Comparison of the aPTT With Alternative Tests for Monitoring Direct Thrombin Inhibitors in Patient Samples

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ABSTRACT

Objectives: *The activated partial thromboplastin time (aPTT) test has been used for years to monitor parenteral direct thrombin inhibitors (DTIs) and unfractionated heparin. Because the aPTT correlates poorly with unfractionated heparin levels, we hypothesized that the aPTT may not be the best test for monitoring parenteral DTIs.*

Methods: *Using 235 excess plasma specimens from 82 adult patients receiving treatment with DTIs (argatroban, bivalirudin, or dabigatran), we compared the aPTT with the ecarin chromogenic assay (ECA), the dilute thrombin time (dTT) test, and the prothrombinase-induced clotting time (PiCT) test.*

Results: *The aPTT correlated poorly with each of the other tests in both bivalirudin- and argatroban-containing samples (r^2 = 0.04-0.23). The ECA and dTT exhibited the best correlations (r^2 = 0.66-0.93). Intermediate correlations were seen when the results of the PiCT were plotted against the dTT or ECA (r^2 = 0.46-0.58). Nineteen specimens obtained from six patients receiving dabigatran showed a good correlation between the dTT and the ECA (r^2 = 0.92).*

Conclusions: *The aPTT does not correlate well with other tests that might be used to monitor parental DTI administration. Further studies are needed to evaluate the clinical usefulness of alternative tests and their correlation with clinical outcomes.*

Upon completion of this activity you will be able to:
- describe the need for laboratories to develop methods for accurately assessing the pharmacodynamic effects of new target-specific anticoagulant therapies in hospitalized patients.
- discuss potential limitations of the activated partial thromboplastin time (aPTT) for monitoring the anticoagulant effects of direct thrombin inhibitors (DTIs).
- compare and contrast the aPTT with alternative tests for monitoring DTIs in hospitalized patients.

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Questions appear on p 754. Exam is located at www.ascp.org/ajcpcme.

Although the classic anticoagulants, warfarin and unfractionated heparin, continue to be widely used, an increasing number of alternative agents have become available for clinical use. In addition to low-molecular-weight heparins and similar synthetic polysaccharides that depend on antithrombin for their activity, newer anticoagulants are antithrombin independent and function either to prevent thrombin generation (the anti–factor Xa inhibitors) or inhibit the proteolytic activity of thrombin (the direct thrombin inhibitors [DTIs]). The newer agents have been developed with the expressed aim of avoiding the requirement for laboratory monitoring that has proven onerous for patients and clinicians. As a result, clinicians and laboratorians do not have a reservoir of data to call upon when asked to assess the plasma content of these drugs; such an assessment may be required in obese patients, those

with renal or hepatic dysfunction, potential overdoses, those with suspected lack of compliance, and/or those who required sudden medical interventions.

The classic clotting tests used to monitor warfarin and unfractionated heparin (the activated partial thromboplastin time [aPTT] and the prothrombin time, have been deemed to be insufficient for drug monitoring of some of the newer anticoagulants.[1] Several groups have found that the anti-Xa assay functions well to monitor the anti-Xa inhibitor, rivaroxaban,[2,3] and may prove to be adequate for the additional anti-Xa drugs that are entering the market. Although the aPTT is accepted by some as a screening test for dabigatran in the plasma, others have found that it may not detect clinically significant levels,[4] and it is not recommended for active drug monitoring.[5] Because dosing of parenteral DTIs is typically monitored by the aPTT, we were interested in learning how well the aPTT correlated with other tests that might be used to adjust DTI dosing in hospitalized patients. We therefore compared aPTT testing with three alternative tests in hospitalized patients receiving DTIs, with an emphasis on patients receiving the parenteral DTIs bivalirudin and argatroban, which are monitored with the aPTT.

Materials and Methods

Plasma Samples

Normal pooled plasma (Cryocheck, Precision BioLogic, Nova Scotia, Canada) was used to establish dose-response curves for each of the three anticoagulants tested. Patient (adult only) specimens were collected from July 1, 2011, to December 31, 2012. Excess citrated (3.2%) plasma sent to the coagulation laboratory at the University of Colorado Hospital (Aurora, CO) for clinically indicated aPTT monitoring of patients receiving DTIs was frozen at –70°C until testing, following a protocol approved by the institutional review board. The specimens were thawed in a 37°C water bath immediately before testing. No testing was performed on refrozen specimens. The results of the tests were not made available to the providers caring for the patients, and no dose adjustments were made based on the results described here.

Calibrators and Control Plasmas

Argatroban and bivalirudin calibrators and control plasmas were obtained from Diagnostica Stago (Parsippany, NJ). Dabigatran calibrators and control plasmas were obtained from Aniara (West Chester, OH).

Instrumentation

All assays were performed on the STA-R Evolution Expert Series analyzer, a large-volume, automated, mechanical end point coagulometer (Diagnostica Stago).

aPTT Testing

Stago STA-PTT-A reagent was used following all manufacturer's instructions. This reagent uses silica as an activator and rabbit brain cephalin, with a final calcium ion (Ca^{++}) concentration of 8.3 mmol/L. The normal range in our laboratory is 25.4 to 33.5 seconds.

Ecarin Chromogenic Assay (ECA)

The ECA assay[6] uses a snake venom enzyme called ecarin to generate meizothrombin from prothrombin, which is added in large excess to the patient plasma. The meizothrombin generated then cleaves a chromogenic substrate that is measured spectrophotometrically. The assay was performed according to the manufacturer's (Diagnostica Stago) instructions. At the beginning of each run, six concentrations of the relevant drug (Diagnostica Stago) were run with a control normal plasma to establish a standard curve. The instrument then calculated the concentration of drug present in each patient sample using the standard curve.

Dilute Thrombin Time (dTT)

Specimens were diluted 1:4 with commercially obtained pooled normal plasma (Cryocheck) by the coagulometer and incubated at 37°C for 4 minutes. Diluted plasma (100 µL) was then mixed with 100 µL of the thrombin reagent (STA-Thrombin, Diagnostica Stago), and the time required for clot formation measured. The thrombin reagent (approximately 1.5 U/mL) contained calcium (concentration not stated). This assay has also been referred to in the literature as the "plasma-diluted thrombin time."[7-10]

Prothrombinase-Induced Clotting Time (PiCT)

The PiCT assay (Pefakit) was obtained from Pentapharm, Basel, Switzerland, and the assay materials were obtained from Centerchem (Norwalk, CT). The PiCT is the time required for plasma coagulation to occur when induced by the prothrombinase complex, consisting of factors Xa and Va assembled on an appropriate phospholipid surface in the presence of calcium.[11] Sample plasma was added to a reagent containing exogenous factor Xa, phospholipids, and the factor V–activating protein, which was purified from the venom of the Russell viper, *Daboia russelli* (RVV-V). A plasma sample of 90 µL was added to 70 µL of a solution containing factor Xa, phospholipids, and RVV-V and incubated at 37°C for 3 minutes, after which 40 µL of a 25-mmol/L calcium chloride solution was added (final added Ca^{++} = 3.45 mmol/L) and the time required for clot formation recorded.[12]

Statistical Analysis

GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA) was used to graph the data and perform linear regression analysis.

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Results

Dose-response curves for each of the three drugs tested were determined by adding varying amounts of the drugs to pooled normal human plasma using four different assays, as shown in **Figure 1**, **Figure 2**, and **Figure 3**. For argatroban, the ECA and dTT were linear within the range of concentrations found in our plasma samples (0-2.2 μg/mL, Figure 1), and the aPTT and PiCT were curvilinear above 2 μg/mL. The bivalirudin dose-response curves showed linearity within the range of concentrations found in our patient samples (≤1.8 μg/mL, Figure 2). The dabigatran dose-response curves were linear within the range of our plasma samples (<150 ng/mL, Figure 3). Others have found higher levels of dabigatran when studying larger populations, and both the aPTT and PiCT tended toward curvilinearity as the upper end of this range.[13]

A total of 235 plasma samples from 82 patients treated with argatroban, bivalirudin, or dabigatran were evaluated in this study. The aPTT, ECA, dTT, and PiCT tests were performed on each plasma sample.

Seventy-six samples drawn from 25 patients receiving intravenous argatroban were tested, as shown in **Figure 4A**. The results of the aPTT correlated poorly with each of the other tests (r^2 = 0.23). The best correlation was obtained by plotting the results of the ECA with the diluted plasma thrombin time (dTT, r^2= 0.93). Intermediate correlations (r^2 = 0.46-0.58) were seen when the results of the PiCT were plotted against the results of the dTT or ECA.

A total of 140 specimens drawn from 51 patients treated with intravenous bivalirudin were analyzed **Figure 4B**. The results of the aPTT did not correlate well with results from any of the other tests. The best correlation (r^2 = 0.66) was obtained when the results of the dTT and ECA assays were plotted against each other.

Nineteen specimens obtained from six patients receiving dabigatran (an oral DTI) were available for study. As shown in **Figure 4C**, the results of the dTT and the ECA correlated with each other better than any other pairing of test results (r^2 = 0.92).

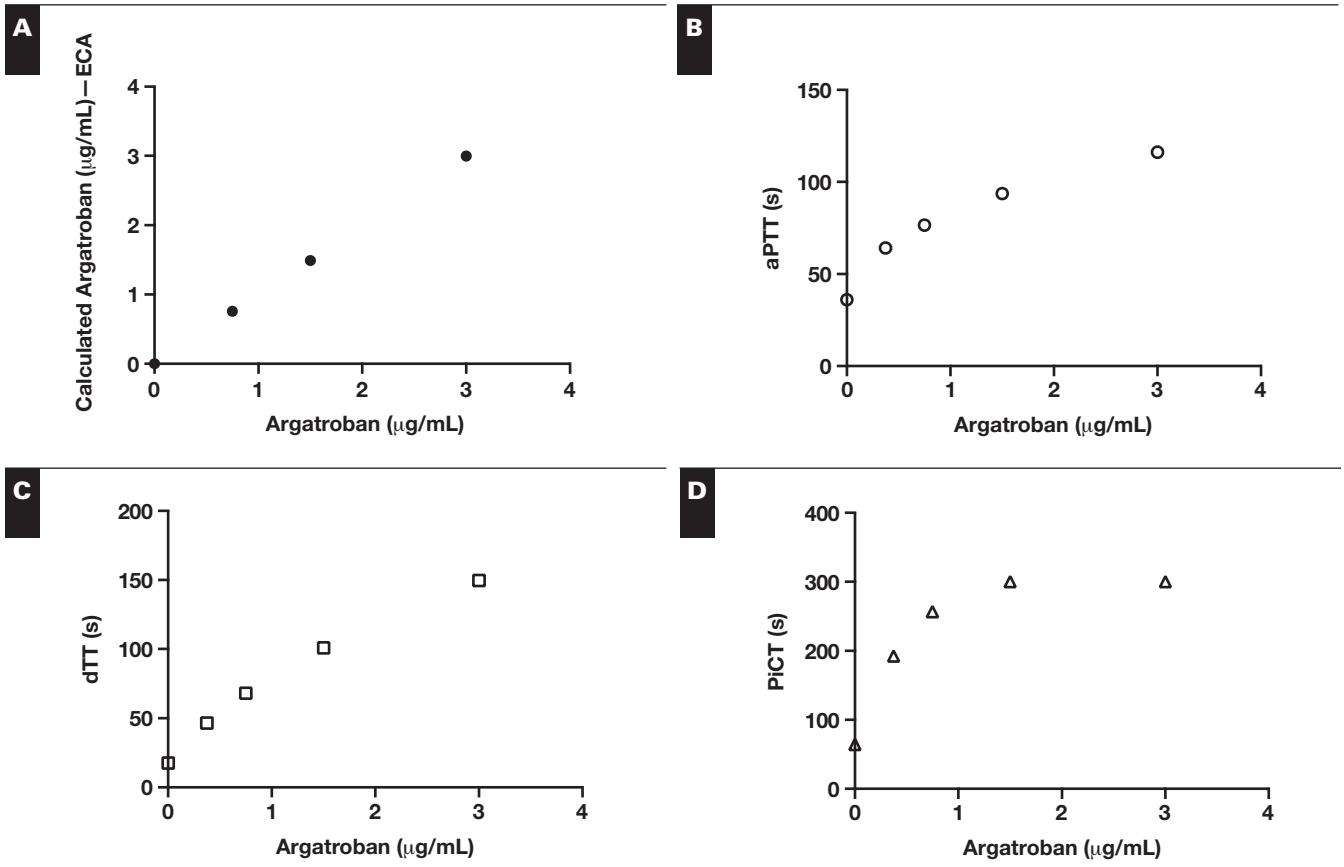

Figure 1 Dose-response relationship between the direct thrombin inhibitors tested and monitoring tests used. Shown is the dose-response relationship between argatroban added to normal pooled plasma and the ecarin chromogenic assay (ECA) (**A**), activated partial thromboplastin time (aPTT) (**B**), dilute thrombin time (dTT) (**C**), and prothrombinase-induced clotting time (PiCT) (**D**). For the dTT, values higher than 200 seconds are graphed as "200." For the PiCT, values higher than 300 seconds are graphed as "300."

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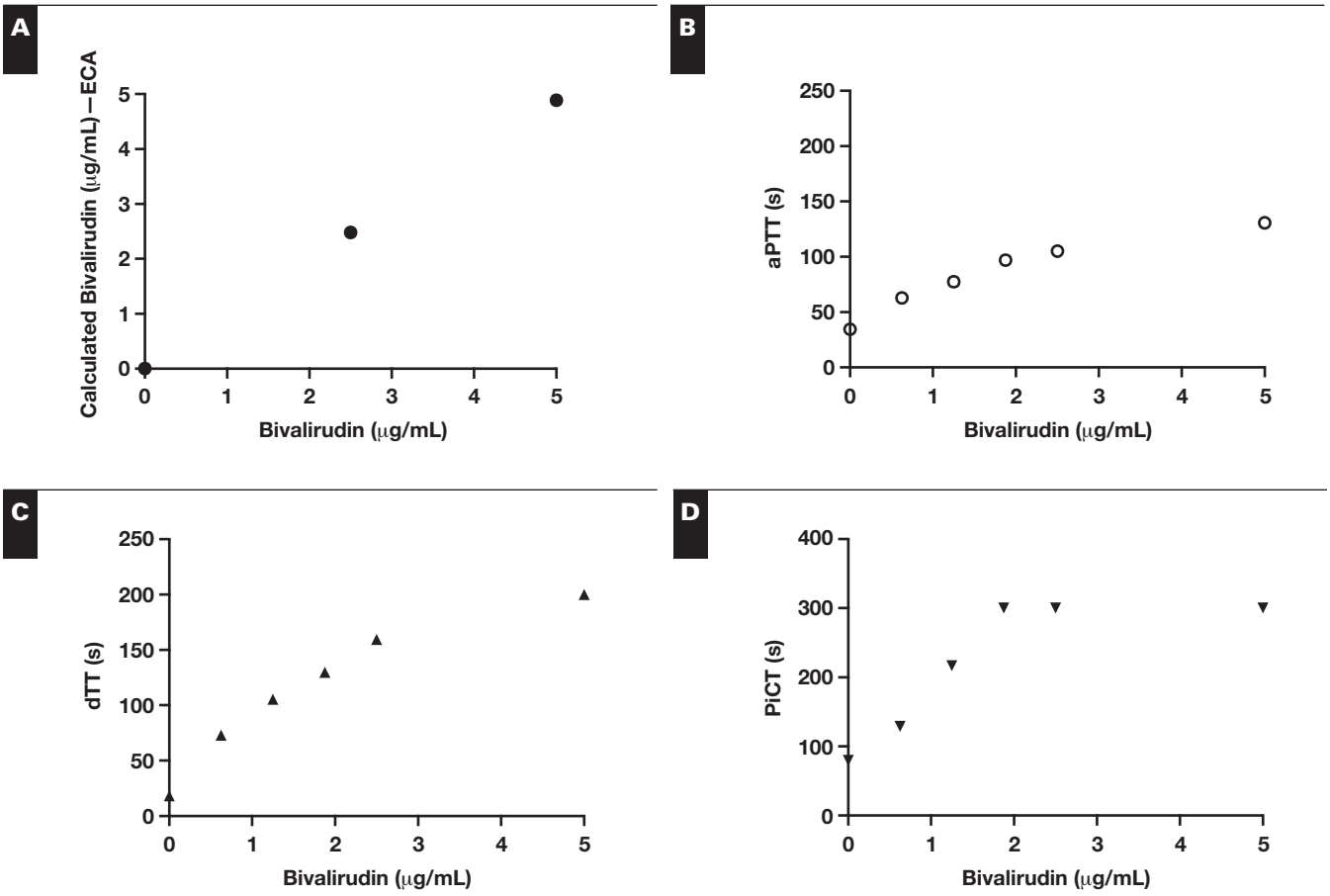

Figure 2 Dose-response relationship between bivalirudin added to normal pooled plasma and the ecarin chromogenic assay (ECA) (**A**), activated partial thromboplastin time (aPTT) (**B**), dilute thrombin time (dTT) (**C**), and prothrombinase-induced clotting time (PiCT) (**D**). For the PiCT, values higher than 300 seconds are graphed as "300."

Discussion

Although the marketing of new anticoagulants often emphasizes that routine monitoring is not required, evolving clinical experience shows that monitoring may be helpful under particular circumstances. When patients taking such drugs experience trauma, require emergent interventions, or experience hemorrhage or thrombosis, laboratories may be asked to assess the degree of plasma anticoagulation. Drug monitoring may also be required when treating very obese patients, those with renal failure, or those using other drugs that affect the kinetics of anticoagulants.[14] One of the classic coagulation tests, the aPTT, which has long been used to monitor unfractionated heparin, was adopted to monitor parenteral DTIs. It has been recognized for some time that differences in the dose-response relationships are observed when different aPTT reagents are used to assess heparin-containing plasma samples.[15] Laboratories attempt to correct for this effect by calibrating their aPTT reagents with an anti-Xa assay.[16] Many laboratories have found, however, that the correlation between the aPTT and anti-Xa level is poor. A recent study has shown that differences in the concentrations of several coagulation proteins (especially factor VIII) in patient plasma samples may explain this variability.[17]

A number of hospitals, including our own, use the aPTT to monitor DTIs administered by continuous infusion. Given the questions raised about the use of the aPTT to monitor unfractionated heparin, there is reason to question whether a different method of monitoring parenteral DTIs should be used. Ideally, one would like to make recommendations about test utilization based on clinical outcomes data, but such data cannot be gathered without large randomized clinical trials. We therefore decided to examine the suitability of the aPTT for monitoring DTIs by comparing it with the results of several alternative tests. Although none of the tests are approved by the Food and Drug Administration for this purpose, the drugs themselves

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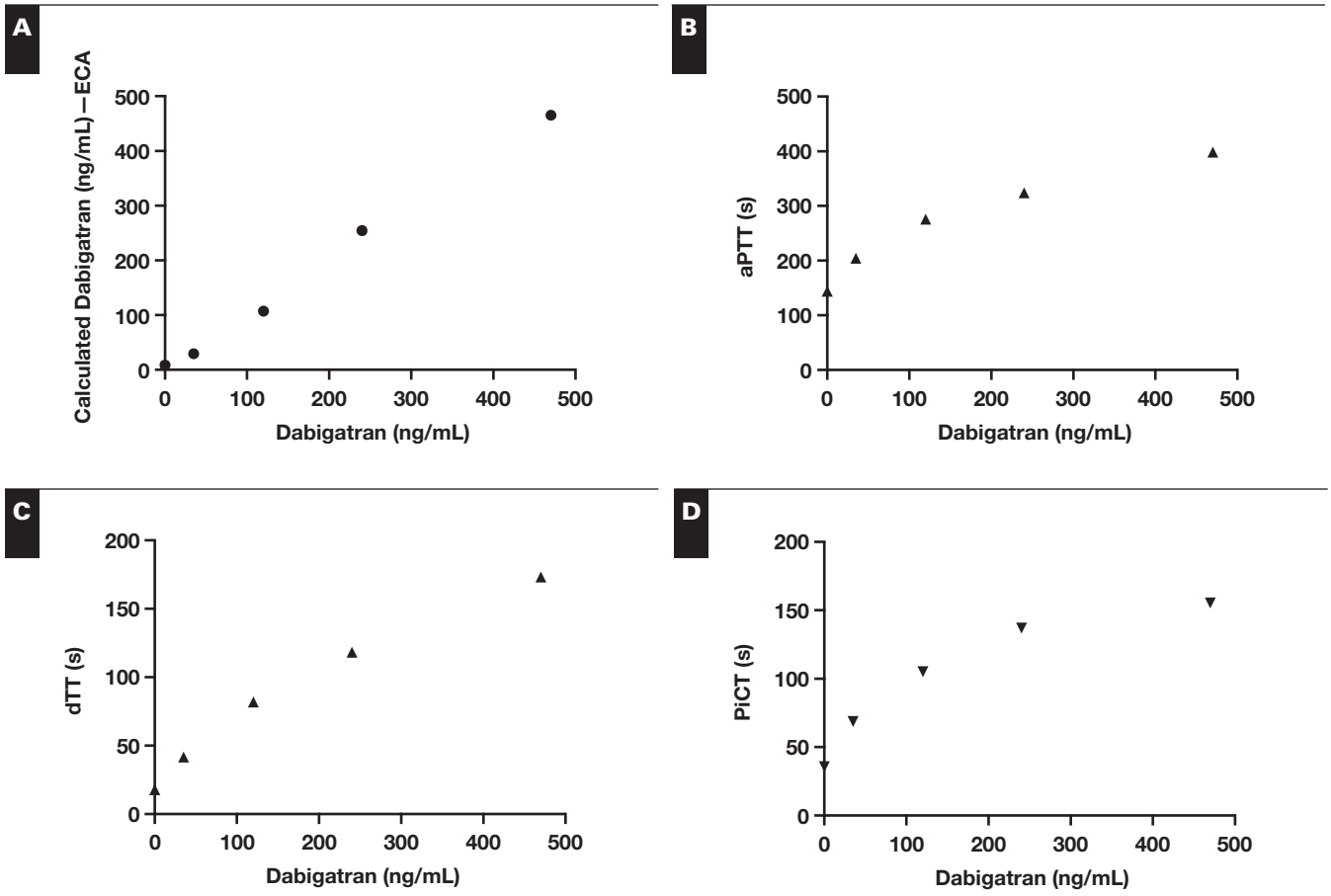

Figure 3 Dose-response relationship between dabigatran added to normal pooled plasma and the ecarin chromogenic assay (ECA) (**A**), activated partial thromboplastin time (aPTT) (**B**), dilute thrombin time (dTT) (**C**), and prothrombinase-induced clotting time (PiCT) (**D**).

are currently available and widely used, and laboratories are asked to assist with patient management on a routine basis.

Theoretically, a number of tests may be used to measure the DTI content of plasma. One class of such tests would depend on the generation of thrombin in the patient's plasma. This might be undertaken by activating the coagulation pathway at the level of factor X (using RVV), by cleaving the patient's prothrombin (factor II) through the generation (or addition) of the prothrombinase complex (which is composed of factors Xa, Va, and appropriate phospholipids) or by using an exogenous protein (such as a snake venom) to proteolyze prothrombin. Ecarin, a protein found in the venom of the Asian saw-scaled viper *Echis carinatus*, has been used most commonly for this purpose. Another type of testing could quantitate thrombin inhibition by adding exogenous thrombin to the patient's plasma. Two different end points could be used with any of these assays—either determination of the plasma clotting time or a colorimetric measurement based on the degree of cleavage of a thrombin-sensitive synthetic substrate. Assays based on the cleavage of synthetic chromogenic substrates are increasingly used in coagulation laboratories, particularly for the measurement of anti-Xa, antiplasmin, and antithrombin activities; however, decades of familiarity with classic coagulation assays has slowed the adoption of other chromogenic assays in many hospitals. Because clot formation, the end point of coagulation assays, can be influenced by many factors, including the concentration of fibrinogen, fibrinogen (or fibrin) degradation products, abnormal concentrations of physiologic plasma constituents, or the presence of drugs, chromogenic assays that are not affected by lupus anticoagulants are *theoretically* superior to clot-based assays. We are not aware of any data, including those presented here, which show that they are in fact clinically superior. Liquid chromatography/mass spectrometry may also be used to measure each drug but requires equipment that may not be readily available in most laboratories.[13,18]

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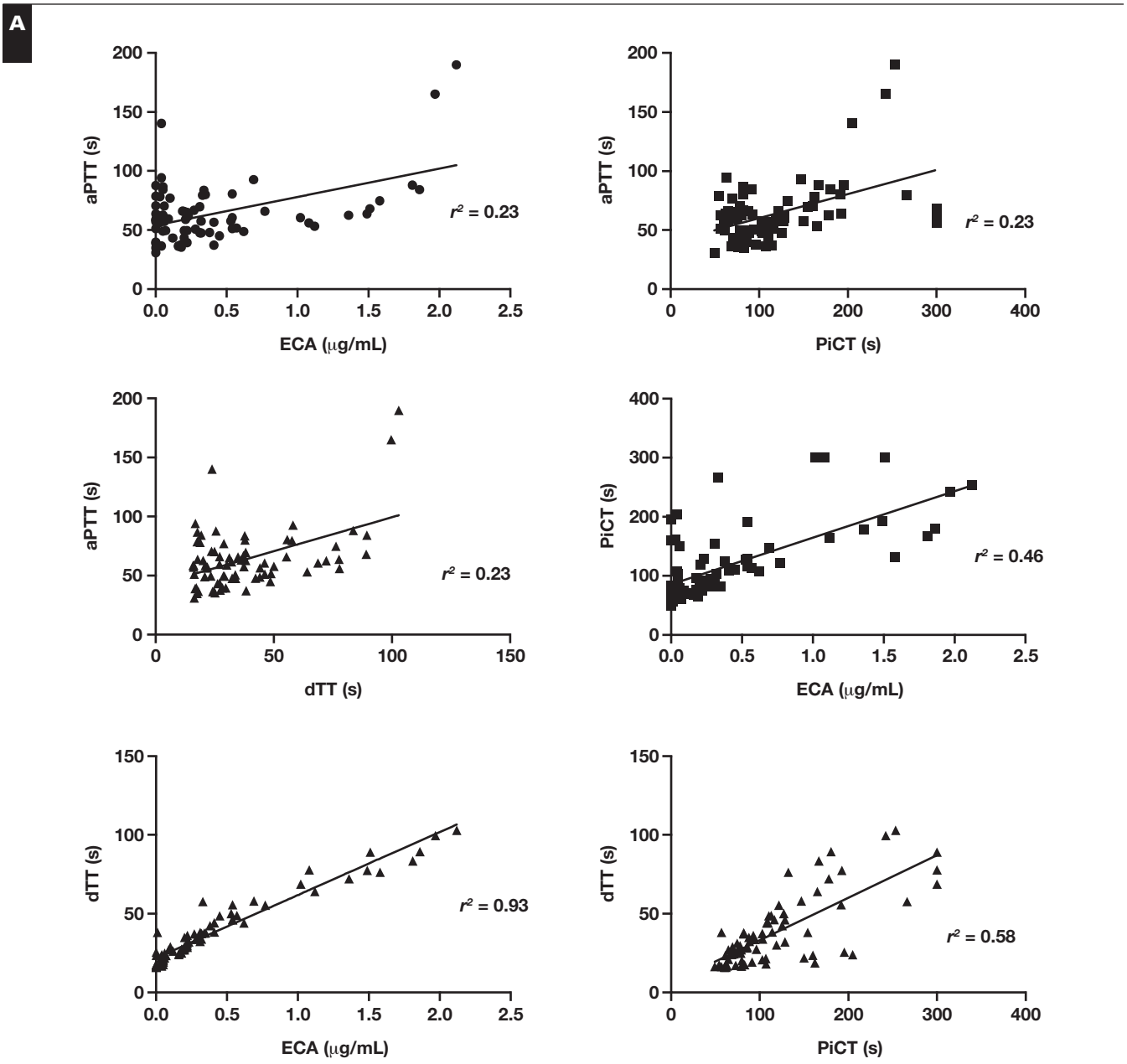

Figure 4 Correlations among monitoring tests performed on patients given direct thrombin inhibitors for therapeutic purposes. **A**, Correlations (linear regression) of monitoring tests performed on 76 samples drawn from 25 patients treated with argatroban.

Efforts are under way at a number of sites to determine which assays are best suited to monitoring the presence of DTIs. Some have used normal plasma with defined amounts of exogenously added DTIs,[19] whereas others have added DTIs to plasma samples drawn from patients with congenital clotting factor deficiencies,[20] acquired coagulopathies such as liver disease, or ingestion of oral vitamin K antagonists.[21] Others have used plasma samples drawn from patients receiving a single DTI (lepirudin).[22] Unlike the other studies, the current study used plasma samples drawn from patients receiving several different drugs at a single institution and is representative of daily laboratory practice.

We collected excess plasma samples from patients receiving three DTIs (argatroban, dabigatran, and bivalirudin) and performed four tests on each sample. Three tests were clot-based coagulation tests, and one used a chromogenic substrate (the ECA). One of the clot-based tests, the PiCT, is not widely used but has been shown to be a reasonable method for monitoring unfractionated and low-molecular-weight heparins in patients.[23] It uses exogenous factor Xa

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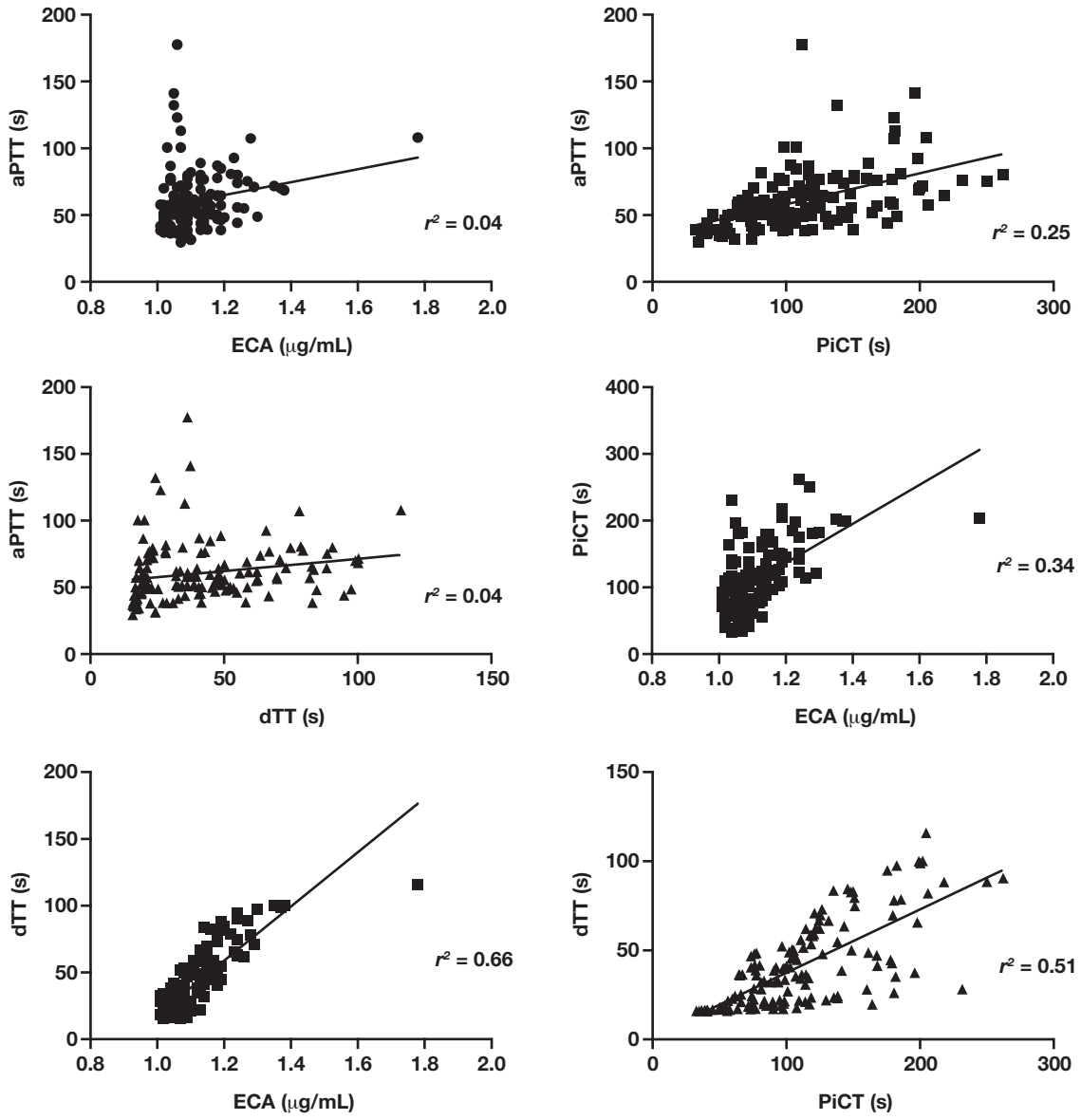

Figure 4 (cont) **B**, Correlations (linear regression) of monitoring tests performed on 140 samples drawn from 51 patients treated with bivalirudin.

and a factor V–activating protein to generate the prothrombinase complex and is not influenced by the plasma content of coagulation proteins other than factor V, factor II, and fibrinogen (although it is affected by lupus anticoagulants).[22] We hypothesized that it might provide a better estimate of plasma DTI content than the aPTT, which may be influenced by the concentration of a number of additional plasma proteins (notably those in the intrinsic and contact pathways). Indeed, the PiCT correlated to a higher degree with both the dTT and the chromogenic ECA than did the aPTT. Our results confirm those of others who found that the PiCT and aPTT are not linearly related when tested with plasma samples from patients receiving lepirudin.[24]

We found that the results of a modified thrombin time (called dTT herein) correlated well with the results of the ECA. One of the shortcomings of the study is that we were not able to measure drug levels directly and thus cannot determine which assay correlates best with clinical outcome. Another is that we only tested one aPTT reagent. A third is that the samples were deidentified, which prevented us from determining

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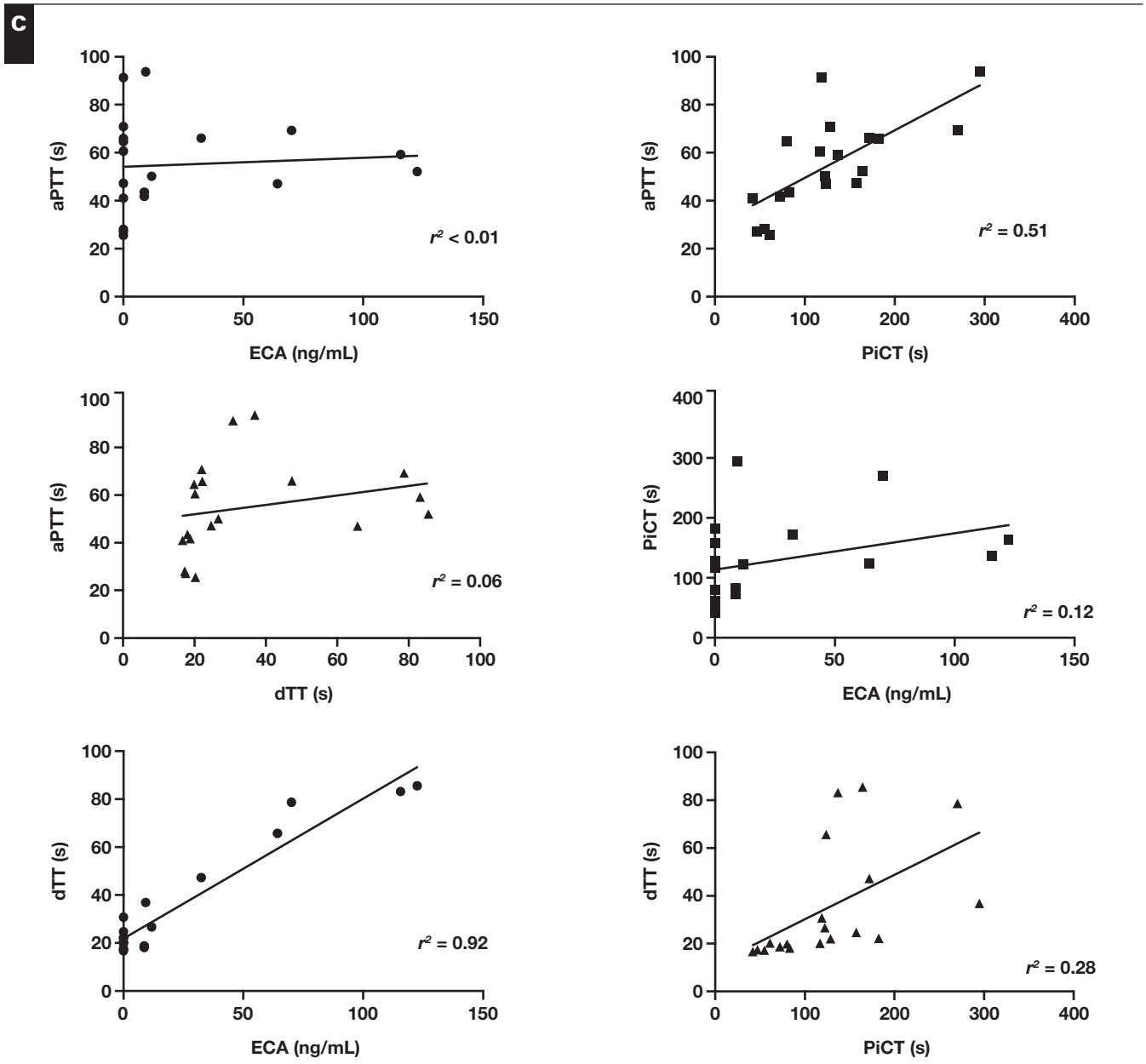

Figure 4 (cont) **C**, Correlations (linear regression) of monitoring tests performed on 19 samples drawn from six patients treated with dabigatran. aPPT, activated partial thromboplastin time; dTT, dilute thrombin time; ECA, echarin chromogenic assay; PiCT, prothrombinase-induced clotting time.

if any of the patients had characteristics (such as liver disease, vitamin K deficiency, or baseline elevated aPTTs/lupus anticoagulants) that might have predicted poor performance of the aPTT as a drug-monitoring tool. With these caveats in mind, our results are consistent with our conclusion that any of the tests used herein are superior to the aPTT for monitoring parenteral DTIs. Because of the limited number of samples obtained from patients taking dabigatran, we consider the data obtained from these samples to be supportive of the findings[13] and recommendations[25] of others rather than definitive. As shown in Figure 4, a prolonged aPTT does not necessarily indicate adequate anticoagulation, and errors in dosing may have significant, currently unappreciated consequences for patients being treated with these anticoagulants. A recent report showed that elevated factor VIII levels can affect aPTT monitoring of argatroban administration.[26]

Modifications of the thrombin time have been proposed for anticoagulation monitoring for many years but have not been widely adopted. One of the first (which used a fibrinogen solution to dilute the patient's plasma) was called the "quantitative thrombin time."[27] Other assays, using a similar principle, involve diluting the sample with normal plasma

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and have carried various names, including *plasma-diluted thrombin time*[7,10] or *diluted thrombin time*.[19] It is important to note that all thrombin time–based tests are not identical and may involve different dilutions of the patient plasma or different thrombin preparations. Calcium concentrations, well known to affect fibrin clot formation,[28] may differ among tests as well. As has been pointed out by others, thrombin time reagents from different sources are not uniform and may have different characteristics when tested in vitro.[29] Adopters of the diluted plasma thrombin time should not assume that all thrombin time reagents (which can be of animal or human origin) have an identical content of thrombin isoforms and will perform identically. Conceivably, thrombin time determinations could be affected by the concomitant administration of plasma expanders or presence of antibodies to bovine thrombin, which can develop the use of topical thrombin in surgical procedures.

The data presented herein provide evidence that the aPTT is a poor measure of anticoagulation with parenteral DTI administration. Institutions that use these drugs for patient management should consider using alternative laboratory methods. Guidelines for therapeutic levels of argatroban and bivalirudin should ideally be determined by clinical outcomes data or a suitable surrogate.

Coagulation reagents were provided by Diagnostica Stago, Parsippany, NJ.

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