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# ANALYSIS OF CANDIDATE ANTICANCER DRUGS BY THERMOSPRAY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY AND BY HIGH-RESOLUTION MASS SPECTROMETRY

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## SUMMARY

Thermospray high-performance liquid chromatography–mass spectrometry (TSP–HPLC–MS) and direct probe high-resolution MS was used to analyze four candidate anticancer drugs. The techniques were used to confirm the identity of the bulk drug and to identify impurities. Analysis by TSP–HPLC–MS resulted in molecular weight information from the separated components using as little as 50 ng of each drug. The high-resolution direct probe MS analysis provided additional structural information and possible empirical formulas for the parent drugs and their impurities. The use of both of these complimentary techniques proved to be very specific for the detection of the anticancer drugs and for postulating the identity of impurities.

## INTRODUCTION

The National Cancer Institute is interested in developing analytical methodology to qualitatively and quantitatively identify candidate anticancer drugs and their impurities. Analytical options are sometimes limited since the compounds are generally not amenable to gas chromatographic (GC) analysis. High-performance liquid chromatography (HPLC) is an ideal separation technique but lacks detectors of sufficient specificity to easily validate the identity of the parent drug or identify impurities[1]. Thermospray (TSP)–HPLC–mass spectrometry (MS) can provide both the specificity and capability for direct identification needed for the analysis[2–7]. TSP is well suited to operation at 1–2 ml/min flow-rates with the high quantities of water often necessary for HPLC separation of polar anticancer drugs[8,9]. Furthermore, TSP ionization is soft, providing molecular weight information from these thermally labile drugs[10]. In many cases the ionization technique is so gentle that only one- or two-ion spectra are detected. The lack of structurally significant fragments can often hinder the ability to validate the identity of the parent drug and identify and impurities detected[11].

High-resolution mass spectrometry (HRMS) has been a useful tool in the iden-

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TABLE I

HPLC CONDITIONS USED IN THE ANALYSIS OF THE LISTED ANTICANCER DRUGS

| *Compound* | *Solvent* | *Column* | *Buffer* | *Flow-rate (ml/min)* |
|---|---|---|---|---|
| Trenimon | Acetonitrile– water (10:90) | RCM $C_{18}$ | 0.1 *M* Ammonium acetate + 0.1 *M* acetic acid | 1.2 |
| 3-Deazauridine | Acetonitrile–water (5:95) | RCM $C_{18}$ | 0.1*M* Ammonium acetate | 1.2 |
| Mitindomide | Acetonitrile–water (5:95) | RCM $C_{18}$ | 0.1 *M* Ammonium acetate + 0.1 *M* acetic acid | 1.2 |
| Terephthalamidine | N,N-Dimethylformamide–formic acid–water (0.7:5:94.3) | Zorbax $C_{18}$ 25 cm × 4.6 cm I.D. | 0.1 *M* Ammonium formate | 1.2 |

tification of unknown compounds[12–14]. The ability to analyze the anticancer drugs by direct probe electron impact (EI) using HRMS enables the calculation of empirical formulas and provides more fragment ions compared to TSP–HPLC–MS. While on-line separation is sacrificed with this technique, HPLC fractions or purified drugs (containing only a few impurities) can be analyzed to yield structural information, not obtainable by TSP–HPLC–MS, necessary to postulate the identity of an impuritie or validate the structure of the parent drug.

The capabilities of combined TSP–HPLC–MS and HRMS were demonstrated for the analysis of several anticancer drugs including 3-deazauridine, terephthalamidine, trenimon, and mitindomide. The complimentary nature of the two techniques is discussed.

## EXPERIMENTAL

### *HPLC–MS*

The TSP–HPLC–MS equipment and operation have been described previously[15,16]. The "filament-on" TSP–HPLC–MS work was performed on a Vestec TSP Interface (Houston, TX, U.S.A.) connected to a Finnigan 4500 quadrupole mass spectrometer. The filament was operated at a 1000 eV potential with a 150 mA emission current. The HPLC conditions used in the HPLC–MS analysis of each anticancer drug are given in Table I.

### *HRMS*

The HRMS work was performed on a MS-902 (AEI, Manchester, U.K.). The instrument was operated at 10K resolution under EI conditions (70 eV electron energy, 0.1 mA emission current). The samples were introduced into the instrument in solid form with a direct probe. The source temperature was elevated slowly until the sample was detected (typically about 250°C). The desired ions were manually peak-matched against perfluorokerosene (PFK) to obtain high-resolution mass measurements. Possible empirical formulas were calculated around a 10 ppm tolerance window from the measured value, using specified heteroatoms, by an off-line IBM-PC micro computer.

## RESULTS AND DISCUSSION

### *Analysis of 3-deazauridine*

The analysis of 3-deazauridine by TSP–HPLC–MS indicated the absence of impurities in the drug formulation. The TSP–HPLC–MS analysis proved to be very sensitive (Fig. 1) with the ability to detect down to 10 ng of analyte under full scan conditions. However, the TSP spectra were very simple, consisting of only an $[M + H]^+$ ion for positive ion detection or an $[M - H]^-$ ion for negative ion detection. HRMS analysis of the drug provided fragment and empirical formula information consistent with the proposed structure of the drug (Table II). Under EI conditions the drug exhibited a molecular ion and several fragment ions, several of which were measured under HRMS conditions to confirm their proposed identity (Table II).

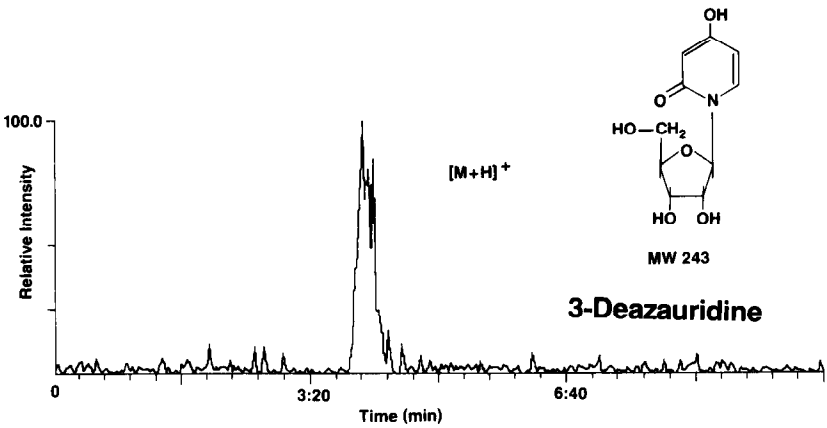
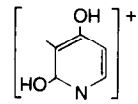



Fig. 1. TSP–HPLC–MS ion chromatogram for 50 ng of 3-deazauridine monitoring the $[M + H]^+$ ion under full scan conditions.

TABLE II

HRMS DIRECT PROBE EI ANALYSIS OF 3-DEAZAURIDINE

Maximum error allowed = 10 ppm. Heteroatoms used: $^{12}C$, atomic weight 12.0000, limiting number 15; $^{14}N$, atomic weight 14.0031, limiting number 1; $^{16}O$, atomic weight 15.9949, limiting number 6.

| *Calculated mass* | *Measured mass* | *Error* | $^{12}C$ | $^{1}H$ | $^{14}N$ | $^{16}O$ | *Identity* |
|---|---|---|---|---|---|---|---|
| 243.0743 | 243.0745 | −0.24 | 10 | 13 | 1 | 6 | $[M]^+$ |
| 170.0453 | 170.0452 | 0.11 | 7 | 8 | 1 | 4 | $[M-HO-CH_2-CH=CH-O]^+$ |
| 151.0269 | 151.0268 | 0.13 | 7 | 5 | 1 | 3 | $[M-HO-CH_2-CH_2-CH_2-OH]^+$ |
| 112.0398 | 112.0398 | 0.04 | 5 | 6 | 1 | 2 |  |



*Analysis of terephthalamidine*

The HPLC–UV analysis (Fig. 2) of terephthalamidine indicated the presence of an impurity of about 6% relative abundance compared to the major peak. TSP–HPLC–MS and HRMS analysis confirmed that the major peak observed on the UV chromatogram belonged to terephthalamidine (Table III). Both HPLC–MS and HRMS were rich in structural information for the parent drug. Initially, however, the impurity was not detected by either technique. This indicated that the impurity's proton affinity was significantly lower than the parent drug, preventing ionization in the "filament off" mode analogous to chemical ionization (CI) by $[NH_4]^+$ in the TSP interface[17]. The impurity was not seen in the direct probe EI analysis as a possible result of its low concentration relative to the parent drug and ion interferences from the parent drug. Subsequently, operation of the TSP interface in "filament on" mode enabled detection of the impurity peak.

The operation of the TSP interface in the "filament on" mode increased the range of compounds amenable to the interface. TSP ionization in the "filament off"

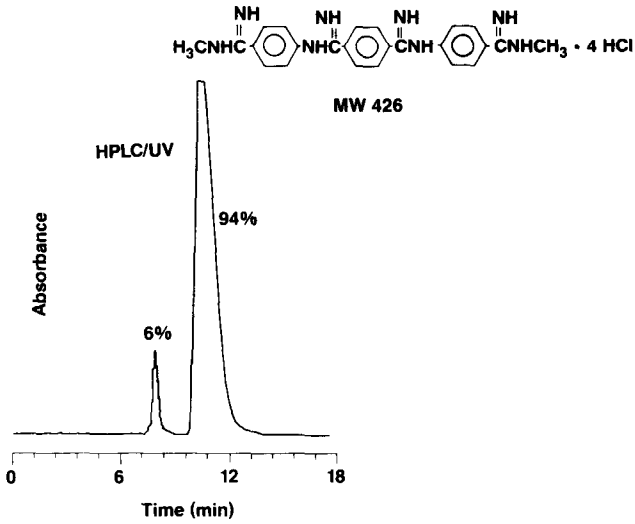



Fig. 2. HPLC–UV (254 nm) chromatogram for the analysis of terephthalamidine.

mode has been demonstrated to resemble $[NH_4]^+$ CI[17]. With the operation of TSP interface in the "filament on" mode the CI reagent ion is changed from $[NH_4]^+$ to $[\text{solvent} + H]^+$ (for this separation the main reagent ion was $[H_3O]^+$). The proton affinity of the solvent reagent ion is lower than $[NH_4]^+$, enabling protonation of many organics not amenable to $[NH_4]^+$ CI[17,18]. The filament is also a good source of electrons for the formation of negative ions by electron-capture processes. This source of electrons is not available in TSP ionization. The HPLC–MS analysis of terephthalamidine in the "filament on" mode using negative ion detection enabled detection of the impurity (Fig. 3) which evidently favored electron-capture anion formation as noted by the strong $[M]^-$ anion. Based on the interpretation of the spectrum, the impurity was postulated to be a synthetic intermediate for terephthalamidine, whose structure is shown in Fig. 3.

*Analysis of trenimon*

The HPLC–MS analysis of trenimon exhibited a peak for the $[M + H]^+$ ($m/z$ 232) ion at the appropriate retention time for the drug (Fig. 4). The TSP spectrum and the HRMS measurements (Table IV) were consistent with the proposed structure for the parent drug shown in Fig. 4. There was also a slight impurity peak (about 5% of the parent drug response) detected in the tail of the parent drug peak (Fig. 4). This impurity appeared to have an $[M + H]^+$ peak at $m/z$ 273 and an $[M + NH_4]^+$ ion at $m/z$ 290, along with a few fragment ions shown in Fig. 5. Since the molecular weight (272) for the compound is even, it is believed that a nitrogen was added to the trenimon parent molecule. The balance of the mass increase of the impurity can be accounted for by the addition of $C_2H_4$, resulting in a net addition of $-N(CH_2)_2$. This addition was proven by a HRMS measurement on $m/z$ (Fig. 5). The proposed structure of the impurity was deducted to be the one shown in Fig. 5.

TABLE III

SPECTRAL INFORMATION GATHERED ON TEREPHTHALAMIDINE BY TSP–HPLC–MS AND HRMS

RI = Relative intensity.

*TSP–HPLC–MS*

| *Positive ion detection* | | | *Negative ion detection* | | |
|---|---|---|---|---|---|
| *m/z* | *RI (%)* | *Identity* | *m/z* | *RI (%)* | *Identity* |
| 427 | 22 | $[M+H]^+$ | 485 | 13 | $[M+\text{Acetate}]^-$ |
| 396 | 18 | $[M+H-CH_3NH_2]^+$ | 425 | 28 | $[M-H]^-$ |
| 365 | 7 | $[M+H-(CH_3NH_2)_2]^+$ | 394 | 45 | $[M-H-CH_3NH_2]^-$ |
| 278 | 100 | $[M+H-NHC_6H_6C(NH)NHCH_3]^+$ | 363 | 14 | $[M-H-(CH_3NH_2)_2]^-$ |
| 247 | 38 | $[M+H-NHC_6H_6C(NH)NHCH_3-CH_3NH_2]^+$ | 276 | | $[M-H-NHC_6H_6C(NH)_2CH_3]^-$ |
| | | | 245 | | $[M-H-NHC_6H_6C(NH)_2CH_3-NH_2CH_3]^-$ |

*HRMS*

| *Calculated mass* | *Measured mass* | *Error (ppm)* | *Empirical formula* | | | | *Identification* |
|---|---|---|---|---|---|---|---|
| | | | $^{12}C$ | $^{1}H$ | $^{14}N$ | $^{16}O$ | |
| 426.2280* | 426.2283 | −0.29 | 24 | 26 | 8 | 0 | $[M]^+$ |
| 426.2253 | 426.2283 | −2.98 | 21 | 28 | 7 | 3 | |
| 426.2267 | 426.2283 | −1.63 | 23 | 30 | 4 | 4 | |
| 276.1249* | 276.1252 | −0.30 | 16 | 14 | 5 | 0 | $[M-NH_2C_6H_6C(NH)_2CH_3]^+$ |
| 276.1262 | 276.1252 | 1.05 | 18 | 16 | 2 | 1 | |
| 276.1236 | 276.1252 | −1.64 | 15 | 18 | 1 | 4 | |

* Best fit for terephthalamidine.

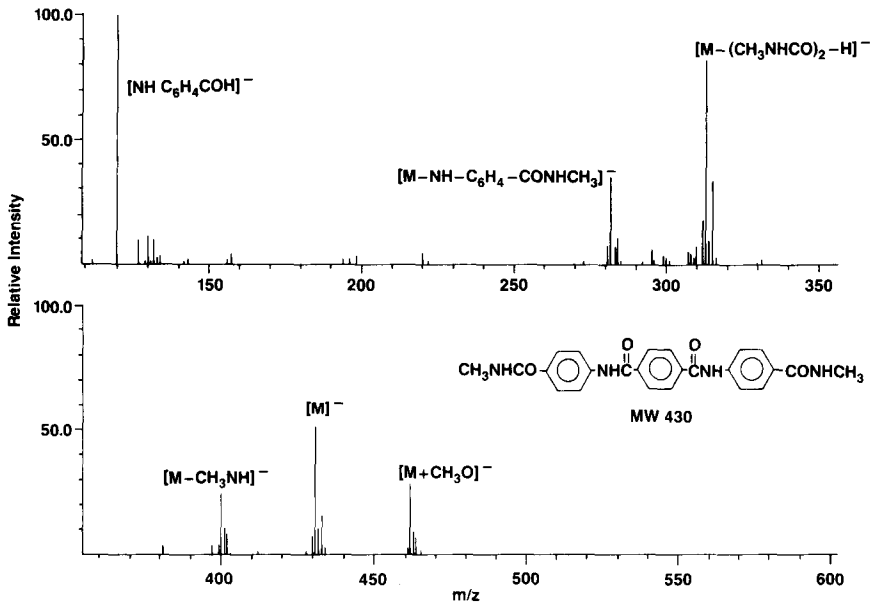



Fig. 3. "Filament on" negative ion spectrum of an impurity found in terephthalamidine.

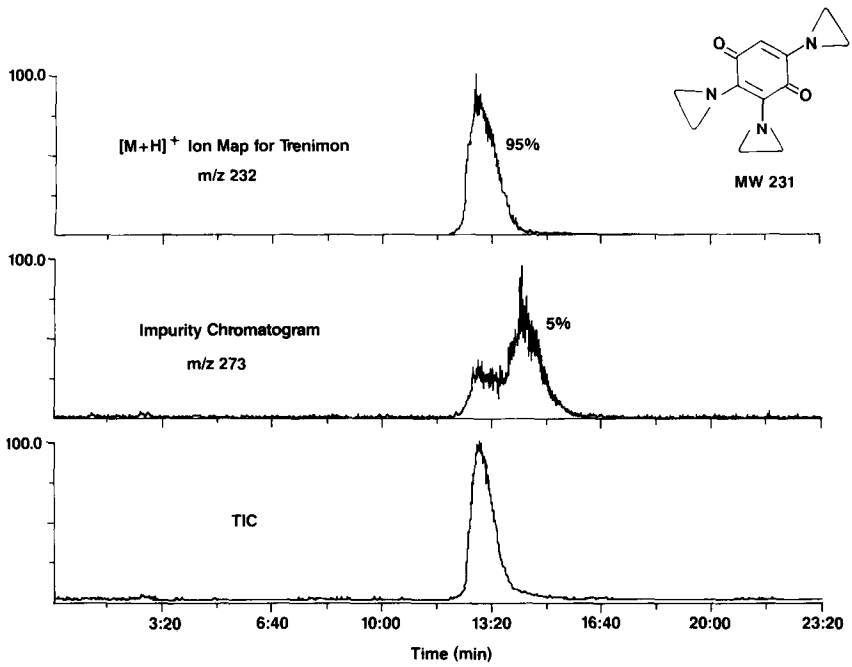



Fig. 4. HPLC–MS analysis of trenimon. The chromatograms for the $[M + H]^+$ ion of trenimon and the apparent $[M + H]^+$ ion for an impurity are shown.

TABLE IV

LISTING OF THE TSP SPECTRUM AND THE HRMS MEASUREMENTS AND ION IDENTIFICATION FOR THE ANALYSIS OF TRENIMON

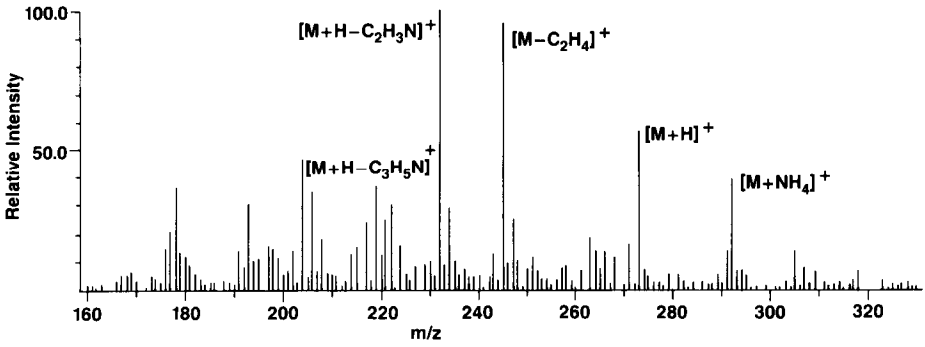
*HPLC–MS*

| *m/z* | *RI (%)* | *Identification* |
|---|---|---|
| 249 | 36 | $[M+NH_4]^+$ |
| 232 | 100 | $[M+H]^+$ |
| 204 | 12 | $[M+H-C_2H_4]^+$ |

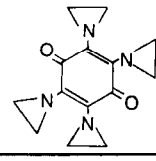
*HRMS*

| *Calculated mass* | *Measured mass* | *Error (ppm)* | *Empirical formula* | | | | *Identification* |
|---|---|---|---|---|---|---|---|
| | | | $^{12}C$ | $^{1}H$ | $^{14}N$ | $^{16}O$ | |
| 231.1008* | 231.1006 | 0.16 | 12 | 13 | 3 | 2 | $[M]^+$ |
| 231.1021 | 231.1006 | 1.50 | 14 | 15 | 0 | 3 | |
| 216.0773* | 261.0771 | 0.18 | 11 | 10 | 3 | 2 | $[M-CH_3]^+$ |
| 216.0786 | 261.0771 | 1.53 | 13 | 12 | 0 | 3 | |
| 203.0695* | 203.0694 | 0.06 | 10 | 9 | 3 | 2 | $[M-C_2H_4]^+$ |
| 203.0708 | 203.0694 | 1.41 | 12 | 11 | 0 | 3 | |
| 147.0307 | 147.0321 | −1.43 | 6 | 3 | 4 | 1 | $[M-(NC_2H_4)_2]^+$ |
| 147.0320* | 147.0321 | −0.09 | 8 | 5 | 1 | 2 | |

* Best fit for trenimon.




| Calculated Mass | Measured Mass | Error | $^{12}C$ | $^{1}H$ | $^{14}N$ | $^{16}O$ | Structure |
|---|---|---|---|---|---|---|---|
| 272.1273* | 272.1277 | −0.40 | 14 | 16 | 4 | 2 | |
| 272.1286 | 272.1277 | 0.95 | 16 | 18 | 1 | 3 | |




*Best fit for proposed compound.

Fig. 5. The TSP–HPLC–MS spectrum of the impurity, mass measurement on the $[M]^+$ ion of the impurity and the proposed structure of the impurity.

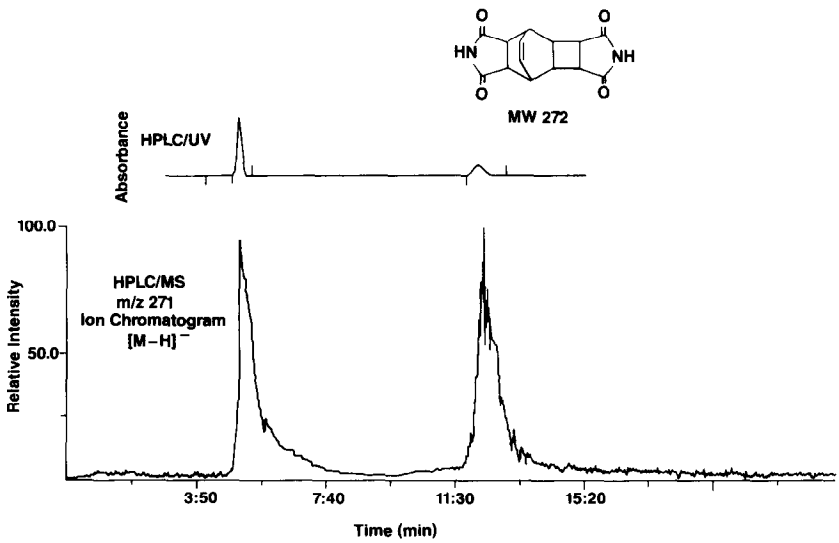



Fig. 6. HPLC–UV chromatogram and HPLC–MS ion chromatogram for the $[M - H]^-$ anion of mitindomide.

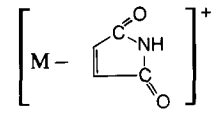
*Analysis of mitindomide*

The HPLC–UV and HPLC–MS analysis of mitindomide indicated the presence of two peaks of nearly equal area (Fig. 6). The TSP spectra of each peak were nearly identical in the positive and negative ion detection mode. The spectrum only consisted of an $[M + H]^+$ ion (positive ion detection mode) or an $[M - H]^-$ anion (negative ion detection mode). The TSP spectra indicated that the compounds were isomers but the spectra does not explain the large difference in retention between the components. The HRMS analysis (Table V) was consistent with the structure of the parent drug. No impurities were detected. Thus, the HRMS information is also consistent with the assumption that the two components are isomers. The other possibility is that the parent drug forms a more polar compound in the solvent system

TABLE V

HRMS DIRECT PROBE EI ANALYSIS OF MITINDOMIDE

Maximum error allowed = 5 ppm. Heteroatoms used: $^{12}C$, atomic weight 12.0000, limiting number 20; $^{14}N$, atomic weight 14.0031, limiting number 2; $^{16}O$, atomic weight 15.9949, limiting number 4.

| *Calculated mass* | *Measured mass* | *Error* | $^{12}C$ | $^{1}H$ | $^{14}N$ | $^{16}O$ | *Identification* |
|---|---|---|---|---|---|---|---|
| 272.0797 | 272.0796 | 0.08 | 14 | 12 | 2 | 4 | $[M]^+$ |
| 244.0848 | 244.0849 | −0.13 | 13 | 12 | 2 | 3 | $[M-CO]^+$ |
| 201.0790 | 201.0791 | −0.13 | 12 | 11 | 1 | 2 | $[M-CONHCO]^+$ |
| 175.0633 | 175.0631 | 0.22 | 10 | 9 | 1 | 2 |  |

which elutes near the solvent front but retains the basic structure of the drug. If such a compound was formed, the polar groups must easily leave the molecule to produce the same TSP spectrum as the parent drug. Also, this type of impurity would not be detected in HRMS analysis since the sample was analyzed in solid form. There was not anough conclusive information to postulate whether the impurity is an isomer or a solvent reaction product of mitindomide.

## CONCLUSIONS

The analysis of these anticancer drugs have demonstrated that HRMS and TSP–HPLC–MS are complimentary techniques, each providing specific information aiding in the validation of compound identity. The same degree of structural information is not obtained where only one techniques is applied.

## ACKNOWLEDGEMENT

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