J. Pharm. Pharmacol. 1989, 41: 512–516
Received April 17, 1989

The Effects of Autoclaving on the Physical Properties and Biological Activity of Parenteral Heparin Preparations

A. R. MENZIES, D. M. BENOLIEL* AND H. E. EDWARDS†

*Research Division, The North East Wales Institute, Deeside, Clwyd, CH5 4BR UK, *Linden House, Ewelme, Oxford, OX9 6HQ and †Coleg Pencraig, Llangefni, Ynys Môn, Gwynedd LL77, 7HY UK*

Abstract—Heparin ampoules have been autoclaved at 115°C, 121°C, 126°C and 130°C for time intervals up to 50 min and the biological potency and physicochemical integrity of the preparations assessed. The anticoagulant activity, determined with the APTT assay, did not change significantly for the autoclaved samples but a decrease was observed using the BP assay. Autoclaving was also associated with a depolymerization process, as confirmed by high performance liquid chromatography, and the formation of 'species' with an absorption in the UV spectrum. However, autoclaving had no detrimental effect on the integrity of the anionic sites on heparin. It is proposed that Maillard-type reactions are responsible for these observations and the results indicate that autoclaving could be used to sterilize parenteral heparin solutions.

Heparin belongs to a group of highly charged anionic polysaccharides called glycosaminoglycans (GAGS) and occurs in the mast cells of most mammalian species. In contrast with other GAGS, heparin is an effective anticoagulant and has been used clinically in the treatment of venous thromboembolism for the past 40–50 years. Latterly advances have been made to explain the structure-function relationship of its mode of action. Pharmaceutically low-dose heparin regimes have now been recognized and currently the advantages of low molecular weight heparins are being evaluated.

The effect of heat on heparin has not been the subject of intensive investigation. Swarte & Huizinga (1964) have shown that heparin solutions could be sterilized by heating with steam at 100°C for 1 h. When the pH of the solution was approximately 7·0, no decomposition could be demonstrated by means of titration with protamine sulphate, toluidine blue (staining), clotting time determination, paper chromatography and UV spectrophotometry. Concurrently, Pritchard (1964) showed that heparin solutions were stable when autoclaved and subsequently for 15 years on storage at 4°C. More recently, Anderson & Harthill (1982) have examined the 'dextrose effect' when heparin is autoclaved. Thomas et al (1984) showed that the 4th (WHO) International Standard for Heparin (freeze-dried) could be stored at elevated temperatures (45°C and 56°C) for over 15 months without any significant loss in anticoagulant activity. When heated to 120°C for 11 days, however, some degradation occurred.

The present study was designed to assess the potential and relevance of autoclaving to the commercial production of sterile heparin solutions. This group (Edwards et al 1985) has already assessed the effect of radiation on dry solid state heparin and has demonstrated that sterilizing doses of 20kGy can be used with only a minimal (~2%) loss in anticoagulant activity. Heparin, however, is not supplied to clinicians in the solid state and when aqueous solutions of heparin are irradiated to doses of 9·2kGy, a 10% decrease in anticoagulant activity occurs (Chawla & Hayward 1980) whilst at higher irradiation (25kGy), greater (22%) losses occur (ABPI 1960). In this study, autoclaving as an 'end of processing' procedure for heparin solutions has been studied at cycles in excess of the MRC recommended sterilization regimes, in order to assess the changes that take place.

Correspondence to: H. E. Edwards, Coleg Pencraig, Llangefni, Ynys Môn, Gwynedd, LL77 7HY, UK.

Materials and Methods

Heparin ampoules (25,000 units in 1 mL) were supplied by Paines and Byrne Ltd. (Batch No. 691006). The ampoules were steam autoclaved (Astell Hearson Swiftlock 2000 autoclave; Model-AAB002) for different autoclaving times at 115, 121, 126 and 130°C where the heating-up period was found to be 34, 37, 40 and 42·25 min, respectively.

The anticoagulant activities of the heparin samples were determined using the British Pharmacopoeia (BP 1980) 1973 method and the 1983 Manchester (National [UK] Reference Laboratory for Anticoagulant Reagents and Control) standardized activated partial thromboplastin time (APTT; Thomson 1980) with the Third International Reference Preparation of Heparin (WHO) being used as standard in both assays. For the BP assay, an aliquot (200 μL) from each ampoule was diluted to 2 units mL^{-1} in distilled water and the non-autoclaved heparin was taken to represent 100%. Bovine blood was obtained from a local abattoir and prevented from clotting by the addition of anhydrous sodium sulphate (7% w/v; 1 volume of anticoagulant to 3 volumes of blood). The blood was subsequently centrifuged (1800 *g* for 2 h at 4°C) to obtain platelet-poor plasma. Thrombokinase extract (thromboplastin) was prepared by the progressive dehydration of freshly collected cow brains (3; freed from all vascular and connective tissues) using acetone and subsequently prepared as described (BP 1980). The BP assay was repeated four times using two concentrations of standard heparin and test heparin. The potency was calculated against the 3rd International Standard (WHO) for heparin and expressed as a percentage of this value with fiducial limits.

For the APTT assay, heparin (50 μL of 0·4 units mL^{-1}) was added to the reaction mixture before the addition of plasma (100 μL). Blood, obtained from six adult male rats

(strain Lister Hooded, 540–600 g) was prevented from clotting by the addition of tri-sodium citrate (0·1 M; 1 volume to every 9 volumes of blood) and centrifuged (1800 *g* for 20 min at 4°C) to obtain platelet-poor plasma. The plasma was pooled and stored at ca −20°C and filtered through glass wool before use. (To remove undissolved plasma proteins as a consequence of freezing (Jackson et al 1968); fresh plasma was always free of particulate matter.) The clotting time for the plasma alone was determined by the addition of distilled water (50 μL), cephalin reagent (100 μL) and resuspended kaolin (100 μL; 0·5 g in 100 mL Owren's buffer, pH 7·35) as activator. All clotting times were recorded in duplicate and expressed to the nearest 0·5 s. Both the standard and test heparins were diluted to 0·4 units mL^{-1} in distilled water. Under these conditions the assay was linear in the range 0·1 to 0·8 units mL^{-1} of heparin in agreement with previous studies (Thomson 1980). The potency was calculated by expressing the clotting time of the test heparin as a percentage of the standard's clotting time (100%).

Molecular weight estimations of heparin were determined by high pressure liquid chromatography (HPLC, Perkin Elmer, USA; Harenberg & De Vries 1983) using a column (Ultrapac TSK G3000 SW, 600 × 7·5 mm i.d., particle size 10±2 μm; LKB No. 2135-360), a detector (Differential Refractometer R401, Waters) and a two-channel recorder. A pre-column (Ultrapac TSK GSWP, 75 × 7·5 mm i.d., 10 μm LKB) was connected between the pump and the main column. The mobile phase was 0·1 M phosphate buffer (pH 7·0; filtered (0·45 μm) and degassed before use). The column was calibrated using heparin fractions of defined molecular weights (provided by Dr Grant Barlow, Abbott Laboratories, USA) and with low polydispersity values. The degassed and filtered 0·1 M phosphate buffer was used to prepare solutions of heparin (5000 units mL^{-1}) which were filtered (0·45 μm) before injecting 60 μL onto the column. The molecular weight parameters were calculated using a Perkin-Elmer Basic program (GPC 2L).

Heparin spectra were measured using a Hewlett Packard 8451A Diode array spectrophotometer. The cell path length was 10 mm and the heparin concentration 500 units mL^{-1}. The pH of the heparins (500 units mL^{-1}) was measured using a Kent E.I.L. 7045/46 pH meter which had previously been standardized at pH 7·0 and pH 4·0.

The chemical analysis of pre- and post-autoclaved heparin samples was determined using a slightly modified version to that outlined by Lam et al (1976) of the original Azure A dye assay of Jacques et al (1949). The heparin sample (50 μL of 4 units mL^{-1}) was added to 1 mL of Azure A dye solution (0·01 mg mL^{-1} in distilled water). The resultant mixture was thoroughly dispersed by vortex mixing and the absorbance measured at 520 nm. The assay was linear in the range 0·5 units mL^{-1} to 8·0 units mL^{-1} of heparin.

Results

The heparin solution (preservative-free) used in this study was supplied in ampoules (25,000 units mL^{-1}) and the activity of the raw material used in its preparation determined by the BP assay to be 154·1 units mg^{-1}. Following autoclaving cycles at 115°C and 121°C for different times, no significant decrease in BP activity was detected (Fig. 1).

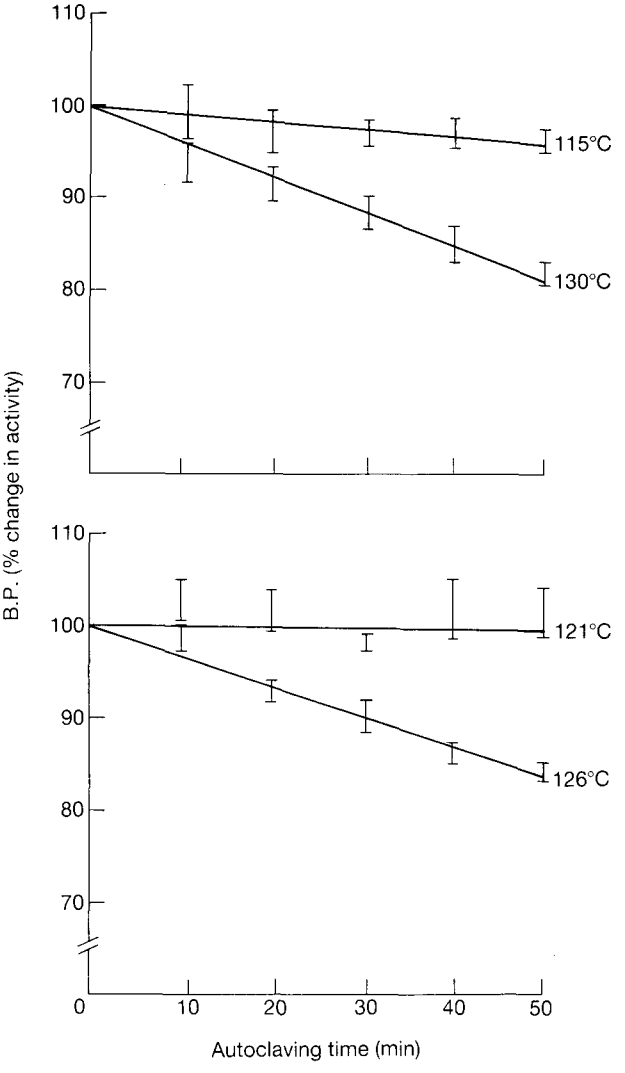

FIG. 1. Changes in the BP anticoagulant activity (with fiducial limits) of heparin following different autoclaving cycles.

However, at the higher temperatures of 126°C and 130°C a greater loss in activity was noted. The decrease was observed immediately at each temperature and a 50 min autoclaving cycle at 130°C resulted in a maximum decrease of 18% in activity.

A different pattern emerged using the APTT assay. Table 1 shows that the anticoagulant activity remains reasonably constant during all the cycles and no significant decrease occurs even at 130°C.

The non-autoclaved heparin could be described as pale yellow in appearance, but following autoclaving cycles at all temperatures, a more pronounced colour was obtained. This varied from the golden yellow colour following autoclaving at 115°C for 50 min, to the dark orange/brown colour at 130°C for the corresponding time. Heparin has an absorption maximum (λ max) at ca 210 nm but when the spectra of autoclaved heparins were measured using non-autoclaved heparin as reference a different pattern emerged (Fig. 2). The maximum absorption occurred at 230 nm and at all temperatures a progressive increase in absorption was noted with

Table 1. The effect of autoclaving on the anticoagulant activity (APTT), chemical activity (Azure A dye) and molecular weight distribution of heparin solutions.

Temperature (°C)	Time (mins)	Anticoagulant[1] Activity (APTT)	Chemical Activity[1]	Molecular weight parameters		
				Mw[2]	Mn[3]	Q[4]
Non-autoclaved	—	100·00%	100·00%	12 050	9680	1·24
115	10	100·00%	98·70%	12 030	9550	1·26
115	20	98·88%	98·90%	11 910	9330	1·28
115	30	101·68%	99·50%	11 900	9590	1·24
115	40	99·44%	97·80%	11 590	9250	1·25
115	50	101·12%	98·90%	11 640	9220	1·26
121	10	104·10%	102·50%	11 670	9210	1·27
121	20	103·28%	102·50%	11 340	8900	1·27
121	30	108·20%	101·70%	11 330	9010	1·26
121	40	103·28%	100·40%	11 140	8860	1·26
121	50	104·10%	103·90%	11 060	8800	1·26
126	10	99·24%	98·40%	11 900	9640	1·23
126	20	96·27%	98·50%	11 450	9110	1·26
126	30	97·73%	98·00%	11 050	8750	1·26
126	40	98·48%	98·48%	10 780	8530	1·26
126	50	100·76%	101·10%	10 770	8550	1·26
130	10	103·74%	101·80%	11 250	8840	1·27
130	20	100·00%	105·60%	10 900	8660	1·26
130	30	103·74%	100·10%	10 940	8790	1·24
130	40	106·54%	101·50%	10 600	8400	1·26
130	50	104·67%	102·30%	10 290	8300	1·24

1. The anticoagulant (and chemical) activities are expressed as a percentage of the non-autoclaved sample.
2. Weight average molecular weight.
3. Number average molecular weight.
4. Polydispersity.

time. Increasing the autoclaving temperature also increased the absorption of the 230 nm peak. Fig. 2 shows the spectra obtained at 115°C and 130°C and similar spectra-profiles were obtained at 121°C and 126°C. At the higher temperatures, a shoulder was detected from 250–300 nm and the 130°C cycle gave values in excess of 0·15 absorbance units at the 30, 40 and 50 min cycles.

The pH of the heparin solutions (500 units mL^{-1}) was recorded after autoclaving. Non-autoclaved heparin had a pH of 6·84 and although a slight decrease was observed for

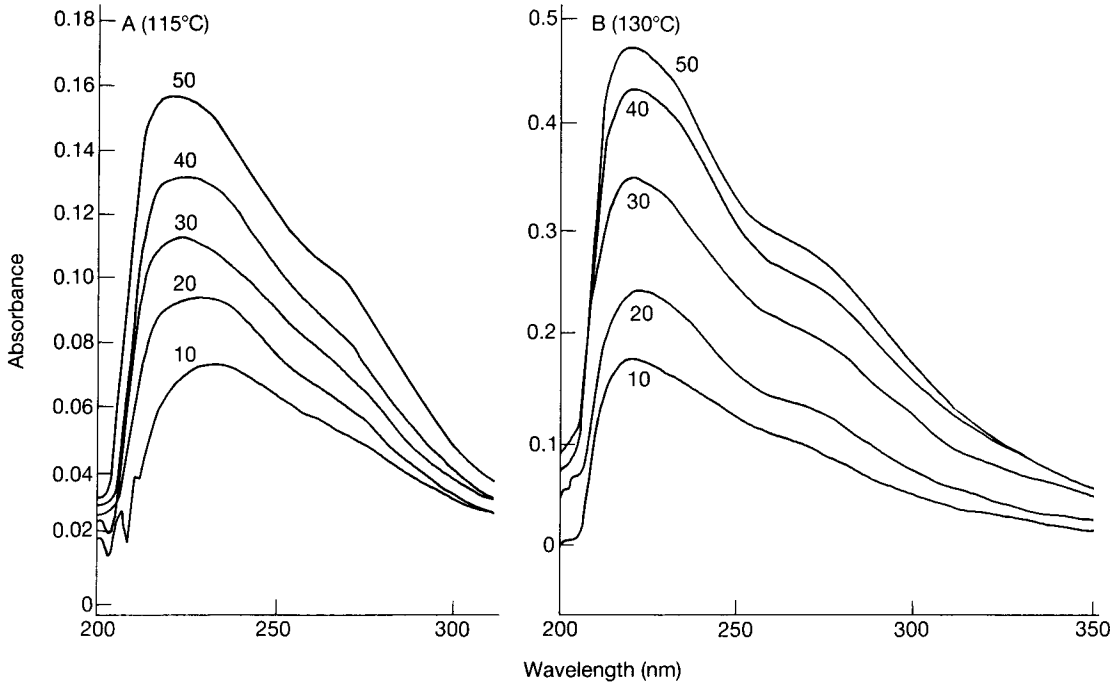

FIG. 2. UV spectra of heparin (500 units mL^{-1}) after autoclaving cycles at (a) 115°C and (b) 130°C for different times (min). Reference was non-autoclaved heparin (500 units mL^{-1}).

all the autoclaved samples the lowest pH recorded was 6·50 for the sample autoclaved at 130°C for 50 min.

When the samples were assayed chemically, the Azure A dye assay showed that no chemical changes had occurred following autoclaving (Table 1). No apparent loss in chemical activity was detected confirming that the anionic groups (iduronic and glucuronic carboxyl, *N*-sulphate and 2-O and 6-O sulphate) on the heparin molecule are not destroyed and can still bind to the dye's amino groups thereby producing comparable metachromasia to that of non-autoclaved heparin. The chemical integrity of the heparin solutions after heat treatment was also confirmed using the carbazole reaction (Bitter & Muir 1962) for hexuronic acid composition (Menzies; unpublished data).

Table 1 shows the changes in the molecular weight parameters and polydispersity for the post-autoclaved heparin samples. The starting material had a weight average molecular weight (M_w) of 12050 Daltons and autoclaving produced a progressive decrease in M_w with increasing temperature. At the 130°C/50 min regime a 14·6% decrease in M_w was noted. This overall trend of limited depolymerization was also observed for the number (M_n) average molecular weight and, as a consequence, no significant change in polydispersity (M_w/M_n) was detected.

Autoclaving produced a progressive shift in the HPLC chromatogram (Fig. 3) to the right indicating the reduction in molecular weight on heating. The net effect of this degradation by heat was attributed to both a reduction in the number of high molecular weight molecules (> 18000 Daltons) and an increase in the number of low molecular weight (< 6000 Daltons) molecules as shown in Table 2. The shape of the chromatogram itself was conserved and hence pre- and post-autoclaving Q values remained constant.

Discussion

This work was initiated to evaluate the possibility of using autoclaving as an 'end of process' sterilization procedure in the commercial manufacture of heparin. Although commercial heparin solutions are currently sterilized by filtration, autoclaving the heparin ampoules would increase the users' confidence in the end product. Several properties of a pharmaceutical preparation have to be considered and assessed in deciding the applicability of a particular sterilizing technique. In the present study the techniques and assays used were selected to provide relevant and comprehensive data concerning the effects of heat on heparin. For example, both a pharmaceutically important assay (BP) and a widely used clinical assay (APTT), were chosen to measure anticoagulant activity. In addition changes in appearance of the solution following autoclaving were recorded spectrophotometrically; the physical state monitored by high performance liquid chromatography and the chemical integrity using the Azure A dye assay. Commercial heparin with an anticoagulant activity of 154·1 units mg^{-1} and at a concentration of 25000 units mL^{-1} was used in the present work. However, other heparins at various concentrations will have to be tested and compared to fully evaluate the effects of heat. The autoclaving cycles used were selected to include a range that would be relevant to the MRC recommended autoclaving cycles. However, it is noted that the f_o factor should also be considered during a cycle to account for the influence of heating up and cooling down times. In addition, the heparin used in this study was free from preservative so that changes after autoclaving could be explained in terms of heparin-heat interactions without the added complication of preservative effects. Indeed, the relevance of previous work (ABPI 1960; Pritchard 1964), in which 0·15% chlorocresol was added as preservative, has also to be considered.

In this study the two biological assays differ in their results. The BP gives a maximum decrease of 18% in activity at 130°C/50 min cycles whilst no change was seen with the APTT assay. The use of filtered rat plasma is unlikely to be the reason for the differences between the BP and APTT assays since in latter APTT assays a freeze dried human plasma (Sigma Chem. Co. Ltd., Dorset, UK) was used by this group and the same trend shown. Although differences between the two assays have previously been observed, at the MRC sterility levels (126°C for 10 min or 121°C for 15 min or 115°C for 30 min), loss of activity would be less than 5%. The

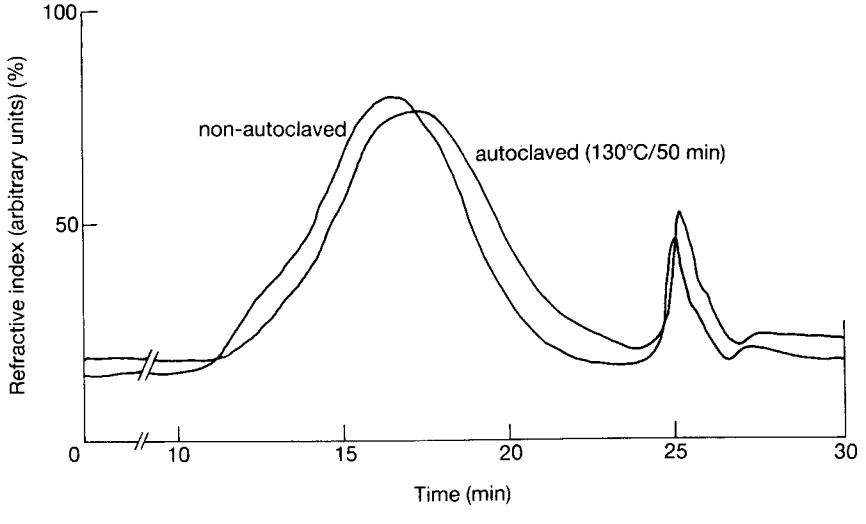

FIG. 3. HPLC chromatograms of non-autoclaved and autoclaved (130°C/50 min) heparin. Mobile phase was 0·1 M phosphate buffer (pH 7·0) and detection by refractive index.

Table 2. Changes in the percentage cumulative weight average and number average molecular weight parameters following autoclaving.

	Molecular weight range			
	Below 6000 % Cumulative		Above 18 000 % Cumulative	
Heparin sample	M_w	M_n	M_w	M_n
Non-autoclaved	4·08%	20·73%	29·71%	6·95%
115°C/50 min	3·70%	16·03%	24·38%	5·81%
121°C/50 min	6·17%	27·87%	24·14%	4·87%
126°C/50 min	6·96%	30·45%	22·43%	4·35%
130°C/50 min	7·71%	31·29%	17·46%	3·32%

difference however between the tests following autoclaving is most likely to be due to the interaction of heparin with the serine proteases of the extrinsic and intrinsic coagulation pathways. The BP assay reflects the extrinsic path primarily and it would appear that autoclaving impairs this interaction.

The HPLC results indicate that some degradation (or depolymerization) is associated with heating of heparin and the decrease in all three molecular weight parameters indicate that glycosidic bond scission is likely. This partial depolymerization would have some effect on the biological activity of heparin since anticoagulant activity measured by global assays is related to the molecular weight. Only the BP assay appears to be susceptible to this 'depolymerization effect' at the high autoclaving temperatures used in this study. The chemical assay using Azure A dye should not be impaired by the depolymerization since it is the integrity and position of the anionic groups (carboxylate and sulphate groups) that is responsible for the metachromasia. A similar result has been reported (ABPI 1960) for heparin samples irradiated and assayed using the toluidine blue test. It can be concluded from these results that autoclaving does not significantly alter the anionic sites.

The UV spectra of autoclaved heparins show that unique species are formed which have an absorption into the visible range. It is proposed that Maillard-type reactions could account for these species whereby aldehydes, ketones and reducing sugars react with amines, amino acids, peptides and proteins. The initial reaction in these reactions is condensation between the carbonyl group of the reducing sugar and amino group. Amino groups are known to remain on the carbohydrate backbone of heparin following *N*-deacetylation and *N*-desulphation but also exist on terminal amino acid residues (Johnson et al 1983; Arai et al 1986). The condensation product could subsequently lose water, become a Schiff base and then undergo cyclization reactions. In heparin, the location of the amino groups necessary for these reactions could impair the reaction rate and different mechanisms and pathways could produce a range of intermediates that could give rise to the 'yellow' colour and UV absorption observed in this study. Such reaction mechanisms require further investigation.

This study has shown that parenteral heparin solutions can be satisfactorily sterilized by steam autoclaving. If standard autoclaving regimes are followed then losses in biological activity and chemical degradation are minimal. However, other factors have also to be considered; these include concentration effects, batch to batch response and an assessment of the stability of post-autoclaved heparin solutions under defined storage conditions.

Acknowledgements

The authors are grateful to the World Health Organisation for the supply of reference heparin and to the National (UK) Reference Laboratory for Anticoagulant Reagents and Control for cephalin reagent. The technical assistance of Miss Clare Bailey is also acknowledged.

References

Anderson, W., Harthill, J. E. (1982) The anticoagulant activity of heparins in dextrose solutions. J. Pharm. Pharmacol. 34: 90–96

Arai, K., Nakamura, Y., Edwards, H. E., Phillips, G. O. (1986) Amino acid end-group in commercial heparin. 1. Determination of the number of amino and hydroxyl end-groups in heparin. J. Polymer Science 24: 911–923

Association of British Pharmaceutical Industry (1960). The use of gamma radiation sources for the sterilisation of pharmaceutical products

Bitter, T., Muir, H. (1962) A modified uronic acid carbazole reaction. Anal. Biochem. 4: 330–334

British Pharmacopoeia (1980) 1973 method, Appendix XIVD A147, London, HMSO

Chawla, A.S., Hayward, C. (1980) Effect of gamma radiation on the molecular weight and the anticoagulant activity of heparin. Pharmacology 20: 224–228

Edwards, H. E., Menzies, A. R., Phillips, G. O., Nakamura, Y., Shimada, M., Takigami, S. (1985) Radiation effects on the biological activity and molecular weight parameters of heparin. Carbohydrate Polymers. 5: 473–478

Harenberg, J., De Vries, J.X. (1983) Characterisation of heparins by high-performance size exclusion liquid chromatography. J. Chromatogr. 261: 287–292

Jackson, C. M., Johnson, T.F., Hanahan, D. J. (1968). Studies of bovine factor X. 1. Large scale purification of the bovine plasma protein possessing factor X activity. Biochemistry 7: 4492–4505

Jacques, L. B., Monkhouse, F. C., Stuart, M. J. (1949). A method for the determination of heparin in blood. J. Physiol. (London) 109: 41–48

Johnson, E. A., Corran, P. H., Paterson, M. (1983) Amino acid content of heparins. J. Chromatogr. 264: 291–296

Lam, L.H., Silbert, J. E., Rosenberg, R. D. (1976) The separation of active and inactive forms of heparin. Biochem. Res. Commun. 69: 570–577

Pritchard, J. (1964) Stability of heparin solutions. J. Pharm. Pharmacol. 16: 487–489

Swarte, H. H., Huizinga, T. (1964) Sterilization of heparin in solution. Pharm. Weekblad. 99: 409–414.

Thomas, D. P., Curtis, A. D., Barrowcliffe, T. W. (1984) A collaborative study designed to establish the 4th International Standard for heparin. Thromb. Haemostas. (Stuttgart) 52: 148–153

Thomson, J. M. (1980) in: Thomson, J. M. (ed.) Blood Coagulation and haemostasis—a practical guide. 2nd edn, Churchill Livingstone, Edinburgh, pp 279–330