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High-performance liquid chromatographic separation and indirect fluorescence detection of thiols

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Abstract

A fluorescent post-column reaction detection scheme has been devised for selective determination of thiols. The post-column reagent is 40 μ*M* Cd^{2+} and 100 μ*M* 8-hydroxyquinoline-5-sulfonic acid (HQS) in non-complexing buffer at pH 10. HQS complexes Cd^{2+} to form a fluorescent product. Thiols in the HPLC effluent compete for complexation of the Cd^{2+}, resulting in a decrease in the fluorescence response. Detection limits of 0.2 μ*M* (0.04 ppm) are achieved for cysteine, homocysteine and glutathione in a 5 min separation. Recoveries from spiked synthetic urine samples are 87–120%.

Keywords: Derivatization, LC; Thiols; Cysteine; Homocysteine; Glutathione; Hydroxyquinolinesulfonic acid; Cadmium; Amino acids; Organosulfur compounds

1. Introduction

Organosulfur compounds include a large variety of molecules and among them, the thiols group is very important. Widely distributed in living cells, as part of proteins or as independent molecules, thiols are involved in numerous biological reactions. Their functions include heavy metal detoxification and transportation, oxidation protection of cells, synthesis of proteins and DNA, etc. They are also found in many drugs. Analysis of thiols then, is extremely important for the biochemical and clinical domains of research in order to understand many physiological and metabolism processes.

Several methods have been reported for thiols analysis using UV–Vis, fluorescence, electrochemical or chemiluminescence detection. Most of them are performed by HPLC and require pre-, post- or on-column derivatization. As reviewed by Shimada and Mitamura [1], these approaches use reagents such as N-substituted maleimides, bimanes, dansylaziridines, or halogenosulfonylbenzofurazans. All the methods described require complex manipulations and are time consuming. For example, Tang et al. [2] determined thiols in water samples using ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F), which belong to the halogenosulfonylbenzofurazans group. More than 1 h of sample preparation was necessary prior to HPLC analysis, and involved numerous manual steps including pre-reduction, reaction at elevated temperatures and quenching of the reaction.

In this paper, thiols are separated by HPLC and detected using a post-column indirect fluorescence

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system. An advantage of post-column reaction detection is that it allows the analytes to be separated in their native form. Thus, it is often possible to employ separation procedures from the literature. The thiol separation used herein is based on that published by Manna et al. [3]. A C_{18} column is used to separate cysteine (Cys), homocysteine (Hcys) and reduced glutathione (GSH) using a mobile phase of 0.01 *M* trifluoroacetic acid in water containing 0.5% methanol. This yields baseline resolution separation of the three analytes in 5 min.

The post-column reactant is a fluorescent complex of cadmium and 8-hydroxyquinoline-5-sulfonic acid (HQS). HQS, like its parent compound (8-hydroxyquinoline; oxine; 8-HQ), forms stable complexes with a wide variety of metals. However, only those metals which have stable electron configurations (i.e. either completely full or empty electron shells) yield fluorescent complexes [4]. Thus HQS can provide sensitive fluorescence detection only for Cd^{2+}, Zn^{2+}, Mg^{2+}, Al^{3+} and La^{3+} [5–10]. The detection limits for these metals, and particularly for Cd^{2+} are very good. For instance, Paull et al. achieved detection limits as low as 0.02 μ*M* (2 μg/l) using an ion-interaction reversed-phase liquid chromatographic method with on-column complexation with HQS [9]. Alternatively, Soroka et al. reported that detection limits well below 10^{-8} *M* and approaching 10^{-9} *M* could be achieved for Cd^{2+} using post-column reaction detection [4].

In addition to direct fluorescence detection of Cd^{2+}, Zn^{2+}, Mg^{2+}, Al^{3+} and La^{3+}, HQS can be used for indirect detection of other species if they perturb the fluorescent metal–HQS complex. For instance, Soroka et al. and Dasgupta et al. demonstrated that Fe^{3+}, Cu^{2+}, Ni^{2+} and Co^{2+} could be detected based on their quenching of the fluorescent Al–HQS complex [4,5]. The post-column detection of thiols used in the present publication is based on the quenching of the strongly fluorescent $Cd(HQS)_2^{2-}$ complex by competitive complexation of the cadmium by thiols to form non-fluorescent Cd–thiol complexes [11]. Alternatively, the weaker fluorescent $Zn(HQS)_2^{2-}$ also provides indirect detection of thiols [12]. However, as the detection limits for $Zn(HQS)_2^{2-}$ are comparable to that for $Cd(HQS)_2^{2-}$, only $Cd(HQS)_2^{2-}$ is studied herein.

After the separation, the effluent is mixed with the post-column reagent containing Cd^{2+} and HQS. When mixed together, Cd^{2+} and HQS form 1:1 and 1:2 complexes. Stability constants for the Cd/HQS system are $\log K_1 = 7.7$, $\log \beta_2 = 14.2$ (Eq. (1)), and HQS $pK_{a2} = 8.23$ [13,14]. As shown by Bishop et al., only the 1:2 complex fluoresces [15].

$$Cd^{2+} + 2\,HQS^{2-} \overset{\beta_2}{\longleftrightarrow} Cd(HQS)_2^{2-} \quad (1)$$

Stability constants for complexation of Cd^{2+} with thiols such as Cys ($\log \beta_2 = 19.6$, [16]) are much greater than that for the complexation of Cd^{2+} with HQS ($\log \beta_2 = 14.2$). Thus, when the thiols (RS^-) eluting from the chromatographic column mix with the $Cd(HQS)_2^{2-}$, the thiols complex the cadmium. This leaves HQS uncomplexed.

$$Cd(HQS)_2^{2-} + 2\,RS^- \longrightarrow Cd(SR)_2 + 2\,HQS^{2-} \quad (2)$$

Since neither HQS^{2-} nor the new complex thiol–cadmium is fluorescent [11], this decomplexation of $Cd(HQS)_2^{2-}$ results in a decrease in the fluorescence background. Presumably, mixed thiol/HQS complexes would also be formed and would be non-fluorescent. However, stability constants are available only for the 1:2 complexes. Therefore, for simplicity Eq. (2) does not include such mixed complexes.

In this work, the HPLC conditions, the concentrations of HQS and Cd, the pH of the post-column solution and the flow-rates are optimized. The specificity of the reaction is studied and the linearity and sensitivity of the method are determined. Artificial urine samples are also analyzed for Cys, Hcys and GSH.

2. Experimental

2.1. Reagents

All solutions and eluents were prepared in Nanopure ultra-pure water (Barnstead, Duburque, IA, USA). L-Cysteine, L-alanine, L-methionine, L-serine, γ-Glu–Cys–Gly (glutathione reduced form; GSH), 2-amino-4-mercaptobutyric acid (DL-homocysteine), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS),

3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), 2-(*N*-morpholino)ethanesulfonic acid (MES) and 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogenorthophosphate was from BDH (UK). 8-Hydroxyquinoline-5-sulfonic acid monohydrate (HQS) was obtained from Janssen Chimica (Beerse, Belgium), cadmium sulfate from Matheson Coleman and Bell (Norwood, OH, USA), Tris ultra pure buffer from Schwarz/Mann Biotech (Cleveland, OH, USA), sodium hydroxide analytical reagent from BDH (Toronto, Canada), trifluoroacetic acid 99% (TFA) from Aldrich (Milwaukee, WI, USA), hydrochloric acid reagent grade from Anachemia (Montreal, Canada) and HPLC-grade methanol and acetonitrile were purchased from Fisher (Fair Lawn, NJ, USA). Artificial urine matrix [17] contained 55 m*M* of sodium chloride (Fisher), 67 m*M* of potassium chloride (EM Science, Gibbstown, NJ, USA), 2.6 m*M* of calcium sulfate dihydrate (Sigma), 3.2 m*M* of magnesium sulfate (Caledon, Georgetown, Canada), 29.6 m*M* of sodium sulfate (BDH, Toronto, Canada), 19.8 m*M* of sodium dihydrogenorthophosphate (BDH, Toronto, Canada), 310 m*M* of urea (BDH, Toronto, Canada) and 9.8 m*M* of creatinine (Sigma).

2.2. Apparatus

The HPLC system consisted of a Beckman System Gold Model 125 dual piston pump (Beckman, Fullerton, CA, USA) operated at 1.0 ml/min, a fluorimetric detector (Model 470, Waters Associates, Milford, MA, USA; $\lambda_{excitation}$: 365 nm; $\lambda_{emission}$: 510 nm; [11] bandwidth, emission: 30 nm, excitation: 18 nm; rise time: 4 s) and a Rheodyne 7120 sampling valve (Rheodyne, Berkeley, CA, USA) fit with a 20-μl loop. Data were collected at 5 Hz with PeakNet 5.2 data software (Dionex, Sunnyvale, CA, USA) interfaced to a 100 MHz microcomputer. Initial optimization studies of the Cd–HQS post-column reagent were performed in the flow injection analysis mode (i.e. no separation column was present). All separations were performed on a C_{18} analytical column (150×4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA).

The post-column reagent was delivered by a LC-600 Shimadzu solvent delivery module (Shimadzu, Kyoto, Japan) at a flow-rate of 1.0 ml/min and using an Upchurch U-609 500 p.s.i. backpressure device (Oak Harbor, WA, USA) (1 p.s.i. = 6894.76 pa). For dynamic reserve, linearity, specificity and sensitivity studies, a constant pressure reagent delivery module was used to deliver the post-column reagent at 1.0 ml/min. An Upchurch P-727 tee (Oak Harbor, WA, USA) was used to connect the post-column solution to the column effluent. Connecting tubing was 0.005 in. I.D. (1 in. = 2.54 cm) polyether ether ketone (PEEK, Upchurch). The post-column reactor was ~5 cm of 0.005 in. I.D. PEEK tubing.

A Corning 445 pH-meter (Corning, NY, USA) with a Corning electrode (3 in 1 Combo P/N 476436) was used in all pH measurements. The instrument was calibrated with Orion NIST certified standard buffers solutions (Beverly, MA, USA).

The software *Solution Equilibra*: *principles and application* (*SolEq*) (1999) from Academic Software (Otley, UK) was used for the construction of theoretical speciation curves for the different equilibria.

2.3. Method

The MeOH–TFA–water mobile phase, the Cd–HQS–buffer post-column as well as all thiols and urine solutions were prepared daily. Thiol solutions were stored at 4 °C. Post-column solutions were prepared by first dissolving the buffer in water on a stirring plate and then adding HQS and cadmium sulfate. The solution was sonicated for 10 to 20 min to dissolve the HQS. The pH was adjusted with NaOH or HCl depending on the buffer.

The HPLC eluent and Tris, MES and MOPSO buffered post-column solutions were degassed by sparging with helium. Since CAPS and CAPSO solutions generate foams, the post-column reagent was not degassed when using these buffers. For background measurements, water was used as the post-column reactant to set the zero fluorescence on the detector.

In the post-column pH study, after equilibration and before injection, about 5 ml of the effluent was collected and $pH_{effluent}$ was determined using the pH meter.

3. Results and discussion

3.1. Post-column reaction

The theoretical limit of detection for indirect fluorescence detection is given by [18,19]:

$$C_{LOD} = \frac{C_P \cdot s_{BN}}{T_R} = \frac{C_P}{T_R \cdot DR} \quad (3)$$

where C_P is the concentration of the fluorescent probe, s_{BN} is the relative standard deviation of the background fluorescence fluctuations (noise), T_R is the transference ratio (the number of probe molecules displaced by one analyte molecule), and *DR* is the dynamic reserve (ratio of the background fluorescence intensity to the noise; $=s_{BN}^{-1}$). To maximize the sensitivity for indirect detection it is desirable to maximize the signal due to the probe so as to maximize *DR* or to allow reduction in the probe concentration. Thus, initial studies focused on optimizing the fluorescence signal from $Cd(HQS)_2^{2-}$.

3.1.1. HQS and Cd concentration

The concentration ratio of cadmium and HQS in the post-column solution is a key factor when considering the background fluorescence intensity. As shown in Fig. 1, for a post-column reagent containing a constant concentration of Cd^{2+} (40 μM), the background (fluorescence signal of $Cd(HQS)_2^{2-}$) increases as the concentration of HQS is increased. The signal then levels off at HQS concentrations greater than about 140 μM (ratios of HQS/Cd higher than 3.5). This behaviour is consistent with that predicted based on literature stability constants. The solid curve in Fig. 1 is the fraction of cadmium in the $Cd(HQS)_2^{2-}$ form ($\alpha_{Cd(HQS)_2^{2-}}$) calculated using *SolEq* based on literature stability constants for Cd^{2+} and HQS (Cd–HQS, Cd–hydroxide species, H_2O [16,20]) and HQS pK_{a2} [14] under the concentration conditions used in Fig. 1. Increasing the HQS concentration increases the fluorescence signal by forming more of the fluorescent $Cd(HQS)_2^{2-}$. At high concentrations of HQS all cadmium is in the $Cd(HQS)_2^{2-}$ form, and so no further increases in background intensity are observed. Thus, on the basis of maximizing the dynamic reserve (*DR*) it would seem reasonable to use HQS concentrations of 140 μM or greater with 40 μM Cd^{2+}.

However, as shown in Fig. 2, the signal observed for Cys, Hcys and GSH increases as the concentration of HQS increases up to only 80 μM HQS for Hcys and GSH and to 140 μM for Cys. At higher concentrations of HQS, the signals gradually decrease. This behaviour can be understood on the basis of the fundamental equilibria governing this post-column reagent (Eqs. (1) and (2)). Firstly, at low concentrations of HQS (<80 μM), only a

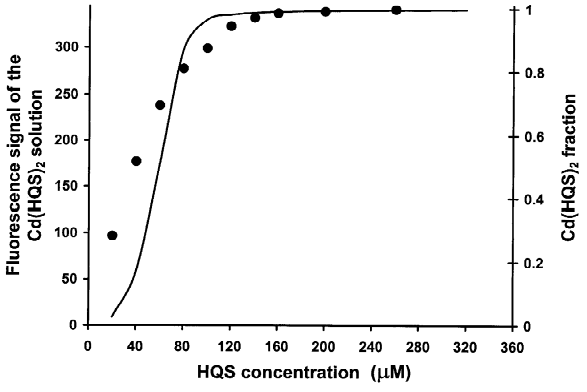

Fig. 1. Fluorescence of $Cd(HQS)_2^{2-}$ complex as a function of increasing HQS concentration to a constant Cd^{2+} concentration. Experimental conditions: post-column reagent: $Cd^{2+}=40$ μM, Tris 100 m*M*, $pH_{effluent}=9.1$. All other conditions are as listed in Section 2.2.

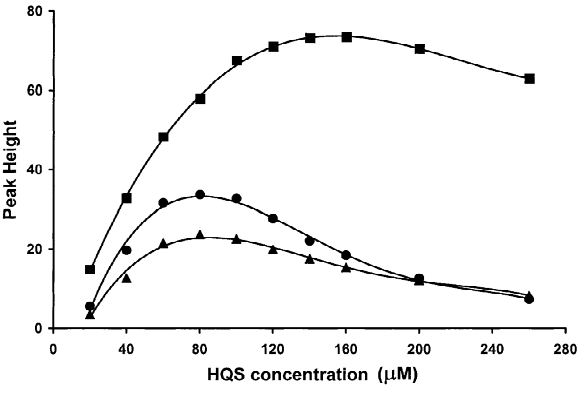

Fig. 2. Cys: ■, GSH: ● and Hcys: ▲ response as a function of increasing HQS concentration to a constant 40 μM Cd^{2+}. Experimental conditions as in Fig. 1. Analyte concentration = 200 μM.

portion of the cadmium is present as $Cd(HQS)_2^{2-}$ (curve in Fig. 1 based on Eq. (1)). Under these conditions thiols eluting from the HPLC column can complex free Cd^{2+}. Thus the thiols will not directly affect the $Cd(HQS)_2^{2-}$ fluorescence. Therefore, under these conditions the transference ratio (T_R) is low, resulting in lower sensitivity. As the concentration of HQS increases above 80 μ*M*, essentially all cadmium is present as $Cd(HQS)_2^{2-}$ (curve in Fig. 1). Under these conditions, the thiol will disrupt the $Cd(HQS)_2^{2-}$ complex, as shown in Eq. (2), with a resultant higher transference ratio. As the concentration of HQS is increased beyond that needed for formation of $Cd(HQS)_2^{2-}$, the excess HQS competes with the thiol for complexation of the cadmium. As a consequence, we observed a decrease in the sensitivity when HQS concentrations are higher than 100 μ*M* (ratio of 2.5) for Hcys and GSH and higher than 180 μ*M* (ratio of 4.5) for Cys. Such decreases in sensitivity at high HQS concentrations were also observed by Wang et al. [11].

The conditional cumulative formation constant for $Cd(Cys)_2$ (log $\beta_2' = 19.6$, based on log $\beta_2 = 19.6$, $pK_a = 8.33$ [14,16] and pH = 9.1) is substantially higher than that of $Cd(GSH)_2$ (log $\beta_2' = 15.2$ based on log $\beta_2 = 15.4$, $pK_a = 8.66$ [13,14] and pH = 9.1) [16]. Cys can then more effectively compete with HQS for cadmium complexation (Eq. (2)) than can GSH. Thus, the optimum fluorescence response occurs at a higher concentration of HQS for Cys than GSH. Unfortunately, no literature formation constants are available for Cd–Hcys.

3.1.2. pH of the post-column solution

The pH of the post-column solution is also an important variable which must be optimized. The fluorescence signal for the $Cd(HQS)_2^{2-}$ complex is pH dependent with maximum intensity at pH 7 according to Soroka et al. [4] and from pH 8–9 based on Wang et al. [11].

Speciation curves were generated using the *SolEq* software for the cadmium–HQS system using literature stability constants (see Section 3.1.1). The curve in Fig. 3 shows the fraction of cadmium present as $Cd(HQS)_2^{2-}$ in a solution containing 40 μ*M* cadmium and 100 μ*M* HQS over a variety of pH. Based on these calculations the background $Cd(HQS)_2^{2-}$ would be expected to exhibit a broad optimum from pH 8–12. Below pH 8 the HQS is incompletely ionized ($pK_{a2} = 8.23$). Thus, the conditional formation constant decreases dramatically as the pH decreases. At high pH, formation of cadmium–hydroxide species competes with the HQS for complexation of cadmium [21]. Under these same solution conditions, the intensity of the $Cd(HQS)_2^{2-}$ fluorescence (background) was experimentally observed (data points in Fig. 3) to increase with pH to a maximum signal between pH 9–11 and then decreased at higher pH. This behaviour is in agreement with that expected (curve in Fig. 3).

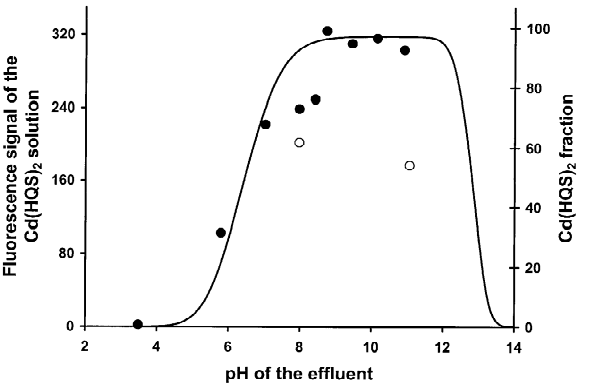

Fig. 3. Fluorescence of the $Cd(HQS)_2^{2-}$ complex as a function of pH of post-column reactant. Experimental conditions: analyte concentration = 200 μ*M*, post-column: HQS 100 μ*M*, Cd^{2+} 40 μ*M* (ratio HQS/Cd = 2.5), Buffers in post-column: ● $pH_{effluent}$ 3.5 = Tris 100 m*M*, $pH_{effluent}$ 5.8 = MES 100 m*M*, $pH_{effluent}$ 7.0 = MOPSO 100 m*M*, $pH_{effluent}$ 7.8–8.5 = Tris 100 m*M*, $pH_{effluent}$ 8.7–9.8 = CAPSO 200 m*M*, $pH_{effluent}$ 10.0–11.0 = CAPS 100 m*M*. ○ $pH_{effluent}$ 7.9 and 11.1 = phosphate 100 m*M*. All other conditions are as listed in Section 2.2.

The effect of pH on the indirect response observed for thiol analytes is shown in Fig. 4. For all of the analytes, optimal signal was observed at about pH 10.3. This is consistent with the results above. To achieve optimal sensitivity, the formation of $Cd(HQS)_2^{2-}$ has to be maximized. For this, the pH has to be higher than 8 (Fig. 3). In addition, the thiols have to be ionized. Thus pH has to be higher than their S–H group pK_a. In this case, the highest pK_a is 8.87 (Hcys). Therefore, the most favourable pH of the post-column solution should be above 8.87. Fig. 4 illustrates this very well.

Also, above pH 10.5 the thiol response decreases. This too is consistent with the studies above (Fig. 3) and results from the formation of cadmium–hy-

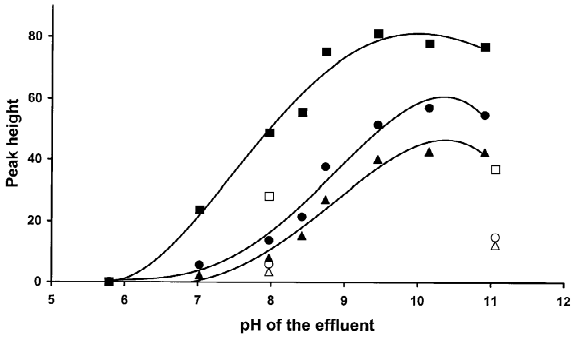

Fig. 4. Cys: ■ □, GSH: ● ○ and Hcys: ▲ △ response as a function of pH of post-column reactant. Experimental conditions as in Fig. 3.

droxides species limiting the amount of $Cd(HQS)_2^{2-}$ in solution, and hence the sensitivity.

3.1.3. Buffers

To obtain the wide range of pH in the studies in Section 3.1.2, a number of buffers were used in the post-column solutions. Care was taken to choose non-complexing buffers [5] such as Tris, MES, MOPSO, CAPS and CAPSO. This was done to ensure that the formation of $Cd(HQS)_2^{2-}$ was not compromised by competitive complexation of the Cd^{2+} by the buffer anion. To illustrate the potential detrimental effects of Cd^{2+} complexation by the buffer, data for a 100 m*M* phosphate buffer is included in Figs. 3 and 4. The use of a phosphate buffer considerably lowers both the $Cd(HQS)_2^{2-}$ background (open symbols, Fig. 3), and as a consequence the sensitivity (open symbols, Fig. 4).

3.1.4. Dynamic reserve (DR)

Eq. (3) shows that the dynamic reserve (background/noise) is inversely proportional to the limit of detection. To maximize dynamic reserve the background of the fluorescent probe has to be as high as possible and the noise in the baseline low.

Firstly, instrumental conditions were adjusted to minimize the baseline noise. Using a dual piston HPLC pump (LC-600 Shimadzu) along with a 500 p.s.i. backpressure device, a periodic oscillation of about 2 mV was evident in the background of an HQS/Cd 100/40 μ*M* post-column reagent. Addition of pulse dampers yielded essentially no improvement. The use of a constant pressure delivery system to deliver the post-column solution reduced the noise to 0.2 mV for the same reagent solution. By reducing the overall noise, dynamic reserves were improved by factors of 5 to 6, which has a direct impact on decreasing detection limits.

The concentrations of HQS and cadmium in the post-column reactant were varied to determine the optimum conditions as defined by a maximum ratio of background to noise (maximum *DR*). The HQS/Cd ratio in the post-column solution was kept constant at 2.5 at a pH of 10.0 with CAPS at 100 m*M*. The responses for Cys, Hcys and GSH were monitored at concentrations (μ*M*) of 50/20, 100/40 and 200/80 for HQS and Cd^{2+}, respectively. As the concentration of Cd–HQS increased, the background increased by an overall factor of 4. However, simultaneously the noise increased by a factor of 3. Thus the overall dynamic reserve increased slightly: 900, 1400 and 1300 for the 50/20, 100/40 and 200/80 solutions, respectively. About a 21% sensitivity was thus achieved by varying the probe (Cd–HQS concentration). In all further studies, the concentration of HQS and Cd were at 100 and 40 μ*M*, respectively.

3.1.5. Reaction rate and flow-rates

The reaction between $Cd(HQS)_2^{2-}$ and the thiols (Eq. (2)) is very fast. The sensitivity observed using a 5 cm segment of straight 0.005 in. I.D. PEEK was comparable to that for a 1.0 m × 0.5 mm I.D. knitted reaction coil (P/N 030805 Waters Associates, Milford, MA, USA). Further, significant band broadening was observed with the longer reaction coil. Thus the 5 cm of tubing was used as the reaction coil in all studies reported herein.

The flow-rate of the post-column solution was varied from 0.5 to 1.5 ml/min while keeping the eluent flow constant at 1.0 ml/min. The resolution between Cys and Hcys was found to be affected when the flow-rate was changed. The best resolution was at a post-column flow-rate between 0.9 and 1.1 ml/min. Therefore, a flow-rate of 1 ml/min was selected for the post-column solution.

3.1.6. Specificity of the reaction

Stability constants for Cd^{2+} with carboxylate, amine and thioether ($RSCH_3$) groups are small (log

$\beta_2 < 5.5$) [13,20,22] relative to the complexation of Cd^{2+} by thiols (e.g. log $\beta_2 = 19.6$ for cysteine). Thus it is expected that Cd–HQS should show good specificity for thiols relative to other biomolecules. To ensure this, high concentration solutions (5000 μ*M*) of serine, alanine and methionine were injected. The post-column solution was HQS/Cd at 100/40 μ*M* in a 100 m*M* CAPS buffer with $pH_{effluent} = 10.14$. When comparing the sensitivity with an almost 27 times lower concentration of Cys, Hcys and GSH (188 μ*M*), response factors (signal/concentration) of serine, alanine and methionine were found to be on average about 300 times lower than those of the thiols (Table 1). This demonstrate that the reaction with $Cd(HQS)_2^{2-}$ is governed by RS^- groups (Eq. (2)).

3.2. Separation conditions, detection limits and determination of thiols in synthetic urine

Manna et al. [3] separated GSH and GSSH using 0.1% TFA (0.01 *M*) in a water–acetonitrile (98:2) mobile phase. However, under these conditions Cys and Hcys were too weakly retained to achieve separation. Decreasing the organic modifier concentration resulted in greater retention of all analytes, and ≤1% acetonitrile or methanol gave adequate separations (Fig. 5). An eluent of 0.5% (v/v) methanol with 0.01 *M* TFA was used in all further studies as it provides a baseline resolution between Cys and Hcys ($R_S = 1.7$). A mobile phase containing 0.5% acetonitrile gave similar retention times as methanol 0.5% but with an inferior Cys–Hcys resolution ($R_S = 1.5$).

Calibration curves for Cys and Hcys are linear

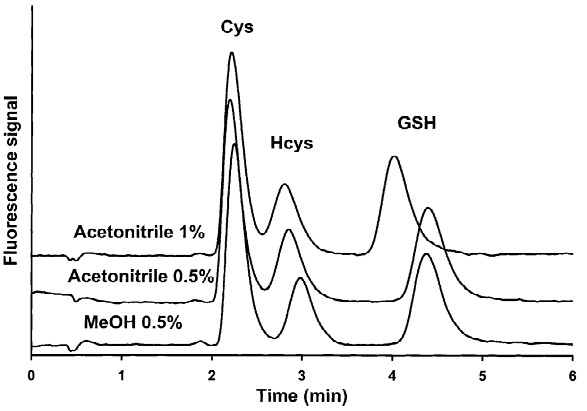

Fig. 5. Cys, Hcys and GSH chromatograms under different separation conditions. Experimental conditions: mobile phase: TFA 0.01 *M*, acetonitrile (1%, 0.5%)–water (99%, 99.5%) and methanol (0.5%)–water (99.5%), post-column: HQS/Cd^{2+} 80/40 μ*M*, Tris 100 m*M*, $pH_{solution}$ 8.5, analyte concentration 200 μ*M*.

($R^2 = 0.9985$ and 0.9987) up to, respectively, 430 and 370 μ*M* with intercepts equal to zero at the 95% confidence limit. Calibrations for GSH however showed a small negative deviation from linearity. The GSH response was well fit by a quadratic expression ($R^2 = 0.9994$) up to 430 μ*M* with an intercept equal to zero at 95% confidence limit. Table 2 shows the detection limits determined by the US Environmental Protection Agency (EPA) method [23]. This method gives the minimum concentration of an analyte that can be reported with 95% confidence to be greater than the noise. The detection limits range from 0.1 to 0.2 μ*M* for Cys, Hcys and GSH. This is an improvement in comparison with Wang's et al. [11] indirect fluorescence method where the cysteine detection limit was 0.4 μ*M* with a narrow dynamic range of 0–18.2 μ*M*. In contrast, Tang et al. [2] achieved 23 and 35 n*M* detection

Table 1
Response factors (signal/concentration) for Cys, Hcys, GSH, Ser, Ala and Met

Compound	Concentration (μ*M*)	Response factor (mV/*M*)
Cysteine	188	414 000
Homocysteine	188	207 000
Glutathione	188	313 000
Serine	5000	1670
Alanine	5000	1300
Methionine	5000	604

Table 2
Detection limits, run to run %RSD and dynamic range for Cys, Hcys and GSH

Thiol	Detection limit		RSD (%)	Dynamic range (μ*M*)
	μ*M*	ppm		
Cysteine	0.1	0.02	2.7	0–430
Homocysteine	0.1	0.02	5.5	0–370
Glutathione	0.2	0.05	3.4	0–430*

* Quadratic fit.

limits for GSH and Cys, respectively, using pre-column derivatization method with SBD-F. However, the derivatization procedure was complex and time consuming (>5 steps over 1.5 h including incubation at high temperature). Another derivatization method [24] using dansyl chloride gave detection limits of 1 pmol (signal-to-noise ratio 2) for GSH and GSSG (oxidized form of GSH). Pre-column derivatization (over 2 h including incubation in the dark) and a 30 min gradient separation were necessary.

Calibration curves were equivalent whether standards were prepared in water or synthetic urine. Synthetic urine [17] samples were spiked with 200 μ*M* of Cys, Hcys and GSH. Recoveries varied between 87 and 120%. Fig. 6 shows a chromatogram of Cys, Hcys and GSH at 200 μ*M* in synthetic urine. The large negative peak at the dead volume is due to the urine matrix and actually indicates an increase in the fluorescence. This increase in fluorescence is surprising and is currently under study. The Cys peak was expected to show poor recovery due to being so close to the negative peak but actually recoveries were near quantitative (95.6–99.7%). Similar quantitative recoveries were observed by Wang et al. [12] for Cys in protein hydrosylate and GSH in human blood serum using Zn–HQS.

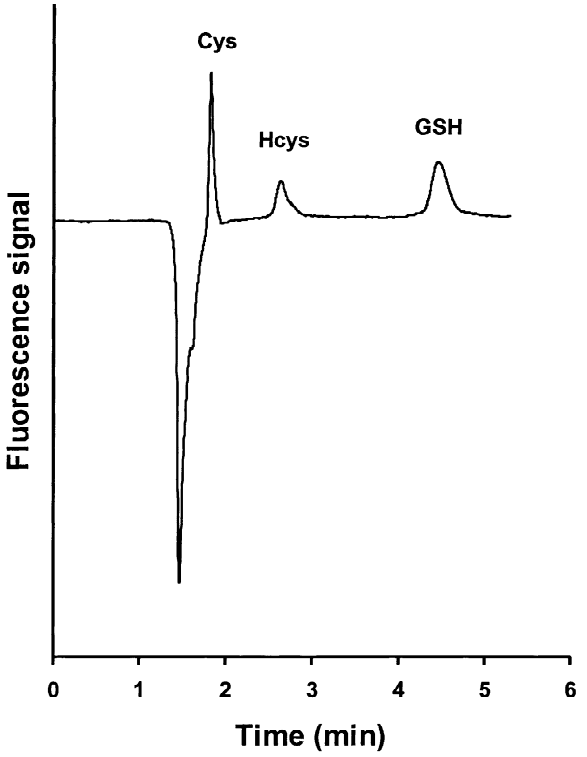

Fig. 6. Separation of Cys, Hcys and GSH (200 μ*M*) in urine matrix. Mobile phase: TFA 0.01 *M*, methanol 0.2%. Post-column: HQS/Cd^{2+} 100/40 μ*M*, Capso 200 mM, $pH_{solution}$ 9.0. Fluorescence detector rise time at 0.5 s.

4. Conclusions

Cys, Hcys and GSH were separated by reverse phase HPLC in their native form and simply detected by the quenching of a fluorescent $Cd(HQS_2)^{2-}$ complex added as a post-column reactant. No derivatization or sample preparation were needed. Detection limits as low as 0.1 μ*M* were achieved and a broad dynamic range of 0–430 μ*M* was obtained.

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