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Conditions for solid-phase extraction of urinary bile acids with octadecylsilane-bonded silica

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Abstract Fast atom bombardment mass spectrometry was used as a detection method in a study of the extraction of urinary bile acids with octadecylsilane-bonded silica. The procedure commonly used in many laboratories was found to result in significant losses of sulfated taurine-conjugated bile acids. Losses of other double conjugates and of bile acid and bile alcohol glucuronides were also seen. The losses were avoided by addition of an equal volume of 0.5 M triethylamine sulfate to the urine before passing it through the sorbent bed. Quantitative elution was then achieved with methanol. Batch variations were observed with sorbents from two different manufacturers. **—Wahlén, E., N. Tamasawa, H. Ichimiya, B. Egestad, and J. Sjövall.** Conditions for solid-phase extraction of urinary bile acids with octadecylsilane-bonded silica. *J. Lipid Res.* 1994. **35:** 1902–1906.

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Sorption on octadecylsilane-bonded (ODS) silica is now the standard method for extraction of bile acids from urine (1). While factors influencing sorption and desorption using the polystyrene resin. Amberlite XAD-2 were investigated in detail by Almé et al. (2), conditions for extraction with ODS-silica have only been studied with a few common bile acids (3).

In the course of studies of bile acids in urine from infants (4, 5), it was noted that the excretion of chenodeoxycholic acid was low in healthy newborn infants and that dihydroxy bile acids might be incompletely eluted from ODS-silica using the standard method of extraction. To evaluate this observation we made a detailed study of the extraction of urinary bile acids with ODS-silica using fast atom bombardment mass spectrometry (FABMS) to monitor the recoveries. The results are reported in this paper.

METHODS

Solvents and reagents

Solvents were of analytical grade and were redistilled before use. Water was deionized and purified in a Milli-Q apparatus (Millipore S. A., Molsheim, France). All reagents were of analytical grade. A 0.5 M solution of triethylamine sulfate (TEAS), pH 7.0–7.4, was prepared with aqueous sulfuric acid and triethylamine (6).

Reference compounds

Chenodeoxycholyltaurine was purchased from Sigma (St. Louis, MO). Mono- and disulfates were prepared by adding limiting amounts of sulfuric acid in dimethylformamide to 1 mg chenodeoxycholyltaurine and 10 mg N,N-dicyclohexylcarbodiimide in 200 μl dimethylformamide (7). After 10 min at room temperature, the reaction was terminated by addition of 1 ml of 48% aqueous ethanol. The mixture was filtered, and the products were separated on a column of Lipidex-DEAP (see below). Chenodeoxycholic acid 3-sulfate was prepared in the same way as sulfochenodeoxycholyltaurine. It was conjugated with [1,2-^{14}C]taurine (3.4 GBq/mmol) and kindly supplied by Dr. J. Zhang. Scymnol sulfate from the Haslewood Collection was kindly donated by Dr. A. R. Tammar.

Sorbents

ODS-silica was from two sources: Analytichem International (Harbor City, CA; Sepralyte and Bondesil) and Waters Associates, Inc. (Milford, MA; Preparative C18). Beds of the silica (200 mg, 40 × 4 mm) were washed with 5–10 ml each of methanol, chloroform–methanol 1:1 (v/v), methanol, and water prior to use. Lipidex-DEAP (Packard Instrument Co., Downers Grove, IL) in acetate form was used for separation of bile acid conjugates (2). Porapak Q and T (ethylvinylbenzene dimethylbenzene copolymers, 100–200 mesh, Waters Associates) were mixed in equal amounts and a 10 × 0.8 cm bed was left overnight in acetone and thoroughly washed with acetone, water, and 0.1 M HCl before use (8).

Abbreviations: ODS, octadecylsilane; FABMS, fast atom bombardment mass spectrometry; TEAS, triethylamine sulfate; GLC, gas–liquid chromatography.

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Fast atom bombardment mass spectrometry (FABMS)

FABMS was performed on a VG 7070E double focusing mass spectrometer equipped with FAB ion source, an Ion Tech atom gun, and a VG 11-250 data system (VG Analytical, Manchester, UK). Aliquots of the samples, corresponding to 50–650 μl of urine, were dissolved in 2–4 μl 70% aqueous methanol before application to the FAB probe. Glycerol was used as matrix and xenon (8 keV) as bombarding atoms. Negative-ion spectra were recorded between *m/z* 800 and 80 at a scan rate of 10 s per decade. The accelerating voltage was 6 kV and the resolution was about 1000 (5% valley). Semiquantitative estimates of bile acids were obtained from the registered intensities of relevant peaks. Exact mass was determined by the VG software peak-matching technique, using narrow and linear accelerating voltage scans covering the unknown peak bracketed by two reference peaks. The data system was operated in the multichannel analyzer mode and the resolution was 2000 (5% valley). The glucuronide of mono(2-ethylhexyl) phthalate ($[M-1]^-$ = 453.1761 Da, (9)) and scymnol sulfate ($[M-1]^-$ = 547.2941 Da) were used to produce the reference peaks.

Gas–liquid chromatography (GLC)

GLC was performed as previously described using fused silica capillary columns coated either with a nonpolar or a polar stationary phase (5).

Subjects

Urine from a 3-month-old boy with neonatal cholestasis of unknown etiology was used to study the extraction of a variety of endogenous bile acids. Recoveries of added sulfated taurine conjugates were studied using urine from a healthy man. Two healthy male infants were investigated at day 4 and at 1 month, respectively. The urine samples were stored at −20°C with no additives.

Analytical procedure

Ten-ml portions of urine were extracted with two types of ODS-silica (Sepralyte and Preparative C18) under four different conditions (I–IV).

I. Urine was diluted with an equal volume of 0.2 M acetate buffer at pH 7 and passed through the ODS-silica at a flow rate of about 1 ml/min, followed by 2 ml of water, which was added to the sample volume.

II. Urine was diluted with an equal volume of water, acidified to pH 3 with 4 M hydrochloric acid and passed through the sorbent followed by 2 ml 0.01 M HCl, which was added to the sample volume.

III. Urine was diluted with an equal volume of water, brought to pH 9 with concentrated (25%) ammonium hydroxide, and passed through the sorbent followed by 2 ml of water, which was added to the sample volume.

IV. Urine was diluted with an equal volume of 0.5 M TEAS and passed through the sorbent followed by 1 ml of 0.25 M TEAS and 2 ml water, which was added to the sample volume.

In all cases, the sorbent beds were washed with two 3-ml portions of water which were freeze-dried separately. Bile acids were then eluted first with 5 ml of methanol, then with 5 ml of methanol–conc. ammonium hydroxide 100:1, and finally with 3 ml of isopropanol. The separate eluates were taken to dryness. The residues of washes and eluates were dissolved in 70% aqueous methanol for FABMS analyses. The sample volumes were acidified and passed through the Porapak bed, which was eluted with acetonitrile and acetone (8). The two fractions were taken to dryness and dissolved in 70% aqueous methanol for FABMS analysis.

RESULTS

To determine the optimal conditions for quantitative extraction and elution, urine was passed through ODS-silica at pH 3, 7, or 9 (2), or after addition of an equal volume of 0.5 M TEAS (6). After washing, elution was performed with methanol, methanol–ammonia (2), and isopropanol. FABMS analyses showed the presence of bile acids in the water washes and in the methanol and methanol–ammonia eluates. The isopropanol fractions and the sample volumes extracted with Porapak did not contain detectable amounts of bile acids. The distribution of bile acids among the fractions differed depending on the conditions of extraction, most notably for a compound giving an ion at *m/z* 498, corresponding to the pseudomolecular anion of dihydroxycholanoyltaurine. Extraction at pH 3 yielded only 35–80% of this compound in the methanol eluate. After sorption at pH 7, 87–93% appeared in this fraction and the remainder was eluted with methanol–ammonia. The relative intensity of *m/z* 498 in the spectrum of the methanol eluate was lower after sorption at pH 9 than after sorption at pH 7. However, the methanol–ammonia eluate in this case only gave peaks of low intensities at *m/z* 528, 624, 611, and 627, corresponding to pseudomolecular anions of sulfated and glucuronidated dihydroxycholanoylglycines and glucuronidated cholestanetetrol and -pentol (10), respectively. When TEAS was added to urine prior to extraction, all bile acids were obtained in the methanol fraction and the relative intensity of *m/z* 498 was high. **Figure 1** shows a comparison of the negative ion FAB spectra of methanol eluates collected after extraction at pH 3 (Fig. 1A) or with TEAS (Fig. 1D).

The nature of the compound(s) giving the ion at *m/z* 498 was studied using material eluted with methanol–ammonia after extraction at pH 3. The base peak in the negative ion FAB spectrum was at *m/z* 498 (Fig. 1B). Exact mass determination gave a value of 498.2904, consistent with the anion of dihydroxycholanoyltaurine (498.2889, calculated

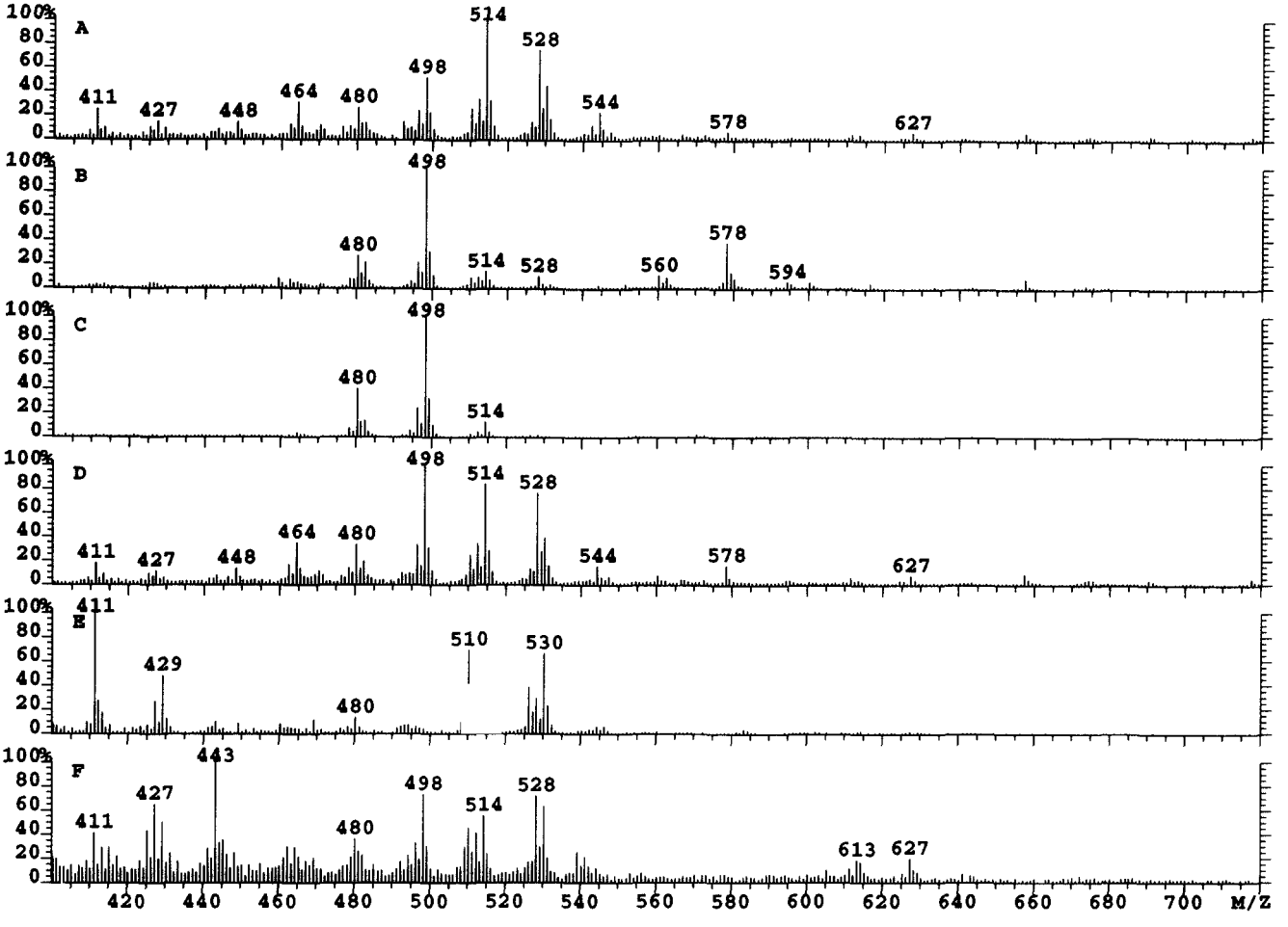

Fig. 1. The high mass regions of the negative ion FAB spectra of the methanol eluate (A) and the subsequent methanol/ammonia eluate (B) after ODS-silica extraction of urine from a cholestatic infant at pH 3. The spectrum of the methanol eluate after extraction in the presence of 0.25 M TEAS is shown in (D) and the spectrum of the taurine-conjugate fraction obtained after solvolysis and group fractionation of the methanol/ammonia fraction (B) in (C). Samples corresponding to 50 μl of urine were applied on the probe. (E) and (F) show the spectra of extracts of urine from a male infant at 4 days (E) and from another at 1 month (F). Samples corresponding to 100 and 650 μl of urine, respectively, were applied on the probe. The peaks at *m/z* 411, 427, 429, and 443 correspond to pseudomolecular ions of C_{21} steroid sulfates (25), those at *m/z* 448 and 464 to dihydroxy- and trihydroxycholanoylglycine, respectively, that at *m/z* 544 to sulfated trihydroxycholanoylglycine, and those at *m/z* 627 and 613 to glucuronides of cholestanepentol and 26-norcholestanepentol, respectively (10). Other peaks are mentioned in the text.

for $C_{26}H_{44}NO_6S$). The peaks at *m/z* 562, 578, and 594 correspond to the anions of sulfated mono-, di-, and trihydroxycholanoyltaurines, respectively, and the peaks at *m/z* 560 and 480 to the anions of sulfated and nonsulfated monohydroxycholenoyltaurines, respectively. After group separation of conjugates on Lipidex-DEAP, the same ions appeared with the same relative intensities in the spectrum of the sulfate fraction, indicating that the ions at *m/z* 480, 482, 498, and 514 were fragment ions of sulfated hydroxycholanoyltaurines which are known to lose SO_3 (80 Da) in the ionization process (11). Solvolysis (12) of an aliquot of the methanol–ammonia fraction yielded a series of compounds eluted as taurine conjugates from Lipidex-DEAP and giving a spectrum (Fig. 1C) consistent with the presence of di- and trihydroxycholanoyltaurines and monohydroxycholenoyltaurine. A minor peak at *m/z* 482 corresponds to monohydroxycholanoyltaurine.

Hydrolysis of another aliquot with cholylglycine hydrolase (13) yielded di- and trihydroxycholanoyl and monohydroxycholenoyl sulfates as shown by a major peak at *m/z* 471 and minor peaks at *m/z* 487 and 453 in the FAB spectrum. After solvolysis, the FAB spectrum showed an intense ion at *m/z* 391, corresponding to the pseudomolecular ion of an unconjugated dihydroxy bile acid. This was identified as chenodeoxycholic acid by GLC of the methyl ester TMS ether derivative. This analysis also showed the presence of 3β-hydroxy-5-cholenoic acid.

These results showed that sulfated taurine conjugates were the major bile acids lost in the conventional extraction procedure. Therefore, the recovery of synthetic sulfo-

chenodeoxycholyltaurine was investigated. When 50 μg of this compound was extracted with ODS-silica from 10 ml acetate buffer, pH 7, 97–98% was recovered in the methanol eluate and only 2–3% in the methanol–ammonia fraction. When the same experiment was performed with urine, pH 7, instead of buffer, 39% of the conjugate required methanol–ammonia for elution. The spectrum of the latter fraction also showed peaks of low intensities at *m/z* 608 and 624, corresponding to glucuronides of mono- and dihydroxycholanoylglycines, and at *m/z* 567 and 583, corresponding to glucuronides of di- and trihydroxycholanoates. When disulfochenodeoxycholyltaurine was extracted, none of the compound was desorbed with methanol and addition of ammonia was required for elution. When tracer amounts of sulfochenodeoxycholyl[1,2-^{14}C]taurine were added to urine, extractions at pH 3 and 7 yielded 76–85% and 89–95%, respectively, in the methanol eluate. Remaining radioactivity could be eluted with methanol–ammonia.

In the presence of 0.25 M TEAS at pH 7, sulfochenodeoxycholyltaurine was quantitatively recovered in the methanol eluate and ammonia was not required for desorption. This was the case both for endogenous bile acids in urine from the cholestatic infant and for unlabeled or labeled compound added to this and normal urine. When different batches of Sepralyte/Bondesil and Preparative C18 were compared, some loss of endogenous sulfochenodeoxycholyltaurine in the water washes (10–20%) was observed with two batches (one of each brand). The bile acids extracted in the presence of TEAS were fractionated on Lipidex-DEAP. The main part of the compounds giving ions at *m/z* 498 and 480 appeared in the sulfate fraction which also showed the expected ions at *m/z* 578 and 560. The peak at *m/z* 514 was small and most of the compound(s) producing this ion was found in the taurine-conjugate fraction. This is consistent with the known predominance in urine of sulfated mono- and dihydroxy bile acids and nonsulfated trihydroxy acids (2).

The extraction technique with TEAS was applied to the FABMS analysis of urinary bile acids from two healthy infants. The sample from the 4-day-old boy gave abundant ions at *m/z* 510 and 530, representing dihydroxy-oxocholenoyl- and tetrahydroxycholanoyltaurine, respectively (Fig. 1E). An ion corresponding to trihydroxy-oxocholenoyltaurine is also seen (*m/z* 526) (14). A peak is present at *m/z* 480 but none at *m/z* 498. After group separation, the ions at *m/z* 510, 526, and 530 appeared in the spectrum of the taurine-conjugate fraction and that at *m/z* 480 in the spectrum of the sulfate fraction. The sample from the 1-month-old healthy boy showed abundant ions at *m/z* 498 and 528 (Fig. 1F). After group separation, these ions appeared in the spectrum of the sulfate fraction together with the ion at *m/z* 480. GLC analysis of this fraction after solvolysis and hydrolysis revealed the presence of large amounts of chenodeoxycholic acid.

DISCUSSION

This study shows that the conditions used in common procedures for solid-phase extraction of urinary bile acids with octadecylsilane-bonded silica (1) can lead to selective losses of bile acids. This was revealed by comprehensive FABMS analyses of fractions collected during the extraction procedure. While it is well established that FABMS is not a quantitative method (11), conditions can be selected that yield semiquantitative results (11). We recorded multiple spectra over an extended time period and ascertained that major changes in relative intensities did not occur (data not presented). However, quenching effects may differ between the fractions and yields of added ^{14}C-labeled sulfochenodeoxycholyltaurine were somewhat higher than when FABMS was used as analytical method. This difference could also be due to the use of different batches of ODS-silica. Regardless of the quantitative accuracy and reproducibility of the results, it is obvious that bile acids were found in fractions that are not collected in the conventional extraction procedures and will therefore be lost. The major loss was due to incomplete elution from the sorbent by methanol, particularly when the sample was applied at an acidic pH. Quantitative extraction and elution of the double conjugates was achieved after dilution of the urine with a solution of TEAS. This has previously been found to be essential for quantitative extraction and elution of unconjugated and conjugated steroids and bile acids in plasma (6, 15, 16). In this case, the extraction is carried out at 64°C to minimize nonionic binding to proteins (18). Many extraction procedures are presently used without consideration of these factors (1). This may explain the results of Nuber, Maucher, and Stange (19) who reported considerable losses, also of common bile acids, when they used published procedures for solid-phase extraction of bile acids in plasma.

Our study has shown that losses can also occur in the absence of protein in extractions of urine. These losses are selective for bile acids conjugated with both taurine and sulfuric acid. However, loss of other double conjugates was also observed, e.g., sulfated (*m/z* 528, Fig. 1B) and glucuronidated (*m/z* 608 and 624) glycine conjugates. Losses of glucuronides of nonamidated bile acids (*m/z* 567 and 583) and bile alcohols (*m/z* 611 and 627) were also noted.

The mechanisms behind the observed losses are not known. The results show that sorption and elution of bile acids depend on structure, pH, and counter-ions. These factors were previously found to be important in extractions with Amberlite XAD-2 (1, 2, 6). Analogous losses were observed with Amberlite XAD-2 and found to be due to the presence of ion-exchanging groups on the sorbent (see ref. 6). Batch variations and time-related changes in properties of Amberlite XAD-2 have been ob-

served and are discussed in ref. 1. While some problems can be overcome by appropriate choice of pH for sorption and desorption, this is more difficult with ODS-silica which is less stable and may lose bonded phase at high or low pH (1, 20). The addition of TEAS results in mild conditions but it is advisable to check the batch of ODS-silica to be used, using either FABMS or addition of a wide range of radiolabeled bile acids to monitor recoveries. Size exclusion chromatography is a possible alternative but more time-consuming method (19) and it has not been evaluated with respect to double conjugates that may be sorbed by ionic interactions with charged groups known to be present in Sephadex gels (21).

Samples from two healthy infants of different age were analyzed using the extraction method with TEAS. The FABMS spectra confirmed the previous conclusion that taurine-conjugated bile acid sulfates are quantitatively important constituents in urine from infants, except during the first days of life (4, 14). Thus, previous studies using uncontrolled extraction methods have probably not resulted in major errors in the evaluation of bile acid composition in the urine of infants (4, 14, 22–24).

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