Radioimmunoassay of Conjugated 1 β -Hydroxycholic Acid in Dried Blood Spots for Diagnosis of Congenital Biliary Atresia

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Radioimmunological measurement of conjugated 1 β -hydroxycholic acid in dried blood spots obtained from newborns at 4–5 d after birth has been carried out to investigate a diagnostic utility for congenital biliary atresia. The method allowed the determination of this bile acid, with a detection limit of 50 pg in one disc (3 mm i.d.), and showed good reproducibility with inter- and intra-assay coefficients with variations of 3.5–7.7% and 6.3–8.8%. With the analysis, a relatively high concentration of this substance was observed in the patients, as compared to normal subjects, averaging 1.63 ng/disc in the former and 0.97 ng/disc in the latter.

Keywords radioimmunoassay; conjugated 1 β -hydroxycholic acid; bile acid; dried blood disc; gas chromatography-mass spectrometry; congenital biliary atresia

Congenital biliary atresia (CBA) is a disorder in which the bile duct is naturally blocked. The incidence of CBA is said to be about 1:10000 live births in Japan.²⁾ These patients have developed irreversible cirrhosis of the liver so that they have a lesser chance of achieving bile drainage following surgery. If the patients undergo a hepatic portoenterostomy before 60 d of age, 70% of them can survive free from jaundice. For earlier detection, measurement of glycocholic acid or total bile acids adsorbed on dried blood spots has been carried out.^{3,4)} However, the efficiency of this screening method for CBA is not good enough. Recent studies have shown that the bile acid metabolism during fetal and neonatal periods is dramatically different from that in adults.^{5–9)} We have previously shown that the predominant bile acid excreted in the urine of newborns is 1 β -hydroxycholic acid (CA-1 β -ol),^{10–13)} which is preferentially conjugated with taurine.¹⁴⁾ The occurrence of this bile acid has been related to the development of a certain form of cholestasis in newborns.^{15,16)} These observations prompted us to investigate the feasibility of conjugated CA-1 β -ol as a new marker for the earlier detection of the CBA. In preceding studies,^{17,18)} we developed a radioimmunoassay (RIA) of this bile acid to study its physiological and pathophysiological role. The present paper deals with the measurement of this substance in dried blood spotted on filter paper by the new RIA in order to assess its diagnostic utility.

MATERIALS AND METHODS

Chemicals and Reagents [2-³H]Tauro CA-1 β -ol (743.7 GBq/mmol) was prepared as previously described.¹⁸⁾ Prior to use, its radiochemical purity was checked by thin-layer chromatography in a system of *n*-butanol–acetic acid–water (25:4:10, v/v/v). The antiserum used in this study was that obtained by immunizing rabbit with *N*-(1 β ,3 α ,7 α ,12 α -tetrahydroxy-5 β -cholan-24-oyl)-2-aminopropionic acid–bovine serum albumin conjugate,¹⁷⁾ and it was used at a 1:3000 working dilution. The specificity of the antibody was tested on cross-reactivity with glycine and taurine conjugates of 1 β -hydroxychenodeoxycholic acid

(3.67 and 3.67%), 1 β -hydroxydeoxycholic acid (5.18 and 5.50%), 1 β -hydroxylithocholic acid (0.23 and 0.36%), and on negligible cross-reactivity (<0.01%) with other bile acids. The reference bile acids were synthesized in our laboratory. Pentafluorobenzyl (PFB) bromide and dimethylethylsilylimidazole were obtained from Tokyo Kasei Kogyo Co. (Tokyo).

Gas Chromatography-Mass Spectrometry (GC-MS) The Capillary GC-MS was performed on a GCMS 9100 MK instrument equipped with a data-processing system, GCMSPAK 1500 (Shimadzu, Ltd., Kyoto, Japan), according to the procedure in the previous paper.¹⁹⁾ Iso-butane was used as a reagent gas. The ionization energy was 100 eV and the emission current was 200 μ A for the chemical ionization mode. A gas chromatographic column (25 m $\times$ 0.2 mm i.d.), a fused silica capillary column bonded with methyl silicone (Shimadzu Hicap CBP-1), was used by coupling it to the mass spectrometer. The carrier gas was helium at a linear velocity of 45 cm/s. The sample was introduced by splitless injection at 300 $^{\circ}$ C. The temperature of the column oven was initially set at 250 $^{\circ}$ C for 2 min, raised to 300 $^{\circ}$ C at 25 $^{\circ}$ C/min, maintained at that temperature for 10 min, and finally raised to 320 $^{\circ}$ C at 2 $^{\circ}$ C/min.

Preparation of Bile Acid-Free Blood The bile acid-free blood was prepared by the partially modified procedure of the National Committee for Clinical Laboratory Standards. Briefly, bile acid-free plasma was prepared by removing bile acids from pooled human plasma by a charcoal treatment. Erythrocytes from apparently normal heparinized blood were washed with isotonic saline, and then recombined with bile acid-free plasma to adjust the hematocrit to 55%, reflecting the higher hematocrit in newborns.

Recovery Test Known amounts of tauro CA-1 β -ol were added to reach final concentrations of 255, 531 and 1062 pg/4 μ l in whole blood. A 50 μ l aliquot of each blood sample was spotted on filter paper and stored at –20 $^{\circ}$ C. One disc (3 mm i.d.) punched from each filter paper was used for the recovery experiments.

Radioimmunoassay Procedure One disc (3 mm i.d.) of

dried blood spots was placed in the assay buffer (0.1–0.3 ml) and was sonicated for 30 min. A 0.1 ml aliquot of the resulting solution was submitted to RIA directly.

All dilutions of the standard, tracer and antiserum were performed in a 10 mM phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.01% sodium azide and 0.9% sodium chloride solutions. The assay was carried out by the method described previously.¹⁸⁾ Control and background were determined by processing samples of the buffer and charcoal-treated bile acid-free blood exactly as above. Values of tauro CA-1 β -ol content were read off a standard curve, which was run concurrently.

GC-MS Analysis of Bile Acid Composition in Dried Blood Spots Three discs (3 mm i.d.) of dried blood spotted on filter paper were placed in a phosphate buffer (pH 7.4, 0.1 ml) and sonicated for 30 min. After the addition of a solution of the internal standard (3 α ,6 β ,7 β ,12 α -tetrahydroxy-5 β -cholan-24-oic acid, 10 ng), the solution was loaded on a Bond Elut C18 cartridge (3 ml) pre-rinsed with methanol (5 ml) and water (5 ml). The cartridge was washed with water (5 ml), and the bile acids were eluted with methanol–chloroform (9:1, 4 ml). After evaporation of the solvent, the residue was solvolyzed at pH 1 with 2 M hydrochloric acid in ethanol–acetone (1:9, 2 ml) at 40 °C for 1 h, then neutralized with 1 M sodium hydroxide and evaporated to dryness. The residue was hydrolyzed with 4 M sodium hydroxide–methanol (1:1, 2 ml) at 80 °C for 16 h, acidified to pH 1 with 6 M hydrochloric acid (0.8 ml) and cooled on ice. The free bile acids were extracted with a Bond Elut C18 cartridge (3 ml) as described above. The residue was dissolved in 90% aqueous ethanol and applied to a piperidinohydroxypropyl Sephadex LH-20 column (6 $\times$ 20 mm) prepared in 90% aqueous ethanol. The column was washed with 90% aqueous ethanol (4 ml) to remove neutral compounds, and bile acids were eluted with 0.1 M acetic acid in 90% aqueous ethanol (5 ml). After evaporation of the solvent, purified bile acids were derivatized to the PFB ester with pentafluorobenzyl bromide–acetonitrile (1:1, 30 μ l) and dimethylethylamine–acetonitrile (1:2, 30 μ l) by warming at 40 °C. After the removal of excess reagents under a stream of nitrogen gas, the DMES ethers were prepared by heating the residue with *N*-dimethylethylsilylimidazole (40 μ l) at 60 °C for 45 min. Excess reagents were removed on a silica gel column (6 $\times$ 20 mm) equilibrated with *n*-hexane–ethyl acetate (3:1, v/v). The derivatized bile acids were recovered in the first 4 ml of effluent and evaporated to dryness under reduced pressure. The residue was redissolved in *n*-hexane and was applied to GC-MS analysis.

RESULTS AND DISCUSSION

In the RIA used in this study, the lowest limit of detection is 50 pg/assay. The serum level of CA-1 β -ol in normal newborns has been shown to be 0.41 μ g/ml. Since one disc, 3 mm in diameter, punched from dried blood spots represents approximately 3.5 μ l of blood, containing 1.43 ng/disc of the bile acid, the sensitivity is sufficient to measure a small amount of this bile acid in the dried

TABLE I. Reliability of the Measurement of Conjugated 1 β -Hydroxycholeic Acid Adsorbed in Dried Blood Spots

Added amount (pg/disc)	Recovery (%) Mean $\pm$ S.D.	C.V. (%)
Inter-assay (<i>n</i> = 10)		
255	96.7 $\pm$ 5.2	5.4
531	99.4 $\pm$ 3.5	3.5
1062	87.8 $\pm$ 6.8	7.7
Intra-assay (<i>n</i> = 10)		
255	93.7 $\pm$ 7.8	6.3
531	100 $\pm$ 7.1	6.5
1061	92.5 $\pm$ 8.1	8.8

Fig. 1. Concentration of the Conjugated 1 β -Hydroxycholeic Acid (CA-1 β -ol) Level in Dried Blood Disc Samples

a) *p* < 0.01.

blood adsorbed on one disc.

Methodological details were determined by using conventional systematic experiments. Extraction of bile acids from the disc was done by ultrasonic treatment in the assay buffer. This procedure made a purification step unnecessary.

Assessment of the assay was carried out by checking the recovery rate and the intra-assay and inter-assay coefficients of variation. A known amount of tauro CA-1 β -ol added to bile acid-free blood was satisfactorily recovered, as shown in Table I. These data indicate excellent concordance between added and measured materials. Inter- and intra-assay coefficients of variation (C.V.) were 3.5–7.7% and 6.3–8.8%, respectively. The blank values obtained with the bile acid-free blood disc were not significant from zero. We evaluated the present RIA method by GC-MS with negative ion chemical ionization detection. The values obtained by the measurement of 12 samples were correlated well (data not shown). Thus, the present method is both precise and accurate.

The RIA was then applied to the measurement of conjugated CA-1 β -ol in dried blood spotted on filter paper obtained from normal newborns and CBA patients at 4–5 d after birth. The results are shown in Fig. 1. The levels from CBA patients were 1.5 times those of the normal subjects, ranging from 1.63 $\pm$ 0.88 ng/disc (mean $\pm$ S.D.) in the former and 0.97 $\pm$ 0.68 ng/disc (mean $\pm$ S.D.) in the latter.

Fig. 2. Distribution Histograms of Conjugated 1 β -Hydroxycholeic Acid (CA-1 β -ol) Values in Dried Blood Disc Samples (a) normal, $n=194$. (b) CBA, $n=34$.

TABLE II. Bile Acid Composition in Dried Blood Spotted on Filter Papers from Normal Infants

Bile acid	ng/disc ^{a)}	% ^{b)}
Cholic acid	5.98	48.0
Chenodeoxycholic acid	1.42	11.4
Deoxycholic acid	1.53	12.3
Lithocholic acid	1.20	9.7
3 β -Hydroxy-5-cholen-24-oic acid	0.34	2.7
3 β ,12 α -Dihydroxy-5-cholen-24-oic acid	0.15	1.2
1 β -Hydroxycholeic acid	0.64	5.1
1 β -Hydroxychenodeoxycholic acid	0.04	0.3
1 β -Hydroxydeoxycholic acid	0.14	1.1
6 α -Hydroxycholeic acid	0.38	2.7
6 α -Hydroxychenodeoxycholic acid	0.33	3.0
2 β -Hydroxycholeic acid	0.29	2.3
Total	12.45	100

a) Each value represents the mean of 4 samples. b) Percent values represent a proportion of each bile acid compared to the total bile acids.

in the latter. The present results support the previous finding that the serum level of CA-1 β -ol is significantly elevated in CBA patients compared to normal populations.²⁰⁾ In an attempt to identify diagnostic parameters, we transcribed the data in a distribution histogram, as shown in Fig. 2. There was significant overlap between the abnormal and normal populations, though the median level (1.56 ng/disc) of the CBA patients was relatively higher than that (0.78 ng/disc) of the normal subjects.

On the other hand, the bile acid compositions of the dried blood spots of 12 normal newborns were analyzed by GC-MS with negative ion chemical ionization detection.¹⁹⁾ As shown in Table II, the predominant bile acids identified were primary and secondary bile acids, accounting for more than 80%. The proportion of CA-1 β -ol represented only 5.1% of the total bile acids. This value was somewhat lower than that obtained by RIA. This may be due to methodological differences. The other 1 β -, 2 β - and 6 α -hydroxylated bile acids, previously found in urine and in the fetal gall bladder, were also presented in small portions. These observations are marked contrast to the urinary bile acid profile, where the predominant bile acids are constituted of 1 β -hydroxylated bile acids.^{11,13)} We therefore suspect that the small proportion of CA-1 β -ol in blood is related to the physiological

immaturity of enterohepatic circulation of bile acids and that the hepatic metabolism of cholic acid is rendered more polar for renal excretion. In these respects, we have identified the uncertainty of the present method as a screening test for CBA utilizing dried blood spots obtained from newborns at 4–5 d after birth.

Further studies are required to determine how to apply the present RIA in order to accurately detect CBA patients earlier.

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