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Analytical measurement of discrete hydrogen sulfide pools in biological specimens

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ABSTRACT

Hydrogen sulfide (H_2S) is a ubiquitous gaseous signaling molecule that plays a vital role in numerous cellular functions and has become the focus of many research endeavors, including pharmacotherapeutic manipulation. Among the challenges facing the field is the accurate measurement of biologically active H_2S. We have recently reported that the typically used methylene blue method and its associated results are invalid and do not measure bona fide H_2S. The complexity of analytical H_2S measurement reflects the fact that hydrogen sulfide is a volatile gas and exists in the body in various forms, including a free form, an acid-labile pool, and bound as sulfane sulfur. Here we describe a new protocol to discretely measure specific H_2S pools using the monobromobimane method coupled with RP-HPLC. This new protocol involves selective liberation, trapping, and derivatization of H_2S. Acid-labile H_2S is released by incubating the sample in an acidic solution (pH 2.6) of 100 mM phosphate buffer with 0.1 mM diethylenetriaminepentaacetic acid (DTPA), in an enclosed system to contain volatilized H_2S. Volatilized H_2S is then trapped in 100 mM Tris–HCl (pH 9.5, 0.1 mM DTPA) and then reacted with excess monobromobimane. In a separate aliquot, the contribution of the bound sulfane sulfur pool was measured by incubating the sample with 1 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride), a reducing agent, to reduce disulfide bonds, in 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA), and H_2S measurement was performed in a manner analogous to the one described above. The acid-labile pool was determined by subtracting the free hydrogen sulfide value from the value obtained by the acid-liberation protocol. The bound sulfane sulfur pool was determined by subtracting the H_2S measurement from the acid-liberation protocol alone compared to that of TCEP plus acidic conditions. In summary, our new method allows very sensitive and accurate measurement of the three primary biological pools of H_2S, including free, acid-labile, and bound sulfane sulfur, in various biological specimens.

Introduction

Hydrogen sulfide (H_2S) is a ubiquitous gaseous signaling molecule that plays an important role in numerous cellular functions and has become the focus of many research endeavors, including pharmacotherapeutic manipulation [1–5]. Hydrogen sulfide is produced predominately from cysteine, by two pyridoxal-5′-phosphate-dependent enzymes, cystathionine-β-synthase and cystathionine-γ-lyase, as well as by 3-mercaptosulfurtransferase. Hydrogen sulfide can diffuse across cellular membranes without the need for a specialized transporter [4,6]. At pH 7.4 and temperature of 37 °C, 18.5% of free hydrogen sulfide exists as H_2S gas and the remainder is almost all hydrosulfide anion (HS^-) with a negligible contribution of S^{2-} [7,8]. It is long been known that the sulfur exists in the body in several forms, ranging from a fully reduced divalent state as sulfide to a fully oxidized hexavalent state as sulfate [1,9,10]. Measurement of biologic sulfur has focused on measuring sulfide, i.e., in the reduced divalent state, in part because of difficulties in accurately measuring other states. Additionally, sulfur equivalents in the reduced divalent state are very reactive within biological matrices, resulting in sulfide equivalents being present in various volatile sulfur pools. It is increasingly clear that these pools, in addition to free hydrogen sulfide, are important in regulating the amount of bioavailable sulfur, with the most relevant being the acid-labile and bound sulfane sulfur pools [10,11]. Fig. 1 illustrates the various volatile sulfide pools with associated chemical species.

Sulfane sulfur refers to divalent sulfur atoms bound only to other sulfur, although they may bear an ionizable hydrogen at some pH values. These include thiosulfate $S_2O_3^{2-}$, persulfides

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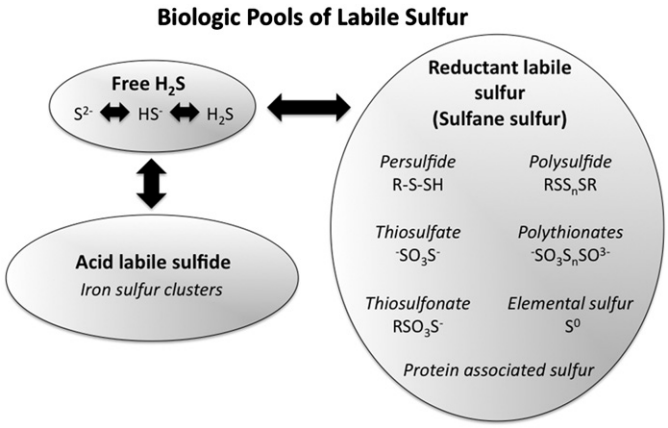

Fig. 1. Biological pools of labile sulfur.

R-S-SH, thiosulfonates R-S(O)–S-R′, polysulfides R-S_n-R, polythionates $S_nO_6^{2-}$, and elemental sulfur S^0 [10]. Acid-labile sulfide, the other major pool, consists of sulfur present in the iron–sulfur clusters contained in iron–sulfur proteins (nonheme), which are ubiquitous in living organisms and include a variety of proteins and enzymes including rubredoxins, ferredoxins, aconitase, and succinate dehydrogenase [10,12]. Some have claimed that the process of acid liberation may also release hydrogen sulfide from persulfides, which have traditionally been classified as sulfane sulfur [13]. This pool of sulfur has been postulated to be a "reversible sulfide sink" and may be an important storage pool, which regulates the amount of bioavailable free hydrogen sulfide that is available [14], though others have suggested that bound forms may be more important in storage and release of exogenously administered sulfide [11].

The Achilles heel of the sulfide field has been the lack of precise methodology for the accurate and reproducible measurement of hydrogen sulfide both in vivo and in vitro. A variety of methods to measure free H_2S have been employed, with divergent results [10,13,15]. These include a spectrophotometric derivatization method resulting in methylene blue formation, variations of this using high-performance liquid chromatography (HPLC) [10], sulfide ion-selective electrodes, polarographic sensors [16], gas chromatography [13,17], and HPLC in conjunction with fluorimetric-based methods using monobromobimane (MBB) to derivatize free H_2S [14,18]. The levels so determined range from nanomolar to hundreds of micromolar concentrations [10,15]. This is due to the fact that the previously favored methylene blue method of hydrogen sulfide detection results in interference from bound sulfide pools coupled with the fact that this method is subject to chemical artifacts and unable to measure bona fide hydrogen sulfide at analytical or physiological concentrations [18]. Compelling arguments against micromolar concentrations reported by older studies [19] are further reiterated by the effect of local nanomolar concentrations affecting biologic function [20]. Indeed, the existence of storage pools of sulfide that can release H_2S along with the volatility and spontaneous oxidation of H_2S may help explain these apparent paradoxes.

Earlier attempts to characterize the various biologic pools of sulfur have utilized MBB in conjunction with dithiothreitol (DTT) as a reducing agent [10,21] or have focused on the free hydrogen sulfide and acid-labile pools alone [11,13]. These study results were limited because of various problematic issues such as pH, volatilization, and oxidation. Here we report a new protocol that measures all relevant biologic hydrogen sulfide pools, namely free hydrogen sulfide, acid-labile sulfide, and bound sulfane sulfur. The results highlight the ability to analytically and comprehensively measure hydrogen sulfide bioavailability in biologic specimens.

Principles

The fluorescent reagent MBB has been widely used to measure various thiol-containing species through alkylation [22]. S-alkylation occurs twice with sulfide under alkaline conditions, forming sulfide dibimane. Our lab has previously published an analytical method of measuring free plasma hydrogen sulfide in vivo and in vitro by derivatization of sulfide with an excess of monobromobimane under alkaline, 1% oxygen, trace-metal-free conditions with RP-HPLC separation and fluorescence detection of the sulfide dibimane product [18].

The release of hydrogen sulfide from the acid-labile pool requires a pH less than 5.4 [11]. Thus the determination of acid-labile sulfide involves acidification of the sample, performed by adding 450 μl of 100 mM phosphate buffer (30 μM H_3PO_4 and 70 μM KH_2PO_4, pH 2.6, 0.1 mM diethylenetriaminepentaacetic acid (DTPA)), causing release of free hydrogen sulfide into the headspace of a Vacutainer tube from the acid-labile pool. After removal of this solution, 100 mM Tris–HCl buffer (pH 9.5, 0.1 mM DTPA) is added and hydrogen sulfide gas is redissolved back into the buffer and the sulfide level is measured by the MBB method. The result reflects both free hydrogen sulfide and hydrogen sulfide released from the acid-labile pool.

The sulfane sulfur component is determined by treatment with tris(2-carboxyethyl)phosphine hydrochloride (TCEP), which cleaves disulfide bonds to liberate the sulfane sulfur atom. Although DTT has been used by others [10,11,21,23] for this purpose, TCEP is water soluble, is nonvolatile, reduces disulfide bonds more rapidly, and has been shown to be very stable across a wide range of pH (2.0–9.5), unlike DTT [24]. TCEP also does not have a thiol moiety and has the additional advantage of not requiring removal before reaction with MBB, in contrast to DTT, which contains a thiol moiety and has been reported to have small amounts of sulfide contaminants [21].

However, reductive dehalogenation of monobromobimane by TCEP with the creation of a fluorescent product has been reported, with the potential for interference [25]; although this product can be extracted by methylene chloride, it is not necessary as HPLC analysis of the reaction mixture separates this product from sulfide dibimane. We found that the product of TCEP and MBB has a retention time of 11.2 min (data not shown), whereas the product of sulfide and MBB, i.e., sulfide dibimane, has a retention time of 16.5 min, and MBB alone has a retention time of 17.6 min. To overcome TCEP consumption of MBB as well as any inhibitory effects on the dye as have been reported with iodoacetamide and maleimide dyes [26], we used a ratio of greater than 10:1 MBB to TCEP.

Persulfide formation normally occurs at alkaline pH at room temperature, and persulfide can be measured by absorbance at 335 nm using a spectrophotometer [27]. Free hydrogen sulfide released into the headspace can react with plasma proteins to form persulfide as reported below using spectrophotometry. Thus, removal of plasma from the reaction vessel after volatilization of hydrogen sulfide into the headspace is necessary before retrapping the volatilized hydrogen sulfide gas in alkaline solution for subsequent reaction with MBB.

Materials

1) Monobromobimane (Sigma–Aldrich, Cat. No. B4380)
2) Sodium sulfide (Alfa Aesar, Cat. No. 65122); this product has been demonstrated to have superior purity compared to other sources [8]