

## A Colorimetric Assay for Chemical Heparin in Plasma

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A simple, rapid colorimetric assay for plasma heparin is presented. The assay employs the metachromasia of azure A when heparin is added. It is useful for 0 to 10 units/ml and does not depend on heparin's anticoagulant activity. Heparin concentrations determined with this assay are not exactly the same as those determined with coagulation assays. This is probably because azure A determines chemical heparin, not anticoagulant active heparin.

Heparin assays in clinical use have been based on heparin's biological activity as an anticoagulant. These range from the simple whole-blood clotting time to the measurement of the accelerating effect of trace amounts of heparin on the neutralization of activated Factor X by its plasma inhibitor (1). Using the biological coagulation system as an amplifier has permitted the determination of very low levels of heparin (0.01 to 1 unit/ml). Doses of heparin much lower than those causing anticoagulation will release lipoprotein lipases from endothelium, adipose tissue, and liver resulting in dramatic clearance of serum triglycerides (2). This lipolytic activity, however, has not been adapted for the assay of heparin. Assays for chemical heparin have been performed by titrating plasma with protamine or polybrene and using a coagulation assay as an end point (3). The direct chemical assay of heparin has been limited to solutions not containing protein. Dietrich determined heparin degradation products by their absorption at 232 nm after treatment with heparinase (4). Scott described the determination of 2-amino sugars after treatment of heparin with nitric acid (5). Colorimetric reactions with various dyes have also been used in non-

protein-containing solutions for determination of heparin without prior degradation (6). The azure A assay has been proposed as a method for standardization of heparin units (7). Jaques has studied the metachromatic reaction of heparin with azure A and toluidine blue most extensively (8). When determining plasma heparin, however, he employs an extraction process first (9). Estes has stated that the metachromatic assays are not suitable for plasma heparin determination (10), mainly due to the interference of proteins and relatively high ionic strength.

We have used the azure A assay effectively in crude bacterial cell sonicates to follow heparinase production by *Flavobacterium heparinum* (11). This encouraged us to investigate its application in plasma heparin determinations.

### MATERIALS AND METHODS

Azure A, certified biological stain, was obtained from Fisher Scientific Company. Ten grams is dissolved in 100 ml distilled water and gravity-filtered through Whatman No. 5 filter paper. This concentrated solution is stored at 4°C for up to 2 weeks. The concentrated solution is diluted by adding 1 ml

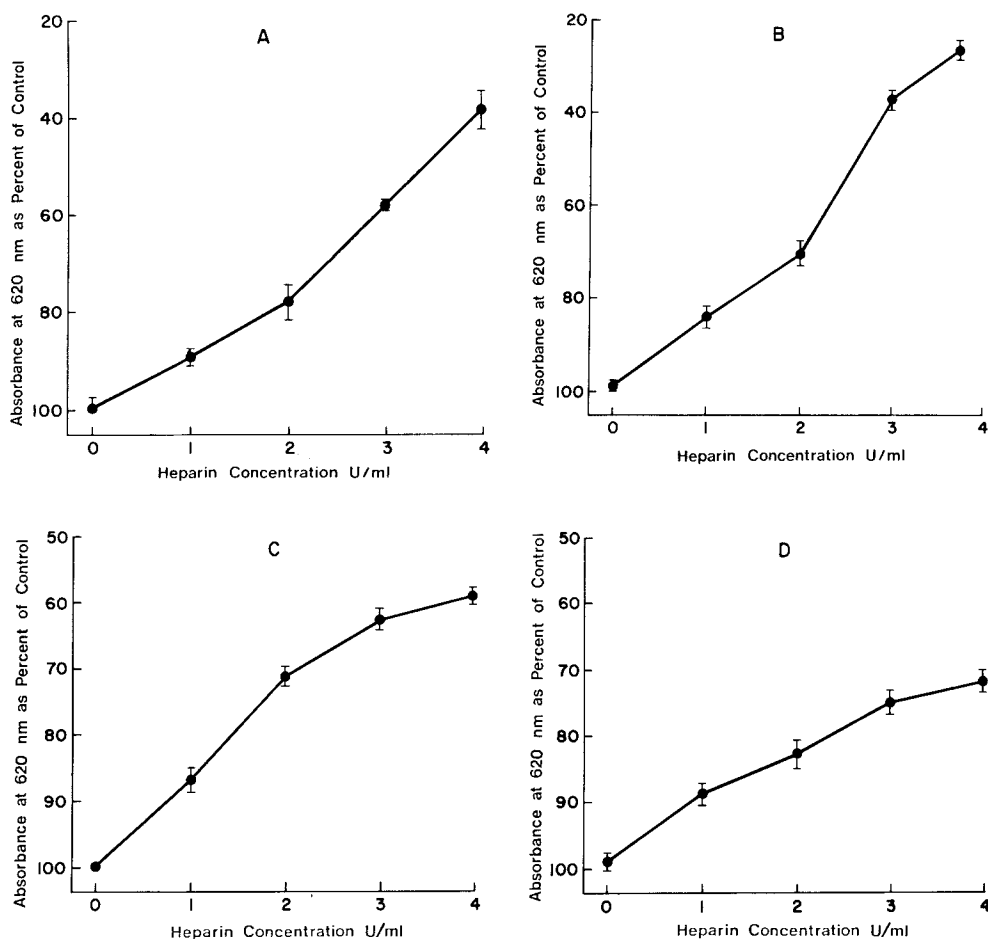


FIG. 1. Standard curves using the 4-unit azure A assay. Five aliquots were assayed from each sample prepared by adding a known amount of commercial heparin to the medium *in vitro*. Limits represent  $\pm 1$  SD. (A) BLH in NS. (B) BLH in 5% BSA in NS. (C) BLH in rabbit plasma. (D) BLH in human plasma.

to 11.5 ml of distilled water resulting in an azure A concentration of 8 mg/100 ml. To perform an assay in the heparin range 0 to 4 units/ml (the 4-unit assay), 1 ml of azure A solution 8 mg/100 ml is added to 1 ml of sample and vortex mixed. This is then read immediately at 620 nm in a Bausch & Lomb Spectronic 88 spectrophotometer. The disappearance of a chromophore is followed so the blank or control consists of the solution (usually plasma) without heparin and the spectrophotometer is set at 1.8.

For an assay with a greater range although less discrimination (the 10-unit assay), concentrated azure A solution is diluted 1 ml in 99 ml of distilled water to yield an azure A solution of 1 mg/100 ml. To perform this assay, 100  $\mu$ l of sample is added to 2 ml of the azure A solution 1 mg/100 ml. This is then vortex mixed and read at 620 nm as in the 4-unit assay.

Wherever feasible, plastic laboratory utensils are used. Glassware is cleaned scrupulously with chromic acid solution by soak-

ing overnight and then washing with water and acetone. The glassware is then siliconized using Prosil-28, PCR Research Chemicals, Inc., Gainesville, Florida.

Beef lung heparin (BLH)<sup>1</sup> was heparin sodium injection USP (beef lung) obtained from the Upjohn Company, Kalamazoo, Michigan, Lot 801HY. Porcine mucosal heparin (PMH) was heparin sodium injection USP (porcine intestinal mucosa) from Organon Inc., West Orange, New Jersey, Lot 2381811B (Liquaemin sodium). Bovine serum albumin (BSA), Fraction V was obtained from Sigma Chemical Company, St. Louis, Missouri. Clinical grade 0.9% sodium chloride injection, USP was used for normal saline (NS) from Abbott Laboratories, North Chicago, Illinois.

The activated partial thromboplastin time (APTT) was determined using the Platelin Activator Assay Kit from General Diagnostics, Morris Plains, New Jersey. Results are reported as the natural log of the APTT in seconds since this represents a more linear relationship between heparin concentration and the APTT (12).

Human plasma was obtained from an informed donor using 3.8 g/100 ml sodium citrate 1:10 as an anticoagulant and centrifugation at 2000 rpm, 4°C for 15 min.

New Zealand White rabbits weighing approximately 2 kg were anesthetized for the animal experiments with rompun, 0.45 mg/kg, subcutaneously, and ketamine, 0.45 mg/kg, intramuscularly. Blood was obtained by catheterization of the right atrium via the right external jugular vein and then diluted 10:1 with 3.8 g/100 ml sodium citrate. Plasma was obtained by centrifugation at 2000 rpm, 4°C, for 15 min. In the *in vivo* experiments, anesthesia was maintained and the catheter was left indwelling in the right atrium for drug administration and blood

<sup>1</sup> Abbreviations used: BLH, beef lung heparin; PMH, porcine mucosal heparin; BSA, bovine serum albumin; NS, normal saline; APTT, activated partial thromboplastin time.

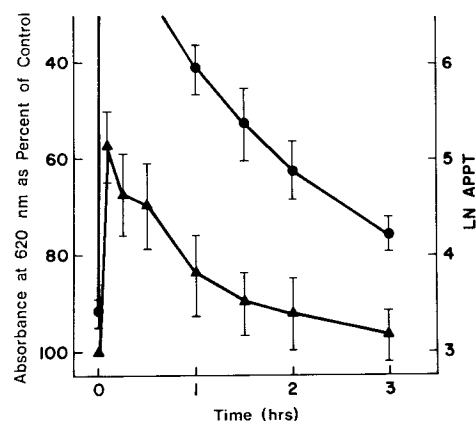


FIG. 2. *In vivo* appearance and disappearance of BLH in rabbits. Six rabbits were given 400 units/kg of BLH intravenously at 1 min. Limits represent  $\pm 1$  SD. Azure A assay (triangles) and ln APTT (circles) were followed.

sampling. An initial blood sample was taken, heparin administered, and then blood samples again taken at timed intervals.

### RESULTS

Standard curves were prepared with BLH in NS, 5% BSA in NS, rabbit plasma, and human plasma. Five aliquots from each tube were determined using the 4-unit assay (Fig. 1). Student's *t* test shows that the differences between each point are significant with *P* values less than 0.05. In a similar experiment assaying known concentrations of heparin every 0.5 unit/ml for a range 0 to 4 units/ml, the difference between each 0.5 unit/ml was always significant with *P* < 0.05. In *in vivo* experiments, 400 units/kg of BLH was administered to six different rabbits and blood samples obtained for determination of the APTT and heparin by azure A assay. These results are presented in Fig. 2. Further *in vivo* experiments are shown in Fig. 3. In separate groups of at least three animals each, either BLH or PMH in doses of 200 and 400 units/kg was administered intravenously. Venous blood was sampled at intervals for 3 h. A standard curve employing BLH in rabbit plasma using the 10-unit as-

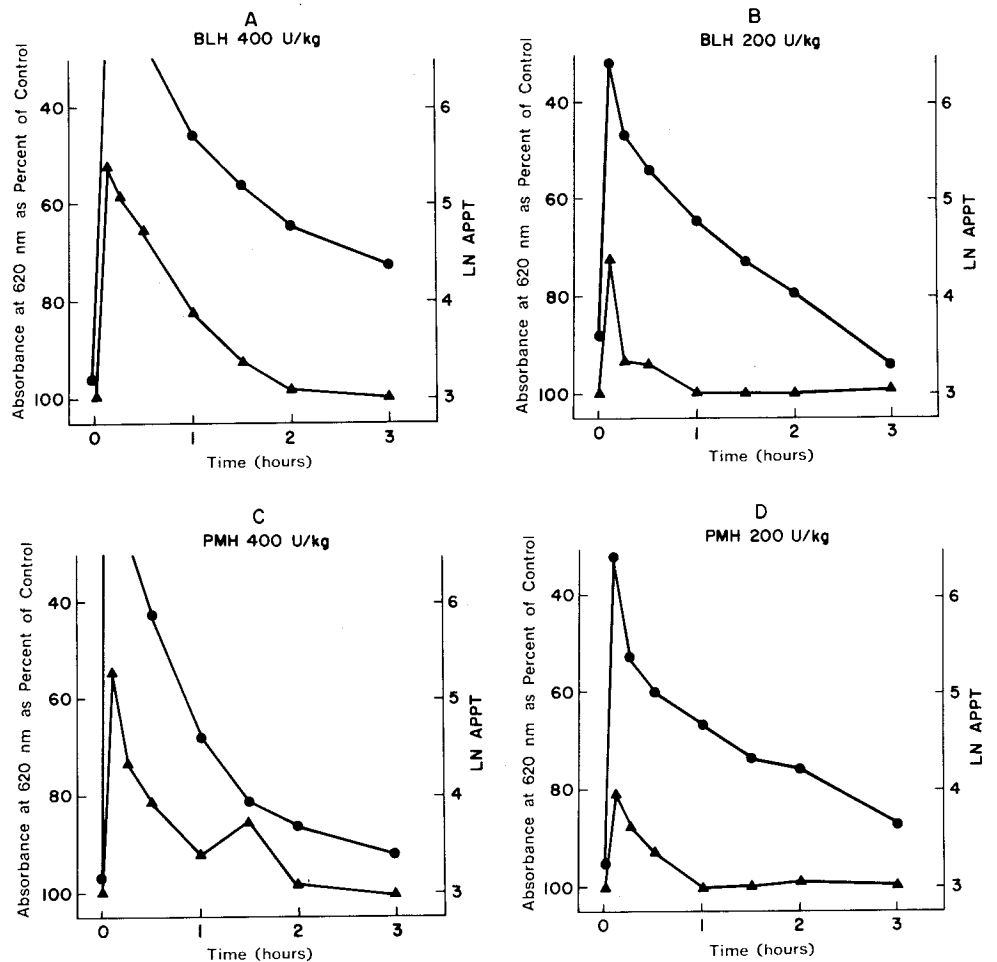


FIG. 3. *In vivo* appearance and disappearance of heparin from two sources at two different doses in rabbits. Each curve demonstrates one representative animal. Azure A assay (triangles) and LN APPT (circles) were followed. Heparin was given intravenously at 1 min. (A) BLH, 400 units/kg. (B) BLH, 200 units/kg. (C) PMH, 400 units/kg. (D) PMH, 200 units/kg.

say is illustrated in Fig. 4. Student's *t* test shows significant differences ( $P$  less than 0.05) between every 2 units of heparin.

#### DISCUSSION

Many investigators using azure A to measure heparin used concentrations of azure A of 10 mg/100 ml and more (6,7,9,13). By using a lower concentration, we obtained less variation in absorbance of the blanks. Other investigators have also examined primarily the change in absorption at 520 nm although

the disappearance of absorption at 620 nm has been noted (13). While following absorbance at 520 nm gave excellent results with heparin in NS and heparin in 5% BSA in NS, both our *in vivo* and *in vitro* assays were not reproducible if we used 520 nm. Attempts to improve the assay with various buffers were futile.

The standard curves in Fig. 1 demonstrate the 4-unit assay's reproducibility in ionic strength of 0.154 (A), in a simple protein solution (B), and in complex plasma systems (C and D). Figure 2 demonstrates the re-

producibility of this assay *in vivo*. The standard deviations are wide, but so are the standard deviations for the APTT. These variations may relate to biological variability in the *in vivo* distribution of heparin between various body compartments (14), but might be due to *in vivo* interconversion of heparin between active and inactive configurations (15). The *in vivo* experiments in Fig. 3 demonstrate the assay's effectiveness for both PMH and BLH at two different doses.

The mechanism of the metachromatic reaction of dyes with heparin has been discussed most fully by Jaques (14). Resonance in the dye molecules allows a partial charge to form on each end of the dimer of the dye. This permits loose interaction with the negatively charged heparin molecule, and thus blocks the amino groups on the dye reducing  $\pi$ -electron delocalization. With less electron mobility the dye molecule absorbs light energy at a shorter wavelength. The same shift can occur with a high salt concentration with no heparin and the dye-polyanion complexes show a critical electrolyte concentration effect. This may explain why buffering negatively affects the assay.

Since the azure A assay performs well in NS and in 5% BSA in NS, a part of the coagulation system is not involved in the reaction. The assay appears to be a direct chemical assay which can determine heparin both free and protein bound in plasma.

The difference in the heparin disappearance curves followed by APTT and azure A in the *in vivo* experiments is interesting. While the curves are similar, azure A shows earlier return of heparin levels toward baseline. This indicates that the azure A assay is measuring something different from the APTT; perhaps heparin amount and not heparin effect. It might also mean that a particular biologically active form of heparin, perhaps that bound to antithrombin III, is not determined by the azure A assay. Or it may be, as suggested by Levy and Jaques (16), that heparin is converted *in vivo* to a more active form. Heparin molecules may

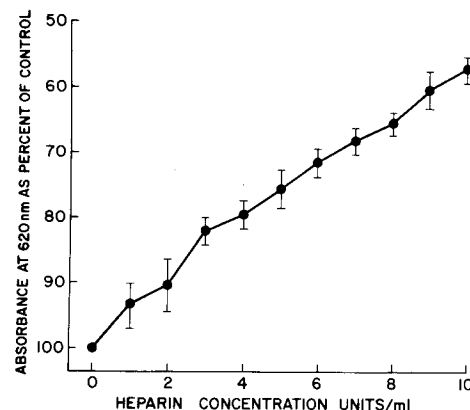


FIG. 4. Standard curve using the 10-unit azure A assay. Five aliquots were assayed from each sample prepared by adding a known amount of commercial BLH to rabbit plasma *in vitro*. Limits represent  $\pm 1$  SD.

be in a particularly inactive configuration *in vitro* as determined by the manufacturer, and are then converted *in vivo* to an active configuration. Heparin itself might cause a change in the proteins of the coagulation system which persists after the heparin has disappeared. Investigation of these hypotheses could provide some answers to questions concerning the fate of intravenous heparin.

The azure A assay's advantages over other chemical assays are its simplicity and rapidity. Its advantage over coagulation assays in clinical use is its ability to rapidly determine high concentrations of heparin.

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