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Spectrophotometric and Voltammetric Studies on the Interaction of Heparin with Crystal Violet and its Analytical Application

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ABSTRACT

The interactions of heparin with crystal violet were studied by absorption spectrophotometry and voltammetry in pH 3.0 Britton-Robinson (B-R) buffer solution. Heparin, which is negatively charged, can easily bind to the positively charged crystal violet to form a supramolecular ion-association complex. Owing to the formation of the new complex, the maximum absorption wavelength of crystal violet at 592 nm decreased and two new absorption peaks appeared at 510 nm and 363 nm after the addition of heparin. The oxidation peak current of crystal violet on glassy carbon electrode (GCE) at + 0.84 V (*vs.* SCE) also changed correspondingly without change of the peak potential, which also indicated that the binding reaction had taken place. Under the selected conditions a new spectrophotometric analytical method was established for heparin with the linear range between 0.10–4.0 mg L^{-1} with a linear regression equation as $\Delta A = -0.002 + 0.227C$ (mg L^{-1}), ($n = 8$, $\gamma = 0.997$). The relative standard deviation for eleven parallel determinations of 0.40 mg L^{-1} heparin was 1.69% and the detection limit (3σ) was 0.09 mg L^{-1}. This new method was further applied to the determination of heparin sodium injection samples with satisfactory results.

KEYWORDS

Heparin, crystal violet, spectrophotometry, voltammetry, ion-association complex.

1. Introduction

Heparin is an important biochemical medicine. It has many biological functions such as anticoagulant, antithrombotic, antilipemic and antiatherosclerosis activities.[1] Research has shown that it has potential therapeutic functions. For example, heparin can interact with many biological proteins such as proinflammatory chemokines, growth factors, extracellular matrix proteins and platelets.[2,3] According to its structure, heparin is a polysaccharide of the glycosaminoglycan (GAG) family, which has linear and strongly negatively charged sugar chains of repeating disaccharide units of duronic/glucuronic acid and glucosamine residues. The molecular mass of commercial heparin products ranges from 3000 to 30 000 Da with an average of 12 000 Da.

Many different methods have been proposed for the assay of GAGs[4–7] and heparin such as a biological method,[8] UV-Vis spectrophotometry,[9] light-scattering technique,[10] HPLC and electrophoresis.[11,12] Among them the most widely used method is UV-Vis spectrophotometry based on the metachromatic interactions with cationic dyes such as toluidine blue, methylene blue and azure A. Jiao *et al.* have applied methylene blue and azure A as spectrophotometric probes for the detection of heparin and investigated the binding mechanism.[13] Recently Liu *et al.* reported the interaction of heparin with some basic diphenylnaphthylmethane dyes such as victoria blue 4R, victoria blue B, night blue and others by resonance Rayleigh scattering technique or fading spectrophotometry.[10,14,15] Owing to the presence of three *O*-sulphate groups, two *N*-sulphate groups and two carboxyl groups per tetrasaccharide unit of heparin, it has a high anionic charge density. Therefore it can interact with cationic dyes to form a heparin-dye supramolecular ion-association complex.

In this paper crystal violet was selected as the bioprobe to investigate the binding reaction with heparin and further used for the detection of micro-amounts of heparin. It is a kind of basic triphenylmethane dye commonly used in the analytical laboratory and can easily be obtained at low costs. Crystal violet which is a cationic dye (molecular structure shown in Fig. 1) has been used for the detection of metal ions. Zhang *et al.* have applied crystal violet for the detection of nucleic acids by resonance light-scattering technique based on its interaction with DNA.[16] In pH 3.0 Britton-Robinson (B-R) buffer solutions, the *O*-sulphate and *N*-sulphate groups in the structures of heparin completely dissociate. The heparin molecule is negatively charged and can easily interact with cationic dyes. Based on this principle, the interaction of heparin with crystal violet was investigated by spectrophotometry and electrochemical methods and further applied to the detection of heparin in heparin sodium injection samples.

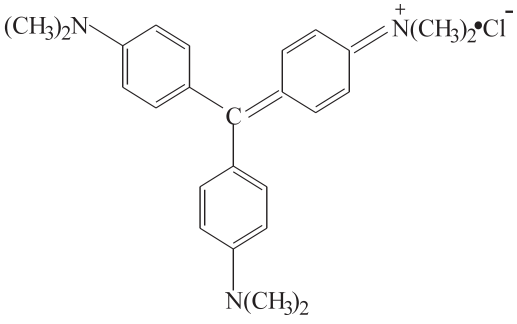

Figure 1 Molecular structure of crystal violet.

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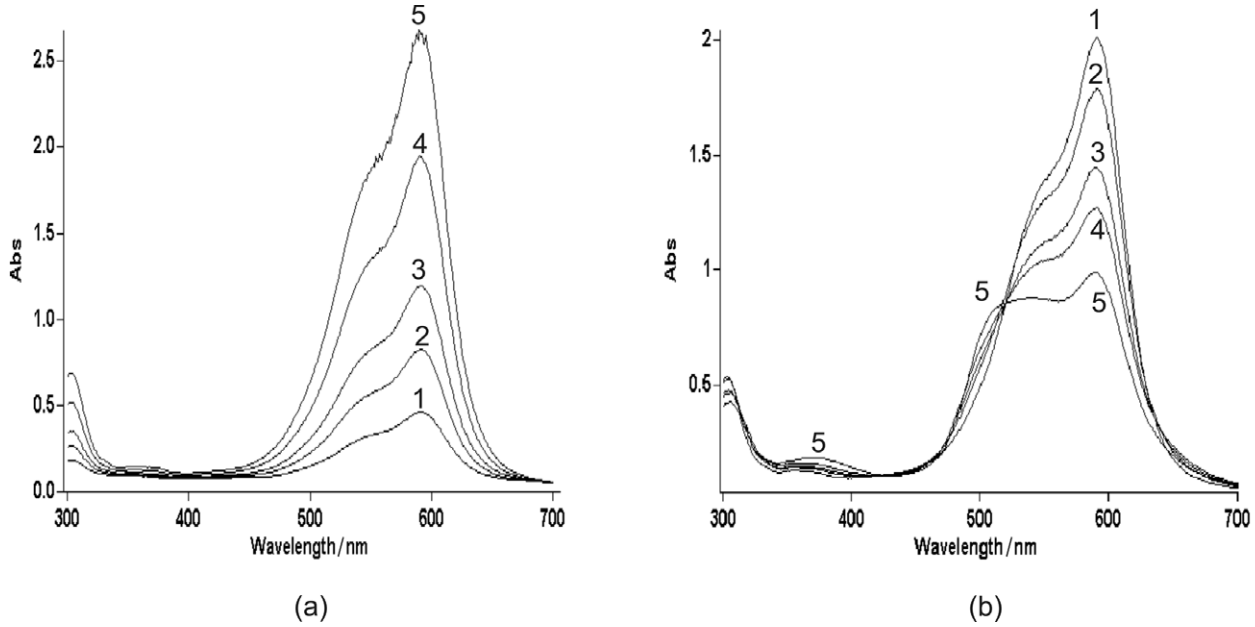

Figure 2 UV-Vis absorption spectra of different concentrations of crystal violet (**a**) and the interaction of crystal violet with different amounts of heparin (**b**) in pH 3.0 B-R buffer solution. (a) 1 → 5: 0.5, 1.0, 1.5, 2.5, 3.5 × 10^{-5} mol L^{-1} crystal violet; (b) 1: 2.5 × 10^{-5} mol L^{-1} crystal violet; 2 → 5: 1 + 1.0, 2.0, 3.0, 10.0 mg L^{-1} heparin.

2. Experimental

2.1. Apparatus

A Cary 50 probe spectrophotometer (Varian, Australia) and a model 752 UV-Vis spectrophotometer (Shanghai No. 3 Analytical Instrument Factory, China) were used for recording absorption spectra or measuring the absorbance at a fixed wavelength, using a 1-cm pathlength cell. Cyclic voltammetric experiments were carried out using a model CHI 832 electrochemical analyser (Shanghai CH Instrument, China) with a glassy carbon electrode (GCE) (Φ = 3.0 mm) as working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode. pH values were measured with a pHS-25 acidity meter (Shanghai Leici Instrument Factory, China). All experiments were carried out at 25 ± 2 °C.

2.2. Reagents

Heparin (sodium salt, 140 IU mg^{-1}, Shanghai Chemical Reagent Plant) was used as received without further purification. The 1.0 mg mL^{-1} stock solution of heparin (140 IU mL^{-1}) was prepared by directly dissolving 0.1000 g of heparin sodium reagent in doubly distilled water from all-quartz still, diluted to 100 mL and stored at 4 °C. The working solutions were obtained by diluting the stock solution with water. 1.0 × 10^{-3} mol L^{-1} crystal violet (Zhongguo Yuanhang Chemical Reagent Factory) was prepared by dissolving 0.04080 g crystal violet in water and diluting to 100 mL. 0.2 mol L^{-1} Britton-Robinson (B-R) buffer solution was used to control the acidity of the reaction solution, which was prepared by mixing 12.35 g boric acid, 13.55 mL 85 % phosphoric acid and 11.80 mL acetic acid diluted to 1000 mL and adjusted to pH 3.0 by 0.2 mol L^{-1} aqueous solution of sodium hydroxide.

All other reagents were of analytical reagent grade and were used without further purification. Doubly distilled water was used throughout.

2.3. Procedure

To a dry 10 mL colorimetric tube, solutions were added in the following order: 1.5 mL of 0.2 mol L^{-1} B-R (pH 3.0) buffer solution, 2.5 mL 1.0 × 10^{-4} mol L^{-1} crystal violet and an appropriate amount of heparin solution or heparin injection sample solution. The mixtures were diluted to 10 mL with water, mixed homogeneously and allowed to stand for 10 min at 25 °C.

For spectrophotometric detection, the absorbances of the solution were recorded at 592 nm against water. The absorbance (A_0) of the blank sample without heparin was obtained under the same conditions, and thus the difference of absorbance ($\Delta A = A_0 – A$) was obtained for measurement. For electrochemical measurements the cyclic voltammetric curves of crystal violet and its mixture with heparin were recorded on GCE and compared with each other in the potential sweep range of 0.5–1.1 V (*vs.* SCE). The changes of peak current of crystal violet were compared.

The reaction conditions were optimized for the spectrophotometric method. The effect of pH on the absorbance was investigated at pH 1.4–4.5 by using different B-R buffers to control the acidity of the reaction solution. The effect of crystal violet concentration on the absorbance was studied with 5.0 mg L^{-1} heparin solutions using different crystal violet concentrations in the range of 0.5 × 10^{-5}–5.0 × 10^{-5} mol L^{-1}. The reaction time was examined by measurement of the absorbance of crystal violet-heparin reaction solution at 592 nm for about 3 h immediately after mixing. The influences of various substances were studied by premixing interfering substances with 2.0 mg L^{-1} heparin solution using the general spectrophotometric procedure.

3. Results and Discussion

3.1. UV-Vis Absorption Spectra of the Crystal Violet–Heparin Interaction System

The absorption spectra of different concentrations of crystal violet in pH 3.0 B-R buffer solution were scanned in the range of 300–700 nm as shown in Fig. 2(a). It can be seen that the maximum absorbance peak appeared at 592 nm and with the increase of the concentration of crystal violet, the absorbance at 592 nm increased correspondingly.

The UV-Vis absorption spectra of crystal violet and its mixture with heparin in pH 3.0 B-R buffer solutions are shown in Fig. 2(b), which were obtained by keeping the crystal violet

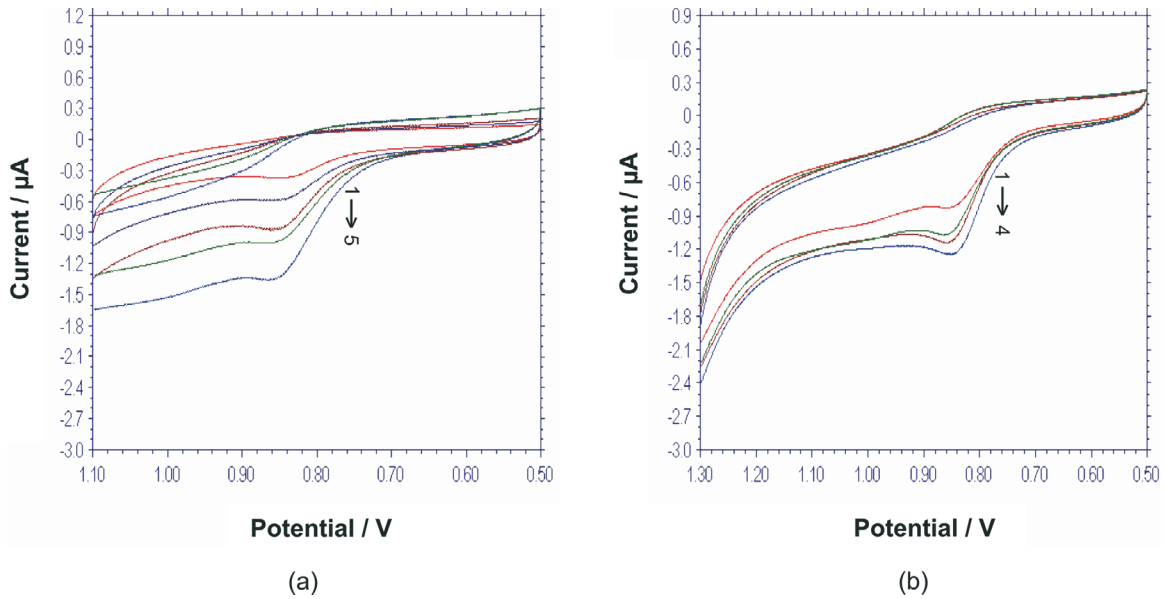

Figure 3 Cyclic voltammograms of different concentration of crystal violet (**a**) and the interaction of crystal violet with different amounts of heparin (**b**) in pH 3.0 B-R buffer solution. (a) 1 → 5: 0.5, 1.0, 1.5, 2.5, 3.5 × 10^{-5} mol L^{-1} crystal violet; (b) 1: 2.5 × 10^{-5} mol L^{-1} crystal violet; 2 → 4: 1 + 1.0, 3.0, 10.0 mg L^{-1} heparin. Scan rate: 50 mV s^{-1}.

concentration and pH value constant and changing the concentration of heparin. In the wavelength range from 300 to 700 nm, heparin showed no absorbance and crystal violet has a maximum absorption at 592 nm (curve 1). On the addition of heparin, the absorption peak of crystal violet at 592 nm decreased while new absorption peaks appeared at 510 nm and 363 nm (curves 2–4), which were attributed to the formation of heparin-crystal violet complex. The absorption peak of the complex formed was apparently different from that of crystal violet because the wavelengths corresponding to both absorbance maxima were different. A well-defined isobestic point is formed at 524 nm. The decrease of absorbance value at 592 nm was proportional to the heparin concentration and can be further applied to the detection of heparin samples.

3.2. Cyclic Voltammograms of the Crystal Violet–Heparin Interaction System

The cyclic voltammetric curves of different concentrations of crystal violet in pH 3.0 B-R buffer solution were also recorded and the results are shown in Fig. 3(a). It can be seen that crystal violet had an oxidative peak at +0.84 V (*vs.* SCE) and did not have any reductive peaks, which indicated that the electrochemical behaviour of crystal violet on GCE was irreversible in pH 3.0 B-R buffer solution. The multiple sweep cyclic voltammetric experiments showed that with the increase of scanning time, the oxidative peak current of crystal violet decreased correspondingly, which was due to the accumulation of the oxidative product of crystal violet on the surface of GCE to form an insulated membrane which interfered with the transfer of electrons. The peak potential of crystal violet shifted negatively with the increase of the pH value of buffer solution, which shows that the electrode reaction involves protons. The peak current of crystal violet increased with the increase of the scanning rate and the plot of the oxidative peak current against the square root of scanning rate was linear in the range from 200 mV s^{-1} to 800 mV s^{-1} with a correlation coefficient of $\gamma = 0.996$, which indicated that the electrode reaction was controlled by diffusion processes.

The cyclic voltammograms of 2.5 × 10^{-5} mol L^{-1} crystal violet solution and its interaction with different amounts of heparin in pH 3.0 B-R buffer solution were recorded and are shown in Fig. 3(b). Crystal violet had an irreversible oxidative peak at +0.84 V (*vs.* SCE) and after the addition of heparin, the oxidative peak current of crystal violet increased without change of the peak potential. No new peak appeared on the cyclic voltammogram of crystal violet-heparin reaction solutions. We therefore conclude that under the selected conditions a supramolecular ion-association complex was formed, which caused the changes of the peak current. The more heparin was added, the more the peak currents changed. The changes of electrochemical responses of crystal violet in the presence and absence of heparin also demonstrate the interaction of crystal violet with heparin to form a crystal violet-heparin complex.

3.3. Optimization of Spectrophotometry Parameters

3.3.1. *Effects of pH and Buffers*

The difference of absorbance values (ΔA) was greatly affected by the pH of the buffer solution and the influence of pH was investigated in the pH range of 1.4 to 4.5. As shown in Fig. 4, ΔA reached its maximum at pH 3.0. Therefore this pH was chosen for the assay. At this pH, crystal violet was positively charged

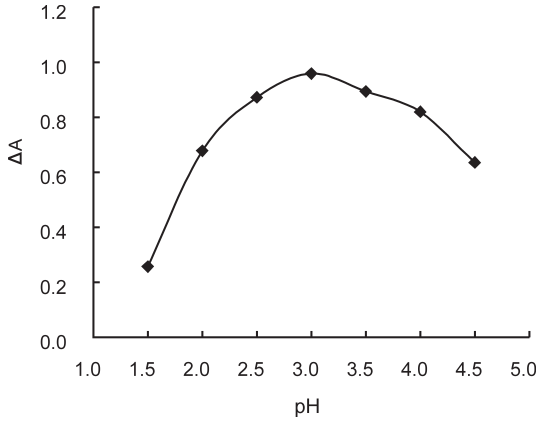

Figure 4 Influence of pH on the crystal violet-heparin interaction 1.5 mL different pH of B-R buffer +2.0 × 10^{-5} mol L^{-1} crystal violet + 20.0 mg L^{-1} heparin.

and heparin negatively charged, so they can strongly interact with each other to form a supramolecular complex. The nature of the buffer also affected the interaction and different kinds of buffers such as B-R, HOAc-NaOAc, NH_3-NH_4Cl and others were tested. In B-R buffer solution the response was maximal. For this reason B-R buffer was used in this paper. The volume range of 0.5–4.0 mL of a 0.2 mol L^{-1} B-R buffer was investigated and the addition of 1.5 mL was selected for the following procedures.

3.3.2. *Effect of Crystal Violet Concentration*

The concentration of crystal violet affected the difference of absorbance values. ΔA increased with the increase of the concentration of crystal violet in the range of 0.5×10^{-5}–2.5×10^{-5} mol L^{-1} and remained constant with further increase of concentration, which indicated that the binding reaction had reached its equilibrium. Therefore a concentration of 2.5×10^{-5} mol L^{-1} of crystal violet was recommended for use in this paper.

3.3.3. *Reaction Time and Temperature*

The stability of the reaction solutions was investigated. After mixing heparin with crystal violet, the binding reaction occurred rapidly. The absorbance differences reached a maximum within 20 min and remained unchanged for at least 2 h. Therefore, this system gave enough time for routine measurements.

The effect of the reaction temperature on the interaction was tested at 15 °C, 25 °C, 30 °C and 37 °C, respectively, and found to be insignificant. A reaction temperature of 25 °C was therefore used throughout.

3.4. Effect of Coexisting Substances

Some substances such as metal ions, amino acids, glucose etc. may exist in biological samples. The possible interferences on the determination were studied by mixing them with 2.0 mg L^{-1} heparin solution. The results are listed in Table 1 and it can be seen that few substances interfere with this assay and good selectivity can be obtained. This method can therefore be applied for the direct determination of heparin in heparin sodium injection samples.

The influences of some surfactants such as sodium dodecyl sulphate (SDS), Tween-20, OP-10, β-cyclodextrin (β-CD), cetyltrimethylammonium bromide (CTAB) and others on the binding reaction were also investigated. The addition of non-ionic surfactants such as Tween-20, OP-10 and β-CD seldom affected the binding reaction, but the addition of SDS and CTAB can greatly influence the value of ΔA, which may be explained by the dissociation of the ionic surfactants in solution and the reaction with heparin to form a surfactant-heparin complex.

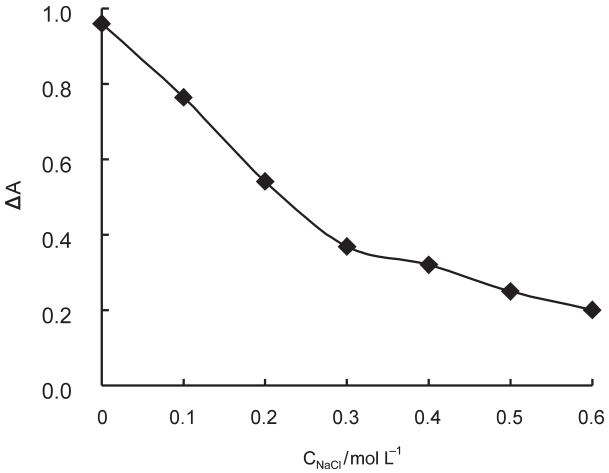

Figure 5 Influence of ionic strength on the difference of absorbance value 2.0×10^{-5} mol L^{-1} crystal violet and 2.0 mg L^{-1} heparin in pH 3.0 B-R buffer solution.

The effect of NaCl concentration in the range of 0.1–0.6 mol L^{-1} on this assay was also examined. The results demonstrate the significant influence of NaCl on the heparin–crystal violet interaction (shown in Fig. 5). The ΔA decreased with increasing salt concentration, which indicated that the interaction of crystal violet with heparin was mainly caused by electrostatic attraction. The increase of Na^+ concentration caused an increasing electrostatic shielding effect and thereby decreased the formation of heparin-crystal violet complex resulting in the decrease of the absorbance signal.

3.5. Calibration Curve for Heparin

Under optimal conditions, a calibration curve was obtained between ΔA at 592 nm and the concentration of heparin with 2.5×10^{-5} mol L^{-1} crystal violet. Good linearity was obtained in the concentration range of 0.10–4.0 mg L^{-1} with the linear regression equation $\Delta A = -0.002 + 0.227C$ (mg L^{-1}), ($n = 8, \gamma = 0.997$). The relative standard deviation for eleven parallel determination of 0.40 mg L^{-1} heparin was 1.69% and the detection limit (3σ) was 0.09 mg L^{-1}. The sensitivity of the crystal violet spectrophotometric method for heparin determination is sufficient for routine detection.

Table 1 Influence of coexisting substances on the determination of 2.0 mg L^{-1} heparin.

Coexising substances	Concentration /mol L^{-1}	Relative error /%	Coexisting substances	Concentration /mg L^{-1}	Relative error /%
Ba^{2+}	1.0×10^{-5}	3.45	L-Leucine	10.0	3.05
Fe^{3+}	1.0×10^{-5}	–1.15	L-Lysine	10.0	3.95
Cd^{2+}	1.0×10^{-5}	–1.15	L-Valine	10.0	–0.72
Ni^{2+}	1.0×10^{-5}	–0.99	L-Glutamic acid	10.0	1.80
Hg^{2+}	1.0×10^{-5}	1.64	L-Arginine	10.0	1.97
Zn^{2+}	1.0×10^{-5}	1.48	L-Cysteine	10.0	2.49
Co^{2+}	1.0×10^{-5}	0.66	L-Tryptophan	10.0	5.18
Ca^{2+}	1.0×10^{-5}	–1.82	L-Serine	10.0	–1.71
OP-10	1.0×10^{-5}	–4.52	Citric acid	5.0	4.31
Tween 20	1.0×10^{-5}	5.17	Glucose	5.0	4.94
β-CD	1.0×10^{-5}	4.93	Alcohol	5.0	–3.98
SDS	1.0×10^{-5}	32.16	Urea	5.0	4.20
CTAB	1.0×10^{-5}	20.74	HSA	1.0	3.68

β-CD, β-cyclodextrin; SDS, sodium dodecyl sulphate; CTAB, cetyltrimethylammonium bromide; HSA, human serum albumin.

Table 2 Determination of heparin in heparin sodium injection sample and recovery.

Sample no.	Specified /mg L^{-1}	Detected /mg L^{-1}	RSD /%	Added /mg L^{-1}	Found /mg L^{-1}	Recovery /%
020911-1	0.893	0.888	1.10	2.00	2.070	103.5
20031003	0.893	0.875	3.43	2.00	1.954	97.7

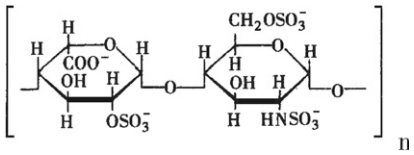

Figure 6 Molecular structure of heparin.

3.6. Sample Determination and Recovery Test

The heparin sodium injection samples were purchased from Anhui Xinli Pharmaceutical Company of China (sample no. 020911-1) and Tianjing Biochemical Pharmaceutical Factory of China (sample no. 20031003) with the specified amount of heparin as 6250 IU mL^{-1}. The procedure for sample assay was as follows: a 1.0 mL portion of heparin sodium injection was pipetted into a 100 mL calibrated flask and was diluted to the mark with water. A 1.0 mL amount of this solution was further pipetted into a 100-mL volumetric flask and then diluted to the mark with water. A 2.0 mL portion of this solution was used in the general procedure with spectrophotometric detection. The results of determinations and recovery tests are listed in Table 2. It can be seen that this new spectrophotometric method was practical and reliable.

3.7. Discussion of the Interaction Mechanism

Heparin is a polysaccharide of the glycosaminoglycan (GAG) family consisting of repeating sulphated and/or carboxylated disaccharide units in its molecular structure, which has three *O*-sulphate, two *N*-sulphate and two carboxyl groups per tetrasaccharide unit. The sulphate groups completely dissociate even below pH 3.0. The carboxyl group is weakly acidic and the pK_a of *D*-glucuronic acid in heparin is 3.6. The carboxyl groups gradually dissociate with an increase of pH and with complete dissociation above pH 5.0.[17] Owing to the presence of sulphate and carboxyl groups and under the selected conditions, heparin exists in a polyvalent anionic state with the anionic groups being mainly $-OSO_3^-$, $-NHSO_3^-$ and $-COO^-$ (as shown in Fig. 6) and with the tetrasaccharide groups linked *via* oxo bridges. In pH 3.0 B-R buffer solution, the *O*-sulphate and *N*-sulphate groups completely dissociate and the whole heparin molecule is negatively charged, while the cationic dye crystal violet species is positively charged. The two species bind *via* electrostatic forces and hydrophobic interaction to form a supramolecular complex, which results in the changes of the spectrophotometric and voltammetric responses of the reaction system. The change in absorbance of the heparin-crystal violet reaction system at 592 nm can be further used to establish a new spectrophotometric method for the detection of micro amounts of heparin and applied to heparin injection samples with satisfactory results.

Acknowledgements

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References

1 S.L Ji and T.M. Zhang., *Chin. J. Biochem. Pharm.,* 1996, **17**, 216–219.
2 S. Ecke, M. Geiger and B.R. Binder, *Eur. J. Biochem.,* 1997, **248**, 475–480.
3 A. Bobik and J.H. Campbell, *Pharmacol. Rev.,* 1993, **45**, 1–42.
4 Q. He and Q.C. Jiao, eds., *Biomedicine Assay*, Chemical Industrial Press, Beijing, P.R. China, 2003, p. 258–259.
5 Q.C. Jiao and Q. Liu, *Anal. Lett.,* 1998, **31**, 1311–1323.
6 N. Volpi, *Anal. Biochem.,* 1996, **240**, 114–118.
7 S. Radoff and I. Danishefsky, *Anal. Biochem.,* 1982, **120**, 373–378.
8 *Pharmacopoeia of the People's Republic of China,* People Health Press, Beijing, P.R.China, 1985, appendix 98.
9 I. Nemcova, P. Rychlorsky, M. Havelcova and M. Brabcova, *Anal. Chim. Acta,* 1999, **401**, 223–228.
10 S.P. Liu, H.Q. Luo and N.B. Li, *Anal. Chem.,* 2001, **73**, 3907–3914.
11 P. Mao, X.L. Huang and C.Z. Li, *Chin. J. Med. Lab. Sci.* 1995, **18**, 358–360.
12 K. Takagaki , H. Munakata, T. Nakamura and M. Endo, *Bunseki Kagaku*, 1995, **44**, 789–790.
13 Q.C. Jiao and Q. Liu, *Spectrochim. Acta A*, 1999, **55**, 1667–1673.
14 S.P. Liu, H. Xu and H.Q. Luo, *Chin. J. Anal. Chem.,* 2002, **30**, 712–715.
15 H. Xu, S.P. Liu and H.Q. Luo, *Chem. J. Chin. Unvi.,* 2002, **23**, 216–218.
16 W.J. Zheng, H.P. Xu and S.Q. Wu, *Analyst,* 2001, **126**, 513–517.
17 T. Katayama, E.I. Takai and R. Kariyama, *Anal. Biochem.,* 1978, **88**, 382–387.