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Note

Sulphate quantification in rabbit tracheobronchial mucus by reversed-phase ion-pair chromatography

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The rheological properties of the mucus secreted into the tracheobronchial lumen of vertebrates seem to be mainly determined by the concentration of the overall glycoproteins (mucins) of this mucus [1]. Each mucin unit involves a protein backbone chain which is covered, over 60% of the length, by short carbohydrate side-chains linked to the peptide region by O-glycosidic bonds. These side-chains are often terminated by fucose or by a sialic acid and may contain, especially the longer ones, internal ester sulphate residues [1,2]. Highly sulphated mucins have been identified in cat, dog, rabbit and human mucus [3]. These mucins seem to be released mainly in response to a pathological stimulation; they appear to be of particular value in mucosal protection against irritants by their acidic properties [3] and to play a role in controlling water content of secretions [2].

So far, evaluation of the sulphomucin content in respiratory mucus has been performed by incorporation of radioactive ^{35}S in animals [3] or, indirectly, after liberation of inorganic sulphates by acidic hydrolysis [4–6] or by pyrolysis [7]. Colorimetric methods [8–10], precipitation with $^{133}BaCl_2$ and determination of sulphate-associated activity in a γ-counting system [11] and ion chromatography [7] have been used for the quantification of the liberated inorganic sulphates. However, colorimetric methods suffer from significant interferences [7], radioactive barium chloride needs special handling, and the ion chromatographic assay requires a dedicated instrumentation.

As a part of our efforts to develop useful tests for screening new potentially mucoactive drugs, we developed a high-performance liquid chromatographic (HPLC) method for the quantification of the sulphate content in rabbit tra-

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cheobronchial mucus (RTBM). A single-pump HPLC instrument was operated in the reversed-phase mode under ion-pair conditions with indirect UV detection of the "transparent" sulphate ions.

EXPERIMENTAL

Sample handling

Aliquots of 100 μl of RTBM, collected as previously described [12], were placed into glass tubes to which 15 μl of 4 *M* hydrochloric acid were added. The tubes were stoppered with PTFE-lined screw-caps and heated at 100°C for 4 h. After cooling, 0.5 ml of acetonitrile (LiChrosolv, E. Merck, Darmstadt, F.R.G.) was added to each sample, and the solution was evaporated to dryness at 60°C under a gentle stream of nitrogen. The residue was dissolved in 0.5 ml of doubly distilled water (containing sulphate levels below the limit of quantitation of our HPLC method) by shaking with a vortex mixer for 30 s. Then, each solution was passed, with a disposable 1-ml syringe, through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.), previously washed with 10 ml of acetonitrile and 10 ml of doubly distilled water. The eluted volume was drawn up with the same syringe and passed through the cartridge again (twice more). Then the cartridge was completely emptied by passing air through it from the syringe. To the final volume was added 0.5 ml of acetonitrile, and the solution was evaporated to dryness at 60°C. The residue was dissolved in 0.3 ml of doubly distilled water and analysed. The same procedure was used when aqueous sulphate samples (100 μl) or different volumes of RTBM were processed.

Standard pig mucosal heparin PM II, 3.676 mequiv./g sulphate, as determined by conductimetry and generously supplied by the authors of this method [13], was used as standard sulphate-containing molecule; 100-μl aliquots of a stock heparin solution (0.37 μequiv./ml SO_3^-, corresponding to 33.5 μg/ml SO_4^{2-}) were carried through the hydrolysis procedure and analysed for sulphates. In order to verify if sulphur-containing amino acids of proteins and other compounds might interfere with the sulphate quantification, bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) and N-acetylcysteine were dissolved in water (5 mg/ml) and processed as described for RTBM.

Instrumentation and operating conditions

HPLC analyses were carried out with a Varian Model 2010 pump using a Rheodyne injector Model 7125 equipped with a 50-μl loop, a Varian Model 2050 variable-wavelength UV detector, set at 280 nm and 0.08 a.u.f.s., and an Omniscribe Model D5116/2 recorder. An RP-8, 5-μm Hibar (E. Merck) column (25 cm × 0.46 cm I.D.), a precolumn (between the pump and the injector) and a guard column (3 cm × 0.46 cm I.D.) tap-packed with Perisorb RP-8, 30–40 μm (E. Merck) were used. The mobile phase contained 0.001 *M* aqueous tetrabutylammonium hydroxide, as pairing agent, with monopotassic phthalic acid, as a UV-absorbing component, to a pH of 6.5. The flow-rate and the chart speed were 1 ml/min and 0.25 cm/min, respectively.

Colorimetric quantification of inorganic sulphates and of acid mucins in RTBM

was also carried out by the sodium rhodizonate and the Alcian Blue standard methods [8,14], respectively, using a Varian Model DMS 100 UV spectrometer.

Quantification

In order to control procedural losses, calibration curves were obtained with both unprocessed and processed standard aqueous solutions (doubly distilled water) containing 5–50 μg/ml sulphate anion from anhydrous disodium sulphate (Merck 6649). Moreover, the internal calibration curve was verified weekly adding scalar amounts (0, 0.5, 1, 1.5, 2, 3, 5 μg) of sulphate from stock solutions (1 or 2 mg/ml) to 100 μl of RTBM samples from a homogeneous pool and processing the spiked mucus.

All the calibration curves were calculated by least-squares regression technique from the plot of sulphate peak heights versus the sample concentrations (area data can also be used), excluding the internal calibration for which the sulphate peak heights were subtracted from the peak height of the endogenous sulphate present in the processed RTBM pool. Evaluation of background interferences was carried out by processing scalar amounts of RTBM (50–125 μl).

RESULTS AND DISCUSSION

We first tried to apply the sodium rhodizonate colorimetric method to the quantification of sulphate anion in RTBM, but we found an increase of the apparent concentration of the sulphate ions (μg/ml) both by increasing the volume (50 to 125 μl) of RTBM-processed pools (Table I) and by adding hydrophosphate or chloride anions to a fixed volume of RTBM (data not shown). We then used reversed-phase ion-pair chromatography with indirect photometric detection of non-absorbing anions, which has recently become a useful alternative technique to the more common ion-exchange chromatography with conductimetric detection [15]. Upon addition of a pairing agent to the mobile phase, the reversed-phase column behaves very much like an ion-exchange column. Moreover, the mobile phase absorbs light, whereas the sample anions do not, giving rise, when eluted from the column, to negative peaks.

Reversed phase ion-pair chromatography requires some time (2–4 h) for column conditioning in order to achieve the best peak shape and reproducibility: for this purpose the column was eluted (0.3 ml/min) by night, and the mobile phase was recycled into the reservoir. Fig. 1 shows three representative chromatograms: sulphate anions have a retention volume (V_R) of 9.6 ± 0.6 ml, whereas potentially interfering anions such as chloride, phosphates and sulphite are rapidly eluted ($V_R < 5$ ml).

The hydrolysed RTBMs were eluted through Sep-Pak cartridges to deplete the samples of the non-ionic or weakly ionic interfering products of hydrolysis. For example, processing bovine serum albumin or N-acetylcysteine did not give rise to quantifiable interfering peaks. Control of sulphate losses during this purification step was evaluated by constructing calibration curves with unprocessed or processed standard aqueous solutions. In both cases, the peak height was directly proportional to the amount of sulphate anion injected. Least-squares regression

TABLE I

SULPHATE LEVELS OBTAINED BY ANALYSING SCALAR AMOUNTS OF RTBM FROM A SINGLE RTBM POOL

Volume of mucus (μl)	Sulphate levels (μg/ml)*	
	Spectrometry	HPLC
50	60.0	43.0
75	93.3	40.5
100	110.0	37.5
125	139.2	39.2

*Mean of two determinations.

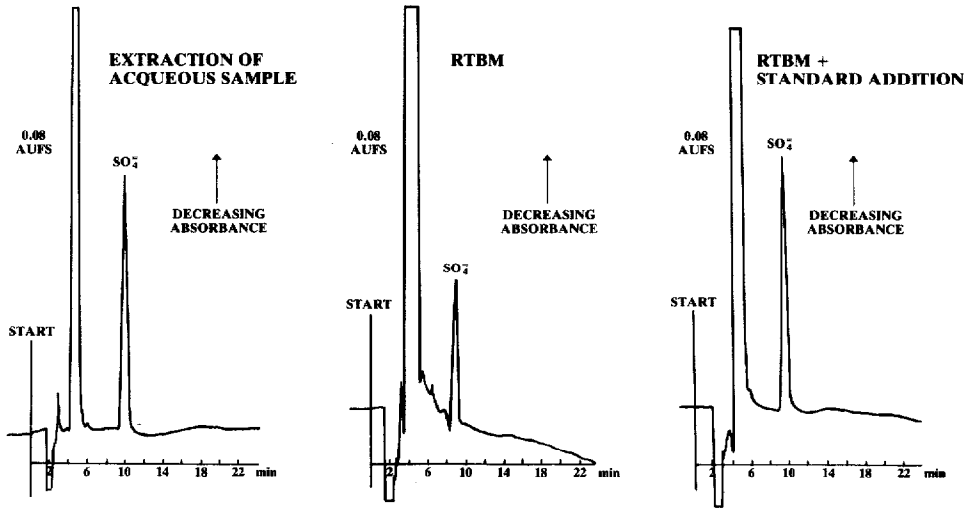

Fig. 1. Reversed-phase ion-pair chromatography of sulphate anions with indirect UV detection (positive peaks due to polarity inversion of the recorder). Processed samples: doubly distilled water spiked with 20 μg/ml sulphate; RTBM containing 10.2 μg/ml sulphate; RTBM spiked with 10 μg/ml sulphate. System peak (not shown) eluted always at ca. 25 min.

analysis gave the calibration lines: unprocessed standards $y=4.45+2.76x$ ($r=0.9978$); processed standards $y=1.80+2.66x$ ($r=0.9966$); where the x is μg/ml sulphate and the y is the sulphate peak height (mm). The procedural recovery of sulphate, calculated as [(slope of the processed curve)/(slope of the unprocessed curve)]·100, was ca. 96%.

Optimal hydrolysis conditions were evaluated by treating RTBM samples with 4 *M* hydrochloric acid for 1–6 h: maximal peak height from RTMB was obtained after 4 h hydrolysis. In addition, treatment of heparin PM II samples for 4 h with 4 *M* hydrochloric acid gave rise to a sulphate recovery of $90.1 \pm 1.7\%$ ($n=4$), confirming the high hydrolysis yield.

Mucus samples without sulphate were not available, so calibration curves for endogenous sulphate in RTBM were constructed using RTBM samples from a homogeneous pool to which scalar amounts of sulphates were added, and processing the spiked mucus. By subtracting the peak of endogenous sulphates, the resulting calibration curves were equivalent to those prepared by using aqueous sulphate solutions. Less than 5 ppm of sulphates are quantifiable in RTBM sam-

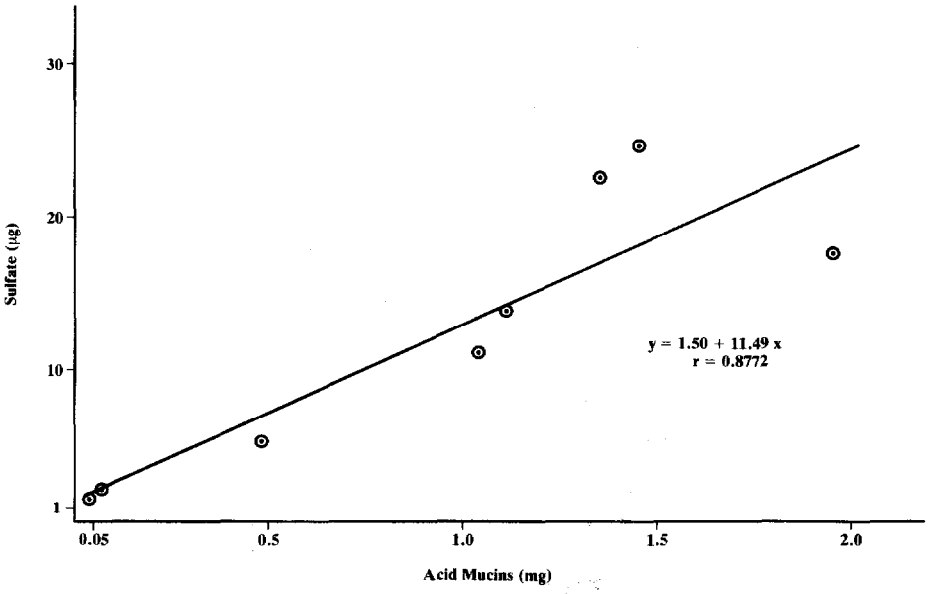

Fig. 2. Correlation between sulphate and acid mucins daily output in RTBM (data from six rabbits).

ples. Moreover, the processing of different volumes of mucus from a RTBM pool showed that this HPLC method, unlike the colorimetric one, does not suffer from background interferences (Table I), and so allows specific quantification of sulphate anions per ml of mucus independently from the volume of RTBM processed.

As an example of application of this method, six male rabbits were cannulated and mucus samples were collected [12] for quantification of sulphates. A good correlation was found between the daily output of acid mucins (dosed via the colorimetric Alcian Blue method) and that of the liberated sulphates (Fig. 2).

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