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Quantitative analysis of *Z*-2-[4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy]ethanol in human plasma using high-performance liquid chromatography

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Abstract

A highly sensitive and precise high-performance liquid chromatography (HPLC) assay was developed and validated for the quantitation of *Z*-2-[4-(4-chloro-1,2-diphenyl-but-1-enyl) phenoxy]ethanol (FC-1271a) in human plasma. Plasma samples (1.0 ml) containing FC-1271a and internal standard (toremifene citrate; Fareston®) were extracted using a 2% 1-butanol, 98% hexane solution with an extraction efficiency of >97%. Samples were reconstituted in methanol, irradiated with high intensity ultraviolet light (254 nm) for 1 min, and injected onto a C_{18} reverse phase column. Samples were eluted isocratically at a flow-rate of 0.5 ml/min with a mobile phase consisting of 6.5% water and 0.5% triethylamine in methanol. The fluorescence of photochemically activated compounds was detected using a fluorometer set at an excitation wavelength of 266 nm and emission wavelength of 370 nm. Under these assay conditions, standard calibration curves were linear through a concentration range of 10–400 ng/ml. In summary, we have developed and validated an HPLC assay to quantitate FC-1271a in human plasma.

Keywords: *Z*-2-[4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy]ethanol; Triphenylethylene; FC-1271a

1. Introduction

A new triphenylethylene (FC-1271a; *Z*-2-[4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy]ethanol), structurally similar to tamoxifen (TAM) and toremifene (TOR) which is devoid of significant estrogenic and antiestrogenic activity [1], is currently being developed for the treatment and prevention of osteoporosis. This compound is a known metabolite of TOR which has been detected in the plasma of humans and non-human primates. FC-1271a has been shown to have positive effects on preventing bone loss in rats while having no estrogenic effects on the uterus. In addition, this compound has positive benefits on serum cholesterol and is devoid of acute and chronic toxicity in non-human primates [2]. Currently there are no published assays for the quantitation of FC-1271a in biological fluids. Because the serum concentrations of FC-1271a seen in recently completed phase I/II clinical trials were generally below the limit of UV detection, we

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developed and validated the current fluorescence-based HPLC assay for the accurate determination of FC-1271a in human plasma.

2. Experimental

2.1. Chemicals and reagents

FC-1271a (*Z*-2-[4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy]ethanol; M_r=379) and toremifene citrate (internal standard; M_r=598.14) were provided by the Orion Corporation, Orion-Pharma, Finland. All reagents (hexane, methanol, butanol, water) were purchased from Fisher Scientific (Pittsburgh, PA), and were of HPLC grade. Triethylamine was obtained through Sigma Chemical Company (St. Louis, MO).

2.2. Chromatographic conditions

The HPLC system was similar to that previously used for TOR [3] and TAM [4] and consisted of a Beckman (Fullerton, CA) Model 320 gradient liquid chromatograph, a Model 420 controller, and two Model 110A pumps. Important differences between the assay systems developed for TAM and TOR and the current system include changes in the mobile phase composition to accommodate the more hydrophilic FC-1271a molecule, a slower flow-rate, and the use of a different internal standard, in addition to other minor differences. The system was equipped with an Applied Biosystems (San Jose, CA) 5-μm reverse phase C18 ODS 4.6×250 mm column, 100-μl injection loop, and a Rheodyne injector. The column was maintained at room temperature for all analyses. Photochemically activated FC-1271a and internal standard were detected with an Applied Biosystems model 980 programmable fluorescence detector set at an excitation wavelength of 266 nm and an emission wavelength of 370 nm. A Spectra-Physics (Santa Clara, CA) 4100 integrator was used for recording retention times and peak heights. The mobile phase consisted of 6.5% H_2O, 0.5% triethylamine, and 93% methanol (v/v) and was degassed under vacuum for 20 min daily prior to column equilibration. Before sample analysis each day, the column was equilibrated for 45 min at a flow-rate of 0.5 ml/min. Upon completion of daily sample analyses, the column was washed with 100% methanol for 30 min at a flow-rate of 1.0 ml/min and then overnight at 0.2 ml/min.

2.3. Standard solutions

FC-1271a standard stock solutions were prepared in 100% methanol at concentrations of 100, 250, 500, 1000, 2000, and 4000 ng FC-1271a free base per milliliter. The internal standard, TOR citrate, was also added to achieve a final concentration of 2000 ng free base/ml. All standard solutions were stored at −20°C in the dark for a maximum of 90 days. Standard stock solutions were found to be stable under these conditions for at least 3 months.

2.4. Sample preparation, extraction, and analysis

Before preparation of FC-1271a plasma standards, stock human plasma (obtained from a local blood bank) was centrifuged for 10 min at 1000 *g* and then gauze filtered. A 5.0 ml amount of each standard stock solution was then added to 45 ml of plasma in a 50-ml volumetric flask yielding final FC-1271a concentrations of 10, 25, 50, 100, 200, and 400 ng/ml. One-milliliter aliquots were then placed in 16×125 mm screw-cap borosilicate glass tubes (Corning Inc., Corning, NY) and stored at −20°C in the dark until the day of extraction/analysis.

All extractions were performed essentially as described previously [3–5]. On the day of sample analysis, 9.0 ml of extraction fluid (2% butanol, 98% hexane; v/v) was added to each 1.0 ml plasma standard. Samples were then vortexed for 30 s and centrifuged at 1000 *g* for 10 min. The organic phase was transferred to a clean 16×125 mm glass tube and evaporated to dryness under a gentle stream of speciality grade nitrogen gas in an analytical nitrogen evaporator at 37°C. This method of liquid phase extraction is simple and provides excellent recovery of FC-1271a (>97%) and internal standard (>88%), which is similar to what we observed with a more complex solid-phase extraction. Each sample was reconstituted with 200 μl methanol, filtered with a syringe filter (0.2 μm, 4 mm, Alltech Assoc. Inc., IL), and placed in a quartz cuvette (Fisher Scientific). The sample was then placed in a lucite box partially

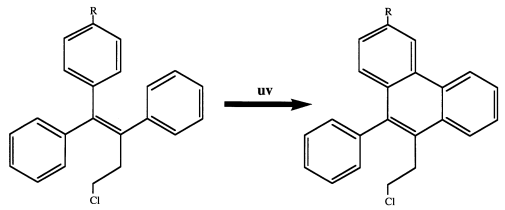

Fig. 1. Activation reaction sequence of FC-1271a and internal standard to their fluorescent phenanthrene ring structures. FC-1271a: R=OCH_2CH_2OH. Internal standard (TOR): R=$OCH_2CH_2N(CH_3)_2$.

lined with aluminum foil and activated to its fluorescent phenanthrene structure at room temperature (see Fig. 1) by irradiation for 1 min with a 15 W mercury vapor lamp (peak wavelength 254 nm, General Electric No. G15T8). After removal from the cuvette, 100 μl of the sample was then injected onto the column. Samples were eluted isocratically at a flow-rate of 0.5 ml/min and retention times and peak heights were recorded. Peak height ratios of FC-1271a to internal standard were calculated. Linear regression analysis of the data yielded the slope, *y*-intercept, and correlation coefficient of each standard curve.

2.5. Validation tests

2.5.1. Initial validation

Two complete FC-1271a standard curves were extracted from plasma as described on each of five consecutive days. One set of standards was analyzed in the morning and another set in the afternoon each day. All samples were assayed in duplicate. The resulting four peak height ratio values for each concentration each day were averaged, with the averages then being used to calculate the regression line.

2.5.2. Precision

Within-run and between-run imprecision for 10 ng/ml, 100 ng/ml, and 400 ng/ml standard concentrations were determined by HPLC analysis of four samples of each concentration on five consecutive days. Within-run imprecision, pooled within-run imprecision, and between-run imprecision for each daily run were calculated as described in Ref. [6].

2.5.3. System specificity and suitability

Retention time, peak resolution, column efficiency, and peak asymmetry calculations were made each day for five consecutive days from the 200 ng/ml plasma standards analyzed during the initial validation. System reproducibility was determined by calculating the coefficient of variation for the peak height ratios of six 200 ng/ml FC-1271a plasma standards. To rule out possible interference from endogenous compounds, 1-ml samples were taken from each batch of human plasma used and extracted and analyzed as described in Section 2.4.

The four retention times recorded for FC-1271a and internal standard each day for five consecutive days were averaged and the standard deviation and range calculated. Peak resolution (R) was calculated as follows: $R=2(t_{R2}-t_{R1})/1.699(W_1+W_2)$, where t_{R2} and t_{R1}=retention times (mm) for TOR and FC-1271a, respectively, and W_1 and W_2=peak widths at half-height (mm) for FC-1271a and TOR, respectively. The column efficiency was evaluated as the number of theoretical plates (n) of the column. The following equation was used to calculate column efficiency: $n=5.54\ (t_R/W_{1/2})^2$ where t_R=retention time (mm) and $W_{1/2}$=peak width at half height (mm). Peak asymmetry was calculated using the following equation: Asymmetry Factor (AF)=$W_{0.05}/2A$, where $W_{0.05}$=peak width (mm) measured at 5% peak height, and A=distance (mm) between perpendicular dropped from the peak maximum and the leading edge of the peak at 5% peak height.

2.5.4. Extraction efficiency

The extraction efficiency of FC-1271a was determined by HPLC. A low concentration (50 ng/ml) and a high concentration (400 ng/ml) of FC-1271a were added to plasma and extracted as described in Section 2.4. For 100% values, the same amount of drug was directly added to glass tubes. Following extraction and centrifugation, the organic phase was carefully removed from the plasma samples to ensure recovery of the entire volume. Peak heights obtained from stock solutions versus those obtained after extraction from plasma were then compared to obtain extraction efficiency values ($n=4$).

2.5.5. Accuracy

The accuracy of the method was determined by preparing and analyzing ten plasma standards each of a low concentration (25 ng/ml), an intermediate concentration (100 ng/ml), and a high concentration (400 ng/ml) as described above in Section 2.4. All samples were analyzed on the day of extraction. A new standard curve was run for each day of accuracy assessment. Peak height ratios were used to estimate the test concentrations of FC-1271a in each specimen based on the standard curve. Accuracy was calculated by averaging the ten found concentrations in each group, subtracting the nominal concentration, and then dividing the difference by the nominal concentration.

2.6. Stability study

Two separate plasma samples (50 ml) were spiked to contain a low concentration (100 ng/ml) and a high concentration (300 ng/ml) of FC-1271a. Toremifene (internal standard) was also added to each to achieve a final concentration of 200 ng/ml. Toremifene is known to be stable at the conditions specified in the stability study protocol [3]. For each concentration, 1.0 ml of stock plasma was pipetted into each of 50 16×125 mm glass extraction tubes. On the same day (day 0), peak height ratios were determined for FC-1271a as controls. Ten tubes of each concentration were then stored at 4°C, room temperature (RT), and 37°C. The remaining tubes were immediately stored at −20°C for analysis after 1 and 3 months to determine long-term stability.

After 1, 3, and 7 days of incubation, three tubes from each of the 4°C, RT, and 37°C storage temperatures were assayed (total of 18 tubes per day) according to the procedure outlined in Section 2.4. After 30 and 90 days of storage (incubation) at −20°C, three tubes from the high (300 ng/ml) and low (100 ng/ml) concentrations were analyzed to determine long-term stability of this compound.

Peak height ratios were calculated for each sample, and then averaged for each concentration at each condition and length of time. The ratios of day 1, 3, 7, 30, and 90 to the day 0 values were calculated for each compound.

2.6.1. Freeze/thaw stability

To assess the stability of FC-1271a under thawing and refreezing conditions, four cycles of freezing and thawing were conducted using a low plasma standard concentration (25 ng/ml), an intermediate concentration (100 ng/ml), and a high concentration (400 ng/ml). Samples were frozen at −20°C and thawed at room temperature. Five samples of each concentration were extracted and analyzed according to the method after each cycle. The internal standard was added immediately prior to extraction. The FC-1271a concentrations in the test samples were determined using a fresh standard curve each day of the analysis. All specimens within each cycle were analyzed the same day. The found concentrations were averaged and then compared to the nominal concentrations.

2.7. Quantitative analysis of FC-1271a in a non-human primate

As an example of how the previously described assay can be used to determine FC-1271a concentrations in biological study specimens, we utilized this method in a preclinical pharmacokinetic study in rhesus macaque monkeys at the California Regional Primate Research Center. Monkeys were given single oral doses of either 60 or 600 mg FC-1271a. In addition to multiple blood draws, liver punch biopsies were taken 48 h post-dosing. The liver biopsies and plasma specimens were extracted and analyzed by HPLC according to the method described above. During extraction, the liver biopsies were weighed, placed in polypropylene 16×100 mm tubes, spiked with internal standard, and homogenized prior to addition of extraction fluid.

3. Results

The chemical structure of FC-1271a and its metabolites are shown in Fig. 2. FC-1271a, itself a metabolite of another triphenylethylene (toremifene; Fareston®), and the metabolites shown have all previously been isolated from human plasma [3]. Two of these, the carboxylated and hydroxylated

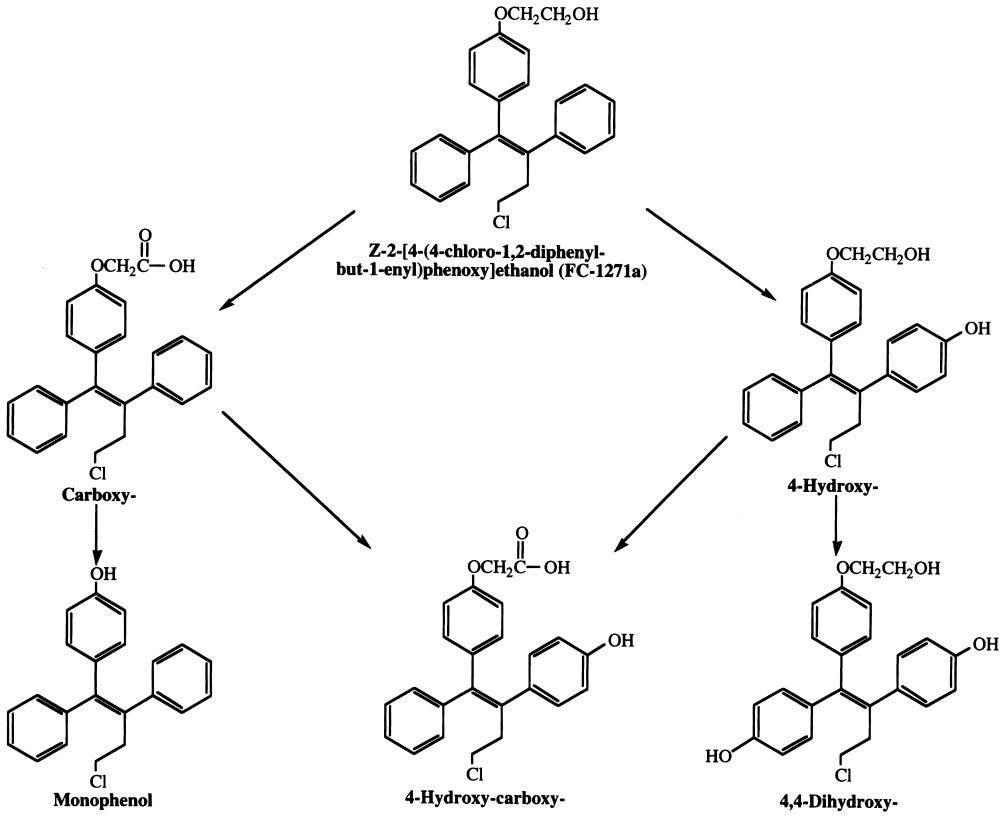

Fig. 2. Chemical structures of FC-1271a and its metabolites.

forms, were detected in rhesus macaque tissue specimens from a preclinical pharmacokinetic study (see below).

3.1. Validation tests

3.1.1. Initial validation

Results from the initial validation study are summarized in Table 1. The FC-1271a standards were linear over a wide range of concentrations (10–400 ng/ml). The average (± standard deviation) *y*-intercept, slope, and correlation coefficient were −1.75±4.23 ng/ml (n=5), 161.07±6.67 ng/ml (n=5), and 0.999±0.001 (n=5), respectively. The limit of sensitivity, or lower limit of quantitation, defined as a 2:1 signal-to-noise ratio, for this drug under the conditions used in the validation study was found to be 5 ng/ml.

Table 1
Summary of linear regression data from initial validation of FC-1271a HPLC assay[a]

Parameters	Day 1	Day 2	Day 3	Day 4	Day 5
Slope	159.72	152.46	169.49	157.96	165.71
y-Intercept	−3.00	−0.88	−6.49	4.92	−3.31
Correlation Coefficient (r)	>0.99	>0.99	>0.99	>0.99	>0.99

[a] Standard curves were calculated each day for 5 days using the average of four peak height ratios per concentration.

3.1.2. Precision

The assay precision data from analysis of 10, 100, and 400 ng/ml FC-1271a plasma standards are summarized in Table 2. Pooled within-run imprecision values [6] for these standard concentrations were 0.004, 0.026, and 0.087, respectively. Between-run imprecision values [6] were 0.001, 0.039, and 0.161, respectively.

3.1.3. System specificity and suitability

The mean retention times (±SD) for FC-1271a and the internal standard were 4.93±0.04 (n=20) and 9.86±0.10 min (n=20), respectively. Peak resolution values, calculated as described in Section 2.5.3, were 3.5, 3.6, 3.5, 3.5, and 3.4 for the 5 days of the initial validation, giving an average (±SD) of 3.5±0.07. Column efficiency values (n theoretical plates) for FC-1271a were 321, 348, 331, 264, and 294, for an average (±SD) of 312±33. For the internal standard, these values were 1453, 2121, 1724, 1697, and 2328, giving an average (±SD) of 1865±353. Peak asymmetry values (AF) for FC-1271a were 1.27, 1.28, 1.29, 1.64, and 1.21, and for the internal standard they were 1.21, 1.12, 1.16, 1.14, and 1.00. This yields average peak asymmetry values (±SD) of 1.34±0.17 and 1.13±0.08. Analyses of human plasma blanks revealed no detectable interference from endogenous compounds.

To assess system reproducibility, the coefficient of variation was calculated from the peak height ratios of six injections of an intermediate standard concentration (200 ng/ml). The six ratios were 1.29, 1.42, 1.32, 1.37, 1.38, and 1.33, giving an average (±SD) of 1.35±0.05. This data yielded a coefficient of variation (SD/mean) of 3.48%.

Table 2
Summary of within-run imprecision data for FC-1271a HPLC assay precision study[a]

Concentration	Day 1	Day 2	Day 3	Day 4	Day 5
10 ng/ml					
Mean	0.080	0.080	0.083	0.077	0.081
S_R[b]	0.004	0.005	0.002	0.003	0.004
100 ng/ml					
Mean	0.622	0.673	0.719	0.618	0.665
S_R	0.010	0.038	0.035	0.019	0.009
400 ng/ml					
Mean	2.465	2.650	2.892	2.544	2.537
S_R	0.062	0.142	0.032	0.120	0.059

[a] Means and run standard deviations were calculated each day for five consecutive days from the peak height ratios of four repetitions of 10, 100, and 400 ng/ml standard concentrations.
[b] S_R=standard deviation of run.

3.1.4. Extraction efficiency

The extraction efficiency of FC-1271a was found to be >97% (n=4) using the extraction procedure outlined above. The extraction efficiency of the internal standard (TOR) under these conditions was previously determined to be approximately 88% when the assay was validated for the analysis of TOR [3].

3.1.5. Accuracy

The accuracy of the assay method was assessed by analyzing ten standards each of the 25, 100, and 400 ng/ml concentrations. The found concentrations and accuracy data are summarized in Table 3. As shown in Table 3, the assay is highly accurate at low and intermediate concentrations. At the highest concentration tested (400 ng/ml), accuracy was still within the pre-set limit of 5%.

3.2. Stability

The results from the stability study show that FC-1271a is a very stable compound. After 7 days at room temperature in plasma, the average peak height ratios (±SD; n=3) for the 100 and 300 ng/ml concentrations were 126.5±4.2 and 119.9±2.7% of control, respectively. Following 1 week of incubation at 4°C, the average peak height ratios for these concentrations were 123.0±0.9 and 107.4±0.7% of control, respectively. Incubation for 7 days at 37°C resulted in peak height ratios of 115.6±8.0 and 111.6±10.6% of control for the low and high concentrations, respectively. Long-term stability results showed that after 30 and 90 days of storage at −20°C, the average FC-1271a peak height ratios were 93.5±5.3 and 106.0±7.6% of control, respectively, for the 100 ng/ml concentration. For the higher concentration, these values were 97.7±1.9 and 115.1±5.9% of control, respectively. Although these percentages are high, the greatest amount of error was observed in those groups which had

Table 3
Summary of FC-1271a HPLC assay accuracy data[a]

Sample #	Found concentrations (ng/ml)		
	Nominal concentration 25 ng/ml	Nominal concentration 100 ng/ml	Nominal concentration 400 ng/ml
1	26.8	97.8	451.3
2	23.5	100.5	398.4
3	22.6	95.9	371.6
4	26.0	103.2	364.8
5	23.5	98.4	360.0
6	27.9	103.3	368.5
7	25.1	98.8	352.4
8	22.9	99.2	387.0
9	22.0	100.2	366.4
10	26.0	97.9	388.1
Mean	24.6	99.5	380.9
SD	2.0	2.4	28.5
Accuracy (%)	−1.5	−0.5	−4.8

[a] Found concentrations were calculated using standard curves prepared on the day of analysis. (n=10).

experienced the most sample evaporation (7 days at room temperature and 37°C, and to a lesser extent 30 and 90 days at −20°C). The group with the least amount of evaporation (7 days at 4°C) showed the smallest amount of error. The controls, analyzed on day 0, experienced no evaporation. In addition, as was observed during the assay precision studies, inter-assay variation can result in small day to day changes in peak height ratio. Most importantly, none of the stability study chromatograms revealed any detectable peaks from potential breakdown products.

3.2.1. Freeze/thaw stability

The stability of FC-1271a in human plasma was also examined following four cycles of thawing and refreezing. Table 4 shows the average found concentrations in these specimens compared to the nominal concentrations. Based on these results, we can conclude that this drug is also fairly stable under conditions of thawing and refreezing, although some variability was seen in the third cycle at the 100 and 400 ng/ml concentrations. The average found concentrations at these levels in the third cycle were somewhat lower than those observed in the accuracy study, but these differences were not seen after the fourth freeze/thaw.

3.3. Photoactivation optimization

The optimum photoactivation time was determined to be 1 min by experimenting with various time exposures to high intensity UV light. Exposure times less than 1 min produced insufficient activation, while longer exposures produced excessive chemical breakdown as determined by the appearance of additional peaks. Activation is most efficient in

Table 4
FC-1271a stability following one to four cycles of freezing and thawing at −20°C and room temperature[a]

Number of freeze/thaws	Found concentrations (ng/ml)		
	Nominal concentration 25 ng/ml	Nominal concentration 100 ng/ml	Nominal concentration 400 ng/ml
1	23.5±1.4	98.2±6.9	430.5±29.3
2	24.2±1.8	100.4±7.9	400.1±61.1
3	24.1±1.5	91.7±3.4	356.3±10.2
4	29.3±2.2	100.3±6.0	392.0±45.6

[a] Found concentrations (mean±SD) were calculated using standard curves prepared on the day of analysis (n=5).

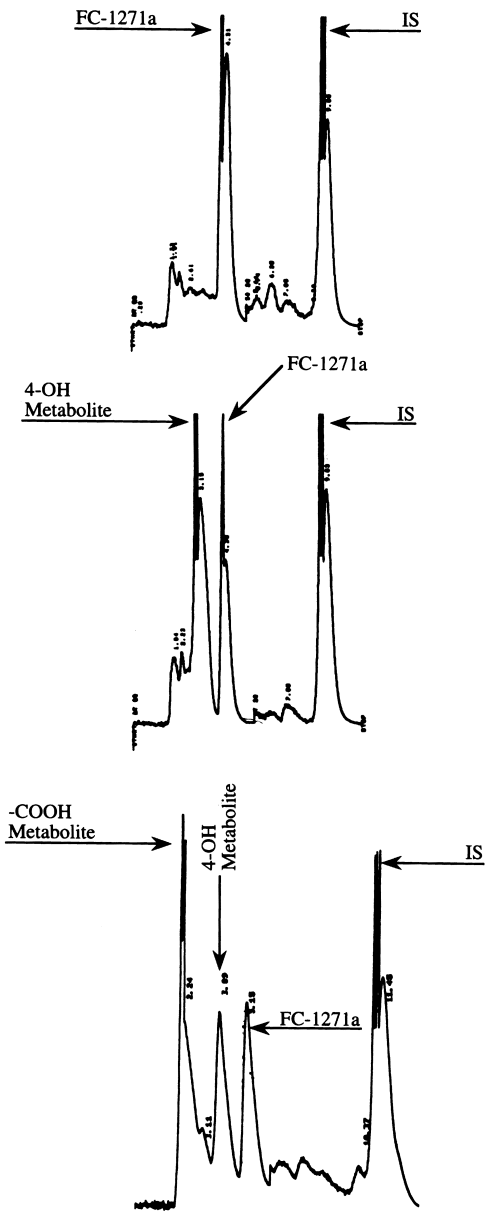

Fig. 3. Chromatograms of a 50 ng/ml FC-1271a plasma standard (top), monkey plasma specimen (center), and monkey liver specimen (bottom) following HPLC analysis. All samples were analyzed at a flow-rate of 0.5 ml/min using a mobile phase consisting of 6.5% water and 0.5% triethylamine in methanol. The liver specimen was assayed using a system equipped with a different column and integrator, thus the slight differences in retention time. All other assay conditions were the same. Retention times are 4.31 and 9.00 min (top), 3.15, 4.30, and 9.03 min (middle), and 2.24, 3.89, 5.15, and 11.45 min (bottom).

methanol and least efficient in aqueous solutions. Although photochemically activated FC-1271a has excellent stability for at least several days at −20°C, all specimens in the validation study were immediately analyzed following activation. This model of fluorescence detection following pre-column photoactivation is simple, rapid, and provides much greater sensitivity than the UV absorbence based assay which we have also developed for this compound.

3.4. Quantitative analysis of FC-1271a in a non-human primate

Fig. 3 shows representative chromatograms of an FC-1271a plasma standard (50 ng/ml), and macaque plasma and liver specimens following analysis using the assay described above. The macaque was given a single, 600-mg oral dose of FC-1271a. Quantitation of these specimens revealed estimated concentrations of 51 ng/ml in the macaque plasma and an estimated concentration of 3.287 μg/g in the macaque liver. As seen in Fig. 3, two metabolites of FC-1271a, determined to be the hydroxylated and carboxylated forms based on comigration patterns, were detected in these specimens. The presence of the carboxy metabolite was interesting, although it was poorly retained in the column. In future, this assay will be refined and revalidated in human plasma to include these two metabolites.

4. Conclusion

In the present study, we developed and validated an HPLC assay for the analytical measurement of FC-1271a in human plasma following pre-column fluorescent activation. It is simple, rapid, highly precise, reproducible, and it displays linearity through a wide range of concentrations. This assay is sensitive to 5 ng/ml plasma and, if necessary, the sensitivity can be further reduced through modification of the chromatographic conditions. Other chromatographic methods which could be used for analyzing FC-1271a include post-column activation, such as that described by Brown et al. for tamoxifen [7], and UV absorbence. However, post-column fluorescent activation is more technically complex than our method, and UV absorbence based systems

do not provide the sensitivity required for the reproducible quantitation of clinically relevant concentrations (<1.0 μg/ml). This method of quantitation of FC-1271a in human plasma will allow for further investigation correlating drug pharmacokinetics to biological activity of this drug in future preclinical and clinical studies.

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