Letters to the Editor

Rapid Fluorometric Determination of Total Bile Acids in Fecal Extracts

To the Editor:

Fecal bile acid excretion represents one of two major pathways for elimination of tissue cholesterol in higher animals. Their analysis is important in studies related to serum cholesterol concentrations, bile acid production and excretion in liver disease, as well as the etiology of atherosclerosis. Analysis of these acids has been difficult, inaccurate and slow, because of a) the diversity of bile acids found in feces, b) destruction of 3-keto-bile acids during analytical workup, and c) the presence of difficult to remove, highly colored acidic pigments in the extracts used for analysis. Because of these problems, photometric and fluorometric methods are inaccurate and have been avoided by substituting accurate, but time-consuming, gas-liquid chromatographic procedures.

We wish to report a major advance in fecal bile acid analysis. We have recently found that it is possible to determine the bile acid content of even crude, highly colored fecal extracts by applying a relatively new fluorometric method: 3α- and 3β-hydroxy-bile acids in fecal extracts (methanolic) reduce β-nicotinamide adenine dinucleotide (β-NAD) in a reaction catalyzed by hydroxysteroid oxidoreductase (HSD). The resulting reduced β-nicotinamide adenine dinucleotide (β-NADH) then reduces nonfluorescent resazurin to fluorescent resorufin in a reaction catalyzed by diaphorase. The overall reaction takes place on a 1:1 molar basis. It has been used to assay dehydrogenases and to determine serum bile acids. Our preliminary evidence suggests that this method can be used to analyze bile acids in highly colored fecal extracts. This is possible because a) the method is exceedingly sensitive and fecal extracts contain relatively high concentrations of bile acids, and b) the pigments found in fecal extracts absorb minimally at 565 nm and 580 nm, the wavelengths used for excitation and fluorescent estimation, respectively, of resorufin.

The extracts used in these experiments were obtained from rat feces according to the following scheme:

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Lyophilized rat feces → extraction (CHCl₃:MeOH) → BH₄ reduction → hydrolysis (2M NaOH; 3 hrs @ 250°F) → Extraction (diethyl ether) → acidify Extraction (petroleum ether) → extraction (diethyl ether) → purified extract
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* Superscript numbers indicate points in the flow diagram where samples were obtained for analysis.

The reagents used in the fluorescent determination contained the following components dissolved in 100 ml of 0.05 M, pH 7.4 phosphate buffer:

**Test reagent:** Two mg resazurin, 4 β-enzyme units (4.2 α-enzyme units) of HSD (1 unit oxidizes 1 μ mole of substrate per minute in the presence of β-NAD at pH 8.9 at 25°C), 48 mg of β-NAD and 33 units of diaphorase (1 unit oxidizes one μ mole of β-NADH per minute at pH 7.5 at 25°C using 2,6-dichlorophenolindophenol as an electron acceptor).
Blank reagents: These reagents had the same composition as the test reagent except that HSD or diaphorase was omitted.

Each tube used for fluorescence measurements contained 2.4 ml of 0.1 M, pH 9 tris buffer, 0.5 ml of test or blank reagent, and 20 μl of sample. A Model 104243 Farrand recording spectrofluorometer was used for measurements. Excitation of fluorescence was at 565 nm and fluorescence was measured at 580 nm. Preliminary tests showed that the method was linear from 0.5 to 5 μg using sodium taurocholate standards. Linear regression correlation coefficients were obtained by determining the fluorescence of 0-20 μl aliquots of rat feces extracts treated with test reagent. The data were plotted and the resulting curves analyzed by the method of least-squares. Correlation coefficients vary from -1 to +1; if the points of a curve exactly fit a straight line, the coefficient is 1. Standard recoveries were determined by adding 1 μg of cholic acid to one of two 10, 16 and 20 μl aliquots of fecal extract. Fluorescent yields were then determined using the test reagent.

<table>
<thead>
<tr>
<th>Type of Extract</th>
<th>Linear Correlation Coefficient</th>
<th>Standard Recovery</th>
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<tbody>
<tr>
<td>1 - Crude</td>
<td>0.990 ± 0.009* (8)**</td>
<td>94.1 ± 4.5*</td>
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<tr>
<td>2 - Partially purified</td>
<td>0.993 ± 0.005 (8)</td>
<td>95.7 ± 3.4</td>
</tr>
<tr>
<td>3 - Purified</td>
<td>0.992 ± 0.006 (8)</td>
<td>95.6 ± 4.3</td>
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* Standard deviation.
** Number of extracts.

The data in the accompanying table summarize our analytical findings. The correlation coefficients show that the method yielded excellent linear results with 0-20 μl aliquots of crude, partially purified, and purified fecal extracts. It would seem valid to argue that such results could be due to nonbile acid fluorescent material. However, when the extracts were treated with blank reagents, lacking HSD and/or diaphorase, no fluorescence developed. That is, the slope of the resulting curves was essentially zero. Since HSD specifically oxidizes 3α- and 3β-hydroxyl groups of 5β-cholan-24-oic acids, it is highly probable that fecal bile acids are exclusively responsible for the fluorescence measured at 580 nm. β-HSD does oxidize the 16- and 17-hydroxyl groups of certain C18, C19, and C24 steroids, but the amounts of these substances in even crude fecal extracts would be exceedingly small, and they would be removed along with other neutral steroids during petroleum ether extraction. Our measurements showed essentially no difference in the apparent bile acid content of partially and totally purified fecal bile acid extracts. The right hand column in the table shows the percentage recovery of standard cholate added to the various extracts. Recoveries were good in all cases.

If the results of the linear regression experiments and those of the standard addition experiments are combined, it appears that it is possible to determine the bile acid content of even crude fecal extracts rapidly by applying the method described above. There is little doubt that the method is precise, but comparisons of results with those obtained using gas-liquid chromatography will be necessary to establish its accuracy. Whether this method can be applied to fecal extracts derived from the excretory products of other species remains to be investigated.

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References