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Note

Development of a polymer-based reversed-phase high-performance liquid chromatographic stability indicating assay for U-78 608, an iron complexing monocarbam antibiotic

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U-78 608 belongs to a new class of β-lactam antibiotics known as the monocarbams (Fig. 1). The monoanionic form of 3-hydroxy-4(1H)-pyridinone, a moiety found in U-78 608, has been shown to form 1:1, 2:1 and 3:1 ligand–substrate complexes with Fe^{3+} (ref. 1). This candidate has shown remarkable activity toward *Pseudomonas*[2–4], presumably because of its ability to complex iron[5]. While the ability to chelate iron appears critical to the anti *Pseudomonas* activity of U-78 608, it also complicates the chromatographic analysis of the compound.

This report discusses the development of a reversed-phase high-performance liquid chromatographic (RP-HPLC) stability indicating assay for the separation of U-78 608 from impurities and degradation products. To avoid trace metal column effects commonly observed with silica-based stationary phases[6], a polymer-based column was used. The assay has been utilized to determine the pH/degradation rate profile of U-78 608.

EXPERIMENTAL

Chemicals

Ethylenediaminetetracetic acid (EDTA) disodium salt was purchased from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC grade) was obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). All other chemicals were reagent grade and water was deionized and double-distilled. U-78 608 was obtained from the Chemical Research Preparation Unit of Upjohn (Kalamazoo, MI, U.S.A.).

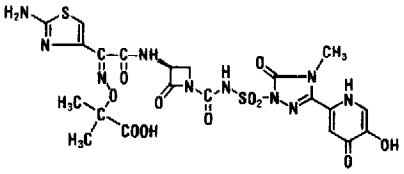

Fig. 1. Structure of U-78 608.

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UV absorbance

Ultraviolet scans of U-78 608 (free acid) in purified water and 0.01 *M* $(NH_4)_2HPO_4$ (pH 8.0)–methanol (80:20, v/v) from 190 to 400 nm were performed using a Perkin-Elmer (Norwalk, CT, U.S.A.) Lamda V UV–VIS spectrophotometer. Sample concentrations of approximately $3 \cdot 10^{-5}$ *M* were used, with the respective solvents as reference solutions.

Ionization constants

The dissociation constants for U-78 608 were determined potentiometrically using a Brinkman (Westbury, NY, U.S.A.) Metrohm 672 automatic titrator. The bulk drug was dried *in vacuo* (*ca.* 27 Torr) at room temperature over 24 h. The material was then weighed and diluted with 0.1 *M* sodium chloride to yield a final concentration of $3.7 \cdot 10^{-4}$ *M*. Duplicate samples were titrated at room temperature with standardized 0.01 *M* sodium hydroxide solution.

HPLC equipment and conditions

A DuPont (Wilmington, DE, U.S.A.) series 8800 quaternary gradient liquid chromatographic system, Perkin Elmer ISS-100 autosampler and IBM (Danbury, CT, U.S.A.) PC/XT with Nelson Analytical (Cupertino, CA, U.S.A.) series 2600 chromatography software were used in conjunction with a Hamilton (Reno, NV, U.S.A.) PRP-1®, 5-μm, 150 mm × 4.1 mm I.D. HPLC column and Brownlee (Santa Clara, CA, U.S.A.) PRP-GU®, 10-μm, 30 mm × 4.6 mm I.D. guard column. The injection volume was 40 μl with UV detection at 280 nm. All components were at room temperature.

Atomic absorption

HPLC fractions from the analysis of U-78 608 were collected and analyzed by atomic absorption for iron content. A Varian (Walnut Creek, CA, U.S.A.) AA-875 spectrophotometer (fitted with Fe hollow cathode lamp), GTA-95 graphite tube atomizer and PSD95 PGRMBL sample dispenser were used for the analysis.

Solution state stability study

Samples of U-78 608 were diluted with low ionic strength ($\mu = 0.01$) buffers[7] ranging from pH 2 through 10. The final concentration of U-78 608 was approximately 10^{-4} *M*. The solutions were stored in glass vials at 25.0 ± 0.1°C and periodically assayed. Rate constants were reported from HPLC stability data through roughly two half-lives. Confidence intervals reported for the rate constants were determined from the slopes of first-order kinetic plots.

RESULTS AND DISCUSSION

UV absorbance

Fig. 2 (curve A) shows UV maxima at 220, 250 and 280 nm for U-78 608 in water (pH 4.5). Ultraviolet detection at 280 nm was chosen for the HPLC analysis since this maximum was not shifted by pH adjustment to pH 8 and the addition of 20% methanol (curve B). Furthermore, the absorption of EDTA below 254 nm prohibited the use of a lower wavelength.

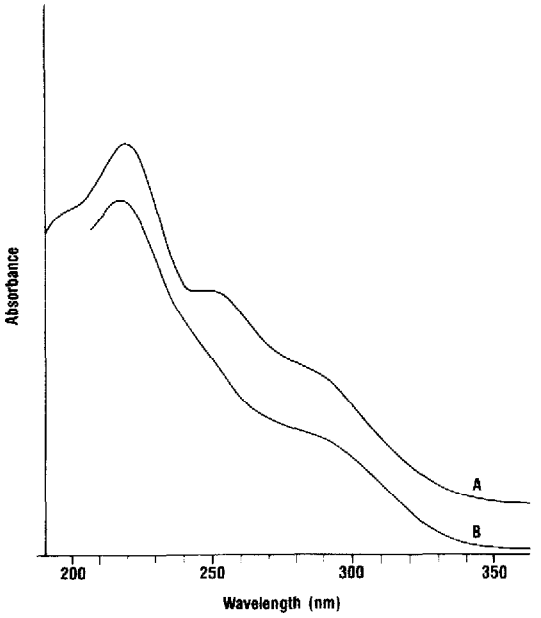

Fig. 2. Ultraviolet scans of U-78 608 in (A) water (pH 4.5) and (B) 20% methanol (pH 8.0).

Potentiometric titration

U-78 608 contains three functional groups capable of losing a proton (carboxylate, amide and hydroxyl). Potentiometric titrations were performed at an ionic strength (μ) of 0.1 to provide information about the species present as a function of pH. This information was useful in choosing the proper chromatographic approach. Fig. 3 displays a potentiometric titration curve for U-78 608. Two endpoints

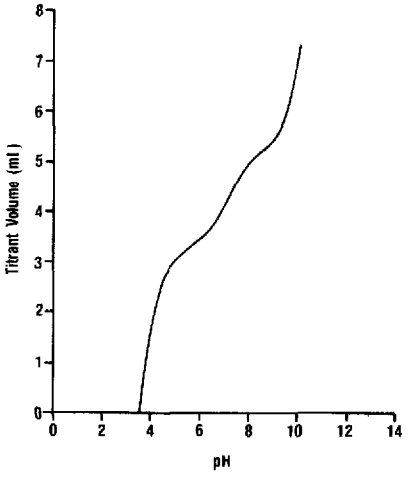

Fig. 3. Potentiometric titration curve of U-78 608 at an ionic strength of 0.1.

TABLE I

ACID DISSOCIATION CONSTANTS OF U-78 608

Univariate 95% confidence intervals in parentheses.

pK_a	*Thermodynamic*	*Apparent* (μ = 0.1 *M*)
1	2.72 (10.9%)	3.17 (3.79%)
2	5.11 (1.96%)	4.76 (1.89%)
3	8.81 (1.25%)	8.21 (1.34%)

were detected at roughly two and three equivalents of base. The apparent and thermodynamic dissociation constants of U-78 608 were determined by fitting the titration data using a least squares non-linear curve fitting program[8] (Table I). Based on these pK_a values, preliminary studies utilized ion-suppression and ion-pairing chromatography.

Early HPLC development

U-78 608 is considered to be a chelating agent because of the hydroxypyridinone moiety. Since conventional silica-based packings are known to contain residual amounts of metal ions[6], a polymeric reversed-phase packing was used throughout the development program. Initially, various mixtures of acetonitrile (10–30%) and pH 5.0 acetate buffer were tested as an isocratic mobile phase. At high organic concentration, U-78 608 eluted near the void volume with little resolution. At low organic composition, the peak was retained on the column (capacity factor, $k' = 2.2$), but the shape was extremely asymmetrical (tailing).

The peak tailing observed may have been due to either secondary equilibrium (*i.e.*, ionization) or metal complexation. The titration data (see Fig. 3) suggest that the compound was present primarily as the dianion at pH 5 in aqueous solutions. With the addition of organic modifier, the apparent pK_a values should be higher than the values measured in aqueous conditions. Thus, if the peak asymmetry noted above was due to secondary equilibrium, reversed-phase ion suppression would appear an appropriate method of chromatography for increasing retention time without tailing. Various mixtures of methanol (25–30%) and pH 3.0 citrate buffer were tested. The drug peak was adequately retained ($k' = 2.0$) although asymmetrical (tailing). A more acidic mobile phase may have reduced the tailing, however, it was likely that acid catalyzed hydrolysis of the β-lactam ring would have created a stability problem.

An alternate approach to reduce the tailing was ion-pair chromatography. Therefore, an isocratic mixture of tetrabutylammonium phosphate in phosphate buffer (pH 7–8) and methanol (10–30%) was tested. The drug peak either eluted near the void volume with insufficient resolution or when further retained was broad with tailing.

Competitive metal complexation

The failure of both ion-suppression and ion-pairing chromatography suggested that the peak broading might be due to metal complexation. HPLC systems which minimize mobile phase contact with stainless-steel are available, however, it was

decided to add EDTA to the mobile phase to compete for any polyvalent metal ions which may be causing the peak asymmetry. A binary mobile phase consisting of (A) 0.005 *M* EDTA and 0.01 *M* $(NH_4)_2HPO_4$ (pH 8.0), and (B) 100% methanol was used. A linear gradient from 100% A to A–B (70:30) over 20 min yielded a symmetrical peak for U-78 608 with a capacity factor of approximately 9. The column was reequilibrated in 10 min at 100% A. This assay was utilized for the remainder of the present study.

Assay linearity, precision and sensitivity

The assay was linear from 5 to 650 μg/ml U-78 608 with a correlation coefficient of 0.998. The precision (coefficient of variation) of six replicate injections of $1 \cdot 10^{-4}$ *M* solutions of U-78 608 was 1.2%. The chromatographic profiles analyzed from two column packing lots were found to be similar. The minimum detectable limit was approximately 0.5 μg/ml at 280 nm.

Interference studies

Stressed samples of U-78 608 in acidic, neutral and alkaline media, as well as aqueous mobile phase, were assayed over time to determine where any potential

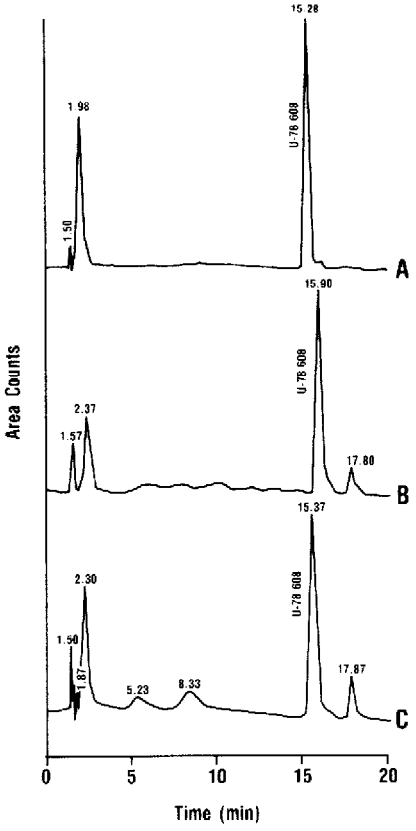

Fig. 4. Typical HPLC chromatograms of degraded U-78 608 under (A) neutral and alkaline conditions (pH 5–10), (B) acidic conditions (pH < 5), and (C) high temperature (> 45°C).

degradation products may elute, and to separate these if necessary from the parent compound. All degradation products appeared well resolved from the parent compound as shown in Fig. 4. After 5 h at pH 9 and 25°C, U-78 608 appeared to degrade to one major degradation product (Fig. 4A). Similar chromatograms were obtained for slightly acidic, neutral and alkaline samples (pH 5–10). Under acidic conditions (<pH 5), additional degradation products were observed (Fig. 4B, pH 3.0, 13 days). Elevated temperatures (>45°C) also resulted in the formation of multiple degradation products. Fig. 4C shows U-78 608 has degraded to multiple products after 48 h at pH 5 and 56°C.

Since base catalyzed hydrolysis of the β-lactam ring was suspected, the stability of samples prepared in aqueous mobile phase (pH 8.0) was of concern. A limited stability study of U-78 608 in the aqueous mobile phase at 25°C was conducted. The pseudo-first order rate constant (and 95% confidence interval) was calculated at 0.117 (10.55%) h^{-1} which corresponds to a t_{90} (time to 10% degradation) of approximately 1 h. Therefore, negligible on column degradation of U-78 608 was expected.

Atomic absorption

Since U-78 608 is suspected of being a strong metal binder, atomic absorption was utilized to determine if any of the observed peaks were due to iron–drug complexes. Sample fractions of the major peaks were found to contain no significant difference in iron concentration than the pure mobile phase. This suggests that U-78 608–iron complexes are not present in the assay.

pH degradation rate profile

The pseudo-first order rate constants (k) at 25.0°C for U-78 608 are plotted as a function of pH in Fig. 5. The maximum stability for U-78 608 in solution at 25°C was near pH 5 (indicated by the minimum in the curve). At pH 5.0, the rate constant with 95% confidence interval and t_{90} were calculated at 0.024 $days^{-1}$ (8.8%) and 4.3 days, respectively.

Above pH 7, the degradation appears to proceed primarily by specific base catalysis. The slope of log k as a function of pH from pH 7 to 10 was approximately

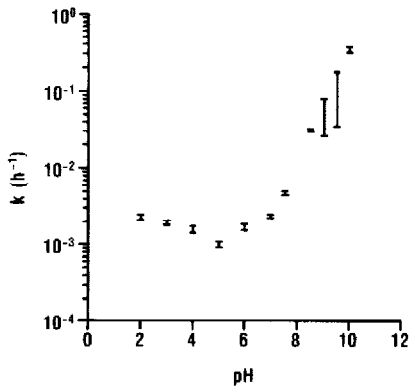

Fig. 5. Solution degradation rate profile for U-78 608 at 25°C and an ionic strength of 0.01 (error bars represent 95% confidence intervals).

0.7, compared to the anticipated slope of unity. Since U-78 608 has a dissociable proton with a pK_a value of 8.81 (see Table I), it appears that ionization of the compound in the pH 7–10 region introduces a small shoulder in the pH to stability curve resulting in an apparent slope of less than unity.

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