Determination of ABT-861 by High-Performance Liquid Chromatography and a Model for Ion-Pair Formation with Trifluoroacetic Acid

N. Simonzadeh*, B. Levison, A. Buko, K. Matuszak, and **M. Hanscom**
Abbott Laboratories, 100 Abbott Park Rd., D-4P3, AP9, Abbott Park, IL 60064-3500

Abstract

ABT-861 is a gonadotropin releasing hormone (GnRH) antagonist candidate drug synthesized at Abbott Laboratories for use in medical conditions responsive to hormonal manipulation (e.g., prostate cancer in elderly males, endometriosis in females, and central precocious puberty in children). A high-performance liquid chromatography (HPLC) method employing gradient elution with ultraviolet (UV) detection is developed for the assay of ABT-861 and determination of impurities in the bulk powder and injectable formulations. The chromatographic conditions employed in this work include the use of a 250 × 4.6 mm, 5-µm ODS Vydac HPLC column at 35°C, an acetonitrile–water (0.1% TFA in each phase, v/v) eluent, and a 60-min run time using UV detection. The chromatographic conditions are used for the determination of ABT-861 and its degradation products and manufacturing impurities in the bulk powder and injectable formulations. The limit of detection is found to be approximately 9 ng at 225 nm. Method validation includes linearity of detector response with amount injected, precision, and standard addition-recovery data. Under the chromatographic conditions employed, diastereomeric and manufacturing impurities and degradation products are separated from ABT-861, demonstrating that the method is stability-indicating. Thus, the current method is seen to be suitable for the routine analysis of ABT-861 and related impurities, providing good selectivity and sensitivity.

Introduction

ABT-861 is a gonadotropin releasing hormone (GnRH) antagonist (1) that suppresses gonadotropin secretion by binding to GnRH receptors. The peptide sequence is as follows:

S-THF-Gly^{0}-D-2Nal^{1}-D-4ClPhe^{2}-D-3-Pal^{3}-Ser^{4}-NMeTyr^{5}-D-Lys(Nic)^{6}-Leu^{7}-Lys(Ipr)^{8}-Pro^{9}-D-Ala^{10}NH$_2$.Acetate

*Author to whom correspondence should be addressed: Senior Author Ninus Simonzadeh.

In animal studies, ABT-861 has been shown to produce sustained testosterone suppression when administered by either subcutaneous infusion or intravenous infusion. It is intended to target diseases that fall into the general category of those responsive to hormonal manipulation.

Peptide drugs have been separated and analyzed by chromatographic methods that include capillary electrophoresis (2–4), reversed-phase high-performance liquid chromatography (HPLC) (5–10), ion exchange chromatography (11–14), and high-performance displacement chromatography (15–16). Other methods of analysis have included titrimetry (17) and bioassay techniques (18–19). In this work, a simple reversed-phase HPLC method that involves an isocratic run to the point just past the elution of ABT-861 followed by a gradient ramp to elute the more strongly-retained degradation products and impurities has been developed. The method has been developed for the assay of the undecapeptide ABT-861 and the determination of related impurities in bulk drug and injectable formulations with no sample pretreatment.

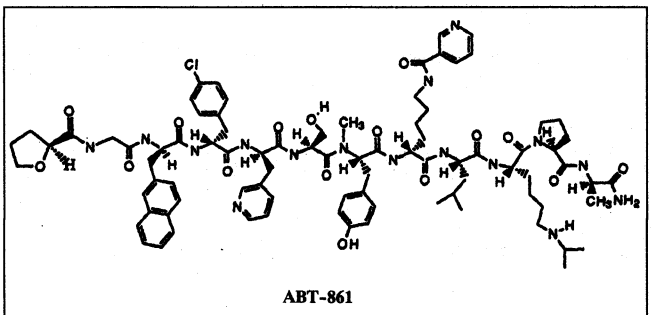
Experimental

Reagents

ABT-861 (Figure 1) was synthesized at Abbott Laboratories (1) and was used in this work in bulk powder form as 3.0-mg/mL injectable formulations. Reagent-grade trifiuoroacetic acid was purchased from Aldrich (Milwaukee, WI), glacial acetic acid was purchased from EM Science (Gibbstown, NJ), and HPLC-grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI). All other reagents used in this work were of the highest purity and used as received.

Preparation of ABT-861 solutions

Solutions of the bulk powder were prepared in 10% (v/v) acetic acid at approximately 1 mg/mL followed by a 2.5-fold dilution



ABT-861

Figure 1. Structure of ABT-861.

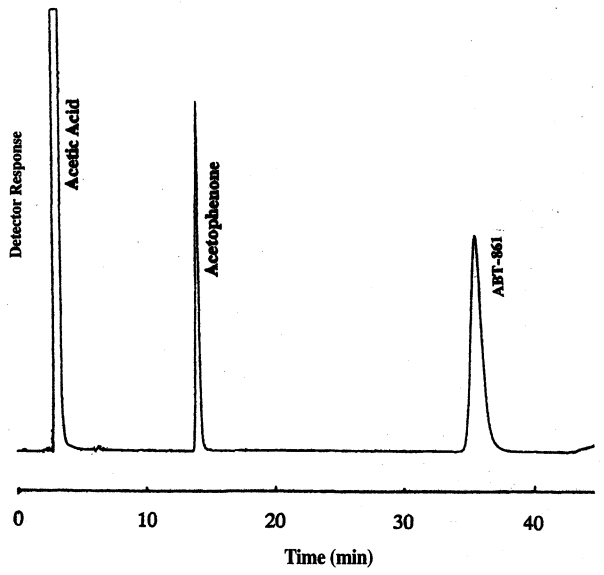


Figure 2. Chromatogram of a standard solution of ABT-861 (0.1 mg/mL) containing acetophenone as the internal standard.

Table I. Typical Chromatographic Conditions

Column:	Vydac ODS (catalog number 201HS54), 250 mm x 4.6 mm, 90 Å, 5-µm particles
Column temperature:	35°C
Wavelength maximum:	225 nm
Working sample concentration:	~ 0.1 mg/mL for assay, 0.4 mg/mL for impurities
Injection volume:	25 µL
Flow Rate:	1 mL/min
Eluent A:	0.1% TFA in CH_3CN
Eluent B:	0.1% TFA in water

Gradient Profile for impurities determination:

Time (min)	% A	% B
0	26	74
40	26	74
60	66	34

Run time:	60 min
Equilibration time before next injection at initial conditions:	approximately 15 min

with 10% acetic acid for the determination of impurities on an area percent basis and a 10-fold dilution (approximately 0.1 mg/mL) for assay versus a reference standard of ABT-861 using acetophenone as an internal standard (Figure 2). The 3.0-mg/mL injectable formulations were diluted to approximately 0.09 mg/mL in ABT-861 with dilute acetic acid (with a final acetic acid concentration of 10%, v/v) and assayed versus a standard of ABT-861 using acetophenone as an internal standard. Characterized lots of ABT-861 of accurately determined purity were used to prepare the reference standard solutions for use in the assay of ABT-861 in the bulk powder and liquid formulations.

Apparatus

The HPLC unit consisted of a Spectra-Physics (San Jose, CA) model UV2000 ultraviolet (UV) detector, model P-4000 gradient pump, model AS-3000 autosampler equipped with a column oven, and a Spectra-Physics Chromjet model 4400 integrator. The analytical column was a Vydac (Hesperia, CA) 5-µm ODS (250 mm x 4.6 mm, 90-Å pore size).

Mobile phase preparation

The mobile phase was prepared by separately diluting 1.0 mL of TFA reagent to 1.0 L with distilled water and acetonitrile. The aqueous and organic components were mixed at the ratio listed in Table I while being continuously sparged with helium.

Chromatographic conditions

Table I lists the chromatographic operating conditions used for the assay and impurities determination for ABT-861.

Results and Discussion

The chromatographic conditions (Table I) were optimized in such a way as to enhance the separation of ABT-861 from the various diastereomeric forms of ABT-861, related compounds, and degradation products. This was achieved by optimizing column temperature, TFA concentration, and the organic/aqueous volume ratio of the mobile phase. It was found that every 10°C increase in column temperature was accompanied by an approximately 7–8 min decrease in the retention time of ABT-861. Similarly, increasing the TFA concentration of the mobile phase resulted in longer retention times, quite possibly due to the ion pairing effect of the trifluoroacetate anion on ABT-861. A 5.0% (v/v) increase in the aqueous component of the mobile phase was also observed to increase the retention time by approximately 10 min. Fitting a mathematical model based on ion-pair formation (explained in the following section) to the retention time data showed the formation of a ~1:1 TFA/ABT-861 ion association complex. A gradient ramp (whereby the organic content of the mobile phase was increased to approximately 66% at approximately 2%/min just after the elution of ABT-861) was used to elute the relatively strongly retained impurities or degradation products that could include trace quantities of benzylated ABT-861 carried over from previous steps in the synthesis. No synthetic precursors were observed, however, in any of the lots of ABT-861 examined thus far.

Ion pair model

The following "local" equilibria are presumed to describe the formation and partitioning of the ion pair formed between ABT-861 and the trifluoroacetate (TFA) anion of the mobile phase:

$$D + n\text{TFA} \overset{Kip}{\rightleftharpoons} (D{\cdot}\text{TFA}_n)_M \overset{Kp}{\rightleftharpoons} (D{\cdot}\text{TFA}_n)_s \qquad \text{Eq. 1}$$

where D is ABT-861, TFA is the trifluoroacetate anion, $(D{\cdot}\text{TFA}_n)_M$ is the ion pair in the mobile phase, and $(D{\cdot}\text{TFA}_n)_s$ is the ion pair in the stationary phase. Because the pKa of trifluoroacetic acid is ≅ 0.3, 98% of TFA exists as the anion at the pH of the mobile phase (pH 2).

Now,

$$k' = \frac{\textit{amount of ABT-861 in the stationary phase}}{\textit{amount of ABT-861 in the mobile phase}}$$

$$= \frac{V_s([D{\cdot}\text{TFA}_n]_s + [D]_s)}{V_m([D{\cdot}\text{TFA}_n]_M + [D]_M)} \qquad \text{Eq. 2}$$

where k' is the capacity factor; V_s and V_M are the volumes of the stationary and mobile phase, respectively; and $[D]_M$ and $[D]_s$ are the concentrations of the free and non-ion-paired ABT-861 in the mobile and stationary phase, respectively.

Ion pair formation in a chromatographic column has generally been viewed to take place via two mechanisms. The ion pair is either formed in the mobile phase, followed by partitioning, or the stationary phase is "coated" with the ion-pairing agent, followed by the formation of the ion-pair in the stationary phase. An assumption that was made at the outset of this work was that $[D{\cdot}\text{TFA}_n]_s >> [D]_s$. This is reasonable inasmuch as the ion pair, as a neutral entity, is presumed to be more lipophylic than ABT-861 and would therefore have greater solubility in the C_{18} stationary phase, and TFA as the ion-pairing agent is present in a large excess in comparison with ABT-861.

Then, for case 1,

$$[D]_M << [D{\cdot}\text{TFA}_n]_M$$

or

$$k' \cong \beta \frac{[D{\cdot}\text{TFA}_n]_s}{[D{\cdot}\text{TFA}_n]_M}; \beta = \frac{V_s}{V_M}$$

$$= \frac{[D{\cdot}\text{TFA}_n]_M}{[D{\cdot}\text{TFA}_n]_M} \beta K_p$$

$$= \beta K_p = K \textit{ (a constant)} \qquad \text{Eq. 3}$$

which would suggest that the capacity factor k' is a constant and independent of the TFA concentration of the mobile phase. This is clearly not the case, because k' was found to increase with increasing TFA concentration in the mobile phase.

For case 2,

$$[D]_M >> [D{\cdot}\text{TFA}_n]_M \qquad \text{Eq. 4}$$

then

$$k' \cong \beta \frac{[D{\cdot}\text{TFA}_n]_s}{[D]_M}$$

$$= \frac{\beta K_p K_{ip} [D]_M [\text{TFA}]^n}{[D]_M}$$

$$= k''[\text{TFA}]^n; k'' = \beta K_p K_{ip}$$

$$\log k' = \log k'' + n \log [\text{TFA}] \qquad \text{Eq. 5}$$

A plot of $\log k'$ versus log [TFA] ([TFA] expressed as %, v/v) was shown to be linear with a value of 0.80 for slope (n = 0.80 from Equation 5), or the ABT-861·TFA ion pair would exist as ABT-861·$\text{TFA}_{0.8}$ (roughly a 1:1 stoichiometry). The plot also yielded a value of 1.96 for the intercept ($\log k''$ = 1.96 from Equation 5).

However, if it is assumed that $[D{\cdot}\text{TFA}_n]_M$ and $[D]_M$ are on the same order of magnitude (case 3), then from Equation 2 comes

$$k' = \frac{\beta K_p [D{\cdot}\text{TFA}_n]_M}{[D]_M + [D{\cdot}\text{TFA}_n]_M}$$

$$= \frac{\beta K_p K_{ip} [\text{TFA}]^n [D]_M}{[D]_M + [D{\cdot}\text{TFA}_n]_M}$$

$$= \frac{k''[\text{TFA}]^n [D]_M}{[D]_M + [D{\cdot}\text{TFA}_n]_M} \qquad \text{Eq. 6}$$

which translates to

$$\log k' = \log k'' + n \log [\text{TFA}] + \log [D]_M - \log ([D]_M + [D{\cdot}\text{TFA}_n]_M)$$

$$= \log k'' + n \log [\text{TFA}] + \log \frac{[D]_M}{([D]_M + [D{\cdot}\text{TFA}_n]_M)} \qquad \text{Eq. 7}$$

A plot of $\log k'$ versus log [TFA] for Equation 7 is not linear because of the dependence of the last term in Equation 7 on [TFA]. However, a plot of $\log k'$ versus log [TFA] was found to be linear, implying that Equation 5 is valid.

At pH 2, ABT-861 has a net charge of +3 with protonation occurring at the Lys(Ipr)[8] (pK_a ≅ 10.7), Pal[3] (pK_a ≅ 5.1), and Lys(Nic)[6] (pK_a ≅ 3.6) residues. Because an approximately 1:1 (TFA/drug) stoichiometry is observed, it may reasonably be assumed that TFA forms an ion pair with the most basic nitrogen containing the highest charge density [Lys(Ipr)[8]]. One may also assume that because of the much less basic nitrogens of the Pal[3] and Lys(Nic)[6] residues, ion pair formation at these sites is weak and not observed.

Solvent for the bulk powder and formulations

The bulk powder was found to be soluble in water, ethanol, and diluted (10%, v/v) acetic acid at a level greater than 3 mg/mL. ABT-861 was observed to have a somewhat higher detector response at its wavelength maximum (225 nm) in a 1:1 ethanol–water mixture than in the individual solvents or in 10% acetic acid (which was subsequently used as the solvent for the various lots of bulk powder and formulations examined).

Acetic acid (10%, v/v) was also found to minimize the possible

interactive effects of formulation pH and excipients on ABT-861's detector response. For a given concentration of ABT-861, the detector response was observed to increase gradually with increasing acetic acid content of a formulation sample preparation, leveling off at just under 10% acetic acid. All formulation and bulk powder samples were subsequently prepared in 10% acetic acid.

Selectivity

In order to demonstrate that degradation products of ABT-861 can be separated from the parent, the bulk powder was thermally stressed under air-oxidative conditions. Thermal stressing was also carried out in order to model conditions used in the stability study for ABT-861 bulk drug lots and to determine the identity of the resulting degradates. According to the mass spectral data, there appeared to be no clear evidence of the existence of the diastereomeric forms of ABT-861 and the C-terminus free acid form (D-Ala^{10}OH) of ABT-861 as trace manufacturing impurities or degradation products in the various lots of ABT-861 examined. Also, according to the mass spectral data, there appeared to be no evidence of the coelution of degradates or impurities with ABT-861. Degradation products and manufacturing impurities at levels greater than or equal to 0.1% were identified by liquid chromatography–mass spectrometry (LC–MS) under the chromatographic conditions described in Table I (see the section on identification of the degradation products and impurities in ABT-861 bulk drug by LC–MS). Figure 3 shows an example of the separation of various degradation products and manufacturing impurities from the intact ABT-861 in a 1-month, 50°C bulk drug stability sample; retention time data and the identification of degradation products and manufacturing impurities are presented in Table II.

To be sure, the acetonitrile content of the eluent or the flow rate may be increased to create somewhat shorter retention times for the parent and the degradates. Care must be taken, however, to avoid interference with the parent peak from closely-eluting impurity or degradate peaks. In this work, a retention time window of 35–40 min for the parent compound was selected in order to have baseline separation of the parent peak from that of a small impurity found in the lots examined. This impurity eluted just before the parent compound (Figure 4). A retention time window of 35–40 min for the parent compound was also used as a system suitability criterion. A second system suitability criterion used was a relative standard deviation (RSD) of 2.0% or less for peak area or peak height ratios for 5 injections of the working standard solution made at the beginning of each run.

The wavelength maximum (225 nm) used for the assay of ABT-861 versus an external standard was also used for the determination of impurities and degradates on a peak area percent basis. The appropriateness of the use of the same wavelength maximum for the 2 assays was shown by demonstrating that the amount of ABT-861 remaining in the stressed samples determined using an external standard closely matched the amount obtained on the peak area percent basis.

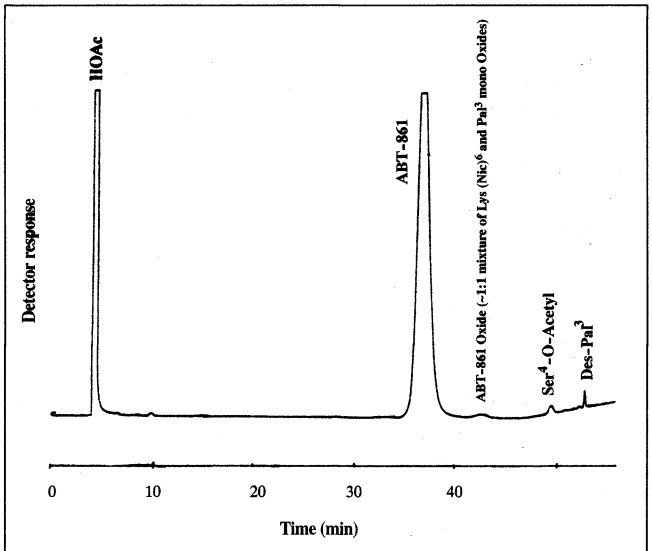


Figure 3. Chromatogram of ABT-861 (0.4 mg/mL) stability sample (1 month at 50°C).

Table II. Retention Time Data and Identification of ABT-861 Degradation Products and Manufacturing Impurities in a Stability Sample (1 Month, 50°C)

Retention time (min)	RRT	Identification	(Molecular mass + 2 H)$^{+2}$/2
36.19	1.00	ABT-861	825
42.08	1.16	Mono oxide of ABT-861	833*
48.91	1.35	Serine^4-O-acetyl ABT-861	846†
52.06	1.44	Des-Pal^3 ABT-861	751†

* Degradation product; identification does not include MS–MS sequence confirmation of locus.
† Manufacturing impurity.

Linearity

Under the chromatographic conditions shown in Table I, the linearity of detector response for ABT-861 base in the 0.009–0.18 mg/mL concentration range was examined. The targeted con-

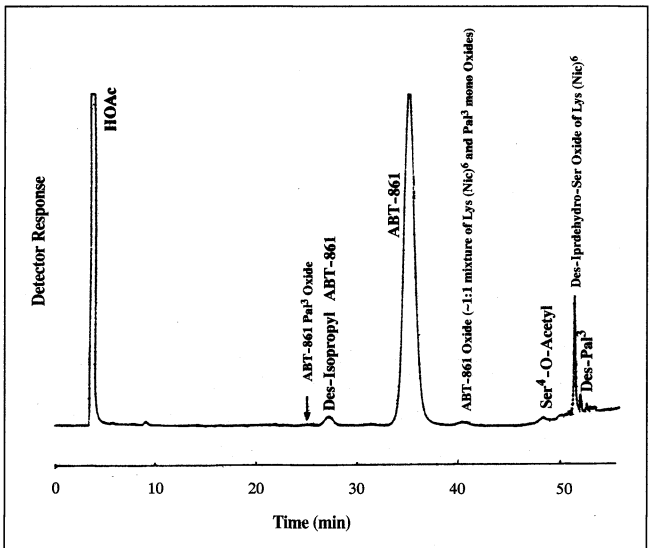


Figure 4. Chromatogram of ABT-861 (0.4 mg/mL) stability sample (1 month at 50°C and 64 h at 90°C).

centration range covers approximately 0.1–2 times the concentration level of ABT-861 (i.e., approximately 0.09 mg/mL) used in the assay of ABT-861. Plots of the peak area ratios (area of ABT-861/area of acetophenone used as internal standard) versus ABT-861 concentration (mg/mL) and peak height ratio versus ABT-861 concentration (mg/mL) yielded correlation coefficients r of 1.0000 and 0.9998, respectively, with lines passing essentially through the origin. The y-intercepts are insignificant, with zero being included in their respective 95% confidence interval; as such, a single point standard can be used for the assay of ABT-861. The values for the y-intercept and the slope for the peak area ratio plot are –0.025692 and 22.373, respectively; for the peak height ratio plot, they are 0.0081973 and 5.87749, respectively.

As part of the method validation for the determination of impurities and degradation products using a low-level standard of ABT-861 (0.003 mg/mL as mono acetate salt), detector response (peak area counts) versus concentration of ABT-861 was found to be linear over the concentration range of 0.0014–0.0068 mg/mL with a slope of 3.511 × 108, a y-intercept of –15831, and a correlation coefficient r of 0.9988.

Limits of detection and quantitation

The limit of detection (LOD) was estimated to be approximately 9 ng for ABT-861 as mono acetate salt. This was based on an approximately 3:1 signal-to-noise ratio and an injection volume of 50 µL. The limit of quantitation for ABT-861 was approximately 8 times that of the LOD, approximately 70 ng (or 0.0014 mg/mL as the mono acetate). This is also the lowest concentration of ABT-861 used in the linearity study described previously. The impurities and degradation products (assuming that they have similar molar absorptivities to that of ABT-861) were estimated versus the ABT-861 low-level (0.003 mg/mL) standard.

Accuracy

Standard addition/recovery experiments were carried out by adding known amounts of ABT-861 to a placebo formulation and analyzing the resulting solutions using the described method. Recovery values are reported in Table III. A mean recovery of 100.1% with a relative standard deviation of 1.73% was obtained for this study. No interferences were observed from the formulation excipients.

Precision

Precision of the method for assay of ABT-861 in bulk drug and in 3.0-mg/mL injectable formulations was determined to be 0.80% RSD and 1.13% RSD, respectively. In each case, separate analysts on separate days made multiple determinations using separate HPLC units, sample and standard preparations, and eluents. The determinations were made at the working sample concentrations described in the section on preparation of ABT-861 solutions (i.e., approximately 0.1 mg/mL for the bulk powder and approximately 0.09 mg/mL for the injectable formulations). However, the established range of quantitation for the bulk powder and the injectable formulations extends from approximately 0.009 mg/mL to approximately 0.18 mg/mL.

The precision for the estimation of degradates and impurities in the bulk powder on an area percent basis was obtained by the analysis of a thermally-stressed lot of ABT-861 for 2 impurities. Two analysts on separate days analyzed 5 sample preparations using separate HPLC units, columns, and eluents. RSDs of 3.8% and 4.3% were obtained for impurity concentrations of 0.30% and 1.32%, respectively.

Table III. Recovery of ABT-861 from the Addition to Formulation Placebo

Effective formulation strength (mg/mL)	Amount added (mg)	Amount recovered (mg)	% Recovery
1.92	5.76	5.73	99.5
1.93	5.79	5.96	102.9
2.56	7.67	7.55	98.4
2.57	7.72	7.78	100.8
3.20	9.59	9.44	98.4
3.22	9.65	9.86	102.2
3.84	11.51	11.48	99.7
3.88	11.63	11.48	98.7
		Mean	100.1%
		Relative standard deviaiton	1.73%

Identification of the degradation products and manufacturing impurities by LC–MS

LC–MS experiments were performed on "regular" and "highly stressed" stability samples of ABT-861. LC–MS runs were made on an ABI 140A HPLC system coupled to a Finnigan MAT TSQ 700 MS with a Finnigan MAT electrospray source. Selected molecular ions were subsequently identified by LC–MS–MS sequencing using collision-induced dissociation of the doubly-charged ions. The strongest molecular ions for ABT-861 and its related impurities and degradation products were the doubly-charged molecular ions $(M + 2H/2)^{+2}$, although in most cases the singly-charged molecular ions were also observed. For the parent compound (ABT-861), the mass-to-charge ratio m/z was 825. Fraction collection was used for smaller peaks, for which LC–MS–MS was found to be inadequate. Here, small peaks were collected offline by repeated injections. The fractions were pooled and then evaporated. Collected fractions were then sequenced by direct injection into the electrospray ionization source.

As discussed previously, Figure 3 depicts the separation of ABT-861 from the various degradation products and manufacturing impurities for a 1-month, 50°C bulk drug stability sample. Figure 4 shows the chromatogram of the same stablity sample that has been further stressed under air-oxidative conditions by heating the bulk powder to 90°C for approximately 64 h with the formation of additional oxidation products and Des-Isopropyl ABT-861. From the MS–MS data, the peak at RRT = 1.16 with m/z = 833 (Tables II and IV, Figures 3 and 4) was identified as a 1:1 mixture of 2 mono-oxides of ABT-861, with oxidation occurring at the Pal^3 and the $Lys(Nic)^6$ residues. The peak at RRT = 0.71 with m/z = 833 (Table IV) was tentatively identified as an early-eluting oxide of Pal^3 residue, possibly a diastereomer of the pyridinyl N-oxide. Retention time data and the identification of

Table IV. Retention Time Data and Identification of ABT-861 Degradation Products and Manufacturing Impurities in a Stability Sample (1 Month at 50°C and 64 h at 90°C)

Retention time (min)	RRT	Identification	(Molecular mass + 2H)$^{+2}$/2
24.88	0.71	Mono-oxide of ABT-861	833*,†
27.10	0.77	Des-isopropyl ABT-861	804‡,*
34.99	1.00	ABT-861	825
40.48	1.16	Mono-oxide of ABT-861	833*,†
47.48	1.36	Serine^4-O-acetyl ABT-861	846‡
50.94	1.46	Des-isopropyl dehydro-Ser^4 oxide of Lys(Nic)^6 ABT-861	803*
51.42	1.47	Des-Pal^3 ABT-861	751‡

* Degradation product.
† Identification does not include MS–MS sequence confirmation of locus.
‡ Manufacturing impurity.

degradation products and manufacturing impurities for this sample are also presented in Table IV.

Conclusion

An HPLC method with UV detection is described for the determination of ABT-861, a GnRH antagonist used in medical conditions responsive to hormonal manipulation. The method involves isocratic elution with gradient ramping to elute the more strongly retained impurities and degradates. The method is shown to be stability-indicating, providing good selectivity with a detection limit of approximately 9 ng for the drug substance. It has been applied to the determination of ABT-861 in the bulk powder and injectable formulations.

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