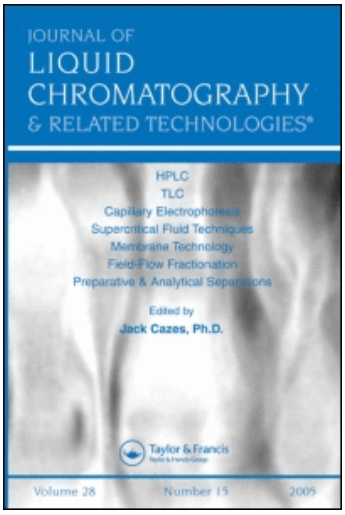

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DETERMINATION OF MINOR IMPURITIES AND DIASTEREOMERS OF 6-[3-[(2-AMINO-1-OXOPENTYL)AMINO]-1-PYRROLIDINYL]-5-FLUORO-3-OXO-3H-PYRIDO[3,2,1-KL]PHENOXAZINE-2-CARBOXYLIC ACID HYDROCHLORIDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Diastereomers of the quinobenoxazine antineoplastic drug Abbott-84441.1 and minor impurities were determined using high-performance liquid chromatography. Manufacturing impurities, degradation products, and diastereomers were separated using a reversed-phase system with gradient elution. Detector response was linear for Abbott-84441.1 to approximately 20 μg/mL which represents 4.0% of the drug concentration. The procedure provides quantitation of impurities to approximately the 0.1% level with precision (relative standard deviations) of 7.3% to 31% in typical bulk drug lots. A variety of reversed-phase columns were evaluated for the assay method with the optimum resolution achieved using a 5-μm Alltima C_{18} packing.

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INTRODUCTION

The synthesis of Abbott-84441.1, a quinobenoxazine antineoplastic drug, has been previously reported [1,2]. Chemically, the drug substance is 6-[3-[(2-amino-1-oxopentyl)amino]-1-pyrrolidinyl]-5-fluoro-3-oxo-3H-pyrido[3,2,1-kl]phenoxazine-2-carboxylic acid hydrochloride. As reported recently [1], this class of compound has in vivo activity against solid tumors and leukaemias of marine origin as well as human tumor xenografts. This paper describes the use of high-performance liquid chromatography (HPLC) for quantitation of minor impurities which can occur in Abbott-84441.1 bulk drug substance. Potential manufacturing impurities and degradation products are determinable by the procedure in addition to the ratio of diastereomers in the bulk drug.

Various methods using HPLC have been reported for the determination of fluoroquinolones and their metabolites[3-7]. The HPLC technique allows the direct determination of these materials without the derivatization which is necessary in gas chromatographic procedures [8-9]. Previously this laboratory has reported the quantitation of impurities in other quinolones using reverse-phase chromatography and gradient elution, but those compounds did not contain the amino acid substituent [10-11]. For Abbott-84441.1 the presence of the L-norvalyl-substituent provides a pair of diastereomers (Abbott-79775.1, (2'S,3S)-6-[3-[(2-amino-1-oxopentyl)amino]-1-pyrrolidinyl]-5-fluoro-3-oxo-3H-pyrido[3,2,1-kl]phenoxazine-2-carboxylic acid hydrochloride and Abbott-79583.1, (2'S,3R)-6-[3-[(2-amino-1-oxopentyl)amino]-1-pyrrolidinyl]-5-fluoro-3-oxo-3H-pyrido[3,2,1-kl]phenoxazine-2-carboxylic acid hydrochloride) which must be controlled. In this work a separation was pursued to adequately resolve the diastereomers and in the same chromatographic run quantitate minor impurities and degradates which can show marked differences in retention times using reversed-phase systems.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model SP-8800 ternary pump and Chromjet data handling system (Spectra-Physics, Santa Clara, CA, USA). A Model 757 variable-wavelength UV detector (ABI Analytical Kratos Division, Ramsey, NJ, USA) and a Model WISP-710B (Waters Associates, Milford, MA) autosampler were used. Chromatographic separations described in this method were made using Alltima C_{18} columns (5 μm, 100Å) measuring 15 cm x 4.6 mm I.D. (Alltech Associates, Deerfield, IL, USA). The following columns were also evaluated for their suitability: Nucleosil C_{18} 4.0 mm x 125 mm, 5 μm, Macherey-Nagel (Bodman Chemicals, Aton PA); CSC-Nucleosil C_{18}, 4.6 mm x 150 mm, 5 μm (Chromatographic Sciences Company (CSC), Resolution Systems, Wilmette, IL); Zorbax SB-C_{18}, 4.6 mm x 250 mm (Mac Mod Analytical, Chadds Ford, PA), 5 μm; Lichrosorb RP-8, 4.6 mm x 250 mm, 5 μm (Alltech); Inertsil C_{18}, 4.6 mm x 250 mm, 5 μm (Metachem Technologies, Redondo Beach, CA). Prior to use, the components of the eluent were filtered through 0.45 μm nylon membranes (Alltech).

Reagents

Acetonitrile and methanol were HPLC grade from EM Sciences (Cherry Hill, NJ, USA). Both sodium citrate dihydrate and citrate acid were reagent grade and were from Fisher Scientific (Fair Lawn, NJ, USA) and Mallincrodkt (St. Louis, MO), respectively. Disodium EDTA and dimethylsulfoxide (DMSO) were reagent grade and were from Aldrich Chemical Co.(Milwaukee, WI, USA). A citrate buffer solution containing 0.01 M citrate and 0.001 M EDTA was prepared by dissolving 2.93 g of sodium citrate·$2H_2O$, 1.96 g citric acid, and

0.75 g EDTA in 2 liters of deionized water and adjusted to pH 3.0 using perchloric acid (Fisher Scientific). All bulk drugs and related impurities were synthesized at Abbott Labs (North Chicago, IL, USA). Isolated compounds were characterized by ^{1}H, ^{13}C, ^{19}F NMR and mass spectrometry. The organic modifier was prepared by mixing 1 liter of acetonitrile and 1 liter of methanol. The diluent was prepared by mixing 1 liter of citrate buffer solution and 1 liter of organic modifier.

Chromatographic Conditions

A linear gradient was used, mixed with the ternary pump as shown in Table I. Other conditions were: flow-rate, 1.0 mL/min; pressure, approximately 1500 psi; detector, 326 nm at 0.10 a.u.f.s., attenuation at 32, and injection volume, 20 μL. All separations were performed at ambient temperature.

Analytical Procedure

Abbott-84441.1 bulk drug samples were prepared by initially dissolving approximately 50 mg of drug substance in 50 mL of DMSO. The solution was diluted with diluent to 100 mL for a sample concentration of approximately 0.5 mg/mL. A 1% standard was prepared by serially diluting the above stock solution 5 mL to 50 mL then 5 mL to 50 mL with diluent. The amounts of impurities were estimated in the sample by comparing the corresponding peak areas in the sample and standard preparations. Impurity content was calculated on the anhydrous basis by correcting the sample concentration for the amount of water contained in the drug substance. The drug substance typically contains approximately 2-4% water, as measured by Karl Fischer titration.

Table I
Linear Gradient for HPLC Eluent

Time (min)	Citrate Buffer (%)	Organic Modifier (%)
0	55	45
30	55	45
60	10	90
75	10	90
76[a]	55	45
90[a]	55	45

[a] Used to re-equilibrate the column to the initial conditions.

RESULTS AND DISCUSSION

Since Abbott-84441.1 has both acid and base functional groups, the chromatographic behavior on reverse-phase columns is dependent on pH as well as the organic modifier of the eluent. The most symmetrical peak shapes for the drug substance (diastereomers) and impurities were obtained in eluents containing aqueous buffers at pH values of approximately 2 to 4. The amount of retention of the drug substance (diastereomers) and impurities did not vary significantly with the type of buffer. The citrate system used has proven rugged in the analysis of a variety of quinolones. Single isocratic eluents failed to adequately resolve the drug from early eluting impurities while still eluting the more strongly retained impurities within a reasonable time. For this reason, a gradient elution system was developed. Single organic modifiers of tetrahydrofuran, methanol and acetonitrile were used in this approach. Again, no single modifier provided optimum resolution of the diastereomers in combination with resolution of both early and late eluting impurities. Acceptable results were obtained using the ternary solvent system described in the text where acetonitrile/methanol (1:1) was

Table II
COMPARISON OF COLUMN PACKING ON THE RESOLUTION OF ABBOTT-84441.1

Packing	Conditions Citrate Buffer/Acetonitrile	Resolution Factor[a]
Lichrosorb RP-8 4.6 mm x 250 mm, 5 μm	60/40	[b]
Inertsil C-8 4.6 mm x 250 mm, 5 μm	70/30	2.2
Zorbax SB-C-8 4.6 mm x 250 mm, 5 μm	65/35	1.6
Nucleosil C_{18} 4.0 mm x 125 mm, 5 μm (Macherey-Nagel)	70/30	4.1
Nucleosil C_{18} 44.6 mm x 150 mm, 5 μm (CSC)	65/35	2.8
Alltima C_{18} 4.6 mm x 150 mm, 5 μm	67/33	5.8[c]

[a]U. S. Pharmacopia XXII, p 1867.
[b]unacceptably Poor Peak Shape.
[c]Typical resolution factors are 7-8 for the method described in the text.

used as the modifier, thereby producing the needed resolution for early and late eluting impurities, as well as for impurities retained close to the diastereomers of Abbott-84441.1. A detection wavelength of 326 nm provides a very similar response for the impurities and drug substance, providing an accurate estimation of unknowns quantitated versus the drug substance. In this procedure EDTA was included as an additive in the eluent to minimize the adsorption of the drug to metal surfaces and to sharpen the peak shape due to trace metals present. Quinolones can form strong complexes with metals [12].

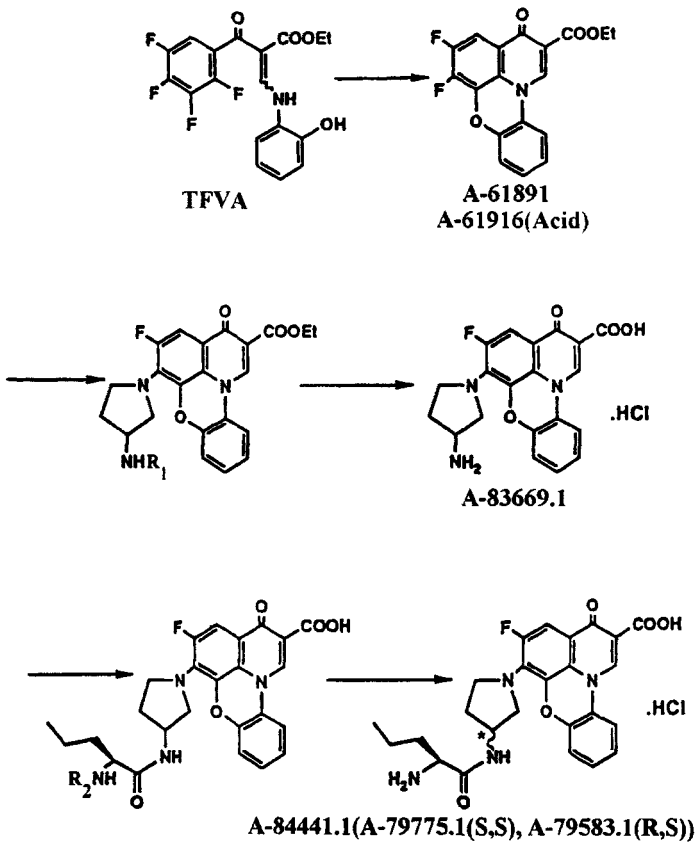

Figure 1. **Synthetic scheme for preparation of Abbott-84441.1**

Route 1: R_1=H, R_2=carbobenzoxycarbonyl (CBZ)

Route 2: R_1=R_2=t-butyloxycarbonyl (Boc)

Several C-8 and C-18 reversed-phase packings were evaluated for the determination of Abbott-84441.1 impurities. For this evaluation, similar isocratic profiles were used as described in the text. However, the initial amount of organic solvents in the starting conditions was adjusted to produce similar retention times for the diastereomers of Abbott-84441.1. The results are

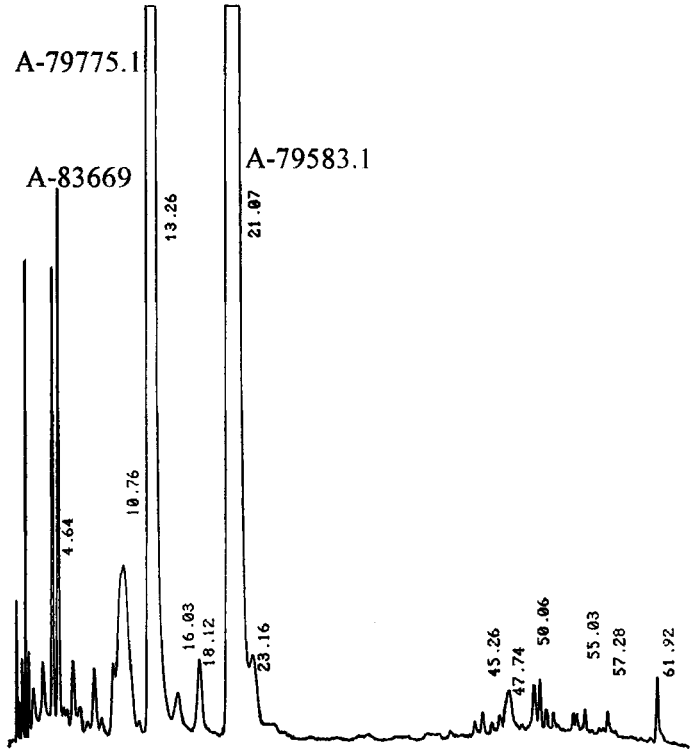

Figure 2. Representative Chromatogram of Abbott-84441.1 Prepared from Route 1

summarized in Table II. The comparision of the resolution factors calculated between the diastereomers of Abbott-84441.1, which is a critical separation in our application, demonstrates that the separation is largely a function of the type of reversed-phase column used. The system used in the procedure is also more useful than others evaluated in the resolving more impurities.

The HPLC conditions described in the text were developed to resolve the drug substance (diastereomers), manufacturing impurities and possible degradation

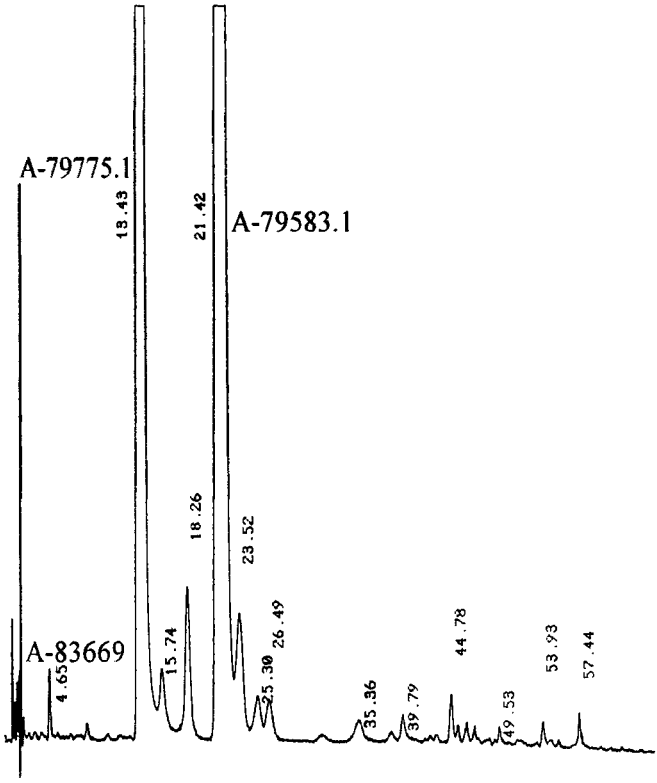

Figure 3. Representative Chromatogram of Abbott-84441.1 Prepared from Route 2

products by different synthetic routes. Shown in Figure 1 are schemes for two synthetic routes used to produce the bulk drug. The routes differ in the method of protection of the L-norvaline amino acid portion and the primary amine on the pyrrolidine ring. Different manufacturing impurities arise from the different synthetic routes.

Shown in Figures 2 and 3, are typical chromatograms of representative lots of Abbott-84441.1 prepared as illustrated. Figure 4 shows an identity

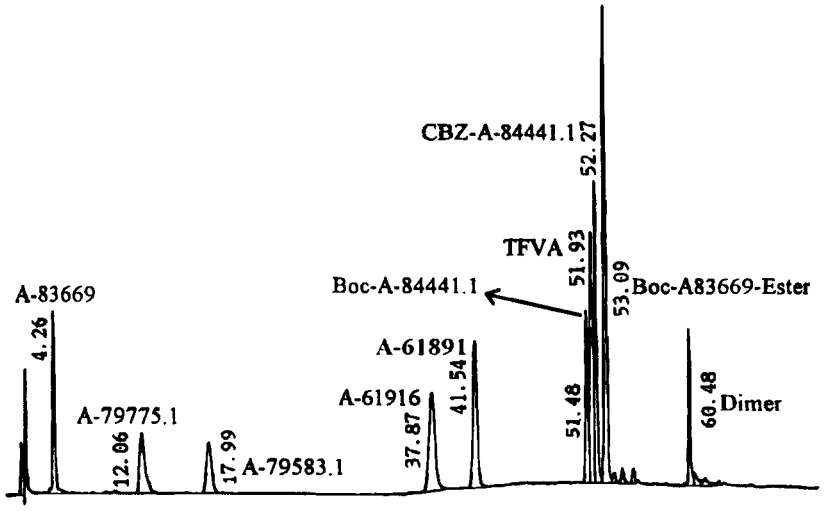

Figure 4. Synthetic Mixture of Typical Impurities Seen in Abbott-84441.1

mixture of various impurities. Comparison of the two chromatograms shows the presence of similar and dissimilar impurities arising from the two synthetic routes. The precursor A-83669 (RT = 4.5 minutes) is present in both samples, although route 1 contains significantly more. Other unknown impurities (RT= 16, 18, and 23 minutes) are formed in both routes. These appear to be isomers of Abbott-84441.1 from the limited LC-MS data obtained. Figure 2 also shows the presence of a dimerized impurity which forms by coupling across the 3-aminopyrrolidine moiety (RT= 61 minutes) and isomers at RT=10.8 minutes. These impurities were identified by LC-MS and NMR techniques. These types of impurities have been observed previously in other quinolones. N-acetal impurities can be detected at RT=30 and 35 minutes. The region between 40 and 58 minutes typically contains peaks at 0.2% or less and is the region where the protected intermediates elute (Figures 2 and 3). These components have not been identified.

Detector response for Abbott-84441.1 was linear to at least 20 μg/mL (correlation coefficient $\geq$ 0.9999). Linearity curve of concentration versus detector response essentially intersected the origin, allowing the use of one-point calibration for quantitation of impurities. Assay precision was assessed by performing the procedure on one lot of Abbott-84441.1. Two analysts performed the determinations on different days. The assay precision (relative standard deviation values) ranged from 31% to 7.3% for impurities having mean values of 0.1% to 1.3%.

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