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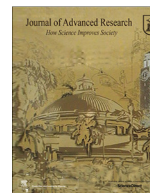

Point-of-care testing and optimization of sample treatment for fluorometric determination of hydrogen sulphide in plasma of cardiovascular patients

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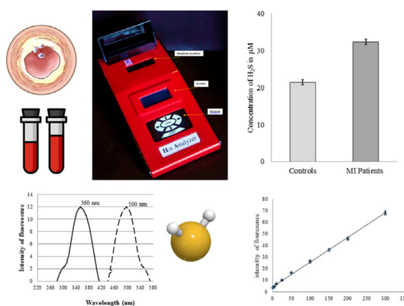
HIGHLIGHTS

- A comparative sample preparation methodology for fluorometric determination of H_2S in plasma.
- Sample treatment protocol is critical to reliable results for determination of gasotransmitter.
- Portable H_2S Analyser was designed, manufactured and verified as POCT for the gas in the ambulance.
- Validation in human plasma proved efficiency of H_2S Analyser in determining H_2S in MI patients.
- H_2S is elevated in MI patients compared to normal controls up to 10 h from emergence of symptoms.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Hydrogen sulphide (H_2S) is one of the gasotransmitters that was reported to have a cardioprotective effect at its physiological levels in blood. Previous determinations of H_2S 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 H_2S 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 λ_{ex} 340 nm and λ_{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 H_2S Analyser was verified in comparison to a benchtop spectrofluorometer where linearity was confirmed in the range of 3–300 µM, LOD being 1 µM, at λ_{ex} 340 nm and λ_{em} 517 nm. Sample

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treatment involving blood centrifugation followed by addition of reagent on plasma produced maximum fluorescence yield. Analysis of blood samples of myocardial infarction (MI) patients and controls showed elevated levels of H_2S in MI patients (28 μM ± 1.111) *vs.* controls (23 μM ± 1.036) at p = 0.0015.

Conclusion: The study is novel in being a POCT approach for selective determination of H_2S molecular form in plasma after simple optimized sample treatment. The study confirms that MI is associated with H_2S elevated levels up to 10 hours from emergence of symptoms.

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Introduction

Hydrogen sulphide (H_2S) 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 H_2S as the third gaseous mediator along with carbon monoxide (CO) and nitric oxide (NO). At physiological pH, 67% of H_2S exist as sulphide ion (HS^-), 33% is un-dissociated as H_2S while S^{2-} is present in negligible quantity [1–4]. One of the most blossoming roles of H_2S 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 H_2S 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 H_2S 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 H_2S, however, it lacks selectivity via interfering coloured substances and formation of dimers and trimmers in addition to its poor sensitivity at physiological levels of H_2S [9]. In the last few years, a number of sensitive fluorescent probes were developed to quantify H_2S, *in-vitro* models and bioimaging [10–20]. One of the high quantum yield synthetic probes is Dansyl azide (DNS-Az), designed by Peng et al. where selective reduction of the azido group by H_2S 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 H_2S determination (with no interference from 18 anions Cl^-, Br^-, I^-, F^-, OH^-, OAc^-, CN^-, N_3^-, NO_2^-, HCO_3^-, HSO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, $S_2O_4^{2-}$, $S_2O_5^{2-}$, HPO_4^{2-} 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 sample pre-treatment methodology for determination of molecular H_2S in human blood using DNS-Az followed by method validation according to ICH Guidelines to represent a sensitive, selective and rapid system for H_2S determination in biological fluids. Moreover, the work presented here applies this methodology on myocardial infarction (MI) patients and control subjects to compare levels of H_2S 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 H_2S 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 (NaN_3) (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 NaN_3 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 ($MgSO_4$, *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 PuriFash 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 H_2S 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 (Na_2S) 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. Na_2S 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 (λ_{em} 517 nm and λ_{ex} 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_2S in laboratory prepared solutions

A 50 mM stock solution of DNS-Az in ethanol and a 10 mM stock solution of Na_2S in deionized water were prepared. Na_2S 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_2S solution was then added to a 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 λ_{em} 517 nm were plotted against Na_2S 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 λ_{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_2S in 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_2S 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_2S 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_2S in PBS were prepared. Na_2S 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_2S stock solution was added to 450 µl plasma to a final concentration of 1–300 µM. The emission spectrum was measured at λ_{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_2S 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_2S.

Method C: Addition of DNS-Az and Na_2S 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_2S on whole blood but no later on plasma.

Method D: Addition of both DNS-Az and Na_2S on plasma separated from whole blood

Blood centrifugation for isolation of plasma was performed followed by addition of 500 µM DNS-Az and Na_2S.

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_2S Analyzer*, IBM SPSS statistics

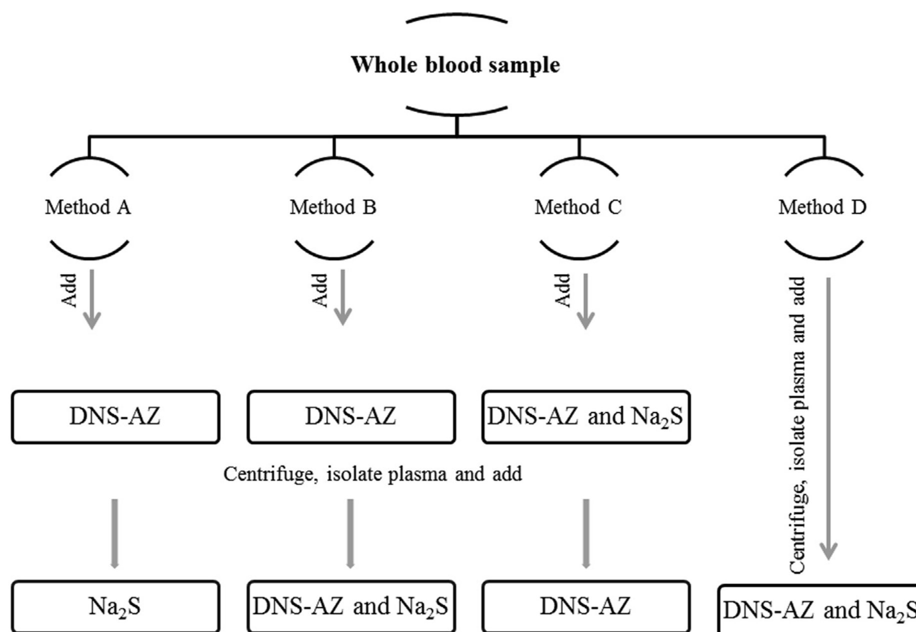

Fig. 1. Summary of methods A, B, C and D for blood sample treatment during the linearity study.

Table 1
Baseline characteristics of subjects involved in the study, data are represented as mean ± SEM.

	MI patients (n = 75)	Normal controls (n = 50)
Age	51.42 ± 1.01	46.11 ± 1.51
Sex (Male/Female)	61/14	41/9
Blood pressure	132/85 ± 6	123/82 ± 3
Serum troponin (ng/ml)	1.247 ± 0.14	0.413 ± 0.15
Diagnosis	58 STEMI, 17 Non-STEMI	Normal

(V. 25.0, IBM Corp., USA, 2017–2018) was used where data 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 Kruskall 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-parameteric 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 λ_{ex} 340 nm for comparison (suppl. Fig. 12A). The probe DNS-Az had no fluorescence intensity unless reduced to P-DNS-Am upon reaction with H_2S. Emission wavelength was found to be 517 nm at excitation at 340 nm. The value of λ_{ex} was confirmed via scanning excitation at λ_{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_2S, the value of λ_{ex} showed a slight bathochromic shift from 340 to 360 nm, while λ_{em} shifted from 517 to 500 nm (suppl. Fig. 12C).

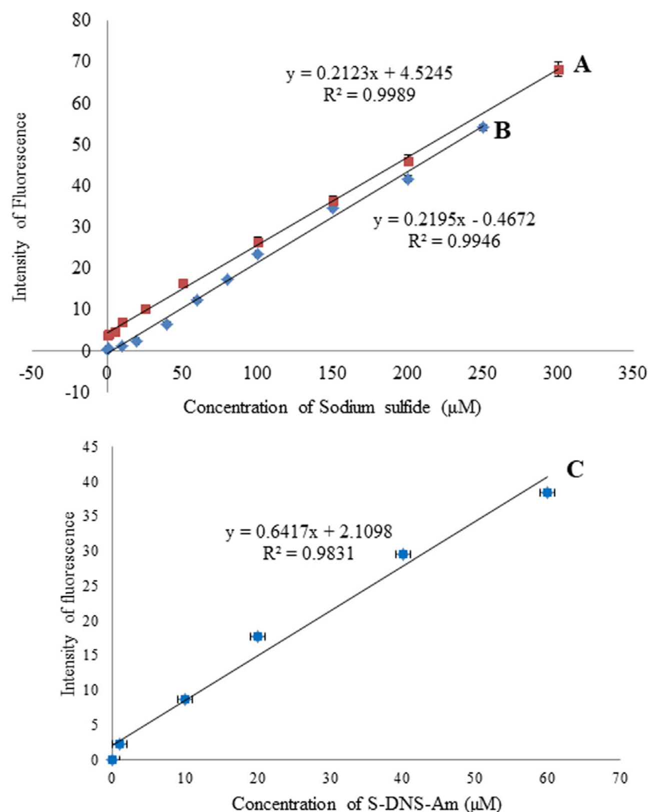

Fig. 2. Calibration curve for determination of (line A) H_2S in PBS/tween 0.5% w/v using DNS-Az, (line B) H_2S by addition of both DNS-Az and Na_2S on plasma (separated from whole blood, method D, and (line C) linearity of standard S-DNS-Am. Each point is the result of measurement of 3 different concentrations. The values plotted represent mean ± SEM.

Linearity for determination of H_2S in using DNS-Az in laboratory prepared mixture

Determination of H_2S 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_2S 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_2S in human blood using DNS-Az

Different calibration curves for determination of H_2S 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_2S (0–300 µM Na_2S) to blood samples showed that the best linearity of response (R^2 = 0.9989) was only possible when both DNS-Az and Na_2S were

added on plasma after centrifugation of blood samples (Fig. 2, line B), where the linearity interval covers by far the physiological range of H_2S (10–100 μM). It is worth mentioning that adding DNS-Az, Na_2S or both to whole blood showed no correlation and consequently no proportionality between concentration of Na_2S and fluorescence intensity. We believe that as H_2S being the third gasotransmitter together tabwith 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 H_2S in whole blood could be attributed to similar reasons where H_2S 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 H_2S 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 H_2S in the blood of 5 mice by adding DNS-Az on both whole blood and plasma after centrifugation of blood samples while adding Na_2S 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 H_2S 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 Na_2S were added on plasma.

Table 2
Validation results for determination of H_2S in human blood using DNS-Az.

Parameter	Results
Excitation wavelength (nm)	360
Emission wavelength (nm)	500
Linearity range (μM)	0–300
Regression equation	y = 0.2123x + 4.5245
Regression coefficient (R^2)	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

Method validation for determination of H_2S using DNS-Az

Validation of the method was undertaken according to ICH Guidelines [24], and results are summarized in Table 2.

Range and linearity

A linear relationship was obtained between concentration of added Na_2S and fluorescence intensity over the range of 0–300 μM with R^2 = 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/v** which is acceptable according to ICH guidelines [24].

Table 3
Precision study for determination of H_2S in human blood using DNS-Az.

Intraday precision study						
Concentration of added Na_2S (μM)	Intensity of fluorescence			Mean	SD	RSD
	Set 1	Set 2	Set 3			
0	3.17	3.84	3.76	3.59	0.36	10.02%
1	3.51	4.17	4.42	4.03	0.47	11.65%
5	3.82	4.68	4.71	4.40	0.50	11.36%
10	6.71	6.47	7.05	6.74	0.29	4.30%
25	9.44	9.89	10.21	9.84	0.38	3.86%
50	16.48	17.43	16.12	16.67	0.67	4.01%
100	29.91	26.23	24.78	26.97	2.64	9.78%
150	37.86	39.41	36.42	37.89	1.49	3.93%
200	49.89	45.25	46.53	47.22	2.39	5.06%
300	72.21	69.12	70.83	70.72	1.54	2.17%
Slope	0.23	0.21	0.21	0.21	0.01	4.76%
Intercept	3.94	4.51	4.12	4.19	0.29	6.92%
R^2	0.9973	0.9958	0.9984	0.9971	0.0013	0.13%
Interday Precision study						
Concentration of added Na_2S (μM)	Day 1 Intensity of fluorescence	Day 2 Intensity of fluorescence	Day 3 Intensity of fluorescence	Mean	S.D	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%
Slope	0.23	0.20	0.19	0.20	0.02	10.00%
Intercept	3.94	5.18	4.68	4.60	0.62	13.47%
R^2	0.9973	0.9959	0.9982	0.9971	0.0011	0.11%

Table 4
Comparison between determination of H_2S in whole blood* (Method A) and in plasma (Method D) using DNS-Az.

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 (H_2S) 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.

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

H_2S is a volatile gas and can be easily metabolized. To ensure that the method used in this study measures H_2S present in the sample and that the process of blood centrifugation to plasma doesn't cause loss of H_2S, 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 H_2S in normal controls versus MI patients

The validated method was applied on both healthy controls and MI Patients. The elevation of H_2S in plasma of MI patients compared to normal control was confirmed in this study. The average level of H_2S 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 H_2S over the range of 2–12 h post MI.

Similar to our findings, Peter et al. showed that the level of plasma H_2S was elevated in patients with any vascular diseases compared to normal controls [25]. In contrast, Li et al. reported that plasma H_2S decreased after acute MI surgery in rats [26]. Elrod et al. concluded that exogenous H_2S administration or modulation of endogenous H_2S decreased myocardial ischemia-reperfusion injury [27]. These debatable outcomes show that the level of serum H_2S in MI patients is still controversial and needs further research.

The reasons behind the elevation of H_2S in plasma of MI patients are still questionable. The endogenous synthesis of H_2S in mammalian tissues is mediated by CSE, CBS and 3-MST enzymes. Concerning the cardiovascular system, it is synthesized

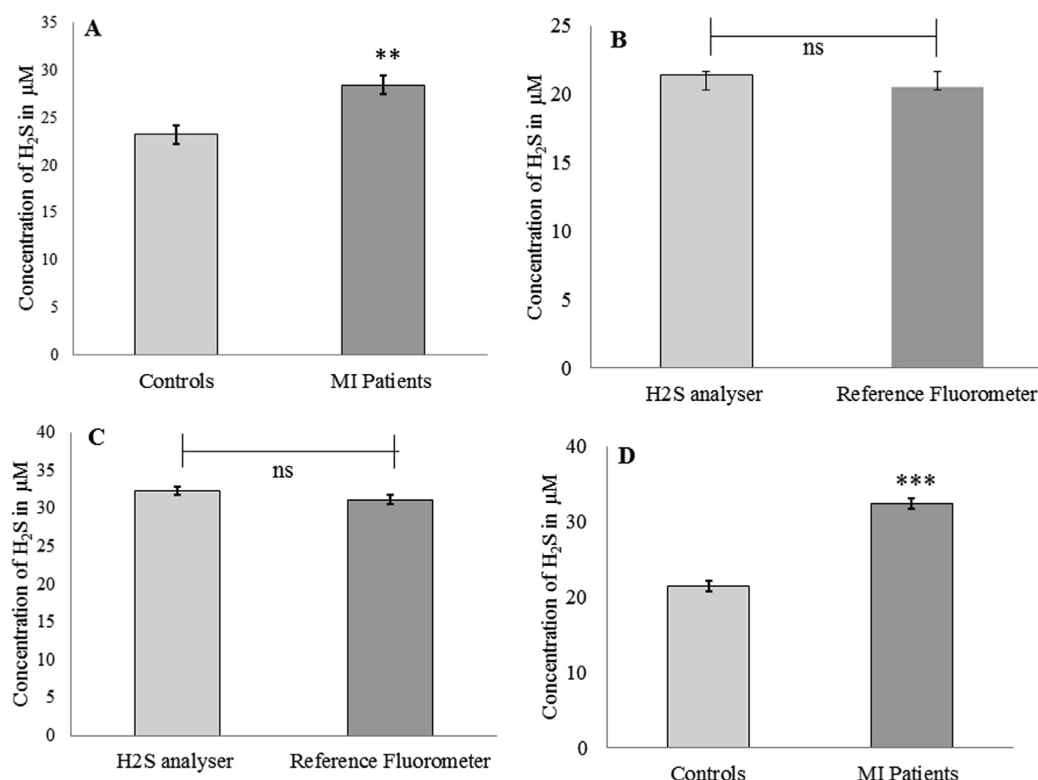

Fig. 3. (A) H_2S levels are elevated in plasma of MI patients compared to normal controls (p = 0.0015) as H_2S is measured after sample treatment with the DNS-Az probe. (B) No significant difference in level of H_2S measured by *H_2S Analyser vs* Reference Fluorometer in normal controls. (C) In MI patients. (D) H_2S levels are elevated in plasma of MI patients compared to normal controls (p = 0.0004) after sample treatment with the DNS-Az probe using *H_2S Analyser*.

Table 5
Comparison between intensity of fluorescence of different S-DNS-Am concentrations for both Reference Fluorometer and *H_2S Analyser.*

Concentration S-DNS-Am (μM)	Intensity of fluorescence of Reference Fluorometer	Intensity of fluorescence of *H_2S analyser*
	Day 1	
10	8.7	7.4
50	34.2	20.2
100	50.5	31.2
250	75.4	58.2
500	148	170.4
1000	328	316.7
	Day 2	
10	8.9	9.2
50	35.7	23.8
100	52.4	36.9
250	77.8	53.2
500	142.2	176.9
1000	335.1	325.8
	Day 3	
10	10.4	8.1
50	38.7	28.9
100	47.6	43.3
250	83.1	50.2
500	153.4	182.4
1000	340.4	330.8

from L-cysteine via CSE enzyme in the heart muscles [28]. It is recently discovered that H_2S 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 H_2S is an important biological mediator that plays a physiological role in different biological systems [29–34].

In-house portable device for determination of H_2S (H_2S Analyser)

After confirming elevation of H_2S 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 H_2S as a cardiac marker in emergency conditions (suppl. Fig. 11).

Design input of the H_2S 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.

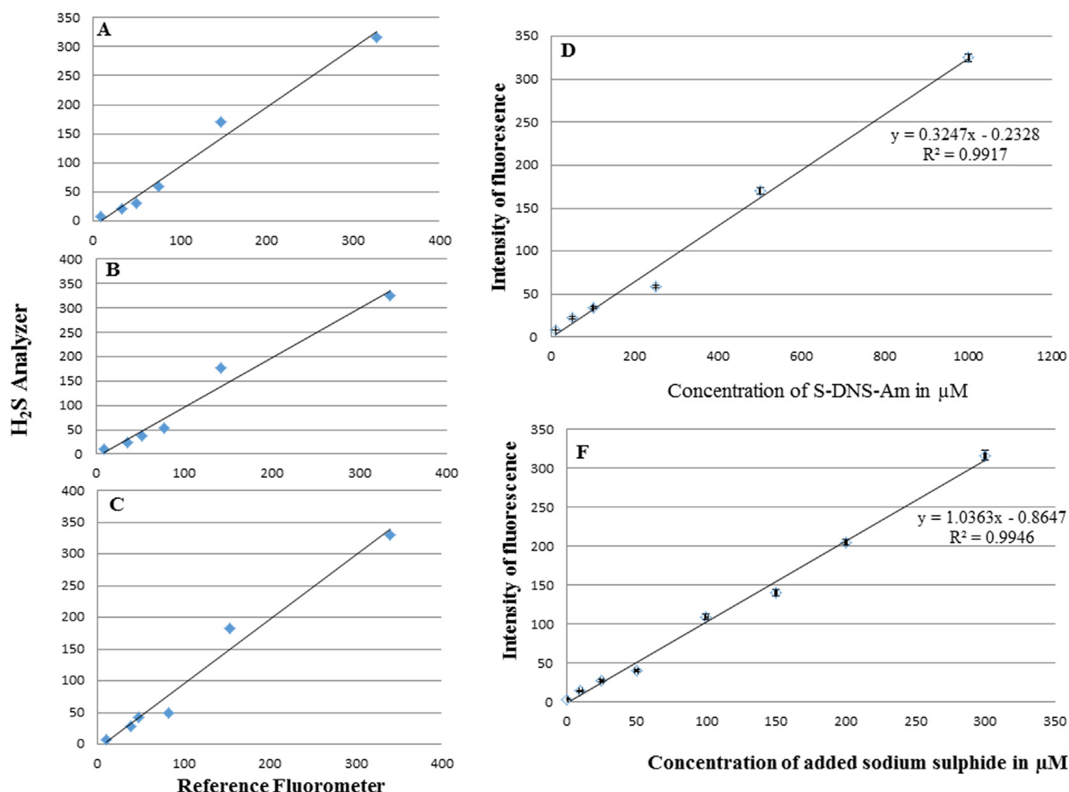

Fig. 4. **(A)** Linear regression analysis showing correlation between *H_2S Analyser* and Reference Fluorometer in day 1 (r = 0.992; a = 10; b = 0.967), **(B)** Day 2 (r = 0.992; a = 9.371; b = 0.952), **(C)** Day 3 (r = 0.987; a = 9.308; b = 0.960). **(D)** Calibration curves for determination of S-DNS-Am using *H_2S Analyser* **(E)** H_2S in human plasma using DNS-Az in *H_2S Analyser.* Each point is the result of measurement of 3 different concentrations. The values plotted represent mean ± SEM.

Table 6
Precision study using the *H_2S Analyser* for both standard Dansyl amide (S-DNS-Am) and for determination of H_2S in human plasma using DNS-Az.

Concentration of S-DNS-Am/or of added sodium sulphide in μM	In intensity of fluorescence (Day1/ or Set 1)	intensity of fluorescence (Day1/ or Set 2)	intensity of fluorescence (Day1/ or Set 3)	Mean	S.D	R.S.D
Intra-day precision for S-DNS-Am						
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%
R^2	0.9915	0.9918	0.9949	0.9927	0.0018	0.18%
Inter-day precision for S-DNS-Am						
10	7.47	8.24	6.82	7.51	0.71	9.45%
100	31.25	36.72	37.48	35.15	3.39	9.64%
250	58.23	60.21	54.16	57.53	3.08	5.35%
500	170.43	184.24	173.45	176.04	7.26	4.12%
1000	316.79	340.71	330.77	329.42	12.01	3.64%
Slope	0.3185	0.3403	0.3324	0.3304	0.0110	3.32%
R^2	0.9915	0.9890	0.9889	0.9898	0.0014	0.14%
Intra-day precision for determination of H_2S in human plasma using DNS-Az						
10	13.89	12.72	16.25	14.28	1.79	12.53%
25	26.55	28.72	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%
R^2	0.9928	0.9936	0.9907	0.9923	0.0014	0.14%
Inter-day precision for determination of H_2S in human plasma using DNS-Az						
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	1.0556	1.0146	1.0360	0.0205	1.97%
R^2	0.9928	0.9837	0.9957	0.9907	0.0062	0.62%

12- 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 H_2S 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 H_2S Analyser

Since this device was designed for selective determination of H_2S 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 H_2S Analyser

The *H_2S 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 *H_2S 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 *H_2S Analyser* and Reference Fluorometer. Correlation charts between the intensity of fluorescence of S-DNS-Am in both devices were plotted (Fig. 4A–C).

Testing the method linearity using H_2S Analyser

Method linearity using *H_2S 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.

The calibration curves for S-DNS-Am in PBS /tween 0.5% for the *H_2S 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 interday precision (intermediate precision) (Table 6) were the relative standard deviation was found to be less than 15%.

Determination of H_2S in human plasma using *H_2S 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_2S in normal controls and MI patients using H_2S Analyser

After verification of the *H_2S Analyser,* the level of H_2S in the plasma of some healthy controls and MI patients measured using the Reference Fluorescence were measured using the *H_2S Analyser*. The concentration of H_2S obtained by both the Reference Fluorometer and *H_2S Analyser* in 24 normal controls and 35 MI patients were compared. There was no significant difference between H_2S levels neither in normal controls nor in MI patients (Figs. 3B and 3C) measured by both fluorometers. Normal controls and MI patients H_2S level was compared with the *H_2S Analyser* and a significant difference was observed where the average level of H_2S 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_2S Analyser* (Fig. 3D), which confirms the same results obtained by the Reference Fluorometer. The *H_2S Analyser* can be used for point-of-care-testing of H_2S 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_2S 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_2S in both aqueous solution and plasma using DNS-Az probe. The POCT methodology was used to compare H_2S 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_2S 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_2S level is in the line the liability of H_2S 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.

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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.

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