Point-of-care testing and optimization of sample treatment for fluorometric determination of hydrogen sulphide in plasma of cardiovascular patients

Ahmed K. Alyan a, Rasha S. Hanafib, *, Mohamed Z. Gadc

a Sepctrum Diagnostics, Industrial Zone, Obour City, 11811 Cairo, Egypt
b Pharmaceutical Chemistry Department, Faculty of Pharmacy and Biotechnology, German University in Cairo, 11835 Cairo, Egypt
c Biochemistry Department, Faculty of Pharmacy and Biotechnology, German University in Cairo, 11835 Cairo, Egypt

HIGHLIGHTS

- A comparative sample preparation methodology for fluorometric determination of H2S in plasma.
- Sample treatment protocol is critical to reliable results for determination of gasotransmitter.
- Portable H2S Analyser was designed, manufactured and verified as POCT for the gas in the ambulance.
- Validation in human plasma proved efficiency of H2S Analyser in determining H2S in MI patients.
- H2S is elevated in MI patients compared to normal controls up to 10 h from emergence of symptoms.

ABSTRACT

Introduction: Hydrogen sulphide (H2S) is one of the gasotransmitters that was reported to have a cardio-protective effect at its physiological levels in blood. Previous determinations of H2S levels in cardiovascular disease (CVD) patients suffered from diversity of analytical methods, different targeted chemical forms of the gas, and multitude of matrices assessed.

Objectives: In this study, a comparative biological sample preparation study is detailed for optimum selective determination of the unionized form of H2S in blood of CVD patients using a new in-house POCT portable spectrofluorometer together with a Reagent-Analyser system.

Methods: Dansyl azide was synthesized to react with hydrogen sulfide in biological matrix to produce the fluorescent dansyl amide. Fluorescence was measured at \( \lambda_{ex} \) 340 nm and \( \lambda_{em} \) 517 nm in the new in-house POCT portable spectrofluorometer. The method was validated according to ICH guidelines. Several blood sample treatments and reaction protocols were compared to achieve maximum fluorescence yield.

Results: The H2S Analyser was verified in comparison to a benchtop spectrofluorometer where linearity was confirmed in the range of 3–300 \( \mu \)M, LOD being 1 \( \mu \)M, at \( \lambda_{ex} \) 340 nm and \( \lambda_{em} \) 517 nm. Sample
Hydrogen sulphide (H₂S) is a colourless gas that has been recently associated with several physiological effects, among which is its pivotal role in regulating several cardiovascular related effects. In fact, many researchers started considering H₂S as the third gaseous mediator along with carbon monoxide (CO) and nitric oxide (NO). At physiological pH, 67% of H₂S exist as sulphide ion (HS⁻), 33% is un-dissociated as H₂S while S²⁻ is present in negligible quantity [1–4]. One of the most blossoming roles of H₂S is its cardio-protective actions on the heart and blood vessels, via an array of cellular and molecular circuits [1–5]. Our research group was able to identify H₂S as a novel early biomarker for detection and diagnosis of myocardial infarction (MI) patients as early as 1–2 h from emergence of angina symptoms in serum of ST-elevated myocardial infarction (STEMI) patients by GC–MS [6]. However, reviewing selective methods of H₂S determination and the associated reported levels of the gas, it appears that assay methodology including sample preparation and focus on the molecular form need further enrichment and improvement. In fact, different research groups reported variable levels of the gas, or did not explicitly define whether the ionic or molecular forms were included in quantitation, all leading to confusion and controversy about levels of this critical molecule in biological systems [7,8]. Although spectrophotometric determination using methylene blue/zinc trap method is generally considered the most common technique for determination of H₂S, however, it lacks selectivity via interfering coloured substances and formation of dimers and trimmers in addition to its poor sensitivity at physiological levels of H₂S [9]. In the last few years, a number of sensitive fluorescent probes were developed to quantify H₂S, in-vitro models and bioimaging [10–20]. One of the high quantum yield synthetic probes is Dansyl amide (DNS-Am), designed by Peng et al. where selective reduction of the azido group by H₂S to amido group leads to the formation of the fluorescent form Dansyl amide (DNS-Am). The probe is characterized by its stability for long time storage, speed of reduction reaction (few seconds), selectivity for H₂S determination (with no interference from 18 anions Cl⁻, Br⁻, I⁻, F⁻, OH⁻, OAc⁻, CN⁻, N₃⁻, NO₂⁻, HCO₃⁻, HSO₃⁻, SO₃²⁻, S₂O₃²⁻, S₂O₅²⁻, HPO₄²⁻ and citrate), and linearity in laboratory prepared solutions [21]. However, no proper quantification in human plasma is reported nor results using this probe in cardiovascular patients. The aim of this study is to provide a proper simple pre-treatment methodology for determination of molecular H₂S in human blood using DNS-Az followed by method validation according to ICH Guidelines to represent a sensitive, selective and rapid system for H₂S determination in biological fluids. Moreover, the work presented here applies this methodology on myocardial infarction (MI) patients and control subjects to compare levels of H₂S in both groups and support that the gasotransmitter can act as an early predictive biomarker for MI. Furthermore, this study aims to design and develop a full point of care system (Reagent and Analyser) for measuring H₂S that could be done as a bedside test where the patient is receiving care.

Materials and methods

Synthesis and spectral characterization of the probe 1, 5 Dansyl azide (DNS-Az)

DNS-Az was synthesized according to the method of Peng et al. by addition of white crystals of sodium azide (NaN₃) (Sorachim, Spain) to Dansyl chloride (DNS-Cl) (Sigma Aldrich, USA) [21]. A suspension of 64.3 mg (0.23 mmol) of DNS-Cl was dissolved in 15 ml ethanol (Sigma, USA) and added to a stirred solution of 64.2 mg (0.98 mmol) of NaN₃ dissolved in 7 ml of water and ethanol mixture and stirred for 3 h at room temperature. The ethanolic layer was evaporated under vacuum to produce an aqueous suspension of DNS-Az, which was extracted by dichloromethane (DCM, HPLC grade, Sigma) in a separating flask and the lower organic phase was filtered over anhydrous magnesium sulphate (MgSO₄, Sigma, Germany). The DCM layer was evaporated under vacuum at 40 °C where light yellow oil was produced. The compound was purified by flash chromatography using PuriFlash 4100 (Interchim, France) with Dichloromethane/ Hexane (1:1) as eluent. For spectral characterization, 50 mM stock solutions of DNS-Cl and DNS-Az in absolute ethanol were prepared (synthetic yield was 40% and purity was 97% as per the peak purity calculation of the UPLC-PDA-MS-MS). The stock solutions were added into 2.0 ml of PBS/Tween 0.5% w/v to make a final concentration of 100 μM. The absorbance spectra were scanned from 250 to 400 nm, followed by scanning emission spectra from 400 to 600 nm upon excitation at 340 nm. In addition, UPLC-ESI-MS-MS (Waters ACQUITY Xevo TQD, USA), and NMR (Varian Mercury 400 plus) were used to confirm the identity of DNS-Az. (Suppl. Figs. 1–9).

Reaction of the probe DNS-Az with H₂S and fluorescence measurement of product DNS-Am (P-DNS-Am)

A 50 mM stock solution of DNS-Az in absolute ethanol and 10 mM stock solution of sodium sulphide (Na₂S) in phosphate buffer saline (PBS, pH 7.4) were freshly prepared immediately prior to any experimental work. The stock solution of DNS-Az was added into 2.0 ml of PBS/tween 0.5% to make a final concentration of 100 μM. Na₂S solution was added to a final concentration of 100 μM. The solution was mixed and kept at room temperature for 5 min in dark. The excitation spectrum of the product P-DNS-Am was scanned from 250 to 400 nm, in addition to its emission spectrum from 400 to 600 nm at excitation wavelength of 340 nm. Alternating scans of excitation (250–410 nm) and emission (400–600 nm) at a fixed wavelength of each (λex 517 nm and λem 340 nm, respectively) were performed to accurately determine the values for maxima. To compare the spectrum of P-DNS-Am to standard Dansyl amide (S-DNS-Am, Sigma aldrich, USA), 50 mM stock solutions of S-DNS-Am in absolute ethanol was prepared, added into 2.0 ml of PBS/Tween 0.5% w/v followed by scanning excitation and emission at same ranges and values of P-DNS-Am.
Linearity of the response for the reaction of DNS-Az with H$_2$S in laboratory prepared solutions

A 50 mM stock solution of DNS-Az in ethanol and a 10 mM stock solution of Na$_2$S in deionized water were prepared. Na$_2$S serial dilutions of concentrations 10–2500 μM were prepared. The stock solution of DNS-Az was added into 2.0 ml of PBS/tween 0.5% to make a final concentration of 400 μM DNS-Az. Na$_2$S solution was then added to make final concentration of 1–250 μM. The solution was mixed thoroughly and kept at room temperature for 5 min in dark. The emission spectrum of the reaction product P-DNS-Am was measured at excitation wavelength of 340 nm, and intensity values at $\lambda_{em}$ 517 nm were plotted against Na$_2$S concentrations to obtain a calibration curve for the product P-DNS-Am.

To compare linearity of fluorescence product P-DNS-Am to standard S-DNS-Am, 1 mM stock solution S-DNS-Am in absolute ethanol was prepared, followed by serial dilutions in PBS/tween 0.5% w/v to final concentrations in the range of 1–250 μM. The emission spectrum was measured at $\lambda_{ex}$ 340 nm, and intensities at 517 nm were plotted against S-DNS-Am concentrations to obtain a calibration curve for the standard S-DNS-Am.

Linearity of the response for the reaction of DNS-Az with H$_2$S in human blood

Four different methods of sample treatment were conducted to select the one that produces linearity of response in biological fluid. In the 4 methods (A to D), DNS-Az and Na$_2$S were added either to whole human blood or to human plasma (Fig. 1).

Method A: Addition of DNS-Az on whole blood followed by Na$_2$S on plasma separated from whole blood

A 50 mM stock solution of DNS-Az in absolute ethanol and a 10 mM stock solution of Na$_2$S in PBS were prepared. Na$_2$S serial dilutions of concentrations 10–3000 μM were prepared. Whole blood was collected on lithium–heparin tubes containing DNS-Az to a final concentration of 500 μM and then centrifuged for 5 min. at 10 °C and 4000 rpm to isolate plasma. Subsequently, 50 μl of each Na$_2$S stock solution was added to 450 μl plasma to a final concentration of 1–300 μM. The emission spectrum was measured at $\lambda_{ex}$ 360 nm. The emission intensities at 500 nm were plotted against sulphide concentrations to obtain a calibration curve.

Method B: Addition of DNS-Az on whole blood, followed by Na$_2$S and DNS-Az on plasma separated from whole blood

The same procedure applied in Method A was followed, with addition of 500 μM DNS-Az on isolated plasma samples prior to Na$_2$S.

Method C: Addition of DNS-Az and Na$_2$S on whole blood, followed by DNS-Az on plasma separated from whole blood

Similar protocol was followed as in Method B with the exception of adding Na$_2$S on whole blood but no later on plasma.

Method D: Addition of both DNS-Az and Na$_2$S on plasma separated from whole blood

Blood centrifugation for isolation of plasma was performed following by addition of 500 μM DNS-Az and Na$_2$S.

Time course study for the stability of fluorescence intensity of P-DNS-Am

The procedure of sample preparation of method D was followed, then monitoring the fluorescence intensity of P-DNS-Am was undertaken every 30 min for 4 h at excitation and emission wavelengths 340 and 517 nm, respectively.

Data processing and statistical analysis

Non-parametric unpaired student-t-test was performed to compare between every two independent groups, where p-value less than 0.05 was considered statistically significant. Data were presented as mean ± standard error of mean (SEM). All the data were statistically analysed using GraphPad Prism 5.00 software. For statistical tests concerning the H$_2$S Analyzer, IBM SPSS statistics...
were expressed as median and percentiles for quantitative non-parametric measures. The following tests were done:

- Comparison between more than 2 patient groups for non-parametric data using Kruskal Wallis test.
- Comparison between two independent groups for non-parametric data using Wilcoxon Rank Sum test.
- Wilcoxon signed rank test for comparison between two dependent groups for non-parametric data.
- Ranked Sperman correlation test to study the possible association between each two variables among each group for non-parametric data.

where p-value < 0.05 was considered statistically significant, and highly significant when it is lower than 0.01 and 0.001.

Subjects included in the study: normal controls and cardiovascular patients

Fifty random healthy subjects were recruited in the study with female: male ratio 9:41 (Table 1). While for CVD patients, 75 unrelated MI patients presenting at the triage of the National Heart Institute (NHI) were recruited. Samples were obtained from patients 1–10 h post chest pain and before admission to the Intensive Care Unit. The study protocol was approved by the Local Ethics Committee and German University in Cairo Ethical Committee (ethical committee number 2017-MZG-04).

Results and discussion

Characterization of DNS-Az probe by liquid Chromatography-Mass spectrometry and NMR

After synthesis of DNS-Az, UPLC-ESI-PDA-MS and NMR were used to confirm identity in addition to absorbance spectra that were sued to discriminate between the starting material DNS-Cl, the probe DNS-Az, and the commercially available S-DNS-Am (Suppl. Fig. 10).

Fluorescence emission of P-DNS-Am

Fluorescence emission spectra of all Dansyl derivatives involved in the study were scanned from 400 to 600 nm at $\lambda_{ex}$ 340 nm for comparison (Suppl. Fig. 12A). The probe DNS-Az had no fluorescence intensity unless reduced to P-DNS-Az upon reaction with H$_2$S. Emission wavelength was found to be 517 nm at excitation 340 nm. The value of $\lambda_{em}$ was confirmed via scanning excitation at $\lambda_{em}$ 517 nm, and locating the global optimum of the spectrum (Suppl. Fig. 12B).

When plasma was the matrix involved in the reduction reaction of DNS-Az with H$_2$S, the value of $\lambda_{em}$ showed a slight bathochromic shift from 340 to 360 nm, while $\lambda_{em}$ shifted from 517 to 500 nm (Suppl. Fig. 12C).

Determination of H$_2$S in PBS/tween 0.5% w/v was linear in the range of 0–250 µM, where the reaction was performed in the dark (Fig. 2, line A). The surfactant 0.5% w/v tween was added to enhance solubilisation of the product P-DNS-Az, and only when DNS-Az was dissolved in PBS before the addition of different Na$_2$S dilutions.

Comparison of linearity range of synthesized P-DNS-Am with the commercially available reference standard S-DNS-Am was undertaken. The two calibrations curves were conducted in the same solvent (PBS/tween 0.5% w/v) and measured at same excitation (340 nm) and emission (517 nm) wavelengths. Both showed linear calibration curves over a high dynamic range of 0–250 µM for prepared P-DNS-Am and 0–70 µM for S-DNS-Am with good correlation coefficients (Fig. 2). The direct and standard addition techniques’ linearity studies showed parallelism (slopes 0.2195 and 0.2123, respectively) which eliminates the probability of matrix and Inner Filter Effects, proving quality of the sample preparation methodology.

Linearity study for determination of H$_2$S in human blood using DNS-Az

Different calibration curves for determination of H$_2$S in human whole blood and plasma were constructed with correspondingly different sample preparation procedures (Fig. 1). The different methods and timings of addition of DNS-Az and Na$_2$S (0–300 µM Na$_2$S) to blood samples showed that the best linearity of response ($R^2 = 0.9989$) was only possible when both DNS-Az and Na$_2$S were
added on plasma after centrifugation of blood samples (Fig. 2, line B), where the linearity interval covers by far the physiological range of H₂S (10–100 μM). It is worth mentioning that adding DNS-Az, Na₂S or both to whole blood showed no correlation and consequently no proportionality between concentration of Na₂S and fluorescence intensity. We believe that as H₂S being the third gasotransmitter together with NO and CO, it could have similar challenges with respect to its analytical determination. NO being detected by Griess method, failed to show linearity in whole blood due to opacity of its matrix and presence of interfering proteins, in addition to suffering from oxidation in whole blood to nitrates [22]. Failure to quantify H₂S in whole blood could be attributed to similar reasons where H₂S is an easily oxidizable molecule. Peng et al were able to develop DNS-Az as a new fluorescent probe and test its efficiency in laboratory prepared solutions. However, their trials to extrapolate the study to human biological sample and consequent sample treatment optimization was missing, which was undertaken in this study followed by validation of this bioanalytical method according to ICH Guidelines. The reported sample treatment here allows measurement of H₂S in human plasma in 2–3 min without the need of the time consuming and costly standard addition method published by Peng et al. Furthermore, the method was tested on 50 normal controls and 75 MI patients. Contradictory to our sample treatment methodology, Peng et al. used DNS-Az to determine H₂S in the blood of 5 mice by adding DNS-Az on both whole blood and plasma after centrifugation of blood samples while adding Na₂S on whole blood only, where a linear calibration curve was obtained [21]. It is worth mentioning that a partial conference poster by Collins et al. in 2013 briefly reports a successful determination of H₂S in human blood; however the full details of measurement were never detailed in the literature [23]. Our study revealed a linear and workable quantification method only when both DNS-Az and Na₂S were added on plasma.

**Table 2**

| Parameter                           | Results            |
|-------------------------------------|--------------------|
| Excitation wavelength (nm)          | 360                |
| Emission wavelength (nm)            | 500                |
| Linearity range (μM)                | 0–300              |
| Regression equation                 | y = 0.2123x + 4.5245 |
| Regression coefficient (R²)         | 0.9989             |
| LOD (μM)                            | 1.0                |
| LOQ (μM)                            | 3.3                |
| Standard deviation (intraday)       | 0.29–2.64          |
| Standard deviation (interday)       | 0.52–5.21          |
| Intraday (% R.S.D)                  | 2.17–11.65         |
| Interday (% R.S.D)                  | 6.06–14.69         |
| Standard deviation of slope         | 0.01               |
| Standard deviation of intercept     | 0.49               |
| Standard error                      | 0.17–1.81          |

**Table 3**

| Concentration of added Na₂S (μM) | Intensity of fluorescence | Mean | SD | R.S.D |
|----------------------------------|---------------------------|------|----|-------|
| 0                                | 3.17                      | 3.84 | 3.76| 3.59  |
| 1                                | 3.51                      | 4.17 | 4.42| 4.03  |
| 5                                | 3.82                      | 4.68 | 4.71| 4.40  |
| 10                               | 6.71                      | 6.47 | 7.05| 6.74  |
| 25                               | 9.44                      | 9.89 | 10.21| 9.84  |
| 50                               | 16.48                     | 17.43| 16.12| 16.67 |
| 100                              | 29.91                     | 26.23| 24.78| 26.97 |
| 150                              | 37.86                     | 39.41| 36.42| 37.89 |
| 200                              | 49.89                     | 45.25| 46.53| 47.22 |
| 300                              | 72.21                     | 69.12| 70.83| 70.72 |
| 400                              | 99.73                     | 95.58| 99.84| 99.971|

**Intraday precision study**

| Concentration of added Na₂S (μM) | Day 1 Intensity of fluorescence | Day 2 Intensity of fluorescence | Day 3 Intensity of fluorescence | Mean | SD | R.S.D |
|----------------------------------|---------------------------------|---------------------------------|---------------------------------|------|----|-------|
| 0                                | 3.17                            | 4.23                            | 4.03                            | 3.81 | 0.56| 14.69 |
| 1                                | 3.51                            | 4.56                            | 4.24                            | 4.10 | 0.53| 12.92 |
| 5                                | 3.82                            | 5.14                            | 4.75                            | 4.57 | 0.67| 14.66 |
| 10                               | 6.71                            | 7.72                            | 6.97                            | 7.13 | 0.52| 7.29  |
| 25                               | 9.44                            | 11.25                           | 10.54                           | 10.41| 0.91| 8.84  |
| 50                               | 16.48                           | 17.31                           | 15.12                           | 16.30| 1.10| 6.74  |
| 100                              | 29.91                           | 27.79                           | 23.26                           | 26.98| 3.39| 12.56 |
| 150                              | 37.86                           | 33.74                           | 34.72                           | 35.44| 2.15| 6.06  |
| 200                              | 49.89                           | 46.81                           | 42.23                           | 46.31| 3.85| 8.31  |
| 300                              | 72.21                           | 67.34                           | 61.79                           | 67.11| 5.21| 7.76  |
| 400                              | 99.73                           | 95.58                           | 99.84                           | 99.971| 0.0013| 0.13% |

**Interday Precision study**

| Concentration of added Na₂S (μM) | Day 1 Intensity of fluorescence | Day 2 Intensity of fluorescence | Day 3 Intensity of fluorescence | Mean | SD | R.S.D |
|----------------------------------|---------------------------------|---------------------------------|---------------------------------|------|----|-------|
| 0                                | 3.17                            | 4.23                            | 4.03                            | 3.81 | 0.56| 14.69 |
| 1                                | 3.51                            | 4.56                            | 4.24                            | 4.10 | 0.53| 12.92 |
| 5                                | 3.82                            | 5.14                            | 4.75                            | 4.57 | 0.67| 14.66 |
| 10                               | 6.71                            | 7.72                            | 6.97                            | 7.13 | 0.52| 7.29  |
| 25                               | 9.44                            | 11.25                           | 10.54                           | 10.41| 0.91| 8.84  |
| 50                               | 16.48                           | 17.31                           | 15.12                           | 16.30| 1.10| 6.74  |
| 100                              | 29.91                           | 27.79                           | 23.26                           | 26.98| 3.39| 12.56 |
| 150                              | 37.86                           | 33.74                           | 34.72                           | 35.44| 2.15| 6.06  |
| 200                              | 49.89                           | 46.81                           | 42.23                           | 46.31| 3.85| 8.31  |
| 300                              | 72.21                           | 67.34                           | 61.79                           | 67.11| 5.21| 7.76  |
| 400                              | 99.73                           | 95.58                           | 99.84                           | 99.971| 0.0013| 0.13% |

**Range and linearity**

A linear relationship was obtained between concentration of added Na₂S and fluorescence intensity over the range of 0–300 μM with R² = 0.9989 (Fig. 2, line B).

**Precision**

The data points of calibration curves were repeated in triplicates on the same day for intra-day precision and on three different days for interday precision (Table 3). Relative standard deviation (RSD) was less than 15% w/w which is acceptable according to ICH guidelines [24].
Limit of detection and limit of quantification

Limit of detection and limit of quantification were found to be 1 and 3.33 μM, respectively.

Robustness of the optimized analytical method towards sample treatment process

H₂S is a volatile gas and can be easily metabolized. To ensure that the method used in this study measures H₂S present in the sample and that the process of blood centrifugation to plasma doesn’t cause loss of H₂S, seven samples of normal controls were tested on both whole blood (method A) and plasma (method D) and intensities were recorded at λ_ex = 360 nm and λ_em = 500 nm. Table 4 shows that variations less than 15% occurred in intensities due to centrifugation of blood, which confirms the robustness of the method during sample treatment.

Stability of fluorescence intensity of P-DNS-Am

The intensity of fluorescence of P-DNS-Am was stable during 4 h, which concludes human plasma matrix does not cause degradation of the product P-DNS-Am over a flexible time frame of measurement (suppl. Fig. 12D).

Levels of H₂S in normal controls versus MI patients

The validated method was applied on both healthy controls and MI Patients. The elevation of H₂S in plasma of MI patients compared to normal control was confirmed in this study. The average level of H₂S in plasma of MI patients was 28.42 μM ± 1.111 compared to 23.19 μM ± 1.036 in normal controls at p = 0.0015 (Fig. 3A). Samples collected from MI patients showed no variation in the concentration of H₂S over the range of 2–12 h post MI.

Similar to our findings, Peter et al. showed that the level of plasma H₂S was elevated in patients with any vascular diseases compared to normal controls [25]. In contrast, Li et al. reported that plasma H₂S decreased after acute MI surgery in rats [26]. Elrod et al. concluded that exogenous H₂S administration or modulation of endogenous H₂S decreased myocardial ischemia-reperfusion injury [27]. These debatable outcomes show that the level of serum H₂S in MI patients is still controversial and needs further research.

The reasons behind the elevation of H₂S in plasma of MI patients are still questionable. The endogenous synthesis of H₂S in mammalian tissues is mediated by CSE, CBS and 3-MST enzymes. Concerning the cardiovascular system, it is synthesized

| Sample | Intensity of fluorescence by adding DNS-Az on plasma (method D) | Intensity of fluorescence by adding DNS-Az on whole blood (method A) | Variation between intensities in the 2 techniques normalized to whole blood (intensity in whole blood – intensity in plasma/intensity in whole blood) * 100 |
|--------|---------------------------------------------------------------|------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| 1      | 7.0                                                          | 7.9                                                              | 11.3%                                                                                                                                 |
| 2      | 12.4                                                         | 11.3                                                             | 9.7%                                                                                                                                 |
| 3      | 8.5                                                          | 7.7                                                              | 10.3%                                                                                                                                 |
| 4      | 9.7                                                          | 10.7                                                             | 9.3%                                                                                                                                 |
| 5      | 7.4                                                          | 8.7                                                              | 14.9%                                                                                                                                 |
| 6      | 11.3                                                         | 10.5                                                             | 7.6%                                                                                                                                 |
| 7      | 8.9                                                          | 10.0                                                             | 11.0%                                                                                                                                 |

* To report variations between 2 methods, it is customary to compare concentrations of analyte (H₂S) rather than fluorescence intensities, however since there no calibration curve is possible in whole blood, and it is incorrect to substitute for intensities in blood in a calibration curve performed in plasma matrix, therefore the table reports intensities rather than concentrations.

Fig. 3. (A) H₂S levels are elevated in plasma of MI patients compared to normal controls (p = 0.0015) as H₂S is measured after sample treatment with the DNS-Az probe. (B) No significant difference in level of H₂S measured by H₂S Analyser vs Reference Fluorometer in normal controls. (C) In MI patients. (D) H₂S levels are elevated in plasma of MI patients compared to normal controls (p = 0.0004) after sample treatment with the DNS-Az probe using H₂S Analyser.
from L-cysteine via CSE enzyme in the heart muscles [28]. It is recently discovered that H2S exerts many physiological effects including anti-inflammatory, neuromodulation, vasoregulation, reducing oxidative stress, protection from reperfusion after myocardial infarction and inhibition of insulin resistance. These findings prove that H2S is an important biological mediator that plays a physiological role in different biological systems [29–34].

In-house portable device for determination of H2S (H2S Analyser)

After confirming elevation of H2S was associated with MI in this study, developing a point of care system for measuring the gas was targeted in collaboration with the “Egyptian company for Biotechnology – Spectrum Diagnostics”, a market leader company in Egypt working in the field of designing, developing and manufacturing of laboratory reagents and portable analysers. This system can be used for measuring H2S as a cardiac marker in emergency conditions (suppl. Fig. 11).

Design input of the H2S Analyser

1- Rapid hand-held easy to use point of care system.
2- Spectrofluorometric detection method.
3- Light source (LED).
4- Portable built-in battery for rural and remote areas.
5- Can be connected to PC through RS232 signal port.
6- Single channel analyser with heating control reading location.
7- Electronic heating through resistance controlled through software.
8- User friendly software.
9- Memory to store calibration curve for direct result display and store up to 250 test results.
10- A simple key pad for selecting the required function during performing the test.
11- Using disposable cuvettes specially designed for fluorometric measurements.

Table 5
Comparison between intensity of fluorescence of different S-DNS-Am concentrations for both Reference Fluorometer and H2S Analyser.

| Concentration S-DNS-Am (µM) | Intensity of fluorescence of Reference Fluorometer | Intensity of fluorescence of H2S analyser |
|---------------------------|---------------------------------|---------------------------------|
|                           | Day 1                            | Day 2                            | Day 3                            |
| 10                        | 8.7                             | 8.9                             | 10.4                             |
| 50                        | 34.2                            | 35.7                            | 38.7                             |
| 100                       | 50.5                            | 52.4                            | 47.6                             |
| 250                       | 75.4                            | 77.8                            | 83.1                             |
| 500                       | 148                             | 142.2                           | 153.4                            |
| 1000                      | 328                             | 335.1                           | 340.4                            |

Fig. 4. (A) Linear regression analysis showing correlation between H2S Analyser and Reference Fluorometer in day 1 (r = 0.992; a = 10; b = 0.967), (B) Day 2 (r = 0.992; a = 9.371; b = 0.952), (C) Day 3 (r = 0.987; a = 9.308; b = 0.960). (D) Calibration curves for determination of S-DNS-Am using H2S Analyser (E) H2S in human plasma using DNS-Az in H2S Analyser. Each point is the result of measurement of 3 different concentrations. The values plotted represent mean ± SEM.
A close system using a reagent with at least one year stability.

13- A kit format for easy handling and performing the test.

Technical specifications of H₂S Analyser

- Channel optical LED method
- Algorithm: based on fluorescent
- Processing: 12 bit ADC
- Autosense: LED intensity controlling and sunlight reduction
- Micro Cuvette volume (min. 300 ul – maximum 1.5 ml).
- Precision: CV < 5%
- Language: English
- Display: 4 line LCD
- Dimensions: L × W × H: (228x 115 × 55) mm
- Weight: 0.55 kg
- Battery: 12v/1.5a
- Charger: DC 12 V/2A

Excitation and emission wavelengths of the H₂S Analyser

Since this device was designed for selective determination of H₂S in human plasma using DNS-Az reagents, the selected excitation wavelength filter was 355 nm and the emission wavelength filter was 505 nm.

Accuracy and precision of the H₂S Analyser

The H₂S Analyser was verified against the Reference Spectrofluorometer (Shimadzu RF-5301-PC) using S-DNS-Am at a range of concentrations of 10–1000 μM, where the intensity of fluorescence of the different concentrations was compared for the two devices on three different days (Table 5).

To assess the performance of the H₂S Analyser compared to the benchtop Shimadzu RF-5301-PC spectrofluorometer, Kruskall Wallis test (suppl. Table 1), Wilcoxon Rank Sum test (suppl. Table 2), Wilcoxon signed rank test (suppl. Table 3), and Ranked Spearman correlation test (suppl. Table 4) were performed. Results (expressed as median and percentiles for quantitative non-parametric measures) concluded that there is no significant difference in intensity of fluorescence of different concentration of S-DNS-Am between the H₂S Analyser and Reference Fluorometer. Correlation charts between the intensity of fluorescence of S-DNS-Am in both devices were plotted (Fig. 4 A–C).

Testing the method linearity using H₂S Analyser

Method linearity using H₂S Analyser was tested using S-DNS-Am in PBS/ tween 0.5% w/v and using the optimized method of addition of DNS-Az on human plasma.

| Table 6 |
|---------|

| Concentration of S-DNS-Am/or of added sodium sulphide in μM | In intensity of fluorescence | intensity of fluorescence | intensity of fluorescence | Mean | S.D | R.S.D |
|-----------|---------------------------|---------------------------|---------------------------|------|-----|------|
| 10        | 7.47                      | 9.41                      | 8.13                      | 8.33 | 0.98 | 11.76% |
| 50        | 20.25                     | 22.79                     | 24.81                     | 22.61 | 2.28 | 10.08% |
| 100       | 31.25                     | 30.48                     | 34.27                     | 32.00 | 2.00 | 6.25%  |
| 250       | 58.23                     | 56.28                     | 64.31                     | 59.60 | 4.18 | 7.01%  |
| 500       | 170.43                    | 159.86                    | 164.47                    | 164.92 | 5.29 | 3.20%  |
| 1000      | 316.79                    | 313.27                    | 325.32                    | 318.46 | 6.19 | 1.94%  |
| Slope     | 0.3185                    | 0.3111                    | 0.3216                    | 0.3170 | 0.0053 | 1.67% |
| R²        | 0.9915                    | 0.9918                    | 0.9940                    | 0.9927 | 0.0018 | 0.18% |

| Concentration of S-DNS-Am | In intensity of fluorescence | intensity of fluorescence | intensity of fluorescence | Mean | S.D | R.S.D |
|---------------------------|---------------------------|---------------------------|---------------------------|------|-----|------|
| 10                        | 7.47                      | 8.24                      | 6.82                      | 7.51 | 0.71 | 9.45% |
| 50                        | 31.25                     | 36.72                     | 37.48                     | 35.15 | 3.39 | 9.64% |
| 100                       | 58.23                     | 60.21                     | 54.16                     | 57.53 | 3.08 | 5.35% |
| 250                       | 170.43                    | 184.24                    | 171.45                    | 176.04 | 7.26 | 4.12% |
| 500                       | 316.79                    | 340.71                    | 336.77                    | 329.42 | 12.01 | 3.64% |
| Slope                     | 0.3185                    | 0.3403                    | 0.3324                    | 0.3304 | 0.0110 | 3.32% |
| R²                        | 0.9915                    | 0.9890                    | 0.9889                    | 0.9898 | 0.0014 | 0.14% |

| Concentration of S-DNS-Am | In intensity of fluorescence | intensity of fluorescence | intensity of fluorescence | Mean | S.D | R.S.D |
|---------------------------|---------------------------|---------------------------|---------------------------|------|-----|------|
| 10                        | 13.89                     | 12.72                     | 16.25                     | 14.28 | 1.79 | 12.53% |
| 25                        | 26.55                     | 27.82                     | 30.46                     | 28.57 | 1.95 | 6.82% |
| 50                        | 39.15                     | 44.47                     | 41.13                     | 41.58 | 2.68 | 6.44% |
| 100                       | 115.41                    | 110.93                    | 118.32                    | 114.88 | 3.72 | 3.23% |
| 150                       | 140.34                    | 153.14                    | 136.68                    | 143.38 | 8.64 | 6.02% |
| 200                       | 203.58                    | 195.63                    | 212.29                    | 203.83 | 8.33 | 4.08% |
| 300                       | 317.57                    | 330.98                    | 305.52                    | 318.02 | 12.73 | 4.00% |
| Slope                     | 1.0378                    | 1.0669                    | 1.0065                    | 1.0376 | 0.0302 | 2.91% |
| R²                        | 0.9928                    | 0.9936                    | 0.9907                    | 0.9923 | 0.0014 | 0.14% |

| Concentration of S-DNS-Am | In intensity of fluorescence | intensity of fluorescence | intensity of fluorescence | Mean | S.D | R.S.D |
|---------------------------|---------------------------|---------------------------|---------------------------|------|-----|------|
| 0                         | 3.84                      | 4.52                      | 3.96                      | 4.10 | 0.36 | 8.78% |
| 10                        | 13.89                     | 15.68                     | 12.17                     | 13.91 | 1.75 | 12.58% |
| 25                        | 26.55                     | 29.49                     | 23.63                     | 26.55 | 2.93 | 11.03% |
| 50                        | 39.15                     | 34.71                     | 42.84                     | 38.90 | 4.07 | 10.46% |
| 100                       | 115.41                    | 104.43                    | 97.92                     | 105.92 | 8.83 | 8.33% |
| 150                       | 140.34                    | 128.71                    | 143.29                    | 137.44 | 7.70 | 5.60% |
| 200                       | 203.58                    | 199.23                    | 215.79                    | 206.20 | 8.58 | 4.16% |
| 300                       | 317.57                    | 330.48                    | 300.26                    | 316.10 | 15.16 | 4.79% |
| Slope                     | 1.0378                    | 1.0568                    | 1.0146                    | 1.0360 | 0.0205 | 1.97% |
| R²                        | 0.9928                    | 0.9837                    | 0.9957                    | 0.9907 | 0.0062 | 0.62% |
The calibration curves for S-DNS-Am in PBS/tween 0.5% for the H$_2$S Analyser was conducted in a range of 10–1000 μM. The calibration curve was linear with $R^2 = 0.9917$ and equation $y = 0.3247x – 0.2328$ (Fig. 4D). Calibration curves points were measured in triplicates on the same day for intra-day precision (repeatability) and on three different days for inter-day precision (intermediate precision) (Table 6) were the relative standard deviation was found to be less than 15%.

Determination of H$_2$S in human plasma using H$_2$S Analyser, involved calibration curves in the range of 0–300 μM, where $R^2$ of 0.9946 with a regression equation $y = 1.0363x – 0.8647$ (Fig. 4E).

The calibration curve was conducted in triplicates on the same day for intra-day precision (repeatability) and on three different days for inter-day precision (Intermediate precision) (Table 6). R.S.D was found to be less than 15%.

**Determination of H$_2$S in normal controls and MI patients using H$_2$S Analyser**

After verification of the H$_2$S Analyser, the level of H$_2$S in the plasma of some healthy controls and MI patients measured using the Reference Fluorescence were measured using the H$_2$S Analyser. The concentration of H$_2$S obtained by both the Reference Fluorometer and H$_2$S Analyser in 24 normal controls and 35 MI patients were compared. There was no significant difference between H$_2$S levels neither in normal controls nor in MI patients (Figs. 3B and 3C) measured by both fluorometers. Normal controls and MI patients H$_2$S level was compared with the H$_2$S Analyser and a significant difference was observed where the average level of H$_2$S in plasma of MI patients was 31.23 μM ± 1.432 compared to 21.5 2 μM ± 1.072 in normal controls at p = 0.0004, while no significant difference existed between same group of subjects when analysed on the Reference fluorometer and the H$_2$S Analyser (Fig. 3D), which confirms the same results obtained by the Reference Fluorometer. The H$_2$S Analyser can be used for point-of-care-testing of H$_2$S in CVD or any other disease conditions. It is a portable small device that can be used in ambulances, rural areas and any campaigns.

**Conclusion**

The study reports a new optimized methodology for human blood sample treatment to determine H$_2$S fluorometrically using DNS-Az probe using a new developed Point-of-Care-Testing fluorometer selective for the gasotransmitter. The analytical method was validated in plasma showing simplicity, selectivity to the unionized form of the gas, stability of the fluorescent product during measurement, sensitivity for detection at physiological levels, and most importantly functionality in aqueous solution and blood samples are reported and discussed. A portable built-in battery spectrofluorometer was designed, developed, manufactured and verified to be used at bed-side test, for simple determination of H$_2$S in both aqueous solution and plasma using DNS-Az probe. The POCT methodology was used to compare H$_2$S levels in CVD patients to normal controls, reinforcing the finding that the gas level is significantly higher in CVD patients. Collectively, our previous study [6] together with this study show that H$_2$S rises in the plasma of MI patients not only as early as 1–2 h after myocardial damage, but it is also significantly higher up to 10 h (the time of collection of blood samples for MI patients in this study). This elevation of H$_2$S level is in the line the liability of H$_2$S usage a predictive biomarker for early detection and diagnosis of MI, therefore better intervention and treatment approaches, especially that simple fluorometric method is now available to medium budget laboratories.

**Compliance with Ethics requirements**

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

**Acknowledgements**

The German University in Cairo and Spectrum Diagnostics Egypt funded the work.

**Declaration of Competing Interest**

The authors have declared no conflict of interest.

**Appendix A. Supplementary material**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2019.11.010.

**References**

[1] Polhemus DJ et al. The cardioprotective actions of hydrogen sulfide in acute myocardial infarction and heart failure. Scientifica (Cairo) 2014:768607.
[2] Reifenstein RJ, Bulpitt WC, Roth SH. Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol 1992;32:109–34.
[3] Richardson CJ, Magee EA, Cummings JH. A new method for the determination of sulphide in gastrointestinal contents and whole blood by microdistillation and ion chromatography. Clin Chim Acta 2000;293:12–21.
[4] Wang Y et al. Role of hydrogen sulfide in the development of atherothrombotic lesions in apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 2009;29(2):173–9.
[5] Calvert JW et al. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. Circ Res 2009;105(4):365–74.
[6] Ali SE et al. A comparative metabolomics approach reveals early biomarkers for metabolic response to acute myocardial infarction. Sci Rep 2016;6:36359.
[7] Fukuto JM et al. Small molecule signaling agents: the integrated chemistry and biochemistry of nitrogen oxides, oxides of carbon, dioxygen, hydrogen sulfide, and their derived species. Chem Res Toxicol 2012;25(4):769–93.
[8] Nagy P et al. Chemical aspects of hydrogen sulfide measurements in physiological samples. Biochim Biophys Acta 2014;1840(2):876–91.
[9] Zheng Y et al. Modified methylene blue method for measurement of hydrogen sulfide level in plasma. Sheng Li Xue Bao 2012;64(6):681–6.
[10] Feng W, Dymock BW. Fluorescent Probes for H$_2$S Detection and Quantification. Handb Exp Pharmacol 2015;230:291–323.
[11] Kang J et al. A high signal-to-background ratio H$_2$S-specific fluorescent probe based on nucleophilic substitution and its bioimaging for generation H$_2$S induced by Ca$^{2+}$ in vivo. Dyes Pigments 2019;171:107755.
[12] Wang JP et al. Based 'successive' nucleophilic substitution mitochondrial-targeted H$_2$S red light emissive fluorescent probe and its imaging in mice. Sensors Actuat B-Chem 2019. doi: https://doi.org/10.1016/j.snb.2019.126773.
[13] Wang JP et al. A highly sensitive fluorescent probe for hydrogen sulfide based on dicyanomethane and its imaging in living cells. Sensors Actuat B-Chem 2019;294:141–7.
[14] Li J et al. High selective distinguishable detection GSH and H$_2$S based on steric configuration of molecular in Vivo. Dyes Pigments 2020;172:107826.
[15] Shi X et al. A dual-sites fluorescent probe based on symmetric structure of naphthalimide derivative to detect H$_2$S. Dyes Pigments 2019;165:38–43.
[16] Zhou N et al. An isoxazole-accelerated nitro oxidation type fluorescent detection and imaging for hydrogen sulfide in cells. Sensors Actuat B-Chem 2019;287:131–7.
[17] Ma T et al. A ‘naked-eye’ ratiometric and NIR fluorescent detection for hydrogen sulfide with quick response and high selectivity for and its bioimaging. Dyes Pigments 2019;165:31–7.
[18] Chao J et al. HS-facilitated sulfur pyran realizing hydrogen sulfide detection and imaging in HepG2 cells and chloroethen. Spectrochim Acta A 2019;214:227–32.
[19] Hsu F et al. A novel isophorone-based red-emitting fluorescent probe for selective detection of sulfide anions in water for in vivo imaging. J Mater Chem B 2017;5:2798–803.
[20] Li J et al. Chromogenic and fluorogenic chemosensors for hydrogen sulfide: review of detection mechanisms since the year 2009. RSC Adv 2015;5(2):191–206.
[21] Peng H et al. A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. Angew Chem Int Ed Engl 2011;50(41):9672–5.
[22] Hunter RA et al. Inaccuracies of nitric oxide measurement methods in biological media. Anal Chem 2013;85(3):1957–63.
[23] Collins N. B.T.D., Development of a lab protocol for the measurement of endogenous hydrogen sulfide in human serum using dansyl azide. In: Society for Neuroscience in Anesthesiology and Critical Care (SNACC); 2013.

[24] Validation of analytical procedures: text and methodology. In: International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use; 2005.

[25] Peter EA et al. Plasma free H2S levels are elevated in patients with cardiovascular disease. J Am Heart Assoc 2013;2(5):e000387.

[26] Li N et al. The H2S Donor NaHS changes the expression pattern of H2S-producing enzymes after myocardial infarction. Oxid Med Cell Longev 2016:6492469.

[27] Elrod JW et al. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci USA 2007;104(39):15560–5.

[28] Chen P et al. Homocysteine metabolism in cardiovascular cells and tissues: implications for hyperhomocysteinemia and cardiovascular disease. Adv Enzyme Regul 1999;39:93–105.

[29] Kolluru GK et al. Hydrogen sulfide chemical biology: pathophysiological roles and detection. Nitric Oxide 2013;35:5–20.

[30] Ali MY et al. Hydrogen sulphide reduces insulin secretion from HIT-T15 cells by a KATP channel-dependent pathway. J Endocrinol 2007;195(1):105–12.

[31] Laggner H et al. The novel gaseous vasorelaxant hydrogen sulfide inhibits angiotensin-converting enzyme activity of endothelial cells. J Hypertens 2007;25(10):2100–4.

[32] Sivarajah A et al. Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. Shock 2009;31(3):267–74.

[33] Yonezawa D et al. A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. Toxicology 2007;241(1–2):11–8.

[34] Zanardo RC et al. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J 2006;20(12):2118–20.