healthy subject is shown in Fig. 1A. The spectrum is very simple because the MALDI technique produces preferentially protonated molecules through ion-molecule reactions occurring in the selvage region close to the solid-state sample. The four peaks composing the spectrum are easily assigned on the basis of their *m/z* values alone. Thus, peaks at *m/z* 15127 and 15868 correspond to protonated α- and β-globin molecules respectively; the less abundant peaks at *m/z* 15289 and 16030 correspond to protonated molecules produced by the condensation of one glucose molecule (180 Da) on the α- and β-globins, respectively (α-globin + glucose $-$ $H_2O \rightarrow 15127 + 180 - 18 = 15289$ Da; β-globin + glucose $-$ $H_2O \rightarrow 15868 + 180 - 18 = 16030$ Da). These results are in agreement with the ESI results: both techniques demonstrate that both globins are glycated to a similar extent.

Under ESI conditions, the higher resolution also allows the identification of $[M + Na]^+$ and $[M + K]^+$ ions *(4)*. Under MALDI conditions, these species are unresolved from the $[M + H]^+$ ions. However, when the delayed extraction method *(15)*, which is not available in our laboratory, is used, the same sample from a healthy subject leads to the spectrum shown in Fig. 1B, composed of the same ions and new ones produced by $[M + Na]^+$ and $[M + K]^+$ species (*m\z* 15149 and 15165 for α-globin, and *m/z* 15890 and 15906 for β-globin).

Quantitative data can be obtained easily by a simple automatic integration procedure of the various peaks in the spectra. Data from the 20 healthy subjects and the 30 diabetic patients were obtained by integrating the peak area; it must be emphasized that, in the case of MALDI measurements, the abundance of a ionic species is related to peak area and not to peak height, as is usual in other mass spectrometric measurements (e.g., ESI). The measured area is proportional to the ion current produced by the different species: the sum of the areas of the various detected species represents the total amounts of nonglycated and glycated α- and β-globins ionized by MALDI. This total area is established as 100, and the amounts of the various species are expressed as percentages of it. The low resolution of MALDI measurements carried out without delayed extraction would not affect, in principle, quantitative measurements. In fact, integration of the area related to the nonglycated α- and β-globin chains includes the $[M + Na]^+$ and $[M + K]^+$ ions revealed in high-resolution conditions, which represent nonglycated species.

In diabetic patients, as expected, a clear increase in the abundance of both glycated components is generally observed.

Linear regression analysis of the percentages of the whole pool of glycated proteins vs HbA_{1c} leads to the straight line shown in Fig. 1C, which crosses the origin of the axis, although its slope is 0.66 and not 1, as is expected. This indicates the different response factor of MALDI and HbA_{1c} in experimental measurements.

In conclusion, the above data show that MALDI/MS may be applied validly to the identification of glycated α- and β-globins. The technique produces clearly reproducible "fingerprints" of globin species, in particular when gridless delayed extraction is available. In our opinion, at this stage MALDI/MS cannot be proposed as a routine tool for HbA_{1c} measurements, mainly because the sample preparation phase is not automated, whereas it is in the proposed ESI-based method. However, it is a valuable tool for the quality control of HbA_{1c} measurements carried out following other principles and may be applied to their standardization.

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Rapid and Accurate HPLC Assay for Plasma Total Homocysteine and Cysteine in a Clinical Laboratory Setting, *Christine M. Pfeiffer,** *Dan L. Huff, and Elaine W. Gunter* (National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341; * author of correspondence: fax 770-488-4609, e-mail cfp8@cdc.gov)

Although several approaches for measuring plasma total homocysteine by HPLC have been described during the last few years *(1–4)*, none combines all the desired fea-

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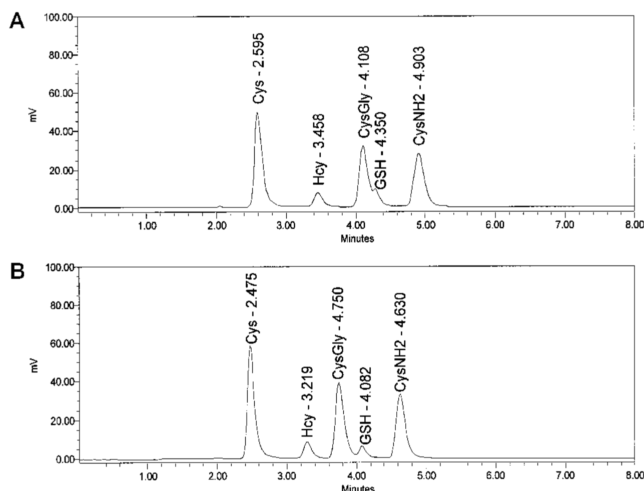

Fig. 1. Chromatograms of a representative plasma sample (12.5 μmol/L tHcy) measured at a mobile phase pH of 5.5 (*A*) and 5.0 (*B*).
Cys, cysteine; *Hcy*, homocysteine; *CysGly*, cysteinylglycine; *GSH*, glutathione; *CysNH2*, cysteamine.

tures for a rapid, user-friendly, and robust assay: (*a*) a stable, efficient, and nonhazardous reducing agent; (*b*) incorporation of a suitable internal standard; and (*c*) rapid, isocratic separation of the thiols of interest, using a mobile phase of mild pH. We have therefore modified the method of Vester and Rasmussen *(5)* by using tris(2-carboxyethyl)phosphine (TCEP), a newer stable, water-soluble phosphine derivative introduced by Gilfix et al. *(6)*, as the reducing agent, cystamine as the internal standard, and isocratic separation of the thiols extracted from only 50 μL of plasma within 6 min.

A mixture of 50 μL of plasma, 25 μL of internal standard, and 25 μL of phosphate-buffered saline (PBS, pH 7.4) was incubated with 10 μL of 100 g/L TCEP (Pierce Chemical Co.) for 30 min at room temperature to reduce and release protein-bound thiols, after which 90 μL of 100 g/L trichloroacetic acid containing 1 mmol/L EDTA was added for deproteinization. After the sample was centrifuged for 10 min at 13000*g*, 50 μL of the supernatant was added to an autosampler vial containing 10 μL of 1.55 mol/L NaOH; 125 μL of 0.125 mol/L borate buffer containing 4 mmol/L EDTA, pH 9.5; and 50 μL of 1 g/L SBD-F (Wako Chemicals) in the borate buffer. The sample was then incubated for 60 min at 60 °C. HPLC was carried out on a 2690 Alliance solvent delivery system and a 474 scanning fluorescence detector (385 nm excitation, 515 nm emission), both from Waters Technologies Corp. Separation of the SBD-derivatized plasma thiols was performed on a Prodigy ODS2 analytical column, 150 × 3.2-mm, 5 μm (Phenomenex) with an Adsorbosphere C_{18}, 3-cm guard column (Alltech Associates), using a 10-μL injection volume and 0.1 mol/L acetic acid-acetate buffer, pH 5.5, containing 30 mL/L methanol as mobile phase at a flow rate of 0.7 mL/min and a column temperature of 29 °C.

L-Homocystine and L-cysteine calibrators (0–50 μmol/L free thiol in 100-μL assay volume) were prepared in PBS, pH 7.4, and in pooled EDTA plasma. The internal standard was cystamine dihydrochloride, which was added to all samples to achieve a final concentration of 10 μmol/L free thiol (in 100-μL assay volume). All chemicals were obtained from Sigma Chemical Co. Calibration was performed daily in PBS and in plasma (standard addition) and was evaluated as both external and internal calibration (area ratios between the thiol and the internal standard).

Plasma specimens from healthy adult volunteers were obtained from whole blood collected into EDTA-containing tubes (Becton-Dickinson) and cooled on ice water; the plasma was separated by centrifugation within 30 min after venipuncture and stored for a maximum of 3 months at −70 °C before being assayed. Blood specimens were collected by the Emory University Hospital Blood Collection Service under an agreement with the CDC (including an omnibus informed consent and Human Subjects Review protocol).

Under the chromatographic conditions described, the retention times of all thiols were very stable, with a CV <2% during 6 months. At pH 5.5, homocysteine, cysteine, and the internal standard were clearly baseline separated from each other and from cysteinylglycine and glutathione (Fig. 1A). Increasing the pH of the mobile phase to 6.0

Table 1. Comparison between the reducing efficiency of TCEP and TBP.[a]

	Internal calibration		External calibration	
	Plasma	PBS	Plasma	PBS
TCEP as reducing agent				
QC low-pool	6.7 ± 0.3[b]	6.7 ± 0.5[b]	6.7 ± 0.1[b]	8.3 ± 0.5[c]
QC medium-pool	13.3 ± 0.8[d]	13.5 ± 0.4[d]	13.2 ± 0.2[d]	15.3 ± 0.7[e]
QC high-pool	29.7 ± 1.4[f]	30.5 ± 0.4[f]	28.2 ± 0.6[f]	31.8 ± 2.0[g]
TBP as reducing agent				
QC low-pool	6.5 ± 0.4[b]	7.6 ± 0.7[c]	8.0 ± 0.2[c]	8.4 ± 0.9[c]
QC medium-pool	13.2 ± 0.5[d]	15.1 ± 1.0[e]	12.8 ± 0.3[d]	13.4 ± 1.3[d]
QC high-pool	28.9 ± 0.4[f]	32.5 ± 1.1[g]	24.8 ± 1.3[h]	25.7 ± 2.3[h]

[a] Mean ± SD plasma HcY of duplicate determinations over the course of 3 days in μmol/L.
[b–h] Values within a row with different superscript letters are significantly different from the other values in that row, $P < 0.05$. Values for each QC pool with the same superscript for both TCEP and TBP are not significantly different. For example, for internal calibration in plasma, the values obtained for the QC low-pool using TCEP were not significantly different from the values obtained using TBP.

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had no significant effect on the separation or retention times. Lowering the pH of the mobile phase to 5.0 and 4.5 improved the separation between cysteinylglycine and glutathione (Fig. 1B). No interfering peaks were observed in plasma or serum samples for any of the thiols measured. Plasma samples with no internal standard added showed no cysteamine peak.

Calibration curves for homocysteine were linear up to 200 μmol/L for samples prepared in PBS ($r^2 = 0.997$) or in plasma ($r^2 = 0.999$). The limit of detection for homocysteine was 0.16 μmol/L.

The mean recoveries (± SD) of L-homocystine added to plasma at five different concentrations (3.13–50 μmol/L free thiol), determined on 10 days, were 98.7% ± 2.5% and 96.7% ± 4.7%, calculated with internal and external calibration, respectively. For cysteine, recoveries were 100.6% ± 1.5% and 98.7% ± 3.5%, calculated with internal and external calibration, respectively.

Two plasma specimens containing high total homocysteine (tHcy) concentrations (28.5 and 360 μmol/L) were diluted with PBS 0- to 8-fold. The ratios of the observed/expected values were between 1.0 and 1.1.

The mean intraassay CVs for 20 plasma samples processed in five replicates on 1 day ranged from 1.1% to 1.8% for tHcy and cysteine (tCys). The mean interassay CV of the same 20 plasma samples processed in one replicate on 5 days was 5.6% and 2.4% for tHcy and tCys, respectively. Analyzed over 20 days, the three in-house plasma quality-control (QC) pools showed a variation for tHcy of 6.7% (low pool, 6.5 μmol/L), 5.0% (medium pool, 12.4 μmol/L), and 4.4% (high pool, 29.9 μmol/L) for internal calibration in plasma. The day-to-day variation was higher when internal calibration was performed in PBS or external calibration was performed in plasma. External calibration in PBS produced significantly increased tHcy values for the low and medium QC pools. The slope of the daily calibration curve demonstrated less variation with internal calibration (4.3% vs 8.5% with external calibration).

The correlation between tHcy concentrations for 38 plasma samples covering tHcy concentrations within and greater than the health-related reference range calculated with PBS calibration and with plasma calibration was very good: internal calibration ($r^2 = 1.0000$; slope = 0.9942; intercept = −0.0063), external calibration ($r^2 = 1.0000$; slope = 0.9838; intercept = −1.3309). However, with external calibration the intercept was significantly higher, which especially affected quantification of low homocysteine concentrations.

We performed a direct comparison of the reducing efficiency of TCEP, the newer reducing agent, and tributyl phosphine (TBP), the older reductant (Table 1). Although the relative fluorescence intensities were lower if TBP was used as the reducing agent (approximately two-thirds of the TCEP value), this difference was not apparent in the calculated concentrations of tHcy because of the calibration. For internal calibration in plasma, tHcy concentrations were indistinguishable between TCEP and TBP. Internal calibration in PBS or external calibration in plasma gave the same results if TCEP was used as the reducing agent. The use of TBP led to measured tHcy concentrations up to 20% different from the values obtained with TCEP. Finally, external calibration in PBS led to significantly increased values for TCEP compared with the other three calculation types. For TBP, we found signifi-cantly increased values for the low QC pool and significantly decreased values for the high QC pool. The slopes obtained with internal calibration were not significantly different for the two reducing agents: 0.034 (TCEP) and 0.035 (TBP) for calibration in plasma, and 0.032 (TCEP) and 0.031 (TBP) for calibration in PBS.

We studied tHcy and tCys plasma concentrations in 70 healthy subjects (27 men and 43 women; mean age, 43.8 ± 10.6 and 40.7 ± 9.0 years, respectively). The mean tHcy and tCys values were 9.1 ± 1.8 and 298 ± 29 μmol/L, respectively, for men, and 7.8 ± 1.7 and 280 ± 32 μmol/L, respectively, for women. For both thiols, men had significantly higher plasma concentrations than women ($P = 0.0112$ and $P = 0.0287$ for tHcy and tCys, respectively).

In conclusion, the protocol described is a robust, user-friendly, rapid assay, suitable for clinical and pediatric settings. The use of cystamine as the internal standard significantly improves the precision of this method and overcomes the matrix effect of plasma.

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Lectin ELISA for the c-*erb*-B2 Tumor Marker Protein p185 in Patients with Breast Cancer and Controls, *David B. Cook,[1*] Abdul A. Bustamam,[1] Ian Brotherick,[2] Brian K. Shenton,[2] and Colin H. Self[1]* (Departments of [1] Clinical Biochemistry and [2] Surgery, Medical School, University of Newcastle upon Tyne, NE2 4HH, United Kingdom; * author for correspondence: fax 44-191-222-6227)

The search for diagnostic and prognostic factors in breast cancer has included several oncogenes, particularly c-*erb*-B2, which encodes a 185-kDa transmembrane glycoprotein receptor, denoted p185, with tyrosine kinase activity

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