ELSEVIER

Journal of Chromatography B, 745 (2000) 413–420

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Liquid chromatographic–mass spectrometric determination of itraconazole and its major metabolite, hydroxyitraconazole, in dog plasma

Alain Carrier*, Josée Parent

RTP Pharma Inc., 1000 Chemin du Golf, Verdun, Québec H3E 1A8, Canada

Received 7 February 2000; received in revised form 12 May 2000; accepted 22 May 2000

Abstract

A fast, reliable and sensitive liquid chromatography–mass spectrometry (LC–MS) assay for the determination of itraconazole and hydroxyitraconazole in dog plasma has been developed. The analysis involves a simple liquid–liquid extraction followed by LC–MS analysis using electrospray ionization in the positive mode. Total separation of itraconazole, hydroxyitraconazole and the internal standard, miconazole, was achieved on a C_{18} column in 3.5 min using an isocratic mixture of acetonitrile and 10 m*M* ammonium acetate. The response was linear over four-orders of magnitude, allowing reliable quantification of each species. This paper describes the development of the method and its validation.

Keywords: Itraconazole; Hydroxyitraconazole

1. Introduction

Itraconazole is a triazole antifungal drug with a broad spectrum of activity against most human fungal pathogens [1–4]. Following its absorption, itraconazole is extensively metabolized by side-chain hydroxylation to form hydroxyitraconazole. This hydroxy metabolite is biologically active and can appear in concentrations nearly twice that of the unaltered drug in the steady state [5]. Fig. 1 gives the chemical structures of itraconazole and its major metabolite, hydroxyitraconazole.

Several biological or chromatographic assays for the quantitation of itraconazole have been reported [6–11]. The non-specificity of the bioassays does not allow for distinguishing between active metabolites and the parent drug [12]. Chromatographic methods are more specific and sensitive. However, only a few methods have been described for the determination of both itraconazole and its hydroxy metabolite [10,11,13,14]. Furthermore, the published methods involve a time-consuming extraction step that limits their use for high throughput analyses. Even though some methods [10,11] use simple protein precipitation that significantly decreases the sample preparation time, total analysis time is usually around 15 to 20 min. Although Compas et al. [8] reported an analysis time of less than 6 min, the method developed had a limited range of sensitivity (0.1 to 3.2 mg/ml).

*Corresponding author. Tel.: +1-514-362-1304 ext. 238; fax: +1-514-362-1172.

E-mail address: acarrier@rtp-pharma.com (A. Carrier).

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00309-1

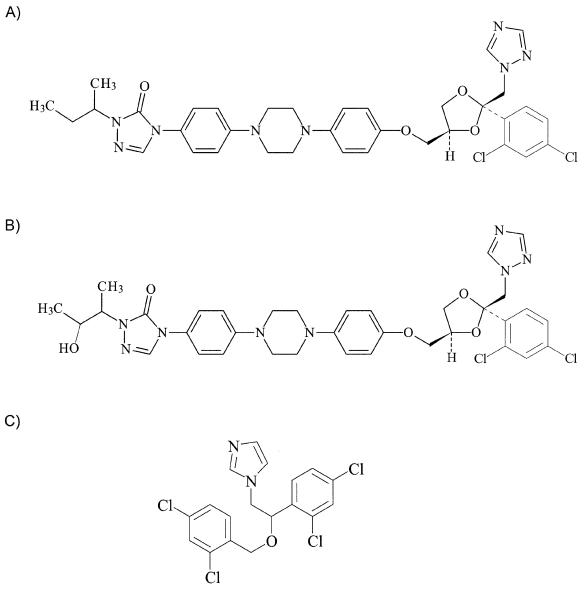

Fig. 1. Chemical structures of (A) itraconazole, (B) hydroxyitraconazole and (C) miconazole.

In order to perform in vivo pharmacokinetic studies, a simple and rugged assay for the determination of itraconazole and its major metabolite, hydroxyitraconazole, in dog plasma was developed using liquid chromatography–mass spectrometry (LC–MS). Liquid–liquid extraction combined with electrospray mass spectrometry in the positive ionization mode allowed analysis of the compounds of interest within 3.5 min. The present work describes results obtained for the validation of the method.

2. Experimental

2.1. Materials and reagents

HPLC-grade acetonitrile (ACN), tetrahydrofuran (THF), methyl-*tert*.-butyl ether (MtBE), analytical-grade dibasic potassium phosphate and triethylamine were purchased from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid was obtained from Fisher (Ontario, Canada). Ammonium acetate and miconazole were purchased from Sigma (Ontario, Canada). Glass distilled water (>18 MΩ), available in the laboratory was filtered through a 0.2-μm filter prior to use. Itraconazole and hydroxyitraconazole were obtained from Research Diagnostics (Flanders, NJ, USA).

2.2. Chromatographic system and conditions

Analysis was carried out using the Agilent Technologies LC–MS system (Kirkland, Canada) composed of the following units: a solvent delivery module, an automatic sample injector (100-well capacity), a controller module, a column oven and a Model series 1100MSD mass spectrometer. The analysis was performed on a Zorbax SB-C_{18} column (3.0 cm×0.46 cm I.D.; 3.5 μm particle size) maintained at 25°C. The mobile phase consisted of acetonitrile–10 m*M* ammonium acetate, pH 3.5 (60:40, v/v). The mobile phase was filtered through a 0.45-μm polypropylene filter and degassed under vacuum. The mobile phase was pumped at 1.0 ml/min and the injection volume was 5 μl.

2.3. Mass spectrometric conditions

MS was carried out on the Agilent 1100 mass spectrometer equipped with an electrospray ionization source in the positive ionization mode. Data handling was performed with a Chemstation V. A.06.01 data handling system. Full scan spectra were obtained by scanning masses between m/z 200 and 800. In the electrospray experiments the chromatographic effluent entered the mass spectrometer through an electrospray capillary set at 3.5 kV. Nitrogen was used both as drying gas (11 l/h) and nebulizing gas (50 p.s.i.; 1 p.s.i.=6894.76 Pa). The temperature of the drying gas was set to 350°C. All the experiments were conducted at a fragmentor voltage of 140 V. Quantitative analyses were conducted in single-ion monitoring (SIM) mode scanning the quasi-molecular ions $[M+H]^+$ of itraconazole, hydroxyitraconazole and miconazole with a dwell time of 100 ms.

2.4. Calibration procedure

A primary stock solution of itraconazole and hydroxyitraconazole was prepared by accurately weighing the analytical standards (approximately

2.5±0.5 mg of each) and dissolving them in 25 ml of THF–water (50:50) in order to obtain a final concentration of 100 μg/ml. Secondary standard solutions were obtained by appropriate dilutions with THF–water (50:50) in order to obtain concentrations of 80, 50, 10, 2.5, 0.5, 0.125 μg/ml. All standards were transferred into glass containers and stored at −20°C. Calibration samples were generated from drug-free plasma by adding 10 μl of the appropriate standards to 0.5 ml of plasma in order to achieve itraconazole and hydroxyitraconazole concentrations of 2000, 1600, 1000, 200 50, 10 and 2.5 ng/ml. Quality control (QC) samples were obtained by preparing another primary stock solution containing both itraconazole and hydroxyitraconazole at approximately 100 μg/ml. Appropriate dilution of these standards was done in THF–water (50:50) in order to achieve concentrations of 40, 5 and 0.250 μg/ml. A 0.5-ml volume of plasma was spiked with 10 μl of these standards in order to obtain final plasma concentrations of 800, 100 and 5 ng/ml for the QC samples. Blank samples were obtained by adding 10 μl of THF–water (50:50) to 0.5 ml of plasma. All standards and spiked plasma samples were transferred into a glass tube and stored at −20°C. Stability data indicated that the material was stable for at least 1 month.

The internal standard stock solution was prepared by weighing 5.0±0.5 mg of miconazole into 100 ml of THF–water (50:50) in order to give a concentration of 50 μg/ml. The 0.02 *M* potassium phosphate solution was prepared by mixing 870 mg of dibasic potassium phosphate, 250 ml of water and 1 ml of triethylamine; pH was adjusted to 8.5 with phosphoric acid.

Peak area ratios (itraconazole/miconazole and hydroxyitraconazole/miconazole) were used for quantitative computations. Calibration lines of chromatographic response versus concentration were determined by least-squares linear regression analysis.

2.5. Sample preparation

Frozen plasma samples were thawed and vortex-mixed before use. To each 0.5 ml of plasma (standards, quality control and samples) 10 μl of a standard solution of miconazole (internal standard, 50 μg/ml) was added followed by 0.5 ml of 0.02 *M* phosphate buffer (pH 8.5) and the samples were vortex-mixed for 90 s. For the extraction, 4 ml of MtBE was added and vortex-mixed for an additional 90 s. After centrifugation (5 min at 1559 *g* rotational centrifugal force (RCF)) the organic layer was transferred to a new tube and evaporated to dryness. The residue was reconstituted in 200 μl of THF–water (50:50, v/v). Samples were vortex-mixed for 30 s and centrifuged for 1 min at 1083 *g* (RCF) prior to loading into the automated injector tray. The LC–MS assays were performed with 10 μl injection. Samples were centrifuged using the Megafuge 1.0R from Heraeus Instruments.

3. Results and discussion

Fig. 2 shows typical mass spectra obtained for itraconazole, hydroxyitraconazole and miconazole using the electrospray technique in the positive ionization mode. Protonated molecules were detected as the major peaks for all compounds studied except for hydroxyitraconazole where the natriated peak was larger. The spectra were obtained with a fragmentor voltage set at 140 V (optimal intensities of all molecular ions).

3.1. Chromatography

Fig. 3 shows three examples of chromatograms, (A) one obtained from blank dog plasma (B) one obtained from dog plasma spiked with itraconazole and hydroxyitraconazole at the limit of quantitation (LOQ) level (2.5 ng/ml) and miconazole at 5 μg/ml and (C) a sample from a dosed dog (t=1 h). Retention times of hydroxyitraconazole, itraconazole and miconazole were 0.8, 1.9 and 2.8 min, respectively. There was no interference from endogeneous plasma components. The specificity of the method was evaluated by injecting separate extracted standards of itraconazole, hydroxyitraconazole and miconazole. Results obtained showed that no interference was observed at their respective masses from the compounds investigated in this study.

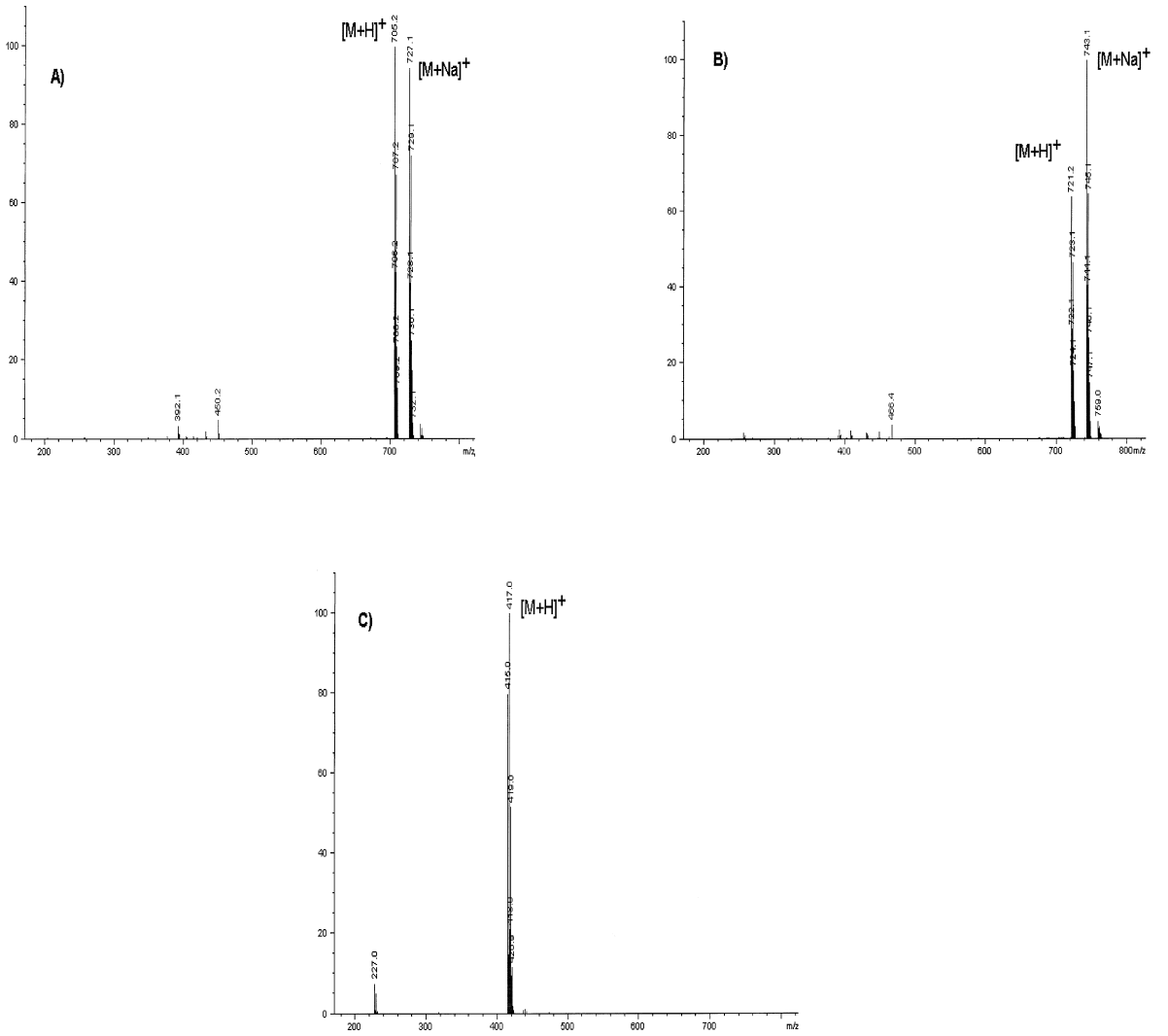

Fig. 2. Positive ion electrospray mass spectra obtained for injections of 2.5 ng of (A) itraconazole, (B) hydroxyitraconazole and (C) miconazole. Fragmentor voltage 140 V, scanning range from m/z 200–800.

3.2. Recoveries from dog plasma

Absolute recoveries were calculated by comparing LC–MS results from samples prepared in organic solvent with ones obtained from spiked extracted plasma samples. The average recoveries of itraconazole and hydroxyitraconazole from plasma were determined at two concentration levels approximately 100 and 800 ng/ml. The observed mean recoveries obtained were 70% for itraconazole and 82% for hydroxyitraconazole. For the internal standard, miconazole, a recovery of 80% was achieved. Details are given in Table 1.

3.3. Linearity

The method was shown to be linear over the concentration range of 2.5 to 2000 ng/ml in plasma for both itraconazole and hydroxyitraconazole.

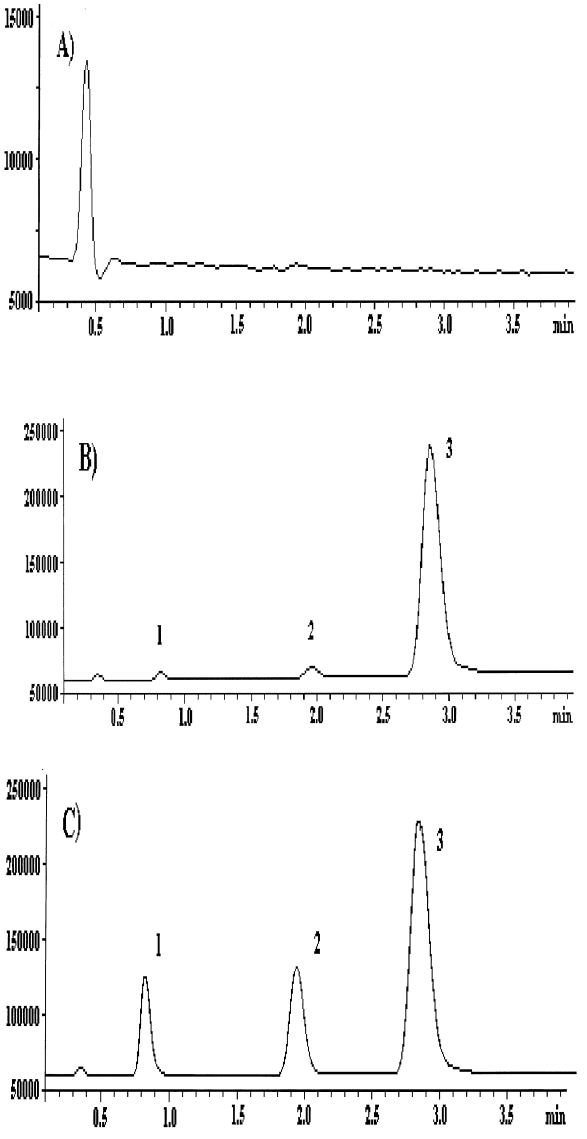

Fig. 3. Representative chromatograms of (A) blank dog plasma, (B) dog plasma spiked at 2.5 ng/ml of itraconazole and hydroxyitraconazole (LOQ) and 4.0 μg/ml of miconazole and (C) sample from a dosed dog (time=1 h).

Least-squares linear regression of itraconazole/miconazole and hydroxyitraconazole/miconazole peak area ratios versus spiked concentrations gave correlation coefficients >0.99 for both compounds. Table 2 gives a summary of the calibration curve regression parameters.

3.4. Within- and between-batch accuracy and precision

Within-batch precision and accuracy of the method was evaluated by analyzing replicate samples each of control dog plasma spiked at three concentration levels, 5, 100 and 800 ng/ml. Relative standard deviations (RSDs) and percent differences between the measured and the nominal concentrations were calculated for each concentration level and were considered acceptable when the RSDs and the percent differences were less than 15%. The results obtained for the within-batch accuracy and precision were, respectively, <13% for itraconazole and <9.5% for hydroxyitraconazole. The results are presented in Table 3 and indicate that the values are within acceptable range and that the method was accurate and precise.

The between-batch precision of the peak response was done by evaluating the RSDs of the slopes of the curves (six) performed for the validation of the method. The results obtained indicated that the RSDs were 10.5% and 11.9% for itraconazole and hydroxyitraconazole, respectively. Data given in Table 2 were found to be within the acceptable range.

3.5. Freeze–thaw stability of itraconazole and hydroxyitraconazole in dog plasma

The stability of itraconazole and hydroxyitraconazole was evaluated by analyzing three replicate samples at one concentration level, 1500 ng/ml, and submitting them to three freeze–thaw cycles (freezing at −20°C: 4–6 h and thawing: 2–4 h at ambient temperature) before the samples were extracted and reanalyzed.

The results obtained after three freeze–thaw cycles demonstrate that 94±4% and 96±4% of the initial content of itraconazole and hydroxyitraconazole, respectively, were recovered and that the analytes were stable under these conditions.

3.6. Stability in dog plasma at ambient temperature

Stability of the itraconazole and hydroxyitraconazole in dog plasma at ambient temperature was also performed. Spiked samples (three/concen-

Table 1
Observed recoveries obtained for itraconazole and hydroxyitraconazole

Compound	Nominal concentration (ng/ml)	Recovery (%)	RSD (%)	*n*
Itraconazole	96	69.0	5.4	12
	768	71.8	2.5	12
Hydroxyitraconazole	104	83.6	5.8	12
	832	81.3	2.4	12
Miconazole	2500	80.1	3.8	12

tration) at 100 and 1000 ng/ml were left at ambient temperature for 18 h. They were then extracted and the results obtained were compared to freshly prepared extracted material at the same level.

The results showed that itraconazole and hydroxyitraconazole were stable for at least 18 h in plasma kept at room temperature. After that period of time, 105±3% of the expected concentration was recovered for both compounds. This indicated that dog plasma could be keep at ambient temperature for at least 18 h without altering the integrity of the samples.

3.7. Stability of reconstituted samples (wet stability)

Stability of the extracted and reconstituted samples at ambient temperature was investigated. Samples spiked at 100 ng/ml were extracted and reconstituted. Reconstituted samples were left for 3, 4.5 or 8 h on the bench at ambient temperature and then analyzed and compared to freshly prepared extracted material at the same concentration.

Results obtained showed that at all three time points tested, 108±7% and 100±6% of itraconazole

Table 2
Summary of calibration curve regression parameters

Run No.	*y*-Intercept	Slope	Correlation coefficient (*R*)
Itraconazole			
1	0.0137	0.0007	1.000
2	0.0158	0.0007	0.999
3	0.0210	0.0006	0.998
4	0.0098	0.0007	0.998
5	0.0216	0.0008	0.999
6	0.0323	0.0008	0.998
Average	0.019	0.0007	0.997
Standard deviation	0.007	$7.5 \cdot 10^{-5}$	0.002
Hydroxyitraconazole			
1	0.0019	0.0004	1.000
2	0.0020	0.0004	0.999
3	0.0013	0.0004	0.996
4	0.0078	0.0004	0.995
5	0.0025	0.0005	0.997
6	0.0033	0.0005	0.995
Average	0.003	0.0004	0.997
Standard deviation	0.002	$5.2 \cdot 10^{-5}$	0.002

Table 3
Within-batch precision and accuracy obtained for itraconazole and hydroxyitraconazole

Compound	Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	% Nominal	RSD (%)	*n*
Run 1					
Itraconazole	768	781	101.7	1.5	6
	96	106	109.9	2.8	6
	4.8	4.7	98.2	11.9	6
Hydroxyitraconazole	832	852	102.4	3.1	6
	104	108	103.7	4.1	6
	5.2	5.5	106.0	11.9	6
Run 2					
Itraconazole	768	796	103.7	1.1	6
	98	109	111.3	2.8	6
	4.8	4.6	94.8	14.1	6
Hydroxyitraconazole	832	885	106.4	1.7	6
	104	111	106.6	1.5	6
	5.2	5.1	98.7	7.2	6

and hydroxyitraconazole, respectively, of the expected concentration was recovered. This indicates that samples could be left on the injector tray for at least 8 h without altering the integrity of the analysis.

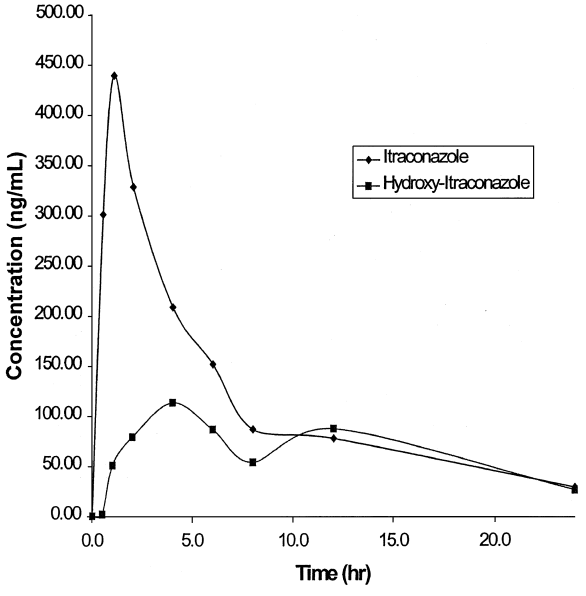

Fig. 4. Mean plasma itraconazole and hydroxyitraconazole concentration–time profiles following a single 2.5 mg/kg oral administration of itraconazole to a dog.

Typically, the analysis of 100 samples will take less than 6 h.

3.8. Biological samples

The method described was successfully used in monitoring itraconazole and hydroxyitraconazole concentrations in dog plasma. Typical concentration–time profiles of itraconazole and hydroxyitraconazole following a 2.5 mg/kg oral dose of one of the itraconazole formulations to a dog is presented in Fig. 4.

4. Conclusion

A fast and sensitive LC–MS method for determining itraconazole and hydroxyitraconazole levels in dog plasma samples was developed. The method provides complete analysis of the two drugs within 3.5 min. Validation experiments support the precision, accuracy and stability of the method over the range 5–800 ng/ml. Recoveries of 70% and 80% were obtained for itraconazole and hydroxyitraconazole, respectively, using a simple liquid–liquid extraction method. The within- and between-run precisions were $<13\%$ (from 5–800 ng/ml). The method was suitable for the determination of it-

raconazole and hydroxyitraconazole concentrations in dog pharmacokinetic studies.

Acknowledgements

The authors wish to acknowledge Dr. Indu Parikh for his critical comments in the preparation of the manuscript.

References

[1] S.M. Grant, S.P. Clissold, Drugs 37 (1989) 310.
[2] D.W. Denning, R.M. Tucker, L.H. Hanson, D.A. Stevens, Am J. Med. 86 (1989) 791.
[3] D.W. Denning, J. Van Wye, N.J. Lewiston, D.A. Stevens, Chest 100 (1990) 813.
[4] W.E. Dismukes, R.W. Bradsher, G.C. Cloud, C.A. Kauffman, S.W. Chapman, R.B. George, D.A. Stevens, W.M. Girard, M.S. Saag, C. Bowles-Patton, Am. J. Med. 93 (1992) 489.
[5] M. Haria, H.M. Bryson, K.L. Goa, Drugs 51 (1996) 585.
[6] D.W. Warnock, A. turner, J. Burke, J. Antimicrob. Chemother. 21 (1988) 93.
[7] R. Woestenborghs, W. Lorreyne, J. Heykants, J. Chromatogr. 413 (1987) 332.
[8] D. Compas, D.J. Touw, P.N.F.C. Goede, J. Chromatogr. B 687 (1996) 453.
[9] D. Law, C.B. Moore, D.W. Denning, Antimicrob. Agents Chemother. 38 (1994) 1561.
[10] P.O. Gubbins, B.J. Gurley, J. Bowman, J. Pharm. Biomed. Anal. 16 (1998) 1005.
[11] S.K. Cox, S. Orosz, J. Brunette, D. Frazier, J. Chromatogr. B 702 (1997) 175.
[12] J.S. Hostetler, J. Heykants, K. Clemons, R. Woestenborghs, L.H. Hanson, D.A. Stevens, Antimicrob. Agents Chemother. 37 (1993) 2224.
[13] C. Lacroix, F. Wojciechowski, P. Danger, Ann. Biol. Clin. 53 (1995) 293.
[14] J.M. Poirier, G. Cheymol, Ther. Drug Monit. 19 (1997) 247.