SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Detection of the signaling molecule H_2S in physiological systems

by Tamás Ditrói

Supervisor: Prof. Dr. Péter Nagy, PhD, DSc

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Supervisor: Prof. Dr. Péter Nagy, PhD, DSc
Kálmán Laki Doctoral School, University of Debrecen

Head of the defense committee:	Prof. Dr. Zoltán Papp, PhD, DSc
Reviewers:	Prof. Dr. Gyula Tircsó, PhD
	Dr. Gábor Sirokmány, PhD
Members of the Defense Committee:	
	Prof. Dr. Béla Juhász, PhD
	Dr. Katalin Ősz, PhD

The PhD Defense takes place at Lecture Hall of building 'A', Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 15:00 on 24th November 2023.

I. Introduction and aim of the study

Hydrogen sulfide is a small gaseous signaling molecule that has gained substantial interest in the last decades, after the discovery of its various functions in biological systems. It has been shown to have important roles in inflammation, vascular regulation, tumor growth and can also protect the cardiovascular system. These functions are generally attributed to its signaling properties and its ability to protect against oxidative stress. Due to these, usage of H_2S for therapeutic purposes is already being investigated. However, it should be stated that in high enough concentration, hydrogen sulfide is a toxic gas which can inhibit cytochrome c oxidase by binding to its heme group, similarly to CO. This results in cell death, via the impediment of the electron transport chain and cell respiration.

Multiple enzymes are capable of producing H_2S, although their contributions are still in debate today. This is mainly due to the fact that the expression levels of these enzymes largely vary in a tissue and organ specific manner. Canonically, the most important sulfide producing enzymes in mammalians are cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST), in combination with aspartate aminotransferase (AAT).

Thanks to its various physiological functions, H_2S is now recognized as an important biological molecule, yet to date its clinical diagnostic value is limited by the difficulty of its accurate measurement in biological systems. This is partially attributed to the fact that the majority of sulfide in the blood and tissue is in bound form that is in constant dynamic equilibrium with the free form, which exists in two protonation states, H_2S and HS^- under physiological conditions Furthermore, reported sulfide concentrations in the blood cover multiple orders of magnitudes, even when similar measurement protocols are used, which imply methodological discrepancies.

The easiest method to measure the sulfide content of a solution is a colorimetric approach, which was developed 150 years ago and still in use today. It relies on the reaction of N,N-dimethyl-p-phenylenediamine (DPD) with H_2S to form methylene blue dye that can be easily quantitated. Due to the simplicity of the method, it appears in numerous publications where it is used on biological samples. However, more and more problems arose regarding this method with the advancement of the field, such as incomprehensibly high measured concentrations in a biological context, formation of dimers and trimers that makes evaluation difficult and the use of a highly acidic media, which liberates H_2S from the bound sulfide pool.

Novel developments gradually helped to overcome some of these issues, but there was still a need for a better analytical method.

Electrochemical techniques, such as sulfide sensitive electrodes, did offer a solution to most of the caveats mentioned above, as they did not interfere with the free-bound sulfide equilibrium and were more sensitive than the colorimetric protocols. However, the presence of the biological matrix and the insufficient specificity for sulfide does pose a new challenge for these electrodes, as physiological systems contain thiols and other sulfur-containing molecules that can interfere with the measurements.

Fluorescent probes can be used as means to minimize the effects of the sample matrix, yet at present moment, such probes are far from flawless. Still, probe development is a constantly improving area, which is expected to bring about a very sensitive and selective detection method that could substantially help the research field.

Chromatographic methods are preferred analytical techniques when the target analyte occurs in complex sample matrices. Such methods entail gas chromatography, which can be used to measure free sulfide levels without derivatization. This however requires a sulfide-specific fluorescent detector and head-space sampling, but its sensitivity is extremely high, well under the nmol/l level. The main drawbacks of this approach are the specialized instrumentation and sample preparation.

Methods employing liquid chromatography usually have a simpler sample preparation procedure. These approaches are based on selective derivatization reactions with an alkylation agent, most frequently with monobromobimane (MBB), whose reaction with sulfide generates the fluorescent sulfide-dibimane. MBB also reacts with any -SH groups, therefore it also fluorescently labels thiols, polysulfides and certain oxidized sulfur species. With a suitable chromatographic separation, sensitive and selective detection can be achieved, with lower detection limit under the nmol/l range. The MBB method is widely used and has been utilized in numerous studies in different biological matrices, although the different reports did not agree on the basal sulfide levels. This might arise from the fact that these works did not use the same exact sample preparation protocols, but employed various experimental setups.

The aim of this work was to shed light on the differences found in the literature by thorough examination of the monobromobimane method and establish a protocol that is easy, accurate, reliable and produces globally comparable results. Our intent was to validate this method by obtaining biologically relevant samples from actual patients and see if the method can be a potential candidate as clinical diagnostic procedure in the future.

II. Methods

Blood samples used for method development were taken from 14 healthy volunteers into Li-Heparine or clot-activator containing blood collection tubes in addition to K_2EDTA and citrate containing tubes. Samples received from international collaborations were taken into Li-heparine tubes. After separation, the collected plasma was stored at -80°C and shipped on dry ice.

Sample preparation and derivatization were carried out in a dark room under red lighting. Precise temperature was achieved by a heating/cooling dry block in amber microcentrifuge tubes, and alkylation times were accurate to the seconds. The pH was set using 200 mM HEPES buffer, and the alkylation was quenched by the addition of 50% trichloroacetic acid (TCA).

Separation of the products was performed on a Thermo Scientific Ultimate 3000 HPLC having a binary pump system, a UV-Vis and a fluorescent detector. LC-MS/MS identification was carried out by connecting a Thermo Scientific LTQ-XL mass spectrometer to the system. The analytes were either separated on an Agilent XDB-C18 (250x4.6mm, 5 μm) column during method development or on a Phenomenex Luna C18(2) (200x4.6mm, 3 μm) column for routine measurements. Both separations applied gradient elution with a mixture of 0.1% TFA/water and 0.1%TFA/ACN.

Hemolysis was followed by recording the UV-Vis spectra of the sera or plasma samples between 400-800 nm in a 384-well plate using a Tecan Spark 10M multimode reader.

Data evaluation and statistical analysis were performed using Microsoft Excel 2016 or Graphpad Prism 5.03, significant differences were determined by Student's t-test, Mann-Whitney test or ANOVA with Tukey post-hoc test.

III. New results of the thesis

III.1. A standardized assay was developed for the measurement of H_2S from blood samples and the most critical parameters affecting the results were identified.

III.1.1. Basic kinetic properties of the reaction between H_2S and monobromobimane were studied.

Using aqueous H_2S standard solutions, an appropriate detection method was set up and the kinetics of the alkylation reaction with monobromobimane was studied. In accordance with our expectations, exponential functions fitted well to the observed kinetic curves and the system follows pseudo-first order kinetics. It can be stated that the reaction reaches completion in 7 minutes regardless of the sulfide concentration in the sample.

Product analysis has also shown, that in contrast to previously published data, out of the two steps of the reaction, the first step (i.e. the formation of sulfomonobimane) is the slower, rate determining step.

III.1.2. We found that sulfide levels determined in blood by the monobromobimane method do not represent free H_2S, rather a mix of free and bound sulfide.

Kinetic experiments were repeated with blood samples instead of standard solutions, and the derivatization was still incomplete after 28 hours.

As all the parameters affecting the kinetics of the reaction were unchanged, the reaction of MBB with free H_2S should have reached completion within 7 minutes. The much slower reaction that we have observed can be attributed to the release of H_2S from the bound sulfide pool. This is supported by the fact that the measured concentrations above 10 μM are considered toxic due to the inhibitory effect of sulfide on cytochrome c oxidase.

Therefore, a simple explanation is the presence of a bound H_2S-reserve consisting of different sources, which can be independently studied as they are liberated upon specific impacts, like reduction or acidification, as discussed in the literature. The sulfide reserves (called pools) play an important buffering role as they can supplement H_2S if necessary, as a slow releasing biological sulfide donor system.

On the other hand, sulfide pools can act as a buffer system and absorb free sulfide in case of a sudden and potentially toxic increase in its level, in order to counteract its harmful effects.

III.1.3. Parameters affecting the reaction between monobromobimane and hydrogen sulfide were identified

As the derivatization reaction does not reach completion due to the constant H_2S release from the pool, apart from the actual concentration of free sulfide, the rates of the reaction and release from the pool will affect our measured level. Parameters affecting the amount of the derivatized product were reagent concentration, pH, alkylation time and temperature.

Alkylation time had a trivial effect: as we let the reaction proceed, we will see more of the product. Overcoming this problem is straightforward, the alkylation has to be precisely timed. We have allowed 1-2 sec of error by timing the reaction precisely using a stopwatch.

We had already assessed the effect of the monobromobimane concentration using standard solutions. With blood samples a similar, linear tendency was observed, higher MBB concentration resulted in higher measured sulfide levels. The linear tendency theoretically allows us to use a simple correction based on deviations in the applied MBB levels measured by the same HPLC method.

pH is an important factor, as it affects the speciation of the H_2S-HS^- equilibrium and simultaneously liberate pH-sensitive pools. As a nucleophile substitution reaction with HS^- as the attacking nucleophile, it is crucial to tightly control the pH during the derivatization reaction. Our experiments showed that with increasing pH, the measured sulfide level also increased, which matches previous findings and shows the importance of pH during the alkylation process.

Temperature is also crucial in studies involving reaction rates. Published methods using monobromobimane all simply refer to „room temperature" which makes it difficult to reproduce these results without knowing the exact temperature. Even national and supranational pharmacopoeias define RT as anything between 15-25°C. However, our experiments have shown a really significant temperature dependence of the reaction rate, highlighting the importance of this loosely controlled parameter.

A difference of 5°C was shown to introduce a 2-fold increase in the measured H_2S level, which is quite significant, as it makes results incomparable and can largely impair the value of entire studies by either introducing artifact differences or masking differences by enlarging errors within groups. Therefore, we strongly recommend the tight and accurate control of this parameter to ensure reliable results.

III.1.4. Chemical, physical and biological parameters of sampling and sample preparation affecting the measured sulfide level were investigated and the method was compared to other published protocols.

Parameters affecting the rate of the reaction are not the only variables that have an effect on the measured sulfide levels. Multiple experimental parameters were identified that can change our measured results, while not affecting the alkylation reaction.

Monobromobimane used as the alkylating agent is a light-sensitive molecule, therefore it is recommended to only work with it in dim or dark environment. We have prepared samples in different light setting from dark rooms to sunlit laboratory benches. During this experiment, the formation of the product SDB was monitored apart from the consumption of MBB. As expected, samples subjected to sunlight had notably decreased MBB and SDB levels, while in samples under fluorescent lightbulbs, only SDB levels were lower. Red and warm-white LED lights did not induce degradation, therefore they are suitable light sources to be used during sample preparation.

Similarly to our method, published protocols with one exception employ acidification to quench the alkylation reaction. Variations are found in the type of acid used (trichloroacetic acid, formic acid, sulfosalicic acid), but their mode of action is essentially the same. At low pH, the reaction rate is diminished due to the shift in the acid-base equilibrium of H_2S, while proteins are also precipitated, allowing us to stop the reaction and remove proteins by centrifugation in the next step. One published method recommend extraction with ethyl acetate, as both SDB and MBB is extracted into the organic layer while proteins stay in the aqueous phase. The organic solvent can be evaporated and the solids redissolved ready to be injected onto the HPLC. We have compared the two quenching methods and found no difference in the results, therefore we recommend the use of acidification to quench the reaction, due to its simplicity and lower chance of experimental error. One advantage of the extraction method is the reduced sample complexity, but this problem is dismissed by the use of high performance liquid chromatography.

Closely related to the pH-dependence we have also investigated the effect of different buffer systems. We have not observed significant differences between HEPES, Tris-HCl and phosphate buffers, although the addition of chelating agents, such as DTPA or EDTA increased the measured sulfide level.

DTPA is usually used in monobromobimane-based methods to inhibit the metal ion catalyzed autoxidation of H_2S. While the theory is valid, we have

not observed any difference when H_2S standard solutions were processed with or without DTPA. However, the presence of a chelator is inevitable when working with plasma collected into EDTA tubes. Comparing different sampling tubes have revealed that Li-heparin, citrate and even serum samples taken from the same subject had the same sulfide level, while the EDTA plasma resulted in a 9x increase in a certain case.

It has been shown before that EDTA can promote hemolysis, which phenomenon was confirmed by our time-dependent spectrophotometric experiment and the associated increase in the sulfide levels. Our experiments also highlighted the need for a precise blood withdrawal method, as the volume taken into the tube (and therefore the effective EDTA concentration) has a direct effect on the sulfide level. It is also important to emphasize that it is only partially caused by hemolysis, as Li-Heparin plasma transferred into EDTA tubes also showed significantly increased measured H_2S concentration.

Based on this finding, we recommend the use of serum or Li-Heparin plasma samples for H_2S determination, as we can prevent a serious and poorly controllable phenomenon from introducing an error to our measurement.

Our aim was also to demonstrate the disagreement between different monobromobimane-based protocols, therefore we have chosen 3 already published methods along with our new method for comparison. As expected, the different protocols gave different results all following the parameter dependences that we have observed before. These result support our notion that a standardized, reliable protocol is necessary to measure blood H_2S levels in a comparable manner.

III.2. Reliability and usefulness of the newly developed method was validated using healthy and patient samples. H_2S levels in patients with homocystinuria were also investigated.

III.2.1. Method reproducibility and possibility of sample storage was demonstrated.

Based on our findings and by tightly controlling every parameter during sample preparation we have demonstrated that measured H_2S concentration values are reproducible with the developed method. With a less than 5% deviation, we have even surpassed another MBB based method using mass spectrometry. Another important finding is that samples kept frozen can be stored for more than a month without perceptible H_2S loss. This gives us the

opportunity for example to study rare diseases where sample collection can take a long time and the whole cohort can be processed together minimizing measurement error. On the other hand, it allows us to take part in collaborations where samples have to be shipped from the collection site to the measuring laboratory.

It was also investigated whether the freeze-thaw cycle introduces any change. We have not observed any difference between fresh and frozen serum and plasma samples, hence we can safely assume that frozen/thawn and fresh samples represent the same, biologically relevant sulfide level.

III.2.2. The method was validated for clinically relevant usage by measuring blood samples from ETHE1 deficient patients with largely increased H_2S levels

To ensure that the method is appropriate to show clinically relevant differences, plasma samples from patients with ethylmalonic encephalopathy was obtained from a Czech collaborator. The defect of the *ETHE1* protein, which is a key enzyme of H_2S catabolism, causes hydrogen sulfide to accumulate in blood. The large increase in the patients' H_2S levels versus healthy subjects was clearly demonstrated by our method supporting its use in the clinical field.

III.2.3. Sulfide-donor property of the novel NSAID drug ATB-346 was demonstrated by our method

We had the opportunity to receive samples through a collaboration from an ongoing phase IIb drug study that investigated the GI safety of a NSAID ATB-346. The drug is a naproxen-derivative that has been enhanced by the addition of a sulfide-donor group developed by the Canadian Antibe Therapeutics Inc. This study focused on the appearance of gastrointestinal safety by monitoring the appearance of ulcers versus naproxen controls. They have found that ATB-346 is not only a more effective analgestic agent than naproxen, but the incidence of ulcers was much lower too.

To confirm its sulfide-donor property, we have received blood samples from both naproxen and ATB-346 groups for H_2S determination. We have observed a significant, 50% increase in H_2S levels in ATB-346 treated patients, thus confirming that the drug is effective and the method is able to identify differences upon sulfide-donor treatment.

III.2.4. Patients with homocystinuria were studied to find connection between H_2S and certain enzymatic defects

Provided by a Czech collaborator, plasma samples from patients with rare diseases affecting the transsulfuration pathway were obtained for H_2S measurement. We have received samples from patients with homocystinuria, from patients with phenylketonuria as controls due to their similar diet and from healthy individuals. In patients with homocystinuria, homocysteine accumulates in their blood causing serious problems in the nervous system. The most common cause of homocystinuria is the defect of cystathionine-β-synthase (CBS) enzyme, where inactivity of the enzyme results in the accumulation of its substrate, homocysteine and consequently methionine. Defect in the remethylation cycle however inhibits the conversion of homocysteine to methionine, therefore increasing the level of the former and decreasing the latter.

As mentioned above, H_2S can be produced by several enzymatic pathways, but most prominent enzymes are the ones in the transsulfuration pathway, mostly CBS. Therefore, presuming that CBS activity is inhibited, one would expect that the loss of a sulfide-producing enzyme would cause decreased H_2S levels. In the case of patients with remethylation defects, the increased homocysteine availability as a substrate for sulfide production would be expected to result in an increase in the sulfide levels.

Our results however did not match our expectations, patients with CBS defects had similar H_2S levels to control patients, while remethylation defect did effectively slightly, but significantly lowered the measured sulfide levels.

While these findings might contradict our hypothesis, in another study while we tried to find correlation between H_2S levels and CBS, we could not see any significant connection. This can be attributed to two factors, on one hand the ratio of the two H_2S producing enzymes of the transsulfuration pathways differs from organ to organ changing their significance in sulfide production. On the other hand, it is suspected that sulfide levels are controlled through its catabolism as we have seen large increase in EE patients.

IV. Potential applications

The main goal of the presented work was to develop a method that can measure H_2S in blood samples in a reliable, sensitive, accurate and reproducible manner. Comparison of sulfide levels in blood based on previous publications is impossible due to the finding presented in this work, but this problem can be resolved using a validated, standard method. Our aim was to make the method as easy as possible, so users with minimal instrumentation can reliably measure sulfide levels, without the need of special sample withdrawal procedure, hypoxic chamber or mass spectrometry.

Due its accuracy, the method presented herein can be applied to study small changes that are of clinically relevant, something that was unattainable earlier, due to low accuracy and the incomparability of the results.

At the moment, measurement of H_2S in blood is not yet introduced into clinical diagnostic practice, due to the lack of a suitable method and sufficient knowledge on the interpretation of the results. However, with the constant advances of the field, H_2S level might become a useful indicator of certain diseases or their progression during treatment. Given that our method is easy, cost effective and rapidly generates results, it has the potential to be used in screening or confirmation rather than implementing more expensive, more complicated diagnostic techniques.

V. Summary

The main objective of the work presented here was to establish a new method for the measurement of hydrogen sulfide in blood samples that is more reliable and reproducible than the ones that are already published. Exact parameters of the sample preparation procedure were systematically studied to assess their effects on the actual measured H_2S concentration. This study shed light on multiple shortcomings of the previously published methods, like the inaccurate description of the temperature used during the alkylation step, which was shown to have an extreme effect on the results. Comparing published methods, we have shown, that these inaccuracies together with the different parameters used during sample preparation can be behind the observed large discrepancies in measured blood H_2S levels.

The primary reason identified behind these findings was the fact, that this method does not actually measure the so-called free sulfide levels (H_2S and HS^-). This can be attributed to the blood sulfide-pool that is in dynamic equilibrium with free sulfide levels, constantly and slowly releasing it as it is consumed by the alkylation agent monobromobimane.

Based on our findings, we have chosen a certain parameter set and developed and validated a method for blood H_2S determination that is reliable and reproducible, ensuring that results produced will be comparable unlike before. We have shown that the reproducibility exceeds those of previous methods and that freezing/thawing does not affect the results, therefore sera/plasma can be collected and analyzed later.

To validate its usability in clinical studies, multiple human samples were provided by our collaborators and assayed using the new method. We had a chance to analyze blood samples from patients suffering from ethylmalonic encephalopathy, where the expected high sulfide levels were unambiguously proven by our method. We were also given a chance to receive blood samples from a Phase 2 study of an NSAID that has a sulfide-donor property. Treated patient blood samples showed a slight but significant increased sulfide level versus the control group proving that the drug has the expected effect and our method is capable of distinguishing such small differences.

The method was also used on samples obtained from patients with rare genetic diseases affecting sulfur metabolism. Our initial hypothesis, that defects in enzymes responsible for H_2S production would translate into changes in bioavailable blood sulfide levels, was however disproved by our measurements. This can be attributed to the more pronounced influence of the catabolic enzymes on H_2S levels as we have seen it in the case of ethylmalonic encephalopathy.

VI. Publications

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Registry number: DEENK/202/2023.PL
Subject: PhD Publication List

Candidate: Tamás Ditrói
Doctoral School: Kálmán Laki Doctoral School
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List of publications related to the dissertation

1. Wallace, J. L., Nagy, P., Feener, T. D., Allain, T., **Ditrói, T.**, Vaughan, D. J., Muscara, M. N., Nucci, G., Buret, A. G.: A proof-of-concept, Phase 2 clinical trial of the gastrointestinal safety of a hydrogen sulfide-releasing anti-inflammatory drug.
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List of other publications

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