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Screening of homocysteine from newborn blood spots by high-performance liquid chromatography with coulometric array detection

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Abstract

Homocystinuria, due to a deficiency of cystationine-β-synthase, refers to the rare inborn error of the metabolism of homocysteine. The identification and prompt treatment of homocystinuria during the neonatal period can prevent or greatly reduce the severity of the clinical consequences. We report a new method for homocystinuria diagnosis from dried blood spots on newborn screening cards, based on high-performance liquid chromatography with electrochemical coulometric array detection. This method shows an excellent linearity ($y=10.36x+0.04$; $r=0.999$), precision (RSDs ranged from 2.7 to 5.8%), recovery (87%) and appears to be a cost-effective approach, being simple, rapid, sensitive and cheap.

Keywords: Electrochemical detection; Detection, LC; Homocysteine; Amino acids

1. Introduction

Homocysteine (Hcy) is the demethylated derivative of methionine, and may be remethylated either back to methionine or catabolised through the transulfuration pathway to cysteine via cystathionine by condensation of serine in an irreversible reaction catalyzed by the pyridoxal phosphate (vitamin B_6) containing enzyme cystathionine β-synthase (CBS).

Homocystinuria (HCU) due to a deficiency of CBS, refers to the rare inborn error of the metabolism of Hcy [1]. Accumulation of Hcy in tissue, blood and urine is the consequence.

Without treatment, the affected subjects usually become mentally retarded, have lens dislocation, skeletal abnormalities and osteoporosis; a high proportion suffer from premature, often fatal thromboembolic disease. The identification and prompt treatment of HCU during the neonatal period can prevent or greatly reduce the severity of the clinical consequences. Subjects with HCU, however, are often not identified during the neonatal period. Not testing for HCU in most neonatal screening programs, where blood samples obtained by heel stick are used, as well as the rate of false-negative results in tested infants are the major reasons for the lack of identification of affected infants. At present, the test for HCU in newborn screening is often performed by analysing methionine concentration in dried blood

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spots using a semi-quantitative bacterial inhibition assay (BIA) [2]. With this method the blood methionine concentration is estimated by comparison of the diameter of the bacterial growth around specimen disks versus the diameter around reference disk containing methionine at multiple concentrations. Although the sequence of metabolic events that causes hyperhomocysteinemia in subjects affected by HCU begins with blocking of the conversion of Hcy to cystathionine by CBS, hypermethioninemia is directly dependent on the subsequent methylation of Hcy to methionine by 5-methylentetrahydrofolate reductase (MTHFR) and other methionine synthases. The mutation of MTHFR is much more common than the rare CBS mutation, therefore in these cases a lower concentration of methionine is the direct consequence.

This method could be affected by different factors such as the lower methionine intake in breast milk (or from some commercial milks) or with earlier newborn specimen collection. Methionine concentration in affected newborns is likely to be only very slightly increased [3], perhaps just above the concentration considered the normal upper limit for plasma methionine (cut-off ~40 μM). Moreover, the possible presence of antibiotics in newborn's blood, could inhibit the bacterial growth. Thus, false-negative results will likely increase when newborn screening for HCU is performed by BIA. For these reasons the cut-off value was lowered to reduce the false negatives, but this has also increased the number of false positives [4].

There was no consensus as to the optimum cut-off for blood methionine concentration from the newborn screening card using BIA and in many countries there was a marked discrepancy between the frequency of HCU as determined from the screening program compared to that of known cases diagnosed clinically [5]. Other methodologies, such as tandem mass spectrometry [6], amino acid analyser [7], thin-layer chromatography [8] or traditional high-performance liquid chromatography (HPLC) [9], may partially overcome some of these difficulties because of much improved sensitivity and precision, but they are not easy to perform, are time consuming [10], expensive and therefore not suitable for a HCU screening program.

We have developed a new method for HCU diagnosis and other hyperhomocysteinemias by analysing total plasma Hcy (tHcy) from dried blood spots on newborn screening cards, based on HPLC coupled with electrochemical coulometric array detection (HPLC–ED). The first practical applications were developed in the neurochemistry field, for different matrices like cerebrospinal fluid (CSF), plasma and tissues [11,12]. Many other applications soon became of public domain, in many different fields, like determination of phenolics and herbicides pollutants, flavonoids in natural and fermented beverages, benzodiazepines and illicit drugs, DNA adducts, etc., [13–16]. This rapid, simple methodology for HCU screening was validated by measuring tHcy of the same subjects with traditional HPLC [9]. Because of the high selectivity and sensitivity of HPLC–ED, the frequency of false-positive and false-negative results is minimised.

2. Experimental

2.1. Chemicals, reagents and standards

Hcy, tri-*n*-butylphosphine (NTB), sodium dihydrogenphosphate, sodium dodecyl sulfate (SDS), acetonitrile, ethylendiaminetetraacetic acid disodium salt (EDTA), sodium chloride were purchased from Sigma–Aldrich (Milan, Italy). All other chemicals were of analytical-reagent grade.

2.2. Sample collection

The specimens tested consisted of dried blood spots on newborn screening cards of healthy infants (by history and analytical data) born in the Fatebenefratelli Hospital (Erba, Italy) and in N.I.C.U. FBF Isola Tiberina (Rome, Italy). Dried blood spots were analysed as part of a pilot newborn screening program. For each newborn a blood sample from heel capillary circulation was collected on the fourth living day during the normal medical inspection in hospital. Before sample collection, newborns were fasting from 1.5 to 3 h. A collection of healthy donor blood spots from the Healthy Donors Section (HDS) of the Clinical Pathology Unit of Fatebenefratelli Hospital, was included as part of a study for tHcy extraction efficiency.

The specimens from HDS were prepared by spiking samples of whole blood with Hcy standard (Hcystd) and spotting them onto filter paper, with the non spiked samples. About 20 μl of whole blood was used to produce each spot.

2.3. Sample preparation

The preparation of reduced tHcy from blood spots consists of a simple reduction reaction which takes about 15 min for a batch of 10 samples. One 5-mm diameter dot was punched from the dried blood spot and placed in a 1.5-ml plastic vial. The paper punched disk was extracted with 200 μl of reducing agent containing 10% NTB in dimethylformamide. The vials, after 30 s of vortex shaking, were incubated at room temperature for 15 min. Samples were then added with 200 μl of 0.3 *M* $HClO_4$, vortex-mixed 30 s and centrifuged at 10 000 rpm for 2 min. About 100 μl of the clear supernatant was transferred to a micro-vial and 30 μl injected into the HPLC system with an autosampler.

The linearity was calculated as follows: aliquots of whole blood from a single donor were spiked with appropriate amounts of Hcystd, to obtain final concentrations of 15, 30, 120 μ*M* plus the basal concentration. These samples were spotted onto filter paper and allowed to dry overnight. The recovery test (extraction efficiency) of tHcy from blood spots was performed adding known Hcystd concentration (15, 120 μ*M*) to 10 aliquots of HDS pooled whole blood. These 20 samples were spotted onto filter paper and allowed to dry overnight. Within- and between-assay precisions were measured by performing 10 different control samples extraction prepared from the pooled HDS blood on the same day, and over a 1-month period, respectively. For this purpose the HDS pooled blood was frozen to lysate the erythrocytes and then was divided in two sets with aliquots spiked with Hcystd to obtain concentrations of 15 and 120 μ*M*; these samples were spotted onto filter paper and allowed to dry overnight. Then the blood spots were extracted as described above.

2.4. Comparison between dried blood spots tHcy and plasma

We compared tHcy concentrations from dried blood spots versus plasma preparing two sets of blood sample from each newborn. One set was spotted onto filter paper and dried overnight. These samples were prepared for injection in HPLC–ED as described above. The other one was immediately centrifuged and the plasma fraction was used to determine the corresponding tHcy values in plasma EDTA with traditional HPLC–fluorescence detection [9].

2.5. Apparatus

To simplify the sample treatment and to remove possible interference of other biological or exogenous species in the present test, it was chosen to use as the HPLC detector, an array of coulometric electrodes. Two packs of four electrodes were placed in series, forming an array of eight electrodes. The capability of the array of multiple electrodes is used to separate complex mixtures of many components and accurately quantify them. This behaviour is used in the Model 5600 coulometric array electrochemical detector to increase the number of molecules simultaneously resolved in a single analytical run.

The instrument used consisted of one Model ESA 582 pump software (ESA, Chelmsford, MA, USA), polyether ether ketone (PEEK) pulse damper, Model ESA 540 autoinjector, coulometric array Model 5600 ESA detector with thermostatic chamber, with an array of eight coulometric electrodes. Separations were performed on a C_{18} column (ESA MD 150, 150×3 mm). A solution of 0.15 *M* sodium dihydrogenphosphate, 1 m*M* of SDS and 10% of acetonitrile, pH 2.9 was used as mobile phase. Samples were eluted isocratically at room temperature at a flow-rate of 0.4 ml/min. Potentials of the detectors were set as follows: V1=0 mV, V2=130 mV, V3=260 mV, V4=390 mV, V5=520 mV, V6=780 mV, V7=800 mV, V8=910 mV. Chromatograms were automatically integrated and concentrations were calculated by the generated calibration curves. The calibration curve was obtained from the analysis of blood spots at variable Hcystd concentrations (15, 30, 60, 90, 120 μ*M*) and automatically calculated as linear regression by subtracting the blank value, corresponding to the endogenous concentration of the blood spot tHcy.

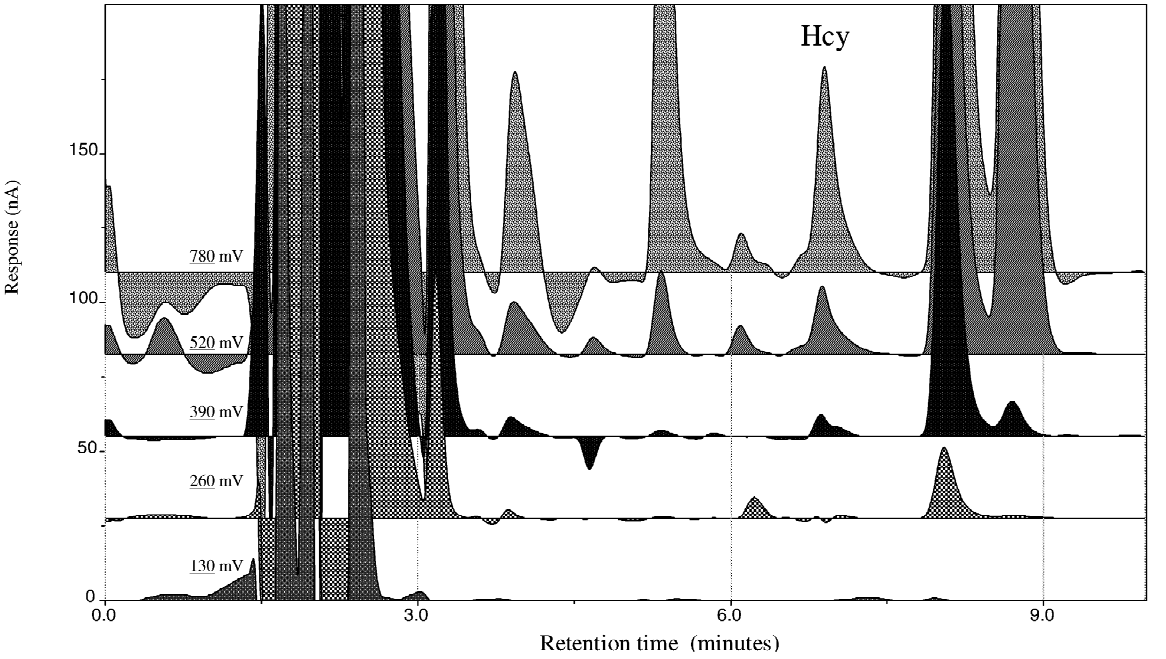

Fig. 1. Multichannel display of a basal blood extracted dot from an healthy donor (HDS specimens): only a few channels are displayed, to simplify the readibility of the chromatograms.

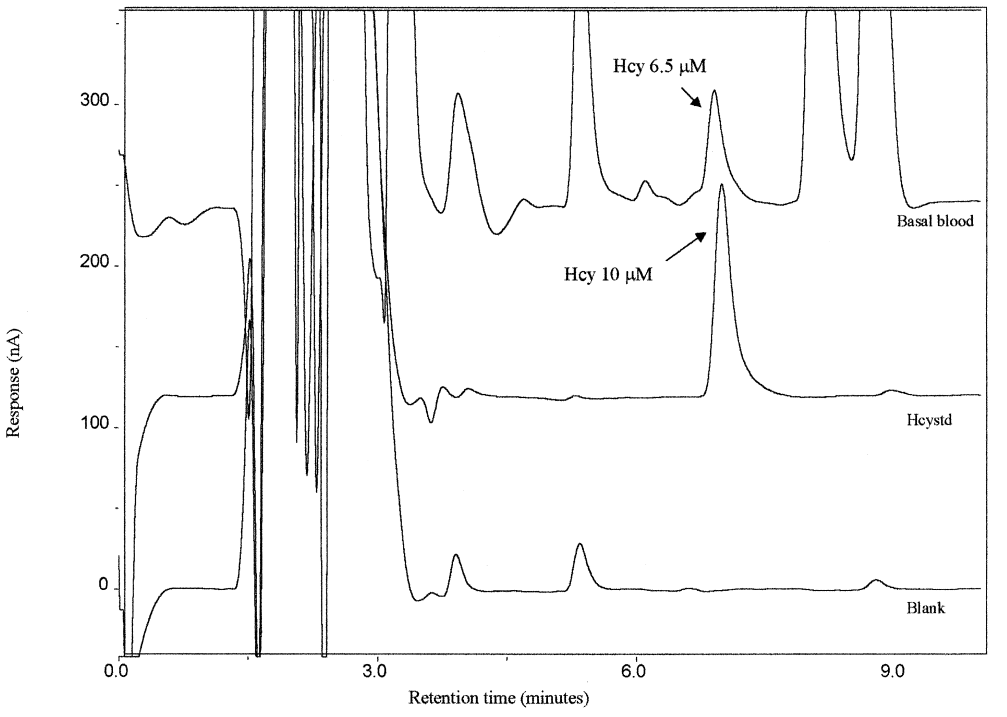

Fig. 2. Comparison of channel 5 (780 mV, dominant channel) for a blank injection with Hcystd and a basal blood.

3. Results

3.1. Assay performance

A typical multichannel chromatogram of a blood sample from a healthy donor (HDS specimens) and chromatographic resolution of tHcy at basal values are shown in Fig. 1. A comparison of the dominant channel (780 mV) used to assay tHcy, among a blank injection, an external standard and a basal blood was made. The tHcy peak was well resolved and without interfering compounds (Fig. 2). In Fig. 3 a blank injection, pushed at the maximum sensitivity usable at the indicated conditions to demonstrate the extrapolated limit of detection is shown. Fig. 4 shows again an injection of a blood from a healthy donor, at lower sensitivity to illustrate the possible simultaneous determination of glutathione (GSH) and of cysteine. Calibration curves for Hcy added to whole blood were linear over the range 0–500 μ*M*.

The linear regression analysis for whole blood yielded $y=10.36x+0.04$ ($r=0.999$), where y was the peak area (μC) and x the concentration of Hcy (μ*M*). The absolute detection limit, based on a signal-to-noise ratio of 3:1, was 0.14 μ*M* of Hcy. The analytical recovery of Hcystd added to blood, calculated by adding the peak areas of the dominant and the subdominant channels, was on average 87% (range 85–90%). The within-assay precisions of tHcy determined as relative standard deviation (RSD) were 4.5% and 2.7% for the samples spiked with tHcy 15 and 120 μ*M*, respectively, while under the same conditions the between-assay precisions determined as RSD were 5.8% and 3.5%, respectively.

The within-assay RSD for the lowest level we measured (1.41 μ*M* of Hcy) was 21.8%, corresponding to a standard deviation of 0.32 μ*M* of Hcy.

3.2. Analysis of tHcy from dried blood spots. Correlation with plasma tHcy

Dried blood spots were analysed as part of a pilot newborn screening program for tHcy. The means

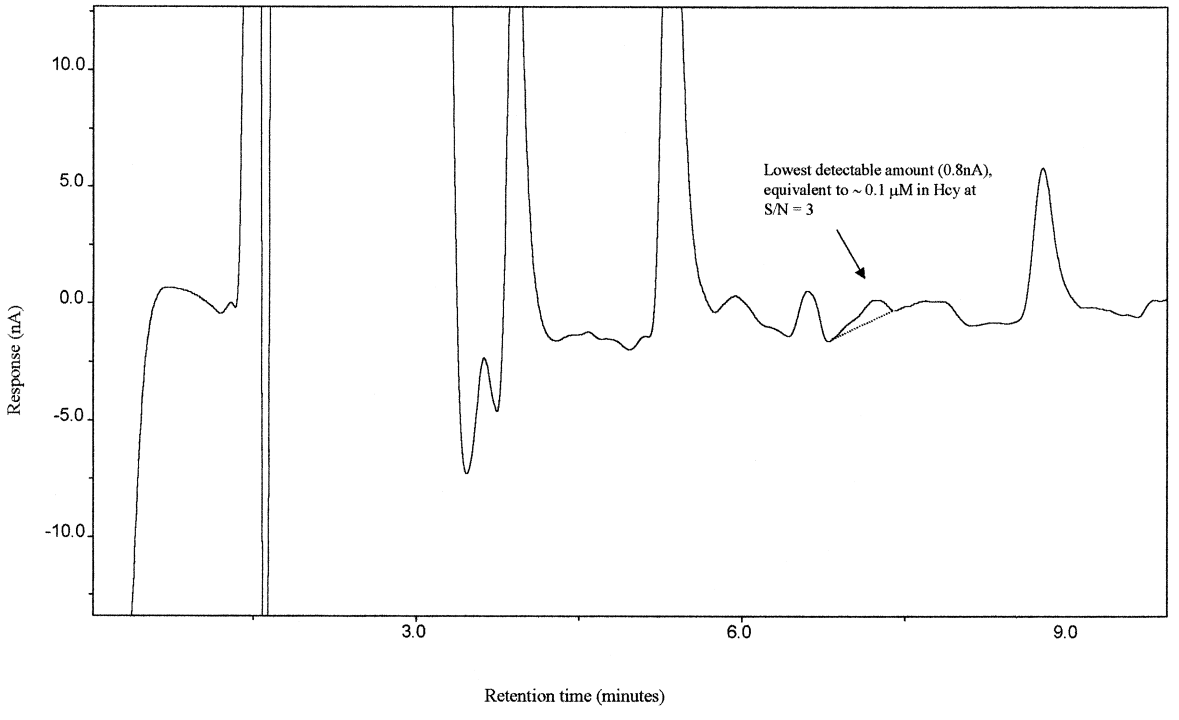

Fig. 3. Channel 5 (780 mV, dominant channel) for a blank injection at highest sensitivity to show the extrapolated limit of detection of 0.14 μ*M* in Hcy at $S/N=3$.

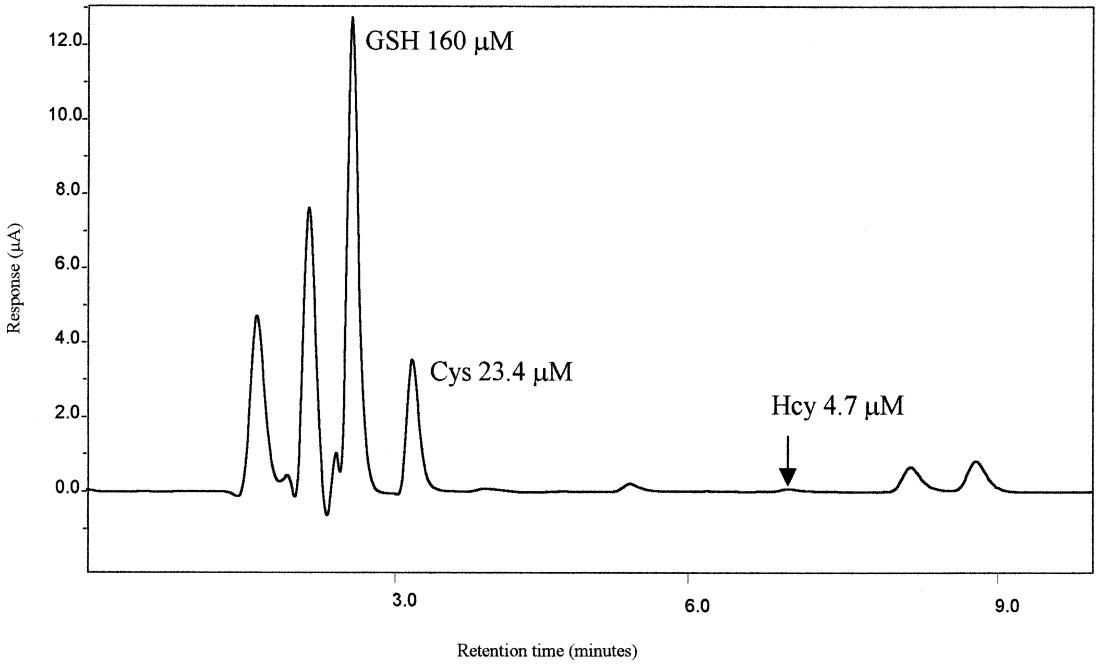

Fig. 4. Chromatogram showing a basal sample, quantitated for Hcy and also for glutathione (GSH) and cysteine (Cys). The wide dynamic range of the linearity and the autorange features of the coulometric array detection allow a simultaneous determination of species at largely different concentrations and response factors.

obtained on 15 samples analysed with both methods were 4.8±0.58 μ*M* and 4.9±0.45 μ*M* for tHcy in dried blood spots and in plasma, respectively, consistent with literature values. The linear regression showed a high correlation coefficient (r=0.903). When 20 μl of whole blood was used to prepare each spot, the punched part of the spot represented 59.1% of the total spot.

4. Discussion

HPLC is considered the confirmatory method more common for plasma tHcy determination. Blood collection and processing are the most important and delicate phases of the pre-analytical steps. Several studies [17,18] show the importance of collecting blood in pre-chilled EDTA-containing tubes followed by immediate centrifugation. Under this condition, the separation of plasma and blood cells will prevent false-positive results which otherwise may reflect the intracellular erythrocytes Hcy synthesis and release [19]. In daily routine clinical analysis, these conditions are difficult to apply, while the use of blood spots drastically reduces this Hcy synthesis and release.

The tHcy is then eluted from the spots, injected into the HPLC system and after a very short chromatographic run, quantitatively detected with coulometric array ED. This HCU analysis can be done by using the blood drop spotted on filter paper during newborn screening by conventional programmes. The difficulty in obtaining urine samples from newborns, suggested to us the selection of blood matrix to perform the HCU neonatal screening test. When an amino acid analyser [7,20] or tandem mass spectrometry [6] were used to measure methionine levels, the analysis was unsuited, resulting in missed diagnoses of HCU: methionine in fact is a less reliable marker of CBS deficiencies than tHcy. These two methods of newborn screening yield a high number of both false-positive and false-negative results associated therefore with an unacceptable number of missed cases. Moreover, tandem mass

spectrometry is more expensive and not readily available for routine use.

The HPLC method shows a good linearity, precision, recovery both at low and high tHcy levels. Specificity for tHcy determination minimises false-positive or false-negative results; moreover, the accuracy of this new method is indicated by correlation of its results with those obtained by a traditional HPLC method, and for all these features, it could be used not only for a rapid, premature HCU diagnosis but also for other hyperomocysteinemias. For the latter it will be necessary to perform prospective studies in a large patient population. These easy sampling and analytical conditions are simple to apply in clinical practice and offer the opportunity to expand and advance newborn screening for inborn errors of metabolism. This test is in line with the goals and strategies of newborn screening: prevention of serious consequences of treatable diseases by early diagnosis and applicability to a large number of samples easily obtained, inexpensively analysed, and quickly processed without false-negative or false-positive results.

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