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DETERMINATION OF FLUCONAZOLE IN HUMAN PLASMA BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract: A rapid, simple and reproducible high performance liquid chromatographic method was developed and validated for determination of fluconazole in human plasma. The separation was performed on MZ C8 column (125 × 4 mm, 5 µm) using acetonitrile – potassium dihydrogen phosphate buffer (15 : 85, v/v), pH 3.0, as the mobile phase at a flow rate of 1.5 mL/min. The wavelength was set at 261 nm. The assay enables the measurement of fluconazole for therapeutic drug monitoring with a minimum quantification limit of 20 ng/mL. The method involves simple, protein precipitation procedure and analytical recovery was complete. The calibration curve was linear over the concentration range 0.1-4 µg/mL. The coefficients of variation for inter-day and intra-day assay were found to be less than 10%.

Keywords: fluconazole, HPLC, UV-detector, pharmacokinetic

Fluconazole [2-(2,4-difluorophenyl)-1,3-bis (1H-1,2,4-triazol-1-yl)-2-propanol] is a broad-spectrum triazole antifungal agent that has emerged as a suitable alternative to amphotericin B in the treatment and prevention of different superficial and systemic fungal infections (1, 2). It inhibits cytochrome P450-dependent enzymes, resulting in impairment of ergosterol synthesis in fungal cell membranes. Fluconazole is well absorbed following oral administration, bioavailability from oral route is approximately 90%. About 80% of a dose is excreted unchanged in the urine and about 11 % as metabolites (3).

Fluconazole is widely used in clinical practice. Its favorable pharmacokinetics facilitates the management of its dosing (4, 5). However, in some situations its pharmacokinetics is difficult to predict, and determination of circulating fluconazole levels is significant to guide its dosing (6–13). The treatment of these infections requires adjustments in fluconazole dosages. Finally, pharmacokinetic studies are significant in experimental treatment models (14). Since changes in the pharmacokinetics of fluconazole are still unknown, it is imperative to consider burn patients' drug levels with a controlled clinical protocol (15).

Several analytical methods including bioassay (16), gas chromatography (17), liquid chromatography (LC) with UV detection (18–22) and LC with tandem mass spectrometry (LC-MS/MS) (23, 24) have been described for quantification of fluconazole in human plasma and serum samples. Most of the presented methods need long chromatographic elution time for analysis of fluconazole in serum and sample preparation is complex and time consuming. The LC-MS/MS method is very sensitive but it is not available for most laboratories because of their apparatus requirement and financial reasons. High-performance liquid chromatography (HPLC) is preferred due to its selectivity and the specificity of the assay. In recent years, there have been wide studies for the different methods of the determination of fluconazole in biological samples by HPLC-UV (25–32). Reversed phase high-performance liquid chromatography (RP-HPLC) is an efficient method for the analysis of drugs.

This study describes a rapid and validated HPLC method using UV detection, which enables

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the determination of fluconazole with good accuracy at low drug concentrations in plasma using single-step extraction procedure. We also demonstrate the applicability of this method for pharmacokinetic studies in humans.

MATERIALS AND METHODS

Chemicals

Fluconazole and methocarbamol (internal standard) were supplied by Pars-Daru Pharmaceuticals (Tehran, Iran). Fluconazole is available as oral capsules containing 150 mg of fluconazole and other inactive ingredients. HPLC-grade acetonitrile and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation process and purified additionally with a Milli-Q system (Millipore, Bedford, MA, USA).

Instruments and chromatographic conditions

The chromatographic apparatus consists of a model Wellchrom K-1001 pump, a model Rheodyne 7125 injector and a model K 2501 UV detector connected to a model Eurochrom 2000 integrator, all from Knauer (Berlin, Germany). The separation was performed on MZ C8 (125 × 4.0 mm, 5 µm) column from Merck (Darmstadt, Germany). The wavelength was set at 261 nm. The mobile phase consisted of acetonitrile – potassium dihydrogen phosphate buffer (15 : 85, v/v), pH 3.0, at a flow rate of 1.5 mL/min. The mobile phase was prepared daily and degassed by ultrasonication before use.

Standard solutions

Stock solution (5 mg/mL) of fluconazole and methocarbamol (10 mg/mL) were prepared in methanol. The working standard solutions were obtained by dilution with methanol.

Sample preparation

To 450 mL of plasma in a glass-stoppered 15 mL centrifuge tube were added 50 mL of methocarbamol as internal standard (10 mg/mL), 500 mL of acetonitrile and 100 mg NaCl. After mixing (30 s), the mixture was centrifuged for 15 min at 8000 rpm. The organic phase was separated and dried under argon. Then, 50 mL of mobile phase was added and 40 mL of supernatant was injected into liquid chromatograph.

Biological samples

Twelve male healthy volunteers were included in this study. The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences and written informed consent was obtained from the volunteers. Fluconazole was administered in a single dose of 150 mg to the volunteers after overnight fasting. Plasma samples were collected at several intervals after dosing and then frozen immediately at –20°C until assayed.

Stability

The stability of fluconazole was assessed for spiked plasma samples stored at –20°C for up to two months and at ambient temperature for at least 24 h. The stability of stock solutions stored at –20°C was determined for up to one month by injecting appropriate dilutions of stock solution in distilled water on day 1, 15, and 30 and comparing their peak areas with fresh stock solution prepared on the day of analysis. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

Plasma calibration curves and quantitation

Blank plasma was prepared from heparinized whole blood samples collected from healthy volunteers and stored at –20°C. After thawing, 50 µL of fluconazole working standards were added to yield final concentrations of 0.1, 0.25, 0.5, 1, 2, 3, and 4 µg/mL. Internal standard solution was added to each of these samples and the samples were then prepared for analysis as described above. Calibration curve was constructed by plotting peak area ratio (y) of fluconazole to the internal standard *versus* fluconazole concentrations (x). A linear regression was used for quantitation.

Precision

The precision of an analytical method describes the closeness of individual measures of fluconazole when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of the biological matrix. The precision is reflected by the standard deviation, while relative precision is calculated as the coefficient of variation (CV), i.e., relative standard deviation (% RSD). Calculations of precision are largely independent of the number of replicates, except that more replicates may give a better estimate of the population variance. The measure RSD can be explaining by: repeatability (intra-day precision) and reproducibility (between laboratories precision). Intra-day precision was determined by repeatability of the method with obtained data on the same day under similar conditions from the precision experiments for five multiple injections at three different

dilutions (0.8, 1.6, and 3.2 µg/mL). The concentration of the sample is then obtained by measuring the peak area and the coefficients of variation were calculated, being within the acceptable criteria of less than 0.1 (Table 1).

Inter-day precision was obtained from reproducibility of the method with obtained data by repeating the assay five times (n = 5) on five different days, by injecting standard fluconazole at three different dilutions (0.8, 1.6, and 3.2 µg/mL). The coefficients of variation value were found to be less than 0.1 (Table 2).

Accuracy

Accuracy is the degree of agreement of a measured value with an accepted reference value. The accuracy of the method is determined by replicate analysis of samples containing known amounts of the fluconazole at three different concentrations equivalent to (0.8, 1.6, and 3.2 µg/mL) of the active pharmaceutical ingredient, by adding a known amount of fluconazole standard, and calculating the recovery of fluconazole with recovery and coefficients of variation for each concentration (Table 3).

Limit of quantification (LOQ) and recovery

The limit of detection (LOD) expresses the lowest concentration of analyte that can be detected for a given type of sample, instrument, and method, but not necessarily quantified under the stated experimental conditions. The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable accuracy (70 – 120%) and precision (< 20%). LOD and LOQ were estimated using the following formulas:

$$LOD = 3.3\sigma/S \text{ and } LOQ = 10\sigma/S$$

where σ represents the standard deviation of the response (estimated based on the calibration curve), and S is the slope of the calibration curve. The LOD and LOQ values were 20 ng/mL and 61 ng/mL,

Table 1. Results of precision study (intra-day) for the fluconazole analysis in plasma (n = 5).

Sample	Concentration (µg/mL)	Injection no.					Mean	SD	CV
		1	2	3	4	5			
Fluconazole	0.8	0.60	0.69	0.66	0.63	0.67	0.65	0.03	0.05
	1.6	1.40	1.44	1.41	1.37	1.19	1.36	0.10	0.07
	3.2	3.22	2.44	2.9043	2.83	2.77	2.83	0.28	0.10

Table 2. Results of precision study (inter-day) for the fluconazole analysis in plasma (n = 5).

Sample	Concentration (µg/mL)	Injection no.					Mean	SD	CV
		1	2	3	4	5			
Fluconazole	0.8	0.60	0.65	0.63	0.61	0.63	0.63	0.02	0.03
	1.6	1.40	1.20	1.42	1.17	1.37	1.31	0.11	0.09
	3.2	3.22	2.58	2.81	2.51	2.83	2.79	0.28	0.10

Table 3. Results of recovery study for the fluconazole analysis in plasma (n = 3).

Sample	Concentration (µg/mL)	Injection no.			Mean	STD
		1	2	3		
Fluconazole	0.8	106.75	107.92	108.36	107.68	0.83
	1.6	110.06	109.46	111.62	110.38	1.11
	3.2	112.21	109.73	110.56	110.83	1.26

respectively. The results of LOD and LOQ supported the sensitivity of the developed method.

Pharmacokinetic analysis

Fluconazole pharmacokinetic parameters were determined by non compartmental methods. Elimination rate constant (k) were estimated by the least-square regression of plasma concentration-time data points lying in the terminal log-linear region of the curves. Peak plasma concentration (C_{max}) and time to peak concentration (T_{max}) were derived from the individual subject concentration-time curves. The area under the plasma concentration-time curve from time zero to the last measurable concentration at time t (AUC_{0-t}) was calculated using the trapezoidal rule. The area was extrapolated to infinity ($AUC_{0-\infty}$) by addition of C_t/K to AUC_{0-t} where C_t is the last detectable drug concentration.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, fluconazole and the internal standard peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Separation was performed on a short reversed-phase C8 column, which allows easy optimizing chromatographic conditions to obtain desirable resolution in a short time. Accordingly, the chromatographic elution step is undertaken in a short time (less than 10 min) with high resolution. Figure 1 shows typical chromatograms of blank plasma in comparison to spiked samples analyzed for a pharmacokinetic study. The average retention times of fluconazole and methocarbamol were 6.1 and 7.8 min, respectively. The peaks were of good shape and completely resolved at therapeutic concentrations of fluconazole. In addition, this separation was obtained using low amount of organic solvent in composition of mobile phase compared to related published methods. In our method, sample preparation involves protein precipitation using acetonitrile. Protein precipitation became more efficient with increasing volumes of acetonitrile. However, greater volumes of acetonitrile diluted the sample, thereby affecting the sensitivity of the assay. To improve the sensitivity, a 1:1 ratio of acetonitrile to plasma was considered for sample preparation. Under this condition, the majority of protein was precipitated and fluconazole and internal standard were free of inference from endogenous components in plasma. The calibration curve for the determination of fluconazole in plasma was linear over the range 0.1 – 4 µg/mL. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (r) for calibration curves were equal to or better than 0.998. The slopes of plasma standard curves in the nine different preparations were practically the same (the coefficients of variation were less than 2% for the slopes of plasma standard curves). For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves. The mean linear regression equation of calibration curve for the analyte was:

$$y = 0.778x - 0.0047$$

where y was the peak area ratio of the analyte to the internal standard and x was the concentration of the analyte. The analytical recovery from plasma at three different concentrations of fluconazole was determined. Known amounts of fluconazole were added to drug-free plasma in concentrations ranging from 0.8 to 3.2 µg/mL. The internal standard was added and the absolute recovery of fluconazole was calculated by comparing the peak areas for extracted fluconazole from spiked plasma and a standard solution of fluconazole in methanol containing internal standard with the same initial concentration. The average recovery was 109.63 ± 1.7% (n = 5) and the dependence on concentration was negligible. The recovery of internal standard, methocarbamol was almost complete at the concentration used in the assay. Using UV detection method, the limit of quantification, as previously defined, was 20 ng/mL for fluconazole. This is sensitive enough for drug monitoring and other purposes such as pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of fluconazole. As shown in Tables 1 and 2, coefficients of variation were less than 10%, which is acceptable for the routine measurement of fluconazole. Stability was determined for spiked plasma samples under the conditions as previously described. The results showed that the samples were stable during the mentioned conditions.

The aim of our study was to develop a rapid and sensitive method for the routine analysis of biological samples in pharmacokinetic fluconazole research. This method is well suited for routine application in the clinical laboratory because of the speed of analysis and simple extraction procedure. Over 350 plasma samples were analyzed by this method without any significant loss of resolution. No change in the column efficiency and back pressure was also observed over the entire study time,

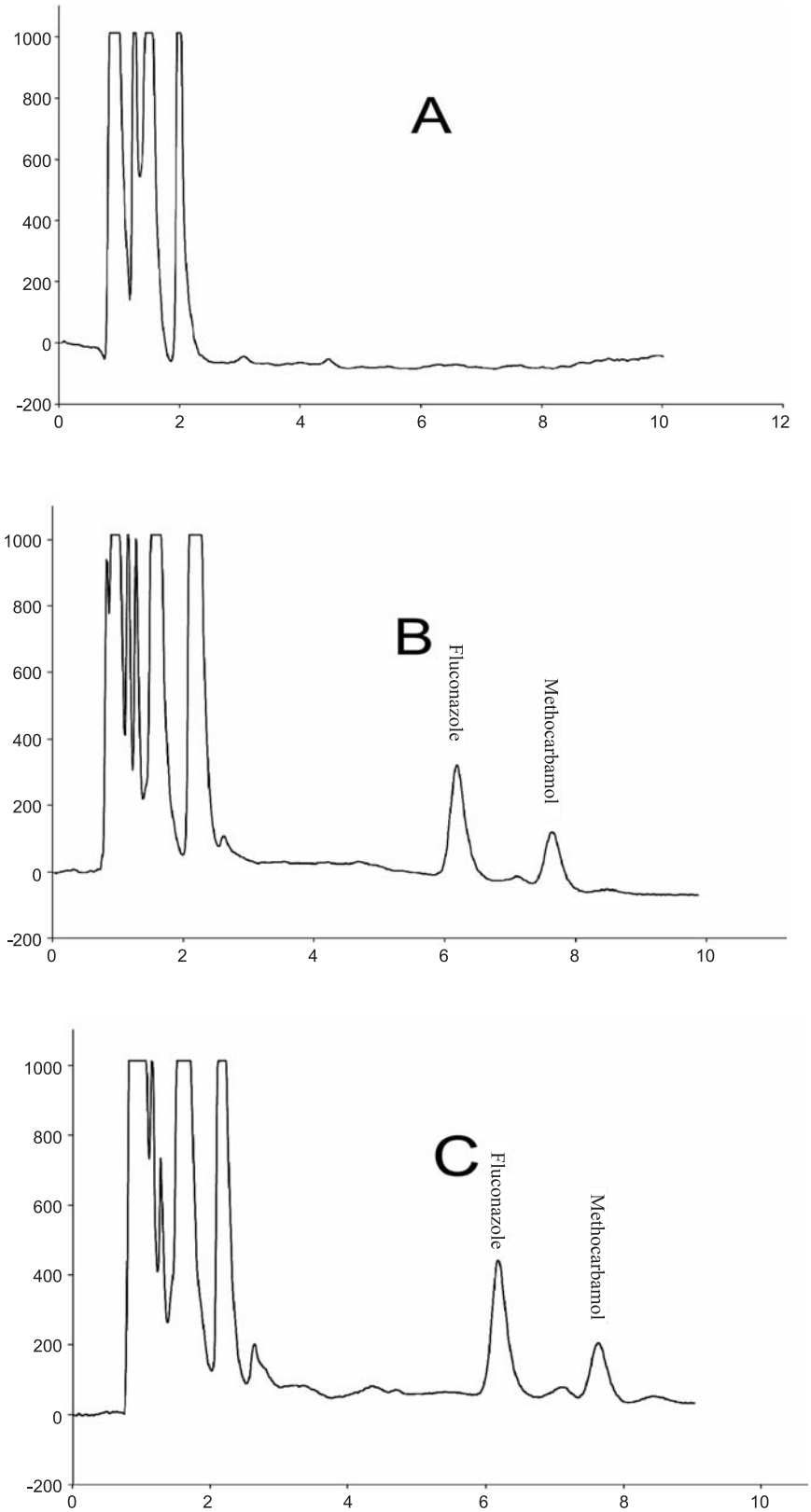

Figure 1. Chromatograms of (A) blank plasma; (B) blank plasma spiked with 1500 ng/mL fluconazole and 500 ng/mL methocarbamol (internal standard); (C) plasma sample from a healthy volunteer three hours after oral administration of 150 mg of fluconazole

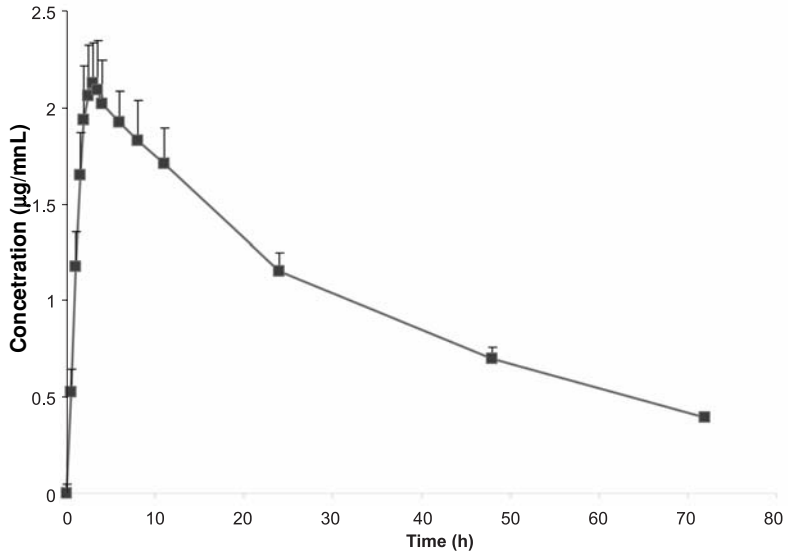

Figure. 2. Mean plasma concentration-time profile of fluconazole in healthy volunteers (n = 12) after a single 150 mg of fluconazole

Table 4. Pharmacokinetic parameters of fluconazole in healthy volunteers following a single oral dose of 150 mg of fluconazole.

Parameter	Result (mean ± SD)
T_{max} (h)	2.62 ± 0.40
C_{max} (µg/mL)	2.22 ± 0.46
AUC_{0-t} (µg×h/mL)	73.26 ± 20.29
$AUC_{0-\infty}$ (µg×h/mL)	91.18 ± 28.54
K_{el} (h^{-1})	0.024 ± 0.004

thus proving suitability of the method. In this study, plasma concentrations were determined in twelve healthy volunteers, who received 150 mg of fluconazole each. Figure 2 shows the mean plasma concentration-time profile of fluconazole. The derived pharmacokinetic parameters of 12 healthy volunteers are summarized in Table 4. These pharmacokinetic parameters are in good agreement with those found previously (4).

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REFERENCES

1. Bennett J.E.: Antifungal Agents, in The Pharmacological Basis of Therapeutics. Goodman-Gilman A., Rall T.W., Nies A.S., Taylor P. Eds., p. 1172, McGraw-Hill, Singapore 1992.
2. Goa K.L., Barredell L.B.: Drugs 50, 658 (1995).
3. Surber C.: in Drug Concentrations in the Skin. Mailbach H.I. Ed., p. 151, CRC Press, Boca Raton 1996.
4. Grant S.M., Clissold S.P.: Drugs 39, 877 (1990).
5. Humphrey M.J., Jevons S., Tarbit M.H.: Antimicrob. Agents Chemother. 28, 648 (1985).
6. Anaissie E., Kontoyiannis D.P., Huls C., Vartivarian S., Karl C., Prince R.A., Bosso J., Bodey G.P.: J. Infect. Dis. 172, 599 (1995).
7. Cameron M.L., Schell W.A., Bruch S., Bartlett J.A., Waskin H.A., Perfect J.: Antimicrob. Agents Chemother. 37, 2449 (1993).
8. Ghannoum M.A., Rex J.H., Galgiani J.N.: J. Clin. Microbiol. 34, 489 (1996).
9. Graybill J.R., Najvar L.K., Holmberg J.D., Correa A., Luther M.F.: Antimicrob. Agents Chemother. 39, 2197 (1995).
10. Louie A., Liu Q.F., Drusano G.L., Liu W., Mayers M., Anaissie E., Miller M.H.: Antimicrob. Agents Chemother. 42, 1512 (1998).
11. Radetsky M., Wheeler R.C., Roe M.H., Todd J.K.: J. Clin. Microbiol. 24, 600 (1986).
12. Rex J.H., Nelson P.W., Paetznick V.L., Lozano-Chiu M., Espinel-Ingroff A., Anaissie E.: Antimicrob. Agents Chemother. 42, 129 (1998).
13. Rex J.H., Pfaller M.A., Galgiani J.N., Bartlett J.A., Espinel-Ingroff A., Ghannoum M.A.,

Lancaster M. et al.: Clin. Infect. Dis. 24, 235 (1997).
14. Craig W.A.: Clin. Infect. Dis. 26, 1 (1998).
15. Santos S.R.C.J., Campos E.V., Sanches C., Gomez D.S., Ferreira M.C.: Clinics 65, 237 (2010).
16. Marchetti O., Majcherczyk P.A., Glauser M.P., Bille J., Moreillon P., Sanglard D.: Antimicrob. Agents Chemother. 45, 696 (2001).
17. Evrard P.A., Derrider G., Verbeeck R.K.: Pharm. Res. 13, 12 (1996).
18. Menacherry S., Hubert W., Justice J.B.: Anal. Chem. 64, 577 (1992).
19. Stahle L., Segersvärd S., Ungerstedt U.: J. Pharmacol. Methods 25, 41 (1991).
20. Rex J.H., Hanson L.H., Armantea M.A., Stevens D.A., Bennet J.E.: Antimicrob. Agents Chemother. 35, 846 (1991).
21. Jacobson I., Sandberg M., Hamberger A.: J. Neurosci. Methods 15, 263 (1985).
22. Mathy F.X., Preat V., Verbeeck R.K.: J. Pharm. Sci. 90, 1897 (2001).
23. Verbeeck R.K.: Adv. Drug Deliv. Rev. 45, 217 (2000).
24. Jovanovic D., Killibarda V., Ciric B.: Clin. Ther. 27, 1588 (2005).
25. Wattananat T., Wiyada A.: Biomed Chromatogr. 20, 1 (2006).
26. Egle H., Trittler R., Kümmerer K.: Ther. Drug Monit. 26, 425 (2004).
27. Ng T.K.C., Chan R.C.Y., Adeyemi-Doro F.A.B., Cheung S.W., Cheng A.F.B.: J. Antimicrob. Chemother. 37, 465 (1996).
28. Cociglio M., Brandissou S., Alric R., Bressolle F.: J. Chromatogr. B 686, 11 (1996).
29. Koks C.H.W., Rosing H., Meenhorst P.L., Bult A., Beijnen J.H.: J. Chromatogr. B 663, 435 (1994).
30. Wallace J.E., Harris S.C., Gallegos J., Foulds G., Chen T.J., Rinaldi M.G.: Antimicrob. Agents Chemother. 36, 603 (1992).
31. Flores-Murrieta F.J., Granados-Soto V., Hong E.: J. Liq. Chromatogr. Relat. Technol. 17, 3803 (1994).
32. Inagaki K., Takagi J., Lor E., Okamoto M.P., Gill M.A.: Ther. Drug Monit. 14, 306 (1992).

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