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# Active drug substance impurity profiling Part II. LC/MS/MS fingerprinting

Edgar C. Nicolas *, Thomas H. Scholz

*The DuPont Merck Pharmaceutical Company, Deepwater, NJ 08023, USA*

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**Abstract**

Drug substance impurities are routinely monitored using HPLC. Because HPLC retention times can vary, uncertainty can arise as to whether a peak at a new retention time is a new impurity. When impurity standards are not available some method is needed to characterize the impurities on-line. This work sought to assess the ability of LC/MS/MS to generate characteristic impurity 'fingerprints', comprised of a precursor ion mass plus at least three product ion masses. MS/MS fingerprints of a drug substance, DuP 941, and three of its impurities were first generated using available standards. Experiments varying collision cell parameters showed that collision energy must be specified in order to reproducibly generate characteristic MS/MS fingerprints. MS/MS fingerprints were also generated on-line for seven impurities in the earliest safety lot of DuP 941. Several subsequent lots of DuP 941 were examined to see how well their impurity fingerprints matched those from the earlier lot. Fingerprint reproducibility was very good for all impurities examined, even down to 0.01 UV area percent for some impurities. MS/MS fingerprinting was able to distinguish two impurities from one another which were known to be positional isomers. It also permitted assignment of tentative structures to the drug impurities. © 1998 Elsevier Science B.V.

*Keywords:* Impurity profiling; HPLC; Mass spectrometry; Impurity fingerprints

## 1. Introduction

The safety of a drug product is dependent not only on the toxicological properties of the active drug substance itself, but on the impurities that it contains. Analytical monitoring of impurities in new drug substances is a key component of the recent guideline issued by the International Conference on Harmonization (ICH) [1]. When the drug substance impurities which were present in early safety and clinical lots are not present in substantially higher amounts in subsequent lots, and no new impurities are seen, then clinical studies may proceed on schedule. However, when a new impurity is observed in a lot of a drug substance, and it cannot be removed, then additional safety studies may be required. It is vital, when monitoring the impurity profile of a drug substance, to be able to determine whether impurities in one lot are the same impurities that were present in earlier lots.

* Corresponding author. Tel.: +1 609 5404702.


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Gradient HPLC with UV detection is the technique commonly used to monitor the impurities in a drug substance. Usually, few of the impurity peaks are identified, and even fewer have available reference standards. Impurities are commonly coded and tracked using a descriptor such as retention time. However, uncertainty can arise if peaks overlap, if they shift in retention time (as can happen in gradient HPLC), or if there are numerous closely spaced peaks.

This paper is Part II of our investigation into the tracking of an active drug substance impurity profile. In Part I [2], we reported that LC/UV diode array spectral matching can be a powerful tool to monitor drug substance impurities even down to levels below 0.1% by area. However, at levels much less than 0.05% by area, it is advisable to obtain additional evidence, such as from mass spectrometry, to confirm that an impurity is the same as in an earlier lot. Impurity isolation and subsequent off-line mass spectrometry, on-line LC/MS, and LC/MS/MS have been used to confirm the identity of known drug process impurities [3–9] and degradates [10,11] by comparison to synthesized reference materials. In all these investigations, the major impurities have been the focus and the many minor impurity peaks, usually at less than 0.1 UV area percent, have not been characterized.

We report here the results of our investigation into the utility of using LC/MS/MS to track not only known major impurities, but also unidentified and unisolated minor impurity peaks. We wanted to determine if LC/MS/MS was sensitive and rugged enough to be used to monitor and differentiate minor impurity peaks present in different lots of a drug substance.

As in Part I [2], experiments were run using DuP 941 (losoxanthrone), an anticancer drug under development at DuPont Merck. Over time, improvements have been made in the DuP 941 synthetic process, and the impurity profile of the various drug substance lots has changed. The structures of DuP 941 and three known impurities are shown in Fig. 1 of Part I [2].

## 2. Experimental

### 2.1. Equipment

The mass spectral data shown in this paper were acquired on a PE Sciex API III Plus triple quadrupole using PE Sciex's Turbo Ionspray interface. Several comparative experiments were also performed on a Finnigan MAT TSQ 7000 equipped with an electrospray interface. All LC/MS and LC/MS/MS experiments used a Hewlett Packard 1090 Liquid Chromatograph equipped with DR5 pumps. A Rheodyne model 7010–082 switching valve and a Jasco model 880-PU pump were utilized as described below and as illustrated in Fig. 1. A Harvard Apparatus model 11 syringe drive was used for MS flow infusion experiments. An additional set of LC/UV chromatograms of the drug lots was acquired using an HP1100 Liquid Chromatograph.

### 2.2. Methods

Ionspray pseudo-molecular ions of DuP 941 and the three known impurities LS, SL, and PC were first generated from 1-ng $\mu l^{-1}$ solutions of each standard infused at 40 $\mu l\ min^{-1}$ to the Ionspray source. For all flow infusion experiments the heated nitrogen stream of the Turbo Ionspray was not used. Each of the standards produced simple spectra consisting mainly of singly and doubly charged pseudo-molecular ions. It was

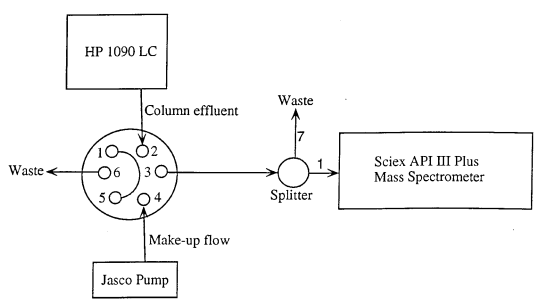


Fig. 1. Schematic diagram of switching valve set-up. When unactivated the column effluent flows to the mass spectrometer and the make-up flow goes to waste. When activated the column effluent flows to waste and the make-up flow goes to the mass spectrometer.

found that the production of the singly charged species, which was used as the precursor ion for MS/MS, could be maximized by setting the orifice potential to 80 V. Flow infusion of the standards was also used in experiments to determine the effect of collision energy and collision gas thickness on the production of an MS/MS fingerprint.

For LC/MS and LC/MS/MS experiments solutions of the different drug substance lots were prepared at a concentration of 5 mg $ml^{-1}$ using the initial mobile phase of the LC gradient.

The injection volume used was 10 μl, resulting in 50 μg being injected on column. The column used was a 4.6 mm × 15 cm Zorbax SB-C8 column with 3.5-μm particles. The initial mobile phase was acetonitrile–water–trifluoroacetic acid (10:90:0.1, v/v/v). A 20-min linear gradient was used, with a final mobile phase composition of acetonitrile–water–trifluoroacetic acid (40:60:0.1, v/v/v). A flow rate of 1.5 ml $min^{-1}$ was used and the UV chromatographic signal was acquired at a wavelength of 240 nm.

The HPLC column effluent was split 7:1 so that only 13% was delivered to the mass spectrometer's Ionspray interface, at a flow rate of 190 ml $min^{-1}$. In addition, a switching valve was used between the UV detector and the splitter to divert the bulk of the drug substance peak to waste (see Fig. 1). While the column effluent was diverted to waste, a make-up flow of 20% acetonitrile in water was supplied to the mass spectrometer by a Jasco pump in order to maintain the ion current. For all LC/MS and LC/MS/MS experiments the following mass spectrometer settings were used: an Ionspray voltage of 4500 V, a Turbo Ionspray temperature setting of 500°C and nitrogen flow of 1.6 l $min^{-1}$, and an orifice potential of 80 V. For the LC/MS experiment to generate the pseudomolecular ions of the unknown drug impurities in lot 3 the mass spectrometer's first quadrupole was scanned from 300–600 Da at a rate of one scan per second. All LC/MS/MS experiments used a collision energy of 25 eV, and a collision gas thickness of $250 \times 10^{13}$ atom $cm^{-2}$.

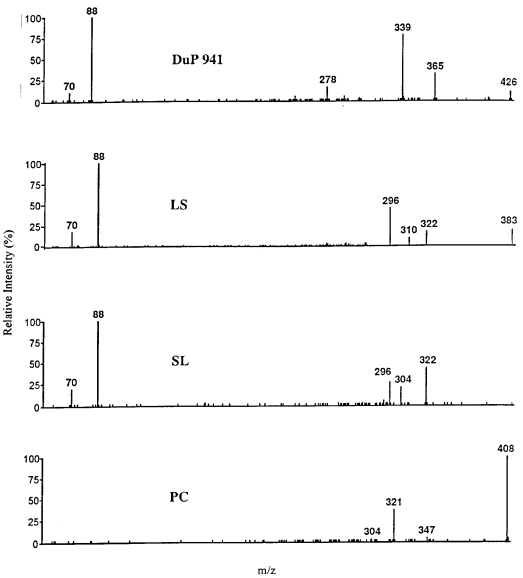


Fig. 2. LC/MS/MS spectra of DuP 941, PC, SL and LS. Precursor ions $m/z$ are: DuP 941, 426; LS and SL, 383; PC, 408.

## 3. Results and discussion

In this study we used LC/MS/MS to characterize a number of DuP 941 impurities. The purpose of this study was to illustrate the utility of the technique; therefore it was deemed unnecessary to investigate all impurities present or to examine all lots. Eight different impurities and four different lots were investigated. Lots 3 and 5 had been prepared using one synthetic route, and lots 7 and 8 using a second route. LC/UV gradient chromatograms for these lots generated using the method described in Section 2 are shown in Fig. 2 of Part I [2].

In order to compare peaks from lot to lot, each peak being examined was given a designation consisting of the lot number and a letter. Letter designations were first assigned to certain impurities in lot 3. For example, the lot 3 peak at 5.8 min was designated '3A'. If an impurity in a subsequent lot was thought to be the same impurity as in lot 3, then the letter used for its designation in lot 3 was also used in the subsequent lot (for example, 5A, 7A and 8A were thought to be

the same impurity as 3A). Any peaks thought to be new impurities in subsequent lots were given new sequential letter designations (for example, 7H).

The peaks in lot 3 labelled as 'B' and 'C' had been previously identified as the impurities PC and LS (see Fig. 1 of Part I [2]). In addition the peak labelled as 'H' in lot 7 had been previously identified as impurity SL. Although reference standards were available for these previously identified impurities, reference standards were not available for any of the other impurities. Therefore the amounts of the impurities present in each lot are reported only in terms of UV chromatogram peak area percent relative to the DuP 941 peak. The impurity peaks ranged in size from 0.23% by area, down to 0.005% by area. If one assumes that the UV extinction coefficient for each of the impurities was the same as that for DuP 941, and that the UV detector response was linear, then the impurities being examined ranged from 2.5 to 115 ng injected on column. With a 7:1 split of the column effluent, the amounts entering the Ionspray interface were actually 300–1400 pg.

### 3.1. MS/MS fingerprinting

Each drug substance impurity, if it ionizes using the ion source employed, can be characterized by an MS/MS 'fingerprint'. This fingerprint could consist of the pseudo-molecular precursor ion, the most intense product ion (base peak), and other product ions of varying intensities. The utility of this approach has been demonstrated for carotenoids [7]. In a poster presented at the 12th Montreux Symposium on LC/MS, Daniel Doerge et al. proposed that on-line structure confirmation requires at least three characteristic product ions whose intensity ratios agree within 20% with those generated by an authentic standard.

For this study MS/MS fingerprints for DuP 941 and three known impurities were first generated from standards using conditions described in Section 2. Fig. 2 shows that for three of the four standards at least five product ions are produced and the precursor ion is present at either low relative intensity or absent altogether. The characteristic MS/MS fingerprint for DuP 941 could be described as a precursor ion of $m/z$ 426, and product ions at $m/z$ 365, 339, 278, 88 (base peak), and 70. The generation of these product ions can be explained by the cleavages shown in Fig. 3. The ion at $m/z$ 88 is produced when a *N*-(2-hydroxyethyl)aminoethyl group is cleaved from the molecule. The $m/z$ 70 fragment probably arises by dehydration of the $m/z$ 88 ion. Loss of the $m/z$ 88 ion, together with hydrogen transfer, can account for the production of the 339 fragment. Loss of a 2-aminoethanol neutral (61 Da) from the precursor ion also occurs, giving rise to the product ion at $m/z$ 365. Loss of both a 61 and 88 fragment from the precursor ion produces, after hydrogen transfer, a product ion with $m/z$ 278.

The MS/MS fingerprints of LS and SL can be seen in Fig. 2 to be almost identical. They both have a precursor ion of $m/z$ 383, although under the collision cell conditions used the precursor ion was observed in the MS/MS spectrum of LS but not in the MS/MS spectrum of SL. Both LS and SL have a base peak at $m/z$ 88, and product ions at $m/z$ 296 and 322 (see Fig. 3 for how these ions might be generated). But there are two product ions in the MS/MS fingerprints of LS and SL that can be used to distinguish them from one another: LS produces a product ion at $m/z$ 310 while SL produces a product ion at $m/z$ 304. The $m/z$ 304 ion in the SL fingerprint might arise from dehy-

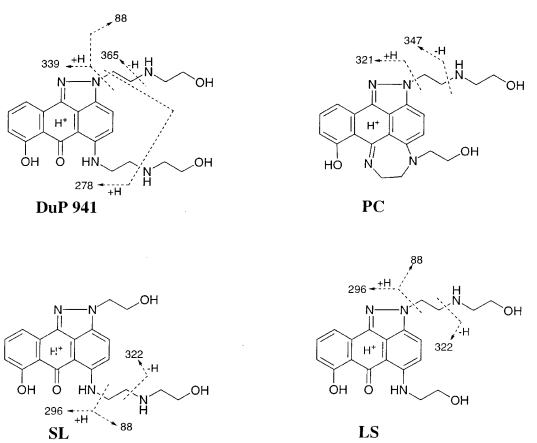


Fig. 3. Structures of DuP 941, PC, SL and LS showing proposed fragmentation sites for the generation of their respective product ions.

dration of the $m/z$ 322 product ion but no simple fragmentation can explain the generation of the $m/z$ 310 ion in the fingerprint of LS. These same distinguishing product ions were also produced in comparative experiments done on a Finnigan MAT TSQ 7000 mass spectrometer. It should be noted that in one subsequent experiment a product ion at $m/z$ 304 was present in an MS/MS spectrum of LS, however, a product ion at $m/z$ 310 was *never* present in any MS/MS spectrum of SL.

Lastly, Figs. 2 and 3 also show that the PC impurity is much more resistant to fragmentation than the other three standards examined. Under the CID conditions employed, the $m/z$ 408 precursor ion was the base peak in the MS/MS spectrum and no $m/z$ 88 product ion was produced. However, a product ion at $m/z$ 321, from loss of 87 from the precursor ion, was produced. The PC fingerprint also showed a low intensity ion at $m/z$ 347 from loss of 61 from the precursor ion. Also present was a very low intensity ion at $m/z$ 304.

### *3.2. Effect of CID variables on MS/MS fingerprints*

MS/MS product ions are generated by collision with argon gas in the mass spectrometer's second quadrupole (Q2). The fragmentation of the precursor ion is affected by two factors: (a) the collision gas thickness (CGT), which is a measure of the cross-sectional concentration of argon gas in the collision chamber, and (b) the collision energy (CE), which is a voltage differential across the collision chamber. In order for an MS/MS fingerprint to be deemed useful and reliable, its ruggedness with regards to CGT and CE needs to be evaluated and established.

The effects of CGT and CE on an MS/MS fingerprint were studied. A solution of DuP 941 was introduced into the mass spectrometer through flow infusion. The interface conditions were set at an orifice potential of 80 V and an Ionspray voltage of 4500 V. Either CGT or CE was varied while holding the other one constant to evaluate changes to the fingerprint.

Table 1 shows the precursor and product ions, and their relative sizes, in the MS/MS spectra of DuP 941, as CGT or CE was varied. CGT was first varied from a setting of 150 to $400 \times 10^{13}$ atoms $cm^{-2}$ while holding CE constant at 20, 25 or 30 eV. Then CE was varied from 10 to 50 eV while holding CGT constant at $250 \times 10^{13}$ atoms $cm^{-2}$.

As shown in Fig. 2, and discussed above, the DuP 941 fingerprint consisted of a precursor ion at $m/z$ 426 and five product ions. At the low CE setting of 20 eV, the fingerprint was reproducible and unaffected by CGT except when CGT was very low (see Table 1, CGT = 150). Based on the consistency seen in the relative ion intensity values, it might be appropriate to include information on relative sizes of the MS/MS ions in the DuP 941 fingerprint. Thus it would be said that at a CE of 20 eV, the ions at $m/z$ 426 and 339 were the 'major' ions (with relative intensities of 80–100%) in the DuP 941 fingerprint. The 'intermediate' ions occurred at $m/z$ 365 and 88 (with relative intensities of 30–40%) and 'minor' product ions occurred at $m/z$ 278 and 70 (with relative intensities less than 10%).

At a higher CE of 25 eV, Table 1 shows that the MS/MS fingerprint was again reproducible for CGT settings of 200–400, but not for a CGT setting of 150. However, the higher collision energy resulted in more fragmentation. The fingerprint had the same ions but different relative ion intensities than when the CE was 20 eV. The product ion at $m/z$ 88 was now a major ion, while the precursor ion at $m/z$ 426, formerly a major ion, was now a minor ion. When the CE was increased to 30 eV even more fragmentation occurred. The $m/z$ 426 ion was almost undetected and the $m/z$ 88 ion was the lone major ion. At this CE setting, as in the lower settings, changes in collision gas thickness did not alter the fingerprint significantly, except at the lowest setting (CGT of 150).

The effect of collision energy on the fingerprint is further illustrated by the fourth group of data at the bottom of Table 1. Here the collision energy was changed from 10 to 50 eV while the CGT was held constant at $250 \times 10^{13}$ atoms $cm^{-2}$. The result was a continuous change in relative

Table 1
Effect of collision energy (CE) and collision gas thickness (CGT) on the MS/MS fingerprint of DuP 941

| CE (eV) | CGT ($10^{13}$ atoms $cm^{-2}$) | Relative ion intensities (%) | | | | | | Base peak counts |
|---|---|---|---|---|---|---|---|---|
| | | 70 | 88 | 278 | 339 | 365 | 426[a] | |
| 20 | 150 | 0 | 5 | 0 | 13 | 6 | 100 | 42 200 |
| 20 | 200 | 1 | 38 | 3 | 77 | 28 | 100 | 26 000 |
| 20 | 250 | 2 | 42 | 5 | 100 | 32 | 82 | 43 400 |
| 20 | 300 | 2 | 38 | 4 | 100 | 30 | 85 | 61 300 |
| 20 | 350 | 2 | 38 | 7 | 100 | 33 | 97 | 59 600 |
| 20 | 400 | 2 | 34 | 7 | 92 | 33 | 100 | 57 000 |
| 25 | 150 | 0 | 30 | 3 | 36 | 20 | 100 | 22 600 |
| 25 | 200 | 7 | 100 | 8 | 80 | 40 | 22 | 20 100 |
| 25 | 250 | 8 | 100 | 16 | 78 | 32 | 10 | 36 000 |
| 25 | 300 | 12 | 100 | 18 | 95 | 40 | 15 | 43 400 |
| 25 | 350 | 10 | 97 | 19 | 100 | 45 | 15 | 45 000 |
| 25 | 400 | 8 | 98 | 21 | 100 | 44 | 20 | 41 500 |
| 30 | 150 | 5 | 74 | 17 | 69 | 40 | 100 | 10 700 |
| 30 | 200 | 16 | 100 | 18 | 27 | 14 | 3 | 21 200 |
| 30 | 250 | 18 | 100 | 22 | 26 | 13 | 1 | 32 600 |
| 30 | 300 | 18 | 100 | 32 | 33 | 18 | 1 | 40 700 |
| 30 | 350 | 17 | 100 | 35 | 40 | 24 | 0 | 37 000 |
| 30 | 400 | 18 | 100 | 33 | 31 | 20 | 0 | 42 700 |
| 10 | 250 | 0 | 0 | 0 | 2 | 0 | 100 | 143 300 |
| 15 | 250 | 0 | 4 | 0 | 20 | 5 | 100 | 102 300 |
| 20 | 250 | 2 | 42 | 5 | 100 | 32 | 82 | 43 400 |
| 25 | 250 | 8 | 100 | 16 | 78 | 32 | 10 | 36 000 |
| 30 | 250 | 18 | 100 | 22 | 26 | 13 | 1 | 32 600 |
| 35 | 250 | 28 | 100 | 18 | 5 | 3 | 1 | 33 600 |
| 40 | 250 | 50 | 100 | 17 | 1 | 0 | 0 | 27 200 |
| 45 | 250 | 77 | 100 | 11 | 0 | 0 | 0 | 19700 |
| 50 | 250 | 100 | 91 | 6 | 0 | 0 | 0 | 12 900 |

[a] $m/z$ of precursor ion.

ion intensities. This occurred to the point that some ions ($m/z$ 339, 365, and 426) that were present at low and intermediate CE settings were no longer observed at high CE settings (40–50 eV). At the same time the product ion at $m/z$ 70 increased from being a low intensity ion to being the base peak in the spectrum. Intermediate collision energy settings (20–30 eV) provided the most definitive MS/MS fingerprints.

To determine if the conclusions on the effect of CE and CGT on the MS/MS fingerprint could be extended beyond DuP 941, the same experiments were conducted on the isolated impurities LS, SL, and PC. The same overall observations were made from these experiments. As noted earlier, for LS and SL, there were five ions that could be used to define the fingerprint. At moderate collision energy values of 20, 25 and 30 eV good fingerprint reproducibility was observed. Product ions at $m/z$ 304 and 310, which differentiated the fingerprints of these two compounds, were generated at each of these collision energy values. In these experiments the product ion at $m/z$ 304, present in the spectra of SL, was not seen in any LS spectrum. Similarly, the product ion at $m/z$ 310, present in the LS spectra, was not seen in any spectrum of SL.

The MS/MS fingerprint of PC was simple and reproducible as long as the CE was between 25 and 35 eV, and as long as the CGT was not too

low. Almost no fragmentation of PC occurred at collision energies below 25 eV. However with collision energies above 35 eV a multitude of product ions began to form giving no distinct MS/MS fingerprint.

### 3.3. Optimization of MS/MS fingerprint sensitivity

There were only two low intensity product ions which differentiated LS from SL. Experiments were performed to assess whether those distinguishing ions would still be visible above the noise when LS and SL were present at low concentrations. For these experiments weighed standards of LS and SL were mixed and then chromatographed on a Zorbax SB-C8, 15 cm × 4.6 mm i.d. column. A distinction between the MS/MS fingerprints of LS and SL could be clearly seen down to 5.0 ng on column of each impurity. The 7:1 split of the HPLC effluent prior to the mass spectrometer meant that the distinction was good down to 625 pg of each impurity reaching the Ionspray interface. However, at 2.5 ng on column the $m/z$ 310 product ion of LS was not observed above the noise and the intensity of SL's product ion at $m/z$ 304 was only slightly greater than two times the noise (see Fig. 4).

Because no product ions are generated between $m/z$ 89 and 295, scanning that mass range produces no useful data but only lessens the scan time available to dwell on the ions of interest. In an attempt to improve sensitivity the mass range scanned was narrowed while still including the three product ions between $m/z$ 295 and 323. The signal-to-noise ratio for the ions of interest improved significantly, as shown in Fig. 5. The LS product ion at $m/z$ 310 could now be clearly detected at about eight times the noise level while the $m/z$ 304 ion in the SL spectrum was present at 15 times the noise level.

### 3.4. Fingerprinting of unisolated impurities

The MS/MS fingerprints for the unisolated impurities had to be generated using two separate chromatographic runs. In the first run the pseudo-molecular ions for five unisolated impurities in lot 3 was determined. Fig. 6A is the LC/UV chromatogram and Fig. 6B is the LC/MS total ion chromatogram from that run. In the LC/MS chromatogram only the tail of the DuP 941 peak is seen because most of it was shunted to waste using a divert valve (see Fig. 1). The five unisolated impurities are labelled A, D, E, F, and G along with the known impurities B and C. For impurities A, D, E, F, and G, the $m/z$ of the singly charged pseudo-molecular ions were determined to be 392, 339, 324, 482, and 558, respectively.

A second experiment was then performed in MS/MS mode in which the retention time and

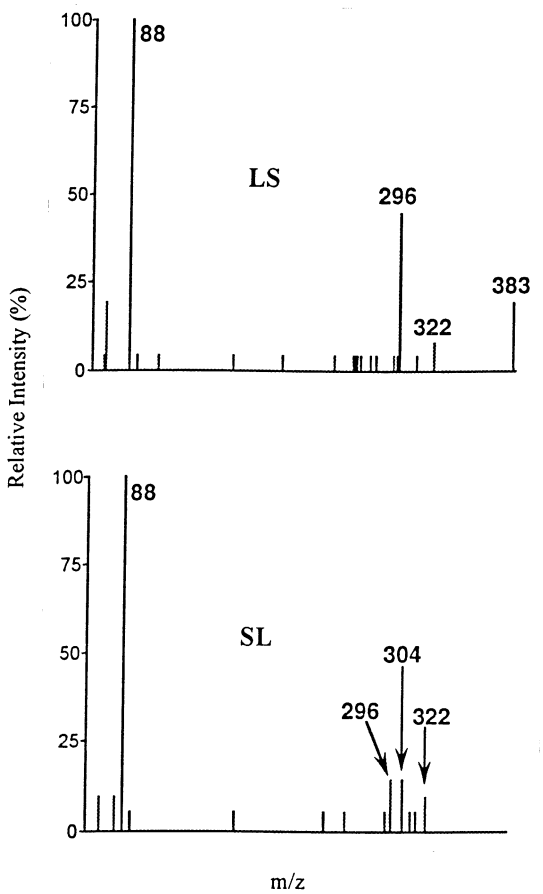


Fig. 4. LC/MS/MS spectra of LS and SL acquired using a 60–385 Da product ion window. 2.5 ng of each component were injected on a 4.6-mm i.d. column using the method described in Section 2.

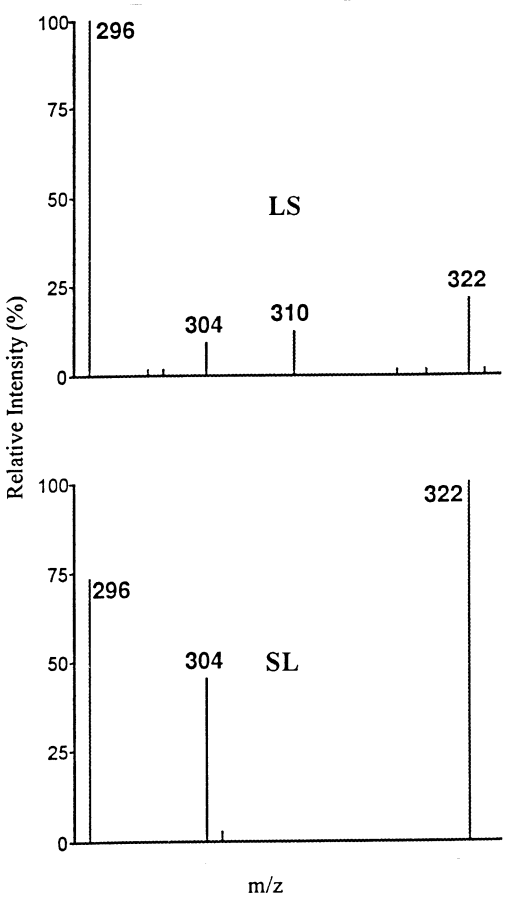


Fig. 5. LC/MS/MS spectra of LS and SL acquired using a 290–325 Da product ion window. 2.5 ng of each component were injected on a 4.6-mm i.d. column using the method described in Section 2.

$m/z$ of the singly charged pseudo-molecular ion for each impurity were used to build a time programmed LC/MS/MS method. A different precursor ion was selected for collision induced dissociation for each time period (the periods were based on the retention times of the impurities). The LC/MS/MS total ion chromatogram obtained is shown in Fig. 6C. As expected, a significant improvement in signal relative to noise is obtained in the LC/MS/MS experiment compared to the LC/MS experiment.

The MS/MS spectra for the five unisolated impurities are shown in Fig. 7. Each of these mass spectra has at least three product ions which, along with the $m/z$ of the precursor ion selected, constitute a fingerprint which can be used to characterize the impurity. For example, the fingerprint of impurity F would consist of a precursor ion at $m/z$ 482, and product ions at $m/z$ 426, 365, 339, 278, and 88. Under the experimental conditions used, the ions at $m/z$ 426, 339, and 88 are the 'major' ions, and the ions at $m/z$ 365 and 278 are the 'minor' ions.

### 3.5. Lot-to-lot tracking of ADS impurities using LC/MS/MS fingerprints

The ability of LC/MS/MS to produce the same characteristic fingerprints ($m/z$ and relative intensities of ions) for impurities from lot to lot is evident from the data in Table 2. The table contains the fingerprints from the eight impurities investigated, seven of which were observed in the earliest safety lot (lot 3) and one (peak H) which

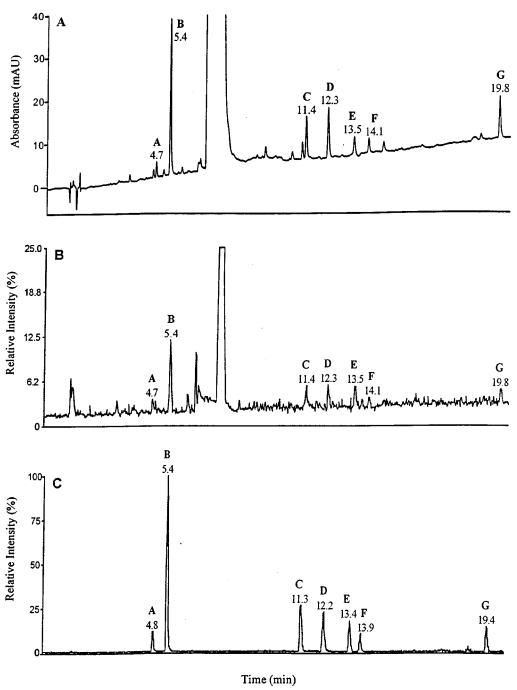


Fig. 6. DuP 941 lot 3 chromatograms: (A) LC/UV; (B) LC/MS; (C) LC/MS/MS. A and B were acquired from a single injection using an HP1090. UV and MS detectors were in series. C was acquired from a subsequent injection.

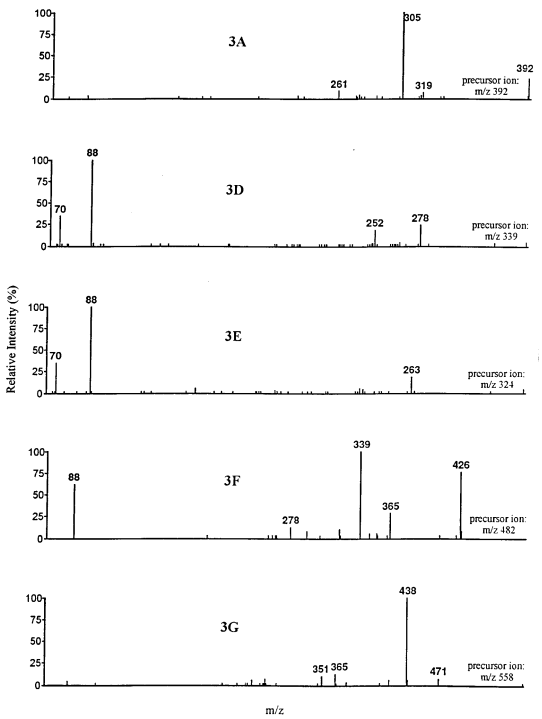


Fig. 7. LC/MS/MS spectra of unisolated DuP 941 impurities.

was observed for the first time in lot 7. The fingerprints generated from the standards of PC, LS, and SL are also given.

The fingerprint data presented in Table 2 strongly indicate that the lot 3 impurity designated as 3B is the known impurity PC, and impurity 3C is the same as known impurity LS. The other impurities in lot 3 (3A, 3D, 3E, 3F, and 3G) are unknown but their MS/MS fingerprints provide a characterization of each against which impurities in subsequent lots can be compared. The next lot analyzed, lot 5, contained peaks that based on their fingerprints, are the same seven impurities present in lot 3. That is, 5A is the same impurity as 3A, 5B is the same impurity as 3B, and so on. The next lot analyzed, lot 7, was observed to contain a peak, 7H, that based on retention time was suspected to be a new peak. Comparing the MS/MS fingerprint of 7H with the seven previously seen impurities strengthens the suspicion that it is a new impurity. Comparing the MS/MS fingerprint of 7H to that of the standard SL allows us to conclude that indeed 7H is a new DuP 941 impurity, and its identity is SL. When lot 8 was analyzed the same impurity fingerprints were observed as in lot 7.

For each of the impurities examined, the $m/z$ of the base peak in the MS/MS spectrum was consistent from lot to lot. The $m/z$ of the other ions produced and their relative intensities was also reproducible from lot to lot. Except for impurity F, the relative ion intensities varied by no more than 10% from lot to lot. For impurity F, in lot 3 the ion at $m/z$ 426 was more intense than the ion at $m/z$ 88, but in lot 5 this relationship was reversed. This result suggests that a description of the characteristic MS/MS fingerprint of an impurity may include a description of which ions are 'major', 'intermediate', and 'minor' ions in relative intensity, but it should not include tight limits on the range of relative intensity values that characteristic ions can have.

### *3.6. Using LS/MS/MS fingerprints to propose impurity structures*

The $m/z$ of the precursor and product ions, together with knowledge of the synthetic routes used to produce the lots, allowed structures to be proposed for the impurities (see Fig. 8). Many of the characteristic ions of impurity A were similar to those of the PC impurity and led us to propose a structure for impurity A that is similar to the structure of PC. Like PC, impurity A did not fragment extensively nor did it produce a product ion at $m/z$ 88 under the CID conditions used. The $m/z$ of impurity A's precursor ion and most intense product ion were both 16 less than the complementary ions in the PC fingerprint. This indicates that impurity A has one less oxygen in its structure than PC does. The impurities also had similar HPLC retention times.

The fingerprints of impurities D and E are similar in two ways. Both have a base peak at $m/z$ 88 in their MS/MS spectrum, and both produce a less intense product ion ($m/z$ 278 in D, and $m/z$ 263 in E) that is 61 Da less than the precursor ion. As with other impurities already discussed, the 61-Da loss is probably due to the loss of a 2-aminoethanol neutral. Other studies have shown

Table 2
LC/MS/MS fingerprints of selected DuP 941 impurities

| Peak[a] | UV area (%) | % Relative intensity | | | | | |
|---|---|---|---|---|---|---|---|
| | | *m/z*: | 261 | 305 | 319 | 392[b] | |
| 3A | 0.09 | | 10 | 100 | 10 | 20 | |
| 5A | 0.01 | | 15 | 100 | 10 | 10 | |
| 7A | 0.02 | | 15 | 100 | 10 | 20 | |
| 8A | 0.06 | | 15 | 100 | 10 | 20 | |
| | | *m/z*: | 304 | 321 | 347 | 408[b] | |
| PC | — | | 5 | 35 | 5 | 100 | |
| 3B | 0.08 | | 5 | 70 | 5 | 100 | |
| 5B | 0.23 | | 10 | 85 | 10 | 100 | |
| 7B | 0.03 | | 10 | 95 | 5 | 100 | |
| 8B | 0.04 | | 10 | 85 | 5 | 100 | |
| | | *m/z*: | 70 | 88 | 296 | 310 | 322 | 383[b] |
| LS | — | | 20 | 100 | 45 | 10 | 20 | 20 |
| 3C | 0.12 | | 25 | 100 | 50 | 10 | 15 | 15 |
| 5C | 0.07 | | 25 | 100 | 55 | 10 | 15 | 10 |
| 7C | 0.05 | | 20 | 100 | 55 | 15 | 20 | 5 |
| 8C | 0.12 | | 20 | 100 | 60 | 10 | 15 | 10 |
| | | *m/z*: | 70 | 88 | 252 | 278 | 339[b] | |
| 3D | 0.12 | | 35 | 100 | 20 | 25 | 0 | |
| 5D | 0.11 | | 30 | 100 | 20 | 20 | 0 | |
| 7D | 0.04 | | 35 | 100 | 20 | 15 | 0 | |
| 8D | 0.04 | | 40 | 100 | 20 | 20 | 0 | |
| | | *m/z*: | 70 | 88 | 235 | 263 | 324[b] | |
| 3E | 0.05 | | 35 | 100 | 5 | 20 | 0 | |
| 5E | 0.04 | | 40 | 100 | 5 | 20 | 0 | |
| 7E | 0.006 | | 45 | 100 | 7 | 30 | 5 | |
| 8E | 0.007 | | 45 | 100 | 7 | 25 | 0 | |
| | | *m/z*: | 88 | 278 | 339 | 365 | 426 | 482[b] |
| 3F | 0.02 | | 55 | 15 | 100 | 25 | 75 | 0 |
| 5F | 0.02 | | 90 | 10 | 100 | 35 | 60 | 0 |
| | | *m/z*: | 351 | 365 | 438 | 471 | 558[b] | |
| 3G | 0.09 | | 10 | 10 | 100 | 4 | 0 | |
| 5G | 0.10 | | 10 | 10 | 100 | 1 | 0 | |
| 7G | 0.005 | | 0 | 0 | 100 | 0 | 0 | |
| 8G | 0.008 | | 5 | 5 | 100 | 0 | 0 | |
| | | *m/z*: | 70 | 88 | 296 | 304 | 322 | 383[b] |
| SL | — | | 20 | 100 | 25 | 20 | 40 | 0 |
| 7H | 0.12 | | 20 | 100 | 30 | 20 | 55 | 0 |
| 8H | 0.04 | | 20 | 100 | 40 | 30 | 55 | 0 |

[a] Peak designation codes: first character is lot number; second character is presumed identity.
[b] *m/z* of precursor ion.

that impurities D and E are photo-decomposition products of DuP 941.

Impurity F also produces a fairly intense product ion at $m/z$ 88 and its base peak has the same $m/z$ (339) as impurity D's precursor ion. Impurities F and D also have a $m/z$ 278 ion (339 − 61) in common. Impurity F has a product ion at $m/z$ 426 (56 Da less than F's precursor ion) that could be generated by loss of a butyl group from the precursor ion. A second 61-Da difference exists between the ions at $m/z$ 426 and 365. Knowing the protecting group chemistry used in the synthesis of lots 3 and 5, and that impurity F was only seen in the LC/MS/MS chromatograms of lots 3 and 5, led us to propose the structure shown. In Part I of our study [2], retention time and UV spectral matching suggested that impurity F was present at low levels in lots 7 and 8. The additional data from LC/MS/MS disproves this.

The very late elution time for impurity G indicated that it must contain a very lipophilic moiety somewhere in the molecule. Its base peak ($m/z$ 438) could arise from loss of a trimethylphenyl group (120 Da) from the precursor ion. Although no product ion at $m/z$ 88 was present in the spectrum, the presence of product ions at $m/z$ 471 (558 − 87) and 351 (438 − 87) does indicate that the long chain present in DuP 941 is also present in this molecule. Again, knowledge of the protecting group chemistry used in the synthesis, along with the MS/MS fingerprint, permits the proposal of the structure shown.

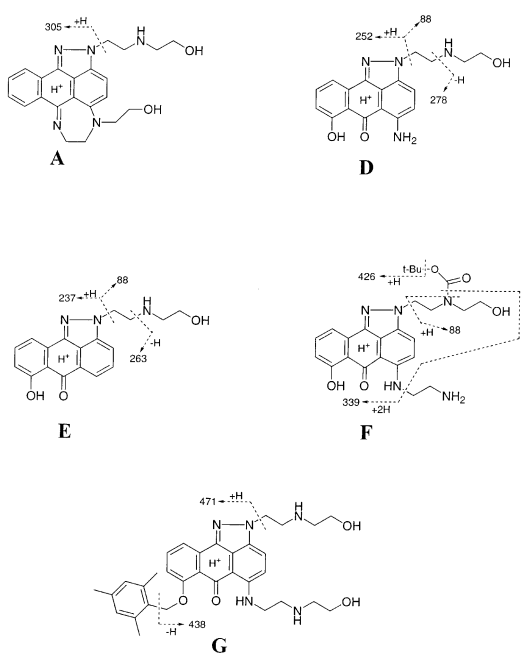


Fig. 8. Possible structures of unisolated DuP 941 impurities showing proposed CID fragmentations.

## 4. Conclusions

LC/MS/MS fingerprinting is a useful tool for the on-line characterization of drug substance impurities. In this study, MS/MS fingerprints, consisting of the $m/z$ of the precursor ion and three or more product ions, exhibited lot-to-lot reproducibility even at impurity concentrations below 0.05 UV area percent. The ability of MS/MS fingerprinting to distinguish isomeric impurities present at low concentration levels was enhanced by scanning a narrower product ion mass window.

Suitable values of collision energy and collision gas thickness, needed to produce a characteristic fingerprint, can be determined by evaluating the effect of CE and CGT on the MS/MS fingerprint of the drug substance. A reproducible MS/MS fingerprint for characterizing a drug substance impurity may require that the collision energy be controlled within a small range of specified values. From our study it appears that the production of a characteristic MS/MS fingerprint is fairly rugged with regards to the setting of the collision gas thickness, except at low CGT settings. Therefore, after a CE setting is chosen, one should use a moderately high CGT setting in order to ensure good reproducibility. When CE and CGT are carefully controlled it may also be possible to specify the relative sizes of the ions in the MS/MS spectrum as part of a characteristic fingerprint. However, tight numerical ranges for the relative ion intensities are probably inappropriate in the absence of reference standards.

Because reference standards for impurities are often unavailable, it is recommended that, if possible, a drug substance lot be analyzed together with retained samples of safety and clinical lots that have been protected from degradation (e.g.,

by storage at very low temperature in the absence of light). When such samples are not available, then having a method such as UV diode array spectral matching or LC/MS/MS fingerprinting to characterize impurities on-line may be essential. Even when retained samples are available, a substantially higher level of assurance as to an impurity peak's identity can be gained, compared to using retention times only, by having a characteristic UV diode array spectrum or MS/MS fingerprint for the impurity. A significant added benefit of LC/MS/MS fingerprinting, as compared to UV diode array spectral matching, is that an MS/MS fingerprint may enable structures to be proposed for the unisolated impurities. This knowledge may make time-consuming isolation of certain impurities unnecessary. It may also assist the manufacturer of the drug substance in devising process changes which might eliminate the impurities.

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