Serum 7-Alpha-Hydroxy-4-Cholesten-3-One as a Marker for Bile Acid Loss in Children

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Objective To establish age-related reference values for 7-alpha-hydroxy-4-cholesten-3-one (C4) in a pediatric population and to investigate bile acid malabsorption in children with short bowel syndrome (SBS).

Study design Serum was obtained between 8:00 a.m. and 11:00 a.m. from 100 healthy children (52% males, 9 months to 18 years of age) after 10 hours of fasting. Pediatric patients with SBS served as disease controls (n = 12). Following solid-phase extraction and purification, C4 was determined by high-performance liquid chromatography using a ultraviolet detector at a wavelength of 241 nm. The upper limit of normal for C4 concentrations was defined as the mean plus 2 SD of the log-normal distribution.

Results The mean concentration and SD of C4 in healthy children was 22.8 ± 15.8 ng/mL with no relation to age or sex and an upper limit of normal of 66.5 ng/mL. Normal C4 values were found in 97 of 100 healthy children, and all 12 patients with SBS had C4 concentrations above 100 ng/mL (mean 299.6 ± 167.8 ng/mL; range 105.7-562.1 ng/mL, $P < .0001$ compared with controls).

Conclusions The determined upper limit of normal for C4 concentration in healthy children corresponds to previously published levels in healthy adults and is independent of age and sex. The consistently elevated C4 concentrations in our patients with SBS confirm the reliability of this noninvasive, nonisotopic method to assess bile acid malabsorption in children. *(J Pediatr 2013;163:1367-71)*.

Bile acid loss syndrome, a consequence of disturbed reabsorption of bile acids in the terminal ileum, manifests as diarrhea, steatorrhea with malabsorption of fat soluble vitamins, and predisposes to formation of gallstones. At risk for bile acid loss are patients with chronic inflammation or surgical resection of the terminal ileum such as patients with Crohn's disease or short bowel syndrome (SBS).[1-3] Other conditions causing bile acid loss include a rapid small bowel transit time in diarrheal diseases of different etiologies such as bacterial overgrowth, blind loop syndrome, carbohydrate malabsorption, dysmotility, or after biliary surgery. Interruption of the enterohepatic circulation of bile acids results in loss of bile acids into the cecum and colon leading to watery osmotic diarrhea. The liver compensates partially by activating the de novo synthesis of bile acids. However, chronic bile acid loss often results in a decreased bile acid pool size and changed bile acid composition.

7-alpha-hydroxy-4-cholesten-3-one (C4) is a semiquantitative serum marker for bile acid synthesis in humans.[4] C4, a precursor of cholic acid in the classic (neutral) pathway of bile acid synthesis from cholesterol, correlates with the time-limiting enzymatic step catalyzed by the 7-alpha-hydroxylase.[4] In patients with bile acid loss, CYP7a1 is upregulated as reflected in a higher serum level of C4, which is relatively stable. In adults, normal values for C4 have been established and serum concentrations higher than 50-60 ng/mL indicate bile acid loss.[2,5-8] Two methods typically have been utilized to assess bile acid loss: the 75-SeHCAT scintigraphy and an assay to measure bile acids in the feces. The latter requires stool collection over 3 days, which is impractical in children.[9] For scintigraphy, the radioactive labeled bile acid 75-SeHCAT is orally administered and after 7 days the remaining 75-SeHCAT is measured. The 75-SeHCAT scan is currently the reference standard as a quantitative method. In adults, a good correlation between the 75-SeHCAT scan and the C4 measurement has been shown.[5,10-12] However, the 75-SeHCAT is cost-intensive, time consuming, and involves radiation exposure, and thus, rarely was used in children. The purpose of this prospective study was to evaluate the "C4 method" to assess bile acid loss in pediatric patients. We determined reference values for C4 in a large cohort of children of different age groups from infancy to 18 years of age. To test C4 as a marker for bile acid loss, we investigated serum samples from children with SBS.

BAM	Bile acid malabsorption
C4	7-alpha-hydroxy-4-cholesten-3-one
HPLC	High-performance liquid chromatography
ICV	Ileocecal valve
PN	Parenteral nutrition
SBS	Short bowel syndrome

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Supported by the Friedrich Baur Stiftung (50/11 to F.F.) and the Deutsche Forschungsgemeinschaft (BE 4490/2-1 to F.B.). The authors declare no conflicts of interest.

 http://dx.doi.org/10.1016/j.jpeds.2013.06.083

Methods

Of the 100 children and adolescents serving as healthy controls, 19 were volunteers and 81 were recruited from the Department of Pediatric Surgery at the Dr von Hauner Children's Hospital. They were scheduled for minor plastic surgeries or elective surgery of the urinary tract or extremities. A structured history was taken for any acute and chronic diseases. Drug intake, weight, length, and body mass index were assessed, and liver enzymes alanine aminotransferase and aspartate aminotransferase measured. Children were excluded if they had by history or laboratory values any evidence of a liver-, pancreatic-, bowel-, renal- or thyroid disease, or if they took any medication over the last 2 weeks.

Patients with SBS were recruited regardless of age from the gastrointestinal department. Exclusion criteria were elevated liver enzymes >2 times the upper limit of the normal or intake of any medication known to affect bile acid synthesis (eg, statins) or ursodeoxycholic acid. The diagnosis of SBS was defined according to the criteria of Wales et al as any child having a laparotomy with a residual small bowel length of less than 25% of the age adjusted small bowel length or the need of postoperative parenteral nutrition (PN) for at least 6 weeks.[13] The age-adjusted length of the small intestine was assessed according to Weaver et al.[14] Anatomic details of the remaining intestine were recorded from surgical records.

Blood samples were obtained in the morning (8:00 a.m.-11:00 a.m.) after overnight fast. In patients with SBS, fasting time was shorter. In 2 patients, it was only 2 hours after the last meal and after stopping PN, and in 4 other patients 3-4 hours. The remaining patients with SBS fasted for at least 8 hours. The blood samples were centrifuged immediately and serum stored at −20°C until analysis.

Written informed consent was obtained from the patient's parents or the patient itself. The study was approved by the local Ethics committee (093-11). The method described by Axelson et al[6] was adapted according to Sauter et al using 7β-hydroxy-4-cholesten-3-one as an internal standard.[5]

Solid-Phase Extraction/Purification

A measurement of 1 mL of serum was diluted with 2 mL of saline, 100 ng of the internal standard 7β-hydroxy-4-cholesten-3-one (Steraloids, Newport, Rhode Island) was added from a 1 mg/1 L stock solution prepared with methanol (LiChrosolv; Merck, Darmstadt, Germany). C4 was extracted in jacketed glass columns, connected to a water bath, using octadecylsilane-bonded silica (Preparative C18, 125Å, 55-105 μm; Waters, Michigan). Columns were prewashed with 2 × 5 mL methanol and 2 × 5 mL of water (LiChrosolv; Merck) and heated to a temperature of 64°C. Samples were sonicated in water for 20 minutes and incubated at a temperature of 64°C for 5 minutes. After loading the cartridges, they were allowed to pass through by gravity (1 mL/min). The extraction procedure was followed by washing the columns with 3 × 5 mL water at a temperature of 64°C and 2 × 5 mL 65% aqueous methanol at room temperature. C4 was then eluted with 2 × 4 mL hexane-chloroform (75:25, v/v, LiChrosolv; Merck and Rotisolv high-performance liquid chromatography [HPLC]; Carl Roth, Karlsruhe, Germany). The samples were dried under nitrogen at 60°C and the extract was reconstituted in 100 μL of methanol.

HPLC-Assay

The HPLC-system consisted of a pump (LC-6A; Shimadzu, Kyoto, Japan), a ultraviolet-detector at wavelength 241 nm, and an integrator (CR-6A; Shimadzu). A reversed phase silica column Nova-Pak C18 column, 3.9 × 300 mm, 4 μm particle size was used with a Nova-Pak C18 guard column, 3.9 × 20 mm, 4 μm particle size (Waters, Milford, Massachusetts). Acetonitril/water (97.5:2.5 v/v, LiChrosolv; Merck) served as mobile phase at constant flow rate of 1 mL/min. The injection volume was 20 μL. C4 was quantified in comparison with the respective peak of the known amount of internal standard.

Statistical Analyses

Results are given as mean ± SD. The D'Agostino and Pearson omnibus normality test was used to test for non-normality in healthy subjects.[15] The upper and the lower level of the reference limits of C4 were determined as mean ± 2 SD of the log-normal distribution. Correlations were tested using Spearman rank coefficient, and sex-dependence via Mann–Whitney U test. The comparison of C4 values in different age groups was performed using Kruskal–Wallis-test/one-way ANOVA. Data analysis was performed using GraphPad Prism 6. P values of <.05 were considered as statistically significant.

Results

C4 Concentrations in Healthy Subjects

The age of the 100 healthy (52 males) controls ranged from 9 months-18 years with a mean age of 9.7 ± 5.1 years and a median of 10.0 years. The mean concentration of C4 ± 1 SD in control children was 22.8 ± 15.8 ng/mL (range 4.7-80.3 ng/mL; median 19.0 ng/mL; 95% CI 19.7-25.9 ng/mL). The 5th to 95th percentile of C4 concentrations in healthy children ranged from 7.3-53.7 ng/mL. Because the serum concentrations of C4 did not follow a normal distribution ($P < .0001$), we used a logarithmic transformation of the C4 values to obtain a normal distribution ($P > .45$). The mean ± 2 SD of the log-normal distributions were 2.92 ± 1.27 ng/mL, resulting in a lower limit of 5.2 ng/mL and an upper limit of 66.5 ng/mL.

Figure 1 shows the C4 values of healthy children for males and females (20.0 ± 11.4 ng/mL for males and 25.9 ± 19.2 ng/mL for females). Age dependency was investigated by dividing into groups (0-<3 years; 3-<6 years; 6-<9 years; 9-<12 years; 12-<15 years; and 15-18 years). Mean in the 6 groups range from 18.2-30.9 ng/mL, medians 14.4-26.1 ng/mL, with no significant differences of C4 values in relation to age (Figure 2).