J. Pharm. Pharmac., 1972, **24**, 858–868 *Received May* 11, 1972

An improved *in vivo* procedure for standardization of heparin preparations

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The WHO International Collaborative Study of the Assay of Heparin has demonstrated the unreliability of present assay methods for heparin. An *in vivo* assay procedure has been redesigned to use standardized dogs in a crossover assay, measuring three coagulation tests and lipoprotein lipase on nine blood samples for each heparin dose. The procedure was tested with five heparin preparations. By the standard method for parallel line assays regression was significant ($P > 0{\cdot}001$) for $\log_2$ dose–$\log_{10}$ response. Analyses of variance were satisfactory and estimation of potency statistically valid. The potency of each heparin in International units varied with the parameter used. Fiducial limits were $\pm 10\%$ for anticoagulant parameters, $\pm 15\%$ for antilipaemic parameter and reproducibility, 97% for anticoagulant parameters, 87·5% for antilipaemic parameter.

There has recently been reported the results of a WHO International Collaborative Study on the assay of heparin (Bangham & Woodward, 1970). The study was for the purpose of deciding whether one or two preparations of heparin were required for the Third International Standard Heparin Preparation. However, the results demonstrated the unreliability of present methods of assay, indicating that the methods for standardization of heparin require re-examination.

Commercially available heparins are assayed by either of the pharmacopoeial methods, which are based on the measurement *in vitro* of only one aspect of the entire anticoagulant action of heparin. Walton, Ricketts & Bangham (1966) suggested, "when the assay system is as complex as the coagulation process, it is desirable that the assay should measure the effect on the over-all process and essential that a positive correlation with the *in vivo* activity be established". There has been no precise indication of the relation of *in vitro* potency to the *in vivo* activity, because there has not been any method of assessing accurately the *in vivo* potency.

Jorpes, Blombäck & Blombäck (1954) described an *in vivo* assay of heparin, in which the maximum clotting time (4 min after heparin injection) was used to detect and measure heparin in blood. Rezansoff & Jaques (1967) studied an *in vivo* assay procedure in which the potencies of various preparations were measured as the area under the response curves for the Lee & White clotting time, partial thromboplastin time, and lipolytic activity. In this study anaesthetized dogs without recovery were used with determination of the relative potency of various heparin preparations on the same dog limited to six to eight doses. Since there is usually a significant difference in response to heparin between dogs (Jaques, 1939), any estimate of potency based on one dog would have wide confidence limits. Further, histological studies by Dr. B. Unger of the animals used indicated congestion, anoxia and dehydration at the end of the experiment. When the test heparins in the International Collaborative study were subjected to this assay, in spite of the limitations just indicated, examina-

tion of the data (which was limited to the clotting time response) showed that when various heparins were assayed against one another and the design of the assay was such that the analysis for parallel line assays could be carried out, the assays were statistically valid in the sense that the regression of the log dose-response line was significant and that the deviations from parallelism were not significant. This suggested that redesign of the experimental conditions used by Rezansoff & Jaques (1967) would produce a satisfactory assay procedure for heparin. For this purpose, heparin has been injected intravenously into conscious animals at two week intervals to allow comparisons of different heparins on individual dogs (i.e. crossover assay).

MATERIALS AND METHODS

Heparin preparations used. Heparin preparations were dissolved in 0·9% sterilized saline containing 0·3% cresol and the solutions divided into suitable aliquots which were frozen in sealed, sterilized vials. Five preparations were tested. These are listed by Jaques, Kavanagh & Lavallee (1967) and Kavanagh & Jaques (1972) as: Intl. Std. and Nos. 59, 58, 19, 48.

International standard for heparin. The 2nd International Standard (established 1958) is a sodium salt of heparin prepared from bovine lung. By definition its potency is 130 International units/mg for all test systems.

Upjohn beef lung heparin. Sodium heparin, U.S.P., Upjohn Co. Lot ZX-320 (Upjohn) prepared from beef lung.

Upjohn pork mucosa heparin. Sodium heparin, U.S.P., Upjohn Co. Lot 145493 (Wilson) prepared from pork intestinal mucosa.

Sheep lung heparin. Sodium heparin prepared from sheep lung by the late Dr. A. Winterstein. Lot 1–2232/665.

Acetic acid-treated heparin. This was prepared, using the procedure of Yosizawa, Kotoku & others (1967). Heparin (Lederle) stood in 40% acetic acid at 37° for 24 h and was then precipitated with 4 volumes of ethanol. The yield was 89·5%.

Collection and examination of blood samples. Blood samples were taken at 5, 10, 15, 30, 45, 60, 90, 120 min after intravenous injection of heparin into a hind leg. Four ml of the blood sample were added to 3·8% sodium citrate. Centrifugation at 2100 rev/min at 4° for 15 min provided plasma for partial thromoboplastin time (PPT), activated partial thromboplastin time (APTT) and lipoprotein lipase (LPL) determination. One ml from the middle portion of the 5 ml blood sample was used for determining the Lee & White clotting time at 37°. PTT and APTT were conducted as outlined by the Diagnostic Division, Ortho Pharmaceutical Corporation, Raritan, N.J., using the Thrombofax reagents. LPL activity was determined according to Dahlbäck, Hansson & others (1968), which measured the glycerol released by LPL. The glycerol was determined by the enzymatic-fluorometric micromethod of Laurell & Tibbling (1966).

Animal care. The principles of animal care laid down by the Canadian Federation of Biological Societies were observed. The male dogs for use in the assay were screened by age (2–3 yr old), weight (10–15 kg), normal Lee & White clotting time (4–6 min) and temperament (reaction to taking of blood sample). Each animal was starved overnight and received a 25 mg tablet of Atravet (Acepromazine maleate) 1 h before the experiment. There was no significant decrease in the haematocrit value of any dog throughout. The only significant change in WBC was in dog 3

with an ear infection for 3 weeks. Platelet, WBC and hematocrit measurements were made in each animal with the injection of the highest dose of heparin. The slight changes in platelet and WBC count observed were not correlated with the heparin injection.

Study of the estimate of relative potency of heparins in vivo

Design. Five heparin preparations were selected. International standard heparin was the reference standard; the two Upjohn preparations were similar to heparins in current clinical use; examples of a modified heparin and a natural heparin of low anticoagulant activity were provided by acetic acid-treated heparin and sheep lung heparin. The responses to three dose levels, 0·15, 0·30, 0·45 mg of each heparin/kg were measured with a two week recovery period between injections. The order of injection of heparin preparation and doses was determined using a table of random numbers (Rohlf & Sokal, 1969).

The experiment was divided into two series:

Series I	*Series II*
A. International standard heparin (standard)	K. Upjohn beef lung heparin (standard)
B. Upjohn beef lung heparin	L. Upjohn pork mucosa heparin
C. Upjohn pork mucosa heparin	M. Acetic acid-treated heparin
D. Upjohn beef lung heparin	N. Sheep lung heparin

For each series, each of five dogs received three doses of four heparins in a multiple cross-over design. International standard heparin and beef lung heparin served as standards in Series I and II respectively. Series I and II were in progress at the same time, using separate groups of five dogs. The reproducibility of these *in vivo* assays was tested in Series I by comparison of heparins B and D which were actually aliquots of a single solution of beef lung heparin. This heparin, having been assayed against the International Standard in Series I, was used to determine the potency, in International units, of the three test preparations in Series II. Reproducibility between series was tested by comparing the responses in two series for (a) B with K, (b) C with L.

Responses to a single injection of heparin. Responses of an unanaesthetized dog to a single intravenous injection of heparin are shown in Fig. 1. Jaques (1941) demonstrated that linear relation with coagulants were evident when clotting times were plotted as log values and Jaques & Ricker (1948) showed this for heparin and that this treatment of data from intravenous administration of heparin gave response curves with essentially equal contributions from degree and duration of hypocoagulability. Values for Lee & White clotting time, PTT and APTT plastin time were recorded on a log scale. Values for these parameters were highest at 5 min after injection, then declined gradually returning to normal at 120 min. The LPL activity was highest at 5 min after injection, and returned to zero in 90 min.

Relation of dose-response. From response curves such as those shown in Fig. 1, the areas under the curves for each parameter were calculated. As Rezansoff and Jaques (1967) had obtained a linear relation between the area under the response curve and heparin dosage, the values so obtained with ten dogs for each of the four parameters for the eight test solutions was examined graphically in several ways. On calculating slopes and intercepts, some plots suggested application of a slope

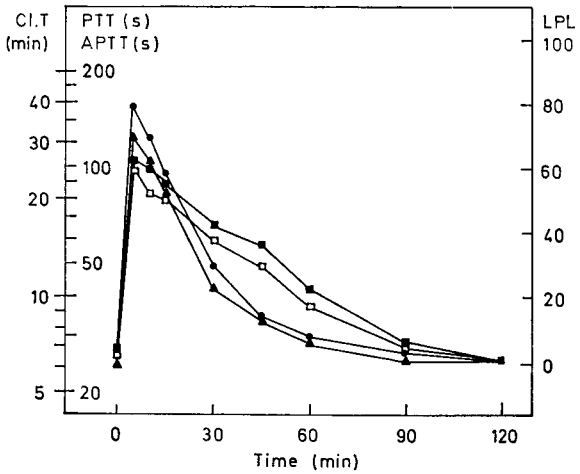

FIG. 1. *In vivo* response to intravenous heparin. 0·3 mg of International Standard Heparin per kg body weight. ●—●, Cl.T. (Lee & White clotting time) (min); ■—■, PTT (partial thromboplastin time) (s); □—□, APTT (activated partial thromboplastin time) (s); ▲—▲, LPL (lipoprotein lipase activity) in units. The release of one μmol of glycerol per litre of plasma per min was used as one unit of LPL.

ratio assay, but closer examination revealed that this would be inadvisable since the lines for a given parameter did not intersect sufficiently close to one point. To determine whether the data could be analysed by an analysis of variance, a test for homogeneity of variances (Bartlett, 1937) was applied. These calculations showed high probabilities in both series for all four parameters with $\log_{10}$ area under the response curve versus $\log_{10}$ dose. Thus homoscedasticity of the data was assured and analyses of variance justified. Examination of these dose-response diagrams suggested that the statistical principles of the parallel line assay could be utilized in the analysis of the data of this multiple cross-over assay. It was decided that for ease of calculation of relative potencies the dose scale should be converted to $\log_2$. The two methods of plotting were compared by calculating U, the sum of the squares of deviations from all individual regression lines. Of the 32 regression lines (4 heparins × 4 parameters × 2 series), U was higher, i.e. less satisfactory, in 17 instances when $\log_{10}$ dose scale was used and was higher in 15 instances when the $\log_2$ dose scale was used. Bartlett's test confirmed the homogeneity of variances for $\log_2$ heparin dose *vs* $\log_{10}$ of the area under the initial response curve. The mean regression lines obtained in this way are shown in Fig. 2. The data for PTT and APTT gave similar results so only the latter is plotted. It can be seen that a linear relation is obtained with each parameter by plotting response on a scale of $\log_{10}$ with heparin dosage on a scale of $\log_2$. A control series was conducted under the same conditions as those of the experimental series on all of the dogs using an injection of 0·3 ml saline-cresol instead of heparin solution. Values for the areas under the response curves of clotting time, partial thromboplastin time, activated partial thromboplastin time and lipoprotein lipase were essentially zero and were ignored in the analysis of data.

Analyses of variance for the double block design. The results for all four heparins of a series were analysed simultaneously according to a multiple assay method (Finney, 1964). The analysis of variance for the data on clotting times in Series I

is presented in Table 1. This consists of simultaneous analysis of the response lines and provides a balance sheet to account for the heterogeneity of the data. The grand mean of all values is $Y = (\bar{Y})/(n) = 1{\cdot}3721$ and the total sum of the squares of the deviations from Y is represented by the total sum of squares (S.S.) = 2·6475. For the most part, the total S.S. is due to the fact that increasing doses of heparin produce prolonged clotting times and most of the total variation from $\bar{Y}$ is accounted for by variation "Between Doses". Variations from Y that are not accounted for by variation between doses or between dogs are relegated to the error term, which will include unknown sources of variations within each dose. A more detailed partitioning of the S.S. due to doses is shown in the upper portion of the table. Some variation is accounted for by the differences in response "Between Preparations".

For maximum precision of the assay the "Between Preparations" S.S. should be small as in Table 1. In a good assay, most of the variation "Between Doses" should be accounted for by the regression relation between dose and response and in both series, the "Regression" S.S. was appropriately large. Deviations of the data above and below the common regression line are represented by the "Deviation from Regression" S.S. In Series I this term was low. The validity of a parallel line assay depends, by definition, upon the degree of parallelism between the response lines, expressed in the analysis of variance by the "Deviations from Parallelism" S.S. This was appropriately small (0·0032).

The *P* values of the analyses of variance are shown in Table 2. The *P* values for Series I for "Between Preparations" are satisfactory except for the LPL data which

Table 1. *Analysis of variance for the data of Series I, from the values for clotting time.*

Nature of variation	d.f.	S.S.	M.S.	F	d.f.	*P*
Between preparations	3	0·0005	0·00016	0·03252	3/44	0·75
Regression	1	2·4129	2·4129	490·4268	1/44	0·001
Deviation from parallelism ..	3	0·0032	0·0011	0·2235	3/44	0·75
Deviation from regression ..	4	0·0143	0·00357	0·7256	4/44	0·50
Between doses	11	2·4309	0·22099	44·91666	11/44	0·001
Between dogs	4	0·0072	0·00180	0·36585	4/44	0·75
Error (within doses)	44	0·2166	0·00492	—	—	—
Total	59	2·6475	0·04487	—	—	—

is just above the 0·05 level. Wide separation of the response lines is not immediately apparent when the LPL data plot is compared with the other plots of Series I data (Fig. 2). When potencies were calculated, it became apparent that Heparin D was responsible for the low *P* value. The *P* values for regression are appropriately low. The values for deviations from parallelism are satisfactory except for the PTT parameter ($P = 0{\cdot}047$) which shows deviations from parallelism that are just significant at the 5% level. The high *P* values for deviations from regression indicate that deviations from regression failed to show significance at the 0·10 level but the value for partial thromboplastin time is the least satisfactory. Interpolation of a table of the *P* distribution gave a *P* value of 0·16. Partial thromboplastin time data shows more variation than those of other parameters. The *P* values for "Between Doses" all show high significance. The *P* values for "Between Dogs", indicate no significant difference between the responses of the dogs used.

When clotting time or accelerated partial thromboplastin time were measured, the analysis of variance indicated that a parallel line assay method was valid to determine

Table 2. P *Values for the analysis of variance.*

Source of variations	Cl.T.	Parameters PTT	APTT	LPL
		Series I (A,B,C,D)		
Between preparations	>0·75	>0·1	>0·25	>0·05
Regression	<0·001	<0·001	<0·001	<0·01
Deviation from parallelism	>0·75	0·047	>0·25	>0·75
Deviation from regression	>0·5	>0·1	>0·25	>0·25
Between doses	<0·001	<0·001	<0·001	<0·001
Between dogs	>0·75	>0·5	>0·25	>0·75
		Series II (K,L,M,N)		
Between preparations	<0·001	<0·001	<0·001	>0·05
Regression	<0·001	<0·001	<0·001	<0·001
Deviation from parallelism	>0·01	>0·001	>0·001	>0·75
Deviation from regression	>0·001	<0·001	>0·001	>0·1
Between doses	<0·001	<0·001	<0·001	<0·001
Between dogs	>0·05	>0·25	>0·25	>0·05

Cl.T. = Lee & White clotting time; PTT = partial thromboplastin time; APTT = activated partial thromboplastin time; LPL = lipoprotein lipase response.

relative potency of the preparations in Series I. The same conclusion applied when LPL activity was measured although the "Between Preparations" S.S. was larger than might be desired. When PTT was measured, the slopes of the response lines (0·36 and 0·37) for Heparin B and D were less than those of Heparin A, 0·43 and C, 0·48. These differences in slope produced S.S. for "Deviations from Parallelism" that were significant at the 5% level ($P = 0·047$), and on the basis of this one assay, calculation of relative potencies using this parameter might very well be rejected. The results of Series II indicate that the lack of parallelism in Series I is fortuitous.

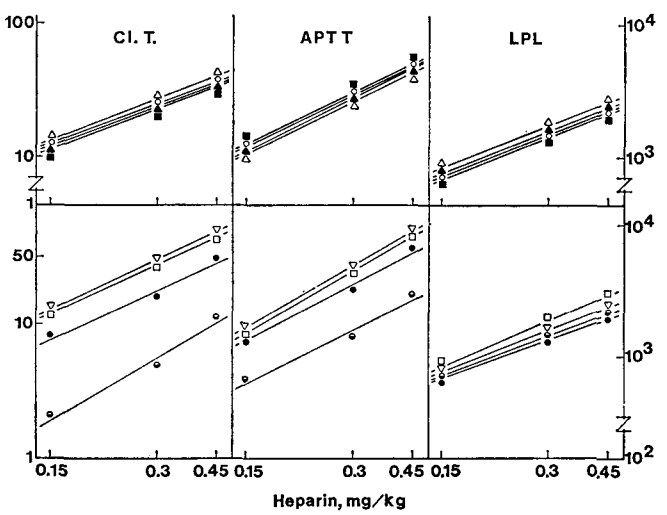

FIG. 2. Regression lines between area under response curve and heparin dose. Ordinate: values for area under response curve plotted as $\log_{10}$. Abscissa: heparin dose (mg/kg) plotted as $\log_2$. Each point is the mean value for 5 dogs; ○, Heparin A; ▲, Heparin B; ■, Heparin C; △, Heparin D; ▽, Heparin K; □, Heparin L; ●, Heparin M; ◒, Heparin N. Cl.T., PTT, APTT, LPL—Values for response by Lee & White clotting time, partial thromboplastin time, activated partial thromboplastin time, lipoprotein lipase.

P values for the analysis of variance for data of Series II are also reported in Table 2. The dose range was quite appropriate when LPL was measured. However, as indicated in Fig. 2, the heparin preparations showed essentially three potency ranges for the anticoagulant parameters. It is evident from Fig. 2 that Heparin N was a prominent source of variation for the clotting parameters.

Since the comparison of Heparin K and Heparin L in Series II is to be matched with the same comparison in Series I, an analysis of variance for this anticoagulant data was made. Only the S.S. for partial thromboplastin time "Between Preparations" shows high significance, $P < 0{\cdot}001$. Of particular interest is "Deviations from Parallelism" for PTT ($P > 0{\cdot}25$) since the corresponding value in Series I was only 0·047. The difference arises from the slope of the response lines for Heparin K, which was 0·45 in Series II compared to 0·36 and 0·37 in Series I. The slopes of the response lines for Heparins C and L were 0·48 and 0·41. Thus, Series II provided a partial check upon Series I and we conclude that the extent of non-parallelism shown by PTT data for Series I was due to the fortunes of random sampling or other sources of error of an unknown nature and cannot be attributed to differences between the biological actions of beef lung and pig mucosa preparations.

The appropriate and reasonable level of significance for "Deviations from Parallelism" should be considered. For the sum of squares for clotting time data, $P = 0{\cdot}09$. The slope of the response lines was 0·30 for Heparins B, C and D, 0·34 and 0·38 for K and L. B, D and K are the same preparation, as are C and L. Because of their small magnitude in the data, the values for the mean square for "Error" tend to magnify the values of *P* and cause the analysis of variance to detect extremely small deviations from parallelism. For these reasons, it is reasonable to accept $P = 0{\cdot}05$ as the approximate criterion of significance for non-parallelism for these assays.

The estimation of relative potencies and their fiducial limits

The analyses of variance for Series I indicated that Heparin B, C and D could be compared to Heparin A by all parameters. For Series II, that the potencies of Heparin M and N could be measured against K for the LPL parameter and L against K for the clotting parameters. Measurements of the potency of Heparins M and N against Heparin K on the basis of coagulation tests and especially PTT and APTT, have less validity. More precise estimates of potency for Heparins M and N require the level of dosage to be changed to reduce the sums of squares for "Between Preparations" and "Deviations from Regression".

Relative potency was estimated by calculation of M and R (Finney's symbols). M is the horizontal distance between two log dose-log response lines and R is relative potency. If two linear regression lines are parallel, $M = X_S - X_T - (Y_S - Y_T)/b$, where X and Y represent the mean response respectively, b is the common slope and subscripts S and T refer to the standard and test preparations. In a parallel line assay, $R = X_S/X_T$. When the dose scale is logarithmic, log R is provided by M which gives $\log X_S - \log X_T$. Calculation in this way gives a relative potency that is fractional when the potency of the test preparation is less than that of the standard. Since our dose scale was logarithmic to base 2, $M = \log_2 R$. Fiducial limits were estimated by calculating M_L and M_U, the lower and upper fiducial limits to M, using Fieller's theorem (Fieller, 1940).

$$M_L \text{ or } M_U = \frac{\left\{M - (X_S - \bar{X}_T) \pm \frac{t}{b}\left[(1-g)\left(\frac{1}{N_S} + \frac{1}{N_T}\right) + \frac{(M - \bar{X}_S + Y_T)^2}{\Sigma\Sigma\,(X - X)^2}\right]^{1/2}\right\}}{(1-g)}$$

where $g = \frac{t^2 s^2}{b^2 \Sigma\Sigma\,(X - \bar{X})^2}$, s^2 = mean S.S. for error in analysis of variance with f degrees of freedom, t = value of Student's t for $P = 0{\cdot}05$ and f degrees of freedom.

When "g" exceeds 1, the data are not suitable to calculate relative potencies or fiducial limits. In this work, all "g" values were less than 0·02 and were included in the calculation of limits, although this is not necessary with such low values.

M_L and M_U were transformed to R_L and R_U, the limits of relative potency as described above. The potency of International standard heparin is 130 units/mg by definition. The potencies of Heparins B, C and D in International units were obtained by multiplying their relative potencies by 130. The mean potency of heparin B and heparin D gave the potency of the reference Heparin (Heparin K) in Series II. Multiplication by the potency of this reference heparin (determined in Series I) gave the potencies of heparin L, M, and N in International units. The potency and fiducial limits of each heparin are shown in International units in Table 3.

Table 3. *Potencies of heparin preparation and their fiducial limits in international units.*

	Cl.T.			PTT			APTT			LPL		
	P	P_U	P_L	P	P_U	P_L	P	P_U	P_L	P	P_U	P_L
A	130	—	—	130	—	—	130	—	—	130	—	—
B	128·6	144·5	114·4	139·8	154·8	126·4	135·3	149·4	122·6	134·0	153·7	116·8
C	129·0	145·0	114·8	144·4	159·9	130·6	142·3	157·1	129·0	128·3	147·1	111·9
D	130·7	146·9	116·3	143·2	158·5	129·5	138·2	152·5	125·2	150·6	172·9	131·4
K	129·7	145·7	115·3	141·5	156·7	128·0	136·7	151·0	123·9	142·3	163·3	124·1
L	135·7	148·8	123·8	167·5	181·3	154·8	141·6	152·6	131·3	126·8	151·1	1·602
M	(88·3)	(97·0)	(80·2)	(119·1)	(128·8)	(110·0)	(120·8)	(130·3)	(112·1)	109·8	130·8	91·6
N	(37·0)	(41·8)	(32·5)	(60·3)	(66·9)	(54·1)	(56·7)	(62·4)	(51·3)	116·0	138·2	96·9

See Table 2 for definition of abbreviations.

For a given heparin, in all cases, potency measured by PTT or APTT was higher than the potency measured by clotting time. When LPL was used as the basis of measurement, the potency of Heparins B, D, M and N were rated higher, C and L were lower compared to the value obtained using clotting time measurements.

The fiducial intervals are satisfactory for this type of assay. Expressing these as percentages, when clotting time was measured, the average interval between R_U and R was 12·4% of R, and the average interval between R and R_L was 11·0% of R. For the first three parameters in both Series, we may conclude that potencies were estimated within ±10% limits. The LPL measurements showed wider limits, averaging about ±15%.

Reproducibility

The reproducibility of this assay method was examined by comparing the relative potency and its fiducial limits of the same heparin preparation within a Series (within the same group of dogs) and between two Series (between two groups of dogs).

These values are reported in Table 4. The reproducibility within a Series was examined by comparing the potency of Heparin B against Heparin D. These were aliquots of the same heparin solution. For all four parameters, the variations of "Between Preparations", "Deviation from Parallelism", "Deviation from Regression" and "Between Dogs" are non-significant; and "Regression", "Between Doses" are highly significant. The relative potency values measured by anticoagulant parameters are less than 1·03; in other words, the reproducibility of this assay system in terms of anticoagulant activity is greater than 97% (clotting time 98·3%, PTT 97·3%, APTT 97·7%). The relative potency measured by LPL is 1·1251, and the reproducibility is 87·5%.

The reproducibility between two Series was checked by measuring the relative potencies of heparin preparations B and D in Series I against K in Series II, and heparin C of Series I against L of Series II. As expected, these comparisons are less satisfactory than those within Series I. In the comparisons for heparins B and D against K, the "Between Preparations" S.S. were somewhat large, giving P values $>0{\cdot}1$, $>0{\cdot}001$, $>0{\cdot}05$ and $>0{\cdot}01$ for the four parameters. In addition, and of more importance, there was significant deviation from parallelism ($P> 0{\cdot}025$) with PTT and APTT. "Deviations from Regression" approached statistical significance

Table 4. *Relative potencies of heparin preparations.*

Heparin preparations		Cl.T.	PTT	APTT	LPL	Mean
B/D	g	0·0068	0·0041	0·0060	0·0086	—
	R	1·0168	1·0272	1·0226	1·1251	1·0479
	R_U	1·1269	1·1132	1·1261	1·2645	1·1577
	R_L	0·9177	0·9480	0·9288	1·0032	0·9494
B/K		0·9245	N.A.	N.A.	0·8415	0·8970
D/K		0·9395	N.A.	N.A.	0·9473	0·9434
C/L		0·8939	0·9803	1·0762	0·9022	0·9631
Mean		0·9193	0·9803	1·0762	0·8970	0·9382

N.A. = Not applicable; R_U and R_L = upper and lower values of R, the relative potency. See Table 2 for abbreviations of other parameters.

($P > 0{\cdot}05$) for PTT. As a consequence of these aberrations, relative potencies for heparins B and D against Heparin K are of less value for PTT and APTT. When heparin C was compared to heparin L, the P values for the clotting time parameter "Between Preparations" was between 0·01 and 0·025 but in other respects the analyses justified the comparison. The relative potencies with one exception, are less than unity, indicating that the dogs of Series II (Heparins K and L) gave average responses that were higher than did the dogs of Series I. The mean relative potency for all tests was about 0·94, which is considered satisfactory, but it will be observed that individual values ranged from 0·84 to 1·07. The mean reproducibility is 93·8%—92% for clotting time, 98% for PTT, 92% for APTT and 89·7% for LPL.

Minimal requirements for reliable assay. For routine application the total procedure as described is laborious and time-consuming. The effect of reduction of numbers of doses, dogs and blood samples was examined by analyses of the data with four dogs (deleting dog 5), two doses (deleting the medium dose) and five blood samples (area under response at 0, 10, 30, 60, 90 min after injection). As shown in Table 5, the P values of analyses of variance on Series I for four parameters are satisfactory.

Table 5. P *values of the analyses of variance for Series I with four animals, two doses and five blood samples.*

	Cl.T.	PTT	APTT	LPL
Between preparations	0·75	9·50	0·50	0·25
Regression	0·001	0·001	0·001	0·001
Deviation from parallelism ..	0·75	0·25	0·50	0·10
Deviation from regression ..	0·75	0·75	0·75	0·75
Between doses	0·001	0·001	0·001	0·001
Between dogs	0·75	0·25	0·25	0·50

See Table 2 for definition of abbreviations.

The fiducial limits are $\pm 16\%$ by coagulation tests and $\pm 20\%$ by measurement of LPL activity. It would appear inadvisable to reduce further the number of subjects. Reducing the number of blood samples below five would not represent a significant technical saving. The APTT test is recommended to replace the PTT test for control of heparin, and very similar results were obtained for the potency of the heparin preparations by these tests, so only the APTT test needs to be done. If the APTT test were to become the generally accepted test for clinical control of heparin, then the Lee & White clotting time determinations might also be dropped. However, there in no great technical saving in this and the immediate information obtained is often useful. As heparinoids and heparins of other species (e.g. whale, dog) have not been examined, these will require the extensive study described here. For such studies with $\log_2$ heparin as the most satisfactory plot, it would make calculation easier if the doses within the range were selected to be in geometrical progession.

DISCUSSION

The assay procedure reported here allows for an accurate assessment since it meets statistical requirements for reproducibility and accuracy described by Bangham & Woodward (1970). In our study, heparin activity values determined by four parameters (clotting time, PTT, APTT and LPL) were analysed by standard statistical methods for parallel line assays and were all found to have a significant ($P < 0{\cdot}001$) regression on a $\log_2$ dose–$\log_{10}$ response plot. Bangham & Woodward reported that while assays of this kind are usually rejected when the slopes of the response line are significantly different at a 1% level, the lack of variation in responses at any one dose level made the analysis of variance in the assays meaningless. They depended on finding for all log dose-response lines a slope of the same sign and on ranking these slopes failing to find a steeper or flatter slope for any preparation. We used the 5% level as a basis upon which to assess deviations from parallelism.

In selecting doses of heparin to be used (as with other drugs) for the statistical tests it is necessary that the responses with the lowest doses be significant and that the responses with the highest doses be measurable (not infinity). Further, for greatest accuracy there should be a close match in the responses for the reference and test preparations. Since the doses required for this could only be determined approximately in preliminary tests, doses were assigned on the basis of equal weights. This gave a satisfactory range of doses for the heparin preparations in Series I and for the LPL parameter for the heparin preparations in Series II. However, for coagulation parameters in Series II, the four heparin preparations were essentially in three potency ranges. Since all were tested in a single dose range, the arrangement

was not well designed to test relative potencies on the basis of clotting parameters. This should be considered as demonstrating the effectiveness of this assay procedure rather than a serious defect. Full details of the experimental protocols, results and statistical treatments in this study have been reported by Kuo (1971).

The statistical analysis has established that we have attained our objective, of improving the *in vivo* assay method for heparin so that it will meet tests of reproducibility and accuracy. It remains to be seen if other methods of assay for heparin can be similarly improved. The method could be considered for the establishment of equivalence values for the Third International Standard Heparin Preparation in terms of the Second International Standard Heparin Preparation. In this regard, the observation that a heparin preparation from beef lung had the same *in vivo* potency as a heparin preparation from pork intestinal mucosa, is significant for assay in control laboratories and for clinical control of heparin in patients.

Acknowledgements

The research work has been supported by research grants to Dr. L. B. Jaques from the Medical Research Council of Canada (MT-2774) and the Ortho Pharmaceutical Company. Beef lung heparins and hog mucosa heparin were supplied by the Upjohn Company. Partial thromboplastin preparations were supplied by Ortho Pharmaceutical Co. We are indebted to Mrs. H. J. Oh, Mrs. Betty Stanley and Mr. Jack Wertman for skilled technical assistance, and to Dr. D. R. Bangham, Division of Biological Standards, National Institute for Medical Research, London, England, for constructive criticism.

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