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Short communication

New method for low molecular weight heparin quantification in tablets by suppressed conductivity detection and cryptand column

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ABSTRACT

Unfractioned Heparins (UFH) and Low Molecular Weight Heparins (LMWHs) are non-chromophoric, high charged sulphated molecules which are difficult to elute and detect with conventional liquid chromatography methods. Moreover, the detection of LMWHs at low concentration level, like in samples coming from dissolution test of tablets formulated with Heparins is problematic due to the weak response by UV or refractive index detection.

According to the *European Pharmacopoeia* requirements, the assay of LMWHs is based on the anti-Xa activity and anti-IIa activity factors, which are biological parameters related to the antithrombotic activity of this compound family. The same assay method could also be applied to determine the dissolution rate of LMWHs contained in tablets, but it is time consuming and expensive test.

The challenge was to develop a simpler and faster alternative method based on Ion Chromatography coupled with Suppressed Conductivity detection, but the particular polycharge interaction of LMWHs with anions exchanger makes the analyte with standard eluents and columns difficult to elute . The analytical problem could be solved by using a particular chromatographic phase with 2,2,2 cryptand sites which gives variable capacity in anion exchange depending on the used eluent. Such phase was very effective for the elution of strongly retained polyanions using simple inorganic eluent phase suitable for suppressed conductivity detection as well.

The developed method allows the elution of a unique peak, which represents the whole polymeric distribution, and therefore it makes easy to quantify quickly and selectively the active ingredient in case of multiple analysis like when a dissolution test is required to characterize and discriminate between different tablets formulations. The method was tested and developed in Cosmo Pharmaceuticals QC labs and the validation results are reported in the present paper. Besides, a comparison between biological and chromatographic methods was carried out as well.

1. Introduction

A new method was developed to quantify Low Molecular Weight Heparins (LMWHs) based on their intrinsic chemical–physical characteristics instead of their biological activity. LMWHs are salts of sulphated glucosaminoglycans having molecular mass-average less than 8 kDa. LMWHs are obtained by either fractionation or depolymerisation of natural heparin [1,2].

Due to their large utilisation in antithrombotic treatments [3] several methods, either biological and chemical, were proposed to determine Heparins in injectable solution, blood and urine.

Existing biological analyses are based on the activity of LMWHs to interact with the enzymes of the coagulation cascade, in particular anti-Xa and anti-IIa activity [4,5]. These enzymatic analyses require long time and show a low accuracy. The FDA accepts deviations from nominal values up to ±15% [6].

The assessment of extended-release tablets containing LMWH with other auxiliary substances assembled with MMX™ technology (Cosmo Technology Ltd., Ireland) requires some dissolution test to be carried out. As per *Pharmacopoeia* [7] requirements, the dissolution test requires the application of at least six replicates with 3 time points for each tablet. In case of out of specification results, further tests on additional 6 or 18 tablets are required.

The enzymatic method accepted by *Pharmacopoeia* [4] is a time-consuming procedure: in addition to the time required by dissolution test, an analyst can analyze not more than two tablets a day.

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The alternative instrumental methods reported in literature and potentially suitable for this target include a large part of separation and detection techniques like fluorimetry [8–10], HPLC with or without various post-column derivatization and different detectors [11–16], capillary chromatography [17], electrophoresis [18–23], flow injection analysis [24], ion-channel sensor [25] and many others. Unfortunately, these methods cannot be used in case of dissolution test where the ionic strength of the medium is high, precluding, for example, the use of mass detector. Furthermore, following the dissolution profile of extended-release tablets containing LMWH, the main difficulty was to quantify the active released, at low concentration in presence of excipients of dissolved tablet and salts coming from dissolution medium, making a method capable of detecting separately every molecular mass fraction unfeasible.

Finally, the required method should be robust, easier, more accurate, economic, less time-consuming and without any treatment of the sample after the dissolution. Moreover, it should be easy to be performed as a routinely test in a pharmaceutical QC laboratory.

Chromatographic approaches to LMWH presented several problems, both in detection because of low response with UV–vis or refractive index detector, and in peaks separation because of the very hydrophilic and polycharged nature of LMWH [26]. Conductimetric detection could analyze this kind of molecules [27], but a suitable stationary phase to separate the LMWH from ions in the medium, in concentration of 10^{-1} M, or others auxiliary neutral polymers enclosed in the matrix of the tablets was needed. Reverse Phase chromatography was unable to elute LMWH and classical anion exchange columns were also ineffective with these too strongly retained polyanionic molecules. Furthermore, it is difficult to obtain a single peak easy to be integrated [28] because of Heparin characteristic mass dispersion.

A polymeric covalent bonded 2,2,2 cryptand chromatographic stationary phase which is able to increase or decrease its capacity depending on the counter ion of the mobile phase [29-31] was tested to elute LMWH. The cryptand stationary phase showed strong affinity to LMWH anions when cryptand sites were in Na^+ form and very poor when in Li^+ form. An inorganic hydroxide solution is normally used to elute from such columns. The hydroxide eluent, where the OH^- is the eluent ion and the cation (Li^+, Na^+) is the capacity agent crypted on column sites, is not expensive and its toxicity is low, moreover it works perfectly with electrochemical suppressor devices used before the conductivity detector. Nevertheless, with the hydroxide we have to consider an important drawback which is the rapid carbonatation of the eluent. Since the carbonate ion is more retained than hydroxide, the column is temporarily carbonate poisoned and it does not work properly anymore. The reproducibility and separation become very poor run after run. Although simple procedures are described to obtain a good hydroxide eluent with low carbonate content, nowadays the better way to obtain ultrapure hydroxide solutions is using an electrochemical eluent generator [32]. Two generator cartridges of LiOH and NaOH were used to obtain a capacity gradient step moving between Na and Li hydroxide.

2. Experimental

2.1. Materials

LMWH was obtained from Opocrin SpA (Corlo di Formigine, Modena, Italy), $Na_3PO_4{\cdot}12H_2O$ from Honeywell (Seelze, Germany), NaOH and HCl from Carlo Erba Reagenti SpA (Rodano, Milano, Italy). H_2O was obtained daily from a Milli-Q system by Millipore Corp (Bedford, MA, USA).

Table 1
Chromatographic conditions

Flow	0.5 ml min^{-1}
Gradient	−12.6 min, NaOH 10 mM, LiOH 0 mM −2.6 min, NaOH 10 mM, LiOH 0 mM −2.5 min, NaOH 0 mM, LiOH 0 mM Injection, NaOH 0 mM, LiOH 0 mM 3.0 min, NaOH 0 mM, LiOH 0 mM 3.1 min, NaOH 0 mM, LiOH 10 mM 7.0 min, NaOH 0 mM, LiOH 10 mM 7.1 min, NaOH 10 mM, LiOH 0 mM
Injection	5 μl
Column temperature	40 °C
Suppressor current	20 mA
Detector temperature	45 °C

Placebo and tablets containing LMWH were manufactured in Cosmo R&D galenical department (Lainate, Milano, Italy)

2.2. Equipments

Dissolution tests were performed with Sotax AT7 Smart apparatus.

Samples were analyzed in IC ICS-3000 Dionex Ion Chromatograph equipped with eluents generator (EG), Cryptand G1 3 mm Guards Column, ASRS 4 mm suppressor and conductivity detector.

2.3. Method

Detection of LMWH dissolved amounts was performed injecting the medium solution directly in IC after filtration on 0.45 μm regenerated cellulose (RC) disposable filters.

The chromatographic conditions are reported in Table 1, the gradient was performed to allow the best separation of LMWH peaks from others anions present in excipients and in dissolution medium. Suppressor was in autoregeneration mode with current of 20 mA. EG allowed on-line generation of hydroxide eluents avoiding carbonatation of the solutions in mobile phase reservoirs.

All the standards solutions and samples used were prepared from LMWH raw material dissolved in pH 7.2 phosphate buffer (6.8 ml HCl 37%, 15.83 g $Na_3PO_4{\cdot}12H_2O$ and 0.75 g NaOH per liter). All the validation samples were prepared adding an amount of placebo powders corresponding to 100% of dissolution while dissolution samples came from a real dissolution test performed in a USP apparatus II. All the solutions were filtered through 0.45 μm RC disposable filter before the injection (full loop, 5 μl) and were quantified with an external standard bracketing calibration.

The method was validated according the ICH procedure [33].

2.4. Specificity

Specificity was evaluated injecting a blank (phosphate buffer), a placebo, and an artificial sample, each dissolved in dissolution medium [Fig. 1].

2.5. Linearity

It was investigated the concentration range corresponding to 1–120% of tablet theoretical dissolution [Table 2].

2.6. Accuracy, range and precision

Accuracy was determined as the recovery of LMWH on theoretical values at three different dissolution concentrations (20, 80 and

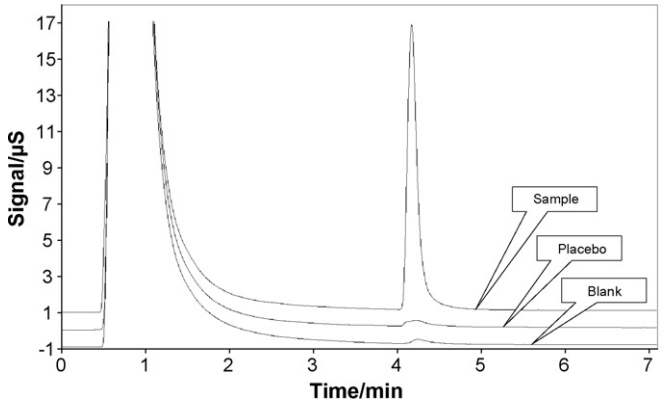

Fig. 1. Specifity test chromatograms.

Table 2
Linearity

Sample	[LMWH] (mg ml^{-1})	Area$_{av}$ (µS min) (n = 3)
Diss 1%	0.0023	0.0244
Diss 10%	0.0228	0.2519
Diss 25%	0.0569	0.6262
Diss 50%	0.1138	1.2388
Diss 80%	0.1821	1.9771
Diss 100%	0.2277	2.4914
Diss 120%	0.2732	2.9529

110%) on three different spiked samples at each point. The precision was evaluated as relative standard deviation of the recoveries of LMWH on the nine injections [Table 3].

2.7. Intermediate precision

Two operators, in different days, analyzed six samples with a concentration of LMWH corresponding to 80% of a tablet theoretical complete dissolution. The precision was calculated as the R.S.D. of the recoveries of LMWH and as difference between the mean values of the recoveries [Table 4].

Table 5
Solution stability

Sample	R.S.D.$_{std}$ (n = 5) (%)	[LMWH]$_{det}$ (mg ml^{-1})	Recovery vs. t_0 (%)
t_0	1.9	0.2180	100.0
24 h	1.3	0.2214	101.6
72 h	0.5	0.2202	101.0

Table 6
Robustness

Method variation	R.S.D.$_{std}$ (n = 5) (%)	[LMWH]$_{det}$ (mg ml^{-1})	Recovery vs. standard method (%)
None	1.9	0.2180	100.0
pH 6.7	0.8	0.2209	101.3
pH 7.7	1.1	0.2196	100.7
Eluents 11 mM	1.6	0.2184	100.2
Eluents 9 mM	0.6	0.2183	100.1
H_2O Step 2.7 min	1.1	0.2165	99.3
H_2O Step 3.3 min	1.2	0.2129	97.7
Column 38 °C	0.9	0.2168	99.5
Column 42 °C	0.5	0.2119	97.2

2.8. Solution stability

A sample solution was analyzed at start time, after 24 and 72 h; every day versus fresh standards [Table 5].

2.9. Robustness

Variations in buffer pH (±0.5), in eluents concentrations (±1 mM), in H_2O gradient step duration (±0.3 min) and in column temperature (±2 °C) were investigated on standard and sample solutions [Table 6]. To evaluate the robustness of the method were considerate the differences in R.S.D.% of five standard injections and

Table 3
Accuracy, range and precision

Sample		[LMWH]$_{theo}$ (mg ml^{-1})	[LMWH]$_{det}$ (mg ml^{-1})	Recovery (%)	Recovery$_{av}$ (%)	R.S.D.$_{Rec}$ (%)
Diss 20%	A	0.0468	0.0459	97.9		
	B	0.0476	0.0471	99.0	98.1	
	C	0.0471	0.0458	97.3		
Diss 80%	A	0.1957	0.1977	101.0		
	B	0.1802	0.1823	101.2	101.4	1.9
	C	0.1805	0.1839	101.9		
Diss 110%	A	0.2513	0.2493	99.2		
	B	0.2546	0.2622	103.0	100.3	
	C	0.2599	0.2568	98.8		

Table 4
Intermediate precision

Sample		[LMWH]$_{theo}$ (mg ml^{-1})	[LMWH]$_{det}$ (mg ml^{-1})	Recovery (%)	Recovery$_{av}$ (%)	R.S.D.$_{Rec}$ (%)	R.S.D.$_{Rec}$ %
Operator A	A	0.1869	0.1884	100.8			
	B	0.1904	0.1872	98.3			
	C	0.1898	0.1921	101.2			
	D	0.1848	0.1866	101.0	101.1	1.6	
	E	0.1878	0.1932	102.9			
	F	0.1889	0.1937	102.5			
Operator B	A	0.1849	0.1906	103.1			
	B	0.1900	0.1944	102.3			
	C	0.1871	0.1921	102.7			
	D	0.1877	0.1887	100.5	101.7	1.2	
	E	0.1856	0.1858	100.1			
	F	0.1888	0.1916	101.5			

Table 7
Methods in comparison

Method	Vessel	% Dissolved		
		t_1	t_2	t_3
Biological	a	13	30	80
	b	12	33	103
	c	10	31	106
	d	13	34	108
	e	19	38	104
	f	17	33	81
	Average	14 (3)	33 (3)	97 (13)
IC	a	15	36	76
	b	18	37	110
	c	12	34	110
	d	17	38	116
	e	23	43	113
	f	18	37	77
	Average	17 (4)	37 (3)	100 (18)

the differences in detected LMWH concentration versus the normal conditions.

Finally, samples from real dissolution tests were simultaneously analyzed with IC and biological methods, and the dissolutions profiles were compared [Table 7]. The applied biological method derives from the *Italian Pharmacopoeia* [34] and was adapted to dissolution conditions. It is slightly different from the European Pharmacopoeia method [4] but both are based on the reactions with antithrombin and with bovine factor Xa before the addition of chromophore substrate. The samples were read in UV spectrophotometer and the regression of the absorbance on log concentration of the test solutions and of the reference ones was calculated. The LMWH dissolved amount was obtained using the compendial statistical method for parallel line assays.

3. Results and discussion

Cryptand columns are able to allow unusual ion chromatography gradient elution condition. Normally the chromatographic phases have a fixed capacity value, and the gradient elution is obtained increasing the ion strength of the eluent along the run. Using cryptand [Fig. 2] the capacity value depends on the counter ion used, it increases increasing the hydrated ionic radius (Li > Na > K) according with the cryptand constant [29]. Hence to obtain a gradient the column is normally conditioned with NaOH and at injection changed to LiOH which decreases the capacity and produces a gradient effect maintaining the same ion strength. In this way cryptand bonded phase is able to perform anion separation in same manner as the classic quaternary ammonium exchanger. Cryptand seems to be excellent with strongly retained analytes like diphosphonate and Heparins.

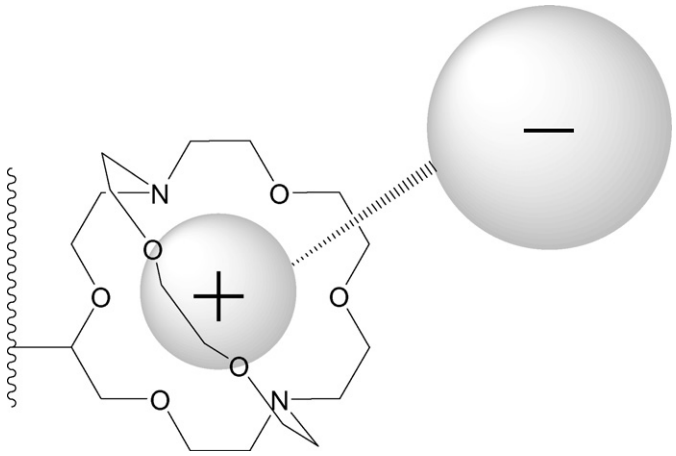

Fig. 2. 2,2,2 Cryptand stationary phase structure and interactions.

In our gradient [Fig. 3] the first step was necessary to charge the cryptand sites of the stationary phase with Sodium cations in order to improve capacity. Before the injection, a water step washed the lines from NaOH which could partially carry the analyte, otherwise strongly bounded with stationary phase, through the column. Water was sufficiently polar to elute the inorganic anions but was unable to elute the LMWH polyanions. The third step of gradient eluted the LMWH fractions decreasing the capacity of the column due to litium cations that replaced sodium in column.

3.1. Specificity

Sample obtained from a placebo spiked with active showed a peak similar to peak obtained by a standard solution.

A small peak but below the calculated LOQ was detected both in Blank and Placebo chromatograms at the same retention time of LMWH [Fig. 1]. The peak areas were respectively 0.0631 and 0.0788 μS min versus a LOQ equivalent area of 0.1122 μS min.

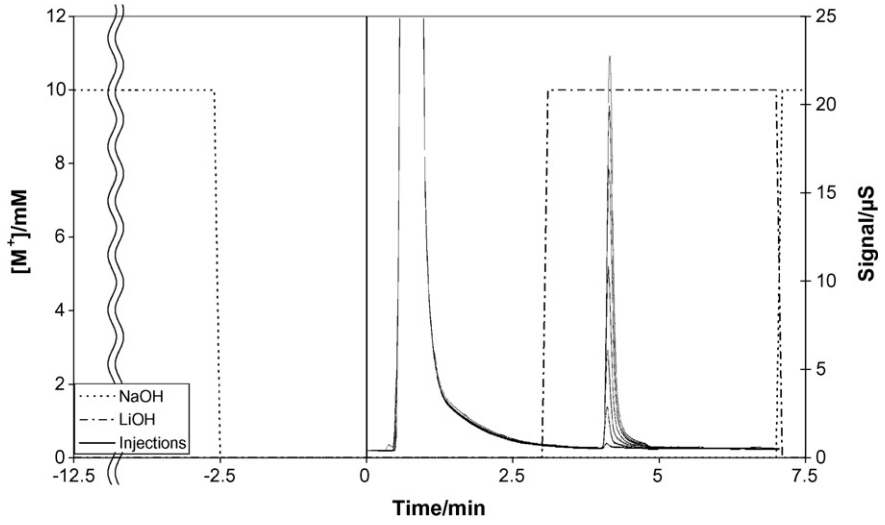

Fig. 3. Linearity test chromatograms overlapped to eluents gradient.

3.2. Linearity

The linear regression over the range established provided the equation: Area = 10.84(4)[LMWH] + 0.005(7), where area is expressed in μS min and concentration of LMWH in mg ml^{-1}. The correlation coefficient is 0.99996 [Table 2].

3.3. Accuracy, range and precision

Recovery values of all the validation samples are in the range 97–103% and R.S.D. (n = 9) is less than 2% [Table 3].

3.4. Intermediate precision

The relative standard deviation of six samples from both operators is less than 2% and the difference between two average values is 0.6% [Table 4].

3.5. Limit of quantitation

LOQ was calculated from the linear regression parameters for linearity test as ten times the ratio s_y (residual standard deviation) on m (slope)[33] and is quantified as 0.0099 mg ml^{-1} equivalent to an area of 0.1122 μS min. This corresponds to 5% of the labeled amount of the drug.

3.6. Solution stability

The differences from the initial determined concentration value were below 2% [Table 5].

3.7. Robustness

All the method variations produced deviations below 3% in comparison with the concentration obtained without method modification. The R.S.D.% of the standard solutions in all the conditions were below 2% [Table 6].

As ultimate validation proof, samples from real dissolution tests were simultaneously analyzed with IC and enzymatic method, and the dissolution profiles were compared. Difference less than 4% of average dissolved value was observed. The t test performed on the two dissolution datasets at every sampling time gave a statistical equivalence of the considered methods at confidence interval of 95% [Table 7].

4. Conclusion

The new presented method, based on an ionic chromatography technique, allows a precise and accurate detection of LMW Heparin. It allows to title easily the LMWH in a strongly ionic medium as a unique peak and not as a dispersion of molecular weights. Our working concentration was function firstly of the dosage of tablets and then of the volume of the medium used in dissolution system, so the range evaluated was between 0.0023 and 0.2800 mg ml^{-1}. It could be possible to have slightly different ranges with different injecting volumes.

The method validation assured a good reproducibility and the method accuracy is definitely higher than existing biological methods and in line with the quality required to a pharmaceutical repertoire.

This method represents therefore a valuable analytical alternative to biological assays in the context of dissolution testing of LMWH tablets.

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