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ANALYSIS OF HEPARINS BY SIZE-EXCLUSION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTO-DIODE-ARRAY DETECTION

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SUMMARY

High-performance liquid chromatography (HPLC) of heparins was carried out with on-line photodiode-array detection. Average molecular weights and molecular weight distribution were calculated using computer data acquisition and handling; size-exclusion chromatography was performed using different selective microparticulate columns and narrow molecular weight distribution heparin standards for calibration; heparin peak purity was investigated using several methods for evaluation. Additional UV-absorbing compounds present in heparin preparations were characterized and quantitated by reversed-phase HPLC. These methods are useful for the analysis of the molecular weight distribution and peak purity of heparins and for the determination of additional drugs, additives or impurities.

INTRODUCTION

In recent years research on the chemical, biochemical and therapeutic properties of heparins has been carried out[1–6], particularly for low-molecular-weight (LMW) heparins[7–9]; these are obtained generally by the fractionation or depolymerization of unfractionated (UF) heparins (molecular weight range 3000–40 000 daltons)[1]. LMW heparins are claimed to possess antithrombotic and anticoagulant properties with reduced bleeding potential, greater bioavailability, longer biological half-lives and duration of therapeutic action compared with UF heparins[7–9]. Heparins are polydisperse heterogeneous sulphated glycosylaminoglycans[1,6] whose anticoagulant properties are related to their molecular weight distributions (MWD)[7]; therefore, the determination of the average molecular weights (MWs) and MWDs are important parameters for their characterization. Heparin solutions are administered parenterally[10,11] and the pharmaceutical products[12–14] may also contain other drugs, additives, preservatives and undeclared compounds; it is therefore necessary to analyse samples for these substances, which may be pharmacologically active or lead to unwanted reactions[15].

We have previously reported[16] the characterization of heparins using size-ex-

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clusion chromatography (SEC) and the determination of their average MWs and MWDs and the different methods for the determination of the MW of heparins were discussed. Although the calculated MWs and MWDs determined by that method[16] were in agreement with those reported by other workers, it had several limitations: the manual quantitative evaluation of SEC data was slow and tedious; the use of tritiated heparin derivatives as calibration standards required special precautions and had restricted LMW ranges; and UV detection at a single wavelength could not be applied to the determination of heparin peak purity or to the characterization of heparin peak purity or to the characterization of additional extraneous chromatographic peaks.

To overcome the above difficulties, we report here the SEC analysis of heparins using high-performance liquid chromatography (HPLC) with on-line photodiode-array detection (DAD), computer data acquisition and handling and narrow-MWD heparin standards for calibration. Reversed-phase HPLC was also used for the qualitative and quantitative analysis of other additional UV-absorbing compounds.

EXPERIMENTAL

Equipment

A Model 1090M liquid chromatograph with a DR5 ternary solvent delivery system, autoinjector, autosampler and a photodiode-array detector (range 190–600 nm, with 4.5-μl cell volume and 6-mm path cell) was used (Hewlett-Packard, Waldbronn, F.R.G.). It was connected to a Model 79994A analytical workstation (Hewlett-Packard) for system control, data acquisition and evaluation and report generation; the installed software was HP 79995A (rev. 4.05) operating software, HP 79997A color view software and HP 79999A (rev. 4.05) GPC software (Hewlett-Packard).

Chromatographic conditions

SEC analysis. Ultropac TSK columns (300 × 7.5 mm I.D.) were used: G 2000 SW (2135-230) or G 3000 SW (2135-230) (LKB, Bromma, Sweden). The eluent was 0.1 *M* aqueous sodium chloride (isochratic), at 0.5 ml/min and the injection volume was 20 μl.

Reversed-phase HPLC. A Hypersil ODS (5 μm) column (100 × 2.1 mm I.D.) and a guard column (20 × 2.1 mm I.D.) (Hewlett-Packard) with the same filling were used. The eluent (flow-rate 0.5 ml/min) consisted of (A) phosphate buffer (0.01 *M*, pH 4.0) and (B) acetonitrile, with a solvent programme of 0–1 min 0% B and 1–10 min to 90% B with a run time of 13 min; the equilibration time was 2 min and the injection volume 2–10 μl. Eluents were filtered and gassed with helium before use.

DAD parameters. The sample wavelengths were set at 206, 220, 240 and 280 nm (4 nm band width) and the reference wavelength at 550 nm (100 nm band width); spectrum storage (range 200–400 nm) was set in the all-acquisition or peak-controlled mode.

Chemicals

All chemicals were of analytical-reagent grade; solvents for HPLC were chromatographically pure. Pharmaceutical preparations of UF and LMW heparins were

commercially available. Heparin USP XXI (Lot 936275), LMW heparin and heparin calibration standards were gifts from Sandoz (Nürnberg, F.R.G.). Reference narrow-MWD heparin calibration standards with known MW were used for SEC calibration (MW 7500, 10 400, 14 500 and 19 000 daltons); their MWs had been determined previously using low-angle laser light scattering (LALLS).

Calculations

SEC analysis. Average MWs and MWDs were calculated from the calibration standards and sample data obtained at 206 nm. Using the GPC software[17], the calibration graph was obtained, and average MWs [M_n, number-average MW; M_w, weight-average MW; M_z, z-average MW; M_{z+1}, $(z + 1)$-average MW; M_{z+2}, $(z + 2)$-average MW; D, polydispersity $= M_w/M_n$] and MWDs (differential and cumulative) were calculated[17–19].

Peak purity. Heparin peak purity was obtained from the sample data and the operating software; comparison of peak spectra, extract chromatograms at different wavelengths and absorbance ratios between two wavelengths were calculated and displayed.

Additional peak analysis. Identification of signals from other peaks was made in a similar manner to that for heparin. Quantitative analysis was performed by integrating the absorbance signals, with internal or external calibration, both for SEC and reversed-phase HPLC.

RESULTS

SEC analysis

Stationary phases were selected according to the nature of the sample; some analyses were run with bimodal column coupling[18]; aqueous sodium chloride was used as the eluent. An LMW heparin was analysed with these columns and the chromatograms are shown in Fig. 1.

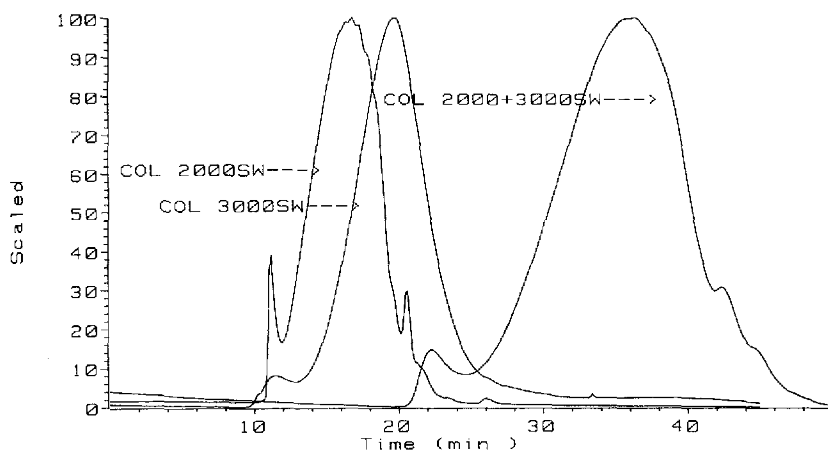

Fig. 1. SEC analysis of an LMW heparin with different columns and column coupling. Maximum absorbance (at 206 nm) scaled to 100.

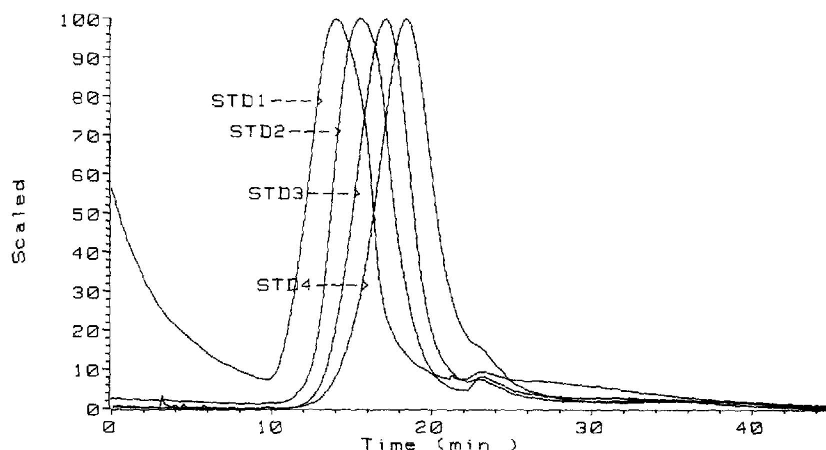

Fig. 2. SEC analysis of narrow-MWD heparin calibration standards (standards 1–4 with MW 19 000, 14 500, 10 400 and 7500, respectively); column, 3000 SW. Maximum absorbance (at 206 nm) scaled to 100.

MW averages and MWD. Narrow-MWD calibration heparin standards with known MW were analysed and the chromatograms are shown in Fig. 2. Data from the analysis of samples and the previously established calibration graph were processed using the GPC software[17–19] to calculate MWDs (cumulative and differential), average MWs and polydispersity. The precision of the average MW determination was better than 1%.

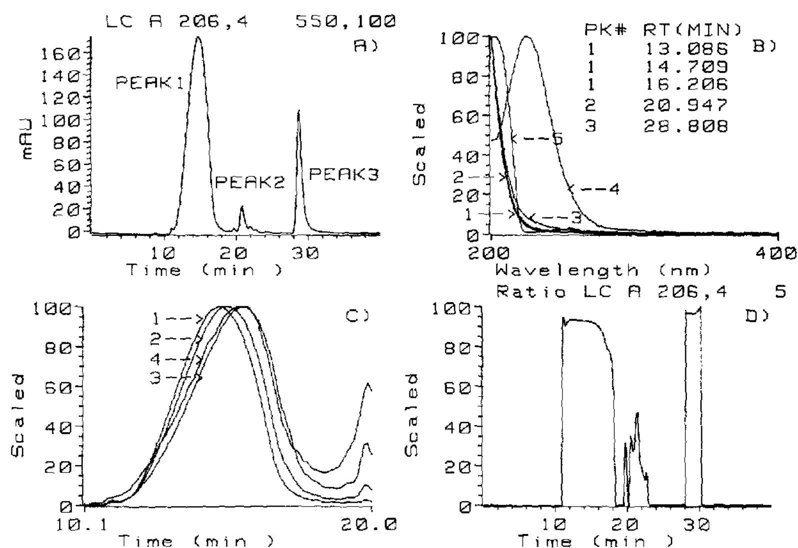

Fig. 3. Analysis of a commercial LMW heparin pharmaceutical product (column 2000SW). (A) Chromatogram at 206 nm; (B) UV spectra of heparin peak 1 (spectra 1, 2 and 3) and peaks 2 and 3 (spectra 4 and 5); (C) merged chromatograms from heparin peak (retention time 10–20 min) with multi-wavelength detection; (D) absorbance ratio 206/220 nm.

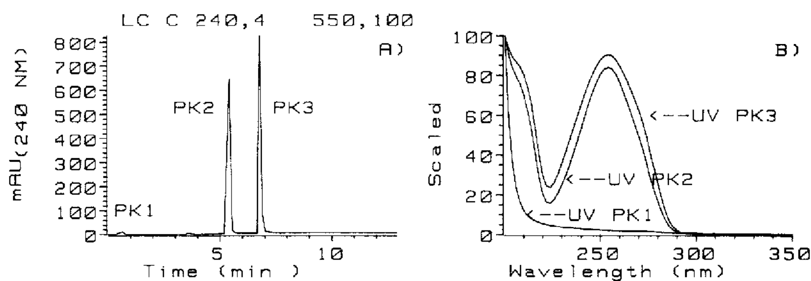

Fig. 4. Analysis of a heparin sample containing methyl (M) and propyl (P) 4-hydroxybenzoates. (A) Chromatogram with reversed-phase column, heparin (peak 1), M (peak 2) and P (peak 3); (B) corresponding UV spectra.

Heparin peak purity. A commercial heparin pharmaceutical product was analysed by SEC and the chromatogram is shown in Fig. 3A; supplementary peaks (peaks 2 and 3) appear apart from that of heparin (peak 1); several criteria for peak purity determination are exemplified in Fig. 3B–D: UV spectra on the ascending, apex and descending portions of the heparin peak (spectra 1, 2 and 3) and those for peaks 2 and 3 are displayed in Fig. 3B; Fig. 3C represents the merged chromatograms at different wavelengths during the heparin peak time interval; and the absorbance ratio between the signals at two wavelengths is shown in Fig. 3D for the same sample.

Reversed-phase HPLC analysis of heparin additives

Heparin samples were analysed by reversed-phase HPLC. Fig. 4 shows the analysis of a commercial heparin sample containing methyl and propyl 4-hydroxybenzoate as preservatives; external calibration allowed the quantitation of the components. Other additives mentioned by manufacturers were analysed in a similar way (retention times in minutes): dihydroergotamine mesylate (7.71); benzyl alcohol (4.97), *p*-cresol, (5.59), xylocaine (7.55) and L-ephedrine (4.54).

DISCUSSION

The use of different SEC columns, narrow-MWD calibration heparin standards, DAD, computer data acquisition and data handling induced us to reinvestigate our previous HPLC methodology for the analysis of heparins. We shall mention the most significant differences from our previous method[16].

The advantages of DAD over conventional UV detection were discussed previously[20–28]; improved analysis of heparins, for the determination of both average MWs and MWDs, peak purity and the analysis of other components result from its use.

SEC analysis

Gel permeation columns with modified microparticulate fillings were used for aqueous SEC analysis; however, compact columns (30 cm instead of 60 cm) were used with no loss of resolution; columns with different operating MW ranges were selected according to the heparin sample, to allow better differentiation of products from

several origins. Heparins were usually analysed with the 3000SW or 2000SW columns; for example, Fig. 1 shows that the 2000SW column is inadequate for the analysis of this heparin sample, as a peak at the exclusion volume is present. Heparins with a wide MWD were analysed with bimodal column coupling, but longer analysis times were needed (Fig. 1). Different salts, buffers and pH did not show advantages over the previously used aqueous sodium chloride[16] as eluent.

Average MWs and MWDs. Compared with our previous technique[16], the present method offers advantages for the determination of average MWs and MWDs as follows. (a) The calibration standards used in this work had the same chemical composition as the samples, narrow-MWD ranges and their MWs had been previously determined by LALLS (Figs. 2 and 3); these conditions and frequent column recalibrations[18,19,29] are essential for the correct measurement of average MWs and MWDs of heparin samples. Previously a mixture of tritiated chemically modified LMW heparins was used for calibration[16]; apart from the restricted range in the LMW region which did not allow extrapolations to higher MW, and the differences in the structures of the standard and the sample, the use of radioactive material afforded special precautions. (b) A wavelength of 206 nm was used, as previously explained[16], as this wavelength also allows the detection of other UV-absorbing components. The advantages of multi-wavelength and spectral scan analysis in this work are detailed below.

Heparin peak purity. Apart from the determination of average MWs and MWDs of heparins by SEC and DAD, the peak purity of heparins was additionally characterized. Fig. 3A shows the chromatogram of a commercial LMW heparin and the peak purity was investigated using several criteria, as follows. (1) The UV spectra at different retention times are displayed and compared in Fig. 3B, which shows the spectra on the ascending, apex and descending portions of the heparin peak (spectra 1, 2 and 3, respectively). Whereas the first two spectra are superimposable, spectrum 3 shows a significantly higher absorption, indicating an impurity; peaks 2 and 3 elute with higher retention times than heparin and indicate the presence of additional components (spectra 4 and 5, respectively); mathematical processing of spectral data such as derivatization or substraction of spectra are additional ways of comparing spectra (not shown). The identification of an unknown peak merely from the retention time and spectral characteristics is difficult in the absence of data from known standards; if these are available they may be stored in a library for comparison with unknown compounds. (2) Fig. 3C shows the merged chromatograms obtained at different wavelengths during the elution of the heparin peak; a pure peak not containing foreign UV-absorbing substances should show four superimposable traces; this does not occur with this sample (Fig. 3C), indicating an impure heparin peak. (3) The ratios of the absorbances at two different wavelengths may be displayed, and pure peaks present a plateau. Absorbance ratios for the heparin sample are displayed in Fig. 3D; the descending part of the heparin peak contains a UV-absorbing impurity and the peak at 20.855 min is in fact a multi-component moiety; the peak at 28.743 min is pure.

These methods (and others such as three-dimensional plots of absorbance, wavelength and time) are useful for the additional evaluation of heparin SEC results.

Reversed-phase HPLC analysis of heparin additives

Additional extraneous LMW substances do not show sharp peaks or are not eluted by SEC; HPLC with reversed-phase columns is more suitable for their qualitative and quantitative analysis. Fig. 4 shows the chromatogram of a commercial heparin containing declared preservatives, which were readily identified and quantitated; optimal UV wavelengths may be selected according to the spectral characteristics of the compounds to be investigated. The use of microbore columns leads to fast analysis with good accuracy and precision.

SEC analysis using differential refractometers or on-line LALLS as detectors have been described recently[29,30] for the determination of average MWs and MWDs of heparins; these methods, however, are insensitive for the analysis of traces of impurities or for the identification of additional compounds. The method described above with DAD is suitable for obtaining more information in this respect.

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REFERENCES

1 B. Casu, *Adv. Carbohydr. Chem. Biochem.*, 43 (1985) 51.
2 R. L. Lundblad, W. V. Brown, K. G. Mann and H. R. Roberts (Editors), *Chemistry and Biology of Heparin*, Elsevier/North Holland, New York, 1981.
3 I. Witt (Editor), *Heparin. New Biochemical and Medical Aspects*, Walter de Gruyter, Berlin, New York, 1983.
4 Abstracts of the XIth International Congress on Thrombosis and Haemostasis, Brussels, 6–10 July, 1987, *Thromb. Haemostas.*, 58 (1987).
5 J. M. Walenga, J. Fareed, D. Hoppensteadt and R. M. Emanuele, *Crit. Rev. Lab. Sci.*, 22 (1986) 361.
6 L. B. Jaque, *Methods Biochem. Anal.*, 24 (1977) 203.
7 J. Hirsh, F. A. Ofuso and M. Levine, in M. Verstraete, J. Vermylen, R. Lijnen and J. Arnout (Editors), *Thrombosis and haemostasis*, Leuven University Press, Leuven, 1987, p. 348.
8 J. Harenberg, J. X. de Vries, E. Weber and R. Zimmerman, *Dtsch. Med. Wochenschr.*, 109 (1984) 951.
9 J. Harenberg, J. X. de Vries, E. Weber and R. Zimmermann, *Med. Welt*, 35 (1984) 1371.
10 A. A.. Sasahara, V. V. Kakkar, R. D. Hull and D. P. Thomas, in A. L. Bloom and D. P. Thomas (Editors), *Haemostasis and Thrombosis*, Churchill Livingstone, Edinburgh, London, 1987, 2nd ed., p. 820.
11 R. Zimmermann and H. Mörl, in G. Schettler and E. Weber (Editors), *Internistische Therapie in Klinik und Praxis*, Georg Thieme, Stuttgart, 1984, p. 466.
12 Bundesverband der pharmazeutischen Industrie, *Rote Liste 1988*, Editio Cantor, Aulendorf, 1988.
13 K. H. Bauer, K. H. Frömmig and C. Führer, *Pharmazeutische Technologie*, Georg Thieme, Stuttgart, 1986.
14 H. P. Fiedler (Editor), *Lexikon der Hilfsstoffe für Pharmazie, Kosmetik und angrenzende Gebiete*, Editio Cantor, Aulendorf, 1981.
15 R. Zimmermann, J. Harenberg, E. Weber, J. X. de Vries, W. Jarrass and W. Schmidt, *Dtsch. Med. Wochenschr.*, 109 (1984) 1326.
16 J. Harenberg and J. X. de Vries, *J. Chromatogr.*, 261 (1983) 287.
17 *HPLC ChemStation. Using GPC Software*, Hewlett-Packard, Waldbronn, 1987.
18 W. W. Yau, J. J. Kirkland and D. D. Bly, *Modern Size-Exclusion Liquid Chromatography. Practice of Gel Permeation and Gel Filtration Chromatography*, Wiley, New York, 1979.
19 J. V. Dawkins, in J. Janca (Editor), *Steric Exclusion Chromatography of Polymers*, Marcel Dekker, New York, 1984, p. 54.
20 T. Owen, *Int. Lab.*, No. 4 (1987) 68.

21 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 273 (1983) 3.
22 A. F. Fell, B. J. Clark and H. P. Scott, *J. Chromatogr.*, 316 (1984) 423.
23 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 282 (1983) 123.
24 G. W. Schieffer, *J. Chromatogr.*, 319 (1985) 387.
25 J. K. Strasters, H. A. H. Billiet, L. de Galan, B. G. M. Vandeginste and G. Kateman, *J. Chromatogr.*, 385 (1987) 181.
26 T. Hoshino, M. Senda, T. Hondo, M. Saito and S. Tohei, *J. Chromatogr.*, 316 (1984) 473.
27 T. Alfredson, T. Sheehan, T. Lenert, S. Aamodt and L. Correia, *J. Chromatogr.*, 385 (1987) 213.
28 F. Overzet, A. Rurak, H. van der Voet, B. F. H. Drenth, R. T. Ghijsen and R. A. de Zeeuw, *J. Chromatogr.*, 267 (1983) 329.
29 W. E. Hennink, J. W. A. van den Berg and J. Feijen, *Thromb. Res.*, 45 (1987) 463.
30 E. A. Johnson, *Thromb. Res.*, 45 (1987) 675.