the range of the reaction by halving the final concentrations of o-phthalaldehyde and naphthylethlenediamine in the final mixture. With these lower concentrations, useful ranges can be obtained with a 4-μL sample and an incubation time of 4 min 30 s. Because of nonlinearity, for concentrations >250 mmol/L we used a quadratic kinetic method with multipoint calibration. Urea concentration and absorbance at 500 nm are related throughout a wide range of concentrations, 0 to 500 mmol/L, without dilution of the sample and with good sensitivity, 1.6 A for 500 mmol of urea nitrogen per liter.

Results by this method correlate well with those by the diacetyl method involving dialysis, and there is no interference from ammonia.

For determination of urinary creatinine, an aqueous standard is preferred because it more closely resembles urine. We obtained results that compared well with those of the continuous-flow method. We used the "low-hydroxide" method (4), which is rapid (no urine dilution), cost-effective, and easy to perform.

For all these analyses, interferences are those described by the authors. So high concentrations of cations such as Li⁺, Ca²⁺, and Mg²⁺ did not interfere with measurements of Na⁺ or K⁺ (6). Likewise, high ammonia values did not interfere with the RA-XT ion-selective electrode for Na⁺ and K⁺ measurements.

For determination of urinary creatinine, the potential interferents—protein, glucose, acetone, bilirubin, urea, ascorbic acid—did not affect results (4). The specificity of the reaction for urea is comparable to that for the diacetyl reaction except when sulfa compounds are present, but these are rarely used at our hospital. Citrulline and β-ureidopropionic acid (3), which interfere with the present reaction, are rarely present in abnormally high concentrations in urine.

For urinary chloride, interferences by bilirubin and hemoglobin are eliminated by the bichromatic procedure and interference by bromide is negligible in this method. As reported by Law and Ertingshausen (2), ascorbic acid, salicylate, cyclophosphamide, digoxin, t-dopa, and caffeine did not produce any interference at therapeutic dosage concentrations.

We thank Nathalie Bonnet for secretarial assistance.

References

CLIN. CHEM. 35/3, 483–486 (1989)

Interference of Coumarin Therapy with the “Heptest” Owing to Declining Prothrombin Concentrations

Johan Fischer, Bert Verbruggen, Hans Wessels, and Clemens Haanen

The Heptest kit (Haemachem, Inc., St. Louis, MO) for quantifying heparin in plasma is based on heparin-mediated inhibition of factor Xa, resulting in prolongation of clotting time. In 19 of 55 plasma samples obtained from 32 patients concurrently receiving coumarin and heparin, Heptest results exceeded true heparin values by more than 0.2 int. unit/mL; four samples showed a deviation exceeding 0.4 int. unit/mL. We show here that these deviations are caused by coumarin-induced decreases of plasma prothrombin. This problem can be circumvented by adding purified prothrombin or normal plasma to the assay mixture.

Therapy with heparin requires its precise and accurate quantification in plasma, to avoid both inadequate antithrombotic treatment and bleeding complications. Several assay procedures have been developed (reviewed in ref. 1) for determining its concentration in plasma.

The "Heptest," a recently introduced commercial test, is based on the inhibition of factor Xa activity mediated by heparin, ultimately resulting in prolongation of plasma clotting time (2, 3). All relevant clotting factors except for antithrombin III and prothrombin are supplied with the kit. Use of Heptest should be restricted to the determination of heparin concentrations in plasma, because in vitro measurement of anticoagulant activity of both heparin and oral anticoagulants cannot be considered to represent the in vivo anticoagulating status.

Heparinization is usually accompanied by therapy with oral anticoagulant (e.g., coumarins) for long-term treatment after cessation of heparin therapy. A decrease in the concentration of prothrombin—one effect of oral anticoagulants—may interfere with proper measurement of heparin concentrations by the Heptest. In the present study, we compared a chromogenic assay with the Heptest to determine the heparin concentration in plasma samples from patients receiving antithrombotic therapy.

Department of Hematology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Received July 15, 1988; accepted December 30, 1988.
Materials and Methods

Plasma Samples

**Normal plasma.** Blood from healthy volunteers was collected into silicone-coated glass tubes finally containing one volume of anticoagulant per nine volumes of blood. The anticoagulant solution contained, per liter, 109 mmol of trisodium citrate, 10 mmol of theophylline, and 0.4 mg of prostaglandin-E1 (to prevent activation of thrombocytes). After centrifugation (15 min, 8000 x g) the separated plasma was stored in 1-mL portions at -70 °C. Heparin standards ("Thromboliquine"; Dioynth, Oss, The Netherlands) were prepared in this plasma.

**Prothrombin-deficient plasma.** Prothrombin-deficient plasma was obtained from DiaMed, Murten, Switzerland.

**Purified prothrombin.** Lyophilized purified prothrombin (Diagnostica Stago, Asnieres-sur-Seine, France) was dissolved in isotonic saline. For relevant experiments, 10 µL of this solution was added to 100 µL of plasma sample before the Heptest was performed.

**Plasma from patients.** The patients in this study had either deep venous thrombosis or pulmonary embolism. They either were undergoing intravenous heparin therapy (continuous infusions for five days) with concomitant oral anticoagulant treatment, or were at increased risk of (recurrent) thrombosis and therefore were receiving therapy with oral anticoagulant only. The patients received no streptokinase or urokinase. Blood samples were anticoagulated as described for normal plasma and stored at -70 °C.

Procedures

**Heptest.** The Heptest (Haemachem Inc., St. Louis, MO) was performed according to instructions of the manufacturer. Briefly: 0.1 mL of factor Xa solution was added to 0.1 mL of test plasma in a tube. After 120 s, 0.1 mL of "Recalmix" (Ca²⁺, cephalin, and a bovine plasma fraction) was added to this mixture and the clotting time was measured in a KC10 coagulometer (Aemelung GmbH, Lieme, F.R.G.).

**Chromogenic heparin assay.** Chromogenic assays of heparin concentrations in plasma were performed according to Telen and Lie (4). After the patient's plasma sample was diluted with an equal volume of isotonic saline, 10 µL of the diluted sample was mixed with 200 µL of assay medium. The assay medium consisted, per liter, of Tris andimidazole, 15 mmol each, 85 mmol of NaCl, and 10 mmol of K₂-EDTA, adjusted to pH 8.4 with 1 mol/L HCl. One volume of this solution was added to a quarter volume of normal plasma and stored at 4 °C. After the mixture was incubated for 5 min at 37 °C, 20 µL of human thrombin (0.12 int. unit) was added, followed 30 s later by 20 µL of chromogenic reagent (S2398; KabiVitrum, Stockholm, Sweden; 1.25 g/L in water). After 120 s the reaction was stopped by adding 50 µL of diluted acetic acid (equal volumes of glacial acetic acid and water). The net change in absorbance was measured at 405 nm (reference wavelength 492 nm) in an Eor 400 FW Easy Reader (SLT-Labinstrumente GmbH, Groedig/Salzburg, Austria) and the concentration of heparin was calculated from a calibration curve constructed from the results for heparin standards in plasma.

"Thrombotest." The Thrombotest (Nycomed, Oslo, Norway) was performed according to the manufacturer's instructions. Briefly, the procedure is as follows. Pre-warm 250 µL of the reconstituted lyophilized reagent to 37 °C. Add 50 µL of anticoagulated blood (nine volumes of blood plus one volume of 109 mmol/L trisodium citrate), then measure the clotting time (we used a Lode coagulometer; Lode, Groningen, The Netherlands).

Thrombotest measures overall clotting activity and is sensitive to deficiencies of clotting factors II, VII, IX, and X and to the presence of PIVKA's (proteins induced by the absence of vitamin K or by vitamin K antagonists such as oral anticoagulants). In our laboratory we find that the results of the test are not clearly affected by the presence of therapeutic concentrations of heparin in plasma.

**Prothrombin content.** The prothrombin concentration was determined by estimation of the prothrombin time. We prepared the standard curve by using various combinations of prothrombin-deficient plasma and pooled citrated plasma from healthy volunteers (one volume of 109 mmol/L trisodium citrate to nine volumes of blood).

Results

In plasma samples from healthy volunteers to which various amounts of heparin were added (range 0.21-0.92 int. unit/mL), the chromogenic assay showed good correlation with the Heptest (r = 0.972, n = 29). No statistically significant differences were observed (P >0.10, Student's paired t-test). In contrast, estimations of heparin concentration as measured by the Heptest showed values exceeding the results of the chromogenic assay by more than 0.2 int. unit/mL ("deviating plasma samples") in 19 of 55 samples from 32 patients. For four samples the deviation was found to exceed 0.4 int. unit/mL.

**Patients receiving an oral anticoagulant.** To investigate the effect of the oral anticoagulant therapy, we used the Heptest to assay heparin-supplemented plasma samples from patients being treated by oral administration of anticoagulants. Large deviations from the linear standard curve of normal plasma were apparent when the Thrombotest was ≤12% (Figure 1). The results of the Heptest could be virtually normalized by the addition of purified prothrombin to the deviating plasma samples (Table 1).

**Patients receiving oral anticoagulant and heparin.** Table 2 presents results for plasma samples from patients concomitantly receiving heparin and oral anticoagulant. A marked difference was seen between heparin concentrations as measured by the Heptest and the chromogenic assay. The

---

Fig. 1. Effect of depletion of the vitamin K-dependent clotting factors on results of the Heptest

Plasma specimens from patients receiving an oral anticoagulant were supplemented with heparin and incubated for 5 min at 37 °C before assay. Normal plasma, O; plasma with Thrombotest results of 90%, D; 12%, E; 10%, V; 8%, @; 5%.
addition of prothrombin-deficient plasma to the assay mixtures did not influence the overestimation of heparin concentrations by the Heptest, whereas after the addition of purified prothrombin the results obtained with the Heptest and the chromogenic assay were in the same range.

Correction by prothrombin and normal plasma. The effect of the addition of different amounts of prothrombin to a deviating plasma sample from a patient who was receiving an oral anticoagulant and heparin is shown in Figure 2. The concentration of endogenous prothrombin in this plasma was 22% of normal. A normal plasma supplemented with heparin to achieve a clotting time equal to that of the deviating plasma sample without supplementation with prothrombin was measured similarly, to serve as a control. Addition of prothrombin did not influence the Heptest clotting times in normal plasma; this contrasted with results for the plasma of the patient. The results of the Heptest obtained in the same patient's plasma, with the addition of various amounts of normal plasma as a source of prothrombin, are shown in Figure 3. The linear relationship represents the heparin dilution effect exerted by the addition of different amounts of normal plasma. An increasing deviation from the dilution curve occurred at concentrations of normal plasma below 300 mL/L.

Discussion

The concentration of prothrombin in patients' plasma appears to be a factor seriously limiting the confident use of the Heptest for monitoring heparin, at least when oral anticoagulant is given concurrently.

This observation is in direct contrast to the conclusions of Baughan et al. (5), who concluded that oral anticoagulants had a negligible effect on results obtained with Heptest. They evaluated this influence in plasma samples without heparin. Indeed, in the absence of heparin, the maximum effect of oral anticoagulant on the clotting time of the Heptest is equivalent to a heparin concentration of 0.04 int. unit/mL (Figure 1). However, in the presence of heparin, large deviations in clotting time, apparently related to the extent of oral anticoagulation therapy, are detected in comparison with normal plasma (Figure 1). Such a deviation will profoundly affect the interpretation of the test results, when one considers that 10 s corresponds to about 0.06 int. unit of heparin per milliliter (Figure 1, normal plasma). The addition of adequate amounts of prothrombin...
Effect of Hematocrit and Added Heparin on Ionized Calcium in Capillary Blood Samples from Neonates

Nina Nelson,1 Sten Öhman,2 and Lasse Larson2

Sampling of capillary blood for determination of ionized calcium (Ca2+) in neonates requires that extra heparin be added to prevent clotting in the sampling tube and (or) in the Ca2+ analyzer. Because the additive dissolves in the plasma compartment, different hematocrit (erythrocyte volume fraction, EVF) values may cause different results for Ca2+. To study the effect of EVF and heparin additive, we repeatedly removed plasma, thereby increasing the EVF. These samples with different EVF's were aspirated into commercial capillary tubes containing heparin and, according to our routine procedure, an additional 10 μL (~0.9 int. unit) of sodium heparin. We found a negative bias of 0.05–0.09 mmol/L in Ca2+ depending on the EVF. Adding saline instead of heparin gave the same effect, indicating that this bias was entirely due to dilution. We suggest compensating for this by adding 0.09 mmol/L to the actual value for ionized calcium when EVF exceeds 70%. The increase in Ca2+ in neonates on days 1 to 5 postpartum is physiological and not an effect of change in EVF.

Additional Keyphrases: erythrocyte volume fraction, age-related effects, sample handling

To avoid time-consuming and unnecessary resampling owing to coagulation in the tube or in the Ca2+ analyzer, additional heparin is needed to capillary tubes used in determination of ionized calcium (Ca2+) in neonates (1). However, the heparin may alter Ca2+ values (2–5), because of (a) formation of a complex with calcium, (b) a direct effect on components in the analytical equipment such as the electrode, or (c) dilution of the sample. Of these, only the first has been thoroughly investigated (6).

References

CLIN. CHEM. 35/3, 486–488 (1989)

Because heparin dissolves in the plasma compartment, the dilution effect, if any, should depend on the hematocrit (erythrocyte volume fraction, EVF). Newborn infants have high EVF's, which decrease during the first nine weeks postpartum (7), so such a dilution effect may vary during this period. We reported earlier an increase in Ca2+ in neonates during the first five days postpartum (8). To investigate if this was a true physiological phenomenon or simply a result of sample handling, we performed experiments to determine the effect of added heparin and EVF on Ca2+. We also examined the relationship between Ca2+ and EVF in a group of newborn infants.

Materials and Methods

Experimental study. Venous blood from 10 adult volunteers was collected into 10-mL evacuated tubes (Becton Dickinson Co., Rutherford, NJ 07070) containing enough dry sodium heparin to give a final concentration of about 16 units/mL in full tubes. We prepared samples with successively higher values of EVF by centrifugation of the evacuated collection tubes (4200 rpm, 5 min) twice. After each centrifugation we removed 0.7 to 2 mL of the supernatant plasma anaerobically. The tube was then rotated to mix the blood components again before analysis.

Before (stage A) and after (stages B and C, respectively) each centrifugation we determined Ca2+ and EVF in samples taken directly from the evacuated collection tube. The same analytes were also determined in blood from the evacuated collection tube aspirated into two 140–160 μL capillary tubes. These capillary tubes (Radiometer, Copenhagen DK-2400, Denmark) contained ~53 units of dry sodium heparin per milliliter (~8 int. units per capillary tube) plus an additional 10 μL of sodium heparin (87.5 int. units/mL, i.e., ~0.9 int. unit of extra heparin per tube) or sodium chloride solution (0.15 mmol/mL). The initial as well as the extra heparin in the capillary tubes was titrated with calcium chloride.