ASSAY-DEPENDENT KINETICS OF HEPARIN: EVIDENCE FOR RAPID *IN VIVO* ACTIVATION OF HEPARIN

F.X. McGOWAN, PATRICIA V. NASH & T.D. BJORNSSON

Division of Clinical Pharmacology, Departments of Pharmacology and Medicine, Duke University Medical Center, Durham, North Carolina 27710, USA

- 1 Multiple blood samples were collected over the first 15 min after an i.v. injection of 25 units/kg of heparin in four healthy subjects. Plasma heparin activity in each sample was determined by a chemical neutralization assay using polybrene and a bioassay based on activated partial thromboplastin time.
- 2 Chemically assayed heparin declined much more rapidly than bioassayed heparin over the first 5-7 min after the dose. Subsequently, both heparin activity vs time curves declined with a similar terminal half-life.
- 3 Slopes of the *in vivo* ln APTT vs plasma heparin activity (determined by chemical neutralization) relationships were significantly greater than those of the corresponding *in vitro* relationships between ln APTT vs heparin activity added to plasma.
- 4 A possible explanation for these heparin assay differences is a rapid *in vivo* enhancement of the anticoagulant effect of heparin. How such enhancement may occur, however, is presently unclear.

Keywords heparin kinetics

Introduction

Although heparin has been in clinical use for over 40 years and its clinical efficacy as an anticoagulant is well established, there are still numerous unresolved issues concerning its pharmacology and optimal use (Bjornsson, 1983; Hirsh *et al.*, 1981; Jaques, 1979; Wessler & Gitel, 1979). This is in part due to the fact that, unlike almost all other drugs, heparin is a relatively crude natural product, consisting of a mixture of molecular species with different chemical and anticoagulant properties. Because of this heterogeneity, the pharmacokinetics of heparin have been studied primarily by determining the activity of the drug in plasma as a function of time by various bioassays and chemical neutralization assays (Bjornsson, 1983; Estes, 1980).

We have recently demonstrated that the pharmacokinetics of heparin in normal subjects are dependent on the heparin assay methodology in addition to being dose-dependent (Bjornsson *et al.*, 1982). The assay-dependence is characterized by lower observed heparin activity in plasma (in unit/ml) when determined by a chemical neutralization assay using polybrene than by bioassays based on activated partial thromboplastin time (APTT) and thrombin time. As a result of these heparin assay differences, both calculated total clearance and apparent volume of distribution are larger when based on chemical heparin than bioassayed heparin, while the biological half-life is similar. Since both the chemical assay and the bioassays are based on *in vitro* reference curves, we have

proposed that these observed *in vivo* differences in heparin activity may be due to an apparent *in vivo* enhancement of the anticoagulant properties of heparin (Bjornsson *et al.*, 1982). Similar heparin assay differences have been observed both in man and animals when heparin activity has been determined by metachromatic assays using toluidine blue or azure A as the chemical assay and by a bioassay based on thrombin time; these studies also demonstrated that heparin can be activated *in vivo* (Levy & Jaques, 1978).

The purpose of the present study in normal subjects was to define the time course describing the development of the assay-dependent kinetics of heparin by using frequent sample collection during the first few minutes after i.v. administration. The first blood samples in our earlier study were collected 5 to 15 min (depending on dose given) after i.v. administration, at which time the difference in observed plasma heparin activities was already complete.

Methods

Subjects and study design

Four young male volunteers participated in this study. They were all in excellent health and none had received prescription or over-the-counter medications for at

least 2 weeks preceding the study. Each subject received an i.v. dose of 25 units/kg of body weight of heparin by rapid injection into an antecubital vein. The heparin used was prepared from bovine lungs (lot no. 434GU, Upjohn Company, Kalamazoo, Michigan). An indwelling catheter (Quik-Cath) was placed in a vein on the opposite arm about 1 h before the dose and occluded with an obturator between sample collections. A total of 10 blood samples were collected after the dose, at 1, 2, 3, 5, 7, 10, 15, 20, 30 and 60 min. The blood was immediately anticoagulated with 3.2% (w/v) sodium citrate to yield 10% (v/v) citrated blood, which was centrifuged at 1000 g for 15 min. The plasma was transferred to test tubes and kept on ice until assayed.

Heparin assays

Plasma heparin activity in each sample was determined by two different heparin assay methods; one is based on the coagulation test APTT (APTT-heparin assay), and the other involves chemical neutralization of heparin using polybrene (PN-heparin assay). The APTT-heparin assay was performed as previously described (Bjornsson *et al.*, 1982), using Automated APTT (General Diagnostics, Morris Plains, N.J.) as the principal reagent. The coagulation times were determined using a Fibrometer coagulation timer (Baltimore Biological Laboratories, Cockeysville, Maryland) and all samples were assayed in duplicate. Briefly, the assay is based on an *in vitro* relationship between APTT and heparin activity added to plasma, yielding final plasma heparin activities ranging from 0.1 to 0.8 unit/ml. This relationship, which is log-linear (Bjornsson & Wolfram, 1982; Bjornsson *et al.*, 1982), was established in each subject before the heparin dose. The intercept baseline APTT values and the slope values of the *in vitro* ln APTT vs heparin activity relationship for the four subjects are listed in Table 1. Heparin activity in plasma samples obtained after the heparin dose is then derived from this relationship based on the observed APTT.

The PN-heparin assay was performed as previously described (Bjornsson *et al.*, 1982) with the following

modifications. The bovine thrombin used (Thrombostat, U.S.P., Parke-Davis, Morris Plains, N.J.) was diluted in 0.0325 M HEPES buffer to yield a final thrombin concentration of 3 NIH units/ml, and 0.0125 M calcium chloride. The plasma volume used in each test was 150 μ l, the heparin standards were 15 μ l, while the polybrene standards and thrombin solution were 50 μ l and 100 μ l respectively. This assay is based on a linear relationship between the heparin present in the sample and the amount of polybrene required for neutralization of heparin. This relationship was established in each subject before the heparin dose, using plasma samples spiked with heparin as was done for the APTT-heparin assay. The average coefficient of determination of the four calibration curves used in this study was 0.991 ± 0.009 .

Pharmacokinetic and statistical analysis

The biological half-life ($t_{1/2}$) of heparin was determined by least-squares linear regression analysis of plasma heparin activity vs time values obtained between 5 and 30 min, after transformation of heparin activities to natural logarithmic values. The total clearance (CL) was calculated as D/AUC, where D is dose and AUC is the total area under the plasma heparin activity vs time curve; AUC was calculated over the range of observed data points using the trapezoidal rule and extrapolated to infinity. The apparent volume of distribution (V_d) was determined as $(CL \times t_{1/2}) / \ln 2$ (Gibaldi & Perrier, 1975).

The statistical significance of individual relationships between ln APTT vs added or measured plasma heparin activity was determined using linear regression analysis. When calculating the slope values of these relationships in the samples obtained after the administration of heparin in the individual subjects, the relationship was forced through the intercept obtained for the respective *in vitro* relationship (Zar, 1974). The paired *t*-test was used to evaluate differences in individual pharmacokinetic parameters obtained using the different heparin assays. Statistical significance was accepted when two-tailed $P < 0.05$. Unless indicated otherwise, results are expressed as mean $\pm$ s.d.

Table 1 Baseline APTT and relationship between ln APTT and heparin activity (unit/ml) added *in vitro* to plasma and measured *in vivo* by PN-heparin assay.

Subject	Baseline (s)	In vitro		In vivo	
		Slope (ml/unit)	r^2	Slope (ml/unit)	r^2
1	32.7	2.214	0.992	3.093	0.926
2	34.7	2.733	0.988	3.327	0.777
3	28.9	1.696	0.993	2.624	0.107
4	23.1	1.322	0.997	2.100	0.832
Mean	29.9	1.991	0.993	2.786	0.661
$\pm$ s.d.	5.1	0.615	0.004	0.543	0.374

Results

Intercept baseline APTT values and slope values of the relationships between $\ln$ APTT vs heparin activity added to plasma *in vitro* are listed in Table 1. Observed baseline APTT values were essentially the same as the intercept baseline values, 29.7 ± 5.3 s. There was a strong positive correlation between individual baseline APTT values and APTT-heparin slope values (r^2 : 0.920; $0.05 > P > 0.02$).

Figure 1 shows a plot of the mean plasma heparin activities vs time, where heparin activity is determined by both the APTT-heparin and PN-heparin assays. There was a rapid initial decline in plasma heparin activity during the first 5–7 min as determined by the chemical neutralization assay whereas plasma heparin activity determined by the bioassay apparently declines monoexponentially from the time of the first sample; the terminal decline in heparin activity with time has apparently the same slopes. The pharmacokinetic parameters of heparin, determined by the two

Figure 1 Average plasma heparin activity after intravenous injection of 25 units/kg in four normal subjects. Heparin activity is determined by two different assays, APTT-heparin ($\square$) and PN-heparin assays ($\circ$). The vertical lines represent 1 s.d.

heparin assays, are listed in Table 2. The $t_{1/2}$ was similar after both assays. Both CL and V_d , however, were statistically significantly or borderline significantly larger when based on the PN-heparin assay than the APTT-heparin assay ($P = 0.075$ and $P = 0.028$, respectively).

Figure 2 shows semilogarithmic plots of the APTT vs plasma heparin activity relationships, both when heparin is added *in vitro* before heparin administration, and when it is measured by the PN-heparin assay in samples collected after heparin administration; samples obtained in the first 5 min after the dose were not included. The anticoagulant effect of heparin observed *in vivo* is significantly greater than the anticoagulant effect observed *in vitro* (Table 1). The slopes of the $\ln$ APTT vs heparin activity relationships were on the average 1.44 ± 0.17 times higher *in vivo* than *in vitro* ($P < 0.001$).

Discussion

The results of this study on the time course of development of the assay-dependent kinetics of heparin have demonstrated that chemically assayed heparin has an apparent initial distribution phase, followed by an apparent elimination phase, while bioassayed heparin has only an apparent monoexponential phase (Figure 1). From about 5 min after the i.v. injection, both chemical heparin and bioassayed heparin in plasma exhibit a similar exponential decline, with a $t_{1/2}$ of about 20 min for the dose used. Since both of the heparin assays utilized are based on *in vitro* reference curves (although gauging different aspects of heparin's chemistry), it is clear that either assay method, or both, measure plasma heparin activity differently *in vitro* compared with *ex vivo*.

The chemical neutralization assay uses polybrene, a synthetic polymerized quaternary ammonium salt, which has been shown to be superior to protamine in neutralization assays of heparin (Godal, 1960). The positively charged polybrene neutralizes the highly negatively charged heparin; heparin owes its negative charges to the carboxyl functions of the uronic acids and the presence of numerous sulphate groups (Jaques, 1979). Since the PN-heparin assay is based on an ionic interaction, changes in this assay would imply a rapid *in vivo* decrease in neutralizing equivalents of heparin. However, changes in the bioassay using APTT, which is based on the anticoagulant effect of heparin via antithrombin III, would imply a rapid *in vivo* apparent increase in the anticoagulant effect of heparin. Of these two possibilities, the latter is more likely to occur and therefore explain the *in vivo* differences in the heparin assays for the following reasons. First, a rapid decrease in neutralizing equivalents would have to involve a substantial loss of sulphate groups. Indeed, based on stoichiometric considerations, the loss would be expected to have

Table 2 Pharmacokinetic parameters of heparin in normal subjects after 25 units/kg dose, based on PN-heparin and APTT-heparin assays.

Subject	$t_{1/2}$		CL ($\text{ml min}^{-1} \text{kg}^{-1}$)		$V_d(\text{ml/kg})$	
	PN	APTT	PN	APTT	PN	APTT
1	18.5	14.7	2.54	2.14	67.7	45.3
2	20.7	21.0	2.52	2.10	75.3	63.6
3	12.7	28.0	3.23	1.32	59.1	53.3
4	16.8	22.6	2.36	1.25	57.2	40.7
Mean	17.2	21.6	2.66	1.70	64.8	50.7
$\pm$ s.d.	3.4	5.5	0.39	0.48	8.3	10.0

to be proportional to the ratio of bioassayed to chemically assayed heparin. Although studies using [^{35}S]-heparin have demonstrated that a substantial fraction of the radiolabel is eventually excreted in the urine as free sulphate, there is no evidence for an initial rapid rate of sulphate excretion (Dawes & Pepper, 1979; Eiber *et al.*, 1960). Second, based on physiological pharmacokinetic considerations, an initial distribution phase is expected to be observed

for all compounds injected intravenously when blood samples are frequently collected (Riegelman *et al.*, 1968; Riggs, 1970). This is because distribution is not instantaneous, even for compounds which distribute primarily within the plasma volume and requires a few recirculations of blood before mixing is complete.

In vivo activation of heparin has been demonstrated in rabbits following s.c. injection of inactivated heparin; anticoagulant activity rose rapidly and

Figure 2 Relationships between APTT and plasma heparin activity in normal subjects *in vitro* and *in vivo*. The circles represent the *in vitro* relationships where heparin is added to plasma samples; the solid lines indicate the best-fitted lines. The squares represent the *in vivo* relationships where heparin is determined by a chemical neutralization assay; the dotted lines indicate the best-fitted lines.

reached a maximum in 3 h (Levy & Jaques, 1978). These studies also showed heparin assay differences in man, dogs and rabbits; heparin determined chemically using a metachromatic assay showed lower heparin activity in plasma than did a bioassay based on thrombin time. The authors concluded that the assay difference was due to an *in vivo* conversion of antithrombin-inactive heparin chains to antithrombin-active heparin chains. The present study provides the first data describing the time course of development of the *in vivo* differences in the heparin assays and it adds further evidence for the hypothesis that administered heparin may be activated *in vivo*. How such activation may occur, however, is presently not clear. The active moiety of heparin, the antithrombin III binding site, involves an oligosaccharide segment having a specific sequence of four to eight monosaccharides with *N*-sulphate, *o*-sulphate, and *N*-acetyl groups being required at specific sites (Björk & Lindahl, 1982; Höök *et al.*, 1981; Lindahl & Höök, 1978; Rosenberg *et al.*, 1981). One theoretical mechanism of an *in vivo* enhancement of the anticoagulant effect of administered heparin might therefore involve acetylation or sulphation at strategic sites, resulting in an active antithrombin III binding site.

The pharmacokinetic parameters obtained in this study are in general agreement with data obtained in

a previous study using the same heparin assay methodologies (Björnsson *et al.*, 1982). Because of the assay differences, both calculated CL and V_d are larger (about 1.7 and 1.3 times larger, respectively) when based on chemical heparin than on bioassayed heparin, while there is no difference in $t_{1/2}$. Also, in agreement with previous findings in normal subjects, there is a strong positive correlation (r^2 : 0.920) between *in vitro* baseline APTT values and APTT-heparin slope values (Björnsson & Wolfram, 1982; Cocchetto & Björnsson, 1982; Whitfield & Levy, 1980).

These studies in normal subjects have suggested that the assay-dependent kinetics of heparin may be due to a rapid *in vivo* activation of its anticoagulant properties. Comprehensive investigations will be required to elucidate the biochemical reactions underlying these changes and possible clinical implications of such processes.

This work was supported by U.S. Public Health Service Grant No. HL-24343. The authors thank Sharon Brown for typing the manuscript and Robert Wagoner for statistical advice. F.X.M. is a recipient of a Pharmaceutical Manufacturers Association Foundation Medical Student Research Fellowship in Pharmacology-Clinical Pharmacology. T.D.B. is a Nanaline H. Duke Scholar.

References

- BJÖRNSSON, T.D. (1983). Clinical pharmacology of heparin. In *Recent Advances in Clinical Pharmacology*, Vol. 3, eds. Turner, P. & Shand, D.G., pp. 129–155. Edinburgh: Churchill Livingstone.
- BJÖRNSSON, T.D. & WOLFRAM, K.M. (1982). Intersubject variability in the anticoagulant response to heparin *in vitro*. *Eur. J. clin. Pharmac.*, **21**, 491–497.
- BJÖRNSSON, T.D., WOLFRAM, K.M. & KITCHELL, B.B. (1982). Heparin kinetics determined by three assay methods. *Clin. Pharmac. Ther.*, **31**, 104–113.
- BJÖRK, I., & LINDAHL, U. (1982). Mechanism of the anticoagulant action of heparin. *Mol. Cell. Biochem.*, **48**, 161–182.
- COCCHETTO, D.M. & BJÖRNSSON, T.D. (1982). Simulation: An underutilized approach to coagulation system model evaluation. *Thromb. Res.*, **28**, 223–236.
- DAWES, J. & PEPPER, D.S. (1979). Catabolism of low-dose heparin in man. *Thromb. Res.*, **14**, 845–860.
- EIBER, H.B., DANISHEFSKY, I. & BORRELLI, F.J. (1960). Studies made with radioactive heparin in humans. *Angiology*, **11**, 40–43.
- ESTES, J.W. (1980). Clinical pharmacokinetics of heparin. *Clin. Pharmacokin.*, **5**, 204–220.
- GIBALDI, M., & PERRIER, D. (1975). *Pharmacokinetics*, pp. 1–43. New York: Marcel Dekker, Inc.
- GODAL, H.C. (1960). A comparison of two heparin-neutralizing agents: Protamine and polybrene. *Scand. J. clin. lab. Invest.*, **12**, 446–457.
- HIRSH, J., TURPIE, A.G.G., KELTON, J.G., HULL, R. & CARTER, C. (1981). Unresolved issues in the use of heparin for the management of venous thromboembolism. In *Chemistry and Biology of Heparin*, eds. Lundblad, R.L., Brown, W.V., Mann, K.G. & Roberts, H.R. North Holland, New York: Elsevier.
- HÖÖK, M., THUNBERG, L., RIESENFELD, J., BACKSTRÖM, A., PETTERSSON, I. & LINDAHL, U. (1981). Structure of the antithrombin-binding sequence in heparin. In *Chemistry and Biology of Heparin*, eds. Lundblad, R.L., Brown, W.V., Mann, K.G. & Roberts, H.R., pp. 271–279. North Holland, New York: Elsevier.
- JAQUES, L.B. (1979). Heparins. Anionic polyelectrolyte drugs. *Pharmac. Rev.*, **31**, 99–166.
- LEVY, S.W. & JAQUES, L.B. (1978). Appearance of heparin antithrombin-active chains *in vivo* after injection of commercial heparin and in amaphylaxis. *Thromb. Res.*, **13**, 429–441.
- LINDAHL, U. & HÖÖK, M. (1978). Glycosaminoglycans and their binding to biological macromolecules. *Ann. Rev. Biochem.*, **47**, 385–417.
- RIEGELMAN, S., LOO, J.C.K. & ROWLAND, M. (1968). Shortcomings in the pharmacokinetic analysis by conceiving the body to exhibit properties of a single compartment. *J. pharm. Sci.*, **57**, 117–123.
- RIGGS, D.S. (1970). *The Mathematical Approach to Physiological Problems. A Critical Primer*. pp. 214–217. Cambridge: The M.I.T. Press.
- ROSENBERG, R.D., OOSTA, G.M., JORDAN, R.E. & GARDNER, W.T. (1981). Mechanism of antithrombin

- action and the structural basis of heparin's anticoagulant. In *Chemistry and Biology of Heparin*, eds. Lundblad, R.L., Brown, W.V., Mann, K.A. & Roberts, H.R. pp. 249–269. North Holland, New York: Elsevier.
- WESSLER, S. & GITEL, S.N. (1979). Heparin: New concepts relevant to clinical use. *Blood*, **53**, 525–544.
- WHITFIELD, L.R. & LEVY, G. (1982). Relationship between concentration and anticoagulant effect of heparin in plasma of normal subjects: Magnitude and predictability of interindividual differences. *Clin. Pharmac. Ther.*, **28**, 509–516.
- ZAR, J.H. (1974). *Biostatistical analysis*, pp. 198–227. Englewood Cliffs, N.J.: Prentice-Hall, Inc.

(Received May 20, 1983,
accepted July 22, 1983)