Development of a High-Pressure Liquid Chromatography Method for Diagnosis of Heparin-Induced Thrombocytopenia

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Abstract

Owing to the disadvantage of radioactivity of the carbon 14 serotonin release assay and the time-consuming procedure of the enzyme immunoassay, we developed a high-pressure liquid chromatography (HPLC) method to detect serotonin released from donor platelets in the presence of heparins and serum samples from patients with heparin-induced thrombocytopenia (HIT).

Samples were analyzed from 60 healthy control subjects, 19 patients with HIT, and 20 patients without HIT after incubation with heparin, low-molecular-weight heparin (LMWH), and danaparoid. Serotonin release was measured from platelets, 300 × 10^3/µL, by HPLC. Serotonin eluted as a single peak from the HPLC column. Serum samples from patients with HIT released 5.5 to 352.5 and 6.6 to 1,533.3 ng/mL of serotonin from platelets in the presence of 0.2 IU/mL of heparin and LMWH, respectively. In the presence of 0 IU/mL of heparin, LMWH, danaparoid, and control samples, less than 2.5 ng/mL of serotonin were released.

The HPLC method permits a rapid, sensitive, and quantitative determination of serotonin released from donor platelets for laboratory confirmation of HIT.

Heparin-induced thrombocytopenia (HIT) is a severe complication of heparin therapy that occurs in 2% to 5% of patients.1 HIT is based on an immunologic mechanism in which antibodies mostly of the IgG subclass are produced against a multimolecular complex of platelet factor 4 (PF4) and heparin.2-4 Symptoms appear after 5 to 10 days of heparin therapy.5 The patients are endangered by severe thromboembolic events.6,7 Because making a diagnosis of HIT based only on clinical symptoms may be difficult, a rapid and reliable laboratory confirmation of HIT is required.

Different functional and antigen assays are available to confirm the disease. Functional assays include the platelet-activation test,8 carbon 14 serotonin release assay (14C-SRA),9 heparin-induced platelet activation assay,10,11 flow cytometric assay,12,13 and enzyme immunoassay (EIA)-SRA.14 Antigen assays detect the PF4-heparin complex by enzyme-linked immunosorbent assay,15 heparin-induced IgG by fluorescent-labeled PF4,16 or by immunofiltration assay.17 The 14C-SRA from platelets is considered the most specific and sensitive test, but it has the disadvantage of using radioactivity.18 The nonradioactive EIA-SRA shows comparable sensitivity and specificity, but it has a time-consuming assay procedure.14 Therefore, we developed an assay system using high-pressure liquid chromatography (HPLC)19 to determine the release of serotonin from donor platelets in the presence of HIT serum samples and heparin, low-molecular-weight heparin (LMWH), and danaparoid.
Materials and Methods

Patients and Samples

The diagnosis of HIT was made according to the following criteria: 20-22 heparin administration; decrease of the platelet count to less than 100 × 10^3/µL (100 × 10^9/L) or to less than 50% of initial value and/or an objectively documented thromboembolic complication during heparin therapy; recovery from thrombocytopenia after cessation of heparin therapy; and exclusion of other causes of thrombocytopenia. A combination of sensitive and specific antigen and biologic assays improves the laboratory confirmation of HIT (heparin-induced platelet activation assay), 10 14 C-SRA, 9 immunofiltration assay, 17 EIA-SRA, 14 and PF4-heparin enzyme-linked immunosorbent assay. 15 Thus, we defined patients as “positive” if one of the biologic or antigen assays gave a positive result.

Samples from 3 groups of patients were analyzed by HPLC-SRA: healthy controls (n = 60), patients with HIT (n = 19), and patients without HIT (n = 20).

From all patients and healthy control subjects, 7.5 mL of blood was obtained in kaolin-containing plastic tubes and was centrifuged at 1,800g/min for 30 minutes at room temperature. Serum was divided into aliquots, shock-frozen in liquid nitrogen, and stored at –80°C. The study was approved by the University of Heidelberg Ethical Committee, Heidelberg, Germany.

HPLC–Serotonin Release Assay

Platelet concentrates were prepared from healthy persons by blood collection into acid-citrate-dextrose solution (vol/vol, 1:6). Platelet-rich plasma was obtained by centrifugation of platelet concentrates for 10 minutes at 1,800g/min and room temperature. The platelets were washed with HEPES-Tyrode buffer containing 0.5 mg r-hirudin (Aventis Pharma GmbH, Bad Soden, Germany), pH 7.4. The platelet count was adjusted to a final concentration of 300 × 10^3/µL.

Then 200 µL of serum containing the IgG fraction was incubated at 56°C for 30 minutes and centrifuged at 1,800g/min for 30 minutes at room temperature. Ten microliters of serum from the middle layer, 37.5 µL of the platelet preparation, and 2.5 µL of the heparin solution (0, 0.2, or 10 IU/mL of heparin [Hoffmann-La Roche AG, Grenzach-Whylen, Germany] and LMWH [Pharmacia, Erlangen, Germany] and 0, 0.02, or 1 IU/mL of danaparoid [Thiemann Arzneimittel GmbH, Waltrop, Germany]) were incubated for 60 minutes at room temperature while gently mixing as described previously. 9 The reaction was terminated by adding 50 µL of a 27-mmol/L concentration of EDTA (Merck AG, Darmstadt, Germany). Samples were centrifuged for 5 minutes at 1,800g/min and room temperature.

For determination and quantitation of the serotonin released from platelets, 80 µL of the supernatant was analyzed by HPLC as described by Gagnieu and colleagues. 23 We used an electrochemical detector (Waters 460, Millipore, Eschborn, Germany), a nucleosil-C18 column (Macherey-Nagel, Düren, Germany) with the corresponding precolumn, a chromato integrator (D-2000, Merck, Darmstadt, Germany), a sample injector (model 231, Gilson-Abimed, Langenfeld, Germany), and a buffer system containing 30 mL of methanol, 4.2 g of citric acid, 0.5 g of sodium-octanesulfone acid, 6.8 g of sodium acetate, 136 µg of dibutylamine, and 37.5 mg EDTA dissolved in 1,000 mL of distilled water and adjusted to pH 2.3. All reagents were of analytic grade and obtained from MedChrom, Heidelberg, Germany. The flow rate was set at 2.5 mL/min and the injection volume at 45 µL. To verify the identity of the serotonin peaks, the retention time of the peaks found was compared with that of the serotonin standard solutions (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). For quantitative determination of the released serotonin, a standard curve was calculated of the means of serotonin standard solutions of 0, 10, 25, 50, and 100 µg/L.

Statistical Analysis

Data are given as mean and SD, if not stated otherwise. The 2.5th and the 97.5th percentiles were determined. The specificity and the sensitivity were calculated in comparison with the EIA-SRA. Furthermore, we calculated the positive and negative predictive values and the confidence interval of the sensitivity. 24

Results

A total of 99 samples were analyzed blindly by the HPLC method. Serotonin released from donor platelets in the presence of HIT serum and 0.2 IU/mL of unfractionated heparin (UFH) or LMWH was eluted from the column as a single peak between 16.2 and 16.9 minutes after application. Figure 1. There were no differences in the results or magnitude of stimulation of platelets dependent on the donor.

The HPLC-SRA was positive in 18 of 19 HIT serum samples incubated with 0.2 IU/mL of UFH or LMWH. When using 10 IU/mL of UFH or LMWH, 1 of 19 samples was positive with each heparin (different samples). All HIT serum samples incubated with 0 IU/mL of UFH or LMWH were negative. After incubation with 0, 0.02, and 1 IU/mL of danaparoid, none of the serum samples was positive. Of 19 HIT serum samples, 15 were positive after incubation with LMWH and donor platelets.

Serum samples from patients with HIT released 5.56 to 352.55 ng/mL of serotonin in the presence of 0.2 IU/mL of
heparin and donor platelets. In the presence of 0.2 IU/mL of LMWH and HIT serum, the serotonin release from donor platelets ranged from 6.67 to 1,533.33 ng/mL. At a concentration of 0 IU/mL of heparin or LMWH, a maximum of 2.42 ng/mL of serotonin was measured. In the presence of HIT serum and 10 IU/mL of UFH or LMWH, the concentrations of serotonin were 0 to 20 ng/mL and 0.00 to 7.94 ng/mL, respectively Table 1 and Figure 2. No release of serotonin could be measured in the presence of danaparoid.

Of 19 patients with positive results in the EIA-SRA, 18 had positive results in the HPLC-SRA in the presence of 0.2 IU/mL of UFH or LMWH. Results of serotonin release from donor platelets as determined by the EIA-SRA and the HPLC method were negative for all 60 healthy control subjects and all 20 patients without HIT.

The sensitivity and specificity of the HPLC-SRA were compared with those for the EIA-SRA and were calculated from all samples. The results are given in Table 2. Compared with the EIA-SRA, the sensitivity and specificity of the HPLC-SRA were 95% and 100%.

The positive predictive value was 1 and the negative predictive value less than 1. The confidential interval of the value of sensitivity ranged from 77% to 99%, calculated with an alpha value of 5%.

Discussion

HIT is a severe complication of heparin treatment. The 14C-SRA from platelets is considered the most sensitive and

Table 1

<table>
<thead>
<tr>
<th>Amount of Heparin (IU/mL)</th>
<th>0.0</th>
<th>0.2</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIT serum samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus unfractionated heparin (n = 19)</td>
<td>≤2.42</td>
<td>5.56-352.55</td>
<td>0.00-20.00</td>
</tr>
<tr>
<td>Plus low-molecular-weight heparin (n = 19)</td>
<td>≤2.42</td>
<td>6.67-1,533.33</td>
<td>0.00-794</td>
</tr>
<tr>
<td>Non–HIT serum samples plus unfractionated heparin (n = 20)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>Normal control samples plus unfractionated heparin (n = 60)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

HIT, heparin-induced thrombocytopenia.

* In ng/mL. Heparin concentrations are related to 300 platelets × 10^3/µL.
specific test, but it has the disadvantage of using radioactivity. In the present article, we describe the detection of serotonin using an HPLC technique. Compared with the EIA-SRA, the results of the HPLC-SRA demonstrate a specificity of 100% and a sensitivity of 95% (Table 2). The method is based on the measurement of serotonin released from donor platelets in the presence of HIT serum and low heparin concentrations. The main differences from the \(^{14}\)C-SRA are that we do not use radioactive materials and the platelets are not preincubated with serotonin. The main difference from the EIA-SRA is the rapid assay procedure.

As Sheridan et al described, low concentrations of UFH induce the release of serotonin from platelets, while high concentrations show an inhibiting effect. The results of a recent study measuring the concentration of serotonin by EIA-SRA confirm these findings, as do those of the present study using HPLC-SRA.

We studied the release of serotonin with 2 different heparins (UFH and LMWH) and a heparinoid (danaparoid). Of 19 HIT serum samples, 15 were positive with LMWH, corresponding to a cross-reactivity rate of 79% and supporting the findings from the literature. After incubation with danaparoid, we found no positive results that can be explained by the low cross-reactivity rate of danaparoid and the low number of samples analyzed. Further studies will identify possible interactions with other glycosaminoglycans. Data from the literature would suggest interactions with other LMWHs and low, if any, interaction with glycosaminoglycans containing saccharide chains of heparins. The turnaround time of the HPLC method itself is only 20 minutes and shorter than the \(^{14}\)C-SRA and the EIA-SRA. The special equipment for HPLC has to be available in laboratories to conduct the assay.

### Conclusion

We present a specific and sensitive assay system to determine serotonin released from platelets in the presence of HIT serum and low concentrations of heparins; the assay system is distinguished by avoidance of radioactive materials and the rapid and easy assay procedure. Results are obtained within 1.5 hours depending on the availability of platelets. The possibility of quantification of serotonin release offers the advantage of standardizing and harmonizing the results. Further validation of this HPLC-SRA system is now to be undertaken.

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**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>HPLC-SRA</th>
<th>EIA-SRA</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
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</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>Sum</td>
<td>18</td>
<td>81</td>
</tr>
</tbody>
</table>

EIA, enzyme immunoassay; HPLC, high-pressure liquid chromatography; SRA, serotonin release assay.

1 Sensitivity, 95%; specificity, 100%.

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**References**


