Quality Control of Heparin Injections: Comparison of Four Established Methods

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Abstract

Heparin is an anticoagulant medication that is usually injected subcutaneously. The quality of a set of commercial heparin injections from different producers was examined by NMR, IR, UV-Vis spectroscopies and potentiometric multisensor system. The type of raw material regarding heparin animal origin and producer, heparin molecular weight and activity values was derived based on the non-targeted analysis of $^1$H NMR fingerprints. DOSY NMR spectroscopy was additionally used to study homogeneity and additives profile. UV-Vis and IR, being cheaper than NMR, combined with multivariate statistics were successfully applied to study excipients composition as well as semi-estimation of activity values. Potentiometric multisensor measurements were found to be an important additional source of information about inorganic composition of finished heparin formulations. All investigated instrumental techniques are useful for finished heparin injections and should be selected according to its availability as well as information and confidence required for a specific sample.

Keywords Spectroscopy, sensors, heparin, injections, principal component analysis
1 Introduction

A finished pharmaceutical product must satisfy certain standards to claim it to be a quality drug. The main criteria for quality of any drug in finished dosage form are its safety, potency, efficacy, stability, patient acceptability and regulatory compliance\(^1\). In this regard, chemical specification is the one of the important stages, and the analytical methodologies involved in the drug testing must be constantly updated. Indeed, in the past decades there were several cases when outdated analytical standards for pharmaceuticals caused human deaths and/or severe adverse effects, because harmful contaminants were overlooked\(^2,3\).

The contamination of the widely used lifesaving anticoagulant drug heparin in 2007-8 is one of such prominent examples, and even now researchers extensively search for new methods to investigate the structure and quality characteristics of this natural product. Nondestructive spectroscopic methods are perfectly suitable for this purpose because they allow to provide analysis without breakdown of heparin polymer molecules and, therefore, all structural information is reflected in their spectroscopic fingerprints. This fact is important as it allows to consider the structural heterogeneity of heparin, which is responsible for its pharmaceutical properties.

Probably, nuclear magnetic resonance (NMR) spectroscopy is the most versatile among the existing approaches for heparin analysis, because with only one sample preparation according to Pharmacopeia and using just four sequential NMR experiments combined with multivariate analysis one can obtain information about purity, assay and provenience of heparin samples\(^4,6\). Moreover, this approach enables correlation with biological activities and macroscopic values such as molecular weight\(^4,7\). Other interesting applications of NMR spectroscopy and multivariate analysis included
investigation of correlation in oligosaccharide composition between parent heparin and daughter low molecular weight (LMW) heparin, monitoring of structural features of crude heparin, identification of adverse contaminants and others.\textsuperscript{8-10}

UV-Vis and fourier-transform infrared spectroscopy (IR) are simple and reliable techniques for identification and quality control of glycosaminoglycans including heparin. IR spectra of glycosaminoglycans are well studied\textsuperscript{11,12} and were used for comparative analysis of their chemical structures\textsuperscript{13,14}, investigation of heparin complexation with cations\textsuperscript{15,16} and its chemical modifications\textsuperscript{17}. Absorbance of \( \nu_{\text{as}} \text{S=O} \) band (1230 cm\(^{-1}\)) was applied for distinguishing the heparin type and quantitative application of heparin in solution\textsuperscript{18}. It should be mentioned that strong absorption of proteins and sensitivity of IR spectrum of heparin prevented the usage of mid-infrared spectral range in some cases\textsuperscript{19}.

**UV-Vis** spectrum of heparin is associated with the presence of carboxylic acid and N-acetyl groups (180-260 nm) and is not directly useful due to overlapping with transition bands of proteins and peptides\textsuperscript{12}. At the same time Lima et al. have shown that chemometric analysis of **UV-Vis** spectral data allowed to differentiate pure heparin samples from crude heparin preparations\textsuperscript{20}. The effect of heparin on the absorbance spectra of other compounds (organic dyes, gold nanoparticles or conjugated polymer) was used to develop quantitative methods for heparin determination\textsuperscript{21,22}.

Besides spectroscopy, another emerging analytical approach for express quality evaluation of pharmaceuticals is so called “electronic tongue”, which is basically an array of cross-sensitive chemical sensors combined with multivariate data processing tools\textsuperscript{23}. Electronic tongues based on different principles (potentiometry, voltammetry, optical sensing) were successfully applied in pharmaceutical tasks to assess the taste of active pharmaceutical ingredients\textsuperscript{24} and taste masking efficacy\textsuperscript{25}, to control the
production of pharmaceutical proteins\textsuperscript{26}, to perform dissolution tests\textsuperscript{27} and in numerous other applications\textsuperscript{28-30}. The main advantages of multisensor approach are fast, simple and inexpensive analysis procedure, which typically requires no complex sample pretreatment stages. A proper choice of sensors for multisensor arrays allows fine tuning of instrument sensitivity and can adjust the performance of a sensor system to the requirements of different analytical tasks. So far heparin has not been tested using such promising instrumental technique in spite of the fact that heparin formulations are aqueous solutions, which are predestined for express quality assessment by multisensor systems.

It can be concluded that heparin active pharmaceutical ingredient (API) was investigated using a broad range of analytical techniques. However, according to our literature search, heparin injections as finished formulations containing a bundle of excipients were previously examined in only several studies\textsuperscript{31-34}.

In this study a representative set of commercial heparin injections was evaluated by different instrumental techniques, including NMR, \textbf{UV-Vis}, IR spectrometry and potentiometric multisensor system. The emphasis was placed on excipients, heparin origin, producer, and activity. Moreover, we demonstrate the usefulness of complementary experimental approaches for the quantification of additives in heparin formulations and discuss the correlation of experimental data with anticoagulant activity.

2 Experimental

\textit{Reagents and chemicals}

A total of eighteen finished formulations, which contained \textit{low-molecular}
weight

heparin (two samples), two heparin calcium (two samples) and heparin sodium (fourteen samples) were studied. The porcine Na heparin samples produced in Belarus S17 were represented by five different batches (S17a-e). The information about investigated samples including solvent and preservatives used as well as formulation activity is summarized in Table S1. All reagents used for sample preparation were of analytical grade.

Apparatus

NMR measurements were performed using Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) with BBO cryo probe equipped with Bruker Automatic Sample Changer (B-ACS 120) at 297 K. $^1$H NMR spectra were recorded with presaturation of water signal using 16 scans (NS) and 4 prior dummy scans (DS). The data of 65k points (TD) were acquired with a spectral width of 19.9947 ppm (SW), an acquisition time of 3.276799 s with an automated receiver gain (RG) adjustment. The following parameters were selected for 2D diffusion-ordered spectroscopy (DOSY) experiments with presaturation: NS=8, DS=8, TD=16k. The data were recorded automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany). All NMR spectra were manually phased and baseline-corrected using Topspin 3.2 (Bruker Biospin, Rheinstetten, Germany).

For sample preparation, 600 µL of a formulation was mixed with 0.2 mL of D$_2$O. This is more convenient sample preparation in comparison with the previously described one, where samples have to be dialyzed to achieve sufficient sensitivity$^{31}$. IR spectra were acquired using IRAffinity-1S FTIR spectrometer (Shimadzu, Kyōto, Japan) in the spectral range 400 – 4000 cm$^{-1}$. To record an FTIR spectrum 10.00 µL of a formulation was placed on a ZnSe crystal, and dried at 55°C for 15 min.
The UV-Vis spectra were recorded using Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyōto, Japan) in the spectral range of 190-350 nm with the resolution of 1 nm. Samples for UV-Vis scanning analyses were prepared by diluting the formulations with bidistilled water (C = 5 IU/ml).

The sensor array employed in this study consisted of 15 potentiometric sensors. 13 electrodes had cation- and anion-sensitive plasticized polymeric membranes, one sensor was polycrystalline Ag$_2$S/AgCl chloride-selective electrode, one sensor was pH glass electrode (“Izmeritel’naya Tehnika”, LLC, Moscow, Russia). The sensors were chosen in order to provide sensitivity towards common excipients that can be found in commercial heparin formulations. The details on sensor membrane compositions are available in Table S2.

Sensor membranes were prepared according to the standard protocol: all the weighted components were dissolved in freshly distilled tetrahydrofurane (THF) and poured into a flat bottomed Teflon beaker for solvent evaporation. The content of polyvinyl chloride (PVC) was about 33%, while plasticizer content was varied around 65% depending on molar mass of the employed membrane active substances. The total membrane weight was 330 mg for all compositions. After THF evaporation the membranes of 8 mm diameter were cut from the parent membrane. Copper wires were attached to the membrane surfaces using the suspension of fine graphite powder in PVC/cyclohexanone mixture (solid electrical contact). The membranes with wires were further glued onto the top of PVC tubes (sensor bodies) with the PVC/cyclohexanone mixture. The general visual appearance of view of Sensor membranes the sensor array is presented in Fig. 1S. (Supplementary materials)

The potentiometric measurements were performed against standard Ag/AgCl
reference electrode ("Izmeritelnaya Tehnika", LLC, Moscow, Russia) in a Teflon sample cell under stirring. All sensors were connected with shielded wires to the multichannel high input impedance digital mV-meter HAN-11 (Sensor Systems, LLC, St. Petersburg, Russia). Sensor potentials were recorded with 0.1 mV precision in a custom made software installed on a Windows PC. Measurement time in each sample was 3 min. Between the measurements in different samples the sensors were washed with three portions of distilled water for 1 min each. Heparin samples were diluted before the potentiometric measurements. 0.3 ml of the sample was added to 30 ml of doubly distilled water and thoroughly mixed with magnetic stirrer right before the analysis.

Chemometrics

Unscrambler X (Camo Analytics, Oslo, Norway) and Matlab 2015a (The Math Works, Natick, MA, USA) were used for multivariate calculations. As a multivariate technique for non-targeted analysis, principal component analysis (PCA) and hierarchical cluster analysis (HCA) were utilized. Partial least squares (PLS) regression was used to correlate spectroscopic profiles with activity values.

In NMR data the region of residual water signal was excluded before modeling. The data were pre-processed by bucketing with 0.01 ppm width. Afterwards the multivariate matrix was scaled to the total intensity in order minimize the intensity differences due to the different heparin content in investigated samples.

FTIR-spectra were preprocessed using the Origin 2016 software (OriginLab Corporation, USA). Preprocessing included baseline correction and smoothing by the Savitzky–Golay method. The data points in the region 400-1700 cm⁻¹ were used for multivariate modelling.
UV-Vis spectra in the range 220-350 nm were modelled without additional preprocessing.

The readings of the potentiometric sensors were employed for the processing as is without further manipulations. The matrix with sensor readings was mean-centered before the multivariate modeling.

3 Results and Discussion

In this manuscript four instrumental techniques (NMR, IR, UV-Vis and potentiometric multisensor measurements) applied to the analysis of heparin injections will be consistently discussed and compared.

NMR spectroscopy

During the last decade, NMR spectrometry has been recognized as the most powerful tool for structure elucidation of heparin polymer structure and impurity composition of heparin and its derivatives. However, as it was noticed in the Introduction, only one study dealt with NMR quality control of commercial heparin injections.

Fig. 1 shows the ¹H NMR spectra of several investigated samples. Heparin injections are usually available as either calcium or sodium salts, which showed considerably different NMR profiles. For example, the Is-1 and Is-5 signals in the sodium salt were remarkably shifted toward lower frequencies (S05 and S04, Fig. 1). The As-4 signal was also notably moved downfield. These findings are in a good agreement with the previous study. The ¹H NMR spectrum of enoxaparin injection (e.g., S01) is relatively complex and, in comparison with heparin samples, showed
additional resonances of ΔU4 (δ 5.98 ppm) and ΔU1 (δ 5.52 ppm) at the upfield region were recognized (Fig. 1). Likewise, heparin preparation S12 obtained from bovine material can be easily differentiated from the samples of porcine origin (S03, S04, S08), which is in agreement with our previous study\textsuperscript{38}. \textbf{The distinguishing of the origin of heparin species of origin by multivariate analysis of NMR spectra is based on the different levels of N-acetylation glucosamine residues and the differences in sulfation at several specific positions (position 6 of glucosamine residues, position 3 of glucosamine, and position 2 of the iduronic acid) \textsuperscript{38}. Therefore, chemometrics analysis of NMR profile can be a useful tool for heparin animal sources control.}

Several compounds such as methyl p-hydroxybenzoate, benzylalcohol and chlorobutanol are often added to heparin injections as preservatives or an agent to keep isotonicity\textsuperscript{31}. The spectra of injections containing some of these additives (i.e., benzyl alcohol at δ 7.4 ppm and chlorobutanol at δ 1.6 ppm) gave characteristic signals due to these compounds, as shown in Fig. 1. The signals of preservatives can be also clearly recognized and identified in 2D DOSY NMR spectra (Fig. S\textsuperscript{24}). Furthermore, DOSY NMR spectroscopy shows the uniformity of polymer heparin material. The contents of such additives can be directly quantified using appropriate internal standard such as potassium methansulfonate\textsuperscript{31}.

PCA was carried out to evaluate the suitability of multivariate statistics for exploratory analysis of the investigated samples (Fig. 2). As expected, heparin formulation prepared from bovine material (S12) as well as two heparin calcium samples (S05 and S11) were outliers along the two first principal components (PCs) PC2 and PC1, respectively. After excluding these samples, two low-molecular weight LMW preparations (S01 and S02) were located separately from a set of sodium heparin injections (Fig. S\textsuperscript{33}). Remarkably, hierarchical cluster analysis HCA was able to
distinguish different producing countries (Fig. S43). It can be seen that German and Austrian preparations as well as the samples produced in the former Union of Soviet Socialist Republics (USSR) have quite similar NMR profiles. Probably, they have the same supplier of the raw material. Clearly, more samples are needed to test this hypothesis.

Anticoagulant activity is the most important heparin property and there are several biological assay methods using fresh bovine blood39. However, since these methods are cumbersome and require proficiency, recent studies were aimed at developing alternative methods that can correlate spectroscopic profiles to anticoagulant activity values4.

To investigate the influence of the heparin structure on the activity of its formulations, 1H NMR spectra of ten sodium porcine heparin preparations were modelled by PCA (Fig. 3). It is obvious that the PC3 axis is important to differentiate heparin injections according to their activity from highest 25000 I.E. at high score values to lowest 1000 I.E. at low score values of PC3.

Thus, the NMR method enabled direct observation of signals of heparin extracted from different animal tissues as well as additives in intact injection samples. It is also promising for the quality check of API type and semi-estimation of anticoagulant activity.

**IR spectroscopy**

As noted above, Fourier-IR spectroscopy has some limitations in direct analysis of glycosaminoglycans and was mostly used to monitor gross changes in heparin12;19. On the other hand, glycosaminoglycans IR spectral signatures allowed to perform a direct molecular distinction of this compounds using chemometric algorithms14. The
applicability of near infrared reflectance coupled with PLS was already demonstrated for the determination of the potency of heparin API\(^{13}\). Samples prepared by mixing of three batches of heparin API samples with known API were used for PLS modeling and obtained model has shown high level of recovery (~100%), good prediction capability and reproducibility.

The general spectral patterns of the FTIR spectra of the studied samples were quite similar and the main differences were in the intensity of spectral bands (Fig. S54). The broad band at 3440 cm\(^{-1}\) was assigned to the hydroxyl stretching vibration of heparin and other presented compounds with the contributions from C-N stretching, the band 2935 cm\(^{-1}\) was ascribed as to C−H stretching vibration. The spectral region 4000-1700 cm\(^{-1}\) was not informative due to high level of noise and was not taken into account in multivariate modelling. Three strong bands were observed in the spectral region 1700-400 cm\(^{-1}\) and were assigned to band of N-acetyl group with contributions of asymmetric vibration of carboxylate group (1617 cm\(^{-1}\))\(^{19}\), stretching vibration of S=O in sulfate esters (1236 cm\(^{-1}\))\(^{18,40}\), and C−O−C stretching vibration in sugar rings (1009, 1045 cm\(^{-1}\))\(^{41}\).

Approximately 90% of variance is described using the first two PCs by the PCA model built from IR spectra (Fig. 4). The samples S02 and S05 located in the right upper corner of the PCA score plot represent low-molecular-weight LMW heparin and heparin calcium, respectively. It can be also seen that PCA was able to catch the tendency of decreasing activity values along PC1. To confirm this funding, PLS model was constructed to relate the IR spectra with the activity of heparin formulations. Root mean square error of cross validation (RMSECV) and \(R^2\) values were found to be 1308 a.u. and 0.96, respectively. This multivariate model can be used for semi-quantitative estimation of activity values based on IR measurements.
**UV-Vis spectroscopy**

The potential of UV-Vis scanning spectroscopy combined with PCA for the analysis of heparin purity in contaminated samples was shown in\(^{20}\). The developed approach allowed to detect as low as 0.1% contaminants in artificially spiked heparin samples.

Fig. S65 shows the UV-Vis spectra of investigated samples. The samples (S03, S06, S08, S09) displayed only one sharp shortwave band (190-210 nm), which is typical for any glycosaminoglycans. UV-Vis spectra of samples S02 (low-molecular weight LMW heparin) and S05 (calcium salt) had an additional broad signal around 240–260 nm. The high intensity bands 250-270 nm (\(\lambda_{\text{max}}=257\) nm) were obtained for the samples S04, S07, S13-S17 and can be attributed to the presence of benzyl alcohol, which was confirmed by \(^1\)H NMR analysis and in a good agreement with the previous study\(^{20}\).

PCA showed that the samples can be separated according to their identity into several groups: LMW heparin S02 was in the region of positive PC2, the samples containing benzyl alcohol (S04, S07, S13-S17) were in the region of positive PC1 values, whereas heparin calcium S05 was located in the negative region of PC1 and positive values of PC2 (Fig. 5). This type of spectroscopic measurements also provided some insights in activity values of finished products: the anticoagulant activity is decreasing from the samples S06 and S03 to the sample S08 (see Table 1 for details).

**Multisensor measurements**

The normalized response of the sensor array in heparin samples is shown in Fig. 6. The differences in the chemical composition of the samples lead to the differences in
registered sensor potentials.

The multisensor array response was employed for PCA. The score plot for the first two principal components (PCs) are shown in Fig. 7. The differences between LMWH-LMW heparin (samples S01 and S02) and heparin clusters were reflected along the PC2 axis.

Sample S03, which was located in the right upper corner of the PCA score plot, contained sulphuric acid. The PC1 reflected the content of hydrochloric acid, which is decreasing from left to right. The sensors with the highest loading values for PC1 were those with pronounced sensitivity to chloride, while the PC2 loadings were high for the sensors with sensitivity towards hydrophilic anions (like, e.g. carbonate and sulfate). No direct correlation between these experimental data and anticoagulant activity was observed, since the response of the potentiometric sensors in the heparin formulations mainly reflected inorganic composition of the investigated samples. Nevertheless, the information derived from the potentiometric sensor array still can be useful for discrimination of the origin of the samples.

The summary of heparin injection analysis are presented in the Table 1. The data have shown that simultaneous analysis by different instrumental techniques combined with chemometrics allows observing of different properties of the samples. This approach can be also applied for other polymer drugs in case of overlapping spectral profiles and when inorganic composition plays important role for their quality control.

Table 1 The results of heparin injection analysis by different instrumental techniques

| Techniques | Main signals | Group classification |
|------------|--------------|----------------------|
| NMR        | Is-1, Is-5, As-4, additional resonances of U4, U1 | Na salt, Enoxaparin, source animal |
4 Conclusions

From the experimental techniques studied, NMR provides the most complete overview of the investigated intact heparin formulations. Our data suggested that apart from the type of the raw material, heparin animal origin and additives profile, other relevant parameters such as producer and anticoagulant activity can be additionally assessed using one simple sample preparation. Other two spectroscopic techniques, UV-Vis and IR, being cheaper than NMR, combined with appropriate multivariate modelling can also supply one with the information about activity level and additives profile. The investigation of the possibility to determine heparin animal origin by IR and UV-Vis spectroscopies is the focus of our ongoing research.

An important finding of this study is that different spectroscopic profiles of heparin injections are correlated with anticoagulant activity. We now collect finished heparin preparations from other sources in order to construct quantitative models to predict this important drug property.

Multisensor measurements are undoubtedly an important additional source of
information about inorganic composition in finished heparin formulations. For example, extremely high (or low) concentrations of sulphuric acid and hydrochloric acid can be easily identified. This is really promising, because currently the qualitative and quantitative characterization of inorganic impurity profiling is an analytical challenge.42

Thus, different instrumental techniques combined with appropriate data modelling provide complementary information about heparin injections. The method used for quality control should be selected according to its availability and information required. Other methods can be also used to check the results of a single method in case where high level of certainty is required.

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6 References

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Figure Captions

Fig. 1  $^1$H NMR spectra of heparin injections of porcine heparin Na (S03, S04, S08), bovine heparin Na (S12), enoxaparin Na (S01) and heparin Ca (S05). Signals of chlorobutanol and benzyl alcohol are marked with a and b, respectively.

Fig. 2 PCA scatter plot based on the NMR spectra of heparin injections.

Fig. 3 PCA scatter plot of Na heparin injections based on NMR profiles. Activity values in I.E./ml are shown on the scatter plot.

Fig. 4 PCA scatter plot of heparin injections based on IR spectra.

Fig. 5 PCA scatter plot of heparin injections based on UV-Vis spectra.

Fig. 6 The heat map of the response of the sensors for the investigated samples normalized by autoscaling procedure.

Fig. 7 PCA scatter plot of heparin injections based on multisensor measurements. Each sample has been measured in triplicate.
Fig. 1
Fig. 2
Fig. 3
Fig. 4

Decrease of anticoagulant activity
Fig. 5

Decrease of anticoagulant activity
Fig. 6
Fig. 7