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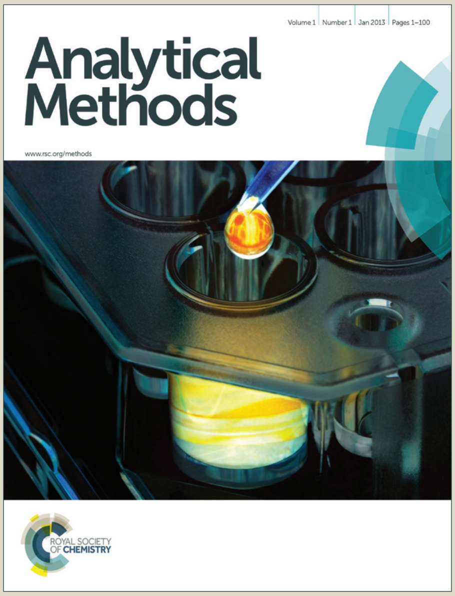

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Analysis of N-methylpyrrolidine in cefepime hydrochloride by ion chromatography using suppressed conductivity detection with solid-phase extraction pre-treatment

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N-methylpyrrolidine (NMP) is the principal hydrolysis product of cefepime hydrochloride, a β-lactam antibiotic. NMP is limited in cefepime hydrochloride by current regulations to not more than 0.3 % w/w. Existing methods for the liquid-phase chromatographic analysis of this impurity fail to separate NMP from the drug substance prior to analysis, with the result that NMP continues to be formed by hydrolysis of cefepime in the final analytical solution. This study proposes a simple, rapid solid-phase extraction procedure that separates NMP from cefepime hydrochloride prior to analysis. NMP was separated from the majority of cefepime in the sample solution using polymeric reverse phase solid-phase extraction. Recovery, precision, sensitivity, linearity and specificity values were adequate to support the use of this method for regulatory purposes. The relative amount of NMP formed in sample extracts processed by solid-phase extraction was at least five times less compared to untreated samples.

1. Introduction

Cefepime (Figure 1) is a fourth-generation cephalosporin antibiotic [1]. It is readily hydrolysed in aqueous solution [2-4], forming N-methylpyrrolidine (NMP; Figure 2) as a degradation product. The rate of hydrolysis is pH-dependent; cefepime is most stable over the pH range 4 to 6 [2]. The United States Pharmacopoeia [USP; 5] specifies a limit of not more than 0.3 % for NMP in cefepime hydrochloride. The test method given in the USP monograph employs ion chromatography with unsuppressed conductivity detection to separate and quantify NMP, using a mobile phase comprising 5 % acetonitrile in 0.01 N nitric acid. Samples are prepared by dissolving cefepime hydrochloride in 0.002 N nitric acid and these solutions are required to be injected immediately after preparation. There are two principal limitations with the current USP method: firstly, the NMP signal is measured against a very high background conductivity (> 3,000 µS/cm) from the mobile phase; secondly, cefepime hydrochloride is unstable in 0.002 N nitric acid (pH 2.7), forming additional NMP as the dissolved samples await injection. The first limitation is of concern because some commonly-used conductivity detectors (including the model used for this study) are not capable of recording conductivity values greater than 3,000 µS. The second problem, concerning sample stability, means that samples must be prepared just before injection. Because NMP is a volatile liquid in its unionised form (boiling point 81 °C, pKa 10.32 [6,7]), however, the use of an acidic diluent is useful to prevent evaporative loss of the analyte from aqueous solutions. The aim of this study was therefore to develop a sample preparation method capable of separating NMP from most of the cefepime present, thereby reducing the relative amount of additional NMP formed in sample solutions awaiting analysis.

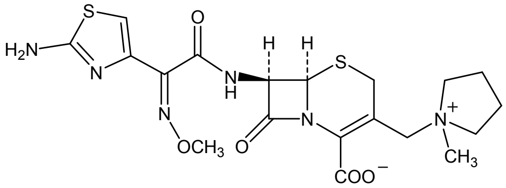

Figure 1. Cefepime.

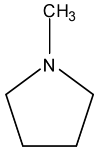

Figure 2. N-methylpyrrolidine

Various chromatographic techniques have been used previously to assay NMP in cefepime. The absence of a chromophore makes HPLC with UV detection impracticable. Chen *et al.* [8] triturated

200 mg of cefepime with chloroform and tested the resulting extract by capillary GC-FID, using pyridine as an internal standard. They achieved a limit of detection of 0.3 ng NMP on-column. The lowest concentration used for evaluation of spiking recovery (n = 3) was approximately 0.17 % w/w with respect to cefepime; recovery was quantitative at this level (100.2 % ± 1.30 %). Whilst this approach succeeds in separating NMP from cefepime, it is limited in that it can only be applied to cefepime hydrochloride in the solid state. It is not suitable for the analysis of NMP in aqueous cefepime formulations, such as Cefepime for Injection USP. Liu and Sunderland [9] evaluated capillary electrophoresis with indirect UV detection using 40 mg/mL cefepime samples. They achieved quantitative NMP recoveries from solutions spiked at concentrations ranging from 0.2 % to 1.6 %, and a limit of detection of 6 µg/mL. An application note published by the Dionex Corporation [10] gave details of a method using ion chromatography with gradient elution (0.006 mol/L to 0.085 mol/L methanesulphonic acid) and suppressed conductivity detection. The limit of detection was 0.03 µg/mL (0.15 ng injected) and quantitative recoveries were achieved from samples spiked with NMP at between 0.26 % and 1.0 %. The stability of cefepime (measured by the rate of formation of NMP) was evaluated over four days in deionised water at -17 °C, 4 °C and 25 °C. An increase in NMP concentration from 0.23 % to 0.27 % was observed after 1 h at 25 °C. At 4 °C, the NMP concentration changed from 0.22 % to 0.29 % over 24 h, but no significant increase in NMP concentration was observed in frozen samples (-17 °C) after four days. Subramanian *et al.* [11] suggested an improved ion chromatographic method designed to overcome the fouling of resin-based ion chromatography columns by cefepime. They used a silica-based cation exchange column with a mobile phase comprising 0.006 mol/L nitric acid with 10 % acetonitrile. A similar mobile phase is used in the current USP assay method (0.01 mol/L nitric acid with 5 % acetonitrile). They reported a limit of detection of 0.15 µg/mL (3 ng NMP injected) and samples spiked with NMP at 0.3 % yielded quantitative recoveries. They specified that sample solutions (prepared in deionised water) must be tested within 30 min if stored at ambient temperature or no later than 6 h if kept below 5 °C.

Solid-phase extraction (SPE) is a well-established technique for isolating and concentrating analytes from a variety of sample matrices [12]. Its principal advantages over liquid-liquid extraction are the different modes of separation available, including ion exchange and mixed-mode (e.g. combined reverse phase and ion exchange) separations, and the fact that the chemistry of the separation is not limited by the requirement for the two phases to be immiscible. A brief study conducted in the early stages of this work established that classical liquid-liquid extraction was not a viable approach for the separation of NMP from cefepime because immiscible solvent systems could not be found in which the solubilities of the two compounds were sufficiently different and in which the rate of cefepime hydrolysis was adequately slow.

2. Experimental

2.1 Reagents

Cefepime hydrochloride USP was obtained from Orchid Chemicals and Pharmaceuticals Limited (Chennai, India). NMP (97 % pure), nitric acid (0.1 N for ion chromatography), methanesulfonic acid (0.1 N for ion chromatography), methanol and acetonitrile (both HPLC grade) were purchased from Sigma-Aldrich Limited (Gillingham, UK). HPLC grade water (> 18 MΩ resistivity) was produced as required using a Purelab Option reverse osmosis system (Elga Process Water, Marlow, UK). The SPE cartridges evaluated were Oasis Plus WCX (mixed-mode reverse phase/weak cation exchange, Part No. 186003518) and Oasis Plus HLB (non-ionic phase with both polar and non-polar functionality, Part No. 186000132), both supplied in a Sep-Pak format by Waters Limited (Elstree, UK).

2.2 Equipment

The ion chromatography system comprised a GP50 quaternary gradient pump, an AS50 autosampler, a LC30 temperature-controlled column compartment (which also housed the conductivity detector flow cell) and an ED50 electrochemical detector (Dionex Limited, Camberley, UK) set up in conductivity mode. The mobile phase was pressurised with nitrogen at 3 p.s.i. and degassed on-line under vacuum. The autosampler was fitted with a 10 µL sample loop which was completely filled during injection.

Development of the sample preparation method was performed using a Metrosep C4 4 mm i.d. x 250 mm cation exchange column with a matching guard column (Metrohm Limited, Runcorn, UK), maintained at 30 °C. The background conductivity of the eluent was reduced post-column by an electrolytic suppressor (model CSRS 300, Dionex, Limited, Camberley, UK) operating at 50 mA; the regenerant was HPLC-grade water at 1.0 mL/min. The mobile phase was 0.01 mol/L nitric acid with 5 % acetonitrile at 1.0 mL/min (consistent with USP test conditions). These parameters were used for sample preparation development work because the Metrosep column is less prone to fouling by strongly-retained organic species than resin-based ion exchange phases.

Once a sample preparation method that was capable of selectively removing cefepime from sample solutions had been developed, the following separation conditions were used. The analytical column was an IonPac CS17 4 mm x 250 mm, with a matching guard column (Dionex Limited, Camberley, UK). The mobile phase was 0.025 mol/L aqueous methanesulfonic acid at 1.0 mL/min. The operating current of the CSRS 300 electrolytic suppressor was increased to 100 mA owing to the greater ionic strength of the eluent. The suppressor was regenerated using recycled mobile phase. Other instrument parameters were as described previously.

A Chromeleon chromatography data system (version 6.8, Dionex Limited, Camberley, UK) was used for data acquisition and processing. Amber glass vials (8 mL) and matching Mininert caps were purchased from Supelco Limited (Brighton, UK) for use in preparing calibration standards. UV spectroscopic data were acquired using an Evolution 300 double-beam spectrometer and Vision Security data system (Thermo Scientific Limited, Hemel Hempstead, UK).

2.3 SPE Elution Profiles – Cefepime Hydrochloride

5.0 mg/mL and 10.0 mg/mL solutions of cefepime hydrochloride were prepared in 0.001 N nitric acid. A weakly acidic solution was used to prevent fugitive losses of NMP. Each SPE cartridge (WCX and HLB) was conditioned with 10 mL of methanol followed by two 2 mL washes with 0.001 N nitric acid. 1.0 mL of sample was then loaded onto each cartridge (n = 2) using a glass microlitre syringe with a Luer tip. Liquid that emerged from the outlet of the SPE cartridges during sample loading was collected in a clean glass vial for subsequent analysis. After sample loading, each cartridge was eluted sequentially with two 2 mL portions of 0.001 N nitric acid containing various amounts of acetonitrile (see Table 1). Each 2 mL eluent fraction was collected in a separate, clean glass vial. The amount of cefepime present in each fraction was determined after adjustment of sample volume by UV spectrometry at 265 nm in a 10 mm path-length quartz cuvette.

Elution Step	Eluent
1 & 2	HPLC-grade water
3 & 4	HPLC-grade water containing 5 % v/v acetonitrile
5 & 6	HPLC-grade water containing 10 % v/v acetonitrile
7 & 8	HPLC-grade water containing 20 % v/v acetonitrile

Table 1. Reagents used for elution profile study.

2.4 SPE Elution Profiles – NMP

A 50 µg/mL NMP solution in 0.001 N nitric acid was prepared by injecting 30.5µL (25 mg) of NMP through a butyl rubber septum into 5 mL of 0.1 N nitric acid in a screw-topped 8 mL vial fitted with a Mininert valve. This NMP concentration was equivalent to 1.0 % w/w with respect to cefepime hydrochloride in a 5.0 mg/mL cefepime solution. The Mininert valve was used to render the vial gas-tight during addition of NMP. The amount of nitric acid present in the vial was more than enough to protonate the NMP added (approximately two-fold stoichiometric excess) thus reducing the potential for evaporative loss. After shaking, this solution was transferred quantitatively, using a glass Pasteur pipette, to a 500 mL volumetric flask filled to the shoulder with HPLC-grade water. At each stage of the procedure, when liquids containing NMP were transferred between containers, the NMP solution was dispensed below the surface of the liquid in the receiving container. Each SPE cartridge was conditioned as described above for the evaluation of cefepime elution profiles, after which 1.0 mL of acidified NMP solution (50 µg NMP) was loaded onto each cartridge. Elution was performed as described previously for cefepime. The amount of NMP present in each eluent fraction was determined, after adjustment of sample volume, by ion chromatography using the conditions for the Metrosep C4 column described earlier.

2.5 Method Validation

2.5.1 Accuracy and Precision

Samples for evaluation of accuracy and precision were prepared by spiking a 5.0 mg/mL solution of cefepime hydrochloride in 0.001N nitric acid with 0.1 % w/w, 0.3 % w/w and 0.8 % w/w NMP. An unspiked 5.0 mg/mL cefepime hydrochloride solution was also prepared. Six extractions at each concentration (including the unspiked solution) were made by loading 1.0 mL of sample onto an Oasis HLB cartridge that had been conditioned with methanol and nitric acid as described earlier. Each cartridge, which was used once only, was eluted with 2 x 2 mL of 0.001 N nitric acid into a 5 mL volumetric flask. The flask was made to volume with 0.001 N nitric acid, the contents mixed and a portion of sample transferred to a polypropylene autosampler vial. Samples were injected using the conditions for the Dionex CS17 column described earlier, and quantified using calibration standards prepared by diluting NMP to 10 µg/mL with 0.001 N nitric acid (equivalent to 1.0 % w/w NMP relative to cefepime hydrochloride).

2.5.2 Linearity and Sensitivity

Solutions containing 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL and 10 µg/mL NMP in 0.001 N nitric acid (covering the range 0.2 % w/w to 1.0 % w/w NMP relative to cefepime) were injected and the resulting NMP peak areas plotted against concentration. An unweighted straight line was fitted to the data points without including or forcing an origin point. The method's limit of detection and limit of quantitation were calculated from the NMP response in an unspiked sample on the basis of the concentration required to produce peaks with a signal:noise ratio of 3:1 and 10:1 respectively, as proposed by ICH Q2 (R1) [13].

2.5.3 Specificity

The method's specificity was evaluated by overlaying a reagent blank chromatogram (derived from a 1.0 mL portion of 0.001 N nitric acid that had been taken through the entire extraction procedure), and unspiked cefepime hydrochloride sample chromatogram, and a calibration standard chromatogram.

2.5.4 Sample Stability

Unspiked cefepime hydrochloride sample extracts that had been prepared for accuracy and precision evaluation were split into two groups. One set of samples (n = 3) was stored at 4 °C and the remaining three samples were left on the laboratory bench (approximately 20 °C to 25 °C). Each sample was retested after 3 days and 6 days.

3. Results

3.1 Elution Profiles

Cefepime elution profiles on the SPE phases evaluated are shown in Figure 3 (5.0 mg/mL cefepime) and Figure 4 (10.0 mg/mL cefepime). Corresponding data for NMP are shown in Figure 5. Elution steps on the horizontal axis correspond with experimental conditions in Table 1.

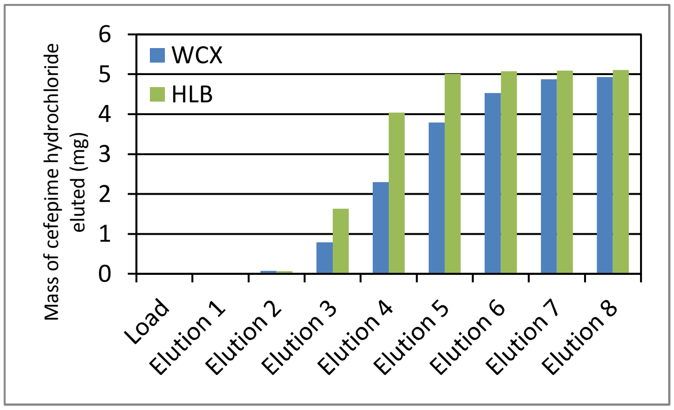

Figure 3. Cumulative elution profiles from SPE cartridges loaded with 1.0 mL of a 5.0 mg/mL solution of cefepime hydrochloride in 0.001 N nitric acid.

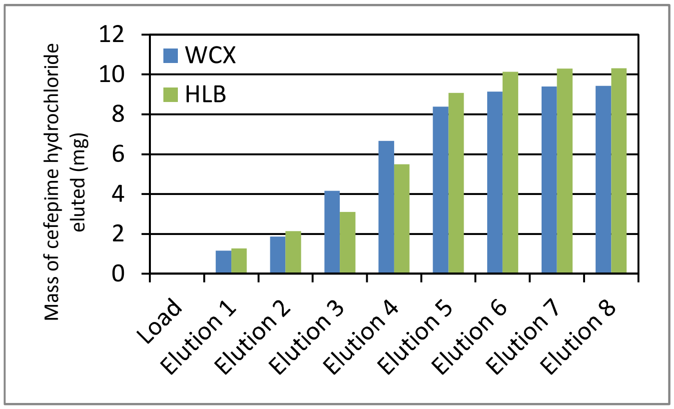

Figure 4. Cumulative elution profiles from SPE cartridges loaded with 1.0 mL of a 10.0 mg/mL solution of cefepime hydrochloride in 0.001 N nitric acid.

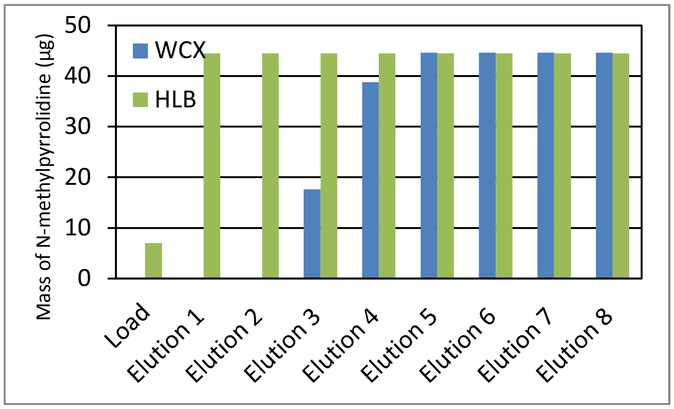

Figure 5. Cumulative elution profiles from SPE cartridges loaded with 1.0 mL of a 50.0 µg/mL solution of N-methylpyrrolidine in 0.001 N nitric acid.

3.2 Method Validation

Accuracy and precision results are summarised in Table 2 and the linearity graph is shown in Figure 6. The y-intercept in Figure 6 is 0.98 % relative to the detector response at 0.3 % w/w NMP (3.0 µg/mL) and the RSD of response ratios is 1.28 %. The overlay specificity chromatogram is shown in Figure 7 and sample stability data are presented in Table 3 and Figure 8. The method's limit of detection and limit of quantitation were 0.033 % w/w NMP and 0.11 % w/w NMP respectively.

	Result (% w/w NMP)			
Replicate	Unspiked	0.1 %	0.3 %	0.8 %
1	0.10	0.19	0.41	0.87
2	0.11	0.21	0.41	0.94
3	0.12	0.23	0.43	0.95
4	0.13	0.25	0.44	1.00
5	0.13	0.24	0.44	0.98
6	0.15	0.24	0.44	0.99
Mean result (% w/w)	0.12	0.23	0.43	0.96
RSD (%)	14.6	9.8	3.4	5.0
Recovery (%)	N/A	110	103	105

Table 2. Accuracy and precision results.

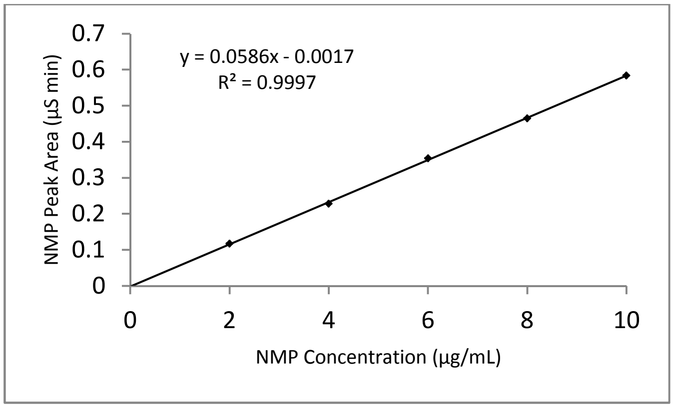

Figure 6. Linearity graph.

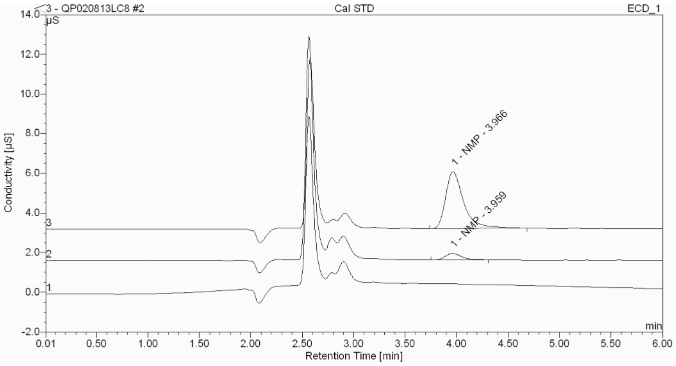

Figure 7. Overlay specificity chromatogram. From bottom: reagent blank; unspiked cefepime hydrochloride sample; calibration standard.

	Mean Result (% w/w NMP; n = 3)	
Time	Refrigerated	Ambient
Initial	0.12	
3 days	0.16	0.43
6 days	0.2	0.68

Table 3. Sample stability results.

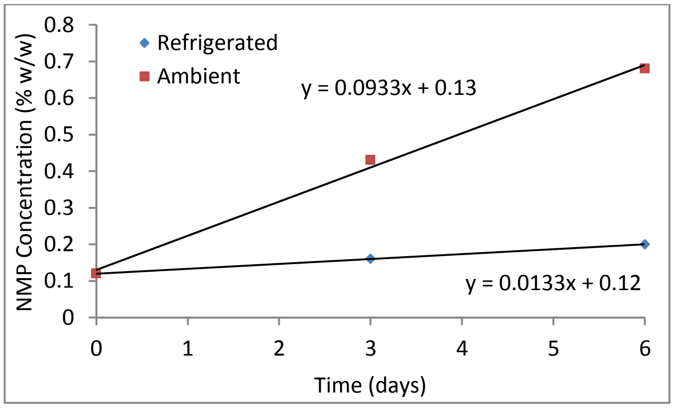

Figure 8. Sample stability graph.

4. Discussion

4.1 Method Development

The polymeric SPE phases used for this study were chosen because of their ability to retain polar compounds and the absence of silanol functionality which might have caused a tailing elution profile for NMP. The Oasis HLB phase, which was ultimately selected for use in method validation, is a copolymer comprising divinylbenzene and N-vinylpyrrolidone. The WCX mixed-mode weak cation-exchange phase has the same polymeric base as the HLB phase, but is functionalised with carboxylic acid. A strong cation-exchange phase (MCX), analogous to the WCX phase but modified with sulphonic acid, was also evaluated during the early stages of the study, but recovery of NMP from this phase would have required strongly basic conditions, the consequences of which could have been evaporative loss of the analyte and a significant increase in the rate of cefepime hydrolysis.

In a purely aqueous eluent, very little cefepime hydrochloride was eluted from SPE cartridges loaded with 5 mg of drug (Figure 3). At 10 mg loading, however, approximately 20 % of the cefepime on the cartridges was eluted by the end of the second aqueous wash (Elution 2 in Figure 4). It is clear that the capacity of both the HLB and WCX phases to retain cefepime was exceeded at the higher loading level. Figure 5 demonstrates that all the available NMP is eluted from the HLB phase at the end of the first aqueous wash (2.0 mL of 0.001 N nitric acid), whilst the WXC phase retained NMP more strongly. This difference in retention behaviour between cefepime and NMP on the Oasis HLB phase forms the basis for their separation.

4.2 Method Validation

4.2.1 Method Performance

Accuracy, precision and linearity data (Table 2 and Figure 6) would meet the acceptance criteria recommended for an impurity assay by Crowther *et al.* [14]. Table 4 shows a comparison between validation results and recommended acceptance criteria relative to the method's limit of quantitation (0.11 %). The method meets the usual requirements of specificity: no interfering impurities are observed in the reagent blank chromatogram. The method's sensitivity is more than adequate to control NMP at 0.3 % relative to cefepime hydrochloride.

Parameter	Validation Result	Acceptance Criterion
Accuracy (% recovery)		
0.1 % spiking level	110 %	70.0 % to 130.0 %
0.3 % spiking level	103 %	70.0 % to 130.0 %
0.8 % spiking level	105 %	70.0 % to 130.0 %
Unspiked samples	14.6	≤ 25.0 %
0.1 % spiking level	9.8	≤ 15.0 %
0.3 % spiking level	3.4	≤ 15.0 %
0.8 % spiking level	5.0	≤ 15.0 %
Linearity		
R^2	0.9997	> 0.98
y-intercept relative to impurity limit (0.3 %)	0.98	± 15.0 %
RSD of response ratios	1.28 %	≤ 10.0 %
Visual assessment	Linear	Linear

Table 4. Evaluation of validation results.

4.2.2 Sample Stability

The principal aim of this study was to improve the stability of sample solutions by reducing the amount of cefepime present in final extracts. Slope values in the sample stability graph (Figure 8) suggest that NMP is produced at the rate of approximately 0.01 %

per day in refrigerated samples and 0.09 % per day under ambient conditions following preparation by SPE. In the study conducted by Dionex [10], the NMP concentration in samples prepared in deionised water increased by approximately 2 % over three days under ambient conditions (0.7 % per day). An increase of 0.07 % was observed in samples stored at 4 °C for 1 day. These data suggest that sample stability is improved by a factor of 7 using the SPE method developed in this study. Also, because the majority of cefepime is excluded from the final sample solution, it is unnecessary to wait for cefepime to elute from the column before injecting the next sample. The run time in this study (6 min) compares favourably with that used in other ion chromatographic methods for NMP in cefepime, e.g. 30 min [10] and 28 min [11]. This five-fold improvement in sample through-put, combined with the seven-fold improvement in stability, means that it is now possible to prepare a number of samples simultaneously for automated injection instead of being obliged to inject samples immediately after preparation, as at present [5]. The use of a refrigerated autosampler would mean that sample solutions would be stable for a number of days. If kept under ambient conditions, the authors recommend that samples should be injected within 12 hours of preparation. This advice is consistent with the limits proposed by Crowther *et al.* for the stability of analytical solutions [14].

5. Conclusions

The sample preparation method proposed in this study for the assay of NMP in cefepime hydrochloride significantly improves the stability of sample solutions. In addition to measuring NMP concentrations in solid-state cefepime, it could be used to monitor the stability, under various conditions, of aqueous cefepime solutions, e.g. Cefepime for Injection USP. Validation data are of a sufficiently high quality to support the use of this method for regulatory purposes.

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Notes and references

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