Highly stable and robust protein S clotting assay validated for an easier laboratory practice

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Introduction

Protein S (PS) is an anticoagulant factor, and its deficiency leads to increased thrombotic risk. Although PS clotting assays are well described and many commercial kits available, pitfalls are met in current laboratory practice, such as limited reagent stability (usually 4h), high variability (sometimes lot to lot for a same reagent) and possible interference from other plasma factors. This new clotting assay for the PS anticoagulant cofactor activity aims to overcome the usual inconveniences observed for current PS assays.

Assay principle and protocol

The diluted tested plasma (1:10) is mixed with a PS deficient « substrate » plasma (R1). A clotting mixture containing optimized concentrations of Activated Protein C (APC), Factor IXa, and synthetic phospholipids (R2) is then added. Following a 3 min incubation step, clotting is initiated with 0.025M Ca++ (CaCl2), and clotting time (CT) is recorded. There is a direct relationship between PS concentration and the corresponding CT.

Water bath protocol:

Assay performed at 37°C

50µl diluted test plasma (1:10) → 1min at 37°C
50µl R1 (PS deficient plasma) → Preincubated at 37°C → 3min at 37°C → Record (CT, sec)
100µl CaCl2 0.025M → Preincubated at 37°C

Materials and Methods

Materials:

- PS deficient plasma: obtained by immunodepletion through anti-Protein S polyclonal antibodies, covalently linked to agarose beads.
- Factor IXa: Highly purified human Factor IX activated to Factor IXa at 37°C, through recycling onto activated contact phase covalently linked to agarose beads.
- Activated Protein C: Highly purified human PC activated by recycling through human thrombin covalently coupled onto agarose beads.
- Normal and pathological citrated plasmas
- Instruments (START4, KC10, STAR)

Method evaluation:

- Linearity of calibration curve over the dynamic range, and stability after reconstitution
- Specificity and interferences
- Precision
- Inter-lots homogeneity and comparison with other commercial assays (Staclot PS)
- Statistics performed with “Analyse-It” software.

Results

PS Calibration curve (START4 semi-automate)

Stability after reconstitution and conservation

(accelerated ageing, 3 weeks at +30°C)

Specificity and heparin effect


Inter-lots consistency and method comparison on patients plasmas (STAR):

N = 40
Range : 31 - 148%
R² = 0.97

Inter-lots consistency was verified on normal and pathological plasmas (deficiencies of PS or other factors, thrombosis risk check up, dicumarol treatment, cirrhosis, FV-L…), and HEMOCLOT Protein S results globally well correlated with another commercial assay (Staclot PS).

Normal and clinical values:

<table>
<thead>
<tr>
<th>PS clotting assay</th>
<th>ZYMUTEST Free PS (Elisa, active form)</th>
<th>HEMOCLOT Protein S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %PS (Range)</td>
<td>SD</td>
<td>Mean %PS (Range)</td>
</tr>
<tr>
<td>Normals Males (N=12)</td>
<td>88 (63-123)</td>
<td>22</td>
</tr>
<tr>
<td>Normals Females (N=6)</td>
<td>118 (91-143)</td>
<td>17</td>
</tr>
</tbody>
</table>

Specificity demonstrated by C4b-BP complexation (inhibition test)

<table>
<thead>
<tr>
<th>Concentration of C4bBP (mg/ml, 1V) added to plasma (9V)</th>
<th>Measured %PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Heparin -</td>
<td></td>
</tr>
<tr>
<td>0,05</td>
<td></td>
</tr>
<tr>
<td>0,1</td>
<td></td>
</tr>
<tr>
<td>0,2</td>
<td></td>
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<tr>
<td>0,5</td>
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Within run repeatability SD was in the range 2.8 - 5.5 (CV 4.2-8.5%) and Total precision SD in the range 3.2 to 11.1 (CV 5.9 to 9.2%), to be precised with additional series.

Conclusions

- Designed with only 2 reagents, this assay is simple, very stable, and fully automatable on the main coagulation analyzers.
- The assay is reliable and accurate, and highly performing at the clinical decision range, for a safe identification of Protein S deficiencies.
- This improved PS clotting assay is proposed as a new efficient and reliable tool for current PS testing, and could introduce easier and safer clinical laboratory practice.
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Aim: Protein S (PS) is an anticoagulant factor, and its deficiency leads to increased thrombotic risk. Although PS clotting assays are well described and many commercial kits available, pitfalls are met in current laboratory practice, such as limited reagent stability (usually 4h), high variability (sometimes lot to lot for a same reagent) and possible interference from other plasma factors. A new PS clotting assay was developed to overcome those limits and facilitate current use.

Method: The diluted tested plasma (1:20) is mixed with a PS deficient « substrate » plasma. A clotting mixture containing optimized concentrations of Activated Protein C (APC), FIXa with trace amounts of FXa, and phospholipids (R2) is then added. Following a 2 min incubation step, clotting is initiated with 0.025M Ca++, and clotting time (CT) recorded.

Results: A linear dose response is obtained in the range 10-200% PS (r²>0.995). PS deficiencies and high PS concentrations are directly and accurately measured. Designed with only 2 reagents, this assay is simple and fully automatable on the main coagulation analyzers. A high stability of at least 48h at 2-8°C, 24h at RT (and possibility to freeze) makes its use easy and consistent with current laboratory practice throughout the working day. Intra and inter assay variability evaluated on STAR were of 4-8% and 7-10% on the range 40 to 130 %. Inter lots consistency was verified (r²>0.98) on N=50 normal and pathological plasmas (deficiencies of PS or other factors, thrombosis risk check up, dicumarol treatment, cirrhosis, Factor V-L…) ranging from 0 to 150%, and results well correlated with another commercial assay (r>0.94). The assay is insensitive to heparin (UFH or LMWH up to 2 IU/ml). Heparinized plasmas at usual therapeutic ranges can be tested.

Conclusions: This improved PS clotting assay is proposed as a new efficient and reliable tool for current PS testing, and could introduce an easier and safer clinical laboratory practice.