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Determination of a new antibacterial agent (Ro 23-9424) by multidimensional high-performance liquid chromatography with ultraviolet detection and direct plasma injection

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ABSTRACT

Analysis of a new antibacterial agent, Ro 23-9424 (I), in plasma has been complicated by the fact that its metabolite, fleroxacin (II), is formed not only *in vivo*, but also nonenzymatically by the hydrolysis of the ester bond of I. In order to minimize sample preparation time and possible hydrolysis during sample preparation, a high-performance liquid chromatographic procedure was developed which features direct injection of plasma and multidimensional chromatography. The first dimension size-exclusion separation allows plasma proteins to elute with the column void volume. The second dimension reversed-phase column provides a high-resolution separation dependent upon the hydrophobicity of the sample species. With a 5-μl injection, the limit of quantitation of the method is 0.35 μg/ml for I and 0.27 μg/ml for II. The method was used to determine steady state plasma *vs.* time profiles for I and II from 750 mg i.v. doses of I administered twice daily.

INTRODUCTION

Ro 23-9424 (I; Fig. 1) is a novel antibacterial agent which consists of a cephalosporin (desacetylcefotaxime) linked by an ester bond at the 3′ position to a fluoroquinolone (II; fleroxacin). *In vitro* and *in vivo* evaluations indicate that I encompasses the antibacterial spectrum of both its components [1–4]. Fleroxacin can be formed *in vivo* after opening of the β-lactam ring, which leads to the expulsion of a 3′ constituent, or by direct hydrolysis of the ester bond. The ester bond hydrolysis can also take place nonenzymatically.

Since the analysis of I and II in plasma was required, studies characterizing the factors which govern the post-sampling hydrolysis of I were performed. These studies included the effects of pH, temperature and stability in plasma at room temperature and at 4°C, with or without solvent, which in previous methods was added to the samples before storage to eliminate the possibility of hydrolysis by esterases [5–6].

Based on the results of the stability studies, a multidimensional HPLC method with direct injection of plasma was developed for the analysis of I and II in order to minimize variability due to hydrolysis during sample preparation and to allow emphasis on the control of temperature. Although procedures with column switching for the direct analysis of compounds in plasma or serum are commonly used [7–15], direct injection of plasma with size exclusion chromatography in the first dimension has not been reported fre-

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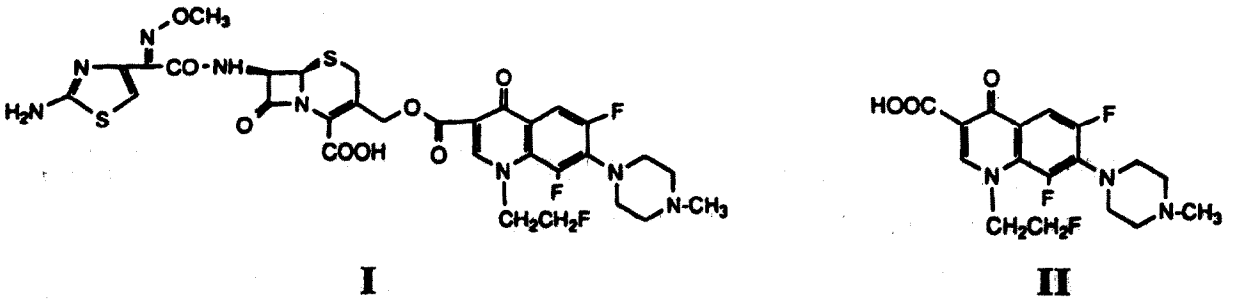

Fig. 1. Structures.

quently [16–18]. Using this technique, we have developed a method which is rapid, reliable and suitable for compounds which are unstable during the extraction procedures.

EXPERIMENTAL

Chemicals

I (Ro 23-9424) and fleroxacin were obtained from Hoffmann-La Roche, Nutley, NJ, USA. Acetonitrile, HPLC grade; phosphoric acid, HPLC grade; and potassium phosphate, dibasic, ACS Reagent Grade were obtained from Fisher Scientific, Fair Lawn, NJ, USA. Isopropanol, HPLC grade, was obtained from Burdick & Jackson, Muskegon, MI, USA. HPLC grade water was purified with a Milli-Q UF Plus water purification unit, Millipore, Bedford, MA, USA.

Apparatus and chromatographic procedure

Column switching was accomplished by collecting a portion of effluent from column 1 into the loop of a 6 port switching valve and backflushing the contents of the loop onto column 2. The HPLC system was constructed from the following components (Fig. 2): a Waters Model 600 HPLC pump (Pump 1); a Waters Model M6000A HPLC pump (Pump 2); a Waters Model 715 WISP autosampler (Autosampler), Millipore, Milford, MA, USA; a LDC SpectroMonitor III detector (Detector 1), LDC Analytical, Riviera Beach, FL, USA; an ABI Model 783A programmable absorbance detector (Detector 2), Foster City, CA, USA; a Rheodyne Model 7060P pneumatically actuated switching valve with a 2-ml loop (Switching Valve), controlled by a 120 VAC Rheodyne Model 7163-031 solenoid valve kit, and a Rheodyne Model 7315 pre-column filter, Rheodyne, Cotati, CA, USA. Data collection was carried out by a P. E. Nelson 3000 Series Chromatography Data System with a Model 60 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 multidimensional chromatographic system was operated as follows: the first dimension column (column 1) was a TSKgel PW_{XL} guard

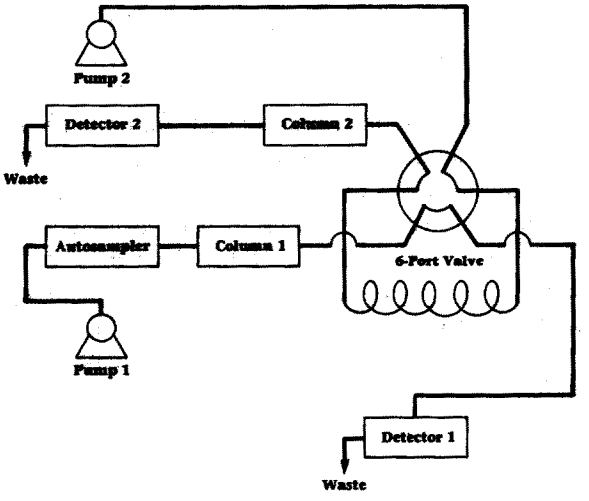

Fig. 2. Schematic of multidimensional system.

column (40 mm × 6.0 mm I.D., 12 μm; Toso-Haas, Philadelphia, PA, USA) equilibrated with isopropanol–0.03 *M* potassium phosphate buffer, pH 6.2 (10:90, v/v), with a flow-rate of 0.6 ml/min. The second dimension column (column 2) was a Zorbax Rx-C8 (250 mm × 4.6 mm I.D., 5 μm; Mac-Mod Analytical, Chadds Ford, PA, USA) equilibrated with acetonitrile–0.05 *M* potassium phosphate buffer, pH 2.7 (18:82, v/v), with a flow-rate of 1 ml/min. A synthetic mixture of 35 μg of I and 8.8 μg of II per ml of water was injected before processing each set of samples to determine correct valve timing, and the events timed by the data system were adjusted accordingly. Both detectors were operated at 287 nm.

Preparation and analysis of standards and samples

During standard and sample preparation, all materials were kept on ice at all times. Standard solutions of I were stored at −70°C.

Calibration standards were prepared in duplicate by mixing 50 μl aliquots of aqueous standard solutions of I or II with 950 μl of control human plasma containing sodium fluoride/potassium oxalate as an anticoagulant. Separate standard curves were needed because even minimal hydrolysis of I during analysis would create an error in the quantitation of II. Standards contained 0.4–40 μg/ml of the dihydrochloride salt of I (equivalent to 0.35–35.01 μg/ml of free compound) or 0.3–10 μg/ml of the monopotassium salt of II (equivalent to 0.27–8.85 μg/ml of free compound).

Control plasma pools containing known concentrations of I or II (quality assurance samples) were stored at −70°C and analyzed in duplicate with every analytical run in order to judge accuracy and precision.

Approximately 250 μl of standards, quality assurance (QA) samples and experimental samples were centrifuged for 5 min at 2500 *g* at 4°C with 0.2 μm cellulose acetate disposable centrifugal microfilters (Schleicher & Schuell, Keene, NH, USA). Aliquots (5 μl) of the filtrates were injected into the HPLC system described above, with the autosampler set at 4°C.

The duplicate calibration standards for I and II were injected at the beginning and end of each sample analysis run. Linear calibration curves were calculated by weighted ($1/y^2$) least squares regression analysis of the peak heights *vs.* concentration data from the calibration standards for I or II. Concentrations of I or II were calculated from the peak heights of samples, with the use of the calibration curve parameters obtained above.

Recovery

The mean of the peak heights from five 1-ml replicate samples spiked with 1.75 μg/ml I or 1.77 μg/ml II were compared with the mean of the peak heights from five replicates consisting of 1 ml aliquots of water spiked at the same level.

Sample stability

Determination of the stability of I or II in plasma after multiple cycles of freezing and thawing was accomplished by analyzing 4 sets of samples (3 replicates per set) which had been frozen and thawed a total of 2, 3, 4 or 5 times. The mean concentrations of the sets were compared to the mean concentration of a set which had been frozen and thawed once before analysis.

After standing for 24 h in the autosampler, QA and freeze/thaw samples were analyzed again with freshly prepared calibration curves to determine the stability of I and II in plasma samples while awaiting injection.

RESULTS AND DISCUSSION

Hydrolysis studies

Preceding method development, studies were performed to determine the factors which contribute to the post-sampling hydrolysis of I and the extent of the effect. In a previous HPLC method [5] and a previous LC–MS method [6], acetonitrile or methanol had been added to plasma before storage as a precaution against hydrolysis by esterases. The 28-h stability of I in plasma, stored at room temperature at 4°C, with and without solvent, was therefore investigated, the assumption being that differences observed between the storage conditions would parallel those

seen over a much longer time period at −70°C. Stability studies were also performed with I dissolved in solvent-deproteinized plasma supernatants and diluted with various buffers in order to determine the effect of solvent, pH and autosampler temperature on hydrolysis.

Data from the stability studies indicated that the most important factor in keeping hydrolysis to a minimum was control of temperature. Methanol did not enhance the stability of I in plasma. Although acetonitrile did decrease hydrolysis somewhat, its addition to plasma before storage was not feasible because of problems with recovery and phase separation at 4°C. With deproteinized plasma, I in supernatant diluted with pH 3 or pH 5 buffer was less stable than in supernatant diluted with pH 8 buffer or water. However, all differences were minimized when the autosampler was set at 4°C instead of room temperature (A. J. Szuna and R. W. Blain, unpublished results). An in-depth discussion of the stability studies is beyond the scope of this paper.

The results indicated that direct plasma injection could be considered, since the important factors in reducing hydrolysis would be reduced sample preparation time and strict temperature control. Low protein binding and expected high concentrations of I and II ($\geq$ 0.2 μg/ml) were additional reasons for the development of a diret plasma injection technique.

Size exclusion chromatography for the direct injection of plasma

For the direct injection of plasma with little or no pretreatment, column switching utilizing one or more pre-columns is commonly used for sample cleanup and trace enrichment [7–12]. When precipitation of proteins is not desired as a pretreatment, various techniques have been used to avoid adsorption of proteins to the initial packing, including the use of a micellar mobile phase with a reversed-phase packing [13] and the use of protein-coated reversed-phase [14], internal surface reversed-phase [15] and size-exclusion supports [16–18].

Because analysis time is an important consideration for an unstable compound, a methacrylate polymer gel pre-column for size-exclusion chromatography, combined with reversed-phase chromatography for the analytical column, was a good choice for I. The use of true multidimensional chromatography with nonredundant separation mechanisms eliminated endogenous plasma peaks, allowing analysis time to be dependent only upon the analyte retention times. The first dimension pre-column was not subject to contamination, circumventing the need for washing and reequilibration steps that could lengthen the run time. A 5-μl injection volume was adequate for the concentrations of analytes found in human plasma; however, since no change in backpressure or chromatography was noted after 560 injections, a larger volume could be used if needed.

Chromatography

After the solvent-front peak due to the first dimension mobile phase, no endogenous plasma peaks were observed (Fig. 3A). Typical second dimension chromatograms of I and II added to plasma and an experimental sample after i.v. administration of I are shown in Figs. 3B, 3C and 3D, respectively.

Sample stability

For I (28.01 μg/ml) and II (7.08 μg/ml) in plasma, the mean concentrations in aliquots that had been frozen and thawed 2 to 4 times ranged from 97.2% to 99.5% and 100.0% to 102.9%, respectively, of the mean concentration in aliquots that had been frozen and thawed once. The data suggest that both compounds are stable in plasma that has been subjected to multiple cycles of freezing and thawing.

The changes in concentrations of I in the QA and freeze/thaw samples after 24 h at autosampler temperature were determined, along with the concentrations of II formed in 24 h. The data suggest that the loss of I in plasma after 24 h is about 11%, while the concentrations of II account for about 4% of the original concentration of I. Under the same conditions, II is stable for 24 h; changes in concentration (−5.5%–1.4%) were probably due to experimental variability.

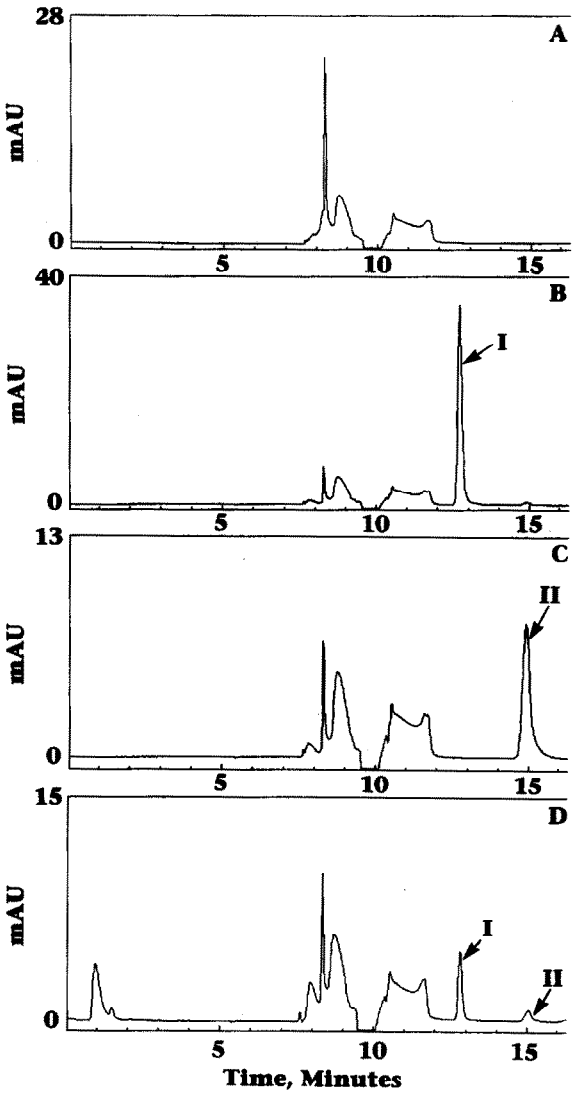

Fig. 3. Second dimension chromatograms: (A) human plasma control blank, (B) I in human plasma–QA sample, 28 μg/ml, (C) II in human plasma–QA sample, 7 μg/ml, (D) I (3.51 μg/ml) and II (0.61 μg/ml) in plasma 3 h after i.v. administration of I.

Precision and accuracy

Since freezing and thawing did not affect the stability of I and II in plasma, analysis of the samples (n = 15) provided intra-day precision data. For I and II, the coefficient of variation was 2.5% and 1.5%, respectively.

The inter-day precision and accuracy of the method were obtained by the duplicate analysis of two concentrations of QA samples on three separate days. For I, the overall C.V.'s for the low (2.63 μg/ml) and high (28.01 μg/ml) concentration QA samples were 4.3% and 3.2%, respectively. The mean of the theoretical values for the low and high concentration QA samples was 98.9% and 105.2%, respectively. For II, the overall C.V.'s for the value of low (1.33 μg/ml) and high (7.08 μg/ml) concentration QA samples were 1.0% and 3.5%, respectively. The mean of the theoretical values for the low and high concentration QA samples was 99.4% and 101.6%, respectively.

Recovery

The overall recovery for I and II after injection of plasma into the HPLC system was 102.5% and 129.2%, respectively. The high recovery of II is probably the result of a matrix effect, due to the presence of plasma proteins on the first dimension column. Since there is no sample preparation, the percent recovery is needed only to demonstrate that the compounds do not elute with the void volume as a result of protein binding.

Application to experimental samples

Steady-state plasma concentrations *vs.* time profiles were determined for I and II after infusion of 750 mg i.v. doses of I over 1 h twice daily. Typical time–concentration curves after the first dose on day 1 are shown in Fig. 4.

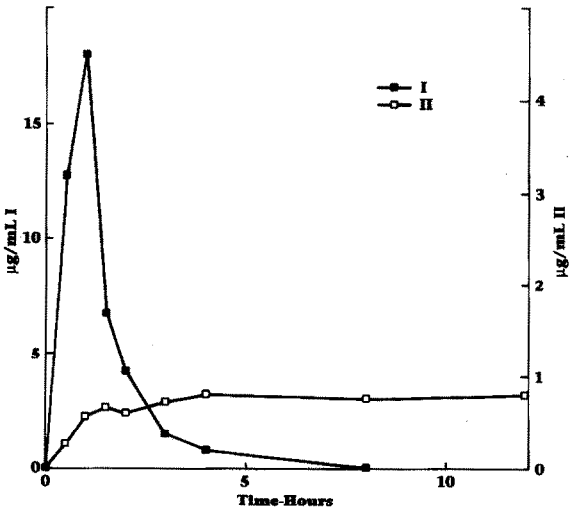

Fig. 4. Plasma concentration *vs.* time profile, day 1 of a multiple dose study, after a 750 mg i.v. dose of I infused over 1 h.

CONCLUSIONS

A method was developed for the analysis of Ro 23-9424 (I) which features direct injection of plasma followed by multidimensional chromatography. Since the ester bond in I is unstable, direct injection reduces sample preparation time and variability due to hydrolysis during extraction procedures. The use of nonredundant separation mechanisms yielded chromatograms with no interfering endogenous plasma peaks. The size-exclusion guard column in the first dimension required a low organic concentration in the mobile phase, which allowed peak compression on the reversed-phase column in the second dimension. The system proved to be reliable, with virtually no change in performance of either column after more than 500 injections. This observation suggests that even larger volumes of plasma can be injected in order to increase sensitivity if needed.

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