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Multidimensional narrow bore liquid chromatography analysis of Ro 24-0238 in human plasma

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Abstract: A highly sensitive LC method has been developed and validated for quantitation of Ro 24-0238 in human plasma using Ro 24-2446 as an internal standard. With 1 ml of plasma, the limit of quantitation of the method was 50 pg ml^{-1} of Ro 24-0238. After solid-phase extraction with C_{18} reversed-phase cartridges, the samples were reconstituted in an acidic buffer solution; under these conditions, Ro 24-0238 and Ro 24-2446 (IS) were converted to their cationic forms. The LC system employed a strong cation exchange column and a narrow bore reversed-phase column, connected via a column switching valve. The cationic analyte and internal standard were separated from most of the endogenous components of plasma on the cation exchange column. A small fraction containing the analyte and the internal standard was transferred by automated valve switching to the narrow bore reversed-phase column, which further resolved the individual components. The chromatography was monitored by UV absorption at 322 nm. The overall intra-assay precision was 3.6% (RSD) and the per cent error was less than ±11%. The overall inter-assay precision was 3.9% (RSD). Linearity was demonstrated in a concentration range of 50–5000 pg ml^{-1}. This method has been applied to pharmacokinetic studies of Ro 24-0238 in man.

Keywords: *Column switching; narrow bore; multidimensional HPLC; cationic exchange; reversed-phase; solid-phase extraction.*

Introduction

Ro 24-0238 [(*R*)-(2*E*,4*E*)-5-(4-methoxyphenyl)-N-[1-methyl-4-(3-pyridinyl)butyl]-2,4-decadienamide], is a potent platelet-activating factor (PAF) receptor antagonist. Ro 24-0238 is a lipophilic organic base (I) (Fig. 1). A highly sensitive analytical method was needed due to the low concentrations of Ro 24-0238 present in plasma following oral administrations of therapeutic doses. In fact, a conventional bore LC method could not provide the required sensitivity for a thorough pharmacokinetic investigation.

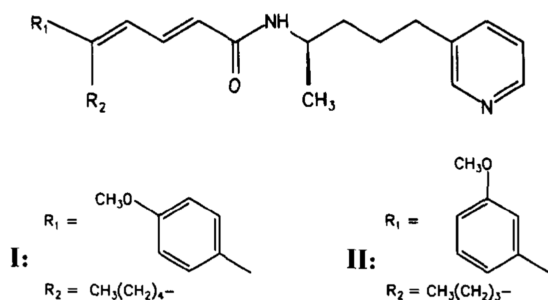

Figure 1
Molecular structures for Ro 24-0238 (I) and Ro 24-2446 (II).

In order to obtain the required limit of quantitation, remarkable sensitivity with HPLC and UV detection (λ_{max} = 322 nm, ϵ = 32,600) has been achieved by combining several techniques. Solid-phase extraction was utilized, followed by LC analysis with column switching [1–7] from a strong cation exchange column to a narrow bore, reversed-phase LC column. This procedure allows quantitation of **I** in human plasma in a concentration range of 50–5000 pg ml^{-1}. Solid-phase extraction was used for pre-concentrating analytes from plasma samples as well as performing significant sample cleanup, thereby minimizing the loading on low capacity LC columns. For additional sample clean-up, cation exchange chromatography was used. Cationic exchange chromatography was coupled via column switching with reversed-phase chromatography, a rarely reported combination [8], and a good choice for lipophilic bases. For the second dimension, narrow bore HPLC was exploited to enhance sensitivity [9, 10]. The problem of limited capacity of narrow diameter columns was overcome via the extensive clean-up in previous steps. Moreover, with compatible separation mechanisms, the difference in the organic modifier contents of the mobile

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phase allowed pre-concentration of the analytes on the head of the second column [11, 12]. This feature essentially eliminated band-broadening during column switching, thereby exploiting the benefit of narrow bore chromatography. Finally, there was sufficient resolution of analytes to allow a conventional, 9 μl, 6 mm pathlength flow cell.

Experimental

Chemicals

Ro 24-0238 (**I**) and Ro 24-2446 (**II**) (Fig. 1) were obtained from the Quality Control Department, Hoffmann-La Roche (Nutley, NJ). Acetonitrile, acetone and purified formic acid (90%) were HPLC grade; ammonium acetate, glacial acetic acid and potassium hydroxide pellets were ACS reagent grade and were all purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was obtained by purifying distilled water with a Milli-Q UF Plus water purification system (Millipore, Bedford, MA). Waters Sep-Pak Plus C_{18} solid-phase extraction cartridges were purchased from Millipore (Milford, MA). Heparinized control human plasma was obtained from Pel-Freeze Clinical Systems (Brown Deer, WI 53223).

Instrumentation

The HPLC system was constructed from a Waters Model 6000A HPLC pump (pump 1); a Waters Model 590 pump (pump 2); a Waters Lambda-Max Model 480 UV absorbance detector (detector 1), operated at 322 nm; a Waters Model 710B WISP autosampler (Millipore, Milford, MA); a Linear Model 206 PHD UV absorbance detector, fitted with a 9 μl, 6 mm pathlength flow cell, operated at 322 nm (detector 2) (Linear Instruments, Reno, NV); an SSI Model 505 Column Oven (Scientific Systems, State College, PA); a Rheodyne Model 7060P pneumatically actuated switching valve, with air actuation controlled by a 120 VAC Rheodyne Model 7163-031 solenoid valve kit; a Rheodyne Model 7315 precolumn filter (Rheodyne, Cotati, CA) and a P.E. Nelson 3000 Series Chromatography Data System with a Model 960 Intelligent Interface and Model 2600 Chromatography Software (Perkin–Elmer, Cupertino, CA). The system configuration and column switching operation are shown in Fig. 2. A relay module consisting of a 5 V power supply and two single pole double throw relays allowed contact closures

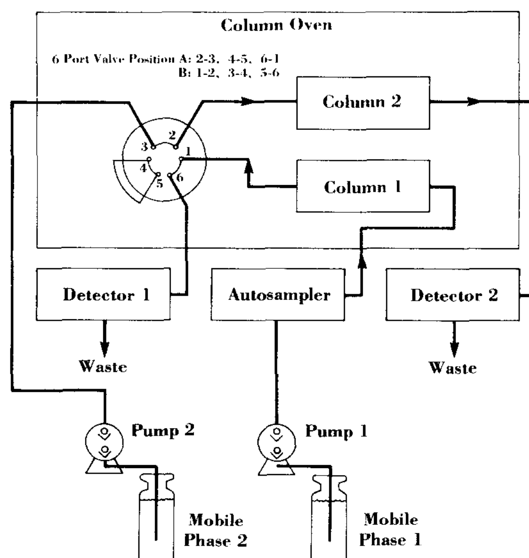

Figure 2
Schematic representation of the multi-dimensional HPLC system.

on the A/D module to control the solenoid valves, and thereby change the position of the switching valve during the chromatographic run.

Chromatographic procedure and conditions

The chromatographic system employed a strong cationic exchange column (column 1), P-SCX, 3 cm × 3 mm i.d. (ES Industries, Marlton, NJ) and a narrow bore reversed-phase column (column 2), Zorbax Rx-C8, 15 cm × 2.1 mm (Mac-Mod Analytical, Chadds Ford, PA). The mobile phase for the first column was acetonitrile–potassium formate buffer (pH 3.0; 0.08 M) (40:60, v/v) (mobile phase 1) with a flow rate of 0.4 ml min^{-1}; for the second column, the mobile phase was acetonitrile–ammonium acetate buffer (pH 4.0; 0.1 M) (80:20, v/v) (mobile phase 2), with a flow rate of 0.2 ml min^{-1}. Both LC columns were operated at 46°C. With the six port valve in position A (Fig. 2), the sample was injected onto the cation exchange column (column 1). Just before the analytes started to elute from column 1, the valve was timed to switch to position B. This allowed the direct transfer of the eluent from column 1 to column 2, the narrow bore reversed-phase column, where the analytes were further separated from endogenous components. As soon as the analytes were completely transferred to column 2, the valve was switched back to position A.

Preparation of standards and samples

Stock solutions of **I** and **II** were prepared at 1 mg ml^{-1} in accetonitrile. The stock solution of **I** was serially diluted with acetonitrile to provide spiking solutions in a concentration range of 0.4–100 pg μl^{-1}. The stock solution of **II** was diluted to provide a spiking solution of 10 pg μl^{-1}. The spiking solutions were stored in amber volumetric flasks at 4°C until use.

Calibration standards were prepared in duplicate by adding 50 μl aliquots of **I** spiking solutions and 50 μl aliquots of **II** spiking solution (internal standard) to 1 ml aliquots of heparinized human plasma, providing a concentration range of 20–5000 pg ml^{-1} of **I** and 500 pg ml^{-1} of **II**. Experimental samples (1 ml aliquots) were spiked with 50 μl aliquots of **II** (internal standard), and 50 μl of acetonitrile. Quality assurance samples were prepared at 140, 200, 1500 and 4000 pg ml^{-1} and stored in amber vials at −20°C until use. These quality control samples (1 ml aliquots) were treated the same as experimental samples. The control blanks were prepared by adding 100 μl aliquots of acetonitrile to 1 ml aliquots of heparinized human plasma.

The calibration standards, experimental samples, quality control samples and the control blanks were deproteinized with 2 ml aliquots of acetonitrile, vortex mixed and centrifuged for 10 min at 3000 rpm at 10°C. The supernatant from each sample was decanted into a culture tube for solid-phase extraction. The SPE extraction was performed on a Milli-Lab 1A Workstation (Millipore, Milford, MA). The extraction procedure was programmed as follows: the supernatant was diluted with 6 ml of water and mixed well; a Sep-Pak Plus C_{18} solid-phase extraction cartridge was conditioned with 3 ml of acetonitrile and then 3 ml of water; the diluted supernatant was loaded on the preconditioned cartridge; the cartridge was washed with 2 ml of water and dried with air, and the analyte and the internal standard were finally eluted from the cartridge with 4 ml of acetone. The extracted samples were evaporated to dryness under a stream of dry nitrogen in a 35°C water bath. If the evaporated samples could not be assayed immediately, they were stored at −20°C and assayed within 36 h. Each sample was reconstituted in 100 μl of mobile phase 1, acetonitrile–potassium formate (pH 3.0; 0.08 M) (40:60, v/v), and filtered through a Whatman 0.2 μm PVDF 13 mm syringe filter (Whatman, Clifton, NJ 07014).

Sample analysis and calculation

One each of the duplicate sets of eight concentration levels of calibration standards was injected at the beginning, and the other was injected at the end of each sample analysis run, bracketing the quality control samples, control blanks and the experimental samples. An 80 μl volume of each sample was injected; about 400 μl (1 min) of the eluent containing analyte and internal standard were transferred from column 1 to column 2 during each column switch cycle. Both detectors were monitored simultaneously.

From the calibration standard concentration versus the peak height ratios of **I/II**, a linear calibration curve was constructed. The parameters of the calibration curve, including slope, intercept, and standard error of the slope and intercept, were calculated using weighted ($1/y^2$) least-squares regression. The peak height ratios of the experimental and quality control samples were interpolated to concentrations using the regression parameters.

Intra-assay precision, inter-assay precision and accuracy were assessed by using quality assurance samples. Five replicates of each of the four quality assurance sample concentration levels were analysed during assay validation to determine intra-assay precision and accuracy. Two replicates of each QA level were run during sample analysis to ensure proper method performance. QA concentration data from both method validation and sample analysis were used to determine inter-assay precision.

Recovery

Absolute recovery of **I** and **II** from plasma was determined by comparing the mean of the peak heights from a set of six replicate extracted samples to a set of six replicate unextracted samples. The extracted samples were prepared by spiking **I** or **II** into plasma at a level of 500 pg ml^{-1} and extracting as described above. The unextracted samples were made by spiking **I** or **II** into extracted control blanks prior to being reconstituted.

Overall recovery (including loss through sample filtration) was evaluated by comparison of the set of six replicate extracted samples to six standard controls which were prepared by

adding identical spiking solutions of **I** or **II** to those used above into empty tubes, evaporating and reconstituting without filtering.

Room temperature stability

To determine stability at room temperature, aliquots of control human plasma spiked with 300 ng ml^{-1} of Ro 24-0238 were left on the bench top exposed to fluorescent lights or shielded from light at room temperature. Five replicates from each portion were taken for processing immediately after preparation and after standing for 6 h. The samples were analysed with the conventional bore method.

Results and Discussion

Chromatography

This method used multidimensional chromatography with transfer from a cation exchange to a reversed-phase column. The chromatographic separation was based on the mechanisms described below: under the first set of mobile phase conditions (pH 3.0), the substituted pyridines **I** and **II** were equilibrated to their conjugate acids (pyridiniums); the pyridiniums had stronger retention than most of the endogenous components on the ion exchange column, which provided a good separation of **I** and **II** from most plasma impurities based on their pK_as. Therefore, during each column switching, only a small and clean fraction from the first dimension column was transferred to the second column. Without the first column separation, a direct injection of a plasma sample to the second column resulted in extreme overloading of the narrow bore column. Upon transfer to the second (reversed-phase) column, **I** and **II** were further separated from each other, as well as from minor endogenous components of plasma, due to differences in lipophilicity. Co-elution of **I** and **II** on column 1 also enhanced the chromatography on the reversed-phase column by minimizing the volume to be switched to the second column, therefore avoiding extra column peak broadening. Furthermore, the analyte was pre-concentrated on the head of the second column, since the first mobile phase had much less organic content than the second mobile phase. This resulted in peak compression, and therefore, increased sensitivity. Because the compressed peak gave very good resolution, it was possible to further enhance sensitivity through improved signal-to-noise by using a relatively long flow cell (6 mm, 9 μl) with a wide bore (1.4 mm).

Figure 3 shows chromatograms of both spiked and experimental samples. A typical run time for plasma samples was 16 min.

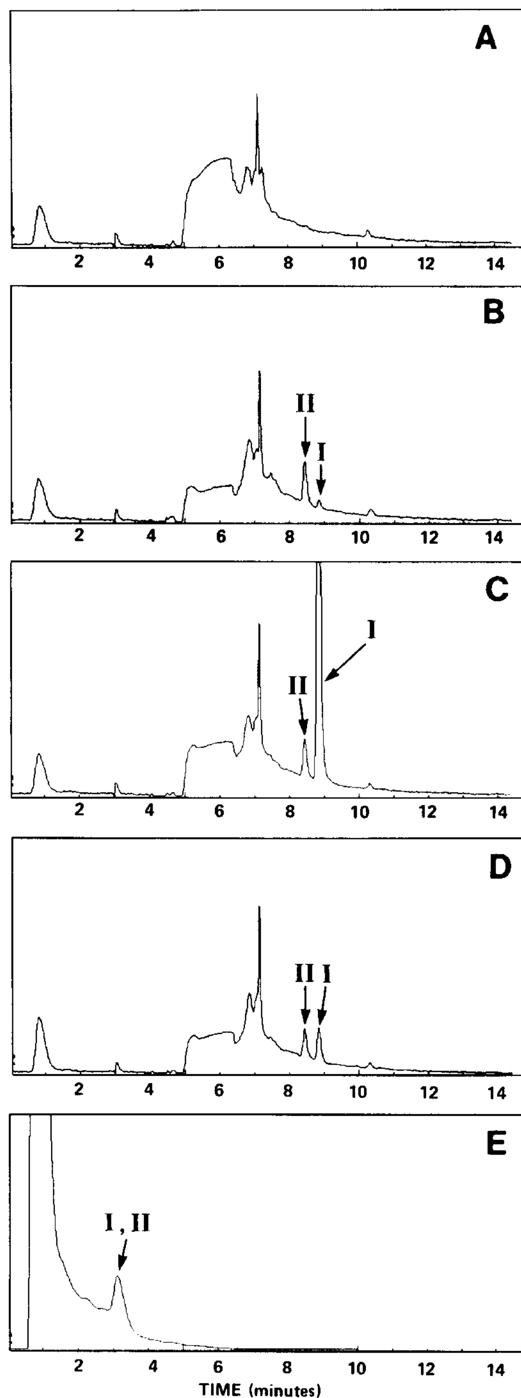

Figure 3

Typical chromatograms of both spiked and experimental samples: (A) plasma blank; (B) 50 pg ml^{-1} of **I** and 500 pg ml^{-1} of **II** in human plasma; (C) 5000 pg ml^{-1} of **I** and 500 pg ml^{-1} of **II** in human plasma; (D) low dose experimental sample; (E) first dimensional chromatogram.

Typical retention times for **I** and **II** on column 2 were 8.8 and 8.4 min, respectively. Control blanks showed no interfering peaks from endogenous components of the plasma matrix, and the analyte peak at the low concentration limit was easily detectable. Baseline resolution of **I** and **II** at the high concentration limit was still obtained. Operation of the column oven at 46°C provided a stable temperature environment and high column efficiency through improved mass transfer.

Sample preparation

Due to high protein binding, it was necessary to treat plasma samples with acetonitrile prior to extraction to obtain good recovery. The solid-phase extraction method was efficient at reducing the plasma impurities to an acceptable level prior to separation on the narrow bore chromatography system. It was essential to use water to wash the plasma matrix impurities off the solid-phase cartridge. If the cartridge was washed with 0.1 N ammonium acetete buffer (pH 4.0) to increase recovery, poor chromatography was observed on the ion exchange column because of the buffer residue remaining in the sample. The potential for degradation of the sample during drying and storage was a concern (described below). Therefore, temperature and time limits during evaporation and sample storage were employed. Filtration of the reconstituted samples to eliminate particles extended column life and performance.

Recovery

The absolute recovery for solid phase extraction of **I** and **II** was 77.1 and 99.4%, respectively. The overall recovery was 70.8% for **I** and 72.6% for **II**. The difference in recoveries was attributed to loss during filtration.

As mentioned above, if 0.1 M ammonium acetate buffer (pH 4.0) was used instead of water to wash the SPE cartridge, the recovery was increased to more than 80%. However, with the use of a small ion exchange column, the ammonium acetate residue tended to saturate the active sites at the front of the column, shifting retention times. Although filtration of the samples reduced recovery, it was necessary to ensure good column performance.

Specificity, linearity and sensitivity

The analyte and the internal standard were well separated from endogenous components of human plasma under the experimental conditions. No interfering peaks were found in four lots of human plasma.

For a typical calibration curve, the slope, intercept, standard error of the slope and the standard error of the intercept were 2.588, 0.061, 0.042 and 0.006, respectively, using a set of seven standards ranging from 50 to 5000 pg ml^{-1}.

With UV detection at 322 nm wavelength, precise and accurate quantitation at 50 pg ml^{-1} was achieved. An analyte concentration of 20 pg ml^{-1} was still detectable, but the reproducibility was limited by the inherent detector sensitivity.

Precision and accuracy

Two of the four concentrations of quality assurance samples were placed close to the limit of sensitivity to assure the accuracy and precision of the method at low concentration levels. As shown in Table 1, the intra-assay precision was acceptable at 3.6% RSD, and the per cent error was less than ±11%. The overall inter-assay precision was 3.9% RSD, which indicated good day-to-day reproducibility.

Stability of plasma samples

The data from the stability experiment demonstrate that Ro 24-0238 is stable in human plasma for up to 6 h at room temperature. Exposure to light did not appear to have an effect on stability. The sample concentrations were found to be 301 ± 2.4 at 0 h,

Table 1
Intra-assay precision, inter-assay precision and accuracy of I from quality assurance samples

Intra-assay precision and accuracy				
Theoretical conc. (pg ml^{-1})	Mean conc. (pg ml^{-1})	RSD (%)	Error* (%)	*n*
140	125	2.2	−10.7	5
200	186	4.6	−8.0	5
1500	1476	5.4	−1.7	5
4000	4159	2.0	+4.0	5
Overall		3.6		

Inter-assay precision				
Theoretical conc. (pg ml^{-1})	Mean conc. (pg ml^{-1})	SD	RSD (%)	*n*
140	126	7.9	6.3	5
200	185	7.7	4.1	5
1500	1506	20.6	1.4	5
4000	4066	153.6	3.8	5
Overall			3.9	

*% Error = [(mean − theoretical)/theoretical] × 100.

296 ± 2.2 after 6 h exposure to light and 300 ± 1.6 after 6 h shielded from light, respectively. Low temperature evaporation and freezer-storage of the samples were suggested in the Sample Preparation section. The reason was that a small, potentially interfering peak (approximately the peak height of 20 pg of **I**) was found from decomposition of the endogenous impurities when the samples were handled improperly.

Column performance

The performance of the narrow bore analytical column was found to be excellent; no evidence of column deterioration was observed after 1000 samples had been injected. The first dimension ion exchange column allowd 200 injections with good column performance. Slowly increasing back-pressure on the first column was observed due to the large amount of endogenous impurities in the sample. In spite of its relatively short life time, the 30 × 3 mm i.d. P-SCX column was reliable and gave good performance compared to other types of columns that were tried, such as a 30 × 3 mm i.d. RP-SCX column and a Pharmacia MONO S HR 5/5 column.

Method application

The present narrow bore LC method has been applied to the analysis of human plasma samples obtained from pharmacokinetic studies. Figure 4 shows the plasma concentrations of **I** versus time determined following a 100 mg dose given to two fed subjects. Prior analysis of these samples by the conventional bore method found measurable levels at only three time points (dashed line in Fig. 4). However, complete plasma level profiles were obtained through the higher sensitivity of the new narrow bore method. The accuracy of both analytical methods is apparent in the three time points where data is available for comparison.

Samples from three subjects given a low dose of **I** (5 mg fasted), which produced no measurable levels with the conventional method, were also analysed. Plasma concentrations of **I** were detected at 0.5 and 1.0 h for two subjects, and at 30 min only for one subject.

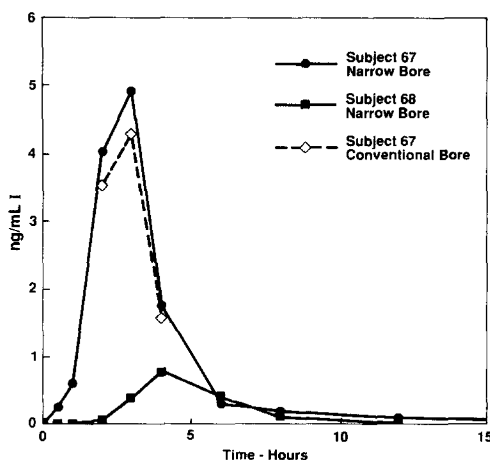

Figure 4
Plasma Ro 24-0238 concentration vs time profile: 100 mg dose given to two fed subjects assayed with narrow bore and conventional bore HPLC methods.

Conclusions

A selective LC methodology with high sensitivity has been developed and validated for quantitation of Ro 24-0238 in human plasma. The use of a highly automated sample preparation system and column switching technology in a narrow bore HPLC system afforded a reliable and sensitive method for assaying low drug plasma concentrations. The method showed good linearity, precision and accuracy over the range of 50–5000 pg ml^{-1} of **I** in human plasma, and it is useful for pharmacokinetic studies, with Ro 24-0238 administered in low oral doses.

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