VIDAS® D-dimer: fast quantitative ELISA for measuring D-dimer in plasma

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VIDAS D-dimer (bioMérieux) is a new quantitative ELISA for D-dimer determination designed for the VIDAS automated system. The test contains single-dose, ready-to-use reagents and is completed within 35 min. Quantitative results are obtained from a calibration curve stored in the software of the system and expressed as fibrinogen equivalent units. The two-step capture/tag test relies on two complementary monoclonal anti-D-dimer antibodies, the second one being labeled with alkaline phosphatase. The upper limit of the measuring range is 1000 μg/L and the lower detection limit is <50 μg/L, which is below the lower limit of the reference interval (68–494 μg/L). Reproducibility (CV) within and between runs ranges from 5% to 7%. There is no interference from heparin, bilirubin, hemoglobin, fibrinogen degradation products, or plasma turbidity. Comparison with a conventional ELISA (y) gave good correlation ($r = 0.91$, $n = 579$) and comparable results ($y = 1.35x - 148$, $S_{y|x} = 750$), especially for D-dimer concentrations ranging from 0 to 1000 μg/L ($y = 1.09x - 10.6$, $r = 0.88$, $S_{y|x} = 170$).

INDEXING TERMS: coagulation • fibrin(ogen) degradation products • thromboembolism

D-dimer fragments are the specific end-products of cross-linked fibrin degradation by plasmin in the presence of calcium [1, 2]. In vivo, however, the action of the endogenous fibrinolytic system induces numerous macromolecular complexes containing D-dimer fragments, but among which D-dimers are not the major fragments [3, 4]. The term D-dimer is often incorrectly used to describe *all* cross-linked fibrin degradation products, for which Mosesson has recently proposed new terminology [5].

The availability of monoclonal antibodies (MAbs) directed against D-dimer [6, 7] has allowed specific assay of these frag-

ments directly in plasma without interference from fibrinogen or its degradation products and has facilitated definition of the physiological or pathological conditions in which their concentrations in plasma are increased [8, 9].[6] Different assay systems utilizing these MAbs have been developed, including semiquantitative and quantitative latex assays and microplate and membrane ELISAs.

D-dimer determinations are usually performed in clinical situations where activation of the coagulation and the fibrinolytic systems is suspected, e.g., in disseminated intravascular coagulation from any etiology. D-dimer determinations have also been a useful diagnostic tool for excluding suspected thromboembolic disease. For this latter purpose, semiquantitative latex assays, although easier to perform than ELISAs, cannot be used safely because of their lower sensitivity [10]. Unfortunately, conventional ELISAs are not adapted for round-the-clock testing of individual emergency cases, which explains why, despite their performance, their use is not as widespread as it should be [10, 11].

Recently, a new method for the assay of fibrin degradation products for use with the bioMérieux (Marcy l'Etoile, France) immunoassay system VIDAS has been developed [12]. Here, we describe the analytical performances of this single, ready-to-use reagent, VIDAS D-dimer, which has the lower detection limit sensitivity of a conventional ELISA and is fully adapted to emergency diagnosis.

Materials and Methods

MATERIALS
Fibrinogen was provided by Laboratoire Français de Biotechnologie (Paris, France), plasmin by Chromogenix (Mölndal, Sweden), bovine thrombin by Miles (Kankakee, IL; cat. no. 82 040), aprotinin by Sigma (St. Louis, MO), and Pefabloc® SC [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride] by Pentapharm (Basle, Switzerland). Alkaline phosphatase (EC 3.1.3.1) was obtained from Boehringer Mannheim (Meylan,

[6] Nonstandard abbreviations: MAb, monoclonal antibody; KIU, kallikrein inhibitor unit; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and SPR, solid-phase receptacle.

France), bilirubin (purim.) from Fluka (Buchs, Switzerland), and injectable heparin solution from Choay (Paris, France).

Separation media Superdex 200 and CNBr-Sepharose were supplied by Pharmacia (Uppsala, Sweden), SW300 by Waters (St-Quentin, France), and ACA34 by Biosepra (Villeneuve-la-Garenne, France). The Asserachrom D-Di assay was obtained from Diagnostica Stago (Asnières, France).

Plasma samples were prepared from citrated blood by centrifugation at 3000g for 15 min. All procedures complied with the ethical standards of the Helsinki Declaration of 1975 as revised in 1983.

PREPARATORY PROCEDURES

Production of MAbs. BALB/c J1Co female mice, 4 to 6 weeks old (IFFA Credo, Les Oncins, L'Arbresle, France), were immunized by intraperitoneal injection with purified D-dimer fragments (150 mg/L), emulsified with an equal volume of Freund's complete adjuvant. Several intraperitoneal injections were performed by using incomplete adjuvant at monthly intervals. Four days after the last injection, spleen cells were harvested and fused according to the Köhler and Milstein method [13, 14] with mouse myeloma Sp2/0-Ag14. After 12–14 days, the culture supernates were screened with an ELISA.

Positive colonies were subcloned twice by limiting dilution. Ascites fluids were obtained from mice primed with a 0.5-mL intraperitoneal injection of Pristane and then injected with ~10^6 hybridoma cells. The two MAbs selected are both IgG1.

Preparation of D-dimer-depleted plasma. A portion (50 mL) of a pool of human plasma containing protease inhibitors aprotinin [100 000 kallikrein inhibitor units per liter (KIU/L)] and Pefabloc SC (0.1 g/L) was eluted on a 1.5 × 5 cm column of Sepharose–anti-D-dimer MAb at a flow rate of 8 cm/h.

Preparation of fibrinogen degradation products. Degradation of human 10 g/L fibrinogen in 50 mmol/L Tris buffer (pH 7.4) containing 5 mmol/L CaCl₂ at 25 °C by 52 U/L plasmin was performed for 5 h to obtain fragment X, and by 130 U/L plasmin for 5 h and 6 h to obtain fragment Y and fragment D, respectively. The reaction was blocked by addition of 250 KIU of aprotinin per unit of plasmin. Fragments X and D were purified by gel-filtration chromatography on a SW300 column in 50 mmol/L phosphate buffer (pH 6.8) containing 150 mmol/L NaCl and 100 000 KIU/L aprotinin. Fragment Y was purified by gel-filtration chromatography on a Superdex 200 column in the same buffer.

Fragment purity was controlled by using 4–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of D-dimer. Human plasma (20 mL) was clotted with thrombin (5000 NIH U/L plasma) in the presence of calcium (20 mmol/L). The clot obtained by centrifugation for 5 min at 20 000g was washed under magnetic stirring for 1 h at room temperature in 5 L of 0.7 mmol/L citrate buffer (pH 7.3) containing 40 mmol/L EDTA and 150 mmol/L NaCl, and then twice in 5 L of demineralized water for 16 h at 2–8 °C. After washing, the clot was minced, suspended in 50 mmol/L Tris buffer (pH 7.8) containing 5 mmol/L CaCl₂ and 2 g/L NaN₃, and degraded for 72 h in the presence of plasmin (400 U per gram of fibrinogen). The reaction was blocked by addition of 250 KIU of aprotinin per unit of plasmin.

The D-dimer fragments obtained were purified by gel filtration on an ACA 34 column in 50 mmol/L Tris buffer (pH 7.3) containing 150 mmol/L NaCl. The purity of the D-dimer obtained was controlled by using 4–15% SDS-PAGE.

ASSAY

With VIDAS D-dimer reagent, fibrin degradation products are assayed with an enzyme-linked fluorescent assay, which combines the ELISA method with a final detection in fluorescence. The test is fully automated on the bioMérieux immunoassay system, VIDAS. The analyzer, which has an extensive menu of immunoassays in the areas of immunochemistry, serology, antigen detection, and infectious disease, consists of three interconnected units: a test-processing unit, a computer, and a graphics printer. Once the sample is loaded, the operation entails no pipetting, no syringes, and no tubing. The protocol that defines the different phases of the reaction is identified by a barcode system on the reagent.

The single-dose, ready-to-use reagent includes a solid-phase receptacle (SPR) and a strip identified by a code with a specific color for each analyte. The SPR resembles a pipetting tip and is used both as a pipetting device and a solid phase with the antibody 10B5E12C9 coated onto its surface. The strip contains all the other ready-to-use reagents: sample diluent, conjugate (alkaline phosphatase-labeled antibody 2C5A10), wash solutions, and substrate (4-methylumbelliferyl phosphate). A barcode identifying the test name, batch number, and reagent expiration date is printed on the strip label.

The operator simply pipettes 200 μL of undiluted plasma into the first well on the strip and then inserts the strip and corresponding SPR into the analyzer. The whole assay is carried out at 37 °C, which allows the time to completion to be reduced to 35 min. The D-dimer concentration of the sample, expressed as fibrinogen equivalents units, is calculated from a calibration curve specific to the reagent batch stored in the software by means of the barcode system. The assay must be recalibrated once every 14 days by running a single calibrator in duplicate. Three controls at concentrations of ~250, 500, and 750 μg/L are performed to validate the new calibration and ensure a daily quality control. D-dimer concentrations >1000 μg/L are obtained after manual predilution of the samples in the diluent provided in the kit. A 10-fold dilution of the sample may be suitable in some clinical conditions.

After a conventional 15-min centrifugation of blood, the test result is thus available in ~55 min. The cost is ~$10 US/test.

ASSAY EVALUATION

Specificity. The cross-reactivity of the two MAbs, 10B5E12C9 and 2C5A10, towards fibrinogen degradation products was assessed on the VIDAS by using the protocol for the VIDAS D-dimer reagent but with the antibody to be tested on the solid phase and an alkaline phosphatase-labeled polyclonal antibody

against fibrinogen fragment D as the label antibody. Increasing concentrations of fibrinogen fragments and D-dimer diluted in the kit dilution buffer were tested.

Cross-reactivity towards fibrinogen was tested by diluting a plasma with D-dimer of ~1000 μg/L either in the kit diluent or in a D-dimer-depleted plasma containing normal concentrations of fibrinogen.

Assay reproducibility. To determine within-run reproducibility, we tested in duplicate with three different batches of VIDAS D-dimer 100 plasma samples with D-dimer concentrations covering the full range of the calibration curve. We then analyzed the data from the 100 duplicates to determine an equation defining assay reproducibility at various concentrations for each batch and a mean equation for the three batches [15]. To determine between-run reproducibility, we tested five plasma samples in 29 successive runs with three different batches of VIDAS D-dimer reagent and calculated their concentrations from the calibration curves specific to each batch stored in the software by the one-point recalibration method. As with within-run reproducibility, we extrapolated a mean equation from these data to define the reproducibility profile of the reagent [15].

Results

Antibody specificity. ELISA binding curves showed that antibody 10B5E12C9, used as the capture antibody in the final reagent, is not fully specific for D-dimer; it shows a low level of reactivity towards fibrinogen degradation products (D, X, and Y) in comparison with that obtained towards purified D-dimer (Fig. 1A). Antibody 2C5A10, which is the labeled antibody in the final reagent, showed a wider specificity; it clearly reacted with fragment X (20% of the response obtained with purified D-dimer, weight basis) and to a lesser extent with fragment D (~2% cross-reactivity) and fragment Y (Fig. 1B).

However, the combination of these two MAbs provided a good reagent specificity: The fibrinogen degradation fragments gave no signal in the final test at the set concentrations (Fig. 1C) and did not compete with D-dimer fragments, even at concentrations 200 times higher (Table 1).

Figure 2 shows dilution curves of a plasma sample with D-dimer at 1000 μg/L diluted with either the kit diluent or a fibrin degradation products-free plasma. The overlap of the curves is an additional proof of the absence of interference from fibrinogen in the assay.

Detection limit. At a 1% risk ($\alpha = 0.01$), the detection limit calculated from 30 determinations of point 0 of the calibration curve was 45 μg/L for the first batch manufactured and 13 μg/L for the two subsequent batches. These values are satisfactory, being lower than the lower limit of the normal reference values (see below).

Hook effect. The absence of a "hook" effect was investigated by assaying increasing concentrations of a solution of cross-linked fibrin degradation products obtained in vitro. Fig. 3 shows the absence of the hook effect at all concentrations tested; that is,
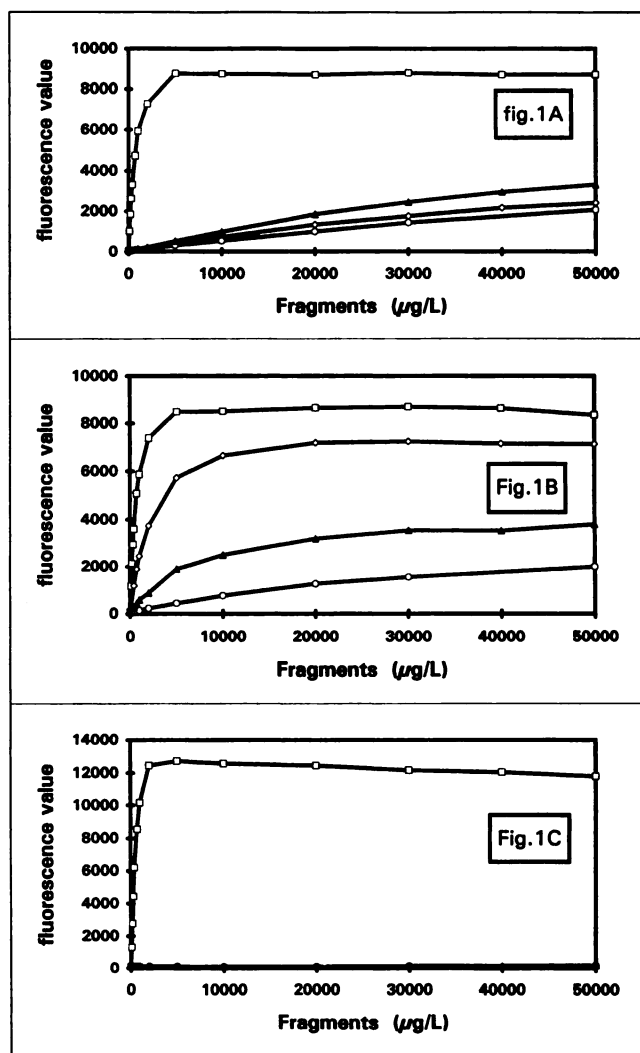

Fig. 1. Specificity of MAbs 10B5E12C9 and 2C5A10 and of VIDAS D-Dimer assay in the presence of increasing concentrations of D-dimer (□), fragment X (◇), fragment Y (○), or fragment D (▲).

The capture antibody is either 10B5E12C9 (*A*) or 2C5A10 (*B*), and the labeled antibody is an alkaline phosphatase-conjugated antifibrinogen fragment D polyclonal antibody. (*C*) 10B5E12C9 is the capture antibody and alkaline phosphatase-conjugated 2C5A10 is the labeled antibody.

above and beyond a certain concentration of D-dimer, the signal remained the same as that at the level of saturation.

Interferences. No interference was observed, even at the highest concentration tested, with hemoglobin (0.3 mmol/L), triglycerides (11 mmol/L), bilirubin (825 μmol/L), rheumatoid factors (435 kIU/L), or heparin (50 kIU/L) (data not shown).

Assay reproducibility. Within- and between-assay reproducibility was determined with three different batches of reagents. This allowed us also to determine intrabatch reproducibility of the reagent. The equations calculated for reproducibility profiles enabled definition of intra- and interassay reproducibility values at all concentrations within the calibration range. Because the assay is a single test, "intraassay" is defined as simultaneous

Table 1. Effect of increasing concentrations of added fibrinogen degradation products X, Y, or D (0–100 000 µg/L) on D-dimer concentration measured in plasma.

Final conc of fragment, µg/L	Fragment added		
	X	Y	D
0	503[a]	432	537
1 000	567	430	497
5 000	537	424	520
10 000	495	423	535
20 000	534	418	533
50 000	514	418	493
100 000	574	405	484

[a] Measured D-dimer, µg/L.

analyses of the same sample. The intra- and interassay CV values are very close and range between 5% and 7% for D-dimer concentrations of 300 to 1000 µg/L. Below 300 µg/L, these values are slightly higher, but remain acceptable (Table 2).

Validation of the one-point recalibration system. The interassay accuracy of the reagent was assessed by measuring four plasma samples and the three controls of the kit in 29 runs performed over 8 weeks, either from an eight-point calibration curve used in each run or from a calibration curve stored in the software. The latter curve was recalibrated every 14 days by assaying a single calibrator in duplicate. The one-point recalibration sys-

Table 2. Reproducibility data (within- and between-run) for VIDAS D-dimer reagent.

D-dimer conc, µg/L	CV, %	
	Within-assay	Between-assay
200	6.5	9.1
300	5.8	7.3
400	5.5	6.4
600	5.3	5.5
800	5.4	5.0
1000	5.5	4.8

See text for details.

tem did not substantially modify either the plasmas' values or assay reproducibility (Table 3).

Between-batch variability. The variability between batches was investigated by measuring 100 plasma samples, the concentrations of which spanned the whole calibration range. The correlation coefficients and the parameters of the standardized principal components lines are shown in Table 4 and indicate good interbatch correlation.

Reference values. Citrated plasma samples (n = 199) were obtained from healthy individuals. The frequency distribution of D-dimer concentrations observed (Fig. 4) was well represented
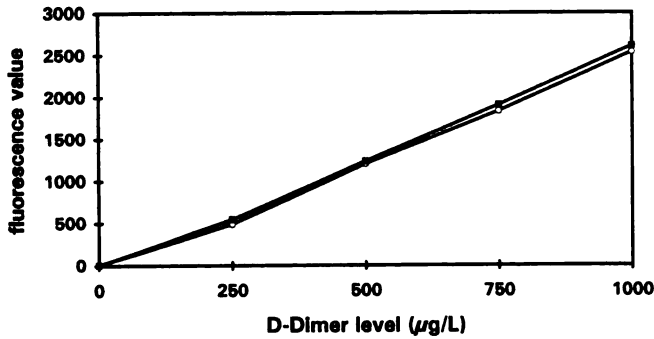

Fig. 2. Cross-reactivity of VIDAS D-dimer assay towards fibrinogen as determined with a D-dimer-containing plasma diluted with the kit diluent (O) or with a D-dimer-free plasma (■).
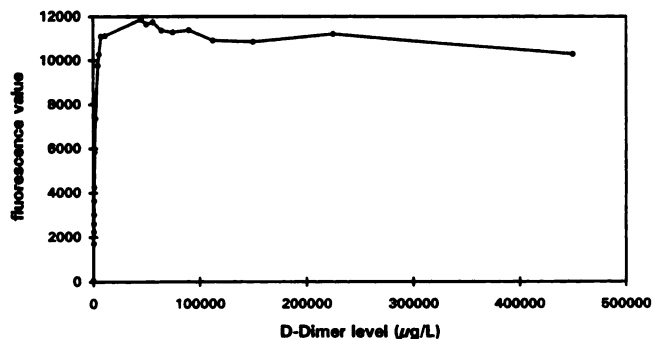

Fig. 3. Effect of increasing concentrations of cross-linked fibrin degradation products on the assay response.

Table 3. Validation of the one-point calibration system recalibrated every 14 days.

Sample[a]	Method A[b]		Method B[c]	
	Mean D-dimer conc, µg/L	CV, %	Mean D-dimer conc, µg/L	CV, %
Patients				
P1	143	11.3	145	10.6
P2	272	6.7	273	5.5
P3	460	5.4	461	6.2
P4	767	4.6	766	5.5
Kit controls				
C1	101	8.4	105	6.9
C2	443	3.1	444	3.5
C3	714	2.6	713	3.4

[a] Each sample and control was assayed singly in 29 successive runs over 8 weeks.
[b] Concentrations determined with an eight-point calibration curve run in duplicate in each assay series.
[c] Concentrations determined with the stored calibration curve, recalibrated once every 14 days with a calibrator run in duplicate.

Table 4. Interbatch reproducibility.

	Batch 1/batch 2	Batch 1/batch 3	Batch 2/batch 3
n	121	101	101
r	0.99	0.98	0.99
Eq.[a]	$y = 1.01 x + 3.5$	$y = 1.06 x - 23$	$y = 1.02 x - 22$

[a] Equations of the standardized principal components lines, where x = values for the first-mentioned batch, y = values for the second-mentioned batch.
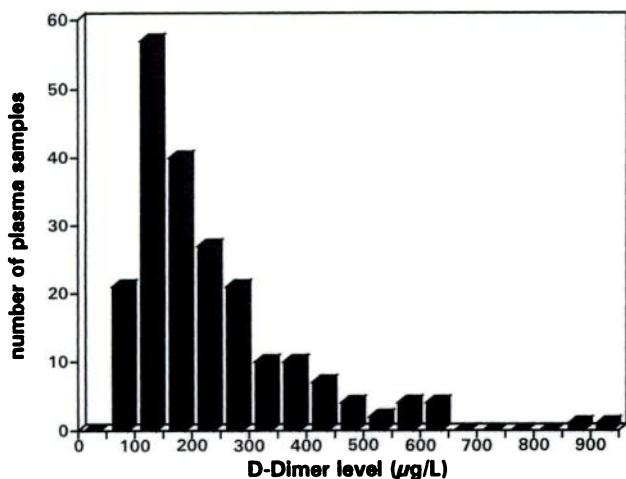
Fig. 4. Normal reference range determined with 199 plasma samples from healthy donors, tested singly with the VIDAS D-dimer reagent.

by a logarithmic gaussian distribution and ranged between 68 and 494 μg/L with an α-error risk of 5% (mean = 207 μg/L).

Correlation with another ELISA. Citrated plasma samples (n = 579) were tested singly with the VIDAS D-dimer method (x) and in duplicate with another ELISA (Asserachrom D-Di; y). Comparison of the two assays by the standardized principal component method yielded r = 0.91, y = 1.35 ($\pm$0.05) x –148 ($\pm$81), and $S_{y|x}$ = 750. The correlation of both methods for concentrations (according to the VIDAS D-dimer measurement) ranging between 0 and 1000 μg/L (n = 404) yielded r = 0.88, y = 1.09 ($\pm$0.05) x – 13 ($\pm$30), and $S_{y|x}$ = 170, which indicates very similar values for both reagents (Fig. 5).

Discussion

The choice of antibodies for a D-dimer assay kit is mainly based on the criteria of minimal reactivity towards fibrinogen and fibrinogen degradation products, such that the assay can be performed on plasma samples with specific detection of cross-linked degradation products [7, 16, 17].

Specificity studies performed with molecules produced in vitro are useful to characterize the antibodies. However, they do not enable characterization of the quality of an assay method.
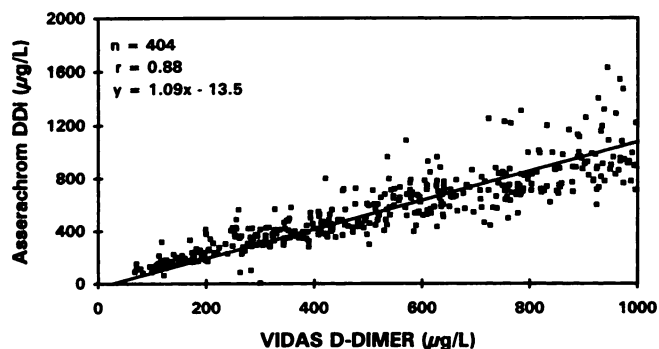

Fig. 5. Comparison of VIDAS D-dimer and a conventional ELISA (Asserachrom D-Di; Diagnostica Stago).

Even if antigens produced in vitro seem identical to those found ex vivo on the basis of their molecular mass, their reactivities often differ according to the methods of production, purification, or storage, and their degree of purity. They are therefore not always representative of the fragments obtained in vivo [7, 18–21].

The methodology used in the assay may also affect the results. In particular, the reaction of an antibody with an antigen may differ substantially, depending on whether the antigen is immobilized or in solution [16, 18]. We therefore chose to perform these assays under conditions most closely resembling those selected for the final reagent. In these conditions, antibody 10B5E12C9, used in immunocapture, shows low reactivity against fibrinogen degradation products (X, Y, and D), whereas antibody 2C5A10 (the labeled antibody) shows a wider specificity. The combination of both antibodies in a two-step ELISA ensures high specificity for D-dimer fragments.

For the assay of plasma samples, we chose a capture antibody with very low cross-reactivity towards fibrinogen. In contrast we gave less importance to the criterion of nonrecognition of fibrinogen degradation products.

Each batch was calibrated with frozen aliquots from eight D-dimer solutions at concentrations ranging between 0 and 1000 μg/L. A plasma bank composed of patients' plasma samples was used to control the validity of the new calibration and the absence of a shift in the concentrations measured.

The use of calibration curves determined with solutions containing fibrin degradation products produced in vitro has proven difficult, leading to interbatch shifts in the D-dimer values of the plasma samples from the plasma bank and hence in the cutoff value to be used with the reagent (not shown). This bias was eliminated, as demonstrated by the excellent correlation between the first three reagent batches, by calibrating on the basis of a pool of plasma samples from patients with thrombotic diseases.

In addition to the unavoidable problems that arise from the differences in antibody reactivity of the various commercial kits [11], these data suggest that standardization of commercial kits with a standard produced in vitro is at present not possible [18, 21].

The results, expressed as fibrinogen equivalents, are comparable with those obtained in a conventional ELISA technique, as shown by the correlation data, especially the standardized principal component line equation. Reference values determined for healthy individuals ranged between 68 and 494 μg/L for the population studied with a 5% risk (95% confidence intervals) and a mean value near 200 μg/L.

The test is particularly adapted to emergency requirements because the use of a single-dose, ready-to-use reagent that does not require daily calibration provides results within 35 min. Automated cycling of the reagent in a thermostatically controlled system shortens incubation times for the different reaction steps while maintaining high analytical performance and, in particular, high reproducibility.

In conclusion, this new reagent for the assay of D-dimer by an ELISA method offers the rapidity, practicability, and analytical

performance required for an emergency test. As demonstrated by two recent studies *[22, 23]*, this test is well suited as diagnostic tool for the exclusion of thromboembolic diseases.

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