nase Ver. 2.0, United States Biochemicals) with the synthesized oligonucleotides as the sequencing primer. When eluted on HPLC, the patient’s hemolsate showed a single, symmetric band with an area of 13.1% of the total at an elution time corresponding to that of HbA1c (data not shown). Isoelectric focusing of the hemolsate detected an abnormal band between the HbA0 and HbF bands (Fig. 1A). Because of the width of this abnormal band, we speculated that it might be derived from β-globin. The molecular structure of the patient’s β-globin was analyzed as follows. The elution pattern of the digested peptides on reverse HPLC showed that the βA1-1 peptide peak at the usual position diminished and a new peptide peak appeared near peptides βT-10 and βT-14. The amino acid composition of this abnormal peptide [Thr 1.03 (1), Glu 2.15 (2), Val 0.13 (1), Met 0.80 (0), Leu 1.93 (1), Lys 1.00 (1), His 0.98 (1), and Pro 1.00 (1), where the number in parentheses shows the theoretical value of the peptide βT-1 indicates that in peptide βT-1, a valine residue at the β-1 position is replaced by a leucine residue and a methionine residue was added. Thus, this abnormal Hb appeared to be Hb Niigata [βN-methionyl-1(NA1)Val→Leu].

To confirm this assumption, a mixture of cloned ssDNA templates was prepared and sequenced. The nucleotide sequence of the segment encoding peptide βT-1 was heterozygous; a G and a T were present at the first position of codon 1 (GTG-TTG); this agrees with the replacement of valine by leucine. The polymorphism of the third nucleotide of codon 2 was T on both the normal and abnormal β genes. Sequencing of the cloned ssDNA templates confirmed that this abnormal β-chain was Hb Niigata (Fig. 1B).

HbA1c used as a clinical marker for glycemic control in diabetic patients (11) can be measured by several methods, including colorimetry, electrophoresis, column chromatography, and immunooagglutination. The HPLC method has been used as a standard method for HbA1c determination. The LA method using a specific antibody to efficiently detect the epitope at the β-chain N-terminal. Thus, this unique mutation on the N-terminal of β-globin seems to cause inappropriately high and low apparent HbA1c titers to be determined by HPLC and LA, respectively. This phenomenon will also occur in Hb Long Island, which has a mutation on the N-terminal of the β-chain.

In conclusion, we should consider the possibility of a mutation of the N-terminal of β-globin, as well as the possibility of rapid turnover of Hb, when an inappropriately low HbA1c titer is found with a LA method.

References


Development of a Rapid Microparticle-enhanced Turbidimetric Immunoassay for Plasma Fatty Acid-binding Protein, An Early Marker of Acute Myocardial Infarction, Markus Robergs, 1 Ferenc F. Van der Hulst, 1 Marc A.J.G. Fischer, 1 Werner Roos, 2 Carlos E. Salud, 2 Hans-Georg Eisenwiener, 7 and Jan F.C. Glatz. 1* (1) Department of Physiology, Cardiovascular Research Institute, Maastricht (CARIM), Maastricht University, The Netherlands; 2 Roche Diagnostics, a Division of F. Hoffmann-La Roche Ltd., Basel, Switzerland; * author for correspondence: fax 31-43-3671028, e-mail glatz@fys.unimaas.nl)
sensitivity of FABP for AMI detection are better than those of myoglobin (6, 7). FABP is also found in skeletal muscle, but the determination of the plasma ratio of myoglobin over FABP allows the discrimination between myocardial and skeletal muscle injury (4, 5).

The application of FABP as an early plasma marker in routine clinical practice requires the availability of a rapid assay system. Several immunochemical assays for FABP have been described, taking an assay time of 2–16 h (2, 3, 8, 9). Recently, a one-step ELISA for plasma FABP with a total performance time of 45 min (10) and an amperometric enzyme immunosensor assay taking 27 min per plasma sample were described (11). However, these assays are of limited use for routine clinical practice. We describe here a microparticle-enhanced turbidimetric immunoassay for FABP that offers the advantages of being precise, easy to perform, rapid, and fully automated.

Latex reagent was prepared by physical adsorption onto carboxylated latex particles of three monoclonal anti-human FABP antibodies that recognize distinct epitopes (12), which were then stored in 10 mmol/L Tris-HCl, pH 8.0, containing 5 g/L bovine serum albumin, 0.01 g/L Tween 20, 1 g/L NaN3, and 50 g/L sucrose. The assay was performed by using a COBAS® MIRA Plus analyzer (F. Hoffmann-La Roche Ltd.). Briefly, 75 µL of latex reagent was mixed with 155 µL of reaction buffer (22 mmol/L phosphate buffer, pH 7.0, 350 mmol/L NaCl, 2 g/L bovine serum albumin, 1 g/L NaN3, 20 mL/L normal rabbit serum (heat inactivated), and 2.57 g/L polyvinylpyrrolidone K90). After incubation at 37 °C for 75 s, 25 µL of sample was added, and the absorbance change of the reaction mixture was measured at a wavelength of 550 nm from about 1 min to ~8 min after the addition of sample. FABP concentration of sample was interpolated automatically from the calibration curve (calibration mode of COBAS® MIRA Plus: LOGIT/LOG 5).

Calibration curves were recorded with tissue-derived human heart-type FABP (13) or recombinant FABP (donated by Dr. T. Börchers, Münster, Germany), diluted with storage buffer to a 1.5 mg/L FABP solution, and stored for up to 2 months at 4 °C.

FABP concentrations in blood samples were also determined by using an earlier established ELISA of the antigen-capture type (10) and recombinant human heart-type FABP as standard. Taking a 25-fold sample dilution into account, the measuring range of this ELISA is between 0 and 300 µg/L FABP with a detection limit of 5 µg/L FABP (10). For the determination of the reference range of FABP in plasma, this assay was used in a more sensitive mode. By a fivefold predilution of the sample, prolonging the sample incubation time to 2 h and the enzyme reaction (coloring time) to 30 min, the analytical range is 30 times lower (0–10 µg/L FABP), and the detection limit (10) is 0.25 µg/L FABP. For method comparison, 163 EDTA plasma samples of patients with a confirmed clinical diagnosis of AMI (14) were analyzed. For the determination of the reference range of FABP sera from 102 blood donors (54 males) were used. Control sera or plasma samples were prepared by adding recombinant human heart-type FABP to a pool of blood from 10 healthy donors. All samples were stored at −80 °C.

A typical five point calibration curve, obtained by automated dilution of the standard solution, is shown in Fig. 1. The analytical detection limit, calculated as the FABP concentration corresponding to the absorbance difference + 2 SD found for the zero calibrator and assessed by analyzing the calibrators 11 times (which was repeated four times), was 1.1 ± 0.3 µg/L FABP. With postdilution, the test range could be extended from 150 µg/L up to 2400 µg/L FABP. With tissue-derived heart-type FABP and the recombinant protein, identical results were obtained (data not shown), confirming the same antigenicity found earlier of both proteins (10).

To analyze whether an antigen excess phenomenon (prozone effect) may affect the test, a dilution series of FABP in storage buffer was prepared and analyzed. As shown in the inset to Fig. 1, FABP concentrations between 150 and 2400 µg/L all yielded a signal that was above the limit defined by the measuring range (more than the test range).

Two pools of serum (controls) with different concentrations of FABP were used to assess intraassay precision by running 11 replicates of each sample pool in a single analytical run. An EDTA plasma sample with a high content of added recombinant human heart-type FABP was also measured. Interassay precision was assessed by measurement of the controls 24 times over a period of 2 weeks. The intraassay CV was 2–6%, and the between-day CV was 3–10% (Table 1).

Analysis of three plasma samples with different concentrations of FABP and diluted (up to 5 times) with 9 g/L NaCl indicated linearity of the assay (data not shown)
shown). Analytical recovery tests were performed by adding a low (7 μg/L) and a high (97 μg/L) concentration of recombinant human heart-type FABP to pools of unaffected plasma (heparinized, citrated, and EDTA plasma) and of serum. The mean recovery was 95%, the range was 90–102%. Additionally, plasma and serum samples with low and high FABP concentrations were mixed in ratios of 0%, 25%, 50%, 75%, and 100%. The mean “mixing recovery” was 101%, with a range of 98–106%, of the calculated concentrations of the mixed samples (data not shown).

For comparison of the latex assay with an established immunoassay (sandwich ELISA), 163 samples from patients with confirmed AMI were analyzed on the same day by both methods. By linear regression analysis, the tients with confirmed AMI were analyzed on the same immunoassay (sandwich ELISA), 163 samples from pa-

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>FABP, μg/L</th>
<th>CV, %</th>
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<tbody>
<tr>
<td>Intraassay</td>
<td></td>
<td></td>
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<tr>
<td>Serum</td>
<td>11</td>
<td>7.7 ± 0.4</td>
<td>5.3</td>
</tr>
<tr>
<td>Serum</td>
<td>11</td>
<td>41.6 ± 0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>EDTA plasma</td>
<td>11</td>
<td>93.4 ± 1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Interassay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>24</td>
<td>7.3 ± 0.7</td>
<td>9.3</td>
</tr>
<tr>
<td>Serum</td>
<td>24</td>
<td>41.4 ± 1.1</td>
<td>2.7</td>
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Intraassay precision was assessed in a single analytical run by analysis of 11 replicates of two control sera and of an EDTA plasma sample with a high concentration of added FABP. The FABP concentrations of the two controls were measured 24 times over a period of 2 weeks to calculate interassay variation. Data are means ± SD of the indicated number of observations.

The turbidimetric latex immunoassay described here represents a substantial improvement over most widely used heterogeneous immunoassays for quantifying human heart-type FABP in serum or plasma. Three distinct monoclonal antibodies directed against FABP are attached to the microparticles. These antibodies show no cross-reactivity with other human FABPs, such as intestinal-type and liver-type FABP (12). With a performance time of 10 min, the assay is much faster than other FABP assays published to date (2, 3, 8–11). The detection limit of 1.1 μg/L FABP is similar to that of reported sandwich ELISAs (9, 10), considering that in the latter, a predilution of the sample is indispensable to avoid matrix effects. The validity of the present latex assay for FABP was verified by experimental results in precision (interassay and intraassay), recovery, and dilution tests. No prozone phenomenon was observed for FABP concentrations up to 2400 μg/L, implying a wide analytical range. The comparison of our latex assay with an earlier published ELISA (10) by measuring FABP in 163 plasma samples of AMI patients showed a good correlation.

The average plasma FABP concentration measured in 102 sera of healthy blood donors (about 1.1 μg/L with the latex assay, 1.8 μg/L with the ELISA) is of similar magnitude as the mean values of 3.65 μg/L (n = 100) reported by Ohkaru et al. (9) and of 1.6 μg/L (n = 79) reported by Wodzig et al. (10). However, applying the ELISA and a fivefold predilution of the samples, we found a reference range between 0.5 and 4.6 μg/L FABP, whereas with the latex assay, only ~80% of samples were in this range, the remainder showing FABP concentrations up to 14 μg/L. This discrepancy could relate to interference in the latex agglutination test by sample components in this homogeneous assay system (no separation steps) or to underestimation by the ELISA from suppressed antibody-antigen interaction as a result of insufficient predilution, which was 5-fold instead of the usual 25-fold, of the samples. It should be noted, however, that for samples from AMI patients and FABP concentrations in the range 0–20 μg/L, we found a good correlation between the latex assay and ELISA. Finally, Ohkaru et al. (9), applying an ELISA method, also reported a FABP concentration >5 μg/L in 15% of cases of healthy volunteers. Unfortunately, the predilution factor used in this study and the reference range were not given (9).

For the detection of AMI, upper reference values of 10 and 12 μg/L FABP have been described (7, 14). With regard to this discriminator value, the latex assay will be a reliable tool for the early diagnosis of AMI, because in only 1 case of 102 sera tested was a slightly higher FABP value (14.1 μg/L) found. In addition, in plasma from AMI patients, FABP concentrations generally show a rapid increase after onset of symptoms (2–4), so that analyses of FABP in two samples taken at a short time interval and expression of the change in concentration may further increase the diagnostic power of this marker.

In conclusion, we found that the simplicity, reproducibility, and full automation in a widely used clinical chemistry analyzer like the COBAS MIRA seem to be factors of choice for the FABP latex immunoassay for routine clinical diagnosis of AMI.
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References

Stabilization of Homocysteine Concentration in Whole Blood, Reiner Probst,1 Richard Brandl,2 Matthias Blümke,1 and Dieter Neumeier1 (1 Klinikum rechts der Isar, Institute for Clinical Chemistry and Pathobiochemistry and 2 Klinikum rechts der Isar, Institute for Vascular Surgery, Ismaninger Str. 22, 81675 Munich, Germany; * author for correspondence: fax 49-89-41404875)

Homocysteine (Hcy) is a cytotoxic (1), sulfur-containing amino acid that increasingly appears to be an independent risk factor for coronary artery disease, stroke, and peripheral occlusive arterial disease (POAD) (2–4) when present at high plasma concentrations. In addition to several physiological factors that can increase Hcy concentration in blood such as vitamin B6, B12, or folate (5) deficiency and renal insufficiency (6), there is an artificial increase of Hcy concentration in whole blood after blood collection. This severe preanalytical problem is due to the well-known, time-dependent release of Hcy from erythrocytes (7) in the isolated blood sample, even when stabilization is attempted with different additives such as EDTA, NaF, or heparin/NaF (8–10). The result of this release is a falsely increased plasma Hcy concentration, unless plasma is separated from blood cells immediately after blood collection. We describe a method to overcome this problem for making the routine determination of Hcy possible for institutions where sample transport is critical.

For this purpose, we used a specially prepared blood collecting system, where whole blood is lysed and Hcy generating and converting enzymes are inhibited at the time of venipuncture, resulting in a stabilization of Hcy for 2 days at room temperature. Preparation of the blood collecting system for Hcy determination in lysate was performed by adding Nonidet P40 (25 ìL, pure), 50 ìL of EDTA disodium salt dihydrate (75 g/L), and 25 ìL of citric acid monohydrate (610 g/L) to a 2.7- mL EDTA-Monovette (EDTA = 1 g/L blood; Sarstedt), which was then used for blood collection. These concentrations of additives enabled sufficient stabilization and caused minimal dilution of the blood sample. After venipuncture, we recommend rigorously shaking the blood collecting system for 5 s to lyse blood cells. Complete lysis was checked microscopically in several samples.

EDTA blood was used for plasma Hcy determination. Centrifugation of blood at 3000g for 10 min and the separation of plasma from the cellular fraction were performed within 30–60 min of blood collection. Sample preparation of EDTA plasma and lysate was performed as described by Vester and Rasmussen (11). The HPLC method was also carried out according to their protocol after minor modification. HPLC measurements of plasma and lysate Hcy were performed by using a C18 reversed-phase 125–4 mm column (Merck LiChrosphere 100, 5-ìm particle) with a guard column [RP-18 (5-ìm) LiChrosphere 100, 4–4 mm], a gradient pump (Merck, L-6200), an autosampler (Merck, AS-2000), and a fluorescence detector (Merck, FL1080). The gradient started with 100% A (acetate buffer, pH 4.2, 20 mL MeOH), which was decreased to 92% over 3 min and then to 88% over the next 2 min at a flow rate of 0.9 mL/min. After an additional 3 min, the system reached its initial conditions; the whole run required 15 min. Eluent B was methanol.

A calibration curve was plotted by using three different concentrations (5, 15, and 40 ìmol/L) of homocysteine dissolved in 5 mmol/L EDTA (pH 7) and added to three lysate or plasma samples (standard addition). Stability measurements were performed by aliquoting blood immediately after venipuncture. These aliquots were stored for different time intervals (0, 1, 2, 4, 6, 24, and 48 h) at room temperature. Then the samples were frozen at −30 °C. Measurements were performed in triplicate. The