The Optimum Number and Types of Plasma Samples Necessary for an Accurate Activated Partial Thromboplastin Time–Based Heparin Therapeutic Range

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• ***Context.*—Monitoring of unfractionated heparin therapy by activated partial thromboplastin time (aPTT) using the ex vivo method for determining the aPTT-based heparin therapeutic range (HTR) is the standard of practice. Many intrinsic and extrinsic factors influence its accuracy.**

***Objective.*—To investigate the optimum number and types of samples acceptable for an accurate ex vivo HTR determination.**

***Design.*—Values from patients receiving unfractionated heparin are used to determine the HTR by published guidelines. The number and types of samples are changed to investigate the effect on HTR parameters.**

***Results.*—Absolute minimum number of samples for an accurate HTR is 20, with fewer than 10% of the samples from the same patient or 50% of the samples with international normalized ratio of 1.3 to 1.5.**

***Conclusions.*—The ex vivo HTR method is the best protocol currently available; however, the number of samples used affects its accuracy. The optimum number of samples is 30 or more but the absolute minimum number is 20. In addition, limitation of specific sample types also affects the HTR parameters. An inaccurate HTR may be calculated if inappropriate sample number or types of samples are used.**

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Unfractionated heparin (UFH) therapy is most commonly used to treat venous thromboembolic disease and acute coronary syndrome.[1] Because of the potential clinical risks for a narrow therapeutic range and significant biological variation, UFH therapy must be monitored.[1,2] The activated partial thromboplastin time (aPTT) is the most common monitoring method; however, it is only a surrogate measure of the plasma heparin concentration.[3]

If the aPTT is used as the monitor for heparin therapy, only the most accurate protocols to determine the heparin therapeutic range (HTR) should be used. A number of protocols have been used that do not accurately reflect the heparin concentration or determine an accurate monitoring range. The fixed-range methods (arbitrary fixed values or ratio-based ranges) and the in vitro or ''spiked curve'' method are not accurate for all reagents.[4–6] The cumulative summary method has been the recommended method but relies on the previous range and small statistical variation of the new reagent compared to the old reagent.[4,7] Finally, the ex vivo method compares the aPTT and heparin level in the plasma of patients receiving UFH.[4,7] This protocol is the most common and recommended method for determining an accurate HTR.[4,7] However with the ex vivo method, significant drawbacks can be encountered: difficulty in obtaining an adequate number of samples, use of the proper types of samples, and integrity of the range, based on a ''best-fit'' linear regression statistical model.[7–9] In addition, significant variation can be due to both the heparin and aPTT values. The causes of variation include (1) differences in the heparin sources (preparation and content), (2) biological variation (heparin binding proteins, coagulation factor levels, and in vivo heparin processing), (3) clinical parameters (international normalized ratio [INR] and optimum clinical concentration), (4) preanalytic variation (sample collection, processing, and storage), and (5) analytic variables (different aPTT reagent sensitivities, components, and concentrations).[4–7,10,11]

Based on these issues, aPTT monitoring of UFH is difficult to standardize even using the ex vivo HTR method.[4,7,8] Many factors can influence both aPTT and heparin values. Few systematic investigations have evaluated the parameters of the ex vivo HTR model.[11–14] No evidence-based guidelines detailing the protocol have been formulated.[4,7] Several small studies[14,15] have evaluated a few aPTT reagents, demonstrating that commercial aPTT reagents have different sensitivities to UFH and hence, HTR. Several groups have proposed criteria and procedures for performing the ex vivo method but in fact did not scientifically establish their guidelines.[4,8,9]

The purpose of this study is to evaluate the number and type of samples affecting the ex vivo HTR method. Since this report is not a comparison of specific reagents per se, the commercial reagents are not identified within the data sets. However we compare different reagents to determine similarity in parameters. This report will present some of

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the required parameters for establishing the ex vivo HTR method.

MATERIALS AND METHODS

Patient Samples and Sample Processing

Patients receiving intravenous UFH over a range of therapeutic doses, but not influenced by other anticoagulants (INR <1.3), were part of this study, unless otherwise stated.[6,7,16,17] The baseline aPTT and prothrombin time values were normal unless otherwise stated. Each sample was initially drawn for the clinical monitoring of heparin. As approved by the institutional review board for human subjects, the residual plasma used in this study was considered discarded and no informed consent was necessary. No identifying information about the patient was available to the research group other than the plasma heparin concentration, aPTT value, initial prothrombin time and APTT values, and if the same individual was in the HTR data set.

All samples were from venous blood specimens collected in 3.2% buffered sodium citrate anticoagulant vacutainers.[6,17] The plasma was prepared within 45 minutes of collection by centrifugation at 2500*g* for 15 minutes according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[7,10] The centrifuged plasma samples contained less than 10 000 platelets/μL.[6,17] Plasma samples were transferred to polystyrene tubes and capped. All aPTT assays were performed on fresh plasma samples within 3 hours of collection. The remaining plasma was frozen at −80°C for heparin determination.[6]

aPTT Reagents and Instruments

The reagents and instruments were obtained from the commercial manufacturers. All aPTT assays were determined by the local hospital or on instruments within the coagulation laboratory per manufacturer instructions (MLA 1400, Instrumentation Laboratory, Bedford, Massachusetts or STA Compact, Diagnostica Stago, Parsippany, New Jersey). The following reagents were evaluated: Actin and Actin FS (Dade-Behring, Marburg, Germany), Synthasil (Instrumentation Laboratory, Lexington, Massachusetts), PTT Automate (Diagnostica Stago), and Auto APTT (Organon Technica, Raleigh, North Carolina). The aPTT reference range (average value, ±2 SD) for each lot of reagent is determined by using a minimum of 20 ''normal'' donors.[6] The HTR is determined by the ex vivo method outlined in the CLSI guideline.[4,6,7]

Heparin Assay

The plasma UFH concentration is determined by using a chromogenic antifactor Xa heparin assay (either HemosIL Heparin reagent kit [Instrumentation Laboratory] on the MLA 1400 analyzer or Rotachrom Heparin reagent kit [Diagnostica Stago] on an STA-R analyzer) per manufacturer instructions.[6] Both assays are calibrated with the manufacturer's calibrators (assigned against the World Health Organization unfractionated heparin standard). In a comparison study of the 2 heparin assays, no significant difference in the heparin values was noted between assay kits ($r = .95$, slope = .99, $P = .86$).[6]

Ex Vivo Heparin Therapeutic Range

Each plasma sample was assayed for both aPTT and heparin level values. With linear regression, the aPTT values (y-axis) are plotted against the heparin concentration (x-axis). The aPTT HTR is calculated with the aPTT lower limit correlated to 0.3 U/mL heparin and the aPTT upper limit is correlated to 0.7 U/mL of heparin.[4,7]

Minimal Number of Samples

To evaluate the minimal number of samples needed for an accurate HTR, large data sets of values (in sets of 5 or 10) of aPTT and heparin levels were used to calculate the heparin therapeutic range. The order of plasma samples used in these studies is sequential from date of draw.

Effect of INR on HTR Determination

In experiments evaluating the effect of oral anticoagulant therapy on the HTR, patients receiving UFH, with an INR up to 1.3, are added to the HTR determinations. In another set of experiments, samples with INR values between 1.3 and 1.5, and 1.5 and 2.0, are also studied for their effect on the HTR.

Effect of the Number of Same Patients

Studies using the same patient (at different times during the heparin therapy treatment period) to determine the HTR were also performed. The number of same-patient samples is included in the HTR determination to evaluate the effect on the HTR.

Statistics

All statistical determinations (descriptive statistics, linear regression, and Student *t* test) were calculated by Prizm 3.02 software (Graph Pad Software, Inc, San Diego, California). Statistical significance was set at $P < .05$. Clinically significant difference was defined as values that vary by 10% or more.

RESULTS

Optimum Number of Samples

Figure 1 demonstrates changes in lower and upper limits of the HTR for 2 different reagents (Figure 1, A and B) when the number of samples is increased. The data in Table 1 show that a minimum of 20 samples will establish an accurate range for the HTR. With 17 lots from 5 different reagents (Table 1), the random variation of changes in the lower and upper limits is clinically significantly different with fewer than 20 samples. The lower limit is clinically significantly different (<10%) for 10 samples in 100% of the lots tested and clinically different in 71% of the lots tested with 15 samples. The variability for both upper and lower limits decreases with 20 or more samples (Figure 2, A and B). The range also significantly varies up to 20 samples but stabilizes at more than 20 samples (Figure 2, C). The *R* value reaches its maximum value at 20 samples (Figure 2, D). There is no significant difference in the amount of variability between reagents or lots (Table 1).

Effect of Warfarin Therapy on the Accuracy of the HTR

Elevations of the INR due to warfarin affect the determination of the HTR in the 2 different reagents studied (Table 2). The most accurate HTR determination occurs when none of the patients have an INR greater than 1.3. However, up to 50% of the samples can have an INR between 1.3 and 1.5 and still generate a clinically significantly accurate HTR; however, a trend of increasing lower and upper limits is observed with an increasing number of samples with elevated INR. Ten percent of the samples with INR greater than 1.5 can be added before a clinically significant change in the HTR is observed, but again there is a trend for increasing ranges at 10% of the samples with INR greater than 1.5.

Variability of Reagent Lots

To eliminate the variables of different patients and different instruments, the HTR is determined for 2 different reagents with 3 different lots, each using the same 20 patients on the same day and the same instrument (Table 3). Although the 2 reagents have different HTRs, the heparin therapeutic ranges are statistically and

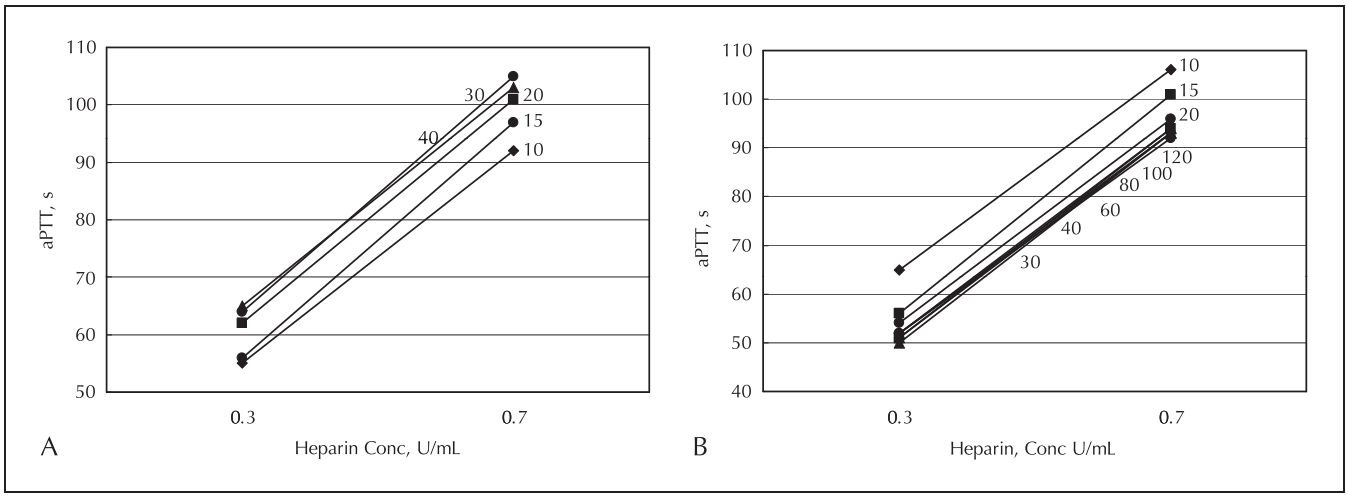

Figure 1. *The calculated activated partial thromboplastin time (aPTT)–based heparin therapeutic range (HTR), with the lower and upper aPTT limits, and the effect of increasing the number of samples on the accuracy of the HTR calculation for 2 different reagents (A and B). Sequential plasma sample aPTT and heparin values are added to the original 10 samples and the upper and lower limits are calculated for that number of samples. The numbers on the graphs represent the number of samples (10, 15, 20, 30, and 40) for that particular line and upper and lower limits. The numerical data are presented in Table 1 (A is D-1 and B is C-1). Abbreviation: Conc, concentration.*

clinically the same for all 3 lots of the same reagent when the same patients and instruments are used (Table 3). This experiment is conducted twice with 20 different patients in each analysis.

Effect of Using Multiple Samples From the Same Patient in the Determination of the HTR

In the evaluation of 2 lots, each from 2 reagents, the HTR significantly changes as the percentage of the number of the same patients (samples drawn at different times during the course of heparin therapy) is increased (Table 4). If the same patient is on the lower side of the average for heparin response, then the patient is usually on the low side for all samples, and the calculated upper and lower limits of the HTR are also lower than the true HTR. The same is true if the patient response is higher than average, causing the HTR lower and upper limits to be higher than the true HTR. As more of the same-patient samples are added to the HTR protocol, more significant deviation is observed. The minimum number from the same patient that can be used is 10% of the total number of samples used to calculate the HTR (Table 4).

COMMENT

Determination of an accurate heparin therapeutic range is one of the most difficult and uncertain protocols in the coagulation laboratory.[4–6,12,18] The most commonly performed method is the ex vivo HTR determination described in several guideline publications.[4,7] Since the HTR method itself creates imprecision, using a standard set of parameters will significantly increase the precision and accuracy of the HTR.[4,7,14,18,19] There are no calibrators

Table 1. Comparison of the Lower and Upper Limits of the Heparin Therapeutic Range (HTR) With Various Numbers of Samples Used to Calculate the Range[a]

	HTR Lower Limit, s					HTR Upper Limit, s				
	No. of Samples					No. of Samples				
Reagent[b]	10	15	20	30	40	10	15	20	30	40
A-1	**63**	**60**	56	51		85	**84**	89	93	
A-2	**65**	**58**	54	53		**102**	95	93	91	
A-3	**63**	**64**	54	54		**85**	91	93	96	
B-1	**48**	**51**	56	58		**80**	**84**	94	94	
B-2	**46**	**48**	54	56	57	90	89	93	92	94
B-3	**51**	58	58	60		95	97	94	92	
C-1	**65**	**58**	54	52	53	**107**	**101**	97	94	93
C-2	**48**	52	55	53		**84**	90	91	93	
C-3	**65**	**64**	53	50		**102**	**96**	91	88	
C-4	**64**	**59**	54	52		**102**	96	95	95	
C-5	**65**	55	53	52	50	**106**	**103**	93	94	93
D-1	**54**	**54**	64	65		**92**	97	104	102	
D-2	**52**	**54**	61	63	65	**81**	85	93	91	94
D-3	**55**	60	62	64	65	**92**	**93**	102	105	103
D-4	**49**	53	56	58		90	96	95	97	
D-5	**50**	**52**	58	60		**82**	92	94	92	
E-1	**61**	**54**	50	46		**79**	81	86	88	

[a] Values in bold have clinically significantly different values from the highest number of samples tested for that reagent lot.
[b] Reagents are listed by reagent type (A, B, C, etc) and individual lot (1, 2, 3, etc).

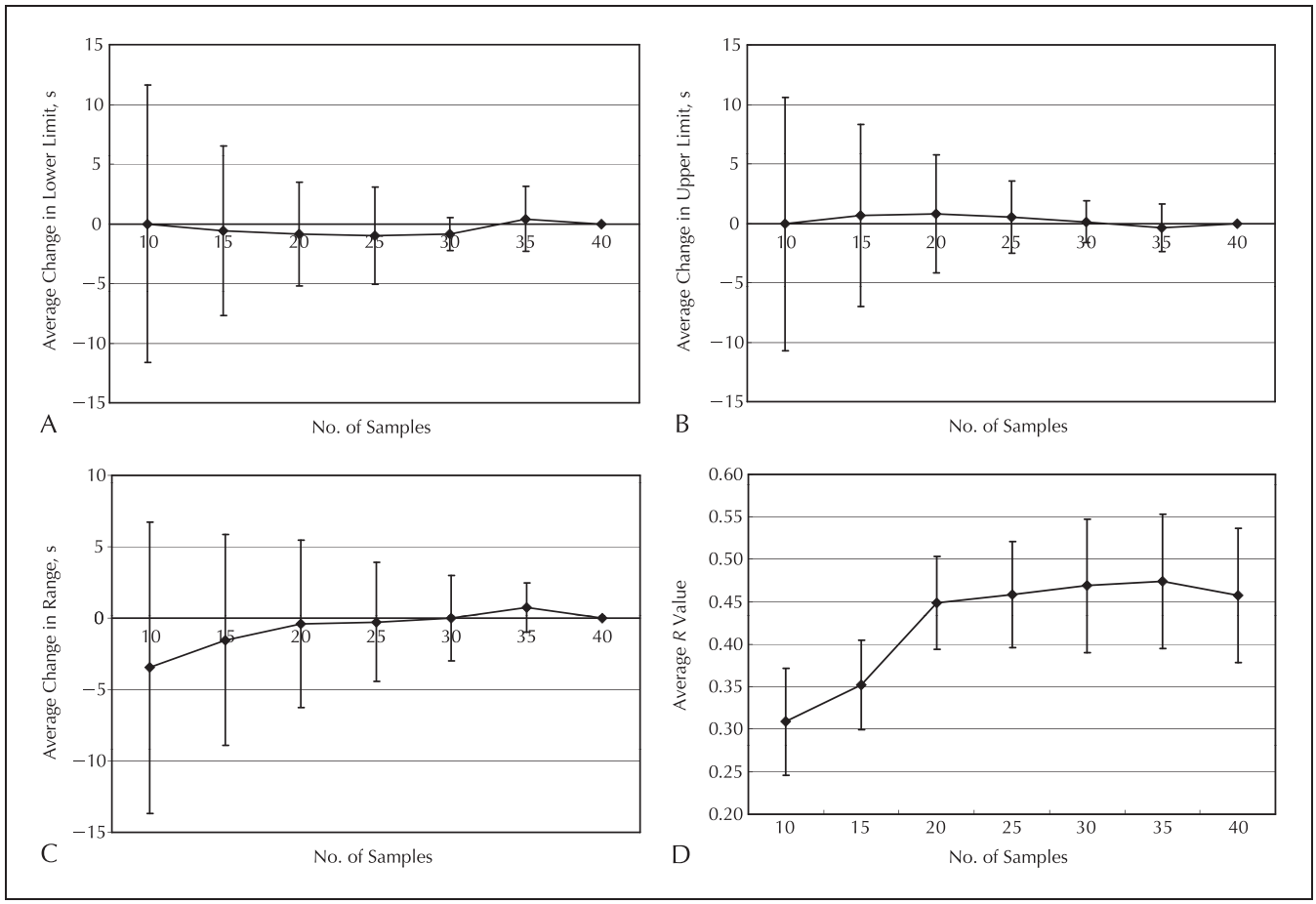

Figure 2. *Statistical comparisons of increasing the number of sample values on the lower limit (A), the upper limit (B), and the range (difference between the upper and lower limits) (C) for all lots of reagents studied. The R values with increasing number of values are shown in D. A sample size of 40 plasma samples is considered the most accurate heparin therapeutic range calculation (graphs A, B, and C). The y-axis is the average difference (in number of seconds) compared to the 40-sample data set. The error bars are the 95% confidence limits. In graph D, the average R value (with error bars) is plotted for the number of plasma samples tested.*

for establishing the HTR; therefore, since the accuracy and precision of the HTR is left up to the individual laboratory, it will need to obtain the appropriate samples and establish the proper method.[7] There are a number of parameters in the HTR method that can create statistically and clinically significant differences in the calculated range.

In this study, a number of common preanalytic variables and methodologic assumptions are investigated. These studies are not being performed on all of the commercially available reagents but are limited to several reagents.[6] However, on the basis of our studies, these reagents generate similar responses for the basic parameters and variables studied.[6] Therefore, the basic findings can be applied to all commercial reagents.

The optimum number of samples is 30 or more for an accurate HTR determination. However, the absolute minimum number of samples is 20. In 17 different lots

Table 2. Comparison of Calculated Heparin Therapeutic Range (HTR; Lower and Upper Limits) With Various Percentages of Patients Receiving Oral Anticoagulant Therapy[a]

	HTR, s			
	INR > 1.3 and <1.5		INR > 1.5 and <2.0	
Percentage	Reagent A	Reagent B	Reagent A	Reagent B
0	64–105	53–93	64–105	53–93
10	64–104	53–93	66–108	56–97
25	65–107	54–95	67–107	58–99
50	68–106	56–98	70–107	60–98
75	67–108	60–103	72–111	65–94
100	70–112	59–100	74–109	66–100

Abbreviation: INR; international normalized ratio.

[a] Each reagent set had 24 patients, and the percentage listed is the number of samples out of 24 samples that have an INR between 1.3 and 1.5 or between 1.5 and 2.0, respectively.

Table 3. Determination of the Heparin Therapeutic Range (HTR) With the Same 20 Patients: Using 2 Vials of Freshly Made Reagent for 2 Different Reagents With 3 Different Lots on the Same Instrument on the Same Day

Reagent Lot	HTR No. 1, s	HTR No. 2, s
Reagent A-lot 1	62–93	63–93
Reagent A-lot 2	63–95	63–95
Reagent A-lot 3	63–94	63–95
Reagent C-lot 1	55–87	55–87
Reagent C-lot 2	58–90	58–90
Reagent C-lot 3	54–83	55–85

from 5 different reagents (4 manufacturers), the HTR did not significantly change when testing 20 samples compared to 30 or more samples (Table 1 and Figure 2). Although variability is still somewhat wide at 20 samples, the accuracy of the HTR is increased with 25 to 40 samples. The changes in the lower and upper limit values are not clinically significantly different at 20 samples compared to 30 samples. Based on these data, the minimum number of samples necessary for the determination of a clinically accurate HTR is 20, but 25 to 40 is better.

Overall, correlations of the aPTT and heparin level are very poor, no matter what the sample size. Even as more samples are added, the *R* values are not routinely above 0.50. When sample numbers of fewer than 20 were correlated, the *R* value was significantly decreased ($P = .02$) as compared to studies with more than 20 samples (Figure 2, D). Based on these studies (and other data not shown), the *R* value should be greater than 0.40 for a valid HTR determination.

The HTR can clinically significantly change when more than 10% of the samples are from the same individual at different time points during heparin therapy (Table 4). The range can shift either higher or lower depending on the overall effect this single patient has on the HTR value, thus making the HTR erroneous (Table 4). The maximum number of samples from the same patient should be no greater than 10%. Because multiple samples can affect the HTR, only a single patient should have multiple samples (<10%) for any HTR determination. If multiple patients with 2 or more samples are included in the HTR determination, the accuracy of the range may be significantly compromised.

An increased number of patients receiving oral anticoagulant therapy, with INR values greater than 1.3, can clinically significantly change the HTR. The HTR increases the most when samples with an INR greater than 1.5 are included in the calculation. Samples with an INR value less than 1.5 should not be used in the determination of the HTR (Table 2). However, fewer than 50% of the samples can have an INR between 1.3 and 1.5 without clinically significantly affecting the HTR upper or lower limits.

Based on the studies presented and work done by others,[4,6,7] minimal requirements for determining the HTR using the ex vivo method are summarized in Table 5. When more samples exceed the minimal number of samples, the accuracy of the HTR is better. A minimum of 20 plasma samples, with no more than 10% of the samples from the same individual and fewer than 50% of the samples with an INR between 1.3 and 1.5, will provide the minimum precision and accuracy necessary for the HTR determination.

HTR Determination Recommendations

The basic procedure is to correlate the heparin concentration and the aPTT, and then to calculate the aPTT HTR with the aPTT lower limit corresponding to 0.3 U/mL of heparin and the aPTT upper limit corresponding to 0.7 U/mL of heparin.[4,7] The aPTT-based HTR is only a guideline for monitoring UFH therapy in patients.[3–6] The standard acceptable HTR determination method (based on College of American Pathologists [CAP] and CLSI guidelines) is the ex vivo UFH plasma method.[4,7,20] Both the CAP and CLSI guidelines make only general recommendations.[4,7,20] However, this method can generate an accurate HTR, if established criteria are adhered to (Table 5). Even when meeting the minimum criteria, this method has some drawbacks for the small to moderate-sized hospitals that do not have access to an adequate number of patients receiving UFH.[6,7] Alternative determination methods must be explored, including the possibility of using frozen plasma samples collected during a 3- to 6-month period. There are several unacceptable methods for HTR determinations, such as setting the HTR by using arbitrary ranges, and ranges used in the past without validation or the manufacturer's suggested range.[4,6] Both the use of ratios and the ''spiked'' plasma method are unacceptable, but unfortunately still commonly used.[4,6] The ex vivo method for the accurate determination of the HTR can be used if the minimal required number and type of samples are used as reported in this article.

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Table 4. Comparison of Calculated Heparin Therapeutic Range (HTR; Lower and Upper Limits) With Various Percentages of Samples From the Same Patient Obtained on Different Days While Receiving Unfractionated Heparin Therapy[a]

Percentage of Samples From the Same Patient	HTR, s			
	Reagent A		Reagent B	
	Lot 1	Lot 2	Lot 1	Lot 2
0 (all different)	65–104	58–101	47–91	54–92
8	65–104	57–101	47–91	54–93
25	63–104	57–103	49–92	54–97
50	63–106	62–105	51–98	57–101
75	58–106	62–109	51–100	58–105
100 (all the same)	60–105	64–109	51–102	59–108

[a] Each reagent set had 24 patients, and the percentage listed is the number of samples out of 24 samples that are from the same patient but were drawn on different days during the course of heparin therapy.

Table 5. Acceptable Parameters for Determination of the Heparin Therapeutic Range (HTR)[a]	
Acceptable Parameters	**Advantages**
≥20 heparinized plasma samples	Easier to obtain 20 samples and still statistically acceptable. Better with 30–50 samples.
<10% of samples from the same patient in HTR calculation	Two samples from the same patient do not influence HTR values.
Sample with INR <1.3	INR value does not influence HTR values.
<50% of samples with 1.3–1.5 INR	INR values do not influence HTR values.
No samples with INR >1.5	INR >1.5 can affect the HTR value.

Abbreviation: INR, international normalized ratio.

[a] The acceptable characteristics pertaining to number and plasma sample are listed with comments regarding the advantages.

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