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MICROASSAY OF INORGANIC SULFATE IN BIOLOGICAL FLUIDS BY CONTROLLED FLOW ANION CHROMATOGRAPHY*

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SUMMARY

The application of controlled flow anion chromatography to the assay of inorganic sulfate in biological fluids is described. The sulfate anion is separated from other anions by ion-exchange chromatography and quantitated conductimetrically. Coefficient of variance is 3.4%, about half that for the barium precipitation assay. Interference from heparin in plasma samples and unknown sources in tissue extract analysis is avoided. Sulfate levels in plasma are not different from those measured in serum after protein precipitation. Normal levels for sulfate concentration in human plasma, cerebrospinal fluid and hepatic tissue extract are reported.

INTRODUCTION

Inorganic sulfate is the substrate for a wide variety of sulfoconjugation reactions, including sulfation of glycosaminoglycans, steroids and drugs [1]. The degree to which homeostasis of inorganic sulfate is achieved and influences the in vivo rate of sulfoconjugation has not been determined. Following drug administration, large demands for inorganic sulfate may occur in liver, lung and intestine, and may exceed the capacity of local stores and local production of sulfate by sulfur amino acid oxidation [2—4]. Conversely, reduced ambient levels of inorganic sulfate may be rate-limiting with regard to sulfation of glycosaminoglycans and drugs [5, 6].

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The presence of specific, energy-requiring absorptive and reabsorptive transport mechanisms in gut and kidney respectively [7—9] provides further evidence that maintenance of adequate inorganic sulfate pools is necessary for homeostasis.

Measurements of free sulfate anion in biological fluids are essential to studies of sulfate metabolism. The anion has been measured by precipitation with barium or organic agents such as benzidine. Although we were able to adapt a radiolabelled barium assay to small volumes of serum [10] formidable difficulties arose when we attempted to apply these methods to tissue extracts, a problem noted by several previous investigators [11—13]. This stimulated us to look at an alternative method.

The technique of controlled flow anion chromatography, by which sulfate is separated from attendant anions and quantitated directly by electrical conductance, represents a new method for sulfate estimation in biological samples [14, 15]. It is inherently more accurate than previous methods and it requires only small sample volumes. When modified for use with biological fluids, this method is an effective analytic tool for determination of free sulfate anion in serum, urine, or cerebrospinal fluid (CSF).

The lack of interference from sulfated glycosaminoglycans and other compounds permits assay of 15—30 μl of heparinized plasma samples and allows accurate assay of tissue extracts. We report values for serum and plasma for humans, levels of sulfate in CSF from infants and children, and hepatic tissue concentrations in mouse and humans.

MATERIALS AND METHODS

Apparatus

The D-10 Ion Analyzer (Dionex, Sunnyvale, CA, U.S.A.) was outfitted with 3 × 50 mm concentrator precolumns containing Dionex anion separator resin®, a 3 × 500 mm anion separator column, also containing Dionex anion separator resin®, and a 6 × 250 mm anion suppressor column, in series. A standard eluent, consisting of 2.4 m*M* Na_2CO_3 and 3 m*M* $NaHCO_3$, was utilized and the flow-rate was adjusted to approximately 140 ml/h.

Sensitivity range of the conductance cell was set to 0—1 μS, which is sufficiently low to assure linear correlation between ion concentration and electrical conductance. Electrical output was fed to a linear strip chart recorder and peak height was estimated from the baseline drawn by free hand using vernier calipers.

Reagents

Reagent grade chemicals were purchased from Fisher Scientific (Montreal, Canada) and prepared in deionized water (resistance $\geq$ 10 MΩ). Disposable polyethylene tubes, syringes, and pipette tips were used, to minimize contamination by exogenous sulfate.

Biological samples

Blood samples, obtained at random by venipuncture from healthy ambulant adults and hospitalized children were centrifuged and the serums frozen at

—20°C until utilized in further studies. Heparinized plasma (100 μl) was obtained from premature infants between 28 and 36 weeks gestation (kindly provided by Dr. Stanley Zlotkin, Dept. of Nutrition, University of Toronto). Matching serum and CSF samples obtained from patients who required lumbar puncture were kindly provided by Dr. Claire Dupont, Department of Clinical Chemistry, Montreal Children's Hospital.

Serum samples from C57B1/6J adult mice were obtained at decapitation; blood was collected from the neck in serum separator tubes (Microtainer, Becton-Dickinson, Toronto, Canada) centrifuged and stored at 20°C until time of assay.

Preparation of sample for injection

Solutions of Na_2SO_4 diluted in 1.5 ml of deionized water were assayed at the beginning of each run to generate a standard curve. Biological samples were diluted with 1 m*M* NaOH to a final volume of 1—1.5 ml and a final sulfate concentration of 5—25 μ*M*.

Effect of other anions on sulfate peak

To evaluate possible interference from other anions, we determined the peak locations relative to sulfate (R_{SO_4}), and maximum conductances, at physiological serum concentrations, of seven other anions: bromide, urate, citrate, oxalate, ascorbate, malate and succinate. The anions, as sodium salts, were diluted with 1 m*M* NaOH, then chromatographed under standard conditions. The elution profiles of 1 m*M* dehydroepiandrosterone sulfate (DHEA-SO_4) and chondroitin sulfate (1%, w/v) were also determined.

Determination of sulfate by barium precipitation

Inorganic sulfate was measured in 22 serum samples by our modification of the radiolabelled barium method [10] and compared with results obtained on the same samples by controlled flow anion chromatography.

Assay of inorganic sulfate in tissue extracts

We studied liver because sulfate metabolism is prominent in this tissue. Livers were removed from C57B1/6J mice immediately after sacrifice, rinsed briefly with saline, blotted on filter paper, minced, and placed in a chilled Potter-Elvehjem homogenizer. Exactly four volumes of chilled 1 m*M* NaOH were added and the tissue homogenized with at least three strokes. The homogenate was then spun for 10 min at 2000 *g* and the supernatant transferred to tubes and centrifuged at 105,000 *g* for 60 min. Aliquots of the supernatant were diluted ten-fold with 1 m*M* NaOH.

Human liver samples were obtained from frozen autopsy material (courtesy of Dr. T.A. Seemayer and Dr. P. Hechtman). After thawing, they were processed as above with the exception that the final extract was diluted only five-fold prior to chromatography.

Determination of hepatic water spaces

Whole mouse livers were weighed after removal (as described above), then placed in a drying oven at 110°C for 24 h and reweighed to obtain tissue dry

weight and the value for total tissue water. The extracellular space was measured with [^{14}C]methoxyinulin (New England Nuclear, Boston, MA, U.S.A.). Animals were given inulin [0.1 ml/kg body weight as 10% solution (w/v) in saline] containing 10 μCi/ml tracer, at zero time by intraperitoneal injection; followed by subcutaneous injections of 5% inulin (containing 5 μCi/ml tracer) at 20 and 35 min. At about 1 h, when plasma inulin was shown to reach steady state, the animal was sacrificed, serum collected and the liver removed. Samples were counted after tissue solubilization with Protosol (New England Nuclear). Results were corrected for quenching. Intracellular water was calculated by subtracting the extracellular volume from total tissue water.

RESULTS

Sulfate standard calibration

Linear correlation of concentration and measured conductance was obtained in the range 0—25 μM with the sulfate standard. The correlation coefficient was greater than 0.99 in all experiments. The method assays absolute quantities of sulfate as low as 50 ng (ca. 0.5 nmol). Occasionally the regression may not pass through the origin, indicating a small amount of contaminating sulfate (always less than 5% of sample values); the latter may originate from surface adsorption of air-borne sulfate particulates.

The relative elution time during controlled flow elution anion chromato-

TABLE I

RELATIVE RETENTION TIMES AND CONDUCTANCES OF SOME PHYSIOLOGICAL ANIONS ASSAYED BY CONTROLLED FLOW ANION CHROMATOGRAPHY

Anion	R_{SO_4}*	Maximum conductance (μS × 10^3)**	Maximum physiological concentration*** (μmol/l)
Urate	—	0	481
Citrate	—	0	135
Succinate	0.52	18	5
Malate	0.54	2§	1
Phosphate	0.56	5960	3000
Bromide	0.65	104	166
Sulfate	1.00	1783	1000§§
Oxalate	1.15§§§	8	31
Ascorbate	1.21§§§	3	80

*Retention time relative to sulfate value of 1.00.

**Conductance for samples of standards at maximum physiological concentrations diluted as for human serum.

***Values for human serum, taken from ref. 16.

§With hemolysis, malate concentration could rise to 5 mmol/l; the corresponding conductance is given.

§§Taken from ref.17.

§§§At high, non-physiological concentrations, these substances emerge in the tail of the sulfate peak (e.g. in urine after ingestion of very large doses of ascorbic acid, unpublished observation).

graphy of seven anions encountered in physiological solution is shown in Table I. Fluoride (a calibration standard) co-elutes with organic anions (results not shown); iodide, another physiological anion, is present only in nanomolar amounts [16] and is therefore not detected by the system. Values for chloride are not shown, since the very large chloride peak is easily identified on all chromatographic runs (Fig. 1).

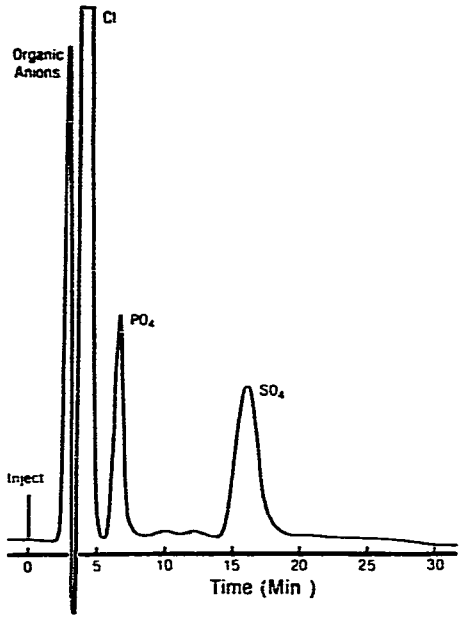

Fig. 1. Elution profile of human infant plasma. A 30-μl aliquot was diluted in 1.5 ml of 1 m*M* NaOH and the whole volume placed in the sample loop. A fraction (100 μl) is injected automatically from the sample loop to the column.

Anions eluting near sulfate do not interfere with its measurement. However, precipitating agents commonly used to remove protein from biological fluids, such as trichloroacetic acid, perchloric acid and sulfosalicylic acid, interfere with the elution profile. In fact, contamination of sulfosalicylic acid with free inorganic sulfate in various lots could be accurately monitored on the ion chromatograph. We omitted protein precipitation and utilized precolumns to prevent pollution of the main separator column and degradation of its resolving power. Precolumns were changed and cleaned after about ten runs (200 samples).

Sulfoesters comprise about 10% of total sulfate in serum, and are presumed to be stable at pH 11 (1 m*M* NaOH) and 24°C. We examined the sulfoesters, DHEA-SO_4 and chondroitin sulfate, and found that both yielded distinct but insignificant peaks co-chromatographing with inorganic sulfate (data not shown). No contamination of the inorganic sulfate elution region was detected at physiological concentrations of sulfoesters (less than 1% of standards chromatographed). We did not detect free sulfate or interfering material in the deionized water effluent from heparinized capillary tubes (Fisherbrand Red Tips) containing porcine intestinal ammonium heparin (2 USP per tube).

Elution profile for human plasma

A typical profile generated by application of dilute human plasma to the D-10 column is shown in Fig. 1. Separate phosphate and sulfate peaks are easily distinguished by their characteristic elution times. Sulfate concentration in heparinized blood plasma is no different from that measured in serum obtained from the same blood sample (data not shown).

Sulfate profiles for CSF, urine and tissue extract were essentially similar (data not shown) although tailing, due to extraneous unidentified compounds, was seen with some samples. Tailing does not effect sulfate quantitation by peak height. We demonstrated this by removing sulfate with excess barium and observing that the extraneous material did not co-eluate at the sulfate peak maximum, but eluated as a small amorphous peak in the tail of the sulfate peak.

Recovery of added sulfate

The average recovery of sodium sulfate added to serum, CSF and tissue extracts was complete (101 ± 3%, mean ± S.E.M., $n = 9$) within the range found in the physiological samples (Fig. 2). Cumulative retention of sulfate on the column during the daily analytical program was not observed.

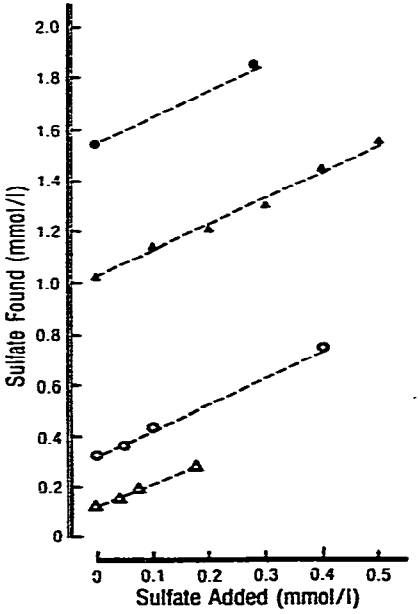

Fig. 2. Recovery of sulfate added to biological fluids. •, Mouse hepatic tissue extract; ▲, mouse serum; ○, human serum; △, human CSF. The dashed lines indicate expected values.

Comparison with barium precipitation method

Measurement of serum sulfate by anion chromatography correlated well with our barium-133 method (Fig. 3) [10] ($r = 0.87$, $p < 0.001$ and not different from unity, $y = 0.987X + 0.003$).

We measured 20 paired samples on different days by the two methods; coefficient of variance was 3.6%, by anion chromatography, and 7.4% for barium precipitation.

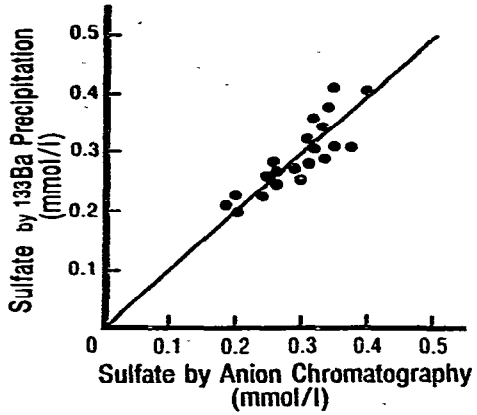

Fig. 3. Comparison of sulfate assayed by the barium-133 precipitation method [10] with assay by controlled flow anion chromatography in 22 serum samples. The line of identity is indicated.

Normal levels of sulfate in CSF and serum plasma

Sulfate concentrations in serum and CSF in man are indicated in Fig. 2, and described for serum and plasma in Table II. Sulfate levels in premature infants were significantly higher than in children and adults ($p < 0.05$, one-way analysis of variance). The data obtained by anion chromatography corroborate those reported earlier for the barium precipitation method [17]. Sulfate levels in CSF were about half those found in the matched serum sample. The difference between the concentrations in the two fluids is highly significant ($p < 0.0001$, paired t-test). Serum sulfate is higher in mouse than in man (Fig. 2) as reported earlier [17].

TABLE II

INORGANIC SULFATE IN HUMAN SERUM, PLASMA AND CEREBROSPINAL FLUID

Sample	n	Sulfate (mmol/l) (mean ± S.D.)
Plasma		
Premature newborns	7	0.41 ± 0.14
Serum		
Children	16	0.33 ± 0.12
Adults	16	0.30 ± 0.05
Cerebrospinal fluid		
Infants and children	9	0.16 ± 0.09
CSF:Serum ratio (matched samples)	9	0.46 ± 0.27

Sulfate concentration in liver

Dry weight of mouse liver was 33 ± 3% wet weight (mean ± S.E.M., $n = 11$); inulin space was 21 ± 6% and cytosol volume, 46 ± 7%. These are expected values. The mean serum sulfate concentration in mice fed normal diet was 1.19 ± 0.03 mmol/l (mean ± S.D.). The corresponding hepatic values ($n = 6$) were:

undiluted tissue extracts, 0.17 ± 0.01 mmol/l; cytosol concentration was 1.23 ± 0.06 mmol/l, or 104 ± 6% of serum. Human liver sulfate (average cytosol concentration) is 0.6 mmol/l (n = 2), or about two-fold greater than the corresponding serum concentration.

DISCUSSION

Controlled flow anion chromatography is a useful assay of free sulfate anion in biological fluids and tissues [15]. Recovery of sulfate at physiological concentrations is satisfactory and the coefficient of variance is half that of the radiolabelled barium method. Absence of interference by other anions and related compounds avoids the difficulties inherent in methods based on barium or benzidine precipitation. The possibility of incomplete precipitation at low sulfate concentrations [10] is avoided. Freedom from the heparin artefact with the chromatographic method permits assay of plasma. The ability to assay small volumes permits study of infants, various body fluids and tissue biopsy material.

We present previously unobtainable data for the premature human infant. Plasma sulfate corresponds to that predicted from our earlier studies [17]. We have also obtained preliminary data on inorganic sulfate in CSF in relation to serum of children. An earlier investigation, in adults only, used a modified benzidine technique [18]; serum levels in that study were unaccountably high.

Liver cytosol sulfate is equivalent to or exceeds extracellular sulfate. Mulder and Keulemans [2] reported that sulfate concentration, determined in rat liver cytosol by an isotope dilution method, was about 94% of the serum concentration, in close agreement with our findings in mouse. Human autopsy liver sulfate exceeds serum levels but we do not yet know whether this is an artefact of the postmortem preparation. Hepatocyte cytosol is normally —60 mV relative to the extracellular space and the Gibbs—Donnan equation predicts an equilibrium intracellular concentration about two orders of magnitude less than that of the extracellular fluid. The relatively high intracellular concentration of sulfate that we and others have found may be the result of active accumulation, compartmentation, binding, or an artefact of methodology. The first alternative is attractive although detailed studies carried out on Ehrlich ascites cells show that equilibrium exchange diffusion serves sulfate uptake and efflux in somatic cells [19, 20]. That tissue sulfate may be over-estimated because of methodological problems seems unlikely. There remains the intriguing possibility that sulfate is accumulated selectively in one intracellular compartment or another. Winters et al. [21] reported that mitochondria rapidly accumulate sulfate, and Crompton et al. [22] characterized the kinetics of transport into those organelles. Whether sufficient quantities could be sequestered by intracellular organelles or bound intracellularly at unspecified sites merits further investigation.

In summary, controlled flow anion chromatography allows accurate assay of inorganic sulfate anion. Analysis of tissue sulfate is also feasible. The method represents a distinct advance over all previous methods. It will allow investigation of sulfate homeostasis in various biological systems in health and disease.

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