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Short Communication

Tissue sulfate determination by ion chromatography

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ABSTRACT

The application of controlled-flow anion chromatography to assay inorganic sulfate in biological fluids and tissues is described. The eluent used in previous methods for analyzing sulfate in biological fluids has been modified by adding 4.5% acetonitrile to separate sulfate from a co-eluting peak. To markedly increase the life of the column, the tissue samples were further diluted, extracted with chloroform, and analyzed at a lower detection range (0.3 μS). The method has been shown to be applicable for determining sulfate in tissues as well as biological fluids.

INTRODUCTION

Intracellular sulfate is derived from several processes: (1) uptake of sulfate from the blood, (2) liberation of sulfate by sulfatase activity, (3) oxidation of sulfur-containing precursors to inorganic sulfate, and (4) lysosomal degradation of sulfated macromolecules [1]. The inorganic sulfate can be used as a precursor for synthesis of the co-substrate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), for sulfate conjugation.

PAPS is the co-substrate for a wide variety of sulfoconjugation reactions, including sulfation of various xenobiotics and many endogenous compounds such as neurotransmitters and steroid hormones. The regulation of *in vivo* sulfoconjugation is not well understood. However, after administration of high dosages of foreign compounds, it is suspected that the availability of the co-substrate, PAPS, is rate-limiting rather than the activity of the sulfotransferase. While energy is required for PAPS synthesis, the hepatic energy state does not appear to be limiting for the synthesis of the co-substrate *in vivo* [2,3]. However, it has been suggested that the availability of inorganic sulfate may be rate-limiting for PAPS formation in the liver and in other tissues [4–8]. This conclusion has arisen from the observation that high doses of drugs that are sulfated lower serum sulfate levels. However, it is not known if tissue sulfate decreases as does serum sulfate after high dosages of drugs that are sulfated. The main reason for the lack of this information is the unavailability of an analytical method for quantitating sulfate levels in the various tissues.

The traditional method for quantitating sulfate in biological fluids is turbidimetric [9]. However, Cole and Scriver [10] reported on a technique of controlled-flow anion chromatography, by which sulfate is separated from attendant anions and quantitated directly by electrical conductance. This method was shown to be superior for the quantitation of sulfate in biological fluids in that it was more specific, less variable, and more sensitive. While this method is good for biological fluids, we found it unacceptable for quantitating tissue sulfate, due to interfering peaks and short column life. Therefore, the pur-

pose of the present study was to modify the method of Cole and Scriver [10] so we could quantitate inorganic sulfate, not only in body fluids but also in tissues, in order that we might monitor the precursor of PAPS under various experimental conditions.

EXPERIMENTAL

Standards

Five-anion standard (fluoride, chloride, nitrate, phosphate, and sulfate) was obtained from Dionex (Sunnyvale, CA, USA). A 1 m*M* sodium sulfate (Sigma, St. Louis, MO, USA) stock solution was prepared in 1 m*M* sodium hydroxide solution. All solutions were prepared in distilled, deionized water (resistance $\geq$ 15 MΩ). Stock solution was stored in a refrigerator at 4°C and kept for a period of up to one week. Five working standard solutions with concentrations of 0.156, 0.313, 0.625, 1.25, and 2.5 nmol/ml were prepared by the appropriate dilution of the 1 m*M* sodium sulfate stock solution.

Sample preparation

Male Sprague–Dawley rats from Sasco (Omaha, NE, USA), weighing 250–350 g, were used for the experiments. Serum and bile samples were diluted 500-fold with 1 m*M* sodium hydroxide, and 100 μl of corresponding aliquots of diluted samples were injected onto the column. Urine was diluted 500- and 4000-fold with 1 m*M* sodium hydroxide, and a 100-μl aliquot was injected onto the column. Liver, kidney, lung, brain, and intestinal homogenates (25%) were prepared in 1 m*M* sodium hydroxide and centrifuged for 20 min at 11 000 *g*. The supernatant was centrifuged again for 60 min at 112 500 *g*. The supernatant from all tissues except brain was further diluted 100-fold, whereas that of brain sample was diluted 25-fold. The diluted supernatant was extracted with an equal volume of chloroform (+99.9%, Fisher Scientific, Fairlawn, NJ, USA) by vortex-mixing for 3 min with a multivortexer, and centrifuged for 10 min at 3000 *g*. A 100-μl volume of the top layer of each tissue supernatant was injected onto the column. The same procedure was employed for blanks (1 m*M* sodium hydroxide).

Ion chromatography system

The analysis was conducted on a Dionex BIOLC ion chromatography system. The system consisted of an eluent-degassing module, a conductivity detector with anion micromembrane suppressor (AMMS-1) and a gradient pump. The system and all containers were carefully cleaned with distilled, deionized water (resistance $\geq$ 15 MΩ) to minimize exogenous sulfate contamination. For anion analysis, an IonPAc AS4A anion-exchange column (25 cm $\times$ 4 mm I.D.) composed of 15 μm polystyrene–divinylbenzene substrate agglomerated with anion exchange latex was employed, protected by an AG4A guard column (Dionex). The aqueous eluent contained 1.8 m*M* Na_2CO_3, 1.7 m*M* $NaHCO_3$, and 4.5% acetonitrile. Sulfate was eluted isocratically at a flow-rate of 2 ml/min. The system was expanded with a Waters 700 Satellite WISP automatic injector (Waters Assoc., Boston, MA, USA). Data were obtained through System Interface Module (SIM) and Digital 380 computer using Waters 840 software.

Recovery study

To determine the recovery of sulfate from samples, an appropriate volume of 1 m*M* Na_2SO_4 stock solution was added to body fluids and tissues to increase the concentrations by 125, 250 and 500 μM.

RESULTS AND DISCUSSION

With this chromatographic system, fluoride eluted at approximately 1 min, nitrate at 3.2 min and phosphate at 5.4 min. Sulfate usually eluted between 6.5 and 7.5 min.

The sulfate standard curve was linear from 0 to 2.5 μM at the 0.3 μS range. The standard curve was reproducible from day to day, but was performed daily to ensure reproducibility and proper quantitation. When the system, containers and solutions were carefully cleaned and prepared with distilled, deionized water, peak area ($\times 10^{-3}$) of blank samples was less than 1000 at the sensitivity of 0.3 μS. The main source of exogenous sulfate contamination was tap water which contained 2.004 $\pm$ 0.335 m*M* sulfate (mean $\pm$ S.D. for fourteen tap water samples).

Fig. 1 illustrates representative chromatograms for sulfate analysis in serum, bile, urine, kidney, and liver. The previous chromatographic method for sulfate [10] was satisfactory in our hands for the quantitation of sulfate in biological fluids (serum, urine, and bile), but not for tissues (kidney and liver). In these tissues the sulfate peak and the peak immediately proceeding sulfate co-eluted. By altering the eluent composition (addition of 4.5% acetonitrile) we were able to adequately separate these two peaks and quantitate sulfate. Using these conditions, it has been possible to achieve baseline separations (Fig. 1). The reproducibility of this assay was good for both biological fluids and tissues.

The method was validated for serum, liver, kidney, and bile. A good linear relationship was obtained between the sulfate added and the sulfate concentration detected (Fig. 2). The recovery of known amounts of sulfate (Table I) ranged from 101 to 115% for serum, 98 to 110% for liver, 66 to 92% for kidney and 93 to 116% for bile.

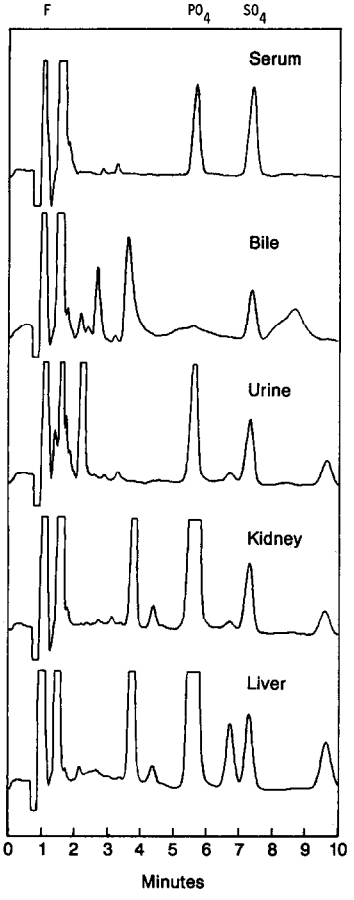

Fig. 1. Typical chromatograms of serum, bile, urine, kidney, and liver diluted 500-, 500-, 4000-, 400- and 400-fold, respectively, with 1 m*M* sodium hydroxide and measured by the conductivity detector at a sensitivity of 0.3 μS.

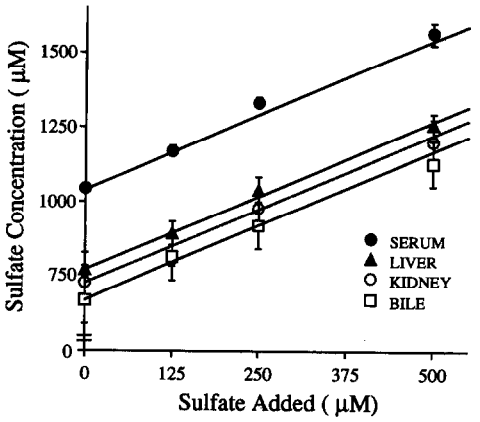

Fig. 2. Recovery of added sulfate to tissues and body fluids. Sulfate was added to increase the body fluids and tissues by 125, 250, and 500 μM. Each value represents the mean ± S.E. for four rats. Solid lines indicate expected concentrations.

Sulfate was originally analyzed at the sensitivity range of 3.0 μS. At this range the guard and analytical column became readily contaminated. The contamination was noted by a decrease in retention time. The column could not be regenerated using Dionex procedures (Document No. 032285). The decrease in retention time was marked with as few as 50 samples. The contamination was probably due to polynucleotides irreversibly binding to the column [11,12]. However,

TABLE I

RECOVERY OF ADDED SULFATE TO TISSUES AND BODY FLUIDS

Sulfate was added to increase the body fluids and tissues by 125, 250, and 500 μM.

Sample	Recovery (mean ± S.E., n = 4) (%)		
	125 μM	250 μM	500 μM
Serum	101 ± 16	115 ± 8.0	104 ± 8.0
Bile	116 ± 7	102 ± 7.0	93 ± 2.0
Liver	101 ± 18	110 ± 8.0	98 ± 6.0
Kidney	66 ± 36	92 ± 8.0	91 ± 7.0

TABLE II

TISSUE SULFATE CONCENTRATIONS IN RATS

Values are expressed as mean ± S.E. for six rats, except for five rats of kidney.

Organ	Sulfate concentration	
	nmol/ml or g tissue	% (compared to serum)
Serum	905 ± 72	100
Intestine	718 ± 36	79
Lung	585 ± 28	65
Liver	563 ± 19	62
Kidney	497 ± 33	55
Bile	343 ± 21	38
Brain	132 ± 8	15

using the sensitivity range of 0.3 μS, a ten-fold further dilution reduced the contaminants enough so that the guard column protected the analytical column. This further dilution, together with the chloroform extraction procedure and the guard column, protects the analytical column. More than 500 tissue samples can be injected into the column without significant alteration in performance. However, the guard column is changed after about every 200 injections.

Table II indicates the concentration of sulfate in various tissues of the control rat. The concentration of sulfate was highest in the serum. The concentration of sulfate in most tissues (liver, kidney, lung, and intestine) was 55–80% of that in serum. The concentration of sulfate in brain is only 15% of that in serum and the concentration in bile is about one third of that in serum.

In conclusion, the described method is the only verified method for the quantitation of tissue sulfate. Sample preparation is rapid and a large number of samples can be analyzed in a day. This improved analytical method should allow progress in the area of sulfate conjugation, due to the availability of a method for quantitating sulfate in tissues where sulfate conjugations occur.

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