Mechanized Amidolytic Technique for Determination of Factor X and Factor-X Antigen, and Its Application to Patients Being Treated with Oral Anticoagulants

Eduard M. van Wijk, Laurens H. Kahle, and Jan W. ten Cate

We describe a mechanized chromogenic assay for factor X, the results of which correlate well with those for the one-stage clotting assays for factor X in which it is activated either via the extrinsic pathway by thromboplastin or directly by Russell's viper venom.

We purified human factor X and raised monospecific antibodies to it in rabbits. We used our chromogenic assay for factor X to develop a factor-X-inhibitor neutralization assay for determination of factor-X antigen.

Patients receiving oral anticoagulant treatment had significantly different factor-X activities after activation via thromboplastin or with Russell's viper venom. The concentration of factor-X antigen, although decreased, significantly exceeded factor-X clotting activity or chromogenic activity in this group of patients. Results of the chromogenic assay for factor X correlated well with results of "Thrombotest," a clotting test introduced by Owren (Lancet II: 754, 1959) to control anticoagulant therapy. For patients taking oral anticoagulant drugs, the therapeutic range by our assay is 180 to 300 units/L.

Additional Keyphrases: clotting abnormalities • expected values in health and disease • chromogenic assay • factor Xa • "Thrombotest" • monitoring therapy with anticoagulants • inhibitor-neutralization assay • Russell's viper venom clotting assay • thromboplastin clotting assay

Chromogenic substrates have been introduced (1, 2) that have various specificities (3) for activated clotting factors. Amidolytic cleavage of the synthetic substrate results in release of p-nitroaniline. The increase in absorbance at 405 nm is proportional to the concentration of activated enzyme. The chromogenic substrate S 2222 is specific for factor Xa (2) and has therefore been used in an assay for factor X in which factor X can either be activated via the extrinsic pathway by thromboplastin (2) or directly by the factor-X-activating enzyme from the venom of Russell's viper (2, 4). Synthesis of factor X is vitamin K dependent (5) and is rapidly diminished when oral anticoagulant drugs are administered. It has recently been suggested that the chromogenic assay for factor X could be used to monitor anticoagulant therapy, because the assay is very suited to mechanization (4, 6). Bergström and Egberg found that factor-X chromogenic activity correlated well with results of "Thrombotest," a clotting test introduced by Owren (7) to control anticoagulant treatment (4). Lämmlé et al. proposed that the therapeutic range for factor X chromogenic activity be between 160 and 280 units/L (8).

Materials and Methods

Materials

Venous blood samples were collected in plastic tubes containing anticoagulant either one volume of trisodium citrate dihydrate (0.12 mol/L) per nine volumes of blood or 15 mg of solid dipotassium ethylenediaminetetraacetate (EDTA) per 10 mL of blood. Test samples were prepared by centrifuging at 2400 × g for 10 min at room temperature, then again at 13 000 × g for 5 min to obtain platelet-poor plasma.

Reference pool plasma was similarly prepared by pooling platelet-poor plasma samples from 40 ostensibly healthy subjects (equal numbers of both sexes). All plasma samples were stored frozen at −70 °C in small aliquots before study. EDTA-treated reference pool plasma was used as reference for EDTA samples, citrate-treated reference pool plasma for citrate samples.

Human factor-X deficient plasma was obtained from Warner Lambert Co., General Diagnostics, Morris Plains, NJ 07950.

Tissue thromboplastin was a commercial rabbit-brain thromboplastin suspension (DADE, Div. American Hospital Supply, Miami, FL 33152; lot no. ALT-317D).

The Russell's viper venom-activated one-stage factor-X clotting assay was performed with "Cofacteurs F. Stuart" from Bio Merieux, Charbonnières-les-Bains, France.

The factor-X-activating enzyme of Russell's viper venom was obtained from Sigma Chemicals, St. Louis, MO 63178 (lot no. 88C 3969).

The chromogenic substrate S 2222 [benzoyl-Ile-Glu-(γ-OR)-Gly-Arg-para-nitroaniline • HCl, where R = 50% H and 50% CH₃] was provided by KabiVitrum, Amsterdam, The Netherlands. One vial (25 mg of substrate) was dissolved in 6 mL of distilled water.

Polypepren, a heparin neutralizer, was from Aldrich Europe, Beersel, Belgium.
Hirudin, a thrombin inhibitor, was from Pentapharm Ltd., Basle, Switzerland.

Sephadex G-50, CNBr-activated Sepharose 4B, and dextran sulfate (sodium salt) were products of Pharmacia Fine Chemicals, Uppasa, Sweden.

Diethylaminoethyl-cellulose was from Merck, Darmstadt, F.R.G.

All other chemicals were of analytical grade (Merck).

Procedures

“Thrombotest” (Nyegaard & Co., Oslo, Norway) was performed according to Owren (7).

The factor-X clotting assay with Russell’s viper venom was performed according to Bachman et al. (14).

The factor-X clotting assay with thromboplastin was performed according to Biggs (15).

Automated chromogenic assay for factor X. Incubate 0.1 mL of test plasma, diluted eightfold with tri(hydroxy-

methyl)aminomethane (Tris)-buffered saline (50 mmol/L Tris and 150 mmol NaCl per liter, pH 7.4), for 6 min at 37 °C with 0.3 mL of reaction buffer. To prepare the reaction buffer, add 5 mL of the activating enzyme (500 units/L), 2.5 mL of CaCl₂ (400 mmol/L), 0.6 mL of hirudin (200 antithrombin kilounits/L), and 0.3 mL of Polybrene (15 g/L) to 51.6 mL of Tris-buffered saline (50 mmol Tris and 500 mmol NaCl per liter, pH 8.6). After this incubation, add 0.1 mL of substrate in distilled water (5.6 mmol/L) to the incubation mixture.

Continuously record the increase in absorbance at 405 nm, calculate the reaction rate after 30 s, and compare it with that of a reference pool plasma. We used an Automated Kinetic Enzyme and Substrate Analyser (Vitatron, Dieren, The Netherlands) for the entire procedure.

Factor-X protein concentration was determined according to Di Scipio et al., with ε280 = 11.6 (16).

For polyacrylamide gel electrophoresis we used a sodium dodecyl sulfate/phosphate system, according to the method of Weber and Osborn (17). We used a gel with 70 g of acryl-
amide and 1.84 g of N,N'-methylenebisacrylamide per liter.

Purification of human factor X. We purified human factor X at 4 °C by a combination of previously described procedures (16, 18). To 800 mL of cryosupernate adjusted to pH 8.6 with NaOH (4 mol/L) we added 64 mL of BaCl₂ (1 mol/L). After a 15-min centrifugation at 1300 × g we reprecipitated the pellet with BaCl₂, after having resuspended it in 800 mL of trisodium citrate (20 mmol/L) and NaCl (150 mmol/L), pH 7.4. This second pellet was suspended again in 300 mL of Na₂EDTA (200 mmol/L) and 300 mL of the trisodium citrate/NaCl buffer. Subsequently we added 400 mL of saturated ammonium sulfate and then stirred for 30 min. After centrifugation for 15 min at 1300 × g the pellet was discarded and another 500 mL of saturated ammonium sulfate was added to the supernate. The solution was stirred for 30 min, then centrifuged (10 min, 39 000 × g). The pellet was resus-
pended in a small amount of trisodium citrate (20 mmol/L, pH 6.0) and the residual ammonium sulfate was removed by gel filtration over Sephadex G-50 on a 1.6 × 40 cm column, with trisodium citrate in the same concentration as elution buffer.

All protein-containing fractions were pooled and subjected to diethylaminoethyl-cellulose column chromatography (column dimensions, 1.6 × 40 cm). The column was washed with trisodium citrate, pH 6.0, until no more protein was eluted, then bound proteins were eluted with a 0 to 0.5 mol/L linear gradient of NaCl. Factor-X activity was eluted at 0.25 mol/L NaCl. We pooled the fractions containing factor-X activity and removed the NaCl by Sephadex G-50 gel filtration. The pooled fractions containing factor-X activity were then chromatographed on dextran sulfate/agarose.

Dextran sulfate in NaHCO₃ buffer (0.1 mol/L, pH 8.3) containing NaCl (0.5 mol/L) was coupled to CNBr-activated Sepharose 4B at a ligand concentration of 4 g/L of packed gel. The suspension was gently mixed for 2 h at room temperature, followed by a second 2-h mixing in Tris (0.5 mol/L, pH 10.0) to block any remaining active groups. The suspension was washed five times each with acetate buffer (0.1 mol/L, pH 4.0) and Tris (0.5 mol/L, pH 10.0) alternately.

The column (1.6 × 25 cm) was first equilibrated with tri-
sodium citrate (15 mmol/L, pH 6.8). Then we applied the sample to the column and washed the column with the same buffer, and eluted with a linear gradient of NaCl 0 to 1.0 mol/L; factor X was eluted at 0.3 mol/L NaCl.

This procedure resulted in a 9300-fold purification of factor X. The characteristics of the purified protein were: a specific chromogenic activity of 130 kilounits/g and a specific pro-
coagulant activity (thromboplastin activation) of 120 kilounits/g. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis revealed a single band when stained for protein with an apparent molecular mass of 66 000 daltons (data not shown). In the reduced form, two protein bands (masses ~49 000 and ~17 000 daltons, respectively) were found (data not shown). These data agree well with those of Di Scipio et al. (16).

Production of antisera. We used this final factor-X prepa-
raration to raise antibodies in New Zealand white rabbits. The monospecific antiserum we obtained verified the complete identity between purified factor X and factor X in plasma (data not shown). Antiserum was absorbed with barium sulfate (100 g/L) and inactivated by heating (56 °C for 30 min). Equal volumes of normal human plasma and 20-fold diluted antiserum exhibited 5% factor-X residual activity after in-
cubation for 1 h at 37 °C. Under the same circumstances there was no substantial loss in activity for prothrombin, factor VII, or factor IX.

Factor-X inhibitor-neutralization assay. This assay was performed essentially according to Brièt et al. (9), except that the mechanized factor-X assay was used to determine residual factor-X activity. Incubate 0.05 mL of test plasma and 0.05 mL of factor X antiserum, diluted in Tris-buffered saline (50 mmol of Tris and 150 mmol of NaCl per liter, pH 7.4) for 30 min at room temperature. Then add 50 μL of reference pool plasma, incubate for another 30 min, then add 0.25 mL of the Tris-buffered saline. We assayed residual factor-X activity of the test sample and of a reference curve with the Automated Kinetic Enzyme and Substrate Analyser, as described above.

Statistical Analysis

The significance of differences between two values was tested by Student’s t-test or, when appropriate, by a paired t-test. To compare the therapeutic range for factor X assayed by the chromogenic method with that for “Thrombotest” we used a McNemar’s test, as described by Swinscow (19).

Results

Optimum Conditions for the Automated Chromogenic Assay

The Kₘ value for the chromogenic substrate S 2222 (Figure 1) was determined from the plot: [S] / vₒ = Kₘ / vₘₐₓ + [S] / vₘₐₓ (20). The amount of S 2222 (substrate) present in a vial was determined by measuring the concentration of p-nitroaniline in solution after alkaline hydrolysis of the substrate, given a molar absorptivity (molar absorption coefficient) for p-nitroaniline of 10.39 L/(mmol-cm) at 405 nm. A substrate concentra-
tion of 1.12 mmol/L (about threefold the Kₘ) was used in the final assay procedure.
The apparent $K_m$ for calcium, calculated in the same way, was 0.91 mmol/L. We used 12.5 mmol of calcium per liter in the assay. EDTA-treated plasma could be assayed at this calcium concentration.

The activation of factor X by the factor-X-activating enzyme from Russell’s viper venom reached a maximum after about 3 min, then remained constant up to 8 min; afterwards activity decreased. We chose an incubation time of 6 min.

Hirudin, a specific thrombin inhibitor, was added to the reaction buffer to prevent fibrin formation. In the absence of hirudin a small amount of fibrin formed, which did not disturb the assay directly. However, in mechanized procedures the fibrin gradually accumulates in the measuring cell, the tubings, and especially around the stirrer. The concentration of hirudin added to the reaction buffer did not influence the reaction between factor Xa and the substrate.

**Analytical Variables**

**Linearity of the mechanized assay and the inhibitor-neutralization assay.** The linearity of the chromogenic assay and the inhibitor-neutralization assay were investigated by testing dilutions of a reference pool plasma. Factor-X activity was linearly related to concentration between 100 and 1500 units/L for the chromogenic assay, whereas for the inhibitor-neutralization assay the relation was linear between 60 and 1000 units/L. Samples exceeding 1000 units/L had to be diluted twofold for assay by inhibitor neutralization.

**Within- and between-assay precision.** The within-assay precision of the various assays is shown in Table 1.

The between-assay precision of the chromogenic assay and of the inhibitor-neutralization assay was calculated by use of a plasma sample that had been stored in small portions, at −70 °C. On 10 subsequent days, one portion was thawed and assayed. The CV for the chromogenic assay was 1.8% (mean 984 units/L, SD 18 units/L); for the inhibitor-neutralization assay it was 9.8% (mean 1036 units/L, SD 102 units/L).

**Analytical recovery.** Purified factor X was added to factor-X-deficient plasma in several concentrations, after which we determined factor-X activity with the chromogenic assay (Table 2). Recovery of factor X was calculated to be complete, indicating that no plasma components were interfering with the assay under these conditions.

**Interference.** Bilirubin, as much as 500 μmol/L, did not affect the chromogenic assay.

Hemoglobin also did not interfere with the chromogenic assay in concentrations up to 300 μmol/L. Greater concentrations, although not directly interfering with the reaction, made it difficult to read the increase in absorbance, because the initial absorbance was too great. Plasma samples of hyperlipemic patients could be assayed, except that those for which the initial absorbance exceeded 1.0 could not, for the reason just mentioned.

Antithrombin III, added to a plasma sample in concentrations up to 4000 units/L (reference interval 800 to 1400 units/L) (6), did not influence results of the chromogenic assay.

Heparin, as much as 10 kilounits/L, did not influence the reaction, because the amount of Polybrene in the incubation mixture sufficed to neutralize this amount of heparin completely. Without Polybrene, heparin interferes with the assay, resulting in a strong decrease in ΔA/min.

**Clinical Results**

**Determination of factor-X activity and antigen in normal persons.** We determined factor-X activity in 149 healthy volunteers (100 men and 49 women) by the automated chromogenic assay (A), and by the one-stage clotting assays involving thromboplastin (B) or Russell’s viper venom (C). Antigen content was determined in 85 volunteers (48 men and 36 women) by the inhibitor-neutralization assay (D). The distribution of the values appeared to be log normal. The mean values (Table 3) are the means of the log-transformed data.

We found no significant sex-related differences (Student’s t-test: A, B, C, p < 0.5, D, p > 0.3). The values for the chromogenic assay and the antigen content did not differ significantly from both clotting assays (paired t-test: in all cases p < 0.5).

**Assays in patients with various illnesses.** Factor-X clotting activity with thromboplastin and factor-X chromogenic activity were determined in 60 patients selected on the basis of having a prolonged prothrombin time. Of these patients, 17 had cirrhosis of the liver, six had diffuse intravascular coagulation, and three had congenital deficiency of factor X. Those patients...
Table 3. Normal Values (Log Normal Distribution Assumed)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mean factor X, units/L</th>
<th>Antilog of (log X) ± 2 SD, units/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor-X thromboplastin clotting activity</td>
<td>1013</td>
<td>998</td>
</tr>
<tr>
<td>Factor-X RVV clotting activity</td>
<td>999</td>
<td>1012</td>
</tr>
<tr>
<td>Factor-X chromogenic activity</td>
<td>1003</td>
<td>991</td>
</tr>
<tr>
<td>Factor-X antigen</td>
<td>1004</td>
<td>949</td>
</tr>
</tbody>
</table>

* 100 men, 49 women.
* 49 men, 38 women.

with a vitamin K deficiency or on oral anticoagulant therapy were not included, but were investigated separately.

Mean values were 707 units/L for the chromogenic assay (y) and 692 units/L for the thromboplastin clotting assay (x). Both assays correlated well, $y = 0.87 \pm 0.04 x + 107$ (SD 36), $r = 0.92$, and did not differ significantly ($p > 0.5$).

Figure 2 shows the correlation between these two assays, for normal subjects and these patients.

**Assays in patients on oral anticoagulant therapy.** Without conscious bias, we chose from the Amsterdam Thrombosis Service 158 patients receiving anticoagulant treatment with coumarin derivatives, and compared results for them in the one-stage clotting assay with thromboplastin and by the automated chromogenic assay. For samples from 50 of these patients the Russell's viper venom clotting assay was performed too, the amount of antigen was determined for 41 of these 158 patients. Table 4 summarizes the results; the correlation coefficients are given in Table 5.

Correlation was good between the automated chromogenic assay and the thromboplastin clotting assay (Figure 3). However, there was a significant difference between the values obtained for both assays in this group of patients (paired t test, $p < 0.001$). The factor-X clotting activity and the factor-X chromogenic activity were consistently lower than the factor-X antigen concentration (paired t test in all cases, $p < 0.001$). The values obtained in the Russell's viper venom clotting assay did not differ significantly from those obtained in the chromogenic assay (paired t-test, $p > 0.5$).

**Comparison of the chromogenic assay and "Thrombotest" in patients on oral anticoagulant therapy.** The values we obtained with the chromogenic assay correlated well with the reciprocal of "Thrombotest" results (Table 5 and Figure 4) (21) in 158 patients on oral anticoagulant therapy. We selected all samples exceeding 63 s "Thrombotest," to exclude patients who had just started therapy or were withdrawn from therapy. From data on 148 patients remaining we developed a therapeutic range for factor-X activity as assayed by the chromogenic method by comparing the data with the therapeutic range proposed for "Thrombotest" (the "Thrombotest" range routinely used in our laboratory is between 190 and 95 s, e.g., between 5.0 and 12.5%). When we used 180 to 300 units of chromogenic factor-X activity per liter as a reasonable therapeutic range, values for 99 patients were also within the

![Fig. 2. Correlation between the factor-X thromboplastin clotting assay and the automated chromogenic assay for factor X in normal persons and in patients with various illnesses](image)

![Fig. 3. Correlation between the factor-X clotting assay involving thromboplastin and the automated chromogenic assay for factor X in coumarin-treated patients](image)
therapeutic range for "Thrombotest" (Figure 5), whereas 27 patients had values outside the ranges of both assays.

A McNemar test evaluating these ranges revealed no significant difference (p >0.3); thus both assays gave about the same diagnostic results (Figure 6).

Effect of Decarboxy Factors

Bas et al. (22) described an inhibitory effect of decarboxy factors on the factor-X clotting activity. Therefore, dilution curves, for samples estimated to contain a constant amount—1.0 and 2.0 parts—of a coumarin-containing plasma (thromboplastin clotting activity, 150 units/L, chromogenic activity, 250 units/L), showed a parallel shift that was proportional to the amount of coumarin-containing plasma added, in the thromboplastin clotting assay as well as in the chromogenic assay (Figure 6). The slope of the curves did not change significantly for either assay upon addition of coumarin-containing plasma (p >0.5 for both assays). This indicates that decarboxy factors interfere with the chromogenic assay system no more than with the thromboplastin clotting assay.

Discussion

The chromogenic assay for factor X is based on the direct activation of factor X by the factor-X-activating enzyme of Russell's viper venom. Activated factor X cleaves p-nitroaniline from the chromogenic substrate S 2222. The increase in absorbance at 405 nm is linearly related to the concentration of factor Xa. The automated method we developed is accurate, having within- and between-assay CVs of less than 2%.

Clotting time, currently used for the factor X assay, is a reciprocal function of the concentration of factor X, such that in the normal and near-normal range a small difference in clotting time gives a great difference in factor X concentration. This lack of sensitivity in the clotting method is compounded by the subjectivity of detecting a clotting point. Moreover in the clotting assay, a whole series of activations is triggered, and unknown interference may occur at any point on the activation pathway. On the other hand, the chromogenic assay for factor X involves a less-complex reaction: a clotting enzyme is activated and the activated enzyme is measured.

Factor X is one of the vitamin K dependent clotting factors, containing 12 γ-carboxyglutamate residues (23). Treatment with vitamin K antagonists competitively inhibits the carboxylation of the precursor factor X (decarboxy factor X) (10) to the zymogen. As described for prothrombin, treatment with vitamin K antagonists results in the appearance of partly carboxylated molecules in the circulation (11-13), which exert activity that depends on the activator used and on the number of γ-carboxyglutamate residues present. In oral anticoagulant therapy, partly carboxylated factor-X molecules may also circulate and may account for the significant differences between the assays with thromboplastin and those with Russell's viper venom. The antigenic activity (including the partly carboxylated and the uncarboxylated factor-X molecules) in this group of patients significantly exceeds the clotting or chromogenic activity, however, beyond the reference interval. Fair et al. (24), using a radioimmunoassay for the determi-
nation of factor-X antigen, found the same result. The pro-
cougulant activity/antigenic activity ratio possibly forms a
measure for the effect of the anticoagulant therapy.

The correlation that we find between the reciprocal of
“Thrombotest” and factor-X chromogenic activity ($r = 0.85$)
is comparable with that reported by Bergström and Egberg
(4). The therapeutic range calculated for factor-X chromo-
genic activity, based on the therapeutic range for “Throm-
botest” values, also compares well with the range proposed by
Lämmlle et al. (8), which is based on the Quick test.

Thus we believe that the chromogenic assay for factor X
may be used to monitor therapy with oral anticoagulants.
Besides the advantages already mentioned for the chromo-
genic assay, results for a test sample are provided immediately
after assaying and may be transmitted to a computer on-line
with the automated analyzer. This implies that a test result
may be directly translated into a dosage by a programmed
dosage scheme. Quality-assurance testing of the chromogenic
assay may be performed with lyophilized factor-X standards,
whereas with “Thrombotest” a whole-blood sample is re-
quired, accompanied by the problems related to the instability
of whole-blood samples.

We thank Misses Rita van Wesep, Marianne Schaan, and Sonja
Brendel for their technical assistance, Mrs. A. Jeltech and Mr. J. L.
Smit of the Amsterdam Thrombosis Service for their cooperation
in providing samples from coumarin-treated patients, and Miss Else
de Haan for typing the manuscript. Further we acknowledge Prof. H.
J. van der Helm for his critical reading of the manuscript, Mr. N.
Nagelkerke for evaluating the statistical data, and Mr. J. Bakker, head
of the central department of photography of the Wilhelmina Gasthuis,
for producing the figures.

References
1. Svendsen, L., Blomnåck, B., Blomnåck, H., and Olsen, P., Syn-
thetic chromogenic substrates for the determination of trypsin,
(1972).
sensitive and highly specific chromogenic substrate for factor Xa.
3. Latallo, Z.S., Uchman, B., and Teisseyre, E., Specificity of chro-
4. Bergström, K., and Egberg, N., Determination of vitamin K sen-
sitive coagulation factors in plasma. Studies on three methods using
synthetic chromogenic substrates. Thromb. Res. 12, 531–547
(1978).
5. Jackson, C. M., and Suttie, J. W., Recent developments in under-
standing the mechanism of vitamin K and vitamin-K antagonist drug
action and the consequences of vitamin K action in blood coagulation.
6. Kahlk, L. H., Schipper, H. G., Jenkins, C. S. P., and ten Cate, J. W.,
Antithrombin III. I. Evaluation of an automated antithrombin III
7. Owen, P. A., Thrombotest: A new method for controlling antico-
8. Lämmlle, B., Eichleiberger, R., Hänni, L., et al., Kontrolle der oralen,
Anticoagulation: Vergleich zwischen Quick- und Kolorimetrischer
Factor-X-Bestimmung bei 107 Patienten. Schweiz. Med. Wo-
Molecular variant of factor VII. Thromb. Haem. 35, 289–294
(1980).
10. Lindhout, M. J., Kop-Klassen, B. H. M., Reekers, P. P. M., and
Hemker, H. C., Demonstration of three proteins induced by vitamin
acid content of human and bovine prothrombin following warfarin
12. Friedman, P. A., Rosenberg, R. D., Hauchka, P. V., and Fitz-
James, A., A spectrum of partially carboxylated prothrombins in
the plasma of coumarin treated patients. Biochim. Biophys. Acta 494,
13. Malhotra, O. P., Purification and characterization of dicou-
marol-induced prothrombin, III Alumina pH 4.6 atypical (2-G1a)
15. Biggs, R., Human Blood Coagulation, Haemostasis and
E. W., A comparison of human prothrombin, factor IX (Christmas
Factor), factor X (Stuart-Prower factor) and Protein S. Biochemistry
determinations by dodecyl sulphate-polyacrylamide gel electrofo-
18. Pepper, D. S., and Prowse, C., Chromatography of human pro-
thrombin complex on dextran sulphate agarose. Thromb. Res. 11,
21. Hemker, H. C., Vermeer, C., and Govers-Rijnsrag, J., Kinetic
aspects of the interaction of blood clotting enzymes. VII. The relation
between clotting time and prothrombin concentration. Thromb.
Haem. 37, 81–85 (1977).
22. Bas, B. M., Muller, A. D., and van der Voort-Beelen, J. M., The
relation between staphylococcal reaction factor and proteins in-
23. Thøgersen, H. C., Petersen, T. E., Sottrup-Jensen, L., et al., The
$N$-terminal sequence of blood coagulation factor $X_2$ and $X_2$ light
chains. Mass-spectrometric identification of twelve residues of
$\gamma$-carboxyglutamic acid in their vitamin K-dependent domains.
and immunochromatogram of normal and abnormal human factor