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Simultaneous analysis of homocysteine and methionine in plasma

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Abstract

The new isocratic cation exchange method separates up to eight different amino thiols. The separated sample components are detected electrochemically using a gold electrode and the integrated pulsed amperometry. The eluent composition is, for example, 0.15 *M* sodium perchlorate, 0.02 *M* perchloric acid and 5% acetonitrile. The report describes the optimization of chromatographic parameters such as column diameter and eluent composition. Quantitative performance is discussed for eight different amino thiols using standards. Also presented is a long term quantitative study for homocysteine and methionine in plasma samples. The preparation of plasma samples is simpler than with the previously reported version of the method. Only a reduction step is required, and neither column switching nor derivatization are necessary.

Keywords: Amino thiols; Homocysteine; Methionine

1. Introduction

Homocysteine has been established as an independent risk factor for cardiovascular disease [1]. More than any other amino thiol, homocysteine has been a focus of intensive analytical method development activities in a number of laboratories.

Several different techniques are in use for analysis of plasma and urine samples at the present time. Two of the HPLC-based techniques utilize fluorescence detection. Two different reagents are recommended for fluorescence enabling derivatization [2,3]. A more recent addition to the HPLC methodology of homocysteine employs stable isotope dilution and electrospray tandem mass spectroscopy [4]. An electrochemical detection technique for aminothiols, the Integrated Pulsed Amperometric Detection (IPAD), was introduced by Johnson and coworkers in 1986 [5]. An application of cation exchange and IPAD for plasma samples was first reported in 1995 [6]. A cation exchange separation followed by ninhydrin derivatization and fluorescence detection [7] has also been applied for homocysteine.

From the non-HPLC techniques, the most common methods are GC–MS [8], enzyme immunoassay [9] and fluorescence polarization immunoassay [10].

In plasma and blood, only ca. 5–15% of the total homocysteine is free. The rest is bound by disulfide bridges, either to other thiols or to various proteins. A reduction step is thus necessary for a meaningful

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monitoring of homocysteine concentration. A variety of suitable reducing agents have been described in the literature. The most important and most widely used disulfide reducing reagent today is the tris(2-carboxyethyl)phosphine (TCEP) [11].

An interlaboratory study involving 14 participants evaluates among-laboratory and among-method variations for the total plasma homocysteine [12].

A simultaneous analysis of other amino thiols together with homocysteine is acquiring importance for better understanding of metabolic pathways and related genetic issues. The 1995 cation exchange/IPAD method [6] made possible simultaneous analysis of methionine and homocysteine in a reduced plasma. The reverse phase/fluorescence technique published in 1999 [3] offers simultaneous analysis of cysteine, cysteinylglycine (CysGly), reduced glutathione (GSH) and cysteamine. A new ion pairing/reverse phase/constant potential coulometry technique also published in 1999 [13], analyzes seven amino thiols simultaneously with homocysteine and homocystine (cysteine, cystine, cystathionine, GSH, CysGly, methionine and oxidized GSH).

In this article, we describe the optimization of the cation exchange/IPAD method, expanding the number of amino thiols detected simultaneously. The column switching for the analysis of homocysteine and methionine in reduced plasma samples is no longer necessary.

2. Experimental

2.1. Chemicals

All standards were purchased from Sigma (St. Louis, MO, USA). Anhydrous sodium perchlorate and concentrated perchloric acid for preparation of the mobile phase were obtained from Aldrich (Milwaukee, WI, USA). The acetonitrile was obtained from Allied Signal (B&J Brand, Muskegon, MI, USA). Tris(2-carboxyethyl)phosphine (TCEP) for the reduction of disulfide bonds was purchased from Pierce (Rockford, IL, USA). All standard solutions, mobile phases and sample preparation reagents were prepared using 18 mΩ deionized water.

2.2. Preparation of plasma samples

We transferred 225 μl of EDTA plasma into a 1.5 ml microcentrifuge vial, added 25 μl of 10% aqueous solution TCEP, vortexed several times and rotated for 30 min. The sample gelled following this step. In the next step, we added 650 μl of mobile phase (0.15 *M* sodium perchlorate, 0.02 *M* perchloric acid and 5% acetonitrile). The sample preparation was completed by a repeated vortexing and centrifugation at 12 000 rpm. The supernatant was passed through a properly preconditioned (10 ml water, 5 ml methanol, 10 ml water, 10 ml air) SepPak C_{18} cartridge vial and injected.

2.3. Cation exchange chromatography and IPAD detection

We utilized a DX-500 liquid chromatograph (Dionex, Sunnyvale, CA, USA). The system consisted of a GP50 pump with on-line degas, an LC30 chromatography oven with the temperature set to 30°C, and an ED40 electrochemical detector that included a thin-layer type amperometric cell. The gold working electrode was 1 mm in diameter. The counterelectrode was the titanium cell body across the 25 μm thin-layer channel from the working electrode. A Ag/AgCl reference electrode was installed downstream from the thin-layer channel. The pulse regime enabling the detection of amino thiols is presented in Table 1.

The chromatography of amino thiols was performed on an OmniPac PCX500 column from

Table 1
Integrated Pulsed Amperometric Detection (IPAD) waveform

Time (s)	Potential (V vs. Ag/AgCl, 3 *M* KCl)	Current integration
0.00	0.10	
0.10	0.10	Start
0.40	1.60	
0.70	0.10	
0.80	0.10	End
0.81	1.60	
0.83	1.60	
0.84	−0.60	
1.04	−0.60	

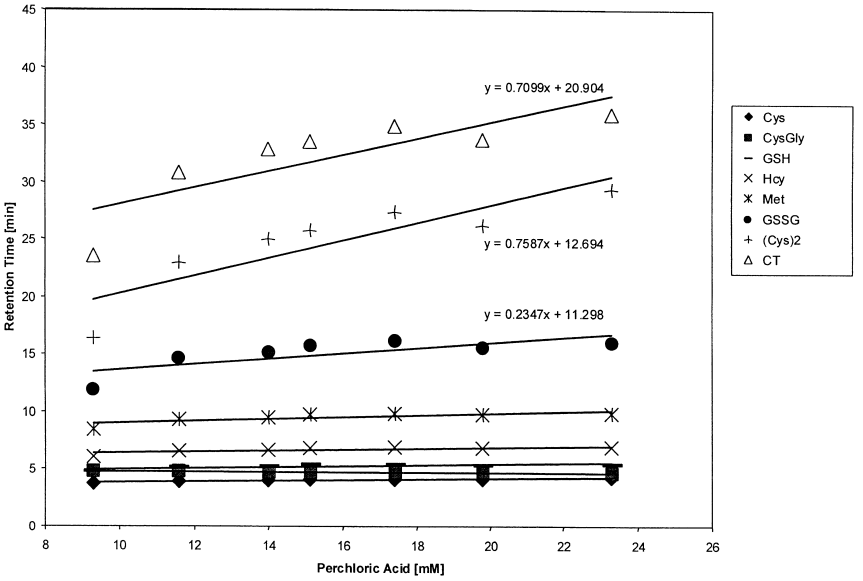

Fig. 1. Retention map of eight physiological amino acids. Column, PCX-500 (250×2 mm I.D.); eluent, 0.15 *M* $NaClO_4$/acetonitrile (5%)/(9.3–23.3 m*M*) $HClO_4$; flow-rate, 0.25 ml/min; Au working electrode; IPAD detection waveform from Table 1. Separation and detection were carried out at room temperature of ca. 25 °C.

Dionex (I.D.×length, 2×250 mm) at a flow-rate of 0.25 ml/min using aqueous mixtures of sodium perchlorate, perchloric acid and acetonitrile as a mobile phase (See Fig. 1 and Results and discussion for the full range of eluent compositions). All chromatographic data were acquired and processed using PeakNet software (Dionex).

3. Results and discussion

3.1. Initial considerations

The original cation exchange/IPAD method [6] for homocysteine in plasma utilized a 4-mm I.D. column (Dionex OmniPac PCX 500 $L=250$ mm) in combination with a shorter 4-mm I.D. column (OmniPac PCX 500 Guard $L=50$ mm) filled with the same cation exchange material as the longer column. The eluent composition (0.1 *M* perchloric acid, 0.15 *M* sodium perchlorate and 5% acetonitrile) was optimized for an ideal resolution of homocysteine from other plasma components eluting near that compound during a cation exchange separation. Some of the plasma components would exhibit a very long retention under those conditions if allowed to enter the longer of the two cation exchange columns. The problem of excessive retention of some of the plasma compounds was solved by the application of the ''heart-cut.'' Only a narrow fraction eluting from the shorter of the two columns was allowed to continue through the main column. The sample components eluting immediately before and after homocysteine were sent directly to waste. This approach was proven to be very practical by one of the authors (J.E.), who was able to analyze thousands of patient plasma samples since the publication of the original paper [6].

Two additional improvements were implemented over the past 5 years. The preparation of samples was simplified by switching from sodium borohydride to TCEP as the reducing agent (See Experimental section for the details of the modified sample preparation procedure). A modified waveform (see Table 1) led to a more stable detector performance.

The original cation exchange/IPAD procedure required two pumps with two separate eluent con-

tainers and a second column switching valve (six-port, Rheodyne 9126). Further improvements of the original procedure became possible by the recent introduction of a narrow bore version of the original column (I.D. 2 mm, 250 mm long, OmniPac PCX 500).

3.2. Chromatographic selectivity

Fig. 1 illustrates the retention characteristics of eight amino thiols on a narrow bore cation exchange column (PCX 500). The eluent composition is the same as in the original report [6], except for the concentration of perchloric acid that is being varied in the range between 9.3 and 23.3 m*M*. In contrast, the original eluent contained 100 m*M* of perchloric acid. Fig. 1 explains why it was not possible to measure the retention of oxidized thiols and cystathionine with the original eluent composition. Using the linear regression equations in Fig. 1 to extrapolate the retention times at the original perchloric acid concentration of 100 m*M*, we calculate 92, 89 and 35 min for cystathionine, cystine and oxidized glutathione, respectively. Injections of the three compounds never produced a peak in the usual time window of 0–30 min under the conditions of the original report and with the 250 mm long column. However, the same three peaks could always be rinsed off to waste from the 5 mm long column with the ''heart-cut'' procedure, provided that the total injection-to-injection time period was at least 30 min long. Oxidized homocysteine is not represented in Fig. 1. It is very strongly retained under the normal conditions. We recommend rinsing the column once a day with an eluent containing 0.9 *M* sodium perchlorate, 20 m*M* perchloric acid and 5% acetonitrile. The homocystine can be removed in under 20 min using such eluent with the increased perchlorate concentration.

Another aspect evident in Fig. 1 is a close co-elution of CysGly and GSH at the lower end of the perchloric acid concentrations. For an optimum separation of the pair, the concentration of perchloric acid must be ca. 20 m*M*.

The resolution of the same peak pair is also controlled by the separation temperature. A 5°C increase in the separation temperature from ca. 25 to 30°C (room temperature vs. column thermostat) affects the resolution of the CysGly/GSH only slightly. At the same time, it reduces the run time of the late eluting peaks by ca. 5–10 min. The column thermostatting at 30°C also improves the retention time reproducibility.

An example of a standard chromatogram obtained with 20 m*M* perchloric acid concentration and at column and detection temperature of 30°C is shown in Fig. 2.

3.3. Reproducibility and calibration

Reproducibility of the new method was evaluated by making six injections of standard solution per day (20 μl of 3.8 μ*M* of all analytes) during a continuous 5-day period. Other chromatographic conditions were the same as for the chromatogram in Fig. 2. The separation and detection temperature was 30°C. The corresponding values of within-run and run-to-run variance are listed as a relative standard deviation in Table 2.

All within-run results are better than 5%. The run-to-run results range from 2 to 7% RSD. Also shown in Table 2 is a summary of calibration data. Cysteine, CysGly were tested at seven levels between 0.19 and 19 μ*M*. GSH was injected at six levels between 0.19 and 9.5 μ*M*. Calibration plots for methionine, oxidized GSH, cystine and

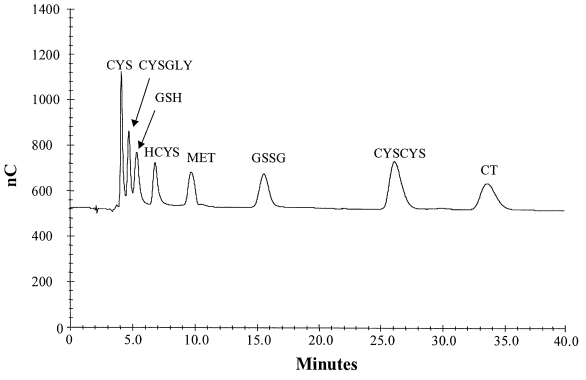

Fig. 2. Standard chromatogram of eight physiological amino acids. Column, PCX-500 (250×2 mm I.D.); eluent, 0.15 *M* $NaClO_4$/acetonitrile (5%)/19.7 m*M* $HClO_4$; concentration of amino acid standards, 3.8 μ*M*; injection volume, 20 μl; flow-rate, 0.25 ml/min; separation and detection temperature, 30 °C; other conditions described in Fig. 1.

Table 2
Quantitative parameters of S-containing amino acids at Au electrodes in 0.15 *M* $NaClO_4$/0.17 *M* $HClO_4$/5% MeCN

Peak number	Amino acid	Limit of detection[a] (pmol)		Linear range/ μ*M*	R^2	RSD (%)	
		pmol	nM			Within-run[b]	Run-to-run[c]
1	Cysteine	0.28	14	0.19–19	0.9998	0.75	2.06
2	Cysteinylglycine	0.92	46	0.19–19	0.9993	1.04	4.10
3	Glutathione (Red)	0.93	47	0.19–9.5	0.9997	2.94	3.66
4	Homocysteine	1.01	51	0.95–19	0.9999	1.38	4.66
5	Methionine	1.01	51	0.95–19	0.9995	1.35	4.43
6	Glutathione (Ox)	1.11	56	0.95–19	0.9997	3.60	4.70
7	Cystine	0.80	40	0.95–19	0.9997	1.73	2.89
8	Cystathionine	1.82	91	0.95–19	0.9998	0.82	6.92

[a] Limit of detection was calculated as $S/N=3$ using the lowest of injected concentrations.
[b] Within-run relative standard deviation was obtained from six injections of each amino acid.
[c] Run-to-run relative standard deviation was obtained during 5 days with six injections each day.

cystathionine were generated with seven standards in the range between 0.95 and 19 μ*M*. All correlation coefficients were greater than 0.999.

Sometimes at the lowest concentrations of aqueous standard, but never in reduced plasma samples, a negative peak caused by the oxygen imbalance between the standard solution and eluent appears in the chromatogram. The nature of that peak was investigated and explained in a 1991 report [14].

The oxygen dip usually elutes very close to the peak of methionine. Since the reproducibility and detection limits for methionine (Table 1) do not significantly differ from those of other peaks (and the oxygen dip never appears in reduced plasma samples), we conclude that the interference by the oxygen dip is negligible from a practical point of view.

3.4. Improved sensitivity

As expected, the sensitivity of detection improves with a smaller column diameter. We compared the relative sensitivities by injecting identical volumes (20 μl) of the eight-component standard on both types of column ($L=250$ mm; 2 and 4 mm I.D.). To achieve comparable linear velocity in both experiments, the volume rates were 1.0 and 0.25 ml/min for 4 and 2 mm I.D., respectively. The peaks of all components exhibited nearly identical retention times under those conditions. The range of peak area ratios (area 2 mm/area 4 mm) was between 2 and 2.8 with a calculated mean value of 2.3. The limits of detection for eight amino thiols are listed in Table 2. All eight compounds can be detected at submicromolar levels allowing easy detection of usual concentration levels even in dilute plasma samples.

3.5. Homocysteine and methionine in TCEP-reduced plasma sample

A typical chromatogram of a reduced plasma sample is shown in Fig. 3. To evaluate the method stability, we carried out an evaluation with 24 blood plasma samples obtained from healthy volunteers (see Table 3). Two injections of each sample were used for within-run calculations ($n=48$). The run-to-run data were collected over 12 weeks during which time each of the 24 samples was injected twice a week ($n=2\times24\times12=576$).

Table 3 also contains data of a 52-week study with two control plasma pools at two different levels for each of the two analytes. To generate the within-run data, each control plasma was injected 20 times within 1 day ($n=20$). For the run-to-run data, two injections of each control plasma were made each week over a period of 52 weeks ($n=2\times52=104$).

The homocysteine results in the three types of plasma samples selected (Control B, Control A, and

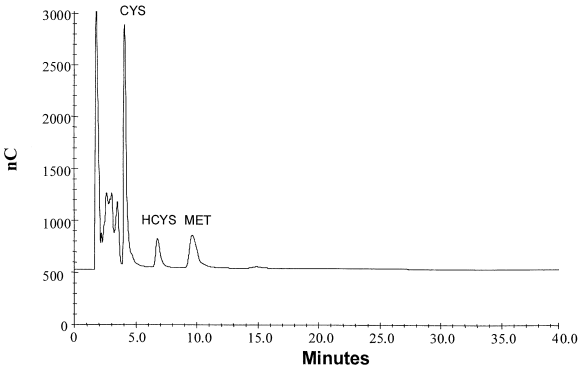

Fig. 3. Chromatogram of a reduced plasma sample. Sample reduction and pretreatment: see Experimental; dilution: 10 times. Column, PCX-500 (250×2 mm I.D.); eluent, 0.15 *M* $NaClO_4$/acetonitrile (5%)/19.7 m*M* $HClO_4$; injection volume, 20 μl; flow-rate, 0.25 ml/min; separation and detection temperature, 30°C; Au working electrode; IPAD detection waveform as described in Table 1.

Volunteers; mean 7.0, 9.7, and 10.8) cover the range of values usually found in healthy control subjects [1].

4. Conclusions

A new method was developed that enables the simultaneous determination of eight different amino thiols in aqueous samples. The reported method avoids derivatization and simplifies the pretreatment of reduced plasma samples.

Unlike the original version of the same technique reported in 1995 [6], the new method does not require two pumps and column switching for the analysis of reduced plasma samples. The method was validated for a simultaneous determination of plasma methionine and homocysteine.

Table 3
Results of a long term study of plasma samples (μ*M*)

	Homocysteine		Methionine	
	Within-run	Run-to-run	Within-run	Run-to-run
Volunteers (24)[a]				
Mean	9.7	9.8	14.0	14.3
Standard deviation	0.7	1.0	1.1	1.2
Range	9.2–10.3	9.0 – 10.2	13.1–14.2	13.0–14.2
Control A (104)[b]				
Mean	10.8	11.3	16.1	16.5
Standard deviation	0.8	0.9	1.1	1.3
Range	10.5–11.2	10.8–11.5	15.3–16.4	15.6–17.0
Control B (104)[b]				
Mean	7.0	7.2	18.9	19.3
Standard deviation	0.6	0.8	1.2	1.3
Range	6.8–7.2	6.7–7.3	18.0–19.2	18.7–19.6

[a] Within-run variability was determined from duplicate analyses per day. Run-to-run variability was measured over 12 weeks with duplicate injections of each volunteer sample per week.

[b] Within-run variability was determined from 20 injections per day. Run-to-run variability was measured over 52 weeks with duplicate injections of each control plasma sample per week.

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