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A simplified screening procedure for determination of total N–NO groups (TNG) and nitrite (NO_2^-) in commercial low-molecular-weight heparins (LMWH) by selective chemical denitrosation followed by high-sensitivity chemiluminescence detection (NO-analyzer, NOA)

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ABSTRACT

Aim of this work was to set up a method for the sensitive and selective determination of nitrite (NO_2^-) and total N-nitroso groups (TNG) in dalteparin and nadroparin, commercial low- molecular-weight heparins (LMWH), prepared by deaminative depolymerization of heparin with nitrous acid. The European Pharmacopoeia VI ed. indicates respectively 5 ppm as the maximum content for contaminant NO_2^- in the former and 0.25 ppm for TNG in the latter and no clear indication is given for N–NO groups in dalteparin, i.e. TNG must be absent because of the specific manufacturing process.

The proposed technique is based on the development of a pre-analytical device, coupled to a chemiluminometer, constituted by three sequentially connected and commercially available purge vessels, where selective reagents are employed for the conversion of NO_2^- and N–NO to nitric oxide (NO). In detail, NO_2^- was determined in the first chamber and non-volatile and volatile TNG in the second and third. This method was validated for selectivity, sensitivity, linearity, accuracy and precision. The method was shown to be selective, with a quantitative linear range of 1–1000 ppb). The bias, intra- and inter-day percent relative error was lower than 1%. The contamination of NO_2^- and TNG in nadreparin was below the limits; for dalteparin NO_2^- fell within the limit, but there was a huge amount of TNG (15.80 ± 0.05 ppm–6.69 ± 0.02 ppm). Preliminary investigation on the solvent-extractable material from dalteparin showed the majority of chemiluminescence retained in the aqueous residue to indicate that this N–NO groups may belong to solvent unextractable material or be tightly bound to the dalteparin backbone.

1. Introduction

Unfractioned heparin (UFH) is one of the oldest drugs still in widespread use for the prevention of thrombosis and maintenance of hemostasis and ever since its discovery in 1916 it has been the drug of choice in the prevention and treatment of venous thromboembolism. Around the 1980s, a series of low-molecular-weight heparins (LMWH) were developed, offering improvements in pharmacological management and treatment with conventional heparins, and for several therapeutic indications LMWH have replaced the parent drug.

LMWH are as effective as UFH in the prophylaxis of deep venous thrombosis, but only one injection a day is needed, instead of three of the parent compound [1]. In addition, in orthopedic surgery, most studies showed a lower bleeding tendency with LMWH than with UFH [2,3]. The mean molecular weight of conventional porcine intestinal mucosal heparins is around 17–20 KDa vs. 4–7 KDa for the LMWH.

LMWH can be in principle prepared by solvent fractionation of UFH, but none of the current LMWH is made by this method. Several partial depolymerization methods are used, the most common being oxidative depolymerization with H_2O_2 (Ardeparin, Normiflo®, Pharmacia and Up John) or Cu^+/H_2O_2 (parnaparin, Fluxum®, Alpha Wasserman), β-eliminative cleavage with heparinase (tinzaparin, Innohep®, LEO Pharma and Logiparin®, Novo Nordisk) or alkaline treatment (enoxaparin, Lovenox® and Clexane®, Sanofi-Aventis), and deaminative cleavage with isoamyl nitrite (Sandoparin®, Biochemie) or nitrous acid (HNO_2) (Dalteparin, Fragmin®, Pfizer; nadroparin, Fraxiparin®, GlaxoSmithKlein) [4].

Commercial LMWH such as nadroparin and dalteparin, obtained by depolymerization of heparin from pork intestinal mucosa, may

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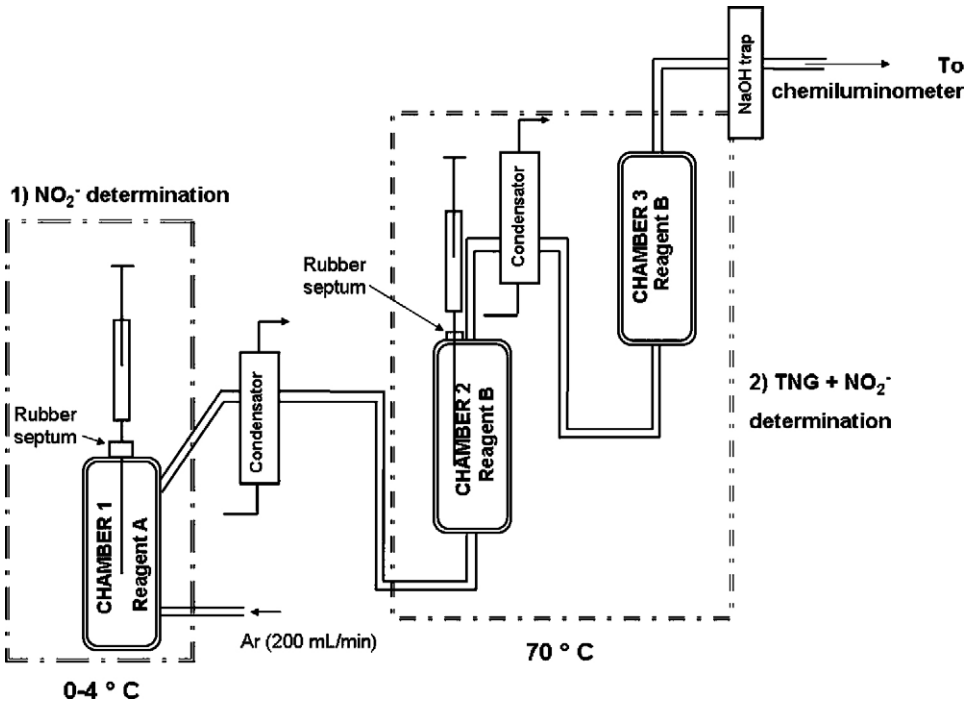

Fig. 1. Graphic representation of the three purging vessel assembly and reagents ((reagent A) 50 mM KI/1 M acetic acid, 0–4 °C; (reagent B) conc. acetic acid/HBr 48%, 5:1, 70 °C) for the selective determination of NO_2^- and TNG in low- molecular-weight heparin.

be contaminated by fragments of protein and DNA which, under strong nitrosative conditions, can give rise to genotoxic N–NO adducts. Alternatively, HNO_2 may potentially induce the formation of N–NO groups at the *N*-acetyl short side chain of the polysaccharide [5]. The European Pharmacopoeia VI ed. (EP) establishes a contamination limit of 0.25 ppm for N–NO in nadroparin; only the NO_2^- limit is reported for dalteparin (5 ppm) while for TNG it is stated that the drug "is produced by a validated manufacturing procedure under conditions designed to minimize the presence of N–NO groups and that the manufacturing procedure must have been shown to reduce any contamination by N–NO groups to approved limits using an appropriate, validated quantification method" [6].

The EP approach for the analysis of TNG in nadroparin, involves the chemical process mediated by HBr in glacial acetic acid with refluxing ethylacetate, originally developed by Downes et al. [7], followed by NO detection by TEA which determines the overall amount of N–NO groups (extractable and not extractable). This methodology requires the use of cold traps (−100 °C, −160 °C, liquid nitrogen) to remove interfering species, and increase selectivity [8]. However, the use of HBr in glacial acetic acid with refluxing ethylacetate may lead to the evolution of NO from the residual inorganic NO_2^- eventually present in the LMWH sample, as demonstrated by Fine et al. in 1975 and by Pignatelli et al. in 1993 [8,9]. Hence, the need of a different methodology (ion-exchange HPLC) for NO_2^- determination, as proposed by the same Pharmacopoeia for dalteparin [6].

In the light of these considerations, aim of this work was to set up a simplified analytical approach, which combining in the same apparatus two well established denitrosating reactions, allows the determination of total (extractable and non-extractable) N–NO groups (TNG) and of NO_2^- in pharmaceutical LMWH preparations obtained by deaminative cleavage with HNO_2. The determination does not involve any sample manipulation, since can be done by direct injection into the pre-analytical apparatus of the liquid matrix stored in the syringe used for administration. For the determination we developed an easy-to-handle pre-analytical device constituted by three commercially available reaction vessels, containing different reagents to carry out selective chemical denitrosation reactions. The assembly was directly coupled to a highly sensitive chemiluminometric detector (NO Analyzer, NOA).

2. Materials and methods

2.1. Apparatus

Fig. 1 shows a diagram of the glassware system connected to the chemiluminometer. It comprises three glass chambers (Sievers Inc., Boulder, CO), connected with Teflon tubes and kept under a constant flow of argon (200 mL min^{-1}), to reach a steady instrumental baseline before the analyses (6–10 mV). The NO released is stripped by inert gas to the detector, where it reacts with ozone to produce a chemiluminescence signal proportional to the analyte concentration, measured with a CLD88 chemiluminometer (ECOMEDICS, Dürten, Switzerland), whose output is connected to a data processing system.

Data were analyzed using Powerchrom™2.2.4 software (2001-11-29, ADIinstruments).

The operative details of the instrumental set up have been reported by Wang et al. [9].

2.2. LMWH samples, chemicals and reagents

LMWH nadroparin (Seleparin®/Seledie®, GlaxoSmithKlein) and dalteparin (Fragmin®, Pfizer) at different dosages were purchased in local pharmacies in Milan. The presence from proteins and DNA was checked according to the EP VI ed [6] by spectrophotometric analysis of the preparation at λ = 260 nm and 280 nm, and no significant absorption indicative of protein and DNA contamination were found. NO_2^-/TNG-free LMWH was from Opocrin s.p.a. (Corlo di Formigine, Modena, Italy): these were prepared by the photolytic procedure reported by Stefan and Bolton [10] followed by addition of 5% SA sulphanilamide (SA, HCl 1 N). All chemicals, *N*-nitroso-pyrrolidine, 1,4-di-nitroso-piperazine, *N*-nitroso-di-propyl-amine, *N*-nitroso-di-isopropyl-

amine, *N*-nitroso-diethanolamine, *N*-nitroso-diphenyl-amine, *N*-nitroso-urea, *N*-ethyl-*N*-nitroso-urea, nimustine hydrochloride, and 1-isoamyl-1-nitrosoguanidine of analytical grade were from Sigma–Fluka–Aldrich (Sigma–Fluka–Aldrich, Milan, Italy); high-purity gases (argon 5.5, oxygen 5.0 and NO) were from Sapio (Monza, Italy). HPLC-grade water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard *N*-nitroso-4-hidroxy-L-proline (N–NO–Hyp) was synthesized in our laboratories as previously reported [11]. Its purity was confirmed by HPLC–ESI–MS analysis carried out on a LCQ advantage™ (Thermo, Rodano, Milan, Italy) instrument, equipped with an electrospray ionization source (ESI) by direct infusion (25 μl min^{-1}) of a 1 mM N–NO–Hyp solution in $H_2O/CH_3CN/HCOOH$ (90/10/01, v/v/v). Analysis was done in positive ion mode under the following conditions: capillary temperature 200 °C; spray voltage 5 kV; capillary voltage 3.44 V; nebulizer gas (nitrogen) flow rate 0.5 L min^{-1}. The chromatographic separation was conducted using a Phenomenex Sinergy polar RP column by an isocratic elution using as mobile phase water/acetonitrile/eptafluorobutirric acid (9/1/0.001, v/v/v) at the flow rate 0.2 mL min^{-1}.

2.3. Standard solutions and calibration

Standard solutions of NO_2^- and N–NO–Hyp were freshly prepared by diluting a 10 mM stock solution of $NaNO_2$ or N–NO–Hyp with MilliQ water; they were stored in the dark at 4 °C until analysis. These solutions were diluted as needed and injected, and the amount of NO was calculated on the basis of the peak area from each injection and used to plot the calibration curves (1–1000 pmol injected, $R^2 = 0.9999$). In parallel the same solutions were added to NO_2^-/TNG-free LMWH, and these spiked LMWH were used to compare the method's figures of merit in the presence of the matrix.

2.4. Determination of NO_2^- and TNG in commercial LMWH

A suitable volume of commercial LMWH (50–200 μL) was injected first into chamber 1 for NO_2^- analysis (reagent A: 50 mM KI/1 N CH_3COOH thermostated at 0–4 °C, ice bath). A second sample was then injected into chamber 2 (reagent B: conc. acetic acid/HBr 48%, 5:1, 70 °C) to measure the total amount of TNG/NO_2^-. The amount of TNG was calculated by subtracting the NO_2^- response from the total peak area, and expressed as ppm (ng_{N-NO}/mg_{LMWH}).

In the case of a TNG/NO_2^- ratio major than 1:120, acidic SA (5% in 1 N HCl) was added to the sample (1:10, v/v). The use of SA [12] was preferred to that of sulphamic acid previously reported [9], since less acidic and hence less destabilizing labile *N*-nitroso compounds and *N*-nitrosoamides (unpublished observations).

2.5. Statistical analysis

Statistical analyses were conducted with the Prism software for Windows package (GraphPad Inc., San Diego, CA, USA). Results are expressed as the mean (S.D) of at least three independent experiments. Student's *t*-test was used; *P*-values <0.05 were considered significant.

3. Results and discussion

3.1. Method set up

A schematic representation of the glassware apparatus and reagents used for the determination of NO_2^- and TNG in LMWH is described in Fig. 1:

- *Chamber 1*: NO_2^- is specifically converted to NO by the cold (0–4 °C) mixture of KI (50 mM) and CH_3COOH (1 M). Gaseous NO is then swept by the argon flow to pass unmodified through chamber 2, chamber 3 and the NaOH trap to reach the chemiluminometer detector (see Fig. 2 and the results discussed below).
- *Chamber 2*: NO_2^- and N–NO groups are quantitatively converted to NO by the stronger reaction conditions of the CH_3COOH/HBr reagent ($T = 70$ °C).
- *Chamber 3*: The third chamber containing 5 mL of reagent B, ensures quantitative entrapping of volatile N–NO groups, which may escape complete conversion to NO due to the high carrier gas flow.

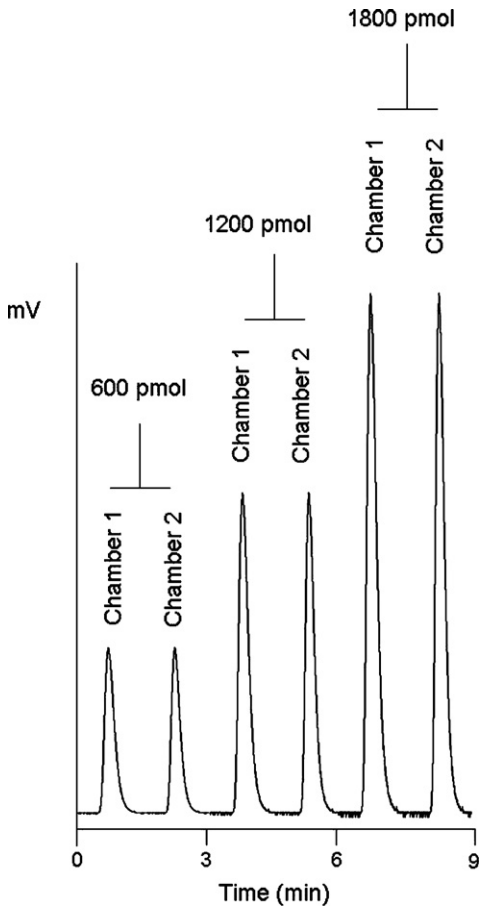

Fig. 2. . Chemiluminometric response from sequential injections of different amounts of NO_2^- in chamber 1 (reagent A) and chamber 2 (reagent B): representative examples.

Then, the amount of TNG is determined by subtracting the peak area of NO generated in chamber 1 from that of total NO peak area (chamber 2 and 3).

The reliability of the method is borne out by the following key points:

(1) NO is generated in the same amounts from NO_2^- in reagents A and B: increasing amounts of NO_2^- injected into chambers 1 and 2 generate identical peak responses, indicating the quantitative conversion of NO_2^- to NO in both reagent systems (Fig. 2);
(2) equal molar amounts of NO_2^- injected in chamber 1, and standard N–NO–Hyp in chamber 2, give identical chemiluminescence responses, indicating that in the two reagents NO_2^- and TNG are quantitatively converted to NO (not shown);
(3) sample injection into chamber 1, in these extremely mild denitrosating conditions, ensures selective determination of NO_2^-. In fact, injection of up to 5 nmol of the prototype N–NO amines (*N*-nitroso-pyrrolidine, 1,4-di-nitroso-

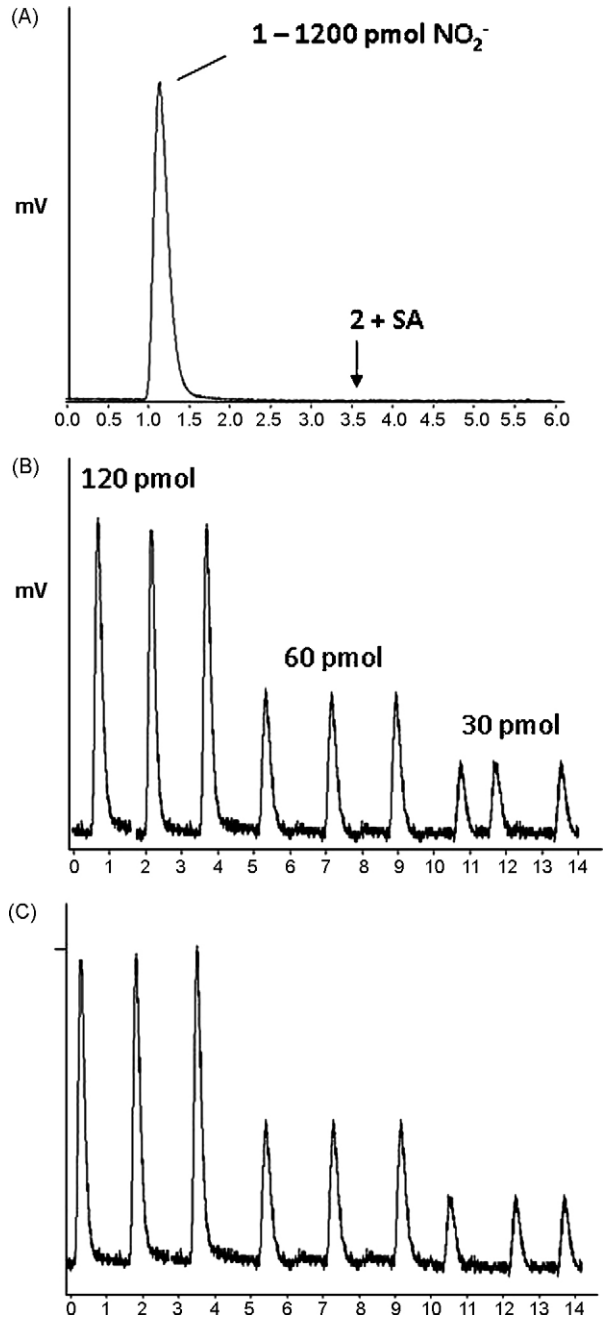

Fig. 3. . Representative example of chemiluminometric response from injection of: (A) 1200 pmol of NO_2^- before and after addition of SA; of 120, 60 and 30 pmol of N–NO–Hyp without (B) and with (C) addition of 1200 pmol NO_2^- and acidic SA to the sample.

piperazine, *N*-nitroso-di-propyl-amine, *N*-nitroso-di-methyl amine, *N*-nitroso-di-isopropyl amine, *N*-nitroso-di-ethanol amine), and of *N*-nitroso-amides (*N*-nitroso-urea), and of *N*-nitroso-guanidine gave no appreciable chemiluminescence signal for N–NO amines, with the instrumental baseline always oscillating within the spectral noise. A minimal conversion to NO (less than 1%) for *N*-nitroso-urea, 1-isoamyl-1-nitroso-guanidine and nimustine was observed (not shown);

(4) to confirm that it was reliable to calculate the TNG content by peak area subtraction, and to check the selectivity of the NO_2^- determination in chamber 1, SA (5% in 1 N HCl) was added to an aqueous solution containing different amounts of standard NO_2^- and *N*-nitroso-amines and injected into chamber 1. As shown in Fig. 3, the NO_2^- response was completely abolished. In parallel, the TNG peak areas (N–NO–Hyp) after injecting the NO_2^--depleted samples into chamber 2 match the corresponding areas of TNG injected alone. The same procedure was applied to specifically prepared LMWH samples free from residual contaminant NO_2^- and TNG, and spiked with known amounts of NO_2^- and N–NO–Hyp, to exclude interference from the matrix (i.e. release of potentially NOA responsive LMWH volatile fragments [8] generated by the strong reaction conditions of reagent B);

(5) to test the reliability of the subtraction method in samples which can be contaminated by high levels of NO_2^-, we injected increasing amounts of N–NO–Hyp (1–120 pmol) mixed with an excess of NO_2^- (1200 pmol). Table 1 shows that the mean areas of the peaks generated by triplicate injections of the mixtures were significantly different for nitrosamine amounts as low as 20 pmol; below this amount it was not possible to quantify the nitrosamine in the presence of the NO_2^- excess.

Table 1
Typical chemiluminometric responses of NO after triplicate injections of 1200 pmol of NO_2^- in chamber 1 (reagent A) and of different amounts of *N*-nitroso-di-isopropylamine into chamber 2 regent B).

Injected (pmol)		Injection (peak area)			
		1	2	3	
N-nitroso-di-isopropylamine	NO_2^-				*P* vs. NO_2^-
0	1200	6785	6790	6740	
120	0	675	679	672	
60	0	341	334	340	
30	0	166	162	163	
20	0	108	112	110	
10	0	55	56	57	
120	1200	7460	7469	7412	0.0001
60	1200	7419	7469	7412	0.0001
30	1200	6947	6956	6903	0.0020
20	1200	6987	6898	6840	0.0128
10	1200	6841	6845	6797	0.0645

3.2. Method validation

This procedure was used to evaluate the analytical figures of merit of the method (Table 2), using N–NO–Hyp as standard. There was a good linear correlation between the chemiluminescence signal and N–NO concentrations in the range from 1 to 1000 ppb with a regression equation of $I = 5.616(\pm 0.0239) \times C_{NO} + 0.9588(\pm 11.49) \times$ (X-intercept = –0.1707, $r^2 = 0.9999$), where C_{NO} is the concentration of NO generated by chemical conversion of standard NO_2^- or N–NO–Hyp to NO. The limit of detection (LOD, calculated as $3\sigma/S$, where σ indicated the standard deviation of the response and S was the sensitivity obtained from the slope of the analytical calibration curve) was 0.2 pmol injected, and the limit of quantification (LOQ) 0.6 pmol.

Table 2
Analytical "figures of merit" of the method proposed for determining NO_2^- and TNG: linearity range (LDR), coefficient of correlation (R^2), N–NO groups limit of detection (LOD), limit of quantification (LOQ), and intra- and inter-day percent relative error (RE%).

LDR (ppb)	R^2	LOD (pmol)	LOQ (pmol)	RE% intra-day ($n = 5$)	RE% inter-day ($n = 25$)	*Accuracy*
1–000	0.9999	0.2	0.6	0.508%	0.584	99.12–01.50 %

Table 3
Level of contamination from NO_2^- and total N–NO groups in 18 different commercial batches of nadroparin and dalteparin. In brackets are reported the TNG contents obtained with the method of Wang et al., described in Ref. [9]. Results are the mean (±S.D.) of five independent determinations.

	Batch	Activity (U.I.)	NO_2^- (ppm)	N–NO (ppm)
Nadroparin				
	1	3800	0.28 ± 0.01	0.007 ± 0.001
	2	3800	0.63 ± 0.02	Under LOQ[a]
	3	3800	0.29 ± 0.01	0.006 ± 0.001
	4	3800	0.62 ± 0.01	Under LOQ[a]
	5	3800	0.44 ± 0.02	0.003 ± 0.001
	6	5700	0.22 ± 0.00	Under LOQ[a]
	7	19000	0.21 ± 0.00	0.003 ± 0.000
Dalteparin				
	1	2500	2.35 ± 0.01	15.80 ± 0.05
	2	2500	2.57 ± 0.01	12.51 ± 0.04
	3	2500	1.42 ± 0.02	12.46 ± 0.03
	4	2500	1.82 ± 0.03	13.32 ± 0.02
	5	2500	2.34 ± 0.02	15.42 ± 0.03
	6	5000	0.98 ± 0.03	6.69 ± 0.02
	7	2500	3.23 ± 0.06	8.52 ± 0.30 (7.99 ± 0.09)
	8	2500	2.74 ± 0.04	9.94 ± 0.10 (9.89 ± 0.15)
	9	2500	2.44 ± 0.02	8.15 ± 0.47 (8.05 ± 0.36)
	10	5000	2.45 ± 0.05	8.60 ± 0.16 (7.62 ± 0.27)
	11	5000	2.38 ± 0.07	8.34 ± 0.21 (7.83 ± 0.09)

[a] Determined after addition of acidic SA.

Precision was evaluated by injecting 10 μL of a standard solution of N–NO–Hyp corresponding to 150 pmol of NO. The calculated relative errors for inter-day ($n = 5$) and intra-day ($n = 20$) replicate measurements were 0.508% and 0.584%.

Accuracy was evaluated spiking increasing amounts of NO_2^- and N–NO–Hyp to different LMWH samples, and the recovery range for both analytes was found to be 99.12–101.50% (not shown).

Finally the method was validated by critical comparison of both (i) the calibration curve with standard N–NO–Hyp and (ii) the TNG contents of five different batches of dalteparin, obtained with the method reported by Wang et al. [9], which is based on a different denitrosating mechanism (CuCl reagent). The results reported in Table 3 and Fig. 4 evidence a satisfactory accordance between the calibration curves and the results obtained using the two methods ($P > 0.05$). The method LOD was found to be more favorable than that reported by Wang (0.2 pmol vs. 1.0 pmol [9]).

3.3. NO_2^- and TNG in commercial nadroparin and dalteparin preparations

The method was employed to determine NO_2^- and TNG in samples of commercial nadroparin ($n = 7$) and dalteparin ($n = 11$), stored in disposable syringes for i.v./s.c. injection. In nadroparin the NO_2^- concentration ranged from 0.28 to 0.63 ppm and TNG from n.d. to 0.007 ppm, well within the limits established by the EP (respectively 5 ppm and 0.25 ppm) (Table 3). By contrast all the dalteparin samples had NO_2^- concentrations below the limit, but TNG was several times higher than the EP limit for nadreparin (15.80 ± 0.05 ppm vs. 0.25 ppm).

This difference can be tentatively explained by the fact that nadroparin and dalteparin are both obtained by depolymerization of heparin with HNO_2, but the different industrial production conditions – reaction temperature and pH, amounts of reagents [4] – can lead to different levels of contamination.

3.4. Origin of TNG in dalteparin

From a toxicological point of view, the result of much relevance achieved with this methodology is that relative to the high levels of TNG in dalteparin preparations (Table 2). This bearing in mind that the different batches we analyzed were from different pharmacies in the city of Milan and were purchased in different years (2006/2008), so the higher content of N–NO groups in dalteparin was not purely a chance finding.

A preliminary investigation, with exhaustive extraction of commercial dalteparin with solvents of increasing polarity (CCl_4, $CHCl_3$, ethyl acetate), and taken up in methanol (NOA-insensitive), gave a modest chemiluminometric response in the combined solvent extracts, accounting for approximately 5% of the TNG response. As expected, the extracted aqueous residue injected in chamber 2 gave a chemiluminometric signal corresponding to approximately 90 % of the total TNG response.

Although only preliminary, these data indicate that most of the denitrosable NOA responsive material may be due to (i) the presence of highly hydrophilic, non-extractable, N–NO material (*N*-nitrosamines/*N*-nitrosamides) or (ii) to the cleavage of N–NO groups covalently and specifically bound to the LMWH backbone, probably to the *N*-acetyl [N(NO)$COCH_3$] short side chains on the

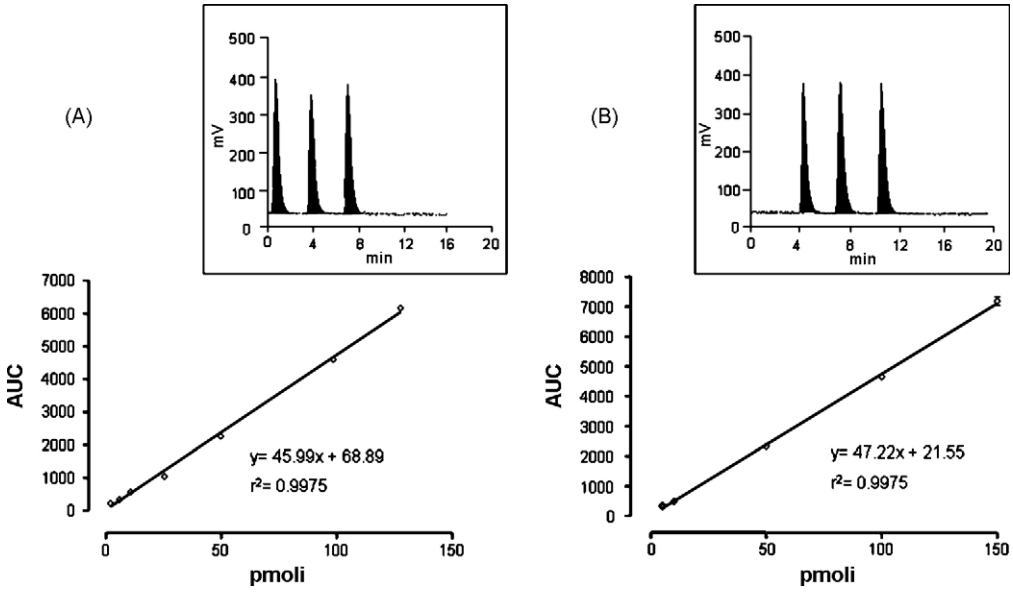

Fig. 4. . Comparison of representative calibration curves obtained using the denitrosation method reported by (A) Downes et al., and (B) Wang et al. (inserts: chromatograms deriving from triplicate injections of 10 μL of a dalteparin sample in the corresponding denitrosating system for TNG analysis).

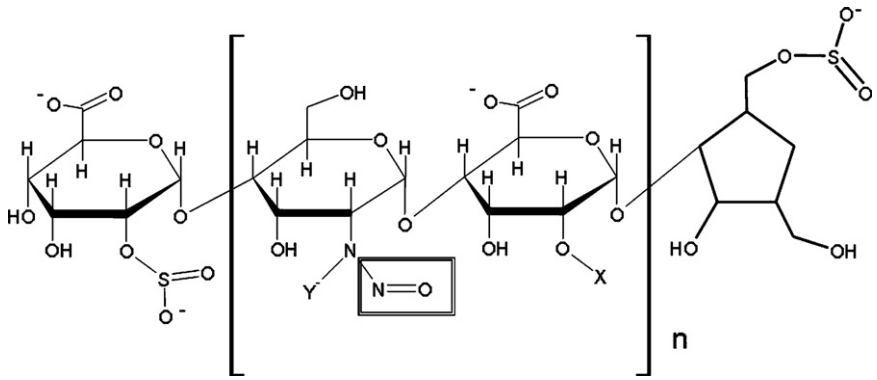

Fig. 5. . Graphic representation of the most probable NO modification site in low-molecular-weight heparin.

surface of the dalteparin structure (Fig. 5). In this last case, that we consider the most likely, taking into account (i) a mean LMWH molecular weight of 5000 Da; (ii) that 150 I.U. correspond approximately to 1 mg of drug, and (iii) that the average TNG content (MW 44 Da) is around 15 ppm, it can be estimated that one in 50 molecules of LMWH is adducted by one NO group.

Hence, under the depolymerization conditions adopted by manufacturer, it is highly probable that N–NO groups are located on the macromolecule structure rather than on hydrophilic N–NO–nucleotides from contaminant DNA, nitrosamine or from proteins. On the other hand, in the LMWH preparations these contaminants were undetectable by UV spectrophotometric analysis.

High-resolution mass spectrometry studies are now in progress to confirm the real increase in the MW of dalteparin and clarify the location of the modification site, in the light of the potential structure-specific harmful health effects of this adduct.

4. Conclusions

The combination of a radical purge vessel with the NOA (formerly employed for NO_2^- and NO_3^- determination [13]) for the analysis of TNG was originally described by Wang et al. [9], using the denitrosating reagent CuCl/HCl in aqueous medium after sulphammic acid addition. However, the chemiluminometric response obtained with this methodology (i) gives badly shaped peaks due to the contamination from NO_3^-; (ii) requires NO_2^- destruction; (iii) suffers of interference/lack of selectivity in the determination of *N*-nitrosamides.

In order to improve the efficiency of their methodology, we developed a pre-analytical device obtained by connecting three commercially available reaction vessels to the NOA. With this apparatus we have been able to measure independently and selectively NO_2^- and TNG in two sequential steps through the combination of two different well-established denitrosating reagents [14,15], to obtain sharp and well-defined chemiluminometric peaks, and to reach a better detectability by a factor of 5 (LOD = 1.0 vs. 0.2 $pmol_{N-NO/NO2^-}$ injected). Presently, this methodology has been specifically set up for the determination of NO_2^- and TNG contamination in pharmaceutical preparations of LMWH obtained by deaminative denitrosation (nadroparin, dalteparin), and validated by critically comparing the results with those obtained with the method reported by Wang et al. [9].

Due to the higher sensitivity, excellent reproducibility and high selectivity (no interference from NO_2^-) and fast analytical response of our methodology, this may be considered as an alternative to the complex and difficult-to-handle pre-analytical apparatus described by the EP [6]. The advantages of the methodology can be summarized as follows: (i) no need to add NO_2^- depleting agents which can destabilize *N*-nitrosamides in acidic aqueous solutions; (ii) no need to disassembly and clean up the apparatus each time the reagent is changed (one piece-device); (iii) a better optimization of the conditions to quantitatively entrap and determine highly volatile *N*-nitroso compounds, and (iv) minimal contamination by NOA sensitive fragments from LMWH degradation.

Perhaps the most significant benefit of the method is its simplicity and cheapness: it affords a considerable time saving in respect to that required by the refluxing ethylacetate methodology [6], and much less operator intervention in sample handling thus to decrease the potential operator error without loss of sensitivity.

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