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Measurement of Serum 7α-hydroxy-4-cholesten-3-one (or 7αC4), a Surrogate Test for Bile Acid Malabsorption in Health, Ileal Disease and Irritable Bowel Syndrome using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Background—Bile acid malabsorption (BAM) is reported in up to 50% of patients with functional diarrhea and irritable bowel syndrome with diarrhea (IBS-D). Serum 7α-hydroxy-4-cholesten-3-one (7αHCO, or 7αC4), an indirect measurement of hepatic bile acid synthesis, has been validated as a measurement of BAM relative to the ^{75}SeHCAT retention test.

Aim—To develop a serum 7αC4 assay, normal values, and compare results from healthy controls, patients with ileal Crohn's disease or resection, and patients with IBS-D or IBS with constipation (IBS-C).

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Methods—Stored serum samples were used from adult men and women in the following groups: 111 normal healthy controls, 15 IBS-D, 15 IBS-C, 24 with distal ileal Crohn's disease, and 20 with distal ileal resection for Crohn's disease. We adapted a published high pressure liquid chromatography, tandem mass spectrometry (HPLC-MS/MS) assay.

Results—The HPLC-MS/MS assay showed good linearity in concentration range 0–200 ng/mL, sensitivity (lowest limit of detection 0.04 ng/mL), and high analytical recovery (average 99%, range 93–107%). The 5^{th} to 95^{th} percentile for 111 normal healthy controls was 6–60.7 ng/mL. There were significant overall group differences (ANOVA on ranks $p<0.001$), with significantly higher values for terminal ileal disease or resection. There were significant differences between health and IBS (ANOVA $p=0.043$) with higher mean values in IBS-D relative to controls (rank sum test, $p=0.027$).

Conclusions—We have established a sensitive non-isotopic assay based on HPLC-MS/MS, determined normal 7αC4 values, and identified increased 7αC4 in IBS-D and in distal ileal resection and disease. This assay has potential as a noninvasive test for BAM in IBS.

Keywords

7αC4; 7αHCO; 7α-hydroxy-4-cholesten-3-one; bile acid retention; ^{75}SeHCAT

INTRODUCTION

Mucosal absorption or secretion of fluids and electrolytes may be abnormal in irritable bowel syndrome (IBS), as was investigated recently using transmural potential difference, which reflects mainly electrogenic chloride secretion (1). Similar results were observed in the jejunum in irritable bowel syndrome with diarrhea (IBS-D) and irritable bowel syndrome with constipation [C-IBS (1)]. Prime endogenous candidates for the induction of colonic secretion are di-α hydroxy bile acids such as chenodeoxycholic and deoxycholic acid. Several studies have documented bile acid malabsorption (BAM) in up to 50% of patients with functional diarrhea or IBS-D (2–5). Moreover, up to 35% of patients with microscopic colitis and diarrhea [which presents with a phenotype that overlaps with IBS-D (6)] have evidence of BAM (5). BAM in IBS may result from accelerated small bowel or colonic transit (7,8). In IBS-D responsive to cholestyramine, Tasleem et al. (9) have observed that there is defective fibroblast growth factor-19 (FGF-19) release from the ileum. FGF-15/19 are peptides that downregulate bile acid biosynthesis in enterocytes and cholangiocytes (10), suggesting that BAM is caused by increased bile acid biosynthesis.

The mechanisms of bile acid-induced colonic secretion include activation of secretory mechanisms [e.g. adenylate cyclase (11)], increased mucosal permeability associated with their detergent effects (12), inhibition of apical Cl^-/OH^- exchange by a process that is dependent on calcium ions and PI_3 kinase but not on IP_3 (13). Oddsson et al. (14) showed that ileal perfusion of bile acid resulted in greater ileal fluid secretion in IBS compared to healthy controls. As in other species, di-αhydroxy bile acids induce fluid secretion in the human colon (15). Bile acids also have been associated with increased colonic contractions; however, there is a wide range in the concentrations that induce propulsive contractions in canine [>20mM (16)] and human [1mM (17)] colon. In humans, a relationship was found between the fecal bile acid excretion and colonic motility; however, in those studies, 40% also had significant steatorrhea (18), a situation that is not typical for IBS.

Experimentally, Kirwan et al. (18) showed that bile acids infused directly into the human sigmoid colon and rectum stimulated colonic motility, and the threshold concentration was >5mM, a concentration that is seldom achieved in the absence of ileal resection. While the bile acid concentration of stool in patients with ileal resection may reach 21mM chenodeoxycholate (19), the concentrations in IBS-D are unclear, and the adaptive responses to bile acid loss

include up-regulation of the ileal active transporter (20) which may conceivably reduce the biological consequences of BAM over time. When bile acid was delivered to the sigmoid in studies of Kirwan et al. (18), it was unclear whether delivery of the same concentration to the proximal colon, as might occur in humans with BAM, would elicit the same effects. Given the observations of abnormal transit in patients with IBS-D (21,22), it is relevant to determine whether BAM is a factor contributing to the phenotype of IBS-D.

Until recently, the measurement of BAM has utilized the ^{75}SeHCAT test, based on bile acid retention of radiolabeled homolog of a natural bile acid, taurocholate (23,24). Regrettably, this test was never approved or used in the USA. However, the measurement of serum 7α-hydroxy-4-cholesten-3-one (7α-HCO or 7αC4) is a method for monitoring the enzymatic activity of hepatic cholesterol 7α-hydroxylase, the rate-limiting and major regulatory enzyme in the synthesis of bile acids (25) and is closely related to the fecal loss of bile acids. Thus, serum 7αC4 is a validated method for detecting BAM (26). In head-to-head comparisons with the ^{75}SeHCAT retention test, increased serum 7α-HCO (normal value: median 17.9ng/ml; mean ± SD 19.6 ±10.4ng/ml) had a sensitivity of 90% and specificity 79% in diagnosing BAM (24). An HPLC method has been developed to measure 7αC4 (27). The aims of this study were to develop a serum 7αC4 assay, ascertain normal values in asymptomatic healthy controls, and compare the results from health with serum from patients with ileal Crohn's disease or resection, and patients with IBS-D or IBS-C.

METHODS

Participants

Stored serum samples were studied from participants who had given permission for future studies using their stored samples at Mayo Clinic. The samples were from 111 healthy volunteers, 15 patients with IBS-D, 15 patients with IBS-C, 24 patients with ileal Crohn's disease, and 20 patients with ileal resection for Crohn's disease. Of the latter group, 4 also had segmental jejunal resection, 9 had cecum ascending colon or right hemi-colon resection, and 4 had extensive colonic resection greater than hemicolectomy. All participants were aged 18–65 years at the time of collection of the samples; samples were collected in the fasting state in the morning. The storage of samples for future research and the use of the samples in this study were approved by the Mayo Clinic Institutional Review Board.

Liquid Chromatography-Tandem Mass Spectrometry Method

We adapted the method described by Honda et al. (27) which measures 7α-hydroxy-4-cholesten-3-one (7αC4) to estimate normal values and levels in IBS and patients with ileal pathology.

Serum Sample Preparation—7α-hydroxy-4-cholesten-3-one was obtained from Steraloids, Inc. (Newport, RI). Deuterium labeled 7α-hydroxy-4-cholesten-3-one (d_7-7αC4) was obtained from Dr. Akira Honda [(27) Center for Collaborative Research, Tokyo Medical University, Ibaraki 300–0395, Japan]. From a 1 mg/mL stock solution, a 9 point calibration curve 0–500 ng/mL was made in 100% methanol. A 25 ng/mL d_7-7α-hydroxy-4-cholesten-3-one working internal standard was made in 100% methanol.

Fifty microliters of d_7-7αC4 internal standard was added to 200 μL of serum, standards and controls. Lipids and proteins were precipitated out by pipetting the following reagents sequentially into the sample: 700 μL of HPLC grade H_2O, 2 mL of acetonitrile, 1 mL of saturated ammonium sulfate at 40 g/100 mL H_2O. The specimen was vortex-mixed for 1 minute and then allowed to incubate at room temperature for 30 minutes. The sample was centrifuged at 3000 rpm (2,101g) for 25 minutes at 4–6 °C without brakes to allow two layers to separate.

One mL of clear supernatant was collected and dried down under nitrogen at 45°C for 15–20 minutes. The extract was reconstituted with 200 μL of 100% methanol and vortex-mix for 30 seconds. Immediately following, the sample was transferred into a microcentrifuge tube and left to incubate for 10 minutes. After precipitation by microcentrifugation at 11,000 rpm (11, 200g) for 1 minute and 30 seconds to promote separation of two phases, 80 μL of supernatant clarified by centrifugation was placed into a 96 well plate for LC-MS/MS analysis.

LC-MS/MS Assay—The LC-MS/MS system consisted of an API 5000 triple-quadruple mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA/Concord, Ontario, Canada) coupled with electrospray ionization (ESI) interface operated in the multiple-reaction monitoring positive ion mode. Chromatographic separation was performed on a Cohesive HPLC System (Thermo Fisher Scientific, Franklin, MA) with a Phenomenex MAX-RP column (150 × 2.0 mm, 4 μm; Phenomenex) operated at a flow rate of 200 μl/ minute and eluted with a linear gradient from 95% to 100% methanol in water. The organic and aqueous mobile phase consisted of methanol and water, respectively, with both containing 0.1% ammonium acetate. The injection volume was 50 μL, and the total analysis time was 14 minutes. The general MS/MS conditions [Q1 and Q3 fragments, declustering potential (DP), enterance potential (EP), collision energy (CE), collision cell exit potential (CXP)] for each metabolite were optimized and are summarized in Table 1.

Statistical Analysis

In all groups, the data were not normally distributed and, therefore, nonparametric statistical analyses were used throughout. Using ANOVA on ranks, we compared the 7αC4 measurements in all groups and separately in IBS versus controls. ANOVA on ranks was followed by Dunn's test to compare the individual disease groups versus health. Dunn's test incorporates a correction for multiple comparisons. Since BAM is potentially relevant in diarrheal disease rather than constipation, we conducted a direct comparison of health versus IBS-D using the Mann-Whitney rank sum test (SigmaStat® Version 2.0, 1997, SPSS Inc., Chicago, IL) and did not need to adjust α of 0.05 for this comparison.

RESULTS

Performance of the LC-MS/MS Method

Linearity and Detection Limits—Linear responses were observed in the concentration range of 0–200 ng/mL. Samples showed good linearity with dilution (Table 2). The observed (experimental) linearity values plotted against the expected values gave slopes and correlation coefficients near 1: range of slopes from 0.9188 to 0.9999 and r^2 from 0.9985 to 1. The detection limit of the assay, defined by an S/N ratio of 10, was 0.04 ng/mL.

Precision and Recovery—The intra- and inter-assay precision data (summarized in Table 3) were assessed for the LC-MS/MS method at concentrations range 0–200 ng/mL by analyzing three serum samples with different 7aC4 content (low, medium, high). The assay was conducted on 5 separate days in triplicate. Intra-assay coefficient of variation (CV) averaged 6.0% and ranged 1.8–13.1%. Inter-assay CVs averaged 6.2% and ranged 3.4–10.6%.

Recovery was determined using a standard spiking technique. Prior to extracting, a pool of serum was divided into four groups and spiked with differing concentrations of 7αC4 (0, 10, 25, and 50 ng/mL). The recovery was determined by measuring the final 7αC4 concentration in the samples after the spike, then dividing by the expected concentration. The average recovery was 99% and ranged 93–107% (Table 4).

Observations in Healthy Volunteers and in Patients with Ileal Disease or Resection

Observations in all groups are summarized in Table 5 and as histogram plots in Figure 1. The 5^{th} to 95^{th} percentile of 7αC4 concentration in normal healthy volunteers was 6–60.7 ng/mL. There were significant overall group differences (ANOVA on ranks $p<0.001$), with significantly higher values for terminal ileal disease or resection ($p<0.05$, adjusted using Dunn's multiple group comparison test).

Identification of BAM in IBS

There were also significant differences between health and IBS (Figure 2, ANOVA on ranks, $p=0.043$), with higher median values in the IBS-D patients relative to healthy controls (rank sum test, $p=0.027$). Three of the 15 IBS-D patients had 7αC4 concentration that exceeded the 60.7ng/mL upper limit of normal (95^{th} percentile).

DISCUSSION

We have established a sensitive non-isotopic assay based on HPLC-MS/MS, determined the normal 7αC4 values in humans, identified increased 7αC4 in a minority of patients with IBS-D, and demonstrated increased levels in patients with ileal resection or disease. This assay has potential for identifying BAM in gastrointestinal diseases including IBS. In order to be completely validated, 7αC4 results should be compared to fecal bile acid measurements. There was previous indirect validation of 7αC4 by the simultaneous assessment of serum 7αC4 and whole body retention using ^{75}SeHCAT (26,28).

The ^{75}SeHCAT retention test had become the gold standard for identification of BAM in ileal disease (29). It was originally validated that the artificial bile acid (tested as the gamma-labeled bile acid analogue ^{75}selenohomocholic acid-taurine) was transported, conjugated absorbed and fractionally excreted as a natural bile acid taurocholate (30). Subsequently, it was also demonstrated that shorter retention half-life of ^{75}SeHCAT is associated with increased level of 7αC4 with a 98% negative predictive value and 74% positive predictive value for diagnosis of BAM (26). Although serum levels of 7αC4 reflect rates of hepatic synthesis of bile acids (31), the latter is influenced by the enterohepatic circulation of bile acids and, hence, loss of bile acids from BAM results in upregulation of the hepatic synthesis rates. This is confirmed by the finding of increased levels of 7αC4 in our positive control groups who had ileal Crohn's disease or ileal resection.

A noninvasive, sensitive assay is needed to identify BAM in clinical and research studies. Investigators have been hampered in this line of investigation since the ^{75}SeHCAT retention test, which was validated, approved and applied in over 20 publications in the prior literature predominantly from European centers, was never approved or licensed for use in the United States. The development of a non-isotopic method to identify BAM fills an unmet need.

In this pilot study, we observed that a minority of patients with IBS-D had increased levels of 7αC4. The prevalence of increased levels of 7αC4 in patients with chronic diarrhea due to IBS-D, collagenous, microscopic and lymphocytic colitis deserves further study in large patient cohorts. This is highly relevant since treatment with bile acid binders such as cholestyramine has been shown to improve diarrhea in 60–76% of patients with BAM who were initially given a diagnosis of functional diarrhea or IBS-D (4,5). Cholestyramine is associated with many gastrointestinal side effects, including constipation, heartburn, flatulence, and has an unpalatable taste, which often leads to discontinuation of the therapy. Newer bile acid binders, such as colesevelam, are better tolerated with less gastrointestinal side effects and greater ease of administration (32,33).

There are potential pitfalls and precautions in the interpretation of the results of 7αC4. First, participants with known chronic liver disease, such as cholestatic liver diseases that are associated with hyperlipidemia, or significant hepatic inflammation with AST or ALT >2 times the upper limit of normal (who might have abnormal bile acid synthesis because of the liver disease) may have abnormal 7αC4. This may lead to a false-positive or negative result in assessment of possible BAM. A second potential pitfall is that concomitant medication therapy, including statin (HMG CoA reductase inhibitor) treatment, may potentially influence bile acid synthesis rates. Among healthy volunteers with high serum levels of 7αC4 (66–85 ng/mL), we reviewed the medical records and confirmed that they were not on treatment with statins or other treatments for hyperlipidemia and their hepatic transaminase levels were within the normal range.

An additional, necessary precaution is to standardize the timing of serum collection, since there is evidence that serum 7αC4 showed two distinct peaks (2- to 4-fold above baseline) during a 24-hour period, the first at 1:00 p.m. and the second at 9:00 p.m. During the night, 7αC4 levels declined, and they returned to baseline levels the next morning (34).

In conclusion, a sensitive non-isotopic assay based on HPLC-MS/MS allows determination of elevated 7αC4 values in humans. This is an indirect marker for BAM and, in the absence of significant liver disease, the assay has the potential to identify BAM in gastrointestinal diseases including IBS and chronic diarrhea. This noninvasive and sensitive measurement has considerable potential in clinical practice and research.

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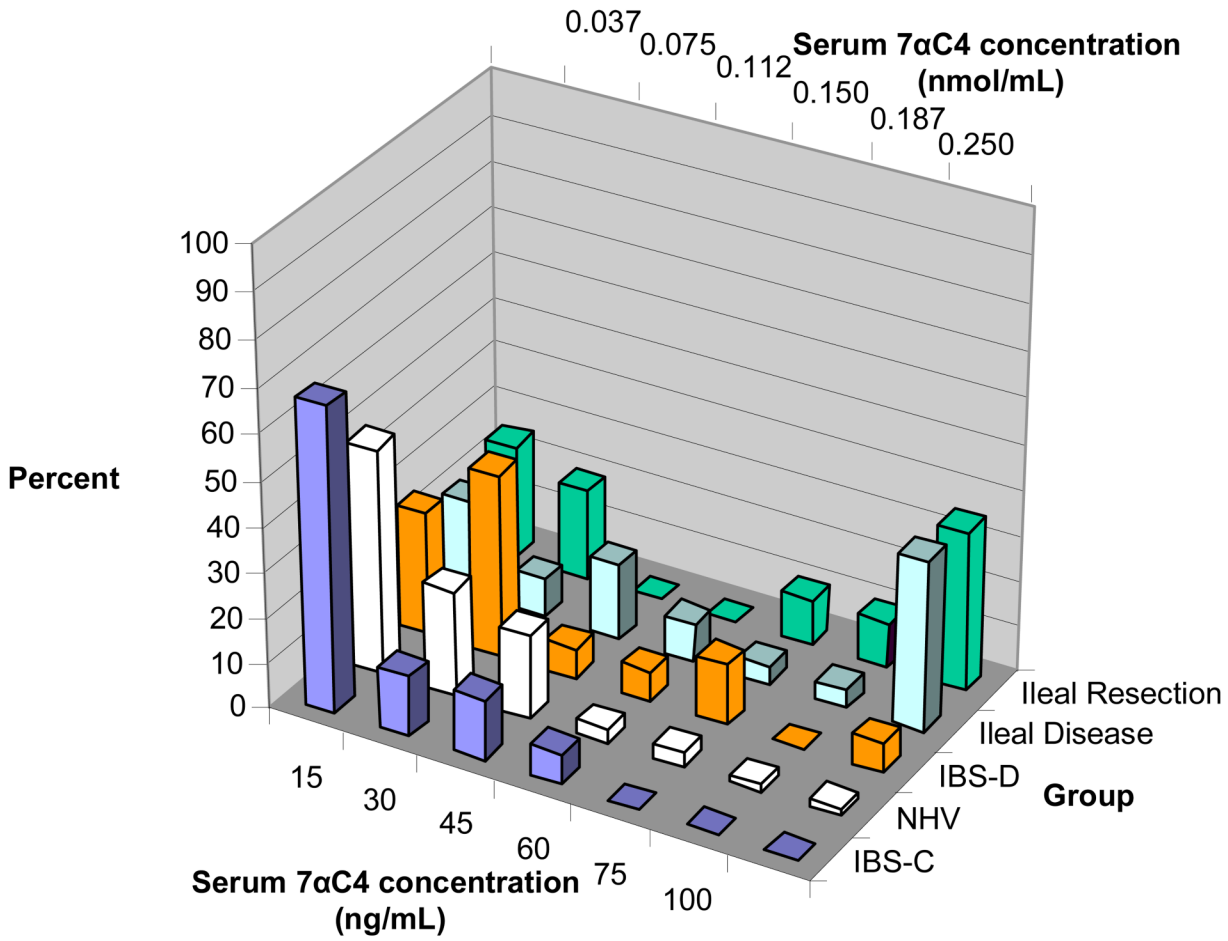

7αC4 (ng/mL)	0-15	16-30	31-45	46-60	61-75	76-100	>100
IBS-C	67	13	13	7	0	0	0
NHV	49	23	18	3	3	2	2
IBS-D	27	40	7	7	13	0	7
Ileal Disease	21	8	17	8	4	4	38
Ileal Resection	25	20	0	0	10	10	35

Figure 1.
Histogram plots of all groups showing the percentage of each group with serum 7αC4 (ng/mL) in different concentrations. Note that values above 60ng/mL fall outside the normal range. Thus, >50% of patients with distal ileal disease or resection and 20% of patients with IBS-D had abnormal serum 7αC4.

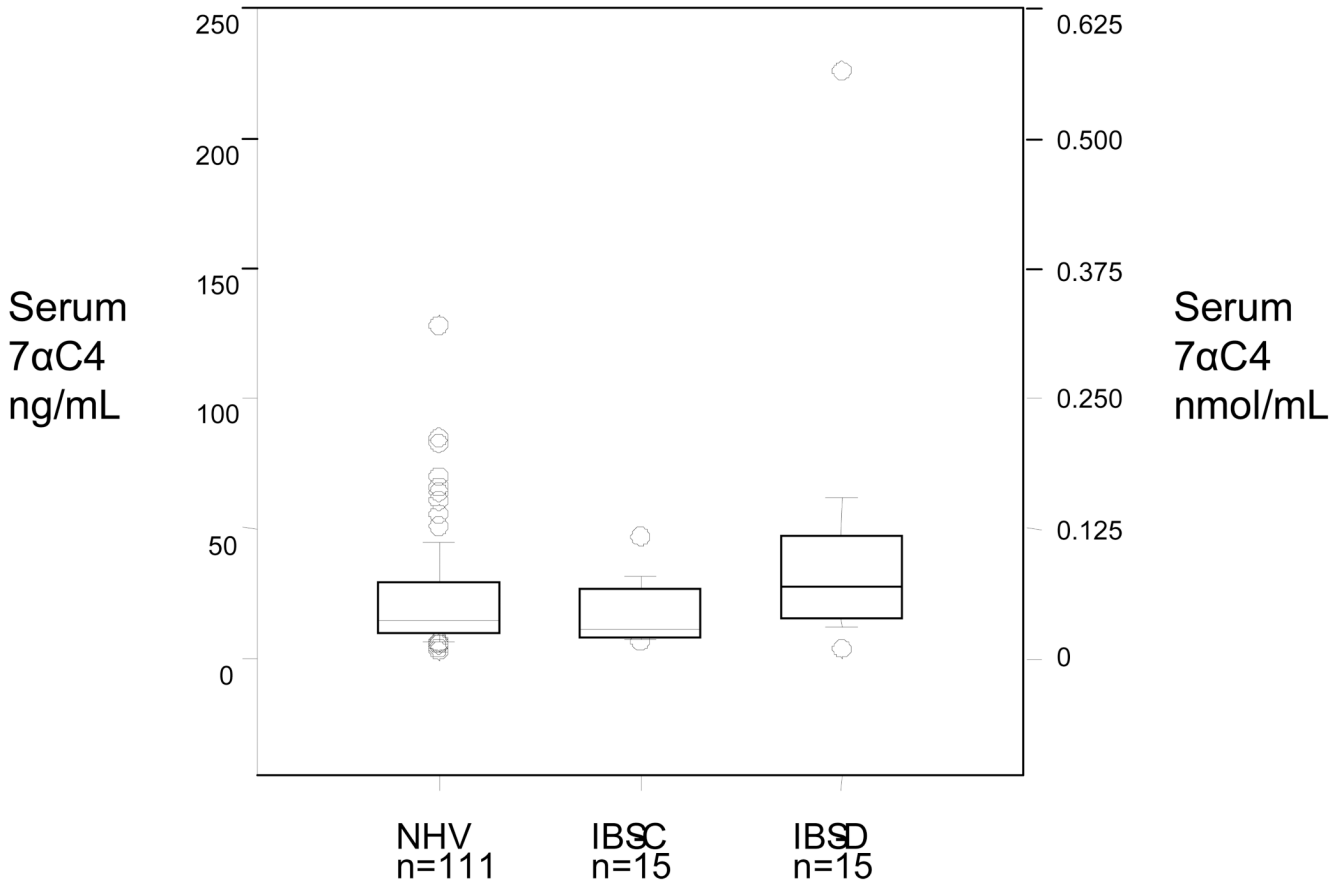

Figure 2.
Comparison of serum 7αC4 (ng/mL) in IBS and healthy volunteers. Data show median and interquartile (box) range, 5th and 95th percentiles and outliers (individual symbols). There are overall significant group differences (ANOVA on ranks, p=0.043), with a borderline significant difference between the healthy volunteer and IBS-D group (Mann-Whitney Rank Sum Test, p=0.027).

Table 1

Monitored Ion Transitions and their Parameter Settings

Analyte (Q1) (m/z, amu)	Fragment (Q3) (m/z, amu)	DP (volt)	EP (volt)	CE (volt)	CXP (volt)
7αC4 (401.32)	383.40	165.00	10.00	26.00	24.00
7αC4 (401.32)	365.40	165.00	10.00	26.50	20.00
7αC4 (401.32)	177.23	165.00	10.00	34.20	24.00
7αC4 (401.32)	97.10	165.00	10.00	40.00	15.00
D_7-7αC4 (408.50)	390.45	165.00	10.00	26.50	20.00

Key: declustering potential (DP), enterance potential (EP), collision energy (CE), collision cell exit potential (CXP)

Table 2

Linearity of 7αC4 in Human Serum

Sample ID	Dilution	Measured ng/mL	Expected ng/mL	Measured/Expected %
1	1:1	35.3		
	1:2	18.2	17.7	103
	1:4	9.7	8.8	110
	1:8	4.8	4.4	109
Mean				107
Sample ID	**Dilution**	**Measured ng/mL**	**Expected ng/mL**	**Measured/Expected %**
2	1:1	117		
	1:2	57.4	58.5	98
	1:4	29.5	29.3	101
	1:8	16.6	14.6	113
Mean				104
Sample ID	**Dilution**	**Measured ng/mL**	**Expected ng/mL**	**Measured/Expected %**
3	1:1	192		
	1:2	90.8	96.0	95
	1:4	46.9	48.0	98
	1:8	24.6	24.0	103
Mean				98
Grand Mean				**103**

Table 3

Intra- and Inter-assay Precision in Human Serum Analyzing 3 Concentration Levels of 7αC4

Intra-assay (within-run)				
Sample ID	**N**	**Mean (ng/mL)**	**SD**	**COV %**
Low	8	3.8	0.50	13.1
Medium	8	42	0.75	1.8
High	8	129	4.1	3.2
Inter-assay (between-run)				
Sample ID	**N**	**Mean (ng/mL)**	**SD**	**COV %**
Low	15	3.9	0.41	10.6
Medium	15	42	1.4	3.4
High	15	127	5.9	4.6

SD= standard deviation; COV = coefficient of variation

Table 4

Recovery of Four 7αC4 Concentrations Spiked into Human Serum

Amount added (ng/mL)	Amount measured (mean ± SD, ng/mL)	COV %	Recovery %
0	26.1 ± 0.4	1.6	
10	38.8 ± 0.3	0.9	107
25	47.7 ± 1.1	2.3	93
50	74.1 ± 1.8	2.4	97
MEAN			**99**

COV = coefficient of variation

Table 5

Descriptive Statistics of Serum 7αC4 (ng/mL) in all Groups of Participants

There are overall significant group differences (ANOVA on ranks, $p<0.001$) with major differences in the ileal disease and resection groups (both $p<0.05$ adjusted) compared to the normal healthy controls (NHV).

Group	Range	Max	Min	Median	25%ile	75%ile	Mean	SD
NHV, n=111	125.1	128.0	2.9	14.3	9.5	29.2	22.3	19.7
IBS-C, n=15	40.1	46.7	6.6	11.5	7.6	26.4	16.8	12.2
IBS-D, n=15	222.4	226.0	3.7	27.6	15.7	46.8	41.9	53.7
Ileal disease, n=24	622.1	627.0	4.9	51.9	21.0	185.0	125.0	162.5
Ileal resection, n=20	955.1	960.0	4.9	65.8	15.0	208.0	169.8	248.1