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A simple, optimized method for the determination of sulphide in whole blood by GC–MS as a marker of bowel fermentation processes

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Abstract

Hydrogen sulphide is produced in human large intestine by anaerobic fermentation and may play a pathogenic role. An analytical method for determination of sulphide in whole blood using an extractive alkylation technique was optimised and validated for this purpose. The sample was mixed with organic phase containing pentafluorobenzyl bromide as an alkylating agent. The benzalkonium chloride was used as a phase-transfer catalyst. The quantitative determination was performed using GC–MS technique in selected ion monitoring mode. The blood levels of sulphide of healthy controls were measured (35–80 μM/l). The method is versatile, reproducible (RSD=2.7%) and suitable for research of anaerobic fermentation in vivo.
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1. Introduction

Hydrogen sulphide is a highly toxic compound occurring in biological samples as well as produced by technological processes. In addition it is also formed in the human large intestine. The sulphate-reducing bacteria are a diverse group of anaerobes, which are uniquely capable of utilizing the sulphate or sulphite ion as an electron acceptor in the dissimilation of organic substrates and are an important source of hydrogen sulphide production within the bowel lumen. Recently, it was found to play an important role in the pathogenesis of inflammatory bowel diseases, namely ulcerative colitis [1,2]. Some analytical methods for the determination of sulphide in the whole blood were published last year, but they are laborious [3] or require special, seldom available reagents [4].

The gas chromatographic method for the determination of sulphide in spring water by extractive alkylation followed by gas chromatography was described [5] and successfully used in forensic analyses of blood samples of poisoning cases lately [6]. However, the sulphide levels were much higher compared to physiological conditions and validation parameters were not shown in the article. Similar methods employing pentafluorobenzyl bromide were

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developed for the determination of other inorganic anions, such as cyanide [7] or azide [8].

All the methods mentioned above suffer from many disadvantages, such as low sensitivity or reproducibility. Owing to this fact we attempted to develop a simple, sensitive and reproducible analytical method for the determination of sulphide in whole blood using GC–MS, what is a widely available instrument in clinical laboratories. This approach makes it possible to analyze large numbers of samples in short periods of time.

2. Experimental

2.1. Reagents

Sodium sulphide nonahydrate, sodium tetraborate decahydrate, benzalkonium chloride and naphthalene were purchased from Sigma (St. Louis, MO, USA). α-Bromo-2,3,4,5,6-pentafluorotoluene (pentafluorobenzyl bromide, PFBBr) was obtained from Fluka (Buchs, Switzerland). Toluene, potassium dihydrogenphosphate and ethyl acetate were obtained from Merck (Darmstadt, Germany). Helium 6.0, used as a carrier gas, and nitrogen 5.0 were purchased from Linde (Düsseldorf, Germany). Distilled water was prepared by reverse osmosis in our laboratory. All reagents and solvents used were of analytical-reagent grade. Oxygen-free water was prepared by bubbling nitrogen into distilled water for 15 min.

2.2. Equipment

The GC–MS analysis itself was performed on a TurboMass (electron ionisation quadrupole mass spectrometer) coupled with a AutoSystem XL gas chromatograph (purchased from Perkin-Elmer, Norwalk, USA). The separation was performed using a capillary column CP-Sil 8 CB-MS (Chrompack) of 30 m length×0.32 mm I.D., film thickness 0.5 μm. Data acquisition and processing were performed by TurboMass software (Perkin-Elmer).

The biological thermostat BT 120 (Laboratorni Pristroje, Czech Republic), laboratory shaker 3006 (GFL, Burgwedel, Germany) and centrifuge Labofuge 400 R (Heraeus Instruments, Hanau, Germany) were used throughout this study.

Pre-evacuated tubes (Vacuette, Greiner Bio-one, 2 ml containing EDTA dipotassium salt as an anticoagulant) for blood sampling were used.

Flat-bottom vials (60×14 mm I.D.) were used for the extractive alkylation procedure and the upper organic phase after the alkylation procedure was transferred into 2 ml amber glass autosampler vials.

2.3. Preparation of standard solutions and whole blood samples

Standard solutions were prepared by dissolving sodium sulphide nonahydrate in oxygen-free water saturated with sodium tetraborate. The solutions for five calibration levels were prepared in concentration intervals of 10–100 μ*M*/l. All subsequent steps were equal for standard solutions and blood samples.

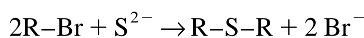
Whole blood samples were drawn using pre-evacuated tubes. The samples were processed or frozen immediately.

2.4. Extractive alkylation procedure

A 1.5-ml volume of 5 m*M* benzalkonium chloride solution in the oxygen-free water saturated with sodium tetraborate and 0.4 ml of whole blood was put into the vial. The content of the vial was gently mixed. Subsequently, 1 ml of 20 m*M* of PFBBr solution in toluene and 1 ml of ethyl acetate containing 10 μ*M*/l of naphthalene (internal standard) were added.

The vials were tightly closed with PTFE-coated caps. The mixture was shaken in the thermostatted oven (55 °C) for 4 h. Then, 2 ml of saturated potassium dihydrogenphosphate solution in redistilled water were added and the mixture was shaken for 1 min at room temperature. The organic and inorganic layers were separated by centrifugation (3500 rpm for 3 min, 0 °C). The upper organic phase was transferred to the autosampler vial and a 1-μl aliquot of the extract was injected onto a GC–MS apparatus.

The alkylation of sulfide is explained by the following scheme:



$$2\text{R–Br} + \text{S}^{2-} \rightarrow \text{R–S–R} + 2\,\text{Br}^{-}$$

where R– = pentafluorobenzyl.

2.5. GC–MS conditions

Helium was used as a carrier gas at a pressure of 100 kPa. The injection port equipped with a 2-mm glass liner (packed with silanized glass wool) was kept at 250 °C. The initial temperature of the column was 100 °C (1 min) programmed at 8.0 °C/min to 250 °C. The splitless period was 1 min.

The transfer line and ionization source were kept at 240 and 250 °C, respectively. The ionization energy was 70 eV. The fragments m/z 128 (for I.S.) and 181 (for bis(pentafluorobenzyl)sulphide) were monitored (dwell 0.2 and 1 s, respectively).

3. Results and discussion

3.1. Internal standard

The internal standard should have similar chemical properties to the sulphide ion, should be derivatized, and must not be present in blood in considerable quantities. Many inorganic anions and organic alcohols were checked, such as cyanide or azide (requiring a different derivatization procedure) or thiocyanate (present in human blood). Despite our efforts to find an optimal internal standard according to the requirements above, naphthalene had to be employed to compensate for autosampler imprecision and instrument instability.

3.2. Optimization of derivatization

The basic factors for extractive alkylation are an extraction solvent, pH for the reaction, reaction temperature and time, shaking parameters, and concentration of phase catalyst and alkylation reagent. Some of the parameters were taken over from Refs. [6–8]: pH for the reaction (9.3), concentration of phase catalyst (5 mM), and alkylation reagent (20 mM).

The influence of temperature on reaction time was thoroughly explored. Results are shown in Fig. 1. The temperature of 55 °C was chosen as an optimum. Also, the effect of continuous shaking was examined. When continuous shaking was omitted, the recovery of 66% and worse reproducibility was found compared to continuously shaken samples.

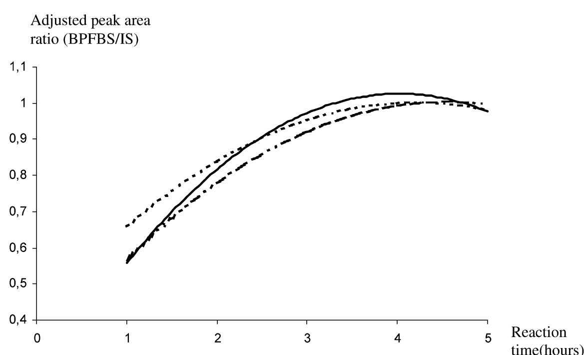


Fig. 1. Relationship of extraction yield on the extraction time and the extraction temperature (- - - 45 °C, · · · 55 °C, — 60 °C). Peak area ratio of BPFBS/I.S. was adjusted as a proportion of average value obtained from samples extracted for 4 and 5 h.

The major problem of this method is a slight increase in the derivative content after the separation of the organic layer. The possible reasons were published previously [6]: interference of glutathione or sulfur-containing amino acids. This process can be omitted by decreasing the pH of the mixture or depletion of the PFBBr. The use of methanol, 0.1 M/l of acetic acid, 0.1 M/l of hydrochloric acid and a saturated solution of potassium dihydrogenphosphate was examined and the results are shown in Fig. 2. The best results were obtained with potassium dihydrogenphosphate.

3.3. GC–MS conditions and calibration

The oven program was optimized to separate quantitated peaks from noise. There were no interfering peaks in real samples (Fig. 3). The mass spectra of I.S. and bis(pentafluorobenzyl)sulfide are shown in Fig. 4. The base mass peaks (m/z) 128 and 181

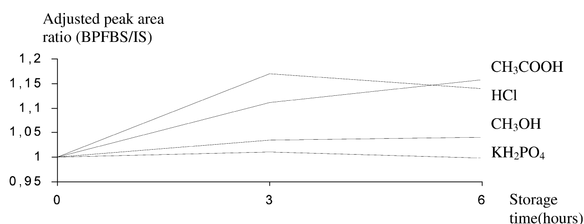


Fig. 2. Discontinuance of the alkylation reaction by various solutions (see text). Peak area ratio of BPFBS–I.S. was adjusted as a proportion of initial value.

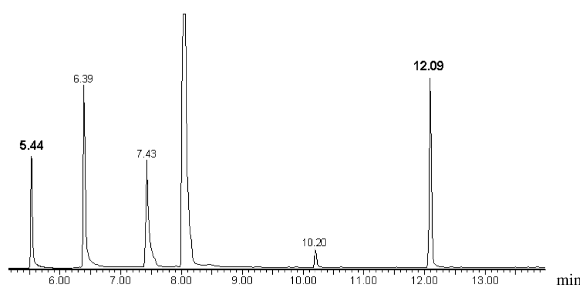


Fig. 3. Chromatogram of I.S. (retention time 5.44 min) and bis(pentafluorobenzyl) sulphide (retention time 12.09 min).

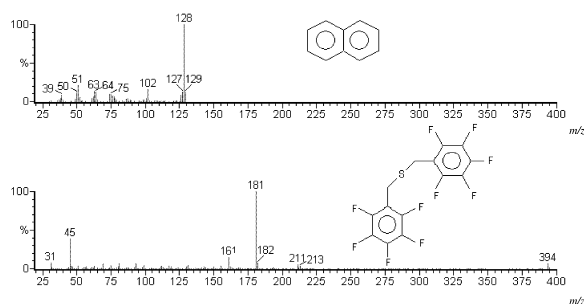


Fig. 4. Mass spectra of I.S. and bis(pentafluorobenzyl) sulphide.

were found to be most suitable for quantification purposes.

Calibration graphs were obtained by plotting the peak area ratio of the derivative of sulfide to the peak area of the I.S. versus the concentration of sulphide. The calibrated concentration range was 10–100 μ*M*/l. The calibration curve was linear, passed the origin and the coefficient of correlation was 0.99 or better. The standard solutions are very unstable and their expiration period was found to be 24 h (if kept in the dark and at 4 °C).

3.4. Precision, accuracy and limit of quantitation

The precision of the analyses was calculated as a relative standard deviation (RSD) 2.69% of heptaplicate of real sample (concentration was 66.9 μ*M*/l). Due to unavailability of reference material for the determination of blood sulphide, the accuracy was determined by the analyses of spiked blood samples. The results are shown in Table 1.

The limit of quantification was determined as a lower limit of the linearity range and was found to be 3 μ*M*/l.

3.5. Storage of samples

Because hydrogen sulphide is such a volatile compound, great attention must be paid to the storage of samples. The concentration of sulphide in blood frozen at −18 °C was found to decrease in days and thus the samples have to be stored at −80 °C. The samples were found to be stable for at least 1 week at this temperature.

4. Discussion

The proposed method was optimised for use in the case of healthy persons or patients suffering from large bowel diseases. Sulphide measurement in blood is difficult because of its volatility, susceptibility of oxidation and binding to organic molecules, probably protein. A set of samples ($n=15$) obtained from healthy controls was analysed and results were in the range 35–80 μ*M*/l. This is in agreement with results obtained by other, more laborious methods [3,9].

Interpretation of results from patients requires further research with a special emphasis to the nature of anaerobic fermentation. It is influenced by many

Table 1
Accuracy of blood sulphide determination (original blood level 53.5 μ*M*/l)

S^{2-} added (μ*M*/l)	Number of determinations	S^{2-} detected (μ*M*/l, mean)	S^{2-} calculated (μ*M*/l, mean)	Deviation from calculated value (%)
12.5	6	64.1	66.0	2.9
25.0	6	76.4	78.5	2.7
37.5	6	88.2	91.0	3.1

factors, such as microbial composition, dietary and genetic factors. Determination of sulphide has to be interpreted with caution in the consequences with other markers: methane or hydrogen.

The supposed use is to determine sulphide production after the oral application of defined dose of dietary fibre (e.g., lactulose, guar gum) or protein. It may also support to determine a long-term effect of dietary fibre supplements.

5. Conclusion

There is an increasing incidence of non-specific inflammatory diseases and colon cancer in many populations throughout the world. Toxic substances generated in the large bowel, such as hydrogen sulphide, are thought to be one of the possible causes. A simple, sensitive method for analysing sulphide in whole blood as a marker compound of anaerobic fermentation in vivo was developed, which enables monitoring of sulphide produced in the large bowel. It enables further research on this topic and it may also support to evaluate an effect of therapy.

6. Nomenclature

BPFBS bis(pentafluorobenzyl)sulfide
EDTA ethylenediaminetetraacetic acid
GC gas chromatography
I.S. internal standard
MS mass spectrometry
PFBBr pentafluorobenzyl bromide

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