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Newborn screening of homocystinuria: quantitative analysis of total homocyst(e)ine on dried blood spot by liquid chromatography with fluorimetric detection

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Abstract

Identification of homocystinuric newborns is hindered by the pitfalls of neonatal screening programs. We propose a fluorimetric HPLC method with a rapid pre-analytical step for homocysteine determination from neonatal dried blood spot cards. Homocysteine in blood spots sampled among 2000 healthy newborns on living day 4, averaged 2.92±2.07 μ*M* (range 0.4–7.5). In eight homocystinuric control children, mean values were 61.71±52.84 μ*M* (range 18.9–145.7). The method showed a good linearity (r=0.999), precision (RSD<7%) and recovery (95%). The correlation between blood spots and plasma samples was r=0.90. This method has all the essential features for a homocystinuria screening program: an easy and rapid pre-analytical step combined with method linearity and precision.

Keywords: Newborn screening; Homocystinuria; Homocysteine; Homocystine

1. Introduction

Homocystinuria (HCU), first described in 1962 [1], is an inborn error of sulphur aminoacid metabolism characterized by the inability to convert methionine to cysteine. The classic form of HCU is due to partial or total deficiency of the enzyme cystathionine-β-synthase (CBS). This alteration is inherited as an autosomal recessive disorder that results in the accumulation of bound and free disulfide homocyst(e)ine (Hcy) and methionine in tissues, blood and urine. Other forms of HCU are caused by methylene-tetrahydrofolate reductase (MTHFR) mutations, which decrease Hcy remethylation to methionine, or by hepatic impairment of cobalamin conversion to its active form. These

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abnormalities cause the inactivation of methionine synthase, the consequent accumulation of Hcy and HCU.

Patients with HCU are susceptible to atherosclerosis, vascular disease, ophthalmologic manifestations like ectopia lentis, skeletal abnormalities such as osteoporosis, and mental retardation. The pathologic consequences of HCU can be avoid by a diet, which should be started as early in life as possible. The diet should be low in methionine, high in cysteine with addition of massive doses of the methyl-group donor choline and/or of vitamins (pyridoxine) [2]. Therefore, early diagnosis is essential to prevent the clinical manifestation of this disorder. Non-identification of homocystinuric subjects is often due to the omission of screening for HCU in most neonatal screening programs. Only few countries have either national or regional screening programs, but many of them have been discontinued [3]. The worldwide incidence of HCU, as derived by screening programs, is approximately 1:335,000 and varies from 1:65,000 (Ireland) to 1:900,000 (Japan) [3]. Clinical studies show incidences ranging from 25 HCU cases among 1.58 million newborns in Ireland to 1:60,000 in New South Wales [3]. In an ophthalmologic setting, Cruysberg et al. [4] identified, over a period of 19 years, 34 HCU patients, 30 of who had been misdiagnosed before age 5. This wide range of incidence and the discrepancy between the number of cases identified through screening programs compared to those diagnosed clinically may be due to poor sensitivity and specificity of bacterial inhibition assay (BIA) [5], the test used in most screening programs.

Aim of this study is to develop a method for HCU screening by Hcy determination from dried blood spots on newborn screening cards. The method is based on HPLC analysis with fluorimetric detection. Moreover, we assessed whether blood spot and plasma Hcy levels are correlated.

2. Experimental

2.1. Chemicals

Homocystine, cysteine, cysteinyl-glycine and glutathione standards (Fig. 1), tri-*n*-butylphosphine,

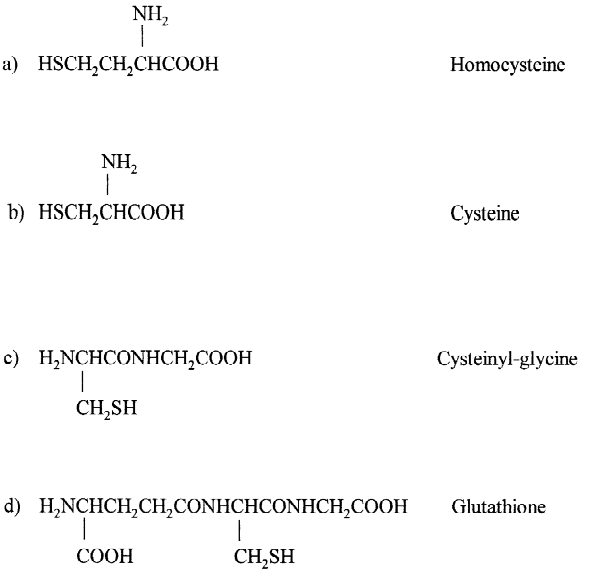

Fig. 1. Chemical structure of homocysteine, cysteine, cysteinyl-glycine and glutathione.

dimethylformamide, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, potassium dihydrogen phosphate, acetonitrile, boric acid, sodium chloride solution, and orthophosphoric acid were purchased from Fluka (Sigma–Aldrich, Milan, Italy). All other chemicals were of analytical-reagent grade.

2.2. Sample collection

The study conformed with the principles outlined in the Declaration of Helsinki and involved 2000 infants born in the Sacra Famiglia Hospital, Erba (Italy) and in San Giovanni Calibita Hospital, Rome (Italy). For each newborn, a blood sample from heel capillary circulation was collected on the 4th living day during routine medical inspection in hospital. The specimens tested consisted of dried blood spots on newborn screening cards that were mailed or delivered to our laboratory.

For further method validation, we also assayed eight homocystinuric children, aged 1–10 years. Four of these patients were identified by genetic analysis: two children were homozygous for CBS and two were homozygous for MTHFR mutations. The remaining four affected controls had abnormalities in cobalamin conversion diagnosed by bio-

chemical analysis of [1-^{14}C]propionate incorporation rate into cultured skin fibroblasts [6]. All controls were chronically administered vitamin supplements: patients who were homozygous for CBS and MTHFR were treated with vitamins B6, B12 and folate, while patients with abnormalities in cobalamin conversion were administered vitamin B12 and betaine.

2.3. Sample preparation

One paper disk (5 mm diameter), containing the dried blood spot, was mixed with 100 μl of borate buffer 1 *M* pH 11 containing 4 m*M* of EDTA, 10 μl of tri-*n*-butylphosphine (10% in dimethylformamide) as reducing agent and 10 μl of ammonium-7-fluoro-benzo-2-oxa-1,3-diazole-4-sulfonate (100 mg in 1 ml of borate buffer 0.125 *M* pH 9.5) in a 1.5 ml plastic vial.

The mixture was incubated at 60 °C for 1 h to convert protein-bound and oxidized Hcy into its free form and, at the same time, to derivatize free SH-groups.

After incubation, 300 μl of orthophosphoric acid (28 m*M*) were added to stabilize the fluorescent adducts [7] and 40 μl of the sample were injected in the HPLC system.

The calibration curves and linearity were obtained using 20-μl aliquots of pooled whole blood (PWB) from single donors, spiked with appropriate amounts of homocystine standard, to obtain final Hcy concentration of 1.87, 3.75, 15, 60, 120 and 240 μ*M*, plus the basal concentration of PWB (6 μ*M*). The homocystine standard was used instead of Hcy to monitor the efficiency of disulfides reduction. In this experimental condition we always obtained full disulfide reduction (data not shown). Samples were then spotted on filter paper and dried overnight.

The Hcy standard concentrations were automatically calculated by linear regression by subtracting the corresponding endogenous concentrations of PWB Hcy.

Within- and between-assay precision was determined by assessment of 10 different control samples prepared from the PWB on the same day, and 10 control samples over a 1-month period. To this purpose the PWB was divided in two sets, spiked with two different Hcy standard concentrations (15 and 120 μ*M*). Twenty replicates from each set were immediately spotted: 10 of them were analysed within the day and 10 over 1 month. Twenty more aliquots were frozen to lysate erythrocytes before spotting.

The analytical recovery of Hcy from blood spots was performed by adding a known amount of Hcy standard (15 and 120 μ*M* final concentrations) to 10 aliquots of PWB, and calculated as:

$$\text{Recovery \%} = 100 \cdot [(\text{Hcy} + \text{Hcy standard}) - \text{Hcy}]/\text{Hcy standard}$$

where Hcy is the basal sample concentration and (Hcy + Hcy standard) is the measured Hcy concentration of the sample, to which a known amount of Hcy standard (15 or 120 μ*M*) has been added.

The absolute detection limit was based on a signal-to-noise ratio of 3:1.

Different volumes of PWB from 20 to 80 μl were spotted in triplicate on the paper to evaluate the influence of blood drop volume on Hcy assay.

Stability of the dried blood spot was assayed during 1 month: 50 μl PWB aliquots were spotted and stored at 4 °C; Hcy was determined after 1, 7, 15 and 30 days.

All the PWB samples used for method validation were spotted onto filter paper and allowed to dry overnight. The blood spots were subsequently extracted as described above.

2.4. Correlation between plasma and dried blood spots Hcy

We tested 106 different control blood samples from 36 healthy and 70 hyperhomocysteinemic adults to compare Hcy concentrations from dried blood spots vs. plasma. One aliquot was spotted onto filter paper, dried overnight and assayed in HPLC as described above. The other one was immediately centrifuged and the plasma fraction was used to determine the corresponding Hcy values with a traditional HPLC method.

2.5. Equipment

The instrument used consisted of two model 422 SV HPLC pumps, coupled to a 465 HPLC auto

sampler (Kontron Instruments, Milan, Italy). Separation was performed on a Discovery C-18 analytical column (250×4.6 mm I.D., Supelco, Sigma–Aldrich). A solution of 0.1 *M* potassium dihydrogenphosphate–acetonitrile (92:8, v/v), pH 2.1, was used as mobile phase. Samples were eluted isocratically at room temperature at a flow-rate of 1 ml/min. Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a Jasco fluorescence spectrophotometer (sensitivity = 1000 and gain = 16) coupled to a Kroma system (Kontron Instruments). Chromatograms were automatically integrated and the concentrations were calculated by the generated calibration curves.

2.6. Statistics

Data are expressed as mean value±SD or as median (range) in case of non-normal distribution. Plasma and dried blood spots were analysed by Deming regression analysis. The influence of different spotted blood volumes (20, 40, 80 μl) on Hcy value was assessed by post-hoc Scheffe's test. Hcy decay on filter paper was analysed by ANOVA for repeated measures. A P value <0.05 was considered statistically significant.

3. Results

3.1. Assay performance

The calibration curves were linear over the Hcy range of 1.87–240 μ*M*. Linear regression analysis yielded $y = 0.985x + 13.7$ ($r = 0.999$), where y was the peak area (mV) and x the Hcy standard concentration. Within-assay precision of Hcy from frozen PWB, determined as relative standard deviation (RSD), was 4.9 and 3.1% for samples spiked with 15 and 120 μ*M* Hcy standard, respectively. Under the same conditions, between-assay precision was 6.7 and 4.25%, respectively. Similar RSD values were found when the spiked PWB was immediately spotted: within-assay precision was 5.1% with 15 μ*M* and 3.6% with 120 μ*M* Hcy standard and between-assay precision 6.3 and 4.8%, respectively. The analytical recovery was on average 95% (range 89–97%). The absolute detection limit of Hcy was 51 n*M*, and blank runs pushed at the highest sensitivity of detection gave no peaks under the same chromatographic conditions (data not shown). We found no significant differences when we increased spotted PWB volumes from 20 to 80 μl. This finding was expected for two reasons: blood diffuses homogeneously on paper card and we used a constant sample quantity (5 mm disk) that was usually taken from the centre of the blood spot.

3.2. Clinical screening

We analysed Hcy levels in 2000 spots from apparently healthy newborns on the 4th living day. Median values were 2.09 μ*M* (range 0.4–7.5), mean values were 2.92±2.07 μ*M*. In the eight homocystinuric children, who served as affected controls, median values were 39.27 μ*M* (range 18.9–145.7), mean 61.71±52.84 μ*M*, respectively. Typical chromatograms of a healthy newborn spot and a homocystinuric subject spot are shown in Fig. 2.

3.3. Plasma and dried blood spots correlation

We correlated the present method with a HPLC method usually used for Hcy measurements in plasma [8]. The Hcy peak from standard, plasma or dried blood spots samples was eluted at the same retention time (5.54 min) and was well resolved without interfering compounds (Fig. 3). A mixture of cysteine, cysteinyl-glycine and glutathione was added to Hcy standard to identify the retention times of the corresponding peaks which were 3.95, 4.56 and 6.83 min, respectively, in plasma and blood spots.

Mean plasma Hcy values were 9.95±1.0 μ*M* in the 36 normal subjects and 19.66±6.01 μ*M* in the 70 hyperhomocystinemic subjects. In the 106 samples analysed by both methods mean, Hcy was 18.34±6.32 μ*M* (range 8.4–44 μ*M*) in plasma and 16.85±4.57 μ*M* (range 7.3–35 μ*M*) in dried blood spots, respectively. The Deming regression analysis showed an equation with a slope of 1.43 (standard error 0.08) and an intercept of −5.72 (standard error 1.33, $r = 0.90$) (Fig. 4). Furthermore, the mean ratio of plasma/blood spot Hcy for all samples was 1.08±0.16.

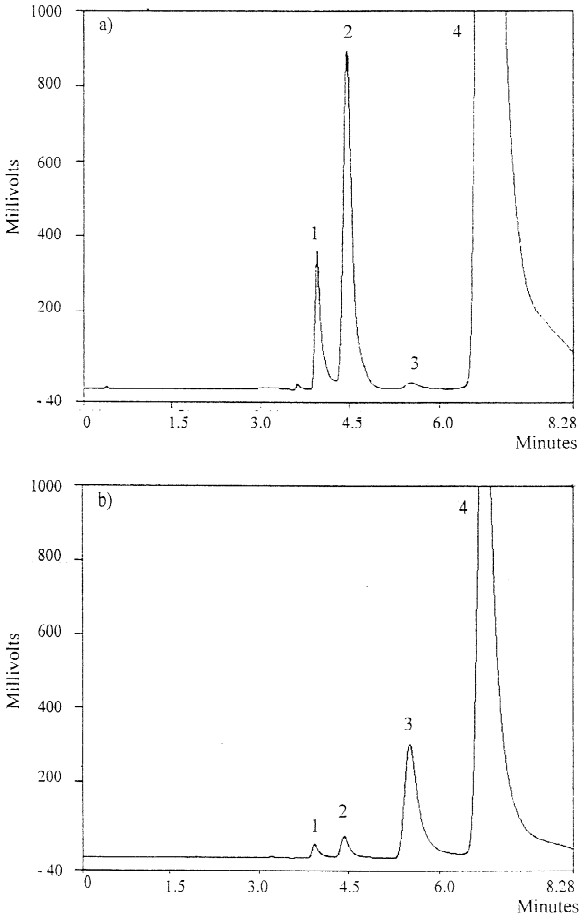

Fig. 2. Typical HPLC chromatograms of main total thiols extracted from dried spot specimens from an unaffected newborn (a) and from a homocystinuric subject (b). Sample from homocystinuric subject was diluted 1:2 with orthophosphoric acid before the injection. Mobile phase: 0.1 *M* potassium dihydrogenphosphate–CH_3CN (92:8, v/v), pH 2.1; flow-rate: 1 ml/min. The corresponding peaks in the elution profile are: 1=cysteine; 2=cysteinyl-glycine; 3=homocysteine; 4=glutathione.

4. Discussion

In the present study, we assessed the feasibility and reliability of a new method for the pre-analytical phase of Hcy analysis, using a blood drop sample spotted and dried on filter paper. Our results indicate that this simple and specific method has the analytical performance features that make it suitable for newborn screening.

Newborn screening of HCU is usually performed by methionine assay in blood spots using a semi-quantitative BIA test. However measurement of methionine has several pitfalls in this context. Newborn intake of methionine is scarce, because of the low methionine content of breast milk and some proprietary formulae. Specimen sampling performed too early after birth will also result in very low methionine blood content and this may affect BIA sensitivity. Presence of antibiotics in newborn's blood could inhibit bacterial growth in BIA test. Another cause of low methionine levels are MTHFR mutations, which are much more common than the rare CBS ones, and result in reduced remethylation of homocysteine to methionine.

Other methods like the cyanide nitroprusside test or its modification, the Spaeth–Barber test [9], can detect increased levels of Hcy in urine, but have low analytical sensitivity. Amino acid analyser [10], thin layer chromatography [11], tandem mass spectrometry [12], traditional liquid chromatography [8,13] or immunoenzymatic assay [14] partially overcome some of these difficulties and have shown greater sensitivity and precision. However, amino acid analyser, thin layer chromatography, tandem mass spectrometry are not easy to perform and are time consuming. Conversely, traditional liquid chromatography and immunoenzymatic assay need plasma amounts of 50–100 μl, that make them unsuitable for a neonatal screening program. In previous works [13,15], we validated a micro method able to process very small amounts of plasma (1–10 μl) collected from heel capillary circulation. However, this method cannot be proposed as newborn screening test for Hcy determination, since recovery of plasma from the capillary tube is difficult and requires a centrifugation step.

The difficulty to obtain urine samples from newborns prompted the selection of the blood matrix spotted on a filter paper, which is routinely used for phenylketonuria screening.

The use of blood spots may prevent the Hcy increase caused by synthesis and release within the erythrocyte [16]. Moreover, we showed that Hcy levels on blood spots are stable for 1 month at 4 °C and do not change with different volumes of blood spotted. This pre-analytical procedure avoids sample centrifugation, necessary to separate plasma from

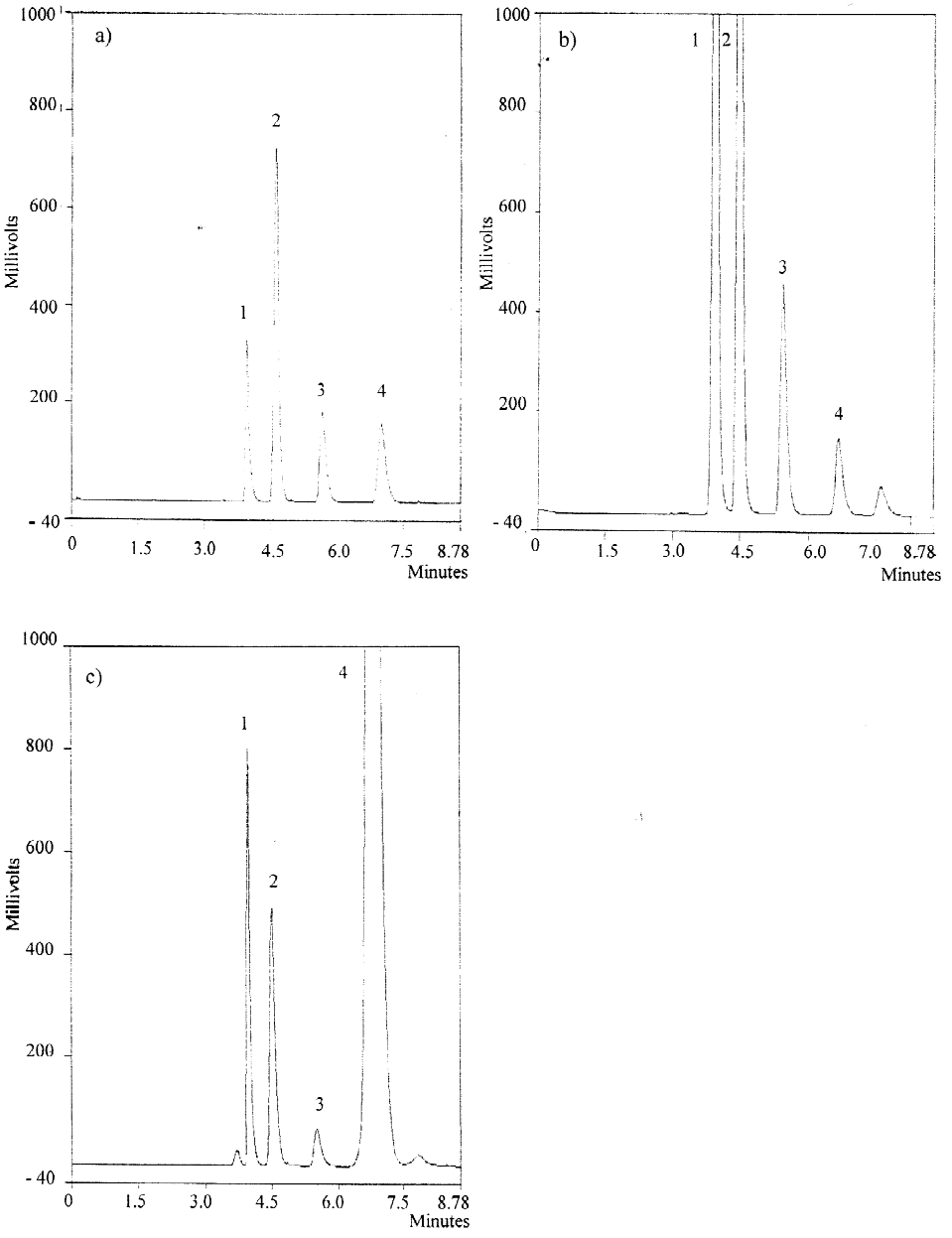

Fig. 3. Chromatographic separation of a standard mixture (a), plasma (b), and dried blood spot (c) from an normal adult subject. Mobile phase: 0.1 *M* potassium dihydrogenphosphate–CH_3CN (92:8, v/v), pH 2.1; flow-rate: 1 ml/min. Standard mixture concentrations are: cysteine = 18.75 μ*M*; cysteinyl-glycine = 7.5 μ*M*, homocysteine = 3.75 μ*M*, and glutathione = 7.5 μ*M*. The corresponding peaks in the elution profile are: 1 = cysteine; 2 = cysteinyl-glycine; 3 = homocysteine; 4 = glutathione.

erythrocytes, and allows Hcy extraction, reduction and derivatization in a single step.

The mean Hcy value (2.92±2.07 μ*M*), obtained from dried blood spot samples of healthy newborns is comparable with data obtained from our previously described micro method [13]. We found a good

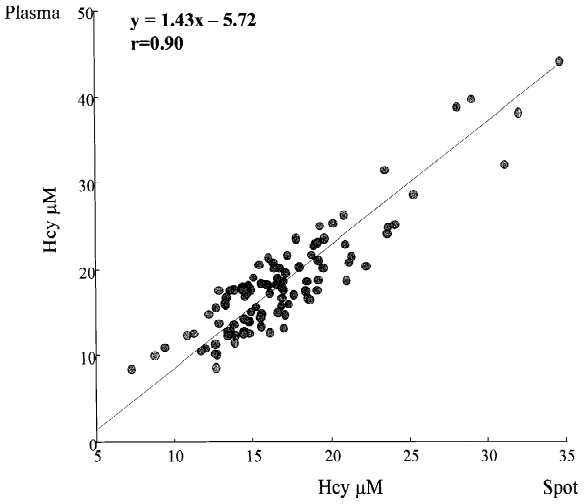

Fig. 4. Deming regression analysis of Hcy concentration in plasma (*y*-axis) and dried blood spot (*x*-axis).

correlation between this spot method and a HPLC method used for routine measurement of plasma Hcy.

Recently, a HPLC method with electrochemical coulometric array detection, without any sample derivatization, was proposed for Hcy determination in dried blood spots [17]. Nevertheless, the method here described appears more suitable for clinical chemistry laboratories than the electrochemical coulometric array, because of the easy availability of the fluorimetric detector.

An important limitation of this study is that we did not find any case of homocystinuria among the 2000 healthy newborns tested, as expected in view of the known incidence of the disease [3,4]. For this reason we used a paediatric control group, with a different age range. However, high methionine levels are known to occur in HCU newborns and high plasma levels of homocystine have also been reported in the literature in newborns 4–56 days of age [18]. Furthermore, in our homocystinuric children, we found mean Hcy values (61.71 ± 52.84 μM) definitely higher than those of healthy newborns and of normal controls of the same age [19,20]. There was no overlap between the upper limit of the newborn series (7.5 μM) and the lower limit of affected controls (18.9 μM).

Another limitation of our study is that the blood spot–plasma comparison study was performed only in adult controls. However, plasma sampling from newborns would be unethical, because of the high sample volume needed. Moreover, the use of adult samples is experimentally reliable for a comparison study.

Our method shows good linearity, precision and sensitivity. The accuracy is good: both recovery and the correlation of results on blood spot samples with those obtained by a traditional plasma HPLC assay are high. Furthermore, the Hcy blood spot/plasma sample ratio is close to the unit with a narrow standard deviation.

These features, coupled to ease of sample collection, fast preparation and short chromatographic analysis, make this quantitative method suitable for newborn HCU screening.

5. Notation

HCU	homocystinuria
Hcy	homocyst(e)ine
BIA,	bacterial inhibition assay
PWB	pooled whole blood
CBS	cystathionine-β-synthase
MTHFR	methylene-tetrahydrofolate reductase
SD	standard deviation
RSD	relative standard deviation

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