

Comparison of radioimmunoassay and enzyme methods for quantification of serum bile acids

B. Porcelli^a, R. Pagani^a, A. Galli^b, L. Barabesi^c, S. Petralia^d, C. Ulivieri^d, L. Terzuoli^a

^aDepartment of Internal Medicine, Endocrine-Metabolic Sciences and Biochemistry, Siena University, Siena

^bDepartment of Laboratory Medicine, Laboratory of Immuno-Haematology, S. Maria alle Scotte Hospital, Siena

^cDepartment of Quantitative Methods, Siena University, Siena

^dDepartment of Laboratory Medicine, Laboratory of Clinical Analysis, S. Maria alle Scotte Hospital, Siena

Summary

Background. Measurement of bile acids in biological matrices has always been problematical. The development of accurate and sensitive methods of analysis of bile acids has therefore been the subject of much research. The best techniques of analysis of major bile acids in normal human serum are currently high-performance liquid chromatography (HPLC) and gas-liquid chromatography (GC), but they are time-consuming, expensive and unsuitable for routine clinical use especially in the Italian reality, in which the probability that small laboratories have GC or HPLC is very low. Moreover, for analysis of first level as total bile acid determination, technique as GC and HPLC are also excessive, while for a first screening are suitable other analytical procedures.

For this reason, in the present paper, we compared two methods suitable for routine quantification of bile

acids in human serum: radioimmunoassay (RIA) and enzyme assay.

Methods. The tests used for analysis of bile acids were commercially available. With regard to the enzyme method, we used two different tests. Precision and accuracy were evaluated by a control serum. Bile acids were also determined in the serum of 160 subjects.

Results. Neither test based on the enzyme method had the accuracy of the radioimmunological test and they overestimated low concentrations of bile acids. Moreover, the tests were not homogeneous.

Conclusions. Our results indicate that the radioimmunological test is valid and reproducible for routine laboratory determination of bile acids in serum. On the contrary, enzyme tests were not satisfactory, at least when performed manually.

Key words: bile acids, radioimmunoassay, enzyme assay.

Introduction

Bile acids, the end products of cholesterol catabolism in the liver, have a major role as detergents in bile and the small intestine. By formation of solubilizing micelles, they facilitate excretion of bile lipids and absorption of dietary lipids¹. The detergent properties of bile acids are further increased by their hepatic conjugation with the amino acids glycine and taurine before secretion into the bile².

Quantification of serum bile acids has been used as a test for liver disease³, since it provides additional diagnostic information with respect to conventional liver tests⁴. However, because of their complex nature (primary, such as cholic and chenodeoxycholic, secondary,

such as deoxycholic and lithocholic, and conjugated, such as glycine- and taurine-conjugated, sulphated and glucuronidated bile acids), measurement of bile acids in biological matrices has always been problematical. A second problem is that bile acids are present in very low concentrations in samples such as plasma and urine.

The development of accurate and sensitive methods of analysis of bile acids has therefore been the subject of much research. Many procedures for bile acid determination, such as thin layer chromatography⁵, gas-liquid chromatography (GLC)⁶, high-performance liquid chromatography (HPLC)⁷, gas-liquid chromatography-mass spectrometry (GLC-MS)⁸ and capillary

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Corrispondenza a: Dott.ssa Brunetta Porcelli, Dipartimento di Medicina Interna, Scienze Endocrino-Metaboliche e Biochimica, Sezione di Biochimica, Polo Scientifico Universitario di San Miniato, Via Alcide de Gasperi 2, 53100 Siena.
Tel. 0577-234293, fax 0577-234285, e-mail: porcelli@unisi.it

electrophoresis⁹ have been described. The best techniques of analysis of major bile acids in normal human serum are currently HPLC and GC, but they are time-consuming, expensive and unsuitable for routine clinical use especially in the Italian reality, in which the probability that small laboratories have gas-liquid chromatography (GC), or high-performance liquid chromatography (HPLC) is very low. Moreover, for analysis of first level as total bile acids determination, technique as GC and HPLC are also excessive, while for a first screening other analytical procedures are commonly used.

For this reason, in the present paper, we compared two methods suitable for routine quantification of bile acids in human serum: radioimmunoassay (RIA) and enzyme assay.

Methods

The tests used for analysis of bile acids, commercially available, were:

- Radioimmunoassay (A) (Conjugated Bile Acids Component System, ICN Pharmaceuticals, Costa Mesa, CA) for the quantitative determination of conjugated bile acids. Tubes coated with antiserum (rabbit) and an [¹²⁵I] tracer containing glycocholic acid derivative in buffer with bovine gamma globulin are used to measure conjugated bile acids. The unlabelled analyte competes with labelled analyte for a limited number of available antibody binding sites. Bound radioactivity is inversely related to the concentration of analyte. The test yields satisfactory correlation with the 3 α -hydroxysteroid dehydrogenase assay. Normal range: less than 6 μ mol/l.
- Enzyme assay (B) (Bile Acids enzymatic colorimetric, Randox, Crumlin, Antrim, UK) for the quantitative determination of conjugated bile acids. 3- α hydroxy bile acids are converted to the corresponding 3-keto-hydroxy bile acids in the presence of NAD⁺ by 3 α -hydroxysteroid dehydrogenase (3- α HSD). The NADH formed reacts with nitroterrazolium blue in a diaphorase catalysed reaction to form a stable blue formazan dye with an absorption maximum at 540 nm. Linearity: up to a concentration of 200 mmol/l. Normal range: 0-6 μ mol/l.
- Enzyme assay (C) (Total Bile Acids, Bio-Stat, Stockport, Cheshire, UK) for the quantitative determination of total bile acids. In the presence of thio-NAD⁺, the enzyme 3 α -hydroxysteroid dehydrogenase converts bile acids to 3-keto steroids and thio-nicotinamide adenine dinucleotide (thio-NADH). Thio-NADH formation is determined by measuring the specific change in absorbance at 405 nm. Linearity: from 1 to 180 μ mol/l. Normal range: 0-10 mmol/l. No interference was observed from ascorbic acid up to 50 mg/dl, bilirubin up to 50 mg/dl, haemoglobin up to 500 mg/dl and lipaemia up to 750 mg/dl of triglyceride.

A control tri-level serum was used to evaluate preci-

sion and accuracy of the three tests (MP Biomedicals, New York, NY). Mean and range of bile acid three levels of (low, medium and high, expressed as μ mol/l) were respectively: 0.73 (0.51-0.95); 16.5 (12.5-20.5); 19.1 (14.5-23.7)

For precision, run to run and day to day were determined six times per day for 10 days. To evaluate the accuracy of the tests control serum was assayed in triplicate.

Serum bile acids were determined by tests (A), (B), (C) and used for statistical analysis. Blood was drawn from 160 subjects with their informed consent, after overnight fasting, and centrifuged within 2 h from collection at 1000 x g for 15 min.

Statistical analysis

Homogeneity of tests (B) and (C) were compared with test (A), used arbitrarily as reference method, analysing the two groups of differences obtained by pairing the measurements of serum bile acids respect to test (A). In this way, verification of homogeneity hypothesis between the tests corresponded to verification of a zero median for both group of differences. Since the two groups of differences were strongly asymmetrical, with many anomalous values, the sign test¹⁰ was used to verify the hypothesis of homogeneity.

Results

Run to run and day to day precision of tests (A), (B) and (C) are reported in Table I. The CVs were: for run to run precision, < 20% (A), (B), (C); for day to day precision, < 20% (A) and (B) and > 20% (C) for low values of bile acids.

The accuracy of test (A) was optimal, while (B) and (C) tended to overestimate low concentrations of bile acids.

With regard to homogeneity between tests, the hypothesis of zero median was strongly rejected for both groups of differences, indicating that the tests were not homogeneous. Specifically, the confidence intervals of the median of the differences (sign test) were 1.38 and 2.16 for differences between tests (A) and (B), and 14.86 and 20.57 for differences between tests (A) and (C).

Discussion

The low concentrations and complex nature of bile acids makes their analysis in biological matrices problematical. Their concentrations in serum, however, are important diagnostic and prognostic indicators of hepatobiliary and intestinal dysfunction.

Bile acids may occur free or conjugated with glycine and taurine, or as sulphates and glucuronides. Free forms are only a small percentage of total bile acids in serum and bile¹¹, and conjugated bile acids show a prevalence of cholic acid derivatives. Healthy subjects and patients with liver disease both show a preponderance

Table I. Precision of radioimmunoassay (A), and enzymatic (B and C) tests.

Bile acids values			
Values of control serum μmol/l	Low 0.73 ± 0.22	Medium 16.5 ± 4.0	High 19.1 ± 4.6
Run to run precision (n = 6)			
Mean ± SD (μmol/l)	0.66 ± 0.01	Test (A) 15.32 ± 0.20	21.45 ± 1.87
CV (%)	1.51	1.30	8.71
Mean ± SD (μmol/l)	2.21 ± 0.29	Test (B) 18.62 ± 0.99	20.73 ± 1.38
CV (%)	13.12	5.31	6.65
Mean ± SD (μmol/l)	3.01 ± 0.42	Test (C) 19.10 ± 2.17	31.24 ± 3.46
CV (%)	13.95	11.36	11.07
Day to day precision (n = 10)			
Mean ± SD (μmol/l)	0.88 ± 0.03	Test (A) 18.55 ± 1.33	24.4 ± 2.3
CV (%)	3.40	7.16	9.42
Mean ± SD (μmol/l)	2.26 ± 0.45	Test (B) 21.16 ± 1.52	25.42 ± 3.26
CV (%)	19.91	7.18	12.83
Mean ± SD (μmol/l)	3.47 ± 1.48	Test (C) 22.81 ± 2.56	33.13 ± 6.45
CV (%)	42.65	11.22	19.47

of conjugated bile acids in serum, with increases in several disorders¹².

Prevalence of non conjugated forms has only been found in certain liver disorders associated with cholestasis¹³ and in cirrhosis¹⁴. This means that simple analytical procedures can be used to assay bile acids without distinguishing qualitative differences. A first screening of patients is therefore possible, monitoring hepatobiliary function by means of these methods. For differential diagnosis, this could be followed by more sophisticated methods such as GC, HPLC and MS.

In the present study we compared two methods that can enable routine assay of bile acids without preliminary extraction of samples, and which can also be performed manually. Three tests based on these methods were compared. The data obtained for radioimmunological method (A) confirmed literature data^{15,16}. The CVs of determinations using a control serum were very low, indicating high precision. Accuracy was also high. Test (A) also offered all the advantages of RIA (having eliminated the problem of radioactive waste generated in our procedures, which was collected by staff from the Radiation Safety Office of Hospital): it used small sample volumes (25 μl) and was sufficiently rapid. Moreover, according to the test instructions, it was not affected by other steroids present in serum. Minor conjugated and unconjugated bile acids had little effect on the results due to their lower serum con-

centrations and/or low cross reactivity with the antibody used in the assay.

With regard to the enzyme method, we used two tests which reveal by two different ways the reaction product obtained by the enzyme 3- α hydroxysteroid dehydrogenase. The enzyme catalyses NADH formation, but direct spectrophotometric determination of NADH is not sufficiently sensitive to measure bile acids in the serum of normal subjects. In test (B), the signal is amplified by the diaphorase-tetrazolium salts system, whereas in test (C) formation of thio-NADH is monitored.

Neither test had accuracy of the radioimmunological test and they overestimated low serum concentrations of bile acids. Statistical analysis showed that the tests were not homogeneous, though (B) was more compatible with (A) than was (C), presumably because its procedure involves using a blank for each sample, largely excluding interference due to various substances. In the case of gross interference, however, the blank did not cancel out the effects. As a result of this phenomenon, which is sample-related, anomalous values were obtained and these caused non homogeneity between tests (A) and (B). This problem was amplified in test (C), probably due to a series of factors such as use of small serum volumes (4 μl), lack of blank for every sample, kinetic reading done in 2 minutes, and, in the specific case of this study, manual

performance of the test. Automatised procedures could probably at least partly solve the problem of the kinetic signal.

Our results confirm data in the literature showing the limited analytical sensitivity of the enzyme method for determination of bile acids¹⁷, despite the specificity of the enzyme 3- α HSD. Fluorescent enzyme methods have been developed to increase sensitivity, though interference by background fluorescence impairs their linearity¹⁸. Tanghoi et al.¹⁹ used an enzyme test similar to (B): however, as found by us, the test was not accurate at low bile acid concentrations between runs or between days.

Our results indicate that the tests based on the enzyme method are not satisfactory for determination of bile acids in serum, at least when performed manually; on the contrary, also if unfortunately it is not possible its automation, radioimmunological test is valid, reproducible and useful for routine assay of serum bile acids, especially when the analysis number is limited and doesn't permit the automation of the method.

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