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# Predictive strategy for the rapid structure elucidation of drug degradants[1]

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**Abstract**

Structural information on drug degradants and impurities can serve to accelerate the drug discovery and development cycle. Traditional structure elucidation methodologies for obtaining this information are often slow and resource-consuming; therefore, LC/MS profiling and LC/MS/MS substructural analysis methodologies have been developed to rapidly and accurately elucidate structures of impurities and degradants. This work is a further development of methodologies used for the elucidation of degradation products of paclitaxel [K.J. Volk et al., Proc. 9th AAPS Ann. Meeting, 1994, p. 29]. In this study cefadroxil was used as a model compound for the evaluation of a predictive strategy for the production and elucidation of impurities and degradants induced by acid, base, and heat, using LC/MS and LC/MS/MS profiling methodology, resulting in an LC/MS degradant database which includes information on molecular structures, chromatographic behavior, molecular weight, UV data, and MS/MS substructural information. Furthermore, libraries such as this can provide a predictive foundation for pre-clinical development work involving drug stability, synthesis, and monitoring.

*Keywords:* Database; Degradant; Impurity; LC/MS; LC/MS/MS; Profile

## 1. Introduction

Increasing demands on the pharmaceutical industry to quickly develop drug candidates for clinical therapy have challenged scientists to expedite the drug discovery and development process. As a result, significant change has occurred in the way pharmaceutical companies are synthesizing and identifying new lead drug candidate com-

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pounds. This change has been brought about by the use of automated synthesis and/or combinatorial synthesis methods. These methods allow for the simultaneous, milligram-scale production of hundreds of new compounds. These new methods are projected to increase the number of drug candidates synthesized by a factor of ten. If these targets are to be achieved, analytical methods which allow for rapid and efficient characterization of these new compounds and their impurities and degradants are essential. Since traditional structure elucidation methodologies for obtaining information about trace level components are time-consuming and resource-intensive, a proactive predictive analytical strategy has been developed which incorporates forced degradation of bulk drug or formulated product followed by LC/MS profiling and LC/MS/MS substructural analysis methodologies to rapidly and accurately elucidate structures of impurities and degradants. These methodologies extend LC/MS profiling methods for the rapid and systematic elucidation of drug metabolites in physiological samples [2] and natural products in crude extracts [3–5]. Analyses combine HPLC separation conditions on-line with an electrospray MS interface to obtain full-scan mass spectra and with tandem mass spectroscopy to obtain structural information. In this way, structural and substructural data for trace components in mixtures are obtained rapidly and systematically without prior fractionation. When obtained prior to the development phase this library of degradants and impurities provides a foundation for future work including the selection of drug candidates, the synthesis of degradants for monitoring purposes, the rapid identification of degradants in samples from development and as a diagnostic tool for the rapid elucidation of new degradation products.

Due to the high sensitivity of mass spectrometry this method was particularly advantageous for application to samples of limited quantity, a situation frequently encountered in pharmaceutical discovery and development research. In addition, LC/MS and LC/MS/MS techniques facilitated the rapid analysis of samples based on the integration of bench-scale mixture analysis methodology (scale-up, fractionation and individual spectroscopic analysis) into one on-line instrumental technique. This predictive methodology was evaluated using the model compound cefadroxil, an orally effective cephalosporin derivative (Fig. 1). Detailed structural information was obtained for impurities and degradants of an expired lot of bulk cefadroxil treated under forced degradation conditions (heat, acid, and base). Treatment of the expired bulk lot of cefadroxil under forced degradation conditions afforded the enhancement of many impurities and degradants as well as the formation of several additional ones. For example, treatment under basic conditions enhanced the presence of cefadroxil isomers, the methyl ester, and cefadroxil +16 Da. These conditions also resulted in the formation of the hydrolyzed lactam, 7-epi cefadroxil, and two piperazinedione isomers.

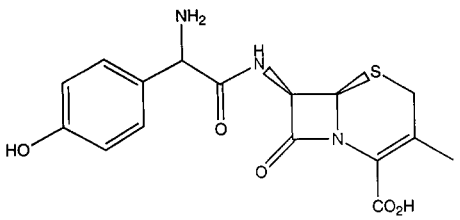


Fig. 1. Structure of cefadroxil.

## 2. Experimental

### 2.1. Sample preparation

An expired bulk lot of cefadroxil monhydrate was subjected to a variety of forced degradation conditions as indicated in Table 1.

Table 1
Cefadroxil degradation conditions

| Condition | Reagent | Reagent concentration (N) | Time (H) | Temperature (°) |
|---|---|---|---|---|
| Acid | HCl | 0.3 | 2 | 24 |
| Base | NaOH | 0.01 | 1.5 | Ambient |
| | | 1 | 0.5 | Ambient |
| Heated solid | | | 6 | 140 |
| Heated solution | $H_2O$ | | 8 | 40 |

Table 2
Profile library of cefadroxil degradant and impurity products

| | | | |
|---|---|---|---|
| 1 | 0.15 | 379 | Cefadroxil + 16 Da |
| 2 | 0.20 | 379 | Cefadroxil sulfoxide isomer |
| 3 | 0.26 | 379 | Cefadroxil sulfoxide isomer |
| 4 | 0.32 | | |
| 5 | 0.40 | 317 | |
| 6 | 0.62 | 363 | Cefadroxil isomer |
| 7 | 0.64 | 363 | $\Delta^2$-Cefadroxil isomer |
| 8 | 0.77 | 233 | |
| 9 | 1.0 | 381 | Cefadroxil with hydrolyzed lactam |
| 10 | 1.0 | 363 | Cefadroxil |
| 11 | 1.98 | 363 | 7-epi-Cefadroxil |
| 12 | 2.12 | | |
| 13 | 2.15 | 363 | Piperazinedione cefadroxil isomer |
| 14 | 2.17 | 377 | Methyl ester of cefadroxil (impurity) |
| 15 | 2.20 | 398 | |
| 16 | 2.25 | 329 | |
| 17 | 2.35 | 512 | Additional side-chain (impurity) |
| 18 | 2.38 | 363 | Isomer of piperazine dione of cefadroxil ($\Delta^2$ or 7-epi) |

### 2.2. Chromatography

A Perkin-Elmer (Norwalk, CT) series 410 pump was used with a LC-234 UV diode array detector. The HPLC conditions used consisted of an Alltech (Deerfield, IL) Lichrosorb RP-18 column 4.6 mm × 250 mm × 5 $\mu$m and 254 nm detection with mobile phase A: 2 mM $NH_4OAC$ and mobile phase B: acetonitrile. The solvent program commenced with 98% A/2% B isocratic for 30 min at 1.0 ml $min^{-1}$, and was then changed to a linear gradient over 10 min to 80% A/20% B followed by a linear gradient over 10 min to 50% A/50% B. 25 $\mu$l of a 5 mg $ml^{-1}$ solution was injected.

### 2.3. Electrospray mass spectrometry

A PE-SCIEX (Thornhill, Ont., Canada) API III tandem quadrupole mass spectrometer equipped with an Ionspray® (nebulizer-assisted electrospray) interface was used on-line with the HPLC system after the UV detector. Since the ionspray interface operates most effectively at flow rates less than 100 $\mu$l $min^{-1}$, the eluent from the UV detector was split approximately 1:10 prior to the interface. The split ratio was easily regulated by adjusting the length of the restriction line (fused silica capillary with 50 $\mu$m i.d. and 160 $\mu$m o.d.). LC/MS experiments were performed while scanning from $m/z$ 150–1000 at a scan rate of 2 s per scan in the positive ion mode. For LC/MS/MS substructural studies, the parent ions were selected in the first quadrupole mass analyser and transmitted into the second quadrupole (collision cell) with a collision energy of 50 eV and an argon collision gas thickness of $400 \times 10^{12}$ molecules $cm^{-2}$.

## 3. Results and discussion

Use of an integrated analytical system incorporating HPLC separation, UV spectroscopy, MS, and MS/MS rapidly provided profiles and substructure information useful for impurity and degradant identification. On-line HPLC chromatographic separation afforded a profile of the impurities and degradants of cefadroxil induced by acid, base, and heat (Table 1), as well as their relative concentrations. HPLC provided a reproducible relative retention time (RRT; cefadroxil = 1.00) using standardized HPLC conditions for the development of an impurity/degradant database and the transfer of data to collaborating laboratories. The reversed-phase HPLC conditions also provided a general measure of the polarity of each compound, useful for interpretation of substructural differences between related compounds. The standard HPLC conditions provided good general separation of those impurities/degradants observed. UV spectra collected on-line provided evidence for general classification and substructures for each compound.

Cefadroxil and related degradants typically produced UV spectra with absorption maxima at approximately 230 and 264 nm. The ionspray interface [6] generated reproducible, abundant ions ($MH^+$), which were the base peaks for all of the

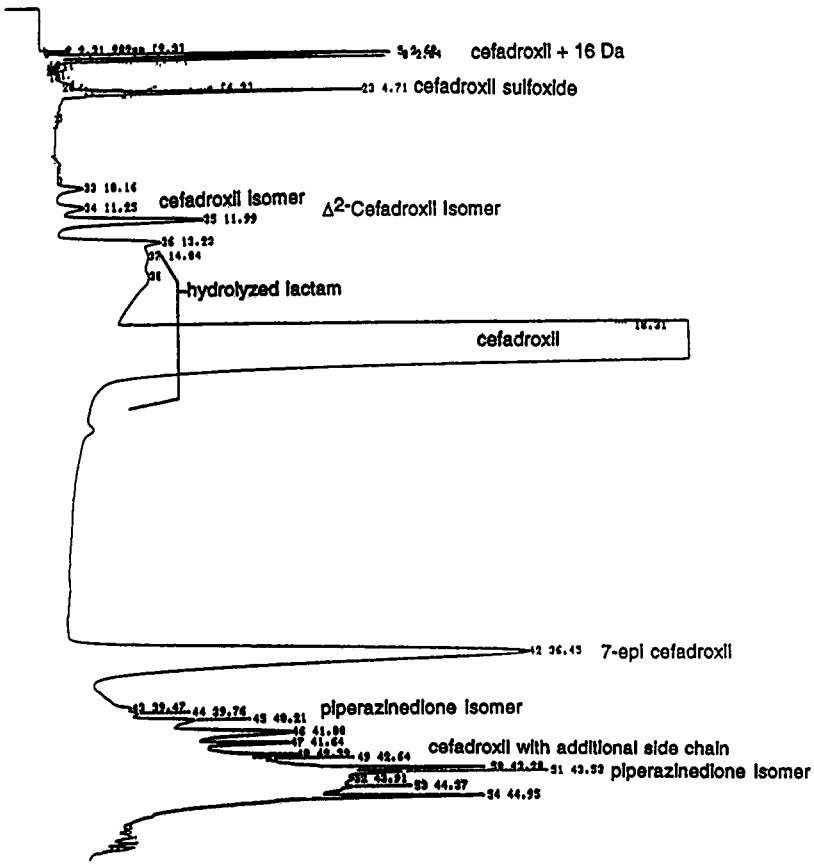


Fig. 2. HPLC chromatogram of base-degraded bulk cefadroxil monohydrate.

impurities/degradants. The $MH^+$ ions provided reliable molecular weight confirmation from the full-scan spectrum, as well as abundant ion current, which is favorable for trace MS/MS analysis.

Chromatographic resolution of co-eluting or unresolved components was not required to obtain product ion data for structural analysis, due to the mass-resolving capability of mass spectrometry. Product ion spectra provided evidence for the substructures of each impurity and degradant. The fragmentation pattern of cefadroxil was used as a substructural "template" for interpretation of the structures of unknown impurities and degradants by the association of specific product ions and neutral losses with specific substructures. This MS/MS comparative method is based on the premise that analogs of cefadroxil in the degraded samples would be expected to retain substructures of cefadroxil. Therefore, cefadroxil-related compounds would be expected to undergo similar fragmentation to cefadroxil, such as cleavage through the $\beta$ lactam to produce product ion at $m/z$ 208. Common MS/MS product ions and neutral losses observed in cefadroxil and in unkown impurities and degradants were evidence for common substructures, and differences were indicative of the variance in these substructures.

Structural data for 18 observed impurities and degradants under various conditions are provided in Table 2, including chromatographic behavior and molecular weight (MW). The mass-resolving capability of mass spectrometry permits independent MS analysis of co-eluting or unresolved compounds with different molecular weights, thus producing an "LC/MS profile" of the sample, including both chromatographic behavior and MW dimensions. Frequently, LC/MS reveals a

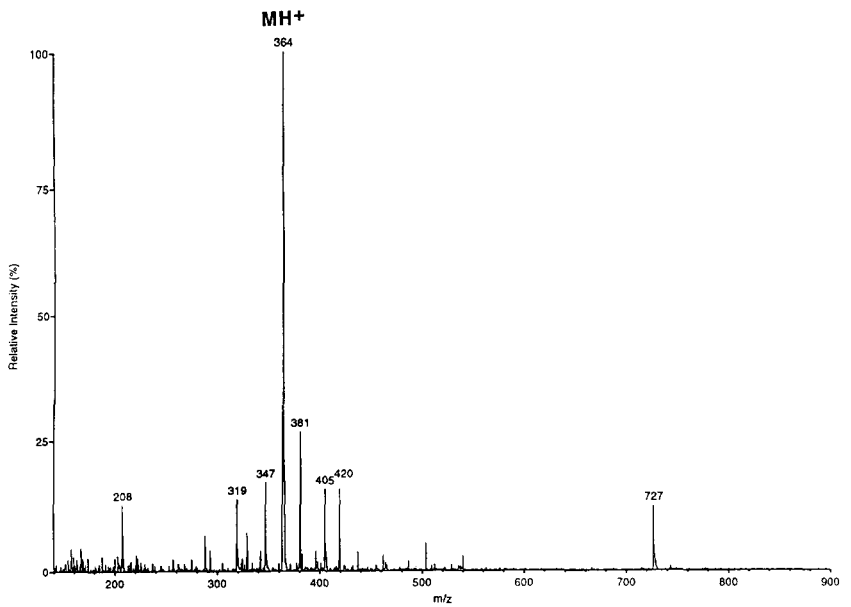


Fig. 3. Full-scan mass spectrum of $\Delta^2$ cefadroxil isomer at $m/z$ 364 ($MH^+$) in base-degraded bulk cefadroxil monohydrate.

greater number of components (which ionize effectively under electrospray conditions) in a sample than HPLC with UV detection alone, such as unresolved components and those insensitive to UV detection. Thus, this integrated method for impurities and degradants rapidly provides a multi-dimensional profile of the components in a complex mixture, containing detailed profiles of the chromatographic behavior, UV spectrum, full-scan mass spectrum, and MS/MS product ion spectrum of individual mixture components.

A representative HPLC profile chromatogram with UV detection (254 nm) of a base-degraded sample of cefadroxil is shown in Fig. 2. A large number of impurities and degradants related to cefadroxil are chromatographically separated in this sample. The molecular weight of each component was obtained on-line from the full-scan ionspray mass spectrum at its retention time as illustrated for $\Delta^2$ cefadroxil isomer (RRT 0.64, $m/z$ 364) in Fig. 3. This information was obtained from the low ionization energy ionspray interface despite other unresolved sample components which produced other ions.

Mass chromatograms (extracted ion current profiles) corresponding to the $MH^+$ ions of selected components (Fig. 4) indicate the specificity of LC/MS for molecular weight differentiation and determination of impurities and degradants of cefadroxil in the base-degraded sample. The difference between the molecular weight of a degradant and cefadroxil is indicative of the substructural differences between the compounds. For example, comparison of the full-scan mass spectra of the hydrolyzed lactam (Fig. 5A) and cefadroxil (Fig. 5B) demonstrates a molecular weight difference of 18 Da, indicative of a substructural difference resulting from hydrolysis.

Following MW determination of each component in the initial HPLC separation, the sample was again subjected to HPLC separation, during which the product ion spectrum of the $MH^+$ ion of each component was obtained at its retention time, providing specific fragmentation information for each component. The structure of each impurity and degradant was proposed based on comparison of product ions and neutral losses observed in the product ion spectrum with the product ions and neutral losses associated with specific substructures of cefadroxil (Fig. 6).

An example of degradant substructural analysis using LC/MS/MS is illustrated with the hy-

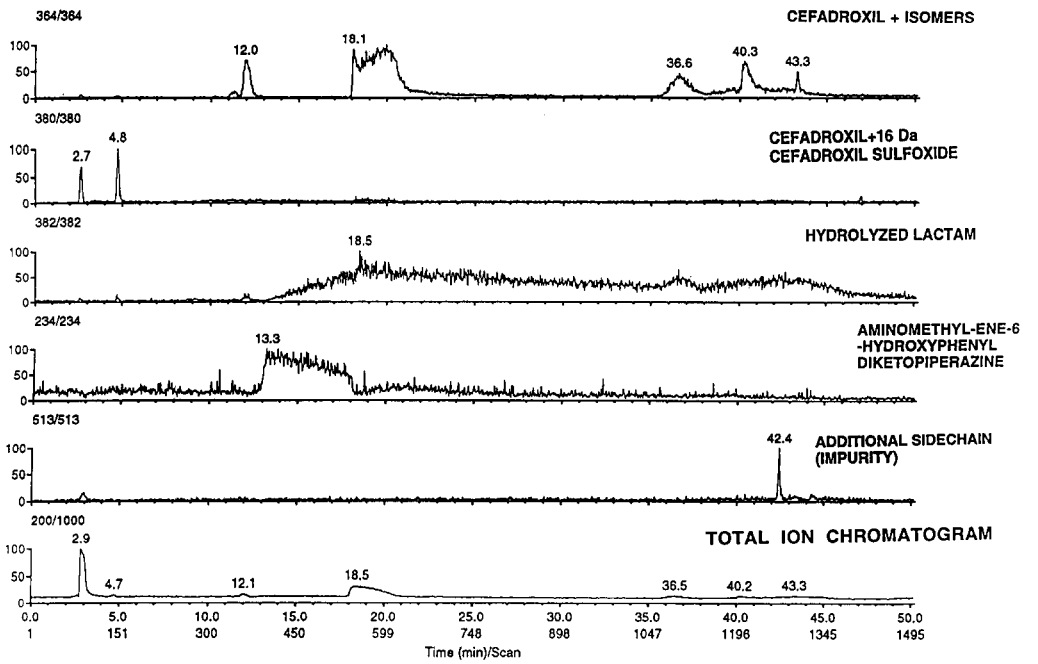


Fig. 4. LC/MS mass chromatograms (extracted ion current profiles) of selected degradants at the $m/z$ values of their $MH^+$ ions in the base-degraded bulk cefadroxil monohydrate.

drolyzed lactam, as shown in Fig. 7. Comparison of the product ion spectrum of the degradant with the MS/MS substructural template of cefadroxil supports the structure shown in Fig. 7. In additionl to the molecular weight difference of 18 Da, the product ion at $m/z$ 208, which is diagnostic of cleavage through the lactam substructure, is absent in the product ion spectrum of the observed degradant. The product ion at $m/z$ 180 in the degradant spectrum is indicative of the dihydrothiazine substructure consistent with a hydrolyzed lactam. This observed product ion at $m/z$ 180 is also absent in the cefadroxil substructural template. The hydrolyzed lactam is further supported by the presence of a product ion at $m/z$ 141, resulting from the neutral loss of $NH_3$.

A consistent pattern of several isobaric components producing multiple chromatographic peaks was observed in the mass chromatograms of the degraded samples. Fig. 4 clearly illustrates the multiple isobaric peaks at $m/z$ 364. These components include cefadroxil as well as several isomers of cefadroxil such as $\Delta^2$ isomer.

The observed impurities and degradants incorporate several consistent structural variations from cefadroxil. However, MS/MS spectra of late-eluting cefadroxil isomers which do not show the characteristic neutral loss of ammonia from the non-cyclized side-chain indicate the cyclized substructure (piperazinedione analog). The RRT 0.20 and RRT 0.25 compounds are consistent with oxidation and can also be generated with $H_2O_2$ treatment. The decrease of the RRT 0.20 degradant and increase of the RRT 0.25 degradant under basic conditions is also consistent with epimerization.

The chromatographic retention characteristics provide an indication of polarity compared to cefadroxil which is useful for elucidation [7]. The $\Delta^2$ isomer and the piperazinedione of similar analogs have been shown to elute earlier and later than the parent compound respectively [8,9]. Differences in the UV spectra of the proposed $\Delta^2$ isomer are consistent with similar degradants of other cephams [8, 9]. The major base degradant, a ring-opened product (RRT 1.0), exhibited poor chromatographic behavior.

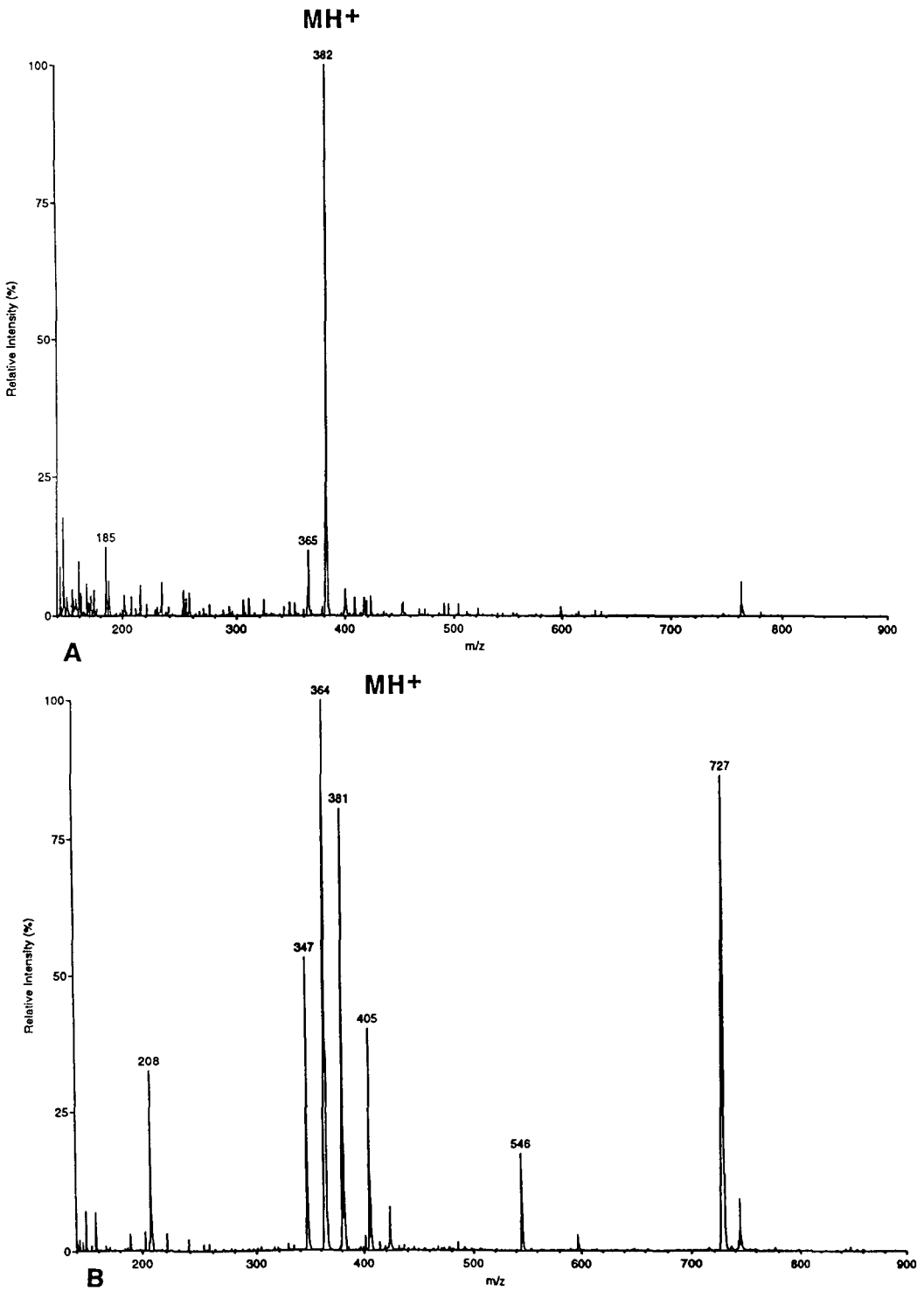


Fig. 5. Full-scan electrospray mass spectra of (A) cefadroxil with hydrolyzed lactam (molecular weight 381) and (B) cefadroxil (molecular weight 363), in the base-degraded bulk cefadroxil monohydrate, indicating a molecular weight difference of 18 Da.

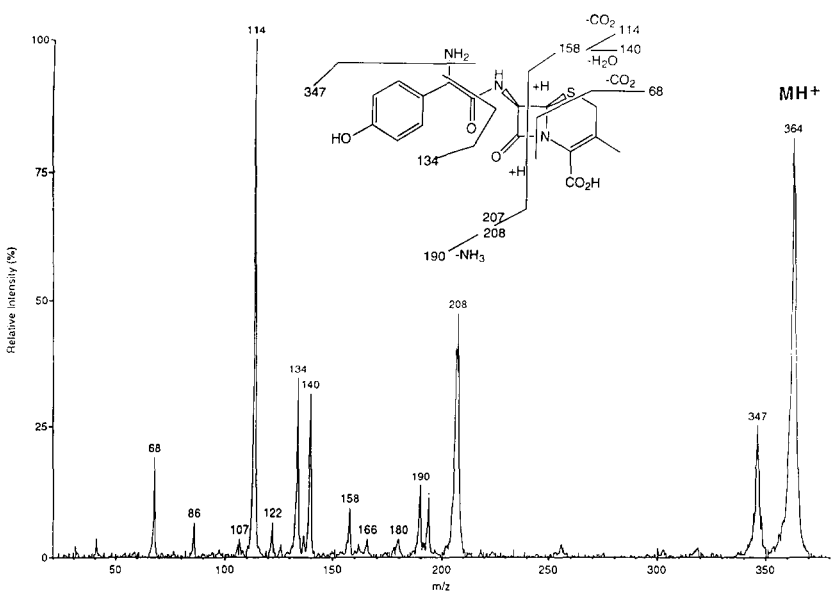


Fig. 6. Product ion spectrum of the ion at $m/z$ 364 ($MH^+$) of cefadroxil used as a template for structural analysis of related impurities and degradants.

chromatographic behavior.

## 4. Conclusions

An important element of this predictive approach requires early lock-in and application of a standard method in drug discovery. A structure database is then created for a potential drug candidate which can be utilized throughout its lifetime. The resulting structure library provides a quick reference for proposed structures, relative retention time, molecular weight, and diagnostic substructure. Libraries such as this can provide a predictive foundation for pre-clinical and development work involving drug stability, synthesis and monitoring. Valuable insights regarding potential problems associated with longterm storage (stability) and scale-up (impurities) can be obtained early in a drug's discovery and development cycle. Libraries can be used to facilitate selection of a drug candidate for development and to correlate structure with activity, bioavailability, lipophilicity or solubility.

The application of a rapid protocol based on systematic LC/MLS profiling and LC/MS/MS substructural analysis for the assignment of impurity and degradant structures in degraded bulk cefadroxil monohydrate has resulted in a detailed impurity/degradant database. This contains characteristic chromatographic (RRT) and mass spectrometric (molecular weight, MS/MS product ions) data indicative of structure.

The use of standardized HPLC conditions can provide reproducible chromatographic data for the rapid identification of impurities and degradants in new samples that have been previously elucidated. The LC/MS profile provided molecular weight data complementary to the LC/UV profile, thus extending the detail of data obtained in mixture profiling to molecular weight, a powerful, specific element in impurity/degradant analysis. In addition, incorporation of LC/MS/MS methodology added substructural data for trace (nanogram to microgram quantity) components without prior fractionation.

The various forced degradation experiments provided a diversity of degradant structures predictive of structures expected to occur under drug processing, storage and physiological conditions. The resulting structural database can be referenced throughout the lifetime of the drug for the

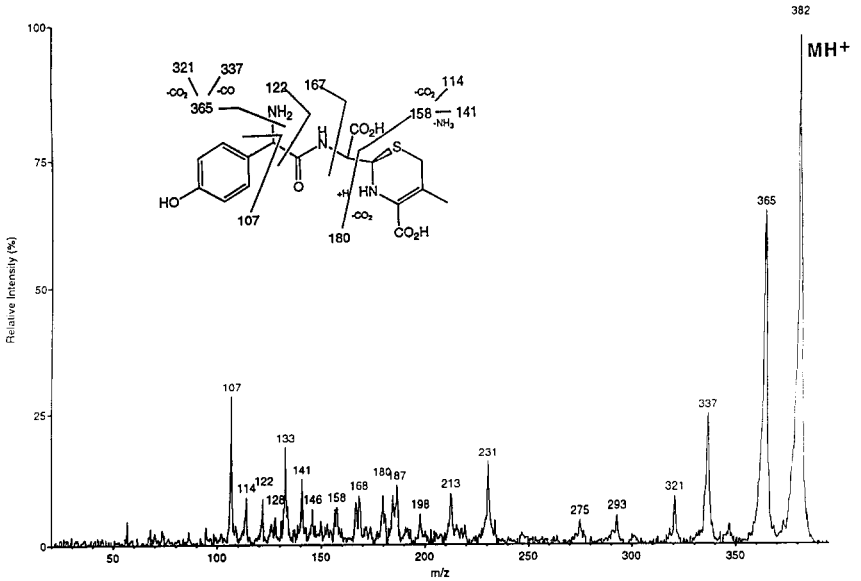


Fig. 7. Product ion spectrum of the ion at $m/z$ 382 ($MH^+$) of cefadroxil with hydrolyzed lactam at RRT 1.0 in base-degraded bulk cefadroxil monohydrate.

rapid identification of impurities, degradants, and metabolites. Elucidation of novel structures will be aided by information in the database. Early knowledge of the structures of these compounds can aid the development of analytical methods for drug monitoring.

Furthermore, the LC/MS profiling and LC/MS/MS substructural analysis methodologies utilized here are not limited to impurity and degradant structural elucidation. They are very useful in elucidation of natural products and metabolities, especially for unfractionated samples containing trace components. In many pharmaceutical research studies, the amount of sample is limited or the process of scale-up and purification is expensive and time-consuming. Thus, research strategies incorporating LC/MS and LC/MS/MS techniques reliably provide rapid identification and confirmation of unknown degradant products, exploration of potentially novel compounds, and primary front-line techniques in a multi-disciplinary structure elucidation strategy utilizing analytical techniques relevant to the available quantity and purity of a sample. In the case of isomers or other compounds difficult to distinguish by tandem mass spectrometry and predictable chemistry, isolation of a few selected compound classes for complementary spectroscopic examination may be necessary for absolute structure confirmation.

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