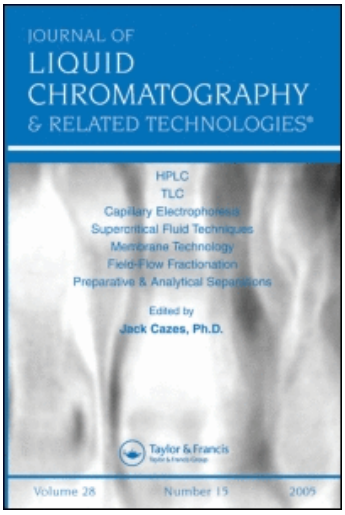

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DETERMINATION OF DEGRADATION PRODUCTS OCCURRING IN ACIDIC SOLUTIONS OF A 21-AMINOSTEROID (TIRILAZAD) USING A GRADIENT HPLC METHOD

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ABSTRACT

A high performance liquid chromatographic (HPLC) method quantitating degradation products of a 21-aminosteroid (tirilazad mesylate) in acidic solutions is described. Mobile phase elution consists of a 58 minute gradient from 5 to 95% mobile phase A, where mobile phases A and B are (81:9:10:0 v/v) and (5.4:0.6:10:84 v/v) acetonitrile, tetrahydrofuran, 0.2 M ammonium formate/ formic acid buffer (pH 3.5) and water. A base deactivated octadecyl silane column thermostatted at 42°C, and fixed wavelength detection at 254 nm are used for this method. The assay is compatible with thermospray mass spectrometry, offering several advantages for the identification of impurities. Validation of the method, along with the use of mass spectrometry for peak identity and purity is discussed.

INTRODUCTION

Tirilazad mesylate (U-74,000F), a 21-amino substituted steroid [16-α-methyl- 17-α-hydrogenpregna-1,4,9(11)-triene-3,20-dione-21-[2,4-bis-(1-

pyrrolidinyl)-6-piperazinylpyrimidine] methane sulfonate], inhibits lipid peroxidation (1,2). Activity in experimental models of head, spinal injury (3,4), and hemorrhagic shock (5) were found. Because the solubility of tirilazad decreases with increasing pH, administration in-vivo uses acidic aqueous solutions (pH 3). Tirilazad degrades in solution and quantitative methods were needed to determine the amounts of these degradation products. Secondarily, the method was used to determine the amounts of process and degradation related impurities in the bulk tirilazad mesylate. In acidic solutions, tirilazad degrades from four reactions: hydrolysis of the piperazine-pyrimidine bond, oxidation of the C-20 ketone, dienone-phenol rearrangement of the steroid A ring and oxidation to produce the amine side-chain (6). Figure 1 shows the major degradation pathways.

Development of a HPLC method that is compatible with thermospray mass spectrometry generally entails the use of volatile buffers and offers several advantages. Information regarding peak identity and purity is obtained. Isolation of unknowns is easier as thermospray mass spectrometry compatible systems use volatile buffers that can be removed via freeze-drying. Compounds with low UV absorbance are detected (assuming sufficient ionization efficiency). Thermospray mass spectrometry compatible systems do have some disadvantages. Peak shape may be compromised because competing amines are not typically used because of their lack of volatility and their affect on sensitivity (compete for protons). In the absence of post-column mixing, mobile phase pH is usually restricted either to acidic pH's or to the use of unbuffered systems. To achieve ionization, acidic pH's are preferred for basic analytes. The buffers may compromise UV detectability as carboxylic acids absorb significantly below 230 nm.

None of the above restrictions significantly affected developing a method for tirilazad. The newer base-deactivated columns made possible the development of high efficiency separations for basic analytes without using competing amines. This report discusses the development of a thermospray mass spectrometry compatible method for degradation

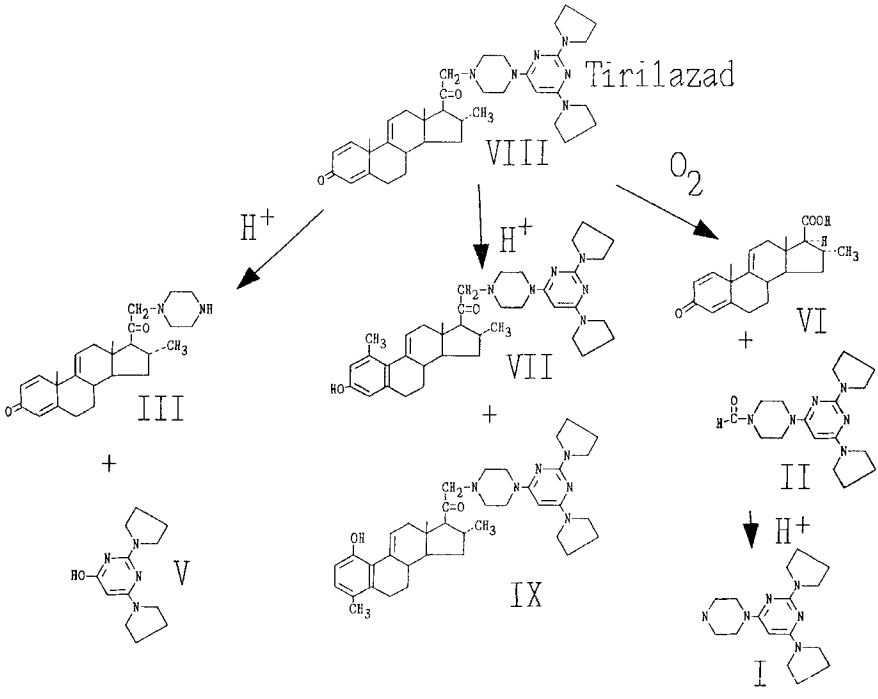

Figure 1. Degradation of Tirilazad mesylate (compounds are numbered to conform with Reference 6).

products in tirilazad, including improvements over the methods used in the identification of the degradation products (6).

MATERIALS AND METHODS

Reagents/Compounds

All solvents were HPLC grade (Burdick and Jackson, Muskegon, MI). Other reagents were analytical reagent grade. Tirilazad mesylate (**VIII**) and compounds **I**, **II**, **III**, **V** and **VI** were prepared in the research labs of The Upjohn Company (6).

Equipment

The gradient pump was a low-pressure, mixing system (Model 2249, LKB, Bromma, Sweden). A line-source detector at 254 nm (UV Monitor III, LDC, Rivera Beach, Fl) was used in the validation experiments. A photo-diode array HPLC detector (Model 990, Waters Assoc., Milford, MA) was used to obtain UV spectra of the degradation products and peak homogeneity of the tirilazad . The column was a 4.6 X 250 mm, 5 µm, octadecylsilane column (Inertsil®, GL Sciences, Tokyo, Japan). Other columns that were evaluated included: YMC-Basic® (YMC, Morris Plains, NJ, USA), Develosil® (DyChrom, Sunnyvale, CA, USA), Rexchrom® (Regis, Morton Grove, IL, USA) and Asahipak ODP-50®, an ODS modified polymeric column (Asahi Chem. Co., Kawasaki-ski, Japan).

An octadecylsilane TLC plate (RP-18, WF254s, EM Science, Cherry Hill, NJ, USA) was used with mobile phase A to evaluate the elution of all peaks from the solvent front. The plates were scanned at 254 nm (Camag, Muttenz, Switzerland) and developed using a starch-iodine mixture (dried at 50 °C for 30 minutes).

Mass spectra were acquired with either a quadrapole MS with thermospray interface (Model 4600, Finnigan, San Jose, CA) or a triple quadrapole system (Model 70, Finnigan, San Jose, CA). The instrument was operated in the discharge mode with a vaporizer temperature of 100 °C, a jet temperature of 250 °C and a repeller voltage of 80 V. To maintain uniform response, a cross-gradient was operated post-column thus maintaining a consistent solvent composition into the interface.

Methods

Column temperature was maintained at 42 ° C. A linear gradient began at 2 minutes and increased from 5% to 95% mobile phase A over a 58 minute period. Mobile phases A and B were (81:9:10:0) and (5.4:0.6:10:84) acetonitrile, tetrahydrofuran, 0.2 M ammonium formate/ formic acid buffer

(pH 3.5), and water. A flow rate of 1.0 ml/min was used. Blank peaks were reduced by passing the aqueous buffer solution through a octadecylsilane cartridge (Sep-pak, Water's Associates, Milford, MA).

Tirilazad mesylate solutions at 1.5 mg/ml were diluted 1:4 with a solution of methanol:water: ammonium formate buffer (used in mobile phase preparation) 50:45:5. A 10 µL injection volume was used.

RESULTS AND DISCUSSION

Method Development

HPLC systems for tirilazad and degradation products of tirilazad (Figure 1) were previously reported using isocratic reversed-phase (7,8) and gradient methods (6). Problems with these methods include insufficient retention of the more polar impurities, inadequate resolution of all known impurities from the tirilazad mesylate, and poor peak shape for tirilazad . Over the past few years, a large number of columns were claimed to give high efficiency for basic analytes. After comparing several of these columns for peak efficiency (Table 1), an Inertsil column was selected for further evaluation. Using the Inertsil column provided a highly efficient chromatographic system without using competing amines.

Using an acetonitrile gradient with pH controlled at 4.0 with ammonium formate as a starting point (6), modifications were made to improve both peak shape and resolution. Adding tetrahydrofuran to the mobile phase improved the separation of the more polar compounds, peaks **I**, **III** and **V**, and reduced peak tailing. The ratio of acetonitrile:tetrahydrofuran was optimized at a ratio of 9:1 and was maintained at that ratio throughout the gradient. The gradient slope, mobile phase pH, and column temperature were then optimized. The resulting chromatography is shown in Figure 2. Table 2 shows the relative retention times, response factors (254 mn), observed UV maximum and molecular ions from LC/MS.

Table 1
Columns Evaluated for use in Method (Parameters measured for tirilazad)

Column Description	Theoretical Plates	Retention Time (min)
YMC-Basic mixed alkyl chain length (4.6 x 150 mm)	3000	25
Develosil, C18 (4.6 x 250 mm)	3100	35
Asahipak ODP-50, C18 modified polymeric column (4.6 x 250 mm)	6500	23
Dupont C8 RX (4.6 x 150 mm)	1400	36
Inertsil C18 (4.6 x 250 mm)	14000	38

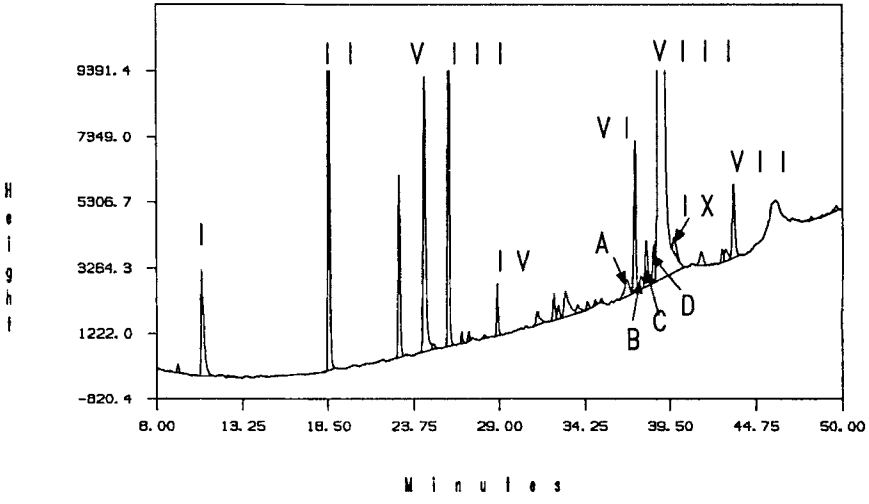

Figure 2. Chromatogram of Degraded Tirilazad mesylate (see Figure 1 for identity of peaks)

Table 2
Chromatographic and Mass Spectroscopic Data on Compounds
(compounds are numbered to conform with Reference 6.)

Compound	UV Absorption Maxima	Relative Response Factor (254 nm)	Relative Retention Time	Molecular Weight	LC/MS Ion (M+H)
I	245, 295	1.23	0.23	302.43	303
II	248, 296	1.76	0.44	330.40	331
III	242	0.48	0.63	408.6	409
IV	242	not known	0.73		339
V	238, 278	0.81	0.58	234.3	235
VI	244	0.73	0.92	326.43	327
VII	251, 293	1.0[b]	1.12	624.87	625
VIII (Tirilazad)	244, 295	1.0	1.0	624.87	625
IX	246, 294	1.0[b]	1.03	624.87	625

[b] Response factors were assumed to be 1.0.

Thermospray Mass Spectrometry

Using the newly developed method, thermospray mass spectrometry was performed on a sterile solution sample (1 month at 47°C, total impurities approximately 8%), an acid-degraded sterile solution (approximately 30%-40% total impurities), and a severely degraded bulk tirilazad mesylate sample (55 months at room temperature, approximately 20%-30% total impurities). Figure 3 shows the UV chromatogram and corresponding thermospray total ion current chromatogram for the degraded sterile solution.

Masses were obtained for significant unidentified impurities (Table 3). From this information, the unidentified impurity at r= 0.81 is the

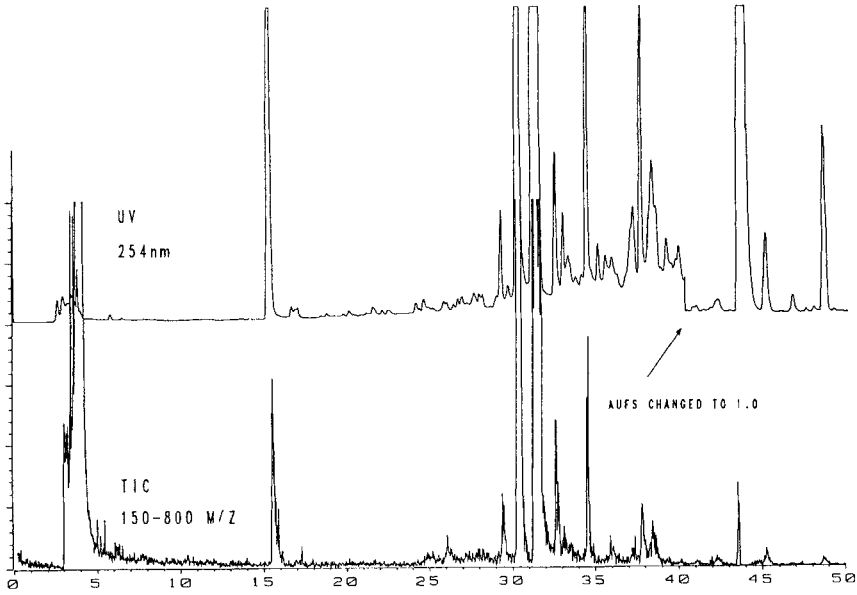

Figure 3. UV and Thermospray Total Ion Chromatograms of U-74,006F Aqueous Degradation Sample

Table 3
Mass Spectral Information for Unidentified Peaks

Relative retention	label	MH+	possible structural characteristics
0.81	IV	339	steroid
0.94	A	640	unknown
0.97	B	642	oxidation product
0.98	C	657	oxidation product
1.03	IX	626	tirilazad isomer

unknown steroid (M/Z = 339) previously discussed in reference 6. Based on the mass (M/Z=625) and the conditions in which the impurity is formed (low pH, high temperature) , the peak was identified as the *para* isomer of the dienone-phenol rearrangement product of tirilazad . Previous studies discussed the possible presence of this impurity, but it was never found (6). The resolution of this impurity is a significant improvement over previous work.

Mass spectrometry data also confirmed the identity and purity of the known degradation peaks (Table 2) and the absence of significant UV transparent peaks. Analysis of the edges of the tirilazad peak in all samples revealed that no extraneous ions were found. This confirms that no impurities with masses differing from the tirilazad were eluting on the front or tail of the tirilazad peak.

Validation

All known process and degradation related impurities were resolved from the tirilazad peak. As previously discussed, MS was used to confirm the absence of extraneous ions eluting under the tirilazad peak. TLC was used to evaluate whether all peaks elute in the chromatogram. No UV transparent peaks were found to be retained at the origin. UV scans of the plates at 254 nm confirmed that all peaks eluted from the solvent front in the bulk tirilazad mesylate sample. For the sterile solution samples, less than 0.2% of the degraded material remained at the origin for a sample that was degraded about 20%.

Chromatographic ruggedness was evaluated by slightly changing mobile phase pH, gradient slope and intercept and column temperature. Changes in pH over the range of 3.4 to 3.7 only cause minor changes in the resolution of the most significant impurities. Decreasing pH increases the resolution of peaks **V** and **III**. The effect of pH is more important for several minor peaks (**A-D**) that elute near tirilazad (see Figure 4). Peaks **A-D** were of little importance for assessing degradation but were important in

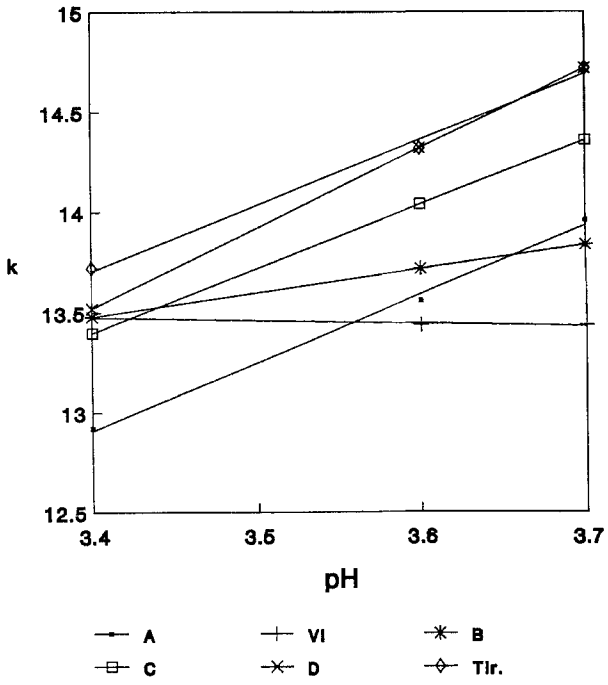

Figure 4. Effect of pH on Retention and Resolution (peaks eluting near tirilazad)

bulk tirilazad mesylate purity. The resolution of these impurities determined the final selection of mobile phase pH. Table 4 shows the effect of gradient slope, initial organic concentration, gradient delay, and temperature on the separation of the most critical peak pairs (tirilazad/**IX**) and (**III/V**). The resolution of tirilazad/**IX** varies from 2.6 to 3.1 over the parameter ranges studied. Only increases in gradient slope cause substantial decreases in resolution of peaks **V** and **III**. Other peak pairs were only moderately affected by these changes.

During the course of validation, some columns were found to cause a substantial increase in an unknown impurity at relative retention 0.97.

Table 4
Effects of Modifying Chromatographic Conditions on Resolution Factors (other variables held constant as specified in Experimental section)

Chromatographic Variable	Resolution Factor for Peak Pair	
	III/V	Tirilazad/IX
Gradient Slope (%/min change in A)		
1.11	10.6	3.1
1.38	7.7	2.9
1.64	5.7	2.8
Column Temperature (°C)		
38	5.1	2.6
40	5.3	2.8
42	5.4	2.8
Initial Percent A		
5	6.6	2.8
10	6.8	2.9
Gradient Delay (min)		
0	6.5	2.8
5	6.8	2.9

When only the column was switched, the same sample would show as much as a 300% increase in the impurity at 0.97 (total amount still less than 0.3%). Other experiments demonstrated that the increased degradation was not caused by the column frits or by instability in the mobile phase. The peak was concluded to be caused by on-column degradation.

Tirilazad peak area response was linear (R = 1.000) over the range of 0.0045 to 4.95 μg (amount injected). The residual relative standard

deviation from the regression was 0.45%. Linearity data provided response factors for peaks **I, II, III** and **V**. Correlation coefficients were >0.999 for all impurities. Repeated analysis of an impurity mix determined the precision for the quantitation of impurities. The relative standard deviations typically varied from 0.3% to 6% for impurities present at 5% to 0.2% (as a percentage of tirilazad plus other impurities). All impurities present at 0.1% or greater in samples stored under nominal conditions were resolved from other impurities.

Sample stability was evaluated over a 48 hr time-frame. The amount of the primary degradation impurity, **III**, increased over time, particularly in the bulk tirilazad mesylate sample. However, total impurity levels did not rise over the same time frame. A limit of 24 hours in a light protected environment ensures sample preparation stability.

For regulatory purposes a limit of quantitation of not more than 0.1% was needed. Root mean square (RMS) noise for seven blank injections was determined at six different 1 minute intervals. From these data, the worst case noise occurs after the onset of the gradient, near the elution of peak **I** (approximately 11 minutes). As compound **I** has the smallest peak height to peak area ratio, the worst case detection limits were calculated for this peak. Using a S/N ratio of 3, the detection limit was 0.007% (as a percentage of tirilazad). The quantitation limit, defined for a S/N ratio of 10, was 0.03%, meeting regulatory requirements.

Batch Analysis and Mass Balance Assessment

Several sterile solution lots stored at temperatures from 4 to 47 °C under various conditions were evaluated for impurities using the new method. The amounts of the degradation products in these lots ranged from 1.2 to 19% and are shown in Table 5. The average mass balance for these solutions was 99.2% with a relative standard deviation of 1.7% . The effect of heat, pH and oxygen on the formation of these degradation

Table 5
Degradation Product Data on Representative Samples (NQ = not quantitated, <0.05%; ND = not detected, approximately <0.02%)

Lot	Conditions	I	III	V	VI	VII	IX	Total Impurities
1	6 months, 8 °C	NQ	0.82	0.27	NQ	ND	ND	1.2
3	6 months, 25 °C	0.11	3.1	1.2	0.10	0.06 3	ND	4.9
4	3 months, 47 °C	1.4	9.9	4.2	0.17	0.62	0.19	19
6	9 months, 25 °C	0.64	4.5	1.9	NQ	0.07 4	ND	7.8
7	36 months, 4 °C	0.23	1.6	0.59	NQ	NQ	ND	3.3
8	6 months, 4 °C	0.11	0.75	0.24	NQ	NQ	NQ	1.9

products was previously discussed (6). The newly identified degradation product (**IX**, ***para*** isomer of dienone-phenol rearrangement) was only found in the sample stored at 47 °C.

CONCLUSIONS

A LC/MS compatible HPLC method for quantifying all significant degradation products in acidic solutions of tirilazad mesylate was developed. The success of this method was evidenced by a mass balance of 99.2%. Development of a method compatible with thermospray mass spectrometry proved advantageous in that molecular-ion information was obtained for nine compound, tirilazad peak homogeneity was evaluated and a significant unknown degradation impurity was identified. Use of an Inertsil column, designed for use with basic molecules, made the development of an efficient method easier as no competing amines were necessary to achieve satisfactory peak shape.

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