ELSEVIER

Journal of Chromatography A, 913 (2001) 309–313

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Simultaneous determination of several amino acids, including homocysteine, cysteine and glutamic acid, in human plasma by isocratic reversed-phase high-performance liquid chromatography with fluorimetric detection

Y.V. Tcherkas*, A.D. Denisenko

Institute of Experimental Medicine, Russian Academy of Medical Sciences, 12 Acad. Pavlov Street, St. Petersburg 197376, Russia

Abstract

A method for the simultaneous measurement of two biologically important thiol compounds cysteine and homocysteine and five amino acids including neurotransmitters aspartate and glutamate is reported. This method utilized derivatization of compounds with *o*-phthalaldehyde in the presence of 2-mercaptoethanol following alkylation of the free sulfydryl group with iodoacetic acid followed by separation using reversed-phase high-performance liquid chromatography. These *o*-phthalaldehyde–2-mercaptoethanol-labeled compounds were separated within 30 min on a Spherisorb ODS-2 column with isocratic elution using 17% methanol, 0.04 *M* sodium phosphate buffer (pH 7.0), 0.002 *M* Na_2EDTA and detected fluorimetrically (excitation 340 nm, emission 450 nm). Using this method, the concentrations of homocysteine, cysteine, glutamic acid, aspartic acid, asparagine, serine and glutamine in human plasma were determined.

Keywords: Amino acids; Homocysteine; Cysteine; Glutamic acid

1. Introduction

Determination of thiol-containing compounds in biological fluids is important in biochemistry and clinical chemistry. Thus, knowledge of homocysteine (Hcy) concentration in plasma is necessary in the diagnosis and monitoring of treatment of homocystinuria and cystathionuria [1]. Besides, a recent study has shown that an elevated plasma level of Hcy is a potent independent risk factor for the development of cardiovascular disease (CVD), cerebrovascular disease, peripheral arterial occlusive disease and thrombosis [2]. It has been suggested that high plasma cysteine (Cys) content might also contribute to atherosclerosis development in hyperlipidemic patients [3]. High plasma levels of glutamic acid (Glu) have been demonstrated in plasma of patients with acute ischemic stroke [4–6].

There is no universal technique for the determination of the mentioned amino acids. The most widely used methods are: (1) direct electrochemical detection after high-performance liquid chromatography (HPLC) separation, (2) pre- or post-column derivatization with monobrombimane (MB) [7], halogenosulfonylbenzofurazans [8,9], *o*-phthalaldehyde (OPA) [10] followed by HPLC. The most widely used of these reagents in the isocratic elution mode is OPA. The OPA method has the advantage of

*Corresponding author. Tel.: +7-812-2344-907; fax: +7-812-2349-489.

E-mail address: tcherkas@mail.ru (Y.V. Tcherkas).

0021-9673/01/$ – see front matter
PII: S0021-9673(00)01201-2

faster derivatization, the lack of need to adjust the pH, isocratic separation. Besides, OPA allows one to carry out evaluation of thiol-containing amino acids as well as other amino acids.

The aim of this investigation was to develop a method for the simultaneous determination of Glu, total Hcy and Cys levels in human plasma.

2. Experimental

2.1. Chemicals and reagents

2-Mercaptoethanol (2-ME), iodoacetic acid, DL-Hcy, DL-Cys, DL-Glu, DL-aspartic acid (Asp), DL-asparagine (Asn), DL-serine (Ser) and L-glutamine (Gln) were obtained from Sigma (St. Louis, MO, USA). OPA was obtained from Fluka (Buchs, Switzerland). Methanol was HPLC grade (PO AZOT, Novgorod, Russia). All other reagents were of analytical or HPLC grade purchased from Riedel-de Haen (Seelze, Germany).

2.2. Equipment

HPLC was performed using a Gilson HPLC system (Gilson, Villiers le Bel, France) equipped with a Gilson 305 pump, a Gilson 805 manometric module, a column heater LC101 (Ecom, Prague, Czech Republic), a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, USA) fitted with a 10-μl sample-loading loop and with a 2-ml loading loop and a fluorimetric detector from Gilson, Model 121. A stainless steel Spherisorb ODS-2 column (250.0× 4.0 mm I.D., 5 μm) Pharmacia (Uppsala, Sweden) protected by a Supelcosil LC-18-DB guard column (20×2.1 mm I.D., 5 μm) Supelco (Bellefonte, PA, USA) was used. Derivatives were measured at excitation and emission wavelengths of 340 nm and 450 nm, respectively.

2.3. Standards and reagent

Stock solutions of Asp, Glu, Asn, Ser, Gln (20 μmol/ml) were prepared in double distilled water. Cys (400 nmol/ml) and Hcy (200 nmol/ml) were solved in filtered 0.05 *M* perchloric acid and stored at −70°C. Aqueous working solutions for Asp, Glu, Asn, Ser, Gln (200 nmol/ml), Cys (200 nmol/ml) and Hcy (100 nmol/ml) were prepared daily.

OPA–2-ME reagent was prepared according to the previously published method [11] with minor modifications. Briefly, 20 μl of 0.4 *M* OPA stock solution (stored at −20°C) was mixed with 40 μl of 2-ME and 140 μl of 0.1 *M* sodium borate buffer (pH 11.5). The mobile phase filtered through a 0.45-μm membrane Millipore (Bedford, MA, USA) was 0.04 *M* sodium phosphate buffer (pH 7.0) containing 17% (v/v) methanol and 0.002 *M* Na_2EDTA. Elution was carried out with at 26°C at a flow-rate of 0.7 ml/min.

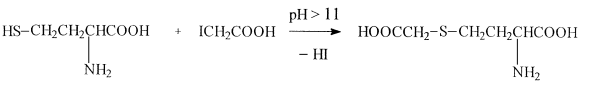
2.4. Sample preparation and derivatization

After overnight fasting, blood samples were collected in sterile tubes, containing Na_2EDTA (1 mg/ml), and gently mixed. Plasma was separated immediately by centrifugation at 3000 *g* for 20 min and stored at −70°C before use. For the reduction disulfide bonds 20 μl of 2-ME were added to 100 μl of plasma sample. The sample was mixed and after incubation for 30 s at room temperature plasma proteins were removed by precipitation with 380 μl of methanol followed by centrifugation at 6000 *g* for 5 min. Then, 20 μl of supernatant or amino acid standard mixture was mixed with 40 μl of iodoacetic acid solution (0.8 *M* in 0.1 *M* sodium borate buffer, pH 10.5) and 120 μl of 0.1 *M* sodium borate buffer (pH 11.5). After incubation for 30 s at room temperature 20 μl of OPA–2-ME reagent was added and after 3 min 10 μl of the reaction mixture was injected onto the HPLC system.

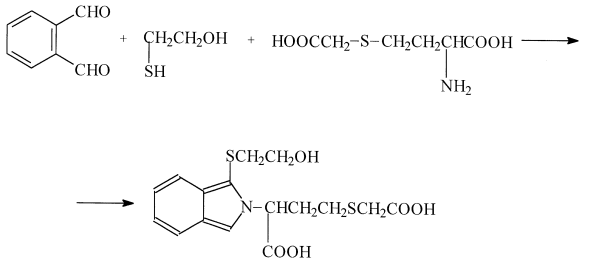
3. Results and discussion

Homocysteine and cysteine are sulfhydryl amino acids and may react with OPA in the same way as the thiol-containing component of the OPA–2-ME reagent. It is known that cysteine yields weakly fluorescent OPA–2-ME derivatives; it may be depend on the presence of sulfhydryl groups on the cysteine molecule [12]. Therefore it was necessary to protect the sulfydryl groups of Hcy and Cys before reaction with OPA. For this purpose we have utilized a reaction with iodoacetic acid, which was added to the standard mixture of amino acids or to the

supernatant of plasma after deproteinization but before the addition of OPA–2-ME [12,13]:



Then OPA–2-ME was added:



Hcy exists in human plasma in various forms; only trace amounts (1%) are in the reduced (sulfydryl) form, the remaining part is oxidized and exists as various disulfides [14]. About 70% Hcy is bound to albumin (via a disulfide bond), whereas the remaining 30% exists as free disulfides, mostly as Hcy–Cys mixed disulfide. Therefore it is necessary to use the reducing agent to release Hcy and Cys. For this purpose 2-ME was added to a plasma sample before protein precipitation. But 2-ME possesses an SH group that can react with iodoacetic acid. Therefore at the further additions of reagents it is necessary to take into account the concentration 2-ME in the assay. In this research the quantity of iodoacetic acid added was four-times more than the quantity of 2-ME in the sample.

Systems that consist of changing methanol concentration (16–19%, v/v), Na_2HPO_4 (0.01–0.03 *M*), NaH_2PO_4 (0.01–0.02 *M*) and Na_2EDTA (0.002 *M*) were used for optimization of conditions for separation of the received derivative. The greatest difficulties were connected with the separation of derivatives of Asn, Ser and Hcy. The retention time of the Hcy derivative was changed under the change of solvent composition to a greater degree, than the retention times of the nearest derivatives – Asn and Ser (data not shown). The methanol concentration in the mobile phase influenced the retention parameters more strongly than concentration of phosphate buffer (data not shown). The retention times of all amino acid derivatives increased with increase of the concentration of phosphate buffer, but the retention time of the Hcy adduct increased much more than other amino acid's retention times. Having defined the optimal concentration of methanol in the eluent, the concentrations of phosphate buffer were varied and the optimal phase for separation of Ser and Hcy derivatives was found (data not shown).

The best separation of derivatives was obtained using the following mobile phase: 17% methanol, 0.03 *M* Na_2HPO_4, 0.01 *M* NaH_2PO_4, 0.002 *M* Na_2EDTA, pH 7.0. The data obtained demonstrate the method of simultaneous measuring of Hcy, Cys and Glu concentrations in blood plasma as OPA–2-ME derivatives in the isocratic elution mode. It took 30 min to separate the OPA–2-ME adducts of Hcy, Asp, Glu, Cys, Asn, Ser, Gln under these conditions. The rest of blood plasma components were washed off by 2 ml of methanol using a second injector. The complete analysis time is 40 min. The chromatogram of the amino acid standard mixture is shown in Fig. 1A. The detection limits (signal-to-noise ratio = 2) were for Asp, Glu, Asn, 0.5 pmol, for Ser, Gln 1 pmol, for Hcy 0.8 pmol, for Cys 0.5 pmol. The response of the detector is linear in a range from 3 up to 250 pmol for all amino acids with correlation coefficients between peak squares and amino acid amounts varying from 0.986 to 0.998 for different amino acids, $P < 0.0001$.

Qualitative analyses of amino acids in samples were carried out on their corrected retention times in standard solutions, quantitative analyses by the method of absolute graduation. The chromatograms of human plasma samples are shown in Fig. 1B and C. Precision of analyses was estimated by a series of replicating inputs of a standard mixture of amino acids ($n = 15$) and sample ($n = 5$) on different days. The areas of Hcy peaks had RSDs 4.3–8.6% for different amounts of amino acid with an average value 6.1% for a standard mixture and 6.7% for human plasma. The standard deviation from the mean value for human plasma Hcy was 1.5 nmol/ml.

We have applied the developed method to quantitative definition of Hcy, Asp, Glu, Cys, Asn, Ser and Gln in blood plasma of healthy donors and CVD patients. The results are shown in the Table 1.

It was found that the Hcy concentration in CVD patients was higher by 41% than that of healthy persons, which is in a good agreement with the data

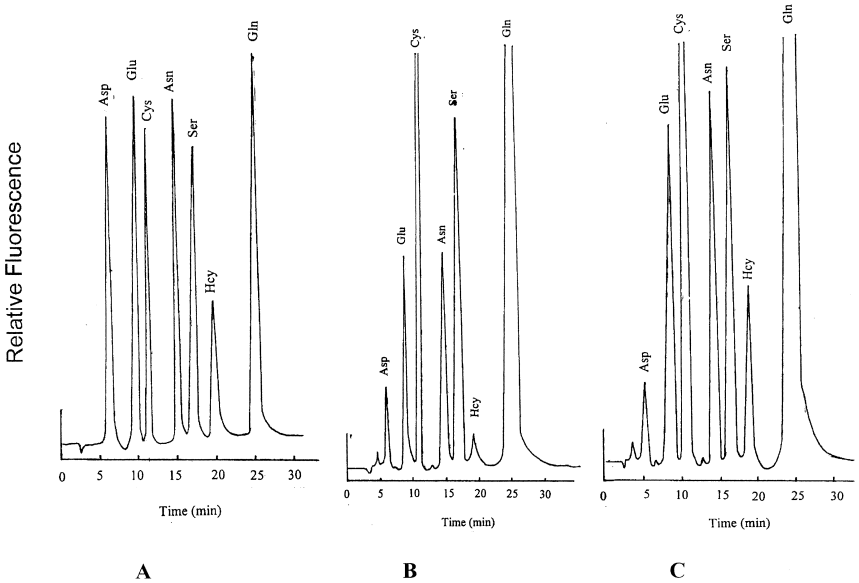


Fig. 1. Elution profiles of an amino acid standard (A), human plasma of healthy donors (B) and CVD patients (C) after derivatization with OPA–2-ME. Chromatographic conditions: column, Spherisorb ODS II (5 μm) (250×4.0 mm I.D.); eluent, 17% methanol in 0.03 *M* NaH_2PO_4, 0.01 *M* Na_2HPO_4, 0.002 *M* EDTA (pH 7.0); flow-rate, 0.7 ml/min; temperature, 26°C; fluorimetric detection excitation wavelength 340 nm, emission wavelength 450 nm; injection volume, 10 μl.

obtained by other investigators [15,16]. CVD patients also have elevated concentration of Glu, whereas the concentrations of Asp, Cys, Asn, Ser and Gln in CVD patients and healthy persons were similar.

4. Conclusions

In summary, we have developed a simple and reliable method for the simultaneous quantitative measurement of Hcy, Cys, Glu, Asp, Asn, Ser and Gln in human plasma by isocratic HPLC with fluorimetric detection. It allows determining in one run two important aminothiols (homocysteine and cysteine) and such neurotransmitters as aspartate and glutamate. It would provide useful information about role of homocysteine, cysteine, glutamate and aspartate in development of both cardiovascular diseases and brain ischemic diseases. The developed method of quantitation of amino acids in blood plasma allows one to carry out the analysis in the isocratic elution mode, which considerably raises the reproducibility of parameters and enlarges the reliability

Table 1
Free amino acid concentrations in plasma (nmol/ml) of healthy subjects and patients with CVD (mean±SD)

Amino acid	Healthy subjects ($n=19$)	Patients with CVD ($n=34$)
Asp	8.3±0.8	9.6±1.3
Glu	32±3.8	41.9±2.5*
Cys	260±11	255±12
Asn	52±2.8	51±2
Ser	88±4.5	82±5
Hcy	8.3±0.5	11.7±1.1*
Gln	649±38	638±21

* Significantly different from control group, $P<0.01$.

of results, and also reduces time and cost of the serial analyses. Although we primarily focused on the analysis of amino acids in plasma, the method could also be applied to the determination of these compounds in other physiological fluids.

References

[1] S. Yap, E.R. Naughten, B. Wilcken, D.E. Wilcken, G.H. Boers, Semin. Thromb. Hemost. 26 (2000) 335.
[2] G.T. Gerhard, P.B. Duell, Curr. Opin. Lipidol. 10 (1999) 417.
[3] N. Jacob, E. Bruckert, P. Giral, M.J. Foglietti, G. Turpin, Atherosclerosis 146 (1999) 53.
[4] A. Davalos, J. Castillo, J. Serena, M. Noya, Stroke 4 (1997) 708.
[5] J. Castillo, F. Martinez, E. Corredera, J.M. Aldrey, M. Noya, Stroke 11 (1995) 2035.
[6] J. Castillo, A. Davalos, J. Naveiro, M. Noya, Stroke 6 (1996) 1060.
[7] D.W. Jacobsen, V.L. Gatautis, R. Green, Anal. Biochem. 178 (1998) 208.
[8] P.E. Cornwell, S.L. Morgan, W.H. Vaughn, J. Chromatogr. B 617 (1993) 136.
[9] A. Araki, Y. Sako, J. Chromatogr. 422 (1987) 43.
[10] K. Shimada, K. Mitamura, J. Chromatogr. B 659 (1994) 221.
[11] B.A. Donzanti, B.K. Yamamoto, Life Sci. 43 (1988) 913.
[12] J.D.H. Cooper, D.C. Turnell, J. Chromatogr. 227 (1982) 158.
[13] K. Hyland, T. Bottiglieri, J. Chromatogr. 579 (1992) 55.
[14] O. Nygard, S.E. Vollset, H. Refsum, L. Brattstrom, P.M. Ueland, J. Intern. Med. 246 (1999) 425.
[15] D.W. Jacobsen, Clin. Chem. 44 (1998) 1833.
[16] H. Refsum, P.M. Ueland, O. Nygard et al., Annu. Rev. Med. 49 (1998) 31.