ELSEVIER

Journal of Chromatography B, 704 (1997) 231–242

JOURNAL OF CHROMATOGRAPHY B

Determination of Ro 48-3656 in rat plasma by reversed-phase high-performance liquid chromatography Comparison of 1.5-μm nonporous silica to 3.5-μm porous silica analytical columns

Brian D. Paasch[a,*], Yvonne S. Lin[b], Seth Porter[a], Nishit B. Modi[b], Timothy J. Barder[c]

[a]*BioAnalytical Methods Development, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA*
[b]*Pharmacokinetics and Metabolism, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA*
[c]*MICRA Scientific, Inc., 1955 Techny Road, Suite 1, Northbrook, IL 60062, USA*

Received 4 April 1997; received in revised form 23 July 1997; accepted 21 August 1997

Abstract

We describe a method for measuring Ro 48-3656 in EDTA rat plasma by neutral pH, reversed-phase high-performance liquid chromatography using a 1.5-μm nonporous silica, C_{18} analytical column and UV absorbance detection to support pharmacokinetic studies. We also describe a comparison of the 1.5-μm nonporous silica C_{18} column versus 3.5-μm porous silica C_{18} columns. The final method using the 1.5-μm nonporous silica column demonstrated good precision (of both quantification and retention time), accuracy and recovery, linearity of dilution and limit of quantification (40 ng/ml Ro 48-3656 using a 20 μl injection). Samples of neat EDTA rat plasma were prepared by ultrafiltration followed by direct injection onto the HPLC column. © 1997 Elsevier Science B.V.

Keywords: Stationary phases, LC; Ro 48-3656

1. Introduction

Ro 48-3657, an orally administered prodrug which is metabolically transformed into an active antagonist of the glycoprotein IIbIIIa, has been considered for clinical development for prevention of secondary thrombotic occlusions [1]. After oral administration, Ro 48-3657 undergoes conversion from its double protected prodrug form principally to an acid, Ro 48-3656, and then to the biologically active zwitterion, Ro 44-3888 (Fig. 1) [2]. In several preclinical studies, rats have been utilized to elucidate the pharmacokinetic behavior of these three molecules. It has been previously demonstrated, using a liquid chromatography–mass spectrometry (LC–MS) method [2], that in rats the intact prodrug, Ro 48-3657, is found in the circulation in vanishingly small quantities briefly after administration and is therefore not routinely monitored (data not shown). To measure Ro 44-3888, the bioactive zwitterion, a IIbIIIa receptor based microplate assay [3] was modified and utilized since it provided an inherent assessment of biological activity and excellent sensitivity (5 ng/ml, data not shown). Finally, an analytical method was needed to measure the inactive intermediate metabolite, Ro 48-3656, in EDTA rat plasma.

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00418-0

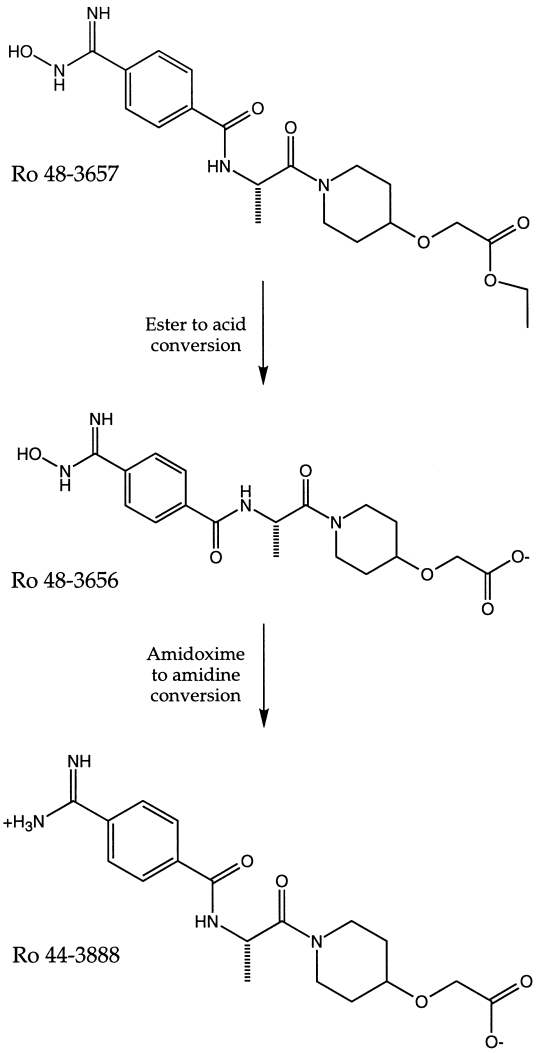

Fig. 1. Molecular diagrams of the double prodrug Ro 48-3657, an intermediate and the analyte of interest, Ro 48-3656, and the active drug, Ro 44-3888.

We used C_{18} reversed-phase high-performance liquid chromatography (RP-HPLC) for separation and quantification of our analyte of interest and we considered columns composed of both porous and nonporous silica. Except for one briefly discussed study where a 3.5-μm porous silica was evaluated [4], previous studies have compared the performance of 1.5–2.0 μm particle nonporous silica columns to columns with porous silica particles of 5-μm to 10-μm [5–11]. The nonporous silica columns consistently outperformed the porous silica columns in several parameters [4–11]. However, among porous silica columns, 3.5-μm particles have been shown to be superior to 5-μm particles [12,13]. For that reason we evaluated the chromatography of our analyte of interest on a 1.5-μm nonporous silica analytical column as well as on two different 3.5-μm porous silica columns. The nonporous silica column demonstrated better selectivity and resolution (removal of interfering matrix peaks), shorter run times, less solvent consumption and greater analyte peak area. Ultimately, we developed a method on the nonporous silica column that was accurate, precise and provided the throughput and sensitivity needed to meet pharmacokinetic study needs.

2. Experimental

2.1. Materials

Analytical grade methanol, acetonitrile, phosphoric acid and sodium hydroxide were used. Water was prepared through a Milli-Q Plus Ultrapure Water System (Millipore, Bedford, MA, USA). Mobile phase components (A, B, C, D) were as follows: (A) Milli-Q water, (B) 400 m*M* sodium phosphate prepared in Milli-Q water from phosphoric acid and adjusted to pH 6.8 with sodium hydroxide and filtered through a 0.2-μm cellulose nitrate filter unit (Nalgene cat 450-0020, Rochester, NY, USA), (C) 30% methanol in Milli-Q water and (D) 100 m*M* phosphoric acid, 50% acetonitrile in Milli-Q water, pH was not adjusted but was approximately 2. These mobile phase components were briefly sparged with helium (1–5 min), then sealed and run under a 0.25–0.40 bar helium blanket. (Mobile phase proportions for the nonporous silica column will be hereafter described in a percent A/B/C/D format). Pooled EDTA plasma from Sprague–Dawley rats was prepared by Harlen Bioproducts for Science (cat 004511, Indianapolis, IN, USA). A crude cocktail of Ro 48-3656 and Ro 44-3888, used for method development, was prepared in DMSO–water from solid source materials provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Ro 48-3656 reference material, at 50.12 μg/ml prepared by the Quality Control department at Genentech, was used for method validation and the preparation of stan-

dards and controls. This reference material was subdivided into single use aliquots and stored at ≤−60°C.

2.2. Instrumentation

The LC system was comprised of modular components by Gilson (Middleton, WI, USA) connected together by GSIOC network: three 306 pumps with 10 series heads, 817 valve actuator with a six-port low-pressure valve, 805 manometric module, 811C dynamic mixer with a 700 μl chamber, 233XL injector with a 401C dilutor fitted with a 500 μl syringe, 832 sample rack temperature regulator, 119 UV–Vis detector with a 10 μl analytical cell and 0.010 in. I.D. (1 in.=2.54 cm) tubing in the cell assembly and a 506C system interface. Mobile phase A was delivered through pump 1, B through pump 2, C and D were swapped via the 817 selection valve and delivered through pump 3. All Gilson component control, data acquisition and data analysis were done through Gilson UniPoint software (versions 1.22 or 1.51). Column temperature was maintained via an Eppendorf TC-50 controller and a CH-30 heater (Eppendorf Scientific, Madison, WI, USA). The system was plumbed from the pumps to the injector with 0.010 in. I.D. polyether ether ketone (PEEK) tubing and from injector to detector with 0.007 in. I.D. PEEK tubing. Two in-line 2-μm titanium frits in holders were included in the fluid path, one between the pumps and injector and the other between the injector and the column (tubing, frits and holders by Upchurch Scientific, Oak Harbor, WA, USA). The injector was equipped with a 100 μl PEEK sample loop (Rheodyne cat 9055-024, Cotati, CA, USA).

2.3. Sample preparation

Depending on available volume, 50–150 μl neat EDTA rat plasma (hereafter, ''plasma'') were transferred to a Microcon-10 filter unit (Amicon cat 42407, Beverly, MA, USA). Samples were centrifuged in an Eppendorf 5415C microfuge with a fixed angle rotor (Brinkmann Instruments, Westbury, NY, USA) at ambient temperature for 15–25 min at approximately 13 800 *g*. Retentates were discarded. Filtrates were injected neat or, if Ro 48-3656 concentration exceeded the standard range, were diluted with Milli-Q water. Previous studies have demonstrated that the original plasma or the prepared filtrates can be stored at ≤−60°C for one year and subjected to several freeze–thaw cycles with no loss of Ro 48-3656 recovery (data not shown).

2.4. Chromatography

Initially, three different columns were evaluated for their utility in this method: Zorbax StableBond SB-C_{18}, 150×4.6 mm column with 3.5-μm, 80 Å pore silica particles (MacMod cat 863953-902, Chadds Ford, PA, USA), Waters Symmetry C_{18}, 150×4.6 mm column with 3.5-μm, 100 Å pore silica particles (Waters cat WAT200632, Milford, MA, USA) and MICRA NPS C_{18}, 53×4.6 mm column with 1.5-μm nonporous silica particles (MICRA Scientific cat 0646MSODS101.5, Northbrook, IL, USA). The separation conditions were similar for all three columns tested. A neutral pH mobile phase was chosen over an acidic pH mobile phase because it was determined that the separation of Ro 48-3656 from Ro 44-3888 was greatly enhanced at neutral pH (data not shown). Numerous method development runs were made varying the percent methanol in the isocratic step comparing the chromatography of a Ro 44-3888/Ro 48-3656 cocktail versus blank plasma filtrate. The separation conditions used for the two porous silica columns were as follows. Mobile phase flow-rate was 1.0 ml/min, the column heater was set to 40°C, the buffer content of the mobile phase was always 100 m*M* sodium phosphate, pH 6.8 and the organic modifier was methanol. The isocratic separation step was 7 min long and the strip step went to 50% methanol, held for 1 min and then returned to starting conditions for re-equilibration. Total run time for one sample was about 16.25 min. During these column comparison tests, absorbance at 240 nm was monitored.

The final method conditions for the nonporous silica column were as follows. Mobile phase flow-rate was 0.75 ml/min and the column heater was set to 40°C. A run sequence consisted of a 1 min equilibration with (72:25:3:0, %) proportioned mobile phase, 1 min of injector activity which concluded with a 20 μl sample injection and commencement of 230 nm absorbance data acquisition, a

3 min isocratic hold at (72:25:3:0, %) a 0.5 min linear gradient to (25:25:50:0, %) followed by a 1.5 min hold of those conditions, a 0.5 min linear gradient back to the initial conditions (72:25:3:0, %) followed by a 2.5 min equilibration hold. Data acquisition concluded at the end of the equilibration hold. Data file storage and software initialization took about 0.25 min so the total run time for a single sample was about 10.25 min. The sample rack temperature controller was set to 4°C. Column back pressure ranged from approximately 265 to 335 bar depending on the percent methanol during the run. Although methanol generates higher back pressure than acetonitrile, methanol was used because, at neutral pH, the retention of Ro 48-3656 was weak on C_{18} columns and methanol provided greater selectivity options (data not shown). At the conclusion of a run set (run sets ranged from a few injections to nearly 200 injections), it was critical for column stability to run a shutdown method where the neutral pH buffer was removed and the column was acidified. The flow-rate was reduced to 0.5 ml/min and the column was rinsed with 10 ml water (100:0:0:0, %), then 10 ml phosphoric acid–acetonitrile (0:0:0:100, %), then again 10 ml water (100:0:0:0, %), and finally 10 ml 15% methanol (50:0:50:0, %) in which the column was stored.

Each run set was preceded by several system suitability runs of a Ro 48-3656 spike in water that were subjectively evaluated by the operator prior to starting the set. Eight standards, serial two-fold dilutions in water prepared from the 50.12 μg/ml stock, were run from lowest concentration, 30 ng/ml, to highest, 3840 ng/ml, with a blank water injection following the highest standard to verify the absence of run to run carryover. Plasma controls were employed which spanned the standard range and a blank plasma control was included to verify that the method did not degrade and result in the inclusion of interfering peaks. Standards and controls were run as a group in duplicate, triplicate or quadruplicate, depending on the size of the sample set, and were placed at intervals throughout the set. Replicate standard peak areas were averaged and calibration curves were generated by plotting mean peak areas versus expected concentrations. Samples were typically injected in singlets and their peak areas were converted to concentration based on the standard curve.

2.5. LC system extra-column volume considerations

Many previous reports have discussed the importance of evaluating the suitability of the HPLC system fluid path volume for running small columns since excessive extra-column volume can result in band-broadening [4,11–13]. Retrospective to other work described in this report, the theoretical suitability of the Gilson LC system for running the small (53×4.6 mm) nonporous silica column was considered. While no changes were made to the system plumbing in this post study evaluation, it was brought to our attention that the UV detector could be better optimized for faster response and data transfer which would have an impact on the observed t_0. The observed t_0 was empirically determined by noting the time of the first detector response after the injection of a plasma filtrate and the injector to detector dead volume was calculated from the observed t_0 and the flow-rate. A comparison was made between the extra-column portion of dead volume to the intra-column fluid volume.

2.6. Method validation

The selection of detection wavelength was made based on spectral, diode array analysis of Ro 48-3656 and of blank plasma filtrate at the retention time of Ro 48-3656 (Model 160 DAD by Gilson).

To validate the limit of quantification (LOQ), a modification of the LOQ test described by Shah et al. [14] was performed where parallel spikes of Ro 48-3656 were made in water and in plasma at 40, 50 and 80 ng/ml. Sufficient volume of each spike was prepared to accommodate ten injections. The water spikes were not processed prior to injection. The plasma spikes were each divided amongst ten ultrafiltration units, processed and injected as discrete samples. This experiment was done twice, once on an old column at the end of its service (~800 injections) and once on a new column.

Intra- and inter-assay contributions to the variability were estimated at each of four (plasma) control levels spanning the standard range using one-way analysis of variance (ANOVA). Data used in this analysis were from five run sets spanning a four month period using two nonporous silica columns where controls were injected one to four times per run set.

The precision of Ro 48-3656 retention time was monitored within each run set and between run sets. Brief consideration was given to Ro 48-3656 retention time agreement between different nonporous silica columns.

Accuracy and recovery were evaluated by preparing parallel spikes of Ro 48-3656 in water and plasma at several concentrations. Water spikes were not processed prior to injection, while plasma spikes were all processed by ultrafiltration.

Linearity of dilution was evaluated by preparing a spike of 3000 ng/ml Ro 48-3656 in plasma which was Microcon-10 filtered. The filtrate was serially diluted two-fold seven times with Milli-Q water and the neat and diluted filtrates were all run in duplicate. A plot of 1/dilution versus mean observed concentration of each dilution was generated to evaluate the results.

Finally, to increase our confidence that the method was indeed reasonably free of matrix interference, a total of ten different blank individual and pooled EDTA rat plasmas were evaluated for the presence of peaks that might interfere with Ro 48-3656 quantification.

2.7. Pharmacokinetic protocol and analysis

A study was performed to characterize the dose linearity of Ro 48-3656 following daily oral administration of Ro 48-3657 for two weeks in rats. Ro 48-3657 was administered as a daily oral gavage to five groups of male Sprague–Dawley rats ($n=6$ per group), weighing 294–356 g. Each animal received an oral gavage of 50, 100, 200, 300 or 400 mg/kg/day Ro 48-3657 in a 0.5% carboxymethylcellulose (CMC)–0.2% Tween 80 suspension (10 ml/kg). Blood samples (0.5 ml) were collected using EDTA as anti-coagulant on day 15 predose, and 1, 2, 4, 8 and 24 h postdose. At each timepoint, three animals were sampled from each dose group. Plasma was harvested and stored frozen at ≤ -60°C until assayed.

For each timepoint, the mean concentration of three animals per dose group was determined and used in the subsequent analysis. Areas under the curve (AUC) for Ro 48-3656 were calculated using a linear trapezoidal method from the first detectable average concentration to the last detectable average concentration. The peak concentration, C_{max}, and the time of peak concentration, T_{max}, are the observed mean values. AUC was normalized to the 50 mg/kg group values to determine dose linearity.

3. Results and discussion

3.1. Column comparison

The goal was to identify a method consisting of an isocratic step for the separation of Ro 48-3656 from matrix interference followed by a higher organic column cleanup step and then re-equilibration at the initial isocratic condition. The chromatograms shown in Fig. 2 and the data in Table 1 highlight the best results observed with each column based on the relative retention times of Ro 48-3656 and potential interfering peaks in the blank plasma as well as Ro 44-3888. The Zorbax column method appears to be free of interfering peaks at the retention time of Ro 48-3656 and the Waters column method is nearly so (additional optimization of the methanol component may have improved the method). The nonporous silica column method provided a wide window of minimal interference around the retention time of Ro 48-3656, and as compared to the porous silica columns, a shorter run time and reduced solvent consumption. The nonporous silica column also yielded larger analyte peak area than that obtained with the porous silica columns. Interestingly, a simple normalization for flow-rate, using the mean peak area observed on the Waters column (Table 1), predicts a peak area of about 141 365 on the nonporous silica column.

(observed analyte peak area, Waters)(1 ml/min)
= (predicted analyte peak area, nonporous silica)
(0.75 ml/min)

However, the observed peak area on the nonporous silica column was 156 293, about 10% more than predicted. Some, or even all, of this difference may be due to experimental variability but we speculate that (1) there may be an issue of mass transfer efficiency where some analyte is lost, temporarily or permanently, in the pores of the porous silica which may not occur in the nonporous silica column; or, (2) there may have been a favorable shift in the extinction coefficient of Ro 48-3656 in the

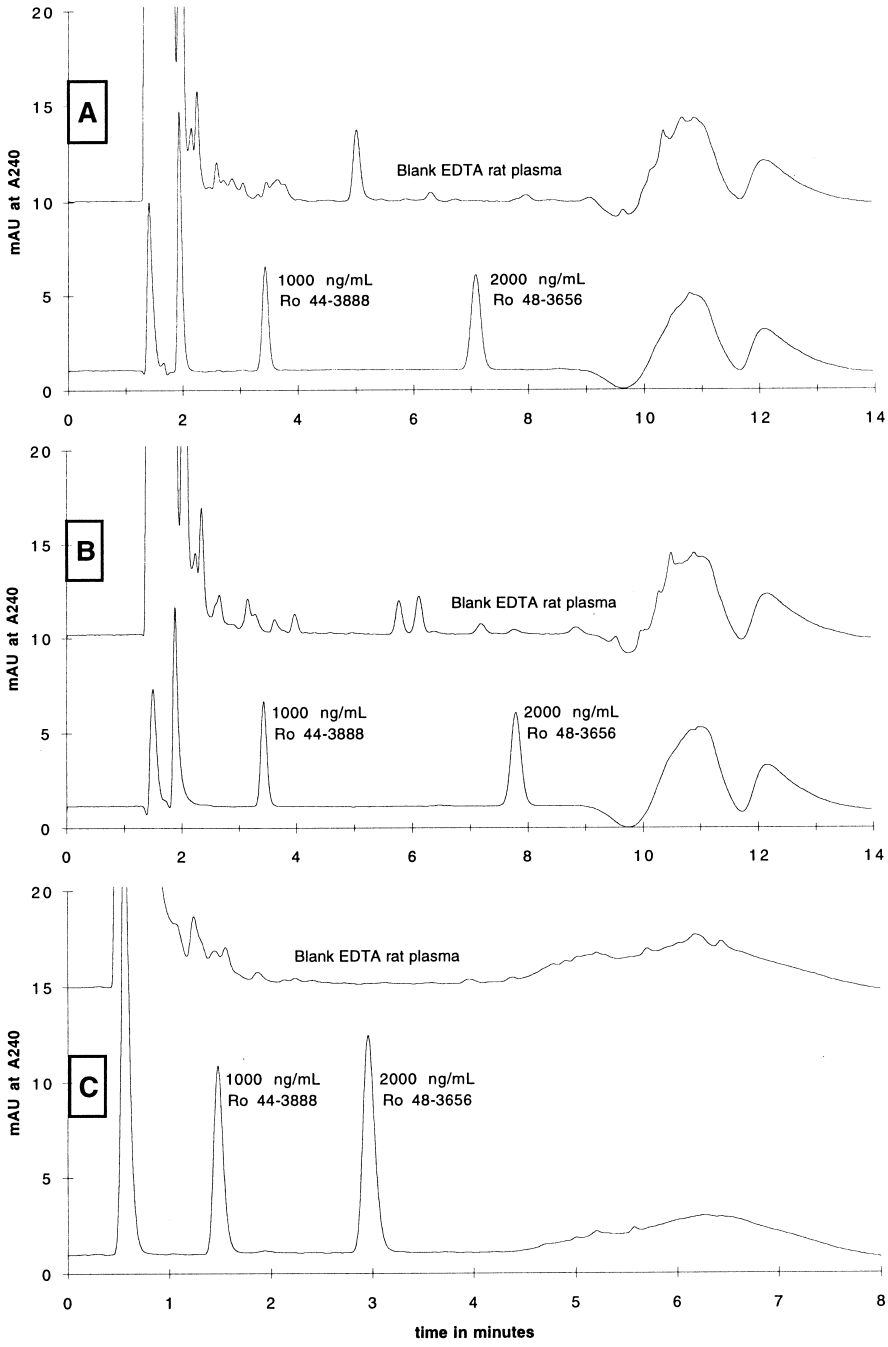

Fig. 2. Comparsion of blank rat plasma to a cocktail of Ro 44-3888/Ro 48-3656 in water on (A) Zorbax, (B) Waters and (C) MICRA columns.

Table 1
Summary of chromatography characteristics for Ro 48-3656 on porous and nonporous silica columns

C_{18} column	Particle/pore	Flow (ml/min)	Total run time (min)	Mobile phase used per run (ml)	Isocratic step (% methanol)	Ro 48-3656 retention time, (min)[a]	Ro 48-3656 (k)[a,b]	Ro 48-3656 peak asymmetry[a]	2000 ng/ml Ro 48-3656 peak area[a]
Zorbax SB	3.5 μm/80 Å	1.0	16.25	16.25	15	7.10 (0.012)	4.46 (0.012)	1.09 (0.059)	101 431 (1961)
Waters Sym.	3.5 μm/100 Å	1.0	16.25	16.25	14	7.80 (0.010)	5.00 (0.006)	1.02 (0.010)	106 024 (5515)
MICRA	1.5 μm/–	0.75	10.25	7.69	0.9	2.95 (0.012)	5.87 (0.026)	1.39 (0.015)	156 293 (626)

[a] Mean (S.D.) from three injections of 2000 ng/ml analyte.
[b] The observed t_0 for these water/analyte injections were 1.3 min on the porous silica columns and 0.43 min on the nonporous silica column.

lesser methanol used on the nonporous silica column versus the porous columns (Table 1). A thorough follow-up of this observation was beyond the scope of this report.

The capacity factor (k) of Ro 48-3656 was almost three-fold more sensitive to changes in the percent organic modifier on the nonporous silica column versus the porous silica columns (Fig. 3). Brizzi and Corradini [15] also reported a sense that k on a nonporous silica column is particularly sensitive to changes in the organic modifier concentration. Jenke [11] noted an average two-fold increase in the sensitivity of k to percent organic modifier on the nonporous silica versus the porous silica when he compared 1.5-μm nonporous silica to 5-μm porous silica for several analytes.

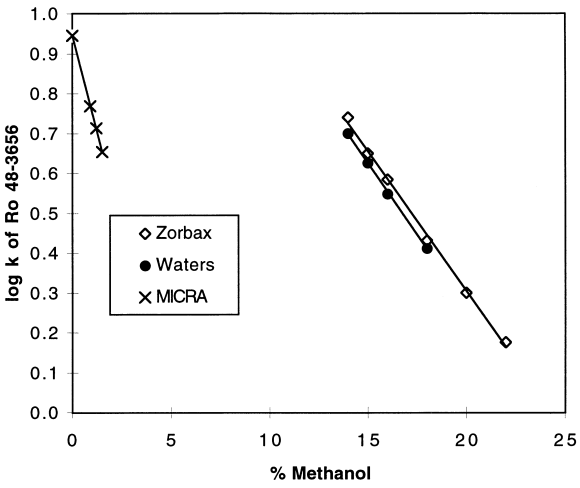

Fig. 3. The capacity factor of Ro 48-3656 was more sensitive to changes in the percent methanol on the nonporous silica column (slope ≈ -0.19) as compared to the porous silica columns (slopes ≈ -0.07).

3.2. LC system extra-column volume

The t_0 from repeated injections of blank plasma was observed to be 0.390 min. Since the flow-rate was 750 μl/min, the total injector to detector fluid volume was approximately 293 μl. The intra-column fluid volume was reported by the vendor to be 241 μl so the extra-column volume was 52 μl, therefore the ratio of extra-column volume to intra-column fluid volume was 0.22. In a report by Barder et al. [4], four different LC systems were empirically evaluated for their suitability to execute small column methods and their data indicated that the extra-column to intra-column fluid volume ratio could be as high as 1.20 and still generate good chromatographic results. Based on these data, our HPLC system extra-column volume was indeed suitable for running the nonporous silica column.

3.3. Method performance

The detection wavelength was identified by doing spectral analysis of the Ro 48-3656 peak and also of blank plasma at the retention time of Ro 48-3656. Under the mobile phase conditions used in this method, Ro 48-3656 had three absorbance peaks, in descending order of intensity, at 206, 229 and 272 nm (data not shown). The blank plasma at the retention time of Ro 48-3656 displayed obvious interference in the low UV which peaked at about 210 and decreased gradually to baseline around 230 (data not shown). Based on these results, 230 nm was selected as the detection wavelength.

A standard range of 30–3840 ng/ml was first identified through empirical investigation. Initially, a simple least squares fit was evaluated for the standards, however line fit and control results (especially the low control) were unacceptably imprecise (data not shown). It is believed that a weighted, $1/x$ or $1/x^2$, least squares fit would have been acceptable for the standards [16]. Since weighted linear fits were not available in UniPoint versions 1.22 or 1.51, nonlinear fits [16] were considered and it was determined that UniPoint's ''cubic fit'' option provided consistent, good quality calibration curves. Ultimately, a brief analysis was done where standard and control peak areas from five run sets were manually transferred to an alternate software package, $1/x^2$ least square fits were applied to the standards and then controls were recalculated from the weighted linear fits (data not shown). Unpaired *t*-tests were done on each of the four controls comparing cubic fit results to $1/x^2$ linear results. The comparison yielded *p*-values for the low, mid I, mid II and high controls of 0.470, 0.767, 0.818 and 0.880, respectively.

The results of the LOQ test at 40 ng/ml are summarized in Table 2. At 40 ng/ml, agreement between plasma and water spikes was excellent, within ±2% of each other. Mean observed values were within ±17% of expected and all coefficients of variation (C.V.s) were less than 10% which are within the specifications recommended by Shah et al. [14]. Based on these results, the reporting range for this method was set to 40–3840 ng/ml Ro 48-3656.

Intra- and inter-assay precision, as determined from the Ro 48-3656 spiked plasma controls (Fig. 4), was very good (Table 3). The estimate of intra-assay precision, ranged from 0.9–13.7%. The estimate of inter-assay precision, ranged from 3.1–4.0%.

As shown in Tables 4 and 5, the Ro 48-3656 retention time within a run set was reproducible. We initially observed a problem with the retention time of Ro 48-3656 between run sets. Prior to the incorporation of the acid wash step at shutdown, the retention of Ro 48-3656 decreased between run sets over time (Table 4). However, once the acid wash sequence was optimized and incorporated, Ro 48-3656 retention time was stable, not only within a run set, but also from set-to-set (Table 5). Under the conditions described, we now expect a column to last for about 1000 injections. We examined the retention time of Ro 48-3656 on four different columns (Table 6). Table 6 summarizes selected retention time data from four different nonporous silica columns. This simple assessment indicated that the retention time of Ro 48-3656 would be reproducible from column to column.

Accuracy and recovery were assessed through parallel spikes of Ro 48-3656 in plasma and water at 40, 50, 80, 300 and 3000 ng/ml. The results of these tests, summarized in Table 7, indicated good agreement between recovery of Ro 48-3656 from water and from plasma. Therefore it was decided that the preparation of standards could simply be done in water without requiring the added time and labor for preparing standards in plasma.

The slope of the line generated from the 1/dilution versus mean observed concentration was 2852 versus an expected slope of 3000 (since the spike was 3000 ng/ml Ro 48-3656) and the r^2 correlation coefficient was 0.997 indicating that the linearity of sample dilution was good. After correcting the raw results

Table 2
Precision and accuracy at the limit of quantification, 40 ng/ml on nonporous silica columns

	Old column		New column	
	Plasma	Water	Plasma	Water
Mean (ng/ml)	34.2	33.6	41.1	41.7
S.D.	3.1	2.9	1.5	3.9
%C.V.	9.1	8.5	3.6	9.4
n	8	9	10	9
% Of expected (40)	85.5	84.1	102.7	104.1
% Ratio plasma/water	101.7	–	98.6	–

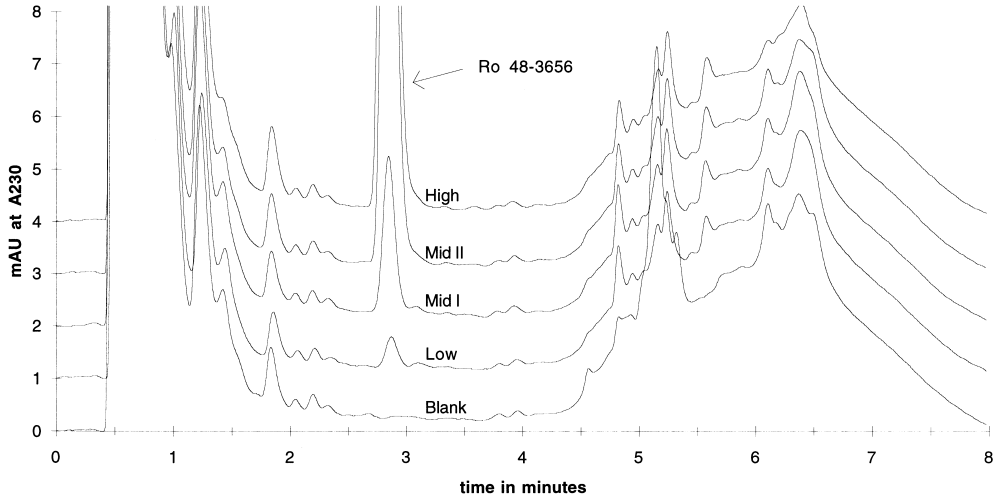

Fig. 4. Chromatograms of five Ro 48-3656 plasma controls. Four controls are Ro 48-3656 spiked rat plasma and one is blank rat plasma. Controls are run at least in duplicate in each run set.

Table 3
Intra- and inter-assay precision estimates from Ro 48-3656 plasma controls

	Low	Mid I	Mid II	High
Expected (ng/ml)	80	350	1900	3400
Mean observed (ng/ml)	74.6	328.7	2060	3368
Total No. of injections	12	12	12	11
No. of assay days	5	5	5	5
Intra-assay C.V. (%)	13.7	6.3	4.6	0.9
Inter-assay C.V. (%)	3.4	3.1	3.6	4.0

Table 4
Retention time summary without column acidification at shutdown

Run set[a]	Mean retention time (min)	%C.V.	No. of injections with Ro 48-3656
1	2.95	0.4	3
2	2.84	2.3	65
3	2.80	0.5	69
4	2.72	0.8	45
5	2.45	0.8	55

[a] Over a three week period.

Table 5
Retention time summary with column acidification at shutdown

Run set[a]	Mean retention time (min)	%C.V.	No. of injections	
			With Ro 48-3656	Total[c]
1	2.93	1.7	112	163
2	2.74	2.1	39	45
3	2.82	1.0	39	46
4	2.81	1.1	38	47
5	2.87	0.8	88	112
6	2.89	1.9	113	146
7	2.93	1.6	133	152
8	n/d[b]	n/d[b]	n/d[b]	75
9	2.87	1.6	24	27

[a] Over a four week period. Acidification method was fully optimized at run set number 5.
[b] Not determined due to corrupted data file error.
[c] 813 total injections before column was damaged in an unrelated experiment.

Table 6
Retention time summary on different nonporous silica columns

Column number	Silica lot number	Stationary phase, C_{18} lot number	Representative Ro 48-3656 mean retention time (min)
13016F08	M011295	0196/95c	2.95
08066F07	M011295	0196/95c	2.93
08066F05	M011295	0196/95f	3.01
28076F05	M081595	0228/96c	2.96

for their respective dilution factors, there was no trend of increasing or decreasing concentration with each dilution (data not shown).

Of the ten blank plasmas evaluated prior to running pharmacokinetic study samples, five had no detectable peak at the retention time of Ro 48-3656. Of the remaining five, the interfering peak area ranged from 3–37% of the peak area of the lowest, 30 ng/ml, standard (data not shown).

3.4. Pharmacokinetic results

Fig. 5 shows the mean Ro 48-3656 plasma concentration versus time data on day 15 following two weeks of daily oral gavage of Ro 48-3657. Estimated pharmacokinetic parameters and normalized parameters are summarized in Table 8. Concentrations of Ro 48-3656 reached a peak at approximately 1 h post-dose. Ro 48-3656 AUC ranged from approximately 5.91 μg·h/ml (50 mg/kg/day) to approximately 44.2 μg·h/ml (400 mg/kg/day). The exposure to Ro 48-3656, as calculated by AUC, is linear between the doses of 50 to 400 mg/kg/day of Ro 48-3657, however the relationship between Ro 48-3656 C_{max} and the dose of Ro 48-3657 is less clear.

4. Conclusions

In this application and with the columns and conditions tested, the nonporous silica column provided superior performance over the 3.5-μm porous silica columns. The method on the nonporous silica column was faster, used less solvent, provided greater analyte peak area and better resolution from interfering plasma peaks. The validated method proved to be accurate, precise and had sensitivity sufficient to meet the needs for analysis of biological samples. Results from the pharmacokinetic study samples were consistent and generally interpretable.

As we used the nonporous silica column, several unique issues and differences relative to our experience with porous silica columns became apparent. (1) An evaluation of the LC system extra-column fluid volume is recommended early in the development process. Retrospectively, we found our system appropriate, however other investigators have described unsatisfactory results with various LC systems [4,11]. (2) The amount of organic modifier needed for a separation may be less on a nonporous silica column than on a porous silica column. For our method and analyte, the organic modifier used in the separation step was reduced by over 90% on the nonporous silica versus porous silica columns; a decrease which appears to be reasonably consistent with other reports [4,11]. Regardless of the magnitude, some reduction of organic modifier is likely needed if transferring a method from a porous silica to a nonporous silica column. (3) Capacity factor

Table 7
Recovery and accuracy of Ro 48-3656 in EDTA rat plasma

Expected (ng/ml)	Observed in plasma, (ng/ml)	Observed in water, (ng/ml)	% Ratio plasma/water	% Ratio plasma/expected
40	41.1	41.7	98.6	102.7
50	51.1	47.7	107.2	102.2
80	78.4	73.7	106.4	98.1
300	254	280	90.9	84.8
3000	2900	2850	101.8	96.7

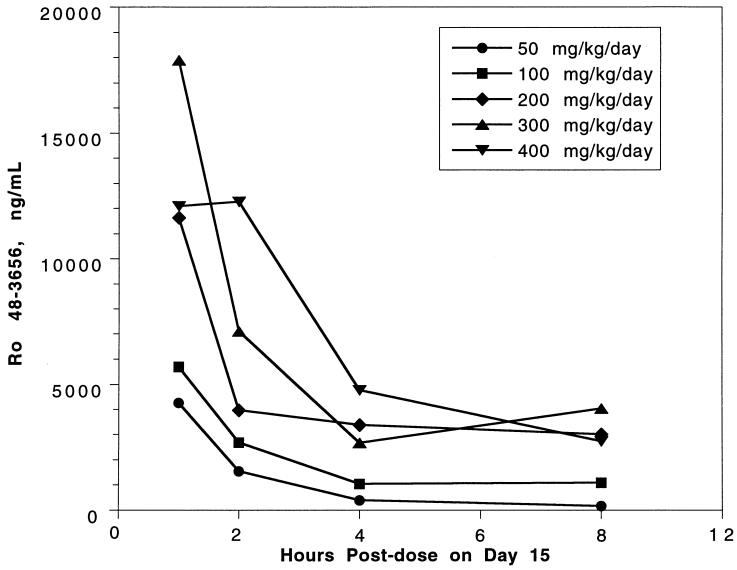

Fig. 5. Mean plasma Ro 48-3656 concentration–time profile on day 15 following daily oral gavage of Ro 48-3657 ($n=3$ per timepoint).

will be more sensitive to changes in organic content. Our data (Fig. 3) supports previous reports of this observation [11,15]. This is relevant when planning very early development experiments and when considering the LC system precision at mixing mobile phase. (4) Depending on overall column dimensions, the back pressure using a 1.5-μm particle column will likely be higher than is traditionally accepted [17]. While some reduction of flow-rate may be necessary, modern HPLC systems should be able to accommodate the higher pressure.

Overall, we realized good performance from the nonporous silica column and found it to be a valuable addition to the various commercial column options available for method development.

Acknowledgements

The authors acknowledge and thank the following for their contributions: Tom Jupille (LC Resources) for thoughtful discussions on peak area differences between columns, Charles ''Chuck'' Frey (Gilson) for an informative discussion on detector settings and Cara Paasch for her editorial review of this paper. Additionally, all from Genentech, Victor Ling for a helpful discussion on column stability at neutral pH, Suresh Kalbag for preparing the Ro 48-3656 reference material, Karen Billeci for help with some statistical analysis, Ellen Gilkerson for recalculating standards and controls using weighted linear regression, Allison Nixon, Anne Walters, Christine Fratino

Table 8
Summary of mean Ro 48-3656 C_{max}, T_{max}, AUC and AUC normalized ratio on day 15 following daily oral gavage of Ro 48-3657

	Dose Ro 48-3657 (mg/kg)				
	50	100	200	300	400
C_{max} (ng/ml Ro 48-3656)	4.27±1.8	5.69±1.0	11.6±4.9	17.9±6.8	12.3±4.8
T_{max} (h)	1	1	1	1	2
AUC (μg h/ml Ro 48-3656)	5.9	12.2	28	36	44.2
AUC normalized ratio[a]	1	2	5	6	7.5

[a] AUC was normalized to the 50 mg/kg dose group value.

and Elizabeth Tomlinson for animal technical support and finally, Zahra Shahrokh and Katrina Brabham for assistance in preparing dosing suspensions.

References

[1] T. Weller, L. Alig, M. Beresinin, B. Blackburn, S. Bunting, P. Hadváry, M.H. Müller, D. Knopp, B. Levet-Trafit, M.T. Lipari, N.B. Modi, M. Müller, C.J. Refino, M. Schmidtt, P. Schönholzer, S. Weiss, B. Steiner, J. Med. Chem. 39 (1996) 3139.
[2] M. Zell, C. Husser, G. Hopfgartner, J. Mass Spectrom. 32 (1997) 23.
[3] N.B. Modi, S.A. Baughmann, B.D. Paasch, A. Celniker, S.Y. Smith, J. Cardiovas. Pharmacol. 25 (1995) 888.
[4] T.J. Barder, P.J. Wohlman, C. Thrall, P.D. DuBois, LC·GC 15(10) (1997) 918.
[5] K.-S. Boos, B. Wilmers, R. Sauerbrey, E. Schlimme, Chromatographia 24 (1987) 363.
[6] G. Jilge, R. Janzen, H. Giesche, K.K. Unger, J.N. Kinkel, M.T.W. Hearn, J. Chromatogr. 397 (1987) 71.
[7] K. Kalghatgi, C. Horváth, Anal. Biotechnol. 434 (1990) 162.
[8] H.J. Wirth, K.K. Unger, M.T.W. Hearn, J. Chromatogr. 550 (1990) 383.
[9] B.d. Collongue-Poyet, C. Vidal-Madjar, B. Sebille, K.K. Unger, J. Chromatogr. B 664 (1995) 155.
[10] A. Rudolphi, K.-S. Boos, D. Seidel, Chromatographia 41 (1995) 645.
[11] D.R. Jenke, J. Chromatogr. Sci. 34 (1996) 362.
[12] J.J. Kirkland, J. Chromatogr. Sci. 31 (1993) 493.
[13] K.M. Kirkland, D.A. McCombs, J.J. Kirkland, J. Chromatogr. A 660 (1994) 327.
[14] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.
[15] V. Brizzi, D. Corradini, J. Pharm. Biomed. Anal. 12 (1994) 821.
[16] H.T. Karnes, G. Shiu, V.P. Shah, Pharm. Res. 8 (1991) 421.
[17] J.W. Dolan, LC·GC 15 (1997) 24.