tion mixture were separated as methyl esters employing Amberlite IRA-400. Yields of the neutral and acidic fractions (isolated as methyl esters) were 197 mg. and 22.1 mg., respectively. The neutral fraction was purified by chromatography on grade I alumina (5 Gm.) when a colorless hydrocarbon fraction (72 mg.) was obtained.

Employing Metal Catalysts—The experiment was repeated with the crude acid (250 mg.), employing 5% Pt on alumina (50 mg.) as the dehydrogenating agent. Analogous processing of the reaction mixture yielded a neutral oil (148 mg.) and methyl esters (41 mg.). Chromatography of the neutral fraction on alumina gave hydrocarbons (58 mg.). I.R. and U.V. analyses showed the hydrocarbon fraction did not undergo any chemical change on further refluxing with 5% Pd-on-carbon (31 mg.) for 2 hr. at 320–350°.

Characterization of the Hydrocarbon Fractions Obtained by Dehydrogenation Experiments—Similar hydrocarbon fractions (I.R. spectroscopy and gas chromatography) were obtained in each of the dehydrogenation experiments. The main constituent (79%) of the hydrocarbon fraction obtained by Se-dehydrogenation of the acids was isolated by gas chromatography on a Reoplex 400 column. It possessed the following characteristics: relative retention times on Reoplex 400 (column temperature, 200°) and SE-30 (column temperature, 185°): 0.49 and 1.76, respectively (reference standard: naphthalene; retention times: 6.70 min. and 1.65 min., respectively). NMR peaks at 1.47, 1.02, and 0.98 δ. Mol. wt. (mass spectrum) 190.

Ozonolysis of Methyl Khusenate—Methyl khusenate (1.00 Gm.) was dissolved in ethyl acetate (5 ml.), cooled to 0°, and ozonized using a Gallenkamp ozone apparatus GE-150. The ozonide was decomposed with hydrogen in the presence of Pd-C and the product treated with Amberlite IRA-400 (5 Gm.) in accordance with the procedure described above. The neutral fraction (918 mg.) obtained was purified by chromatography on grade III alumina (50 Gm.) using hexane–benzene mixture (1:1) as solvent and recrystallized from ethanol. Yield: 52% (by GLC), m.p. 102–103°.

Anal.—Calcd. for $C_{15}H_{22}O_3$: C, 71.95; H, 8.86. Found: C, 71.97; H, 8.87.

Infrared absorption bands (CCl_4 solution) at 1728, 1708, 1458, 1430, 1388, 1356, 1300, 1195, 1164, and 1042 cm.$^{-1}$.

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Drug Standards

New Electromechanical Method for the Assay of Heparin *In Vitro*

By WILLIAM F. SEIP, THEODORE R. CARSKI, and DAVID N. KRAMER

A rapid, simple, and accurate *in vitro* assay for heparin employing a diatomaceous silicate modified plasma and a precision coagulation timer is reported. The method reveals standard deviations of ±2.5 sec. from the average clotting times with a coefficient of variation of about 2 per cent. Sodium heparin U.S.P., heparin reference standard lot H, and the second international standard for heparin have been successfully assayed by this method. The assays have also revealed the stability of sodium heparin to autoclaving and high energy electron irradiation.

WALTON *et al.* (1) have recently reviewed current methods of heparin assay. One of the most commonly employed methods is that of Reinert and Winterstein (2) which is the basis for the U.S.P. heparin assay procedure. A second method is that of Adams (3) which is prescribed by the British Pharmacopoeia. There exist numerous other methods which are essentially modi-

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fications of the original Howell procedure (4) upon which the various current heparin activity units are based.

Elliott (5) reported the use of a coagelometer in the determination of heparin concentration. A linear relationship was observed at heparin concentrations of 0 to 2.0 mcg./ml. of whole blood with coagulation times varying from 12 to 30 min. The coagelometer employed, based on a photoelectric readout of the coagulation, was complex and no details were given as to the reproducibility and reliability of the method employed. No data were presented on the assay of heparin employing plasma.

The accepted procedures employed in the assay of heparin are generally time-consuming (a few hours to several days) and subjective to a large extent. It was considered desirable to simplify the assay procedure and to obtain a more precise end point determination by detecting clotting times by the use of the Fibrometer[1] precision coagulation timer. Recently Barnett and Pinto (6) and Fletcher *et al.* (7) presented data describing the performance of the instrument in the determination of coagulation times.

Attempts to employ the standard U.S.P. heparin assay procedure using the precision coagulation timer were unsuccessful in our hands.

The use of a diatomaceous silicate[2] in the partial thromboplastin time assay by Struver and Bittner (8) prompted an investigation of the effect of this "activating" agent on plasma clotting times. As a result, the necessary conditions for a simple and reliable assay for heparin activity were obtained by modification of the U.S.P. assay procedure by the addition of the diatomaceous silicate. This report summarizes the new *in vitro* assay procedure, its assets, and limitations.

EXPERIMENTAL

Apparatus—The precision coagulation timer and standard accessory equipment were employed. The automatic pipet was set to deliver 0.1 ml. The thermal Prep-Block precision heating unit was used to provide a constant supply of disposable plastic Fibrotube[1] cups preheated to 37 ± 0.5°.

Freezing point depression readings were obtained on 0.9% sodium chloride solution using a Beckman molecular weight apparatus to assure isotonicity.

Chemicals and Reagents—Dry sodium heparin preparations were obtained from three sources; these preparations were stored at −20° in their original containers over desiccant; the units/mg. are their assigned potencies.

(*a*) Second international standard for heparin, described by Bangham and Mussett (9), from the National Institute for Medical Research, Mill Hill, England: 130 units/mg.

(*b*) U.S.P. sodium heparin reference standard; lot H, U.S.P., New York, N.Y.: 2.8 units/mg.

(*c*) Sodium heparin; Pharmaceutical Division, Wilson Laboratories, Chicago, Ill: 143 units/mg.

A stock diatomaceous silicate reagent was made by suspending 1.5% of the diatomaceous silicate (w/v) in 0.025 *M* calcium chloride in distilled water and stored at room temperature.

Citrated plasma was obtained by bleeding healthy sheep as directed under *assay* in the U.S.P. monograph entitled "Sodium Heparin" (10). Various plasma lots, from individual sheep, were obtained in approximately 100-ml. volumes and stored in the frozen state at −20°. The 100-ml. volumes of plasma were subsequently thawed, thoroughly mixed, subdivided into 10-ml. portions in screw-capped borosilicate vials, and stored at −20°.

Assay Solution Preparations—A fresh heparin sample (tablet or powder) was used for each assay. Dry heparin was weighed and appropriate dilutions made in 0.9% sodium chloride for stock concentrations as follows: 0.33, 0.36, 0.39, 0.42, 0.45, and 0.48 units/ml., according to labeled potency. The heparin assay solutions were maintained at room temperature during the assay period.

A 10-ml. portion of frozen plasma was thawed, mixed, brought to 37°, and decanted into 10-ml. borosilicate glass-stoppered volumetric flasks, then placed for use in an ice bath at 0°. A freshly thawed 10-ml. portion of plasma was prepared daily for each assay. One 10-ml. portion is more than sufficient to complete an assay as presented in Table III.

The diatomaceous silicate reagent was maintained at 37° ± 0.5° in a water bath.

Clotting Time Assay—The control clotting time (CCT) of citrated sheep plasma was determined by delivering 0.1 ml. of 0.9% sodium chloride into each of three or four disposable plastic cups which were then equilibrated at 37° for 3 min. or longer. One-tenth milliliter of plasma was then added to the first plastic cup placed in the center well of the coagulation timer. Precisely at the end of 30 sec., 0.1 ml. of the diatomaceous silicate reagent, preheated to 37° ± 0.5° and well mixed by a magnetic stirring bar, was delivered, with moderate force, into the 0.9% sodium chloride–plasma mixture and the timer started automatically.

When the coagulation timer digital read-out mechanism stopped, the probe assembly was immediately removed, the probes rinsed with fresh 0.9% sodium chloride, brushed gently with a soft-bristle toothbrush, rinsed once again, and blotted dry. The clotting time, in seconds, was recorded. The digital timer was reset to zero and the probe assembly reinserted into the instrument and the next determination immediately made using the second plastic cup containing 0.1 ml. of 0.9% sodium chloride. The procedure was repeated again until three or four determinations were obtained. Each determination required approximately 4 min. to complete.

For the assay of heparin standards and unknowns 0.1-ml. volumes of the various heparin concentrations were substituted for the 0.9% sodium chloride solution.

[1] Fibrometer and Fibrotube are registered trademarks of the Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.
[2] Celite 315 diatomite mineral filler. Celite is a registered trademark of Johns-Manville, New York, N. Y.

Plasma control clotting times (CCT) were determined before and after heparin assay. Times were averaged and subtracted from the clotting times (HCT) obtained from the heparin determinations.

RESULTS

At a constant calcium chloride concentration (0.025 *M*), as seen in Fig. 1, a concentration of 1.5% (w/v) of the diatomaceous silicate was found suitable for the determinations since the least variation of plasma clotting times was observed at this value.

It was apparent that the average CCT was dependent upon the plasma lot employed, as shown in Table I. In practice it was found that the CCT was constant to within ± 5 sec. of the average during the assay period. Qualitative observation on plasma stored in the frozen state for a period of 1 year did not reveal apparent changes in clotting time. For example, lot No. 1 plasma assayed initially gave an average CCT value of 123.6 ± 8 sec. for 15 assays; 11 months later, the average CCT value of 125.4 ± 8 sec., for 7 assays, was obtained.

A study was made to determine the optimum plasma concentration for use in the assay in order to establish the plasma control clotting time (CCT). Various dilutions of plasma were assayed and the results are shown in Fig. 2. A concentration of 0.33 ml. plasma per ml., in the final assay mixture, was chosen for experimental convenience and reliability. The clotting times obtained on the same lot of plasma on assays performed on *different days* are summarized in Table II. The change observed in Table II (9/7/66 to 12/15/66) is attributed to the introduction of a new preparation of the diatomaceous silicate reagent as indicated. The average value of 7 CCT determinations on lot No. 3 of sheep plasma, performed on the *same day* (12/19/66), during a regular assay was 92.8 sec., with a standard deviation of ± 2.02 sec., and a coefficient of variation of 2.19% (Table III). It was observed that thawed

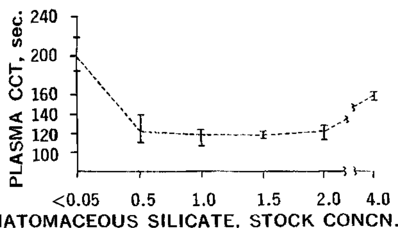

Fig. 1—Effect on the control clotting time (CCT) of citrated sheep plasma, in seconds, due to varying the concentration of the diatomaceous silicate in 0.025 M *calcium chloride. The concentration of citrated sheep plasma and 0.9% sodium chloride diluent were held constant.*

Table I—Variation of CCT Between Sheep Plasma Lots

Plasma Lot	Av. CCT
1	125.4 ± 8 sec. (7 assays)
2	94.4 ± 8 sec. (7 assays)
3	100.2 ± 8 sec. (6 assays)

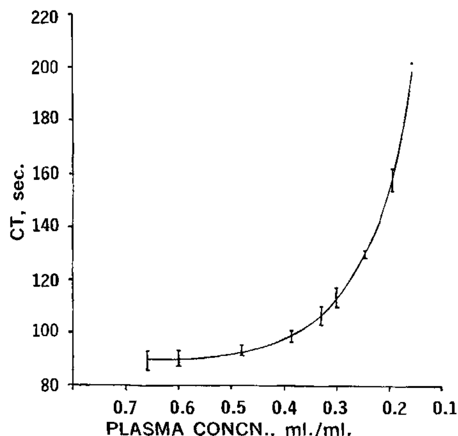

Fig. 2—Effect on the clotting time, in seconds, due to varying the final concentration of citrated sheep plasma. The diluent (0.9% sodium chloride) and the 1.5% diatomaceous silicate–calcium chloride reagent were held at constant concentration.

Table II—Variation of Clotting Times Within the Same Sheep Plasma Lot No. 3

Day	Av. CCT, sec.
8/25/66	107.6
9/2/66	105.8
9/7/66	106.0
12/15/66	96.0[a]
12/16/66	93.3
12/19/66	92.8

[a] New preparation of diatomaceous silicate–calcium chloride reagent introduced.

plasma, maintained at 0° (ice bath), displayed a continuous increase in clotting time as a function of the duration of storage as shown in Fig. 3. Attempts to correct for this effect of plasma inactivation, on standing during the assay procedure, provided no significant improvement in linearity of response.

For the assay of heparin potency, concentrations of the various heparin preparations were chosen which would result in a range of clotting times from 110 to 250 sec. corresponding to approximately 0.11 to 0.16 heparin units/ml. in the final assay mixture. A comparative assay was performed between U.S.P. heparin reference standard lot H (curve U), and the second international standard for heparin (curve I), Fig. 4. From the curves (Fig. 4) there were obtained slope values corresponding to about 0.001 Δ heparin units of activity/second. Additional slope values of calibration curves plotted for eight heparin assays employing sheep plasma lot No. 3, ranged from 0.0007 to 0.0016 units/second and together averaged 0.001 Δ units/second. The assay precision was shown in obtaining clotting times having a standard deviation of ± 2.77 sec. and a coefficient of variation of 1.71%, Table III. Details of Table III are also presented to illustrate sample data collection in the assay method.

The results of comparative assays employing U.S.P. heparin lot H and the second international standard for heparin are summarized in Table IV. The U.S.P. reference standard lot H is approxi-

TABLE III—HEPARIN ASSAY (FIG. 4)[a]

Heparin source	CCT	Clotting Times (HCT) — U.S.P.						Clotting Times (HCT) — Second International						CCT
	—													—
Order of determination	1	2	4	6	8	10	12	3	5	7	9	11	13	14
Final unit concn./ml.	—	0.11	0.12	0.13	0.14	0.15	0.16	0.11	0.12	0.13	0.14	0.15	0.16	—
	92.7	133.3	148.2	151.7	158.8	186.2	191.3	124.2	134.8	143.7	150.8	163.2	177.3	97.7
t, sec.	89.8	130.7	149.8	147.2	159.8	187.8	194.7	125.8	132.8	141.3	161.7	170.7	180.7	96.2
	88.8	138.3	148.8	150.8	158.8	190.8	203.2	123.8	132.3	143.3	152.7	167.2	185.7	94.2
	87.3			157.7			198.7							
Av.	89.6	134.1	148.9	151.8	159.1	188.2	196.9	124.6	133.3	142.8	155.1	167.0	181.2	96.0
CT (HCT–CCT[b])	—	41.3	56.1	59.0	66.3	95.4	104.1	31.8	40.5	50.0	62.3	74.2	88.4	—

[a] Date: 12/19/66; U.S.P. lot H, tablet weight: 22.1 mg. (61.9 units); second international standard, sample weight: 12.5 mg. (1625 units); sheep plasma, lot 3; Fibrometer No. 03695; assay starting time: 10:00 a.m., completion time: 1:23 p.m., time lapse: 3 hr. and 23 min. [b] Average CCT time: 92.8 sec.; combined average standard deviations: ±2.46 sec (CCT: ±2.02, HCT^{U}: ±2.77, HCT^{I}: ±2.58 sec.); combined average coefficient of variation: 1.92% (CCT: 2.19; HCT^{U} 1.71; HCT^{I} 1.86%).

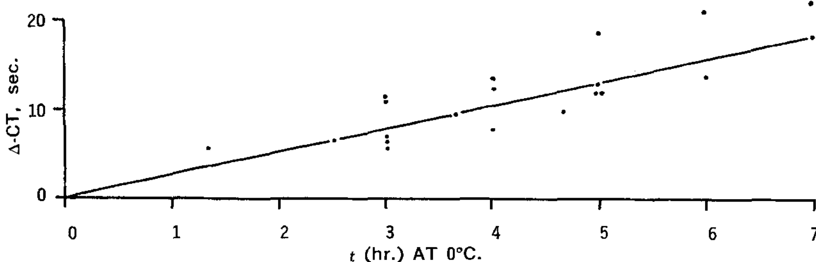

Fig. 3—Spontaneous inactivation curve (change in clotting time: Δ CT) of citrated sheep plasma, thawed, then stored at 0° in an ice bath. The slope of the curve shows a change of 0.0418 sec. in clotting time per minute of storage. The curve, illustrated, represents the average of seven assays, the slopes being 0.0309, 0.0342, 0.0366, 0.0437, 0.0445, 0.0474, and 0.0560 sec./min., respectively.

mately 10% higher in potency when compared to the second international standard for heparin.

To arrive at the percentage difference, shown in Table IV, the assumed potency in units/ml., of the unknown is divided into the difference in units/ml., between the unknown and the extrapolated potency derived from the standard curve. Reference to Fig. 4 will show that an assumed heparin potency of 0.120 units/ml. (curve U) is equivalent to an extrapolated value of 0.132 units/ml. (curve I), or a difference of 0.012 units representing a 10% higher potency.

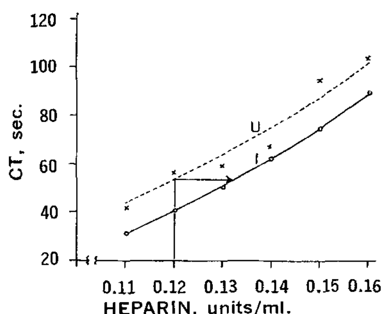

Fig. 4—Comparative assay of heparin potency. The U.S.P. heparin reference standard, curve U. The second international standard for heparin, curve I. Clotting time (CT) in seconds, ordinate, were obtained by subtracting the plasma control clotting time (CCT) from the clotting time values obtained on the addition of heparin (HCT).

TABLE IV—PER CENT POTENCY DIFFERENCE (U.S.P. HEPARIN *Versus* SECOND INTERNATIONAL STANDARD HEPARIN)

Assay Date	U.S.P. % Higher
8/2/66	18.0
8/3/66	11.3
8/4/66	8.0
8/5/66	6.4
8/25/66	7.2
9/2/66	11.0
12/15/66	11.5
12/19/66	10.0
	Av. 10.4%

Finally, it was of interest to determine the effect of autoclaving and irradiation on heparin potency. Fifty-milliliter solutions containing commercial samples of sodium heparin, in concentrations ranging from 0.5 mg./ml. to 16.0 mg./ml., were autoclaved at 121° for 45 min. Appropriate dilutions were made and the assays of these solutions revealed a negligible change from the control heparin activity.

Vacutainer[3] specimen tubes (3200KA), each containing about 140 units of dry sodium heparin, were irradiated by high energy electrons in a Hughes linear electron accelerator at dosages of 5 and 8 Mrads, at 6 to 7 Kw., 9 Mev., and 500 pulses per second. The assay revealed a loss in potency of approximately 15% as compared to appropriate controls.

[3] Vacutainer is a registered trademark of Becton, Dickinson and Co., Rutherford, N. J.

DISCUSSION

The accepted assay procedures for heparin are generally complex and subjective. The results of this investigation have led to the development of a rapid, simple, and objective *in vitro* procedure. Considerable effort was expended in obtaining plasma having reproducible and consistent control clotting times. It was found that, employing the precision coagulation timer and a diatomaceous silicate modified sheep plasma reagent, clotting times of approximately 100 sec. were obtained with a standard deviation of ± 2.5 sec. and a coefficient of variation of 2.0%. This was accomplished by the addition of 0.025 *M* $CaCl_2$, containing 1.5% of the diatomaceous silicate, to citrate sheep plasma. Just as in the Struver and Bittner method for the assay of thromboplastin activity (8), the diatomaceous silicate was found to serve as an excellent anion glass-activator for obtaining the useful clotting times. The effect of the diatomaceous silicate was not only to accelerate the clotting time but also to yield reproducible and predictable clotting time behavior without apparent interference with the heparin assay. Citrated sheep plasma, when stored in the frozen state for as long as 1 year, was found to be useful in the assay.

The spontaneous inactivation curve of citrated sheep plasma (Fig. 3) stresses the importance of assaying "standard" heparin preparations when comparisons are being made with unknown heparin preparations. The daily variations in clotting times, within the same lot of plasma, are difficult to interpret; however, these variations could be attributed to foreign contaminants, to subtle difference in concentrations of platelets, or to changes in other clotting factors.

In order to assay heparin, the differences in clotting time between the control and the samples containing an unknown heparin were obtained. A calibration plot was made of the differences in clotting time *versus* the heparin concentration in units/ml. (Fig. 4). No attempt was made to plot either curve by regression analysis because of the curvilinear characteristic. Curve I, based on observed CT values obtained with the second international standard for heparin, was amenable to visual plotting. No explanation, however, can be offered for the scatter of the observed U.S.P. heparin standard CT values used to draw curve U. This scatter, although random (Fig. 4), was observed with several of the comparative assays employing the U.S.P. sodium heparin reference standard; the second international standard for heparin gave very little scattering. Final potency values calculated from the illustrated curves, or from linear curves based upon a semilog plot, were the same.[4] Linearity was achieved as a function of heparin concentration in the range of from 0.11 to 0.16 units/ml. At lower concentrations of heparin, the curves become asymptotic and hence not suitable for assay, while at concentrations higher than 0.16 units/ml. there was an exponential increase in clotting times, rendering the curves inaccurate. Assay of heparin concentrations higher than 0.16 units/ml. required that dilutions be made to achieve clotting time values in the range of 110 to 250 sec.

U.S.P. heparin lot H exhibited a potency 10% greater than the second international standard for heparin by the method described; however, Burck (11), using the U.S.P. procedure for sodium heparin assay, noted a similar difference. It should be pointed out that the U.S.P. heparin reference standard is dispensed in the form of tablets with the units based on the weight of the tablet. A small variation in the composition of each tablet mixture may also contribute to the difference in reported activity as shown in Table IV. This observation was also noted by Burck (11).

It was of interest, in the course of this study, to determine the effect of autoclaving and irradiation on the stability of heparin preparations. Assays of sodium heparin solutions, autoclaved at 121° for 45 min., demonstrated no significant deterioration in the potency. This corroborates the findings of Pritchard (12) and Hejgard (13), who reported that autoclaving had little effect on the anticoagulant activity of heparin. Dry sodium heparin, irradiated at dosages of 5 and 8 Mrads, showed a 15% loss in potency indicating the relative stability of the material to high energy electrons.

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[4] *Note:* The data, Table III, when plotted as log $\frac{HCT - CCT}{CCT}$ as a function of heparin concentration, show a linear relationship, reflecting a pseudo first-order dependence on heparin concentration.