Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 265–275
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Identification of low-level degradants from low dose tablets

Andrew Blanchard [a,*], Carlos Lee [a], Beverly Nickerson [a], Linda L. Lohr [a], Andrew J. Jensen [a], Karen M. Alsante [a], Thomas R. Sharp [a], Dinos P. Santafianos [a], Ronald Morris [a], Kimberly D. Snyder [b]

[a] *Analytical Research and Development, Pfizer Global Research and Development, Eastern Pt. Road, Groton, CT 06340, USA*
[b] *Commissioning Agents Inc., San Mateo, CA 94403, USA*

Received 17 December 2003; received in revised form 4 May 2004; accepted 10 May 2004
Available online 13 September 2004

Abstract

A multifaceted approach was successfully used to identify three of four unknown degradants in degraded low dose tablets. Accelerated solvent extraction (ASE) was found to be an invaluable tool in this multifaceted approach. ASE was capable of extracting four individual degradants of an active pharmaceutical component from 10 tablets into 15 mL of solvent with approximately 100% recovery for each degradant. Using ASE instead of manual extraction led to the extraction and isolation of the degradants in 1 day instead of 7 days. One of the degradants was extracted by ASE, isolated by semi-prep HPLC, and identified by LC-MS and NMR spectroscopy. The structures of two of the remaining three degradants were confirmed by synthesis of authentic samples, while the fourth degradant is yet to be identified.

Keywords: Accelerated solvent extraction; ASE; Degradants; Degradation products; Low dose tablets; Extraction; Identification

1. Introduction

Impurity and degradation product identification is an important activity during the development of a drug candidate that aids in impurity monitoring and tracking. A challenge in impurity identification is isolating sufficient material for structure confirmation. This quantitative challenge is especially difficult for low dose drug products such as tablets containing active pharmaceutical ingredient (API) at 10 mg per tablet or less. Isolation often requires multiple extractions to isolate sufficient quantities of impurities. The isolated impurities are often dissolved in large volumes of solvent. Concentrating this solution can be difficult if the solvent is non-volatile. Heating is not desirable in the concentration step, since further degradation of the impurities/degradants of interest can occur. Due to the low concentration of the impurity in the extract, numerous preparative HPLC runs are required to isolate sufficient quantities for analysis.

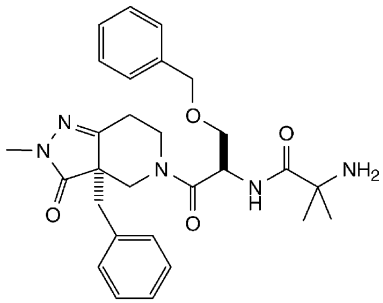
Accelerated solvent extraction (ASE), also referred to as pressurized fluid extraction or pressurized liquid extraction, is an automated extraction technique that uses elevated temperatures and pressures to extract components from solid and semi-solid samples with organic and/or aqueous solvents. Using ASE, degradation products can be extracted from tablets with less time and effort than that required for manual extraction. A more concentrated extract can be obtained, requiring fewer preparative HPLC runs to isolate individual degradants in quantities sufficient for identification. In some cases, mass spectroscopy (MS) and/or nuclear magnetic resonance (NMR) analysis of the concentrated ASE extract itself can provide information that aids in the identification of the degradants.

ASE was first described in the mid 1990s [1–3]. Since then, numerous environmental and food-related applications of this technique, and a number of review articles summarizing these applications, have been published [4–8]. Several pharmaceutical applications of ASE have been reported in the literature, including extraction of active ingredients from tablets [9–11], transdermal patches [11,12] medicinal plants/natural products [13–18] and drugs from animal feed [11,14,19,20] and bovine liver [14,21]. These pharmaceutical applications focused on extraction of active ingredients from various sample matrices. To our knowledge, no applications using ASE to extract impurities or degradation products of active ingredients from formulations could be found

* Corresponding author.

0731-7085/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.jpba.2004.05.012

in the literature. In this paper we describe the first use of ASE to extract degradation products from a drug product formulation. ASE was successfully used as part of a multifaceted approach to extract, isolate, and characterize unknown degradants of the API, **1**, a potential candidate for the treatment of musculoskeletal frailty [22,23] and congestive heart failure [24,25].



(**1**)
API
$C_{28}H_{35}N_5O_4$
r.m.m. 505

2. Experimental

2.1. Degradant formation

The low dose tablets (10 mg or less) of the API, **1**, were placed in an open dish in a Hotpack (Philadelphia, PA) benchtop humidity chamber (Model No. 435304) set at 70 °C and 75% relative humidity for 6 weeks. The degraded tablets were then stored at 5 °C until extractions were performed.

2.2. Accelerated solvent extraction (ASE)

Extractions were performed on an ASE 200 accelerated solvent extractor system (Dionex Corporation-Marlton, NJ). Commercial grade nitrogen (99.99% purity) was used for purging all the liquid from the ASE extraction cells. Hydromatrix (high purity diatomaceous earth) was used in the ASE extraction cell to fill any remaining volume left in the cell to reduce the extract volume. The Hydromatrix (Part No. 0019-8003) was supplied by Varian Sample Preparations Products (Palo Alto, CA).

2.3. Sample preparation for ASE tablet extraction

Degraded tablets were wrapped in Whatman (Clifton, NJ) No. 2 filter paper disks (7.5 cm diameter) and crushed with a mallet. The crushed tablet and filter paper were then transferred to the extraction cell by two different procedures (Fig. 1). One degraded tablet was wrapped in a filter paper disk and crushed. In the first procedure, the crushed tablet, still wrapped in the filter paper, was placed into an extraction cell. In the second procedure, the crushed tablet was unwrapped and transferred into the extraction cell, and the used filter paper was placed on top of the crushed tablet. In both procedures, the remaining cell space was filled with Hydromatrix. Tablets transferred into the cell using these two procedures were then extracted using identical methodology.

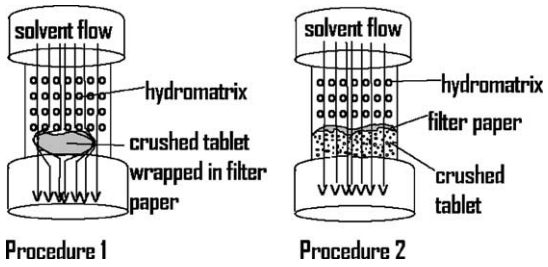


Fig. 1. Comparison between sample preparation procedure 1 and procedure 2. Notice that in procedure 1 the flow of solvent through the cell does not diffuse through the matrix because the path of least resistance would be to flow around the wrapped filter paper containing the crushed tablet. Dispersing the tablet at the bottom of the cell (procedure 2) forces the flow of solvent to diffuse through the tablet matrix.

2.4. Manual tablet extraction

Individual tablets were placed in a 100 mL volumetric flask. The flask was half filled with dissolving solvent (90/10/0.1, v/v/v, water/acetonitrile/aqueous H_3PO_4). The sample was sonicated with an Aquasonic model 250T sonicator for 5 min, or until the tablet was completely dispersed. The flask was filled to volume with dissolving solvent. The solution was filtered with a Whatman Autovial 0.45 μm nylon filter.

2.5. Analytical HPLC

Analyses of the ASE extracts were performed on a Waters (Milford, MA) 2690 Alliance HPLC system with a Waters 996 photodiode array detector. The HPLC column was a 3 μm ODS−AQ (4.6 mm × 150 mm) from YMC Inc. Chromatographic data were acquired and processed using Millennium 32 software (version 3.05.01, Waters Corporation). HPLC grade acetonitrile (J.T. Baker – Phillipsburg, NJ), reagent grade potassium dihydrogen phosphate (J.T. Baker) and reagent grade phosphoric acid (J.T. Baker) were used. Milli-Q water was supplied from a Millipore (Billerica, MA) gradient A10 water purification system installed in the laboratory.

The HPLC method utilized to assay the degradants after extraction contained a segmented binary gradient. The mobile phase components were 0.1% aqueous H_3PO_4 and acetonitrile. The flow rate was 1.0 mL/min. The detection wavelength was 210 nm. The initial gradient started at 4% acetonitrile, increased to 26% acetonitrile in 7 min, followed by a 10 min isocratic hold at 26% acetonitrile. The second

gradient increased from 26% acetonitrile to 82% acetonitrile during the next 19 min, followed by a 12 min equilibration back to initial conditions. Total run time was 48 min. Percent recoveries of all degradation products were calculated against a manually extracted degraded tablet containing API.

2.6. Semi-preparative HPLC of degradants

Semi-preparative HPLC isolations were conducted using a Kromasil C_4 column (Column Engineering – Ontario, CA), 19 mm × 199 mm, packed with 5 μm particles. The column was equilibrated at room temperature. UV–vis detection was performed at 210 nm. For isolation of the ASE extracted tablet solutions, a mobile phase flow rate of 8 mL/min was used. Injections of 1500 μL were made using a run time of 25 min. An isocratic mobile phase consisting of 70% aqueous 0.05 M KH_2PO_4, pH 3, and 30% acetonitrile was used.

2.7. Mass spectrometry

Electrospray LC-MS experiments were performed on a Micromass Instruments Quattro II triple quadrupole system, with an attached Agilent Technologies (Palo Alto, CA) 1100 modular HPLC system. LC mobile phases, which were not directly amenable to LC-MS experimentation, were modified by replacing the nonvolatile components, such as H_3PO_4, with volatile components, such as trifluoroacetic acid (TFA).

2.8. Nuclear magnetic resonance spectroscopy

99.9% Deuterated dimethyl sulfoxide (DMSO-d_6) and 99.9% deuterated chloroform ($CDCl_3$) were used as supplied from Cambridge Isotopes Labs Inc. (Andover, MA). Samples were dissolved in 0.8 mL solvent and analyzed by liquids NMR. All NMR spectra were collected at 298 K using a Bruker-Biospin (Billerica, MA) 5 mm gradient broadband inverse probe, with a *Z* axis gradient, on a Bruker-Biospin Avance DRX 500 (^{13}C 125.71) MHz NMR spectrometer. The following spectra were acquired: 1-D proton, 1-D carbon with proton decoupling, gradient selected distortionless enhancement by polarization heteronuclear single quantum coherence (gs-DEPTHSQC) [26], gradient selected BIRD-filtered heteronuclear multiple bond correlation (gs-BIRDHMBC) [27] and gradient selected proton double quantum-filtered correlation spectroscopy (gs-DQFCOSY) [28]. Short-range ^{1}H–^{13}C scalar couplings were set equal to 145 Hz, and corresponding long-range couplings were set equal to 10 Hz. Similar spectra for the API were collected for comparison (Table 1, Fig. 2). Proton chemical shifts were referenced to the solvent, 2.50 ppm for dimethyl sulfoxide or 7.27 ppm for chloroform. Carbon chemical shifts were referenced to the solvent, 39.51 ppm for dimethyl sulfoxide or 77.23 ppm for chloroform. Data processing was performed with a SGI/O2 Unix workstation using XWINNMR (Bruker-Biospin) software version 2.7. The tabulated NMR chemical shifts for the API, **1**, are shown in Table 1.

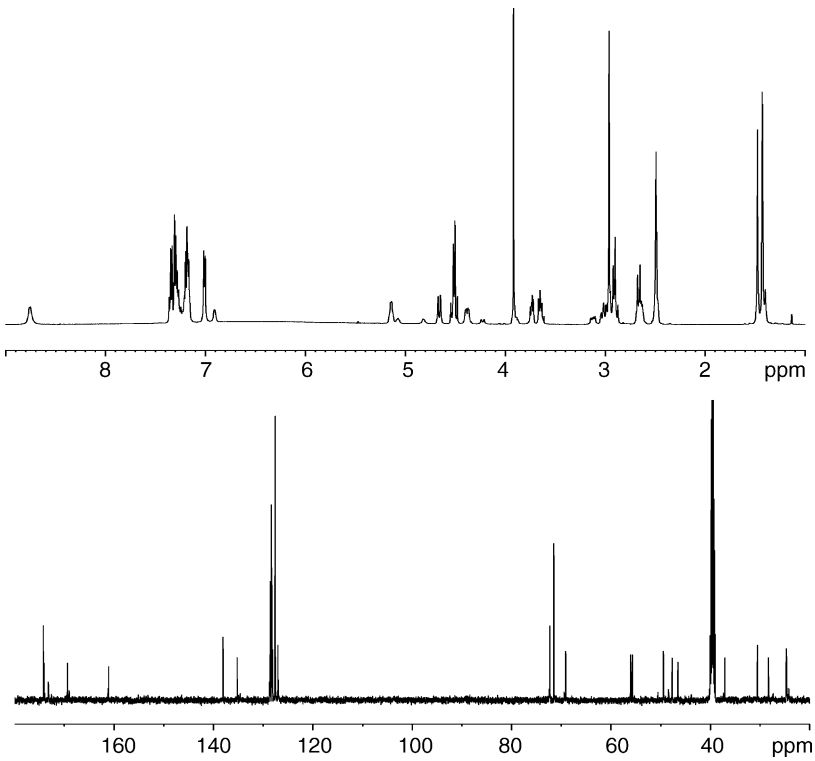
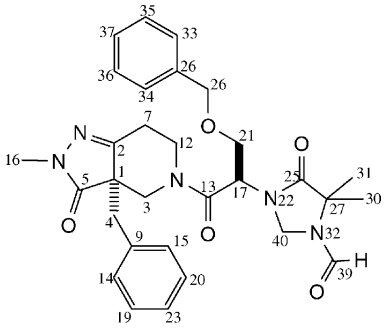


Fig. 2. NMR spectra of API (**1**). The top spectrum shows the proton spectrum. The bottom spectrum shows the proton decoupled carbon spectrum.

Table 1
Chemical shifts for the protons and carbons of the API, **1**, and degradants **2**, **3**, and **4**

Carbon #	Parent/API							
	(**1**) r.m.m. 505		(**2**) r.m.m. 517		(**3**) r.m.m. 533		(**4**) r.m.m. 545	
	Carbon (ppm)	Proton (ppm)	Carbon (ppm)	Proton (ppm)	Carbon (ppm)	Proton (ppm)	Carbon (ppm)	Proton (ppm)
1	55.96 (55.65)		56.75		55.74		56.90	
2	161.02		161.25		161.54		161.28	
3	47.67	4.66, 2.67	48.89	5.01, 2.48	47.70	2.63, 4.68	48.97	4.99, 2.48
4	37.03	2.91, 2.89	37.83	3.16, 2.80	36.78	2.85, 2.98	37.73	3.15, 2.81
5	164.05		174.51		174.11		174.61	
7	28.27	2.49	29.23	2.97, 2.61	27.92	2.43, 2.96	29.18	3.03, 2.61
9	135.20		134.85		135.38		134.79	
12	46.51	4.38, 3.01	48.10	4.52, 2.97	46.44	2.98, 4.46	48.10	4.39, 3.03
13	169.28		169.35		169.80		168.90	
14	128.58	7.01	128.97	7.07	128.67	7.06	128.92	7.06
15	128.58	7.01	128.97	7.07	128.67	7.06	128.92	7.06
16	30.49	2.96	31.01	3.06	30.42	2.96	31.08	3.07
17	49.36	5.15	51.25	5.32	48.82	5.08	51.64	5.38
19	128.17	7.20	128.81	7.18	128.03	7.18	128.60	7.19
20	128.17	7.20	128.81	7.18	128.03	7.18	128.60	7.19
21	69.03	3.73, 3.65	66.99	3.86, 3.78	69.30	3.58, 3.76	66.45	3.88
23	127.00	7.17	127.45	7.18	126.85	7.17	127.50	7.18
25	173.12		178.63		173.80		174.19	
26	72.26	4.50	73.66	4.60, 4.52	72.18	4.49	73.86	4.57
27	55.65 (55.96)		59.67		55.60		60.06	
29	138.02		137.30		138.19		136.88	
30	24.67 (24.55)	1.47	23.67 (23.27)	1.33 (1.25)	25.10 (24.94)	1.41 (1.39)	26.13 (25.99)	1.59 (1.48)
31	24.55 (24.67)	1.43	23.27 (23.67)	1.25 (1.33)	24.94 (25.10)	1.39 (1.40)	25.99 (26.13)	1.48 (1.59)
33	127.55	7.30	128.14	7.30	127.40	7.29	128.22	7.30
34	127.55	7.30	128.14	7.30	127.40	7.29	128.22	7.30
35	128.30	7.35	128.57	7.37 (7.36)	128.21	7.35	128.92	7.36
36	128.30	7.35	128.57	7.36 (7.37)	128.21	7.35	128.92	7.36
37	127.54	7.28	128.39	7.18	127.40	7.29	127.50	7.18
39					160.76	7.92	159.75	8.33
40			59.05	4.73, 4.40			58.11	5.32, 5.06
NH 22						8.21		
NH 32						8.16		



Reference Structure
Degradant (**4**), MW=545
$C_{30}H_{35}N_5O_5$

2.9. Synthesis of degradation products

2.9.1. Synthesis of N-[2-(3a-benzyl-2-methyl-3-oxo-2,3,3a, 4,6,7-hexahydro-pyrazolo[4,3-c]pyridin-5-yl)-1-benzyloxymethyl-2-oxo-ethyl]-2-formylamino-2-methyl-propionamide (3)

The free base of the API (0.0023 mol) was added to a 25 mL 3-neck round bottom flask containing ~7 mL of acetic anhydride, 1 mL (0.027 mol) of formic acid, and ~0.5 g of 4 Å activated molecular sieves (Aldrich – Milwaukee, WI). The reaction mixture was refluxed for ~3 h, then brought to room temperature. Approximately 20 mL of CH_2Cl_2 was added and the solution was filtered. The filtrate was transferred to a separatory funnel and 0.1 M NaOH was added until the solution was basic to litmus paper. The organic layer was collected, dried with anhydrous $MgSO_4$, and the solvent evaporated under reduced pressure. The crude reaction product was purified on a silica gel column using ethyl acetate. The recovered yield was 79%. The expected 533 Dalton relative molecular mass was confirmed by electrospray mass spectrometry and NMR spectroscopy. The tabulated NMR chemical shifts for degradant (**3**) are shown in Table 1.

2.9.2. Synthesis of 3-[2-(3a-benzyl-2-methyl-3-oxo-2,3,3a, 4,6,7-hexahydro-pyrazolo[4,3-c]pyridin-5-yl)-1-benzyloxymethyl-2-oxo-ethyl]-5,5-dimethyl-4-oxo-imidazolidine-1-carbaldehyde (4)

2.5 g of the API (free base) was placed in a 125 mL Erlenmeyer flask equipped with a stirring bar. 10 mL of 96%

formic acid was added to the flask together with ~10 drops of 5 M KOH. The mixture was repeatedly heated to dryness (under nitrogen) on a hotplate. Additional amounts of formic acid were added as the mixture evaporated to dryness. The above process was repeated as needed over a 2 h period until a brownish-yellow solution was obtained. The resulting crude reaction mixture was purified by semi-preparative HPLC, yielding ~9 mg of material. The expected 545 Dalton relative molecular mass was confirmed by electrospray mass spectrometry. The semi-preparative HPLC used a mobile phase flow rate of 10 mL/min and the injection volume was 550 μL. The run time was 20 min. The mobile phase components were 0.1% aqueous trifluoroacetic acid (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The initial gradient started at 20% (B), increased to 50% (B) in 20 min, followed by a 5 min isocratic hold at 50% (B). The second gradient decreased from 50% (B) to 20% (B) during the next 1 min, followed by a 9 min equilibration back to initial conditions. The total run time was 30 min. The tabulated NMR chemical shifts for degradant (**4**) are shown in Table 1, while the proton and carbon spectra are shown in Fig. 5.

3. Results and discussion

The low dose tablets of the API, **1**, subjected to accelerated stability conditions and to thermal and thermal/humidity forced degradation conditions, generated seven major degradants. Three of the degradation products were identified by HPLC retention time comparisons with existing standards and confirmed by mass spectroscopy. Relative molecular masses of the remaining four unknowns were determined by LC-MS. These unknown degradants are described in Table 2. Mass spectral data alone were insufficient to permit identification or proposal of structures for the unknown degradants. Tablet degradation experiments were scaled up to generate sufficient quantities of the unknowns for isolation. An ASE extraction method was developed to provide enriched starting materials for semi-preparative HPLC isolation.

Traditional methods of extraction were impractical to isolate the four degradants of interest. The manual extraction procedure required approximately 20 min of sonication time per tablet and 100 mL of extraction solvent per tablet. This procedure could be modified to extract one tablet in 10 mL of extraction solvent in 20 min to increase the sample concentration. Therefore, the highest achievable concentration for the least abundant degradants was 0.04 mg/mL. Using semi-preparative liquid chromatography to isolate degradation products from these resulting extraction solutions would require a significant number of injections and an overall increase in the total volume of each isolated sample. ASE was an excellent alternative to the conventional manual extraction method yielding concentrations of approximately 0.2 mg/mL for the least abundant degradants. ASE aided the semi-preparative HPLC purification step, which yielded samples that gave more useful mass spectrometry fragmentation (MS-MS fragmentation) than non-enriched samples. Based on this data several structures were proposed and led to synthetic attempts to generate some of the degradants to confirm the identity of the unknowns.

3.1. Optimum ASE parameters for extraction of degradants

A typical ASE extraction cycle or static run consists of six steps: (1) loading the sample into the extraction cell, (2) filling the extraction cell with solvent, (3) heating and pressurizing the cell, (4) allowing the sample to interact with the solvent at the desired temperature and pressure for a set length of time (referred to as static hold), (5) flushing the cell by adding clean solvent, and (6) purging the solvent from the cell using a suitable gas. The ASE procedure delivers a filtered extract for subsequent analysis by a suitable method. The parameters that can be optimized on the ASE are: extraction cell configuration, temperature, extraction solvent, pressure, extraction time, flush volume, and purge time. As described below, sample preparation in the extraction cell, temperature, and extraction solvent were the major factors found to affect extraction efficiency for this project.

3.1.1. Sample preparation

Two sample preparation techniques were examined (Fig. 1). The remaining ASE parameters were set as recommended by the vendor: three static runs (each static run lasting 5 min), cell pressure of 1000 psi, 70 °C cell temperature, and a flush volume of 60% of the cell volume.

Table 2
Thermal/humidity forced degradation products for tablets of API (**1**)

Condition	Relative retention time	Degradant amount (mg per tablet)	Relative molecular mass	Change of mass from API
Thermal/humidity (70 °C/75% RH)	1.15	0.6	563	+58
Thermal/humidity (70 °C/75% RH)	1.26	1.5	517	+12
Thermal/humidity (70 °C/75% RH)	1.64	1.0	533	+28
Thermal/humidity (70 °C/75% RH)	1.78	0.4	545	+40

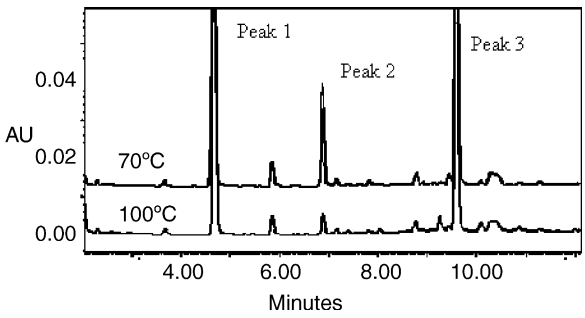


Fig. 3. Chromatograms of thermally challenged tablets extracted at 70 and 100 °C. At 100 °C peak 2 further degraded leaving only 25% remaining from the original amount.

The first sample preparation procedure, in which the crushed tablet remained wrapped in filter paper, gave poor extraction efficiencies (12–55% recovery) compared to manual extraction due to the inability of the solvent to diffuse through the wrapped filter paper. The second procedure gave good extraction efficiencies (90–100% recovery) because the solvent was able to freely diffuse through the matrix and interact with the solutes. This second method was chosen for subsequent experiments.

3.1.2. Temperature

Elevated temperature during the extraction increases the kinetics of extraction by enhancing analyte solubility, lowering solvent viscosity, increasing the efficiency of diffusion into the matrix, and increasing desorption kinetics. A disadvantage of increasing temperature during extraction is the potential for degradation of the component of interest.

Single tablet samples were extracted at 40, 70 and 100 °C to evaluate extraction efficiency and analyte stability. Samples extracted at 70 °C gave slightly better recoveries than samples extracted at 40 °C. Samples extracted at 100 °C gave slightly better recovery for some components; however, one of the components of interest further degraded and its recovery was approximately 25% of that recovered during manual extraction. A temperature of 70 °C was therefore selected because of the higher extraction efficiency compared to 40 °C with no additional breakdown of the degradation products. Percent recoveries for the thermally labile degradant at extraction temperatures of 70 and 100 °C are graphically represented in Fig. 3.

3.1.3. Extraction solvent

The active ingredient in the tablets is very soluble (> 1 g/mL) in aqueous solution at pH 3 and lower. Based on this information, the initial extraction solvent used for ASE was 90% (v/v) of 0.1% aqueous H_3PO_4 and 10% (v/v) acetonitrile. This solvent, used to extract tablets subjected to stress conditions, consistently gave recoveries greater than 98% when compared to manual extraction of the active drug and its four degradants. The two most hydrophobic degradants, however, had recoveries of less than 98%. Changing the extraction solvent to 80% (v/v) of 0.1% H_3PO_4 and 20% (v/v) acetonitrile increased the recoveries of the two hydrophobic degradants without decreasing the extraction efficiencies of the other two degradation products or of the API, **1**. The extraction efficiencies for the API and the four degradants are graphically displayed for the two different extraction solvents in Fig. 4.

3.1.4. Number of cycles

An extraction cycle consists of (1) filling the extraction cell, (2) heating and pressurizing the cell, (3) a static holding time, and (4) flushing with clean solvent. All of the experiments performed up to this point used 3 cycles and yielded extract solutions of approximately 15 mL. Since the main objective was to obtain extremely concentrated solutions of the degradation products, it was thought that using 1 cycle would decrease the final volume by a factor of three and therefore might achieve higher concentrations, even if 100% recovery was not achieved. Surprisingly, the final volume after collecting 1 cycle was approximately 12 mL and did not yield any significant increase in the concentration of the degradants. This experiment indicated

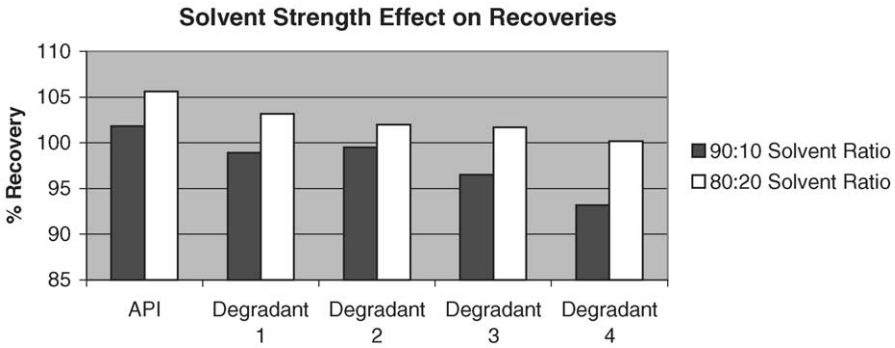


Fig. 4. Extraction efficiencies using 0.1% H_3PO_4/acetonitrile at ratios of 90:10 (v/v) and 80:20 (v/v). Degradants **3** and **4** are the two most hydrophobic components.

that the majority of the volume might be from the initial filling of the cell. A 3-cycle approach yielded the best combination of high recovery and small volume of solution.

3.1.5. Additional parameters

The pressure, static time, flush volume, and purge time were found to have negligible effect on the extraction recoveries when each parameter was evaluated at two different extremes. The following ranges were evaluated: 3 and 5 min static hold times (3 min is the lowest allowed); 20 and 60% flush volume (percentage of the volume of solvent needed to fill the cell); 1000 and 2000 psi cell pressurization; and 60 and 100 s purge time. No significant differences in the recoveries of the degradants were observed using these different parameters.

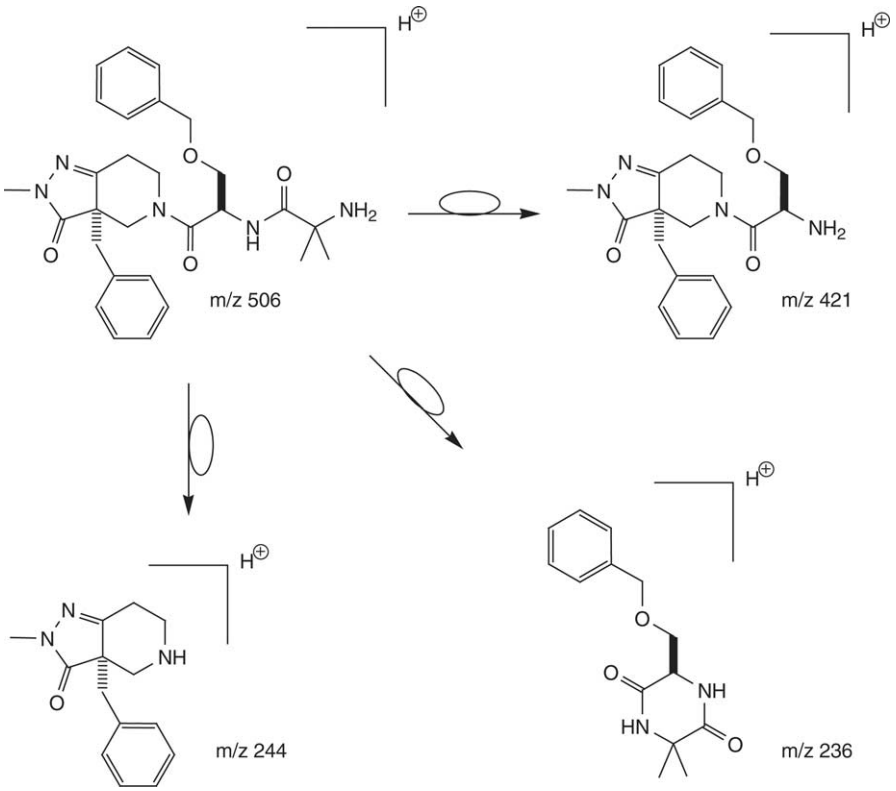
3.1.6. Increased number of tablets for extraction

All experiments described up to this point were performed extracting only one tablet. This does not offer any significant advantage over manual preparation, which is capable of extracting one tablet into 10 mL of solvent. A 10-tablet extraction was attempted in which 10 crushed tablets were transferred to the extraction cell and all remaining space in the cell was filled by the filter paper. This experiment resulted in approximately 100% recoveries for all of the degradants in a 15 mL volume, yielding a concentration that is approximately seven times greater than what could be achieved through manual extraction. The highly concentrated solution obtained by ASE reduced the semi-preparative HPLC isolation step from 7 days to 1 day.

3.1.7. Isolation and identification of degradants

While key pieces of information (Table 2) about the four unknown degradants could be determined by direct LC-MS observation of dissolved tablets, the information was insufficient to determine their identities. The API fragments in the mass spectrometer (see Scheme 1) such that structural changes of analogous structures can be localized to particular regions of the molecule. Mass spectral comparisons of the degradants with the API localized the structural changes of the impurities of interest to involvement with the amide side chain.

The identification process began with the purposeful degradation of tablets to generate working quantities of the unknown degradation products. Single, isolated compounds are much easier to identify than mixtures or formulations. Because of this, each degradation product not only needed to be extracted from the surrounding tablet matrix, but the degradation products also needed to be separated from each other and the API. Traditional extraction methods, as previ-



Scheme 1. Mass spectral fragmentation of API.

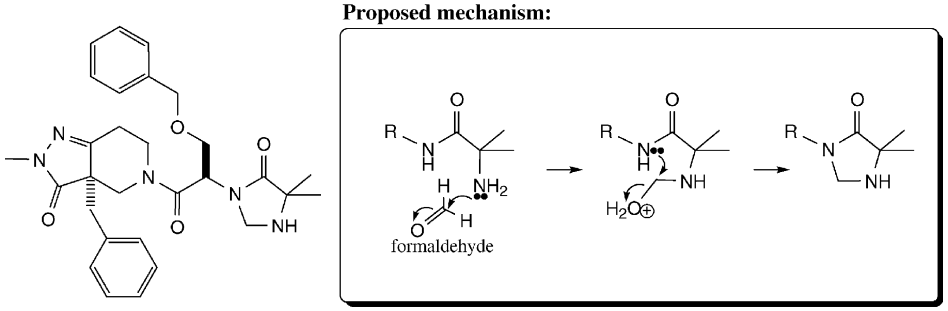
ously mentioned, generate large volumes of solution. This is not ideal for semi-preparative scale HPLC isolation as a maximum of 2 mL of solution can be injected for each run. The ASE process allowed for injection of very concentrated solutions, which in turn kept the collected volumes of each degradation product to a minimum.

The semi-preparative HPLC methodology allowed separation of all four unknown degradation products. Unfortunately, while separated from the other degradation products, the 563 Dalton degradant co-eluted with the API. Several attempts were made to improve the chromatography, but the 563 Dalton degradant could not be separated from the API main band and still maintain separation of all three of the other unknowns. A Gilson 206 fraction collector was used for the isolation. The three separated degradation products were collected individually on each injection and the 563 Dalton degradant was collected along with the API main band. Since the LC/MS profile and the NMR spectrum of the parent API were well known, it was possible that the structure of the 563 Dalton degradant could be determined after subtracting the known data from the API.

Each collected fraction was transferred to a round bottom flask and the solvent stripped using a Roto-Vapor apparatus. The four individual isolates were analyzed by LC-MS and NMR. All isolated material was stored at 5 °C. Details of the analyses of each of the four degradation products are discussed below.

3.1.8. The 517 Dalton degradant

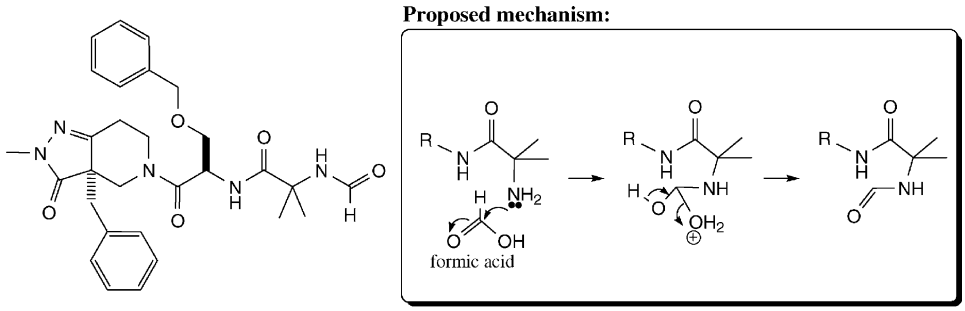
Semi-preparative HPLC was used to isolate the 517 Dalton degradant from the ASE extract. The aqueous fraction collected for the 517 Dalton degradant, was shown to be 100% pure by analytical HPLC. The aqueous isolate was further extracted with methylene chloride, dried with anhydrous $MgSO_4$, and the organic solvent was removed via rotary evaporation to give ~9.5 mg of isolate. MS-MS and NMR analyses supported structure **2** in which a methylene group had been added to the aminoacyl side chain of the API, which enabled the formation of a five-membered ring.



(2)
$C_{29}H_{35}N_5O_4$
r.m.m. 517

3.1.9. The 533 Dalton degradant

LC-MS analysis of the isolated degradant confirmed an increase of 28 Daltons over that of the API and suggested formation of a formamide adduct, **3**. This potential adduct was synthesized and compared with the properties of the degradant.



(3)
$C_{29}H_{35}N_5O_5$
r.m.m. 533

Approximately 27 mg of the sample was isolated through synthesis/semi-preparative HPLC and analyzed by NMR and MS. Both NMR and MS confirmed the structure of the degradant as the formamide adduct, **3**.

3.1.10. The 545 Dalton degradant

Comparison of the spectral properties of the previously identified degradants, **2** and **3**, suggested that this 545 Dalton

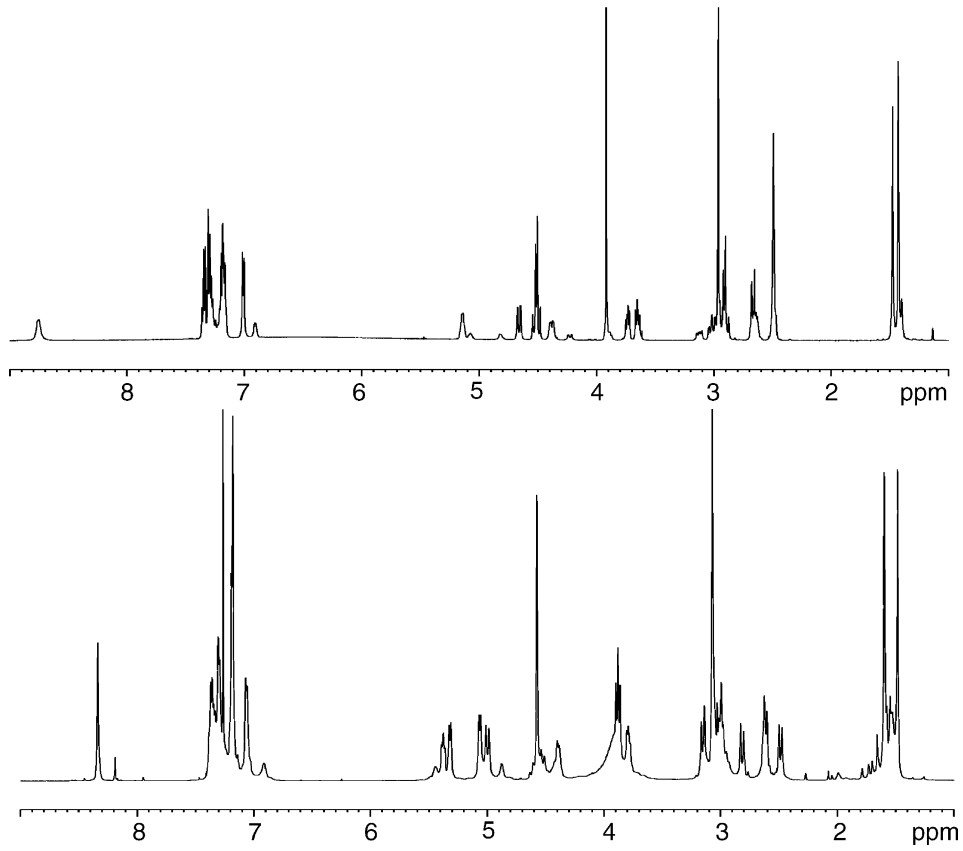
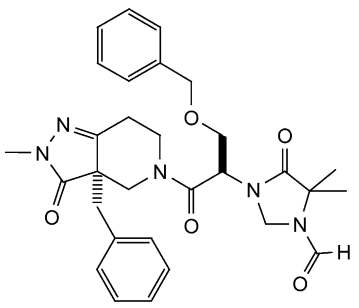


Fig. 5. Comparison of the API proton spectrum (top) with the proton spectrum of degradant structure (**4**) (bottom). Unlike in the API proton spectrum, the degradant (**4**) proton spectrum shows the presence of a new aldehyde resonance at 8.33 ppm as well as two new resonances at 5.06 and 5.33 ppm attributed to a methylene.

degradant was the result of formylation and a ring closure analogous to that of **2**. This target structure, **4**, was synthesized and 24 mg was isolated through semi-preparative HPLC. NMR data helped to confirm the additional methylene and aldehyde groups (Fig. 5). In Fig. 5, the aldehyde proton of **4** is observed at 8.33 ppm, while the new methylene protons are observed at 5.06 and 5.33 ppm. The data for the synthesized material matched the corresponding NMR and MS data for the targeted degradant thereby confirming the structure.

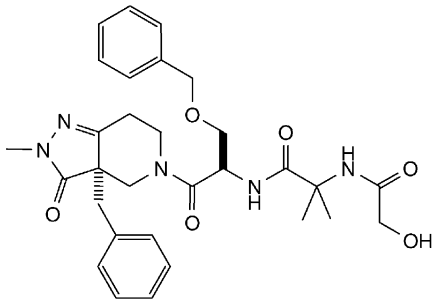


(4)
$C_{30}H_{35}N_5O_5$
r.m.m. 545

3.1.11. The 563 Dalton degradant

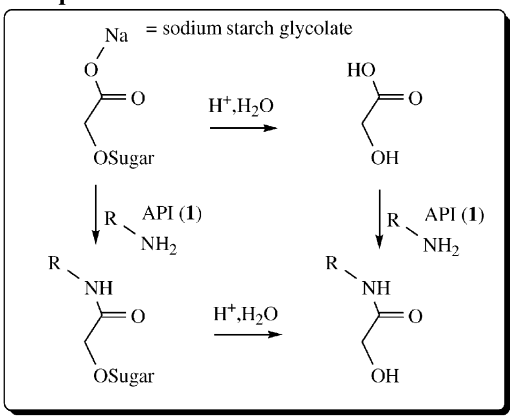
Attempts were made to isolate the lowest level degradant, the 563 Dalton degradant, for analysis by LC-MS and NMR. Since the 563 Dalton degradant co-eluted with the parent compound using the trifluoroacetic acid method discussed in the experimental section, this method was not scaled up for semi-preparative HPLC analysis. A method using the Kromasil C_4 semi-preparative column was developed using a mobile phase consisting of 70% 0.05 M KH_2PO_4 (pH 3.0) and 30% acetonitrile. The details of this method are discussed in the experimental section. Unfortunately, co-elution with the parent main band, combined with excessive contamination of residual buffer from the semi-preparative HPLC mobile phase, prevented useful NMR analysis of this degradant within the time restrictions of the project.

Spectral data suggested the +58 Dalton degradant might be due to an addition of glycolic acid originating from sodium starch glycolate to the aminoacyl side chain of the API. The potential glycolic acid adduct, **5**, was synthesized and its structure was confirmed by NMR analysis. However, HPLC retention time comparisons showed that the degradant found in the tablets was in fact not the glycolic acid adduct. The structure of the 563 Dalton degradant is yet to be determined.



(**5**)
$C_{30}H_{37}N_5O_6$
r.m.m. 563

Proposed mechanism:



4. Conclusions

Isolation and identification of degradation products in low dose tablets can be challenging due to the low quantity of degradation products present in the tablets. In this case, a multi-faceted approach was used to identify unknown degradation products, present at 0.4–1.5 mg each per tablet, from low dose tablets of a pharmaceutical candidate. Tablets were first subjected to accelerated heat/humidity conditions to increase the levels of the degradants of interest. ASE was optimized and used in combination with semi-preparative HPLC to successfully extract and isolate one of the four unknown degradants from the degraded tablets. NMR and MS were used to confirm the structure of the degradant. The enriched extract samples afforded by ASE, provided high quality MS data of the remaining degradants. The above, together with confirmation of the structure of the first degradant, allowed for the proposal of tentative structures for the three remaining degradants. The proposed structures for two of the three remaining degradants were synthesized and purified by column chromatography and semi-preparative HPLC. These structures were confirmed by NMR and MS to be the degradants observed in the tablets. Attempts to identify the fourth degradant have so far been unsuccessful.

This work outlines a multi-faceted approach to identifying degradants, which includes the novel application of ASE. In the future, ASE may play a larger role in the pharmaceutical industry due to its automated sample extraction capabilities and ease of method development.

References

[1] B.E. Richter, J.L. Ezzell, D. Felix, K.A. Roberts, D.W. Later, Am. Lab. 27 (1995) 24–28.
[2] B.E. Richter, B.A. Jones, J.L. Ezzell, N.L. Porter, N. Avdalovic, C. Pohl, Anal. Chem. 68 (1996) 1033–1039.
[3] J.L. Ezzell, B.E. Richter, D. Felix, S.R. Black, J.E. Meikle, LC-GC 13 (1995) 390–398.
[4] H. Giergielewicz-Mozajska, L. Dabrowski, J. Namiesnik, Crit. Rev. Anal. Chem. 31 (2001) 149–165.
[5] A. Hubert, K.-D. Wenzel, W. Engelwald, G. Schüürmann, Rev. Anal. Chem. 20 (2001) 101–104.
[6] P.L. Buldini, L. Ricci, J.L. Sharma, J. Chromatogr. A 975 (2002) 47–70.
[7] V. Camel, Analyst 126 (2001) 1182–1193.
[8] B.E. Richter, LC-GC 17 (6S) (1999) S22–S28.
[9] E. Björklund, M. Järemo, L. Mathiasson, L. Karlsson, J.T. Strode III, J. Eriksson, A. Torstensson, J. Liq. Chromatogr. Relat. Technol. 21 (4) (1998) 535–549.
[10] M. Ashraf-Khorassani, S. Hinman, L.T. Taylor, J. High Resolut. Chromatogr. 22 (5) (1999) 271–275.
[11] Application Note 327, Dionex Corporation, Sunnyvale, CA, 1997.
[12] F. Hoefler, R. Neufang, B. Raffelsberger, O. Staehler, LaborPraxis 23 (1999) 52–54.
[13] B. Benthin, H. Danz, M. Hamburger, J. Chromatogr. A 837 (1999) 211–219.
[14] R. Kawamura, Y. Kikuchi, T. Ohira, M. Yatagai, J. Nat. Prod. 62 (1999) 244–247.
[15] E.-S. Ong, S.-O. Woo, Y.-L. Yong, J. Chromatogr. A 313 (2000) 57–64.
[16] Application Note 335, Dionex Corporation, Sunnyvale, CA, 2000.
[17] A. Brachet, S. Rudaz, L. Mateus, P. Christen, J.-L. Veuthey, J. Sep. Sci. 24 (10–11) (2001) 865–873.
[18] H. Danz, D. Baumann, M. Hamburger, Planta Med. 68 (2002) 152–157.
[19] J.R. Williams, E.D. Morgan, B. Law, Anal. Commun. 33 (1996) 15–17.
[20] Extraction of drugs from animal feeds using accelerated solvent extraction (ASE), Application Note 326, Dionex Corporation, Sunnyvale, CA, 2000.
[21] R. Draisci, C. Marchiafava, L. Palleschi, P. Cammarata, S. Cavalli, J. Chromatogr. B: Biomed. Sci. Appl. 753 (2001) 217–223.
[22] L.C. Pan, P.A. Carpino, B.A. Lefker, J.A. Ragan, S.M. Toler, J.C. Pettersen, D.O. Nettleton, O. Ng, C.M. Pirie, K. Chidsey-Frink, B. Lu, D.F. Nickerson, D.A. Tess, M.A. Mullins, D.B. MacLean, P.A. DaSilva-Jardine, D.D. Thompson, Endocrine 14 (2001) 121–132 [erratum, Endocrine 14 (2001) 437].
[23] P.A. Carpino, B.A. Lefker, S.M. Toler, L.C. Pan, J.R. Hadcock, E.R. Cook, J.N. Dibrino, A.M. Campeta, S.L. DeNinno, K.L. Chidsey-Frink, W.A. Hada, J. Inthavongsay, F.M. Mangano, M.A. Mullins, D.F. Nickerson, O. Ng, C.M. Pirie, J.A. Ragan, C.R. Rose, D.A. Tess, A.S. Wright, L. Yu, M.P. Zawistoski, P.A. Dasilva-Jardine, T.C. Wilson, D.D. Thompson, Bioorg. Med. Chem. 11 (2003) 581–590.
[24] M.K. King, D.M. Gay, L.C. Pan, J.H. McElmurray III, J.W. Hendrick, C. Pirie, A. Morrison, C. Ding, R. Mukherjee, F.G. Spinale, Circulation 103 (2001) 308–313.

[25] J.T. MacAndrew, S.S. Ellery, M.A. Parry, L.C. Pan, S.C. Black, Eur. J. Pharmacol. 432 (2001) 195–202.
[26] W. Wilker, D. Leibfritz, R. Kerssbaum, W. Bermel, Magn. Res. Chem. 31 (1993) 287–292.
[27] R. Burger, C. Schorn, P. Bigler, Magn. Res. Chem. 38 (2000) 963–969.
[28] I.M. Brereton, S. Crozier, J. Field, D.M. Doddrell, J. Magn. Res. 93 (1991) 54–62.