notes on methodology

Determination of bile acid pool size in man: a simplified method with advantages of increased precision, shortened analysis time, and decreased isotope exposure

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Summary A simplified isotope dilution method for measurement of the bile acid pool size in normal subjects is described and compared with the traditional method of Lindstedt (*Acta Physiol. Scand.* **40:** 1–9, 1957). Advantages of this simplified method include a four- to eightfold reduction of isotope dose, facilitation of analytical procedures, and a reduction in the required number of duodenal intubations. In 15 human subjects who had two separate estimates of pool size by this method, precision averaged 2.6%. In 16 comparisons, pool size measured by this method averaged 13.7% higher than simultaneous estimates by the Lindstedt method. Factors affecting accuracy (as opposed to precision) in both methods are discussed.

Supplementary key words isotope dilution · bile acid metabolism

Clinical investigation of biliary physiology and pathophysiology has often necessitated measurement of bile acid pool size (1–3). Quantitation of the bile acid pool by the traditional method of Lindstedt (4) requires isolation of a specific bile acid (usually cholic acid) as well as measurement of cholic acid mass, radioactivity, and bile acid composition in at least four samples (4–6). From the research subject's point of view, this procedure entails ingestion of a relatively large amount of radioactivity and at least four duodenal intubations.

In the present investigation, an alternative method for measurement of the bile acid pool without measurement of bile acid kinetics is reported. This technique requires one-eighth to one-fourth the radionuclide dose of the Lindstedt method and only one duodenal intubation, and it is precise to ±2.6%. The necessity for isolation of cholic acid and determination of bile acid composition is obviated by this technique.

Methods. 16 Caucasian and 10 American Indian volunteers, 19–34 yr old, were studied on the metabolic ward of the Phoenix Clinical Research Section, NIAMDD; 13 were female and 13 were male. None had evidence of hepatobiliary or other abnormality as judged by history, physical examination, and screening laboratory tests. Informed consent was obtained from all subjects prior to study.

[*Carboxy*-^{14}C]cholic acid and [2,4-^{3}H]cholic acid were obtained commercially (New England Nuclear, Boston, Mass.) and repackaged as a sterile 50% ethanol solution by the National Institutes of Health radiopharmacy. Radiopurity was greater than 98% as judged by thin-layer chromatography.

6 hr after a light supper, each subject swallowed a single-lumen polyvinyl tube weighted with a stainless steel aspiration tip and mercury bag. The isotope, 1–5 μCi of ^{14}C or 3–15 μCi of ^{3}H, in 1–10 ml of 50% ethanol was then either injected intravenously via a running infusion set containing normal saline with 175 meq/l of sodium bicarbonate or, more frequently, infused through the polyvinyl tube, which was subsequently flushed with 50 ml of bicarbonate solution (880 meq/l). Food was withheld overnight until completion of the study. In the morning, the tube was positioned radiographically in the descending portion of the duodenum. Gallbladder contraction was stimulated by infusion of 5% protein hydrolysate (Cutter Laboratories, Berkeley, Calif.) through the tube, which was clamped for 10 min and then drained by gravity for 30–60 min. Usually, 40–60 ml of dark brown bile was obtained; 3–6 ml of bile was retained and the remainder was returned via the tube. For storage, bile samples were mixed with an equal volume of methanol and refrigerated at 10°C.

For determination of bile acid specific activity, an aliquot of bile–methanol solution was made to 70% methanol and extracted with 2.5 vol of petroleum ether. The petroleum ether was discarded, and an aliquot of the methanol phase was evaporated and counted in Aquasol (New England Nuclear) and water 10:1 with a Packard model 3320 liquid scintillation counter, using external standardization to correct for quenching. If highly colored, the methanol solution was decolorized under blue light for 24 hr prior to evaporation and counting. Bile acid mass was determined on a corresponding aliquot of the methanol phase using an automated modification of Talalay's enzymatic assay (7).

Because the isotopic cholic acid is distributed randomly in the entire bile acid pool, total bile acid pool size can be calculated from the following formula:

$$P_T = \mathrm{dpm}_A(B_T/\mathrm{dpm}_T)$$

where P_T is the total bile acid pool, dpm_A is the number

Abbreviations: SA_c, cholic acid specific activity.

of disintegrations per minute administered, B_T is the concentration of total bile acids, and dpm_T is the concentration of radioactivity.

The reproducibility (precision) of this simplified method was tested by performing two separate measurements of pool size at intervals of 1–23 days in each of 15 subjects using first ^{3}H-labeled then ^{14}C-labeled cholic acid.

On 16 occasions in 13 subjects bile acid pool size was simultaneously determined by the present method and by the Lindstedt technique (4). In these subjects duodenal bile was obtained by siphonage as described above on four consecutive mornings beginning 12 hr after isotope administration. Bile acid composition and cholic acid specific activity were determined in each of these samples as follows. Bile salts were deconjugated by a 3-hr hydrolysis in 1.25 N NaOH at 250°F and 15 psi. The hydrolyzate was acidified and extracted with $CHCl_3$–MeOH 8:3. An aliquot of this extract was used to determine bile acid composition by gas–liquid chromatography of the acetate derivatives of the bile acid methyl esters on 1.5% QF-1. The remaining extract was applied to thin-layer chromatography plates, which were subsequently developed in isooctane–ethyl acetate–acetic acid 5:5:1. The cholic acid band was identified with iodine vapor and eluted with $CHCl_3$–MeOH. One aliquot of the eluate was assayed for radioactivity. Another aliquot was evaporated and redissolved in a known volume of 0.01 N NaOH for enzymatic determination of cholic acid mass. These eluates were periodically analyzed by gas–liquid chromatography to ensure that they contained only cholic acid. This method is accurate to ±3.8% as determined on standard mixtures of bile acids of known cholic acid specific activity (SA_c).

A least squares regression line of ln (SA_c) vs. time was calculated, and the hypothetical SA_c at time zero was obtained by extrapolating this regression line to time zero. This allowed calculation of the cholic acid pool by the Lindstedt method from the equation:

$$P_c = dpm_A/SA_c(0)$$

where: P_c is the Lindstedt cholic acid pool, dpm_A is the number of disintegrations per minute administered, and $SA_c(0)$ is the cholic acid specific activity extrapolated to time zero.

Lindstedt total bile acid pool was calculated by dividing the Lindstedt cholic acid pool by the fraction of bile acids corresponding to cholic acid.

The effect of route of isotope administration on pool size obtained by the simplified method was evaluated in four subjects by simultaneous intravenous injection of [^{3}H]cholic acid and intragastric infusion of [^{14}C]cholic acid. The ^{3}H and ^{14}C bile acid specific activity in bile obtained the following morning was determined using standard double-label counting techniques.

Results. In the 15 subjects who had two separate estimates of bile acid pool size by our 1-day method (**Table 1**), the difference in the two estimates averaged 5.2% (range 0.4–14.9%). This corresponds to a mean precision of 2.6% and indicates that the method provides a reproducible estimate of bile acid pool size.

In the 16 individual comparisons, bile acid pool size measured by the 1-day method averaged 13.7% higher than by the Lindstedt method (percentage of the mean of the two estimates, **Table 2**). This discrepancy is a predictable result of the extrapolation of cholic acid specific activity to time zero in the Lindstedt measurements. The magnitude of the discrepancy will vary directly with the fractional turnover rate of cholic acid; however, assuming an average cholic acid half-life of 2.5 days (4, 8), this extrapolation should theoretically result in a difference of 13.9% between estimates of pool size by the two methods. This predicted difference agrees well with the observed difference of 13.7%.

In the four subjects who received, simultaneously, [^{3}H]cholic acid intravenously and [^{14}C]cholic acid orally, the 1-day estimate of pool size calculated on the basis of the orally administered isotope was within 4.7%, 5.9%, 3.5%, and 4.1%, respectively, of the pool size calculated on the basis of the intravenously administered isotope. This suggests that the route of isotope administration does not significantly affect the measurement of pool size and provides evidence that both isotopes were completely mixed with the bile acid pool.

Discussion. The method reported here provides a highly reproducible estimate of bile acid pool size (precision 2.6%, Table 1). To our knowledge no calculated estimate of reproducibility for the Lindstedt method has been reported; however, Danzinger et al. (5) have used that method to measure total bile acid pool size (μmoles/kg) on two occasions in each of six healthy controls. Precision for the Lindstedt method calculated on the basis of their data is 7.9%. The difference in precision between the two methods may be a result of the more complex analytical techniques, such as gas–liquid chromatography, required for the Lindstedt method (4).

The difference between bile acid pool size measured by the simplified technique and the Lindstedt technique observed in the present study is a predictable result of extrapolation to time zero in the Lindstedt determination. Because the true pool size is not known, it cannot be ascertained which of these two answers is the more accurate. If bile acid synthesis and removal rates are constant throughout the study period, the Lindstedt method should be more accurate; however, experiments in animal models suggest that both bile acid synthesis and removal are lower during fasting than during feeding hours (9–11). If this diurnal variation holds true for man, extrapolation of cholic acid specific activity across the 12-hr fasting interval between isotope administration and sampling might

TABLE 1. Reproducibility of bile salt pool size measurements by the 1-day method

Subject	Days between Measurements	Pool 1	Pool 2	$\lvert P_1 - P_2 \rvert$	Precision[a]
		mg	*mg*	*mg*	%
CW	14	4545	4663	118	1.28
TL	3	1566	1530	36	1.16
MM	23	3167	2730	437	7.41
ST	21	2643	2587	56	1.07
TF	21	2465	2360	105	2.18
WR	1	5350	5779	429	3.86
GG	1	2723	2346	377	7.44
FB	1	3128	3083	45	0.72
CP	1	2998	3011	13	0.22
NP	1	1534	1488	46	1.52
LJ	1	2472	2745	273	5.24
RR	1	3407	3065	342	5.28
DH	1	1540	1550	10	0.32
TM	1	2593	2618	25	0.48
SK	1	2051	2080	29	0.70
Mean					2.59

[a] Precision is calculated by the standard formula:

$$\text{Precision (\%)} = \left(\frac{|P_{avg} - P_1|}{P_{avg}} + \frac{|P_{avg} - P_2|}{P_{avg}}\right) \cdot 0.5 \cdot 100$$

where P_1 is pool 1, P_2 is pool 2, and $P_{avg} = (P_1 + P_2)/2$.

provide a time-zero specific activity that is slightly too high and an estimated pool size that is falsely low. The lower the rate of bile acid synthesis and removal during the fasting hours, the more accurate would be the simplified estimate of pool size, because that method involves no extrapolation. Conversely, it is likely that the simplified method will overestimate pool size under conditions in which fractional turnover rate is accelerated. It is possible that administration of the isotope 24 hr prior to sampling (instead of 12 hr) in the Lindstedt determination would narrow the discrepancy between measurements by that method and the simplified method because extrapolation across a 24-hr interval would tend to average any diurnal changes in bile acid synthesis and removal rates. In any case, the discrepancy between estimates of pool size by the two methods probably is no greater than the observed 13.7% difference.

One of the most important benefits derived from the present method of bile acid pool size determination is decreased radiation exposure of the research subject. He is spared three-fourths to seven-eighths of the dose of radionuclide required for the Lindstedt method for two reasons. First, sampling is done close to the time of isotope administration so that little, if any, isotope has been removed from the pool. This alone can reduce the isotope dose fourfold because sampling in the Lindstedt method is carried out for about two cholic acid half-lives. Second, the present method does not require thin-layer chromatographic isolation of cholic acid. This step in the Lindstedt method limits the absolute amount of cholic acid available

TABLE 2. Comparison of bile salt pool size estimated by the 1-day method and by the method of Lindstedt

Subject	Total Bile Salt Pool		Difference		r^a
	Lindstedt Method	1-day Method			
	mg	*mg*	*mg*	%[b]	
TL	1470	1566	96	+6.32	0.950
LD (^{3}H)[c]	3062	3745	683	+20.07	0.999
LD (^{14}C)	2718	3525	807	+25.85	0.999
RB	2252	2535	283	+11.82	0.997
MB (^{3}H)[c]	2100	2420	320	+14.16	0.993
MB (^{14}C)	1980	2321	341	+15.86	0.997
JE	2958	2998	40	+1.34	0.991
AM	2451	2636	185	+7.27	0.965
WA	2555	2791	236	+8.83	0.993
FT	2461	2703	242	+9.37	0.993
NP	1945	2576	631	+27.91	0.971
FBr	2851	3613	762	+23.58	0.998
MD	2742	2810	68	+2.45	0.997
LH	2484	2742	258	+9.87	0.997
FBa	1948	2170	222	+10.78	0.990
RN	1740	2193	453	+23.04	0.998
Mean				+13.66	

[a] Correlation coefficient for ln (SA_c) vs. time in the Lindstedt determination.

[b] Percentage difference is calculated by dividing difference (mg) by the average pool by the two estimates. Plus sign indicates Lindstedt estimate smaller than estimate by our method.

[c] Subjects LD and MB both received [^{3}H]- and [^{14}C]cholic acid simultaneously.

for radioassay and by our estimates increases the necessary isotope dose about twofold. Although [^{14}C]- and [^{3}H]cholic acids are weak beta emitters, the high concentration of bile salts in the gallbladder results in significant exposure of that organ. For [^{14}C]cholic acid, we calculate a gallbladder dose of 98.4 mrads/μCi (see Appendix). Thus, any reduction in the amount of radioactive bile acid administered may represent a significant reduction in health hazard.

Two reports include measurement of bile acid pools by a method similar to ours except that sampling was done 3 hr after intravenous administration of isotope (12, 13). Incomplete mixing of the isotope with the bile acid pool could lead to large errors by this method, but no estimate of the completeness of mixing was provided in these reports. In contrast, the high precision of the present method on repeat studies in individual patients strongly suggests that mixing is complete after a 12-hr waiting period. Moreover, the fact that the 12-hr ln (SA_c) forms a nearly perfect line with subsequent points on the Lindstedt curve (correlation coefficient >0.990 in 13 of our 16 determinations, Table 2) provides additional strong evidence for the completeness of isotope mixing in the first 12 hr.

Where data on individual bile acid kinetics are not essential, measurement of bile acid pool size by the simple and precise method reported here should facilitate investigations of the determinants and the importance of bile acid pool size in man.

APPENDIX

Because the highest concentration of bile acids is found in gallbladder bile, it is the gallbladder that receives the highest radiation exposure from radiolabeled bile acids. For purposes of calculating the exposure to the gallbladder resulting from 1.0 μCi of [^{14}C]cholic acid, we make the following assumptions. (*a*) The concentration of bile acids in gallbladder bile is about 50 mg/ml (14). (*b*) The gallbladder mucosa is continuously coated with a layer of bile that is at least as thick as the penetrating ability of the ^{14}C beta particle (about 1 mm). (*c*) The bile acid pool size is about 2000 mg. (*d*) About 20% of the radioactive cholic acid is converted to absorbed deoxycholic acid (15). (*e*) The half-life of both cholic acid and deoxycholic acid is about 2 days (16, 17). (*f*) Because of 2-π geometry, the gallbladder is irradiated by only half the emitted beta particles.

The standard formula for calculation of exposure from a beta emitter (18) is:

$$\text{Exposure in rads} = 73.8(\bar{E}_B)(t_{1/2})(C_0)$$

where $\bar{E}_B$ is the average energy of the emitted beta particles (about 0.045 MeV for ^{14}C), $t_{1/2}$ is the effective half-life of the labeled substance in days, and C_0 is the concentration of radioactivity in the tissue (μCi/g) at time zero. Calculating from assumptions *a* and *c*, 1.0 μCi of [^{14}C]cholic acid resulted in a C_0 of approximately 0.025 μCi/g.

Multiplying by a factor of 1.2 to correct for deoxycholic acid formation and absorption and by a factor of 0.5 to correct for 2-π geometry, the gallbladder exposure from 1.0 μCi of [^{14}C]cholic acid is

$$\text{Exposure} = (0.5)\,(1.2)\,(73.8)\,(0.045)\,(2)\,(0.025) = 0.0984 \text{ rads, or } 98.4 \text{ mrads.}$$

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