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Quantitation of free sulfate and total sulfoesters in human breast milk by ion chromatography

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SUMMARY

A method for the assay of free sulfate and total sulfoesters in human breast milk by ion chromatography is described. After incubation in 1 *M* hydrochloric acid at 95°C for 90 min, hydrolytic cleavage of sulfoester standards was essentially complete. The increase in free sulfate after hydrolysis was used as a measure of total acid-labile sulfoesters. We found that this fraction [222 ± 16 μmol/l (mean ± standard error), $n=29$] comprised 87% of the total sulfate in mature milk. Free sulfate (35 ± 3 μmol/l) therefore makes only a small contribution to the total sulfate pool available to human infants.

INTRODUCTION

Breast milk is a complex biological fluid which provides a composite of nutritional, immunological and growth-promoting substances to the recipient infant. The links between the biochemistry of human milk and the physiological significance of some of the more recently identified components remain to be clarified [1,2]. One such constituent, inorganic sulfate, is essential for the biosynthesis of the various sulfoesters intrinsic to normal growth and development [3,4]. Free sulfate is usually derived from the enzymatic catabolism of sulfur amino acids. This is of particular significance in newborns since they may initially lack sufficient enzymatic activity to convert methionine to cysteine and then metabolize cysteine to taurine and sulfate. Hence, early in life, the ability of the infant to synthesize sulfoester molecules may be limited [4,5].

Sturman et al. [6] found very little ^{35}S-labelled inorganic sulfate in human or rat milk after administration of ^{35}S-labelled taurine to the mother. Instead,

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the label was found in the form of an acid-labile sulfate ester, N-acetylneuramin lactose sulfate, which was subsequently hydrolyzed in the stomach of the offspring. Significant amounts of sulfoester were also found in human milk, but these were not quantitated. In our hands, the determination of total sulfoesters in milk by assay of liberated sulfate was unreliable using the conventional chemical precipitation methods, presumably because of marked matrix effects. We have therefore adapted an ion chromatographic method [7,8] and quantitated, for the first time, the free and esterified sulfate fractions of human milk.

EXPERIMENTAL

Reagents

Sulfate ester standards were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade chemicals were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Acetonitrile (HPLC grade) was obtained from BDH (Toronto, Canada). $Na^{35}SO_4$ (> 10 Ci/mol) was obtained from New England Nuclear (Montreal, Canada).

Milk samples

Twenty-nine human milk samples representing a lactational stage between 15 and 46 days post-partum were collected from 21 mothers giving birth at Chedoke-McMaster Hospitals (Hamilton, Canada). Mothers were instructed to completely empty each breast four to six times per day after cleansing their breasts with sterile water and using breast pumps [washed with 10% (w/v) nitric acid and rinsed three times with deionized water] provided by the researchers. Each 24-h collection of milk was pooled, the volume measured and an aliquot removed for analysis and stored at −20 °C until analysis. A pool of expressed breast milk (PEBM) from several donors was used for method development and quality control.

Sample preparation

Breast milk samples were equilibrated for 1 h in a 37 °C water bath and stirred to emulsify the lipid and ensure a homogeneous preparation. Samples were then centrifuged at 2500 *g* for 30 min at 4 °C, allowing the lipid layer to separate from the aqueous infranatant. A 500-μl aliquot of the aqueous layer was extracted by needle aspiration, added to 500 μl of acetonitrile and vortex-mixed for 15 s. After centrifuging at 2500 *g* and 4 °C for 10 min, the protein-depleted supernatant was removed and re-centrifuged yielding a clear supernatant. An aliquot of the supernatant was diluted five-fold with distilled, deionized water (resistance > 10 MΩ/cm) prior to chromatography.

Recovery analysis

To assess recovery of free sulfate during sample preparation, radiolabelled sulfate was added to a sample of whole pooled milk. Three aliquots of this milk were removed and counted by liquid scintillation (Beckman Instruments, Toronto, Canada). The milk was then defatted and three aliquots of infranatant were removed and counted using Aquasol-2 scintillation fluid (New England Nuclear, Boston, MA, U.S.A.). Following acetonitrile deproteinization, a further three aliquots of supernatant were removed and counted.

Acid hydrolysis procedure

To determine the time required for hydrolysis, 300 μl of defatted, deproteinized sample were placed in a 2.0-ml vial with a sealed screw cap (Sarstedt Canada, St. Laurent, Canada). A 300-μl aliquot of 2.0 *M* hydrochloric acid was added and the mixture vortex-mixed for 15 s. The vials were then placed in a water bath at 95°C for 90 min. The resulting hydrolysate was allowed to cool to room temperature and then centrifuged at 2500 *g* and 4°C for 10 min. A clear brown supernatant was obtained and then filtered through a nylon high-performance liquid chromatography (HPLC) syringe filter (Micron Separators, Honeye Falls, NY, U.S.A.). The first three drops were discarded to eliminate any contamination from the filter devices. With a distilled, deionized water blank, this procedure yielded a flat chromatographic baseline. The filtrate was then diluted with 20 volumes distilled, deionized water and chromatographed. The time required for complete hydrolysis was investigated by assaying free sulfate in aliquots taken every 30 min over 3 h.

To test the efficacy of our hydrolysis procedure, standards consisting of sulfoesters of a variety of parent compounds were added to water and to breast milk and the recoveries determined.

Ion chromatography

Quantitation of free inorganic sulfate and acid-labile sulfate esters was carried out using the following instrument configuration: a Waters 590 solvent delivery system equipped with a Waters WISP 710B autosampler and a Waters 430 conductivity detector (Waters Chromatography Division, Millipore Canada, Mississauga, Canada). Separation of sulfate ions was carried out on AG-2 and AS-2 nitrate retaining columns (Dionex, Sunnyvale, CA, U.S.A.), as they provided the best resolution of the sulfate peak. The nitrate-retaining columns were protected by a Waters IC-PAK pre-column filter. Background conductivity of the eluent was reduced using a Dionex micro-membrane suppressor.

A 100-μl aliquot of diluted sample was injected onto the column. The system was run at a flow-rate of 2.00 ml/min using an eluent consisting of 3.0 m*M* $NaHCO_3$ and 2.4 m*M* Na_2CO_3. The conductometer was used at a sensitivity of

1 μS full scale. The micro-membrane suppressor was continuously regenerated with 13 mM sulfuric acid at a flow-rate of 4 ml/min.

To test whether there was any non-specific binding of sulfate to the ion chromatography columns, tracer amounts of $Na_2{}^{35}SO_4$ were added to a sample of human milk and the sample was chromatographed. Effluent fractions (approximately 1 ml) were collected by a Gilson FC-100 fraction collector (Mandel Scientific, Ville St. Pierre, Canada) over a period of 50 min and assayed by liquid scintillation counting.

RESULTS

Ion chromatography

Fig. 1 shows the chromatographic profiles for sulfate in authentic K_2SO_4 standard (A), sulfoester hydrolysate (B), breast milk (C) and hydrolyzed breast milk (D). In hydrolysates (B and D), there is excellent separation of

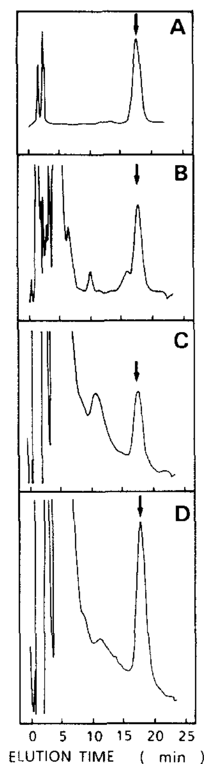

Fig. 1. Ion chromatography of sulfate and sulfoester hydrolysates. (A) Potassium sulfate (10 μmol/l) in aqueous solution. (B) Pooled expressed breast milk (1:10 dilution). (C) Ascorbic acid 2-sulfate (25 μmol/l) after hydrolysis. (D) PEBM after hydrolysis (1:80 dilution). The sulfate peaks are denoted by arrows.

the sulfate from the large chloride peak due to hydrochloric acid and there was good separation between the sulfate and other anionic components in unhydrolyzed milk (C).

The retention time for sulfate (17 min) was unaffected by sample matrix, as evidenced by identical times for elution in all samples. For human milk, however, other anions with earlier retention times contribute significant background conductivity, but the sulfate peak is well separated and readily quantitated in much the same way as similarly tested samples of saliva and sweat [7,9].

Identity of the sulfate peak was confirmed by examining the elution pattern of labelled sulfate added to milk. When the delay attributable to transit time from conductivity cell to fraction collector is taken into account, the elutions of label and conductivity are co-incident (Fig. 2). Summing of counts from all fifty fractions in each of three separate injections showed virtually complete recovery of the injected label (101.2±1.4%). Only a single radiolabelled peak was observed and there was no carry-over to subsequent injections.

Sample preparation

As shown in Table I, the method used for preparation of samples for ion chromatography was efficacious. Mean recoveries after defatting step were complete, and losses on deproteinization were insignificant.

Hydrolysis conditions

The increase in free sulfate due to hydrolysis of endogenous milk sulfoesters is shown in Fig. 3. Under the hydrolysis conditions we used, free sulfate con-

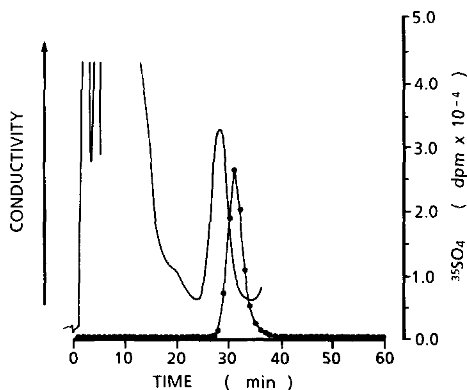

Fig. 2. Chromatogram of PEBM containing ^{35}S-labelled sulfate. A lower flow-rate (1 ml/min) was utilized for fraction collection, resulting in longer elution times. The single peak of radioactivity, as detected in the eluted fractions, was delayed relative to the conductivity peak by the time required for transit from conductivity cell to fraction collector.

TABLE I

RECOVERIES OF LABELLED SULFATE DURING SAMPLE PREPARATION

Sample	Added (mean ± standard error, $n=3$) (cpm)	Recovery (%)
Whole milk	15094 ± 401	—
Fat-free infranatant	16087 ± 161	106 ± 2.0
Acetonitrile extract	15824 ± 24	98.4 ± 0.6

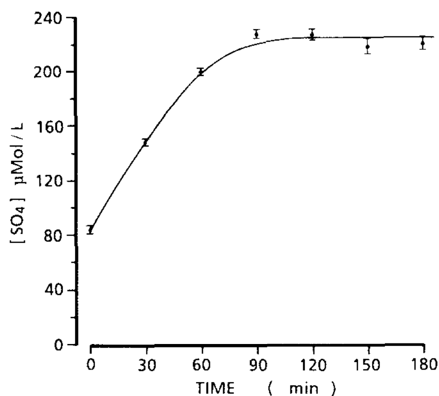

Fig. 3. Time course for PEBM hydrolysis. The value at zero time represents the initial free sulfate content. Mean ± standard error for three replicates is shown.

centrations were unchanged after 90 min. All subsequent analyses used a 90-min incubation time.

The recoveries of sulfate from various parent alcohols are shown in Table II. These compounds included carbohydrates, aliphatic and aromatic alcohols and hydroxysteroids, representing most of the common biological sulfoesters. Recoveries ranged from 91 to 99% in aqueous solutions – not significantly different from those obtained for milk matrix (87–101%). The microheterogeneity of chondroitin sulfate, a variable sulfated glycosaminoglycan, made it impossible to determine the sulfate content directly. However, based on an aqueous solution containing an estimated 50 mg of sulfate per l, the recovery from the milk pool was 105%.

Sulfate and sulfoester content of human milk

Precision estimates were derived from a single sample of pooled expressed breast milk containing 65 μmol/l free sulfate and 109 μmol/l total acid-labile sulfoester. The within-assay ($n=6$) and between-assay ($n=10$) coefficients

TABLE II

RECOVERIES OF SULFOESTER STANDARDS FROM AQUEOUS SOLUTION AND POOLED EXPRESSED BREAST MILK

Compound	Milk matrix			Aqueous matrix		
	Added (μmol)	Recovered (μmol)	Recovery (%)	Added (μmol)	Recovered (μmol)	Recovery (%)
L-Ascorbic acid 2-sulfate	0.733	0.693	94.5	7.33	6.98	95.2
α-D-Glucosamine 3-sulfate	0.484	0.457	94.4	24.2	23.1	95.2
N-Acetylglucosamine 3-sulfate	0.404	0.396	98.0	26.9	25.1	93.3
Ethanolamine O-sulfate	0.496	0.499	101	9.92	9.56	96.3
3-Indoxyl sulfate	0.400	0.395	98.8	4.00	3.81	95.3
Myoinositol hexasulfate	0.416	0.390	93.8	0.208	0.206	98.8
Cholesterol 3-sulfate	0.380	0.350	92.1	0.524	0.477	91.0
Lithocholic acid 2-sulfate	0.430	0.373	86.7	0.877	0.796	90.8

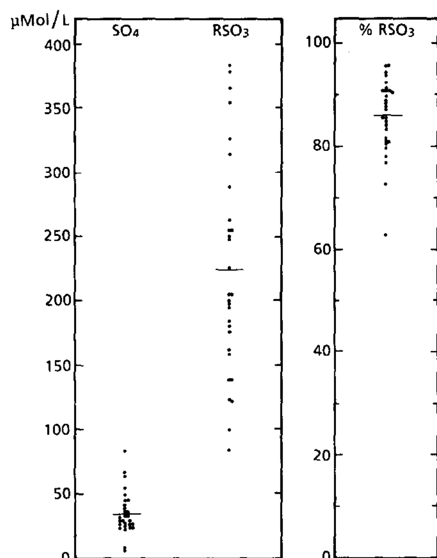

Fig. 4. Free and esterified sulfate concentrations in various samples of mature human milk. Means are indicated by horizontal lines.

of variation were 8 and 12% for free sulfate and 5 and 8% for total acid-labile sulfoesters respectively.

Mature milk samples collected from 21 different mothers were found to contain 34.7 ± 2.63 μmol/l (mean ± standard error) of free sulfate. The total acid-labile sulfoester content was more than five-fold higher (221.5 ± 15.8 μmol/l) and the sulfoester fraction made up 87.1 ± 8.0% of the total sulfate found in breast milk (Fig. 4).

DISCUSSION

The accurate detection and measurement of free inorganic sulfate in biological fluid has been a problem for many years [7,8]. The barium turbidimetric procedures have been widely used for assay of serum sulfate concentrations [10–12], but these methods and others employing benzidine precipitation are highly susceptible to matrix interferences [13–15]. Controlled-flow ion chromatography with conductometric detection [7,8,16] is an alternative method that appears ideally suited for sulfate determination in biological fluids. It has been successfully used in the assay of serum and other biological fluids, including cerebrospinal fluid, hepatic tissue extracts, salivary and sweat secretions [7,9,17,18].

Milk contains large proportions of lipid, protein and carbohydrate, and relatively higher concentrations of inorganic ions (such as calcium, magnesium and phosphate) than found in serum or other biological fluids – all of which complicate analysis by published chemical precipitation methods. Our previous experience with ion chromatography led us to expect that a defatting/deproteinizing step would permit accurate assay of sulfate in human milk without further preparation. Indeed, we found that removal of the lipid and protein yields a 'clean' sample with virtually no loss of analyte. Studies with ^{35}S-labelled sulfate showed further that the label co-eluted with the sulfate standard and there was no loss of label during chromatography.

This method for determination of free sulfate is not markedly different from that we reported for other biological fluids [7,9]. However, correspondingly reliable methods for sulfoester determination have not been described. Assays for sulfoester sulfate liberated by acid hydrolysis have used a polyethylene glycol stabilizer and a preformed barium sulfate seed and produce satisfactory results in large urine samples [19,20]. A barium radiometric assay has been adequate for small volumes of urine in our hands [5], but the limited sensitivity and specificity of the barium precipitation assay for low sulfate concentrations in complex matrices is even more limiting in these procedures. On the other hand, ion chromatography has been used to measure trace anion in hydrolysates containing comparative large amounts of acid and conjugate anion (e.g. hydrochloric acid, nitric acid) [21,22]. Moreover, the concentrations of acid we used for hydrolysis (0.1–1.0 *M* hydrochloric acid) are such that tailing of chloride into the sulfate peak causes only minimal interference (Fig. 1).

It has been suggested that hydrolysis of sulfate esters by chemical means is an unsatisfactory method of measuring sulfoesters due to the resistance of some compounds to the usual acid mixtures [23]. This is not likely to be significant in our milk analysis for several reasons. First, the sulfate moiety of the physiologically significant sulfoester found in milk is attached to the C-6 hydroxyl of galactose – a bond that is notably acid-labile [6,24]. Second, our recovery of sulfate from various standards representing these and other classes

of sulfoesters was better than 90%. Finally, it is unlikely that the sulfate esters that are mildly resistant to acid hydrolysis, i.e. certain steroid and alkyl sulfates [25], make up a significant proportion of esters in milk. It has also been suggested that the subtraction method for RSO_3 determination is unacceptable due to the large amounts of endogenous sulfate present in the matrix [23].

Our results show that the esterified fraction is far greater than the free fraction in human milk. The average free sulfate concentration of 35 ± 3 μmol/l (mean ± standard error, $n = 29$) is less than 15% of that found in adult serum (299 ± 25 μmol/l, $n = 17$ [9]) and only one half of that in human saliva (72 ± 4 μmol/l, $n = 17$ [9]). It is therefore unlikely that free sulfate could be quantitated by any of the barium methods, whose lower limit of sensitivity is about 100 μmol/l [12]. Acid-labile sulfoester concentrations ranged from 84 to 378 μmol/l (mean, 222 ± 16 μmol/l), constituting 87 ± 2% of the total sulfate.

In conclusion, ion chromatography is a satisfactory method for the analysis of sulfate and sulfoesters in breast milk. Results of our preliminary survey indicate that there is very little free sulfate present in milk. As suggested by Sturman et al. [6], the amount of sulfate available through hydrolysis in the infants's gastrointestinal tract is potentially much larger but the physiological significance of this source of sulfate in infancy requires further study.

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