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Full Length Research Paper

Evaluation of the i*n vitro* and i*n vivo* therapeutic equivalence of two pharmaceutically equivalent heparin products

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The current study compares the anticoagulant effects of a reference heparin product, heparin Leo® USA to a test product, heparin Hikma, Jordan. Both products are usually administered intravenous (IV) or subcutaneous (SC) and bioequivalence requirements are typically waived. Hence, it is pivotal to compare the therapeutic equivalency of the two products without jeopardizing the health of human volunteers. We have compared the anticoagulant effects of the two products in male albino rats by comparing the aPTT values 2 h following a single subcutaneous dose of either Hikma or Leo heparins. The average aPTT values for both Hikma and Leo heparins were 101.2 and 102.8 s, respectively. Further, the USP heparin assay test was performed to determine the ID_{50} of the Hikma and Leo heparins. The average volume of heparin that caused 50% inhibition of plasma *in vitro* was 0.431 and 0.439 ml for Hikma and Leo heparins, respectively. There was no statistically significant difference between the two products. The study concludes that the Hikma Heparin is therapeutically equivalent to the Leo heparin. Both products could be used interchangeably.

Key words: Hikma heparin, Leo heparin, therapeutic equivalence, heparin assay test, anticoagulant effects, aPTT.

INTRODUCTION

Heparin sodium is the sodium salt of sulfated glycosaminoglycans present as a mixture of heterogeneous molecules varying in molecular weights. Heparin is composed of polymers of alternating derivatives of D-glycosamine (N-sulfated, O-sulfated, or N-acetylated) and uronic acid joined by glycosidic linkages (Figure 1). The molecular weights of these molecules range from 5000 to 30,000 Da, with a mean of 15,000 Da. Unfractionated heparin (UFH) has a potent anticoagulant activity (McLean, 1916). It prolongs the clotting time of blood. The anticoagulant effects of heparin occur mainly through the formation of a complex with the plasma protein antithrombin (AT), a serine protease that is also known as heparin cofactor. The heparin-AT complex increases the affinity of AT to thrombin (IIa) by approximately 100 to 1000 folds (Hirsh and Raschke, 2004; Colman et al., 2006; Haines and Bussey, 1997). The heparin-AT complex recognizes and binds factor Xa via a pentasaccharide sequence and thrombin via an 18 saccharide sequence and then irreversibly inactivates these factors. In addition, at higher doses heparin forms another complex with heparin cofactor II which augments the anticoagulant effects of the heparin-AT complex and further deactivates circulating thrombin and a number of clotting factors such as IXa, Xa and XIIa. Further, through its action on thrombin, the hepain-AT complex also inhibits the thrombin-induced activation of factors V and VIII (Hirsh and Raschke, 2004).

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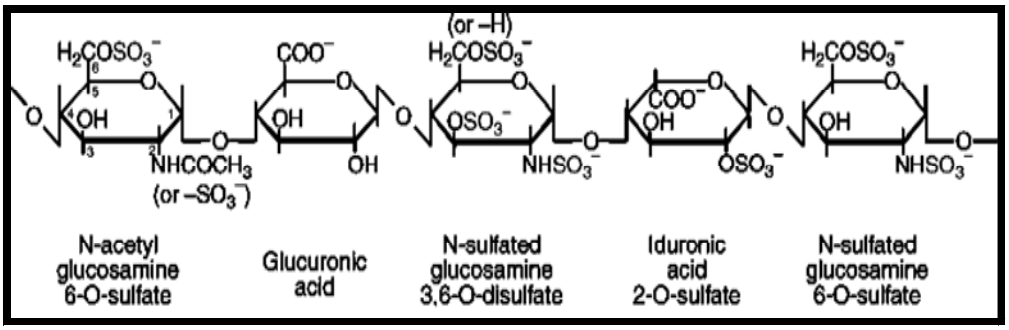

Figure 1. Chemical structure of major repeat units of heparin.

Unfractionated heparin is commercially extracted from the porcine intestinal mucosa or beef lungs. It has been clinically utilized for more than 70 years in the acute management and prevention of thromboembolic events frequently observed in acute coronary syndromes or after major surgeries such as pelvic and orthopedic surgeries (Leyvraz et al., 1983; Poller, 1985). Unfortunately, because of its origin, a potential anaphylactic reaction is always a possibility when administering UFH (Kishimoto et al., 2008). The risk for the latter event could be augmented if a heparin pharmaceutical product is approved to be marketed without proper validation of its content and uniformity. In addition, the anticoagulant profile and clearance of each UFH molecule vary based on its length; the smaller chains are cleared less rapidly than longer ones, this leads to substantial inter- and intra-subject variabilities observed in the anticoagulation response to UFH (Hirsh and Raschke, 2004).

Despite the clinical significance of discrepancies in the composition of heparin, its products are usually waived from bioequivalence studies as parental dosage forms. Hence, the UFH bioavailability of a new product is not typically compared to the products of the originator. Consequently, the biological activities of generic heparin products are not routinely evaluated.

In the cur0rent study, we compared the anticoagulant properties of heparin manufactured by Hikma pharmaceuticals (5000 IU/ml) to a reference heparin product (manufactured by Leo, USA). Both the *in vitro* and the in *vivo* clotting inhibition of both products were evaluated. The *in vitro* testing aimed at comparing the anticoagulant activities of the Hikma and Leo heparins when the same dose of heparin is exposed to the same amount of sheep plasma as outlined in the USP heparin assay test. The increase in aPTT with the addition of heparin, compared to no treatment, is an indirect measure of the heparin potency. On the other hand, the *in vivo* testing aimed at comparing the anticoagulant activities of Hikma and Leo heparins following subcutaneous injection of the same dose of either drug into male rats as discussed in the materials and methods section. Differences in the anticoagulant activities would likely reflect differences in heparin bioavailability between the Hikma and Leo products.

Another method of comparing the anticoagulant effects of heparin is the anti-Xa activity assay. The Anti-Xa activity is measured using a chromogenic titration assay that is expensive and of limited availability. In addition, the aPTT assay is still the most common tool to monitor heparin therapy and adjust its dosing in most clinical settings. Hence, a decision was made not to pursue the anti-Xa activity assay for heparin comparisons in the current study.

MATERIALS AND METHODS

In vivo study

Animals

Specific pathogen-free inbred male albino rats (250 to 300 g weight) were used. The animals were obtained from the University of Jordan Animal Facility, a nationally accredited facility, and fed standard chow pellets and water *ad libitum*. All rats were acclimated for one week before commencing the experiments. All the animals were treated according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, 1996).

Treatments

Animals were fasted for 6 h prior to treatments. Heparin products were diluted with phosphate buffer saline solution (PBS, pH = 7.4) to get a final concentration of 500 IU/ml. 15 rats were equally divided into three groups, 5 animals in each group. First group was the no-treatment control and received only PBS. The second group received test product (Hikma-heparin®,5000 IU/ml), and the third one received the heparin reference product (Leo-Heparin®, 5000 IU/ml). The two treatment groups received heparin solution at doses of 500 U/kg. Doses given to rats were calculated based on the body weight measured immediately prior to each dose. All treatments were given via subcutaneous injection. Subcutaneous bioavailability of UFH is dose-dependent and ranges from 30% at low doses to 70% at high doses. Higher doses presumably saturate protein-binding sites, thereby allowing a larger proportion to reach the systemic circulation (Hirsh and Raschke, 2004). The onset of anticoagulant effect is usually evident within 1 to 2 h after subcutaneous injection (Hirsh and Raschke, 2004). We have used a high dose of the heparin (human loading dose is usually 80 to 100 units/kg for a max of 10000 units) in order to improve the

Table 1. aPTT values for different treated groups.

	aPTT of group I (s)	aPTT of group II (s) (Test)	aPTT of group III (s) (Reference)
Rat 1	23	106	110
Rat 2	28	98	96
Rat 3	25	108	102
Rat 4	30	92	98
Rat 5	27	102	108
Mean ± SD	26.60 ± 2.08	101.20 ± 6.42	102.80 ± 6.10

bioavailabilities of both products and minimize host-related interferences (Hirsh and Raschke, 2004).

Anticoagulant action measured by activated partial thromboplastin time

Two hours after treatment, blood was collected from the retro-orbital plexus of each rat using plain capillary tubes. For analysis, a 0.5 ml of blood was collected in a sterile (sodium citrate) anticoagulated tubes (Minicollect®, Greiner, Germany) with a blood to anticoagulant ratio of 9:1 v/v. Tubes were mixed and centrifuged immediately after collection for 10 min at 4000 rpm. Then, plasma was transferred into a clean test tube and the activated partial thromboplastin time (aPTT) assay was performed immediately as described previously in the literature (Struver et al., 1962; Lenahan et al., 1966).

Briefly, 0.1 ml of plasma was mixed in a clean siliconized glass tube (10 X 75 mm) with 0.1 ml of cephaloplastin reagent and incubated at 37°C for 3 min. Then, 0.1 ml of calcium chloride (0.02 M, pre-warmed at 37°C) was added and the time required for clotting was recorded.

In vitro study

Preparation of plasma

Fresh blood was collected from sheep and mixed immediately with 8% sodium citrate solution by gentle agitation for a final ratio of one volume sodium citrate to 19 volumes of blood. Plasma was obtained by centrifuging the blood (4000 rpm for 4 min at 8°C), and pooling the separated plasma. The pooled plasma was then subdivided into portions of 60 ml each, then immediately frozen and stored in liquid nitrogen until use. The frozen plasma was thawed for 20 min using a water bath at a temperature of 37°C. Any particulate matter present was removed by straining the thawed plasma using a coarse filter. Screening of the plasma samples was carried out before using in the *in vitro* heparin assay. The sample was considered suitable for use if a solid clot is formed within 5 min after the addition of 0.2 ml of calcium chloride solution (1% w/w) to 1 ml portion of the pooled plasma in a clean test tube.

Preparation of heparin solution for *in vitro* testing

The concentration of both the test and the originator was 5000 IU/ml. Both heparin products were diluted with PBS to obtain a final concentration of 2 IU/ml. This concentration was selected after running a series of preliminary trials. It is defined as the minimum concentration of UFH which maintains the fluidity of 1 ml of prepared plasma for 1 h after the addition of 0.2 ml of calcium chloride solution. Heparin working solutions were freshly prepared and used in the same day of the assay.

Heparin assay procedure

The heparin potency assay procedure measures the volume of heparin required to cause a 50% inhibition of clot formation as described in USP pharmacopoeia (USP, 2008). Briefly; graded volumes of heparin working solution (test or reference, of 2 IU/ml) were added to meticulously clean 13 to mm × 100 mm test tubes as follows. A set of 16 test tubes was used for each heparin product (Hikma and Leo). Heparin volumes ranging from 0 to 0.8 ml per tube were added as outlined in Tables 2 and 3. To each tube so prepared, sufficient saline was added to make the total volume 0.8 ml (heparin plus saline total volume of 0.8 ml; for the tube with no heparin, 0.8 ml saline was added; for the tube with 0.8 ml heparin, no saline was added) (Tables 2 and 3). 1.0 ml of prepared plasma was then added to each tube. Finally, 0.2 ml of calcium chloride solution (1% w/w) was added and time was immediately recorded. The contents were then mixed by inverting three times in such a way that the entire inner surface of the tube became wet. The procedure was repeated three times for each of the test and the reference heparin products (3 sets of 16 tubes for each heparin product).

The extent of clotting was recorded one hour after the addition of calcium chloride and was classified into five grades (0.00 0.25, 0.50, 0.75 and 1.0); where zero indicates no clotting, 1 represents full clotting that rendered the whole sample one solid mass. Between these 2 extremes, samples were classified in stages 0.25, 0.50 and 0.75 depending on the volume of the final clot and the ability to withstand a vigorous three-time shaking of the tube. For mathematical calculations, the series contained at least 2 tubes graded more than 0.5 and 2 tubes graded less than 0.5. All of the observations were initially recorded by the same investigator who was blinded to the tested samples. Results were then independently verified by a second investigator.

RESULTS AND DISCUSSION

In vivo results

The effects of both test and reference products on the *in vivo* clotting inhibition were quantitated using the activated partial thromboplastin time (aPTT) assays in male albino rats. The aPTT assay is a sensitive tool for the comparison and evaluation of heparin products (Struver et al., 1962; Lenahan et al., 1966). Table 1 shows the aPTT values for different treated groups. Our results showed that both Hikma and Leo heparin products increased the clotting time significantly when compared to the untreated control group. Both products caused similar prolongation of the clotting time and they

Table 2. *In vitro* determination of grade of clotting following the addition of heparin Leo (2 IU/ml) to 1 ml of sheep plasma.

Tube no.	Volume of heparin (ml)	Volume of saline (ml)	Volume of plasma (ml)	Volume of $CaCl_2$ (ml)	Total volume	Grade of clotting*
1	0.8	0	1	0.2	2	0
2	0.75	0.05	1	0.2	2	0
3	0.7	0.1	1	0.2	2	0.25
4	0.65	0.15	1	0.2	2	0.25
5	0.6	0.2	1	0.2	2	0.25
6	0.55	0.25	1	0.2	2	0.25
7	0.5	0.3	1	0.2	2	0.25
8	0.45	0.35	1	0.2	2	0.25
9	0.4	0.4	1	0.2	2	0.5
10	0.35	0.45	1	0.2	2	0.5
11	0.3	0.5	1	0.2	2	0.75
12	0.25	0.55	1	0.2	2	0.75
13	0.2	0.6	1	0.2	2	1
14	0.15	0.65	1	0.2	2	1
15	0.1	0.7	1	0.2	2	1
16	0	0.8	1	0.2	2	1

* Grading was as follows: Grade 0: No clotting observed or small clots less than 1 cm in length after 1 h. This was arbitrarily defined as 100% inhibition of clotting after 1 h of $CaCl_2$ addition. Grade 0.25: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a single shaking via inversion of the tube. This was donated 25% inhibition of clotting. Grade 0.5: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a two-time shaking of the tube. Grade 0.75: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a three-time shaking of the tube. Grade 1: A stable clot more than 1 cm in length was formed in the tube that remained intact after a three-time shaking of the tube. This was arbitrarily defined as zero percent inhibition of clotting after one hour of $CaCl_2$ addition.

Table 3. *In vitro* determination of grade of clotting following the addition of heparin Hikma (2 IU/ml) to 1 ml of sheep plasma.

Tube no.	Volume of heparin (ml)	Volume of saline (ml)	Volume of plasma (ml)	Volume of $CaCl_2$ (ml)	Total volume	Grade of clotting*
1	0.8	0	1	0.2	2	0
2	0.75	0.05	1	0.2	2	0
3	0.7	0.1	1	0.2	2	0.25
4	0.65	0.15	1	0.2	2	0.25
5	0.6	0.2	1	0.2	2	0.25
6	0.55	0.25	1	0.2	2	0.25
7	0.5	0.3	1	0.2	2	0.25
8	0.45	0.35	1	0.2	2	0.5
9	0.4	0.4	1	0.2	2	0.5
10	0.35	0.45	1	0.2	2	0.75
11	0.3	0.5	1	0.2	2	0.75
12	0.25	0.55	1	0.2	2	1
13	0.2	0.6	1	0.2	2	1
14	0.15	0.65	1	0.2	2	1
15	0.1	0.7	1	0.2	2	1
16	0	0.8	1	0.2	2	1

* Grading was as follows: Grade 0: No clotting observed or small clots less than 1 cm in length after 1 h. This was arbitrarily defined as 100% inhibition of clotting after 1 h of $CaCl_2$ addition. Grade 0.25: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a single shaking via inversion of the tube. This was donated 25% inhibition of clotting. Grade 0.5: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a two-time shaking of the tube. Grade 0.75: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a three-time shaking of the tube. Grade 1: A stable clot more than 1 cm in length was formed in the tube that remained intact after a three-time shaking of the tube. This was arbitrarily defined as zero percent inhibition of clotting after one hour of $CaCl_2$ addition.

Table 4. Statistical analysis of Leo heparin clotting times.

Tube no.*	Volume of heparin (ml)	Grade of clotting	log V	x	y
7	0.5	0.25	-0.30	-0.35	0.33
8	0.45	0.25	-0.35	-0.40	0.42
9	0.4	0.5	-0.40	-0.46	0.58
10	0.35	0.5	-0.46	-0.53	0.67
11	0.3	0.75	-0.52		
12	0.25	0.75	-0.60		

x: Paired average of log volumes; y: Paired average of degree of clotting. * Grading was as follows: Grade 0: No clotting observed or small clots less than 1 cm in length after 1 h. This was arbitrarily defined as 100% inhibition of clotting after 1 h of $CaCl_2$ addition. Grade 0.25: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a single shaking via inversion of the tube. This was donated 25% inhibition of clotting. Grade 0.5: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a two-time shaking of the tube. Grade 0.75: At least 1 cm long clot mass was formed in the tube, but was re-suspended after a three-time shaking of the tube. Grade 1: A stable clot more than 1 cm in length was formed in the tube that remained intact after a three-time shaking of the tube. This was arbitrarily defined as zero percent inhibition of clotting after one hour of $CaCl_2$ addition.

Table 5. Statistical analysis of Hikma heparin-5000 clotting times.

Tube no.	Volume of heparin (ml)	Grade of clotting	log V	x	y
6	0.55	0.25	-0.26	-0.30	0.33
7	0.5	0.25	-0.30	-0.35	0.42
8	0.45	0.5	-0.35	-0.40	0.58
9	0.4	0.5	-0.40	-0.46	0.67
10	0.35	0.75	-0.46		
11	0.3	0.75	-0.52		

x: paired average of log volumes; y: paired average of degree of clotting.

increased the clotting time from 26.60 s for the control group into 101.20 and 102.80 s for the test and reference groups, respectively.

In fact, there was no statistical difference between the effects of Leo product on the clotting time when compared to the Hikma product (P> 0.05). In a clinical setting, aPTT should be measured 6 h after the administration of the heparin dose and no dose adjustments should be attempted prior to 6 h (Koda, 2009). For product comparisons, however, the anticoagulant effects of heparins would be apparent in 1 to 2 h following SC administration (Hirsh and Raschke, 2004). Hence, measuring the aPTT after 2 h of the SC heparin dose in our study was appropriate for the intended purpose of product comparison.

In vitro results

The *in vitro* clotting inhibition by the two pharmaceutically equivalent products was used as a surrogate indicator of *in vitro* bioequivalence of the generic and originator products. This *in vitro* assay is described in the US Pharmacopeia as one of the recommended heparin assay tests (USP, 2008). The idea of the test is to compare the minimum volumes of prepared heparin test and standard solutions that produce 50% inhibition of sheep plasma clotting as outlined in the protocol. Plasma was prepared by mixing the freshly collected blood with sodium citrate which chelates the calcium from that blood and allows us to harvest the plasma.

Tables 2 and 3 show the results of the effects of escalating serial volumes of Leo heparin (Table 2) and Hikma heparin (Table 3), which had been previously diluted to 2 IU/ml, on the extent of clotting. The latter is estimated 60 min after the addition of calcium chloride solution ($CaCl_2$). The $CaCl_2$ solution was added to initiate the clotting process. The aforementioned procedure was repeated three times and the data is the average of the three runs.

Calculation and statistical analysis of the results

The volumes of standard or test preparations used in the successive 5 or 6 tubes that bracket a grade of clotting of 0.5 were logarithmly transformed for both Leo heparin and Hikma heparin (Tables 4 and 5, respectively). These tubes included at least 2 tubes with grades larger than 0.5 and two tubes with grades less than 0.5.

Each tube was numbered and listed serially, and the grades of clotting were recorded as described in the

Table 6. Summary of statistical analysis of the *in vitro* clotting inhibition by Hikma or Leo heparins.

	Vu (test)	Vs (Reference)	Test/reference*100
Run I*	0.423	0.372	113.7
Run II	0.423	0.472	89.6
Run III	0.448	0.472	94.9
Average	0.431	0.439	99.4

V: Antilog value of the heparin volume that caused 50% inhibition of plasma clotting.* Each run consisted of 16 tubes for Hikma or Leo heparins as explained in the materials and methods.

materials and methods.

Statistical analysis of results was performed with Minitab 4. Each value is the average of three runs as previously described. Table 6 summarizes the average results for the three runs. Statistical analysis using two-sample t-test confirmed that there was no statistically significant difference between the median volumes of test and reference that caused 50% plasma clotting. The 95% confidence interval for the ratio of the means (85.1 to 112.1) with a point estimate of 99.4% further indicated that there were no statistically significant differences between the components of the pair (test and reference).

Conclusion

The current study concluded, based on both *in vivo* and *in vitro* comparisons, that the Hikma-Heparin® 5000 IU/ml is pharmaceutically and therapeutically equivalent to the reference product, heparin LEO®. Hence, both products are expected to be bioequivalent and can be used interchangeably.

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