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Determination of plasma aminothiols by high performance liquid chromatography after precolumn derivatization with *N*-(2-acridonyl)maleimide

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ABSTRACT

Design, synthesis and properties of new derivatization reagent *N*-(2-acridonyl)-maleimide (MIAC) for thiol groups is presented. The reaction of MIAC with aminothiols is specific, very fast and yield highly fluorescent products. The HPLC method for determination of homocysteine, cysteine and glutathione based on utilization of MIAC is developed. A baseline separation of derivatives is achieved by isocratic elution on reverse phase column within 6 min. The method is linear in the range of 0.5–25 μM for homocysteine and glutathione, and in the range of 0.5–200 μM for cysteine. The limits of detection for homocysteine, cysteine and glutathione are 1.2, 1.4 and 2.0 pmol, respectively, per 20 μl injection. Within and between-run precision expressed as relative standard deviations are in the range of 1.35–4.38% and 0.89–4.13%, respectively.

1. Introduction

The biological aminothiols, such as cysteine, homocysteine, glutathione and their disulfides, are critical cellular components which play important roles in metabolism and homeostasis. Their altered levels in plasma have been linked to specific pathological conditions [1–4]. Measurement of aminothiols in physiological liquids is important for diagnosing of several human diseases as well as for drug monitoring. These facts impede the examination of the aminothiol contents in biological fluids and searching for high sensitive methods for analysis.

Several methods for quantification of aminothiols have been developed. They can be classified mainly in two groups: chromatographic methods and immunoassay methods. Chromatographic methods include GC-MS, ion-exchange chromatography and HPLC with different detection techniques [1,5,6]. Immunoassay methods include Enzyme Immuno Assay and Fluorescence Polarization Immuno Assay [7]. The HPLC method is one of the most popular procedures for quantification of aminothiol compounds in biological matrices and the fluorescent detection is preferred by most researchers.

Although a number of techniques were employed to assay these aminothiols, some problems still arise due to the operation of sophisticated instrumentation or the complexity of the procedure and its quantification reproducibility, particularly in case of such multi-component samples as biological fluids. The most frequently used method for total plasma aminothiol measurement is RP-HPLC with fluorescent detection after derivatization. Commonly used fluorogenic reagents include monobromobimane (mBrB) [8,9], 9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl)-3-oxo-3H-naphtho[2,1-*b*]pyran (ThioGlo) [10], ammonium 7-fluorobenzo-2-oxa-1,3 diazoloe-4-sulfonate (SBD-F) [11,12], 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F) [13,14], *N*-(1-pyrenil) maleimide (NPM) [15], dansylaziridine [16]. These reagents are not totally satisfactory. Some of them generate multiple fluorescent products, which interfere with quantification of thiols, require long derivatization time and elevated temperatures. Light sensitivity and photounstablility of the reagents or derivatives is another problem [5,7]. Thus, the development of sensitive, selective, and reactive derivatization reagents is still needed.

There are many reports in the literature describing highly fluorescent acridine and acridone compounds as effective derivatizing reagents and fluorescent markers for labeling [17]. These

Abbreviations: PyBOP, (benzotriazol-1-yl-oxy)tripyrrolidinophosphonium hexafluorophosphate); DIEA, *N*,*N*-diisopropylethylamine; DCCI, dicyclohexyl carbodiimide; TCEP, tris(2-carboxyethyl)phosphine; NMP, *N*-methyl-2-pyrrolidone.

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compounds display specific photophysical properties, i.e. high fluorescent quantum yield and high molar absorption coefficients. Several studies have been reported utilizing 2-aminoacridone for derivatization of simple sugars and a number of linear and branched polysaccharides [18]. Acridone-*N*-acetyl chloride was successfully used for pre-column fluorescence derivatization and determination of amino acids and *N*-nitrosamines [19,20]. A method for quantification of amino compounds has also been published based on 9-(2-hydroxyethyl)acridone as derivatization reagent after formation of its activated amide intermediate 9-(2-acridone)-oxyethylcarbonylimidazole [21]. Acridone-9-ethyl-*p*-toluenesulfonate was applied for determination of free fatty acids and acridone-9-*N*-acetyl-benzene-disulfonate for determination of alcohols [22]. *N*-(9-acridinyl)maleimide was originally developed as thiol reagent [23] but utilized more frequently as reagent for sulfite determination [24]. In view of these facts, we designed a new thiol specific derivatization reagent with acridone structure and maleimide moiety attached to the acridone ring in its second position—*N*-(2-acridonyl)maleimide (MIAC). MIAC reagent displays high affinity for thiol groups and the reaction is very fast and simple. The fluorescence intensity of the MIAC is extremely low but after reaction with thiol compounds yield highly fluorescent products, which is another advantage of the presented new derivatizing reagent. The reaction products show good stability due to their specific configuration—large and planar structure of the acridone ring.

In the present paper, we describe the design, synthesis and properties of new thiol derivatizing reagent *N*-(2-acridonyl)maleimide and its application for quantification of aminothiols in human plasma. The developed method is fast, simple, sensitive and precise. The MIAC-thiol derivatives were baseline resolved and quantified by means of RP-HPLC for less than 6 min.

2. Experimental

2.1. Chemical and reagents

L-Homocysteine, L-cysteine, glutathione, TCEP·HCl and 2-aminoacridone were purchased from Sigma (Germany). Dibutylamine, trichloracetic acid (TCA) and perchloric acid were purchased from Merck (Germany). The acetonitrile of gradient grade was obtained from Sigma (Germany). The water used for buffer preparations was purified by Purelab UHQ II system (ELGA, Vivendi Water Systems, Netherlands). All other reagents were of the highest purity available.

2.2. Synthesis of derivatization reagent

To a solution of 2-aminoacridone (42 mg, 0.2 mmole) in 2 ml mixture of acetonitrile and *N*-methyl-2-pyrrolidone (9/1, v/v), maleic anhydride (39.2 mg, 0.4 mmole) was added. After 1.5 h stirring at room temperature, DIEA (51 μl, 0.6 mmole) and PyBOP (78 mg, 0.3 mmole) were added to the reaction mixture. The time course of the reaction was followed by means of HPLC. After 2.5 h complete cyclization of the starting intermediate was observed. Acetonitrile was evaporated under vacuum and residue dropped to 30 ml of 5% aqueous sodium hydrogencarbonate. The formed fine orange-brown colored precipitate was filtered off, washed with water and dried under vacuum over KOH. Recrystallization from acetonitrile/acetic acid results in orange-yellow fine needles. Yield 54 mg (93%). ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 11.95 (s, 1H), 8.25 (d, J = 8.0 Hz, 1H), 8.18 (s, 1H), 7.77 (t, J = 7.5 Hz, 1H), 7.70 (dd, J = 8.8, 1.7 Hz, 1H), 7.65 (d, J = 8.8 Hz, 1H), 7.58 (d, J = 8.3 Hz, 1H), 7.30 (t, J = 7.4 Hz, 1H), 7.22 (s, 1H); MS (ESI, m/z 290.15, cal. 290.27).

2.3. Standards and samples

Individual stock solutions of Cys, Hcy and GSH were prepared in deionizated water. Working standard solutions with different concentrations were prepared from the stock solutions by appropriate mixing and diluting with PBS buffer with pH 7.4 according to previously described analytical procedure [25].

Samples of human plasma used during this study were provided by the Clinical laboratory of the University Hospital for Neurology and Psychiatry at Medical University of Sofia.

2.4. Analytical procedures for determination of aminothiols

To 100 μl of plasma sample 10 μl of reducing reagent TCEP (10 g l^{-1}) were added. The sample was incubated at 40 °C for 10 min and 20 μl of precipitation agent TCA (100 g l^{-1}) were added. After incubation for 10 min at room temperature the precipitated protein was separated with centrifugation for 15 min at 13,000 × *g*. To an aliquot of 50 μl of supernatant 50 μl of 0.3 M phosphate buffer with pH 8.2 and 10 μl of derivatizing reagent MIAC (10 mM in DMSO) were added. The reaction mixture was thoroughly vortexed for a minute and was acidified with concentrated HCl (5 μl). 20 μl injection volumes were used for HPLC analysis.

2.5. Chromatographic analysis

2.5.1. Instrumentation

Analyses were carried out on HPLC system consisted of P2000 binary gradient pump (ThermoSystem, USA), fluorescent detector FL3000 (ThermoSystem, USA) and 20 μl manual injector model 7125NS (Rheodyne, USA). The fluorescence of MIAC derivatives was measured using 260 nm for excitation and 416 nm for emission wavelengths, respectively. Data acquisition and presiding was achieved with CW 1.7 chromatographic software (DataApex, Czech Republic). As the concentrations of the different thiol compounds in the plasma samples are in different range, we used two channels detector working at different range of sensitivity.

2.5.2. Chromatographic conditions

The separation of MIAC-derivatives of Hcy, Cys and GSH was carried out on Nucleodur Sphinx100-5 C18 ec (125 × 4) column (Masharey-Nagel, Germany) at flow rate of 1.5 ml min^{-1}.

Elution solvent A was 20 mM ammonium acetate and 1 mM dibutylamine in water/methanol (95/5%, v/v) mixture. The pH of the buffer was adjusted to 2.4 with 35% perchloric acid using pH meter (Schott, Germany) equipped with SenTix 41 electrode (WTW, Germany).

Elution solvent B was a mixture of acetonitrile and buffer A (95/5%, v/v).

Both buffers were filtered on nylon 0.45 μm membrane and degassed in sonic bath under vacuum before use.

The elution profile consisted of: (i) separation isocratic step 20% B for 6 min; (ii) washing step 20–70% B for 1 min, followed by 70% B for 3 min and (iii) equilibrium step of 4 min at initial conditions. The total analytical time was 15 min.

3. Result and discussion

3.1. Design, synthesis and properties of MIAC

In this paper, we present design and synthesis of new thiol specific derivatizing reagent.

In order to develop an effective derivatizing reagent that allows measuring of very low thiol concentrations, it is necessary to find

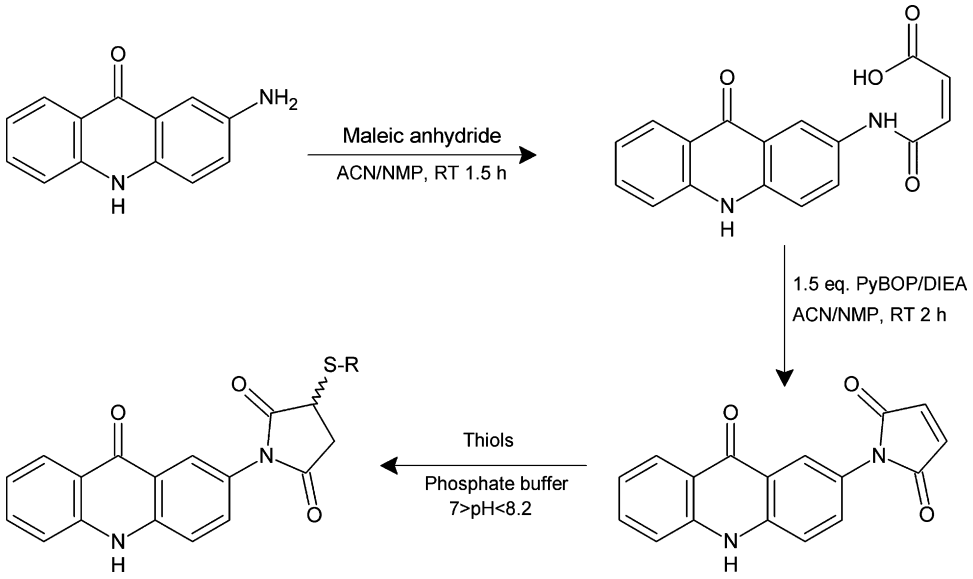

Fig. 1. Synthesis of MIAC and its reaction with aminothiols.

a compound selective to thiols and with high fluorescent properties of its derivatives. 2-Aminoacridone was found to be suitable for these goals, because of its excellent fluorescent properties and high quantum yield. According to the literature [26] and our experimental investigations of acridone compounds, the substitutions in the second position of the acridone structure have favourable effect on the fluorescence properties of these compounds. Moreover, another advantage of these compounds is the high stability of their structure. They undergo no chemical changes in strongly acidic or basic media. Maleimide moiety was attached to the amino group in the second position of the acridone ring as a reactive to thiols group because of its specific reactivity towards sulfhydryl groups. The reaction of the maleimide double bond with thiol proceeds rapidly, for less then a minute, even at room temperature.

Synthesis of MIAC is shown in Fig. 1. The final product was obtained in two-step procedure with high yield and purity. After acylation of 2-aminoacridone with maleic anhydride, the intermediate maleamic acid was cyclized to maleimide using PyBOP as condensing reagent [27]. During the development of the synthesis, we found that PyBOP is the superior reagent for ring closure over other tested "classical methods"—acidic dehydratation and DCCI cyclization [28,29]. Reaction with PyBOP is fast, with practical yield and free of isomaleimide isomer. According to our knowledge, such utilization of PyBOP has not been demonstrated until now. The obtained MIAC reagent was investigated for its spectral properties. The absorption and emission spectra of MIAC and its Hcy adduct are presented in Fig. 2. The relative fluorescence of MIAC was measured for 10 μl of 10 mM MIAC solution but after whole sample pretreatment without addition of thiol compounds. The fluorescence of HCy-MIAC was measured for 10 μM HCy after derivatization with 10 μl 10 mM MIAC and also after whole sample preparation. According to the obtained data, the presented new derivatizing reagent shows little fluorescence before reacting with thiols. Contrary to the reagent the thiol derivative is highly fluorescent.

The rate of the reaction between MIAC and the thiol compounds, with concentrations of 5, 10 and 15 μM for HCy, GSH and Cys, respectively, was elucidated varying the time of the derivatization. The reaction was carried out for 10, 30 s, 1, 3 and 5 min, respectively. Hcy and Cys were completely derivatized for only 10 s. The reaction for GSH was slower than for the other thiols. A reaction time of 1 min was found to give completely derivatized GSH product. As it could be seen in Fig. 3, the obtained contents for all thiols remain constant after a minute reaction time.

3.2. Analysis of aminothiols in human plasma

3.2.1. Sample preparation

The Hcy, Cys and GSH contents were determined in human samples in a few steps: (a) reduction of disulfides with TCEP, (b) deproteinization with trichloracetic acid, (c) derivatization of reduced aminothiols with highly fluorescent reagent MIAC, (d) separation of aminothiol—MIAC derivatives by reversed-phase HPLC with fluorescent detection.

In biological systems, thiols are presented as reduced forms, mixed disulfides with other low-molecular weight thiols and cross-linked to proteins. Measurement of various forms is sometimes of diagnostic importance because the oxidation chemistry of thiol compound depends on the presence of the thiol groups. By modifying the sample preparation procedure our method allows measurement of the total content, the disulfide forms and also the reduced free forms of the thiols although they are presented at picomolar concentrations or less.

The accurate thiols determination requires reduction of disulfide bonds of their oxidized forms. In the presented study, TCEP reagent was chosen for reduction of thiol compounds [30,31]. The excess of the reducing agent reacts with MIAC but in our chromatographic conditions the retention time of the TCEP-MIAC adduct was greater than other thiols so it did not interfere with the quantitative analysis.

The next step of the thiols assay is the removal of protein species usually carried out by protein precipitating agent as sulfosalicylic acid, methanol, acetone, perchloric acid, trichloroacetic acid, metaphosphoric acid. According to our observations, organic solvents were not effective enough to remove the existing proteins from the biological samples. The perchloric acid was found to be an efficient deproteinizating agent from the group of inorganic acids, but it deteriorated to some extent the analytical recovery of the thiol compounds (data not shown), probably due to the oxidation of the sulfhydyl groups. Effective protein precipitation was achieved with TCA without any losses of the compounds of interest.

The reaction between the maleimide group and the sulfhydryl group is fast and complete when the pH of the reaction media

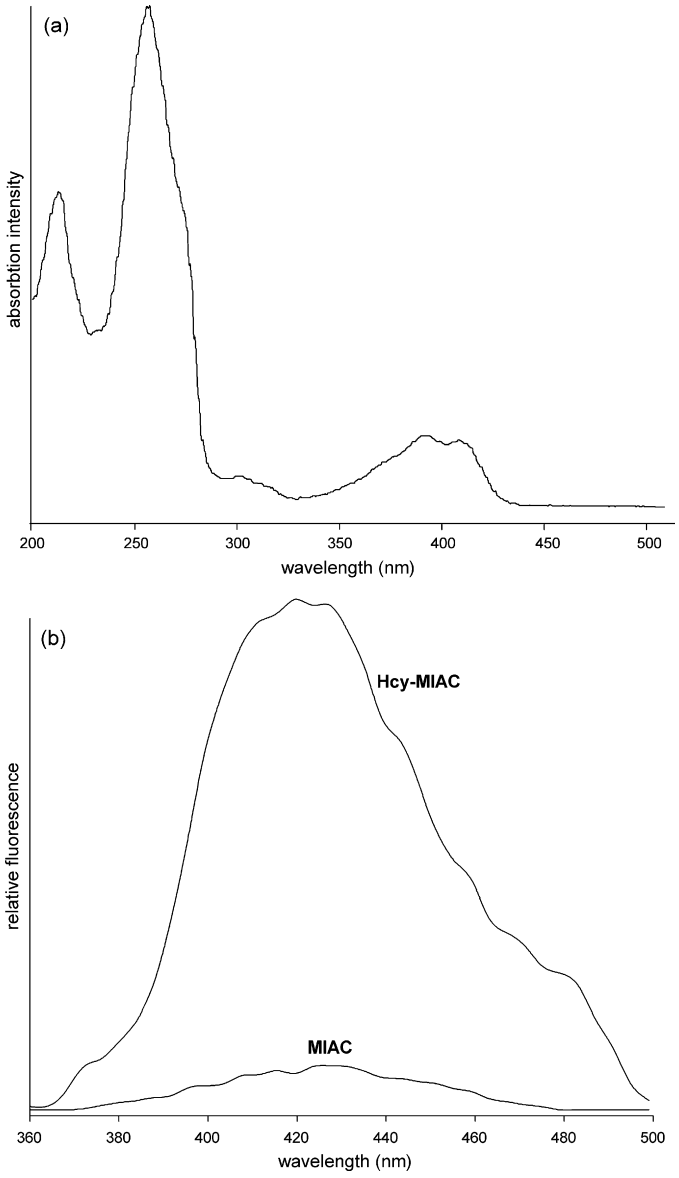

Fig. 2. Spectral properties of MIAC and derivatives. (a) Absorption spectra of MIAC in chromatographic mobile phase and (b) emission spectra of MIAC and Hcy-MIAC adduct after excitation at 260 nm. (for details see the text).

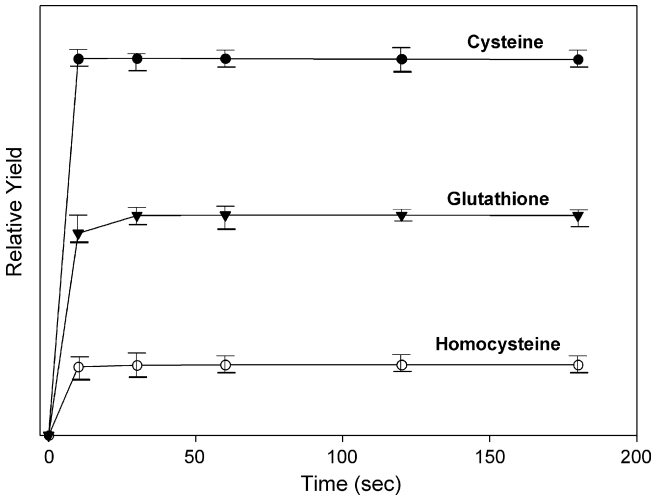

Fig. 3. Kinetics of derivatization reaction of aminothiols with MIAC. The concentrations of the aminothiols were 5, 10 and 15 μM HCy, GSH and Cys, respectively. The measurements were performed in seven independent experiments.

is in the pH 7–8. After acidic protein precipitation, the pH of the samples needs to be adjusted to the desired pH interval so high capacity buffer is required. Our initial experiments were carried out with carbonate buffer. It is a high capacity buffer for complete neutralization of TCA and maintenance of the desired pH of the reaction. Unfortunately, some side products and fluorescent impurities of derivatization reagent were observed. The use of borate and TRIS buffers were also not expedient for the derivatization reaction. They all have low capacity and the reaction could not be performed completely. 0.3 M phosphate buffer with pH 8.2 was found to be optimal for complete derivatization reaction of the thiol compounds with no hydrolysis byproducts.

3.2.2. Chromatographic analysis

Hcy, Cys and GSH derivatives were baseline separated in reverse phase mode of HPLC by isocratic elution within 6 min with composition of the mobile phase—80% B and 20% A (described in Section 2.5.2). Gradient of organic eluent up to 70% was included in the HPLC analysis for elution of the reducing reagent TCEP, the excess of derivatizing reagent and some other hydrophobic plasma components. The chromatographic conditions were successfully chosen, so the area of the chromatogram where aminothiol derivative peaks appeared was free of impurities and reagent hydrolysis product peaks. All compounds were eluted from the column in 15 min, with the column being ready for another injection (Fig. 4). In addition, presented chromatographic conditions allow also determination of *N*-acetyl-cysteine (NAC) and dipeptide glutathione derivatives (Glu-Cys and Cys-Gly), but due to the absence of high quality standards they were omitted in the present study.

Table 1
Within-run and between-run precision data of the analytical procedure expressed as relative standard deviation (RSD, %)

	Within-run precision		Between-run precision	
	Concentration (μmol l^{-1}) (n = 6)	RSD (%)	Concentration (μmol l^{-1}) (n = 6)	RSD (%)
Homocysteine	3	2.08	2	3.18
	10	1.73	5	1.19
	15	1.35	10	0.89
Glutathione	3	2.65	2	3.17
	10	1.78	5	4.13
	15	2.14	10	2.38
Cysteine	20	1.76	10	1.83
	100	4.38	50	1.06
	200	2.94	100	2.03

Table 2
Recovery data for Hcy, GSH and Cys

	Plasma content (μmol l^{-1})	Spiked amount (μmol l^{-1})	Measured amount (μmol l^{-1})	Recovery (%)
Homocysteine	5.11	1.5	6.48	91
		2	6.99	94
		3	7.89	93
Glutathione	2.11	1.5	3.38	85
		2	4.02	96
		3	4.90	93
Cysteine	48.39	15	62.11	91
		20	66.93	93
		30	74.12	86

3.2.3. Figures of merit

The method linearity was tested in the range of 0.5–15 μM for Hcy and GSH and in the range of 0.5–200 μM for Cys. The calculated correlation coefficients were 0.9998, 0.9995 and 0.9996 for Hcy, GSH and Cys, respectively.

Within-run precision for the method was obtained by injecting the same sample six times consecutively and comparing the peak areas for the derivative peaks. Between-run precision was determined by preparing six times, independently the same sample according the whole sample procedure. The derivatized and acidified samples were stored at 4 °C until HPLC analysis. The calculated relative standard deviations (RSD, %) are shown in Table 1. The derivatized samples were found to be stable for more than 2 weeks at −20 °C.

For recovery calculation different levels of Hcy, GSH and Cys [(1.5, 2, 3 μM of Hcy, GSH and 15, 20, 30 μM Cys)] were added to aliquots of a plasma sample. The samples were analyzed after the whole sample pretreatment. The results are shown in Table 2.

The detection limits for Hcy, GSH and Cys (at signal/noise ratio of 3:1) were 1.2, 2.0 and 1.4 pmol, respectively, per 20 μl injection.

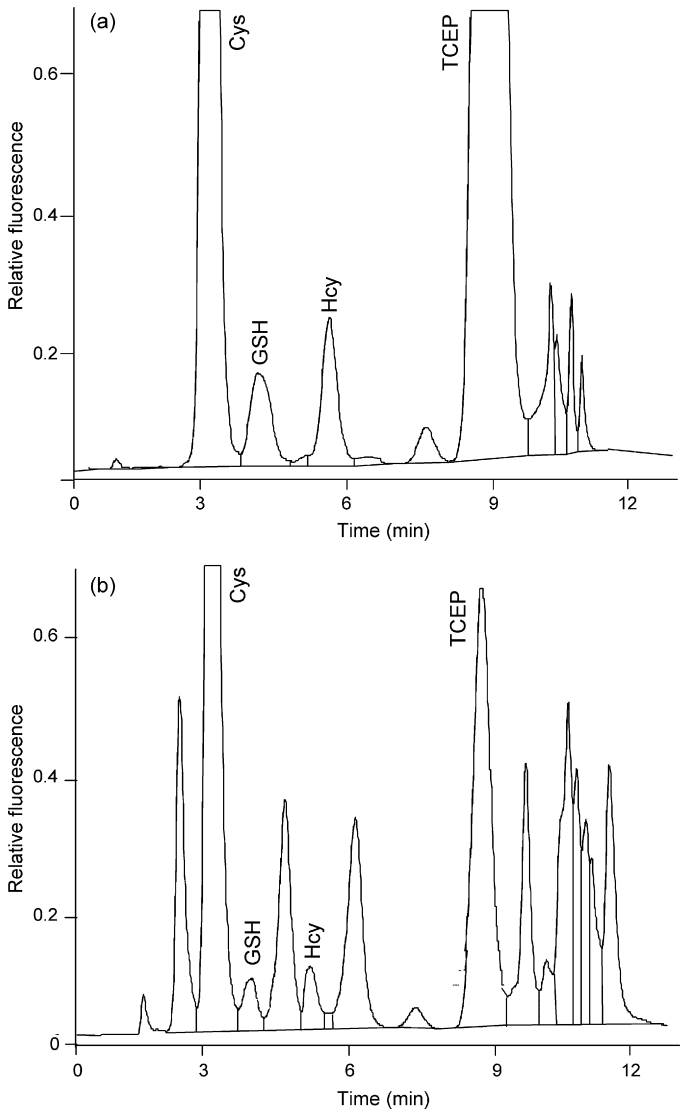

Fig. 4. Representative chromatograms of (a) standard mixture containing 10 μmol l^{-1} of Hcy, GSH and 100 μmol l^{-1} of Cys and (b) real plasma sample.

3.2.4. Method application

The assay was applied to determine the Hcy, Cys and GSH content in real plasma samples. The quantification was achieved by comparison to standard curves prepared by plotting peak area versus concentration of known standard solutions. The results of this survey with respect to the diagnostic and medical impacts of the obtained data, will be presented as a separate publication.

4. Conclusion

A new derivatizing reagent for aminothiol determination, *N*-(2-acridonyl) maleimide was developed. It presents low fluorescence intensity in comparison with its highly fluorescent thiol derivatives, shows high specificity and very fast reactivity to thiol groups in mild and simple derivatization condition. The described method based on MIAC as derivatization agent is fast, simple, highly sensitive and precise. It allows measuring of very low thiol concentrations in human plasma samples.

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