THE ESTIMATION OF HEPARIN IN BLOOD

BY

M. BASSIOUNI

From the Special Unit for Juvenile Rheumatism, Canadian Red Cross Memorial Hospital, Taplow, Bucks

(RECEIVED FOR PUBLICATION AUGUST 18, 1952)

The estimation of heparin in blood by a quantitative chemical method has been attempted by Jaques (1939), Jaques, Monkhouse, and Stewart (1949), and Monkhouse and Jaques (1950), but several difficulties were encountered. The method of Charles and Scott (1933) needs a very large quantity of blood and is not practical for use in man or small laboratory animals.

A method has therefore been devised which gives satisfactory results with 2 ml. of blood: it is based on a preliminary extraction of heparin from plasma with alkali, then removal of proteins by ammonium sulphate and heat, and finally precipitation of heparin with a basic dye (Holmgren and Wilander, 1937; MacIntosh, 1941), which is then estimated colorimetrically.

Methods

Reagents.—The following are required:—

- (1) *Sodium citrate*, 3.8 g./100 ml.
- (2) *Sodium hydroxide*, 2N.
- (3) *Ammonium sulphate*, 5.5 g.
- (4) *Dye solution*, dimethyl thionin or Azure A (Revector, Hopkins & Williams, Ltd.), 100 mg. dissolved in 100 ml. distilled water.
- (5) *Standard heparin*, calcium salt of heparin 99 units/mg. (Evans).
- (6) *Ammonium sulphate solution*, 90% saturated at room temperature.
- (7) *Buffered solvent*, 6 ml. made up from 2 ml. acetone, 2 ml. distilled water, 2 ml. glycine-sodium hydroxide buffer consisting of 20 parts of molar glycine solution and 80 parts of normal sodium hydroxide solution.

Extraction.—Blood, 2 ml., is drawn, added immediately to 1 ml. 3.8% sodium citrate, and centrifuged at 2,000 r.p.m. for 10 minutes; the plasma is then pipetted off, the cells washed with 2 ml. saline, and the wash added to the plasma. The plasma is made up to 6 ml. with normal saline in a conical pyrex flask of 25-ml. capacity; 1 ml. 2N NaOH is added, the flask is corked and warmed at 50° C. in a hot water-bath for 40 minutes, 5.5 g. ammonium sulphate is then added, the flask recorked, shaken vigorously, and left for 20 minutes: it is then warmed quickly in a boiling water-bath to 75° C., vigorously shaken

again and transferred rapidly to fluted filter paper (Whatman No. 4, 9 cm. diameter). The filtrate is received in a thoroughly cleaned test tube (16-ml. capacity) and is made up with about 7 to 8 ml. of distilled water to 15 ml., i.e., a final 50% saturated ammonium sulphate solution. This is then transferred to a vacuum pump and the pressure reduced gradually to remove the hydrogen sulphide and ammonia which interfere with the next step. After all gas is driven off the filtrate is made up to 15 ml. again with distilled water, avoiding splashing.

Preparation of Standard Solutions and Blank.—A blank is prepared with 7.5 ml. saturated ammonium sulphate, 1 ml. 2N NaOH, and 6.5 ml. distilled water.

Standards are prepared with solutions of standard heparin containing concentrations ranging from 0.01 mg. to 0.06 mg. per ml. To 1 ml. of 2N NaOH, 1 ml. of the standard heparin solution is added together with 5.5 ml. distilled water and 7.5 ml. saturated ammonium sulphate, giving 15 ml. of 50% saturated ammonium sulphate solution and with the same pH as the unknown. The solution is heated to 75° C., gas bubbles quickly removed *in vacuo*, and made up as before to 15 ml.

Precipitation of Heparin and Estimation of Heparin-dye Complex.—To all tubes 0.3 ml. exactly of dye solution is added, mixed, the tubes covered with caps, and left for 48 hours at room temperature. The tubes are centrifuged while capped for 45 minutes at 3,000 r.p.m., then the supernatant fluid is pipetted off by a fine pipette attached to the suction pump. This should be done cautiously so as not to disturb the precipitate at the bottom. Then 2 ml. of 90% saturated ammonium sulphate is added to the precipitate, it is shaken, and the tubes centrifuged (capped to prevent evaporation) at 3,000 r.p.m. for 20 minutes. The supernatant fluid is pipetted off and 6 ml. of acetone buffer added to dissolve the precipitate.

Estimations are made electrocolorimetrically in a double-cell absorptiometer using green-blue filter No. 623. A calibration curve is made from the readings of the standards, subtracting the blank reading from each, and taking the mean of four readings.

Results

Recoveries.—Using 2 ml. amounts of plasma, to which heparin is added in amounts from 0.01 mg. to 0.15 mg., about 90% of the added heparin is recovered (Table I and Fig. 1).

TABLE I
RECOVERIES OF ADDED HEPARIN

Amount Added (mg.)	Amount Recovered (%)
0.01	96
0.01	100
0.02	92
0.02	90
0.03	67
0.03	90
0.04	80
0.04	85
0.05	88
0.05	86
0.06	96
0.07	100
0.150	92

Normal Blood Levels.—Estimations of heparin-like substances in blood from 10 apparently healthy individuals are shown in Table II. Owing

TABLE II
HEPARIN LEVELS IN TEN APPARENTLY NORMAL INDIVIDUALS

	Age	Sex	Mg./100 ml. Blood
1	39	M	2.3
2	44	F	1.2
3	42	M	1.6
4	10	F	1.0
5	60	F	0.25
6	25	F	1.7
7	40	M	0.3
8	30	F	0.5
9	35	F	1.7
10	28	M	2.2 2.3 2.0

* Three samples of the same specimen.

to the fact that other substances such as chondroitin sulphuric acid can be precipitated by the dye, true heparin levels in blood may be lower than these figures.

Heparin Levels in Blood after Intravenous Injection.—Following the intravenous injection of 7,500 international units (Pularin Evans) in an adult weighing 75 kg., the highest level of heparin was found at the five-minute reading, thereafter declining gradually to reach the normal level again after two and a half hours (Fig. 2).

Summary

A method is described for the extraction and estimation of heparin in 2 ml. of blood.

Adequate recoveries were obtained from amounts added to plasma ranging between 0.01 and 0.15 mg. per 2 ml. of plasma. The estimation of apparent heparin in blood of apparently

FIG. 1

FIG. 2

healthy individuals shows amounts varying from 0.25 mg. to 2.3 mg./100 ml. The real values of heparin in blood may be lower as other inactive, heparin-like substances may be precipitated by Azure A from blood.

The intravenous injection of 7,500 international units of heparin gave a maximum level at the first reading taken after five minutes, declining gradually until the pre-injection level was reached by about two to three hours.

I wish to express my gratitude to Drs. E. G. L. Bywaters and L. E. Glynn for encouragement and helpful criticism, and to Mr. Reading for technical assistance. Evans Laboratories kindly supplied the standard heparin.

REFERENCES

- Charles, A. F., and Scott, D. A. (1933). *J. biol. Chem.*, **102**, 431.
 Holmgren, H., and Wilander, O. (1937). *Z. mikr.-anat. Forsch.*, **42**, 242.
 Jaques, L. B. (1939). *Amer. J. Physiol.*, **125**, 98.
 ———, Monkhouse, F. C., and Stewart, M. (1949). *J. Physiol., Lond.*, **109**, 41.
 MacIntosh, F. C. (1941). *Biochem. J.*, **35**, 776.
 Monkhouse, F. C., and Jaques, L. B. (1950). *J. Lab. clin. Med.*, **36**, 782.