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Determination of homocysteine in human plasma by micellar electrokinetic chromatography and in-capillary detection reaction with 2,2′-dipyridyl disulfide

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Abstract

We present a new method for homocysteine quantitation in human plasma based on in-capillary reaction of homocysteine with 2,2′-dipyridyl disulfide. Homocysteine is in this so-called thiol-exchange reaction quantitatively transformed in mixed disulfide concomitantly with formation of an equimolar amount of 2-thiopyridone that is further separated by micellar electrokinetic chromatography and determined specifically at 343 nm. The concentration of homocysteine is thus estimated indirectly from the result of 2-thiopyridone determination. The linear detection range for concentration versus peak area for the assay was from 0.03–3 m*M* (correlation coefficient 0.994) with a detection limit of 6 μ*M* and a limit of quantitation 20 μ*M*. The inter-day reproducibility of the peak area and the migration time were 1.37% and 0.05%, respectively. The method is simple, relatively rapid and can be easily automated. Moreover the common capillary electrophoresis apparatus with a UV detector can be used to distinguish between normal and pathological hyperhomocysteinemia plasma samples.

Keywords: Derivatization, electrophoresis; Homocysteine; Dipyridyl disulfide

1. Introduction

Homocysteine is an endogenous sulfhydryl amino acid, which is generated by the demethylation of methionine. Once formed, homocysteine is either irreversible catabolized by transsulfuration to cysteine or remethylated to methionine. These transformations are controlled by enzymatic reactions.

In humans, 15–20 mmol of homocysteine are synthesized each day, but most of them are converted to cysteine, under the enzymatic control of the cystathionine β-synthase, or to methionine, under the control of methionine synthase and methylenetetrafolate reductase. The level of plasma homocysteine between 5 and 15 μ*M* is considered normal, with a mean level of about 10 μ*M*. Abnormal concentrations termed as hyperhomocysteinemia are classified as moderate (16–30 μ*M*), intermediate (31–100 μ*M*), and severe (>100 μ*M*) [1–6].

An elevated plasma homocysteine level may occur as the result of inherited disorders of the main enzymes of its metabolism, but also as the result of: (i) nutritional deficiencies of the vitamin co-factors (B6, B12 and mainly folates); (ii) diseases (chronic renal failure, malignancies, acute lymphoblastic leukemia, anemia, hypothyroidism, diabetes); (iii) physiological factors (age, sex); (iv) medications (methotrexate, phenytoin, carbamazepine, nitrous

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oxide, theophylline, metformin, colestipol, niacin, penicillamin, thiazide diuretics, etc.) and (v) lifestyle determinants (smoking, coffee consumption, alcoholism, physical activity) [7]. Therefore, the routine determination of homocysteine plasma level is often desirable.

At physiological pH, homocysteine exists in plasma in trace amount in reduced form whereas most of it is present as various disulfides. About 70% is bound to albumin, the remaining 30% forms disulfides with itself or with other thiols. Consequently, the determination of free homocysteine has been largely abandoned and the determination of total homocysteine is performed. A lot of methods are now available for homocysteine determination [8–10]. High-performance liquid chromatography (HPLC) is at present the most widely used [12–22] but capillary electrophoresis (CE) becomes increasingly popular.

CE is a high-efficiency analytical technique that has had a great impact as a tool in clinical and forensic practice in the last 10 years [23]. Even the analytical and forensic tasks related to the World Trade Centre Tragedy have been solved by means of CE [24]. The CE advantages with respect to other analytical techniques: the required very small sample volume, rapid analysis, great resolution power and low costs, have made this technique ideal for the analysis of numerous endogenous and exogenous substances present in biological fluids including homocysteine and other biologically important thiols, such as cysteine, glutathione, etc. However, the absence of chromophore for a sensitive detection is a problem with these analytes. As in the case of HPLC, two approaches can be used to resolve this point: direct electrochemical detection or derivatization coupled with spectrophotometric or spectrofluorimetric detection. Consequently, the different modes of CE have been combined with different detection techniques such as electrochemical, UV-absorbance and laser-induced fluorescence detection to determine homocysteine in plasma, serum, urine, microdialysate, etc. [25–33]. All these methods usually require expensive derivatization reagents and/or special equipment.

Recently, a method using in-capillary detection reaction of 2,2′-dipyridyl disulfide (DPDS) (Fig. 1) for the specific detection of thiols during capillary micellar electrokinetic chromatography (MEKC) has been published [34]. Its principle is very simple. DPDS and a sample containing thiols are injected consecutively in the capillary as two discrete plugs separated by a short plug of a background electrolyte, the length of which must be sufficient for the preseparation of thiols before the reaction with DPDS. Due to the differences in the mobilities of the DPDS and thiols, the zones of thiols are individually approaching the zone of DPDS and on column mixing and reactions occur. Thiols are thus transformed quantitatively into mixed disulfides concomitantly with formation of the equimolar amounts of 2-thiopyridone that are further separated by MEKC and detected spectrophotometrically at 343 nm. Their concentrations are so estimated indirectly from the results of the 2-thiopyridone determinations. In this paper, we propose its application for the determination of total homocysteine in human plasma. As a result, homocysteine even at a moderate hyper-

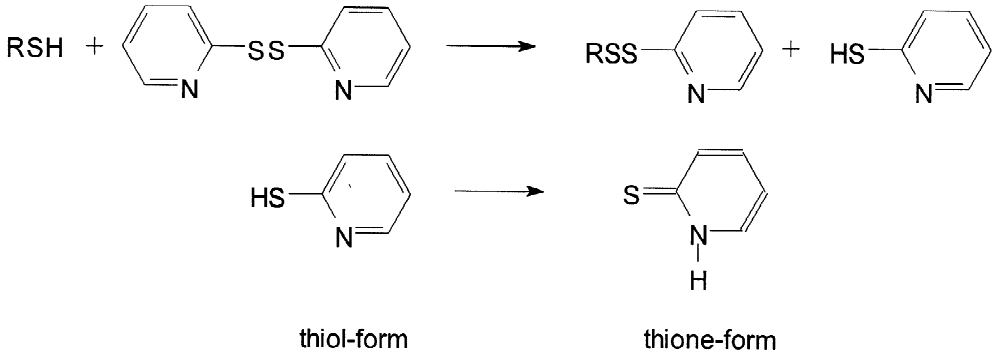

Fig. 1. Reaction of 2,2′-dipyridyldisulfide with thiol-RSH.

homocysteinemia level can be easily and simply determined by means of CE apparatus equipped with common UV detection.

2. Experimental

2.1. Materials and reagents

2,2′-Dipyridyl disulfide (DPDS), dithiothreitol (DTT), reduced glutathione (GSH), cysteine hydrochloride and EDTA disodium salt were obtained from Sigma (St Louis, MO, USA), homocystine from Calbiochem (San Diego, CA, USA). Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) was from Molecular Probes (Leiden, The Netherlands). Homocysteine, tri-*n*-butylphosphine (TBP), ammonium-7-fluoro-2-oxa-1,3-diazol-4-sulfonate (S-BD-F), trichloroacetic acid (TCA), perchloric acid (PCA) and all other chemicals and solvents were of analytical reagent grade, supplied from Fluka (Buchs, Switzerland). Fifty millimolar phosphate buffer (pH 7.5) was prepared by the mixing of 50 m*M* solution of monosodium phosphate and 50 m*M* solution of disodium phosphate. Standards of thiols were prepared in water and stored at −20 °C. Stock solution of 0.5 *M* TCEP was prepared freshly by dissolving an appropriate amount of the compound in water each day. DPDS was prepared as a 0.1 *M* stock solution in acetonitrile that was diluted 20 times with the background electrolyte before the use. All solutions were prepared with Milli-Q Academic water (Millipore, Milford, WA, USA) and filtered through a 0.45 μm membrane filter.

2.2. Instrumentation

A Hewlett–Packard (HP) ^{3D}Capillary Electrophoresis System (Waldbronn, Germany) with a diode-array UV–VIS detector was used to carry out all separations. Data were collected on an HP Vectra VL 5 166 MHz personal computer using the HP ^{3D}CE ChemStation Software. A HP extended light path capillary [64.5 cm (58.5 cm effective length) 50 μm I.D.] was used for all analyses. The capillary was washed with 0.1 *M* NaOH for 1 min, deionized water for 1 min and the background electrolyte for 3 min before each run and washed with deionized water for 1 min after each run. The in-capillary detection reaction was performed by injection of DPDS solution, the background electrolyte, a sample, and finally the background electrolyte, consecutively for a specified time as given in the text below. Separations were performed at 28 kV (positive polarity). Samples were detected by means of a diode-array detector at 343 nm with a bandwidth 10 nm. Spectra were also collected during the runs for the peak identification. In addition, spiking samples with the standard further supported the identity of homocysteine peak.

HPLC was performed by means of the pump LCP 4100.2 (ECOM Ltd., Prague, Czech Republic) equipped with the fluorescence detector RF-535 (Shimadzu, Kyoto, Japan). The CSW software (DataApex, Prague, Czech Republic) was used for system control, data acquisition and processing. The separations were carried out on the 150×4.6 mm I.D., Supelcosil LC-18 column packed with 5 μm particles (Supelco Park, Bellefonte, PA, USA) protected with the 8×4 mm guard column. Other chromatographic conditions were as previously reported [11].

2.3. Blood collection and subject

Specimens were taken from volunteers in a fasting state of different ages. Fresh bloods were drawn into tubes containing EDTA, cooled on ice and centrifuged under standard conditions (10 min at 1000 rpm, within <20 min after collection). The plasma supernatant was stored at −20 °C until analysis.

2.4. Sample preparation

A 450 μl of control or real plasma samples was pipetted into a 1.5 ml centrifuge tube with 50 μl of 0.5 *M* TCEP. The reaction mixture was vortex-mixed for 1 min and incubated at 37 °C for 20 min to allow the total reduction. After cooling, 50 μl of 3 *M* trichloroacetic acid solution containing 1 m*M* EDTA was added. The tube was immediately capped and vortex-mixed at room temperature for 5 min. Precipitated proteins were removed by centrifugation at 13 000 rpm for 10 min. The supernatant was filtered through a 0.45 μm filter, transferred to a vial and used for further analysis.

For the HPLC analysis, the determination of total homocysteine was carried out after TBP reduction, protein precipitation by PCA and SBD-F derivatization [11].

3. Results and discussion

Although the described in-capillary detection system has been demonstrated specifically for GSH, recently, its applicability has been further expanded on the monitoring of DTT in biological and chemical systems [35] and on the determination of cysteine in human urine [36]. While the entire method has been used sufficiently well for the determination of DTT in different samples, a slight modification of sampling parameters has been necessary for the cysteine determination—the intermediate plug of the background electrolyte between the DPDS and sample plugs has been extended to get suitable resolution of homocysteine and cysteine. Additional optimization of sampling parameters was also performed in the case of plasma homocysteine determination. In order to get a lower detection limit the DPDS plug was enlarged five times.

As mentioned in the Introduction, most of the homocysteine is present in plasma as oxidized species, and thus, the determination of total homocysteine in plasma requires the reduction of the disulfide bound between homocysteine and other thiols or albumin. Besides, only the reduced form of homocysteine is active in thiol-exchange reaction with DPDS. Several reductants—sodium borohydride [12], DTT [16], TBP [14] and recently proposed TCEP [22] were tested. Among these four possibilities, the best results were achieved by TCEP. The reduction yield with sodium borohydride was low and also the formation of gas caused problems with current interruptions during MEKC. Sulfhydryl reagent DTT, actually its unreacted fraction, gave the identical reaction with DPDS like homocysteine and cysteine and the corresponding peak overlapped the peaks related to homocysteine and cysteine. It was especially evident at the high concentrations of DTT that had to be used for reduction. TBP did not cause problems mentioned for borohydride and DTT but it has a highly disagreeable odor and is an irritant. On the other hand, TCEP being soluble in aqueous solutions is fully compatible with an applied detection system based on MEKC separation in contrast with poorly water-soluble TBP. The reduction by means of TCEP was further optimized with respect to the different parameters. The time and temperature of incubation and the concentration of TCEP were examined in order to get complete reduction of homocystine. The results indicated that the highest reduction yield was reached after 20 min incubation at 37 °C and with 50 μl of 0.5 *M* TCEP added to the reaction mixture (Fig. 2). The established conditions were adopted for analysis of plasma homocysteine.

Membrane filtration and acid precipitation followed by centrifugation were evaluated to remove proteins from the samples. The precipitation by acetonitrile was omitted since the presence of organic solvents in sample matrix would have a strong influence on separation in MEKC [37,38], especially in the case of hydrophilic components such as thiols. The main advantage of membrane filtration is the absence of sample dilution. Unfortunately, this procedure was found relatively expensive and time consuming, what is more, it gave also poor recovery. Therefore, removal of proteins by acid precipitation using TCA was found to be convenient, easy and economical.

Fig. 3 shows an electropherogram of 1.5 m*M* homocystine standard in blank plasma under the optimal conditions of the background electrolyte 50 m*M* SDS in 50 m*M* phosphate buffer (pH 7.5); separation voltage 28 kV (positive polarity); temperature of capillary 25 °C; detection at 343 nm and sampling parameters: 5 m*M* DPDS in acetonitrile 50 mbar for 100 s; the background electrolyte 50 mbar for 360 s; the sample 50 mbar for 2 s; the background electrolyte 50 mbar for 2 s in described order. The plasma sample was processed according to the optimized protocol as described above. It is important to emphasize that the shown peak corresponds to 2-thiopyridone formed in the reaction of homocysteine with DPDS (see the spectrum inside Fig. 3).

The quantitative parameters of the developed method were estimated by homocysteine standards in blank plasma. Each standard was analyzed in triplicate. The calibration graph for the peak area was

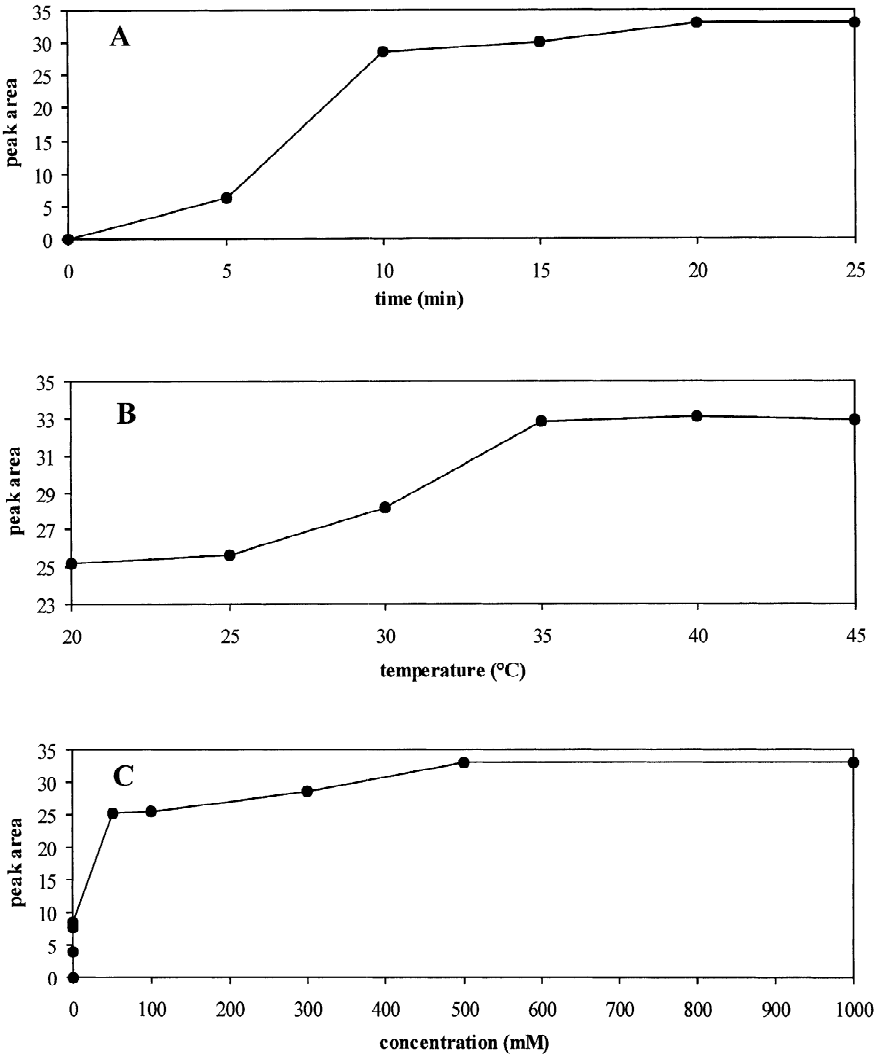

Fig. 2. TCEP reduction reaction yield as a function of time (A) and temperature (B) of the incubation and concentration of TCEP (C). The samples were prepared as described in the Experimental section with the exception that the optimized parameter was varied, whereas two others parameters were kept on constant values (time 30 min, temperature 50 °C and 1 *M* concentration of TCEP).

linear over the range of 0.03–3 m*M* of homocysteine in sample with the correlation coefficient 0.994. The detection limit was in the range 6 μ*M* at a signal-to-noise ratio of 3, the limit of quantitation 20 μ*M* at a signal-to-noise ratio of 9. The sensitivity of the method is thus sufficient for the determination of total homocysteine in the plasma samples even with moderate hyperhomocysteinemia level. The improvement in the sensitivity even for normal concentration range should be obtained using Agilent ''High Sensitivity Cell''. This research is now in progress.

The recoveries were tested by the analyses ($n=10$) of the plasma samples with known concentration of endogenous homocysteine spiked with 50 μ*M* homocysteine. The recovery of homocysteine by the MEKC method was estimated to be between 97% and 105%. The same samples were also used for the study of reproducibility. The results of replicated

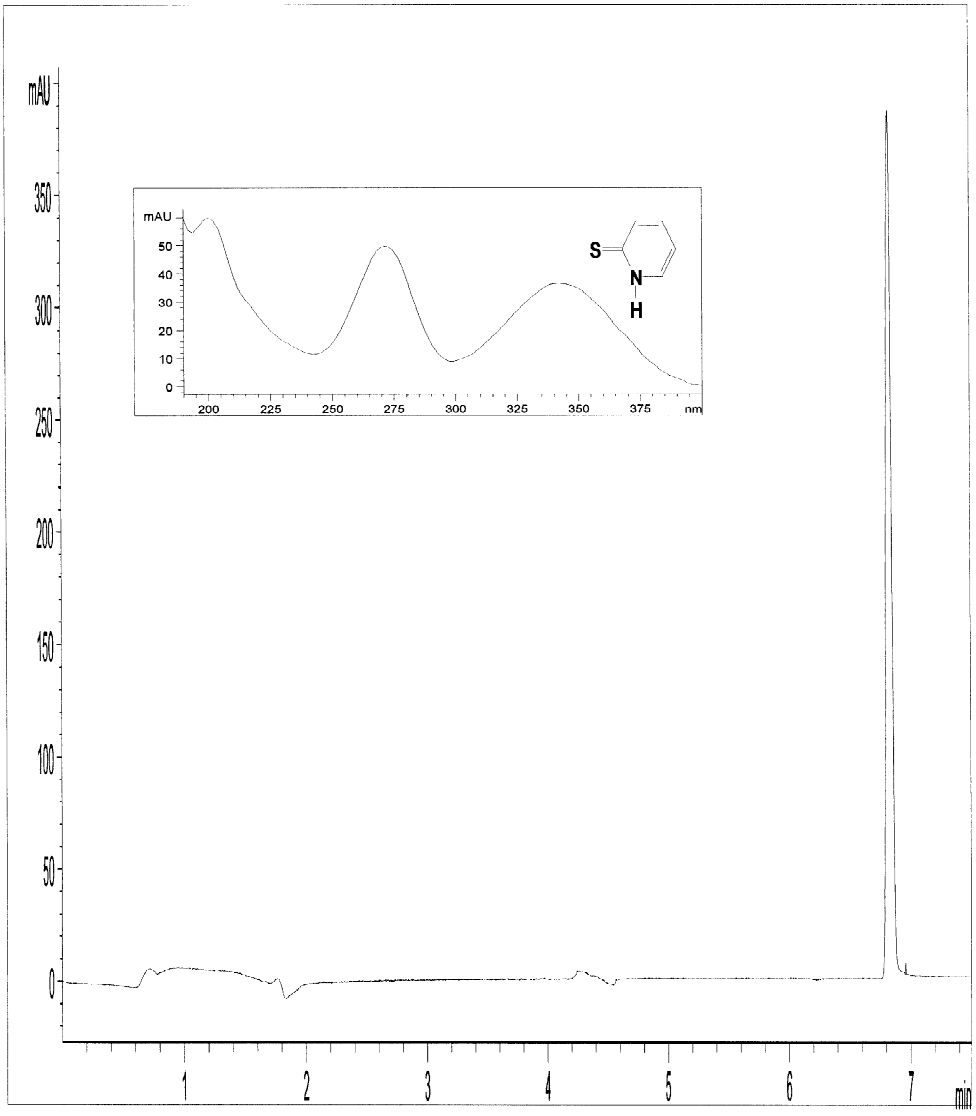

Fig. 3. Electropherogram of 1.5 m*M* homocystine standard in blank plasma. Insert – the spectrum of 2-thiopyridone. The sample was prepared as described in the Experimental section. Separation conditions: the background electrolyte 50 m*M* SDS in 50 m*M* phosphate buffer (pH 7.5), separation voltage 28 kV (positive polarity), temperature of capillary 25 °C, detection at 343 nm and sampling: 5 m*M* DPDS in acetonitrile 50 mbar for 100 s; the background electrolyte 50 mbar for 360 s; the sample 50 mbar for 2 s; the background electrolyte 50 mbar for 2 s.

analyses ($n=10$) showed good reproducibility obtained for peak area (<1.37%) and excellent reproducibility obtained for migration time (<0.05%).

The applicability of the developed method was demonstrated by analyses of real plasma samples. Representative electropherogram is shown in Fig. 4A. The identity of the homocysteine peak was confirmed by spiking the sample with 75 μ*M*

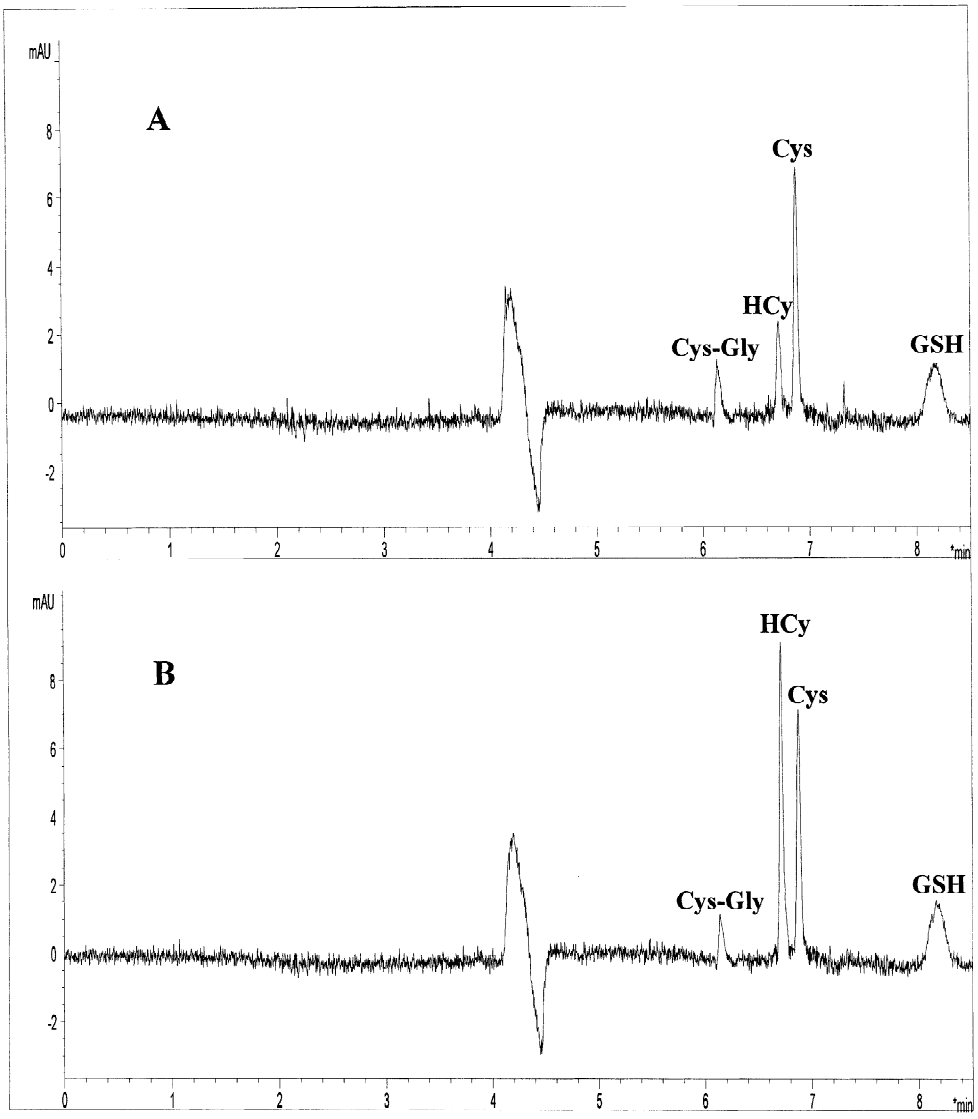

Fig. 4. Electropherograms from the determination of homocysteine in human plasma. Plasma sample (A) and the same sample spiked with 75 μ*M* homocysteine standard (B). Cys–Gly – cysteinylglycine; Hcy – homocysteine; Cys – cysteine and GSH – glutathione. The plasma was prepared as described in the Experimental section; other conditions as in Fig. 3. The concentration of total homocysteine found was 32.0±1.35 μ*M*.

homocysteine (Fig. 4B). As it can be seen good resolutions among homocysteine and other endogenous thiols were achieved. The concentration of total homocysteine found was 32.0±1.35 μ*M*. This value was confirmed by HPLC analysis of the sample.

4. Conclusion

A method for the specific determination of homocysteine using MEKC and in-capillary reaction with DPDS was developed. The method is relatively

rapid, simple and can be easily automated. Its detection limit covers the concentration range at which homocysteine is present in pathological plasma samples. Moreover, the small sample consumption predicts this method for analysis of small volume, for example pediatric samples.

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