not change, although active PAI-1 changed to latent PAI-1 as a result of freezing and thawing.

Although it is thought that measuring active PAI-1 concentrations is the best way to evaluate the inhibitory ability of tPA, active PAI-1 quickly loses its activity with time as a result of conformational changes. We therefore believe that the activity assay does not reflect the real PAI-1 activity of plasma, especially in cases of retesting or multisample testing, which are influenced by the loss of activity with time. Furthermore, the plasma must be separated immediately after blood sampling, and active PAI-1 concentrations must be measured immediately after plasma separation, which is a cumbersome protocol for clinical laboratories. In our assay, total PAI-1 was stable at 10–25 °C for 5 days after separation of plasma, making it suitable for clinical laboratory use.

PAI-1 is also present in platelets and is released by stimulation. Our assay recognizes this platelet-derived PAI-1, which causes the total PAI-1 concentration to increase. We must therefore either use platelet-poor plasma or avoid activating the platelets in plasma samples.

In conclusion, we report the development of a fully automated LPIA for total PAI-1 that is simple, reproducible, and overcomes the drawbacks of other assays in terms of dynamic range, time, and specificity. Because this assay can detect all three forms of PAI-1 present in plasma with equal efficiency, it can estimate the exact amounts of PAI-1 that are released from endothelial cells by injury or stimulation and from adipose tissue as a result of hypertriglyceridemia or an insulin-dependent mechanism. This assay could therefore be a useful tool for investigating the pathophysiologic role of PAI-1.

References

Detection of Mutations in the Hepatitis B Virus Polymerase Gene
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Antiviral treatment of chronic hepatitis B infection aims to reduce viral replication and/or to affect the immune response to the virus and virus-infected cells. The development of reverse transcriptase inhibitors such as lamivudine, which has been shown to be a safe and potent inhibitor of hepatitis B virus (HBV) replication (1, 2), has facilitated major advances in the antiviral treatment of chronic hepatitis B. Today, lamivudine is a first-line
therapy for prophylaxis of HBV recurrence in decompensated cirrhotic patients and liver transplant recipients.

A major problem with lamivudine treatment is the emergence of drug resistance, which increases with extended duration of therapy (3). Resistant variants have been localized in the reverse transcriptase (rt) region of the HBV polymerase gene. Lamivudine-resistant amino acids have been described at positions rt180 (rtL180M) and rt204 (rtM204V/I/S) (4, 5). Methods for the identification of mutations in the HBV polymerase gene include conventional DNA sequencing, restriction fragment length polymorphism analysis, and reverse hybridization (6, 7). In the past, conventional direct DNA sequencing, which is the gold standard method, was the most labor-intensive and time-consuming method (6). Recently, however, a standardized and largely automated HBV polymerase gene-sequencing assay, the Trugene™ HBV Genotyping Kit, version 1.0 (Bayer/Visible Genetics, Toronto, Ontario), became commercially available. This assay may be suitable for routine diagnostic laboratory work and clinical trial applications. In this preliminary study, we evaluated the performance of the new HBV genotyping assay. Patients undergoing lamivudine treatment were retrospectively investigated for emergence of specific mutations.

Serum samples from five HBV DNA-positive (serum load >1000 HBV DNA copies/mL) patients undergoing lamivudine therapy for more than 6 months were analyzed retrospectively. Blood had been collected in 9.0-mL tubes (Vacuette™; Greiner Bio-one GmbH), and after centrifugation, sera had been aliquoted and stored at −70°C. Alanine aminotransferase (ALT) concentrations had been determined with the ALT assay for Roche/Hitachi analyzers (Roche Diagnostics), and aspartate aminotransferase (AST) concentrations had been determined with the AST assay (Roche). If the ALT concentration exceeded 23 U/L, it was considered abnormal, and the corresponding value for AST was 19 U/L. Serum HBV load had previously been measured with the Cobas Amplicor™ HBV Monitor Test (Roche Diagnostic Systems) according to the manufacturer’s instructions. This molecular assay has a detection limit of 2.0 × 10^2 HBV DNA copies/mL. Testing had routinely been done at 3-month intervals beginning at month 9 after the start of therapy. Between months 9 and 15 after the start of therapy, all patients had an increase in HBV DNA load of at least 1 log.

For testing mutations in the HBV polymerase gene, an aliquot was thawed, and HBV DNA was obtained according to the extraction protocol included in the Cobas Amplicor™ HBV Monitor Test protocol. Subsequent steps were done according to the manufacturer’s protocol for the Trugene™ HBV Genotyping Kit, version 1.0. Initially, a 1.2-kb sequence of the HBV polymerase gene, representing the central portion of the rt domain, was amplified by PCR, and sequencing reactions were then performed on this amplification product with the CLIP™ sequencing (Visible Genetics) technology. CLIP sequencing allows both directions of the amplification products to be sequenced simultaneously in the same tube with use of two different dye-labeled primers for each of the four sequencing reactions.

Electrophoresis and subsequent data analysis were performed automatically with the automated OpenGene™ and GeneObjects™ DNA sequence analysis system (Bayer/Visible Genetics). Data were acquired with the GeneLibrarian module of GeneObjects software by combination of the forward and reverse sequences. The query sequence was compared with the consensus sequences of HBV genotypes A to G in the Trugene HBV Module of the OpenGene software to determine the HBV genotype of the sample. Mutations in the rt gene as well as in the overlapping surface antigen (HBsAg) gene were also automatically detected and reported. According to the manufacturer, the detection limit of this system is ~2.0 × 10^9 HBV DNA copies/mL, and all viral variants present at concentrations ≥20% of the total can be detected.

Four patients were found infected with HBV genotype A and one patient with HBV genotype D. In four of the five patients, one or more characteristic mutations were detected in the rt region of the viral polymerase gene (Table 1). In two of the four patients, mutations had developed within the first year of lamivudine therapy; in the remaining two patients, mutations had developed within the second year of lamivudine therapy. Mutations were found at positions 173 (V173L), 180 (L180M), 204 (M204I and M204V), and 207 (V207I). In three patients, mutations appeared during lamivudine therapy together with a significant increase (minimum of 3 logs) in serum HBV load. In the fourth patient, the M204I mutation was found although viral load was rather low (month 9 after start of therapy; Fig. 1). In this patient, lamivudine therapy had been continued, and by month 12, the serum HBV load had increased by 1 log. Analysis at this time point revealed the appearance of the V207I mutation in addition to the existing M204I mutation. After discontinuation of therapy, serum HBV load increased by 3 logs within 3 months, and the mutant HBV strains had almost disappeared, whereas the wild-type virus had reappeared. Although the M204I mutation was no longer detectable, the V207I mutation was still detectable but showed an R (G or A with ~25% A) instead of the expected G at this position (Fig. 1).

The Trugene HBV Genotyping assay could be performed within 6 h. Amplification of the polymerase gene of HBV took 2 h, followed by a 2.5-h sequencing reaction including a 0.5-h manual pipetting. Finally, 1.5 h was needed for electrophoresis and analysis of data.

In this preliminary study, the Trugene HBV Genotyping assay was used in a routine diagnostic laboratory. The assay is mainly automated and can easily be performed by a trained medical technologist. In contrast to conventional direct DNA sequencing, this sequencing assay provides automated generation of the genotyping report. Manual sequence analysis is time-consuming and difficult, especially if detection of both the rt and HBsAg mutations is required in addition to the genotyping. Moreover, because of the sensitive CLIP technology, the
Trugene HBV Genotyping assay does not require a nested PCR step, which might be prone to contamination. The Trugene HBV Genotyping assay thus meets standardization requirements of the routine diagnostic laboratory.

Methods such as restriction fragment length polymorphism analysis and reverse hybridization have been proposed to identify mutations in the HBV genome (6, 7). Both methods seem to be sensitive but identify only known variants. In contrast, sequencing is the only method currently available that enables identification of new mutants that could be related to resistance (5). Because it is possible that more variants will arise during lamivudine therapy, sequence analysis should always be one of the diagnostic tools.

In this study, we found mutations at position rt204 in all patients with one or more characteristic mutations and a mutation at position rt180 in one of those patients. Both of these mutations have been associated with lamivudine resistance (4, 5). Mutations at positions rt207 (in two patients) and rt173 (in one patient) were also found. Both of these are secondary mutations and have previously been described as associated with famciclovir treatment (4, 8). Although lamivudine-resistant HBV strains have been shown to have impaired replication capacity compared with the wild type, their clinical emergence often leads to deterioration of liver function, which occasionally may be severe or even fatal. It is therefore of major importance to detect mutations as soon as possible. This could be guaranteed by frequent (every 3 months) determinations of serum HBV load and sequence analysis in the case of a significant increase. If one or more characteristic mutations are present, alternative therapies such as adefovir dipivoxil may be indicated (9).

The region of the HBV genome that is associated with the development of lamivudine resistance is also classically used to differentiate HBV genotypes. The Trugene HBV Genotyping assay automatically analyzes the sequence and compares it with genomic reference sequences; it is therefore able to provide HBV genotype and resistance information from the same data. The HBV genotype may correlate with different clinical features of HBV infection. Recent data suggest that Eastern Asian patients with HBV genotype C are more likely to have severe liver disease, whereas those with genotype B are more likely to develop hepatocellular carcinoma (10, 11). In India, HBV genotypes A and D were found to be predominant, and HBV genotype D is associated with more severe liver disease and may predict occurrence of hepatocellular carcinoma in younger patients (12).

In summary, patients undergoing lamivudine therapy who show a significant increase in serum HBV load should be tested for the emergence of drug resistance. The Trugene HBV Genotyping Kit is useful for the routine diagnostic laboratory and provides important molecular information to allow optimal therapeutic management of patients with chronic HBV infection.

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**Table 1. Patient data, biochemical values, and results obtained by molecular assays.**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, years</th>
<th>Sex</th>
<th>Months after start of lamivudine therapy</th>
<th>ALT, U/L</th>
<th>AST, U/L</th>
<th>Serum HBV DNA load, copies/mL</th>
<th>Mutation(s) detected in the rt region of the DNA polymerase gene</th>
<th>HBV genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>F</td>
<td>9</td>
<td>32</td>
<td>36</td>
<td>$5.2 \times 10^4$</td>
<td>None</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>M</td>
<td>9</td>
<td>18</td>
<td>15</td>
<td>$4.1 \times 10^4$</td>
<td>M204I</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>M</td>
<td>9</td>
<td>29</td>
<td>12</td>
<td>$1.8 \times 10^3$</td>
<td>None</td>
<td>D</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>F</td>
<td>9</td>
<td>18</td>
<td>12</td>
<td>$2.1 \times 10^4$</td>
<td>M204I</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>M</td>
<td>9</td>
<td>29</td>
<td>16</td>
<td>$4.7 \times 10^7$</td>
<td>None</td>
<td>A</td>
</tr>
</tbody>
</table>

*BDL, below the detection limit.*

*V207I mutation was detectable but showed an R (G or A with ~25% A) instead of the expected G at the position.*

Fig. 1. Appearance of resistance mutations in the rt region of the HBV polymerase gene in a patient (patient 4) with HBV genotype A infection.
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References


Standardization of C-Peptide Measurements in Urine by Method Comparison with Isotope-Dilution Mass Spectrometry, Colette Fiorens, Dietmar Stöckl, Dimitri Baetens, André P. De Leenheer, and Linda M. Thienpont

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In the past, standardization of measurements of diagnostically important polypeptides and proteins was hampered by the noncommutability of primary standards (1). One way to overcome this problem is to establish a method comparison with a reference measurement procedure. Until now, reference measurement procedures, such as isotope-dilution mass spectrometry (ID-MS), have been scarce. Recent developments in the MS field, however, have made the technique easily applicable to the analysis of polypeptides and proteins (kinetic studies, sequence analysis, and determination of molecular mass and posttranslational modifications). To the best of our knowledge, only two groups have used ID-MS for the quantitative determination of a specific polypeptide/protein. One of these groups described the measurement of apolipoprotein A-I after enzymatic digestion (2), the other described offline ID-liquid chromatography (LC)-MS assays for serum proinsulin, insulin, and C-peptide (3, 4). Neither group, however, examined the potential of ID-MS for standardization of the respective routine test systems (usually, immunoassays).

Here we report on the use of an ID-MS measurement procedure for standardization/recalibration of C-peptide measurements in urine by use of a method-comparison study with split-sample measurements. In view of the model character of the study for future applications, we chose urinary C-peptide over the clinically more important serum C-peptide because of the ease of sample collection and MS measurement. The measurement procedure applies online ID-LC-electrospray tandem MS (ID-MS) and is described in detail elsewhere (5).

For calibration, it makes use of a commercial C-peptide preparation with a peptide content of 89% and a purity by HPLC of >99% (according to the manufacturer’s information). This purity was taken into account for calculation of the C-peptide content in the calibrators. The C-peptide preparation was delivered in a vial containing 250 µg of freeze-dried material; a calibration solution was prepared by carefully weighing the added volume (~1 mL) of a solution containing, per liter, 10 g of protease-free bovine serum albumin (BSA). The exact dissolution volume of the vial contents could be derived from the gravimetric data and the density of the BSA solution. From this stock solution, we prepared a series of 1:5 gravimetrically diluted solutions in the same BSA solution to obtain working solutions of respectively 50 and 10 mg/L. Immediately after preparation, the working solutions were divided in 100-µL portions in plastic vials and frozen at ~20 °C until the day of analysis. Each day of analysis, an aliquot was thawed and gravimetrically diluted 1:10 with the 10 g/L BSA solution to obtain working solutions of ~1 mg/L. Once diluted, the aliquots were never reused.

The C-peptide immunoassays evaluated in the study (see the acknowledgements) were all performed in the application laboratories of the respective manufacturers/distributors for Belgium with the exception of one manual test, which was performed in the routine clinical laboratory of one of the authors. All of the manufacturers/laboratories strictly followed the prescribed assay protocols, with particular care for adequate internal quality control. The results are therefore considered representative for the respective assays.

Random urine specimens were collected during 4 weeks from 45 apparently healthy male and female volunteers between 15 and 65 years of age, according to the standards of the Committee for Medical Ethics of the Ghent University. The C-peptide concentrations in the collected samples were spread across a wide range. With respect to the collection conditions, we had to adhere to the assay instructions, which do not foresee special sam-