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Determination of Ro 23-7637 in dog plasma by multidimensional ion-exchange–reversed-phase high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

Ro 23-7637 (I) is a new drug under development for the treatment of metabolic diseases. A high-performance liquid chromatographic–ultraviolet detection (HPLC–UV) analytical procedure for its analysis in dog plasma was developed and is reported here. Following C_{18} solid-phase extraction, the sample is applied to a strong cation-exchange column in the first dimension. The analyte and internal standard, Ro 24-4558 (II), are transferred to a base-deactivated C_{18} reversed-phase column in the second dimension (orthogonal separation mechanism), with UV detection at 254 nm. The reversed-phase solid-phase extraction provides a gross isolation of the drug, based on hydrophobicity. The first-dimension ion-exchange separation allows neutral species and anions to elute with the column void volume, while separating basic species according to pK_a. The second dimension provides a high-resolution separation dependent upon the hydrophobicity of the sample species. The rationale for using orthogonal multidimensional chromatography was that an exhaustive examination of reversed-phase and normal-phase columns invariably resulted in co-elution of I with endogenous plasma components, which limited the sensitivity of the method. We have utilized C_{18} solid-phase extraction, followed by multidimensional HPLC–UV, to develop an accurate and precise analytical method whose limit of quantitation, 5 ng/ml using 0.5 ml of plasma, is determined by inherent detector sensitivity. Increased sensitivity can be readily achieved by using more sample or by using microbore HPLC on the second dimension.

INTRODUCTION

Column switching is an exceptionally versatile tool for the HPLC analysis of complex samples [1,2]. HPLC analysis of drugs in biological fluids, using column switching as a means of sample clean-up with direct injection or limited sample pretreatment [3–8], is a commonly accepted tool for analysis of drugs in complex biological fluids. There is a strong theoretical justification for using true multidimensional chromatography with non-redundant separation mechanisms [9,10]. Its usefulness for separating the analyte from trace endogenous components with or without selective off-line sample clean-up, such a solid-phase or liquid–liquid extraction, has been widely recognized [11–17]. However, the percentage of methods utilizing ion-exchange coupled with reversed-phase chromatography for compounds in biological samples is relatively small [18–20]. An analytical procedure for the analysis of Ro 23-7637 (I, Fig. 1) in dog plasma was needed to support the development of I. A method utilizing orthogonal multidimensional chromatography was required after numerous reversed-phase and

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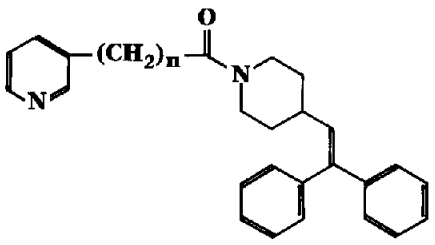

I: n= 8
II: n=10

Fig. 1. Structures of I and II.

normal-phase columns failed to separate I from co-eluting endogenous plasma components. We report a reversed-phase solid-phase extraction–strong cation-exchange chromatography–reversed-phase chromatography multidimensional separation for the analysis of I in dog plasma in a concentration range of 5–2000 ng/ml with Ro 24-4558 (II, Fig. 1) as the internal standard.

EXPERIMENTAL

Chemicals and materials

Ro 23-7637, 4-(2,2-diphenylethenyl)-1-[1-oxo-9-(3-pyridinyl)nonyl]piperidine (I), and Ro 24-4558, 4-(2,2-diphenylethenyl)-1-[1-oxo-11-(3-pyridinyl)undecyl]piperidine (II), were obtained from the Quality Control Department, Hoffmann-La Roche (Nutley, NJ, USA). Ammonium acetate (ACS reagent grade), sodium hydroxide (ACS reagent grade), hydrochloric acid (ACS reagent grade), 90% formic acid (purified), acetonitrile (HPLC grade), and acetone (ACS reagent grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Glacial acetic acid (HPLC grade) was obtained from J. T. Baker (Phillipsburg, NJ, USA). Distilled water was purified with a Milli-Q UF Plus water purification unit (Millipore, Bedford, MA, USA). Waters Sep-Pak Plus C_{18} solid-phase extraction cartridges were obtained from Millipore (Milford, MA, USA). Dog plasma was obtained from Rockland (Gilbertsville, PA, USA).

Instrumentation

Solid-phase extraction was performed on a MilliLab 1A Workstation (Millipore). A liquid chromatograph capable of collecting a portion of the effluent from one HPLC column in the loop of a six-port switching valve and backflushing the contents of the loop onto a second HPLC system was constructed from the following components (Fig. 2) (items in parentheses refer to labeled items in Fig. 2): a Waters 600 gradient HPLC pump (pump 1); a Waters M6000A HPLC pump (pump 2); a Waters Model 441 UV absorbance detector, with 254-nm lamp and filter (detector 1); a Waters Model 712 WISP autosampler (Millipore); an ABI Model 783A programmable absorbance detector (detector 2) (Applied Biosystems, Foster City, CA, USA); an SSI Model 505 HPLC column oven (Scientific Systems, State College, PA, USA); a Rheodyne Model 7060P pneumatically actuated switching valve with a 2-ml loop, controlled by a 120 VAC Rheodyne Model 7163-031 solenoid valve kit, a Rheodyne Model 7315 precolumn filter (Rheodyne, Cotati, CA, USA); pre-cut 0.007″ and 0.005″ I.D. × 1/16″ O.D. stainless-steel tubing (Upchurch Scientific, Oak Harbor, WA, USA); 0.005″ I.D. × 1/16″ O.D. PTFE tubing (Beckman Instruments, Fullerton, CA, USA). Data collection was carried out by a P. E. Nelson 3000 Series chromatog-

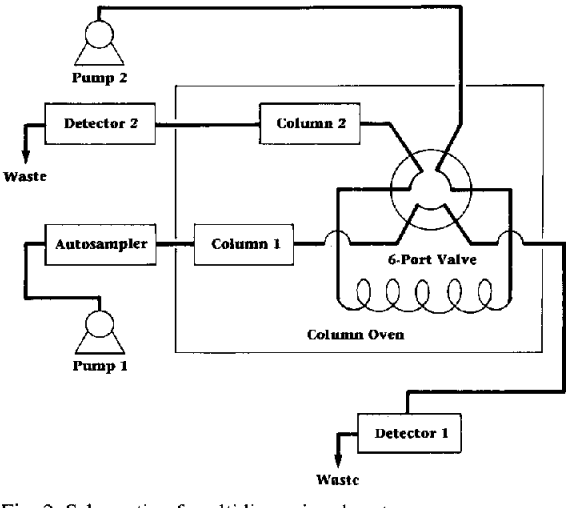

Fig. 2. Schematic of multidimensional system.

raphy data system with a Model 960 intelligent interface and Model 2600 chromatography software (Perkin Elmer Nelson Systems, Cupertino, CA, USA). A relay module consisting of a 5-V power supply and two single-pole double-throw relays allowed contact closures on the A/D module to control the solenoid valves, and thereby change the position of the switching valve during the chromatographic run. The precolumn filter was connected directly to the injector module on the autosampler with a 10-cm section of 0.007″ I.D. tubing; the filter outlet was connected to the heat exchanger on the oven with a 20-cm section of 0.005″ I.D. tubing. Internal connections in the oven were made with the shortest practical lengths of 0.005″ I.D. stainless-steel tubing; detector 1 was connected to the switching valve and detector 2 was connected to column 2 with the shortest practical lengths of 0.005″ I.D. Teflon tubing.

Sample preparation

Calibration standards were prepared in duplicate by adding 0.5-ml aliquots of heparinized dog plasma, 50-μl aliquots of I spiking solutions and 50-μl aliquots of II (internal standard) working solution (in acetonitrile) to 100 mm × 16 mm culture tubes and vortex-mixing. Standards contained 5, 10, 20, 50, 100, 200, 500 and 2000 ng/ml I and 250 ng/ml II. For experimental samples, 50 μl of II (internal standard) working solution and 50 μl of acetonitrile were added.

Sample precipitation/extraction

Aliquots (2 ml) of acetonitrile were added to each calibration standard, control blank, quality assurance sample, and experimental sample. The samples were vortex-mixed vigorously for 10 s and centrifuged for 10 min at 2500 *g*. The supernatant from each sample was decanted into a clean 100 mm × 16 mm culture tube. These tubes were placed into a MilliLab automated sample processor rack for extraction. For each sample and standard, the following extraction procedure was employed: a Sep-Pak Plus C_{18} solid-phase extraction cartridge was conditioned with 3 ml of acetonitrile and washed with 3 ml of 0.1 *M* ammonium acetate buffer, pH 4.0. A 4-ml volume of 0.1 *M* ammonium acetate buffer, pH 4.0 was added, and the sample was mixed by the MilliLab workstation. A 6.5-ml sample was applied to the solid-phase extraction cartridge. The cartridge was washed with 3 ml of 0.1 *M* ammonium acetate buffer, pH 4.0, and 4 ml of air were forced through the cartridge. The analyte and the internal standard were eluted from the cartridge with 5 ml of acetone into a 100 mm × 16 mm culture tube. The samples were evaporated to dryness under a stream of dry nitrogen in a 35°C N-Evap water bath. Each sample was reconstituted with 400 μl of acetonitrile–0.1 *M* HCl (50:50, v/v) and the samples were vortex-mixed for 2 min at maximum speed on an S/P multitube vortex mixer. The samples were transferred to autosampler vials with limited-volume inserts.

Sample analysis

The multidimensional chromatographic system was operated as follows: the first-dimension column was a Mac-Mod Zorbax 300 SCX column, 150 mm × 4.6 mm I.D., equilibrated with acetonitrile–0.03 *M* sodium formate, pH 3 (50:50, v/v), with a flow-rate of 1 ml/min. The second-dimension column was a Mac-Mod Zorbax Rx-C_{18} column, 250 mm × 4.6 mm I.D. (Mac-Mod Analytical, Chadds Ford, PA, USA), equilibrated with acetonitrile–0.1 *M* ammonium acetate buffer, pH 4 (88:12, v/v), with a flow-rate of 1 ml/min. The column oven was operated at 42°C; both detectors were set for detection at 254 nm. A synthetic mixture containing 1000 ng of I and 125 ng of II per 400 μl of reconstitution solvent was injected before each set of samples to determine correct valve timing, and the data system-timed events file controlling the relays was modified accordingly. Valve timing was set so as to minimize collection of the first-dimension eluent before and after the elution of the peaks of interest.

One each of the duplicate set of calibration standards was injected at the beginning and the end of each sample analysis run, bracketing the quality assurance standards, control blanks, and experimental samples. From the peak-height ra-

tios of I to the internal standard, a linear calibration curve was constructed, and the slope and the intercept of the calibration curve were calculated by using weighted ($1/y^2$) linear regression. Concentrations of I were calculated from the peak-height ratio of samples with the use of the parameters obtained above.

Sample stability

The stability of I in dog plasma at room temperature was determined by adding I to plasma and analyzing 0.5-ml aliquots immediately (three replicates), after 3 h (five replicates), and after 6 h (four replicates).

The relative stability of I and II in the reconstitution solvent was determined by comparing the peak-height ratio before and after 24 h at room temperature.

Recovery

Absolute recovery of I and II from plasma was determined by comparing the mean of the peak heights from two sets of six replicate blank plasma samples spiked at 400 ng/ml I or 500 ng ml II to the mean of the peak heights from six replicates consisting of extracted control blanks which were spiked with analyte or internal standard at the same level as above before reconstitution.

RESULTS AND DISCUSSION

Method development

Sample preparation was performed using reversed-phase solid-phase extraction. Due to high protein binding, it was necessary to treat plasma samples with acetonitrile prior to extraction to obtain good recovery. In addition, acetone was needed to elute I from the solid-phase extraction cartridge, due to its high lipophilicity. This allows effective washing of the cartridge to remove polar molecules without loss of the analyte. The solvent used to reconstitute the sample and the reconstitution volume were crucial to obtaining good reproducibility and recovery.

HPLC with UV detection was chosen as the analytical method because the drug does not possess sufficient volatility for gas chromatography; nor does it possess native fluorescence, or easily oxidizable or reducible functional groups for electrochemical detection. UV detection at 254 nm was used (I and II both have absorption maxima at 252 nm and molar absorptivities of 18 594 and 18 586, respectively). The initial, single-dimensional chromatographic system using alkyl silane-bonded reversed-phase columns, polybutadiene-coated alumina reversed-phase columns, as well as normal-phase chromatography on silica gel failed to separate I from endogenous components. Co-elution of endogenous components with the analyte limited sensitivity to at best 50 ng/ml, with 1 ml of plasma. Fig. 3A shows a typical chromatogram.

The analytical method was converted to multidimentional chromatography. Ion exchange was chosen as the first-dimension separation mechanism to exploit ionization of the pyridinium group at low pH, providing a good class separation. Anions and neutral species elute with or close to the solvent front, while bases are separated relative to their pK_a values. The use of solid-phase extraction sample clean-up was fortuitous, since it reduced the ionic strength of the sample, allowing preconcentration on the head of the ion-exchange first-dimension column. Fig. 3B and C show first-dimension chromatograms containing both I and II in dog plasma without and with activation of the switching valve, respectively. The analyte and the internal standard are separated on this column from both early- and late-eluting peaks. Using an analyte and an internal standard which differed only in the number of carbon atoms in the alkyl chain linking the two ring systems insured that both compounds could be collected together in the switching valve loop.

The Zorbax Rx-C_{18} column used in the second dimension, which separates analytes based on their lipophilicity, provides a non-redundant separation mechanism relative to the first-dimension column. Mechanisms such as ion exchange and gel permeation provide highly compatible first-dimension mechanisms when reversed phase is used in the second dimension, since they require little or no organic content in the mobile phase.

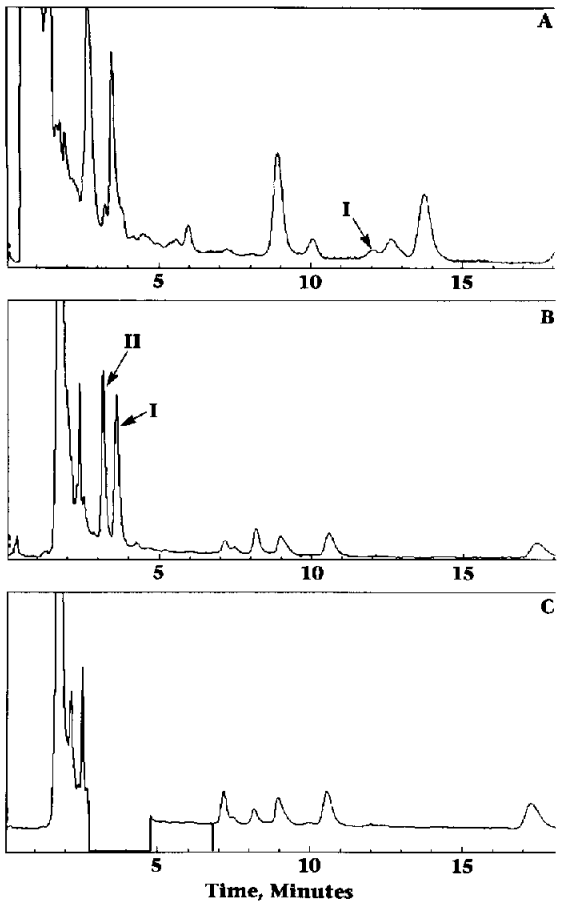

Fig. 3. (A) Chromatogram of I in plasma using a single-dimensional reversed-phase system. Column: Waters Assoc. Nova-Pak C_{18} 75 mm × 3.9 mm I.D.; mobile phase: acetonitrile–0.1 *M* ammonium acetate buffer, pH 4.0 (60:40, v/v) at 1 ml/min; detection: UV absorbance at 254 nm. (B) Chromatogram of I in dog plasma, first dimension (cation-exchange). (C) First-dimension chromatogram showing collection of analyte and internal standard into sample loop.

In this case, there is sufficient difference between the organic content in the first- and second-dimension mobile phases to achieve peak compression in the second dimension. It is also advantageous to backflush the loop onto the second-dimension column. There is a retention order reversal on the second-dimension column between the analyte and the internal standard, relative to the first dimension. Backflushing the loop preserves the separation achieved in the first dimension when the peaks are applied to the second-dimension column. There is only a 10% loss in peak height when the 2-ml sample is injected onto the second-dimension column, relative to injecting the same mass of analyte in 150 μl of reconstitution solvent.

Fig. 4A shows a typical second-dimension chromatogram of I and II. Fig. 4B and C show a chromatogram of a 5 ng/ml standard of I in dog plasma, and an expanded chromatogram of that standard, respectively, while Fig. 4D shows a dog plasma control blank containing neither the drug nor the internal standard. In these chromatograms, the analyte elutes at 13.2 min, while the internal standard, II, elutes at 16.9 min. There are no endogenous peaks in the blank that interfere

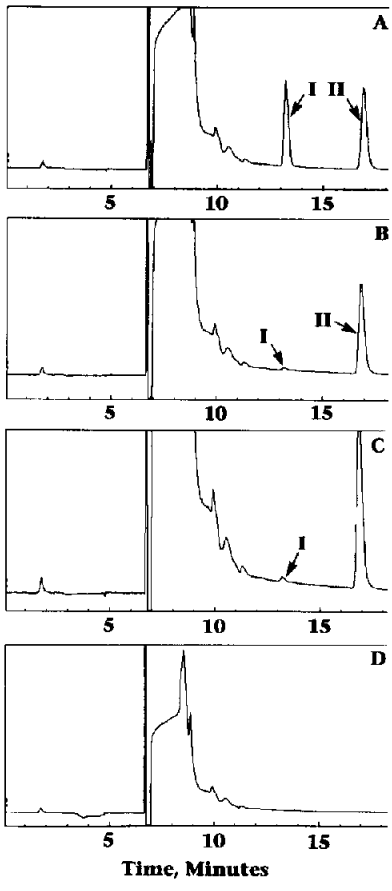

Fig. 4. (A) Chromatogram of I in dog plasma, second dimension, 200 ng/ml standard. (B) Chromatogram of I in dog plasma, second dimension, 5 ng/ml standard. (C) Expanded view of B. (D) Chromatogram of dog plasma control blank.

with either the analyte or the internal standard in the second-dimension chromatograms. The current sensitivity, 5 ng/ml with 0.5 ml of dog plasma, is limited by the inherent detector signal-to-noise ratio and by the amount of sample extracted, rather than by interferences from endogenous components. It is estimated that by extracting 1 ml of plasma and by using a microbore column in the second dimension, the detection limit could be reduced to below 0.5 ng/ml.

Recovery

Recovery was 86.5% for I and 83.1% for II. The recovery of I and II was improved by reconstituting the extracts in acetonitrile–0.1 *M* HCl (50:50, v/v) instead of first-dimension mobile phase. Increasing the aliquot size of the reconstitution solvent from 0.2 to 0.4 ml further increased recovery.

Precision

The intra-assay precision was estimated by performing twenty replicate analyses on a plasma sample fortified with 200 ng/ml I and 250 ng/ml II (internal standard). The relative standard deviation of the peak-height ratio was 6.6%, providing acceptable reproducibility.

The inter-assay precision was estimated by determining the mean and the relative standard deviation (R.S.D.) for the values obtained for the high and low quality assurance samples on three separate days. The R.S.D. for the low (80 ng/ml) quality assurance sample was 2.03%, while the R.S.D. for the high (800 ng/ml) quality assurance sample was 1.04%. The overall R.S.D. (average of R.S.D.s for high and low quality assurance samples) was 1.54%, indicating excellent day-to-day reproducibility.

Reproducibility was further enhanced during routine sample analysis by substituting water for ammonium acetate buffer in the solid-phase extraction procedure. There was evidence that, occasionally, residual ammonium acetate left after evaporation interfered with the chromatography on the ion-exchange column, causing improper collection of the analytes into the loop.

Linearity

The response factor [analyte peak height/(internal standard peak height × analyte concentration)] differed by less than 5% from the mean response factor for all calibration levels, indicating good linearity for the method.

Stability

Analysis of plasma which had been allowed to stand for 0, 3 and 6 h at room temperature yielded mean ± S.D. values of 471 ± 4.2 ng/ml (n = 3), 478 ± 6.4 ng/ml (n = 5) and 480 ± 4.8 ng/ml (n = 4). These data demonstrate that I is stable in dog plasma for 6 h at room temperature. The concentration of I in dog plasma stored at −20°C for 231 days remained essentially unchanged.

A full autosampler tray of 48 samples requires over 16 h to analyze, so the relative stability of the drug in reconstitution solvent was a concern. The average ± S.D. of the change in the peak-height ratio from a calibration curve run immediately and after standing 24 h was +1.3 ± 3.2%. These data demonstrate that the relative stability of I and internal standard is good.

Application to experimental samples

The plasma concentration *versus* time curves for three male beagle dogs given a 30-mg oral dose of compound I are shown in Fig. 5.

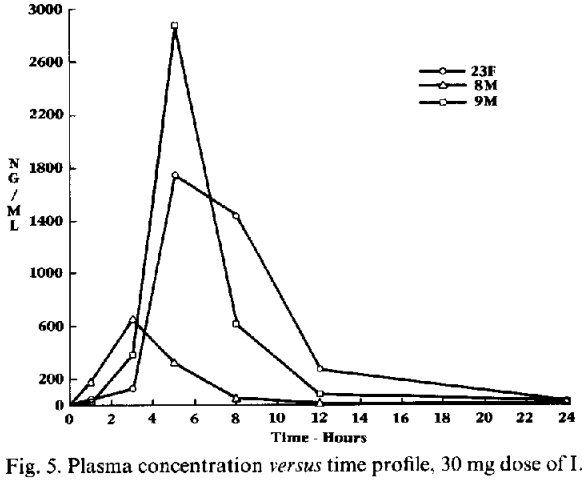

Fig. 5. Plasma concentration *versus* time profile, 30 mg dose of I.

Reliability

No problems in reliability were observed related to the increased complexity of the multidimensional system. Changing the precolumn filter element after every second tray was required to keep backpressure low on the first-dimension system. A benefit from solid-phase extraction was good column life, with over 300 sample injections obtained per column.

CONCLUSIONS

A specific HPLC analytical method with sensitivity limited only by the inherent signal-to-noise ratio of the detector has been developed for I by exploiting the resolution benefits of multidimensional chromatography with non-redundant separation mechanisms. The use of a robotic system for solid-phase extraction clean-up of the samples prior to HPLC analysis affords a method that is both robust and non-labor-intensive. The method showed good linearity over the range 5–2000 ng/ml I in dog plasma, based upon a 0.5-ml aliquot of sample. Further reductions in detection limits should be possible using a larger sample aliquot or employing microbore chromatography in the second dimension.

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