A simple HPLC method for measuring sulfate in ultra filtered sheep plasma

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ABSTRACT

Plasma sulfate concentration is a useful indicator of sulfur amino acid oxidation. Measurement of sulfate in water and other fluids has been made by turbidimetric, ultra-violet (UV) absorption or ion chromatography HPLC methods. The turbidimetric method is variable and suffers from interferences and both turbidimetric and UV methods are less sensitive than ion chromatography. We describe a quick and simple HPLC method, where sample preparation is a centrifugal filtration and the sulfate measurement is by single column HPLC using one buffer at a constant flow rate. Recoveries from spiked samples were 99.5 % (RSD 1.8 %) and the RSD of repeated standards over 6 weeks was 0.7 %. Using this method, we measured plasma sulfate levels in both growing lambs with intestinal parasites and in sheep fed fresh *Lotus corniculatus* containing condensed tannins (CT) or other fresh forages. The CT in *Lotus corniculatus* decreased ($P < 0.05$) plasma sulfate concentration while parasite infection did not ($P > 0.05$). Our method provided quick and reliable data that could be transferable for measuring sulfate in blood, serum and other biological fluids and could be used as a biomarker of whole body sulfate status under various physiological conditions in farm animals.

Keywords: sheep; ovine; plasma sulfate concentration; analytical method; amino acid metabolism.

INTRODUCTION

Sulfur has essential roles in the catalytic pathways of biomolecules in cells. These include sulfate conjugation as one step in the detoxification of many compounds, activation of many biological molecules (eg heparin, gastrin) and as a component of the essential amino acids methionine and cysteine as well as peptides (eg glutathione) proteins, vitamins, cofactors and structural components (eg glycosaminoglycans) (Pelis & Renfro, 2004).

Plasma sulfate levels are controlled largely by the kidneys and normal plasma sulfate concentrations in vertebrates range from 0.3-1.8 mmole/L (Pelis & Renfro, 2004). Mammals require transport pathways to absorb sulfate from the intestine and intracellular sulfate comes from three main sources; hydrolysis of sulfate conjugates, oxidation of organic sulfur containing compounds (eg methionine and cysteine) and transport (Markovich, 2001).

Sulfate has previously been measured by turbidimetry, ion chromatography and as sulfur by inductively coupled plasma atomic emission spectrometry (ICPAES). A dual ion exchange column separation method has been used to remove proteins, amino acids and other interfering substances and the sulfur containing fractions measured as sulfur by ICPAE (Sun *et al*., 1994). This method is slow and the separations unnecessary if the other (non sulfate) fractions are not needed.

The ideal analytical method for plasma sulfate, or indeed anything, is to measure it directly on the original sample with no preparation at all. Plasma proteins, however, often interfere with the measurement of other components and a clean up step is usually required to remove them. Ultra filtration could possibly be used as this preparation step. It has been used effectively to measure sulfate in milk samples, with detection by suppressed conductivity (Gaucheron *et al.*, 1996). A single column HPLC method to measure sulfate in filtered and diluted human urine and serum has been used by Hoffman *et al*. (1991).

The aim of this research was to develop a fast, simple sulfate analysis method for routine use with plasma. We have focused on sulfate only in this study, ignoring the other anions in plasma as long as they did not interfere with the measurements. We proposed that the ultra filtered sample prepared for plasma amino acid analysis could also be used for measuring sulfate. This new method was then used to measure plasma sulfate as part of estimating sulfur amino acid oxidation using $^{35}SO_4$, ^{35}S-Cysteine and ^{35}S-Methionine in lactating ewes and growing lambs.

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MATERIALS AND METHODS

Analytical method selection

We tested a number of possible methods for measuring plasma sulfate that used plasma "as is" and varying degrees of sample preparation. The advantages and disadvantages of each method are summarised in Table 1.

Although the turbidimetric method using a Cobas Fara II autoanalyser (F. Hoffmann-La Roche Ltd, Basel, Switzerland; method revision date 2/89) used plasma "as is", the relative standard deviation (RSD) for some duplicates was very high. The zwiterionic buffer HPLC method of Hu *et al.* (2001) measured sulfate on serum and urine "as is" but we found poor resolution of cations and anions with the reported conditions. We also found that phosphate and sulfate co-eluted and that use of a non-suppressed system meant that increasing the sample sodium concentration decreased the sulfate peak area.

Other HPLC methods use considerable sample preparation to remove proteins and/or other interferences. One of the simplest of these is the addition of acetonitrile to the plasma to precipitate protein (Morris & Levy, 1988). We found too much variability using this method and later found the water/acetonitrile separated into two phases over time. When the layers were sampled and analysed separately and the remaining sample remixed, we found the sulfate concentration varied with the depth of the sample.

As shown in Table 1, ultra filtration followed by HPLC analysis had many advantages and this method was chosen to measure sulfate in our samples.

Selected method - sample preparation and analysis

Blood samples were collected using sodium heparin as anticoagulant and centrifuged at 4 °C for 15 minutes at 3270 g and the plasma collected. As many of the samples were originally taken for amino acid analysis, dithiothreitol (DTT) in phosphate buffer was added to these plasmas as a reducing agent and nor leucine (NL) in 0.1 % phenol as an internal standard for the amino acid analysis (Bermingham *et al.*, 2006). 0.5 mL of sample was centrifuged at 11000 g for 45 minutes in a vivaspin unit (PES membrane; 10000 molecular weight cut off; Vivascience, product number VS0102) and the filtrate frozen at -85 °C until analysed. A 50 μL sample of the filtrate was put into an auto sampler vial, capped and frozen at -20 °C until analysed for sulfate.

TABLE 1: A summary of method advantages and disadvantages showing the reasons for choosing the selected method.

Method	Advantages	Disadvantages	Decision
Dual ion exchange columns	Many S fractions	Slow	Reject
	Knowledge of S metabolism	Complex	
		Relatively expensive	
		S recoveries can be variable	
Turbidimetric	No preparation	Poor repeatability	Reject
	Quick		
	Cheap		
Zwitterionic HPLC	Simple	$^{3-}PO_4$ co-elutes with $^{2-}SO_4$	Reject
	No preparation	↑ $[Na^+]$ gives ↓ $[^{2-}SO_4]$	
Acetonitrile extract; HPLC	Relatively simple	Variable recoveries	Reject
		$^{2-}SO_4$ not evenly distributed in extract	
		Peak not well resolved	
		Toxic, flammable solvent	
Ultra filtration; HPLC	Simple		Accept
	Repeatable		
	Good duplication		
	Minimal preparation		
	Relatively quick		
	Relatively cheap		

The HPLC method was based on Alltech data sheet U269029 for the Wescan Anion/R column (#269.001; 10 µm packing; 4.1 mm x 250 mm). A guard column was fitted and the column and conductivity detector (mounted in the column oven) were run at 35 °C. The mobile phase was 5 mmole/L p-hydroxy benzoic acid at pH 8.4 at a flow rate of 1.6 mL/min. Sample injection volume was usually 5 µL and a standard (1 mmole/L K_2SO_4) in 1:1 water:mobile phase was run every 10 samples. Injection volumes from 5 to 20 uL were run to check linearity and sensitivity in the concentration range of interest (0.3 to 2 mmole/L).

Repeatability was assessed on standards run on the same and on different days to measure short and long term drift. The first batch of samples was run in duplicate to confirm the results for the standards and later analyses were not duplicated.

Animal trials

The selected method was used to measure plasma sulfate concentrations to determine specific radioactivity for sulfate as an estimate of sulfate clearance rate and sulfur amino acid oxidation (as part of a larger data set) for the following animal trials.

Trial 1

To examine the effects of fresh forages on amino acid metabolism, 16 ram lambs 16 to 18 weeks old were fed pasture, Lucerne (*Medicago sativa*), Sulla (*Hedysarum coronarium*) or a 1:1 Sulla/Lucerne mix hourly from overhead feeders and infused with $^{35}SO_4$ (about 5 MBq/h; 40 TBq/mmol, with an initial bolus of 5 MBq) and ^{35}S Cysteine (about 4 MBq/h; 37 TBq/mmol, with an initial bolus of 4 MBq) (Burke, 2004; Burke *et al*., 2004). Blood was sampled continuously from 5 h and samples removed at 6, 7 and 8 hours. There were 4 lambs per treatment.

Trial 2

To examine the effects of condensed tannin (CT) on tissue amino acid metabolism in lactating sheep, 12 ewes were surgically modified with an abomasal cannula; permanent catheters in the mesenteric (M, x2) portal (P) and hepatic (H) veins and mesenteric artery (A) and a transonic flow probe around the pudic artery. On day 40, a temporary catheter was inserted in the mammary (V) and jugular veins.

After 2 weeks recovery from surgery, the animals were fed *Lotus corniculatus* hourly from overhead feeders (2000 g DM/day; 80 g CT/day). Six sheep were drenched orally 3-4 times/day with polyethylene glycol (PEG; 160 g/d in water) to inactivate the CT. The other six were drenched with water to act as controls.

On day 42 (after surgery; at about 6 weeks lactation) $^{35}SO_4$ was infused into a jugular vein (about 4 MBq/h; 37 TBq/mmol, with an initial bolus of 4 MBq) blood sampled continuously from A and samples removed at times 0, 2, 4, 6, 8, 10 and 12 h. On day 45, ^{35}S-Methionine was infused (about 6 MBq/h; 37 TBq/mmol, with an initial bolus of 6 MBq) blood sampled continuously from 6 h and samples removed at times 8, 10, and 12 h.

Trial 3

To examine the effects of parasite infection on tissue amino acid metabolism, twelve 33 kg wether lambs were surgically modified as for Trial 2 with the addition of abdominal aorta and vena cava catheters (to measure hind limb metabolism) and without the mammary vein catheter or flow probe. They were fed 800 g DM/day (0.6 to 0.8 maintenance) fresh Sulla, hourly from overhead feeders. Six animals were given 6000 *Trichostrongulus colubriformus* L3 larvae/d orally for 6 days and the other six were drenched once to act as controls (Bermingham *et al.*, 2006). $^{35}SO_4$ and ^{35}S-cysteine were infused on days 42 and 45 respectively, blood sampled continuously from 4 h and samples removed at times 6 and 8 h.

All animal manipulations for these trials were reviewed and approved by the AgResearch Animal Ethics Committee, Palmerston North, New Zealand, as required by the Animals Protection Act (1960) and Animals Protection Regulations (1987) and their amendments.

Calculations and statistics

Sulfate concentrations (relative to a 1 mmole/L standard solution run every 10 samples) averages, standard deviations and RSD's were calculated in Excel spreadsheets. Genstat (8th edition; VSN International, Herts, UK) was used for statistical significances by General Analysis of Variance.

RESULTS AND DISCUSSION

Initial method tests

Data for the rejected methods are included here to demonstrate why those methods were not chosen. Duplication for the turbidimetric method was mostly acceptable (average RSD 3.8 %; n = 7; range 1.6% to 7.4%) but some were much more variable (average RSD 36 %; n = 3; range 27% to 47%). There were samples and standards in the latter group and, as there was no apparent reason for the variation, this method was rejected.

Initial runs with the acetonitrile method showed plasma sulfate concentrations around 0.2 mmole/L, outside the expected range of 0.3 to 1.8 mmole/L. There was also a gradual increase in sulfate peak area over time within an analytical run and the sulfate concentrations varied with which part of the acetonitrile extract was sampled; 0.21, 1.03 and 0.01 mmole/L for mixed sample, top and bottom of the vial respectively. The autosampler rack temperature was increased from 4 °C to 20 °C to avoid this apparent separation which reduced the variability somewhat. The major problem with this method, however, was the large negative peak that eluted immediately after the much smaller sulfate peak, making it difficult to determine exactly where the sulfate peak ended. Because of these uncertainties, this method was also rejected.

Selected method validation and results

For the chosen method (ultra filtered plasma sample; HPLC) injection volumes from 0 to 20 μL gave a linear response (R^2 of 1.00) and additions of known amounts of sulfate standard to plasma samples gave good recoveries (99.5 %, RSD 1.8 %). The RSD of repeated standards measured over 6 weeks was 0.7 % (range 0.4 % to 2.2 %). These first analytical runs showed the variability for both samples and standards to be low. The average RSD for 99 duplicated samples was 1.77% with a median of 1.67% and a range from 0.02% to 8.3%. The addition of DTT, NL and phenol did not result in corresponding peaks and these compounds are probably not retained on the column. The addition of phosphate (as pH buffer for the DTT) increased the existing phosphate peak which typically eluted 8 minutes before the sulfate.

Feeding Sulla produced significantly higher plasma sulfate concentrations than pasture or Lucerne during the cysteine infusion as shown in Table 2 (P = 0.04) and gave higher concentrations than Lucerne during the sulfate infusion (P = 0.1) indicating that something in the Sulla was affecting plasma sulfate concentrations.

Meier & Schmidt-Kessen (1978) found that human circulating sulfate levels are maintained fairly constant (+/- 10 %) that fasting sulfate levels increase with age and that other differences are largely due to diet. Oral water, protein or inorganic sulfate were found to increase serum levels temporarily and that an oral sulfate loading (eg a high protein diet) could double plasma sulfate concentrations, with the excess sulfate removed over 12 hours.

Sulla contains CT which are known to improve protein flow out of the rumen and provide more protein (including sulfur amino acids) to the small intestine for absorption of amino acids, especially essential amino acids (Waghorn *et al.*, 1987). A Sulla diet could therefore be thought of as "high metabolically available protein" compared to pasture and Lucerne and this could account for the higher sulfate levels.

TABLE 2: Mean sulfate concentrations (mmole/L) in sheep plasma showing the effects of collection time and feed on ram lambs (Trial 1; pasture, Lucerne, Sulla or a Sulla/Lucerne mix (Mix)) during i.v. ^{35}S sulfate and ^{35}S cysteine infusions. LSD at 5%; (df) is degrees of freedom.

Infusion	Time	Treatments					Significances		
	(h)	Pasture	Lucerne	Sulla	Mix	LSD (df)	Trt	t	Trt*t
Sulfate	6	1.21^{a}	1.22^{a}	1.68^{b}	1.44^{ab}	0.41 (12.4)			
	7	1.23^{ab}	1.19^{a}	1.62^{b}	1.44^{ab}	0.41 (12.4)			
	8	1.24^{ab}	1.20^{a}	1.63^{b}	1.48^{ab}	0.41 (12.4)			
LSD (df)		0.06 (24)	0.06 (24)	0.06 (24)	0.06 (24)		0.1	0.56	0.42
Cysteine	6	$1.20^{a\,x}$	$1.14^{a\,x}$	$1.69^{b\,x}$	$1.43^{ab\,x}$	0.36 (12.5)			
	7	$1.10^{a\,y}$	$1.09^{a\,xy}$	$1.53^{b\,y}$	$1.42^{ab\,x}$	0.36 (12.5)			
	8	$1.04^{a\,y}$	$1.05^{a\,y}$	$1.48^{b\,y}$	$1.42^{b\,x}$	0.36 (12.5)			
LSD (df)		0.06 (24)	0.06 (24)	0.06 (24)	0.06 (24)		0.04	<	0.003

^{a,b} Treatment means with a common superscript do not differ at 5 % level.

^{x,y} Time means with a common superscript do not differ at 5 % level.

Significances are P values for Treatment (Trt), Time (t) and Treatment*Time.

< signifies P <0.001.

Lotus corniculatus also contains CT and could also be considered "high metabolically available protein". As seen in Table 3, however, inactivating the *Lotus* CT with PEG actually increased the plasma sulfate concentration for both infusions (although this was not significant for the methionine infusion). Different forages contain different types of CT and some CT are more effective at protecting protein than others (Leng, 1997). This may account for the different behaviour of the CT containing diets. While PEG can contain sulfate, both PEG 3350 and sulfate, when administered orally, are considered to be poorly absorbed from the gastrointestinal tract (Brady *et al.,* 1986). Given that an oral dose of sulfate temporarily increases serum concentration (Meier & Schmidt-Kessen, 1978) and that the administration of PEG and sulfate together has no effect (Brady *et al.,* 1986) we suggest that, even if there was sulfate in the PEG, it is more likely that the PEG would reduce the availability of the sulfate for absorption and similarly reduce the plasma concentration.

Perhaps more interestingly, there was a significant time effect, indicating that sulfate concentrations adjusted more quickly than we expected. Meier & Schmidt-Kessen (1978) found there was a circadian rhythm in human sulfate serum concentration with a minimum before noon and a maximum in the afternoon and evening. While the blood samples in our studies were taken in the afternoon, we saw a decrease in sulfate concentrations with time, indicating that the amount of blood sampled (up to 10 % of the total blood pool) possibly affected sulfate concentration, that serum and plasma sulfate concentrations differ or that there is a difference in plasma sulfate concentrations between sheep and human through the day.

While there was a decrease in plasma sulfate concentration between parasite free and parasitised animals infused with ^{35}S cysteine in trial 3, it was not significant (1.4 vs 1.2 mmole/L respectively; LSD 0.50 with 10 degrees of freedom; P = 0.38).

Comparing Table 3 (trial 2) with the other trials, the sulfate concentrations for trial 2 are roughly half of those obtained in trials 1 and 3. The lactating sheep used for trial 2 probably require more sulfate, both for milk - about 1 mmole/L in cow's milk (Fox & McSweeny, 1998) and tissue structure (particularly for the mammary gland). Similarly, the higher food intake needed to maintain lactation could also mean a greater toxin load from the feed and a higher requirement for sulfate for detoxification. These factors could account for the lower circulating sulfate levels in trial 2 and indicate that plasma sulfate concentrations could reflect whole body sulfate status.

This study shows that sulfate can be measured reliably and without duplication on ultra filtered plasma. This filtrate can also be used to measure amino acids, resulting in the additional sample preparation time for sulfate being zero. Although outside the scope of this paper, it seems likely that this sample preparation method could be used to measure other anions by HPLC in plasma as well as sulfate and/or other anions in other samples.

Given the differences in plasma sulfate concentrations observed in sheep under varying conditions, it is possible that sulfate concentration could be used as an indicator of whole body sulfate status.

TABLE 3: Mean sulfate concentrations (mmole/L) in sheep plasma showing the effects of collection time and condensed tannins (CT) in lactating ewes fed *Lotus corniculatus* (Trial 2) during I.V. ^{35}S sulfate and ^{35}S methionine infusions. LSD at 5%; (df) is degrees of freedom.

Infusion	Time	Treatments		LSD (df)	Significances		
		-PEG (CT active)	+PEG (CT inactive)		Trt	t	Trt*t
Sulfate		0.50	0.78	0.2 (10.4)	0.005	0.33	0.23
Methionine	8	0.53^{x}	0.64	0.2 (9.2)			
	10	0.51^{xy}	0.64	0.2 (9.2)			
	12	0.49^{y}	0.64	0.2 (9.2)			
LSD (df)		0.02 (18)	0.02 (18)		0.17	0.06	0.1

^{x,y} Time means with a common superscript do not differ at 5 % level.

Significances are P values for Treatment (Trt), Time (t) and Treatment*Time.

ACKNOWLEDGEMENTS

Many thanks to John Koolaard and Zaneta Park-Ng for their timely assistance with genstat and the interpretation of results. This research was funded by the Foundation for Research, Science and Technology.

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