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(54) Title: NEW METHODS FOR THE *IN VITRO* MEASUREMENT OF THE BIOLOGICAL ACTIVITY OF AN ULTRA LOW MOLECULAR WEIGHT HEPARIN SAMPLE

(57) Abstract: The present application concerns Ultra Low Molecular Weight Heparins (ULMWH) and the assays for measuring their biological activity.



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**New methods for the *in vitro* measurement of the biological activity
of an Ultra Low Molecular Weight Heparin sample**

The present application concerns Ultra Low Molecular Weight Heparins (ULMWH) and the assays for measuring their biological activity.

Low Molecular Weight Heparins are defined in the European Pharmacopoeia (European Pharmacopoeia 6.0 (01/2008:0828, 2041-2043)) by their molecular mass (less than 8000 Daltons) and their potency (an anti-factor Xa activity not less than 70 IU per milligram and a ratio of anti-factor Xa activity to anti-factor IIa activity not less than 1,5).

By contrast, semuloparin, developed by Sanofi-Aventis (AVE5026), belongs to a new generation of hemisynthetic heparins. It is a new ultra-low molecular weight heparin (ULMWH), with a mean molecular weight of 2400 Daltons and a novel antithrombotic profile resulting from high anti-factor Xa activity (~160 U/mg) and residual anti-factor IIa activity (~2 U/mg), as described by C. Viskov *et al.* in *Journal of Thrombosis and Haemostasis*, 2009, vol. 7, 1143-1151. Semuloparin, in the form of its sodium salt, is in clinical development for venous thromboembolism prevention.

Semuloparin is obtained by selective and controlled depolymerization of heparin, as described in the patent application WO 2004/033503 and in *J. Thromb. Haemost.* (*ibid*). It was shown that its unique structural features result from the highly selective depolymerization of heparin by the phosphazene base that protects the antithrombin (AT)-binding site from destruction.

Generally, for Low Molecular Weight Heparins (LMWH), the anti-factor Xa and anti-factor IIa activities are measured by using an International Standard as defined by the European Pharmacopoeia (European Pharmacopoeia 6.0 (01/2008:0828, 2041-2043)).

In particular, the patent application WO 2004/033503 teaches to measure the anti-factor Xa activity of semuloparin by the amyolytic method on a chromogenic substrate as described by Teien *et al.*, *Thrombosis Research*, 10, 399-410 (1977), using as a standard the first International Standard for low molecular weight heparins. Similarly, it teaches to measure its anti-factor IIa activity by the method described by Anderson L. O. *et al.*, *Thrombosis Research*, 15, 531-541 (1979), with as a standard the first International Standard for low molecular weight heparins.

WO 2005/010051 discloses new heparin-derived oligosaccharide mixtures characterized by a mean molecular weight comprised between 1800 and 2400 Daltons, an anti-factor Xa activity comprised between 190 IU/mg and 450 IU/mg, and virtually no anti-factor IIa activity. Said oligosaccharide mixtures are obtainable by a

re-depolymerization process of an ULMWH. The anti-factor Xa activity of these mixtures is measured relative to a reference ULMWH having an anti-factor Xa activity of 140 to 180 IU/mg (on dry basis) and an anti-factor IIa activity of 2,1 IU/mg, the activity of this reference ULMWH being measured relative to the International Standard for low molecular weight heparins.

However, the inventors have now shown that the International Standard generally used in assays for Low Molecular Weight Heparins, herein called "LMWH" or Low Molecular Mass Heparins (LMMH) International Standard, is not suitable for Ultra Low Molecular Weight Heparins, such as semuloparin. More specifically, it was surprisingly shown that in determining potency of semuloparin against said LMWH International Standard using 5-parameters data reduction model, the whole curves for samples were not found parallel, thus failing to comply with the parallelism pre-requisite.

The parallelism requirement between sample and standard is commonly used for the bioassays data reduction (R.G. Das *et al.*, *BioProcess International*, 2008, 46-56) and recommended by the Pharmacopoeia: comparison of the high and low asymptotes and the slope of curves is recommended in chapter 5.3 of the European Pharmacopoeia and in part <111> of the US Pharmacopoeia. In particular, the methodology developed by Dunn *et al.* (Measuring Parallelism, Linearity and Relative Potency in Bioassay and Immunoassay Data, *Journal of Biopharmaceutical Statistics*, 2005, 15:437-463; The five-parameter logistic: A characterization and comparison with the four-parameter logistic, *Analytical Biochemistry*, 2005, 343:54-65, both incorporated herein by reference) involves *inter alliae* measuring parallelism by comparing two "dose-response" curves for mathematical similarity of shape, determination of logistic curves by using the 4 or 5-parameters model, determination of the distance of two sets of data from "perfect" parallelism.

In view of the lack of parallelism obtained for semuloparin, in particular when measuring the anti-factor IIa activity, it appears that the LMWH International Standard is not suitable as a unique reference standard for measuring the biological activity of an ULMWH, as this leads to a lack of parallelism, and, hence, a failure in the statistical analysis of the potency estimation results, as well as a lack of compliance towards the methodology set forth in the European Pharmacopoeia for anti-factor IIa and anti-factor Xa titrations.

There is therefore a need to provide straightforward and reproducible methods of measuring the biological activities of ULMWH.

More particularly, there is a need for a standard suitable for determining relative potencies of ULMWH samples such as semuloparin samples.

The inventors have identified a standard suitable for the *in vitro* measurement of the biological activity of an ULMWH sample, where said standard comprises said ULMWH sample.

According to a first object, the present invention thus concerns a method for the *in vitro* measurement of the biological activity of an ULMWH sample, wherein said method is carried out relative to a standard comprising the same ULMWH.

ULMWH is used herein to refer to Ultra Low Molecular Weight Heparins, as defined by the European Pharmacopoeia in force, *supra*. In the framework of the instant invention, it includes heparin derivatives having a molecular weight of 2000 to 4000 Daltons. ULMWH include in particular semuloparin.

Semuloparin has a mean molecular weight of 2000-3000 Daltons (about 2.4 kDa on average), a high anti-factor Xa activity (approx. 160 U/mg on dry basis), a low anti-factor IIa activity (approx. 2 U/mg on dry basis) and a high anti-factor Xa / anti-factor IIa ratio (>30).

The term "semuloparin" as used herein is used to refer to semuloparin in the free form or any of its salts, including semuloparin sodium salt.

The term "standard" as used herein refers to the standard, or calibrator used for measurement of *in vitro* activity to assign the potency of a sample.

The standard is generally expressed in units (U).

The standard comprising the same ULMWH is herein referred to as "the ULMWH standard".

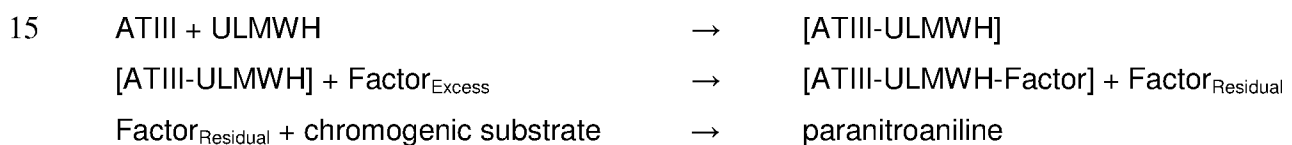
According to the instant invention, the ULMWH standard is a preparation of semuloparin. Said preparation is preferably a solution of semuloparin.

According to a preferred embodiment, the ULMWH standard has a pre-determined biological activity; more particularly, the ULMWH standard has a pre-determined anti-factor Xa activity, generally an anti-factor Xa activity of around 160 U/mg on dry basis and an anti-factor IIa of around 1 to 5 U/mg on dry basis.

According to a preferred embodiment, the method of the invention is a dilution assay, wherein the ULMWH sample to be measured is supposed to have the same potency as the ULMWH standard.

5 The measurement of the biological activity of an ULMWH sample is carried out by determination of the relative potency(ies) of said sample. This determination requires the parallelism of the curves of the ULMWH sample to be measured and those of the ULMWH standard.

10 The measurement of said biological activity includes the measurement of the anti-factor Xa activity and/or the measurement of the anti-factor IIa activity of an ULMWH sample. These assays determine the ability of said sample to accelerate the inhibition of factor Xa (anti-factor Xa activity) and/or of factor IIa (or thrombin), by antithrombin III (ATIII), according to the following reactions:



20 The amount of the paranitroaniline, measured at 405 nm, is inversely proportional to that of the ULMWH sample.

The term “curves” above refers to the graphic representation obtained by plotting the optical density measured in the above reaction as function of the concentration of the sample.

25 The measurement of said biological activity is preferably made by adapting the method described in the monograph on LMWHs of the European Pharmacopoeia in force, such as European Pharmacopoeia 6.0 (01/2008:0828, 2041-2043).

30 Generally, said method comprises an amidolytic assay on a chromogenic substrate, according to the method described in the monograph on LMWHs of the European Pharmacopoeia in force, as above.

35 Said chromogenic substrate depends on the activity to be measured. Suitable chromogenic substrates for measuring the anti-factor Xa activity may be chosen from the group consisting in S-2222TM (Bz-Ile-Glu(g-OR)-Gly-Arg-pNA·HCl) and S-2765TM (N-α-Z-D-Arg-Gly-Arg-pNA 2HCl also referred to as N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-

arginine-4-nitroanilide dihydrochloride) both available from Chromogenix; CBS 31.39 (CH₃-SO₂-D-Leu-Gly-Arg-pNA) available from Stago; BIOPHEN® CS-11(22) (Mixture (50%-50%) of Bz-Ile-Glu (γOCH₃)-Gly-Arg-pNa (form 1) and Bz-Ile-Glu (γOH)-Gly-Arg-pNa (form 2)) available from Hyphen BioMed; BIOPHEN® CS-11(32) (Suc-Ile-Gly (γ Pip)-Gly-Arg-pNa, HCl) available from HYPHEN BioMed; BIOPHEN® CS - 11(65) (D-Arg-Gly-Arg-pNA, 2HCl) available from HYPHEN BioMed. More preferably, said chromogenic substrate for measuring the anti-factor Xa activity is S-2765™.

Suitable chromogenic substrates for measuring the anti-factor IIa activity may be chosen from the group consisting in S-2238™ (H-D-Phe-Pip-Arg-pNA·2HCl also referred to as D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride) available from Chromogenix, CBS 34.47 (H-D-CHG-But-Arg-pNA) and CBS00.68 both available from Stago, BIOPHEN® CS-01(81) (Tos-Gly-Pro-Arg-pNa, AcOH) and BIOPHEN® CS-01(38) (H-D-Phe-Pip-Arg-pNa, 2HCl) both available from HYPHEN BioMed. More preferably, said chromogenic substrate for measuring the anti-factor IIa activity is S-2238™.

The method of the invention may be conducted in solutions of biological samples such as plasma, or in buffer solutions, preferably in buffer solutions.

Buffers may be chosen from any known buffers, in particular those having a pH close to biological media, such as those buffers having a pH comprised between 6.5 and 9. Buffer solutions include in particular tris(hydroxymethyl)aminomethane sodium chloride pH 7.4, tris(hydroxymethyl)aminomethane-EDTA pH 8.4, and tris(hydroxymethyl)aminomethane-NaCl-PEG pH 7.4. They may be prepared according to routine, well-known procedures.

Generally, the method of the invention comprises the following steps:

- a) preparing said standard comprising said ULMWH, and preparing said sample,
- b) determining the calibration curve for the biological activity of said ULMWH standard,
- c) determining the curve for the biological activity of said sample,
- d) confirming standard and sample parallelism,
- e) determining the biological activity of the ULMWH sample against the calibration curve obtained in step b).

Step a

The ULMWH standard, in a form such as a water for injection solution, may generally be prepared by diluting a given amount of the ULMWH into a given amount of the chosen solution such as buffer solution. The given amounts are advantageously expressed in volumes.

Said ULMWH standard and ULMWH sample are generally processed in the same way, in the same medium, e.g. buffer solution.

The ULMWH sample may be prepared in solution of water for injection, by weighing an appropriate quantity in order to obtain activities in U/mL equivalent to those of the ULMWH standard. The sample is then diluted in the same medium (e.g. buffer solution) as the ULMWH standard.

Generally, there is a loss of weight occurring on drying, due to the absorption of water molecules by the sample; if applicable, this loss is to be determined by methods known in the art, so that the activity may be expressed on dry basis (U/mg).

Steps b and c

A potency (e.g. anti-factor Xa or anti-factor IIa activity) expressed in U/mL is assigned to the given amount of the ULMWH, before dilution. A corresponding potency may thus be calculated for the diluted sample, depending on the dilution rate.

Several diluted samples may be prepared, with for each a corresponding potency.

From 4 to 14 calibration points may be performed. Particularly, the method of the invention involves 4 or 14 calibration points depending on whether a routine procedure or experimentation procedure is carried out, respectively. The procedure may be carried out according to the 5-parameters methodology (in particular for the 14 calibration point method) or the parallel lines methodology (in particular for the 4 calibration point method).

The assay for carrying out the method of the invention may be performed by known procedures, such as on a micro-plate reader and dispenser.

The calibration curve may thus be obtained by correlating the calculated potencies with the obtained Optical Density (OD).

The activity in U/mL of the ULMWH sample may be obtained by correlating the activity and the obtained Optical Density (OD), according to the calibration curve obtained with the ULMWH standard.

Generally, the anti-factor Xa activity assigned to the ULMWH standard before dilution is 100 U/mL. Preferably, the points of the calibration curve are comprised between approximately 0.002 and 0.8 U/mL for the anti-factor Xa activity curve in the 14 calibration

points method. The range of the calibration points for the 4 point calibration curve is generally comprised within the above range, such as $0.04\text{-}0.07 \pm 0.002$ U/mL.

Generally, the anti-factor IIa activity assigned to the ULMWH standard before dilution is 1.8 U/mL. Preferably, the points of the calibration curve are comprised between 0.0007 and 0.3 U/mL for the anti-factor IIa activity curve in the 14 calibration points method. The range of the calibration points for the 4 point calibration curve is generally comprised within the above range, such as $0.007\text{-}0.012 \pm 0.001$ U/mL.

Step d

For the 14 calibration points method the parallelism between standard and sample is assessed by using the methodology developed by Dunn *et al.* (Measuring Parallelism, Linearity and Relative Potency in Bioassay and Immunoassay Data, Journal of Biopharmaceutical Statistics, 2005, 15:437-463; The five-parameter logistic: A characterization and comparison with the four-parameter logistic, Analytical Biochemistry, 2005, 343:54-65, both incorporated herein by reference).

For the 4 calibration points method the parallelism between standard and sample is assessed using the statistical analysis described in the chapter 5.3 of Ph.Eur for parallel line methodology.

Step e

The biological activity (relative potency) of the ULMWH sample is calculated from the calibration curve obtained with the ULMWH standard.

According to a further object, the present invention also concerns a standard suitable for measuring the *in vitro* biological activity of an ULMWH sample, which comprises the same ULMWH than the sample to be measured.

Preferably, said standard comprises semuloparin.

Said ULMWH standard is suitable for conducting the method of the invention.

Said ULMWH standard is preferably obtained by diluting a sample of the ULMWH to be measured, which is calibrated by comparison with a recognized standard of LMWH, such as the second international standard of LMWH, currently available. The ULMWH standard is generally assigned an anti-factor Xa activity around 160 U/mg on dry basis and an anti-factor IIa of around 2 U/mg on dry basis.

FIGURES

Figures 1 and 2 illustrate the anti-factor Xa activity of bemiparin and semuloparin according to the 14 calibration points procedure.

Figures 3 and 4 illustrate the anti-factor IIa activity of bemiparin and semuloparin according to the 14 calibration points procedure.

Figures 5, 6, 7 and 8 illustrate the anti-factor Xa activity and anti-factor IIa activity of semuloparin compared with a standard of Low Molecular Mass Heparin for assay Biological Reference Preparation batch 5 (BRP5) according to the 14 calibration points procedure.

Figures 9, 10, 11, 12 illustrate the anti-factor Xa activity and anti-factor IIa activity of semuloparin compared with a heparin standard (K5, USP357) according to the 14 calibration points procedure.

Figure 13 and 14 illustrate the anti-factor Xa activity and anti-factor IIa activity of semuloparin and a standard comprising semuloparin (3000ET) according to the 4 calibration points procedure.

The following examples are given for representative and non limiting illustration of the invention.

EXAMPLES

I - THE 5-PARAMETERS METHODOLOGY (14-calibration points procedure)

Anticoagulant activity of semuloparin was determined *in vitro* by chromogenic assays measuring its ability to accelerate the inhibition of factor Xa or factor IIa by anti-thrombin III.

The curve profile of biological activity of the semuloparin was compared to that of bemiparin, the LMWH reference standard of the European Pharmacopoeia BRP5, and the heparin standard (K5, USP357).

The logistic curves were determined to assess parallelism of the products using the 5-parameters model.

The anti-factor IIa and anti-factor Xa activities were measured by assays according to the following protocols:

PREPARATION OF REAGENTS

The reagents used below are all commercially available.

Tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4

- 5 Dissolve 6.08 g of tris(hydroxymethyl)aminomethane, 8.77 g of sodium chloride in 500 mL of distilled water. Adjust the pH using hydrochloric acid. Dilute to 1000 mL with distilled water.

Tris(hydroxymethyl)aminomethane sodium chloride PEG buffer solution pH 7.4

- 10 Dissolve 6.08 g of tris(hydroxymethyl)aminomethane, 8.77 g of sodium chloride in 500 mL of distilled water. Add 0.5 g of polyethylene glycol 6000. Adjust the pH using hydrochloric acid. Dilute to 1000 mL with distilled water.

Tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4

- 15 Dissolve 6.06 g of tris(hydroxymethyl)aminomethane, 2.8 g of ethylenediamine tetracetic acid (EDTA) 10.24 g of sodium chloride in 500 mL of distilled water. Adjust the pH using hydrochloric acid. Dilute to 1000 mL with distilled water.

Acetic acid

- 20 Dilute 420 ml of glacial acetic acid to 1000 mL with water.

Antithrombin III solution

- Reconstitute antithrombin III as directed by the manufacturer and dilute with tris(hydroxymethyl)aminomethane sodium chloride PEG buffer solution pH 7.4 to 1 IU/mL.

25

Factor Xa solution, bovine

Reconstitute as directed by the manufacturer and dilute with tris(hydroxymethyl)aminomethane sodium PEG chloride buffer solution pH 7.4 to 1.8 nKat/mL.

30

Thrombin solution, human

Reconstitute human thrombin as directed by the manufacturer and dilute with tris(hydroxymethyl)aminomethane sodium chloride PEG buffer solution pH 7.4 to 5 NIH/mL.

35

Chromogenic substrate for anti-Xa (S2765)

Dissolve N- α -benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride in water to give a 0.003 M solution. Dilute in tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R to 0.0005 M before use.

5

Chromogenic substrate for anti-IIa (S2238)

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in water to give a 0.003 M solution. Dilute before use in titrating in tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R to give a 0.0005 M solution.

10

EQUIPMENT, MATERIALS, ANALYTICAL CONDITIONS

Equipment is given indicatively – any other equipment validated for this method can be used.

15

PACKARD MULTIPROBE II STD: Automated liquid handling system (dilution robot).

MR 7000 or DIAS from LABSYSTEM: Microplate reader, incubator and dispenser.

20

PREPARATION OF SOLUTIONS TO BE ASSAYED

Prepare the semuloparin solutions in order to obtain activities in U/mL equivalent to those of the standard currently used (stock solution in water for injection).

If possible, determine loss on drying while the substances are being weighed, in order to express the activity on the dried basis; if not, express it on the as-is basis.

25

The following dilutions are performed in the Tris-NaCl pH 7.4 buffer.

PREPARATION OF STANDARDS

Semuloparin, the LMWH standard of the European Pharmacopoeia (BRP5), the heparin standard (K5 USP 357) and the semuloparin reference standard (3000ET) are diluted in the same way, and the ranges in U/mL or UI/mL are given in Tables 1 and 2.

30

Anti-factor Xa activity

Table 1- Standard range for anti-factor Xa activity (geometric progression of 1.6)

	Activity in U/mL
first calibration point (S1)	0.8
last calibration point (S14)	0.002

5 **Anti-factor IIa activity**

Table 2- Standard range for anti-factor IIa activity (geometric progression of 1.6)

	Activity in U/mL
first calibration point (S1)	0.3
last calibration point (S14)	0.0007

ASSAY

10 The assay was performed using MR7000 or DIAS (dynex) from LABSYSTEM.

ANTI-FACTOR Xa ACTIVITY:

15 After dispensing 20 μ L of solution to analyze, add 20 μ L of Antithrombin III (ATIII), mix and equilibrate for 1 minute at 37°C or at room temperature and add 40 μ L of bovine factor Xa solution. Incubate 1 minute and add 100 μ L of chromogenic substrate for anti-FXa (S2765). Stop the reaction after 4 minutes by adding 100 μ L of acetic acid. Measure the absorbance at 405 nm.

ANTI-FACTOR IIa ACTIVITY:

20 After dispensing 20 μ L of solution to analyse, add 20 μ L of ATIII, mix and equilibrate for 1 minute at 37°C or at room temperature and add 40 μ L of human thrombin solution. Incubate 1 minute and add 100 μ L of chromogenic substrate for anti-FIIa (S2238). Stop the reaction after 4 minutes by adding 100 μ L of acetic acid. Measure the absorbance at 405 nm.

25

RESULTS INTERPRETATION

Establish the parallelism using the 5-parameters methodology, described by Dunn *et al. (ibid)*.

30 The anti-factor Xa and anti-factor IIa experiments were performed 6 times (mean of 6 curves).

According to the above procedures, semuloparin was compared to a lower, second-generation LMWH (bemiparin - fig. 1 to 4), a LMWH international standard (BRP5 - fig. 5-8) and a heparin reference standard (K5, USP357 - fig. 9-12).

5 1. Comparison of bemiparin and semuloparin

Results are illustrated in Figures 1 and 2 for the anti-factor Xa activity and in figures 3 and 4 for the anti-factor IIa activity.

According the methodology of Dunn et al (ibid) the statistical analysis and parallelism were assessed. Anti-FXa concentration response curves indicated that the constrained and the unconstrained models were equivalent, suggesting that the curves are similar and then the in vitro anti-FXa behavior of bemiparin and semuloparin could be considered as equivalent. In contrast anti-FIIa concentration response curves showed that the constrained and the unconstrained models were not equivalent, suggesting that the curves are not similar, reflecting a non parallelism between the 2 curves. Finally, the in vitro anti-FIIa behavior of bemiparin and semuloparin could not be considered as equivalent (see also Jeske W.P. et al., "A common standard is inappropriate for determining the potency of ultra low molecular weight heparins such as semuloparin and bemiparin", Thromb. Res., 2011, 128(4), 361-367).

20 2. Comparison of Low Molecular Mass Heparin standard (BRP5) and semuloparin

Results are illustrated in Figures 5 and 7 for the anti-factor Xa activity and in figures 6 and 8 for the anti-factor IIa activity

Using the same statistical methodology, it was showed that for both anti FXa and anti-FIIa the curves are not similar between the LMWH (BRP5) standard and semuloparin, suggesting both the in vitro anti FXa and anti FIIa behavior of LMWH and semuloparin could not be considered as equivalent.

30 3. Comparison of heparin standard (K5, USP357) and semuloparin

Results are illustrated in Figures 9 and 11 for the anti-factor Xa activity and in figures 10 and 12 for the anti-factor IIa activity.

Same conclusion as above, both the in vitro anti FXa and anti FIIa behavior of heparin standard and semuloparin could not be considered as equivalent, after the assessment of the parallelism using the 14 points calibration methodology.

It is apparent that semuloparin can not be compared with either bemiparin, the LMWH standard or the heparin standard.

In conclusion, the above results using 14 calibration points methodology clearly and unexpectedly show that semuloparin could not be validly compared with the usual LMWH standard.

II - THE 4-CALIBRATION POINTS METHODOLOGY

This method may be used as a routine procedure. Anticoagulant activity of semuloparin was determined *in vitro* by chromogenic assays. The methods were adapted from those recommended by the European Pharmacopoeia for low molecular weight heparin (LMWH).

The ability of semuloparin to accelerate the inhibition of factor Xa or factor IIa by anti-thrombin III was measured.

The biological activity of the sample was compared to that of a well characterised in-house semuloparin reference standard.

The potency of semuloparin was expressed by comparison with the standard preparation, using the usual statistical methods for parallel-line assays (§5.3 of the European Pharmacopoeia).

The anti-factor IIa and anti-factor Xa activities were measured by assays according to the following protocols:

PREPARATION OF REAGENTS

Same specifications as described above under the 14-calibration points procedure.

PREPARATION OF STANDARDS

The standard and test samples are processed in the same way. Any dilution method is appropriate, provided that the ranges in U/mL are identical to those given in Table 3 and Table 4.

Anti-factor Xa activity

Table 3 - Standard range for anti-factor Xa activity (geometric progression of 1.2)

Activity in U/mL	
1st calibration point (S1)	0.04
4th calibration point (S4)	0.07

Anti-factor IIa activity

Table 4- Standard range for anti-factor IIa activity (geometric progression of 1.2)

	Activity in U/mL
1st calibration point (S1)	0.007
4th calibration point (S4)	0.012

ASSAY

5 The assay is performed using MR7000 or DIAS from LABSYSTEM,

ANTI-FACTOR Xa ACTIVITY:

10 After dispensing 20 µL of solution to analyse, add 20 µL of ATIII, mix and equilibrate at 37°C or at room temperature for 1 minute and add 40 µL of bovine factor Xa solution. Incubate 1 minute and add 100 µL of chromogenic substrate for anti-FXa (S2765). Stop the reaction after 4 minutes by adding 100 µL of acetic acid. Measure the absorbance at 405 nm.

15 Calculate the regression of the absorbance on log concentrations of the test and standard solutions and calculate the potency using the usual statistical methods for parallel-line assays (§5.3 European Pharmacopoeia) for each serie. For each serie the validity criteria must be fulfilled and the final potency is calculated in Units of anti-factor Xa activity per milliliter (or per milligram) by an arithmetic mean of the two preparations.

ANTI-FACTOR IIa ACTIVITY:

20 After dispensing 20 µL of solution to analyse, add 20 µL of ATIII, mix and equilibrate at 37°C or at room temperature for 1 minute and add 40 µL of human thrombin solution. Incubate 1 minute and add 100 µL of chromogenic substrate for anti-FIIa (S2238). Stop the reaction after 4 minutes by adding 100 µL of acetic acid. Measure the absorbance at 405 nm.

25 Calculate the regression of the absorbance on log concentrations of the test and standard solutions and calculate the potency using the usual statistical methods for parallel-line assays (§5.3 European Pharmacopoeia) for each serie. For each serie the validity criteria must be fulfilled and the final potency is calculated in Units of anti-factor IIa activity per milliliter (or per milligram) by an arithmetic mean of the three preparations.

30 RESULTS INTERPRETATION

For each run, the analysis of variance must be valid and the C test less than 1.2. This may be done by the parallel line method according to the European pharmacopoeia or according to the Dunn *et al.* (see above) method.

EXPRESSION OF RESULTS

The relative potency is expressed in U (anti-FXa or anti-FIIa)/mg on the dried basis.

$$\text{Potency on dried basis} = \frac{\text{Potency as-is} \times 100}{100 - H}$$

where H = loss on drying (%).

The loss on drying is measured according to Chapter 2.2.32 of the European Pharmacopoeia.

According to the above procedures, semuloparin was compared to a standard comprising semuloparin (3000ET).

Comparison of semuloparin with a standard comprising semuloparin (3000ET)

Results are illustrated in Figures 13 and 14.

According to the usual statistical methods for parallel line assays (§5.3 European Pharmacopoeia), the analysis is considered valid and both products are considered to have the same *in vitro* behaviour for the anti-factor Xa and anti-factor IIa activity.

In conclusion, the above results clearly show that semuloparin can be validly compared with a standard comprising semuloparin.

III – *IN VITRO* MEASUREMENT OF THE BIOLOGICAL ACTIVITY OF ULMWH SAMPLES

The anti-FXa and anti-FIIa activities of semuloparin containing samples were determined according to the 4-points calibration methodology disclosed above, using a standard comprising semuloparin.

Results are summarized in the table below:

Samples	Activity in U/mg on dry basis	
	Anti-FXa	Anti-FIIa
1	154	2.8
2	151	2.5
3	149	2.4
4	159	2.7
5	159	2.6

NB : the loss on drying was measured at 70°C according to process d) of Chapter 2.2.32 of the European Pharmacopoeia

CLAIMS

1. A method for the *in vitro* measurement of the biological activity of an Ultra Low Molecular Weight Heparin (ULMWH) sample, wherein said method is carried out relative to a standard comprising an ULMWH.

2. Method according to claim 1 wherein said standard comprises semuloparin.

3. Method according to claim 1 or 2, wherein said ULMWH is semuloparin.

4. Method according to claim 1, 2 or 3 wherein said standard comprises the same ULMWH as the ULMWH sample.

5. Method according to anyone of the preceding claims comprising the measurement of the anti-factor Xa activity of said ULMWH sample.

6. Method according to anyone of the preceding claims, for the measurement of the anti-factor IIa activity of said ULMWH sample.

7. Method according to anyone of the preceding claims, wherein said method is an amidolytic assay on a chromogenic substrate.

8. Method according to claims 5 and 7, wherein said chromogenic substrate is chosen from the group consisting in S-2222TM (Bz-Ile-Glu(g-OR)-Gly-Arg-pNA·HCl); S-2765TM (N-α-Z-D-Arg-Gly-Arg-pNA 2HCl also referred to as N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride); CBS 31.39 (CH₃-SO₂-D-Leu-Gly-Arg-pNA) available from Stago; BIOPHEN® CS-11(22) (Mixture (50%-50%) of Bz-Ile-Glu (γOCH₃)-Gly-Arg-pNa (form 1) and Bz-Ile-Glu (γOH)Gly-Arg-pNa (form 2)); BIOPHEN® CS-11(32) (Suc-Ile-Gly (γ Pip)Gly-Arg-pNa, HCl); BIOPHEN® CS - 11(65) (D-Arg-Gly-Arg-pNA, 2HCl).

9. Method according to claims 6 and 7, wherein said chromogenic substrate is chosen from the group consisting in S-2238TM (H-D-Phe-Pip-Arg-pNA·2HCl also referred to as D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride);

CBS 34.47 (H-D-CHG-But-Arg-pNA); CBS00.68; BIOPHEN® CS-01(81) (Tos-Gly-Pro-Arg-pNa. AcOH); BIOPHEN® CS-01(38) (H-D-Phe-Pip-Arg-pNa, 2HCl).

10. Method according to anyone of the preceding claims, wherein the sample
5 is in buffer solution.

11. Method according to anyone of the preceding claims, comprising the
following steps :

a) preparing said standard comprising said ULMWH, and preparing said sample

b) determining the calibration curve for the biological activity of said ULMWH
10 standard

c) determining the curve for the biological activity of said sample,

d) confirming standard and sample parallelism

e) determining the biological activity of the ULMWH sample against the calibration
15 curve obtained in step b).

12. Method according to claim 11 wherein the calibration curve is obtained
from 14 calibration points.

13. Method according to claim 11 wherein the calibration curve is obtained
20 from 4 calibration points.

14. Standard suitable for measuring the *in vitro* biological activity of an
ULMWH sample, which comprises an ULMWH.

15. Standard according to claim 14 wherein said standard comprises the same
ULMWH as the sample to be measured.

16. Standard according to claim 14 or 15, comprising semuloparin.

AMENDED CLAIMS

received by the International Bureau on 18 April 2012 (18.04.12)

1. A method for the *in vitro* measurement of the biological activity of an Ultra Low Molecular Weight Heparin (ULMWH) sample, wherein said method is carried out relative to a standard comprising an ULMWH, wherein said ULMWH is semuloparin and said standard comprises semuloparin.

2. Method according to claim 1 comprising the measurement of the anti-factor Xa activity of said ULMWH sample.

3. Method according to anyone of the preceding claims, for the measurement of the anti-factor IIa activity of said ULMWH sample.

4. Method according to anyone of the preceding claims, wherein said method is an amidolytic assay on a chromogenic substrate.

5. Method according to claims 2 and 4, wherein said chromogenic substrate is chosen from the group consisting in S-2222™ (Bz-Ile-Glu(g-OR)-Gly-Arg-pNA·HCl); S-2765™ (N-α-Z-D-Arg-Gly-Arg-pNA 2HCl also referred to as N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride); CBS 31.39 (CH₃-SO₂-D-Leu-Gly-Arg-pNA) available from Stago; BIOPHEN® CS-11(22) (Mixture (50%-50%) of Bz-Ile-Glu (γOCH₃)-Gly-Arg-pNa (form 1) and Bz-Ile-Glu (γOH)Gly-Arg-pNa (form 2)); BIOPHEN® CS-11(32) (Suc-Ile-Gly (γ Pip)Gly-Arg-pNa, HCl); BIOPHEN® CS - 11(65) (D-Arg-Gly-Arg-pNA, 2HCl).

6. Method according to claims 3 and 4, wherein said chromogenic substrate is chosen from the group consisting in S-2238™ (H-D-Phe-Pip-Arg-pNA·2HCl also referred to as D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride); CBS 34.47 (H-D-CHG-But-Arg-pNA); CBS00.68; BIOPHEN® CS-01(81) (Tos-Gly-Pro-Arg-pNa, AcOH); BIOPHEN® CS-01(38) (H-D-Phe-Pip-Arg-pNa, 2HCl).

7. Method according to anyone of the preceding claims, wherein the sample is in buffer solution.

8. Method according to anyone of the preceding claims, comprising the following steps :

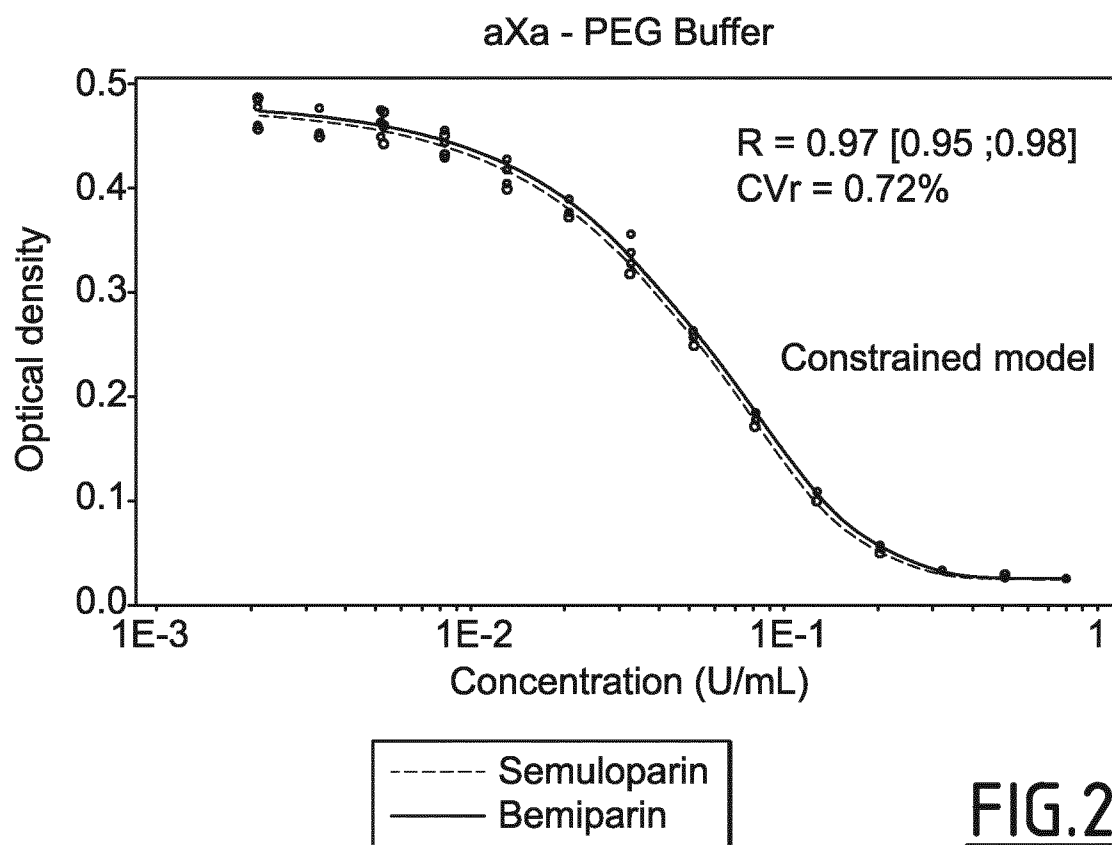
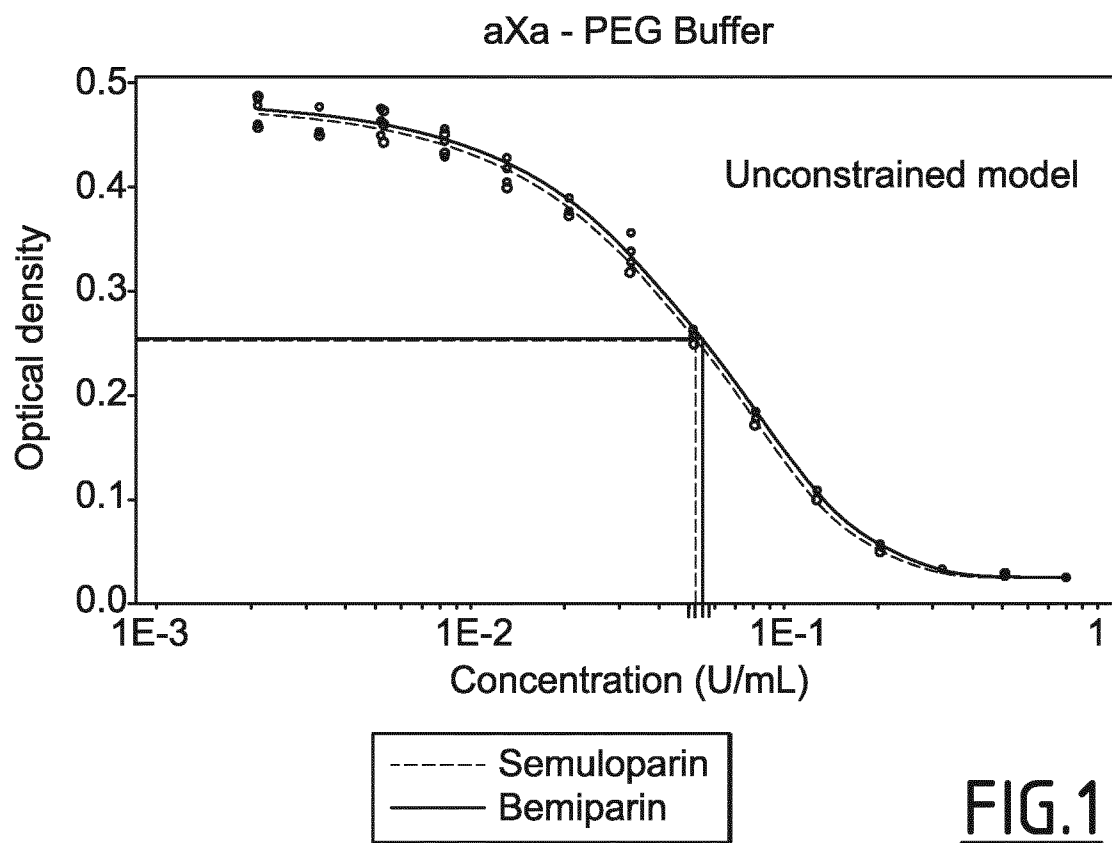
- a) preparing said standard comprising said ULMWH, and preparing said sample
b) determining the calibration curve for the biological activity of said ULMWH standard
c) determining the curve for the biological activity of said sample,
5 d) confirming standard and sample parallelism
e) determining the biological activity of the ULMWH sample against the calibration curve obtained in step b).

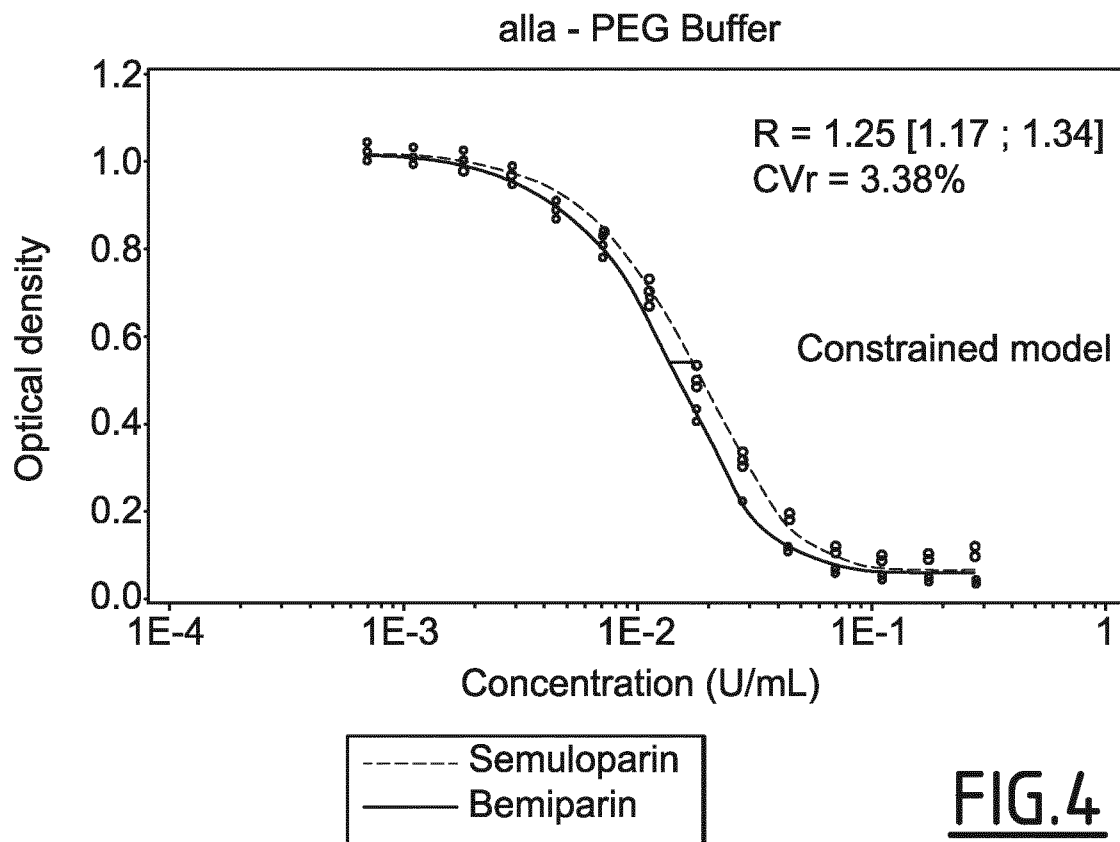
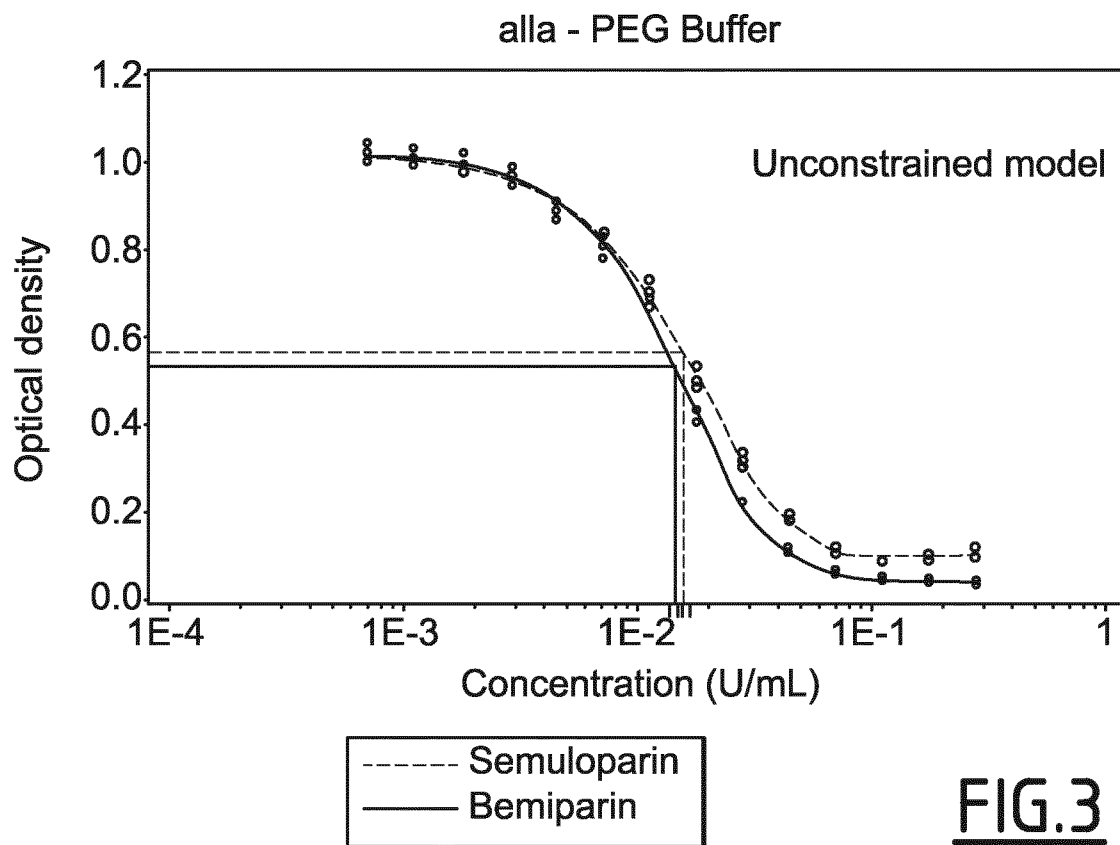
9. Method according to claim 8 wherein the calibration curve is obtained from
10 14 calibration points.

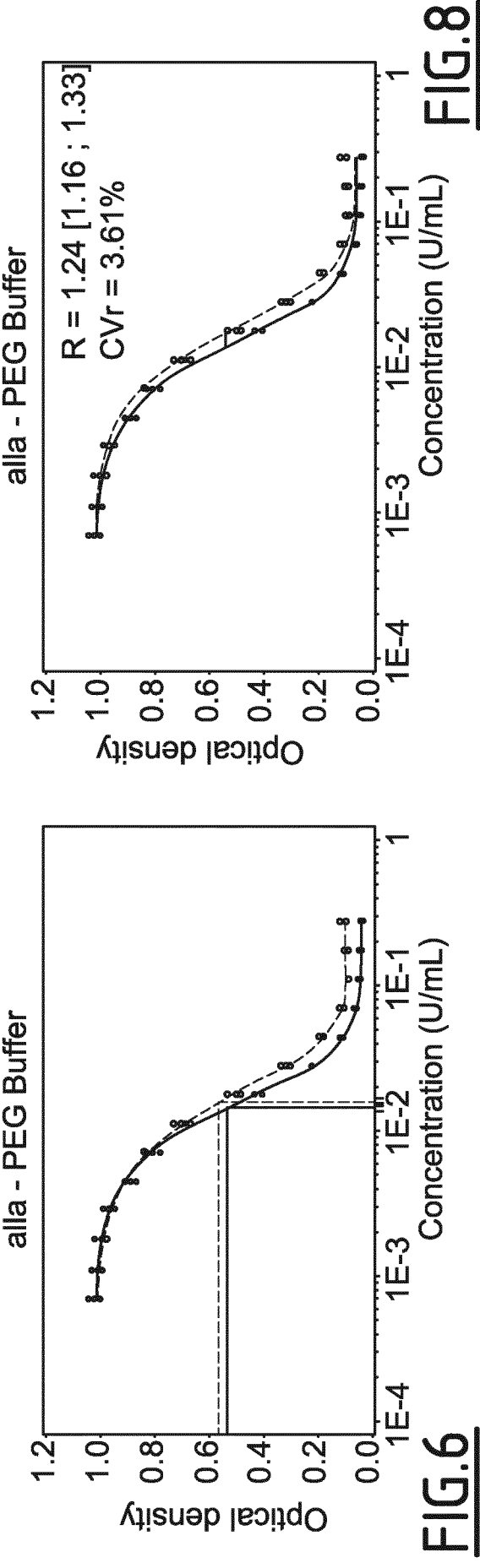
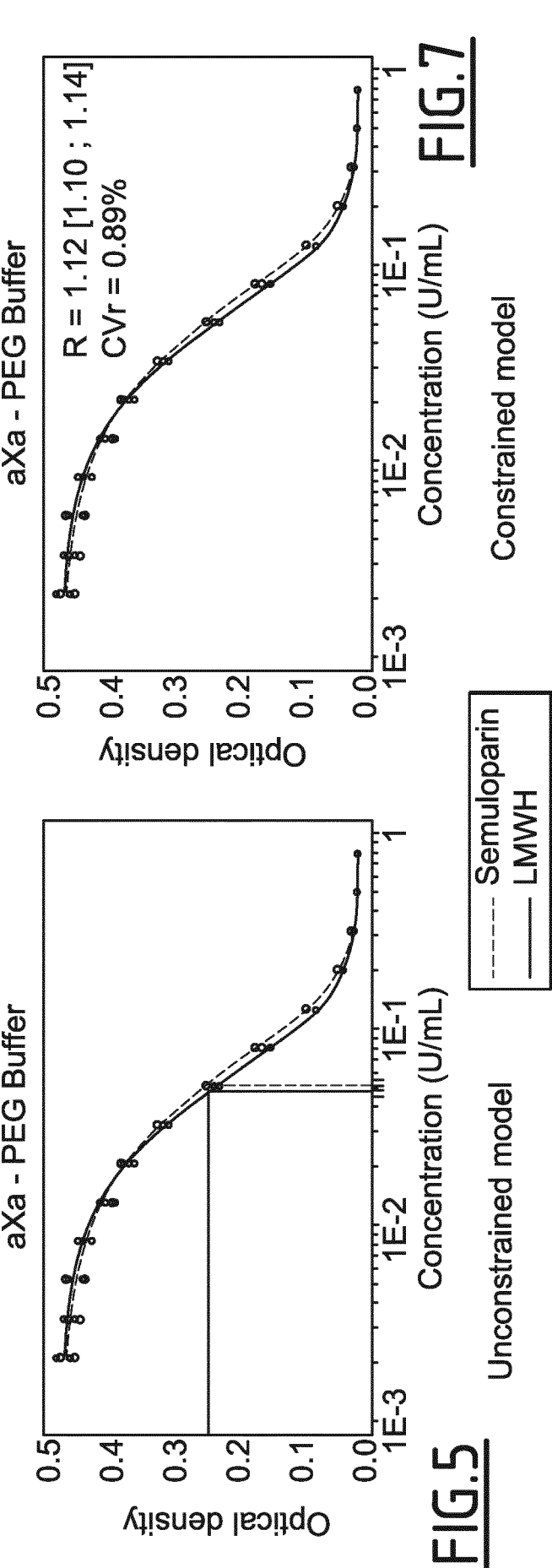
10. Method according to claim 8 wherein the calibration curve is obtained from
4 calibration points.

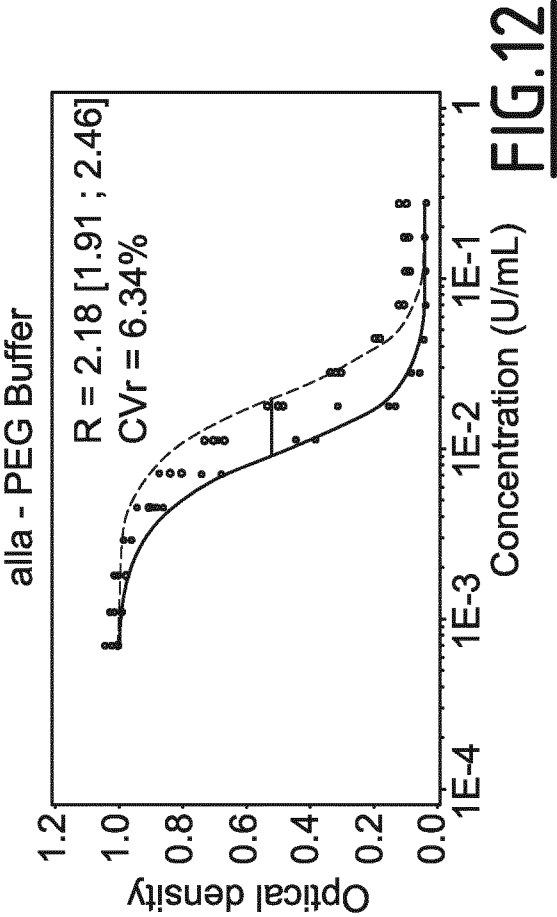
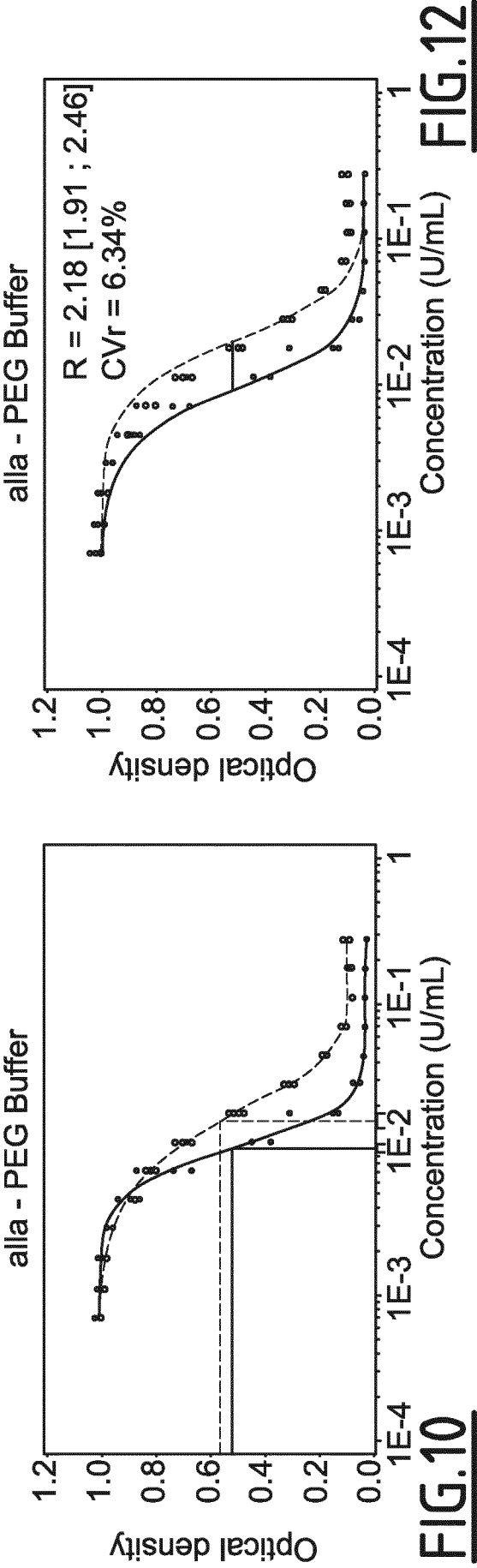
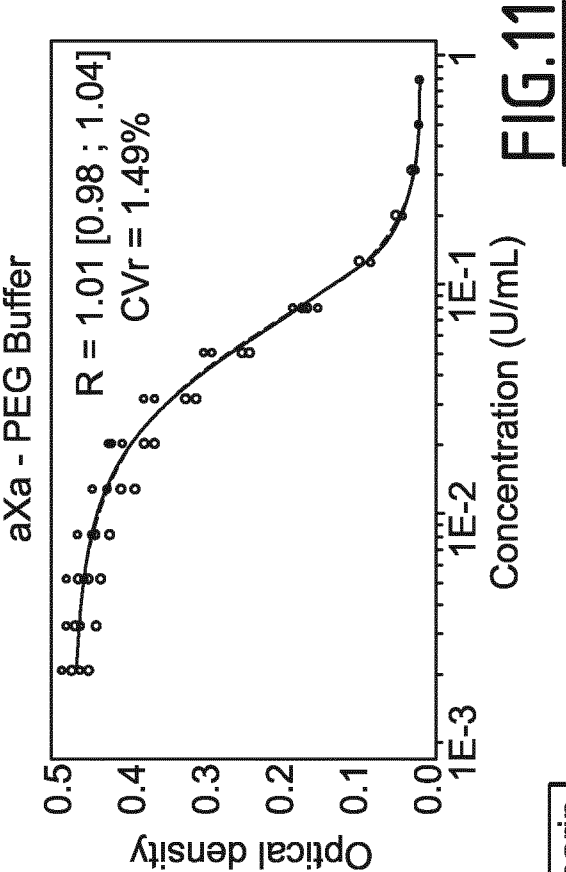
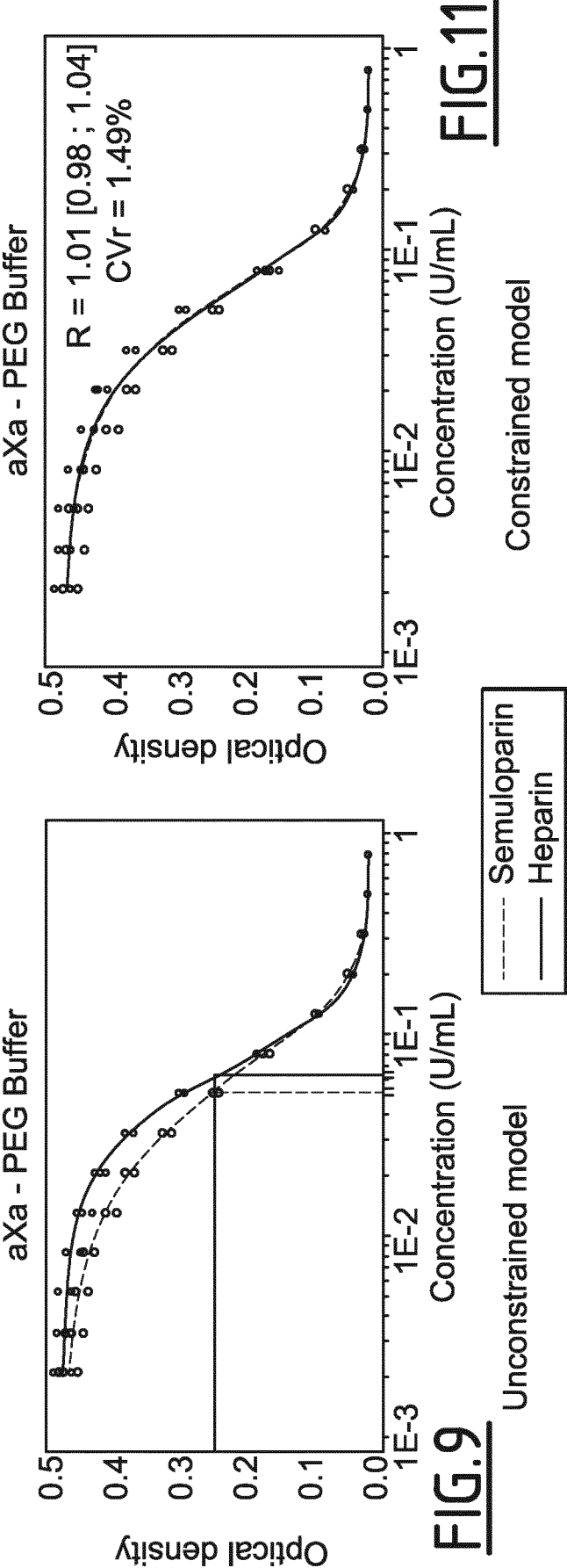
15 11. Standard suitable for measuring the *in vitro* biological activity of an ULMWH sample, which comprises an ULMWH, wherein said ULMWH is semuloparin and said standard comprises semuloparin.

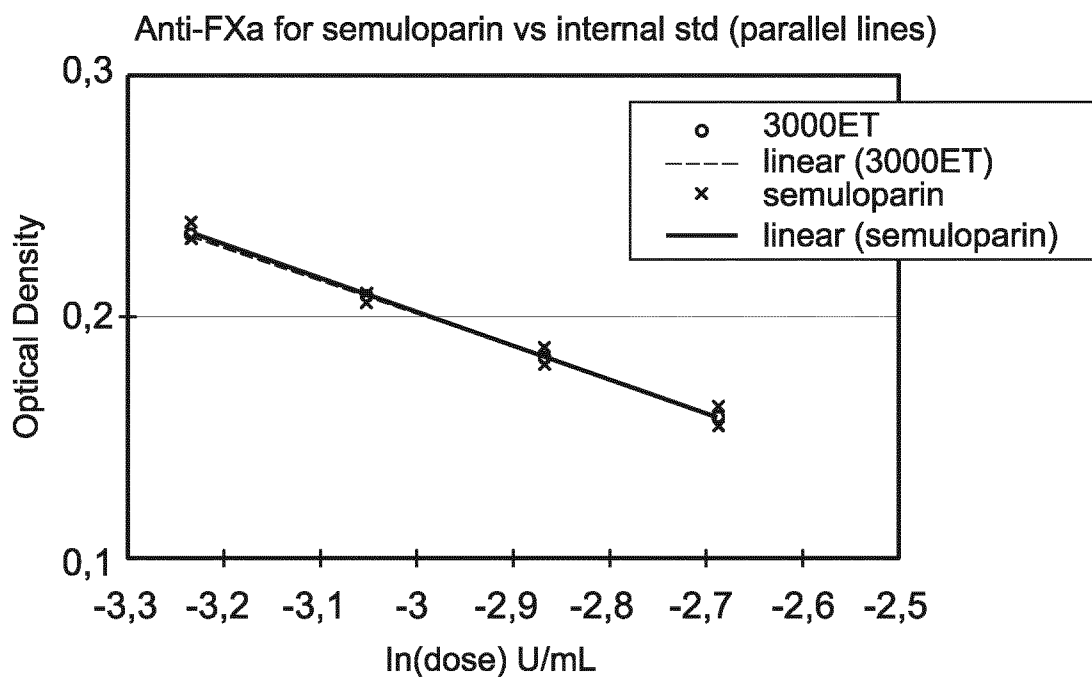
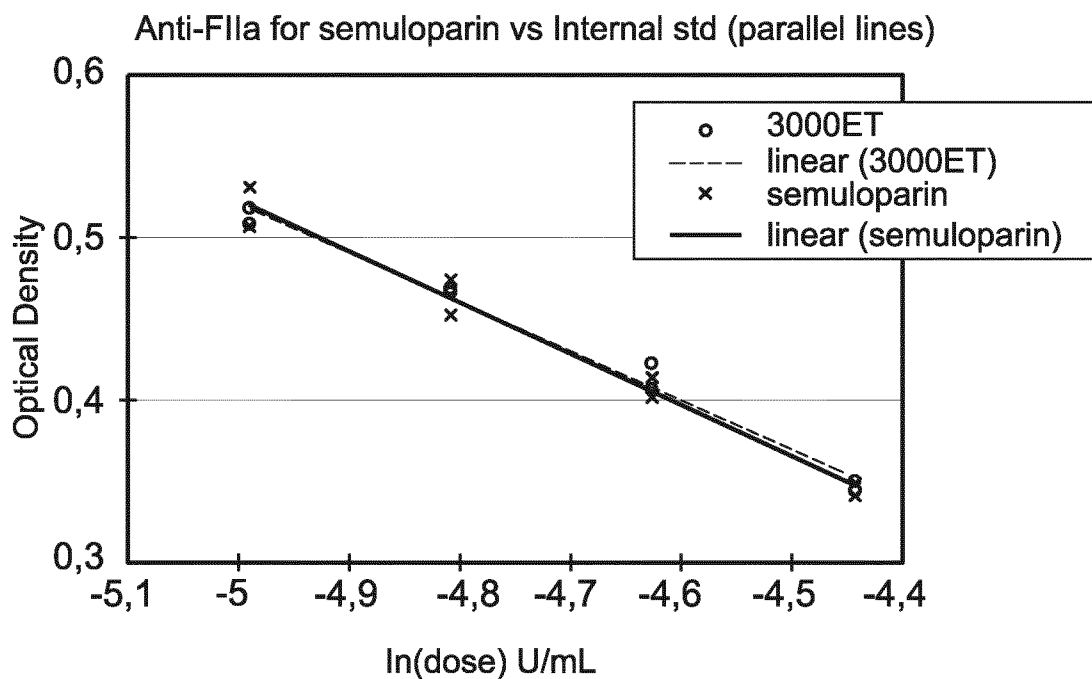
20









FIG.13FIG.14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/071633

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/86
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	EP 1 070 503 A1 (ROVI LAB FARMACEUT SA [ES]) 24 January 2001 (2001-01-24) par 0001, 0007-0008. ----- -/--	14,15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

27 February 2012

Date of mailing of the international search report

29/03/2012

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/071633

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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International application No

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