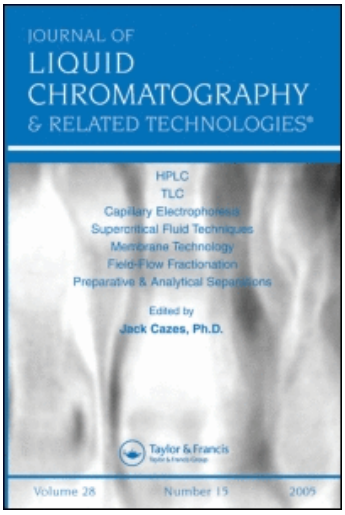

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Determination of 6,7-Difluoro-1-(2,4-difluorophenyl)-1,4-dihydro-4-oxo-3-quinilinecarboxylic Acid and Related Compounds by High Performance Liquid Chromatography

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DETERMINATION OF 6,7-DIFLUORO-1-(2,4-DIFLUOROPHENYL)-1,4-DIHYDRO-4-OXO-3-QUINILINECARBOXYLIC ACID AND RELATED COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

6,7-Difluoro-1-(2,4-difluorophenyl)-1,4-dihydro-4-oxo-3-quinilinecarboxylic acid (Abbott-72931) and related compounds are determined by high performance liquid chromatography (HPLC). A two-part assay procedure quantitates both the drug substance and the related compounds. The chromatography used is based on a Bakerbond® C-4 column with a 0.04 M citrate buffer (pH 4.4) eluent modified with THF. Detection is at 254 nm. Quantitation of Abbott-72931 is achieved using an external standard method and an Abbott-72931 reference standard. An isocratic HPLC system is used with a 75% buffer/25% THF eluent. Assay precision (RSD values) for two lots of Abbott-72931 ranged from ± 0.66% to ± 0.97%. Quantitation of related compounds in Abbott-72931 is achieved by an external standard method using the same chromatography, with gradient elution to remove the more strongly retained impurities from the column. Unhydrolyzed Abbott-72931 ethyl ester is quantitated using an Abbott-72931 ethyl ester reference standard. Other compounds are quantitated versus the Abbott-72931 reference standard.

Assay precision of related compounds determined in two lots of Abbott-72931 at levels of 0.04% to 1.0% ranged from ± 58% to ± 6.8%. Detector response for Abbott-72931 and Abbott-72931 ethyl ester is linear to at least 25.4 and 9.9 μg/mL, respectively (correlation coefficients > 0.999, essentially intersecting the origin). Recovery of known related compounds of Abbott-72931 ranged from 94.4 to 107.5% when added to the drug substance at levels of 0.2% to 2.0%.

INTRODUCTION

The use of Abbott-72931, which is chemically 6,7-difluoro-1-(2,4-difluorophenyl)-1,4-dihydro-4-oxo-3-quinilinecarboxylic acid and closely related compounds in the synthesis of a variety of fluoroquinilone antibacterials, has been described (1-3). It is necessary to control the purity of this material in order to eliminate undesired side reactions and carry over of impurities contained in the intermediate into the final bulk drug. This paper describes the use of high performance liquid chromatography (HPLC) for the quantitation of Abbott-72931 and related compounds.

HPLC was used in this work because it potentially offered the desired combination of speed, sensitivity and specificity. In addition, the technique eliminates the derivatization step necessary in gas chromatographic assays for these types of compounds (4-5). A number of HPLC procedures have been reported for fluoroquinilones. Previously reported methods have largely dealt with determining the active drug substance and metabolites (6-10). Quantitation of Abbott-72931 as an impurity in temafloxacin has been reported by this laboratory (11). The large differences in retention and chromatographic properties between Abbott-72931 and finished fluoroquinilone drugs

necessitated the development of a different separation which is more appropriate for Abbott-72931 and its related compounds.

MATERIAL AND METHODS

Reagents

Sodium citrate dihydrate and citric acid were reagent grade from Fisher. A 0.04 M citrate buffer at pH 4.4 was prepared by dissolving 12.0 g of sodium citrate dihydrate and 8.0 g of citric acid in 2 liters of deionized water. Acetonitrile and tetrahydrofuran (THF) were HPLC grade from EM Science. Abbott-72931 and Abbott-72931 ethyl ester were prepared in-house.

Liquid Chromatograph and Conditions

The liquid chromatographic system used consisted of a Spectra-Physics Model 8800 pump and a Model Spectra-100 UV detector (Spectra-Physics, San Jose, CA). A Shimadzu Model SIL-9A autosampler and Model C-R4A data system were used (Shimadzu Corporation, Kyoto, Japan). An HPLC column heater maintained at 40°C was used (Jones Chromatography, Littleton, CO). Chromatographic separations were achieved using a Bakerbond® C-4 reverse phase column measuring 4.6 mm x 250 mm (5 μm particle size), (J.T. Baker, Phillipsburg, NJ). Flow rates of 1.5 mL/min., UV detection at 254 nm and 50 μL injection volumes were used throughout this work. The HPLC eluent was 75% citrate buffer/25% THF for the Abbott-72931 assay. For the

TABLE I
Gradient Profile Used for Abbott-72931 Related Compounds

Time (min.)	% Buffer	% THF
0*	75	25
13	75	25
35	50	50
50	50	50
55**	75	25
70**	75	25

* Initial Conditions
** Used to re-equilibrate the column to initial conditions

determination of Abbott-72931 related compounds, gradient elution was used to 50% THF. The gradient profile is shown in Table I.

Abbott-72931 Assay

Standard and Sample Preparations

Approximately 15 μg/mL solutions of Abbott-72931 were prepared by dissolving 150 mg of either Abbott-72931 standard or sample in acetonitrile. The stock acetonitrile solutions were further diluted in the eluent.

Procedure

Replicate injections of standard and sample preparations were made to obtain integrated peak areas with calculated relative standard deviations of $\leq$ 2%. The Abbott-72931 content in each sample was calculated by the external standard method using the following equation:

% Abbott-72931 (anhydrous, solvent free basis) =

$$\frac{\textit{avg. peak area (spl.)}}{\textit{avg. peak area (std.)}} \times \frac{\textit{std. wt. (mg)}}{\textit{spl. wt. (mg)}} \times \textit{std. purity}^{*} \times \frac{100}{[\ 100 - \%LOD^{**}\ ]} \times 100\%$$

* House Standards with > 99% purities were used
** Loss on Drying at 60°C for two hours

Abbott-72931 Related Compounds Assay

Standard and Sample Preparation

A mixed standard solution containing 1.5 μg/mL each of Abbott-72931 and Abbott-72931 ethyl ester was prepared in buffer/THF (75:25). Each Abbott-72931 sample was prepared by dissolving 150 mg of material in 100 mL of acetonitrile, then diluting to 150 μg/mL concentration in buffer/THF (75:25).

Procedure

Replicate injections of the mixed standard preparation were made using the initial conditions shown in Table I to obtain integrated peak areas of Abbott-72931 and Abbott-72931 ethyl ester with RSD values ≤ 5%. Using the gradient elution program shown in Table I, a blank solution of buffer/THF (75:25) and each sample preparation was injected. The chromatograms were traced to 50 minutes in order to insure complete elution of the related compounds. The percent Abbott-72931 ethyl ester was calculated using the Abbott-72931 ethyl ester reference standard. Each additional related compound not detected in the blank solution was calculated using the Abbott-72931 reference standard. The following equation was used:

% Impurity (anhydrous, solvent free basis) =

$$\frac{\textit{peak area spl.}}{\textit{avg. peak area std.}} \times \frac{\textit{std. conc. mg/mL}}{\textit{spl. conc. mg/mL}} \times \textit{std. purity}^{*} \times \textit{RRF}^{**} \times \frac{100}{[100 \times \%\textit{LOD}^{***}]} \times 100\%$$

* House Standards with 99% purities were used
** RRF (Relative Response Ratios) for related compounds is the peak area response of Abbott-72931 divided by peak area response for the impurity.
*** Loss on Drying at 60°C for two hours

The relative response ratios used for determining known related compounds of Abbott-72931 are summarized in Table II.

RESULTS AND DISCUSSION

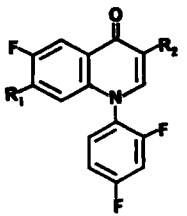
Shown in Figure 1 are typical chromatograms for the Abbott-72931 determination. Detector linearity was demonstrated by chromatographing standard solutions containing 0.53 μg/mL to 25.4 μg/mL of Abbott-72931. The plot of Abbott-72931 concentration versus integrated peak area is shown in Figure 2. The linear regression data for this plot is included in Figure 2. These data show that the plot is linear (correlation coefficient > 0.9999) and essentially intersects the origin (y-intercept = -210 counts).

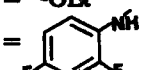
To determine the assay precision, two lots of Abbott-72931 were determined on separate days by different analysts. As shown by the data in Table III, the assay precisions (RSD values) were ± 0.66% and ± 0.97%.

Figure 3 is a chromatogram of a synthetic mixture of Abbott-72931 and known related compounds. Peak identities in Abbott-72931 samples can be made by comparing relative retention times for related compounds with

TABLE II

Relative Response Ratios of Known Abbott-72931 Related Compounds



	R_1	R_2	Name	Relative Retention Time	RRF*
1 =	-OH	$-CO_2H$	7-hydroxy-substituted Abbott-72931	0.63	0.826
2 =	-OEt	$-CO_2Et$	7-ethoxy-substituted Abbott-72931 ethyl ester	0.71	0.768
3 =	-F	$-CO_2Et$	Abbott-72931 ethyl ester	0.74	-------
4 =	-F	$-CO_2H$	Abbott-72931	1.0	1.0
5 =	-OEt	$-CO_2H$	7-ethoxy-substituted Abbott-72931	1.2	0.637
6 =		$-CO_2H$	7-(2,4-difluoroanilino)-substituted Abbott-72931	2.0	3.48
7 =	------	--------	Abbott-72931 ethyl ester Precursor	2.7	2.38

** For unknown impurities, use RRF = 1.0.

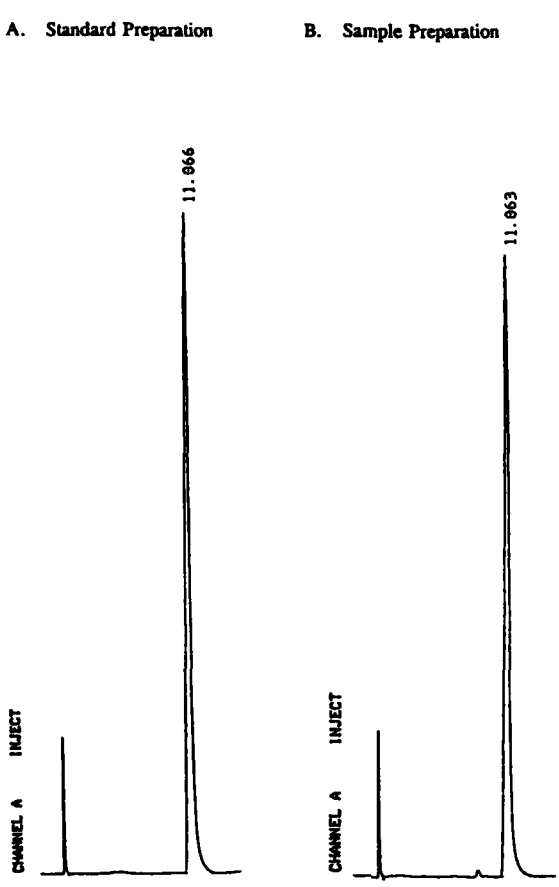


Figure 1. Typical Chromatograms for Abbott-72931 Assay (HPLC Conditions Stated in Text)

those from a synthetic mixture. To accurately estimate the concentration of known related compounds, differences in response between Abbott-72931 and related compounds are corrected by applying relative response factors. These values were obtained by ratioing the slopes for calibration curves of Abbott-72931 to those of the individual related compounds. Four-point curves at concentrations of approximately 0.20 μg/mL to 2.0 μg/mL were used.

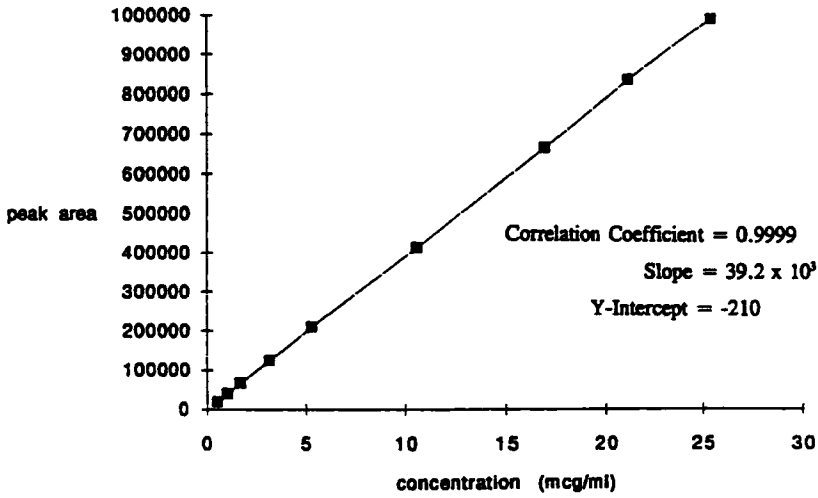


Figure 2. Linearity Curve for Abbott-72931 Assay

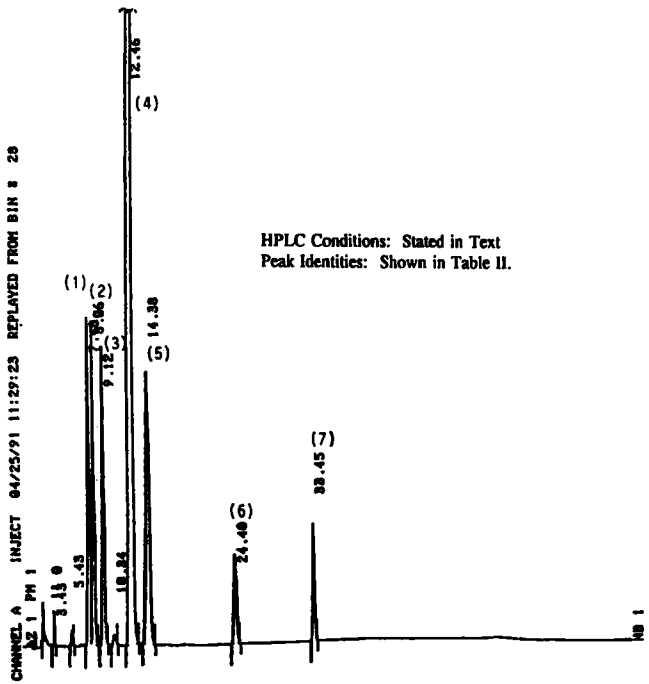


Figure 3. Chromatogram of a Synthetic Mixture of Abbott-72931 and Known Impurities

Table III
Abbott-72931 Assay Precision Data

Lot 1			Lot 2		
Day	Analyst	Wt.(%)	Day	Analyst	Wt.(%)
1	1	98.9	1	1	98.7
1	1	97.7	1	1	97.7
1	1	97.8	1	1	97.1
2	2	96.4	2	2	97.2
2	2	98.9	2	2	96.8
2	2	97.4	2	2	97.6
	Mean	97.9	2	2	98.1
	SD	± 0.95		Mean	97.6
	RSD	± 0.97%		SD	± 0.65
				RSD	± 0.66%

Shown in Figure 4 are typical chromatograms for the standard and sample preparations. Detector linearity for Abbott-72931 ethyl ester was demonstrated by chromatographing standard solutions containing 0.59 μg/mL to 9.91 μg/mL of analyte. The plot of Abbott-72931 ethyl ester concentration versus integrated peak area is shown in Figure 5. The plot is linear (correlation coefficient > 0.9999) and essentially intersects the origin (y-intercept = 212 counts).

Assay precision was determined by quantitating related compounds in two lots of Abbott-72931. Two analysts performed the determinations on separate days. The precision data are presented in Tables IV and V. As shown, relative standard deviations ranged from ± 6.8% to ± 58% for individual compounds present at the 1.0 to 0.04% levels.

Standard addition and recovery experiments were performed for related compounds of Abbott-72931. Authentic impurity samples were used to

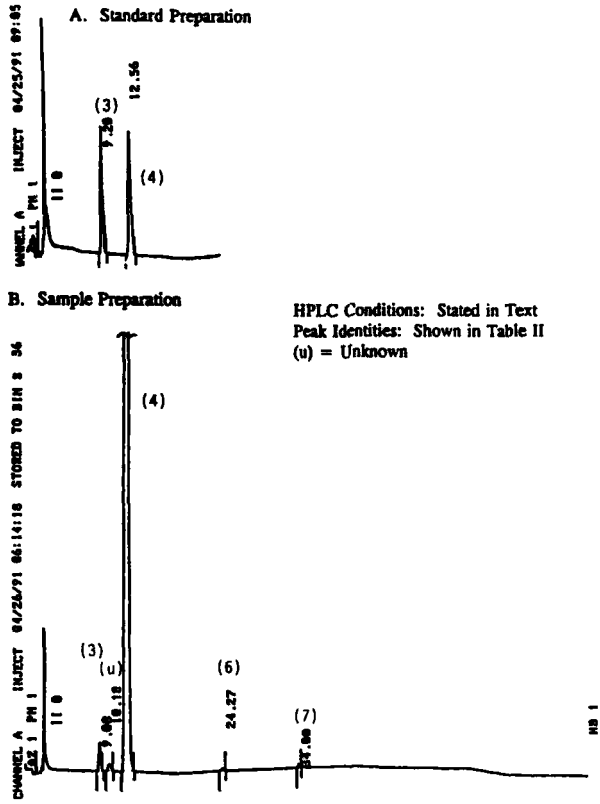


Figure 4. Typical Chromatograms for Abbott-72931 Impurity Determination

spike the Abbott-72931 sample preparation. Known additions were made at approximately 0.2 to 2.0% levels. Recoveries were calculated versus authentic impurity standard preparations. These data are presented in Table VI. As shown, recoveries ranged from 94.4 to 107.5%, which is quantitative within the precision of the method.

TABLE IV
Precision Data for Determinations of Related Compounds in Abbott-72931, Lot 1

Day	Analyst	Unknown 1	Abbott-72931 Ethyl Ester	Unknown 2	7-(2,4-difluoro-anilino)-substituted Abbott-72931	Precursor to Abbott-72931 Ethyl Ester
1	1	0.02	0.93	0.12	0.10	0.33
1	1	0.04	1.23	0.16	0.31	0.45
1	1	-----	0.86	0.06	0.21	0.33
1	1	0.03	0.82	0.11	0.21	0.31
1	1	0.01	0.84	0.07	-----	0.31
2	2	0.06	0.97	0.13	0.24	0.40
2	2	0.07	1.04	0.14	0.24	0.14
2	2	0.06	1.09	0.16	0.35	0.43
	Mean =	0.04	0.97	0.12	0.24	0.34
	SD =	± 0.023	± 0.14	± 0.038	± 0.08	± 0.09
	RSD =	± 58%	± 14%	± 32%	± 32%	± 28%

TABLE V
Precision Data for Determinations of Related Compounds in Abbott-72931, Lot 2

Day	Analyst	Abbott-72931 Ethyl Ester	Unknown 2	7-(2,4-difluoro-anilino)-substituted Abbott-72931	Precursor to Abbott-72931 Ethyl Ester
1	1	0.89	0.27	0.28	0.19
1	1	0.94	0.27	0.31	0.19
1	1	0.93	0.26	0.24	0.17
2	2	1.11	0.23	0.28	0.21
2	2	1.11	0.24	0.31	0.21
2	2	1.12	0.24	0.28	0.24
	Mean =	1.0	0.25	0.28	0.20
	SD =	± 0.11	± 0.017	± 0.026	± 0.021
	RSD =	± 11%	± 6.8%	± 9.3%	± 10%

TABLE VI
Standard Addition and Recovery Data for Abbott-72931 Related Compounds

	0.2% LEVEL			0.5% LEVEL			1.0% LEVEL			2.0% LEVEL		
IMPURITY*	µg/mL Added	µg/mL Rec	% Recovery	µg/mL Added	µg/mL Rec	% Recovery	µg/mL Added	µg/mL Rec	% Recovery	µg/mL Added	µg/mL Rec	% Recovery
1	0.398	0.394	99.0	0.795	0.750	94.4	1.59	1.56	98.2	3.18	3.11	97.7
2	0.358	0.349	97.4	0.715	0.753	105.3	1.43	1.45	101.6	2.86	2.90	101.6
3	0.402	0.400	99.5	0.805	0.809	100.5	1.61	1.61	100.2	3.22	3.24	100.6
5	0.348	0.355	102.0	0.695	0.728	104.7	1.39	1.44	103.8	2.78	2.80	100.6
6	0.370	0.350	94.5	0.740	0.702	94.9	1.48	1.42	95.7	2.96	3.18	107.5
7	0.410	0.416	101.5	0.820	0.777	94.8	1.64	1.59	96.9	3.28	3.22	98.3

*Impurity structures shown in Table II.

1 = 7-hydroxy-substituted-Abbott-72931
2 = 7-ethoxy-substituted-Abbott-72931 Ethyl Ester
3 = Abbott-72931 Ethyl Ester
5 = 7-ethoxy-substituted-Abbott-72931
6 = 7-(2,4-difluoroanilino)-substituted-Abbott-72931
7 = Ring opened precursor to (3)

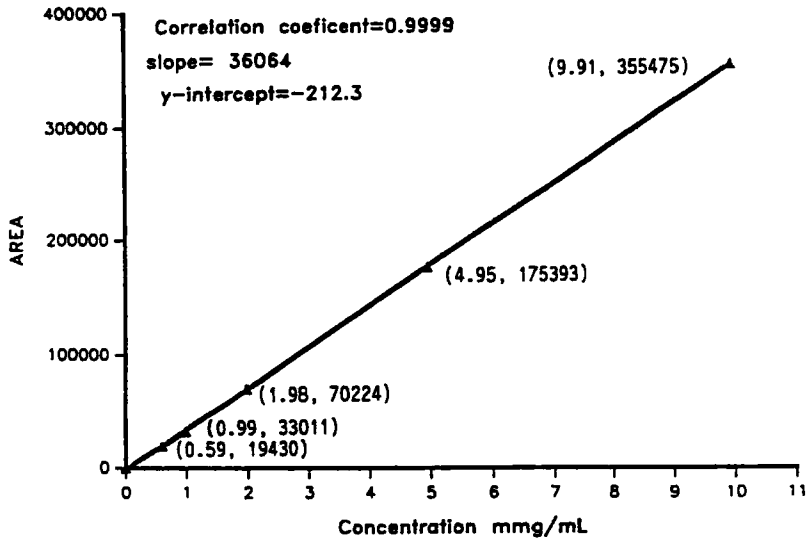


Figure 5. Linearity Curve for Abbott-72931 Ethyl Ester

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