trations for EtG. The peak values for both compounds were obtained in the 4-h collection, and both compounds were still measurable in the sample collected at 29 h, but not at 32 h.

Among 54 clinical urine samples selected at random from those sent to the laboratory for routine testing of recent alcohol consumption, all 31 samples with a detectable EtG (mean, 427 μmol/L; range, 1.7–3162 μmol/L) were also positive for EtS (mean, 257 μmol/L; range, 1.1–2095 μmol/L), and 2 others were positive only for EtS (0.6 and 2.4 μmol/L, respectively). There was a good correlation between EtS and EtG \((r^2 = 0.839; P < 0.0001)\) with somewhat higher mean concentrations for EtG (mean EtG:EtS ratio, 1.5; range, 0.3–3.0). The remaining 21 samples were negative for both EtS and EtG. No EtS was detected in 25 urines collected on separate days from two healthy individuals who had abstained from ethanol for several days before sampling, according to self-reports.

These results demonstrate that sulfation conjugation is a metabolic pathway for ethanol in humans and that EtS is a common constituent in the urine after alcohol intake. Sulfotransferases constitute an important inactivation and detoxification enzyme system for xenobiotics and small endogenous molecules (13). However, based on comparison with previous data on the relative importance of EtG to overall ethanol metabolism (1), it appears that only a very small fraction (≤0.1%) of the ethanol ingested undergoes sulfate conjugation in humans. Being a direct derivative of ethanol, EtS appears to be a specific indicator of recent alcohol consumption and, as for EtG, also shows a much longer window of detection than the parent compound, implying a higher sensitivity. This means that urinary EtS could be a new promising candidate marker to disclose recent alcohol consumption even when ethanol is no longer measurable in body fluids. Whether there is any advantage in measuring EtS instead of the other markers of acute alcohol consumption remains to be elucidated. Potential applications include verification of abstinence or detection of relapse drinking during outpatient treatment of alcohol-dependent individuals and in forensic toxicology to determine whether the ethanol identified originates from alcohol ingestion before death or sampling or was generated artfactually (14).

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References


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Homocysteine (Hcy) is a sulfur amino acid and a metabolite of the amino acid methionine. Hcy is very reactive because it contains a free sulfhydryl (thiol) group, and it readily oxidizes to form various disulfides. The fraction of free Hcy in plasma is therefore <2% of total plasma Hcy (tHcy) (11).

Increased Hcy has been associated with cardiovascular, cerebrovascular, and peripheral vascular disease (2–4) and is recognized as an independent risk factor. The need for a simple automated assay is therefore increasing, and various analytical methods have become available. Currently the two most widely used techniques are HPLC and immunochemistry.

We implemented a new automated enzymatic method for the measurement of tHcy on two different routine clinical chemistry analyzers and compared these assays with HPLC and the AxSYM immunoassay.

Reagents and calibrators for the enzymatic method...
were obtained from Catch Inc. In this method Hcy and L-serine form cystathionine, which is then converted to Hcy, pyruvate, and ammonia. The enzymes involved are cystathionine β-synthase and cystathionine β-lyase, respectively. tHcy is measured by the production rate of pyruvate by inclusion of lactate dehydrogenase and NADH in the reaction mixture. Reagent 1 contains serine, NADH, and lactate dehydrogenase; reagent 2 contains the NADH in the reaction mixture. Reagent 1 contains cystathionine β-synthase and cystathionine β-lyase. The calibrators have Hcy concentrations of 0.0 and 26.5 μmol/L (5). The enzymatic assay was implemented on two Synchron LX-20 and CX-5 analyzers (Beckman Coulter, Inc.).

The HPLC method is a modification from Spaapen et al. (6), as obtained from Instruchemie. Plasma samples were pretreated with tri-n-butylphosphine for reduction and deproteinized with trichloroacetic acid. Derivatization was performed with 7-fluorobenzo-2-oxal,3-diazole-4-sulfonic acid. 2-Mercaptoethylamine was used as an internal standard. Separation was obtained by use of an Inertisil ODS-3 column with sodium acetate buffer as the mobile phase. Assay recovery was optimized by use of samples obtained from the Dutch Foundation for Quality Assessment in Medical Laboratories.

The immunoassay is based on the fluorescence polarization immunoassay technology of the AxSYM immunochemistry analyzer (Abbott Laboratories) (7).

A total of four Dutch clinical chemistry laboratories participated in the study. Laboratory 1 was the laboratory of the BovenIJ Hospital (315 beds) in Amsterdam, equipped with both the CX-5 and LX-20 analyzers. Laboratory 2 was the laboratory of the Medical Center Alkmaar (800 beds) in Alkmaar, equipped with the CX-5 analyzer and a HPLC. Laboratory 3 was the laboratory of clinical chemistry of the Elkerliek Hospital (524 beds) in Helmond, equipped with the LX-20 analyzer. Laboratory 4 was the laboratory of clinical chemistry of the Gemini Hospital (320 beds) in Den Helder, equipped with the AxSYM. All laboratories participated in the comparison study, and laboratories 1, 2, and 3 participated in the analytical evaluation.

Samples for testing linearity were prepared in laboratories 1, 2, and 3, and samples for the interference study were prepared in laboratories 1 and 3. Samples for the comparison study were collected in laboratory 2 from patients screened for tHcy by a fasting plasma measurement or an oral methionine loading test. Samples (anonymized) were used for the study only if patients gave their informed consent, according to the guidelines of the board of directors of the hospital. A total of 100 samples were used. The patient demographics were as follows: males, n = 42 patients; mean age, 50 years (range, 32–70 years); mean tHcy, 19.5 μmol/L (range, 5.3–110.6 μmol/L); females, n = 58 patients; mean age, 44 years (range, 20–79 years); mean tHcy, 20.2 μmol/L (range, 4.1–107.0 μmol/L). Blood was drawn in tripotassium EDTA tubes (Vacutainer; Becton Dickinson BV) and kept on ice; plasma was separated within 30 min and stored at −20 °C. The frozen aliquots were distributed, thawed, and measured in the participating laboratories on the same days.

Imprecision was assessed in laboratories 1, 2, and 3 by use of commercial control samples, all prepared from human serum, obtained from three suppliers: Bio-Rad Laboratories BV; UTAK Laboratories Inc.; and the Dutch Foundation for Quality Assessment in Medical Laboratories.

Within-run imprecision (CV) was determined by measuring six different control samples 21 times in one run. Total imprecision was established according to the EP5 protocol (8) and linearity according to the EP6 protocol (9). The lower limit of detection was defined as the concentration corresponding to a signal 3 SD above the mean (n = 20) for calibrator 0, which was free of Hcy. The influence of bilirubin, hemoglobin, and lipids was evaluated according to the CERMAB protocol (10).

We compared the results obtained with the three methods by debiased regression according to Passing and Bablok (11). The mean difference was calculated by the paired t-test and displayed graphically as recommended by Bland and Altman (12). All statistical analyses were done with Analyze-It software.

The enzymatic assay was linear from 1.5 to 90.0 μmol/L on both the LX-20 and CX-5 analyzers. The lack of linearity was rejected at P < 0.01. The limits of detection on the LX-20 and CX-5 were 0.21 and 0.41 μmol/L, respectively. The results from the imprecision study are presented in Table 1.

### Table 1. Imprecision of the tHcy measurement with the Synchron CX-5 and LX-20.

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Measured tHcy, μmol/L</th>
<th>Synchron CX-5</th>
<th>Synchron LX-20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6.6*</td>
<td>9.9*</td>
</tr>
<tr>
<td>Imprecision (CV)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-run, %</td>
<td></td>
<td>2.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Between-run, %</td>
<td></td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Between-day, %</td>
<td></td>
<td>2.4</td>
<td>6.1</td>
</tr>
<tr>
<td>Total, %</td>
<td></td>
<td>4.1</td>
<td>6.7</td>
</tr>
</tbody>
</table>

* Commercial human-based control samples from *Bio-Rad Laboratories, *UTAK Laboratories Inc., or *Dutch Foundation for Quality Assessment in Medical Laboratories.
Interference studies showed that hemoglobin up to 0.97 mmol/L (1600 mg/dL) and bilirubin up to 267 μmol/L (15.6 mg/dL) did not interfere in the assay. Triglycerides >9.7 mmol/L produced a nonlinear significant decrease in measured tHcy concentrations of more than twice the total imprecision of the test on both analyzers.

Bland–Altman plots comparing the enzymatic methods on both the CX-5 and the LX-20 with HPLC are shown in Fig. 1. Passing–Bablok regression analysis revealed that both the LX-20 and CX-5 measured tHcy concentrations that were significantly higher than HPLC results. The calculated slopes (95% confidence intervals) for the LX-20 in laboratories 1 and 3 were 1.060 (1.04 – 1.085) and 1.034 (1.014 – 1.053), respectively, and for the CX-5 in laboratories 1 and 2 were 1.047 (1.030 – 1.065) and 1.022 (1.004 – 1.042), respectively. For all comparisons, the intercepts were not significantly different from 0.0 μmol/L. When compared with the AxSYM, the calculated slopes for the LX-20 in laboratories 1 and 3 were 1.078 (1.054 – 1.100) and 1.044 (1.014 – 1.073), respectively, and for the CX-5 in laboratories 1 and 2 were 1.076 (1.049 – 1.097) and 1.050 (1.031 – 1.076), respectively. The calculated intercepts were −0.322 (−0.575 to −0.009), −0.289 (−0.715 to 0.010), −0.461 (−0.702 to −0.163), and −0.485 (−0.793 to −0.252) μmol/L, respectively.

Until recently the measurement of tHcy in plasma was limited to laboratories equipped with highly specialized instrumentation such as gas chromatography–mass spectrometry or HPLC. The introduction of an immunoassay (7) brought homocysteine testing within reach of routine clinical chemistry laboratories. Recently a new enzymatic tHcy test has become commercially available that is easy to implement on a routine clinical chemistry analyzer, which is preferable from a logistic point of view.

The linear range of the enzymatic assay is adequate. Samples with tHcy concentrations >90 μmol/L should be diluted, but concentrations that high are seldom found in clinical practice.

The within-run imprecision was good with exception of the LX-20 measurements at low concentrations in laboratory 1. The results obtained with the same tHcy concentration in laboratory 3 led us to conclude that the performance was analyzer dependent and thus can be optimized.

Total imprecision (CV) ranged from 2.5–3.5% (high controls) to 4.1–7.0% (low controls). According to Fraser et al., (8) the AXSYM has a slightly higher within-run imprecision compared to the LX-20 and CX-5.

Fig. 1. Difference (mean and 95% confidence intervals) between plasma tHcy measured by HPLC and AxSYM (A), LX-20 in laboratory 1 (B), and CX-5 in laboratory 2 (C). (A), mean (95% confidence interval) difference, −0.12 (−0.49 to 0.25) μmol/L; (B), mean (95% confidence interval) difference, 1.47 (0.94–2.00) μmol/L; (C), mean (95% confidence interval) difference, 1.10 (0.65–1.56) μmol/L. Not shown (but comparable) are the results obtained with the LX-20 in laboratory 3 (mean difference, 0.98 μmol/L; 95% confidence interval, 0.55–1.40 μmol/L) and the CX-5 in laboratory 2 (mean difference, 0.75 μmol/L; 95% confidence interval, 0.42–1.08 μmol/L).
al. (13), objective goals for total imprecision can be calculated based on the CV_{within-subject}. They proposed a desirable performance for imprecision as 0.5 × CV_{within-subject}. The reported within-subject variation of tHcy is 7.0–40%, with a value of ~8.5% reported most frequently (14–19).

It therefore can be concluded that the total imprecision of this enzymatic assay does not meet the criterion of desirable performance at all concentrations. Several studies on interlaboratory variation of tHcy measurements have been published (20–23). Compared with the results reported in those studies, the total imprecision of the enzymatic method seems to be better than that for HPLC but somewhat worse than the imprecision of the AxSYM assay.

From the patient-comparison study it can be concluded that both the CX-5 and LX-20 analyzers report higher tHcy concentrations than HPLC, whereas the AxSYM results are lower. None of the calculated intercepts for regression comparison of the enzymatic assays with HPLC were significantly different from 0.0 μmol/L. This highlights the fact that the differences between the two methods are probably caused by differences in the calibrators, which is supported by the observation that with the HPLC method, the measured tHcy concentration of standard solution 2 of the enzymatic test was 7.5% higher than the value stated by the manufacturer. Standard solution 1 did not contain Hcy.

In Fig. 1 two data points catch the eye, at 43.9 and 80.8 μmol/L tHcy. For both data points, the tHcy values measured by the LX-20 and CX-5 analyzers were much higher than the results obtained by HPLC and the AxSYM. These two samples were obtained after methionine loading, and the tHcy concentrations of the matching fasting samples were very similar with all three formats. After a methionine load the Hcy metabolism is stressed in a nonphysiologic way, potentially leading to increased concentrations of intermediates of the methionine Hcy pathway. In our study 26 paired samples from a methionine load test were collected. To determine whether methionine loading influences the enzymatic measurement, we compared the differences (expressed as the percentage relative difference) between the enzymatic assay and HPLC for the fasting samples with the differences between the enzymatic assay and HPLC for the post-methionine load samples. We found no significant difference between the fasting differences and post-methionine load differences [t-test, fasting mean (SD), 6.12 (6.59)%; post-methionine load mean (SD), 6.49 (6.64)%; difference between the means, -0.37% (P = 0.805; n = 26)].

In conclusion, from the point of practicability, this new, easy to implement enzymatic method is useful in a routine clinical chemistry laboratory setting. However, to meet the criterion of desirable performance, total imprecision could be improved by measuring the plasma samples in replicate. Nevertheless, compared with the imprecision of the widely used HPLC method, the enzymatic method is more precise. Moreover, the tHcy concentrations measured by this assay are slightly but significantly different from those measured by HPLC and immunochemistry, although we found a strong linear correlation between the methods. With regard to the small differences found, calibration of the test with samples provided by Institutes for External Quality Assessment could easily correct this problem.

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References
Increased S100B in Cerebrospinal Fluid of Infants with Bacterial Meningitis: Relationship to Brain Damage and Routine Cerebrospinal Fluid Findings,

Diego Gazzolo,† Dariusz Grutzfeld,‡ Fabrizio Michetti,§ Amelia Toesca,¶ Mario Lituania,‖ Matteo Bruschetti,‖ Anna Dobranska,§ and Pier-luigi Bruschetti‖ (†Department of Pediatrics and Obstetrics and Gynecology, Giannina Gaslini Children’s University Hospital, Genoa, Italy; ‡Department of Neonatology, Children’s Memorial Health Institute, Warsaw, Poland; §Institute of Anatomy and Cell Biology, Catholic University, Rome, Italy; ¶address correspondence to this author at: Institute of Anatomy and Cell Biology, Catholic University, Largo Francesco Vito, 1, I-00168 Rome, Italy; fax 39-06-30154813, e-mail fabrizio.michetti@rm.unicatt.it)

Perinatal infections such as bacterial meningitis (BM) are one of the major factors associated with perinatal brain damage (1–4). Despite accurate monitoring, the early stages of meningitis are crucial because brain damage may occur at a subclinical stage when ultrasound assessment is still silent (5, 6). Laboratory assessment is based on chemical analysis of cerebrospinal fluid (CSF) and the detection of bacteria, and the possibility of detecting cases at risk of brain damage is to date limited. The measurement of brain constituents able to diagnose subclinical lesions at this stage could therefore be useful.

S100B is a calcium-binding protein primarily present in nervous tissue (7–9). Increased S100B in biological fluids has been shown to be a marker of brain damage both in adults and during the antenatal and postnatal periods (10–16).

The present case–control study is aimed at investigating whether the measurement of S100B in CSF could also be useful in infants with BM for the early detection of cases at risk of encephalitis.

Samples of CSF were collected from infants consecutively admitted between April 1998 and June 2000 to our tertiary referral center for intensive care for infectious diseases. For the present study we identified from our database 44 patients with BM and matched them for gestational age at sampling with 44 patients without BM (1 BM case vs 1 control). We retrieved clinical, laboratory, and routine CSF test data and CSF S100B concentrations.

Eligibility criteria for infants with BM were as follows: clinical (respiratory distress, lethargy, presence/absence of minor/major neurologic symptoms, feeding and abdominal distension problems, temperature instability or increases, unexplained recurrent hypoglycemia, poor vascular perfusion) and laboratory signs of sepsis with altered CSF results (leukocyte count, protein, glucose, visible bacteria) (17). Causative bacteria were gram-positive cocci (Streptococcus agalactiae) in 19 cases, unidentified gram-positive cocci in 12 cases, and gram-negative rods (Haemophilus influenzae or Escherichia coli) in 13.

For ethical reasons, the healthy group consisted of infants in whom CSF samples had been collected to investigate confirmed or probable meningitis. Infants were included in the control group when CSF findings of meningeal inflammation such as CSF leukocyte count and protein and glucose concentrations were normal and CSF culture or bacterial antigen test results were negative (17).

An additional criterion for admission to the control group was that the ultrasound patterns were negative for encephalitis and for central nervous system diseases. Exclusion criteria were fetal or neonatal central nervous system malformations, chromosomal abnormalities, perinatal asphyxia, and congenital heart disease.

In the control group sepsis was identified (15 of 44 with unidentified gram-positive cocci, 22 of 44 with Staphylococcus epidermidis, and 7 of 44 with Staphylococcus species).

All recruited infants were delivered at term without apparent perinatal complications and/or clinical history of neurologic abnormalities or comorbidities between birth and admission to our neonatal intensive care units.

Cerebral ultrasound (US) was assessed at the time of CSF sampling, at 72 h after admission, and on discharge from hospital. Cerebral computerized tomography (CT) was performed, in the infants with BM plus encephalitis (BME), for the detection of the presence and extension of encephalitis when US was already suggestive of brain lesion.

Local Ethics Committees approved the study protocol, and the parents of the infants gave signed and informed consent that an aliquot of the CSF obtained at the time of sampling be used for research purposes.

At sampling, 100 μL of CSF for S100B measurement was obtained by lumbar puncture for study. Samples were immediately centrifuged at 900×g for 10 min, and the supernatants were stored at −70 °C.

The S100B protein concentration was measured by use of a commercially available immunoluminometric assay (Lia-mat Sangtec 100; AB-Sangtec Medical) (10, 18, 19).

Each measurement was performed in duplicate according to the manufacturer’s recommendations. The limit of detection of the assay was 0.02 μg/L, the intraassay imprecision (CV) was <5%, and the interassay CV was <10%. S100B measurements were performed by a single expert who did not know the infants’ clinical conditions.

S100B concentrations are reported as medians and interquartile ranges. Statistical analyses were performed with the Kruskal–Wallis one-way ANOVA for comparisons between groups and the Mann–Whitney U-test when data did not follow a gaussian distribution. The Fisher exact test was used to compare the incidences of abnormal