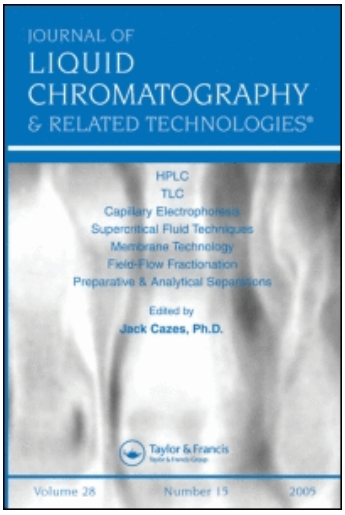

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SIMULTANEOUS HPLC DETERMINATION OF FREE, CONJUGATED, AND SULFATED BILE ACIDS

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ABSTRACT

An HPLC method has been developed to separate the 18 major bile acids in a single analytical run (cholate, chenodeoxycholate, deoxycholate, lithocholate, sulfolithocholate, ursodeoxycholate and their glycine and taurine conjugates). Bile acids are rapidly extracted from bile, urine or peritoneal fluid over a C18 cartridge and resolved on a reverse-phase column. Peaks at 210 nm are quantitated by comparison with the peak area of commercial standards using 7α, 12α-dihydroxycholanic acid as a retention standard. Standard regression lines are linear over the range of 2-40 μg for conjugated acids and 50-800 μg for unconjugated acids (correlation coefficient range, r=0.962 to 0.989; coefficient of variation, CV=7.8%). Limits of detection are 1 and 20 μg respectively. In a preliminary evaluation, the analysis of urine demonstrated profile shifts in conjugation pattern and total bile acid concentration between patients with hepatitis, biliary obstruction and nonhepatic illness. Abnormal urine profiles from another patient with chronic cholecystitis changed toward a normal pattern in the weeks post-cholecystectomy. This method can thus provide a rapid, sensitive means of analyzing bile acid profiles in a variety of body fluids.

INTRODUCTION

Analyzing the bile acid profile of serum (1-7), bile (8), urine (9), feces (10) and body fluid (11) can provide a sensitive indicator of hepatobiliary and gastrointestinal disease. Alterations in total amount and profile shifts in individual acid ratios correlate with both the severity and mechanism of the disease process (1-5,7,9,10). Yet, bile acid analysis remains an esoteric test in most clinical laboratories, mainly due to the limitations and complexity of current analytical methodologies (12). Immunologic and enzymatic assays provide only a total quantitation and cannot detect many of the major acids (13). Gas chromatography/mass spectrometry (GC/MS) can yield a comprehensive profile but requires extensive sample preparation and multiple analytical runs (13,14). High-pressure liquid chromatography (HPLC) offers the advantage of profile analysis in a single run and has become the current standard for examining bile acids. However, none of the contemporary assays are capable of concurrently resolving the three principle forms of bile acid in biological specimens: the conjugated, free and sulfated acids (15-21). These HPLC assays also require extensive specimen preparation, have long analysis times, and rely on derivatization or enzymatic detection.

We have developed a reverse-phase HPLC method capable of rapidly resolving the major bile acids in a single run. With spectrophotometric detection, sulfolithocholate conjugates can be measured in addition to the other free and conjugated acids. We have applied this method to the analysis of bile acid profiles in urine, bile, and body fluid and have investigated its ability to detect hepatocellular and biliary disease-induced changes in urine bile acid profile.

MATERIALS

Apparatus

The chromatographic system consisted of two 510 HPLC pumps, a 680 automated gradient controller, a 994 programmable photodiode array detector and a 5200 printer/plotter (Waters Instruments, Milford, MA). A reverse-phase, 4.6 x 250 mm ultrasphere ODS-C18 column with 5 μm-diameter particle size was used for the separations (Beckman Instruments, San Ramon, CA). Disposable filter membranes, Durapore 0.45 μm, were used to prepare buffers (Millipore Corp., Bedford, MA).

Reagents and standards

Sodium acetate (Aldrich Chemical Co., Milwaukee, WI), phosphoric acid (J.T Baker, Inc., Philipsburg, NJ), sodium hydroxide (American Research Products Co., Solon, OH), acetone (Fisher Scientific, Fairlawn, NJ), methanol and hexane (Baxter, Muskegon, MI) were all AR, ACS or HPLC grade reagents. A standard mixture for column calibration was prepared to a final concentration of 1 mg/ml conjugated acids, 20 mg/ml unconjugated acids and 10 mg/ml internal standard in methanol: glycocholic acid (GC), glycochenodeoxycholic acid sodium salt (GCDC), glycodeoxycholic acid (GDC), glycolithocholic (GLC), glycolithocholic acid 3-sulfate disodium salt (GSLC), cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (LC), lithocholic acid 3-sulfate sodium salt (SLC), ursodeoxycholic acid (UDC), taurocholic acid sodium salt (TC), taurochenodeoxycholic acid sodium salt (TCDC), taurodeoxycholic acid sodium salt (TDC), taurolithocholic acid sodium salt (TLC), taurolithocholic acid 3-sulfate disodium salt (TSLC), and tauroursodeoxycholic acid sodium salt (TUDC) (Sigma Chemical Co., St. Louis, MO); glycoursodeoxycholic acid sodium salt (GUDC) and 7α,12α dihydroxy-5β cholanic acid as internal standard (Calbiochem, La Jolla, CA).

METHODS

Sample preparation

Bile specimens were obtained by post-surgical drainage tube. Random urine specimens were obtained from Mayo Clinic patients with nonhepatic complaints or from hospitalized patients with diagnosed hepatobiliary disease. Normality was defined by having liver enzyme and routine chemistry values in the reference range for our clinic population. The body fluid specimen was collected by biopsy needle from the peritoneal cavity surrounding the liver in a patient with biliary obstruction. All samples were stored frozen at -20°C until analysis.

Internal standard, 500 μg, was added to 10 ml urine, 1 ml body fluid or 0.1 ml bile. Bile acids were extracted by modification of previously described methods (15,20). Samples were diluted 1:4 with 0.1N NaOH, mixed and heated for 5 minutes at 65°C to denature all protein-bile acid complexes. After cooling to room temperature, the sample was passed over a C18 disposable column, Bond-Elut® (500mg, Varian, Harbor City, CA) or Sep-Pak® (360mg, Waters, Milford, MA), that had been preconditioned with methanol and equilibrated with 0.1N NaOH. Columns were washed according to the manufacturers directions. The Bond-Elut® columns were washed with 10 ml water and eluted with 5 ml methanol. The Sep-Pak® cartidges were washed with 10 ml water, 10 ml 10% acetone, 10 ml water and eluted with 5 ml methanol. Sep-Pak® cartridges were also tested using only 10 ml water as a wash with no significant improvement in recovery. The methanol eluants were dried under nitrogen at 40°C, redissolved in 250 μl initial column buffer,and injected onto the column, 5 to 100 μl.

Chromatographic conditions

Bile acids were separated in a 40 minute nonlinear gradient elution. Column was conditioned to initial

conditions of 66% methanol:34% sodium actetate, 30 mM pH 3.4. After injection, methanol was increased to 67% over 7 minutes, then to 76% over the next 14 minutes. The gradient was held at 76% methanol for 4 minutes, increased to 90% methanol over 8 minutes and held at 90% for 7 more minutes. The column was washed for 5 minutes in 100% methanol and reequilibrated to initial conditions. Column effluent was monitored at 210 nm. Peak identities were confirmed by absolute and relative retention times (ratio to internal standard). UV spectra was used to determine peak purity by comparison to commercial bile acid spectra. Questionable peaks were also confirmed by spiking the specimen with standard and evaluating the peak shape and overall retention time.

RESULTS

Assay variance

The reverse-phase column was calibrated using a mixture of 17 commercial bile acids and internal standard (without SLC). This mixture was injected consecutively at a constant 10μl volume to determine within-run precision. Day-to-day precision was determined by injection of varying amounts of standard mix over a 20 day period. Table 1 presents the average and total recovery of all bile acids. The range indicates the spread between individual bile acids. Day-to-Day variation was found to be larger than the within-run variation (coefficients of variation, CV=6.25% and 11.23% respectively, Table 1), reflecting an increased error when varying the volume of injection.

Regression lines for bile acid quantitation are shown in Figure 1 and regression equations are summarized in Table 2. Correlation coefficients ranged from r=0.962 to 0.989. The assay is linear over a range of 2-40 μg/injection for conjugated acid and 50-800 μg/injection

TABLE 1

Bile Acid Fractionation: Variance Overview
Comparison of Bond-Elut® and Sep-Pak® C18 Cartridges

	PERCENT OF STANDARD RECOVERED		
	MEAN (range)	CV (range)	N
Injection of Standards			
Consecutive 10 µl Runs	99.6 (91-106)	6.3 (3.4- 8.5)	12
Day to Day (2-30 µl)	99.4 (91-106)	11.2 (7.7-15.8)	20
Bond-Elut® Extraction			
Standards Alone Bile/Urine Method	84.1 (75- 98)	12.0 (2.9-18.5)	4
Standards from Spiked Bile	81.2 (51-100)	7.8 (2.5-22.1)	3
Standards from Spiked Urine	96.4 (66-113)	7.8 (2.9-15.5)	7
Sep-Pak® Extraction			
Standards Alone Bile/Urine Method	77.5 (41-102)	11.5 (1.5-23.1)	2
Standards from Spiked Bile	78.3 (24-109)	15.0 (1.6-31.5)	2

for unconjugated acid, with a detection limit for individual bile acids of 1 µg and 20 µg acid respectively. An average of 99.4% of the injected, multicomponent standard was recovered.

The regression lines were validated by the injection of up to 5 standard runs per week over a 10 month period. The slope and intercepts were found to vary by less than 3.7% over the lifetime of a single column, but could shift by as much as 9.8% between columns. The construction of new regression lines is thus recommended with each new column.

Extraction method

The recovery of bile acid through the extraction procedure was examined using the multicomponent standard mixture in both water and a specimen matrix. Overall

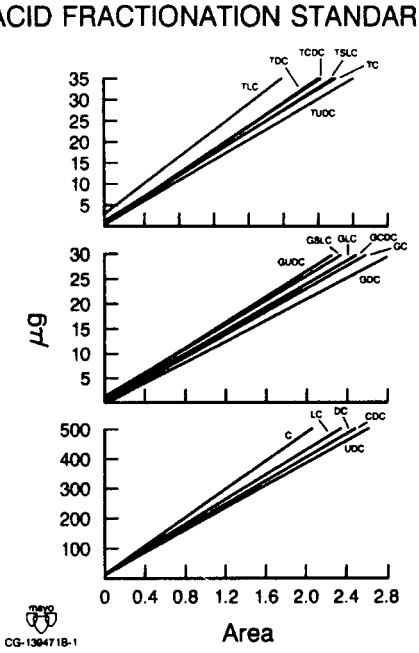

FIGURE 1
Regression lines for quantitation of bile acids by HPLC. Abbreviations listed in the Materials section.

recovery was calculated as [quantitated bile acid] ÷ [concentration spiked into the specimen]. Two different C18 columns were tested, Bond-Elut® and Sep-Pak®. Results are compared in Table 1. Recovery was higher overall with Bond-Elut® columns, from either water or sample. Use of acetone:water or only water in the Sep-Pak® wash procedure provided no improvement in recovery, especially for SLC conjugates (GSLC and TSLC), 20-28% compared to 50-70% with Bond-Elut®. In the presence of a specimen matrix, Bond-Elut® had better recovery yield and about half the variation of Sep-Pak® (Table 1).

TABLE 2

Regression Equations for Bile Acid Quantitation[a]

	Regression Equation (μg)	r[b]	$S_{y \cdot x}$
TC	15.07 X + 1.20	0.978	1.31
TCDC	16.36 X + 0.64	0.982	1.13
TDC	16.53 X + 0.54	0.987	0.90
TLC	18.60 X + 2.92	0.965	1.56
TSLC	15.41 X + 0.73	0.983	0.98
TUDC	14.24 X + 0.36	0.983	1.03
GC	13.44 X + 0.23	0.986	0.99
GCDC	13.69 X + 0.89	0.988	0.88
GDC	12.47 X - 0.34	0.962	1.64
GLC	13.82 X + 0.63	0.989	1.00
GSLC	14.28 X + 1.59	0.974	1.29
GUDC	15.02 X + 1.26	0.963	1.36
C	253.9 X + 15.0	0.978	22.8
CDC	194.9 X + 11.9	0.988	17.1
DC	196.8 X + 10.6	0.989	16.0
LC	206.4 X + 16.2	0.989	16.4
SLC	1343 X + 3.10	0.944	52.0
UDC	186.4 X + 11.4	0.989	16.8
IS	155.2 X + 27.3	0.966	14.6

[a] Number of determinations ≥ 70
[b] r = Correlation Coefficient

Assay resolution

Baseline separation of all six major free bile acids and their glycine and taurine conjugates was achieved in less than 40 minutes as demonstrated in Figure 2. The absorbance of SLC at 210nm was insufficient to allow quantitation at physiological levels. Although the SLC peak separates from the other bile acids (elution between UDC and GCDC, retention ratio = 0.72 with respect to the internal standard), the detection limit of 1000 μg did not allow the practical inclusion of SLC in the assay. Free SLC is therefore not included in the Figure 2 chromatograms. Typical chromatograms of bile and urine are included in Figure 2 to demonstrate the ability of

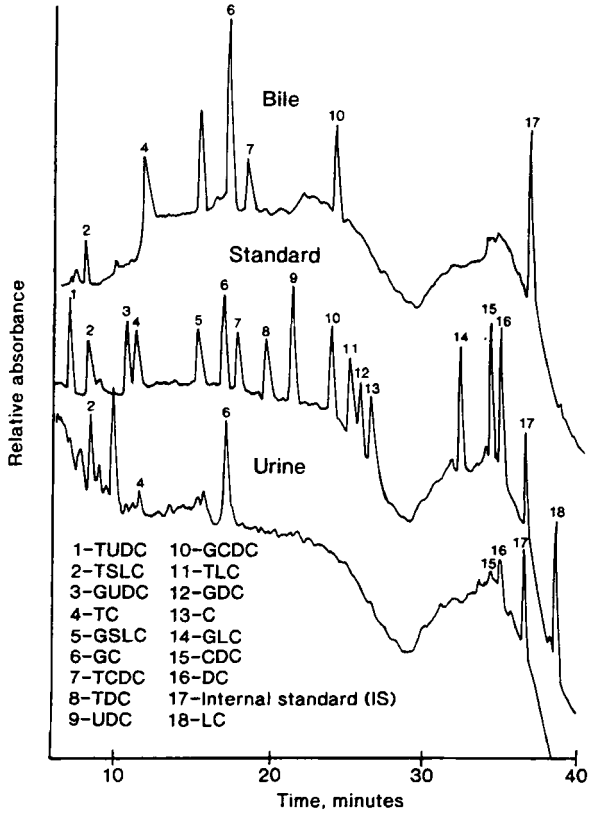

FIGURE 2
Chromatograms of bile acid separated by HPLC. Human bile collected by biliary drainage tube, commercial standard bile acid mixture and human urine sample injections as described in text.

this method to determine profiles in bile and urine. The wide variation in the amount of individual acids in the bile specimen required analysis of at least two dilutions to obtain peak areas within the linear range. The method also resolved the profile of peritoneal fluid in a patient with obstruction and biliary rupture. This profile was very similar to bile and was thus not

included in Figure 2. However, the additional quantity of protein in this specimen did not interfere with the determination.

Preliminary applications

In order to investigate the clinical utility of determining bile acid profiles by our method, the urine profiles of seven patients were examined. Two were diagnosed with liver disease, hepatitis C and biliary obstruction, while the other five patients were being seen for nonhepatic illnesses and displayed normal levels of liver enzymes. The profiles are compared in Table 3. The amount of individual bile acid or groups of bile acids are presented as percentages of the sample total. Due to similarity, only one normal profile is included as a representative of normal profiles. The total amount of bile acid increased 20-25 fold in hepatic disease. The conjugated acids predominate in the primary (TC,GC,TCDC) and tertiary classes (TSLC,GSLC,TUDC,GUDC). Both patients with liver disease demonstrate shifts from glycine to taurine conjugation. The patient with hepatitis shows a shift from C to CDC conjugates, while the obstructed patient demonstrates a primary to tertiary shift resulting from the loss of cholate to sulfolithocholate conjugates, mostly TSLC.

The urine profiles, over an eight week period, of a patient with chronic cholecystitis were also examined (Table 4). The first urine specimen, pre-cholecystectomy, was collected 3 days past an attack and 5 days prior to the subsequent attack of pain. The later specimens were collected 3 and 7 weeks post-cholecystectomy. The patient's liver enzyme and routine chemistry test values were within normal range. Diagnosis of chronic cholecystitis due to gall stones was made on the basis of abnormal ultrasound and delayed ^{99m}Tc HIDA clearance. The

TABLE 3

Urine Bile Acid Profile
Patient Comparison: Normal vs Diagnosed Liver Disease

INDIVIDUAL BILE ACID (PERCENTAGE OF TOTAL)	NORMAL	HEPATITIS	OBSTRUCTION
Taurocholic	20.3	0	15.1
Taurochenodeoxycholic	0	40.6	0
Taurodeoxycholic	0	0	0
Taurolithocholic	0	0	0
Taurosulfolithocholic	35.7	34.7	56.1
Tauroursodeoxycholic	0	19.4	10.4
Glycocholic	21.8	5.3	6.0
Glycochenodeoxycholic	0	0	0
Glycodeoxycholic	0	0	0
Glycolithocholic	0	0	0
Glycosulfolithocholic	0	0	3.7
Glycoursodeoxycholic	22.2	0	8.6
Cholic	0	0	0
Chenodeoxycholic	0	0	0
Deoxycholic	0	0	0
Lithocholic	0	0	0
Sulfolithocholic	0	0	0
Ursodeoxycholic	0	0	0
TOTAL (μg bile acid/ml)	3.1	66.1	73.4
BILE ACID GROUP			
Conjugated	100.0	100.0	100.0
Glycine	43.9	5.3	18.4
Taurine	56.1	94.7	81.6
Unconjugated	0	0	0
Cholic	42.1	5.3	21.1
Chenodeoxycholic	0	40.6	0
Deoxycholic	0	0	0
Lithocholic	0	0	0
Sulfolithocholic	35.7	34.7	59.9
Ursodeoxycholic	22.2	19.4	19.0
Primary Bile Acids	42.1	45.9	21.1
Conjugated	42.1	45.9	21.1
Unconjugated	0	0	0
Secondary Bile Acids	0	0	0
Conjugated	0	0	0
Unconjugated	0	0	0
Tertiary Bile Acids	57.9	54.1	78.9
Conjugated	57.9	54.1	78.9
Unconjugated	0	0	0

TABLE 4

Urine Bile Acid Profile Comparison: Cholecystitis over Time in a Single Patient

INDIVIDUAL BILE ACID (PERCENTAGE OF TOTAL)	PRE	--POST-CHOLECYSTECTOMY-- 3 WEEKS	7 WEEKS
Taurocholic	23.9	29.3	12.2
Taurochenodeoxycholic	0	0	0
Taurodeoxycholic	0	0	0
Taurolithocholic	0	0	0
Taurosulfolithocholic	41.3	50.1	27.8
Tauroursodeoxycholic	34.8	0	6.4
Glycocholic	0	0	33.6
Glycochenodeoxycholic	0	0	0
Glycodeoxycholic	0	0	0
Glycolithocholic	0	0	0
Glycosulfolithocholic	0	0	0
Glycoursodeoxycholic	0	20.6	20.0
Cholic	0	0	0
Chenodeoxycholic	0	0	0
Deoxycholic	0	0	0
Lithocholic	0	0	0
Sulfolithocholic	0	0	0
Ursodeoxycholic	0	0	0
TOTAL (μg bile acid/ml)	25.7	16.0	27.0
BILE ACID GROUP			
Conjugated	100.0	100.0	100.0
Glycine	0	20.6	53.6
Taurine	100.0	79.4	46.4
Unconjugated	0	0	0
Cholic	23.9	29.3	45.8
Chenodeoxycholic	0	0	0
Deoxycholic	0	0	0
Lithocholic	0	0	0
Sulfolithocholic	41.3	50.1	27.8
Ursodeoxycholic	34.8	20.6	26.4
Primary Bile Acids	23.9	29.3	45.8
Conjugated	23.9	29.3	45.8
Unconjugated	0	0	0
Secondary Bile Acids	0	0	0
Conjugated	0	0	0
Unconjugated	0	0	0
Tertiary Bile Acids	76.1	70.7	54.2
Conjugated	76.1	70.7	54.2
Unconjugated	0	0	0

pre-cholecystectomy profile (Table 4) demonstrates similarities to the obstructive profile (Table 3). The pattern of conjugation is 100% taurine with a shift to tertiary acids (TSLC,TUDC) and a concurrent loss of cholic conjugates. This profile changes after surgery toward a normal profile at 7 weeks. During this period, there is an increase in glycine conjugation and a general shift back to primary cholic conjugates. The total quantity of bile acid (27.0 μg/ml) remained elevated compared to normal patients (3.1 μg/ml) but was lower than the obstructive patient (73.4 μg/ml). This total did not change over the 8 weeks that the profile was monitored.

DISCUSSION

Bile acids exist in a balance dependent on the status of an individual's enterohepatic circulation. Diseases of the liver, bile duct and gastrointestinal tract can affect the synthesis, flow and absorption of bile acids, resulting in changes in both the total amount and ratio of individual acids in all body fluids: bile, serum, feces, urine and body secretions (1-11,22). Analysis of bile acid can provide a sensitive indicator to disruption of enterohepatic circulation. Yet, the clinical value of this test has been restricted by the limitations of current methodologies, the best of which only provide partial profiles (13,14) or requires GC/MS. Each procedure does not necessarily provide the same information, making clinical correlations between methods difficult to perform (12). The HPLC method described here offers several advantages over previous technologies.

The specimen can be prepared and analyzed in less than 90 minutes with our assay. Only a single extraction over a disposable C18 column is needed prior to HPLC

separation, compared to other assays requiring multiple solid phase extractions (15,19,21), thin layer chromatography (19) or enzymatic hydrolysis and derivatization (21). We noted dramatic differences in recovery between manufacturers of C18 preparative columns despite their matrix similarities. Bond-Elut® columns were preferred due to improved recovery of SLC conjugates.

The use of ultraviolet absorbance provides a simple means of monitoring the elution of bile acids and eliminates the need for long derivitization and hydrolysis steps. Ultraviolet spectrophotometric quantitation is not as sensitive as fluorescent derivatization (21) or 3α-HSD enzymatic detection (17,18), due to a 20 fold difference in absorptivity between free and conjugated acids (20). Although ultraviolet detection is not capable of detecting free acids at the concentrations found in normal serum, this method is sensitive enough to detect free acids, other than SLC, in bile and urine, confirming previous studies conducted on gastric fluid by similar methodology (16). The method can also determine conjugates of SLC (TSLC,GSLC) simultaneously with other physiologically important acids. Sulfated acids are known to constitute more than 50% of the serum or urine bile salts in cases of extrahepatic obstruction, but few current HPLC methods are capable of detecting them (6,12-14). The methods that can resolve the sulfated acids also fail to determine free acids or to apply their method to samples other than bile (20). This method thus offers determination of SLC conjugates with improved recovery and precision comparable to other HPLC methods.

Comparison of urine profiles from normal patients and patients with liver disease demonstrated a total concentration increase and profile changes characteristic

of either hepatocellular or obstructive disorders. While many authors have noted a total increase in serum bile acid concentration during the progression of liver disease (3-5,13,22), others have seen shifts in CDC to C ratios (1,2,22), increases in serum sulfated bile acids (12,13) and alterations in conjugation pattern from glycine to taurine (23) that are similar to those changes noted in our urine specimens. We were also able to demonstrate the normalization of a urine profile during the weeks after surgery in a patient with chronic cholecystitis. However, the elevated total quantity of bile acid did not change over the period monitored. Although these studies were only preliminary, they furnish evidence that our method can be applied to urine samples to provide diagnostic information that compares to previous studies conducted on serum. Larger studies analyzing the profiles of different patient groups are currently being conducted.

This HPLC method thus has the ability to simultaneously analyze 17 of the 18 major bile acids found in body fluids. Both a total quantitation and individual acid profile of the specimen are obtained. This method can also provide a correlation of bile profiles from different body fluids. The ability to rapidly determine comprehensive profiles in multiple body fluids at single collection timepoints should produce more thorough diagnostic information and provide for wider applications of the method to clinical investigations.

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