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Evaluation of an Ion Chromatography Method for Quantitating Sulfate in Plasma, Serum and Urine

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Abstract

Sulfate is the fourth most abundant anion in human circulation and is essential for numerous metabolic and cellular functions. Remarkably, the analytical utility for measuring sulfate in clinical settings has not been reported, despite the physiological importance of sulfate being documented over the past several decades. In this study, we evaluated the utility of a previously described method for quantitating sulfate in human plasma, serum and urine in a clinical setting. Human serum, plasma and urine samples, and anion standards were analysed by ion chromatography using a hydroxide eluent generator coupled with suppressed electroconductivity detection. The method was validated using the following sulfate parameters: linearity of detection (0-1041 µmol/L) showing R^2=1.000; limit of detection 9 µmol/L; limit of quantification 27 µmol/L; within run imprecision of 0.8% for plasma and 5.0% for urine; between run imprecision of 6.1% for plasma and 4.3% for urine; mean analytical recoveries of 100-103%; inter-operator variability of 0.0-5.7%; stability of sulfate at room temperature for 1 day showing R^2=0.966 for serum and R^2=0.999 for urine; stability of sulfate in serum, plasma or urine at 4°C or -20°C up to 30 days showing R^2=0.985-0.999; comparison of plasma versus serum sulfate showing R^2=0.983-0.992; and robust lack of interference from other anions. The validated method provides capability for future clinical investigation of sulfate homeostasis in human health and disease.

Keywords: Sulfatemia; Sulfate assay; Ion chromatography

Abbreviations: CV_a: Coefficients of Variations; HREC: Human Research Ethics Committee; ICS: Ion Chromatography System; LOD: Limit of Detection; LOQ: Limit of Quantification; NSAIDS: Nonsteroidal Anti-Inflammatory Drugs; OMIM: Online Mendelian Inheritance in Man; QC: Quality Control; SD: Standard Deviation; SLC: Solute Linked Carrier; STD: Standard.

Introduction

Sulfate (SO_4^{2-}) is an important nutrient for human growth and development [1]. Sulfate conjugation (sulfonation) of glycosaminoglycans, such as heparan sulfate and chondroitin sulfate, is required to maintain the normal structure and function of tissues [2,3]. In addition, sulfonation detoxifies xenobiotics and certain pharmacological drugs, including acetaminophen [4,5]. Sulfonation also inactivates steroids and thyroid hormone, and contributes to the biotransformation of bile acids, catecholamines and cholecystokinin [6-10].

To date, more than 20 disorders have been linked to genetic defects that perturb sulfonation or sulfatase capacity [11]. For example, defects in several sulfatase genes are linked to metachromatic leukodystrophy (OMIM 250100), X-linked ichthyosis (OMIM 308100), chondrodysplasia punctata 1 (OMIM 302950), mesomelia-synostoses syndrome (OMIM 600383) and mucopolysaccharidoses types II (OMIM 309900), IIIA (OMIM 252900), IIID (OMIM 252940), IVA (OMIM 253000) and VI (OMIM 253200). In addition, mutations in the solute linked carrier 26A2 gene (*SLC26A2*) lead to under-sulfonation of proteoglycans in chondrocytes and cause a range of mild to lethal forms of chondrodysplasias: multiple epiphyseal dysplasia (OMIM 226900), diastrophic dysplasia (OMIM 222600), atelosteogenesis Type II (OMIM 256050) and Achondrogenesis Type IB (OMIM 600972) [12]. Collectively, these disorders highlight the physiological consequences of perturbed sulfate homeostasis.

In adults and children, approximately two thirds of daily sulfate requirements are met from the intracellular metabolism of sulfur-containing amino acids [13,14]. Sulfite oxidase deficiency, a disorder with severe neurological consequences, perturbs the ability to generate sulfate from amino acid oxidation [15,16]. In addition, the human foetus lacks the ability to derive sulfate from amino acid oxidation and relies on sulfate supplied from maternal circulation via the placenta [17]. During human and animal gestation, maternal circulating sulfate levels increase by approximately 2-fold to meet the high fetal demands for sulfate [18,19]. Interest in human circulating sulfate level has expanded following studies that link hyposulfatemia to animal pathologies [11].

In humans, circulating sulfate levels are maintained at approximately 300 to 400 µmol/L [20]. The kidneys filter sulfate in the glomerulus and then reabsorb the majority (approximately 80%) of filtered sulfate back into circulation [21]. The first step of sulfate reabsorption is mediated by the SLC13A1 sulfate transporter on the apical membrane of the renal proximal tubule, and the second step via SLC26A1 on the basolateral membrane [22]. Loss of *Slc13a1* and *Slc26a1* in mice leads to renal sulfate wasting, hyposulfatemia, growth retardation, and behavioural abnormalities [21,23-25]. The depleted sulfate levels in circulation reduce intracellular sulfonation capacity, as shown in the *Slc13a1* and *Slc26a1* null mice [23,26]. In addition, genetic defects in SLC13A1 have been linked to hyposulfatemia, growth retardation and osteochondrodysplasias in dogs and sheep [27,28]. More recently, genetic variants in *SLC13A1* were linked to renal

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sulfate wasting and hyposulfatemia in humans [29,30]. Furthermore, *SLC13A1* gene expression is down-regulated by vitamin D depletion, hypokalaemia, metabolic acidosis, NSAIDS and glucocorticoids [31]. Collectively, the above studies have shown the dietary, genetic and physiological contributions to altering circulating sulfate levels and renal sulfate excretion. Those findings, together with animal studies showing the adverse physiological consequences of renal sulfate wasting and hyposulfatemia [21,23], warrant investigation of sulfate levels in humans.

It is remarkable that sulfate is not routinely measured in clinical settings, even though it has been recognised as an essential analyte in human physiology for more than six decades [32]. Since the 1950's, several studies have tested various methods for measuring sulfate in biological fluids, including benzidine or barium precipitation, capillary electrophoresis, electrospray tandem mass spectrometry (ESMS-MS) and ion chromatography [20]. The benzidine precipitation assay has produced unaccountably high levels of sulfate, and this method has since been discontinued due to its carcinogenic potential. An early study comparing barium precipitation and ion chromatography showed these methods produce similar sulfate levels from the same samples [33]. More recently, Cole and Evrovski reported that the ion chromatography method has significantly less variation in serum sulfate measurements (304 ± 5 µmol/L, p=0.006, unweighted mean ± SD of data from six studies) when compared to nine studies that used the barium precipitation method (334 ± 43 µmol/L) [20]. In addition, the ion chromatography method has negligible interference by other anions, when compared to the barium precipitation method that can over-estimate sulfate levels by approximately 3-10% [34]. Based on these findings, we assessed the analytical validity of an ion chromatography method for measuring sulfate in plasma, serum and urine, including sulfate parameters on analytical sensitivity, linearity, recovery, imprecision, specificity, stability of extract, and interference.

Materials and Methods

Instruments and reagents

The following items were purchased from Thermo Scientific Dionex: Dionex ICS-2000 Ion Chromatography System (ICS-2000); Analytical IonPac AS18 4mm column (Part No. 060549); Guard column IonPac AG18 4mm (060551); Elugen™ hydroxide cartridge (053921); and an Anion standard (057590). Sodium thiosulfate ($Na_2S_2O_3.5H_2O$) was purchased from AJAX (Catalogue No. A517). Aliquots of Urine Control (Bio-Rad Lyphochek Quantitative Urine Control, Cat. No. 377) were stored at -20°C up to 12 months. Plasma control samples were prepared in-house and stored at -80°C up to 12 months. Deionized H_2O (dH_2O, Millipore system) was obtained on the day of analysis for dilution of controls and test specimens.

Blood and urine collection, processing and storage

Urine samples (no preservative added) were collected at approximately the same time as a blood sample in either a lithium heparin tube (for plasma) or a serum separator tube (for serum) from healthy volunteers and patients. The minimum and preferred volumes were 100 µL of urine, plasma or serum, that were stored at room temperature, 4°C or -20°C. The Chairperson of the Mater Health Services Human Research Ethics Committee (EC00332) assessed this research as meeting the conditions for exemption from the requirement of full HREC review and approval in accordance with section 5.1.22 (a) and (b) of the National Statement on Ethical Conduct in Human Research (2007) updated 2015.

Calibrators and procedure

The calibrator sulfate solutions were prepared on the day of analysis by diluting the Anion standard containing 1041.0 µmol/L sulfate: 1/2 (STD1, 500 µL Anion Standard+500 µL dH_2O, 520.5 µmol/L sulfate), 1/10 (STD2, 100 µL Anion Standard+900 µl dH_2O, 104.1 µmol/L sulfate) and 1/100 (STD3, 100 µL STD2+900 µL dH_2O, 10.4 µmol/L sulfate). Calibrator solutions, diluted samples of urine (20 µL urine+980 µL dH_2O) and plasma or serum (40 µL plasma/serum+360 µL dH_2O), and sodium thiosulfate-spiked anion standard STD1 (final concentration 500 µmol/L sodium thiosulfate) and urine (20 µL urine+980 µL of 1 mmol/L sodium thiosulfate) were added to an auto-sampler vial and assayed by ion chromatography with suppressed conductivity detection using a Dionex ICS2000 with the program parameters shown in Supplemental Table 1. Data management and analyses were performed using Chromeleon® software version 6.5 SP2.

Results

Specificity

Typical elution profiles of sulfate and its separation from other inorganic anions by ion chromatography are shown in Figure 1. Sulfate was detected as a single peak in the chromatographs with a retention time of 7.00-7.40 minutes. Sulfate peaks in the chromatographs for serum, plasma and urine, correlate to the sulfate peak in the anion standard, and do not elute near other detectable anions, including chloride, nitrate and phosphate.

Analytical sensitivity

The limit of detection (LOD, 9 µmol/L) and quantification (LOQ, 27 µmol/L) were calculated from 3.3 and 10 times, respectively, the SDs of responses: LOD=3.3 σ/S and LOQ=10σ/S, where the slope of the calibration curve (S)=0.022 and the SD of y intercepts of regression lines (σ)=0.061 (Figure 2A). The LOD was low enough for the detection of sulfate, while the calculated LOQ was precise and accurate for the

Sample	sulfate spike (µmol/L)	Recovery sulfate (%)				
		Run 1	Run 2	Run 3	Mean	SD
Plasma*	50	102	105	102	103	1.8
	100	100	102	100	101	1.4
	250	98	102	102	101	2.4
	500	100	103	103	102	2
Urine*	10,000	101	105	100	102	2.8
	25,000	98	102	103	101	2.5
	50,000	102	102	95	100	4.1
	1,00,000	98	103	100	100	2.8

*Recovery dilutions were 1/5 for plasma and 1/50 for urine

Table 1: Recovery of sulfate.

quantitation of sulfate in diluted serum, plasma and urine samples.

Linearity and recovery

Measurement of sulfate standards ranging from 0-1041 µmol/L showed a linear response, with R^2=1.000 (Figure 2A). Overall recovery of sulfate ranged from 95-105% for plasma and urine spiked with 50-500 µmol/L sulfate and 10-100 mmol/L sulfate, respectively (Table 1). Cumulative retention of sulfate on the column between runs was not observed.

Imprecision

Within run and between run imprecision data are shown in Table 2. The within run imprecision coefficients of variations (CV_a) were 0.8% for plasma and 5.0% for urine samples. The between run imprecision CV_a were 6.1% and 4.3% for plasma and urine, respectively. To assess the inter-operator variability, two independent operators tested the same samples in triplicate within a 2-hour window (Table 3). The results [mean (SD)] were 213 (2.1) µmol/L for plasma and 3944 (171.8) µmol/L for urine, showing <5% analytical variability. In addition, CV_a values were 0.0%, 2.0% and 5.7% for the inter-operator analyses of 520.5 µmol/L, 104.1 µmol/L and 10.4 µmol/L sulfate standards, respectively, indicating the greatest imprecision for samples at the lowest concentration.

Stability of extracts

To determine the stability of the serum extracts, initial measurements at <2 hours of blood collection and serum extraction were compared to those from serum stored at room temperature for 1 day and at 4°C for 7 days (Figure 2B and 2C). Serum sulfate levels were unchanged at 1 and 7 days, showing R^2=0.966 and 0.985, respectively. Urine stability was assessed after 1 day at room temperature, as well as 7 and 30 days at -20°C (Figure 2D, 2E and 2F). Urinary sulfate levels at all three time points were similar to measurements within 2 hours of urine sampling, showing R^2=0.999. Plasma extracts were also stable when stored at 4°C for 7 days, with R^2=0.985 (Figure 2G). Measurements of sulfate in plasma and serum were similar on the day of extraction, R^2=0.992 and when stored at 4°C for 7 days, with R^2=0.983 (Figure 2H and 2I).

Interference

Anion standard and urine samples were spiked with sodium thiosulfate to ascertain possible thiosulfate interference with sulfate detection. Interference (>5% of the peak response) was not observed in either sample at or near the retention times of other anions, including sulfate (Figure 3).

Discussion

In this study, we validated an ion chromatography method for measuring free inorganic sulfate levels in plasma, serum and urine in a clinical setting. Our validation includes specificity, analytical sensitivity, linearity, recovery, imprecision, stability and interference. In addition, our data also show that sulfate levels are similar in plasma and serum extracts stored up to 7 days at 4°C. Collectively, this information demonstrates the clinical utility of the ion chromatography method and validates its use for establishing reference intervals of sulfate levels in healthy individuals. While the requirement for measuring sulfate in humans is largely underappreciated, its physiological significance has been highlighted in animal studies [21,23,26,35,36] that are relevant to renal sulfate wasting and decreased serum sulfate levels in humans with loss-of-function variants in the *SLC13A1* sulfate transporter gene [29,30].

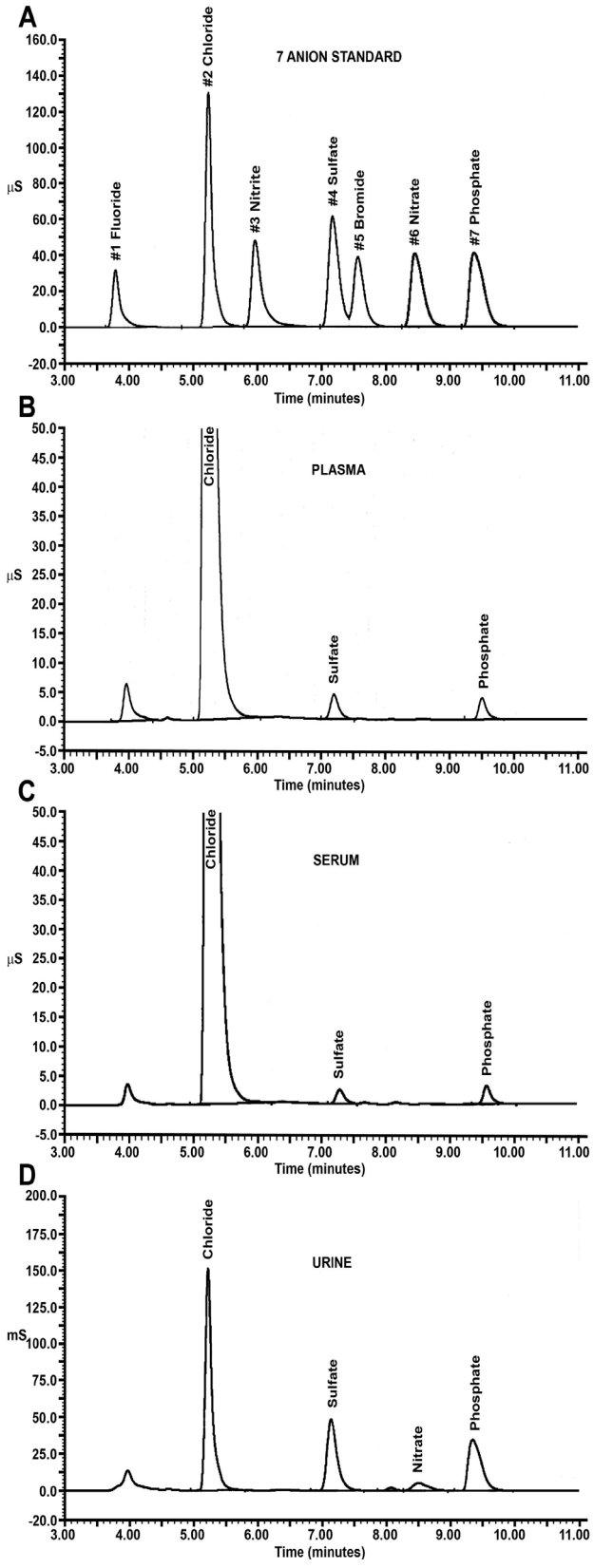

Figure 1: Analysis of sulfate by ion chromatography. Representative chromatographs of (A) anion standard, (B) plasma, (C) serum and (D) urine showing the separation of sulfate from other inorganic anions.

We show the ion chromatography method to consistently separate the sulfate peak from other anions and to produce a chromatograph of the seven anion standards that is similar to the manufacturer's data. Earlier studies also demonstrated that other anions, including bromide, urate, citrate, oxalate, ascorbate, malate, succinate, iodide, chloride, sulfite, thiosulfate, borate and persulfate, do not interfere or co-elute with sulfate [33,34]. Similarly, we show distinct separation of sulfate from thiosulfate, which is relevant to elevated urinary thiosulfate excretion in those individuals with sulfite oxidase deficiency and molybdenum cofactor deficiency [37]. In addition, sulfoesters, including dehydroepiandrosterone sulfate and chondroitin sulfate, yield insignificant and distinct peaks from sulfate [33]. Furthermore, we show no difference in sulfate levels between plasma and serum, indicating no effect of the lithium heparin in plasma samples. Collectively, these findings demonstrate that the ion chromatography method produces a reliable elution profile for sulfate in plasma, serum and urine, which is distinct from other anions at physiological concentrations.

We show linear detection of sulfate within the range of 0-1041 µmol/L, with LOQ of 27 µmol/L. Free inorganic sulfate in human serum and plasma is approximately 300 to 400 µmol/L [20], which is well within the linear concentration range and above the LOQ when diluted 1:10. The required 40 µL of serum and plasma minimize the sampling volume of blood, which is relevant for sulfate measurements in neonates and children. For urine, sulfate concentrations are within the millimolar range, which requires dilution of 1:50 to achieve measurements within the linear range and above the LOQ. These findings demonstrate the relatively small volumes of plasma, serum and urine that are required for sulfate measurements using ion chromatography.

In our stability studies, we compared measurements from fresh extracts with those extracts stored at room temperature, 4°C or -20°C for periods of time that exceed the typical storage time of less than 2 hours at room temperature in a pathology setting. This is relevant to situations where samples may need to be stored or transported prior to analysis. Intracellular sulfate concentrations are low [38] and thereby are unlikely to contribute to extracellular levels. However, there is potential for removal of sulfate from endogenous molecules via sulfatases that could potentially lead to false elevated sulfate measurements. Our data indicate that sulfate levels in plasma, serum and urine after 1 day at room temperature, as well as longer term storage at either 4°C or -20°C, are not different to those levels found in fresh extracts. These findings extend a previous study showing that storage of serum at -70°C for 2, 8 or 60 days had no significant effect on the measured sulfate concentration [39], presumably due to the negligible effect of sulfatase activity in plasma, serum and urine. Together, these findings support the practicality of performing the analysis of plasma, serum and urine sulfate levels at a convenient time post sampling.

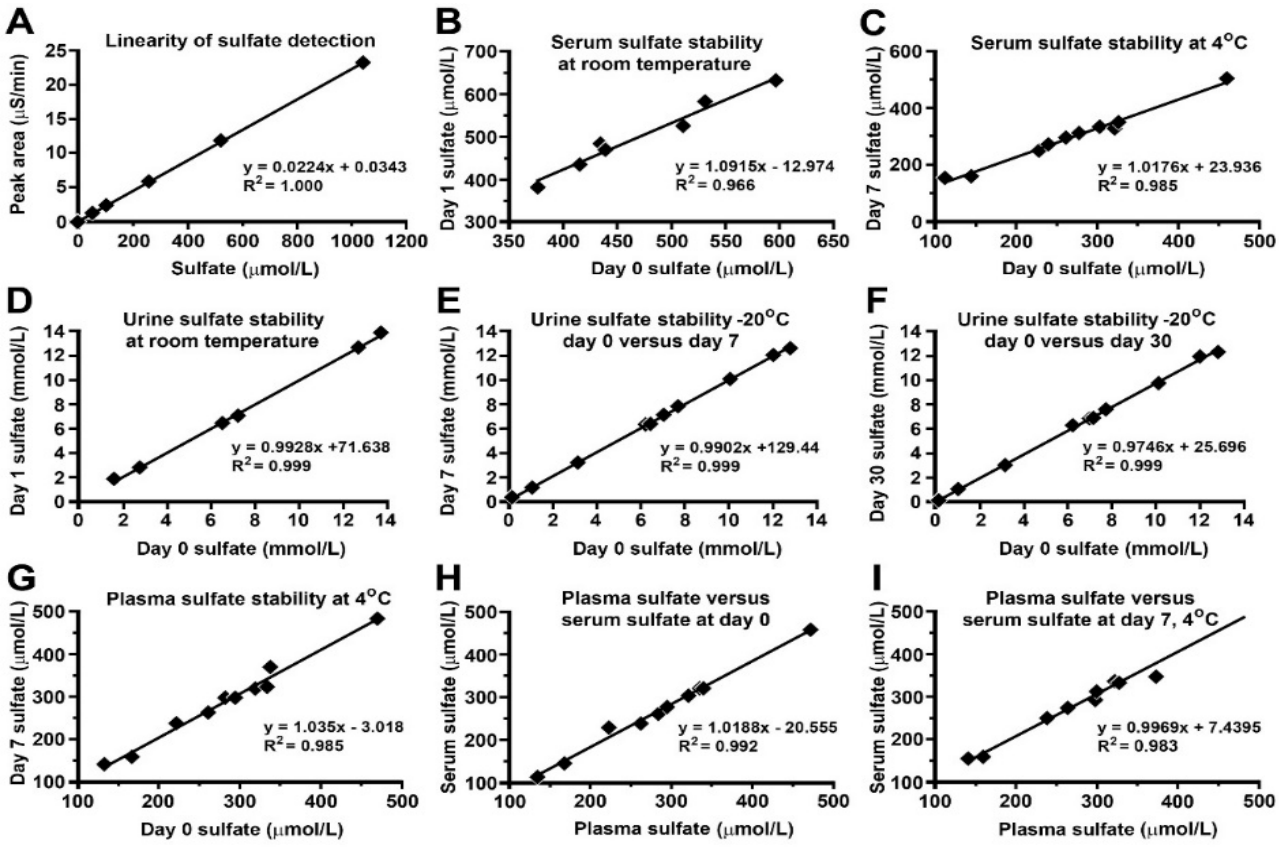

Figure 2: Linearity of sulfate detection and stability of sulfate in extracted samples. Individual data points are the mean of triplicate assays (n=15 to 30 samples assayed for each panel A-I). Day 0 corresponds to sulfate measurements on fresh extracts <2 hours of sampling.

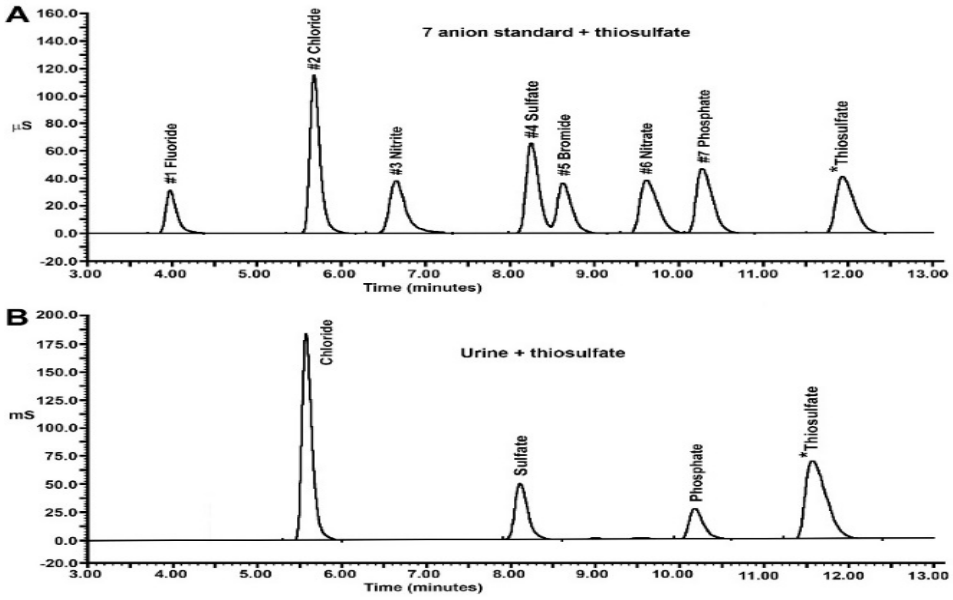

Figure 3: Thiosulfate interference was not observed at or near the retention times of other analytes. Representative chromatographs of thiosulfate-spiked (A) anion standard and (B) urine showing the separation of sulfate from thiosulfate and other inorganic anions.

	Sample	N	Mean (µmol/L)	SD (µmol/L)	CV_a (%)
Within run imprecision	**Plasma**	8	147	12	0.8
	Urine	16	4102	203	5
Between run imprecision	**Plasma**	21	169	10	6.1
	Urine	40	3996	171	4.3

Table 2: Imprecision of sulfate measurements.

Sample	Operator 1 (µ mol/L)	Operator 2 (µ mol/L)	Mean (µmol/L)	SD (µ mol/L)	CV_a (%)
Plasma QC	215	212	213	2.1	1
Urine QC	4066	3823	3944	171.8	4.4
STD 1	520	520	520	0	0
STD 2	107	104	105	2.1	2
STD 3	13	12	12.5	0.7	5.7

Table 3: Inter-operator variability.

Conclusion

Sulfate has important roles in human physiology, and perturbed sulfate homeostasis has adverse health outcomes. Methodologies for measuring sulfate in human biological fluids have been reported in research settings over many years but none of these have been validated for their analytical utility in clinical settings. Despite this importance, and possibly due to the unreliability of previous methods, sulfate determination is rarely undertaken as a clinical investigation. In this study, we validated an ion chromatography method for measuring sulfate in serum, plasma and urine. Our study demonstrates that the ion chromatography method has utility for investigation of sulfate levels in human health.

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Competing Interests

The authors declare that they have no competing interests.

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