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Journal of Chromatography B, 696 (1997) 99–115

JOURNAL OF CHROMATOGRAPHY B

Profiling degradants of paclitaxel using liquid chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry substructural techniques

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Received 14 January 1997; revised 24 March 1997; accepted 24 March 1997

Abstract

A rapid and systematic strategy based on liquid chromatography–mass spectrometry (LC–MS) profiling and liquid chromatography–tandem mass spectrometry (LC–MS–MS) substructural techniques was utilized to elucidate the degradation products of paclitaxel, the active ingredient in Taxol. This strategy integrates, in a single instrumental approach, analytical HPLC, UV detection, full-scan electrospray MS, and MS–MS to rapidly and accurately elucidate structures of impurities and degradants. In these studies, degradants induced by acid, base, peroxide, and light were profiled using LC–MS and LC–MS–MS methodologies resulting in an LC–MS degradant database which includes information on molecular structures, chromatographic behavior, molecular mass, and MS–MS substructural information. The stressing conditions which may cause drug degradation are utilized to validate the analytical monitoring methods and serve as predictive tools for future formulation and packaging studies. Degradation products formed upon exposure to basic conditions included baccatin III, paclitaxel sidechain methyl ester, 10-deacetylpaclitaxel, and 7-epipaclitaxel. Degradation products formed upon exposure to acidic conditions included 10-deacetylpaclitaxel and the oxetane ring opened product. Treatment with hydrogen peroxide produced only 10-deacetylpaclitaxel. Exposure to high intensity light produced a number of degradants. The most abundant photodegradant of paclitaxel corresponded to an isomer which contains a C3–C11 bridge. These methodologies are applicable at any stage of the drug product cycle from discovery through development. This library of paclitaxel degradants provides a foundation for future development work regarding product monitoring, as well as use as a diagnostic tool for new degradation products. © 1997 Elsevier Science B.V.

Keywords: Paclitaxel

1. Introduction

Paclitaxel, known commonly as taxol, is a member of the taxane family of natural products and is currently used in the treatment of ovarian and breast tumors [1]. Paclitaxel and other related taxanes are extracted from the bark and leaves of various species of the *Taxus* family [2–6]. The low concentration of paclitaxel in yew trees, approximately 0.01% in dry bark, presented many challenges during the pharmaceutical development process [7,8]. Detailed studies conducted in our laboratory involving HPLC profiles of extracts and process intermediates from *Taxus brevifoliia* and *T. baccata* biomass indicated the

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PII S0378-4347(97)00208-9

presence of many related taxane compounds for which identification was desirable. To rapidly facilitate this goal as well as to provide detailed structural information about the trace level impurities observed in the bulk drug substance, we developed LC–MS and LC–MS–MS strategies based on previous methods for the rapid and systematic elucidation of drug metabolites in physiological fluids and natural products in crude extracts [7–10]. Analyses combined optimized HPLC separation conditions on-line with an electrospray MS interface to obtain molecular mass information from the full scan mass spectra (LC–MS) and structural information from the tandem mass spectra (LC–MS–MS). Using these methodologies, structural and substructural data for trace taxane components in mixtures were obtained rapidly and systematically without prior fractionation. In addition, 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. As a result, an analytical database of over 50 taxanes has been compiled from the studies of natural extracts for taxanes in preparations from *Taxus brevifolia* and *Taxus baccata* and includes: chromatographic characteristics using a standard HPLC system, molecular mass, and collision induced dissociation (cid) tandem mass spectrometry (MS–MS) product ion spectra [7,8].

During the course of drug development, the bulk drug and drug formulation are studied under different stress conditions such as temperature, acid, base, oxidizing conditions, and light. The stressing conditions which may cause drug degradation are utilized to validate the analytical monitoring methods and serve as predictive tools for future formulation and packaging studies [11]. Traditional methods involving process scale-up, isolation, and purification of trace components such as degradants and impurities are expensive and time-consuming. Therefore, the use of methods such as LC–MS profiling which permit the rapid cataloguing and identification of potential degradants are attractive alternatives and serve to decrease the drug product development cycle time.

In addition to previously described studies [7,8] involving the identification of taxanes related to paclitaxel in natural and process related extracts using LC–MS and LC–MS–MS methodologies, our laboratories have also been involved in degradation studies of bulk paclitaxel. The previously described methodologies and resulting database proved to be extremely useful during the development process of paclitaxel and were applied to rapidly characterize degradation products of paclitaxel. In this paper, the profile information obtained during forced degradation studies of paclitaxel will be discussed. These degradation studies were carried out in acidic, basic, high intensity light, as well as exposure to oxidizing (peroxide) conditions to gain an assessment of stability and potential degradants which may be encountered during the pharmaceutical development process of paclitaxel.

2. Experimental

2.1. Chromatography

The HPLC system consisted of a Perkin Elmer Series 410 pump (Norwalk, CT, USA) coupled to a Perkin Elmer LC-235 diode array detector. UV spectra and chromatograms (230 nm) were also obtained. A modified version of an assay for paclitaxel was used and consisted of a pentafluorophenyl reversed-phase column (4.0 mm×250 mm, 5 μm, ES Industries, Marlton, NJ, USA) with a mobile phase of 65:35 buffer (2 m*M* ammonium acetate, pH 6.9): acetonitrile held isocratic for 26 min followed by a 25 min linear gradient to 20:80 (buffer–acetonitrile) at a flow-rate of 2.0 ml/min [12]. Typically, 25 μl of sample were injected with a Model 7125 Rheodyne injector (Cotati, CA, USA).

2.2. Degradation protocols

Degradation samples were prepared from a stock solution of paclitaxel (2 mg/ml in acidified methanol, pH 5.5). Base degradation was performed by incubating 1.0 ml of paclitaxel stock solution with 0.1 ml of aqueous Na_2CO_3 (0.05 *M*) for 10 min to produce a pH 8 solution, followed by re-establishment of pH 5.5 by addition of 1–2 μl of concentrated HCl. Acid degradation was performed by incubating 2.0 ml of paclitaxel stock solution with 0.8 ml of aqueous HCl (2.4 *M*) for 240 min,

followed by the addition of 0.38 ml of concentrated ammonium hydroxide to re-establish a pH 5.5 solution. Oxidative degradation was performed by incubation of 1.0 ml of paclitaxel stock solution diluted with 3 ml of 30% hydrogen peroxide to a concentration of 0.5 mg/ml. The formation of a white precipitate was observed since paclitaxel has limited solubility in aqueous solutions. Light degradation was performed by exposing bulk paclitaxel to 1000 foot candle white light for 92 days. A different lot of bulk paclitaxel was used in the light degradation studies than for acid, base, and oxidative degradation studies.

2.3. Electrospray mass spectrometry

A PE-SCIEX (Concorde, Canada) API III triple quadrupole mass spectrometer equipped with an ionspray (pneumatically assisted electrospray) interface was used on-line with the HPLC system and UV detector described above. Since the ionspray interface operates most effectively at flows less than 200 μl/min, the eluent from the UV detector was split approximately 1:20 prior to the interface. The split ratio was regulated by adjusting the length of the restriction line (fused-silica capillary with 50 μm I.D. and 160 μm O.D.). LC–MS experiments were performed while scanning from m/z 250 to 1300 at a scan rate of 2 s/scan. For LC–MS–MS substructural studies, the parent ions were selected in the first quadrupole mass analyzer and transmitted into the second quadrupole (collision cell) with a collision energy of 50 eV and argon collision gas thickness of 400×10^{12} atoms/cm^2. The final mass analyzer was scanned from m/z 50 to an m/z value approximately 20 Da higher than the parent $(M+NH_4)^+$ ion to record the product ions.

3. Results and discussion

3.1. Base degradation

Due to the high sensitivity of mass spectrometry, implementation of LC–MS profiling methods was found to be particularly advantageous for the rapid characterization of impurities and low level degradants. The chromatographic method used in these and other paclitaxel profiling studies provided good resolution and reproducible relative retention (RRT, relative to paclitaxel) times using standardized HPLC conditions [7,8]. This has resulted in the development of a taxane database containing chromatographic and structural information about related taxanes, impurities, and degradants and the ability to transfer data to collaborating laboratories. In addition, the reverse phase HPLC conditions also provided a general measure of lipophilicity of each compound, useful for interpretation of substructural differences between related taxanes.

The structure of paclitaxel shown in Fig. 1 illustrates the ester attachment of the paclitaxel sidechain to the tricyclic (baccatin) core as well as the various sites of acetylation on the diterpene core. Fig. 2 illustrates the HPLC–UV chromatogram of bulk paclitaxel used in these degradation studies. Several low level impurities are observed in bulk paclitaxel. Since paclitaxel is a natural product, it is not surprising that trace level impurities are carried through the large scale purification process. The identification of these low level taxanes has been the subject of separate papers [7,8]. As shown in Fig. 3, dissolution of paclitaxel in basic aqueous/methanolic solutions results in the rapid formation of several degradants. Three early eluting degradants and a single late eluting degradant are formed upon exposure of paclitaxel to basic conditions. The elution order of the three early eluting degradants indicates they are less lipophilic than paclitaxel. Based on the

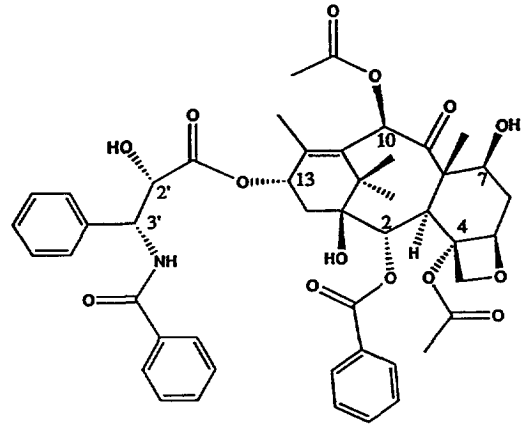


Fig. 1. Structure of paclitaxel.

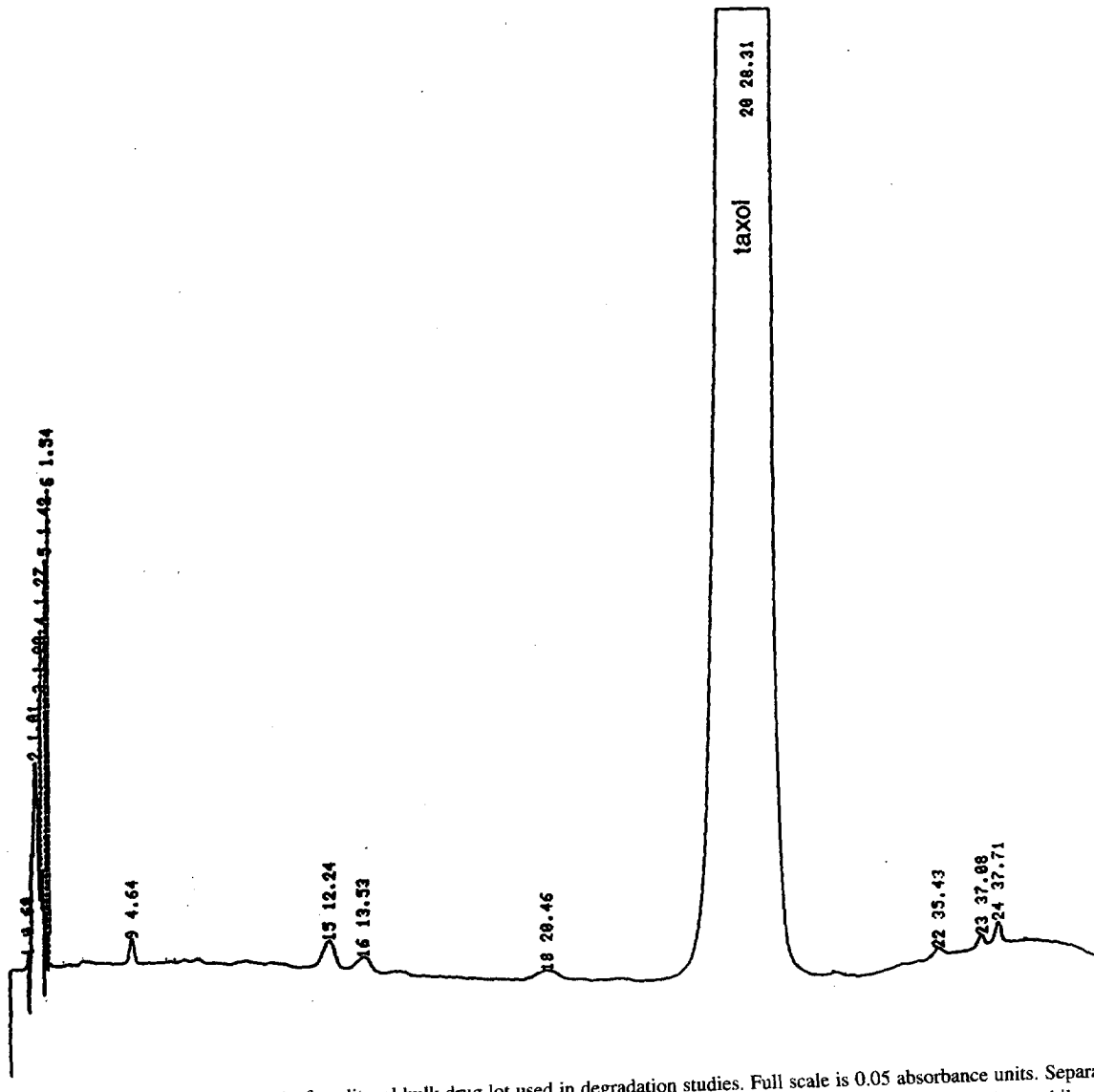


Fig. 2. HPLC chromatogram (230 nm) of paclitaxel bulk drug lot used in degradation studies. Full scale is 0.05 absorbance units. Separation achieved using a ES Industries pentafluorophenyl reverse phase column (4.0 mm×250 mm×5 μm, 60 Å, ES Industries) with a mobile phase of 65:35 buffer (2 m*M* ammonium acetate, pH 6.9)–acetonitrile held isocratic for 26 min, followed by a linear gradient to 20:80 buffer–acetonitrile over 25 min at a flow-rate of 2.0 ml/min.

structure of paclitaxel (Fig. 1), a favorable chemical process with basic conditions would involve hydrolysis of the ester linkages. In fact, information obtained on-line during LC–MS profile studies of the base induced degradants indicates molecular masses consistent with the hydrolyzed sidechain and core.

The electrospray LC–MS interface utilized in these studies is an extremely soft ionization process and produces primarily pseudo-molecular ions such as $(MH)^+$ or $(M+NH_4)^+$ ions, providing definitive molecular mass information. Due to the lack of either an acidic or basic functional group, taxanes generally produce ammoniated molecular ions $(M+NH_4)^+$ when ammonium ions are present in the mobile phase. The LC–MS mass chromatograms corresponding to the ammoniated molecular ions of

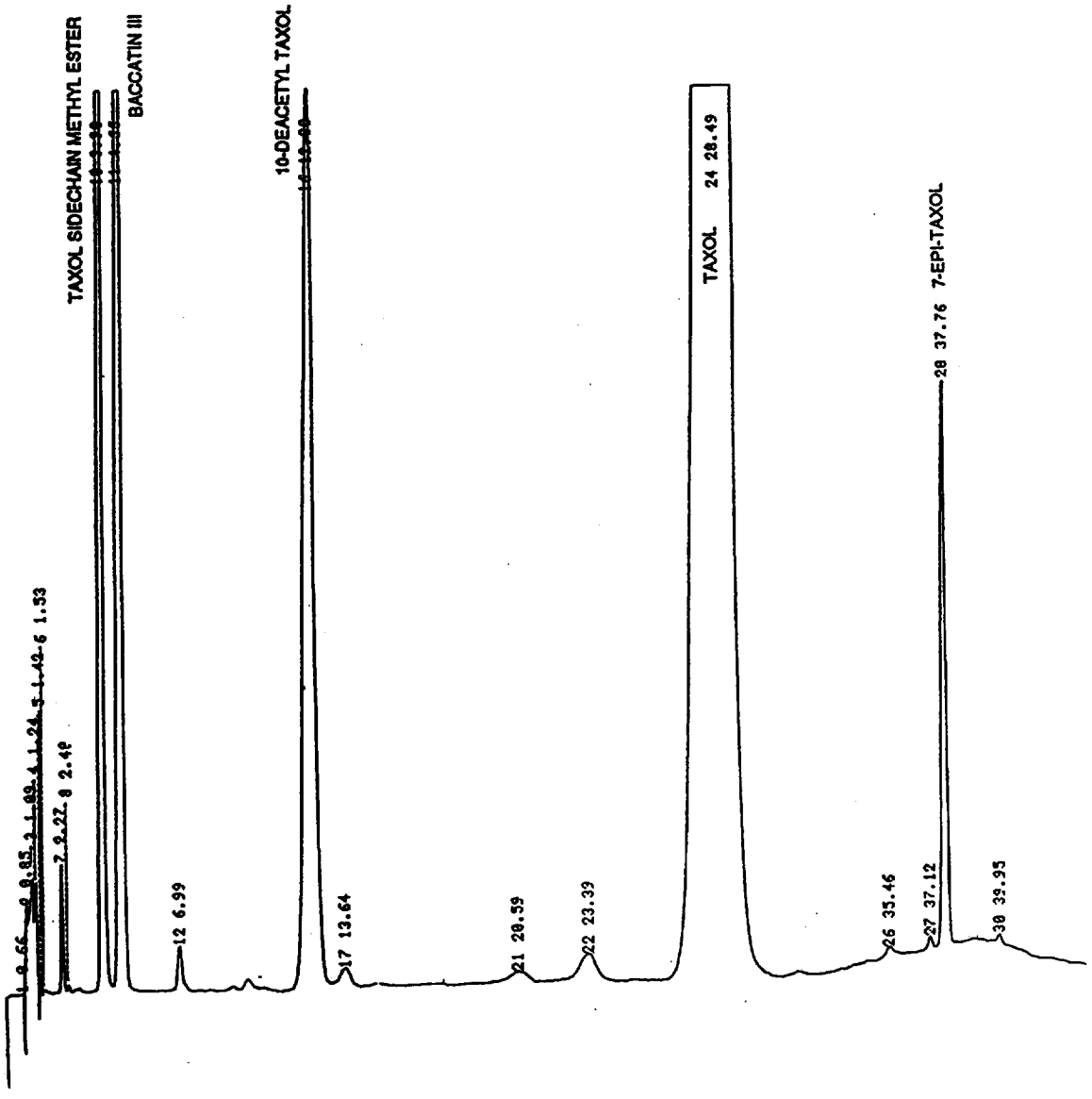


Fig. 3. HPLC chromatogram (230 nm) of base-induced degradants of paclitaxel.

paclitaxel and its base-induced degradants are shown in Fig. 4. The difference between the molecular mass of paclitaxel and the degradant is indicative of the substructural differences between the compounds. For example, comparison of the molecular mass difference between paclitaxel (MW 853) and the degradant at 12.2 min (MW 811) demonstrates a molecular mass difference of 42 Da, which is commonly indicative of an acetyl substructure in taxanes. The observed molecular masses of the degradants are consistent with the methyl ester of the paclitaxel side chain, baccatin III (paclitaxel core), deacetylated paclitaxel, and an isomer of paclitaxel.

When dealing with complex mixtures, the production of only molecular ions is an ideal situation for molecular mass confirmation. Unfortunately, the lack of fragmentation information in the full scan mass spectrum is detrimental from the structure

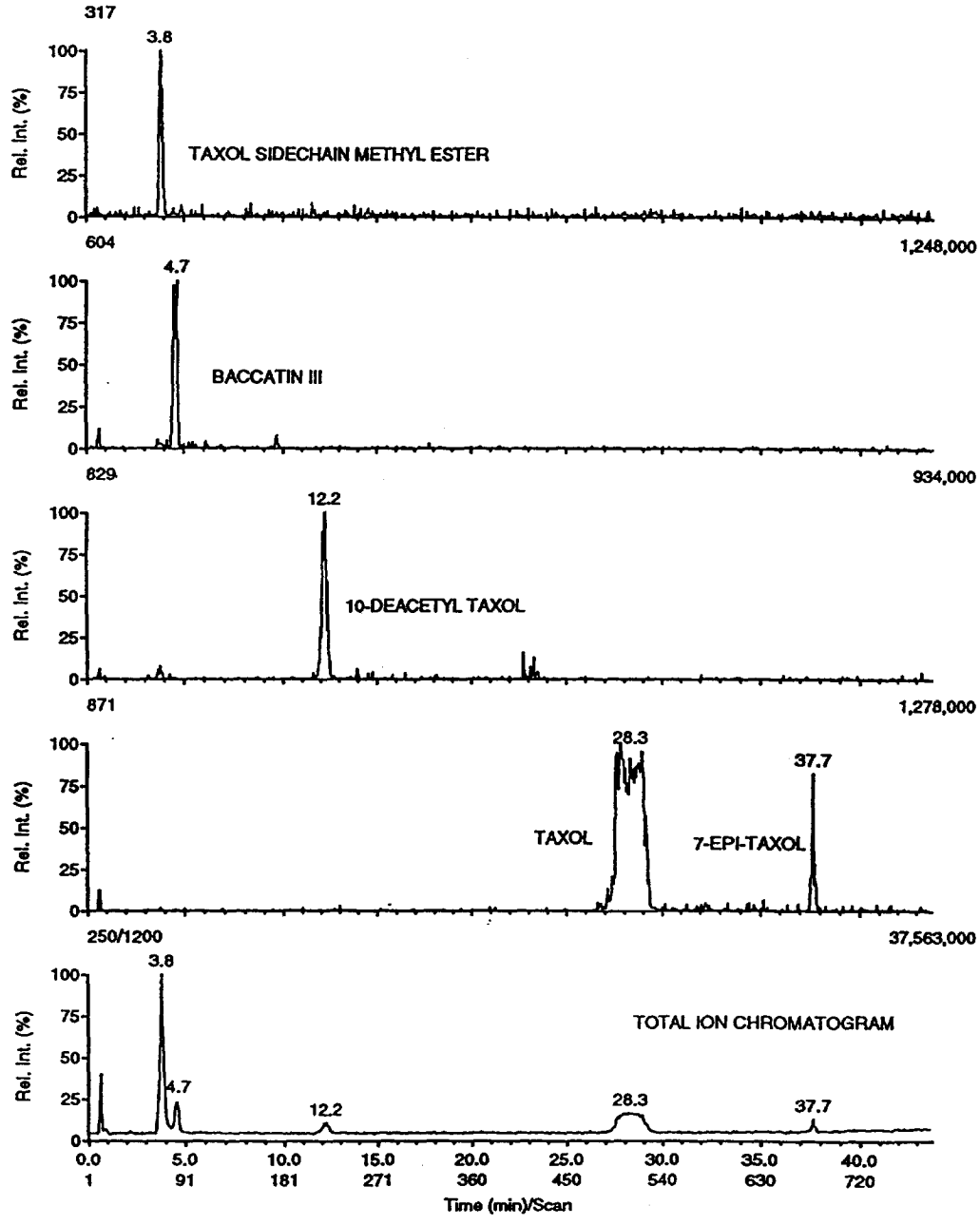


Fig. 4. LC–MS mass chromatograms (extracted ion current profiles) of paclitaxel treated with sodium carbonate for 10 min, at the m/z ratio of their $(M+NH_4)^+$ ions, in the degraded sample shown in Fig. 3. The m/z ratio of the mass chromatograms plotted are shown at the left of each trace.

elucidation perspective. However, when electrospray ionization is coupled on-line with a tandem mass spectrometer, detailed structural information for each component can be obtained [7–10,13–15]. In these studies, we relied heavily on these unique taxane fragmentation patterns and our previous taxane data-

base to assist in the structural determination of the degradation products. In addition to chromatographic retention characteristics and molecular mass information, structures of degradants were proposed based on the comparison of their fragmentation patterns to those of paclitaxel and other well characterized taxanes which were used as substructural templates. Interpretation of the structures of unknown degradants proceeded by the association of specific product ions and neutral losses with specific substructures. This MS–MS comparative method is based on the premise that the targeted degradants would be expected to retain much of the original paclitaxel structure such as the taxane core substructure. Therefore, paclitaxel-related degradants would be expected to undergo similar fragmentations to paclitaxel and other taxane standards. Common MS–MS product ions and neutral losses observed in paclitaxel and the unknown taxanes were evidence for common substructures and differences were indicative of variance in those substructures.

Fig. 5A illustrates the MS–MS product ion spectrum of the base induced degradant at m/z 829 ($M+NH_4^+$). Fig. 5B illustrates the product ion spectrum of paclitaxel at m/z 871 ($M+NH_4^+$). Comparison of the fragmentation template of paclitaxel to that of the product ion spectrum of the degradant indicates a great deal of similarity. Diagnostic product ions indicative of the paclitaxel side chain at m/z 286, 268, and 240 are present in both spectra indicating the structural difference in the degradant is not located on the side chain. However, a key product ion at m/z 569 indicative of the paclitaxel core is noticeably absent in the product ion spectrum of the degradant. Instead, a product ion at m/z 527 is observed. This 42 Da mass difference is due to deacetylation in the degradant. Based on this structural information as well as molecular mass and chromatographic retention information, the degradant was assigned as 10-deacetyl paclitaxel.

During the development of paclitaxel and our studies involving the identification of taxanes related to paclitaxel in natural and process related extracts, we noticed a consistent pattern of two isobaric components producing two distinct chromatographic peaks in the mass chromatograms of *Taxus* extracts [7,8]. This pattern was the result of epimerization of paclitaxel analogs at the 7-position. The later eluting isomer had the 7-β-configuration and their identities, in several cases, were confirmed using synthesized or isolated standards. The formation of these epimers was also found to be pH dependent. At neutral pH and above, epimerization was favored. With these previous insights, the late eluting paclitaxel isomer observed in the base degradation study, can be assigned as 7-epipaclitaxel. This same diagnostic comparative process was used to identify the other base-induced degradants as the methyl ester of the side chain and baccatin III. Formation of the methyl ester of the side chain resulted from esterification with methanol present in the sample diluent. Fig. 6 summarizes the base degradation pathway determined for paclitaxel.

3.2. Acidic conditions

Fig. 7 illustrates the HPLC–UV chromatogram of bulk paclitaxel incubated in an aqueous/methanolic solution of HCl for 240 min. The two primary degradation products elute prior to paclitaxel, indicating their polar characteristics versus paclitaxel. The earlier eluting degradant at 13.0 min has the same relative retention time and molecular mass as 10-deacetylpaclitaxel. Apparently, the acetate group at the 10-position is fairly labile as evidenced by the presence of 10-deacetylpaclitaxel in bulk paclitaxel, base-degraded paclitaxel, and acid degraded paclitaxel. The molecular mass of the later eluting, 19.1 min degradant, was determined to be 871 Da. This molecular mass difference corresponds to an 18 Da increase relative to paclitaxel. Examination of the product ion spectrum of this degradant shown in Fig. 8A indicates the diagnostic product ions indicative of the paclitaxel side chain at m/z 286, 268, and 240 are present indicating the structural difference in the degradant is not located on the side chain. Product ions characteristic of the core were observed at m/z 587 and m/z 527, indicative of the 18 Da increase. In addition, the typical core ions observed for paclitaxel at m/z 569 and m/z 509 are also present resulting from loss of water. Based on the structure of paclitaxel (Fig. 1), a chemical process with acidic conditions would involve the addition of water (18 Da) to the paclitaxel core, possibly opening of the oxetane ring. This information coupled with preliminary NMR data led to the proposal of an oxetane

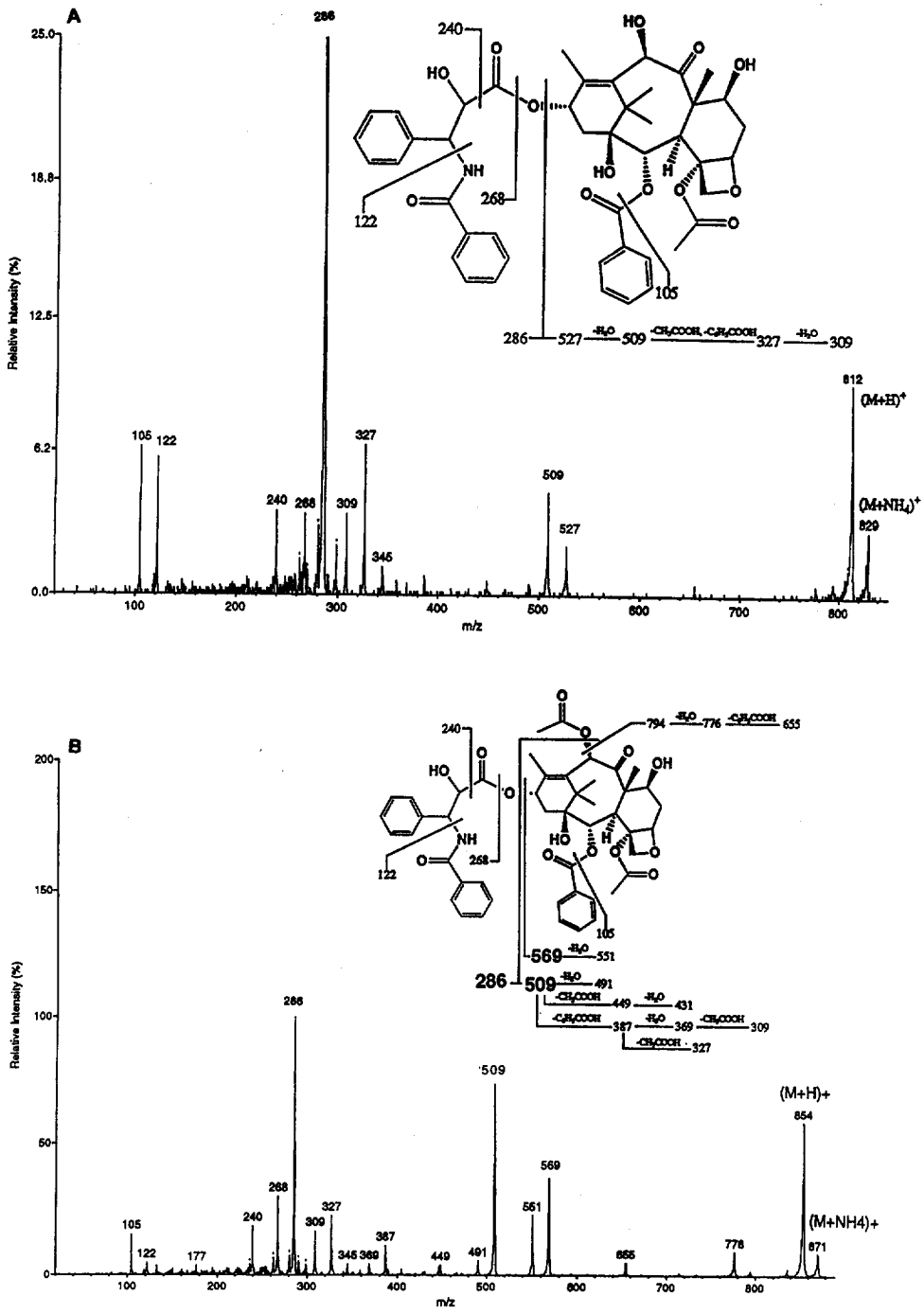


Fig. 5. Product ion spectrum of the ion at m/z 829 $(M+NH_4)^+$ of the base-induced degradant. Product ions, neutral losses and their correspondence to specific substructures are indicated. (B) Product ion spectrum of the m/z 871 $(M+NH_4)^+$ ion of paclitaxel used as a template for structural analysis of related taxanes.

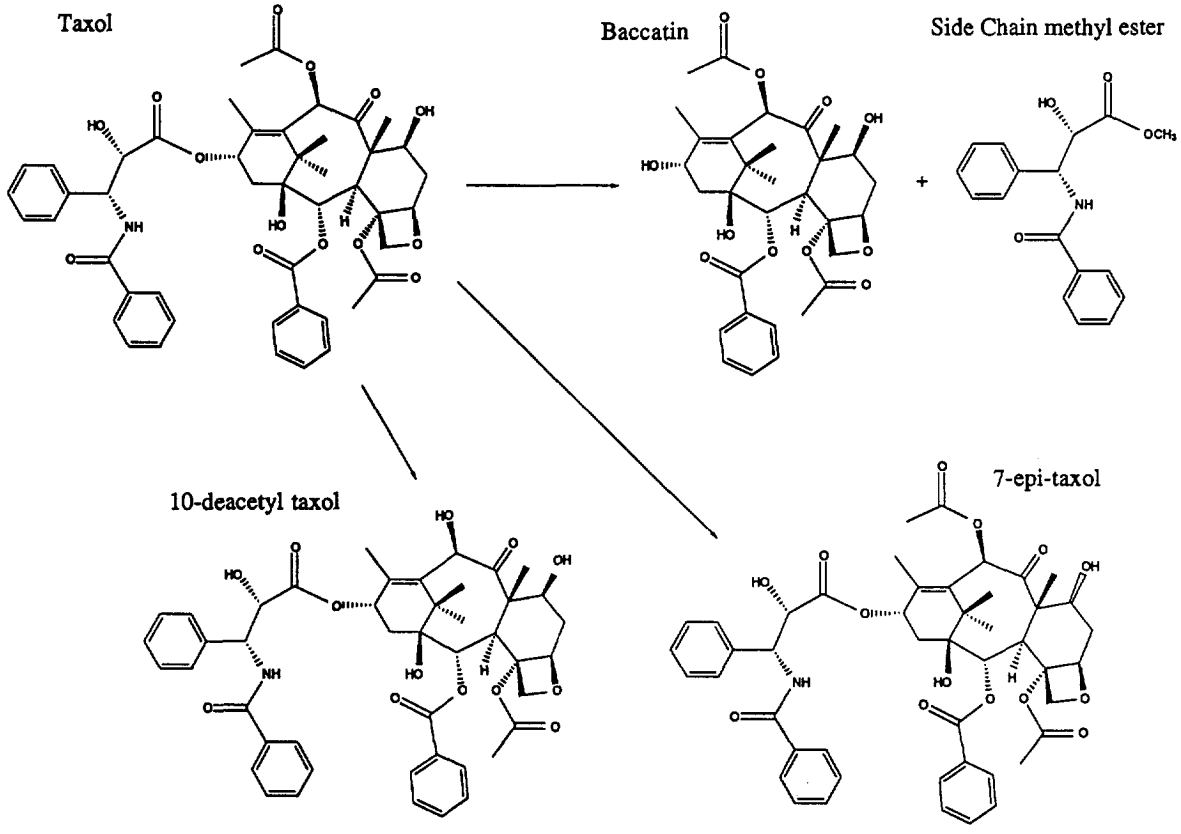


Fig. 6. Base-induced degradation pathway of paclitaxel determined by LC–MS profiling.

ring opened product which also suggested migration of the C-4 acetyl group to the oxetane oxygen. The proposed degradant was synthesized (BMS-190301). Comparison of the chromatographic and spectroscopic profile of BMS-190301 (Fig. 8B) to the chromatographic and spectroscopic profile of the acid degradant of paclitaxel indicated an exact match. Based on the minimal degradation observed with the relatively harsh acidic conditions, paclitaxel appears to be quite stable in an acidic environment.

3.3. Oxidizing conditions

Fig. 9 illustrates the HPLC–UV chromatogram of bulk paclitaxel incubated in an aqueous solution of hydrogen peroxide. Only a single minor degradant was observed eluting prior to paclitaxel. This degradant at 13.0 min has the same relative retention time and molecular mass as 10-deacetylpaclitaxel previously observed in the acid and based degraded samples. The lower UV response is due to the protocol used for dissolution and incubation of paclitaxel in an aqueous solution of hydrogen peroxide and resulted in less paclitaxel injected on-column compared to the acid and base degradation studies. Based on the minimal degradation observed with these oxidizing conditions, paclitaxel also appears to be relatively stable in an oxidizing environment.

3.4. Exposure to high intensity light

Fig. 10 illustrates the HPLC–UV chromatogram of bulk paclitaxel exposed to 1000 FC white light for 92 days. A number of light induced degradants were observed. From a stability perspective, the largest and most interesting degradant corresponded to the 14.8 min peak which had a molecular mass identical to paclitaxel (MW 853). Other lower level photo-

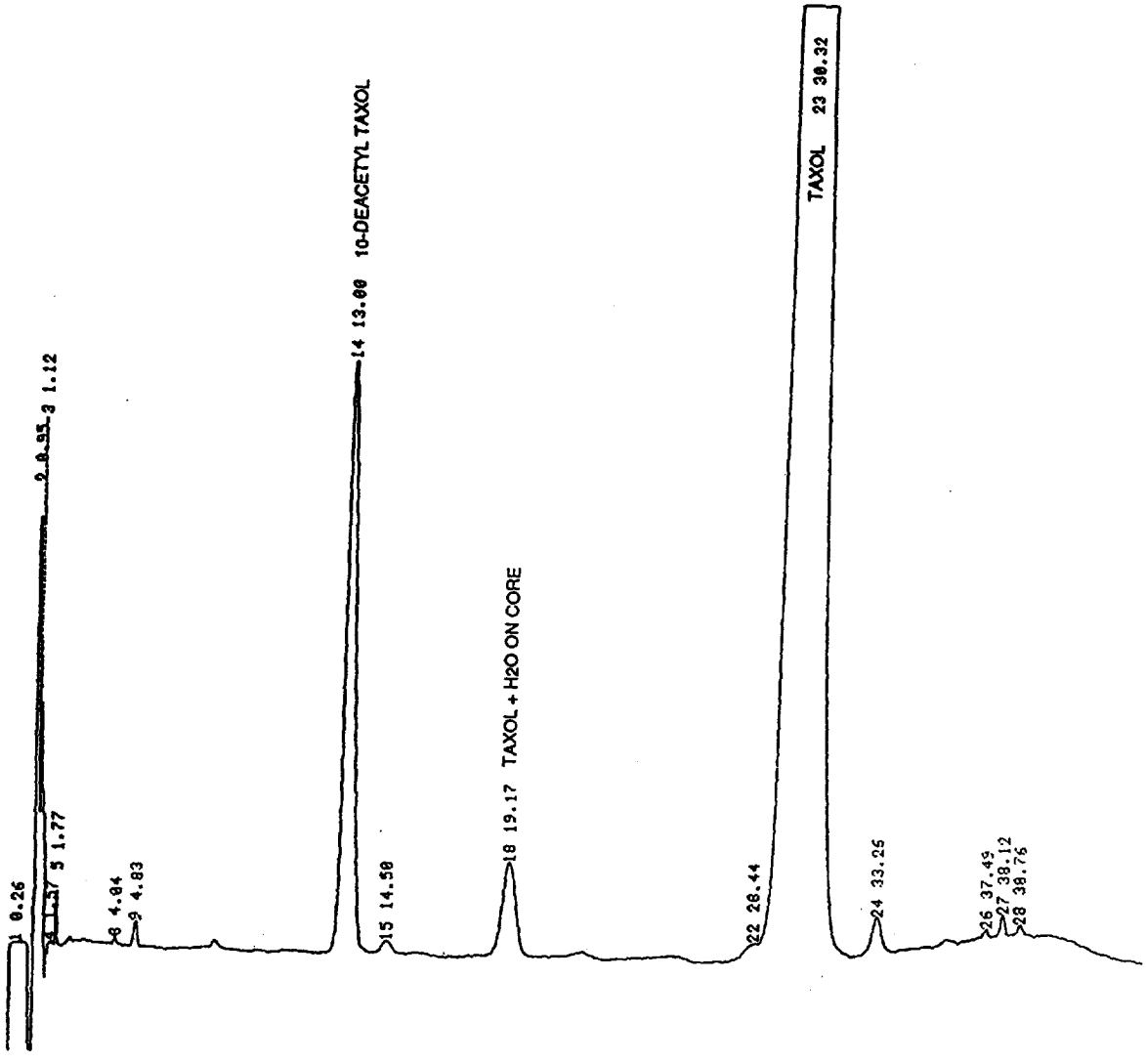


Fig. 7. HPLC chromatogram (230 nm) of the acid induced degradants of paclitaxel. Separation conditions described in Fig. 2.

degradants observed in this profile included a second early eluting paclitaxel isomer at 9.9 min, and three isomers of paclitaxel minus a core carbonyl at retention times of 27.8, 30.2, and 36.4 min. The product ion spectra of each of the photodegradants observed indicated a modification of the paclitaxel core. The MS–MS product ion spectrum of the 14.8 min paclitaxel isomer is shown in Fig. 11. Examination of the product ion spectrum of this primary photodegradant indicates the same diagnostic product ions indicative of the paclitaxel side chain at m/z 286, 268, and 240 are present. In addition, the product ion at m/z 509, indicative of the deacetylated core, is also present. The absence of the product ion at m/z 569 indicates facile loss of a single molecule of acetic acid is favored for this paclitaxel isomer. This is also consistent with the observation of a new product ion at m/z 794 corresponding to loss of acetic acid from the protonated molecule. In addition, a new product ion at m/z 467 is observed and possibly resulting from consecutive loss of ketene (CH_2CO) from the m/z 509

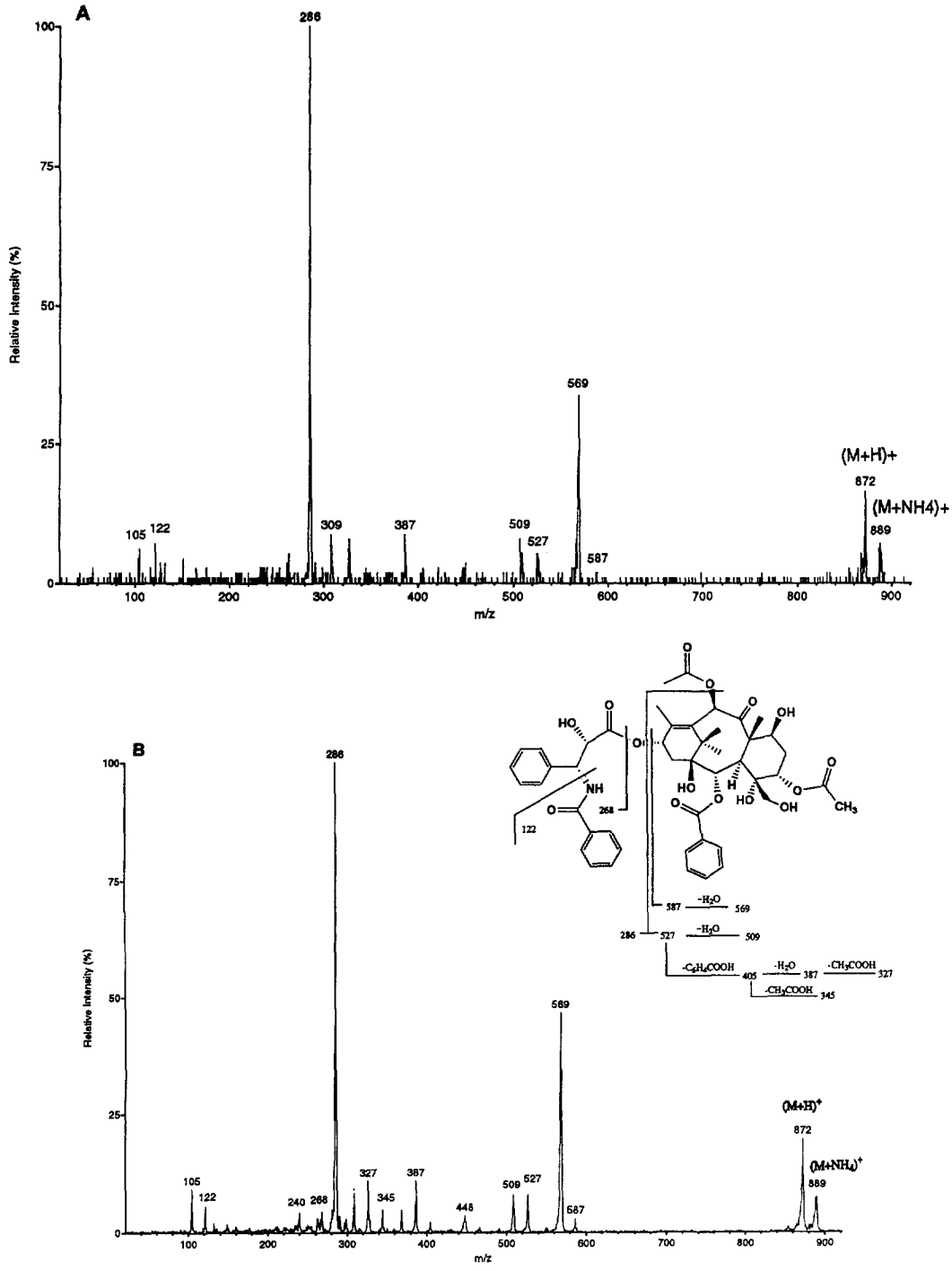


Fig. 8. Product ion spectrum of the acid degradant of paclitaxel at m/z 889 $(M+NH_4)^+$. (B) Product ion spectrum of BMS-190301, reference standard of acid degradant, at m/z 889 $(M+NH_4)^+$. Product ions, neutral losses, and their correspondence to specific substructures are indicated. The structure shown is based on detailed spectroscopic studies of synthesized material.

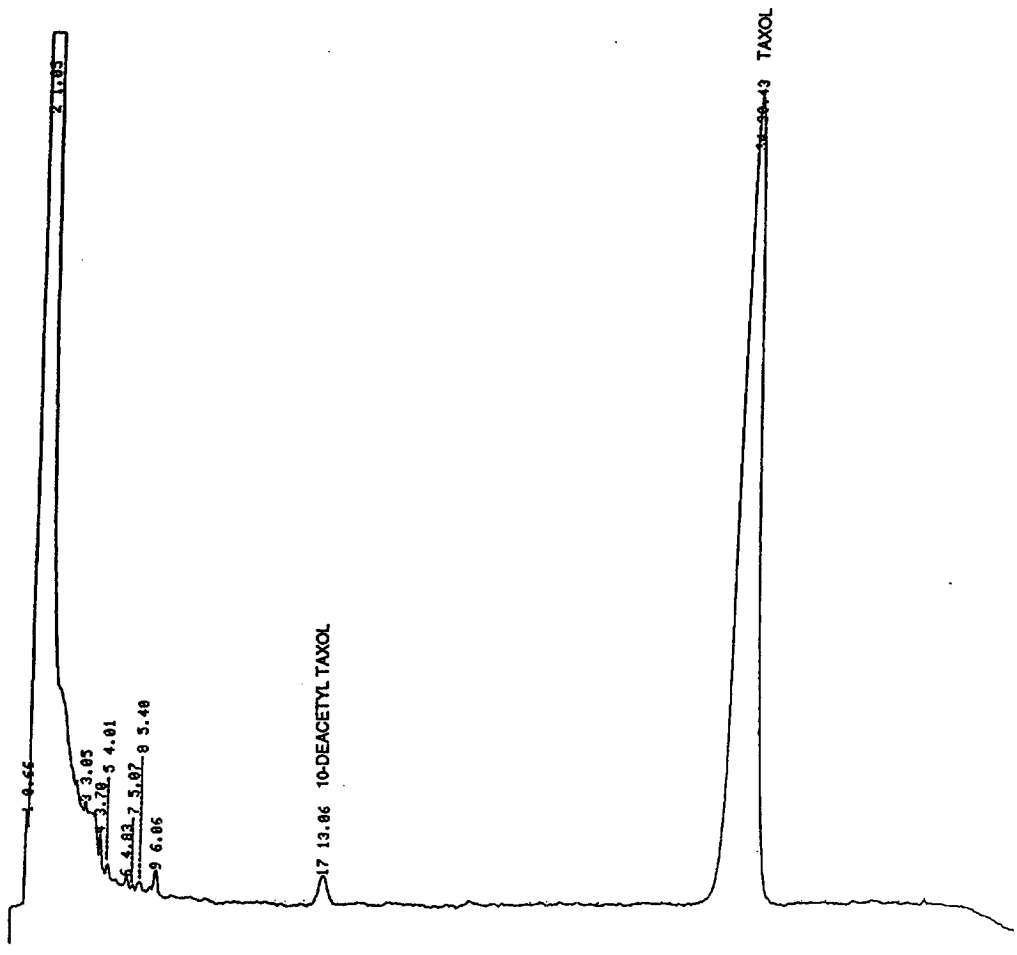


Fig. 9. HPLC chromatogram (230 nm) of the degradation caused by incubation of paclitaxel with aqueous hydrogen peroxide for 15 min. Separation conditions described in Fig. 2.

product ion. The changes in the product ion spectrum of this photodegradant relative to product ion spectrum of paclitaxel suggested a structural difference (rearrangement) near an acetate on the core of this particular photodegradant. Small amounts of this photodegradant were isolated and characterized by proton NMR which indicated the C3–C11 bridge shown in Fig. 11 [16]. The susceptibility of taxanes to skeletal rearrangements via radical intermediates has been well documented [16–18]. This knowledge coupled with the complexity of the possible free radical initiated rearrangement products and the lack of carbon–carbon bond cleavage throughout the paclitaxel core in the product ion spectra made structure proposals difficult for most photodegradants. Due to the large number of potential free radical rearrangement products which may be formed, additional complementary NMR spectroscopic studies are planned to fully elucidate several of the other photolysis products. However, a summary of all degradation products, including major photolysis products, observed in the in these LC–MS profile studies are tabulated in Table 1. Based on the light induced degradation profile, paclitaxel appears to be susceptible to a variety of photolytic reactions. As a result, this database serves as a valuable library

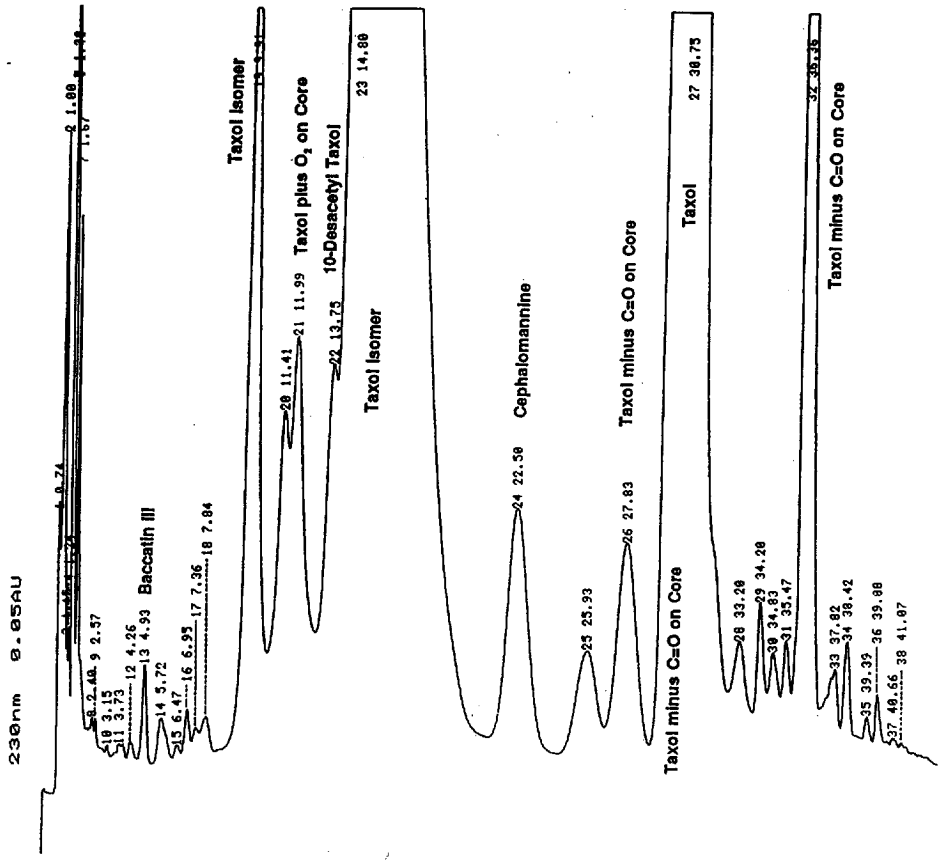


Fig. 10. HPLC chromatogram (230 nm) of bulk paclitaxel exposed to 1000 FC white light for 92 days.

to reference any new degradants observed in long-term storage stability samples or new process impurities.

4. Conclusions

The high sensitivity of mass spectrometry is particularly advantageous for application to samples which contain trace impurities and degradants, a situation frequently encountered in pharmaceutical discovery and development research. A strategy involving the use of LC–MS profiling and LC–MS–MS substructural analysis has been shown to be capable of providing a highly sensitive and specific method for rapidly obtaining molecular mass and structural information about low level impurities and degradants.

Degradation products were elucidated on the basis of their chromatographic relative retention times using standardized HPLC conditions, molecular mass information obtained from the full scan mass spectrum acquired during LC–MS profiling, and the product ion spectrum acquired during LC–MS–MS substructure analysis studies. Degradation products formed upon exposure to basic conditions included baccatin III, paclitaxel sidechain methyl ester, 10-deacetylpaclitaxel, and 7-epipaclitaxel. Degradation products formed upon exposure to acidic conditions included 10-deacetyl paclitaxel and the oxetane ring opened product. Treatment with hydrogen peroxide produced only 10-deacetylpaclitaxel. Exposure to high intensity light produced a number of degrad-

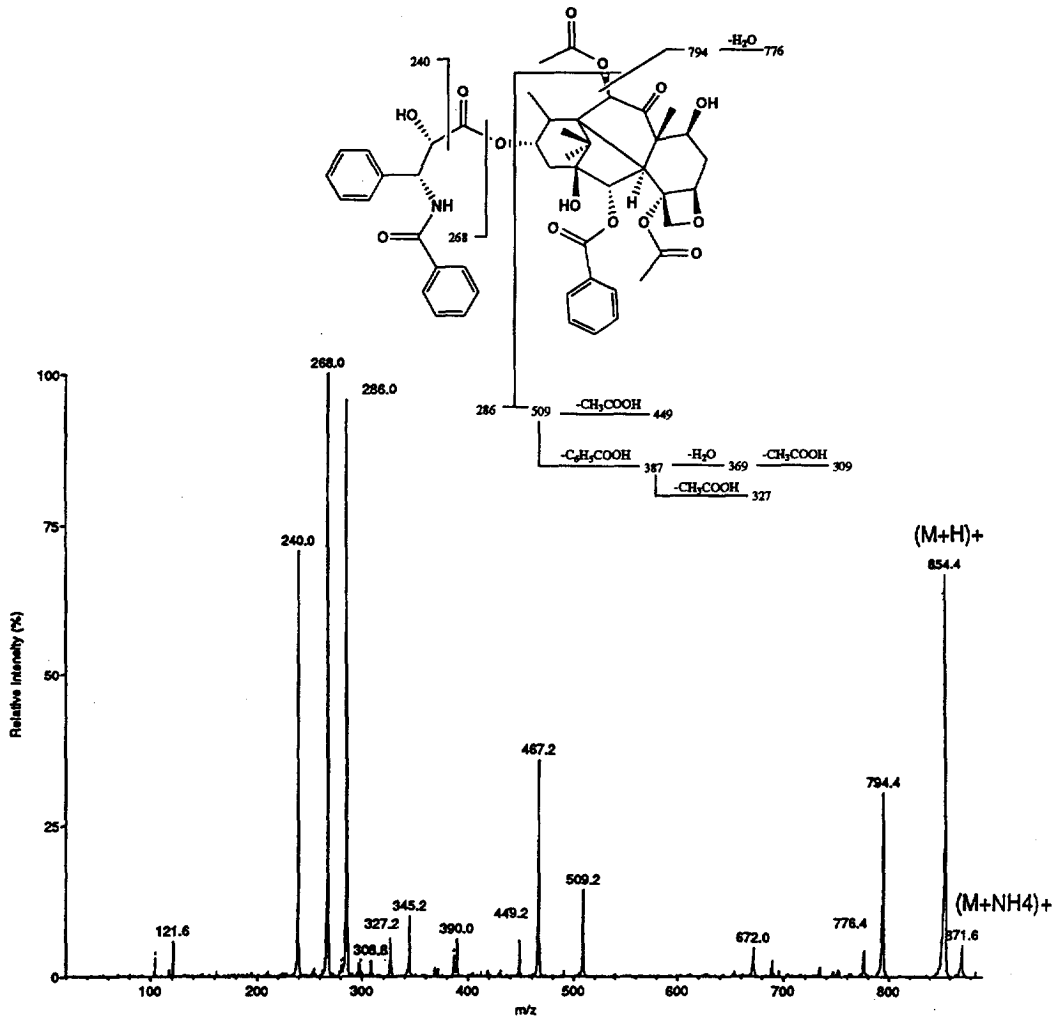
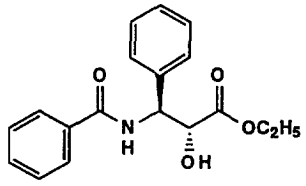
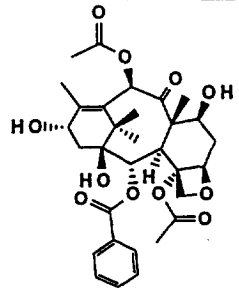
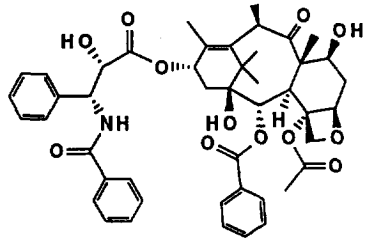
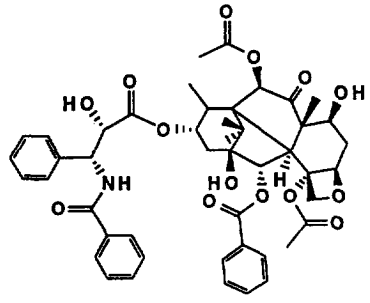


Fig. 11. Product ion spectrum of the photodegradant paclitaxel isomer, t_R 14.8 min at m/z 871 $(M+NH_4)^+$.

ants. The most abundant photodegradant of paclitaxel corresponded to an isomer which contains a C3–C11 bridge. Other lower level photodegradants observed in this profile included a second early eluting paclitaxel isomer and three isomers of paclitaxel minus a core carbonyl. Based on these studies, an LC–MS degradant database, including information on molecular structures, chromatographic behavior, molecular mass, and substructure-specific MS–MS product ions of the components has been developed and added to the impurity database.

LC–MS and LC–MS–MS techniques can be used to facilitate 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. Using this methodology, detailed structural information is typically obtained for potential degradants in less than one day. Most importantly, these predictive studies will provide a foundation for future work involving the analysis of new paclitaxel degradation products.

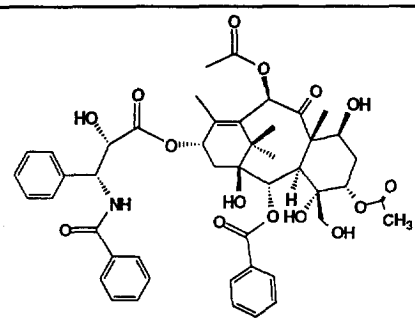
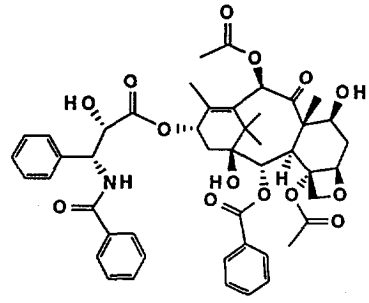
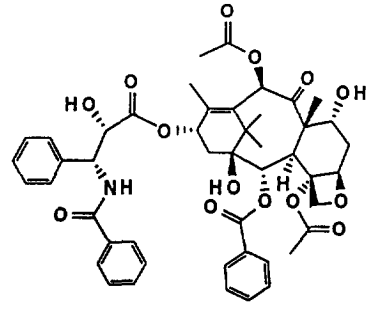
Table 1
Paclitaxel degradant database

RT	RRT	MW	Name	Structure	Sample Source
3.9	0.14	313	sidechain methyl ester		base
4.6	0.16	586	baccatin III		impurity base
9.9	0.35	853	paclitaxel isomer	unknown	light
			paclitaxel		base acid peroxide
14.8	0.52	853	paclitaxel isomer (C3-C11 bridge)		light

Acknowledgments

The authors wish to gratefully acknowledge the contributions of collaborators in this project. Steven Klohr consulted on chemical structures, spectral interpretation and provided assistance in editing the manuscript. Ira Rosenberg and Jerry Allison encouraged and supported these studies and provided valuable resources. Sample degradation conditions and discussions on results were contributed by Mike Saleh, Mohamed Abdelnasser, Venkatapuram Palaniswamy, W. Larry Parker, Richard Partyka, and

Table 1. Continued

19.2	0.67	871	oxetane ring opened		acid
27.8	0.97	825	paclitaxel minus CO	unknown	light
28.6	1.0	853	paclitaxel		bulk drug
30.2	1.06	825	paclitaxel minus CO	unknown	light
36.4	1.27	825	paclitaxel minus CO	unknown	light
37.8		853	7-epi paclitaxel		base

Vincent Warren. Bernard Floor and John Woolever contributed to development of the chromatographic method.

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