Determination of lipid-bound sulfate by ion chromatography and its application to quantification of sulfolipids from kidneys of various mammalian species

Keiko Tadano-Aritomi,* Toshiyuki Hikita,* Atsushi Suzuki,† Hidenao Toyoda,† Toshihiko Toida,† Toshio Imanari,† and Ineo Ishizuka[1,*]

Department of Biochemistry,* Teikyo University School of Medicine, Kaga 2-11-1, Itabashi-ku, Tokyo 173-8605, Japan; and Faculty of Pharmaceutical Sciences,† Chiba University, 1-33 Yayoi, Inage, Chiba 263-8522, Japan

Abstract **A variety of procedures have been developed for determining the sulfate ester content of various biomolecules. Ion chromatography (IC), that is, quantitation of ionic substances by ion conductimetry after separation by anion-exchange chromatography, has been increasingly utilized for the determination of inorganic sulfate in clinical and environmental samples. We adopted suppressed-mode IC to the determination of lipid- or glycolipid-bound sulfate released by acid hydrolysis and found that it has the advantage of increased precision for wide concentration ranges (30 pmol to ~μmol) and lack of interference from other lipids. To minimize deterioration of the separation column, the lipophilic constituents in the acid hydrolysate were removed by a two-phase partition system of chloroform-methanol-water. The inorganic sulfate was quantitatively extracted into the aqueous phase by replacing water with an alkaline buffer. By this method, the concentration of sulfolipids was determined in the kidney of mammals with various body mass. Sulfolipids were more concentrated in the kidney of smaller animals, which have higher maximum urine concentrating activity per gram of body mass, supporting the hypothesis of the function of sulfolipids as an ion barrier on the luminal surface of renal tubules.—** Tadano-Aritomi, K., T. Hikita, A. Suzuki, H. Toyoda, T. Toida, T. Imanari, and I. Ishizuka. **Determination of lipid-bound sulfate by ion chromatography and its application to quantification of sulfolipids from kidneys of various mammalian species. *J. Lipid Res.* 2001.** 42: **1604–1608.**

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Sulfoglycolipids[2] have attracted interest as a potential participant in the ion barrier on the cell surface (1), and they are present abundantly in the brain, testis, and kidney of mammals. Whereas the brain and testis mainly express GalCer I^3-sulfate (SM4s) and Galβ-3alkylacylGro-I^3-sulfate (seminolipid, SM4g) (2, 3), respectively, our studies have shown that rat kidney contains 11 species of sulfoglycolipids that belong to the ganglio- series (4–6) and the isoglobo- series (7, 8). Recently, it was reported that mutant mice lacking SM4s due to deficient synthesis of its precursor, GalCer, showed increased expression of more polar sulfoglycolipids in their kidneys, although some common parameters related to renal function were in the normal range (9).

To elucidate the roles that each sulfolipid plays in vivo, a simple and reliable method applicable to quantitative determination of various sulfolipids is required. Kean (10) reported a rapid nonhydrolytic method for the quantitative determination of SM4s based on the ability of its sulfate ester to form a blue ion pair with a cationic dye, azure A, which is extractable into organic phase. Although variants of Kean's method have been developed for determination of SM4s (11) and more polar sulfoglycolipids (12), they are not applicable to sulfolipids in general without the use of relevant standard compounds because the color yield depends on the nonpolar nature of the whole molecule.

In principle, determination of inorganic sulfate liberated after acid hydrolysis of sulfolipids can be a method of choice when hydrolysis is complete, and conventionally, it was determined colorimetrically, for example, by chelating barium ions with rhodizonate (13, 14). Compared with conventional methods, ion chromatography (IC) has demonstrated increased specificity and sensitivity as well as the inherent capacity for simultaneous determination of various anions, and it has been applied to samples from biological, environmental, and industrial origin (15). Re-

[2] Abbreviations for sulfoglycolipids follow the modifications of the Svennerholm system (*J. Neurochem.* 10: 613–623, 1963), and the designation of the other glycolipids follows the IUPAC-IUB recommendations (*Eur. J. Biochem.* 257: 293–298, 1998).

Abbreviations: HSO_3-Chol, cholesterol 3-sulfate; IC, ion chromatography; SB1a, Gg_4Cer II^3,IV^3-bis-sulfate, (HSO_3-3)Galβ3GalNAcβ4(HSO_3-3)Galβ4GlcCer; SM2a, Gg_3Cer II^3-sulfate, GalNAcβ4(HSO_3-3)Galβ4GlcCer; SM3, lactosylceramide sulfate (lactosylsulfatide), LacCer II^3-sulfate; SM4s, galactosylceramide sulfate (galactosylsulfatide), GalCer I^3-sulfate.

[1] To whom correspondence should be addressed.
e-mail: ii@med.teikyo-u.ac.jp

cently, the sulfate content in glycoproteins or glycosaminoglycans has been successfully determined by IC (16, 17). In the present study, we extended suppressed-mode IC to the detemination of lipid- or glycolipid-bound sulfate after quantitative liberation from parent compounds. By this method, the concentrations of sulfolipids in the kidney of various mammals were determined. The unexpected behavior of inorganic sulfate in chloroform-methanol-water partition systems was also discussed.

MATERIALS AND METHODS

Materials

Analytical grade chemicals from various suppliers were used throughout this work. High-purity water was prepared by a Puric water purification system (Organo). Stock solutions (10 μmol/ml) of inorganic anions, chloride (Cl^-), fluoride (F^-), bromide (Br^-), iodide (I^-), nitrate (NO_3^-), phosphate (PO_4^{2-}), and sulfate (SO_4^{2-}) were prepared from their analytical grade sodium or potassium salts by dissolving appropriate amounts in water.

SM4s, Gg_3Cer II^3-sulfate (SM2a), and Gg_4Cer II^3,IV^3-bis-sulfate (SB1a) were isolated from rat kidney (4, 5), and LacCer II^3-sulfate (SM3) was isolated from human kidney (18). Cholesterol 3-sulfate (sodium salt; HSO_3-Chol) was purchased from Sigma (St. Louis, MO). Bovine kidneys were collected at a local slaughterhouse. Dog, cat, and rabbit kidneys were kindly provided by J. Takaya (Radioisotope Research Center, Teikyo University School of Medicine), and guinea pig kidneys were provided by Dr. H. Tanaka (Toho University). These kidneys, together with those of mouse (C57BL) and rat (Wistar), were extracted with chloroform/methanol (19), and acidic lipids were eluted sequentially from a DEAE-Sephadex column by a concave gradient of ammonium acetate, as described previously (4–9), typically in the following order (concentration of ammonium acetate given): fraction 1, monosialosyl gangliosides (0.1 M); fraction 2, monosulfated glycolipids (0.4 M); fraction 3, disialosyl gangliosides (0.6 M); fraction 4, cholesterol sulfate (0.8 M); fraction 5, bis-sulfated glycolipids (1.7 M). The several minor monosulfated glycolipids, including GlcCer I^3-sulfate (20), SM3, and the isoglobo-series sulfoglycolipids (7, 8), were present in rat kidney and eluted simultaneously with SM4s in the monosulfated glycolipid fraction. However, their concentrations were <1% of SM4s (7, 8, 20), except for SM3 in mouse kidney, which accounted for ~10% of SM4s (9). Fractions 2, 4, and 5 containing SM4s+SM3, HSO_3-Chol, and SB1a, respectively, were used for analysis.

Assay procedure

Purified sulfolipids or acidic lipid fractions containing 1–10 nmol of sulfolipids were dissolved in 200 μl of 1.0 M HCl and heated at 100°C for 3.5 h. After the solvent was evaporated in a SpeedVac (Savant Instrument, Inc.), the residue was treated three times with 0.5 ml of water followed by evaporation in the SpeedVac to minimize residual HCl in the reaction mixture. The dried residue was alkalinized by adding 0.5 ml of sodium carbonate buffer containing 1.42 mM $NaHCO_3$ and 1.5 mM Na_2CO_3 (pH 7.8), vigorously mixed with 2.0 ml of chloroform/methanol (2:1, v/v) (21) and centrifuged at 3,000 rpm for 5 min. A 0.8-ml aliquot of the 1-ml upper phase was dried in the SpeedVac and redissolved in 50–100 μl of water, and a 10-μl portion was injected into the IC system.

Ion chromatography

Sulfolipids were determined as the sulfate liberated after acid hydrolysis by anion exchange HPLC, called ion chromatography (15). The system consists of a LC10AD double plunger pump, a sample autoinjector (model SIL-10 AXL), and a conductivity detector (model CDD-6A; Shimadzu, Kyoto, Japan). A TSK-gel IC-Anion PW (4.6 × 50 mm) separation column (TOSOH, Tokyo, Japan) was tandemly connected to a cation exchange Dowex 50WX8 (4.6 × 150 mm, 200–400 mesh) suppresser column to reduce the conductivity of the eluent. The system was preequilibrated with sodium carbonate buffer containing 1.42 mM $NaHCO_3$ and 1.5 mM Na_2CO_3 (pH 7.8), and the same buffer was used as the mobile phase at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Optimization of conditions

Sulfolipid sulfates have usually been liberated by acid hydrolysis or solvolysis (1). When several sulfolipids, including SM4s, SM4g, SM2a, SB1a, and HSO_3-Chol, were treated with 1 M HCl at 100°C, liberation of sulfates was complete within 1–3 h (data not shown). Thus, treatment with 1 M HCl at 100°C for 3.5 h was adopted for hydrolysis.

Originally, ion chromatography was developed for the determination of various ions in hydrophilic materials. To apply this method successfully to amphipathic compounds, the lipophilic constituents released by hydrolysis should be removed before analysis, because hydrophobic compounds are retained by the separation column and could result in deterioration of resolution and reproducibility. For this purpose, the acid hydrolysate was partitioned in the two-phase system of Folch, Lees, and Sloane Stanley (21) after removal of HCl by evaporation. Unexpectedly, recoveries of sulfate ion from the first upper phase of the Folch partition was variable, and a considerable amount of sulfate was recovered from the second upper phase.

As the control for correcting recovery, several anions, which do not occur naturally, were added to each sample. Among them, fluoride and bromide eluted too early to be separated from a faster eluting chloride peak derived from HCl. Iodide had a retention time similar to that of sulfate. Nitrate showed an adequate retention time but behaved differently than sulfate in the partition system; whereas some sulfate always remained in the lower phase, almost all nitrate added was recovered from the first upper phase.

For the quantitative determination of lipid-bound sulfate without the use of internal standards, liberatead sulfate must be extracted completely into the upper phase. It has been reported that 97.6% of the inorganic sulfate liberated by hydrolysis of SM4s was extracted in the first two upper phases (22). Because the trace of residual HCl used for hydrolysis could possibly lower the pH of the partition system and consequently lower the recovery, the partition was tried under alkaline conditions. By adding NH_4OH to the partition system at a final concentration of ~1 M, the recovery was greatly increased to ~95%, although some tubes, for example, one out of three to four tubes, still gave unexpectedly low recoveries. The best recovery of ~98% was achieved with a mild alkaline buffer, 1.42 mM $NaHCO_3$/1.5 mM Na_2CO_3 (pH 7.8), which was used for the mobile phase as well, without causing interfering peaks on HPLC (**Fig. 1**). The recoveries were determined

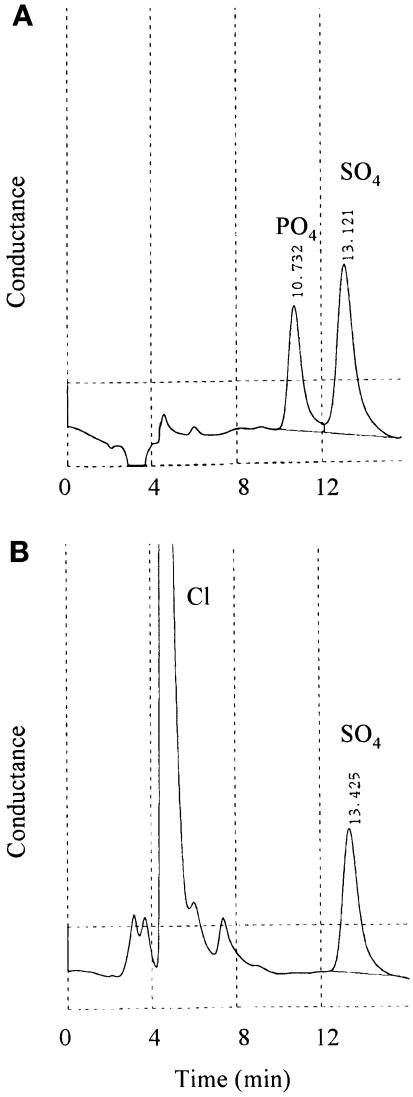

Fig. 1. Separation of anions by ion chromatography. A: A standard mixture of $NaHPO_4$ (1.0 nmol, 10.7 min) and Na_2SO_4 (0.5 nmol, 13.1 min). B: SM4s from bovine kidney containing 0.5 nmol of sulfate (13.4 min). The fractions from DEAE-Sephadex containing SM4s were pooled, and an aliquot was assayed by the present method.

on known amounts of purified SM4s added to the crude acidic lipid fractions from DEAE-Sephadex (**Table 1**). The precision of the method was established by six separate determinations using 1–10 nmol of purified sulfolipids (SM4s, SB1a, and HSO_3-Chol) as well as the acidic lipid fractions containing individual sulfolipid. The coefficients of variation were 0.5–2%. The detection limit was 0.03 nmol for sulfate (10 μl injection volume) at a signal-to-noise ratio of 3.

Although the behavior of the sulfate ion in the partition system was unexpected and could not be fully understood, the possibility is that the protonation of sulfate ions (23), which could occur under acidic conditions, may keep them in the organic phase.

Comparison of the IC method with the azure A method

Quantities of purified sulfolipids, SM4s, SM4g, SM3, SM2a, SB1a, and HSO_3-Chol, determined by the present method, were in good agreement with those obtained by the azure A method after acetylation (**Fig. 2**). The detection limit of the present method is ~10 times lower and the dynamic range is much wider (30 pmol to μmol) than those of the azure A method (0.5–10 nmol) (12).

The azure A assay is the method of choice for the determination of SM4s and SM4g owing to its sensitivity and simplicity. We have successfully applied this method to more polar sulfoglycolipids with two to five sugar residues by using peracetylated derivatives (12). However, sulfoglycolipids in crude lipid mixtures give erroneously higher optical densities because gangliosides and acidic phospholipids present in a large quantity interact, though much more weakly, with azure A. Moreover, an authentic standard is necessary for the exact determination of purified sulfolipids because color yields closely depend on the behavior of the dye-sulfolipid complex in the solvent phase

TABLE 1. Recovery of sulfate from the upper phase

SM4s Fraction (A)	SM4s Added (B)	Determined (C)	Recovery
	Sulfate (nmol)		%
6.1	0	6.1 ± 0.5[a]	
6.1	1.0	7.1 ± 0.6	103 ± 4[b]
6.1	5.0	11.0 ± 1.7	98 ± 4
6.1	25.0	30.7 ± 1.5	98 ± 2

The total lipid extract from bovine kidney was applied to a DEAE-Sephadex column, and the fractions containing SM4s were pooled. An aliquot (A) was mixed with the known amount of purified SM4s (B) and assayed by the present method (C).

[a] Values are the mean ± coefficient of variation of four separate experiments.

[b] Recovery = [(C − A)/B] × 100.

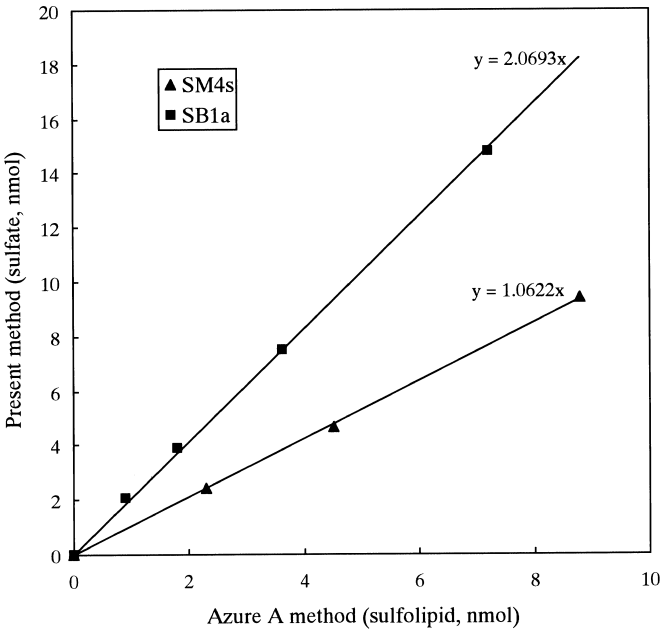

Fig. 2. Comparison of the quantitative assay using IC with the azure A method. Purified SM4s and SB1a were assayed as described in the text. Bis-sulfated glycolipid SB1a gave values approximately twice as high as monosulfated glycolipid SM4s. The coefficient of variation for four separate determinations was <4%.

partition. The present method, in contrast, can be a versatile method for the quantitative determination of lipid- or glycolipid-bound sulfate esters using sodium or potassium sulfate as the standard as long as each sulfolipid can be separated clearly from the others.

Determination of sulfolipids in the acidic lipid fractions of the kidney

The present method was applied to the quantification of sulfolipids from the kidneys of various mammals, using an aliquot of pooled fractions from DEAE-Sephadex (4–6) containing 1–10 nmol of sulfolipids (**Table 2**). Even the fractions containing a relatively large amount of phospholipids or other lipids produced no interfering peaks around the region of sulfate. When total lipid extracts were analyzed, however, sulfate peaks could not be separated from large amounts of faster eluting peaks. The blank values, obtained with samples before hydrolysis, usually yielded trace or no peaks, indicating that endogenous inorganic sulfate was negligible, at least in the acidic lipid fractions eluted from DEAE-Sephadex.

The concentrations of SM4s in bovine and rat kidneys determined by the present method were in good agreement with those reported previously (4, 20, 24), and the high concentration of HSO_3-Chol in the kidney of rabbit (25) was also confirmed. SB1a was detected only in rat and mouse kidneys (Table 2). The concentration of SB1a in rat kidney was in good agreement with our previous value determined by the azure A method (5).

The glycolipid-bound sulfate was determined in the kidney of several mammalian species. The body mass of these animals differs over 24,000-fold, ranging from 25 g (mouse) to 600 kg (bovine) (Table 2). As shown in **Fig. 3**, there was a significant negative correlation between body mass and the concentration of glycolipid sulfate: the smaller the animal, the higher the concentration, which was characterized by an allometoric scaling law (26, 27). These data confirmed our hypothesis that sulfated glycolipids, one of the components of ion barrier at the cell membrane on the luminal surface of the renal tubules, are more concentrated in the kidney of smaller animals, which have higher maximum urine concentrating activties per gram of body mass (1, 28). Why values for canine and rabbit kidneys, the only exceptions, deviated from the theory of allometry will be determined by future studies.

In the kidney of SM4s-deficient mice, the concentrations of SM3 and SB1a determined by the present method were increased, indicating upregulated biosynthesis of more polar sulfoglycolipids to compensate for the lack of SM4s (9). Although the functions of sulfolipids are not fully understood, their accurate determination in various mammalian tissues may provide the basis for clarifying the roles of sulfolipids in vivo.

TABLE 2. Concentrations of sulfolipids in the kidneys of various mammals

	Body Mass	Fractions from DEAE-Sephadex[a] SM4s[c]	HSO_3-Chol	SB1a	Total of Sulfoglycolipids[b]
	g	*Sulfate nmol/g wet tissue*			
Bovine	600,000	92	15	n.d.	92
Dog	25,000	271	9	n.d.	271
Rabbit	2,600	92	123	n.d	92
Cat	2,300	146	16	n.d.	146
Guinea pig	580	234	52	n.d.	234
Rat	250	188	64	15	203[d]
Mouse	25	275	51	20	295

n.d., not detected.

[a] Fractions 2, 4, and 5, containing SM4s+SM3, HSO_3-Chol, and SB1a, respectively, were used for analysis as described in Materials and Methods.

[b] The sum of SM4s, SM3, and SB1a.

[c] GlcCer I^3-sulfate (20), SM3, and other minor monosulfated glycolipids, if present, elute in this fraction (see Materials and Methods). The concentration of SM3 was <2 nmol/g in the kidneys of all animals tested except for mice, which contained 22 nmol/g by TLC densitometry after staining with azure A (9).

[d] Because rat kidney also contains SM2a (24 nmol/g) (4) and SB2 (22 nmol/g as sulfate) (29) as significant sulfoglycolipids, the total amount of glycolipid sulfate should be 249 nmol/g.

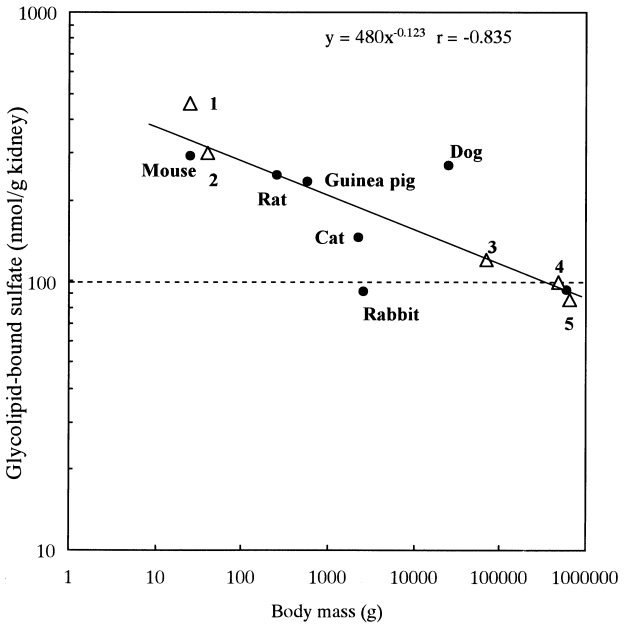

Fig. 3. Relation between the renal concentration of glycolipid-bound sulfate and the body mass of mammals. The total concentration of sulfoglycolipid sulfate in the kidney (nmol/g wet tissue) is plotted against body weight (g). Solid circle, the sum of sulfoglycolipids (without cholesterol sulfate) determined by the present method. Open triangle, data from previous papers; 1, mouse (C57BL/6J, 3 weeks) (30); 2, house musk shrew (*Suncus murinus*; 28); 3, human (31); 4, horse (32); 5, bovine (33).

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