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Sodium Heparin Determination: Comparison of an Instrumental Method with the USP Method

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Abstract □ An instrumental method for determining sodium heparin in aqueous solutions is described that has the advantages over the USP grading procedure of being simpler and quicker but just as reproducible. It is based on the principle that heparin concentration in solutions can be determined by measuring the recalcification clot time of heparinized sheep plasma by mechanical means. The concentration is read from a standard curve of clot time *versus* concentration of standard heparin.

Keyphrases □ Heparin—determination using instrumental method, compared to USP method □ Sodium heparin—determination using instrumental method, compared to USP method □ Blood clotting time—used in instrumental method for determination of sodium heparin in aqueous solutions, instrumental method compared to USP method

The USP (1) procedure for sodium heparin assay is rather cumbersome in both operation and calculation. This paper describes an instrumental method which offers ease of operation, calculation, and reliability.

The USP procedure is based on the increase in recalcification clotting time of sheep plasma with increasing concentration of heparin. The test is performed by comparing grades of clotting in assay samples with grades of clotting in a set of standards. Both sets of samples must be prepared within 20 min of each other, and the end-points are checked 1 hr after addition of the calcium chloride reagent.

With the instrumental method, standardized sheep plasma is used to develop a standard curve of known sodium heparin concentrations plotted against clotting time measured by the instrument. From this standard curve, sodium heparin concentrations can be read directly using clotting time obtained with assay samples.

The instrumental method differs from the USP method by measuring the time at which the clot forms rather than by grading the extent of clotting after 1 hr. The principle of the instrument is shown in Fig. 1.

EXPERIMENTAL

Each test vial contains a magnetic stainless steel ball. A drive motor moves the vial up and down in the reaction well. Before a clot forms, the stainless steel ball is held stationary by two calibrated permanent magnets as the vial moves. While in this stationary position, the ball interrupts a light beam directed through the vial at a photocell. As the vial moves up and down relative to the suspended ball, the test fluids flow back and forth around the ball, ensuring continuous and uniform mixing. When calcium chloride solution is added to the moving vial, an automatic timing device is activated. When clot formation occurs, the ball is pulled out of the magnetic field. Displacement of the ball more than 0.8 mm in either direction from its original suspended position permits the light beam to strike the photocell, stopping the timer.

The nominal bore diameter for the vial is 0.505 cm. The spherical nominal diameter of the stainless steel ball is 0.475 cm, having a weight of 0.4 g. The nominal clearance of the ball suspended in the vial is 0.015 cm on either side. Silicone oil standards of varying viscosities are used to factory calibrate each instrument. In brief, a master clot timing instrument is initially set up by using the most viscous plasma sample obtainable. A set point for the magnetic field is established with the viscous plasma. Silicone oil standards are prepared with the master instrument, which enable future adjustments to be made on subsequent production instruments.

One silicone oil standard has a viscosity that enables the photocell system to trip intermittently a timing motor relay circuit. This

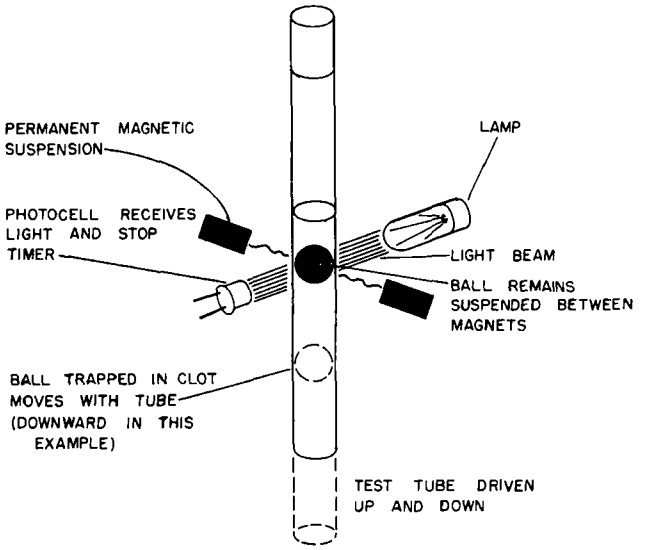

Figure 1—*Instrument for measuring the time at which blood clot forms.*

indicates that the magnetic field is weak enough. Another standard is made with a viscosity that does not enable the photocell system to trip the relay. This then indicates that the magnetic field is not too weak. The movement of the vial is two complete excursions per second with an absolute timing accuracy of ±0.2%

Apparatus—The following were used: a clot timing instrument[1] equipped with autoprecision pipeter, test vials of 5.0-ml capacity containing magnetic stainless steel balls, disposable plastic pipeter tips, graduated 2.0-ml pipets, and Whatman No. 1 filter paper. All glassware, stainless steel balls, and pipeter tips must be meticulously clean. If reused, they should be acid cleaned and thoroughly rinsed.

Reagents—The following were used: USP heparin sodium reference standard; normal saline, 9.00 g NaCl in distilled water, diluted to 1 liter; 0.02 *M* $CaCl_2 \cdot 2H_2O$[2]; and frozen sheep plasma[3], prepared for heparin assay in aliquots. All reagents must be equilibrated to 37° before use.

Standardization of Sheep Plasma—Each lot of sheep plasma exhibits a different clotting time and must be standardized against sodium heparin reference material. This was done by testing the sheep plasma against several concentrations of standard sodium heparin solution. The goal was to determine the concentration of sodium heparin that will give clotting times with the sheep plasma in the range of 100–150 sec. This clotting time range falls on the linear portion of the curve relating log of clotting time in seconds against sodium heparin concentration in units per milliliter.

The standardization procedure employed freshly thawed sheep plasma filtered through Whatman No. 1 filter paper and a sodium heparin standard solution prepared on the day of the assay. USP heparin sodium reference standard was made with normal saline to

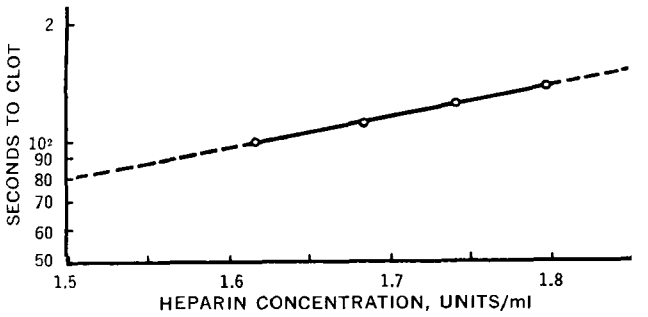

Figure 2—*Preparation of heparin standard curve.*

[1] Clotek system, Hyland Laboratories, Inc., Glendale, Calif.
[2] Chemical reagents are supplied with the Hyland Clotek system.
[3] Wilson Laboratories, Park Forest South, IL 60466

Table I—Comparison of Clot Timing Method with USP Method Using Range of Concentrations

Theoretical Concentration, Units/ml	Clot Timing Method, Mean[a] Values, Units/ml	USP Method, Mean[a] Values, Units/ml
68.38	66.15	66.22
70.13	70.36	68.72
72.07	73.55	69.56
74.20	75.51	71.08
76.15	77.04	73.38
78.03	78.74	76.07
80.14	81.50	80.90
82.16	83.70	82.64

[a] Mean of two determinations.

a concentration of 3 USP units/ml. Several dilutions of this sodium heparin solution were made to achieve the desired concentrations; a concentration of 1.80 units/ml gave a clotting time of 133.5 sec. This solution was further diluted as discussed under *Assay Procedure* to construct a standard curve.

Instrumental Operation—Empty test vials are placed in the reagent well containing one stainless steel ball each. Using the automatic pipeter, 0.1 ml of sheep plasma and 0.1 ml of a sodium heparin solution were added. The disposable tip was changed, and 0.1 ml of 0.02 *M* $CaCl_2$ was added while completely depressing the pipeter button to activate the timer. When the timer stopped, the time in seconds was automatically recorded.

Assay Procedure—Three dilutions were made from the heparin stock solution, described under *Standardization of Sheep Plasma,* which had a clotting time of 133.5 sec. Each standard dilution (1.80, 1.74, 1.68, and 1.62 units/ml) was tested twice with 0.1 ml sheep plasma. When plotted against the log of time in seconds (Fig. 2), a straight line was obtained for the clotting time range of 100–150 sec.

Test samples were diluted appropriately to bring their concentrations within the range of the standard curve. A 0.1-ml aliquot of the diluted sample was added to the test vial, and clotting time was determined with 0.1 ml sheep plasma. From the standard curve, the sodium heparin concentration of the test sample was calculated as follows: sodium heparin units per milliliter in sample equals sodium heparin units per milliliter in standard (from curve) times dilution of sample.

Equivalency—Eight samples of sodium heparin solution were prepared in duplicate spanning concentration ranges used in commercial sodium heparin blood collection units. These samples were assayed by both the USP and instrumental methods. The means and theoretical values for each sample are given in Table I.

In addition, 12 production batches of sodium heparin blood collection units were assayed by both methods. The results are given in Table II.

Reproducibility—Reproducibility of the clot timing instru-

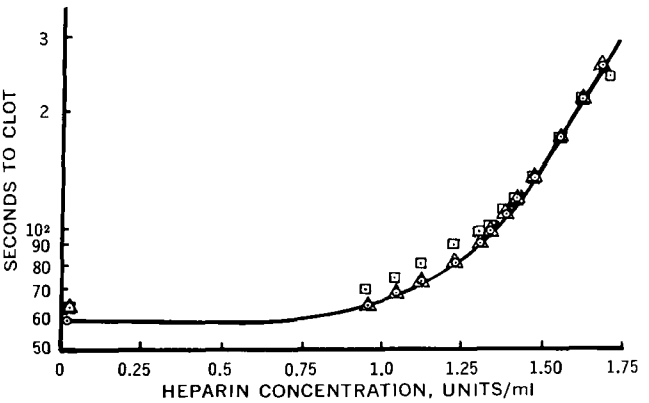

Figure 3—*Reproducibility of clotting times using the clot timing instrument. Key:* ⊡, *Stock Solution I tested on day stock solution was prepared;* ⊙, *Stock Solution II tested on day stock solution was prepared; and* ◬, *Stock Solution I tested 24 hr after stock solution was prepared.*

Table II—Comparison of Clot Timing Method with USP Method Using Production Batches at 75.0 Units/ml

Batch	Clot Timing Method			USP Method		
	Number of Determinations	Mean ± *SD*, Units/ml	Range	Number of Determinations	Mean ± *SD*, Units/ml	Range
1	7	78.66 ± 0.88	77.40–79.79	4	80.38 ± 5.36	75.80–83.06
2	5	79.38 ± 1.17	78.56–81.41	4	78.91 ± 3.05	75.80–81.53
3	5	79.68 ± 0.60	79.08–80.45	4	77.75 ± 3.86	73.90–82.80
4	7	77.09 ± 0.58	76.27–78.00	4	74.44 ± 2.68	71.07–76.60
5	6	76.71 ± 0.25	76.40–76.99	4	71.30 ± 4.36	66.90–75.14
6	5	71.89 ± 0.46	71.27–72.45	4	71.68 ± 2.64	69.06–75.19
7	5	70.80 ± 0.67	70.22–71.81	4	69.55 ± 2.36	66.80–71.83
8	8	73.48 ± 0.86	72.27–74.67	4	70.21 ± 1.87	67.52–71.53
9	5	74.57 ± 0.78	73.53–75.33	4	70.98 ± 1.02	69.87–72.13
10	8	73.55 ± 1.31	71.80–75.27	4	76.44 ± 1.35	74.73–77.67
11	7	74.05 ± 2.21	70.47–76.13	4	73.80 ± 1.02	72.37–74.57
12	8	75.23 ± 0.82	74.27–76.87	4	72.72 ± 0.91	71.85–73.87

Table III—Clot Timing Assays

ml Stock Solution/100 ml in Saline	Heparin Concentration[a], Units/ml	Stock Solution I[b] Dilutions, sec	Stock Solution I[c] Dilutions, sec	Stock Solution II[b] Dilutions, sec
Blank (saline)	0.0	61.0	61.0	58.8
1.30	0.921	67.9	64.4	64.6
1.50	1.063	74.5	70.8	70.5
1.60	1.134	80.5	75.1	75.8
1.70	1.205	—	—	—
1.75	1.240	88.8	85.0	86.8
1.80	1.276	—	—	—
1.85	1.311	97.2	95.5	96.0
1.90	1.347	103.4	103.2	102.6
1.95	1.382	110.4	111.5	112.0
2.00	1.418	120.8	120.4	119.6
2.10	1.488	140.0	141.8	141.9
2.20	1.559	166.9	167.7	168.9
2.30	1.630	206.7	207.7	—
2.40	1.701	243.2	265.5	—

[a] Stock Solutions I and II each contained 113.3 mg/250 ml in normal saline using USP heparin sodium reference standard (156.4 units/mg). [b] Tested on day when stock solution was prepared. [c] Tested 24 hr after stock solution was prepared.

ment method was established by preparing two stock solutions of sodium heparin (I and II) from which several dilutions of each were prepared with normal saline. These dilutions provided a final concentration range of 0.921–1.70 sodium heparin units/ml (Table III). On the day of preparation, both sets of diluted stock solutions were assayed by the clot timing instrument (Table III and Fig. 3). Twenty-four hours later, the instrumental assays were repeated on a second set of stock solutions made from Stock Solution I (Table III and Fig. 3).

RESULTS AND DISCUSSION

Table I shows that the estimates of reproducibility have 8 degrees of freedom each [(2 − 1) × 8]. The within-standard deviation for the USP method is 0.98, and it is 0.59 for the instrumental method. These values are not significantly different from one another, and the methods compare favorably in terms of reproducibility.

Table II shows that the clot timing instrument gave slightly higher values for sodium heparin concentration except for one instance. The differences between the two methods are significant for the eight samples in Table I and for the total of the eight in Table I plus the 12 in Table II in terms of accuracy. The mean difference between the two methods was 1.75 units or 2.3% at 75 units/ml. The instrumental method tended to overestimate the theoretical but not significantly (0.67 unit). The USP method underestimated the theoretical (1.59 units), giving a value significantly different from the zero bias.

The data in Table III show that the clotting time increases nonlinearly with increasing heparin concentration. Since it is desirable in a regression analysis that variability be equal at all concentrations, the log of time was analyzed as the dependent variable with heparin concentration as the independent variable. This log transformation equalized the variation, although it did not completely linearize the data for all heparin concentrations (Fig. 3). However, as seen in Fig. 2, the curve was linear with heparin concentrations that produced clotting times of 100–150 sec, and this portion was used as the "working curve."

Figure 3 indicates a small shift where stock dilutions were analyzed on succeeding days, but the average difference between the assays on the 2 days was not different from the zero bias.

The instrumental method required fewer and simpler dilutions and a shorter incubation period than the USP method. The instrumental assays were completed in about 25% of the time needed for the USP method.

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