Measurement of bile acid synthesis by three different methods in hypertriglyceridemic and control subjects

William C. Duane
Department of Medicine, GI Section (111D), Veterans Affairs Medical Center, 1 Veterans Drive, and University of Minnesota, Minneapolis, MN 55417

Abstract In hypertriglyceridemic subjects, bile acid synthesis measured by isotope dilution is consistently higher than synthesis measured by fecal acidic sterol output. To see which of these two measurements was the more accurate, we compared them to synthesis measured by release of \(^{14}\)C\(_2\)O\(_2\) from \([2\text{6,}^{14}\text{C}]\)cholesterol. In 14 hypertriglyceridemic subjects, mean \(\pm\) SEM synthesis by the \(^{14}\)C method was 1540 \(\pm\) 199 pmol/day, similar to values by fecal acidic sterol output (1660 \(\pm\) 295). Both were significantly lower than values by isotope dilution (2520 \(\pm\) 269, \(P = 0.0001\) and 0.0015, respectively). In 12 normolipidemic controls, mean \(\pm\) SEM synthesis by the \(^{14}\)C method was 1230 \(\pm\) 189 pmol/day, nearly identical to synthesis by fecal acidic sterols (1220 \(\pm\) 187). Both were somewhat less than synthesis by isotope dilution (1590 \(\pm\) 153), but in neither case were the differences statistically significant \((P = 0.098\) and 0.129, respectively). In 3 hypertriglyceridemic subjects, synthesis measured by the \(^{14}\)C method increased by 42%, 44%, and 109% after 4 days of biliary sampling, suggesting that the isotope dilution procedure actually stimulated synthesis. Fraction of bile acid not absorbed during daily enterohepatic cycling was 8.4 \(\pm\) 1.4% in the hypertriglyceridemic subjects compared to 4.9 \(\pm\) 0.8% in the normolipidemic controls \((P = 0.037)\). We suggest that during sampling of bile for isotope dilution measurements, the terminal ileum is abruptly presented with a large bolus of unadulterated bile acid, both because of artificial stimulation of gallbladder contraction and return of surplus collected bile to the subject. In hypertriglyceridemia, because of an inefficient absorptive mechanism, this may result in unusual loss of bile acid with consequent stimulation of bile acid synthesis.—Duane, W. C. Measurement of bile acid synthesis by three different methods in hypertriglyceridemic and control subjects. J. Lipid Res. 1997. 38: 183–188.

Supplementary key words bile acids and salts • bile • triglycerides • cholesterol • enterohepatic circulation

The ability to measure bile acid synthesis in human subjects has contributed to our understanding of several diseases including cholesterol cholelithiasis and hyperlipidemia. It has also helped characterize the action of drugs such as cholestyramine and the fibric acid derivatives. Measurement of bile acid synthesis in healthy human subjects is difficult and necessarily somewhat in-direct. The two oldest and best established methods for accomplishing this measurement are fecal acidic sterol balance and isotope dilution (1, 2). In general, these two methods have provided comparable results when performed in healthy subjects (3, 4). However, a perplexing observation, first made 20 years ago, is that in patients with hypertriglyceridemia, isotope dilution yields a value for bile acid synthesis that is 30–160% higher than the value measured by acidic sterol balance (3, 5, 6).

Moreover, there is little basis to judge which of the two methods yields the more accurate value for bile acid synthesis in hypertriglyceridemic subjects. In only one study have the two methods been compared to a third method in hypertriglyceridemic subjects. In that study, Davidson et al. (6) found that synthesis by isotope dilution was higher than by fecal, acidic sterol balance in four hypertriglyceridemic subjects. Synthesis measured by release of \(^{3}\)H\(_2\)O from \([24,25-^{3}\text{H}]\)cholesterol was similar to that measured by isotope dilution in three subjects, but in the fourth was nearly identical to the value by fecal acid sterol output. Thus, while this study suggested that fecal acidic sterol balance provided a falsely low value for bile acid synthesis in hypertriglyceridemia, the study was not conclusive because of small numbers and inconsistent results.

In the present study we have measured bile acid synthesis by both isotope dilution and acidic sterol balance in 14 subjects with hypertriglyceridemia and 12 normolipidemic controls. In all subjects the results were compared to values for synthesis measured by a third method, namely conversion of \([26-^{14}\text{C}]\)cholesterol to \(^{14}\)CO\(_2\). This technique has been used extensively in our laboratory for more than 10 years (7–10). We have previously shown that in normal subjects this method pro-

Abbreviations: SEM, standard error of the mean; CO, carbon monoxide; VLDL, very low density lipoprotein; BMI, body mass index.
TABLE 1. Anthropometrics and serum lipids of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Hypertriglyceridemia</th>
<th>Normolipidemia</th>
<th>PValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (inches)</td>
<td>69 ± 1</td>
<td>70 ± 1</td>
<td>0.203</td>
</tr>
<tr>
<td>Weight (lbs)</td>
<td>229 ± 10</td>
<td>189 ± 7</td>
<td>0.021</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.7 ± 1.3</td>
<td>27.7 ± 0.7</td>
<td>0.002</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>197 ± 5</td>
<td>187 ± 8</td>
<td>0.781</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>322 ± 21</td>
<td>115 ± 8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values given as mean ± SEM; BMI, body mass index.

provides values for bile acid synthesis that correlate well with values determined by isotope dilution (11). Moreover, in baboons with an intact enterohepatic circulation, this method has been shown to yield values of bile acid synthesis nearly identical to those determined by fecal acidic sterol output (12).

METHODS

We studied 26 healthy male volunteers ranging in age from 37 to 73 years (Table 1). Twelve subjects had neither hypertriglyceridemia nor hypercholesterolemia and served as controls. The other 14 subjects had hypertriglyceridemia (serum triglyceride level >200 mg/dl) without hypercholesterolemia. In 4 of these (#’s 8, 9, 12, and 14) we had sufficient information, including lipids and apoprotein B levels in very low density lipoproteins (VLDL) as well as lipid levels in family members, to meet our previously published criteria for familial hypertriglyceridemia (13). The other 10 could not be classified with certainty, usually because of absence of sufficient information in family members. All subjects were in good health by previously published criteria (14), although the hypertriglyceridemic subjects, on average, were more obese than the controls (Table 1). All had biliary ultrasonography, which demonstrated gallstones in 2 control subjects (#’s 16 and 19) and 1 hypertriglyceridemic subject (#8), but absence of gallstones in the other 23 subjects. Serum lipids and isotope dilution measurements of bile acid synthesis for many of the subjects (#’s 8, 9, 10, 13, 14, 15–21, and 23–25) have been published previously in a study of bile acid absorption in hypertriglyceridemia (13). Informed consent was obtained from each subject, and all study procedures were approved by the committee overseeing use of human subjects in research at the Minneapolis VA Medical Center.

The isotope dilution measurement of bile acid synthesis was performed as described in previous publications (13, 15). Briefly, in the evening following a light supper, each subject swallowed a small polyvinyl tube weighted with mercury bag and metal aspiration tip. About 5 μCi each of [24-14C]cholic acid and [24-14C]chenodeoxycholic acid were administered via this tube which was rinsed with isotonic sodium bicarbonate. The tube was left in place overnight to permit migration into the duodenum. The next day gallbladder bile was collected via the tube using intravenous cholecystokinin octapeptide (Kinevac, Squibb & Sons, Inc., Princeton, NJ) to stimulate gallbladder contraction. Bile was collected on ice with light shielding for both the tube and collection vessel. A small sample of bile, usually between 1 and 4 ml depending on color, was placed in methanol for analysis. The remaining bile was returned to the subject via the tube. After sampling, the tube was removed and the subject are normally. Gallbladder bile was collected similarly for an additional 3 consecutive days. Each of the four bile samples was analyzed for specific activity of cholic acid and chenodeoxycholic acid as described in previous publications (4, 13, 15, 16). The specific activity measurements were used to calculate bile acid synthesis by the method of Lindstedt (2). Throughout the sampling procedure subjects resided on our metabolic ward where they were given a diet designed to simulate their customary home diet as judged by careful dietary history.

For measurement of fecal acidic sterol output, subjects ingested three capsules per day, each containing 200 mg chromic oxide as a nonabsorbable marker. Marker ingestion continued for a 20-day period during which the subjects resided on the metabolic ward consuming their customary diets as indicated above. During the last 10 days of marker ingestion stool was quantitatively collected. Collections for each of the five 2-day intervals were homogenized with an equal volume of water. Aliquots of these homogenates were analyzed for acidic sterols by gas–liquid chromatography as previously reported (17). Briefly, after saponification, addition of hyocholic acid as an internal standard, and extraction of neutral sterols, the bile acids were acidified and extracted into chloroform–methanol. Quantitation was then accomplished by gas–liquid chromatography of the acetate derivatives of the bile acid methyl esters. This method is a modification of that originally described by Subbiah et al. (5). Total acidic sterol output was calculated by multiplying daily chromium intake times the mean concentration ratio of acidic sterol/chromium in stool. When this procedure was done after the isotope dilution sampling, we allowed a rest interval of at least 4 weeks. During this time subjects were not residents of the metabolic ward but maintained their usual, customary diets as outpatients. Subjects’ weights were monitored to assure that no substantial change occurred between measurements.

Determination of bile acid synthesis as release of 14CO2 from [26-14C]cholesterol was accomplished as described in previous publications (7, 10). Briefly, about 30 μCi of (26-14C)cholesterol (New England Nuclear, Boston, MA) was orally administered at least 7 days
prior to sampling. Breath CO₂ was quantitatively collected by having the subject sit with his head in a Plexiglas hood through which air was drawn into three serial traps containing phenethylamine in a scintillation cocktail. Synthesis was calculated by dividing ¹⁴CO₂ output on the breath by the specific activity of free cholesterol determined on a serum sample obtained at the time of breath sampling. All sampling was done between 8:00 and 10:00 A.M. to eliminate effects of circadian periodicity. Again, when this procedure was done after the isotope dilution sampling, we allowed a rest interval of at least 4 weeks, during which the subjects maintained their customary diets as outpatients.

For purposes of determining absorption of bile acids, we also measured biliary secretion rate of bile acid. On the four samples of bile obtained during the isotope dilution procedure, we measured concentration of total bile acids and bilirubin. Also on at least two separate occasions subjects underwent measurement of output of CO on breath to estimate bilirubin production (18). These measurements were used to calculate rate of bile acid secretion into bile by a method we have recently described (18). This method utilizes the fact that CO production rate reflects both bilirubin production and secretion into bile. Biliary secretion for any bile constituent can then be determined from the ratio of constituent/bilirubin measured in gallbladder bile multiplied by the endogenous CO production rate. The method is more reproducible than marker perfusion, but it provides an estimate of bile acid secretion that is about 25% lower than that measured by marker perfusion. It is uncertain which of the two estimates is more accurate; however, because all subjects in the present study were studied by the same method, the comparison of controls and hypertriglyceridemic subjects should be valid and informative.

Fractional bile acid absorption was calculated by the formula: \[1 - \frac{[A/(O \times 24)]}{O} = \text{daily output of bile acid in the stool measured as described above and O = hourly output of bile acid into bile.}\]

Statistical testing was done using SAS (SAS Institute, Cary, NC) on a Northgate personal computer equipped with a 486DX microprocessor. Comparisons were done by paired t-test except when the hypertriglyceridemic group was compared to the control group, in which case unpaired t-tests were used. Regression analysis and the analysis of variance calculation of P values for the regression were also performed with SAS.

RESULTS

Individual values for bile acid synthesis by all three methods in the hypertriglyceridemic subjects are presented in Table 2. Mean ± SEM synthesis by fecal acidic sterol output was 1660 ± 295 μmol/day, similar to the mean of 1540 ± 199 μmol/day measured by release of ¹⁴CO₂ from [26-¹⁴C]cholesterol. In contrast, mean synthesis measured by isotope dilution in the hypertriglyceridemic subjects was 2520 ± 269 μmol/day, which by paired t-test was significantly higher than both the mean for fecal acidic sterol output (\(P = 0.0015\)) and the mean for the ¹⁴CO₂ method (\(P = 0.0001\)).

For the normolipidemic subjects, mean synthesis by fecal acidic sterol output was 1230 ± 189 μmol/day, which was nearly identical to the mean for synthesis measured by the ¹⁴CO₂ method of 1220 ± 187 μmol/day (Table 2). In these subjects, mean synthesis measured by isotope dilution was 1590 ± 133 μmol/day. The difference between this value and that for either fecal acidic sterols or the ¹⁴CO₂ method did not reach statistical significance (\(P = 0.129\) and 0.098, respectively). Nevertheless, because of these fairly low \(P\) values, we cannot rule out the possibility that even in these normal subjects, isotope dilution may have somewhat overestimated bile acid synthesis.

Mean bile acid synthesis was significantly different for normals compared to hypertriglyceridemic subjects when measured by isotope dilution (\(P = 0.006\)), but not when measured by either fecal acidic sterols (\(P = 0.229\)) or the ¹⁴CO₂ method (\(P = 0.251\)). Here again it is possible that with larger numbers of subjects these latter two differences might have become statistically significant. Other studies have demonstrated higher output of fecal acidic sterols in hypertriglyceridemic subjects (19, 20), although these differences tend to disappear when corrected for body weight (6, 19) because hypertriglyceridemic subjects tend to be obese, as they were in the present study (Table 1).

To assess the possibility that the isotope dilution procedure artificially increased bile acid turnover by removal of bile acid in the sampling process, we calculated the amount of bile acid removed each day. For the hypertriglyceridemic subjects the mean ± SD μmol of bile salt removed each day for the first 3 days of sampling was 121 ± 40. The corresponding values for normal control subjects were 138 ± 53. When these numbers were expressed as fraction of the bile acid pool, calculated by standard Lindstedt kinetics (2), the mean ± SD fraction of the pool removed per day was 0.013 ± 0.007 for the hypertriglyceridemic subjects and 0.015 ± 0.009 for the normolipidemic subjects. These values represent a negligible part of the normal fractional turnover of bile acid, which is on the order of 0.6 pools/day in hypertriglyceridemia and 0.4 pools/day in normals (13).

To determine whether bile acid synthesis was actually being stimulated by the isotope dilution sampling procedure in subjects with high triglycerides, we performed...
4 consecutive days of gallbladder bile sampling in three hypertriglyceridemic subjects and measured synthesis by the $^{14}$CO$_2$ method the day before sampling was started and the day sampling was completed. Compared to the baseline value prior to sampling, synthesis by the $^{14}$CO$_2$ method increased by 42%, 44%, and 109% after 4 days of bile sampling in the three subjects.

To assess the possibility that impaired absorption of bile acid contributed to the overestimation of bile acid synthesis provided by the isotope dilution method, we determined bile acid absorption using fecal acidic sterols to estimate daily loss of bile acid and the CO$_2$ method of measuring bile acid secretion to determine total bile acid presented to the duodenum (see Methods). Five hypertriglyceridemic subjects could not be included in these calculations because they did not have measurements of bile acid secretion. The results from the remaining subjects are presented in Table 2 and demonstrate a significantly lower mean (±SEM) fractional absorption of bile acid in the hypertriglyceridemic subjects (0.916 ± 0.014) than in the normolipidemic subjects (0.951 ± 0.008). Thus, of the bile acid presented to the duodenum, a mean (±SEM) of 8.4 ± 1.4% was not absorbed in the hypertriglyceridemic subjects compared to 4.9 ± 0.8% in the normolipidemic controls ($P = 0.037$).

**DISCUSSION**

In agreement with studies from other laboratories (3, 5, 6), we found that isotope dilution provided a substantially higher value for bile acid synthesis than did fecal acidic sterol output in subjects with hypertriglyceridemia. Although as a group our hypertriglyceridemic subjects tended to be obese (Table 1), most previously reported subjects with this disparity have not been obese.
(3, 5, 6) suggesting that elevation of triglycerides, rather than obesity, is the primary factor associated with discrepant values for bile acid synthesis by the two methods.

To assess which method provided the more accurate value, we also measured synthesis by a third method, namely release of $^{14}$CO$_2$ from $^{26}$[14C]cholesterol. In an earlier study we found that this CO$_2$ method provided values of bile acid synthesis similar to values by isotope dilution in normal lipidemic subjects (11). Moreover, studying both baboons and rhesus monkeys, Redinger, Chow, and Grace (12) found close agreement for bile acid synthesis measured by the CO$_2$ method compared to synthesis measured by fecal acidic sterol.

In the present study, we also found mean synthesis by the CO$_2$ method was nearly identical to mean synthesis by fecal acidic sterol output. Moreover, for the 26 subjects in this study, there was a highly significant correlation between synthesis measured by the CO$_2$ method versus synthesis measured by fecal acidic sterol output ($r = 0.67, P < 0.0002$). In contrast, mean synthesis by both the CO$_2$ method and fecal acidic sterol output was about 40% lower than mean synthesis by isotope dilution. It appears, therefore, that in hypertriglyceridemia isotope dilution yields a value for bile acid synthesis that is higher than the true unperturbed level of synthesis. In normal lipidemic controls there appeared to be a similar tendency for isotope dilution to overestimate bile acid synthesis; however, the discrepancy was much less marked than in hypertriglyceridemia, and the difference between isotope dilution and the other two methods did not reach statistical significance (Table 2).

We also found that the 4-day sampling procedure actually increased synthesis measured by the $^{14}$CO$_2$ method in three hypertriglyceridemic subjects (see Results) rather than simply providing inaccurate, measurement of unperturbed synthesis. This is consistent with previous studies evaluating factors that might cause inaccuracies in the isotope dilution technique. Two such potential problems, possible incomplete absorption of orally administered isotopic tracer and possible inappropriateness of modeling with first order kinetics, both appear quite unlikely in view of earlier studies (3, 21).

Thus, removal of bile acid in the samples is an unlikely explanation for stimulation of bile acid synthesis by the 4-day sampling procedure in our hypertriglyceridemic subjects. Moreover, removal of too much bile in samples would not account for the difference between normal and hypertriglyceridemic subjects with respect to inaccuracies of the isotope dilution method (Table 2).

A notable difference between hypertriglyceridemic subjects and normal controls is in fractional absorption of bile acid. We and others have previously reported impaired bile acid absorption in hypertriglyceridemics (13, 22), although both studies were based on the isotope dilution method. Nevertheless, in the present study we confirmed impaired absorption of bile acid in our obese hypertriglyceridemic subjects using fecal acidic sterol output (Table 2). The isotope dilution sampling procedure may put unusual demands on the intestinal absorption apparatus because a considerable amount of undiluted, unadulterated gallbladder bile is presented to the duodenum, as a result of artificially induced gallbladder contraction with re-infusion of surplus collected bile. A reasonable hypothesis would be that this sampling procedure causes some loss of bile acid because of a rapid passage of a large bile acid bolus by the terminal ileal absorptive site. Such an artifically induced loss of bile acid would presumably be greater in hypertriglyceridemic subjects because they have a less efficient absorptive mechanism.

We have previously reported that, in normal subjects, isotope dilution measurements of bile acid synthesis yield values similar to those obtained by both fecal acidic sterol output (4) and the $^{14}$CO$_2$ method (11). In the normal subjects of the present study we also did not find a significant difference in synthesis measured by these three methods, although there was a tendency for isotope dilution to provide the highest value of the three (Table 2). It should be noted that our previous comparison of isotope dilution with fecal acidic sterol output actually showed the opposite trend, namely for the isotope dilution value to be lower than fecal sterol output (4). Although this discrepancy may simply represent statistical variability, it could have resulted from different methods of inducing gallbladder contraction. Thus, in the previous study we used intraduodenal infusion of an amino acid solution, not intravenous infusion of cholecystokinin octapeptide, to stimulate gallbladder contraction. It is likely that intraduodenal amino acids were a less potent stimulator of gallbladder contraction (indeed we switched to cholecystokinin octapeptide to improve sample collection).

The findings of the present study clearly indicate the need for caution in the use of the isotope dilution procedure in hypertriglyceridemic patients. It is also possible that given more study subjects we might have found that the isotope dilution procedure using cholecysto-
kinin octapeptide provided a statistically significantly higher value for bile acid synthesis than the other two methods, even in normal subjects (Table 2). Thus, in our opinion, when isotope dilution measurements of bile acid synthesis are undertaken in any subject population, it would seem prudent to minimize artificial induction of gallbladder contraction and to confirm the accuracy of the measurement procedure by comparison to an alternate method of measuring synthesis.

We wish to acknowledge the help of Ms. Cathy Pinther-Evans and Linda Hartich, without whom these studies could not have been performed. Financial support was provided by grants from the Department of Veterans Affairs and the National Institutes of Health (R01-DK42433).

REFERENCES


