

Estimation of bile acid pool sizes from their spillover into systemic blood

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Abstract We have examined the possibility of assessing primary bile acid pool sizes from the spillover of the bile acids into systemic blood after intestinal exposure to the total endogenous bile acid pool; the studies were carried out in 16 healthy subjects. Bile acid spillover was calculated as the integrated area under the curve of bile acid conjugates in serum of each primary bile acid class in response to a well-defined sustained cholecystokinin-induced stimulus of the enterohepatic circulation for 55 min causing complete gallbladder emptying. Serum levels of each species of primary bile acid conjugates were measured by two specific and sensitive radioimmunoassays, one for conjugated cholate and one for conjugated chenodeoxycholate. Primary bile acid pool sizes determined with [24-¹⁴C]cholic acid and [24-¹⁴C]chenodeoxycholic acid according to Lindstedt (1957. *Acta Physiol. Scand.* 40: 1-9) served as reference. Bile acid conjugates of both species reached a peak 70 min after the start of the cholecystokinin infusion, probably reflecting simultaneous intestinal absorption of both primary bile acids in this model. Highly significant linear correlations were found between the integrated areas under the curve and primary bile acid pool sizes, which were closer for chenodeoxycholate ($n = 16$, $r = 0.81$, $P < 0.001$) than for cholate ($n = 16$, $r = 0.74$, $P < 0.005$). Although these linear correlations did not allow accurate prediction of individual primary bile acid pool sizes from serum bile acid measurements, identification of populations with small (under $25 \mu\text{mol} \cdot \text{kg}^{-1}$) or large (over $50 \mu\text{mol} \cdot \text{kg}^{-1}$) chenodeoxycholate pool sizes was possible as judged from the 95% confidence intervals of the linear regression lines. The utility of this approach of cholate pool size assessment proved to be far less. Apart from the integrated areas under the curve, peak levels of each bile acid conjugate at 70 min were also noted to be closely correlated with primary bile acid pool sizes, again with greater significance with respect to chenodeoxycholate ($n = 16$, $r = 0.84$, $P < 0.001$) than to cholate ($n = 16$, $r = 0.52$, $P < 0.05$). Assessment of chenodeoxycholate pool size could be made with similar accuracy from bile acid peak levels as from the integrated area under the curve, but not from basal fasting bile acid levels in the serum. We conclude that our results confirm the suggested, but hitherto unproven, relationship between primary bile acid pool size and primary bile acid spillover into systemic blood. Since such a relation will be valid only if enterohepatic circulation of the total bile acid pool occurs, stimulation by cholecystokinin infusion and normal contractility of the gallbladder appear to be essential prerequisites. Measurement of responses of chenodeoxycholy conjugates in serum offers a potential alternative for the estimation of chenodeoxycholate pool sizes by isotope dilution in bile in population studies, thus circumventing the use of radioisotopes and

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Supplementary key words gas-liquid chromatography • radioimmunoassay • cholic acid • chenodeoxycholic acid • serum bile acids

Isotope dilution techniques in bile have been applied frequently to quantify bile acid pool size and turnover since their introduction by Lindstedt in 1957 (1). Various simplified modifications of the original method have been published (2, 3). However, all involve collection of duodenal bile after administration of radioactive bile acids. This procedure is time-consuming and not well tolerated, and carries some radiation hazard for the subject. Alternatively, when bile acids with an abundance of stable isotopes are used as markers, sophisticated and very expensive analytical techniques like combined gas-liquid chromatography-mass spectrometry are required. The hitherto commonly used methods preclude, therefore, a more widespread use of measurement of bile acid pool sizes in population studies. Nevertheless, such studies may be of interest since small bile acid pool sizes, especially of chenodeoxycholate (CDC), have been noted frequently in cholelithiasis (4, 5).

The present investigations were designed to evaluate a simple alternative method to estimate primary bile acid pool sizes by measurement of bile acids in serum that would be applicable to population studies in man. The established Lindstedt method (1) in duodenal bile was used as a reference. Our hypothesis was that changes of the levels of conjugates of each primary bile acid in serum in response to the endogenous bile acid load may be used to assess primary bile acid pool sizes, at least under well-defined conditions of a standardized cholecystokinin (CCK) infusion which mobilizes the complete bile acid

Abbreviations: CDC, chenodeoxycholate; CCK, cholecystokinin; C, cholate.

pool for one single enterohepatic circulation. This idea originates from the knowledge that intestinal input of bile acids into the portal blood is the major determinant of nonfasting systemic levels of serum bile acids (6, 7).

It has been convincingly demonstrated that even in the presence of liver disease fractional hepatic uptake of various loads of bile acids remains constant during physiological changes of the enterohepatic circulation and that differences between healthy subjects in this respect are relatively small (7–10). In view of the high efficiency of bile acid absorption in healthy subjects without intestinal disorders (11), evacuation rate of the biliary tract and individual differences in bile acid pool size should be considered as the main potential variables of the actual bile acid input into the portal circulation and the consequent serum bile acid profile.

This report describes a comparative study of primary bile acid pool sizes and serum levels of conjugates of each bile acid class measured by separate sensitive and specific radioimmunoassays in healthy subjects with proven normal gallbladder emptying to test the above-mentioned hypothesis. The predictive power of basal serum bile acid levels and the serum bile acid response to an effective, sustained CCK stimulus of the enterohepatic bile acid circulation regarding primary bile acid pool size was analyzed. The results indicate that this procedure may render assessment of chenodeoxycholate pool sizes a more feasible option than it used to be.

METHODS

Subjects

Sixteen healthy subjects (4 males, 12 females), 33–72 years of age, agreed to participate in the study after informed consent. All had a normal bowel function and were free of intestinal or metabolic disorders. Eligibility required a noncompromised hepatic function and fasting normolipidemia according to conventional laboratory tests (serum triglycerides and cholesterol, bilirubin, alkaline phosphatase, γ -glutamyl transpeptidase and transaminases). All had normal cholecystograms and virtually complete gallbladder emptying during CCK infusion (1.2 U \cdot kg⁻¹ \cdot hr⁻¹ for 55 min) as ascertained by a validated method of quantitative cholescintigraphy (12). Gallbladder residue was $4 \pm 3\%$. During the study all subjects were admitted to the clinical study unit and took a standard hospital diet. The study protocol was approved by the Ethical Committee on Research in Human Subjects at the Municipal Hospital of Arnhem on December 2, 1980.

Experimental design

Serum bile acid responses to CCK infusion. After an overnight fast, a saline drip was inserted into the antecubital

vein to administer CCK (Kabi-Vitrum, Studsvik, Sweden) intravenously by means of an infusion pump (1.2 U \cdot kg⁻¹ \cdot hr⁻¹ for 55 min). From the opposite arm, 19 serial blood samples were taken for serum bile acid measurement from an indwelling needle kept patent with a slow flow of saline solution (at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 105, 120 and 150 min after the start of the CCK infusion).

Bile acid kinetics. On four consecutive mornings concentrated bile samples were aspirated through a duodenal polyvinyl tube with a terminal mercury bag following a 12-hr overnight fast and intravenous gallbladder stimulation by intravenous injection of CCK-octapeptide, sincalide (Kinevac, E. Squibb, Princeton, NJ). In the evening prior to the first bile sampling at 6:00 PM, 5 μ Ci of [24-¹⁴C]cholic acid (sp act 52 mCi/mmol) and 5 μ Ci of [24-¹⁴C]chenodeoxycholic acid (sp act 50 mCi/mmol) (New England Nuclear, Boston, MA) had been administered intravenously. The four bile samples were used to calculate primary bile acid pool sizes according to Lindstedt (1).

Analyses

Serum bile acids. Blood samples were allowed to clot at room temperature for 1 hr. Serum was pipetted from the glass tubes after centrifugation (3000 rpm for 5 min) and stored at -20°C for subsequent analysis by radioimmunoassay by two different specific antibodies (Nordic-lab[®], glycocholic acid (GC), and glycochenodeoxycholic acid (GCDC) RIA kits, Oulunsalo, Finland) (13).

Precision studies of the radioimmunoassays, each specific for conjugates of one bile acid class, produced the following results. The intra-assay coefficient of variation of the GC-RIA ranged from 0.1 to 5.7% for concentrations of 6–15 $\mu\text{mol} \cdot \text{l}^{-1}$. The interassay coefficient of variation of the GC-RIA was 8.0–8.3% at concentrations of cholyglycine or cholytaurine of 3 $\mu\text{mol} \cdot \text{l}^{-1}$. The GCDC-RIA had an intra-assay coefficient of variation from 0.4 to 4.5% concentrations of 9.0–15.0 $\mu\text{mol} \cdot \text{l}^{-1}$ and an interassay coefficient of 8.5–12.9% at concentrations of 3 $\mu\text{mol} \cdot \text{l}^{-1}$ chenodeoxycholyglycine or chenodeoxycholytaurine.

Cross-reactivity of GC-antibody with cholytaurine was 150% as compared with cholyglycine at concentrations of 1–5 $\mu\text{mol} \cdot \text{l}^{-1}$, with chenodeoxycholytaurine and chenodeoxycholyglycine, 4.9 and 0.0%. Cross-reactivity of GCDC antibody with chenodeoxycholytaurine was 126% as compared with chenodeoxycholyglycine at concentrations of 1–5 $\mu\text{mol} \cdot \text{l}^{-1}$, whereas no activity of GCDC-antibody with choly conjugates was detected. Primary bile acids when tested as unconjugated compounds were bound to the same extent by each of the antisera as their respective glycoconjugates (cross-reactivity 100%). Deoxycholic acid, conjugated or unconjugated, shows no binding with GCDC-antibody (cross-reactivity < 0.1%) negligible binding to GC-antibody (cross-reactivity

tivity of deoxycholyglycine 1.1%, deoxycholytaurine 3.7%, deoxycholic acid 1.0%.

Bile acid kinetics. Duodenal bile samples were stored at -20°C immediately after collection for later analysis. After enzymatic deconjugation as described previously (14), we prepared a methanolic extract of bile acids using Sep-Pak C18 cartridges (15). Methylation of bile acids was achieved by adding an acidified (0.05 ml of concentrated HCl) mixture of 1 ml dimethoxypropane methanol 1:1 (v/v) (both Merck, Darmstadt, FRG), which was evaporated under a nitrogen stream at room temperature after 30 min reaction time (16). The methylated cholic acid (C) and chenodeoxycholic acid (CDC) fractions were separated by thin-layer chromatography using precoated silica gel 60 plates (layer thickness 0.25 mm) (Merck, Darmstadt, FRG), and iso-octane-ethylacetate-acetic acid-N-butanol 10:5:1.5:1.5 (v/v) as solvent system (17).

The methylated C and CDC bands were scraped off separately and eluted with diethylether-methanol 1:1 (v/v). Specific activities of C and CDC methylesters were determined from their mass and radioactivity after conversion into trifluoroacetates. These methods, which have been described previously (18), involve liquid scintillation counting (using a universal xylene-based scintillation cocktail, Instagel, Packard-Becker, Groningen, Netherlands and a Philips liquid scintillation counter PW 450) and gas-liquid chromatography. Bile acids were quantified against 7-ketodeoxycholic acid as an internal standard using a gas chromatograph type Becker 402 (Delft, Netherlands) fitted with a U-shaped all-glass column, $1.00\text{ m} \times 4\text{ mm}$ i.d. packed with 1% OV-210 on Gas Chrom Y, 100-120 mesh (Chrompack, Middelburg, Netherlands).

Statistics

Semilogarithmic specific activity decay curves of C and

CDC in bile allowed calculation of the pool size of these primary bile acids from the ratio of the administered amount of radioactivity to specific activity at the time of administration, as obtained by extrapolation (1) (time points 0.5, 1.5, 2.5, and 3.5 days, linear correlation coefficients of decay curves $r = 0.992 \pm 0.009$ (mean \pm SD) for C and $r = 0.995 \pm 0.006$ for CDC). Pool sizes have been expressed in micromoles per kilogram body weight.

We used a programmable Hewlett-Packard computer (HP-9830) to calculate the integrated area under the serum bile acid curve during the time course from 0 minutes until a minimum was reached after termination of CCK administration (Fig. 1). Correlations between bile acid pool size and both fasting levels of C and CDC conjugates and their response to CCK infusion (peak concentration and area under the curve) were evaluated by the method of least squares. Their significance was derived from Pearson's correlation coefficient r (19).

Furthermore, 95% confidence intervals of these linear regression lines have been computed to define the upper and lower limits of the serum bile acid responses [area under curve (AUC) and serum peaks] characteristic for populations with small or large bile acid pools (20). Arbitrarily, we considered pools under $25\text{ }\mu\text{mol} \cdot \text{kg}^{-1}$ to be small and pools over $50\text{ }\mu\text{mol} \cdot \text{kg}^{-1}$ to be large.

RESULTS

The average response of systemic levels of primary bile acid conjugates during CCK infusion is given in Fig. 1. Peak levels for both classes of bile acid conjugates were reached simultaneously, 70 min (confidence limits 60-80 min) after the start of CCK infusion, followed by a mini-

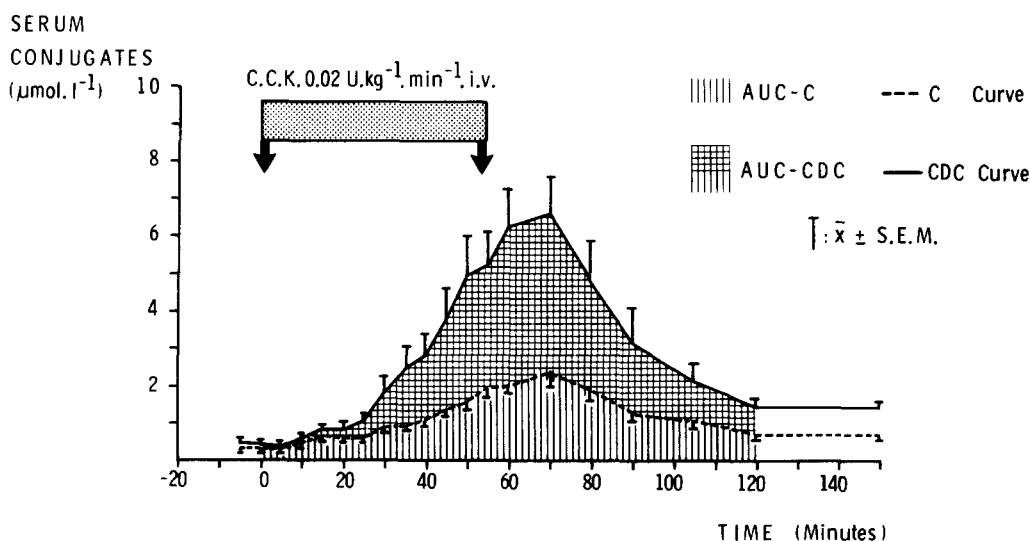


Fig. 1 Response of serum bile acid conjugates to a sustained infusion of cholecystokinin in 16 subjects. Shown are mean serum concentrations of cholic acid (C) and chenodeoxycholic acid (CDC) conjugates \pm SEM at each data point. AUC, area under curve; CCK, cholecystokinin.

mum at 120 min (confidence limits 105–150 min). C pool size was 9.9 to 55.7 $\mu\text{mol} \cdot \text{kg}^{-1}$; the CDC pool was 14.4 to 82.8 $\mu\text{mol} \cdot \text{kg}^{-1}$. The means \pm SD were $27.0 \pm 11.5 \mu\text{mol} \cdot \text{kg}^{-1}$ and $33.5 \pm 16.0 \mu\text{mol} \cdot \text{kg}^{-1}$ for C and CDC, respectively. Fasting levels of C and CDC conjugates in 16 healthy subjects are depicted as a function of their respective primary bile acid pool sizes in Fig. 2.

No significant correlations were found ($n = 16$, $r = 0.31$ and $r = 0.34$ for C and CDC). In contrast, the response of C and CDC conjugates in serum to the sustained CCK stimulus expressed as integrated area under the curve showed a significant linear relationship which was closer with respect to CDC pool size ($n = 16$, $r = 0.81$, $P < 0.001$) than to C pool size ($n = 16$, $r = 0.74$, $P < 0.005$) (Fig. 3). Using the 95% confidence intervals of the regression line, it was possible to define the lower and upper limits of the area under the serum curve of populations of individuals with large or small primary bile acid pool sizes according to preset criteria (e.g., 25 and 50 $\mu\text{mol} \cdot \text{kg}^{-1}$ as shown in Fig. 3). The utility of these limits appeared to be far better for CDC pools (1 misclassified subject out of 16) than for C pools (5 misclassified subjects out of 16).

Alternatively, linear regression analysis of peak levels of bile acid conjugates during sustained CCK stimulation as a function of primary bile acid pool sizes revealed similar significant correlations ($n = 16$, $r = 0.52$, $P < 0.05$ and $n = 16$, $r = 0.84$, $P < 0.001$ for C and CDC, respectively) (Fig. 4). Fourteen out of the 16 subjects investigated were assigned to the correct CDC pool size category using the 95% confidence intervals of the latter regression lines as compared to 11 out of 16, who were classified in the proper C pool size category (Fig. 4).

DISCUSSION

The main finding of the present study, which was confined to healthy persons with intact intestinal, gallbladder, and liver functions, is that measurement by radioimmunoassay of the rise of serum bile acid conjugates in response to a well-defined CCK stimulus can provide a fair estimate of CDC pool sizes in a population.

This did not hold true for C pool sizes, which may be explained by a distinction in hepatic extraction and in intestinal absorption of both primary bile acid conjugates. Estimated fractional uptake of CDC-conjugates by the liver has been reported to be less efficient than that of C-conjugates, whereas CDC-conjugates are absorbed better from the intestinal lumen (21, 22). As a consequence, enterohepatic circulation of the bile acid pool can be expected to cause a greater and more predictable rise of CDC-conjugates than of C-conjugates in peripheral blood.

The correlations between bile acid pool size and serum response of bile acid conjugates confirm that primary bile acid pool sizes are major determinants of their own spill-over into systemic blood (Figs. 3 and 4). Intra-individual reproducibility of the response of C-conjugates in serum to a liquid meal as reported by LaRusso et al. (6) also supports this statement. Among healthy normal subjects, variation of intestinal absorption, hepatic uptake, and glycine-taurine ratio will be relatively less important factors in this regard; however, they apparently impair reliable prediction of individual primary bile acid pool sizes.

It is of interest that pool size estimation from the rise of bile acid conjugates in serum, expressed either as integrated area under the curve or as peak level at 70 min,

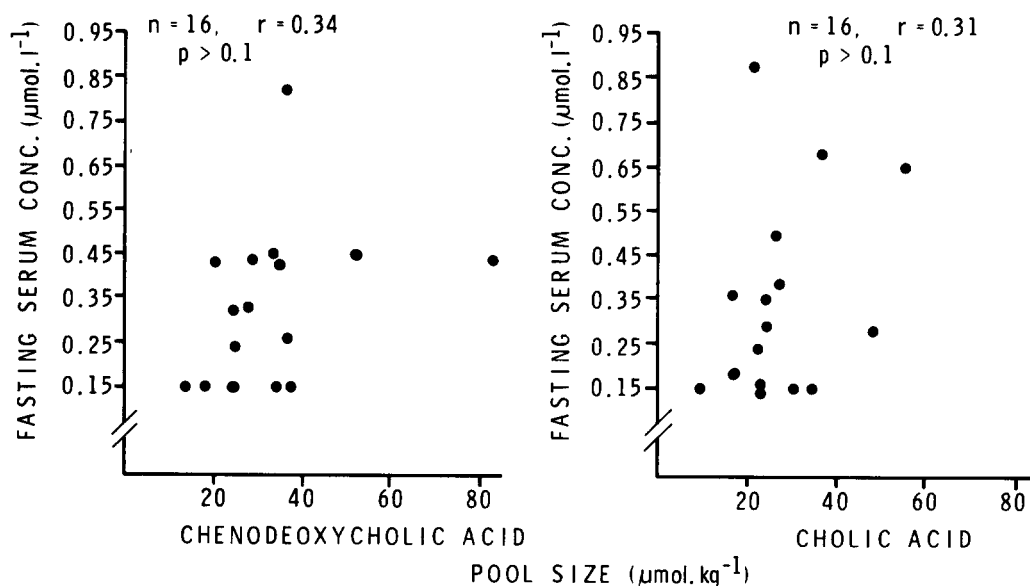
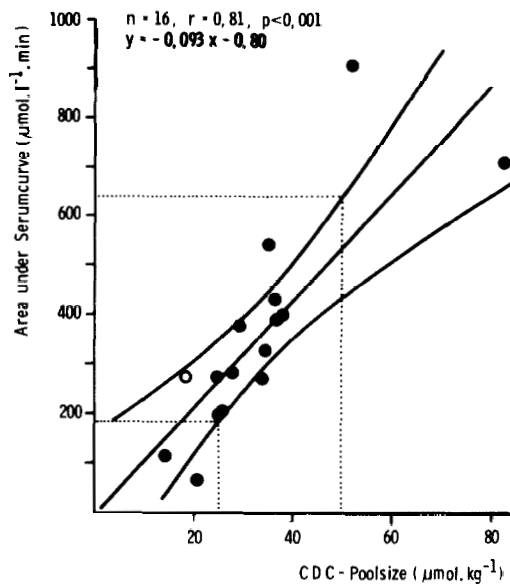


Fig. 2 Fasting levels of serum conjugates of primary bile acids as a function of primary bile acid pool size (as analyzed by isotope dilution and measurement of specific activity in bile) in 16 healthy subjects.

CDC-CONJUGATES



C-CONJUGATES

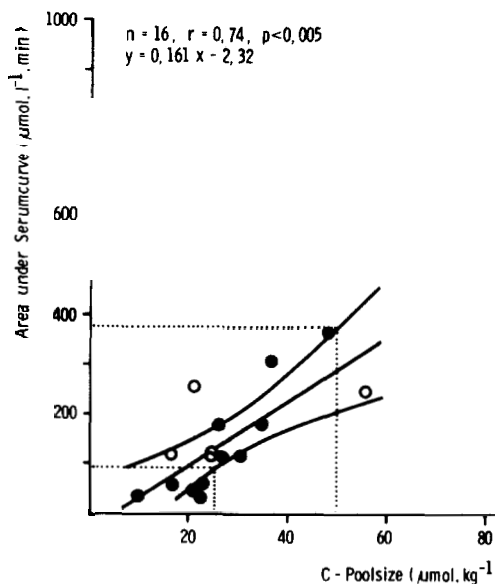


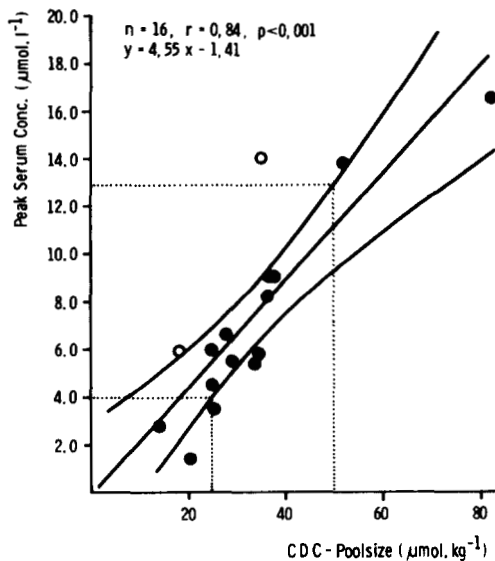
Fig. 3 Responses of primary bile acid conjugates in peripheral blood to a sustained CCK infusion expressed as AUC and plotted as function of chenodeoxycholate (CDC) and cholate (C) pool size in 16 healthy subjects. Linear regression lines and 95% confidence levels are depicted. Open circles denote subjects who are not correctly classified into categories of subpopulations with large and small bile acid pools according to upper and lower 95% confidence intervals, respectively. AUC, area under curve; CCK, cholecystokinin.

produced comparable results (Fig. 4). The latter approach may be preferable for the use in population studies.

Our data showed that, in contrast with peak levels, fasting levels of serum bile acid conjugates did not correlate

with primary bile acid pool sizes in our hands (Fig. 2). These findings are at variance with those of Angelin, Björkhem, and Einarsson (23) who described a linear relationship between total fasting C concentrations in serum and C pool size in normal subjects, but not be-

CDC-CONJUGATES



C-CONJUGATES

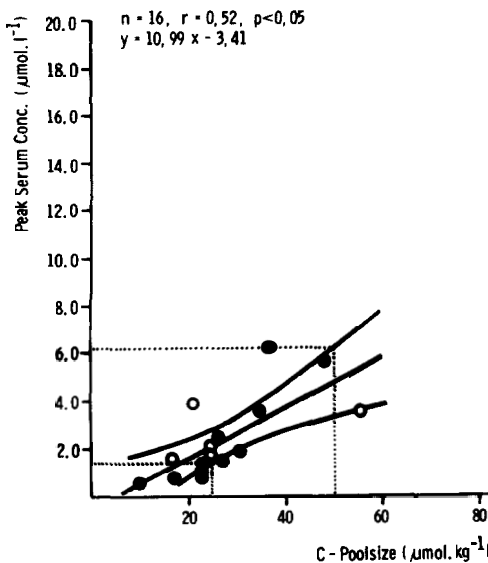


Fig. 4 Responses of primary bile acid conjugates in peripheral blood to a sustained CCK-infusion expressed as peak concentration and plotted as function of chenodeoxycholate (CDC) and cholate (C) pool size in 16 healthy subjects. Linear regression lines and 95% confidence levels are depicted. Open circles denote subjects who are not correctly classified into categories of subpopulations with large and small bile acid pools according to upper and lower 95% confidence intervals. CCK, cholecystokinin.

tween fasting CDC levels in serum and CDC pool size. This discrepancy cannot be due to a lack of specificity of the radioimmunoassays employed measuring one of both classes of primary bile acids, conjugated as well as unconjugated. Unconjugated bile acids may be a considerable fraction of serum bile acids in the fasting state (24). A better explanation may be the lack of sensitivity of these radioimmunoassays in respect to fasting serum bile acids in the normal range.

A remarkable feature of the profiles of serum bile acid conjugates in this study was the simultaneous occurrence of peak levels of C- and CDC-conjugates at 70 min (Fig. 1). This finding seems to differ from reports on the serum response of conjugates of both bile acid classes to a meal or a bolus injection of CCK causing a later peak of C-conjugates than of CDC-conjugates in systemic blood (25). These apparently conflicting results may be attributable to differences in study design.

Administration of a strong sustained CCK stimulus was aimed at mobilizing the total endogenous bile acid pool. Absorption of such a large bile acid load might depend more on ileal transport sites, and is further enhanced by an increase in intestinal motility due to CCK. This may explain the absence of the early peak of CDC-conjugates reflecting preferential jejunal absorption after a meal or after a CCK bolus injection (6, 22, 25, 26). Another consideration can be that sustained CCK infusion induces pancreatic biliary bicarbonate secretion without having an effect on gastric acid secretion as intake of a meal would have. CCK will also impair transpyloric transport of gastric contents. Such an unopposed increase in intestinal pH can be expected to prevent jejunal absorption of CDC-conjugates.

In this study, we preferred an infusion of CCK over a 55-min period to a fatty test meal, in order to avoid inter-individual variations of gastric emptying and to assure a single circulation of the total bile acid pool.

The use of radioimmunoassay measurement of conjugates of both primary bile acids has the advantage of potential application of the method on a wide scale. Although variable but relatively small amounts of unconjugated bile acids can be demonstrated in serum (24), it is not likely that exclusion of that fraction will ameliorate the assessment of bile acid pool sizes, since hepatic extraction of free bile acids is less efficient, as compared to that of conjugated bile acids (10).

However, estimation of primary bile acid pool sizes from serum levels of bile acid conjugates will be unreliable in patients who suffer from small intestinal bacterial overgrowth because of increased bile acid deconjugation in their duodenum and proximal jejunum (27).

A second limitation of this method of bile acid pool size assessment can be expected in conditions that are associated with abnormal portosystemic shunting. This will cause a disproportional high serum bile acid response

and, therefore, overestimation of primary bile acid pool sizes (7).

Another pitfall may be a decreased effect of CCK on gallbladder contractility, as has been described in patients with gallstone disease investigated by quantitative cholecintigraphy (12, 28). Using this technique we found virtually complete gallbladder evacuation in the participating subjects during sustained CCK infusion (55 min), which assured us that the total bile acid pool had circulated indeed.

In conclusion, we have demonstrated that measurement of serum bile acid conjugates after a well-defined stimulus of the enterohepatic circulation can be a practical method to estimate chenodeoxycholate pool size in large study populations of subjects with noncompromised intestinal, hepatic, and gallbladder functions. Only a minimum of analytical effort (investigation of three serum samples) is required once the relationship between CDC pool size and the response of CDC-conjugates in peripheral blood has been established. ■■

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