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The use of high-performance liquid chromatography with diode array detector for the determination of sulfide ions in human urine samples using pyrylium salts

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ABSTRACT

Hydrogen sulfide is a toxic gas involved in the regulation of some essential biological processes. A novel, precise, accurate and rapid method based on high-performance liquid chromatography with diode array detection for the determination of sulfide ions in human urine sample is proposed. The method involves the derivatization of sulfide with pyrylium salts – (2,4,6-triphenylpyrylium hydrogensulfate(VI) (L1) and 4-[p-(N,N-dimethylamino) phenyl]-2,6-diphenylpyrylium chlorate(VII) (LN1). The separation occurs on InfinityLab Poroshell 120 EC C_{18} column using acetonitrile and phosphate buffer as a mobile phase. The detectors utilized a wavelength of 371 or 580 nm. The calibration curves were linear in the range of 2–150 μmol L^{-1} and 1–50 μmol L^{-1} for L1 and LN1 derivatives, respectively. The samples were found to be stable from sample collection to final analysis. The method was successfully applied to samples from apparently healthy volunteers.

1. Introduction

Hydrogen sulfide is an endogenous gasotransmitter in the cardiovascular system which plays a similar role to nitric(II) oxide and carbon (II) oxide [1,2]. Hydrogen sulfide is synthesized in various mammalian tissues such as in the brain, cardiovascular system, liver, and kidney [3] by the desulfurization of L-cysteine [4] or L-methionine [5]. While it is widely distributed in a variety of organs, hydrogen sulfide plays interesting roles in the kidneys [6,7]. It participates in the control of renal function and increases urinary sodium. Abnormal levels of H_2S have been associated with various disease states. Deficiency of H_2S has been implicated in diabetic kidney injury and its associated complications: hypertension, diabetic nephropathy and hyperhomocysteinemia [8]. Likewise, in the liver, abnormal levels of H_2S are correlated with liver cirrhosis [9]. Hydrogen sulfide is produced in high quantities in the kidneys [10] and is associated with many functions in renal physiology [7,11]. It participates in the control of renal function and increases urinary sodium. Deficiency of it has been implicated in diabetic kidney injury and associated with hypertension, diabetic nephropathy and hyperhomocysteinaemia [8].

Due to the link between hydrogen sulfide and disease states, it is necessary to develop methods for selective and sensitive detection of H_2S [12]. Currently, separation methods like gas chromatography (GC) (including headspace extraction applied to gas chromatography [13]), capillary electrophoresis (CE) [14–16], inductively coupled plasma-mass spectrometers (ICP-MS) [17], ion chromatography [18] coupled to a UV-VIS (post-column reaction) [19,20], conductivity [16], amperometric [21–23] and fluorescence [24] detection and high-performance liquid chromatography (HPLC) coupled to a UV-VIS [25] detection are used for H_2S determination. GC with flame photometric ionization detector [26,27] or mass spectrometry is generally suited for the selective determination of H_2S in natural gas samples. GC analysis of sulfide was also performed after derivatization to bis(pentafluorobenzyl)sulphide [28]. A HPLC [29,30] based on pre-column fluorescence derivatization with monobromobimane [31] or N,N-dimethyl-p–phenylenediamine [32] have been developed for the determination of trace amounts of sulfide. Thin-layer chromatography (TLC) procedure for H_2S determination was also proposed [33–35]. As far as we know, there is no method for determination of sulphide ions in urine.

In this work, pyrylium salts: (2,4,6-triphenylpyrylium hydrogensulfate(VI) (L1) and 4-[p-(N,N-dimethylamino)phenyl]-2,6-diphenylpyrylium chlorate(VII) (LN1) were utilized for the determination of sulphide ions in human urine. Conversion of the derivatization reagents into the corresponding 2,4,6-triphenylthiopyrylium (L3) and 4-[p-(N,N-dimethylamino)phenyl]-2,6-diphenylthiopyrylium (LN3) cations was

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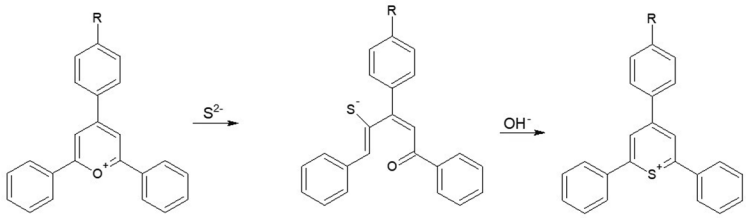

where:

R= H	L1	L3
R= $N(CH_3)_2$	LN1	LN3

Fig. 1. Reaction scheme of sulfide with 2,4,6-triphenylpyrylium compounds.

described in other works (Fig. 1) [36,37]. For instance, for LN1 such transformation involves a blue-shift of the absorption band from 540 nm for the pyrylium derivative to 580 nm for LN3 [36]. This is a highly selective reaction and only hydrogen sulfide and sulfide anions can induce such a colour change from magenta (LN1) to blue (LN3). This method was found to have benefits when compared to the other procedures mentioned above, such as high selectivity, relatively simple sample preparation, and short determination time.

2. Materials and methods

2.1. Chemicals and materials

Chemical reagents such as sodium hydroxide, sodium sulfide nonahydrate, 35–38 wt% hydrochloric acid, sulfuric(VI) acid, tris(hydroxymethyl)aminomethane, acetonitrile and sodium dihydrogen phosphate monohydrate were supplied from the POCH (Gliwice, Poland). 70% chloric(VII) acid, ethanol and 10 wt% trichloroacetic acid were purchased from the CHEMPUR (Piekary Śląskie, Poland). Other chemical reagents (2,4,6-triphenylpyrylium hydrogensulfate(VI), creatinine and methanol, p-N,N-dimethylaminobenzoic aldehyde, acetophenone) were received from the Sigma-Aldrich (St. Louis, MO, USA). All of the chemical reagents used for testing were analytical or HPLC grade. Deionized water was degassed before use.

Chemical compound 4-[p-(N,N-dimethylamino)phenyl]-2,6-diphenylpyrylium chlorate(VII) was synthesized at the Department of Organic and Applied Chemistry of the Faculty of Chemistry of the University of Lodz [36]. 0.1 mol of p-N,N-dimethylaminobenzoic aldehyde and 0.2 mol of acetophenone were introduced into a round-bottomed flask. 6 mL of concentrated sulfuric acid(VI) was added dropwise for half an hour while the solution was mixed on a magnetic stir plate at room temperature. Stirring continued for over 60 min at 100 °C, after which 200 mL of ethanol and 10 mL of 70% chloric acid (VII) were added. The resulting mixtures were kept at room temperature for 24 h to crystallize. The crude mixture was filtered, and the powder was further purified from the synthesis intermediates (acetic acid) and dried. Product was identified using NMR, IR and MS.

2.2. Preparation of standard solutions

Sodium hydroxide solutions (c = 1 mol L^{-1}) were prepared by weighing solid sodium hydroxide and transferring to a 50 mL volumetric flask. The flask was then filled with deionized water up to the mark and stirred.

TRIS buffer solutions (tris(hydroxymethyl)aminomethane) (c = $1{\cdot}10^{-1}$ mol·L^{-1} and appropriate pH) were prepared by dissolving 1.2114 g of TRIS in 80 mL of water and adjusted to the appropriate pH with 36% hydrochloric acid or sodium hydroxide solution (c = 1 mol L^{-1}). The pH was measured with a glass electrode calibrated with standards. The final solution was diluted with the appropriate amount of water to a total volume of 100 mL.

Sodium sulfide stock solution (c = $2.6{\cdot}10^{-3}$ mol·L^{-1}) was prepared by dissolving 0.0624 g of $Na_2S{\cdot}9H_2O$ in 100 mL of degassed water using an ultrasonic cleaner. The exact concentration of the sodium sulfide stock solution was determined by iodometric titration.

Sodium sulfide working solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) was prepared by placing 0.2 mL sodium hydroxide solution (c = 1 mol·L^{-1}) and 3.8 mL of sodium sulfide solution (c = $2.6{\cdot}10^{-3}$ mol·L^{-1}) in a 10-mL volumetric flask. The solution was further diluted with degassed water and mixed by an ultrasonic bath cleaner.

Stock solutions of L1 and LN1 (both at $1{\cdot}10^{-2}$ mol·L^{-1}) were prepared by dissolving the proper amount of L1 or LN1 in acetonitrile within a 10 mL volumetric flask. Working solutions of both L1 and LN1 (c = $1{\cdot}10^{-3}$ mol·L^{-1}) were prepared by dilution with acetonitrile.

Phosphate buffer solution (c = $1{\cdot}10^{-1}$, $1{\cdot}10^{-2}$ or $2{\cdot}10^{-2}$ mol·L^{-1} and the appropriate pH) was prepared by dissolving of sodium dihydrogen phosphate monohydrate in water and adjusting the solution to the appropriate pH with phosphoric acid (glass electrodes were calibrated with buffer standards, and used to measure the pH).

Creatinine solution (c = 1.5 mol·L^{-1}) was prepared by dissolving a mixture of 950 µL of 0.5 mol·L^{-1} hydrochloric acid and 50 µL of 36–38% concentrated hydrochloric acid. The solution was placed for 30 s on a vortex shaker operating at 3500 rpm/min to completely dissolve the creatinine.

2.3. Instrumentation

Chromatographic studies were performed on an Agilent 1260 Infinity High Performance Liquid Chromatograph (Agilent Technologies, CA, USA), equipped with an online degasser, autosampler (G7129A), quaternary pump (G1311B), column oven compartment (G7116A) and diode detector (G7115A). Chromatographic separation was achieved using an InfinityLab Poroshell 120 EC C18 (4.6 × 150 mm; 4 µm) analytical column (Agilent Technologies). The OpenLAB LC ChemStation software (Version C.01.04) was used to control the instrument, acquire data and data analysis (Agilent Technologies, CA, USA). Calibration curves and analytical figures of merit were obtained using Excel. During the experiments, the following laboratory equipment was also used: deionizer the POLWATER (Kraków, Poland); glass electrode euroSENSOR (Gliwice, Poland); ultrasonic bath POLONIC (Warszawa, Poland); pH meter CP-315 M ELMETRON (Zabrze, Poland); analytical balance accurate to 0.01 mg OHAUS (Nänikon, Switzerland); low temperature laboratory freezer cooling up to –80 °C PANASONIC (Amsterdam, Netherlands); laboratory fridge cooling up to 4 °C LIEBHERR (Bulle, Switzerland); freezer in a laboratory refrigerator cooling to –20 °C LIEBHERR (Bulle, Switzerland); MIKRO 120 laboratory centrifuge Hettich (Legnica, Poland); MULTI SPEED VORTEX MSV-3500 vortex shaker BioSan (Riga, Latvia). The spectrums of the synthesized LN1 were recorded on the apparatus Bruker Avance III 600 MHz (NMR ^{1}H and ^{13}C spectra), Nexus

FT-IR Thermo Nicole (spectrometer IR) and Varian 500-MS Ion Trap (spectrometer MS).

2.4. Specimens and urine collection

The non-fasting urine samples were collected from healthy volunteers between 23 and 70 years old of both sexes (regardless of dietary intake) . Urine samples were used immediately or stored at –80 °C, if needed. A research experiment using human urine samples was accepted by the University of Lodz Bioethics Research Commission to conduct this project (6/KBNN-UŁ/II/2019), and informed consent was obtained from all individuals.

2.5. Analytical procedures

2.5.1. Analytical procedures – Standards solutions

L3 analysis: 1 mL of TRIS buffer solution (c = $1{\cdot}10^{-1}$ mol·L^{-1} and pH 9), 3 mL of acetonitrile, 300 µL of sodium sulfide solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) and 600 µL of L1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) were mixed. 200 µL of chloric(VII) acid (70%) was added after 10 min. Lastly, the solution was adjusted to a volume of 10 mL with degassed water.

LN3 analysis: 0.5 mL of TRIS buffer solution (c = $1{\cdot}10^{-1}$ mol·L^{-1} and pH 10), 3 mL of acetonitrile, 300 µL of sodium sulfide solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) and 600 µL of LN1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) were mixed. After mixing for 2 min, 400 µL of chloric(VII) acid (70%) was added. The solution was then adjusted to a final volume of 10 mL with degassed water.

2.5.2. Analytical procedures – A human urine sample

L3 analysis: 1 mL of TRIS buffer solution (c = $1{\cdot}10^{-1}$ mol·L^{-1} and pH 9), 3 mL of acetonitrile, 1 mL urine (immediately after elimination) and 3000 µL of L1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) was mixed. 200 µL of chloric(VII) acid (70%) was added after 10 min of mixing. The solution was adjusted to a final volume of 10 mL with degassed water. The resulting reaction mixture was transferred to 2 mL tube and centrifuged at 14,000 rpm for 10 min.

LN3 analysis: 0.5 mL of TRIS buffer solution (c = $1{\cdot}10^{-1}$ mol·L^{-1} and pH 10), 3 mL of acetonitrile, 1 mL urine (immediately after defecation) and 3000 µL of LN1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) was mixed. 400 µL of chloric(VII) acid (70%) was added after 2 min of mixing. The solution was then adjusted to a final volume of 10 mL with degassed water. The resulting reaction mixture was finally transferred to 2 mL tube and centrifuged at 14,000 rpm for 10 min.

Creatinine concentration was determined in each of human urine samples as naturally occurring sulfide ions, according to the method described by Kuśmierek et al. [38].

2.6. Derivatives stability

The short- and long-term stability of L1 and LN1 derivatives in standard solutions and real samples. Replicate QC samples were prepared at a concentration of 30 nmol·mL^{-1} and kept at –80, –20, 4, and 25 °C. At room temperature, stability was measured over a 24-hour period. At other temperatures assessed, stability was measured over 3 days. Freeze-thaw stability was studied at three temperatures (–80, –20 °C) in the QC samples for three consecutive days.

2.7. Validation of the method

2.7.1. Validation of the method – Standards solutions

In order to validate the method's range and determine its accuracy and precision, a series of solutions were prepared in six replications, as follows.

L1 analysis: An appropriate volume (20, 50, 100, 150, 300, 500, 750, 1500 µL) of sodium sulfide solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) was mixed with an appropriate volume (40, 100, 200, 300, 600, 1000, 1500, 3000 µL) of L1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) and other chemical reagents listed in Section 2.5.1.

LN1 analysis: an appropriate volume (10, 20, 50, 100, 150, 300, 500 µL) of sodium sulfide solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) was mixed with an appropriate volume (20, 40, 100, 200, 300, 600, 1000 µL) of LN1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) and other chemical reagents listed in Section 2.5.1. The tests were carried out for three consecutive days and fresh solutions were prepared daily.

2.7.2. Validation of the method – Solutions with the addition of a human urine sample

Validation was carried out on standards containing urine in order to check if matrix components are affected in the designated range. For this purpose, a series of solutions was prepared in six replications, as follows. For method 1, the appropriate volume (0, 20, 50, 100, 150, 300, 500, 750, 1500 µL) of sodium sulfide solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}), 3000 µL of L1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) and other chemical reagents listed in Section 2.5.2 were mixed. Whereas for method 2, the appropriate volume (0, 10, 20, 50, 100, 150, 300, 500 µL) of sodium sulfide solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}), 3000 µL of LN1 solution (c = $1{\cdot}10^{-3}$ mol·L^{-1}) and other chemical reagents listed in Section 2.5.2 were mixed. The tests were carried out over three consecutive days with fresh solutions prepared daily. A morning urine sample from one volunteer, a 25 year old woman, was chromatographed for three consecutive days. After introducing the urine sample into a series of reaction mixtures, it was placed in a low-temperature laboratory freezer (–80 °C) and thawed to room temperature before each chromatographic analysis.

2.7.3. Precision and accuracy

The precision and accuracy of the proposed method was determined by analysing urine samples containing known amounts of analyte standards. Three concentrations representing the entire range of the calibration curves were investigated: low, high and one near the centre concentration of the linear concentration range on three separate occasions using six replicates. The spiked samples were processed according to the analytical procedure described in Section 2.7.2. Accuracy was calculated as a percentage of analyte recovery using the following formula: $\text{Accuracy}(\%) = \frac{(\text{measured amount} - \text{endogenous content})}{\text{added amount}} \cdot 100\%$.

3. Results and discussion

The use of L1 and LN1 in determination of sulfide ion content has been shown previously in spring water [37]. The goal of this study was to extend the above derivatization scheme for the determination of sulfide ions in urine samples. Therefore, individual conditions for each stage of the analytical method were optimized.

3.1. Optimisation of the chromatographic process

Chromatographic conditions were initially set according to previously developed method in our research group [37]. To maximize the performance of the assay, the following chromatographic parameters were optimized for both methods (L3 and LN3): percentage of acetonitrile in the mobile phase, flow rate of the mobile phase, pH of the phosphate buffer solution, concentration of the phosphate buffer solution and wavelength detection. To choose the optimal value for each of the aforementioned parameters, particular attention was paid to the shapes of the peaks, their surface areas, retention times, and to the complete separation of the derivatives tested from the excess of derivatizing reagent.

Several mobile phase compositions (percentage of acetonitrile and phosphate buffer solution) with different C_{18} columns were tested to ascertain the optimum HPLC conditions for the separation of thiopyrylium derivatives (L3, LN3) from excess reagent (L1 and LN1) and other

matrix components. An acceptable separation of the L3 and LN3 derivatives was obtained only on InfinityLab Poroshell 120 EC C18. The influence of acetonitrile content on the separation was investigated within the range of 25–85% for L3 and LN3 methods. As expected, an increase in the acetonitrile content in the mobile phase, from 25 to 80%, resulted in an increase in peak area (corresponding to L3 derivative) from 350 to 1100 mAUs. For the L3 method, the best separation of L1 and L3 peaks was obtained when the mobile phase contained 40% acetonitrile. For the LN3 method, the best separation of LN1 and LN3 peaks was obtained for a mobile phase which contains 65% of acetonitrile. An increase in acetonitrile content in the mobile phase from 25 to 65% resulted in increase in peak area from 374 to 483 mAUs. Satisfactory results were achieved when the mobile phase consisted of acetonitrile and phosphoric buffer solution at the ratio of 40:60 and 65:35 (*v/v*) for L3 and LN3, respectively.

Mobile phases with addition of varying buffer were evaluated during optimization and the phosphoric buffer was chosen for further studies. The influence of phosphate buffer concentration (within the range of 0.005–0.05 mol·L^{-1}) and pH (within the range of 2.3–4.5) on the separation was also studied. The results showed that different concentration of phosphate buffer did not have a significant impact the separation of L3 or LN3 from an excess of derivatization reagents. The chosen concentration of phosphate buffer was 0.01 mol·L^{-1} for L3 and LN3 derivatives. Moreover, the pH of the phosphate buffer was found to affect the separation process. The pH of the mobile phase had a minor impact on the retention and selectivity of L3 and LN3 derivatives. The value of the retention factor changed within the range of 6.5–7.1 and 5.1 for the studied conditions for L3 and LN3, respectively. Based on the maximum area of the L3 and LN3 peaks, the optimum phosphate buffer pH was 3.0 and 3.5 for both L3 and LN3, respectively.

Method optimization also involved changes to the flow rate to ensure that the method can efficiently separate all sample components. The influence of the flow rate on the separation and peak area of L3 and LN3 derivatives was checked between 0.4 and 1.0 mL·min^{-1}. As expected, an increase in peak area from 0.4 to 1.0 resulted in a decrease of retention factor from 7.0 to 6.2. Based on these findings, a flow rate of 0.9 mL·min^{-1} was chosen for the L3 method. For the LN3 method, the maximum retention factor was obtained for 0.55 mL·min^{-1}.

Table 1 shows the optimum chromatographic conditions for the two reagents. Typical chromatograms obtained for the free-sulphide and sulphide spiked urine samples (as a pyrylium derivative L3 and LN3) are presented in Figs. 2 and 3. One of the most important factors limiting the throughput of HPLC analysis is column turnaround time. In our procedure, this time was ca. 9 and 6 min for L3 and LN3, respectively.

Table 1
Optimum chromatographic conditions for L3 and LN3 derivatives determination.

Parameter	L3	LN3
Column	InfinityLab Poroshell 120 EC-C18 (4.6 × 150 mm; 4 μm)	
Composition of the mobile phase (acetonitrile:phosphate buffer solution, *v/v*)	40:60	65:35
pH phosphoric buffer	3.0	3.5
Concentration of phosphate buffer solution [mol·L^{-1}]	0.01	0.02
Mobile phase flow rate [mL·min^{-1}]	0.90	0.55
Volume of injection [μL]	25	
Autosampler temperature [°C]	4	
Column temperature [°C]	25	
Analysis time [min]	9	6
Wavelength [nm]	371	580

3.2. Optimization of derivatization reaction conditions

The reaction of sulfide ions with L1 or LN1 takes place in two steps [36,37] leading to the conversion of the derivatization reagents into thiopyrylium cations. There are different experimental variables that may affect each step of the derivatization reaction efficiency. As such, to determine the best reaction conditions for urine samples, many reaction variables were evaluated. These variables included for the first step: (1) the type of a buffer, (2) pH of reaction solution, (3) concentration of buffer solution and acetonitrile, (4) excess of L1 and LN1 derivatizing reagent and (5) derivatization reaction time. While the following parameters were optimized for the second step of derivatization reaction: (1) the type of acid and (2) volume of the acid used.

Reactions between sulfide and pyrylium cations described in previous reports were carried out in an alkaline solution [36,37]. Due to the poor solubility of L1 and LN1 in water, acetonitrile must be added into the derivatization mixture [37] and the volume of this organic solvent has a clear effect in the yield of the derivatization reaction.

In order to find the best buffer for this method, both TRIS and phosphate buffers were evaluated. Higher peaks were obtained when TRIS solutions were used for L1 and LN1 methods. TRIS buffer solutions in the 7.0–11.0 pH range were prepared to determine the optimum pH of the reaction mixture. The results in Table 2 show that the pH of reaction mixture increased the peak areas for L3 and LN3 derivatives at pH 9 and 10, respectively. Different concentrations of buffer solutions (within the range 25–300 mmol·L^{-1}) containing acetonitrile (in the range 0–70%) were also investigated. The peak intensities were higher for buffer solutions of 100 and 50 mmol·L^{-1} for L1 and LN1 methods, respectively. Based of these findings, 1 mL and 0.5 mL of 0.1 mol·L^{-1} TRIS solution was selected as an optimum for L1 and LN1 method, respectively. The largest peak areas for both L3 and LN3 derivatives were obtained from the samples with 3.0 mL of acetonitrile, which amounted to 30% of the reaction solution volume.

In the first stage of quantitative determination of sulfide ions in human urine samples, the appropriate volume of L1 and LN1 derivatizing reagent solutions were optimized to maximize efficiency. An excess of derivatizing agent was studied within the range of 1:1 to 1:50 (number of sulphide ions mole to number of pyrylium agent). The results showed that the use of a 2-fold molar excess results in greater repeatability of peak area for both methods. The data also showed that both derivatizations require longer reaction times for urine samples than for spring water [37].

The course of the derivatization reaction (and formation of cyclic thiopyrylium cation) is terminated by the addition of acid. An experiment was devised to check whether the type of acid (chloric(VII), trichloroacetic, hydrochloric) and amount of acid added (in the range of 100–700 μL) influence the product formation efficiency for L3 and LN3. Acids used in the deproteinization of urine samples can be used as an alternative to hydrochloric acid used in previous studies [37]. Thus, any proteins present in the urine should precipitate when the derivatization reaction is completed. The best results (peak area and height) were obtained in tests with chloric(VII) acid for the L3 and LN3 method. Chloric(VII) acid content in the samples was selected at 2 and 4% for the L3 and LN3 methods, respectively (Figs. 2 and 3).

3.3. Validation

The proposed methods were validated for 1) selectivity, 2) linearity, 3) limit of detection and quantitation, precision, recovery, stability and matrix effect according to the guidelines for analytical methods [39,40]. It was assumed that the concentration of L3 and LN3 compounds is equal to the initial concentration of the sulfide ion.

Selectivity: In our studies, selectivity is defined as the capability of an analytical method to distinguish and quantify the analyte in the presence of additional components in urine samples. Selectivity was determined from six different samples of urine with and without spiked

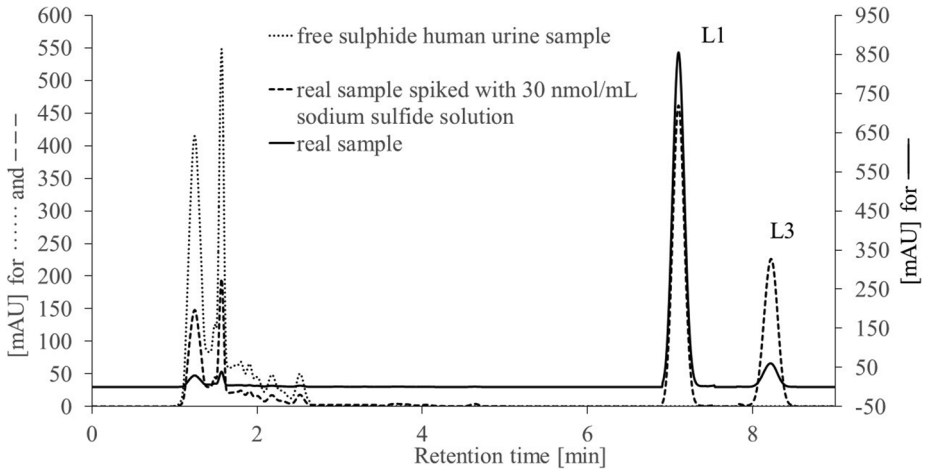

Fig. 2. An example of chromatograms obtained for L3 derivative.

in sulfide ions by comparison of chromatograms. No significant interferences from endogenous substances were observed. Furthermore, examination of peak purity showed that peaks which correspond to L3 or LN3 were not attributable to more than one compound.

Linearity: The relationship between detector response and L3 or LN3 concentrations was continuous and repeatable. It was demonstrated using an eight-point and seven-point calibration curves, respectively. The calibration curves were linear in the tested range from 2 to 150 μmol·L^{-1} and from 1 to 50 μmol·L^{-1} for total L3 and LN3, respectively. The equations for the linear regression line were $y = 2.0247x + 90.175$ for L3 and $y = 2.7643x - 31.349$ for LN3. The coefficients of correlation for the calibration regression were 1 and 0.9999 for both calibration curves.

Limit of detection and quantitation: Urine samples can contain endogenous sulfide ions, LOD and LOQ were estimated according to the following equations [39] $LOD = 3S_a/b$ and $LOQ = 10S_a/b$ where S_a is the SD of the y-intercept of regression line and b is the slope of the calibration curve. The values of the LOD and LOQ are presented in Table 3. To the best of our knowledge, this data has not been previously reported.

Precision and accuracy: Precision and accuracy were determined by analysing spiked urine samples, as described in Section 2.7.2. The values of precision and accuracy are presented in Table 3. The values obtained in this study fulfil the criteria for accuracy and precision of analytical methods [39].

Stability of analyte: The developed method was successfully used to study the stability of sulfide ions with L3 and LN3 in the presence of urine components. Bench-top stability indicated reliable stability behaviour during the experimental runs. The findings of the freeze/thaw stability test showed that the L3 and LN3 were stable in human urine in two freeze–thaw cycles when stored at –80, −20 °C and thawed to room temperature. The processed sample stability of L3 and LN3 samples showed that the analytes were stable for 24 h at room temperature. The auto-sampler stability tests showed the stability of L3 and LN3 in an auto-sampler (25 °C) at room temperature for 24 h. Finally, the auto-sampler 4 °C was applied to prevent the urine samples degraded in room temperature. The results from the long-term test indicate that storage of urine samples at −80, −20, 4 °C is adequate when maintained for 3 days.

Table 2
Optimum conditions for the derivatization reaction for the L1 and LN1 reagents.

Parameter	L3	LN3
pH of the TRIS buffer solution	9	10
Concentration of TRIS buffer solution in the reaction mixture [mmol·L^{-1}]	100	50
Content of acetonitrile in the reaction mixture [%]	30	30
Content of concentrated chloric(VII) acid (70%) in the reaction mixture [%]	2	4
Molar ratio sulfides to pyrylium salt [n_s^{2-}:n_{L1}or n_{LN1}]	1:2	1:2
Time of the derivatization reaction [min]	10	2

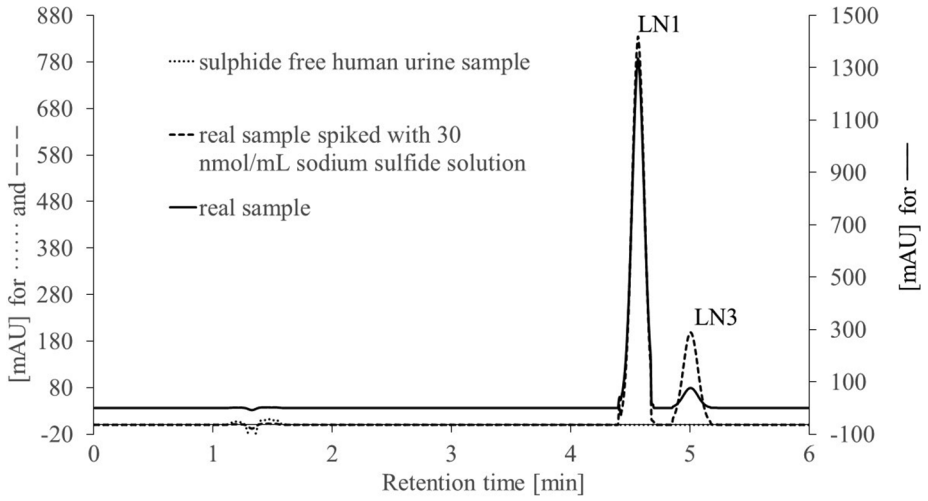

Fig. 3. An example of chromatograms obtained for LN3 derivative.

Table 3
Calibration data.

Analyte	Method	Regression equation	Linear range ($\mu mol_{added} \cdot L^{-1}$)	R^2	LOD ($\mu mol \cdot L^{-1}$)	LOQ ($\mu mol \cdot L^{-1}$)	Precision (%)	Accuracy (%)
Human urine	L3	y = 2.0247x + 90.175	2–150	1	5.6	16.9	0–0.4	99–104
	LN3	y = 2.7643x – 31.349	1–50	0.9999	3.3	10	0.03–0.33	95–128

4. Real sample analysis

The validated analytical procedure was applied to the analysis of 20 urine samples derived from apparently healthy volunteers. Sulphide ions were not detected in some samples. Average concentrations of sulfide ions in urine samples were 6.7 ± 4.05 $\mu mol \cdot mol_{creatinine}^{-1}$ and 5.7 ± 3.95 $\mu mol \cdot mol_{creatinine}^{-1}$ for L1 and LN1 methods, respectively. These results are consistent with those reported previously [9,41,42], and indicate that this assay can be successfully used in large populations. The assay is safe for the operator and environmentally friendly, meeting the needs of biological sample analysis.

5. Conclusion

The derivatisation methods were adopted for HPLC-DAD analysis of sulfide ions. The use of pyrylium cations was useful for analysis of sulfide ion content in human urine. Results from the analysis of sulfide in biological samples using the proposed methods are highly reproducible, reliable and similar to the results from other published methods. HPLC-DAD combined with pyrylium cations derivatisation can now be regarded as viable alternatives to GC [13,26–28], HPLC [25,29–31], IC [18] and EC [14–16] methods if the concentration of sulfide in the sample is high enough.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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