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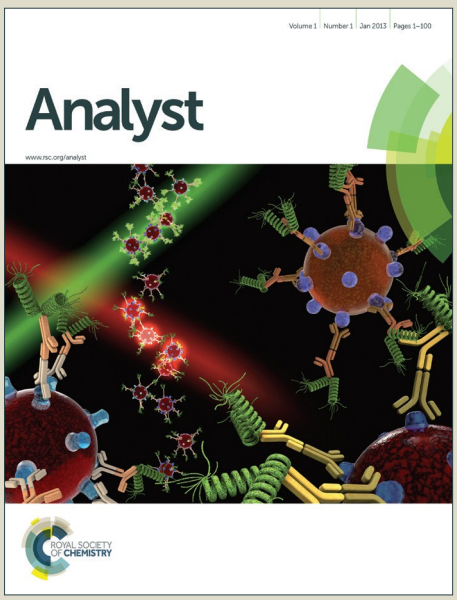

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Quinones as novel chemiluminescent Probes for sensitive and selective determination of biothiols in biological fluids

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ABSTRACT

Altered plasma aminothiol concentrations are thought to be a valuable risk indicator which interestingly utilize for routine clinical diagnosis and monitoring of various metabolic disorders and human diseases, and accordingly there is a need for accurate and reliable assay capable of simultaneously determining aminothiols including, glutathione (GSH), *N*-acetylcysteine (NAC), homocysteine (Hcys), and cysteine (Cys) in human plasma. Herein, a highly sensitive, selective, and very fast HPLC-chemiluminescence (HPLC-CL) coupled method is reported, exploiting for the first time the strong nucleophilicity and high reactivity of aminothiols toward quinones for CL assay. The unique redox-cycling capability of MQ and/or Michael addition adducts, thioether-quinone conjugates, were utilized to establish a novel analytical method based on the reaction of adduct with dithiotheritol (DTT) to liberate reactive oxygen species (ROS) which are detected by a luminol-CL assay. Specimen preparation involved derivatization of aminothiols with menadione (MQ) for 5 minutes at room temperature. A unique green chemistry synthesis of thioether-quinones in HEPES buffer (pH 8.5) was introduced by our reaction methodology; without needing to hazardous organic solvent or catalyst. The aminothiol-MQ adducts were separated using solid-phase extraction followed by isocratic elution on an ODS column. Linearity was observed in the range of 2.5-500, 5-500, 10-1500, 20-2000 nM with detection limits (S/N of 3) of 3.8, 4.2, 8, and 16 (fmol/injection) for GSH, NAC, Hcys, and Cys, respectively. The method was successfully applied for selective determination of aminothiols in human plasma from healthy people and patients with rheumatic arthritis and diabetes mellitus. The obtained results postulated the usefulness of our method for investigating the relationship between aminothiols metabolism and related human disorders.

INTRODUCTION

The physiological significance of low molecular weight thiols such as homocysteine (HCY), cysteine (CYS), glutathione (GSH), and *N*-acetylcysteine (NAC) (Figure 1) is well recognised with the levels of these compounds within biological fluids such as plasma and urine serving as valuable biomarkers in a number of clinical situations and a wide variety of diseases[1,2]. For instance, elevated levels of these aminothiols have been linked to Alzheimer's, Parkinson's, rheumatic arthritis, and cardiovascular diseases and increased levels of GSH and CYS have been noted in AIDS-related dementia[3,4]. Depletion of

intracellular aminothiols has been associated with liver disease, cervical cancer, leukemia, diabetes mellitus and several other disorders[3,5]. Consequently, the monitoring aminothiol levels in physiological systems are needed to further investigate their specific role as biomarkers. While there is an urgent need to monitor aminothiols and indeed several procedures proffered[6-10], considerable scope remains for the event of fast protocols that require minimal sample pre-treatment.

The main stumbling block in the development of measurement assay has been the unfavorable physicochemical properties of aminothiols. In fact, they lack a strong absorption in UV–vis regions or native fluorescence, are easily oxidized to disulfides, and are generally highly polar and water soluble, which makes their measurements an overwhelming challenge face the analyst and biologist[8-10]. Accordingly, the development of novel measurement protocols is particularly attractive. Among the various detection techniques, fluorescence detection is the most widely used technique owing to its sensitivity and selectivity[7-10]. Several fluorescent reagents have been employed, including *o*-phthaldialdehyde (OPA), monobromobimane (mBBr), *N*-substituted maleimides, iodoacetamido-containing reagents, difluoro-4-bora-3a, 4a-diaza-s-indacene (BODIPY), and benzofurazan derivatives[8]. Although the fluorescence derivatization assay overcame many obstacles, most of fluorescent reagents exhibit certain difficulties[9-12]. Therefore, new derivatizing reagents are still desirable to simplify the detection process and improve the sensitivity and selectivity of the detection methods.

The choice of derivatizing probes is remarkable not only for the selective and sensitive detection, but also for improvement of chromatographic properties and stabilization of thiols. The optimum reagent should react with thiol group specifically and rapidly at lowest possible temperature. The sulfhydryl group of aminothiols has strong nucleophilicity and high complexation activity which utilized in a verity of discipline[9]. On one hand, the high reactivity of thiols as strong nucleophiles towards α-β unsaturated compounds has been recognized from long time. On another hand, the important roles of quinones in biology can be attributed to their versatile oxidative and electrophilic properties which promote electron transfer in living systems and Michael addition with cellular thiols, such as free cysteine, glutathione, and cysteine residues of proteins[13]. Consequently, the nucleophilic addition of thiols to quinone moieties is well established, providing the basis of the synthesis of many biologically active compounds[14], numerous spectroscopic and pre-column chromatographic derivatisation as well as electrochemical protocols[15-19] which have been assessed and

successfully presented the fundamental characterization technique for the analytical determination of biological thiols. However, the reasonability of these conventions has remained a matter of theory. New measurement techniques thus are required exploiting the nucleophilicity of biothiols towards electrophiles.

The application of chemiluminescence (CL) assay for determination of trace and ultra-trace concentration of organic and inorganic compounds has interestingly increased owing to the simplicity and selectivity of CL measurements[13]. Several subsidiary articles have described the CL determination of thiols using batch or flow injection methods[20-22]. These methods lack the selectivity required to accurate measurement of thiols in complex biological matrices. Although many attempts to address this issue are recently reported[23-25], the sensitivity was not sufficient to ultra-trace concentration of thiols in some biological matrices. Michael-addition-type probes have been actively developed in recent years for the design of chromo-and fluorogenic probes for thiol sensing and methods using CL probe have yet to be published [9]; nevertheless, CL detection is a highly sensitive technique with several advantages over fluorescence and UV detection.

Up to date, there is no methodology that utilizes merits of aminothiols nucleophilicity and CL assay for biothiols measurement. Recently we have reported many novel and highly sensitive CL methods for determination of quinones and quinone-adducts. The proposed methods exploited the unique photochemical or redox-cycling ability of quinones to liberate powerful oxidizing agents including superoxide anion, hydroxyl radical, and hydrogen peroxide which measured by luminol-CL assay[13, 26-28]. Menadione (MQ), a representative quinone, can rapidly conjugate with plasma thiols after intraperitoneal administration forming thioether adducts[26]. Although the thioether adducts might be considered a part of quinone metabolic fate, the adducts retain the redox reactivity of parent quinones (Figure 1). In view of this fact, quinones can be exploited as a highly selective electrophile which could be attacked by aminothiols rapidly at room temperature giving thiol conjugates which retain quinone moieties and enter redox-cycling more efficiently than parent quinones (Figure 1)[26]. Therefore, the objective of our work is the development of novel and simple analytical methodologies that employ quinones as redox indicators for *in vitro* and *in vivo* assay of aminothiols.

EXPERIMENTAL

Chemicals and Reagents

All chemicals and solvents were of extra pure grade. MQ, acetonitrile (ACN), and methanol (HPLC) grade were supplied by Kanto Chemical Company (Tokyo, Japan). DTT, tetra-*n*-butylammonium bromide (TBAB), nitric acid, Hcys were from Nacalai Tesque (Kyoto, Japan). Luminol and phosphate buffer saline (PBS) powder (0.01 mol/L) were from Wako Pure Chemical Industry (Osaka, Japan). *Tris*(2-carboxyethyl) phosphine (TCEP), NAC, and Cys were from TCI (Tokyo, Japan). GSH was from KOHJIN Co. Ltd (Tokyo, Japan). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from Sigma Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) was purchased from Merck (Darmstadt, Germany). *Tris* (hydroxymethyl) aminomethane (Tris) was obtained from INC biomedical Co (Eschwege, Germany). SPE (solid phase extraction) cartridges, Oasis® hydrophilic lipophilic balance (HLB) 1cc/30 mg was from Waters (Milford, MA, USA).

MQ-GS, MQ-Hcys, MQ-Cys, and MQ-NAC were synthesized in our laboratory as described previously and the structures confirmed by MS and Elemental analysis[26]. Milli-Q water was purified by a Simpli Lab-UV (Millipore, Bedford, MA, USA) an ultrapure water system. Standard stock solutions of GSH, Hcys, Cys, and NAC (1 mmol/L) were prepared in purified water and of MQ (100 mmol/L) in ACN were kept at -30 ^{o}C until used. Working solutions were prepared each day from stock solution by appropriate dilution with purified water. All other chemicals were prepared in purified water unless otherwise indicated.

Instrumentation

The HPLC system (Figure 2) consisted of three LC-10AS liquid chromatographic pumps (Shimadzu, Kyoto), a Rheodyne 7125 injector (Cotati, CA, USA) with a 20-µL sample loop, a CLD-10A CL detector (Shimadzu), and SIC chromatorecorder (Tokyo, Japan). PTFE tubing (15 m×0.5 mm i.d., GL Sciences, Tokyo) was used as reaction coil. Chromatographic separation was performed on Cosmosil® 5C_{18}-MS-II (250 x 4.6 mm, i.d., 5µm, Nacalai Tesque) that used as stationary phase. Isocratic elution with a mixture of 10 mM Tris-HNO_3 buffer (pH 8.8) and ACN (65:35, v/v %) containing 30 mM TBAB was used as mobile phase. The eluent from the column was mixed with 1 mM of DTT in ACN and 1 mM of luminol in 25 mM NaOH aqueous solution, simultaneously. The flow rates of the mobile phase, DTT, and luminol solutions were set at 0.50, 0.25, and 0.25 mL/min,

respectively. Lumat LB-9507 luminometer (Berthold) was applied for time profile CL measurement, and HORIBA F22 pH meter was used to adjust buffer pH.

Sample preparation

One hundred microliters of human plasma or calibration solution, diluted with 500 mmol/L HEPES buffer, pH 8.5 to approximately 300 µL, was mixed with 10 µL of TCEP solution (100 mmol/L in HEPES buffer, pH 8.5) and allowed to react at room temperature for 15 min. 20 µL of MQ solution (100 mmol/L in ACN) was added and the sample was spin for 5 min at room temperature. Oasis HLB 1 cm^3/30 mg cartridges were used to isolate the resulting adducts from each biological sample. The cartridges were conditioned with 0.5 mL of methanol and equilibrated with 0.5 mL of purified water. The samples were passed through individual cartridges, after which the cartridges were washed two times with 250 µL of purified water. The target analytes were eluted with 150 µL of 40% ACN, followed by 150 µL of neat ACN. Each mixture was vortex mixed, diluted ten times, and 20 µL was then injected into the HPLC-CL system.

Method Validation

The method was validated by evaluation of the following parameters: specificity and selectivity was assessed by comparing chromatograms of plasma and plasma incubated with MQ. Sensitivity was termed by limit of detection (LOD) and limit of quantitation (LOQ) that were defined as the concentration with a signal-to-noise (S/N) ratio of at least 3 and 10, respectively. Linearity, expressed by the correlation coefficient (*r)*, was evaluated with calculation of a least-squares regression line. Linearity of each analyte was determined with at least 6 concentration levels, not including the blank on 3 separate days. In order to assess the intra- and inter-day precision and accuracy, three quality control (QC) samples at low, middle, and high concentrations of GSH, Hcys, Cys, and NAC were prepared as described above. The intra-day precision was assessed by calculating the % RSD for the analysis of the QC samples in triplicates; and inter-day precision was determined by the analysis of the QC samples on three separate days. The recovery for GSH, Hcys, Cys, and NAC were determined by comparing the peak height ratios of the analytes in human plasma at the QC concentrations to those in purified water at equivalent concentrations and expressed in percentage.

RESULTS AND DISSCUSION

Most of aminothiols are metabolically related and their disturbance can correspond to metabolic disorders, the simultaneous measurement of all the cited thiols thus presents a great interest. In this context, we applied for the first time the Michael-addition reaction of aminothiols to quinone for CL determination of aminothiols in various matrices after chromatographic. The thioether conjugates can react with dithiothreitol (DTT) which mimic NADH in biological systems liberating powerful oxidizing agents. The oxidizing agent can oxidize luminol giving 3-aminophtalate which shows high CL response. Factors affecting our methodology were carefully studied to achieve optimum derivatization, separation, and CL conditions as follow:

Optimization of derivatization condition

In this study, the nucleophilic addition of thiols to quinone was utilized to establish the first CL assay of aminothiols in biological systems. The principal of the proposed method is the reaction of the resulting conjugates with reductant to liberate powerful oxidizing agents which measured by luminol-CL assay. Factors affecting derivatisation reaction were carefully studied to achieve the high sensitivity required for ultratrace determination of aminothiols. Foremost, quinone type; many quinone compounds including 1,4-naphthoquinone, 2-bromo-1,4-naphthoquinones, 1,2-naphthoquinone, 1,2-naphthoquinone-4-sulfonate, MQ, and plumbagin were studied under our investigation to select the best quinone which showed the highest selectivity and sensitivity (Figure 3). Although 1,2-naphthoquinone and 1,4-naphthoquinone showed high arylation capability with nucleophiles[29], in this study MQ was selected as the optimal quinone because it showed the highest CL intensity and has one site for arylation and thus cross-link arylation would be cancelled, i.e. each aminothiol reacts with MQ giving one product correspond to one peak instead of two products in case of others unsbistituted quinones. The reaction of aminothiols with conjugated carbonyls, MQ, involves the addition of nucleophile RS^- to β-carbon of double bond followed by a proton transfer reaction[30]. For a given reaction, the observed rate constant found to be depended on pH and type and concentration of buffer. The reactive species is RS^-, thus the reaction rate and yield increase with pH, approaching a maximum at a pH where the SH group is completely ionized, i.e. as the pK_a of aminothiols more or less nearness to 8.5~9, the optimum pH was 8.5 (Figure S-1, supporting information). The catalytic effects of different buffers were compared, HEPES buffers turned out to be the most efficient, as compared to, for example

phosphate, borate, boric/borax, and Tris buffers (Figure S-2, supporting information). This is in agreement with Broensted catalysis low that the catalytic activity of a proton donor depends on its dissociation constant[31]. On the other hand, the concentration and the volume of buffer in the reaction media could affect the derivatization reaction. The reaction rate and yield were increased with elevation of HEPES molarity until 0.5 M which selected for further study (Figure S-3, supporting information).

Recently, the application of water as a reaction media has been highlighted and many of discoveries in green chemistry have been done. On one hand, water is regarded an environmentally friend solvent. On another hand, water is very cheap and safe solvent[31]. In our reaction, it is possible that water could increase the electrophilicity at the carbon center through hydrogen bond formation with carbonyl oxygen. Besides, the nucleophilicity of sulfhydryl group of aminothiols could also increase via the same mechanism. This explanation was clearly observed in our experiment, where the increasing of organic solvent in reaction media affected both reaction yield and rate (Figure S-2, supporting information).

The ratio of MQ/aminothiol in the reaction media was regarded as a significant factor which could affect reaction rate. Increasing the ratio of MQ to particular aminothiol was corresponded to accelerate the reaction rate until reach a plateau at molar ratio 10 which selected as optimal ratio (Figure 4). The use of a considerable excess of MQ effectively ensures that sufficient indicator is present to scavenge the aminothiol and prevent the side reaction. Under the chosen condition, the addition of thiols to MQ was quantitavely completed with reproducible manner in comparison with authentic specimen of synthesized conjugates, MQ-GS, MQ-Hcys, MQ-Cys, and MQ-NAC. Time course studies on the derivatization reactions of GSH, Hcy, Cys, and NAC with MQ were carried out at first separately and subsequently with a mixture of the thiols. As shown in Figure 5, the S/N ratio of the peak height vs. time indicated that the derivatization reactions proceeded to completion in a conveniently short time, 5 minutes in authentic specimens or biological fluids.

Under described conditions, aminothiols showed the highest reactivity compared to basic, acidic, neutral, aromatic, and hydroxyl containing amino acids (Figure S-4, supporting information). Aminothiols contains both sulfhydryl and amino group which can attack *α,β*-unsaturated compounds. However, under optimum reaction condition, mercaptide ions are about 300 times more reactive than amino groups[32].

Optimization of separation conditions

The vast majority of separation techniques applied for aminothiols employs pre-column derivatization and very few methods utilizes post-column derivatization after aminothiols separation in their native forms because of the instability and liability of sulfhydryl group to oxidation on analytical column. Hence, the derivatization of aminothiols by MQ could improve the stability of target analytes. The different nature between conjugates and parent MQ made the separation a challenge process. So, many columns and mobile phases comprising different organic modifiers, as well as buffers at different pH values, were studied to achieve good separation within feasible time between aminothiols conjugates of MQ and MQ itself. Satisfactory separation was achieved using reversed phase column, isocratic elution, and tetra-*n*-butylammonium bromide (TBAB) as an ion-pair reagent. The mobile phase was a mixture of 10 mM Tris-HNO_3 buffer (pH 8.8) and ACN (65:35, v/v %) containing 30 mM TBAB at flow rate 0.5 mL min^{-1}. The separation power and neutral effect of TBAB on CL response recommended it as optimum ion pair reagent.

Optimization of CL conditions

ODS column eluent containing MQ, MQ-GS, MQ-Hcys, MQ-Cys, and MQ-NAC was mixed with DTT and luminol and allowed to react in the reaction coil before CL detection (Figure 2). A typical chromatogram (Figure S-5, supporting information) showed that MQ-GS, MQ-Hcys, MQ-NAC, MQ-Cys, and MQ eluted at 15, 17, 18.5, 35, and 45 min, respectively. Many factors including CL probe, its concentration, and its dissolving agent can seriously influence CL response and sensitivity of target analytes. The optimum CL probe was luminol compared to other CL probes. This is important because luminol is a general CL probe exhibiting high CL sensitivity for superoxide anion radicals and other ROS. The influence of luminol concentration on CL intensity and S/N ratio was also examined and it was found that CL intensity increased linearly as the concentration of luminol increased; however, the background noise also increased. The best S/N ratio was obtained at 1 mM luminol (Figure S-6, supporting information). Moreover, the luminol solvent also significantly affected CL intensity. The highest CL response was achieved when NaOH was used at 25 mM, compared to carbonate or borate buffer solutions. In a preliminary study using a batch method, we explored the effect of different reductants; adequate CL response was only achieved with the dithiol compound, DTT. Consequently, DTT was used for further investigation by HPLC. Study of the influence of DTT on CL intensity and S/N ratio showed that the best CL response and S/N ratio were obtained at 1 mM DTT (Figure S-7 supporting information). The flow rate of both luminol and DTT also influenced the S/N ratio;

increasing or decreasing the flow rate than 0.25 mL min^{-1} showed low S/N ratio. As our reaction mechanism is glow-type, the mixed solution should pass through a PTFE reaction coil before CL detection. Using of reaction coil longer than 15 m showed high CL intensity and peak broading, thus 10 m was selected as optimal length (data not shown).

Method validation

The proposed method was validated according to the criteria described in the experimental section and based on the U.S. Guidance of Industry on Bioanalytical Method Validation (2001)[33]. Under optimum experimental conditions, a linear relationship was observed by plotting relative CL intensity versus target analyte concentration. The calibration curve prepared using quinone-thioether conjugates standard solutions was linear in the range 2.5-500 nM for GSH, 5-500 nM for NAC, 10-1500 for Hcy, and 20-2000 nM for Cys, with excellent correlation coefficients (r) > 0.990. The detection limits (S/N of 3) obtained were 3.8, 4.2, 8, and 16 (fmol/injection) for GSH, NAC, Hcys, and Cys, respectively (Table S-1, supporting information). Our results show that, for quantification of aminothiols in biological fluids, this method is faster, more sensitive, and proceed at room temperature than those using halogenbenzofurazans, other fluorescence reagents including BODIPY, maleimides and iodoacetamido containing probes[34-42] and CL[24,25] methods which reported previously. Method precision, both within day and between days, was assessed at low, middle, and high concentrations of GSH, NAC, Hcys, Cys as shown in Table 1. The intraday precision was below 0.1% as relative standard deviation percent. While the interday precisions for the aminothiols were lower than 2%, and thus the proposed method was achieved reasonable reproducibility required for aminothiols determination.

Determination of aminothiols in human plasma

The measurement of total aminothiols concentration in biological fluids should proceed carefully owing to their instability in aqueous medium and their tendency to oxidize to disulphide. In freshly prepared plasma or serum, almost all aminothiols are in the disulfide form-either bound to protein or to low-molecular-mass sulfhydryls, and only traces are found in free sulfhydryl form[43]. Thus, the reduction of disulphide bonds is one of the most critical steps in the sample pre-treatment procedure before the derivatization. Trialkylphosphines, including *tris*(2-carboxyethyl (TCEP), represented the most powerful reductant. As the concentration of total thiols was of interest, various concentrations of TCEP were tested at different time intervals to select an optimum reduction conditions. It was found that the

incubation of human plasma in presence of 100 mmol/L of TCEP at room temperature for 15 minutes was enough to obtain high reproducible results as mentioned in experimental section.

The removal of protein is a serious step to improve the performance of analytical procedure and extend the instrumentation lifetime. Acidification, addition of organic solvent, and solid phase extraction (SPE) are widely employed as means of proteins elimination. Although acidifications using trichloroacetic (TCA) acid or perchloric acid are preferable, acidic conditions of the sample are often not suitable to other sample pre-treatment such as reduction and derivatization steps[10]. Furthermore, the utilization of TCA to protein precipitation might interfere at time with the peaks of interest like in ours CL assay. Organic solvents including ACN, acetone, or methanol are favourable to mass spectroscopy [10], however, the large volume of organic solvent in our reaction media can affect both reaction yield and rate.

SPE was selected as optimal extraction technique to remove the proteins and others interfering substances. The derivatization of aminothiols with MQ might improve their physicochemical properties, high polarity, which intricate their extraction from biological fluids. In our previous study[26], Oasis® HLB sorbent (polydivinylbenzene-co-*N*-vinylpyrrolidine) was selected because it delivers highly reproducible recoveries and binds hydrophilic compounds, thioether conjugates of MQ specifically and selectively as shown in chromatograms (Figure 6). An HLB cartridge was washed and equilibrated, and the sample was loaded, washed and eluted as described in the experimental section. Many elution systems and elution volumes were studied to obtain high recovery. The best system is 150 µL 40% ACN followed by 150 µL 100% ACN. Under described condition, recovery was excellent: 93-97%, 74-109%, 97-100%, and 89-97% for GSH, NAC, Hcys, and Cys, respectively, at three different concentrations (Table 2). Thus, SPE provided the high recovery required for the detection of low aminothiols concentrations in human plasma. As shown in Figure 6 (A), peaks of aminothiol were eluted without interference from other biological substances.

The concentrations of aminothiols in human plasma (n= 3) were determined as follow: 1.87µmol/L, 0.093µmol/L, 2.03 µmol/L, and 174.9 µmol/L for GSH, NAC, Hcys, and Cys, respectively. These values were in good agreement with those reported in previous studies [34,35,38-40]. The extra-peaks found in chromatogram might be other unstudied aminothios which reported to be found in human plasma such as cysteinylglycine and γ-

glutamylcysteien. The proposed method was also extended to determine aminothiols in rheumatic arthritis (Figure 6 (B)) and diabetic patients (Figure 6 (C)); comparing with health control, the concentration of aminothiols were elevated two or three folds in plasma of rheumatic patient and decreased five to six folds in plasma of diabetic patients. This finding confirms the applicability of total aminothiols concentration as valuable biomarkers for the diagnosis of various human disorders. Hopefully, our method could be generalized for determination of sulfhydryl-containing compounds that of biological, pharmacological, and toxicological effects.

CONCLUSION

The potential exploitation of quinones as CL labels for the determination of thiols has been briefly assessed for the first time with MQ shown to promote a highly selective and sensitive response towards Cys, Hcys, NAC, and GSH. The present method offers a simple, rapid, sensitive, and reproducible HPLC method that has been fully validated for simultaneous determination of total concentrations of the aminothiols in human plasma after derivatization and SPE. Furthermore, the procedure was successfully applied to the determination of aminothiols in plasma of rheumatic and diabetic patient raising the utility of aminothiols as precious biomarkers of various human disorders. Moreover, the novel proposed method might succeed in determining aminothiols in other biological fluids including, urine, saliva, and cerebrospinal fluids. The convention presented here plainly provides a sound footing from which advance studies can be progressed and extended to the quantification of other sulfhydryl thiols and matrices. The large numbers of structurally diverse and commercially available quinones mark them as a promising CL reagent for thiols and other compounds to be introduced in analytical clinical chemistry.

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TABLES

Table 1. Method comparison with previously reported methods

Method	Labelling reagent	LOD (nM)				Reaction time (min)	Temperature (^{o}C)
		GSH	Cys	Hcys	NAC		
HPLC-FL[34]	SBD-F	0.8	1.5	0.8	0.3	60	60
HPLC-FL[35]	SBD-BF	20	10	100		30	RT
HPLC-FL[36]	ABD-F	100	500	500		10	50
HPLC-FL[37]	IAP	1	1	2.3	2	15	35
HPLC-FL[38]	TMPAB-I	0.3	0.3	0.7	0.3	20	45
HPLC-FL[39]	TMMB-Br	0.2	0.8	0.3	0.2	35	30
HPLC-FL[40]	MIAC	100	70	60		1	RT
HPLC-FL[41]	MIPO	0.17	0.75		0.75	35	40
HPLC-FL[42]	mBrB	50	50	50		1	RT
HPLC-CL[24]	Mn/HCHO	70	50	50	70	online	RT
HPLC-CL[25]	Gold nanoparticles	22.5	14.6	19.7		online	RT
HPLC-CL	Menadione	0.02	0.08	0.04	0.02	5 min	RT

Table 2. Method recovery and precision in human plasma

Thiols (added/µmol l^{-1})	Concentration, µmol l^{-1} (mean ± SD)	Precision (RSD, %)	Recovery, %
GSH			
0	1.87 ± 0.08	0.02	
0.05	1.90 ± 0.02	0.01	96.7
0.1	1.96 ± 0.05	0.01	97.1
0.3	2.04 ± 0.06	0.01	93.4
NAC			
0	0.093 ± 0.01	0.06	
0.05	0.106 ± 0.01	0.03	74.3
0.1	0.180 ± 0.03	0.05	93.4
0.4	0.538 ± 0.02	0.06	109.1
Hcys			
0	2.03 ± 0.04	0.03	
0.1	2.09 ± 0.03	0.02	97.9
0.5	2.45 ± 0.05	0.03	97
1	3.04 ± 0.06	0.03	100.2
Cys			
0	174.9 ± 0.4	0.03	
0.1	180.9 ±0.2	0.02	97.9
0.25	188.2 ±0.1	0.01	94.1
0.5	200.6 ±0.3	0.02	89.2

FIGURE LEGENDS

- **Figure 1**. (A) Chemical structures of studied aminothiols and (B) the schematic reaction of aminothiols with MQ.

- **Figure 2.** Effect of quinones type on S/N ratio under optimal derivatization condition. 100 µl calibration solutions, 10 µl TCEP (10 mM), and 170 µl HEPES (0.5M, pH 8.5) were mixed and incubated for 15 min at RT, then 20 µl represented quinones (600 µM) was added. The mixture was spinned for 5 min at RT, and 20 µl was diluted with mobile phase and injected to HPLC-CL system.

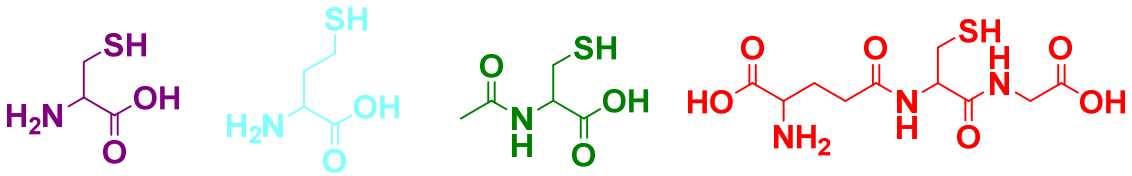
- **Figure 3**. Effect of MQ/thiol ratio on S/N ratio under optimal derivatization condition. 100 µl calibration solutions, 10 µl TCEP (10 mM), and 170 µl HEPES (0.5M, pH 8.5) were mixed and incubated for 15 min at RT, then 20 µl of different concentrations of MQ was added. The mixture was spinned for 5 min at RT, and 20 µl was diluted with mobile phase and injected to HPLC-CL system.

- **Figure 4**. Time courses of derivatization reaction of thiols with MQ
  100 µl calibration solutions, 10 µl TCEP (10 mM), and 170 µl HEPES (0.5M, pH 8.5) were mixed and incubated for 15 min at RT, then 20 µl of different concentrations of MQ was added. The mixture was spinned for various time intervals at RT, and 20 µl was diluted with mobile phase and injected to HPLC-CL system.

- **Figure 5.** Schematic diagram of HPLC-CL system

- **Figure 6**. Chromatogram of aminothiols in plasma of (A) health human, (B) rheumatic patient, and (C) diabetic patient treated with TCEP and MQ as described under our experiment.

(A)



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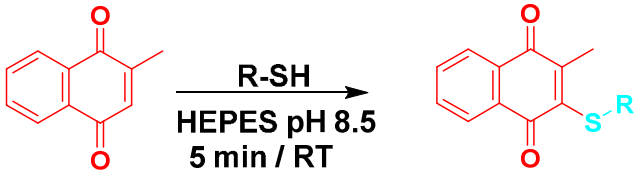

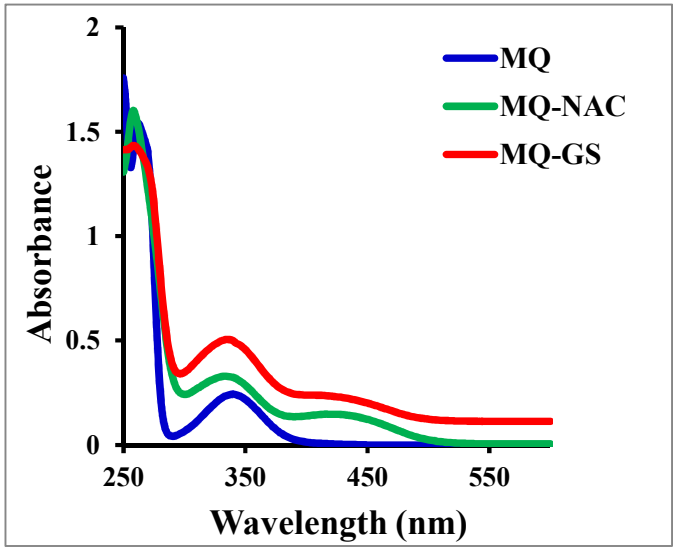

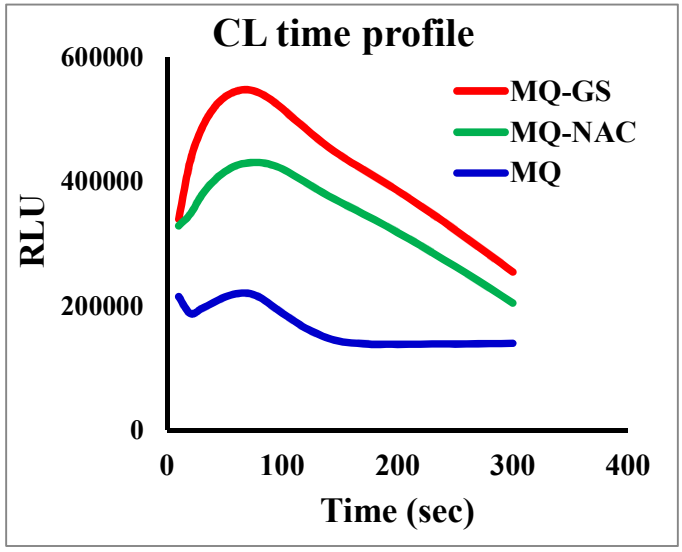

Figure 1

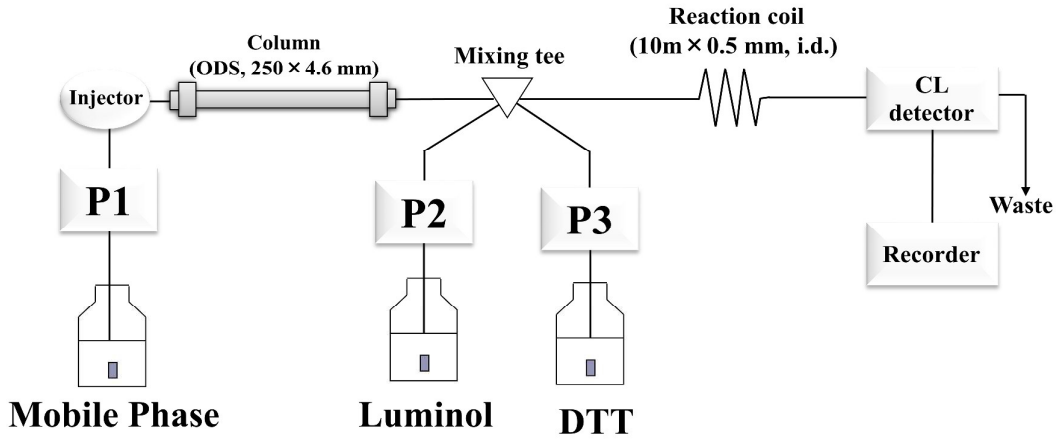

Figure 2

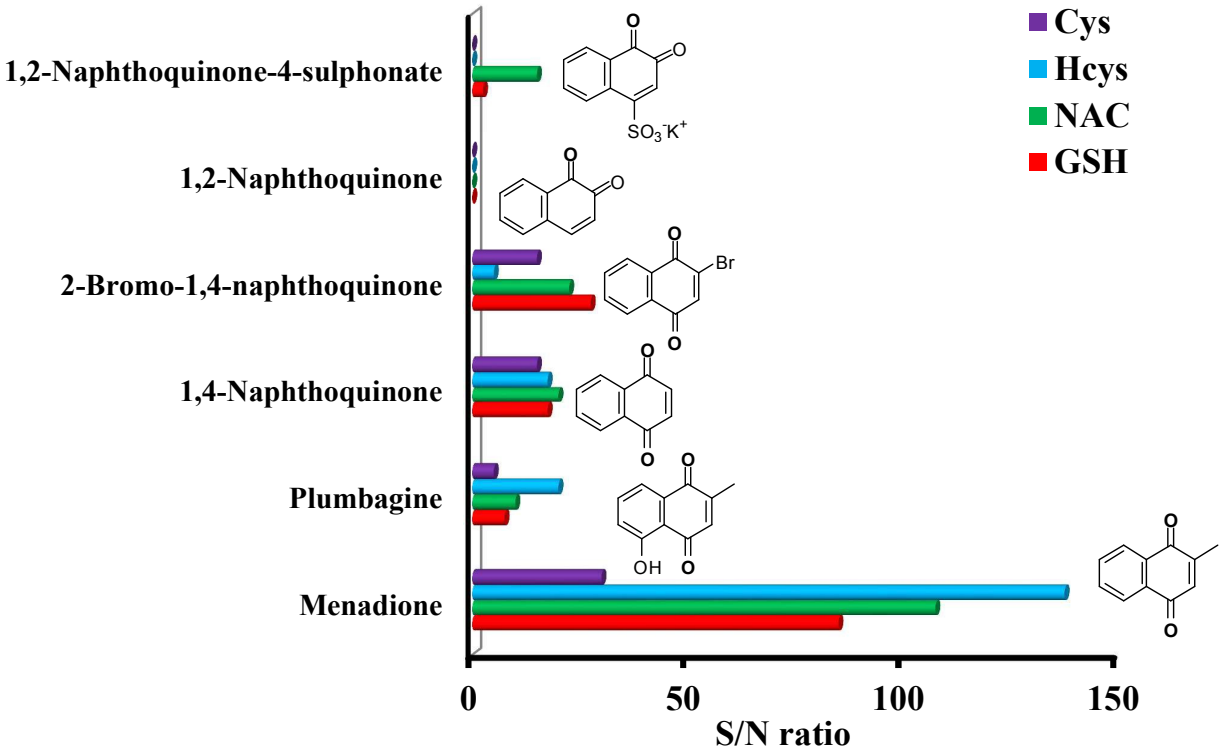

Figure 3

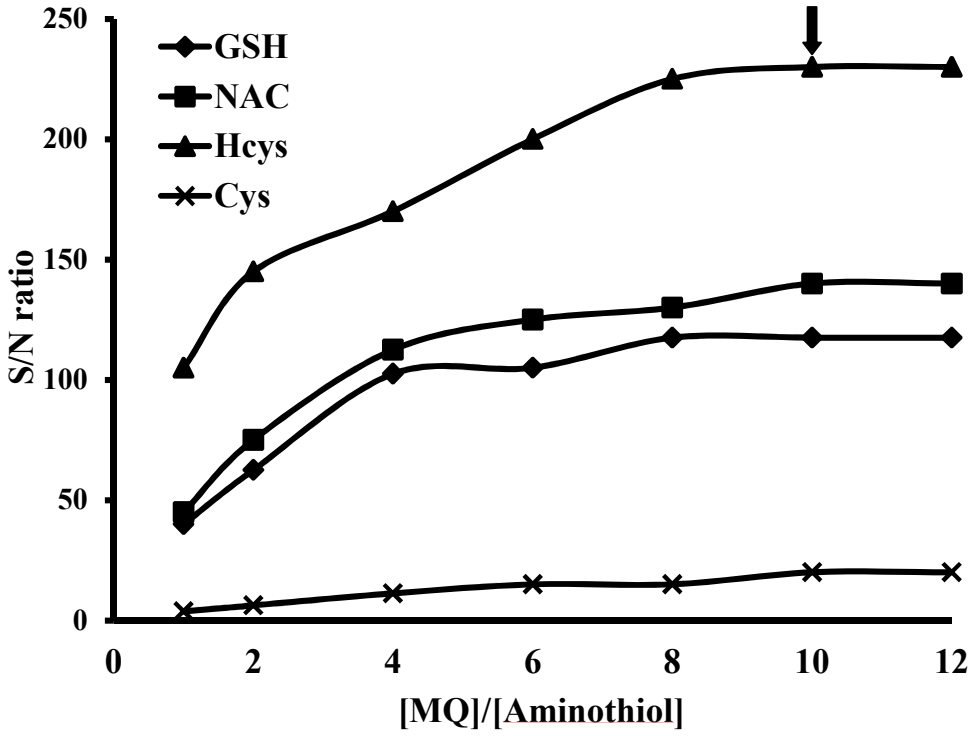

Figure 4

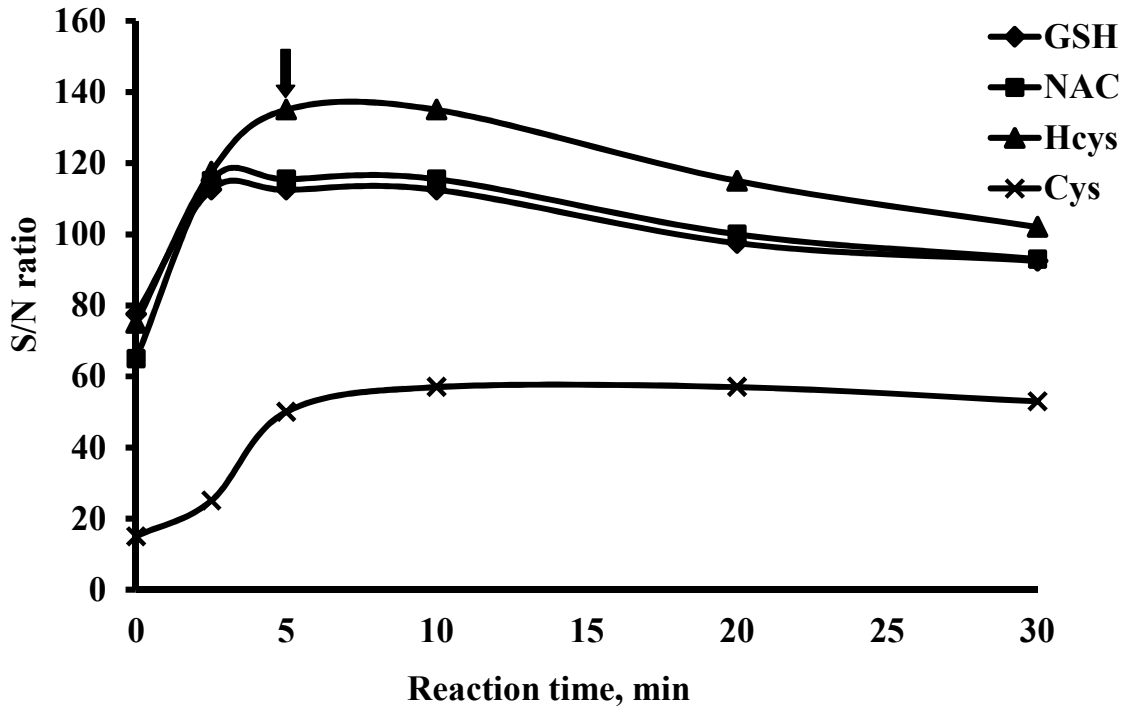

Figure 5

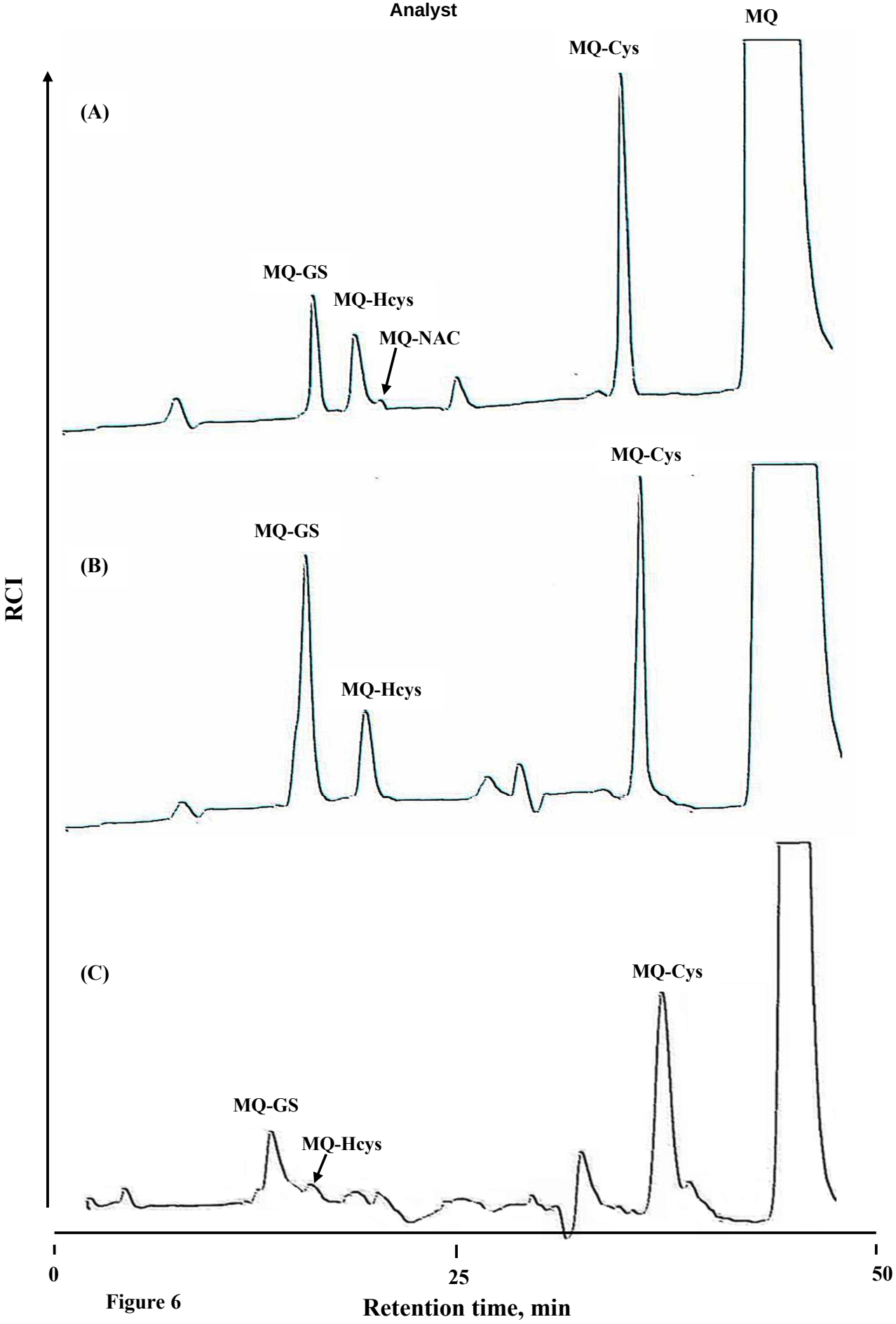

Figure 6