A Clinical Evaluation of Automated Chromogenic Tests as Substitutes for Conventional Prothrombin Time and Activated Partial Thromboplastin Time Tests
A. Duncan,1 E. J. W. Bowle, C. A. Owen, Jr., and D. N. Fass

Automated procedures involving a chromogenic substrate sensitive to thrombin-sarcosine-Pro-Arg p-nitroanilide were compared with conventional tests for prothrombin times and activated partial thromboplastin times (APTT) and with specific assays for factors V, VII, VIII, IX, X, XI, and XII. The reproducibility and sensitivity of the chromogenic tests were compared with those of the clotting tests. Further, we have confirmed that the chromogenic test for APTT is sensitive to factor VII deficiency, unlike the clotting test for APTT. This might be an advantage in monitoring orally anticoagulated patients. The ready availability of the automated equipment or performing the chromogenic tests suggests their potential or routine use. However, some discrepant results in certain patients with liver disease and in others with factor VIII inhibitors warrant caution.

Additional Keyphrases: coagulation assays • thrombin • variation, source of • synthetic substrates • reference interval

The use of chromogenic substrates for assaying thrombin, plasma coagulation, factor Xa, and antithrombin III is well established (1) and clinical applications in patients receiving oral anticoagulants have been described (2, 3). We report the potential usefulness of a new substrate, sarcosine-Pro-Arg-pNA, for measuring prothrombin times (PT) and activated partial thromboplastin times (APTT) as well as for determining deficiencies of specific coagulation factors.2 As in other amidolytic assays, thrombin cleaves the p-nitroanilide (pNA) moiety from its arginine (Arg) bond, and the free pNA is quantified spectrophotometrically at 405 nm.

Materials and Methods

Conventional clotting tests were performed as previously described (4). Citrated plasma was prepared from nine volumes of freshly drawn blood mixed with one volume of sodium citrate (3.8 g/L) in polypropylene tubes. After centrifuging the blood (10 min, 1000 × g), we usually used this fresh plasma for the assays; otherwise, the citrated plasma was frozen at −70°C in polypropylene plastic vials. Samples were thawed at 37°C before assay.

Reagents

"Quantichrome" reagents were supplied by Abbott Laboratories, North Chicago, IL; exact reagent concentrations were not specified.

Quantichrome-PT reagent is a lyophilized mixture of sarcosine-Pro-Arg-pNA, rabbit brain thromboplastin, and calcium, buffered to pH 7.4 with Tris · HCl-saline. It is reconstituted with 20 mL of water just before use.

Quantichrome-PTT reagent consists of three components: ellagic acid, the activating reagent; rabbit brain phospholipid (5) and calcium in Tris · HCl-buffered saline, pH 7.4, furnished as a lyophilized powder; and the lyophilized chromogenic substrate, sarcosine-Pro-Arg-pNA, plus soybean trypsin inhibitor in Tris · HCl-buffered saline, pH 7.4. All three components are reconstituted with water 15 min before use and are stable for at least 4 h at 4°C. Before use, 2 mL of reconstituted ellagic acid reagent is vortex-mixed with 18 mL of reconstituted phospholipid-calcium reagent.

Normal plasma was citrated plasma collected from 30 apparently healthy men, pooled, and frozen at −70°C in 1-mL aliquots in plastic vials.

Coagulation-factor-deficient plasmas were commercially obtained (George King Biomedical, Overland Park, KS) and were from persons with severe congenital deficiencies of factors VII, VIII, IX, X, XI, or XII. Factor V-deficient plasma was prepared from normal plasma from which factor V had been removed by affinity chromatography with a monoclonal antibody coupled to Sepharose (6).

Automated analyzer. We used an ABA 100 automated spectrophotometer (Abbott Laboratories) with an auxiliary reagent dispenser and a temperature-controlled cuvette kept at 37°C. Absorbance was measured at 405 nm.

Procedures

To assay specific coagulation factors, we diluted normal plasma (100% coagulation factor activity) at 0°C with a factor-deficient plasma (<1% activity) to yield final activity concentrations of 80%, 40%, 20%, 10%, and 5% of normal for the particular factor. After adding 25 µL of each diluted sample to 100 µL of the appropriate factor-deficient substrate (another fivefold dilution), we placed 50 µL of each mixture, normal plasma, or deficient plasma in separate sample cuvettes. From each, 5 µL was automatically aspirated and mixed with 500 µL of the PT reagent for measuring factors V, VII, and X, or with 500 µL of the APTT reagent for measuring factors VIII, IX, XI, and XII. For PT, the difference in absorbance immediately after mixing and after 5 min of incubation at 37°C was taken as the endpoint. APTT samples were incubated with APTT reagent for 5 min, then 200 µL of chromogenic substrate was added; ΔA was the difference in absorbance between the start and the endpoint 5 min later. Unknown patients' plasmas were diluted fivefold in the appropriate factor-deficient plasmas and processed like the control samples. Percentage concentrations of the coagulation factor were determined by interpolation of the observed absorbance. All samples were measured in duplicate.

Figure 1 shows the typical standard curve for assay of factors V, VII, and X by either a PT or APTT technique, and for factors VIII, IX, and XII by the APTT technique.

1 Section of Hematology Research, Mayo Clinic and Foundation, Rochester, MN 55905.
2 Nonstandard abbreviations: pNA, p-nitroanilide; PT, prothrombin time; APTT, activated partial thromboplastin time.

Received June 11, 1984; accepted March 21, 1985.

CLINICAL CHEMISTRY, Vol. 31, No. 6, 1985 853
Results

To determine the reference interval for normal values, we studied plasmas from 30 apparently healthy men. Our usual clotting prothrombin time (4) ranged from 17 to 19 s. The chromogenic “prothrombin time” had an absorbance range of 0.72 to 1.04 ΔA. The correlation coefficient between the two tests was 0.68.

The normal range for the clotting APTT was 32 to 38 s (4) and for the chromogenic assay was 0.75 to 1.2 ΔA. The correlation coefficient between the two was 0.74.

Variability (CV) of results for 12 plasma samples measured for chromogenic prothrombin time was 2% intra-assay and 7% interassay. Corresponding values for the chromogenic APTT for 17 samples were 3.7% and 11%, respectively.

The sensitivity of the two chromogenic assays was defined as the greatest concentration of a clotting factor that yielded absorbance at least 0.10 A below the lower limits of normal (0.72 for PT and 0.75 for APTT). This turned out to be 35% of normal for PT assays for factors V, VII, and X. By the APTT method, maximal sensitivity was 30% of normal for factors VII, X, XI, and XII and 40% for factors V, VIII, and IX. These are approximately the same sensitivities observed in clotting assays (7).

Finally, more than 200 plasma samples randomly collected from various patients (Table 1) being studied in the Special Coagulation Laboratory were assayed by both the clotting and the chromogenic tests (both APTT and PT tests). Of these, 145 samples exhibited significant abnormalities—deficiencies of factors VIII (30), IX (16), X (11), XI (seven), VII (five), XII (three), and V (three)—or were from patients with assorted coagulation factor inhibitors (15), liver disease (13), disseminated intravascular coagulation (11), oral anticoagulant treatment (18), heparin therapy (seven), or miscellaneous (six). Except for two patients with liver disease and three with inhibitors to factor VIII, the results of all the paired clotting tests and chromogenic tests agreed within the CV limits noted above.

In one of two patients with cirrhotic liver disease, the clotting APTT was distinctly abnormal, exceeding the upper limit of normal for our laboratory by 8 s, while the chromogenic result was normal. The opposite was true of a second patient with mild liver disease. These discrepancies persisted for replicate samples of blood. Three of 11 patients with factor VIII inhibitors indicated by clotting assays (12, 20, and 1800 Bethesda units) had no demonstrable factor VIII by clotting assays, but all had demonstrable factor VIII by chromogenic assays. Two of these patients had been recently transfused with cryoprecipitate.

As expected, the chromogenic APTT was satisfactory for assay of factors V, X, VIII, IX, XI, and XII, and the chromogenic PT test for the assay of factors V, VII, and X. However, unlike the clotting APTT test, the chromogenic-substrate APTT test was also sensitive to factor VII deficiencies, as previously described (8).

Table 2 compares the agreement of results for the various factors by the automated chromogenic system and the conventional manual-clotting systems. Except for the factor XII assays, correlations between the two techniques were excellent (r ≥ 0.85). If the first two factor XII assays are excluded, the correlation here is also excellent. Results for factors V, VII, and X by both tests exceeded correlation coefficients of 0.90, 0.88, and 0.94, respectively.

Discussion

The inability of the clotting APTT to detect a deficiency of factor VII in plasma is compatible with the concept that factor VII has a function limited to the extrinsic clotting cascade, whereas the APTT measures the intrinsic system. However, we have confirmed that the chromogenic APTT was just as sensitive to a deficiency of factor VII as it was to deficiencies of factors V and X. This sensitivity was true whether the factor VII was lacking for congenital or acquired reasons.
required (oral anticoagulant or liver disease) reasons. Perhaps at the high dilution of plasma samples in the activation mixture of the chromogenic APTT test (5 and 500 µL) the interactions between the intrinsic and extrinsic cascades are exaggerated (9-11).

The conventional PT test reflects the activity of only three of the four vitamin K-dependent procoagulant proteins (prothrombin and factors VII and X), as does the clotting APTT (prothrombin and factors XI and X). Thus, the chromogenic APTT may have greater sensitivity for controlling oral anticoagulant therapy because it reflects the activities of all four of the factors involved (II, VII, IX, and X).

We encountered one technical problem in the use of the chromogenic substrates. For single-factor deficient plasmas the recommended addition of one part of the patient's plasma to seven parts of substrate plasma mixture was satisfactory. However, for samples with multiple plasma deficiencies eightfold dilution was too great. We found that fivefold dilution was entirely satisfactory for both single- and multiple-factor-deficient samples.

The validity of the use of a plasma-based substrate lacking a specific coagulation factor depends upon the absence of that factor. If the substrate contains 3% of the normal concentration of factor VIII, with this substrate a patient's plasma will seem to have no factor VIII when the actual concentration with conventional clotting techniques is also 3% of normal. One of four factor VIII-deficient plasmas and one of two factor IX-deficient substrate plasmas, obtained commercially, were unsatisfactory for use in the chromogenic assays.

The precision when the chromogenic substitutes for the PT and APTT were used was as good as for the clotting assays; however, the samples required for the chromogenic tests are much smaller, and the automated instrument used is already widely found in clinical laboratories. Thus, the chromogenic tests should be given serious consideration as possible routine tests for the clinical laboratory, particularly in the management of patients receiving oral anticoagulants, pediatric patients, and patients receiving therapy with heparin. In our studies, no information was available for a cost comparison between the chromogenic and the clotting tests. In the past few months, however, several new chromogenic factor assays have become available that are cost competitive with the clotting-based tests. Nonetheless, until the discrepancies observed for the patients with liver disease and for the three patients with anti-factor VIII activity are resolved, the coagulation laboratory must continue to use the conventional tests.

References