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Determination of sulphated glycosaminoglycans by automated potentiometric titration with simple coated-wire electrodes

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Abstract: A method for the determination of sulphated glycosaminoglycans is based on their precipitation with (1-ethoxycarbonyl)pentadecyltrimethylammonium bromide (Septonex), the excess of which is back-titrated with sodium tetraphenylborate. The titration is monitored by a simple coated-wire ion-selective electrode with a plasticized poly(vinyl chloride) membrane on aluminium wire. Under certain conditions the results are almost independent of the relative molecular mass of glycosaminoglycans. The method has been applied to the determination of the active ingredient in the pharmaceutical preparation, heparon injection.

Keywords: *Glycosaminoglycans determination; heparon; heparin; coated-wire electrodes; potentiometric titration; ion-pair formation-based titrations.*

Introduction

Sulphated glycosaminoglycans (SGAGs) represent the components of the extracellular matrix that has a substantial importance in the structure and function of connective tissues; these poly-dispersed systems of heteropolymeric non-identical molecules behave as linear anionic polyelectrolytes. In aqueous solution, the strong negative charges create repulsion between parts of a chain so that there can be neither association nor coiling, and the chains must be extended. Criteria for their classification are based largely on the relative molecular mass and on the presence of contaminants [1]. The range of relative molecular mass of heparin is 6000–30,000 dalton, and that of heparon 7000–11,000 dalton. Heparin prepared from beef lungs comprises at least 20 chains of polysaccharides the main components of which are 2-sulphoiduronic acid (**1**), 2-deoxy-2-sulphamino-α-D-glucose-6-sulphate (**2**), β-D-glucuronic acid (**3**), 2-acetamido-2-deoxy-α-D-glucose (**4**), and α-L-iduronic acid (**5**). The basis of the polymer connection is a glycosidic bond between compounds (**1**) and (**2**) [2]:

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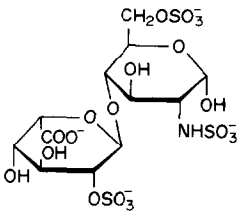

Heparin strongly complexes cations (especially polycations), e.g. K^+, Ca^{2+}, alkylamines, alkaloids, basic dyes, quaternary ammonium salts and basic proteins. SGAGs have a wide range of biological activity; typical effects are enzyme activation, enzyme inhibition and inhibitor activation. During the last 40 years a series of studies has been published on the isolation, clinical testing and chemical nature of various SGAGs [3–5].

Simultaneously with the increasing production of SGAGs it was necessary to develop reliable analytical methods. The papers of Jaques [1] and Johnson [6] are considered to be the most important on this topic. One of the classical analytical methods is elemental analysis — determination of total nitrogen — together with colorimetric determination of the total content of hexosamine and uronic acid. The water content is also of concern since SGAGs are notably hygroscopic. Sulphate and carboxylate estimations are two of the most important modes of characterizing SGAGs. A reliable procedure is that of Dodgson and Price [7], which is based on the turbidimetric determination of precipitated barium sulphate, stabilized with gelatin. Terho and Hartiale (see ref. 8) used rhodisonic acid for the determination; however, the quality of its commercially available forms varied too much. The most convenient method to estimate sulphation is the conductometric titration procedure of Casu and Guenaro [9]. Spectrophotometric methods were reported for the oldest SGAGs; these methods are mostly based on acid hydrolysis and on determination of total uronic acid and hexosamines [10]. For such determinations various dyestuffs are used, such as Azure A or Toluidine Blue.

The ratio of iduronate to glucuronate or 2-amino-2-deoxy-D-galactose is often of more interest or concern than the total content, which should be easily predictable in a sample free from non-glycolaminoglycans materials. The iduronate/glucuronate ratio may be determined non-destructively by ^{13}C-NMR [11].

A disadvantage of the majority of these assays is that standards are required which may be difficult to obtain. During the development of the pharmaceutical preparation, heparon injection, a necessity arose for the quantitative determination of the active ingredient and its identification. For this purpose a new method was developed which is based on the precipitation of SGAGs with a cationic substance (Septonex), the excess of which is then back-titrated with sodium tetraphenylborate using a coated-wire electrode. Experience with the potentiometric titration method was obtained during the development of a control method for the determination of local anaesthetics in parenteral preparations [12]. Techniques for such ion-pair formation-based titrations were reviewed by Vytřas [13].

Experimental

Electrodes

The coated-wire electrode with a membrane formed by poly(vinyl chloride) plasticized with 2,4-dinitrophenyl-*n*-octyl ether on aluminium wire was prepared as described earlier

[14, 15]. The saturated calomel electrode of the Metrohm 6.0702.110-type with a double junction filled with 0.01 M sodium nitrate was used as a reference half-cell.

Apparatus

The voltage values of the measuring cell were measured using a Potentiograph E 436 with a Dosimat E 436 D (Metrohm); constant magnetic stirring was used.

Reagents and materials

The materials were sodium tetraphenylborate (Lachema), thallium(I) nitrate (Fluka), (1-ethoxycarbonyl)pentadecyltrimethylammonium bromide (Septonex) (Slovakofarma Hlohovec), heparon (VÚFB Praha), heparin (Léčiva Praha) and a sulphated defined amylose fraction of maize starch (VÚFB). 0.04 M Sodium tetraphenylborate ($NaBPh_4$) was prepared as described earlier [14] and standardized by potentiometric titration of thallium(I) nitrate [16, 17] the content of which was controlled bromometrically. Septonex (0.04 M) was prepared and standardized by potentiometric titration with 0.04 M $NaBPh_4$ using the coated-wire electrode versus a saturated calomel electrode.

The preparations of heparon, heparin and a sulphated amylose fraction were first dried as described previously [6] and kept for 16 h in a thin layer under vacuum over P_2O_5. The solutions contained 10 mg ml^{-1} of SGAGs.

Potentiometric titration of SGAGs

A volume of SGAGs solution containing 20–60 mg of SGAGs was pipetted into a titration vessel; 10 ml of 0.04 M Septonex was added and the solution was diluted with water to 50 ml. The excess of Septonex was back-titrated with 0.04 M $NaBPh_4$ using the potentiometric cell. Titration curves were evaluated with the help of a template (Metrohm).

Results and Discussion

Titration curve shape

Titration curves of heparon are given in Fig. 1. It is obvious that with the increasing

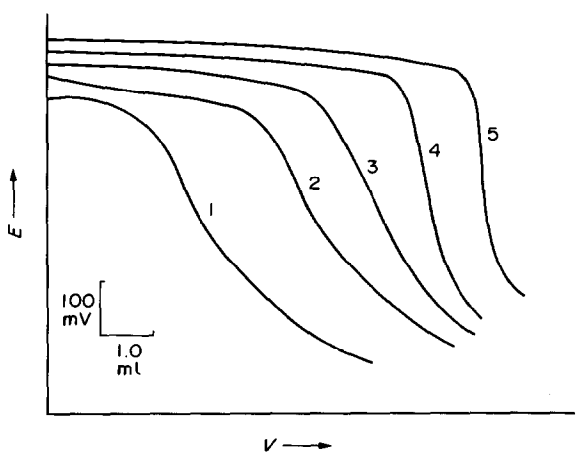

Figure 1
Determination of heparon. Titration curves of excess Septonex with $NaBPh_4$ for (1) 62.03, (2) 44.30, (3) 35.44, (4) 26.58 and (5) 17.72 mg of heparon.

amount of heparon (and, consequently, with the decreasing concentration of Septonex) the slope of the titration curve decreases considerably in the vicinity of the equivalence point, and the accuracy of the end-point reading decreases as well as the accuracy of the whole assay. This fact is probably related to the establishment of equilibrium in the system formed by SGAGs polyanion, Septonex and tetraphenylborate.

The possibility of a direct assay of SGAGs was also checked. However, when the SGAGs solution was directly titrated with Septonex, precipitation occurred, but a potentiometric titration curve could not be recorded. In the case of the back-titration, dependence on the sequence of the addition of the substances was observed; it was not acceptable to add water before Septonex because the results were not proportional to the concentration of SGAGs. For an acceptable assay performance, the solutions of heparon (the volume should not exceed 7 ml) and Septonex should be pipetted into a titration vessel; after the SGAGs-Septonex ion-pair complex has been formed, the distilled water is added and the mixture is titrated with the $NaBPh_4$ solution.

Verification of the dependence of the results on the relative molecular mass of the SGAGs

As has been pointed out, heparon is not a uniform substance but a fraction of SGAGs with a relative molecular mass of 7000–11,000 dalton. Therefore, it was necessary to verify the possible dependence of the results on the molecular mass of the SGAGs. Two further substances of known molecular mass (M_r) — heparin with M_r ~16,000 and sulphated amylose with M_r ~100,000 — were selected for the verification.

The titration curves for small amounts of heparin (25–40 mg) were nearly identical to those for heparon. At a higher concentration (about 50 mg), a second inflection appeared on the curve (Fig. 2). The titration curves of sulphated amylose did not differ from the heparon curves for the smallest amount of the substance (25 mg); however, a second inflection appeared when the amount was about 35 mg, and more inflections appeared at higher amounts (Fig. 3). This evidence suggests that the results do not depend on the M_r value over a broad range (700–100,000 dalton in the tests) if small amounts of SGAGS (20–30 mg) are used for the analysis.

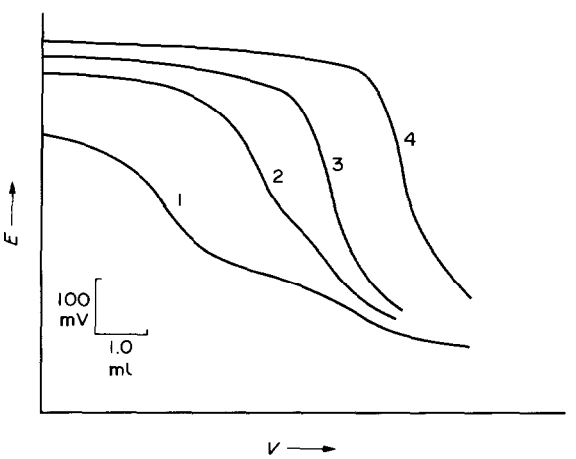

Figure 2
Determination of heparin. Titration curves of excess Septonex with $NaBPh_4$ for (1) 54.92, (2) 45.77, (3) 36.62 and (4) 27.46 mg of heparin.

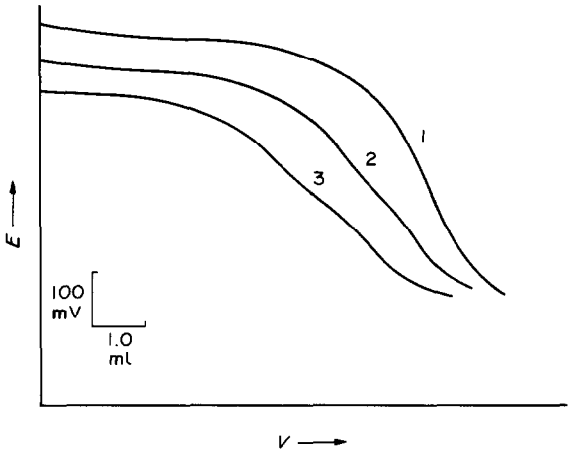

Figure 3
Determination of a sulphated amylose fraction of maize starch. Titration curves of excess Septonex with $NaBPh_4$ for (1) 26.63, (2) 35.51 and (3) 44.38 mg of amylose fraction.

Evaluation of results

The results obtained for the titrations of various amounts of SGAGs were evaluated statistically [18]. All the results were related to the amounts of Septonex actually consumed during the SGAGs-Septonex precipitation.

Because of the chemical nature of SGAGs, use of a calibration curve was chosen for evaluation of the results. Amounts of heparon were plotted against the amounts of Septonex bound in the precipitation with SGAGs (Fig. 4). The calibration curve $y = a + bx$ was determined by the linear regression method; x represents the amount of Septonex in millimols, y is the amount of SGAGs in milligrams. The parameters of the regression line were calculated: $a = -1.9$, $s_a = 0.7$, $b = 210.1$, $s_b = 4.4$, $s_{yx} = 0.4$ and $r_k = 0.9996$. The amounts found of heparon, heparin and sulphated amylose were calculated from the experimental results according to this equation. As shown in Table 1, the differences

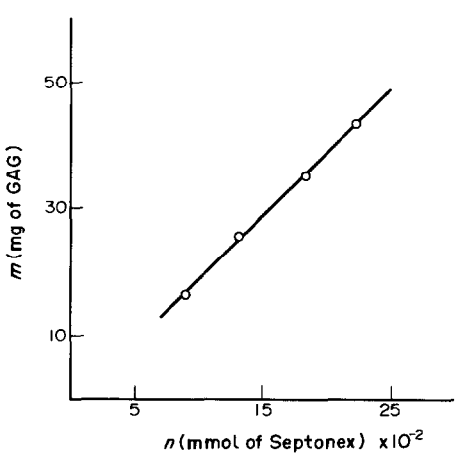

Figure 4
Calibration graph for the determination of SGAGs.

Table 1
Results of the determination of heparon, heparin and a sulphated amylose fraction by potentiometric back-titration

SGAGs added (mg)	Consumption of Septonex* (mmol)	SGAGs found (difference)* (mg)
Heparon first batch		
62.03	0.2941	59.89 (−2.14)
44.30	0.2185	44.01 (−0.29)
35.44	0.1801	35.94 (+0.50)
26.58	0.1356	26.59 (+0.01)
17.72	0.0929	17.62 (−0.10)
Heparon second batch		
48.52	0.2380	48.10 (−0.42)
29.11	0.1481	29.22 (+0.11)
46.22	0.2260	45.58 (−0.64)
Heparin		
54.92	0.3084	62.89 (+7.97)
45.77	0.2247	45.31 (−0.46)
36.62	0.1855	37.07 (+0.45)
27.46	0.1404	27.60 (+0.14)
Sulphated amylose fraction		
26.63	0.1345	26.36 (−0.27)
35.51	0.1812	36.17 (+0.66)

*The results are mean values of four measurements.

between the taken and found amounts of SGAGs do not usually exceed ±1.5%, if the taken amounts are smaller than 50 mg.

Choice of cationic compound

The possibility of determination of SGAGs by means of other cationic compounds was also tested. Septonex can be replaced by cetylpyridinium chloride, cetyltrimethylammonium chloride or other cationic compounds containing one or more long-chain alkyls. The use of procaine hydrochloride (procainium chloride) instead of Septonex cannot be recommended.

Conclusions

Potentiometric titration using simple coated-wire ion-selective electrodes was found useful for determination of SGAGs in parenteral preparations as well as for the verification of the content of the substance. The electrodes are easy to manipulate, have a long life and can be easily renewed. Compared with the proposed gravimetric assay, in which SGAGs are precipitated by ethanol, the method of determination described is rapid and reliable. Therefore, the method can be recommended for the determination of heparin and other SGAGs and for the assay of the pharmaceutical preparation, heparon injection. To determine the active ingredient in heparon injection (50 mg in 1 ml) the use of an amount corresponding to one half of an ampoule is recommended.

References

[1] L. B. Jaques, *Pharm. Sci.* **3**, 289–291 (1982).

[2] L. B. Jaques, *Meth. Biochem. Anal.* **24**, 203–312 (1977).
[3] L. B. Jaques, *Science* **206**, 528–533 (1979).
[4] L. B. Jaques, *Pharmac. Rev.* **31**, 99–166 (1979).
[5] L. B. Jaques and J. Mahadoo, *Semin. Thromb. Hemos.* **4**, 298–325 (1978).
[6] E. A. Johnson, *Pharmac. Res. Commun.* **14**, 289–319 (1982).
[7] K. S. Dodgson and R. G. Price, *Biochem. J.* **84**, 106–110 (1962).
[8] L. Roden, J. R. Baker, J. A. Cifonelli and M. B. Mathews, *Meth. Enzym.* **28**, 73–140 (1972).
[9] B. Casu and U. Gennaro, *Carbohydr. Res.* **39**, 168–176 (1975).
[10] E. V. Chandrasekaran and J. N. BeMiller, *Methods Carbohydr. Chem.* **8**, 89–95 (1980).
[11] L. Ayotte, E. Mushayakarara and A. S. Perlin, *Carbohydr. Res.* **87**, 297–301 (1980).
[12] J. Dohnal and K. Vytřas, *Farm. Obzor* **54**, 405–411 (1985).
[13] K. Vytřas, *Ion-Sel. Electrode Rev.* **7**, 77–164 (1985).
[14] K. Vytřas, M. Dajková and M. Remeš, *Cesk. Farm.* **30**, 61–67 (1981).
[15] K. Vytřas, *Microchim. Acta* **III**, 139–148 (1984).
[16] K. Vytřas, V. Říha and S. Kotrlý, *Sb. Ved. Pr., Vys. Sk. Chemickotechn., Pardubice* **35**, 41–56 (1976).
[17] K. Vytřas, *Am. Lab.* **11**, 93–105 (1979); *Int. Lab.* **9**, 35–46 (1979).
[18] K. Eckschlager, I. Horsák and Z. Kodejš, *Evaluation of Analytical Results and Methods* (in Czech), pp. 85–92. SNTL, Prague (1980).

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