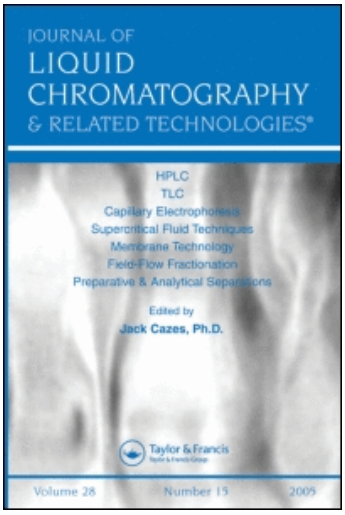

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 17 April 2002

To cite this Article Kim, D. H. , Lee, S. K. , Park, Y. H. and Lee, D. W.(2002) 'DETERMINATION OF CIPROFLOXACIN AND ITS METABOLITES IN URINE, USING ON-LINE SPE', Journal of Liquid Chromatography & Related Technologies, 25: 3, 433 — 443
To link to this Article: DOI: 10.1081/JLC-120008758
URL: http://dx.doi.org/10.1081/JLC-120008758

J. LIQ. CHROM. & REL. TECHNOL., 25(3), 433–443 (2002)

DETERMINATION OF CIPROFLOXACIN AND ITS METABOLITES IN URINE, USING ON-LINE SPE

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ABSTRACT

An isocratic high-performance liquid chromatographic method with column switching and a direct injection system was developed to determine ciprofloxacin, a kind of quinolone derivative and its four kinds of metabolites in urine. A manually actuated system equipped with six port valves allowed a clean-up step with 0.01 M NaH_2PO_4 (pH 7.0) and the transfer of the analyte by a backflush mode. Also, the sample pretreatment condition for spiked urine was investigated to determine the optimum SPE condition for all analytes. All analytes showed over 92% recovery, using C18(10 μm, 30−50 μm), XAD-2 and XAD-4 as precolumn.

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INTRODUCTION

Quinolones are an important group of antibiotics with bactericidal action, used in the treatment of a variety of bacterial infections. Ciprofloxacin is a kind of quinolone carboxylic acid derivative from Gram-negative and Gram-positive bacteria that are resistant to penicillins, cephalosporins, aminoglycosides, β-lactams, and tetracyclins (1–4). Recently, ciprofloxacin has been widely used in the treatment of urinary and respiratory tract infections, and also in gastrointestinal diseases. It is known to react with DNA gyrase inhibition, similar to nalidixic acid and norfloxacin (5). The structures of ciprofloxacin and its metabolites are depicted in Figure 1.

Much research has been conducted about the separation and detection methods of quinolones and their metabolites in biological fluids by use of chromatographic methods, including HPLC (High Performance Liquid

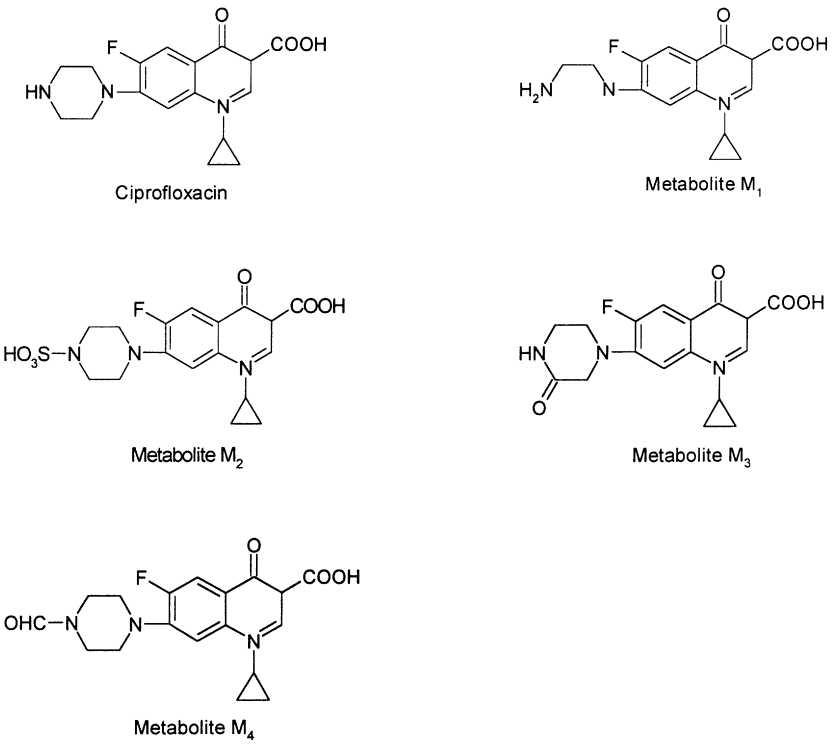

Figure 1. The structures of ciprofloxacin and its metabolites.

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Chromatography) and CE (Capillary Electrophoresis). As a sample pretreatment procedure, most methods involved LLE (Liquid Liquid Extraction) or SPE (Solid Phase Extraction) for the clean-up of biological samples. Pou-Clave et al. proposed an extraction with methylene chloride at serum pH, evaporation of the organic layer and re-extraction of the residue with methylene chloride in the presence of phosphoric acid (6). Weber et al. proposed deproteinization with acetonitrile and direct injection of the supernatant (7). Manceau et al. made simultaneous determination of enrofloxacin and ciprofloxacin in animal biological fluids by HPLC (8). They used Sep-Pak C18 cartridge for sample preparation. Posyniak et al. investigated the effects of the matrix and sample preparation on the determination of fluoroquinolone residues in animal tissues (9).

In this process, they have optimized conditions for SPE, varying the concentration of acetonitrile, trichloroacetic acid. Barron et al. determined the residues of enrofloxacin and its metabolites, ciprofloxacin in biological materials using SPE, followed by capillary electrophoresis with photodiode array detection (10). Hernandez et al. used capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECK) to investigate the separation and determination of 10 kinds of quinolones (11). In the process of sample pretreatment, they used C_{18} Sep-Pak cartridge, a kind of solid phase extraction to remove the sample matrix.

Recently, the clean-up and separation steps have been joined to reduce analysis time and loss of analytes during sample handling with minimum contamination. This analytical system is so called as column switching or on line SPE system. Ba. et al. first developed the fully automated HPLC of ciprofloxacin with direct injection of plasma and Muller-Hinton broth for pharmacokinetic studies (12).

In previous works, we have determined the optimum separation condition for ciprofloxacin and its four kinds of metabolites in urine, involving C18 Sep-Pak cartridge as sample preparation work (13). In this study, we devised a manually actuated column switching system, coupled with HPLC to separate ciprofloxacin and four kinds of its metabolites, spiked to urine samples. In order to minimize the band broadening effect, we allowed the clean-up of matrix and transfer of analytes by a backflush mode. The optimum SPE condition was determined to eliminate the matrix interferences and quantify the analytes. In this process, we investigated the effect of pH of sample and washing buffer on the matrix interferences and band broadening. Also, the recovery of analytes was studied with the change of adsorbents. Using the column switching system, it was possible to determine five kinds of analytes in urine in 20 minutes.

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EXPERIMENTAL

Chemicals

Ciprofloxacin (CFX) was purchased from Sigma, and its four kinds of metabolites (M1, M2, M3, M4) were obtained from Bayer AG (Wuppertal, Germany). The solvents used in this experiment were all HPLC grades. The heptanesulfonic acid (HSA) and tetrabutylammonium bromide (TBABr) were obtained from Sigma. Sodium dihydrogen phosphate and sodium hydrogen phosphate were obtained from Merck.

Chromatographic System and Conditions

A schematic diagram of the column switching system is described in Figure 2. A Waters 600E liquid chromatograph (Waters, Milford, U.S.A.) equipped with an isocratic pump delivered mobile phase 1. The pump 2 was a peristaltic pump, delivering the washing buffer. A manually actuated switching system is composed of an injection valve and a six port switching valve. The HPLC system was composed of a model 600 solvent delivery system (Waters, Milford, U.S.A.), a model 600 UV detector (Waters, Milford, U.S.A.), and a model 7725 Rheodyne injection valve with a 20 μL sample loop (Rheodyne, Cotati, CA, U.S.A.). UV detection was performed at 276 nm.

The chromatograms were collected using a program Autochrowin (Younglin, Seoul, Korea). Chromatography was performed on CAP-CELL PAK C18 column with dimensions of 250×4.6 mm i.d. (Shiseido, Tokyo, Japan). Column temperature was maintained at 40°C using a column oven (Samsung, Seoul, Korea).

The mobile phase 1 was volume to volume ratios of isopropanol to acetonitrile to 0.007 M HSA to 0.005 M TBABr, at a ratio of 11 : 5 : 42 : 42 plus 0.05% triethylamine with a pH of 3.0 adjusted with 99.9% phosphoric acid. The mobile phase 1 flow rate was 1.2 mL/min, the column temperature was 40°C, and the wavelength of UV detection was 276 nm. The mobile phase 2 was 0.01 M NaH_2PO_4, adjusted to different pH values with H_3PO_4. The mobile phase 2 flow rate was 1.2 mL/min.

The switching system included the following four steps:

Step 1: Injection of the sample. The analytes were transferred to the precolumn where they were retained (0–1 min, valve 1 and valve 2 in load position).

Step 2: Purging of precolumn with mobile phase 2. The unwanted compounds were eliminated throughout this step (1–3 min, valve 1 in load position and valve 2 in inject position).

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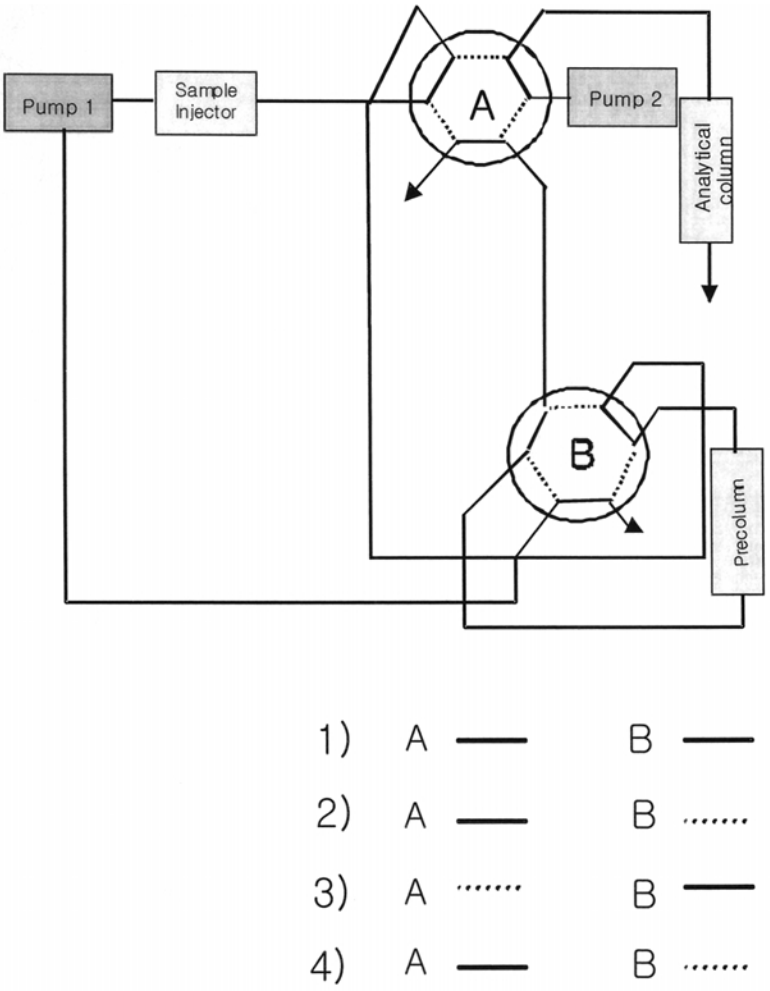

Figure 2. The schematic diagram of column switching system.

Step 3: Desorption of the analytes from precolumn to analytical column. The mobile phase with high elution strength allowed the retained analytes to go to the analytical column in backflush mode (3–20 min, valve 1 in load position and valve 2 in inject position).
Step 4: Preconditioning of precolumn with mobile phase 2. The precolumn was equilibrated with mobile phase 2 (20 − 23 min, valve 1 in inject position and valve 2 in load position).

The injection volume was 50 μL. The adsorbent used for the sample pretreatment was C_{18} Sep-Pak(10 μm, 30–50 μm), XAD-2(150–200 mesh), XAD-4 (150–200 mesh) from Waters.

Sample Preparation

The stock solutions were prepared to 100 ppm by dissolving 1 mg of each sample in 2.0 mL of acetonitrile, diluting with 0.5 mL of 99.9% phosphoric acid and afterwards with water to a final volume of 10 mL. After that, the stock solution was diluted one after another with 0.1 N phosphoric acid to 50, 20, 10, 5, 2, 1, 0.5, 0.1 ppm. After the urine sample was diluted to 1 : 100 with water, 1 mL of diluted urine was diluted again with 4 mL of 0.01 M sodium hydrogen phosphate buffer solution, and then spiked with 0.1 mL of 10 ppm standard solution.

Quantitation

Peak area ratios were used to quantify ciprofloxacin and its metabolites. Quantification of ciprofloxacin and its metabolites was based on calibration curves obtained with external standard samples containing different concentrations of each sample. The concentration of each sample ranged from 0.1 ppm to 50.0 ppm.

RESULTS AND DISCUSSION

Effect of the Change of pH of Spiked Sample on Matrix Interference

In a previous paper, we reported that the optimum separation condition for ciprofloxacin and its metabolites, volume to volume ratios of isopropanol to acetonitrile to 0.007 M HSA to 0.005 M TBABr, was 11 : 5 : 42 : 42 plus 0.05% triethylamine with a pH of 3.0 adjusted with 99.9% phosphoric acid. We investigated the effect of pH for spiked urine sample on matrix interferences. As shown in Figure 3, the interfering peak appeared between CFX and M3 at the condition of pH 3.0 and pH 5.0. However, as the pH of spiked urine sample was increased up to 7.0, this effect was found to be negligible, making the quantification of M3 possible. The reason for this phenomenon is not clear. This might be related to the elution of unknown endogeneous components, similar to the polarity of CFX or M3, depending on pH condition of spiked sample. Therefore, we spiked five kinds of analytes to urine at the condition of 0.01 M NaH_2PO_4 (pH 7.0).

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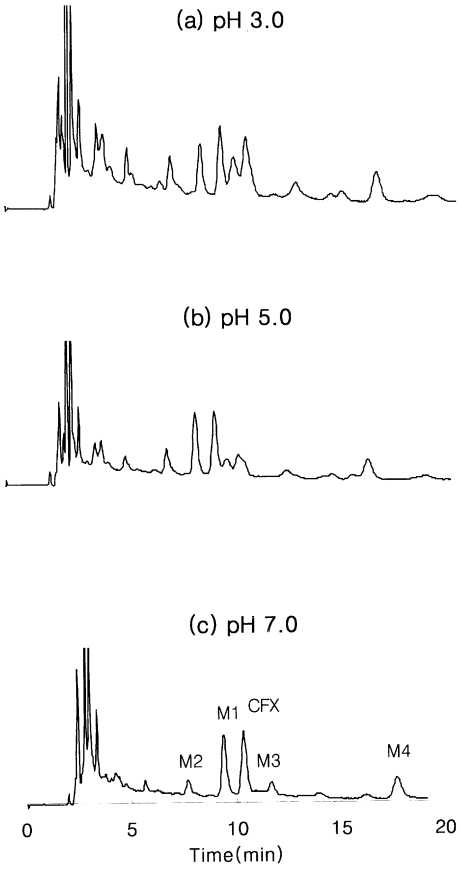

Figure 3. The chromatograms of ciprofloxacin and its metabolites in spiked urine at different pH, backwashed by 0.01 M NaH_2PO_4, adjusted to pH 3.0. (a) pH 3.0; (b) pH 5.0; (c) pH 7.0.

Effect of the Change of pH of Washing Buffer on Band Broadening and Matrix Interference

We investigated the effect of the pH change of washing buffer on the elimination of matrix components and band broadening. A backward washing mode was more effective in reducing band broadening than forward washing. It was expected that the band broadening effect would be minimized by making the pH of washing buffer equalized to that of mobile phase condition. As shown in Figure 4, this expectation turned out to be true. As the pH value of washing buffer

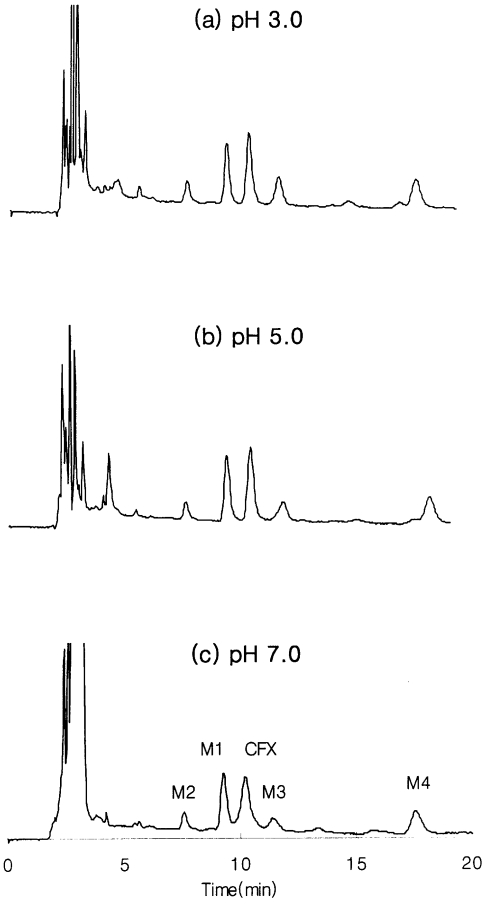

Figure 4. The chromatograms of ciprofloxacin and its metabolites in spiked urine at pH 7.0, backwashed by 0.01 M Na_2HPO_4; (a) pH 3.0; (b) pH 5.0; (c) pH 7.0.

was more acidic, the band broadening was more reduced. This phenomenon can be explained by considering the pKa values of analytes.

Under the acidic mobile phase condition (pH 3.0), spiked analytes at pH 7.0 would usually exist as zwitterionic or neutral form (for ciprofloxacin; pKa $1 = 6.0$, pKa $2 = 8.8$) as well as protonated form. This is the reason why band broadening is more pronounced at pH 7.0 than at the more acidic conditions. Also, the effect of the elimination of urine matrix was more pronounced at the acidic condition. Therefore, we have determined the optimum washing condition as 0.01 M Na_2HPO_4, adjusted to pH 3.0 with backflush mode.

The Effect of Various Adsorbents on Recovery of Analytes

Based on the optimum SPE condition, four kinds of adsorbents, C18 (10 μm), C18 (30–50 μm), XAD-2 and XAD-4 were used to compare the extraction efficiency of analytes, ciprofloxacin, M1, M2, M3, and M4. Especially, XAD-2 and XAD-4 resins were included, here, to investigate the adequacy of extraction of quinolones in urine, for they are shown to be good adsorbents for hydrophobic organic compounds. As shown in Figure 5, there was no significant difference in the recovery of analytes among four kinds of adsorbents. On the whole, the average recovery of all analytes was over 92% and no special interferences from these packing materials were found. The particle size of C18 packing material did not make a big difference in the recovery of analytes. Among the adsorbents, C18 (10 μm) was somewhat superior to the other packing materials.

Validation

The recoveries of ciprofloxacin, M1, M2, M3, and M4, extracted from the spiked urine were calculated by pre-made calibration curves. Good linearity was observed for all analytes in the range from 0.1 ppm to 50.0 ppm ($r^2 > 0.99$). All analytes were extracted with high efficiency (> 89%) from spiked urine. Tables 1 and 2 show the results of intra-assay and inter-assay of the method. Average variabilities for all types of assays were calculated at less than 10.8%.

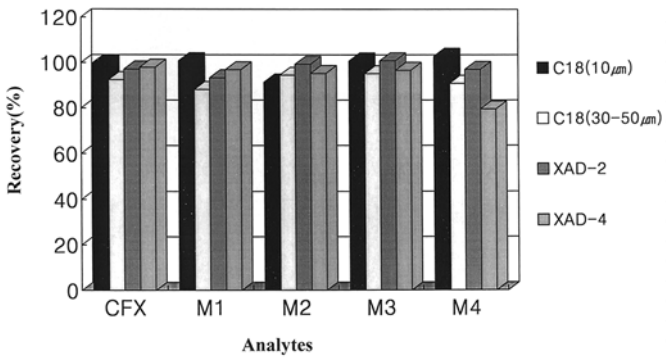

Figure 5. The recovery of ciprofloxacin and its metabolites on different kinds of adsorbents.

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Table 1. Recovery of Ciprofloxacin and Its Metabolites, Spiked to Diluted Urine, Using C18 (10 μm) as a Precolumn (Inter-Day Assay)

	Recovery (%); $n = 3$		
Analyte	1.0*	2.0*	5.0*
Ciprofloxacin	100.1 ± 0.4	107.7 ± 0.3	107.0 ± 0.6
M1	101.0 ± 0	109.5 ± 0.3	108.2 ± 0.7
M2	87.0 ± 10.8	117.1 ± 0.6	108.4 ± 0.8
M3	83.2 ± 2.4	102.0 ± 8.1	83.6 ± 0.1
M4	88.9 ± 7.3	103.3 ± 0.7	106.8 ± 1.1
Average	**92.0**	**107.9**	**102.8**

*Concentration of spiked analyte (μm/g).

Table 2. Recovery of Ciprofloxacin and Its Metabolites, Spiked to Diluted Urine, Using C18 (10 μm) as a Precolumn (Intra-Day Assay)

	Recovery (%); $n = 3$		
Analyte	1.0*	2.0*	5.0*
Ciprofloxacin	90.9 ± 6.2	106.3 ± 3.6	106.0 ± 2.7
M1	91.7 ± 1.9	113.8 ± 1.2	110.5 ± 4.2
M2	93.8 ± 2.0	117.8 ± 0.6	110.2 ± 3.8
M3	84.0 ± 3.2	92.7 ± 8.5	105.3 ± 9.1
M4	87.9 ± 2.0	87.3 ± 1.2	94.1 ± 1.3
Average	**89.7**	**103.7**	**105.2**

*Concentration of spiked analyte (μm/g).

CONCLUSION

In this study, five kinds of analytes, ciprofloxacin, M1, M2, M3, and M4, which were spiked to urine samples, were baseline separated using an on-line SPE system with backflush mode. The backflush mode was more effective for reducing band broadening than the forward flush mode. Also, as the washing buffer was more in acidic condition, the matrix interferences were eliminated more. Among the spiked pH conditions for sample preparation, pH 7 was adequate to quantify the analytes. The recovery of all analytes were over 92%, using precolumn as C18 (10 μm, 30–50 μm), XAD-2 and XAD-4. This column switching system is expected to be applicable to the analysis of other quinolone derivatives in various biological fluids in a simple way.

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ACKNOWLEDGMENT

This work was supported by KOSEF.

REFERENCES

1. Norris, S.; Mandell, G.L. In *The Quinolones*; Academic Press: London, 1988, 4.
2. Level, M. Pharmacotherapy **1998**, *8*, 3.
3. Borner, K. J. Clin. Chem. Clin. Biochem. **1986**, *24*, 325–331.
4. Gau, W.; Kurz, J.; Petersen, U.; Ploschke, H. J.; Wuensche, C. Arzeim-Forsch/Drug Res. **1986**, *36* (10), 1545–1549.
5. Awni, W. M.; Clarkson, J.; Guay, R. P. J. Chromatogr. **1987**, *419*, 414–420.
6. Pou-Clave, L.; Campos-Barreda, F.; Pascual-Mostaza, C. J. Chromatogr. **1993**, *563*, 211.
7. Weber, A.; Chaffin, D.; Smith, A.; Opheim, K. E. Antimicrob. Agents Chemother. **1985**, *27*, 531.
8. Manceau, J.; Gicquel, M.; Laurentie, M.; Sanders, P. J. Chromatogr. B **1999**, *726*, 175–184.
9. Posyniak, A.; Zmudzki, J.; Semeniuk, S. J. Chromatogr. A **2001**, *914*, 89–94.
10. Barron, D.; Jimenez-Lozano, E.; Cano, J.; Barbosa, J. J. Chromatogr. B **2001**, *759*, 73–79.
11. Hernandez, M.; Borrul, F.; Calull, M. J. Chromatogr. B **2000**, *742*, 255–265.
12. Kim, D. H.; Lee, S. K.; Park, Y. H.; Lee, D. W. J. Liq. Chrom. & Rel. Technol. **2000**, *23* (20), 1069–1081.
13. Ba, D.; Ducint, D.; Fourtillan, M.; Saux, M. C. J. Chromatogr. B **1998**, *714*, 317–324.

Received July 27, 2001
Accepted August 24, 2001
Manuscript 5632

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