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Aqueous high-performance size-exclusion chromatographic assay for high-molecular-weight impurities in ceftiofur sodium

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ABSTRACT

An aqueous high-performance size-exclusion chromatographic assay for high-molecular-weight (HMW) impurities in ceftiofur sodium bulk drug is described. The assay uses a sodium dodecyl sulfate micellar mobile phase to provide complete recovery of the analyte from a glycerylpropyl-bonded silica column. Using 254-nm absorbance detection corrected for relative detector response, the assay provides a linear response and complete recovery of HMW impurities. The relative standard deviation for repeated assay of a single bulk drug lot is 2.5%, with systematic variation of column, mobile phase batch, analyst, laboratory, instrument and day. The detection limit is 0.03%.

INTRODUCTION

The β-lactam antibiotics, which include both the cephalosporins and the penicillins, have long been known to contain autologous high-molecular weight (HMW) impurities [1,2]. These HMW impurities are considered potential antigens, but have been reported to be weakly antigenic, at most, when administered parenterally [3]. In contrast, HMW conjugates between β-lactams and proteins have been reported to induce allergic reactions.

Formation of HMW impurities in aqueous solutions of penicillins or cephalosporins is well documented [4,5] and these materials have been characterized chemically and immunologically. HMW impurities from penicillins have been reported to be amide-linked polymers of β-lactam ring-opened degradation products of the parent antibiotic [4–7]. We have found no published data on the structure of cephalosporin-related HMW impurities, although efforts to identify such materials are occasionally mentioned parenthetically. High-performance aqueous size-exclusion methods have been developed to determine HMW impurities in both penicillins and cephalosporins [8–10].

Ceftiofur sodium (Sterile Powder Naxcel) is a cephalosporin antibiotic marketed by Upjohn for the treatment of bovine respiratory disease. During analytical development, thin-layer chromatography (TLC) indicated the presence of HMW impurities in ceftiofur sodium bulk drug which were not detected using a reversed-phase gradient high-performance liquid chromatographic (HPLC) assay. A high-performance size-exclusion chromatographic (HP-SEC) method was developed to determine the HMW impurities, using an aqueous mobile phase to provide adequate analyte solubility. Competing separation mechanisms typical of aqueous SEC assays were encountered, including ion exclusion, adsorption and salting-out effects [11]. Addition of micellar sodium dodecyl sulfate (SDS) to the mobile phase was required in order to obtain complete recovery of HMW impurities from the analytical column. Previously published methods [8–10] were investigated; none provided complete recovery of ceftiofur HMW impurities from the column.

This paper describes the development of a quantitative aqueous HPSEC assay for these HMW impurities in ceftiofur sodium bulk drug, with particular attention to optimizing resolution and achieving complete recovery. Validation of the method for use in pharmaceutical quality control is also described.

EXPERIMENTAL

Reagents

Ceftiofur sodium and ceftiofur hydrochloride bulk drug lots were provided by Upjohn (Kalamazoo, MI, USA). Deionized water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA). HPLC-grade solvents were obtained from Baxter, Burdick and Jackson (Muskegon, MI, USA). Other reagents were of analytical-reagent grade obtained from Mallinckrodt (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA). All reagents and solvents were used as received.

Chromatography

The assay uses a 250 mm × 4 mm I.D. LiChrospher 100 DIOL (5-μm particle size) glyceryl-propyl (diol) silica column with 100 Å pore size (EM Science, Cherry Hill, NJ, USA). Other chromatographic experiments utilized 250 mm × 4 mm I.D., 5μm particle size silica columns: LiChrospher Si 100 silica (EM Science), Zorbax TMS C_1 bonded phase (MAC-MOD, Chadds Ford, PA, USA), Vydac Protein C_4 bonded phase (Separations Group, Hysperia, CA, USA), Zorbax C_8 bonded phase (MAC-MOD), Zorbax ODS C_{18} bonded phase (MAC-MOD), Zorbax CN cyano bonded phase (MAC-MOD), Brownlee aminopropyl-bonded phase (Brownlee Labs., Santa Clara, CA, USA) and a TSK 2000 SW aqueous size-exclusion column with proprietary inactivation chemistry (Phenomenex, Torrance, CA, USA).

The HPLC system consisted of a Beckman (Fullerton, CA, USA) Model 110A isocratic pump, a Rheodyne (Cotati, CA, USA) Model 7135 loop injection valve with pressure bypass and either an LDC UV-Monitor III 254 nm line source detector (LDC/Milton Roy, Riviera Beach, FL, USA) or a Waters Model 410 differential refractive index detector (Millipore–Waters, Milford, MA, USA). Chromatographic data were collected and analyzed using an in-house VAX-based (Digital Equipment, Maynard, MA, USA) chromatographic data system. Data analysis was performed using SAS Version 5.16 (SAS Institute, Cary, NC, USA).

The mobile phase for the optimized HPSEC assay procedure was made by preparing a 0.005 *M* potassium orthophosphate solution, adjusting the pH to 7.5 with the addition of concentrated potassium hydroxide solution, adding 1% (w/v) SDS, stirring until completely dissolved and vacuum filtering/degassing through a 0.45-μm nylon-66 filter (Rainin, Woburn, MA, USA). Other mobile phases were prepared similarly, as indicated under Discussion. The pH was measured using a Sargent-Welch (Skokie, IL, USA) pH 6000 with an S-30072-15 combination electrode.

Samples for assay were dissolved at *ca.* 0.1 mg/ml in mobile phase and 20 μl were injected, avoiding contact with plastic vial caps or tubing (*ca.* 2 μg on-column). The flow-rate of the system was maintained at 1.0 ml/min. Elution volumes of totally included and totally excluded analytes were approximated using sodium nitrate and blue dextran, respectively. The peak-area percentage of the HMW impurities was determined, and this was divided by a relative response factor (*RRF*) of 0.81 to account for the relative detector response. Correction for co-eluting low-molecular-weight impurities, if any, is accomplished using data from a separate low-molecular-weight impurities assay. The weight percentage of HMW impurities can be calculated, if desired, from the peak-area percentage result (corrected for *RRF*) using as-is major component potency and low-molecular-weight impurities data from separate HPLC assays.

Preparation of isolated HMW impurities

Isolated HMW impurities were prepared by dissolving 10 g of a ceftiofur sodium lot containing HMW impurities in 200 ml of 0.1 *M* sodium phosphate buffer (pH 7). The solution was filtered through a 45-mm Diaflo Model YM2 1000-dalton (nominal) ultrafiltration membrane (Amicon, Danvers, MA, USA) fitted to an Amicon Model 8050 stirred ultrafiltration cell. The material retained on the membrane was rinsed with two 50-ml portions of water, dissolved from the top of the membrane in *ca.* 5 ml of water, frozen in liquid nitrogen and

freeze-dried using a Labconco (Kansas City, MO, USA) Model 75035 vacuum freeze-drying apparatus. The purity and HPSEC elution profile of the isolated material were determined using the HPSEC impurities assay.

Absolute recovery from column

Recovery of HMW impurities from the column was investigated by directly comparing the amount eluted from the column with the amount injected. Filled loop injection was used to control the injection volume precisely. First, 20 μl of a solution of isolated HMW material (8.5 μg) were injected onto the column and the total effluent was collected. Then the same volume was injected without a column in-line and the effluent was collected. The amount of each collected solution was determined gravimetrically, the absorbance at 254 nm was measured for each solution and the percentage recovery was calculated.

Relative response factor

The *RRF* is the ratio of the chromatographic detection response of the analyte per unit weight to that of ceftiofur. The *RRF* for HMW impurities relative to ceftiofur was determined using two approaches. In the first, the absorbance at 254 nm was determined using a line source detector for separate mobile phase solutions containing a known concentration of either the HMW impurities or ceftiofur. The resulting mass absorptivities were then ratioed to determine the relative response. In the second approach, the slope of the recovery study uncorrected amount found *vs.* amount added data is taken as the *RRF*. Absorption spectra of ceftiofur and isolated HMW impurities were measured using a Hewlett-Packard (Palo Alto, CA, USA) Model 8450A diode-array spectrophotometer or a Hewlett-Packard Model 1090A chromatograph with a diode-array detector.

Recovery study

A bulk drug lot of ceftiofur hydrochloride containing 1.86% of HMW impurities was spiked with isolated HMW impurities up to 7.86%. The peak-area percentage of HMW impurities was determined using the optimized assay with an *RRF* of 0.81 and the percentage of recovery was calculated for each point.

RESULTS AND DISCUSSION

During early analytical development for ceftiofur sodium, a non-migrating impurity was detected in a silica TLC impurities screen. Visible amounts of a non-eluting impurity were also noted at the inlet of a reversed-phase HPLC column used for ceftiofur impurities assays. These materials were hypothesized to be HMW impurities, and this was confirmed by their retention on an ultrafiltration membrane with a 1000-dalton nominal molecular weight cut-off, about twice the 522.56-dalton molecular weight of ceftiofur free acid. However, ultrafiltration does not provide definitive molecular weight information because retentivity depends on molecular size, shape and hydration, and at low molecular weights the correlation between retentivity and molecular weight is not as great as at higher molecular weight. Because the HMW impurities could not be assayed using existing methods, a separate chromatographic assay was developed.

Several types of stationary phase were screened to determine the most suitable chromatographic mode for the separation, inlcuding C_1, C_4, C_8 and C_{18} bonded-phase columns, a silica column, cyano and aminopropyl bonded-phase columns, and two different aqueous size-exclusion columns. For each column, mobile phase pH, organic content and buffer concentration were varied in attempts to obtain an acceptable separation. Both ceftiofur sodium and the HMW impurities show high aqueous solubility and generally low non-aqueous solubility, and ceftiofur solution stability is reduced at basic or acidic pH, focusing consideration on aqueous-based near-neutral mobile phases. Aqueous size-exclusion chromatography was the only investigated chromatographic mode in which the HMW impurities were eluted from the column at mobile phase pH below 8. The LiChrospher DIOL size-exclusion column (100-Å pore size) was selected for further assay development. This silica-based column is inactivated by a glycerylpropyl-bonded phase, and has a reported fractionation range of about 10^3–10^5 dalton [11].

The assay development goals were optimization of the mobile phase to achieve complete recovery of both the HMW impurities and ceftiofur from the column and acceptable resolution between the HMW impurities and ceftiofur, determination of an

appropriate UV absorbance *RRF* for the HMW impurities and validation of the assay for use in pharmaceutical quality control.

Mobile phase optimization: selectivity and absolute recovery

In aqueous SEC, non-size-exclusion mechanisms can contribute to retention, potentially affecting both recovery and resolution. Non-elution of the HMW impurities from both reversed- and normal-phase HPLC systems suggested that adsorption might make recovery difficult. Additionally, ceftiofur's negative charge at neutral pH makes ionic contributions to the separation mechanism likely.

The ionic strength of the mobile phase is critical to selectivity. Without salt, the HMW impurities and ceftiofur eluted together in the totally excluded volume. Under these conditions, negatively charged compounds such as ceftiofur tend to be retained less than neutral species on silica-based SEC columns because of coulombic repulsion from residual silanols near the stationary phase pores. This effect is mitigated by increasing the mobile phase ionic strength [11], which provides a shielding effect. With increasing salt concentration, ceftiofur was more strongly retained and separation from the HMW impurities was achieved. At salt concentrations above 10 m*M*, the retention volume of ceftiofur peak became greater than totally included volume of the column, suggesting an increasing contribution from adsorption.

The recovery of both the HMW impurities and ceftiofur decreased with increasing salt concentration. Although acceptable resolution between the HMW impurities and the ceftiofur peak and complete recovery of ceftiofur were achieved with a pH 7.5 mobile phase containing 5 m*M* potassium phosphate, HMW impurities recovery from the column was less than about 75%.

Complete recovery and acceptable resolution were achieved by increasing the mobile phase solvent strength by adding micellar SDS. The 1% (w/v) SDS in the final assay procedure is about 35 m*M*, exceeding the SDS critical micelle concentration (*ca.* 5 m*M* in the 13 m*M* ionic strength buffer). Using this micellar mobile phase, an absolute recovery of HMW impurities of 100.9 ± 5.7% [95% confidence limit (CL)] was determined at *ca.* 8.5 μg of HMW material injected, as described under Experimental. The addition of organic modifiers such as acetonitrile, methanol or tetrahydrofuran to the aqueous mobile phase was alternatively attemped, but did not improve the recovery, and degraded the resolution between ceftiofur and the HMW impurities at higher concentrations (*e.g.*, > 25% acetonitrile).

The separation obtained using the final HPSEC assay procedure is shown in Fig. 1. The HMW impurity peaks elute before the major component peak at retention times of *ca.* 1–2.2 min. Ceftiofur and most other low-molecular-weight impurities elute at about 2.7 min. The resolution between ceftiofur and the closest eluting HMW impurity peak is *ca.* 3.6, and is sufficient for accurate determination of the HMW impurities.

Several impurities with molecular weights similar to that of ceftiofur, which are determined using a separate HPLC assay, co-elute with the HMW impurities. This was determined by assaying isolated HMW peaks from the HPSEC separation using the separate assay for low-molecular-weight impurities. The co-elution is speculated to result from interaction of the co-eluting low-molecular-weight impurities with the SDS micelles, which have a hydrodynamic radius of about 30 Å (aggregation number near 60), and would therefore elute between the excluded peak and the included peak on the LiChrospher DIOL 100 Å column. The peak areas of the co-eluting low-molecular-weight impurities can be subtracted from the total peak area found in the HPSEC procedure, but significant amounts of these are rarely observed.

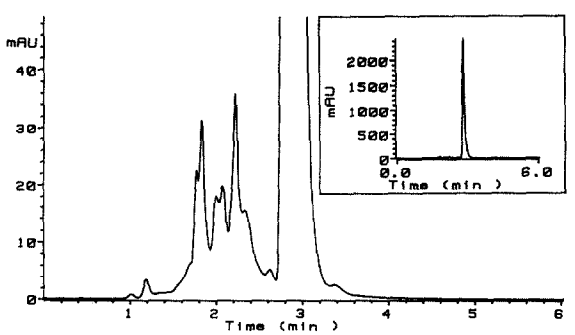

Fig. 1. Chromatogram of ceftiofur sodium bulk drug (2.5 μg on-column). Column, EM Science LiChrospher 100 DIOL, 5 μm (250 mm × 4.6 mm I.D.); mobile phase, 0.005 *M* KH_2PO_4 (pH 7.5) containing 1% SDS; flow-rate, 1.0 ml/min; detection, 254 nm.

Linearity, recovery and response factor determination

The detector peak-area response is linear over the range 0.005–9.25 μg of isolated HMW impurities injected (r^2 = 0.999999; residuals apparently random), which corresponds to about 0.25–460% of the amount of bulk drug injected in the assay procedure. This range encompasses the amount injected in the absolute recovery experiment (8.5 μg) and thus allows complete absolute recovery to be inferred over the entire linear range. An insignificant *y*-intercept (−1110 μV s: estimated standard deviation (ESD) = 5716) shows the absence of additive bias and is consistent with complete recovery. The same mobile phase without SDS provided a lower recovery throughout the investigated range of 0.1–14.46 μg HMW impurities injected (75.3% at 13.25 μg of HMW material injected), and progressively lower recoveries with lower levels of HMW impurities injected on to the column.

HMW impurities present in ceftiofur sodium are determined on a peak-area percentage basis relative to the major component peak using UV absorbance detection at 254 nm. The difference in chromatographic detector response between the analyte and the major component is accounted for by dividing the area percentage result by an *RRF*, the ratio of the mass response of the analyte to that of the major component.

The *RRF* used in this work is 0.81 ± 0.01 (95% CL), the least-squares regression slope from the recovery study discussed below. The value agrees with an *RRF* of 0.80 measured directly by rationing the 254-nm absorbance of isolated HMW impurities in mobile phase solution to that of ceftiofur. These experimental values are near 1.0, consistent with a possible structural relationship between the HMW impurities and ceftiofur suggested by the qualitative similarity between their absorbance spectra.

These approaches require a knowledge of the purity of both the isolated HMW impurities and the ceftiofur lot used for comparison. Because insufficient isolated HMW material was available for complete material balance assays, it was assumed that these purity values were identical. Additionally, it was assumed (throughout this work) that the isolated HMW impurities are structurally representative of those present in bulk drug lots. The HPSEC elution profile of the isolated HMW impurities is shifted to earlier elution times than that of the impurities normally found in bulk drug lots. However, the diode-array detector absorbance spectra of the HMW impurity peaks in bulk drug lots are similar to those of the isolated materials, suggesting that the structure of the isolated HMW impurities is similar to that of the impurities normally observed.

These assumptions were tested by using two different approaches to determine the *RRF* directly for materials naturally present in bulk drug, with no assumptions regarding analyte purity. A set of measurements using TLC with similar-response detection based on spray-reagent derivatization gave an *RRF* of 0.87 ± 0.17 (95% CL) [12]. Data from a separate experiment using the HPSEC assay with refractive index detection proved more difficult to interpret because both positive and negative peaks occurred in the HMW impurities region. An *RRF* of 0.85 was calculated by integrating both positive and negative peaks with respect to the same baseline. These results are consistent with the *RRF* value assigned using isolated HMW impurities, and thus support the assumptions.

The spiked recovery is acceptable over a range of 0.3–6.0 wt.% HMW impurities added. In this study, the peak-area percentage of HMW impurities was determined for a bulk drug lot of ceftiofur hydrochloride that had been spiked with isolated HMW impurities. This bulk drug lot contained the lowest level of HMW impurities that had been observed at that time. If the relative detector response difference is not taken into account, the least-squares regression slope for these data is 0.81 ± 0.01 (95% CL). This slope was assigned as the assay *RRF* value, as discussed above. Recovery data are given in Table I, the "HMW impurities found" values being calculated using an *RRF* of 0.81. At each spiking level, the percentage of HMW impurities recovered is within 1 assay standard deviation (see below) of the amount added. The recovery is linear, as shown by a least-squares r^2 value of 0.9997 and apparently random residuals. The slope (found *vs.* added) is 1.002 ± 0.005 (ESD), and the *y*-intercept is not significantly different from zero (0.08 ± 0.04 ESD) when the data are corrected for the 1.86% ± 0.10% (95% CL) HMW impurities found in the ceftiofur hydrochloride bulk drug lot used in the spiking study.

TABLE I

RECOVERY OF SPIKED HMW IMPURITIES

Percentage found data are corrected for mobile phase blank and a detection *RRF* of 0.81 (see text). The adjusted percentage found is the difference between the actual percentage found and the percentage of HMW impurities assayed in the ceftiofur hydrochloride lot used in the spiking study (1.86%).

HMW impurities added (%)	HMW impurities found (%)		Regression residuals (% HMW impurities)
	Actual	Adjusted	
0.27	2.18	0.32	−0.031
0.26	2.21	0.35	0.007
0.56	2.49	0.63	−0.007
0.56	2.47	0.61	−0.029
0.55	2.56	0.70	0.069
1.16	3.14	1.28	0.030
1.11	3.09	1.23	0.041
1.13	2.99	1.13	−0.080
3.02	4.94	3.08	−0.026
3.01	4.97	3.11	0.016
3.01	4.97	3.11	0.009
5.97	7.88	6.02	−0.040
5.90	7.84	5.98	−0.009
5.86	7.86	6.00	0.051

Limit of detection

The limit of detection (L_D) can be defined by the expression $L_D = Y_{bl} + 6S_{bl}$ [13,14], where Y_{bl} is the average signal of the baseline and S_{bl} is the standard deviation of the baseline. Using this definition, L_D is the smallest peak-height value that can be distinguished from the highest probable excursion of the baseline with 99.86% certainty, assuming a normal noise distribution. A standard deviation of $15.3 \cdot 10^{-6}$ absorbance units was measured for the chromatogram baseline from an entire run, excluding only the mobile phase disturbance discussed below. This corresponds to an L_D of 0.03% for HMW impurities, based on peak height.

Precision and ruggedness

The recovery of HMW impurities and the resolution of ceftiofur from the closest eluting HMW peak were studied as a function of mobile phase composition by assaying a ceftiofur sodium bulk drug lot using the same column with systematically varying mobile phase compositions, as detailed in Table II. The selected mobile phase is on relatively flat area of both the resolution and recovery response surfaces, predicting acceptable ruggedness with respect to small variations in mobile phase composition. The relative 95% confidence limit on individual recovery results is *ca.* ±5.6% at the HMW impurities level present (5.4%), based on relative assay variability (see below). Recovery values significantly greater than 100% are due to decreasing resolution, which results in the assignment of an increasing portion of the ceftiofur peak area to the HMW impurities.

Precision and ruggedness were further investigat-

TABLE II

MOBILE PHASE RUGGEDNESS

Conditions are given under Experimental. Mobile phase S is used in the assay.

Mobile phase	SDS (%)	Phosphate (%)	Resolution	Recovery (%)
1	0.1	1.0	3.05	81
2	0.1	5.0	4.01	84
3	0.1	10.0	4.93	69
4	1.0	1.0	2.68	106
5	1.0	5.0	3.60	101
6	3.0	1.0	1.65	112
7	3.0	5.0	1.63	110
8	3.0	10.0	1.43	110

ed by assaying a single ceftiofur sodium bulk drug lot using five different columns on six different days and in three separate laboratories. The individual assay value standard deviation is 0.16% for the 60 assays, corresponding to a relative standard deviation of 2.5% and a relative 95% confidence limit of $\pm 5.6\%$ [using $t(\alpha = 0.975; n = 59)$ and assuming a single assay value]. Analysis of variance (ANOVA) showed no significant effect on assay results at the 95% confidence level due to different columns, days, laboratories or analysts.

Mobile phase blank

A series of small peaks reproducibly elutes at the totally excluded volume when a blank injection is made, as shown in the top chromatogram in Fig. 2. Although a blank correction can be performed, the presence of a significant blank would compromise the ruggedness of the method, and extensive efforts were made to eliminate this blank.

The artifact peaks were observed only with mobile phases containing micellar SDS, and only when using Rheodyne Model 7125 or 7126 injection valves, which do not include a pressure by-pass. The blank peaks are reduced by an order of magnitude (to *ca.* 0.05% of the total peak area) by replacing the stator and stator face assembly in a Model 7126 with an assembly incorporating a pressure by-pass, as shown in the bottom chromatogram in Fig. 2.

The modified valves contain the same solvent

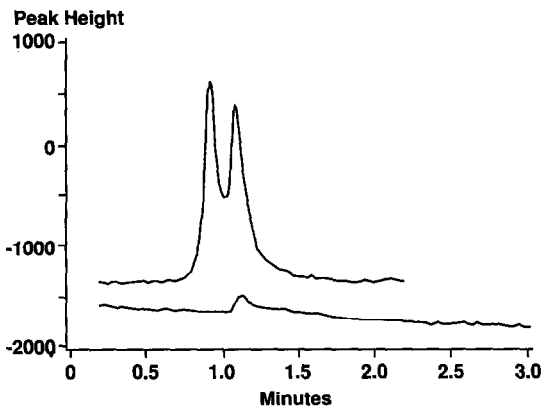

Fig. 2. Chromatograms of mobile phase blanks injected (bottom) with an injection valve equipped with a pressure by-pass and (top) with an injection valve not equipped with a pressure by-pass. The ordinate (peak height) is in μV; 1 V = 1 absorbance unit.

contact materials as the unmodified valves. Exhaustive cleaning of the dismantled injection valve with organic solvents and (separately) nitric acid failed to reduce the magnitude of the blank, and the size of the artifact peaks remains constant even after many repeated injections. Identical blanks are observed for manual injections and autosampler injections for a given valve, ruling out other autosampler components as a primary blank source. These results suggest that the blank somehow results from the high-pressure transient during valve switching. However, high-pressure extraction of components of the injection valve materials does not appear to explain fully the observed blank peaks. Micellar solubilization has been reported to be a relatively weak function of pressure [15,16]. Either increased or decreased solubility of less than about 10% has been reported, depending on the solute, surfactant structure and applied pressure. This contrasts with the order of magnitude decrease in the blank obtained by incorporating the pressure bypass.

No explanation for the blank peaks has been proposed to date which is consistent with all of the available information. Use of Rheodyne Model 7135 and 7125-075, which have the by-pass equipped stator and stator face assembly, or Rheodyne Model 7125 and 7126 valves rebuilt with stator and stator face assemblies with pressure by-pass is recommended to limit the magnitude of the blank. Extractable material from plastic to which the sample may be exposed before injection can also contribute to the blank peak, and plastic autosampler sample path tubing and plastic vial caps are therefore avoided.

CONCLUSIONS

The described size-exclusion chromatographic assay of HMW impurities in ceftiofur sodium bulk drug is suitable for use in pharmaceutical quality control. The method exhibits linear response and complete recovery of HMW impurities, with a relative standard deviation of 2.5%.

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