Journal of Chromatography, 452 (1988) 399–408
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 754

QUANTITATIVE MICROANALYSIS OF BILE ACIDS IN BIOLOGICAL SAMPLES

COLLABORATIVE STUDY

FUMIO NAKAYAMA*
Kyushu University, Faculty of Medicine, Department of Surgery I, Fukuoka (Japan)

SUMMARY

The analysis of bile acids in biological samples has always presented a problem because of their complex nature and low concentration. Recently, newer analytical procedures for bile acids have become available, including enzymatic analysis, radioimmunoassay, thin-layer chromatography (TLC), gas chromatography, high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) with selected ion monitoring (SIM). However, they differ greatly with respect to specificity, sensitivity, accuracy and simplicity. On the other hand, the choice of analytical procedure differs according to the specific aims and the nature of biological samples to be analysed. These newer procedures have been compared in a double-blind fashion by distributing bile, plasma and urine samples to seven participating laboratories. GC–MS–SIM was found to be the most sensitive and reliable, but it requires other procedures for preliminary clean-up and fractionation steps. Enzymatic analysis is simple and gives small analytical errors but tends to overestimate plasma bile acids. Radioimmunoassay gives variable results but is useful as a screening procedure for large numbers of plasma samples. TLC gives reliable results for biliary bile acids in experienced hands, except for differentiation between conjugated dihydroxycholanoic acids. HPLC, whether using derivatization or with fixed 3α-hydroxy steroid dehydrogenase detection, is suitable for the analysis of major bile acids in normal human serum but not for the identification of unknown minor peaks.

INTRODUCTION

The analysis of bile acids** in biological samples has presented problems first because of the complex nature of the bile acids present, *i.e.*, primary, such as cholic

* This paper has been prepared by F. Nakayama on behalf of the participants of the comparative study on quantitative microanalysis of bile acid analysis. The participants were: F. Nakayama, T. Hoshita, S. Ikawa, T. Osuga, T. Nambara, I. Makino and H. Miyazaki.

** In this paper, bile acids include mono-, di-, tri- and gtetrahydroxycholan-24-oic and -cholen-24-oic acid and -23-nor-bile acid and -keto-bile acid.

0021-9673/88/$03.50

and chenodeoxycholic, and secondary, such as deoxycholic and lithocholic, and unconjugated and conjugated, such as glycine and taurine conjugated, sulphated and glucuronidated. Second, bile acids are present in very low concentrations in some biological samples such as plasma and urine. Therefore, the development of accurate and sensitive methods of analysis of bile acids has been the subject of intensive research. Recent advances in instrumental analysis seem to have solved the problem to some extent. At present colorimetric, spectrophotometric, paper chromatographic, thin-layer chromatographic (TLC), gas chromatographic (GC), high-performance liquid chromatographic (HPLC) and GC–mass spectrometric with selected ion monitoring (GC–MS–SIM) techniques, in order of historical development, are available. However, they differ greatly with respect to specificity, sensitivity, accuracy and simplicity. The choice of analytical procedure differs according to the specific aims and the nature of biological samples to be analysed. In this work methods of quantitative microanalysis of bile acids such as radioimmunoassay (RIA), TLC, HPLC and GC–MS–SIM were compared a double-blind fashion with respect to various biological samples such as bile, plasma and urine. Seven leading laboratories in Japan participated in the study.

EXPERIMENTAL

As no single laboratory had sufficient expertise on all the analytical procedures to be compared, a collaborative study was necessary. This study was designed to include leading laboratories specializing in bile acid research, and Kyushu University Faculty of Medicine, Department of Surgery I, Fukuoka (Professor Fumio Nakayama, Dr. Jiro Yanagisawa and Dr. Hitoshi Ichimiya), Hiroshima University School of Medicine, Department of Pharmaceutical Sciences, Hiroshima (Professor Takehiko Hoshita), Tottori University School of Medicine, Steroid Research Institute, Yonago (Professor Shiro Ikawa), University of Tsukuba School of Medicine, Department of Internal Medicine, Ibaraki (Professor Toshiaki Osuga and Dr. Yasushi Matsuzaki), Tohoku University, Pharmaceutical Institute, Department of Analytical Chemistry, Sendai (Professor Toshio Nambara and Dr. Junichi Goto), Hirosaki University School of Medicine, Department of Internal Medicine III, Hirosaki (Associate Professor Isao Makino) and Nihon Kayaku Research Institute, Tokyo, Japan (Dr. Hiroshi Miyazaki) participated.

Each laboratory was asked to perform particular analyses according to their expertise. The comparative study was carried out in a double-blind fashion. A set of the same bile, urine and plasma samples was distributed to each participating institution, identification of the samples being known only to the principal investigator (Prof. Fumio Nakayama). The bile samples used were either puncture aspirated gallbladder bile at the time of cholecystectomy or hepatic bile collected via external drain from obstructive jaundice cases. A total of ten bile samples were distributed, of which three were prepared in triplicate from the same bile sample. As large amounts of human serum could not be obtained, frozen plasma (with sodium tartrate, citric acid and glucose added) from a blood bank and human plasma obtained at the time of plasmapheresis was used. Ten plasma samples were distributed, of which three were prepared in triplicate from the same plasma sample. Urine was obtained from by 24-h collection from patients with cholelithiasis, obstructive jaundice and gastric cancer.

Ten urine samples including three triplicate samples prepared from the same sample were also distributed.

In order to avoid the variances arising from differences in the bile acid standard used, ursodeoxycholic acid, *ca.* 99% pure by GC, obtained from Tokyo Tanabe (Tokyo, Japan) was also shipped to each participating laboratory.

Analytical procedures

Enzymatic analysis (EA). 3α-Hydroxysteroid dehydrogenase (3α-HSD) and 7α-HSD and 3β-HSD were used[1,2]. In some cases, bile acids in plasma were analysed enzymatically after fractionation into free and glycine- and taurine-conjugated bile acids by piperidinohydroxypropyl-Sephadex LH-20 (PHP-LH-20) column chromatography[3].

RIA. Glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA) RIA methods were used, with antisera prepared in the laboratory or obtained commercially (Dainabott or Eiken Immunochemicals, Tokyo, Japan)[4,5].

GC. After extraction of biological samples and hydrolysis, bile acids were derivatized into either methyl ester trimethylsilyl or ethyl ester dimethylethylsilyl ethers and subjected to GC using packed[6,7] or capillary[8] columns. For plasma samples, solvolysis preceded hydrolysis.

TLC. After separation on thin-layer plates and colour development, free and glycine- and taurine-conjugated bile acids were quantitated densitometrically[9].

HPLC. Bile acids in bile or plasma were extracted with a Sep-Pak C_{18} column[10] followed by fractionation on a PHP-LH-20 column into free and glycine- and taurine-conjugated bile acids. Each group was separated by HPLC with various detection systems: (1) direct UV detection, (2) prelabelling with 1-anthroyl nitrile[11] and (3) UV detection after passage through a 3α-HSD column[12]. An ethanol extract of bile was injected directly into the HPLC instrument when profile analysis was desired[13]. The columns and separation conditions used have been described previously[3,11–13].

GC–MS–SIM. A capillary column gas chromatograph coupled with a multiple ion detector was used with five deuterated bile acid standards[14].

Comparison of methods. Each method provides a variety of information. Therefore, in order to compare the accuracy and precision of the various methods tested, values for total bile acids, the ratio of trihydroxy- to dihydroxy-bile acids and of glyco- to tauro-bile acids and 7α-hydroxylated bile acids (cholic and chenodeoxycholic acids) were used as common denominators. Total bile acids in bile and plasma was the sum of lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids for TLC, GC, HPLC, HPLC with 3α-HSD detection and GC–MS–SIM, whereas for urine it was the sum of all peaks identified as bile acids by GC and GC MS. For RIA the values for conjugated cholic and chenodeoxycholic acids were calculated and compared.

RESULTS

Bile acids in bile

By statistical evaluation of the analysis of the triplicate samples[15], the analytical errors arising from the sample preparation and those from the determination

TABLE I

TOTAL BILE ACID CONCENTRATION IN TRIPLICATE BILE SAMPLE

C.V. = Coefficient of variation; 3α-HSD = 3α-hydroxysteroid dehydrogenase.

Method	*Laboratory*	*Mean ± S.D. (mM) (n = 3)*	*C.V. (%)*	*Variance**
3α-HSD	C	(99.2 ± 1.1) (*n* = 1)**		
	D	92.9 ± 3.3	3.5	NS
	F	(103.9 ± 8.2) (*n* = 1)**		
TLC	E	97.0 ± 11.5	11.8	NS
GC	A	(68.9 ± 4.3) (*n* = 1)**		
	E	73.3 ± 8.0	10.9	NS
	F	114.2 ± 16.3	14.3	S (1%)
HPLC	B	97.9 ± 7.5	7.7	S (1%)
GC–MS–SIM	G	102.2 ± 8.6	8.5	NS

* The analytical errors were divided into two sources, *viz.*, sample preparation or measurement itself. S = Error during sample preparation was significant; NS = not significant.

** Single sample was used, but three determinations were made.

itself could be distinguished. The concentration of total bile acids obtained ranged from 70 to 110 m*M* and the inter-laboratory variations seem to be large (Table I). 3α-HSD gave only a small inter-laboratory variation. Those obtained by TLC resembled closely those obtained by GC–MS–SIM. The coefficient of variation was also small. The underestimation by GC in laboratories A and E was considerable but could be corrected by using an appropriate internal standard.

Differences between the various analytical methods in determining the bile acid composition was evaluated using triplicate samples (Table II). With respect to the percentages of cholic acid (CA) and chenodeoxycholic acid (CDCA) compared with total bile acids (TBA), TLC was found to be inadequate because it gave a poor resolution of the conjugated dihydroxycholanoic acids present in bile, *i.e.*, the conjugates of CDCA from deoxycholic acid (DCA). As shown in Table II, percentage of CDCA and CA in TBA ranged from 36 to 43% for CDCA and from 42 to 49% for

TABLE II

COMPOSITION OF BILE ACIDS IN TRIPLICATE BILE SAMPLE

Tri = Trihydroxycholanoic acid; Di = dihydroxycholanoic acid.

Method	*Laboratory*	*Mean ± S.D. (%) (n = 3)*		*Tri/Di*
		CDCA	*CA*	
TLC	E	–	48.8 ± 3.0	1.03 ± 0.06
GC	A	(36.8 ± 1.5) (*n* = 1)*	(44.0 ± 1.7) (*n* = 1)*	(0.79 ± 0.05) (*n* = 1)*
	E	37.3 ± 1.6	45.1 ± 0.4	0.82 ± 0.02
	F	41.9 ± 1.4	41.9 ± 0.8	0.73 ± 0.02
HPLC	B	36.3 ± 2.2	47.8 ± 1.9	0.91 ± 0.07
	F	37.2 ± 0.7	44.8 ± 1.4	0.82 ± 0.04
GC–MS–SIM	G	42.6 ± 0.4	49.1 ± 0.2	0.99 ± 0.01

* Single sample was used, but three determinations were made.

TABLE III

GLYCINE/TAURINE RATIO OF CONJUGATED BILE ACIDS IN TRIPLICATE BILE SAMPLE

Method	*Laboratory*	*Mean ± S.D. (n = 3)*	*C.V. (%)*	*Variance**
TLC	E	4.59 ± 0.77	16.9	NS
HPLC	B	3.07 ± 0.25	8.1	S (1%)
	F	3.07 ± 0.09	2.9	NS

* The analytical errors were divided into two sources, *viz.*, sample preparation or measurement itself. S = Error during sample preparation was significant; NS = not significant.

TABLE IV

TOTAL BILE ACID* CONCENTRATION IN TRIPLICATE PLASMA SAMPLE

Method	*Laboratory*	*Mean ± S.D. (μM) (n = 3)*	*C.V. (%)*	*Variance***
3α-HSD	A	(4.9 ± 0.5) (*n* = 1)§§		
	D	7.3 ± 0.3	4.6	NS
PHP–3α-HSD***	B	7.0 ± 1.6	22.9	S (1%)
	C	5.3 ± 0.3	5.7	NS
GC	A	(0.1 ± 0.1) (*n* = 1)§§		
HPLC–3α-HSD§	C	(3.5 ± 0.3) (*n* = 1)§§		
GC–MS–SIM	G	5.2 ± 0.2	3.7	NS

* Sum of free, glyco- and tauro-bile acids.

** Analytical errors were divided into two sources, *viz.*, sample preparation or measurement itself. S = Error during sample preparation was significant; NS = not significant.

*** Fractionation on PHP-Sephadex LH-20 followed by 3α-HSD detection.

§ HPLC connected with 3α-HSD column[12].

§§ Single sample was used, but three determinations were made.

TABLE V

TOTAL BILE ACID* CONCENTRATION IN PLASMA SAMPLE

Method	*Laboratory*	*Mean ± S.D. (μM) (n = 3)*		
		Sample S-8	*Sample S-9*	*Sample S-10*
3α-HSD	D	16.1 ± 0.7	14.4 ± 0.3	19.1 ± 0.6
HPLC–D**	B	6.7 ± 0.1	4.0 ± 0.1	16.7 ± 0.2
HPLC–3α-HSD***	C	4.6 ± 0.1	4.1 ± 0.6	15.1 ± 0.0
GC–MS–SIM	F	6.0 ± 0.3§	3.2 ± 0.1	12.3 ± 1.1

* Sum of free, glyco- and tauro-bile acids.

** HPLC with detection of derivatized bile acid[11].

*** HPLC connected with 3α-HSD column[12].

§ Including sulphated bile acid formation.

TABLE VI

DETERMINATION OF 7α-HYDROXYLATED BILE ACIDS WITH 7α-HYDROXYSTEROID DEHYDROGENASE (7α-HSD) DETECTION *VERSUS* SUM OF CHOLIC AND CHENODEOXYCHOLIC ACIDS BY GC–MS–SIM

Triplicate determinations for each sample. Results in μM.

Sample	*7α-HSD*		*GC–MS–SIM**	
	Laboratory A	*Laboratory D*	*Laboratory F*	*Laboratory G*
S-1				
S-2	3.8 ± 1.0	4.2 ± 0.3	–**	4.0 ± 0.1
S-3			–	
S-4	113	98.1 ± 3.4	–	112 ± 1
S-5	3.9	4.2 ± 0.1	–	3.8 ± 0.1
S-8	–	7.9 ± 0.3	4.9 ± 0.1	–
S-9	–	5.3 ± 0.2	1.8 ± 0.1	–
S-10	–	11.3 ± 0.3	11.7 ± 0.9	–

* Including sulphated fraction.
** Not determined.

CA. The coefficient of variation for the percentage of CA or CDCA in the triplicate samples using the same analytical procedure was below 6.0%, much less than the 15% (Table I) in quantitative analysis by GC, HPLC and GC–MS–SIM. The minimum coefficient of variation for bile acid composition of less than 1% was found using GC–MS–SIM. Close agreement was obtained for CA/TBA and CDCA/TBA between GC in laboratories A and E and HPLC in laboratories B and F.

The trihydroxycholanoic acid/dihydroxycholanic acid ratio varied greatly, from 0.73 by GC (laboratory F) to 1.03 by TLC (laboratory E). However, the coefficient of variation using the same method in the same laboratory on the triplicate samples was 1—8%. The glycine/taurine conjugation ratios agreed well with those obtained by the HPLC in laboratories B and F (Table III). However, the value obtained by TLC was 50% higher than those by HPLC.

Analysis of bile acids present in bile in minute amounts, *i.e.*, lithocholic (LCA) and ursodeoxycholic acids (UDCA), gave large coefficients of variation from 50 to

TABLE VII

RIA *VS.* OTHER METHODS: CONJUGATED CHOLIC ACID LEVEL IN PLASMA

Triplicate determinations. Results in μM.

Sample	*RIA (laboratory A)*	*HPLC–D* (laboratory B)*	*HPLC–3α-HSD** (laboratory C) (n = 1)*	*GC–MS–SIM (laboratory F)*
S-8	0.99 ± 0.18	0.51 ± 0.00	0.47	–
S-9	0.95 ± 0.09	0.47 ± 0.03	0.45	–
S-10	4.45 ± 0. 13	9.10 ± 0.11	7.92	7.28 ± 0.36***

* HPLC with detection of derivatized bile acid[11].
** HPLC connected with 3α-hydroxysteroid dehydrogenase column[12]. Single determination.
*** After group separation on PHP-Sephadex LH-20 column.

TABLE VIII

RIA *VS.* OTHER METHODS: NON-SULPHATED CHENODEOXYCHOLIC ACID LEVEL IN PLASMA

Triplicate determinations. Results in μM.

Sample	*RIA* (laboratory A)*	*HPLC–D** (laboratory B)*	*HPLC–3α-HSD*** (laboratory C)*	*GC–MS–SIM (laboratory G)*
S-1		–	2.1	
S-2	2.4 ± 0.9	–	2.1	3.6 ± 0.2
S-3		–	1.5	
S-4	70.7 ± 11.0	–	36.2	53.4 ± 1.5
S-5	2.7 ± 0.4	–	2.2	3.1 ± 0.1
S-8	2.0 ± 0.1	4.9 ± 0.1	3.5	–
S-9	1.7 ± 0.2	1.9 ± 0.0	1.9	–

* RIA system detected non-sulphated fraction of unconjugated, glyco- and tauro-chenodeoxycholic acid.

** HPLC with detection of derivatized bile acid[11].

*** HPLC connected with 3α-hydroxysteroid dehydrogenase column[12]. Single determination only.

14% (the latter by GC–MS–SIM). It was not possible to determine LCA and UDCA quantitatively by TLC.

Plasma bile acids

Results for total bile acids in the triplicate plasma samples are summarized in Tables IV and V. The coefficient of variation was smallest with GC–MS–SIM, in-

TABLE IX

URINARY BILE ACID DETERMINATION

Triplicate determinations. Results in μM.

Sample	*GC (laboratory A)*	*GC–MS–SIM (laboratory F)*	*GC–MS–SIM (laboratory G)*
Total bile acids in sulphated and non-sulphated fractions:			
U-1	0.32*	3.10 ± 0.44	3.80 ± 0.08
U-2**	47.1		
U-3**	70.2	90.8 ± 8.2	94.8 ± 5.0
U-4	45.5	(C.V. 9%)	(C.V. 5%)
U-5	7.3	16.6 ± 2.7	22.4 ± 0.5
Sulphated and non-sulphated CDCA:			
U-1	0.20*	1.24 ± 0.29	1.44 ± 0.02
U-2**	5.22		
U-3**	1.53	25.2 ± 1.3	29.6 ± 2.1
U-4**	1.63	(C.V. 5%)	(C.V. 7%)
U-5	2.45	5.98 ± 1.09	8.15 ± 0.21

* Single determination

** Triplicate sample.

TABLE X

URINARY BILE ACID DETERMINATION: ENZYMATIC METHODS *VS.* GC–MS–SIM

Sample	*Total bile acids (μM)**	
	*Enzymatic method***	*GC–MS–SIM (n = 3)*
U-6	8.65	2.30 ± 0.17
U-7	12.23	0.87 ± 0.12
U-9	23.92	80.95 ± 1.98

* Sum of sulphated and non-sulphated bile acids.

** Determined with 3α-hydroxysteoid dehydrogenase (3α-HSD), 3α-HSD and 7α-HSD after solvolysis.

dicating the necessity to use an internal standard to correct for the losses occurring during the sample preparation.

The 7α-HSD method for the determination of primary bile acids, *i.e.*, CA and CDCA, was compared with GC–MS–SIM (Table VI). Analyses of triplicate samples showed that the value obtained with 7α-HSD was comparable to those given by GC–MS–SIM the but coefficient of variation was fairly large (26%) and sometimes an excessively high value was obtained (laboratory D).

The values given by RIA for triplicate samples showed good agreement with those obtained by the other methods (Tables VII and VIII). However, the values from other samples were variable by 200–50%. Therefore, the analytical error when using RIA seems to be unacceptably large.

Urinary bile acids

Enzymatic analysis (EA), GC and GC–MS–SIM were compared for the determination of urinary bile acids (Tables IX and X). In contrast to the good agreement obtained by GC–MS–SIM in two laboratories, EA gave variable results in spite of the refinement made in the clean-up steps such as inclusion of solvolysis. Therefore, EA is not suitable for the analysis of urinary bile acids (Table X).

DISCUSSION

It is relatively easy to analyse bile acids in bile as there are fewer compounds in bile that interfere with the determination. However, when bile has a high viscosity, the sampling errors could be large unless due precautions are taken, *i.e.*, a blow-out pipette should be used to minimize the error. During fractionation by methods such as PHP-LH-20 column chromatography prior to HPLC , GC or GC–MS–SIM, the use of a suitable internal standard is essential to ensure adequat e reproducibility and to compensate for the sample losses during the clean-up steps. Even with a relatively simple analytical procedure such as TLC a reasonably high accuracy can be obtained, although the separation of individual conjugated dihydroxycholanoic acids is not satisfactory. The inter-laboratory variation was relatively small when using EA, GC and HPLC. However, GC with a packed column and HPLC without specific detec-

tion are unsuitable for the determination of minor bile acids such as LCA and UDCA because the peaks are broad and even with microcomputer control the reproducibility is not satisfactory. One must resort to GC–MS–SIM for the analysis of minor bile acids.

As the concentration of bile acids in normal human serum is very low, except for GC–MS–SIM the usual analytical methods such as TLC, HPLC and GC are unsatisfactory. EA using 3α-HSD is simple yet sensitive for the analysis of total bile acids but its major drawback is its inability to determine sulphated bile acids, which are present at levels of 10–50%[16]. The analytical error by EA is small, even with low concentrations such as in normal serum, compared with the other analytical procedures but tends to overestimate. Therefore, double checking by other analytical methods such as GC–MS–SIM is recommended. EA with 7α-HSD is useful for determining primary bile acids, *i.e.*, the sum of CA and CDCA. For preliminary fractionation by PHP-LH-20 column chromatography followed by enzymatic determination with 3α-HSD to be successful, a correlation must be made for the losses occurring in the clean-up and fractionation steps. RIA applied to conjugated CA and CDCA gave variable values compared with those obtained by other methods but is useful as a screening procedure for large numbers of samples because of its simplicity. The sensitivity of GC with a packed column was found to be insufficient for the analysis of bile acids present in normal serum at very low concentrations but is adequate for serum with a high bile acid content such as in obstructive jaundice. The use of a suitable internal standard is essential. However, GC with a capillary column and solventless[14] or splitless injection mode may have sufficient sensitivity for the determination of major bile acids in serum. HPLC using UV detection is not sufficiently sensitive to be applicable to the analysis of bile acids present in normal serum. HPLC coupled with prelabelling of bile acids (HPLC-D)[11] was found to have sufficient sensitivity and accuracy and could be used for the analysis of bile acids present in low concentrations such as in normal human serum. With the fixed HPLC with 3α-HSD detection, it usually gives lower values than those obtained by HPLC-D or GC–MS–SIM and the sensitivity is lower than that given by HPLC-D. However, with the use of a suitable internal standard, it may well be suitable for the analysis of major bile acids in normal human serum. However, with HPLC procedures there certain problems have to be solved such as the determination of sulphated bile acids and elucidation of the nature of unknown peaks. On the other hand, GC–MS–SIM with deuterated bile acid standards has been found to be satisfactory with regard to specificity, accuracy and sensitivity in analysing serum bile acids and is specially useful for the determination of minor bile acids and the characterization of unknown peaks. However, in order to obtain information on conjugate forms, it preliminary fractionation steps by other procedures are necessary.

For the analysis of urinary bile acids, EA is totally unsuitable because of the presence of various keto bile acids. Even GC is unreliable. Satisfactory corrections cannot be made because of the complex preliminary fractionation steps involved, and the sensitivity of GC is not sufficient for the analysis of bile acids present in low concentrations in urine. Although there is a small difference in the values obtained by GC–MS–SIM in two laboratories, their coefficients of variation were less than 9%, as shown in Table IX.

Urinary bile acids are sulphated to a greater extent than serum bile acids and

some portion of urinary bile acids is glucuronidated. Unusual orientations of hydroxy and keto groups have been found to be present. Therefore, more complex but practicable preliminary clean-up and fractionation steps with good reproducibility and recovery are necessary. At present, GC–MS is the only reliable analytical procedure for urinary bile acids.

ACKNOWLEDGEMENT

This work was aided by Resarch Grant 56370042 (principal investigator: F. N.) from the Ministry of Education, Science and Culture, Japanese Government.

REFERENCES

1 F. Mashige, K. Imai and T. Osuga, *Clin. Chim. Acta*, 70 (1976) 79.
2 S. Ikawa, H. Kawasaki, Y. Yamanishi, T. Mura and M. Miyake, *Tohoku J. Exp. Med.*, 145 (1985) 185.
3 J. Goto, M. Hasegawa, H. Kato and T. Nambara, *Clin. Chim. Acta*, 87 (1978) 141.
4 W. J. Simmonds, M. G. Korman, V. L. W. Go and A. F. Hofmann, *Gastroenterology*, 65 (1973) 705.
5 I. Makino, A. Tashiro, H. Hashimoto, S. Nakagawa and I. Yoshizawa, *J. Lipid Res.*, 19 (1978) 443.
6 I. Makino, H. Hashimoto, K. Shinozaki, K. Yoshino and S. Nakagawa, *Gastroenterology*, 68 (1975) 545.
7 S. M. Grundy, E. H. Ahrens, Jr., and T. A. Miettinen, *J. Lipid Res.*, 6 (1965) 397.
8 W. Van der Linden, B. Katzenstein and F. Nakayama, *Cancer*, 52 (1983) 1265.
9 A. Kibe, T. Kuramoto and T. Hoshita, *Anal. Biochem.*, 100 (1979) 146.
10 K. D. R. Setchell and A. Matsui, *Clin. Chim. Acta*, 127 (1983) 1.
11 J. Goto, M. Saito, T. Chikai, N. Goto and T. Nambara, *J. Chromatogr.*, 276 (1983) 289.
12 S. Onishi, S. Itoh and Y. Ishida, *Biochem. J.*, 204 (1982) 135.
13 F. Nakayama and M. Nakagaki, *J. Chromatogr.*, 183 (1980) 287.
14 J. Yanagisawa, Y. Akashi, H. Miyazaki and F. Nakayama, *J. Lipid Res.*, 25 (1984) 1263.
15 G. W. Snedecor and W. G. Cochran, *Statistical Methods*, Iowa State University Press, Iowa, 5th ed., 1956, p. 237.
16 I. Makino, H. Hashimoto, K. Shinozaki, K. Yoshino and S. Nakagawa, *Gastroenterology*, 68 (1975) 545.