Enzymic Fluorimetric Determination of Sulphated and Non-sulphated Primary Bile Acids in Urine Using a Rapid Solvolysis Technique

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A simple and rapid enzymic fluorimetric method for the determination of sulphated and non-sulphated primary bile acids in urine has been developed. Octadecylsilane-bonded silica cartridges (Sep-Pak C_{18}) are used for the solid-phase extraction of bile acids (BA) from urine samples. Sulphated BA are solvolysed before measurement with an improved rapid solvolysis procedure. The measurement is based on the reaction of 7α-hydroxylated BA with β-nicotinamide adenine dinucleotide (β-NAD^+) in the presence of the enzyme 7α-hydroxysteroid dehydrogenase (7α-HSD). The NADH generated is monitored fluorimetrically. The mean relative standard deviation of the method was 8% ($n = 32$), the detection limit 0.4 µM and the mean recovery of added BA 99%. The solvolysis step in this procedure takes about 4 h and is faster than the conventional procedures which are usually carried out in an 18-h protocol. Sulphated and non-sulphated primary BA were determined in urine from 16 healthy persons and 67 hospitalised patients suffering from various hepatobiliary diseases. The reference range for 7α-hydroxylated urinary BA was in agreement with previously published results. The method is simple and suitable for routine clinical use. About 30 urine samples can be analysed in one working day.

Keywords: *Bile acids determination; 7α-hydroxysteroid dehydrogenase; fluorimetry; urine analysis; enzymic method*

In recent years there has been increasing interest in bile acid (BA) analysis because such determinations are valuable for the diagnosis of various hepatobiliary diseases.[1] Methods for the determination of BA in biological fluids have been reviewed.[2] In a recent report,[3] the predictive value of urine BA analysis for various liver diseases and its usefulness for screening and for following up liver disease has been stated. Especially when the urine flow is corrected by using the BA to creatinine ratio, the predictive value of urinary BA analysis is equal to or exceeds that of fasting plasma BA and routine liver tests.[3] In addition, urine samples are preferred to blood because (a) there is a greater risk of AIDS infection from blood samples, (*b*) renal excretion of BA reflects more the steady state than do short interval changes in plasma BA induced by gall-bladder contraction,[3] (c) the simplicity of urine sampling has an advantage especially in screening for liver disease and for outpatient follow-up and (d) it is easier to take urine samples from young infants and newborn babies.

BA are usually determined in urine by high-performance liquid chromatography (HPLC),[4] gas - liquid chromatography (GLC),[5] radioimmunoassay (RIA)[6] or gas chromatography - mass spectrometry (GC - MS).[7] More recently two enzymic spectrophotometric methods for the determination of BA in urine have been developed.[8,9] Most of these methods give detailed information on BA and can be used in metabolic studies, but they are time consuming and require many preparatory steps, so it is difficult to use them as routine screening procedures.

As sulphated BA constitute the great majority of bile acids excreted into the urine of patients suffering from hepatobiliary diseases, their determination is of great diagnostic importance. In addition, inadequate solvolysis techniques for the 7-sulphated bile acids can lead to the underestimation of their concentration levels in biological fluids.[10]

In this paper, a simple and rapid enzymic fluorimetric method for the determination of sulphated and non-sulphated BA in urine is described. Octadecylsilane-bonded silica cartridges (Sep-Pak C_{18}) are used for the solid-state extraction of BA from urine. An improved solvolysis step is used for the rapid (4 h) solvolysis of 7-sulphate esters of BA. Free BA are then determined after reaction with β-nicotinamide adenine dinucleotide (β-NAD^+) in the presence of the enzyme 7α-hydroxysteroid dehydrogenase (7α-HSD). The NADH generated is measured fluorimetrically after the end of the reaction. The use of 7α-HSD instead of 3α-HSD is advantageous in terms of selectivity, as most of the 7α-steroids in urine are primary BA.

The detection limit of the method was 0.4 µM and the average relative standard deviation about 8%. Recovery experiments gave a mean of 99% for various concentration levels. The reference range for total primary BA in urine with this method was 1.3–1.8 µM (n = 16), which is in agreement with previously published results.[9]

The method was applied to the determination of sulphated and non-sulphated BA in urine from 67 patients and 16 healthy individuals. Statistical evaluation of the results by non-parametric tests (Wilcoxon's rank sum test, Lehman index)[11] showed a statistically significant difference between the healthy and various diseased groups. Sulphated BA are the predominant components (>65%) of total BA.

The proposed method is simple to perform, rapid (30 samples per working day) and reliable. It can be used as a screening method for diagnostic purposes. To our knowledge, this is the first enzymic fluorimetric method for the determination of primary BA in urine.

Experimental

Apparatus

All measurements were performed using a Perkin-Elmer M512 fluorescence spectrophotometer with a 150-W xenon lamp and a 1.000 cm path length cuvette with continuous stirring and at constant temperature (25.0 ± 0.1 °C).[12]

The following instrumental settings were used for the measurement: ratio mode; dynode voltage, 750 V; excitation wavelength, 340 nm; excitation slit width, 20 nm; emission wavelength, 455 nm; and emission slit width, 20 nm. The sensitivity scale factor used to measure fluorescence intensity was at the ×10 position. The instrument was calibrated against quinine sulphate standard solutions. The fluorimetric signals were recorded on a Sargent-Welch XKR potentiometric

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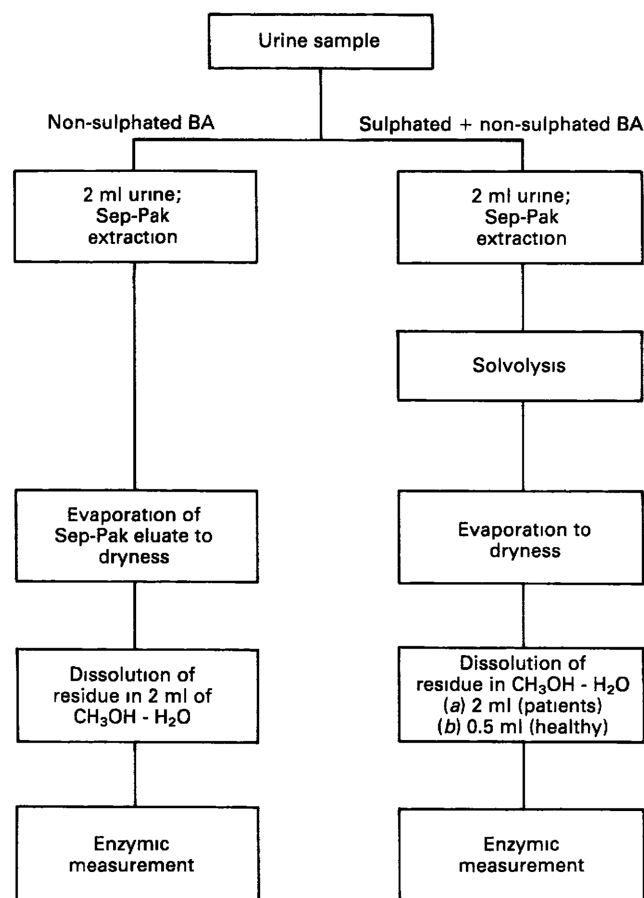

Fig. 1. Schematic diagram of the procedure for urinary BA determination

recorder. The sonicator used was a Metason H 50–60 from Struers.

Reagents

All solutions were prepared in doubly distilled, de-ionised water from analytical-reagent grade materials.

7α-HSD (*E.C.* 1.1.1.159), 0.50 U mg^{-1} (*Worthington*). This was supplied as powder obtained from *E. coli*. A stock enzyme solution of 1.0 U ml^{-1} in 0.020 M Tris buffer (pH 7.2) containing 2 mM EDTA was prepared. This solution was stored at −20 °C, was stable for at least 1 week and was kept in an ice-bath when in use.

β-NAD$^+$. A 10.0 mM solution was prepared by dissolving 0.0300 g of β-NAD$^+$ (grade A; Calbiochem) in 5.00 ml of water. This solution was kept in a refrigerator and was stable for at least 2 weeks.

Glycine (1.0 M) - *hydrazine* (0.40 M) *buffer* (*pH* 9.5). Prepared by dissolving 37.6 g of glycine, 26.0 g of hydrazine sulphate and 0.93 g of $Na_2EDTA.2H_2O$ in about 400 ml of water, adjusting the pH to 9.5 with NaOH solution and diluting to 500 ml.

BA solutions. Sodium salts of cholic acid (CA) and chenodeoxycholic acid (CDCA) were purchased from Sigma and were chromatographically pure (TLC).[13] Stock solutions of BA (1.00 mM) were prepared by dissolving appropriate amounts of the sodium salts in water.

Sulphated BA solutions. Sodium salts of CDCA 3-sulphate and CA 3-sulphate were kindly donated by Dr. Kirck (Steroid Reference Collection, London). 3,7-CDCA disulphate was synthesised as described by Parmentier and Eyssen.[14] All sulphate esters of BA were chromatographically (TLC) homogeneous[13] and enzymatically inactive to both 3α-HSD and 7α-HSD. The solid compounds were dried under vacuum at 40 °C for 48 h before use. Stock solutions of sulphated BA (1.00 mM) were prepared by dissolving appropriate amounts of these BA in water.

Samples

Urine samples (24 h) were collected from 67 patients with various hepatobiliary disorders and from 16 healthy persons. All urine samples were kept at −20 °C until use.

Procedure for the Determination of BA in Urine

The procedure consists of three main steps (Fig. 1): extraction of BA, solvolysis and enzymic fluorimetric measurement.

Extraction of BA

A Sep-Pak C_{18} cartridge (Waters Associates) is washed with 3 ml of methanol and 6 ml of water, then 2.00 ml of a urine sample is applied and passed through at a rate of about 1 drop s^{-1}. The cartridge is washed with 2 × 2 ml of dilute acetone solution (1 + 4). The BA are then eluted with 2 × 2 ml of absolute methanol. The eluate is evaporated to dryness at 40 °C under nitrogen and the residue dissolved in 0.50 ml (for normal urine samples) or 2.00 ml (for patients' urine samples) of dilute methanol solution (1 + 1). This extract is used for the determination of non-sulphated primary BA in urine. The same extraction procedure is used for the determination of total sulphated and non-sulphated primary BA. In that event the methanolic eluate from patients' and normal urine samples is subjected to solvolysis.

Chemical solvolysis

The 4-ml methanolic extract obtained from the Sep-Pak is mixed with 4 ml of dimethoxypropane (DMP), 2 ml of diethyl ether and 1 drop of concentrated HCl. The mixture is then sonicated for 4 h at 40 °C and evaporated to dryness under a stream of nitrogen. The residue is dissolved in 2.00 ml of methanol - water (1 + 1) for patients' samples or 0.50 ml for samples from healthy subjects and the solution obtained is used for the determination of total primary BA, sulphated and non-sulphated.

Enzymic fluorimetric measurement of BA

A 1.60-ml volume of glycine - hydrazine buffer solution (pH 9.5), 0.100 ml of 7α-HSD solution and 0.200 ml of sample or standard are placed in a cuvette. The stirrer is started and, after the fluorescence signal has stabilised, the reaction is initiated by injecting 0.100 ml of β-NAD$^+$ solution. The final fluorescence signal is recorded after the completion of the reaction.

Calibration Graphs and Calculations

Two calibration graphs (fluorescence intensity *vs.* concentration) were constructed, (A) using as a standard free CDCA solutions in BA-free urine, not submitted to the solvolysis procedure, for the determination of non-sulphated primary BA, and (B) using as a standard CDCA 3,7-disulphate solutions in BA-free urine treated according to the whole analytical procedure, for the determination of sulphated BA. The concentration range of the standards was between 10 and 80 μM.

The measurement of the fluorescence intensity (F_1) which corresponds to the urine samples not subjected to solvolysis was used for the calculation of the free (non-sulphated) primary BA from calibration graph A.

The measurement of the fluorescence intensity (F_2) obtained from the urine samples subjected to solvolysis corresponds to the total primary BA in urine. The difference $F_2 - F_1$ is used to calculate the sulphated BA concentration from calibration graph B.

Table 1. Effect of the concentration of sulphate esters on the determination of free primary BA with 7α-HSD

Sulphate ester	Molar ratio (sulphated : non-sulphated)	Recovery, %	
		CDCA	CA
CA 3-sulphate . .	0	100	100
	6.2	—	—
	8.3	110	102
	12.5	105	102
	25.0	100	108
	Mean ± SD:	104 ± 5	103 ± 4
CDCA 3-sulphate . .	0	100	100
	6.2	80	89
	8.3	97	96
	12.5	99	98
	25.0	96	95
	Mean ± SD:	94 ± 8	96 ± 4
CDCA 3,7-disulphate . .	0	100	100
	6.2	85	88
	8.3	94	98
	12.5	99	97
	25.0	97	95
	Mean ± SD:	95 ± 6	96 ± 5

Statistics

For the calibration graphs linear regression analysis was used. For the statistical evaluation of the results for urine samples Wilcoxon's non-parametric test was used[11] and the Lehman index[11] was determined for each group of patients.

Results and Discussion

General considerations concerning optimum pH, temperature, effect of β-NAD$^+$ concentration on the reaction rate, NADH monitoring and other measurement details were similar to those reported previously.[12,15]

Extraction of BA from Urine

Unextracted urine exhibits a high background fluorescence even when diluted 10-fold, so it is not suitable for the direct measurement of BA with a fluorimetric tehnique. The solid-phase extraction procedure that was employed is a simple and effective way of reducing the background fluorescence and at the same time provides pre-concentration of the urine samples when low BA concentrations are present, *e.g.*, in normal urines. It was found that most of the fluorescence compounds in urine are removed during the acetone washing step. The background fluorescence of 12 urine samples was significantly reduced (about 95%) after the Sep-Pak extraction. The recovery of free CA and CDCA and conjugates from the Sep-Pak extraction was found to be 90%, in agreement with previous observations.[16] Complete dissolution of the residue after extraction is facilitated with methanol - water (1 + 1).

Solvolysis

For the determination of total primary BA, sulphated and non-sulphated, a solvolysis step is essential. Both the 3α- and 7α-positions must be hydrolysed, because 7α-HSD does not catalyse the oxidation of the non-sulphated 7α-position when the 3α-position is esterified with sulphate. This effect, which is presumably due to steric hindrance,[9] can lead to a false negative determination of 7α-BA. However, non-esterified BA can be measured accurately in the presence of sulphated BA (Table 1).

It is well known that solvolysis of the 3-sulphate esters of BA is completed in only 3–4 h whereas 7-sulphated esters of BA are resistant to chemical solvolysis methods reported so far and require 18–24 h for complete solvolysis.[10] The long time required for complete solvolysis of 7-sulphated esters of BA is a great disadvantage of many published analytical techniques[4,6,8,9] as they cannot be used as routine screening methods.

Table 2. Time required for complete solvolysis of the 3- and 7-sulphated esters of CDCA by four different methods

Method	Reference	3-Position Reaction time/h	3-Position Solvolysis, %	7-Position Reaction time/h	7-Position Solvolysis, %
1	10	4	98	24	98
2	14	18	100	18	90
3	17	4	81	24	82
4	This work	4	97	4	84

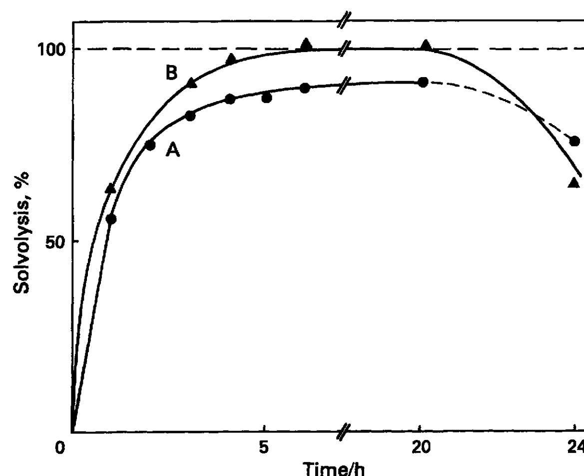

Fig. 2. Effect of time on the solvolysis of 60 μM of CDCA disulphate solution at 40 °C (each point in triplicate). (A) 7α-Position (determined by 7α-HSD); and (B) 3α-position (determined by 3α-HSD)

Our modified proposed solvolysis method shortens the time required for complete solvolysis of the 7-sulphate esters of BA from 18 and 24 h[10,14] to 4 h. A comparative study of the time required for complete solvolysis of the 3- and 7-sulphate esters of CDCA by the proposed and three established methods[10,14,17] is presented in Table 2. Our modified rapid solvolysis procedure is based on the principles described by Burstein and Lieberman.[18] The solvolysis mixture consists of the methanolic Sep-Pak extract, of DMP for the removal of the water content as it reacts with water to form a methanol - acetone mixture[19] and diethyl ether, which, according to Burstein and Lieberman,[18] accelerates the solvolysis significantly. One drop of concentrated HCl is sufficient to acidify the solvolysis mixture.

Sonication is used here for the first time in order to facilitate better mixing and acceleration of the reaction. It was observed that the sonication time has an important effect on the rapidity of solvolysis of the 7-sulphate ester of CDCA. It was found that sonication should be carried out during the whole solvolysis time in order to obtain the best results. The time dependence of the solvolysis for both the 3α- and 7α-positions is shown in Fig. 2. It can be seen from Fig. 2 that solvolysis for the 3α-position is more rapid than that for the 7α-position. Extension of the solvolysis time to 24 h failed to increase the yield of solvolysed BA; in fact, the recovery decreased. These findings are in accordance with those of Dommes *et al.*[17]

The temperature dependence of the solvolysis was studied. The higher the temperature the more complete was the

solvolysis for both the 3α- and 7α-positions, but an upper temperature limit of 40 °C was set because of the volatility of the organic solvents used in the solvolysis mixture. Solvolysis for the 3α-position is more rapid than that for the 7α-position. CDCA disulphate was used as a standard to check the solvolysis procedure.

Enzymic Fluorimetric Method

The enzymic fluorimetric method used for the determination of primary BA was first reported by Haslewood *et al.*,[20] and many applications and modifications of this method have been reported for the determination of primary BA in bile and serum.[15,16] This method has now been applied for the first time to urine samples and many problems had to be solved, as urine is a highly fluorescent biological sample.

Calibration graphs with free CDCA sodium salt (A) and CDCA disulphate solutions in BA-free urine (B), which were subjected to the whole procedure, are linear at concentrations up to 80 μM and cover the whole range expected to be found in urine from normal persons and patients with hepatobiliary disorders. The following equations were used: (A) $y = 0.25 + 1.13x$ ($r = 0.998$, $n = 8$) and (B) $y = 1.00 + 0.92x$ ($r = 0.999$, $n = 8$), where y is the fluoresence intensity and x is BA concentration. The relative standard deviation for urine samples subjected to the whole analytical procedure varied from 4.8 to 11.4% for CDCA disulphates and from 7.9 to 9.0% for the non-sulphated CDCA for high and low concentrations, respectively (eight runs per concentration).

The accuracy of the method was tested with recovery experiments on the whole procedure by adding appropriate amounts of BA to a urine sample from a healthy individual. The average recovery of added free BA was 96% (range 87–108%) and of sulphated BA 102% (range 96–110%). Analytical recoveries are shown in Table 3. The detection limit [the analyte concentration giving a signal equal to the blank signal (normal urine) plus three standard deviations of the blank] was 0.4 μM.

The proposed method was applied to the determination of sulphated and non-sulphated BA in urine samples from patients with various hepatobiliary disorders ($n = 67$) and healthy individuals ($n = 16$). The patients were divided into nine different groups according to their disease.

The statistical evaluation of the results by Wilcoxon's rank sum test revealed a statistically significant difference from normal values in the groups with Chron's disease ($P<0.005$), liver cirrhosis ($P<0.005$), liver cirrhosis - cholestasis ($P<0.001$), chronic hepatitis ($P<0.001$) and alcoholic hepatitis ($P<0.05$), whereas no statistically significant differences from normal values were observed in the groups with ulcerative colitis ($P>0.5$), various cancers ($P>0.05$), cured alcoholic hepatitis ($P>0.1$) and other non-hepatic diseases ($P>0.1$). The Lehman index value correlated well with these results. The statistical evaluation of the results is given in Table 4. The results are summarised in Fig. 3. It can be seen from Fig. 3 that sulphated BA comprised the majority of BA excreted in the urine in the groups tested, that is, (a) liver cirrhosis, 79%; (b) liver cirrhosis - cholestasis, 65%; and (c) chronic hepatitis, 77%.

The proposed method does not discriminate between free BA and their glycine or taurine conjugates as do all enzymic methods. Further, only the primary BA, CA and CDCA and

Table 3. Analytical recoveries of different mixtures of CDCA and CDCA 3,7-disulphate added to urine samples

Added/μM		Recovered‡/μM		Recovery, %	
CDCA* +	CDCA disulphate†	CDCA +	CDCA disulphate	CDCA +	CDCA disulphate
30	30	28	33	93	110
20	40	18	41	90	103
20	60	17	63	85	105
15	60	15	59	100	98
15	75	15	78	100	104
20	—	22§	—	110	—
80	—	78§	—	98	—
—	20	—	19§	—	95
—	80	—	79§	—	99
		Mean ± SD	..	96 ± 8	102 ± 5
		n	..	7	7
		RSD, %	..	8.4	4.9

* From equation (A).
† From equation (B).
‡ Mean value of two determinations.
§ Mean value of six determinations.

Table 4. Statistical evaluation of the results of the determination of primary BA in urine

Diagnosis	n	Range/μM	Median value/μM	P	Lehman index	Statistical significance*
Healthy	16	0–4.3	0.25	—	—	—
Ulcerative colitis	17	0–36.9	1.1	>0.5	0.39	NS
Chron's disease	11	0–269	5.4	<0.005	0.16	S
Various cancers	11	0–11.9	2.9	>0.05	0.37	NS
Alcoholic hepatitis	2	6.5–10.9	8.7	<0.05	0	S
Alcoholic hepatitis, cured	2	0	0	>0.1	0.75	NS
Liver cirrhosis	7	1.1–30.5	5.4	<0.005	0.11	S
Liver cirrhosis - cholestasis	5	27.1–83.4	39.0	<0.001	0	S
Chronic hepatitis	8	6.8–89.7	16.3	<0.001	0	S
Non-hepatic diseases	4	0	0	>0.1	0.75	NS

* NS = not statistically significant; S = statistically significant.

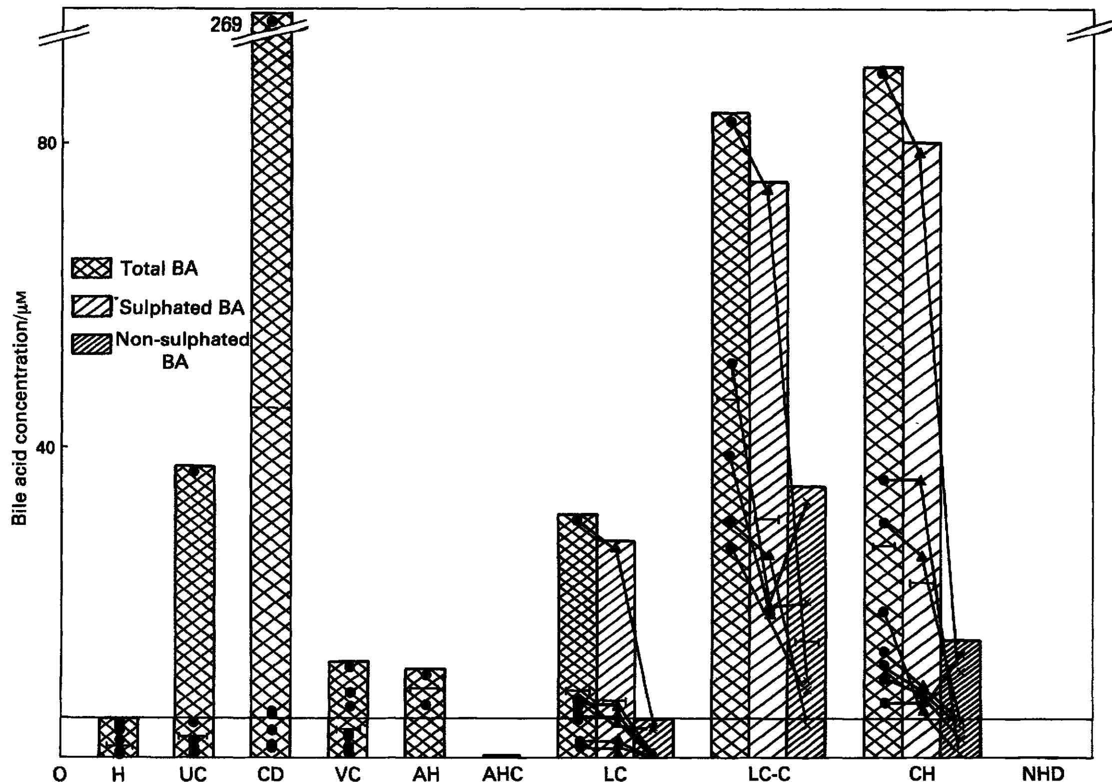

Fig. 3. Distribution of urine BA levels by diagnostic group in 83 patients. (H) Healthy; (UC) ulcerative colitis; (CD) Chron's disease; (VC) various cancers; (AH) alcoholic hepatitis; (AHC) alcoholic hepatitis, cured; (LC) liver cirrhosis; (LC-C) liver cirrhosis - cholestasis; (CH) chronic hepatitis; and (NHD) non-hepatic diseases

their conjugates are measured. However, this is not a disadvantage as most of the 7α-substituted compounds in urine are 7α-BA and other 7α-substituted compounds are rarely found, as in cases of cerebrotendinous xanthomatosis.[21]

The major merits of the present method are the new rapid solvolysis step and the simplicity and ease of the sample preparation, which can be also applied as a rapid preparatory step in many other analytical techniques, such as HPLC, GC-MS and enzymic - spectrophotometric assays. The method does not require expensive instruments as in HPLC and RIA units, but only simple filter fluorimeters, and can be used in small clinical chemistry laboratories. Thirty urine samples can be analysed for BA in one working day. It will be useful as a routine screening method for the determination of BA in many hepatobiliary diseases, especially when the urine BA to creatinine ratio is used, which reliably reflects the presence and degree of liver disease even when it is mild, with an accuracy that equals or exceeds that of routine liver tests.[3]

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