Point-of-care fibrinolytic tests: the other side of blood coagulation

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Point-of-care (POC) coagulation tests with paramagnetic iron oxide particles have provided alternatives to testing previously done only in the laboratory. With this technology, POC fibrinolytic tests have followed quietly the trail blazed by POC clotting tests and have found specific applications. These include rapid verification of in vivo thrombolytic drug action by in vitro testing with subsequent quantitative drug monitoring of the systemic lytic state, and also the determination of in vitro thrombolytic drug response before the drug is actually administered, to individualize therapy by selection of the most appropriate drug. Other applications include POC coagulation factor assays associated with fibrinolysis, and most recently the POC screening of patients with fibrinolytic defects. In this latter application, plasma from cardiac catheterization (n = 19) and venous thrombosis (n = 47) patient groups were tested. Controls consisted of two independent donor pools (n = 10, n = 21) as negatives and two plasma samples with known genetic defects in the fibrinogen molecule (Aα554 Arg → Cys) as positives.

Blood coagulation testing at the point of care (POC) now exists in many hospitals. POC testing has been driven by economic factors and by improved technology [1]. In particular, dry chemistry coagulation testing at the POC with paramagnetic iron oxide particles (PIOP) has provided alternatives to testing previously done only in the laboratory and has opened the door to convenient fibrinolytic testing [2–4]. With this technology, POC fibrinolytic tests have followed quietly the trail blazed by POC dry chemistry clotting tests and have found specific applications. For example, during and after thrombolytic drug administration, a quantifiable measure of a patient’s systemic lytic response can be determined rapidly at the POC to verify drug action [5]. Furthermore, the response of a patient’s in vitro-generated clot to a thrombolytic drug can be determined before the drug is actually given, allowing therapy to be individualized by selecting the most appropriate drug [6]. Both of these fibrinolytic applications are being pursued in multicenter clinical trials in several countries. Two additional potential clinical applications of POC fibrinolytic testing are the ability to perform fibrinolytic factor assays [4] and to screen for fibrinolytic defects, which will be discussed further in this report.

The PIOP/dry chemistry technology may be used with whole blood or citrated plasma, yielding similar results for most tests. After placement of the test card that contains appropriate dry reagents and PIOP in the instrument, the card is warmed by a 37 °C heat source. The sample is then added to the test card. The liquid sample dissolves the reagents and frees the PIOP. The PIOP then organize into ensembles or “stacks” under the influence of orthogonal magnetic fields. The field perpendicular to the test card surface oscillates, causing the particles to oscillate [3, 7]. The motion of PIOP is monitored in the near infrared throughout the test. When the sample clots, the PIOP become entrapped in the gel clot, and their motion diminishes gradually. The peak PIOP oscillation signal before the decay in amplitude is the typical end point for clotting time measurements that correlates well with conventional laboratory methods. A variety of conventional and new clotting tests are possible with this technology. For example, a PIOP/dry chemistry test [ecarin clotting time (ECT)] was developed for antithrombin drugs. Fig. 1 shows a plot of clotting time vs the concentration of the thrombin inhibitor polyethylene glycol (PEG)–hirudin, a therapeutic drug being developed as a

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Nonstandard abbreviations: POC, point of care; PIOP, paramagnetic iron oxide particles; ECT, ecarin clotting time; PEG, polyethylene glycol; LOT, lysis onset time; t-PA, tissue plasminogen activator; and TAS, Thrombolytic Assessment System.
replacement for heparin in patients with conditions such as heparin-induced thrombocytopenia. This test is based on ECT [8, 9]. Ecarin is a snake venom product from *Echis carinatus* (EC 3.4.99.27) that provides a test specific for thrombin inhibitors.

Ecarin, a protein prothrombin activator, causes coagulation of citrated whole blood or citrated plasma by the calcium-independent activation of prothrombin. Ecarin catalyzes the hydrolytic cleavage of the 323Arg–324Ile bond in the human prothrombin molecule, whereby thrombin activity is generated without the release of a zymogen fragment. This form of active prothrombin has been termed meizothrombin and is inhibited by r-hirudin or PEG–hirudin, but surprisingly, not efficiently by the heparin–ATIII complex. Ecarin has been used as the basis to develop a sensitive analytical method for the determination of thrombin inhibition by antithrombin drugs such as r-hirudin or PEG–hirudin and is based on the method of Nowak and Bucha [8, 9]. The PIOP/dry chemistry ECT test card is intended to determine the concentration of an antithrombin drug in citrated whole blood and can be used to monitor patients receiving antithrombin drugs such as r-hirudin or PEG–hirudin.

The data shown in Fig. 1 are for citrated whole-blood samples from healthy donors, supplemented with increasing concentrations of PEG–hirudin (Knoll) and (or) porcine heparin (Sigma) and tested in the dry chemistry format. The ECT test cards were prepared from ecarin (Centerchem), calcium, PIOP, buffers, and stabilizers. As the in vivo concentration of antithrombin drug increases, the clotting time increases in a linear dose-dependent manner with essentially no effect of heparin on the clotting time end point, a potentially important clinical consideration when determination of only the anticoagulant effects of the direct thrombin inhibitor is desired.

For fibrinolysis (thrombolysis) measurements, where the clot subsequently dissolves, a new end point known as lysis onset time (LOT) was established [10]. LOT is the point at which the PIOP oscillation amplitude starts to increase again (Fig. 2), eventually resulting in full restoration of movement and is detected well before the clot dissolves, which provides the earliest indication of fibrinolysis. LOT can be precisely measured and is inversely proportional to the concentration of plasminogen activator present in the system [3]. A mathematical model for LOT was developed that is supportable by experimental data [7]. Fig. 2 shows a PIOP oscillation curve vs time for a lytic test. The peak event corresponding to the induced clotting time is observed along with the subsequent rise event corresponding to LOT. Increased concentrations of thrombolytic agent incorporated in the test card dry chemistry decrease the clotting time in the oscillation curve. For high concentrations of a thrombolytic agent such as thrombin or batroxobin, clotting time can be reduced to a few seconds. Increased concentrations of thrombolytic agent in the test card or in the sample being tested shorten the LOT in the oscillation curve. For high concentrations of thrombolytic agent, LOT can be <90 s. In this report, data are shown to demonstrate that two new potential applications of LOT may be achievable: fibrinolytic factor assays and fibrinolytic defect screening.

Fig. 1. Heparin effects on ECT test cards.

Solid line denotes response of ECT test to PEG–hirudin in the absence of heparin. Dotted line denotes the response of the ECT test to PEG–hirudin in the presence of 500 U/L porcine heparin.

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\[5\] LOT differs from clot lysis time, an older term found in hematology textbooks. Clot lysis time is related to the time required to dissolve an in vitro clot in tests such as whole-blood clot lysis time. Whole-blood clot lysis time is used as a simple functional screening test to determine lytic tendency and consists of allowing a tube of whole blood to clot and examining the tube periodically to determine how long it takes for the clot to dissolve. In healthy individuals, the clot remains undissolved, even after 24 h. In individuals with abnormal antiplasmin activity or in some fibrinolytic states, the clot is observed to dissolve in a few hours [11]. It should be recognized that the visual determination of clot lysis time cannot be made until a substantial amount of lysis of the thrombus occurs. Euglobulin clot lysis time is a variation of this test.
Materials and Methods

PLASMINOGEN ASSAY
Plasminogen-free citrated plasma was prepared by passing pooled normal citrated plasma (Specialty Assayed Reference Plasma, George King) through a lysine–Sepharose column (Pharmacia). This treatment selectively removes plasminogen without affecting other coagulation proteins. Plasminogen (Sigma) was then added to the plasminogen-depleted citrated plasma to yield the different concentrations used in the assay. Dry chemistry plasminogen test cards were prepared by assembling the test card device and injecting 24 μL of a solution [containing 1500 U/L thrombin, 500 000 U/L tissue plasminogen activator (t-PA), 500 000 U/L urokinase, PIOP, salts, buffers, and stabilizers] into the card chamber, freezing the card/reagent mixture, and lyophilizing the test card to yield the dry chemistry formulation. Test cards were placed in a Thrombolytic Assessment System (TAS) analyzer (Cardiovascular Diagnostics), and 30 μL of patient or normal donor citrated plasma were added to the test card. The TAS analyzer was programmed to detect and display the LOT value.

SCREENING FOR FIBRINOlytic DEFECTS
Citrated plasma samples were obtained from stockpiled venous thrombosis patients and patients undergoing cardiac catheterization. CHIII and Dusart are samples from well-studied thrombosis-prone families with abnormal fibrinogens known to exhibit impaired fibrinolysis [12–16]. The defect in both families consists of the same amino acid substitution of cysteine at the Aa554 arginine site [15]. To provide “normals,” samples were obtained from local donors at Cardiovascular Diagnostics, and from donors at a major blood center. All citrated plasma samples were maintained frozen at −85 °C before analysis and thawed at 37 °C before use. Dry chemistry test cards suitable for screening patient and normal samples were prepared as follows: 24 μL of a solution containing 1500 U/L thrombin, t-PA at a concentration of 200 000, 400 000, 800 000, or 1 000 000 U/L, PIOP, salts, buffers, and stabilizers were injected into an assembled test card device, frozen, and lyophilized to yield the dry chemistry formulation. The formulation included heparin neutralizers, which negated the effects of heparin, if present. Results shown are for a t-PA concentration of 200 000 U/L. The test cards were placed in the TAS analyzer, and 30 μL of patient or normal donor citrated plasma were added to the test card. The TAS analyzer was programmed to detect and display the LOT value.

Results
PLASMINOGEN ASSAY
A set of increasing responses forming a curve resulted when LOT values were plotted vs plasminogen concentration in the sample (Fig. 3). The resulting LOT values ranged from 90 s at the higher plasminogen concentrations (120 to 160 mg/L) to 250 s at 45 mg/L. Plotting the LOT results of the plasmin(ogen) assay vs the inverse of the plasminogen concentration results in a linear LOT response (Fig. 4).

SCREENING FOR FIBRINOlytic DEFECTS
When the low-concentration t-PA (200 000 U/L) test data were obtained, we observed that the venous thrombosis, cardiac catheterization, and two normal groups exhibited a wide range of individual responses. The mean LOT values and their standard deviations (in s) were: 432 ± 102 for the venous thrombosis group (n = 47), 329 ± 88 for the cardiac catheterization group (n = 19), 283 ± 76 for the local “normal” donors (n = 10), and 319 ± 54 for the blood center “normal” donors (n = 21). The LOT values obtained for the Dusart and CHIII samples were 875 and 670 s, respectively. All measurements are means of duplicates. LOT measurements, with low-concentration t-PA, are generally within ± 10%. Precision approaches ± 5% as the amount of t-PA reaches 500 000 U/L, the concentration used to generate the data in Fig. 2.
Discussion

The calibration curve for plasminogen indicates that it should be possible to provide a POC test for low-range plasminogen in patient samples. This test would actually measure total available plasmin and provide the composite fibrinolytic effect of plasminogen and free plasmin, if any. Typically, plasminogen would be measured. In the case of a patient undergoing extended thrombolytic therapy, e.g., for deep vein thrombosis, it may be important to determine periodically that the patient has sufficient plasminogen reserves for the therapy to continue to be effective. A low-range test for total available plasmin is well suited to meet this objective. Normal- and higher-range plasminogen (concentrations >200 mg/L) is not a critical care issue and can be determined with a laboratory test in a routine fashion.

A linear LOT response is obtained by plotting LOT vs the reciprocal of the plasminogen concentration (Fig. 4). This linearization had previously been established for plasminogen activators [3]. This depiction of the LOT response allows easier conversion of LOT into plasminogen concentration. Our test may be suitable for use with thrombolytic therapy patients to assess plasminogen reserves at the POC.

In screening for fibrinolytic defects, it is important to establish a normal range. Extensive testing was not undertaken to do this, but selected random samples were used from two different donor pools. Whether all of the samples in each pool are “normal” was not addressed by our study. Similarly, some of the cardiac catheterization patients are probably normals. For example, patients with heart valve problems but without vessel disease might have normal LOT values. However, when the means of each “normal” pool and their standard deviations are different, a general range of probable normals may be inferred from the data, as depicted graphically in Fig. 5. Similarly, the known abnormal samples, Dusart and CHIII, fall well outside of this general range of probable normal values. In fact, LOT values for both Dusart and CHIII are more than 5 SD from the mean of either normal group. Therefore, other samples falling well beyond the normal ranges would be expected to be abnormal as well. It is remarkable, therefore, that the venous thrombosis group contains two or four individuals whose LOT values are >5 SD from the mean value of the “normals,” depending upon which “normal” group is used. Therefore, we have been able to demonstrate impaired fibrinolysis in vitro for some of the venous thrombosis patients. Specific

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Fig. 3. Plasminogen calibration curve.

Plasminogen assay calibration curve generated by addition of plasminogen to pooled plasminogen-depleted citrated plasma added to PIOP/dry chemistry test cards.

Fig. 4. Plasminogen calibration curve; inverse response.

Plasminogen assay calibration curve generated by addition of plasminogen to pooled plasminogen-depleted citrated plasma added to PIOP/dry chemistry test cards plotting LOT vs inverse plasminogen concentration and showing the resulting linear relation.
fibrinolytic defects were not detected and correlated to clinical outcomes, as that was beyond the scope of the present feasibility study.

Venous thrombosis is caused by a variety of defects, at least one subset of which are fibrinolytic defects. Finding or identifying this subset of patients is not easily accomplished. Two methods that have been used are euglobulin lysis time and whole-blood clot lysis time. Both methods are labor intensive and difficult to standardize. For the individual samples tested here, the origin of the fibrinolytic defects is unknown. However, the simple, rapid test methodology, as described, should enable convenient screening and identification of abnormal individuals who could undergo further testing to determine the exact cause of the fibrinolytic defect.

Table 1 tabulates POC clotting time measurements and LOT measurements for different end uses. For the end use of lytic state monitoring, clotting time end points, such as in fibrinogen and thrombin time tests, provide an indirect measure of the systemic lytic state. LOT provides a direct measure [3, 5]. For drug response, clotting time end points provide a measure of in vitro anticoagulant effects after administration of an anticoagulant drug. With appropriate test chemistry, LOT provides a measure of in vitro fibrinolytic effects before the administration of a thrombolytic drug [6]. In factor assays, clotting time and LOT end points (as discussed previously) may be used to determine concentrations of clotting and fibrinolytic factors, respectively. Finally, clotting time end points in tests such as prothrombin time and activated partial thromboplastin time enable screening for clotting defects, and fibrinolytic tests such as the low-concentration t-PA response test described in this report enable screening for fibrinolytic defects.

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