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Note

Improved method for the determination of sulphate in human serum using ion chromatography

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Sulphate conjugation plays an important role in the metabolism of a variety of endogenous compounds and of drugs [1–5]. It has been demonstrated by several authors that the availability of sulphate affects the extent to which these compounds are sulphate-conjugated [1,3,6–8]. Sulphate is the precursor of the high-energy phosphoadenosine phosphosulphate (PAPS), the cosubstrate of sulphate conjugation. A knowledge of the turnover of sulphate as reflected by serum sulphate concentration and urinary sulphate excretion might increase our understanding of the role that sulphate plays in sulphate conjugation.

Several methods have been introduced for measuring sulphate in biological fluids [9–12], but all of them are very time-consuming and are not suitable for a routine clinical or research laboratory dealing with a large number of samples. Ion chromatography is now becoming a routine method for measuring sulphate in biological fluids [13–16], but a disadvantage is the deteriorating effect of proteins on the matrix of the ion-exchange column. This causes day-to-day changes in the working conditions and is very expensive. Protein-rich samples should be precipitated prior to application to an ion-exchange column. Using acetonitrile to precipitate proteins (the method advised by the manufacturers [17]), we regularily observed a deterioration of the ion-exchange column within two months.

We have now adapted the method of protein precipitation by perchloric acid [18] for use in ion chromatography. This was achieved by the counter-precipitation of perchloric acid by potassium carbonate. This method enabled us to measure sulphate in protein-rich biological fluids using ion chromatography in a routine clinical laboratory.

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EXPERIMENTAL

Chemicals

Sodium sulphate, sodium hydrogen carbonate, sodium carbonate, perchloric acid, potassium carbonate and acetonitrile were of analytical-reagent grade (E. Merck, Darmstadt, F.R.G.).

Blood samples

Blood samples were collected without anticoagulants in 10-ml syringes (Safety-Monovette, Sarstedt, Nümbrecht, F.R.G.) and processed for routine clinical blood tests.

Perchloric acid precipitation

Perchloric acid used to precipitate serum proteins interferes in the determination of sulphate by ion chromatography. Therefore, the perchlorate was precipitated following the protein precipitation. Serum (1 ml) was added to 1.0 ml of ice-cold 0.665 mol/l perchloric acid, vortexed and kept at 4 °C for 10 min to allow precipitation to be completed. The mixture was then centrifuged at 3100 *g* at 0 °C for 15 min and 1.0 ml of the clear supernatant was added to 1.0 ml of ice-cold 0.7 mol/l potassium carbonate, vortexed and centrifuged at 3100 *g* a 0 °C for 15 min. The clear supernatant was diluted with distilled water to a 200-fold final dilution. For the determination of sulphate 80 μl were injected on to the column.

Acetonitrile precipitation

To 1.0 ml of serum, 3 ml of acetonitrile were added followed by 6 ml of distilled water. The mixture was shaken by hand for 10 s and then centrifuged at 7700 *g* at 20 °C for 30 min. The clear supernatant was diluted ten-fold with distilled water and 80 μl were injected for measurement of sulphate concentration.

Protein determination

Protein was determined by a routine clinical laboratory method (Biuret) in the supernatant after precipitation [19].

Ion chromatography

The sulphate concentration of the serum was determined using an Ionen-Chromatograph 2000i with a continuous suppressor system (Dionex, Sunnyvale, CA, U.S.A.). The AS 4 anion-exchange column (250 mm×4 mm I.D.) (Dionex) contained surface-sulphonated polystyrene–divinylbenzene resin beads with cross-linked latexed anion-exchange beads (particle size 15 μm). The MPIC-NG 1 pre-column (40 mm×4 mm I.D.) (Dionex) contained divinylbenzene beads (particle size 10 μm). The chart recorder used was an SE 120 (BBC, Ludwigshafen, F.R.G.) and the sensitivity was set at 3 μs full scale. The eluent used was a mixture of 2.8 mmol/l sodium hydrogen carbonate and 2.25 mmol/l sodium carbonate at a flow-rate of 2 ml/min. The regenerant was 0.0125 mol/l sulphuric acid [17]. Each sample was chromatographed in duplicate using an injection loop (80 μl).

TABLE I

PROTEIN CONCENTRATION REMAINING IN IDENTICAL SERUM SAMPLES AFTER PRECIPITATION

Sample	Protein concentration (g/dl)	
	Precipitated by acetonitrile	Precipitated by perchloric acid
1	1.10	0.40
2	0.87	0.19
3	0.70	0.20
4	0.86	0.20
5	0.70	0.20

Statistics

Statistical parameters were calculated on a programmable calculator (HP 41 CV).

RESULTS

Protein precipitation

The protein concentration after precipitation was determined in five serum samples, which were each split and precipitated using either perchloric acid or acetonitrile. The protein concentrations remaining after acetonitrile precipitation were approximately four times higher than those with perchloric acid precipitation (Table I). No adverse effect on the separation characteristics of the ion-exchange column was observed with the small remaining protein concentrations after perchloric acid precipitation.

Effect of precipitation time

Five serum samples were split into five aliquots and were incubated with perchloric acid for 4, 7, 10, 15 and 20 min and then further processed. No change was found in the serum sulphate concentration with increasing incubation time. This indicated that under the conditions used no liberation of sulphate by perchloric acid from sulphate conjugates was found.

Influence of dilution

Pooled serum was precipitated by perchloric acid and diluted stepwise to a final concentration ranging from 80-fold to 640-fold. A linear decrease in the sulphate peak height was found for dilutions from 1:160 to 1:640 (Fig. 1). A final dilution of 1:200 was chosen for all further experiments. Standard solutions of sulphate were prepared ranging from 0.7 to 56.2 μmol/l. The detector response was linear for concentrations ranging from 1.4 to 42.1 μmol/l ($r = 0.998$). Calibration graphs for sulphate were obtained daily for concentrations ranging from 1.8 to 14.0 μmol/l.

Coefficient of variation

Pooled serum was divided into eight samples and each was precipitated by perchloric acid and was further processed. The sulphate concentration in each

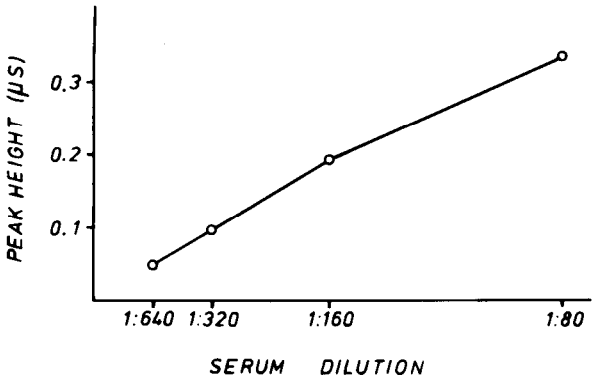

Fig. 1. Sulphate peak height in pooled human serum after protein precipitation by perchloric acid. A linear increase was found for dilutions from 1:640 to 1:160 ($r=1.000$).

sample was then determined. The average sulphate concentration was 0.252 mmol/l with a standard deviation of 0.013 mmol/l. The coefficient of variation was 5.3%.

Recovery

Six randomly chosen serum samples were split into two 1.0-ml aliquots. In each of the first samples the basal sulphate concentration was determined. To each of the second samples 0.141 mmol of sulphate was added and the sulphate concentration was determined. The recovery of the added sulphate (mean 99.8%, standard deviation 12.8%) ranged from 86 to 113% (Table II). The percentage of sulphate recovered was independent of the basal sulphate concentration.

Effect of storage time

Five serum samples were divided into six aliquots and the sulphate concentration was determined either immediately or after the samples had been stored at −70°C for 2, 8 or 60 days. No significant change in the measured serum sulphate concentrations was found over this period of time. Because of increasing varia-

TABLE II

RECOVERY OF ADDED SULPHATE IN NORMAL HUMAN SERUM

Sulphate added to 1 ml of serum (A) (mmol)	Sulphate concentration (mmol/l)		Recovery $[A/(C-B)]\cdot 100$ (%)
	Before addition (B)	After addition (C)	
0.141	0.244	0.403	113
0.141	0.278	0.403	89
0.141	0.297	0.424	90
0.141	0.254	0.414	113
0.141	0.334	0.455	86
0.141	0.334	0.486	108

tion of the measured sulphate concentration with increasing storage time, a maximal storage time for serum samples of two days was tolerated.

Effect of protein precipitation on column characteristics

The retention time of sulphate under the conditions used is 5.0 min with a baseline separation from the preceding nitrate peak that was regularly observed in serum samples. After application of approximately twenty serum samples precipitated by acetonitrile, the retention time of sulphate decreased continuously and baseline separation from the preceding nitrate peak was lost. So far no method has been found that will restore the separation characteristics of the ion-exchange column. After perchloric acid precipitation the retention time did not decrease, and baseline separation persisted. Using one AS 4 ion-exchange column and one MPIC pre-column we have now over a period of six months injected 1000 serum samples precipitated by perchloric acid. There was no change in retention time or in the separation characteristics of the sulphate peak.

Handling of samples

We compared the number of serum samples that could be handled by a trained technician in a routine clinical laboratory using perchloric acid or acetonitrile precipitation. In a 8-h working day about 40 serum samples could be handled after perchloric acid precipitation and assayed in duplicate. In the same period only twenty samples could be assayed after acetonitrile precipitation. The limiting step was the continuous decrease in the retention time of the sulphate peak and a loss of baseline separation. This required a new eluent with lower concentrations of sodium hydrogen carbonate and of sodium carbonate to restore the initial retention time and the baseline separation for the sulphate peak. However, this caused a broadening of the sulphate peak and also required a new calibration graph for sulphate.

Serum sulphate concentration in normal subjects

Using this perchloric acid precipitation we determined the sulphate concentration in serum samples from 41 normal adults. The mean serum sulphate concentration was 0.302 mmol/l, with a standard deviation of 0.057 mmol/l, ranging from 0.216 to 0.498 mmol/l. There was no statistically significant difference between males and females. This is comparable to serum sulphate concentrations found by other workers using different assay methods (Table III).

DISCUSSION

Protein precipitation of serum samples with perchloric acid was found to be very effective in reducing the protein concentration to an extent that does not affect ion-exchange columns. The counter-precipitation of perchlorate by potassium carbonate eliminated the perchlorate, which itself interferes in the separation of sulphate. Using this method of protein precipitation we did not find any change in the separation characteristics of an ion-exchange column in regular use for over eight months. This has enabled us to measure sulphate concentrations

TABLE III

MEAN SERUM SULPHATE CONCENTRATIONS IN NORMAL SUBJECTS DETERMINED BY DIFFERENT METHODS

Reference	Method	Mean concentration (mmol/l)
9	Turbidimetry	0.330
10	Atomic absorption spectrometry	0.290
11	Spectrophotometry	0.190
13	Ion chromatography (NaOH precipitation)	0.300
14	Ion chromatography (no precipitation)	0.325

in a large number of serum samples in a routine clinical laboratory.

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