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

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SUMMARY

Thermospray high-performance liquid chromatography–mass spectrometry was used to confirm the identity of five bulk anticancer drugs, and in some cases, to identify drug impurities. Analysis resulted in both molecular weight and structural (fragment ions) information obtained from the full scan spectra of as little as 50 ng of each drug. The technique was also used to evaluate the chromatographic specificity of corresponding ultraviolet or refractive index high-performance liquid chromatographic detection in the presence of drug degradation products.

INTRODUCTION

The Pharmaceutical Resources Branch (PRB) of the National Cancer Institute (NCI) has the responsibility for providing drugs effective in cancer chemotherapy to programs involved in laboratory, preclinical, and clinical research and treatment of cancer. Inherent in this responsibility is the need to obtain detailed analytical information demonstrating that bulk batches of such drugs are completely suitable for the manufacture of pharmaceutical dosage forms and that such pharmaceutical dosage forms are themselves suitable for use in research and clinical programs. Specifically, analytical methodology must be developed and validated in order to identify qualitatively and quantitatively significant drug impurities (synthetic intermediates, by products, degradation products). The analytical options are sometimes limited since most of the subject drugs are not amenable to gas chromatographic (GC) analysis. High-performance liquid chromatography (HPLC) is an ideal separation technique but lacks detectors of sufficient specificity to identify uniquely the parent drug or impurities[1]. HPLC combined with mass spectrometry (HPLC–MS) can provide both the specificity and capability for direct identification needed for analysis and assay validation. Of the HPLC–MS techniques available, thermospray (TSP) appears to offer the greatest capability for direct, high sensitivity application to existing drug assays based on HPLC. First, the high flow-rate capability of TSP (0.5–2.0 ml/min) eliminates the need for splitting the HPLC effluent with conventional analytical col-

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TABLE I

THERMOSPRAY HPLC–MS CONDITIONS

Compound	*Solvent*	*Column*	*Buffer*	*Column flow (ml/min)*	*Aerosol temp. (°C)*
Tetrahydrouridine	100% water	RCM C_{18} 10 cm × 8 mm I.D. 10 μm	0.1 *M* ammonium acetate	1.6	195
Azolastone	50% methanol in water	RCM C_{18} 10 cm × 8 mm I.D. 10 μm	0.5 *M* ammonium acetate added post column at 0.3 ml/min 5% acetic acid	1.3	185
Triazone	100% water	Zorbax C_8 25 cm × 4.6 mm I.D.	0.1 *M* ammonium acetate	1.6	195
Aphidicolin glycinate	55% methanol in water	Zorbax C_8 25 cm × 4.6 mm I.D.	0.1 *M* ammonium acetate	1.6	182
Sulfamic acid derivative	30% acetonitrile in water	RCM C_{18} 10 cm × 8 mm I.D. 10 μm	0.1 *M* ammonium acetate	1.4	162

umns[2–6]. Second, TSP can operate with the large amounts of water and buffer often necessary for the HPLC separation of polar, ionic chemicals such as anticancer drugs. Finally, TSP ionization is very soft, providing molecular weight information from these thermally labile drugs[7] which cannot be obtained by off-line or thermal desorption HPLC–MS techniques.

The capabilities of TSP–HPLC–MS were demonstrated for the analysis of several anticancer drugs including a triazinone, tetrahydrouridine, aphidicolin glycinate, azolastone, and a sulfamic acid derivative. The specificity of the TSP–HPLC–MS technique, its use in the validation of HPLC drug assays, and the identification of drug impurities are also discussed.

EXPERIMENTAL

The HPLC consisted of two Model 6000A pumps (Waters Assoc., Milford, MA, U.S.A.) controlled by a Model 720 system controller (Waters Assoc.). A Model 709 pump (Milton Roy, St. Petersburg, FL, U.S.A.) was used for the post-column addition of buffer. The anticancer drugs were introduced into the system with a Model U6K injector (Waters Assoc.), and chromatographed using the conditions in Table I. A switching valve before the column allowed direct analysis (sample loop mode) of samples or standards on the TSP–MS system without HPLC column separation. The sample loop mode of operation was used in the initial evaluation of the interface for single component injections. A coaxial mixing tee located between the column and UV detector was used to add aqueous buffer (required for TSP operation) when the presence of such buffer in the HPLC mobile phase interfered with the chromatographic separation of drug component[8]. A Model 440 UV detector (Waters Assoc.) set at 254 nm and a 2-μm filter (Rainin Inst., Woburn, MA, U.S.A.) were also used in-line between the HPLC and the TSP interface.

The TSP interface (Finnigan MAT, San Jose, CA, U.S.A.) was installed on a Finnigan 4500 quadrupole mass spectrometer. The interface included a temperature controller which allowed monitoring of the vaporizer, a source-jet, and aerosol temperature zones. Control of the source and vaporizer assembly temperatures was achieved using cartridge heaters. The HPLC effluent was introduced into the interface without splitting and continuously removed from the source using a liquid nitrogen cold trap prior to a mechanical rough pump. The mass spectrometer was operated with an ion entrance voltage of -15 V, an extractor voltage of -30 V, a lens voltage of 0 V, and a quadrupole entrance of -48 V in the positive ion mode. The instrument was scanned from m/z 150 to m/z 550 at a rate of 2 sec per scan. The presence of numerous solvent-buffer cluster ions limited the lower mass range available to the technique[8]. The data were collected and processed with an INCOS data system (Finnigan MAT).

The TSP interface was optimized by tuning the instrument parameters to produce the highest stability and intensity for solvent–buffer ions in the mass range of 30–150 m/z (oscilloscope). Previous experiments have shown that the temperature of the analyte signal correlates closely with the signal for these solvent–buffer ions[9]. The optimal aerosol temperatures determined for each separation are given in Table I. Mass calibration and ion intensities were checked with polypropylene glycol (AMW 3000) before each analysis to verify acceptable TSP operation.

TABLE II

TABULAR LISTING OF THE MASS SPECTRA FOR FIVE ANTICANCER DRUGS

Tetrahydrouridine			*Azolastone*			*Triazone*			*Aphidicolin glycinate hydrochloride*			*Sulfamic acid derivative*		
MW 248			MW 242			MW 245			MW 431			MW 290		
m/z	*%Ri*	*Identif.*	*m/z*	*%Ri*	*Identif.*	*m/z*	*%Ri*	*Identif.*	*m/z*	*% Ri*	*Identif.*	*m/z*	*% Ri*	*Identif.*
461	4	$[2M+H-2H_2O]^+$	260	30	$[M+NH_4]^+$	263	100	$[M+NH_4]^+$	471	28	—*	308	100	$[M+NH_4]^+$
249	52	$[M+H]^+$	243	8	$[M+H]^+$	246	20	$[M+H]^+$	456	68	$[M+H-HCl+acetate]^+$	271	40	—*
231	100	$[M+H-H_2O]^+$	224	35	$[M+NH_4-HCl]^+$	210	38	$[M+H-H_2O]^+$	414	14	$[M+NH_4-HCl]^+$	229	13	—*
213	2	$[M+H-2H_2O]^+$	164	100	$[M+H-CONH_2Cl]^+$	190	12	$[M+H-C_2N_2O]^+$	396	100	$[M+H-HCl]^+$			
						168	48	$[Ribose + NH_4]^+$	338	35	$[M+H-COCH_2NH_2]^+$ $-HCl$			

* Composition unknown.

RESULTS AND DISCUSSION

Confirmation of identity

A primary requirement in the assay of bulk drugs and formulations is the qualitative confirmation of the drug identity. Non-chromatographic spectroscopic techniques (*e.g.* infrared, NMR, MS) often fulfill this function when interference from impurities in the drug sample is not of concern. Application of the TSP–MS technique using simple loop injection (without an HPLC column) provides additional access to the mass spectrometer for non volatile and/or thermally labile drugs. However, in the presence of significant impurities, the interpretation of results from such non-chromatographic methods can be ambiguous. In such cases, TSP–HPLC–MS provides the only means of obtaining specific confirmation of drug identity in a single step.

The TSP–HPLC–MS spectrum obtained from each of the subject drugs provided a highly complete and specific means of chromatographic characterization. In all cases, the TSP spectrum was shown to correlate with the proposed structure (Table II), providing both molecular weight (molecular ion) and structural (fragment ion) information.

Two of the drugs, aphidicolin glycinate hydrochloride and a sulfamic acid derivative, also produced fragment ions which apparently formed from thermal decomposition within the thermospray source during analysis. For example, TSP–HPLC–MS analysis of the sulfamic acid derivative yielded an $[M+H]^+$ ion with good chromatographic peak shape (base peak width *ca.* 50 sec) as shown in Fig. 1.

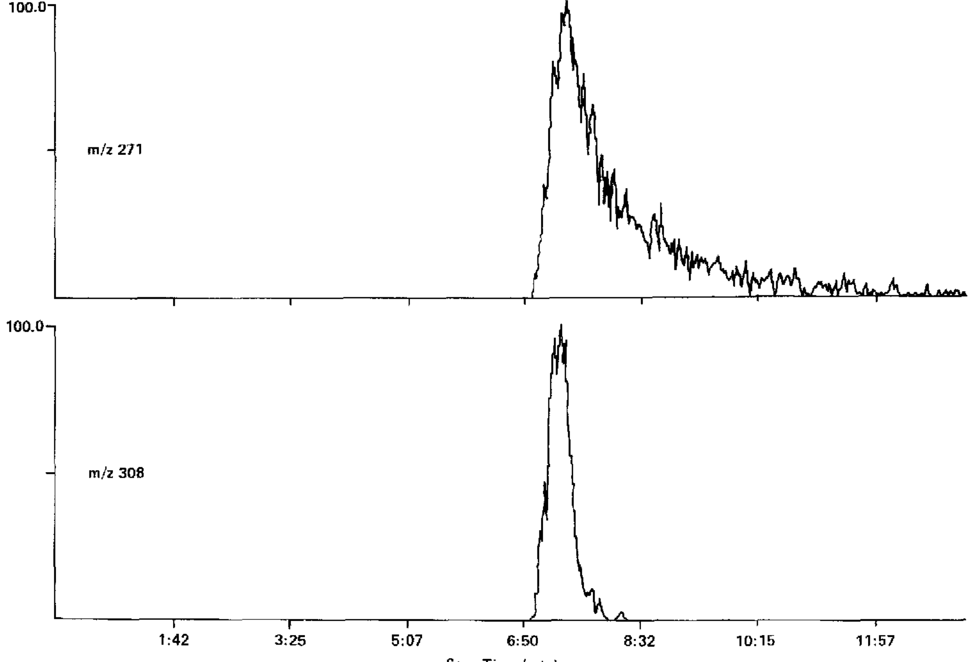

Fig. 1. Ion chromatograms for *m/z* 308 $[M+NH_4]^+$ ion of sulfamic acid derivative and a source decomposition ion *m/z* 271.

However, the fragment ions at m/z 271 and m/z 229 maximized *ca.* 5 sec later and exhibited extreme tailing (base peak width *ca.* 4 min). A probable explanation for this behaviour is condensation of the analyte aerosol in the source block with subsequent thermal decomposition of the parent drug.

Identification of drug impurities

The combined chromatographic–structural delineation capabilities of TSP–HPLC–MS was demonstrated to be particularly useful for the identification of significant impurities in bulk anticancer drugs. Such identification is laborious and often difficult using spectroscopic or chromatographic techniques alone.

Both the HPLC–RI and HPLC–UV analyses of tetrahydrouridine had demonstrated the presence of several unknown impurities at levels ranging from 0.5 to 5.0% of the total detector response. Subsequent TSP–HPLC–MS analysis of the drug (Fig. 2) allowed assignment of tentative but reasonable structures to each of the three impurities. The identity of impurity 1, ribose, was confirmed with an authentic standard (Fig. 3). Identification of impurities 2 and 3 as deoxyuridine and deoxytetrahydrouridine, respectively, could not be directly confirmed although deoxytetrahydrouridine is a known by-product in the synthesis of the parent drug (Fig. 4). Struc-

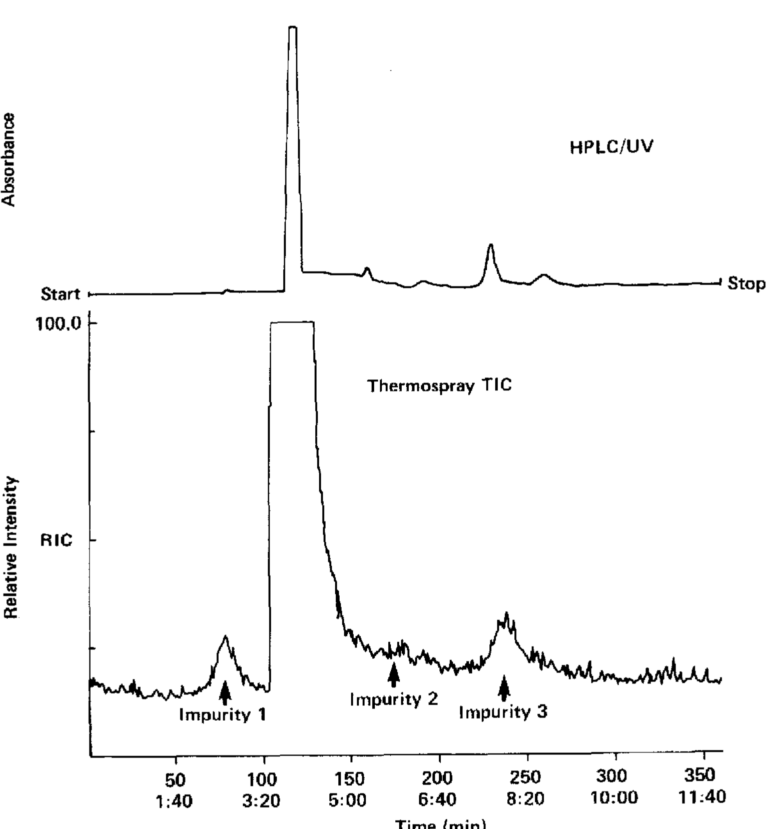

Fig. 2. HPLC–UV and TSP–HPLC–MS chromatograms for the analysis of tetrahydrouridine.

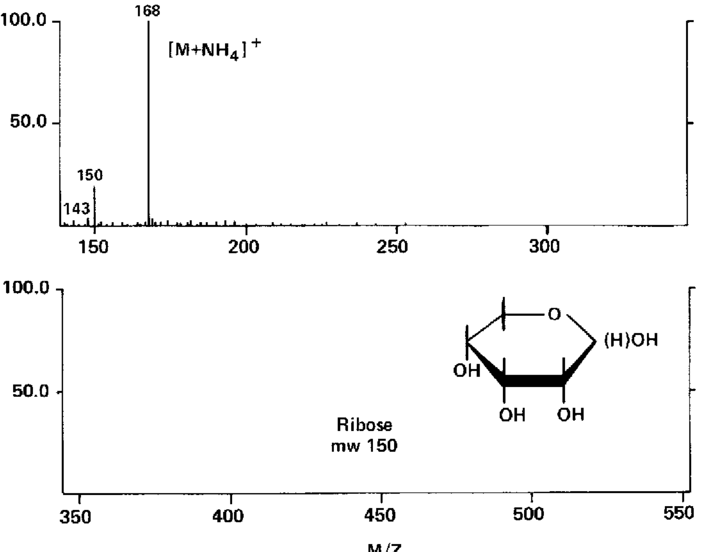

Fig. 3. TSP spectrum of impurity 1 in tetrahydrouridine, identified as ribose.

tural assignments were based on m/z 231 $[M+H]^+$ and m/z 158 $[M+OAc-\text{ribose}]^+$ for deoxyuridine, and m/z 233 $[M+H]^+$ and m/z 160 $[M+OAc-\text{ribose}]^+$ for deoxytetrahydrouridine (Fig. 5).

Both HPLC–RI and TSP–HPLC–MS (Fig. 6) analysis of aphidicolin glycinate hydrochloride (retention time *ca.* 6 min) detected the presence of at least one significant impurity eluting at *ca.* 9 min. The parent drug exhibited ions at m/z 396 $[M-HCl]^+$, m/z 414 $[M+NH_4-HCl]^+$, m/z 455 $[M+OAc-HCl]^+$, and m/z 456

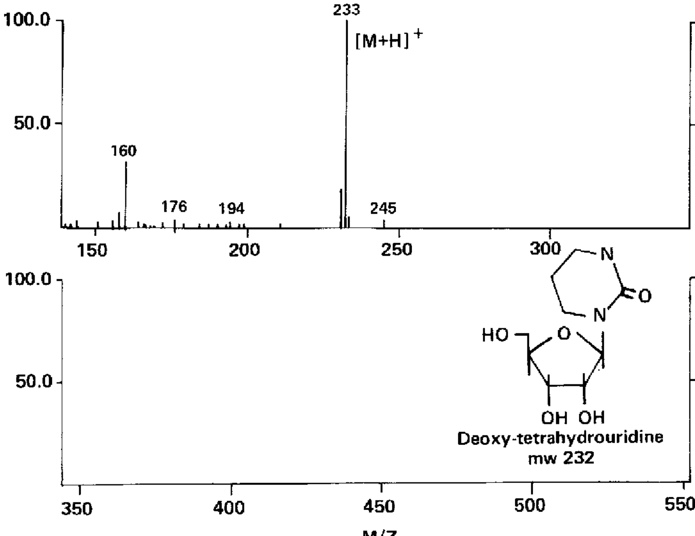

Fig. 4. TSP spectrum of impurity 3 in tetrahydrouridine, identified as deoxytetrahydrouridine.

Tetrahydrouridine

Deoxy-tetrahydrouridine

Fig. 5. Synthesis of tetrahydrouridine.

$[M+HOAc-HCl]^+$. The impurity exhibited major ions at m/z 356 $[M+NH_4]^+$ and m/z 416 $[M+HOAc]^+$ indicative of aphidicolin, a synthetic precursor. The structure of the impurity was subsequently confirmed with an authentic standard (Fig. 7).

Validation of stability-indicating HPLC assays

The validation of drug assays has always presented the problem of adequately demonstrating assay specificity in the presence of degradation products formed under a variety of conditions. The exact nature of these degradation products is not always known nor are authentic standards often available. One method of demonstrating the specificity of HPLC assays is used for anticancer drugs and involves forcing the

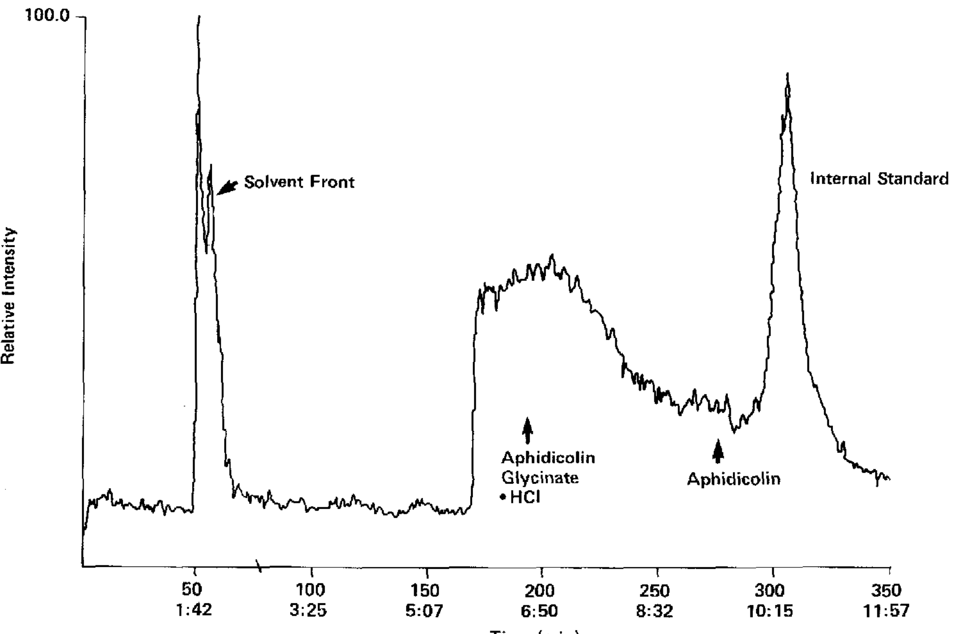

Fig. 6. TSP-HPLC MS total ion current chromatogram for the analysis of aphidicolin glycinate hydrochloride.

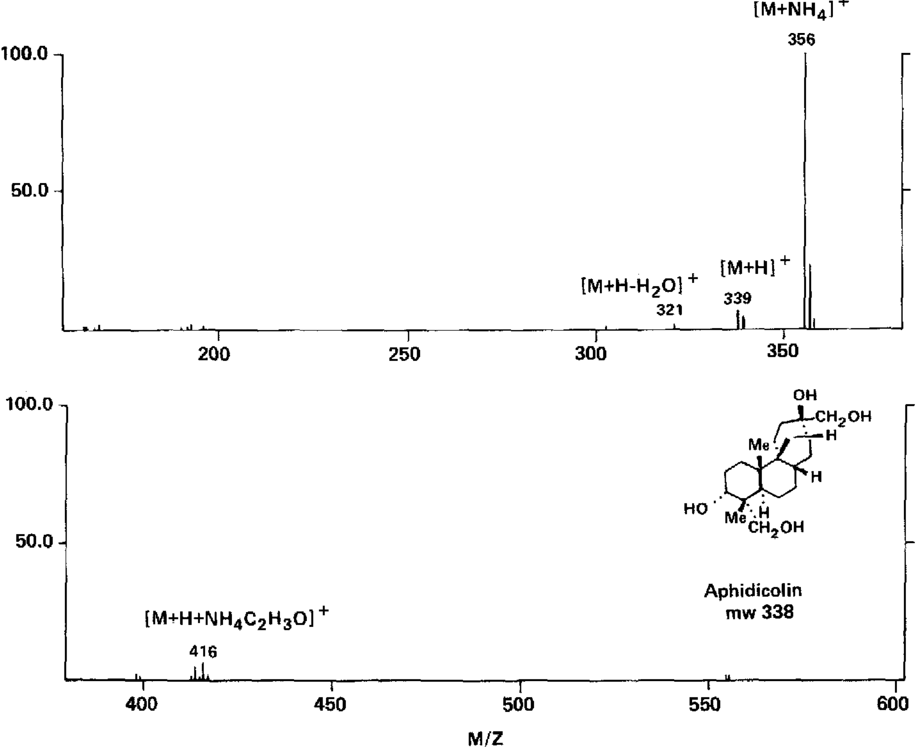

Fig. 7. TSP spectrum of aphidicolin, a precursor in the formation of aphidicolin glycinate hydrochloride.

degradation of parent drug under a variety of accelerated conditions (*e.g.* light, moisture, heat, acid, and base) as shown in Table III. Analysis of the remaining drug using dual detectors based on different compound response properties (*e.g.* RI and UV) provides a response ratio which will remain constant, compared with undegraded standard, so long as the peak composition also remains constant (*i.e.* contains a single component). This of course assumes that the individual response of each detector is linear in the range used.

The lack of a UV detector response for the "sulfamic acid derivative" (SAD)

TABLE III

DETERMINATION OF POSSIBLE INTERFERENCES IN THE HPLC ANALYSIS

Samples stored at stated conditions for 24 h.

Sample No.	*Temperature (°C)*	*hv*	*Solvent*
1	25	Dark	Dry
2	25	Dark	H_2O (2.0 ml)
3	25	Room light	H_2O (2.0 ml)
4	90	Dark	H_2O (2.0 ml)
5	25	Dark	1 *N* NaOH (2.0 ml)
6	25	Dark	1 *N* H_3PO_4 (2.0 ml)

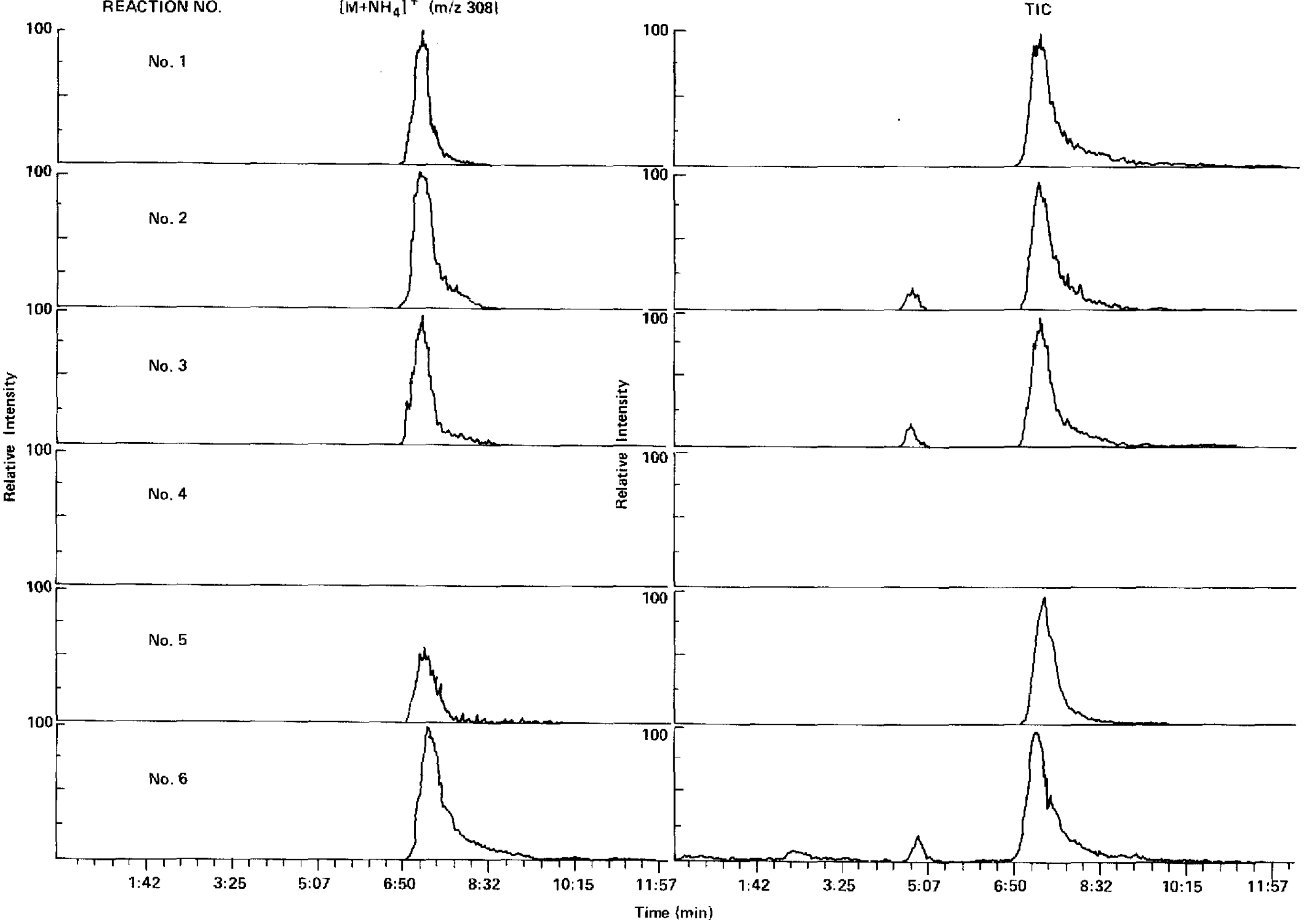

Fig. 8. The total ion current and $[M+NH_4]^+$ (*m/z* 308) ion chromatograms for the analysis of forced degradation Conditions No. 1–6 of SAD.

TABLE IV

THE AREA RATIO BETWEEN THE TOTAL ION CURRENT AND SELECTED ION CURRENT (*m/z* 308) AND THE STATISTICAL PROBABILITY THAT NO COELUTING INTERFERENCES ARE PRESENT FOR THE ANALYSIS OF THE FORCED DEGRADATION OF SAD

Condition No.	*Area ratio TIC (m/z 308)*	$P^{\star}$
1	2.13	0.76
2	1.97	0.65
3	1.82	0.56
4	0.00**	0.00
5	37.0**	0.00
6	1.35	0.35

* If *P* is greater than 0.1, then there is no statistical difference between the response ratio of the analyte in the degraded solution *vs.* the standard.

** Analyte completely degraded (HPLC–RI).

precluded using the UV/RI response ratio in demonstrating the specificity of the routine HPLC–RI assay. However, the availability of TSP–HPLC–MS provided a highly selective alternate in terms of the ratio of the total ion current (TIC) and selected ion current at *m/z* 308 $[M+NH_4]^+$ (Fig. 8). Conditions No. 1–3 and No. 6 (Table III) exhibited the same TSP spectrum as the standard and the TIC/selected ion current (*m/z* 308) ratios (Table IV) were statistically identical (component to standard), indicating no coeluting interference was present in the peak. Condition No. 4 exhibited no signal by either HPLC–MS (Fig. 8) or HPLC–RI, indicating no drug was left under these degradation conditions. Condition No. 5 exhibited an inconsistent area ratio by TSP–HPLC–MS and no response by HPLC–RI indicating the drug had decomposed and that a degradation product eluted at the retention time of SAD. However, this degradation product was transparent to HPLC–RI, and the HPLC–RI assay remained analyte specific.

TSP–HPLC–MS also provided the means for identification of the components formed from SAD degradation. Conditions No. 2, No. 3, and No. 6 all exhibited a peak in the TIC at 4.6 min (Fig. 8). This peak showed a TSP spectrum which consisted of a base peak at *m/z* 229 and a simple fragment ion at *m/z* 212 (8% relative intensity). The peak at *m/z* 229 was also observed in the TSP spectrum of SAD, as a thermal degradation product. No structural assignment has been made for this degradation product.

The TSP spectrum of the degradation product from Condition No. 5 (Fig. 9) contained numerous ions also observed in the thermal degradation of the SAD standard. Most of the ions in the spectrum could be rationalized by simple cleavages in the molecule and will require additional information by other techniques in order to postulate a definitive structure.

Thermospray sensitivity

The TSP–HPLC–MS analysis of all the above candidate anticancer drugs exhibited adequate sensitivity with typical analytical concentrations in the range of 100–200 ng (injected). These amounts resulted in good full scan spectra (signal-to-noise $>$ 20 for the base peak).

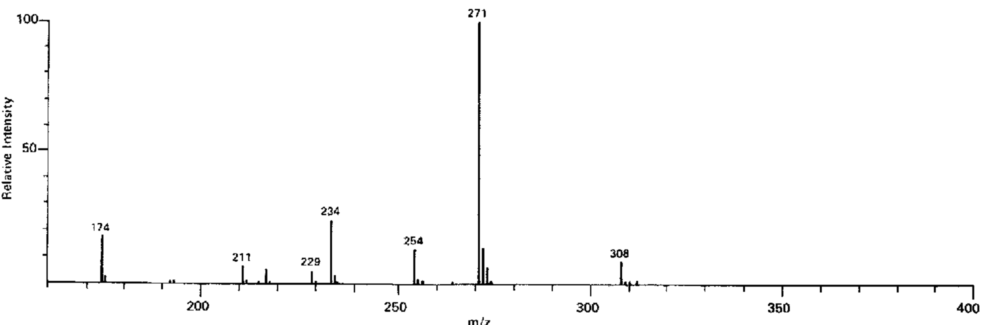

Fig. 9. TSP–HPLC–MS spectrum of the degradation product from Condition No. 5 that is transparent to HPLC–RI detection.

CONCLUSIONS

TSP–HPLC–MS has proven useful in the confirmation of anticancer drug identity, in the identification of drug impurities, and in the validation of routine HPLC assays. This HPLC–MS technique provided specificity unavailable using conventional UV or RI detectors.

ACKNOWLEDGEMENT

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