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Rapid Analysis of N-methylpyrrolidine in Cefepime with Thermal Desorption Ion Mobility Spectrometry

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Abstract

N-methyl-pyrrolidine (NMP) a potential impurity in the cephalosporin antibiotic cefepime is analysed using a rapid thermal desorption – ion mobility spectrometry (TD-IMS) method. The thermal desorption approach is shown to be capable of rapidly extracting NMP from the cefepime at 80 °C without causing thermal degradation of the cefepime. The ion mobility method has an analysis time of 1 minute and demonstrates good linearity over a range from 0.3 – 3.0 $\mu\text{g ml}^{-1}$ of NMP, with limits of detection and quantification of 0.056 and 0.1875 $\mu\text{g ml}^{-1}$ respectively. The developed method was applied to the analysis of a cefepime sample and determined that NMP was present in a cefepime sample at a level of 0.0376% with a percentage relative standard deviation (n=6) of 3.2%. This was compared with a LC-UV method which was in close agreement measuring NMP at 0.0384% in the cefepime sample with a percentage RSD (n=6) of 5.7%. These results show that the TD-IMS method gives comparable data to the established LC methods and demonstrates the potential of TD-IMS for rapid measurement of volatile compounds in pharmaceutical matrices.

Introduction

Impurities in drug active pharmaceutical ingredients (APIs) may arise from a range of different sources, including starting material residues, unwanted side products of the synthetic process and from degradation of the API itself. Since these impurities may be toxic or have unknown effects on the human body, their presence is of great concern to both the pharmaceutical industry and regulatory bodies to ensure that pharmaceutical products are safe for use. To limit the risk that impurities pose to the consumer there are stringent regulations imposed that require drug APIs to be tested to ensure that impurities are below certain levels, if the level of impurity is above the control level then the API is deemed not safe for human consumption and cannot be used in drug formulations [1].

Cefepime (I) is a fourth generation cephalosporin antibiotic (Figure 1). It has been used widely as a broad spectrum antibiotic and is effective against both Gram-positive and Gram-negative bacteria [2], [3] and has been shown to be particularly effective against Gram-negative species compared to other cephalosporin antibiotics. [4] Cefepime is therefore often employed for the treatment of severe systemic and nosocomial bacterial infections including pneumonia,[5] bone and soft tissue infections [6] and febrile neutrophenia.[7] The antibiotic is usually formulated as the hydrochloride salt, with L-arginine added to control the pH between pH 4-6 where the cefepime is most stable.[8] The lyophilised powder is then reconstituted and administered either intravenously or intramuscularly as a short infusion (<30 minutes) or as a continuous infusion (>24 hours).[9]

A disadvantage of cefepime is that it is intrinsically unstable and will slowly degrade, even when stored at 4 °C. Viaene *et. al.* (2002) reported that when stored at temperatures of 30 °C and higher, aqueous solutions of cefepime, exhibited a colorimetric change from light yellow to dark red.[10] This colour change was accompanied by a significant increase in the pH of the solution, thus, suggesting the presence of alkaline degradation products. Fubara *et. al.* studied buffered solutions of cefepime under different temperature conditions and showed that temperature played a significant role in the rate of degradation, with 10% of the cefepime in solutions degrading after ~2 days [11]. The instability of cephalosporins in aqueous media is as a result of the highly reactive beta lactam ring, which is susceptible to base catalysed hydrolysis.[10] Degradation studies of cefepime conducted by Sprauten *et al* (2003) indicated that ring opening of the beta lactam moiety occurred initially, followed by cleavage of the C3 substituent, *N*-methylpyrrolidine (NMP, II), an acyclic tertiary amine.[4]

As NMP is a recognised degradation product, the United States Pharmacopoeia (USP) states that in cefepime for injection the limit of *N*-methylpyrrolidine 'must not exceed more than 1.0% w/w relative to cefepime [12] and in cefepime hydrochloride 'must not exceed more than 0.3% w/w relative to cefepime.[13]

The analysis of NMP in cefepime poses a challenge to the analyst due to the labile nature of cefepime in aqueous solution. Liquid chromatographic separations have been previously used to determine NMP in cefepime using both reversed phase [10,14] and ion chromatography [15] methodologies. Separation of NMP from cefepime has also been accomplished using capillary electrophoresis. [16] However, a significant issue with these techniques is the sample run times, all of these liquid phase techniques require a run time of several minutes to obtain a result. Since cefepime is unstable in solution the more rapidly a sample can be

analysed the better, to avoid giving an artificially high level of NMP and 'false positive' results. Gas chromatography has also been explored as alternative, however for GC analysis NMP needs to be extracted from the cefepime before analysis to prevent cefepime breaking down into NMP in the injector which complicates the sample preparation. [17] The lability of the NMP ion in the gas phase is also shown to be a problem, as cefepime readily fragments to give NMP in the low pressure region of the mass spectrometer interface giving an artificially high level of NMP. A novel approach to this problem was described by Smith *et al.* who explored using a differential ion mobility separation hyphenated to mass spectrometry to determine NMP in cefepime. [18] Here a cefepime solution was infused directly into the mass spectrometer without any chromatographic separation. Using the differential mobility separation it was possible to differentiate between NMP present in the sample and NMP produced by fragmentation of cefepime in the mass spectrometer interface enabling a more accurate determination of NMP in the cefepime sample with an analysis time of 2 minutes per sample.

Ion mobility spectrometry (IMS) offers a very rapid alternative to using a chromatographic separation. IMS separations typically take place on the millisecond timescale enabling very fast analysis,[19] and can also be hyphenated to mass spectrometry to give additional mass-to-charge information. As a standalone instrument, IMS has been primarily used for two applications in pharmaceutical analysis. The first application is cleaning validation, and a number of groups have published work where IMS has been used to check the surfaces of process equipment to ensure that contaminants from previous syntheses and cleaning products are not present before starting a new reaction.[20] IMS is particularly attractive here since swabs can be taken from the surface and then thermally desorbed enabling rapid analysis and potential identification of contaminants by their drift time.[21] The second application is the detection of active pharmaceutical ingredients and a number of drug substances both pharmaceutical and illicit, have been characterized by IMS. Opiates, including heroin, fentanyl, codeine and morphine, have been studied by IMS.[22] In addition, the implementation of IMS instruments in the workplace has provided a non-invasive technique for environmental monitoring. This monitoring is critical in the pharmaceutical industry to ensure that workers exposure to APIs is kept to a minimum. [23] Desorption electrospray ionization (DESI) has been used with a standalone IMS system by Harris and coworkers [24] to screen antimalarial drugs. Roscioli *et al.* also coupled DESI with a standalone drift tube IMS system enabling the analysis of codeine, Ritalin and Effexor standards, however this study determined that further work was required to fully enable studies of pharmaceutical formulations.[25] IMS has been used to screen for adulterants in weight loss supplements and a study by Dunn *et al.* found that supplements were adulterated with a number of undeclared APIs including erectile dysfunction drugs and anti-depressants.[26]

For the detection of drug impurities, ion mobility separations have been used in conjunction with mass spectrometry, the additional dimension offered by ion mobility proving useful in separating active pharmaceutical ingredient ions from excipient ion species such as polyethylene glycols. [27, 28, 29] Standalone ion mobility has also been used to determine trace impurities in cosmetic intermediates. This approach used thermal desorption to determine the level of a volatile impurity (dimethylaminopropylamine DMAPA) in stearamidopropyldimethylamine which is an intermediate used in the industrial production of cosmetic oils. This

technique was shown to be able to effectively quantify DMAPA over a range from 0.030-0.500 µg/mL.[30] The use of thermal desorption as demonstrated here by Zamora *et. al.*, has significant potential for analyzing NMP in a cefepime sample. NMP is very volatile with a boiling point of 81 °C and can be readily thermally desorbed, while cefepime as a quaternary ammonium ion is very involatile and will not thermally desorb. The work presented here explores using thermal desorption to rapidly extract NMP from a cefepime sample prior to rapid (1 minute) IMS analysis.

Materials and Methods

Thermal Desorption - Ion Mobility Spectrometry (TD-IMS)

Sample Preparation

N-methyl pyrrolidine standard solutions were prepared by dissolving 0.1 g of NMP (Sigma-Aldrich, Gillingham UK) in 50 ml of dichloromethane (DCM) to make a 2 mg/ml stock solution. This stock solution was then diluted further with DCM to produce a 10 µg/ml standard solution which was then used to create a series of standards over a range from 0.3 to 10 µg/ml (0.3, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 10 µg/ml).

Cefepime hydrochloride (Quay Pharmaceuticals) samples were prepared by taking 15 mg of cefepime into a 30 ml glass scintillation vial and adding 10 ml of DCM. The sample was manually shaken for 2 minutes and then 1 ml was passed through a 0.45 µm PVDF syringe filter to remove any undissolved cefepime and other particulates from the solution prior to analysis.

TD-IMS analysis method

TD-IMS samples were analysed using a Smiths Detection Ionscan LS linear ion mobility spectrometer. Both samples and standard solutions were analysed in surface analysis mode where the autosampler picks up a volume of sample, deposits it on a PTFE surface, and then uses a heated anvil with a nitrogen gas flow to desorb the volatile analyte from the surface into the ion mobility spectrometer for analysis. This enables the involatile cefepime to be left behind on the surface while the volatile NMP is sampled.

All samples were analysed in the positive ion mode using 300 ml/min nitrogen as a drift gas. Instrumental conditions were as follows; drift heater 220 °C, inlet heater 280 °C, desorber temperature 80 °C, shutter grid width 0.2 ms, scan period 25 ms, analysis duration 60 s. 1 µl of sample was injected onto a PTFE membrane for all of the analyses performed. Ion mobility spectra were obtained in the positive ion mode using nicotinamide as a carrier gas dopant. The use of nicotinamide as a dopant has been shown to reduce the amount of fragmentation observed in the spectrum compared to hydronium ions and also provides an internal standard of known mobility to compare and reference with analyte peaks.[31]

Complementary HPLC-UV and LC-MS experiments

LC-UV analysis method and sample preparation

A stock solution of 10 mg/ml NMP was prepared by dissolving 100 mg of NMP into 10 ml of 10:90 methanol: water + 0.05% formic acid, this was then shaken for 5 minutes to ensure complete dissolution. 100 µl of this solution was then diluted into 100 ml of 10:90 methanol:water + 0.05% formic acid to give a solution with a concentration of 10 µg/ml. Standard solutions were prepared over a range from 0.4 µg/ml to 4 µg/ml (0.4, 0.5, 1, 2, 3 and 4 µg/ml) by diluting this solution down with 10:90 methanol:water + 0.05% formic acid. Cefepime hydrochloride samples were prepared as follows, the cefepime hydrochloride bottle was taken out of the freezer and left to stand for 15 min to acclimatize to room temperature. Measurement precision was calculated by preparing six repeat samples of 1.5 mg/ml cefepime hydrochloride in 10:90 methanol:water + 0.05% formic acid. Cefepime samples were then sonicated for 5 minutes to ensure full dissolution of cefepime in the 10:90 methanol:water + 0.05% formic acid. All cefepime samples were analysed immediately after preparation to prevent the formation of excess NMP from cefepime degradation in solution. HPLC-UV analysis was performed on an Agilent 1200 series LC system, separation took place on an Agilent Eclipse XDB-C₈ column using an isocratic method of 10:90 methanol:water + 0.05% formic acid at a flow rate of 1.0 ml/min. 10 µL of sample was injected and the column was maintained at 25 °C. NMP was monitored at a wavelength of 194 nm

HPLC-UV Stability study

A HPLC-UV experiment was performed to determine the stability of cefepime in solution. A scanning UV experiment revealed that NMP has its absorbance maxima at 194 nm and analysis of NMP standards demonstrated a linear relationship between NMP concentration and absorbance at this wavelength. The stability study was performed by preparing 10 ml of a 1.0 mg/ml cefepime solution in 10:90 methanol:water + 0.05% formic acid. Following dissolution of the cefepime, the solution was aliquoted out into 1 ml aliquots, which were then left at room temperature for different time periods before HPLC-UV analysis. The 1.0mg/ml cefepime samples were then sequentially analysed at 0, 30, 60, 90, 120, 150, 240 and 300 minutes post preparation. The cefepime samples were then analysed using the LC method described above.

UPLC-MS Analysis method

Cefepime samples were analysed using a Waters Synapt HDMS quadrupole time-of-flight mass spectrometer interfaced to a Waters Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Manchester, UK). An isocratic LC method was used for the separation of NMP from cefepime hydrochloride. Samples were injected on to a Agilent Eclipse XDB-C₈ column (4.6 mm x 150 mm, 5 µm particle size) at a flow rate of 1 ml/min with the solvent composition at 10:90 methanol: water + 0.05% formic acid, the column was maintained at a temperature of 25 °C and the injection volume was 10 µL, the eluent flow was split post column with ~200 µl/min going into the mass spectrometer ion source and ~800 µL/min diverted to waste. Total run time was 5 minutes.

All mass spectrometric experiments were performed using positive ion mode electrospray ionisation, a mass range from m/z 80 to 650 was monitored, source conditions were as follows; capillary voltage 3.0 kV, sample

cone voltage 20.0 V, extraction cone voltage 4.0 V, source temperature 120 °C, desolvation gas temperature 350 °C, cone gas flow 50 l/hr, desolvation gas flow 300 l/hr.

Results and Discussion

Thermal Desorption - Ion Mobility Spectrometry

Initial TD-IMS experiments were performed by injecting 1 µl of NMP standards onto a teflon membrane and thermally desorbing the NMP into the IMS system. The ion mobility spectra presented in Figure 2 show that only a single large peak is observed for nicotinamide (drift time 9.675 ms, $K_0 = 1.878 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$) with an otherwise very clean spectrum when a blank DCM injection is performed. When an NMP standard in methanol is injected onto the Teflon membrane and desorbed a second peak is observed with a drift time of 8.700 ms ($K_0 = 2.089 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$) which correlates to protonated NMP. Only the NMP monomer ion was observed at all of the concentrations analysed in this work and no dimer formation was noted.

The thermal desorber temperature was then optimised. As a quaternary amine, cefepime has very low solubility in DCM, however in practice it was found that some cefepime always makes it on to the Teflon membrane, either from fine particles in solution (despite filtering with a 0.45 µm PVDF filter) or from small amounts of dissolved cefepime in solution. An experiment was performed over a range of different thermal desorber temperatures and showed that the amount of NMP detected increased dramatically at higher temperatures and could be detected in repeated runs of the same sample. These observations imply that cefepime on the membrane was thermally degrading to release NMP. By reducing the thermal desorber temperature to 80 °C it was observed that a strong peak for NMP could be detected in the first run but was absent in subsequent analyses from the same sample, indicating that all of the 'free' NMP had been desorbed and no additional NMP was being formed from degradation of cefepime on the surface.

Standards were then analysed over a range from 0.3 to 10 µg ml⁻¹ NMP (correlating to 0.3 – 10 ng of NMP loaded onto the Teflon membrane). A linear response was observed between 0.3 – 3 µg ml⁻¹, at the higher concentrations the calibration graph was observed to deviate from the line and increase non-linearly (Figure 3). Limits of detection and quantification were calculated using the signal-to-noise ratio obtained for the 0.3 µg ml⁻¹ standard. The obtained signal to noise ratio of 16:1 gave limits of detection and quantification of 0.056 and 0.1875 µg ml⁻¹ respectively. These data show that the TD-IMS approach has a relatively narrow linear dynamic range of just over a single order of magnitude. However, for this type of application where samples need to be compared to a control level this is not a major problem as long as samples are prepared at the appropriate concentration so that the control level is in the linear dynamic range.

TD-IMS Cefepime analysis

Six 1.5 mg ml⁻¹ cefepime samples were prepared as described in the methods section. These were then analysed by TD-IMS. NMP was detected in all of the samples. Figure 4 shows the NMP response obtained from a 1.5 mg ml⁻¹ cefepime sample. The average amount of NMP detected in the replicate cefepime samples was

0.0376% with a % relative standard deviation calculated from the 6 measurements of 3.2% (Table S1). The data obtained is significantly below the control level of 0.3% demonstrating that the TD-IMS approach possesses the requisite sensitivity for the analysis, and that the cefepime hydrochloride sample is suitable for use.

LC-UV and LC-MS analysis

LC-UV analysis was performed to cross validate with the TD-IMS method and compare the levels of NMP detected in the cefepime sample with the two methods to see if the result achieved using TD-IMS was in agreement with that obtained with the established methodology. Initially separations were performed on a C18 column, however the peak shapes obtained were unsatisfactory for quantitative analysis so a C8 Zorbax column was used which gave significantly improved peak shape and shorter retention times. The LC-UV trace for a cefepime sample is shown in Figure 5a, cefepime gives a very strong response at 2.30 minutes with NMP identified at a retention time of 1.37 minutes. There were some additional background responses present in the LC-UV trace, so as a confirmatory step the analysis was also performed as an LC-MS experiment using the same column and method on a Waters Synapt HDMS mass spectrometer and compared with a NMP standard. The LC-MS chromatogram shown in Figure 5b shows a similar profile to the LC-UV trace and the mass spectrum of the peak at 1.32 minutes (Figure 5c) is observed to give an intense peak at m/z 86.1 which correlates to the $[M+H]^+$ ion of NMP, positively identifying this peak as NMP. The mass spectrum obtained from the broad cefepime peak (Figure 5d) shows the expected $[M]^+$ ion of cefepime at m/z 481.2 and a large number of fragment ions at lower m/z including the NMP $[M+H]^+$ ion at m/z 86.1, m/z 396.1 which is $[Cefepime-NMP+H]^+$ and m/z 324.1 which is $[Cefepime-NMP-C_2O_3+H]^+$ [32]. These data show how labile cefepime is in the mass spectrometer interface and demonstrates that it is not possible to accurately determine NMP in cefepime using MS analysis without an associated separation technique.

Degradation study

Since cefepime degrades in common reverse phase solvents a study was performed to determine how rapidly cefepime degrades in solution to ensure this does not impact the data obtained with the LC method used. Figure 6 shows the level of NMP measured from cefepime samples left to degrade in the 10:90 methanol:water + 0.05% formic at room temperature for various periods of time between 0 and 300 minutes. This figure shows that there is relatively little difference between $t=0$ minutes and $t=30$ minutes, but that after this period the level of NMP increases significantly, the NMP level at $t=60$ minutes is ~15% higher than it is at $t=0$ minutes and by $t=300$ minutes the NMP level is approximately 33% higher than at $t=0$ minutes. These data show that cefepime is stable in solution for up to 30 minutes and that the 5 minute LC runs used in this experiment should not be significantly affected by degradation of cefepime in solution, providing that cefepime samples are analysed immediately after preparation and not allowed to degrade.

LC-UV cefepime analysis

A calibration for NMP was set up using NMP standard solutions between 0.4 to 4.0 $\mu\text{g ml}^{-1}$ to cover a similar range as that used in the TD-IMS experiments. The calibration graph obtained (Figure S1) was linear across the entire range with a correlation coefficient of >0.99 . The amount of cefepime detected in the replicate

cefepime samples was 0.384% with a relative standard deviation calculated from the 6 measurements of 5.7% (Table S1). This demonstrates a close agreement with the TD-IMS analysis and this was confirmed by performing a two tailed paired t-test between these two sets of data. This gave a p-value of 0.4663 suggesting there is no statistically significant difference between the two techniques and confirms that the TD-IMS method gives a statistically similar result to the gold standard LC-UV technique. Comparison of the two methods (Table 1) shows that TD-IMS can yield a similar result in 1 minute compared to 5 minutes and with slightly better precision of 3.2% RSD vs 5.7% RSD.

Conclusions

The data presented show that the ion mobility test developed for analysing NMP in cefepime hydrochloride samples is capable of detecting NMP at levels below the control level with a lower limit of quantification of 0.013% from a 1.5 mg/ml solution of cefepime (equivalent to 187.5 ng/ml of NMP). The percentage RSD precision of the ion mobility experiment is 3.2%, which is acceptable for a fast screening technique for rapid drug batch testing where suspect samples can be flagged up for further investigation. The main limitation of the IMS method is the limited quantitative range of the technique ($0.1875 - 3 \mu\text{g ml}^{-1}$ NMP) meaning that samples must be prepared at the correct concentration to ensure a quantitative response is obtained. The thermal desorption temperature must also be carefully regulated, since at higher temperatures additional NMP may be produced via thermal degradation of cefepime. At a desorber temperature of 80 °C all of the free NMP is desorbed, while cefepime is not observed to degrade to produce any additional NMP. These data show that the fast IMS method is fit for purpose for NMP screening in cefepime. The IMS method is fast and straightforward with sample analysis times of 1 minute compared to the 5 minute run time plus autosampler cycle time of the LC-UV and LC-MS methods. Given the relative instability of cefepime in solution this will reduce the likelihood of 'false positive' results where cefepime has degraded in solution to give NMP. These data demonstrate that IMS has significant potential as a fast and rugged analyser for determining volatile compounds in pharmaceutical matrices.

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Figures

Figure 1: Degradation pathway of cefepime (I) forming N-methylpyrrolidine (II).

Figure 2. TD-IMS spectrum of a) DCM blank b) 5 $\mu\text{g/ml}$ NMP standard in DCM

Figure 3. Calibration curve for NMP obtained using TD-IMS in surface analysis mode

Figure 4. TD-IMS spectrum of a filtered extract from a 1.5 mg/ml solution of cefepime hydrochloride

Figure 5. A: HPLC-UV chromatogram (194 nm) from a 1 mg/ml cefepime hydrochloride sample B: LC-MS extracted ion chromatogram (m/z 86.1) from 1.5 mg/ml cefepime hydrochloride sample C: mass spectrum from the NMP peak at 1.32 minutes D: mass spectrum from the cefepime peak at 2.51 minutes.

Figure 6. Graph showing the formation of NMP from 1 mg/ml of cefepime hydrochloride dissolved in 10:90 methanol:water +0.05% formic acid over time, monitored using HPLC-UV (194 nm).

Table 1. Comparison between TD-IMS and HPLC-UV analysis of NMP in cefepime

	Mean amount of NMP detected in cefepime sample % (n=6)	Percentage RSD	Run time
TD-IMS	0.0376	3.2%	1 minute
HPLC-UV	0.0384	5.7%	5 minutes

Figure S1. HPLC-UV NMP calibration curve.

Table S1. TD-IMS and HPLC-UV quantification data of NMP in cefepime

Method	Percentage NMP in cefepime sample (%)						Average	Std Dev	% RSD
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6			
LC-UV	0.0425	0.0372	0.0393	0.0373	0.0373	0.0368	0.0384	0.0022	5.73%
TD-IMS	0.0378	0.0373	0.0373	0.0374	0.0360	0.0397	0.0376	0.0012	3.19%