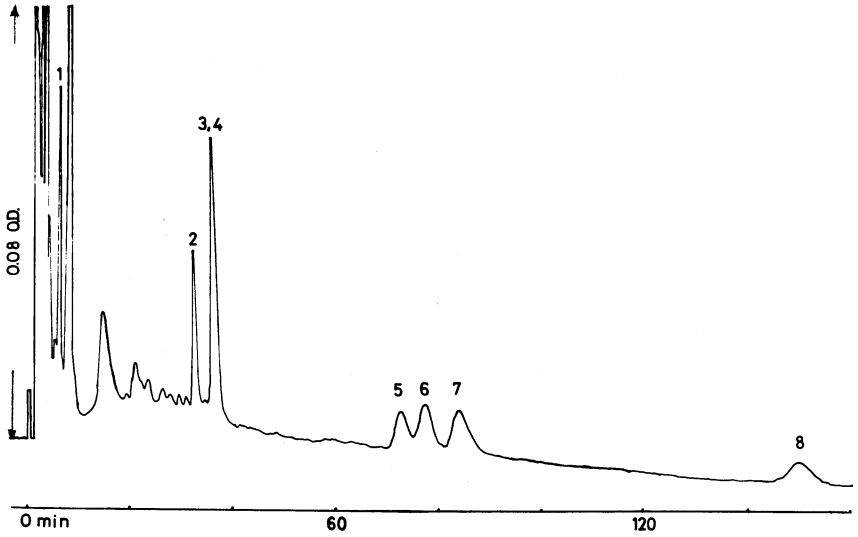


Fig. 1. Liquid chromatographic analysis of p-nitrobenzyl esters of bile acids; 1: lithocholic acid, 2: glycolithocholic acid, 3: deoxycholic acid, 4: chenodeoxycholic acid, 5: glycodeoxycholic acid, 6: glycochenodeoxycholic acid, 7: cholic acid, 8: glycocholic acid.

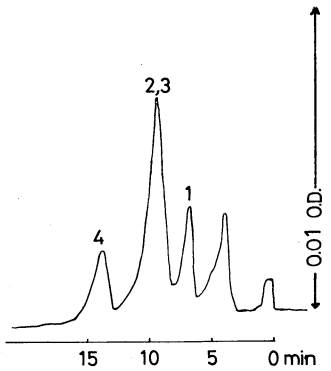


Fig. 2. Liquid chromatographic analysis of taurine-conjugated bile acids; 1: taurolithocholic acid, 2: taurodeoxycholic acid, 3: taurochenodeoxycholic acid, 4: taurocholic acid.

The procedure described has the distinctive performance which makes possible the individual separation of the conjugated and free bile acids, compared with gas-liquid chromatography. However, in order to complete the separation between deoxycholic acid and chenodeoxycholic acid in free and taurine-conjugated, it should be considered to examine the selection of mobile phase and column. As a matter of fact, this method was applicable for the analysis of bile acids in the biological samples, i.e. bile and serum. The analytical data on the biological samples will be described elsewhere. The development of the quantitative analysis of individual conjugated and free bile acids by the high-performance liquid chromatography may extend the value of the studies of bile acid metabolism in the clinical investigation of hepatobiliary disease.

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