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Determination of heparin on intraocular lens surfaces by ion chromatography

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Abstract

A sensitive and selective method has been developed for the determination of heparin on heparin coated PMMA, poly(methyl methacrylate), intraocular lenses. Heparin was hydrolysed to glucosamine and glucuronic acid, and the content of glucosamine was determined using ion chromatography with pulsed amperometric detection. In order to verify that a complete hydrolysis was obtained for the heparin on the coated intraocular lenses, electron spectroscopy for chemical analysis (ESCA) was used for analysing traces of sulphur on the lens surfaces. The sensitivity of the method allows quantitative determination of 150 ng of heparin on one individual lens. The new method was compared to a standard spectrophotometric method, measuring the colour intensity of a heparin toluidine blue complex. Correlation between the methods was shown for samples prepared from PMMA lenses coated with different amounts of heparin.

Keywords: Lens, intraocular; Heparin

1. Introduction

Heparin is a water soluble, highly sulphated, linear polysaccharide consisting of alternating *N*-acetyl-D-glucosamine and D-iduronate residues (Fig. 1). It is a constituent in various tissues, e.g. in the lining of arterial blood vessels, lungs and liver. The polymer plays an important role in the blood coagulation process, being a very strong inhibitor to blood clotting. Pure heparin solutions are used to prevent blood clotting, e.g. for preventive purposes during surgical operation. Another important medical application for heparin is surface modification of plastic materials for implants, making the material more biocompatible. One example of this application is heparinized intraocular lenses, which has been successfully used for many years in replacing the clouded natural lens in cataract surgery [1]. Also other types of implants have been heparin surface modified in order to increase biocompatibility, for example pericardial patches [2], subconjunctival

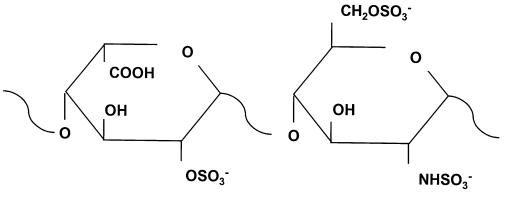

Fig. 1. Repeating unit of heparin.

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implants [3], intraaortic balloons [4] and vascular shunts [5].

In the quality control testing of the heparinized intraocular lenses, a toluidine staining method (TOL) have been used to verify the success of the heparinization process. The TOL method is based on staining of the lens with toluidine blue and measuring the absorbance at 550 nm directly through the lens. However, this staining method is not a quantitative method for heparin, and the range of linearity is very uncertain. In order to obtain quantitative information on the amount of heparin on the individual lenses, a new method had to be developed. Expecting very low amounts of heparin on the lenses, it was essential for the new method to be very sensitive.

Most methods described in the literature were rejected due to lack of sensitivity, selectivity or precision. However, it has been shown that the use of ion chromatography in combination with a pulsed amperometric detection (PAD) on acidic hydrolysates of heparin in solution offered the desired sensitivity and selectivity [6]. In this paper we describe the further development of this method, applied on very small amounts of heparin, immobilised on the surface of intraocular lenses.

The development of this method also made it possible to determine the correlation between the TOL method and the amount of heparin on individual lenses. This correlation is also presented as a part of this study.

2. Experimental

All glass vials were silylated with a solution of 5% 1,1,1,3,3,3-hexamethyl disiloxane in hexane at room temperature over night. This procedure was performed in order to minimise adsorption of glucosamine to the inner surfaces of the vials.

2.1. Chemicals, standards and samples

Sodium hydroxide (50% w/w), hydrochloric acid (37% w/w), sodium chloride, sodium acetate and toluidine blue, of analytical reagent grade quality, were obtained from Merck. Sodiumborate p.a. was from Mallinckrodt. 1,1,1,3,3,3-hexamethyl disiloxane, >99% was obtained from Merck-Schuckardt. For the standard solutions, D-(+)-glucosamine hydrochloride with purity >99% were obtained from Sigma, and heparin was obtained from Franklin (OH, USA). Water from a Millipore Milli-Q system was used for all preparations.

Prior to preparation of the standard solutions, glucosamine hydrochloride was dried in vacuum at 40°C. The heparin substance was stored at controlled conditions (20°C, 50% relative humidity). The water content was found to be 10%, determined by Karl Fischer titration.

The samples were prepared from intraocular PMMA lenses, equivalent to the commercial Heparin Surface Modified (HSM) lenses of Pharmacia. The total surface area of the lenses was calculated to be 85 mm^2, including the haptics. The calculations were done using a CAD program.

For this study, the lenses had been specially prepared with different amounts of heparin. The heparin-coated lenses were prepared using the method for end-point attachment of heparin on IOLs developed by Larsson et al. [7], which gives a surface modification that is chemically equivalent to that of the commercial Pharmacia HSM lenses. The amount of heparin was controlled by applying the procedure for different numbers of cycles. The same batch of heparin was used for heparinization of the lenses and for preparation of standard solutions.

In order to be able to correlate the TOL method to the amount of heparin on the lenses, toluidine blue absorbance values were acquired for the heparin-coated lenses. The toluidine blue staining method is a spectrophotometric method indicating the amount of heparin on the lens. The method is based on staining the lens with Toluidine Blue and measuring the absorbance at 550 nm directly through the lens.

The heparin coated lens was stained with an alkaline solution of toluidine blue dye (20 mg toluidine blue in 100 ml of 0.0125 *M* borate buffer) binding to the heparin on the surface. After 10 min, the lens was removed from the staining solution and carefully rinsed. The absorbance of the heparin–toluidine complex was measured in a spectrophotometer directly through the lens at 550 nm. The amount of dye uptake, and so the absorbance, was expected to increase with the amount of heparin on the surface, due to the interaction between the dye and the heparin.

2.2. Procedure

The determination of the amount of heparin was performed after hydrolysis of heparin into glucosamine and glucuronic acid. The glucosamine was used to generate a standard curve for detector response against amount of glucosamine. The heparin was used to relate the amount of glucosamine that was obtained in the hydrolysis, to the amount of heparin in the sample.

The heparin substance was hydrolysed in 600 μl of 4.8 *M* hydrochloric acid during 24 h at 98°C in a sealed headspace vial. The same conditions were used also for the lenses. An aliquot of the hydrolysate was then evaporated until dryness, using a low-speed vacuum centrifuge. The residue was dissolved in water and injected into the chromatographic system. In order to verify that a high recovery was obtained in the hydrolysis step, the hydrolysed lenses were analysed by ESCA for traces of sulphur.

2.2.1. Experimental procedure for one lens

One lens was placed into a 10 ml headspace vial, and 600 μl of 4.8 *M* HCl was added. The vial was sealed and placed in a heating chamber at 98°C for 24 h.

After cooling, an aliquot of 500 μl was taken out and transferred into a silylated 2 ml vial. The vial was placed in a low speed vacuum centrifuge and the solution was evaporated to dryness at low temperature. The residue was dissolved in 500 μl of water.

The chromatography was performed according to description below.

2.2.1.1. Instrumentation

Pump: Dionex gradient pump, GP40; Detector: Dionex electrochemical detector, ED40; Autosampler: Spectra physics autosampler AS3500; Chromatography system: Dionex Compaq, Hp, Peaknet system; Column: Dionex CarboPac PA1, 4*250 mm, and CarboPac PA1-Guard (10–32).

2.2.1.2. Instrumental conditions

Flow-rate: 1.0 ml/min; Mobile phase: 95 m*M* sodium hydroxide/10 m*M* sodium acetate; Regime: Isocratic elution; Injection volume: 60 μl; Detector, ED 40: Gold electrode; Operating mode: Integrated amperometry; ED40 Waveform: E1=+0.05 V, t1=400 ms; E2=+0.75 V, t2=200 ms; E3=−0.75 V, t3=400 ms

The ESCA experiments were done on a Physical Electronics 5000 LS x-ray photoelectron spectrometer equipped with a monochromatic Al Kα source. All spectra were acquired in the Constant Analyser Energy (CAE) mode, and charging was controlled using a low energy flood gun set at 3 eV.

Before being mounted to the sample holder, the lens was rinsed in water in order to remove any residuals from the hydrolysis solution. A survey scan (0–1100 eV, 1 channel/eV) was taken at a pass energy of 178 eV, 45° exit angle, and a nominal spot size on the sample of 1 mm diameter.

3. Results and discussion

3.1. Determination of heparin as glucosamine using ion chromatography

The hydrolysis conditions were optimised to achieve the maximum amount of glucosamine in the hydrolysate, when starting from heparin substance. The optimisation was designed as a reduced factorial experiment, with HCl concentration, temperature and time at three levels (Table 1). The reduced design resulted in 17 experiments, where each lens was analysed in duplicates.

It was found that the highest temperature setting yielded the best results, and the results also suggested that even higher temperatures would give better results. For practical reasons, however, the temperature was not raised further. Optimum hydrolysis conditions for the heparin standard were found to be 4.8 *M* HCL for 24 h at the chosen temperature 98°C. These conditions were used also for hydrolysis of the heparin on the lenses.

Table 1
Parameter settings for factorial design optimization

Setting	HCl concentration (*M*)	Temperature (°C)	Time (h)
High	6	98	24
Mid	4	91	20
Low	2	84	16

The chosen hydrolysis conditions were found to balance incomplete hydrolysis of the heparin on one hand, against degradation of the glucosamine on the other hand. At these settings, the amount of glucosamine obtained from the heparin substance was 20% of the heparin dry weight. Other sources of heparin were investigated for comparison, and yielded 15–30% glucosamine at the same conditions. As a precaution against possible batch differences, the same batch of heparin was used both for standards and samples.

The retention time for the glucosamine peak was approximately 3.4 min. No interfering peaks were found in the chromatograms from lenses without heparin coating (Fig. 2). The other product of the hydrolysis, the glucuronic acid, was strongly retarded, and could not be detected on the chromatographic system.

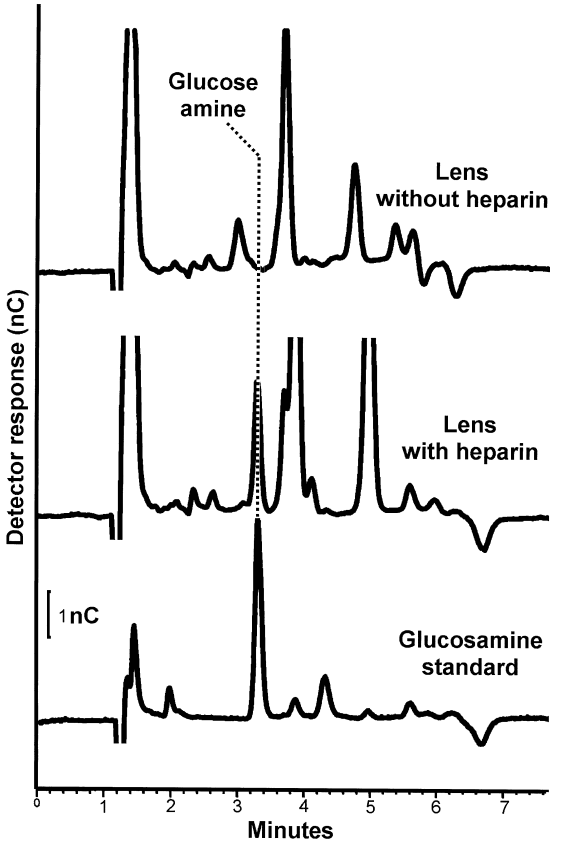

Fig. 2. Typical chromatograms obtained for a glucose amine standard and samples prepared from PMMA lenses, coated with heparin and not coated. All samples were treated identically and according to the described method.

Peak area responses of glucose amine were used to quantitate the amount of heparin for standards and samples (heparin coated lenses). Peak area response was found to be linear with glucose amine concentration over the range 0.05–0.7 μg/ml or calculated as heparin, over the range 0.25–3.5 μg/ml. Correcting for the 500 μl that were taken from the 600 μl volume, and the 60 μl that were injected, it follows that 150 ng of heparin can be quantitatively determined on one individual lens.

The intermediate relative standard deviation was better than 10% for two heparin standards analysed on five different occasions over a period of a few months.

After hydrolysis, the lenses were examined with ESCA analysis. The analysis revealed no detectable levels of sulphur (Fig. 3), indicating complete hydrolysis and removal of the heparin from the lens surface. Small amounts of chlorine and silicon were found as residues from the hydrolysis process.

3.2. Estimation of heparin using toluidine blue (TOL) staining

Lenses coated with different amounts of heparin were analysed with the TOL method. The lenses contained from 0.2 to 6.5 μg of heparin each with haptics included. The same lenses were subsequently analysed individually, using the ion chromatography

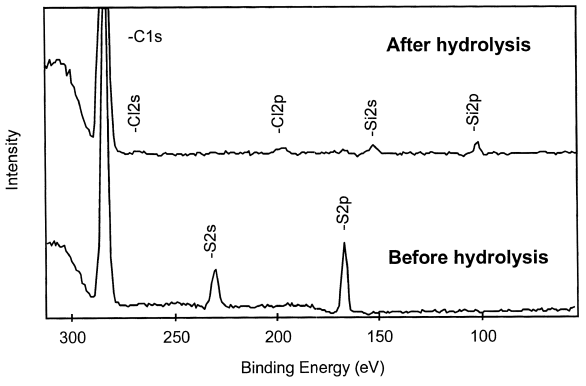

Fig. 3. ESCA spectra from the lens surface showing surface chemistry before and after hydrolysis. Spectra baseline shifted for clarity.

method to determine the amount of heparin on each lens. In this manner, it was possible to determine the correlation between the TOL absorbance values and the amount of heparin on the lenses.

The presence of TOL had no impact on the chromatographic method, i.e. no additional peaks were detected.

The correlation was found to be reasonably linear at low TOL absorbance values. At very high absorbance values, though, the relation is significantly non-linear and fits well to a second order polynomial (Fig. 4). The non-linearity in the correlation is due to the non-linearity of the TOL in the high loading region. At the typical loading, however, linearity is sufficient.

The chromatographic method measures the total amount of heparin on the lens. This means that using e.g. larger lenses than the ones used in this study would give larger amounts of heparin per lens at the same surface coverage. Commercial Pharmacia HSM lenses of the same size as used in this study were found to contain 0.6 μg of heparin, with a variability of 5%, based on TOL measurements.

At the limit for quantitative determination of 150 ng of heparin per 85 mm^2 of lens surface, the surface coverage would be only 1.8 ng/mm^2. Applying the chromatographic method for larger implants would allow for much smaller surface coverages to be quantitatively determined.

The TOL method, on the other hand, measures the surface coverage, and not the total amount of heparin on the lens. However, using the correlation between TOL values and the amount of heparin that was established here, it is possible to use the simple and straightforward TOL method as a production quality control tool, while still being able to estimate the amount of heparin on the lenses. It may be noted that although the described TOL method is not quantitative, the sensitivity of the method is in the same range as the chromatographic method when used on intraocular lenses of this size. An obvious limitation of the TOL method is that the samples have to be transparent, which is not the case of most implants.

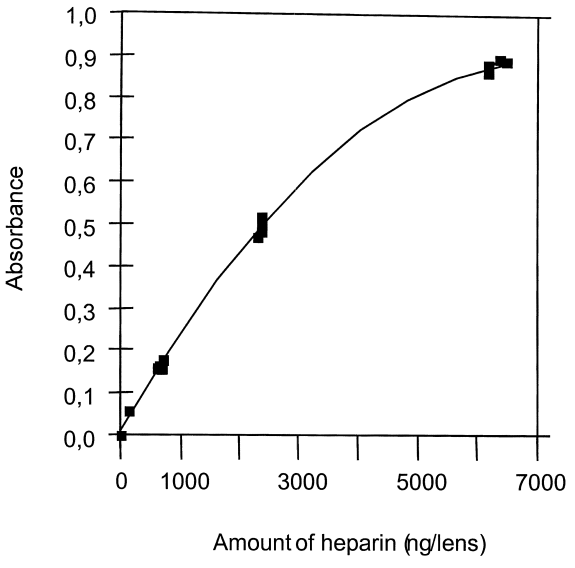

Fig. 4. Correlation between TOL absorbance values and amount of heparin per lens. A second order polynomial fit is shown. Polynomial fit results, Abs=0.0128+0.000246*ng−1.71e−8*ng^2.

4. Conclusions

A sensitive and selective method has been developed for the determination of heparin on heparin coated PMMA lenses. The intermediate relative standard deviation was better than 10% for two heparin standards analysed on five different occasions over a period of a few months. The sensitivity of the method allows quantitative determination of 150 ng of heparin on one individual lens.

A correlation between the new method and the toluidine blue staining method has been established for individual PMMA lenses coated with different amounts of heparin. The correlation was found to be reasonably linear at low TOL absorbance values, but significantly non-linear at very high absorbance values. With the aid of this relationship, it is possible to use the simple and straightforward TOL method as a production quality control tool, while at the same time being able to estimate the amount of heparin on the lenses. The TOL method also provides means to confirm visually that the desired surface coverage uniformity is achieved.

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