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Quantitative determination of nystatin in human plasma using LC–MS after inhalative administration in healthy subjects

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Abstract

The antifungal polyene antibiotics nystatin was tested in a clinical trial to describe pharmacokinetics and safety after repeated administration of Nystatin "Lederle" sterile powder in healthy volunteers. To monitor the nystatin concentration–time profile in plasma we developed a sensitive method in the range of 1–100 ng/ml based on liquid chromatography coupled with tandem mass spectrometry. The target substance was separated from the biological matrix on C_{18} solid-phase extraction cartridges with methanol. The Chromatography was performed isocratically using a reversed phase Caltrex Resorcinearene column. The mobile phase consisted of 5 mM ammonium formate buffer and acetonitrile (40:60, v/v). The mass spectrometer works with electrospray ionization in its positive selected ion monitoring (SIM) mode using the respective MH^+ ions, *m/z* 926.6 for nystatin and *m/z* 924.4 for amphotericin B as internal standard. The method validation was performed according to the demands and international criteria for validation of bioanalytical methods and was successfully applied to the quantification of nystatin in human plasma in the pharmacokinetic trial.

Keywords: Nystatin; Inhaled aerosol; Drug assay; LC–MS

1. Introduction

The macrolide antibiotics nystatin was isolated from the actinomycete species *Streptomyces noursei* in 1950 and launched into antifungal therapy just five years later [1]. Nystatin is an efficient antimicrobial agent against a broad spectrum of saprophytic and pathogenic fungi [2,3]. Its current clinical use, however, is limited to topic applications on the skin and mucous membranes because of its low tolerability after parenteral administration [4]. Nystatin and the structurally related amphotericin B are lactones consisting of a hydroxylated polyene macrolide backbone connected by a glycosidic bond with mycosamine (Fig. 1). Both drugs are not absorbed from the gastrointestinal tract, skin, or vagina [3,5]. Nystatin is on the market in several pharmaceutical formulations for oral treatment of gastro-intestinal mycoses and for many other topical applications in dermatology, gynecology or otology [3]. Nystatin "Lederle" sterile powder (ICN Pharmaceuticals, Frankfurt/Main, Germany) is on the marked for aerosol therapy in pulmology. Contrary to inhaled amphotericin B, there is to our knowledge no information on disposition and safety of nystatin after pulmonary administration [6]. So far, a few HPLC methods are available to measure nystatin plasma concentration–time profiles after parenteral administration of high doses in animals [7–9]. The limits of quantification of these methods (above 50 ng/ml) are not sufficient to quantify the drug in human plasma after pulmonary administration of therapeutic doses. Therefore, we described in this paper the development and validation of a novel LC–MS method for nystatin measure plasma concentrations up to the detection limit of 1 ng/ml in a pharmacokinetic study after chronic inhalation of therapeutic doses of the drug in man.

2. Methods

2.1. Quantitative assay of nystatin in plasma

Nystatin was provided by ICN Phamaceuticals (Frankfurt/Main, Germany) as a yellow crystalline powder. Because

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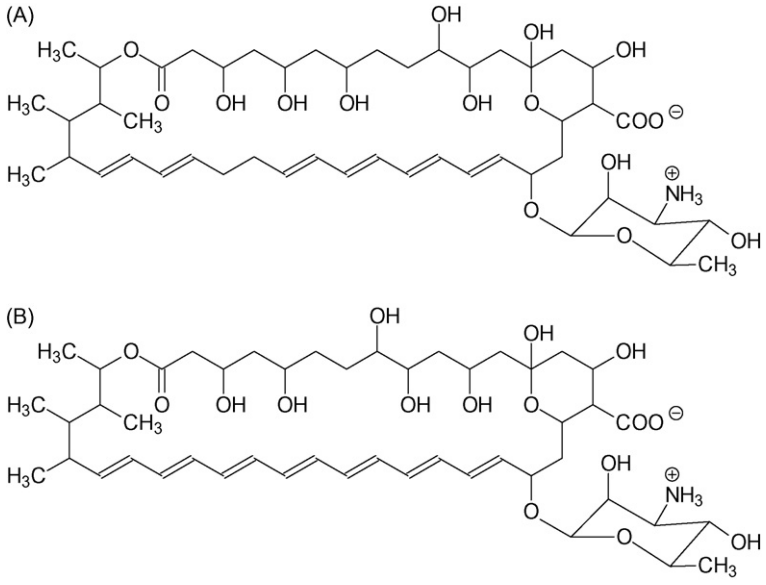

Fig. 1. Chemical structure of nystatin (A) and amphotericin B (B).

no stable isotope-labeled nystatin was available, the structurally related amphotericin B was used as internal standard (Sigma–Aldrich, Taufkirchen, Germany). Amphotericin B differs from nystatin by transition of the OH-group and an additional double bond (Fig. 1). Ammonium formate and formic acid were purchased from Merck (Darmstadt, Germany), acetonitrile and methanol (LC–MS Chromasolv®) from Baker (Gross, Gerau, Germany). Deionized water (conductance: ≤0.055 μS/cm, pH 5.0–6.0) was generated using the SG system RF 40 EZ (Hamburg, Germany).

2.1.1. Sample preparation

To 1.0 ml plasma (sample from the clinical study, calibrator or quality control sample), 25 μl of the internal standard solution (1.0 μg/ml amphotericin B) and 1.0 ml water were added. One milliliter of this solution was extracted with C18 SPE Bond Elut C_{18} cartridges (Varian, Darmstadt, Germany) using the Gilson Aspec XL apparatus (Abimed, Langenfeld, Germany). The cartridges had been preconditioned with 1.0 ml methanol. After extraction, cartridges were washed with 1.0 ml water and dried with compressed air. Then, the analytes were eluted twice with 0.4 ml methanol. The combined elutes were evaporated to dryness at 30 °C under a gentle air stream and dissolved in 50 μl mobile phase. Aliquots of 10 μl were injected into the LC–MS system.

2.1.2. Chromatography and mass spectrometry

The LC–MS/MS system consisted of the API 2000 triple quadrupole mass spectrometer equipped with the electrospray ionization (ESI) source TurboIonSpray™ (Applied Biosystems, Darmstadt, Germany), a binary pump with integrated degasser (Hewlett-Packard Series 1100, Waldbronn, Germany), the column oven L-5025 (Merck-Hitachi, Darmstadt, Germany) set at 25 °C, an autosampler equipped with the Peltier cooling system set to 15 °C (Perkin Elmer Series 200, Darmstadt, Germany), and the Caltrex Resorcinearene® column (125 mm × 2 mm, particle size 5 μm; Caltrex, Greifswald, Germany). Caltrex Resorcinearene® is a new separation phase consisting of condensed alkyl substituted diphenols which are bound to silicagel carrier material.

The chromatography was performed isocratically using 5 mM formate buffer/acetonitril (40:60, v/v) as mobile phase with a flow rate of 0.3 ml/min.

The mass spectrometer operated in the single ion monitoring (SIM) mode by TurboIonSpray™ ionization. Peak areas of the respective protonated molecules were measured (nystatin, *m/z* 926.6; amphotericin, *m/z* 924.4). Details of the optimized TIS and MS parameters are given in Table 1.

The chromatograms were evaluated with the internal standard method using peak-area ratios for calculation of the calibration function and linear regression analysis weighted by $1/x$ (x = concentration). For data acquisition and statistical evaluation, the device-specific software Analyst 1.2 (Applied Biosystems, Darmstadt, Germany) and the program package Microsoft® Office Excel 2003 (Microsoft Cooperation, Redmond, USA), respectively, were used.

2.1.3. Method validation

Validation of the quantitative assay was performed according to the accepted international recommendations [10–12].

Table 1
Optimized TIS and MS parameters for the determination of nystatin

ESI parameters	MS parameters
Nebulizer gas: 50 psi	Declustering potential: 20 V
Auxiliary gas: 85 psi	Focusing potential: 400 V
Temperature: 200 °C	Entrance potential: 10 V
Curtain gas: 20 psi	Capillary voltage: 5000 V

Nitrogen was used as nebulizer, auxiliary and curtain gas (1 psi = 6894.8 Pa).

Selectivity was verified by comparing chromatograms of five pooled blank plasma with five plasma samples which were spiked with 50 ng/ml nystatin and the internal standard amphotericin B.

The calibration function range between 1.0 and 100 ng/ml was chosen because of the low plasma concentrations expected after inhalative administration of nystatin. One calibration batch for validation consists of six calibrators, one double blank sample without analyte and without internal standard, one blank sample spiked with internal standard and three quality control samples.

Recovery of nystatin was estimated by comparing the peak area signal of nystatin after extraction from plasma with the peak area signal of nystatin which has been dissolved in methanol. Independently prepared samples with 3, 50 and 80 ng/ml nystatin were measured six-fold.

Stock solution stability of nystatin and the internal standard was verified by daily measurement of three aliquots of the stock solutions over the period of three weeks before and during validation. Stock solutions were stored in darkness at 4–6 °C.

Post-preparative stability in open sample vials in the autosampler (15 °C) was assessed by repeated tracing the respective MS signals from freshly prepared samples in mobile phase (3, 50, 80 ng/ml, five times) over a period of 8 h.

Resuming investigations to the long-term, short-term stability and freeze-thaw stability were not accomplished.

Accuracy and *precision* were assessed with six sets of calibrator samples containing 1, 5, 10, 30, 60, and 80 ng/ml nystatin and quality control samples spiked with 3, 50 and 80 ng/ml nystatin (representative for the low, medium and high segment of the calibration function). All samples were prepared using pooled human blank plasma and stored at −20 °C until use on test days. Accuracy was assessed by comparing the concentrations measured in quality control and calibrator samples with the respective nominal concentrations and expressed as relative error. Precision was evaluated by measuring six separately prepared and measured calibrators and/or quality control samples and expressed as relative standard deviation of the mean concentrations.

To avoid matrix effects, all calibrator samples and as quality control samples were prepared in blank human plasma, a formiate buffer containing mixture was used for elution and an internal standard for quantification, and the chromatographic flow of the first minute was discarded to prevent that constituents of the matrix reach the ESI source.

2.2. Pharmacokinetic study with inhaled nystatin

12 healthy white subjects (7 women, 5 men, age 20–35 years, body mass index 19.9–25.4 kg/m^2) were included after giving written informed consent. The subjects were ascertained to be of good health by means of histories, physical and routine laboratory examinations, 12-lead electrocardiograms and body plethysmography. All abstained from alcohol, took no medication except hormonal contraceptives and were on standard diet during hospitalization. The study had been approved by the local ethics committee.

2.2.1. Study protocol

500,000 IU (100 mg) of nystatin (Nystatin "Lederle" sterile powder, ICH Pharmaceuticals, Frankfurt/Main, Germany) were suspended in 5 ml saline and inhaled for 5 days in two portions of 2.5 ml at 08:00 a.m. and 08:00 p.m. (duration 5 min) using the ultrasonic nebulizer OPTINEB®-ir (Nebu-Tec, Eisenfeld, Germany). Blood from a cubital vein (5 ml) was sampled before dug administration on the first treatment day (plasma blank) and before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h after last administration in the morning of the 5th treatment day. The samples were centrifuged at 2000 × *g* and at 4 °C for 10 min using the centrifuge LABOFUGE-400 R (Heraeus Instruments, Hanau, Germany). Plasma was stored in polypropylene tubes (Nalgene, Rochester, USA) at least at −20 °C until quantitative analysis.

2.2.2. Pharmacokinetic and statistical evaluation

Maximum (C_{max}) and minimum plasma concentration (C_{min}) were obtained from the concentration–time curves of nystatin. Areas under the concentration–time curves ($AUC_{0–12h}$) were assessed using the trapezoidal formula and average concentrations (C_{av}) were derived from $AUC_{0–12h}$/12 h. Peak-trough fluctuations (PTF) were calculated by $(C_{max} - C_{min})/C_{av}$. Arithmetic mean ± standard deviations (SD) were given as appropriate.

3. Results and discussion

3.1. LC–MS analysis

Both nystatin and the internal standard amphotericin B showed mass spectra with main peaks of the protonated molecules at *m*/*z* 926.6 for nystatin and at *m*/*z* 924.4 for amphotericin B. Besides of the protonated molecule peak of nystatin, two additional ions occurred with *m*/*z* 908.4 representing the loss of one water molecule and with *m*/*z* 948.6 representing the sodium salt adduct. Under these conditions no fragment ions could be observed. Amphotericin B showed the same ionization pattern (Fig. 2).

In former HPLC methods, RP-18 columns were used [7–9,13]. However, with the "Caltrex Resorcinarene" colums, nystatin could be more separated from the internal standard than with commercially RP-18 columns. The retention time of nystatin was 2.0 min, of amphotericin B 2.4 min after isocratic elution with ammonium formate buffer/acetonitrile (40:60, v/v). These chromatographic conditions (binary mixture, isocratic elution, 40% water) were similar to the conditions under which nystatin has been separated in other liquid chromatographic assays [9,13].

3.2. Validation

The method was selective for nystatin as shown by absence of the nystatin-specific signal in six different plasma blank samples (Fig. 3). The recovery in plasma ranged in dependency of the concentration between 67% (at 3 ng/ml) to 103% (at 80 ng/ml) (Table 2). Therefore, it may be that recovery of nystatin from plasma is limited by binding to the material used for extrac-

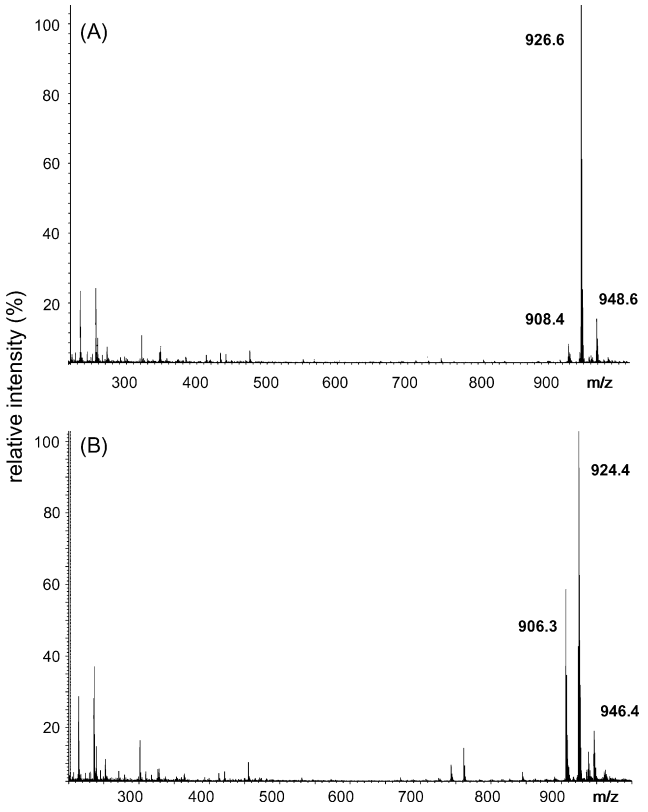

Fig. 2. Mass spectra of nystatin (A) and the internal standard (B) obtained with positive TurboIon® spray ionisation.

Table 2
Recovery of nystatin from human plasma and accuracy and precision of calibration and quality control of the LC–MS assay. Accuracy is given in percent of the respective nominal concentration and precision as standard deviation in percent of the respective concentration mean

(A) Quality controls (between-day; $n=6$)			
Concentration spiked (ng/ml)	3.0	50.0	80.0
Mean concentration measured (ng/ml)	3.1	45.4	76.4
Standard deviation	0.18	1.78	3.07
Relative error (accuracy) (%)	3.2	−10.2	−4.7
Relative standard deviation (precision) (%)	5.6	3.9	4.0

(B) Quality controls (within-day; $n=5$)			
Concentration spiked (ng/ml)	3.0	50.0	80.0
Mean concentration measured (ng/ml)	3.0	51.1	80.8
Standard deviation	0.25	5.33	5.57
Relative error (accuracy) (%)	1.3	2.1	1.0
Relative standard deviation (precision) (%)	8.1	−10.4	6.9
Recovery (%)	67.2	80.7	102

(C) Calibrators ($n=6$)						
Concentration spiked (ng/ml)	1.0	5.0	10.0	30.0	60.0	100
Mean concentration measured (ng/ml)	1.0	5.1	9.8	28.4	58.4	103
Standard deviation	0.04	0.30	0.76	0.97	1.74	2.23
Relative error (accuracy) (%)	4.0	2.5	−2.1	−5.8	−2.7	3.1
Relative standard deviation (precision) (%)	3.6	5.9	7.7	3.4	3.0	2.2

tion and separation. The linearity of the calibration curves and the quality of the assay, however, were not influenced by the concentration dependence of recovery.

Nystatin was stable in the autosampler (post preparative stability) over a period of 8 h at 15 °C. Stock solutions of nystatin and amphotericin B were stable at −20 °C at least for three months. With these stock solutions, working solutions were prepared freshly every week. In all cases, the deviations from the initial values were lower than 12%. Therefore, stock solution and post-preparative stability were considered to be acceptable because the deviations were within 85–115% of the expected value (Table 2).

The calibration function in human plasma was linear between 1.0 and 100 ng/ml nystatin as verified with six independently prepared and measured calibration sample sets. The coefficients of correlation were between 0.9974 and 0.9999 and the calibration function showed a homoscedastic distribution. The residual plot clearly showed that individual errors were randomly distributed around the concentration axis (linear regression model, weighting factor $1/x$, x = concentration). The F-test revealed no significant difference between the variances ($F_{exp} \times 4.001 < F_{tabl} \times 15.98$ for a 99% confidence level) [14]. The individual deviations from the nominal concentrations of all calibrator samples and quality control samples were lower than 12%.

Within-day accuracy and within-day precision as well as between-day precision were better than 15% of the nominal values (accuracy) and the respective means (precision), respectively (Table 2). Therefore, the method fulfilled the international

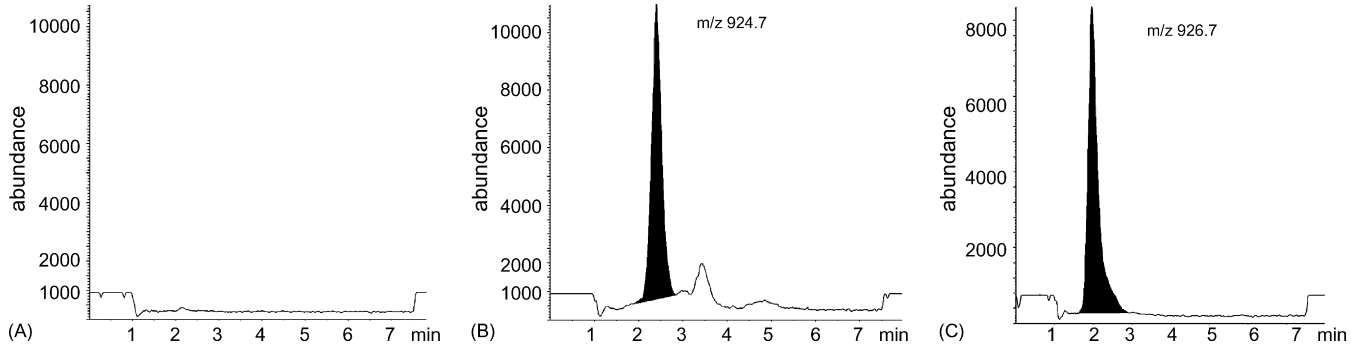

Fig. 3. Mass chromatograms of extracts from blank human plasma sample (A) spiked with the internal standard amphotericin (B) and nystatin (C), respectively.

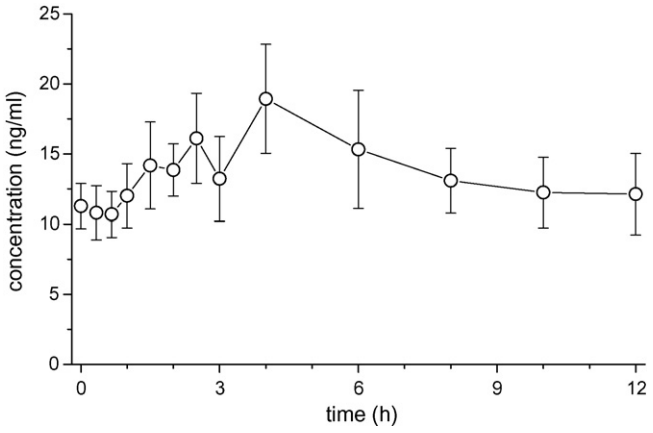

Fig. 4. Plasma concentration–time curve of nystatin in 12 healthy subjects on the 5th day of repeated inhalation of 250,000 IU (100 mg) twice daily. Means and standard errors of means are given.

Table 3
Pharmacokinetic characteristics of nystatin in healthy subjects measured on the 5th day of repeated inhalation of 250,000 IU twice daily

Volunteer	AUC_{0-24h} (ng × h/ml)	C_{max} (ng/ml)	C_{min} (ng/ml)	PTF (percentage)
1	65	11.5	3.0	157
2	35	4.6	1.3	114
3	123	17.6	3.2	141
4	185	23.7	11.4	80
5	142	16.3	8.0	70
6	352	51.1	6.7	151
7	50	14.4	1.0	322
8	142	44.2	1.0	366
9	145	20.7	8.6	100
10	359	44.2	17.9	88
11	224	40.6	11.9	154
12	194	19.8	7.8	74
Mean	168	25.7	6.8	151
SD	105	15.2	5.2	96
Minimum	35	4.6	1.0	70
Maximum	359	51.1	17.9	366

acceptance criteria for bioanalytical methods which should be below 15% (below 20% at LOQ) [10–12].

3.3. Pharmacokinetics of inhaled nystatin

After chronic pulmonary administration of 500,000 IU (100 mg) daily, nystatin appeared slowly in plasma with a maximum peak concentration of about 50 ng/ml. The maximum trough level has been 18 ng/ml. The individual concentration–times curves were highly variable (Fig. 4, Table 3). Comparison of these data with plasma levels and tolerability of intravenous liposomal nystatin in patients with human immunodeficiency virus infections revealed that the systemic exposure with nystatin in our study was about three-potencies lower [6]. Therefore, systemic effects are not expected after daily inhalative administration of an aerosol with 500,000 IU of nystatin sterile powder. In our study, there was no any drug relelated adverse reaction after repeated administration of inhaled nystation.

4. Conclusions

A quantitative assay for nystatin was developed to measure human plasma concentrations between 1.0 and 100.0 ng/ml after repeated inhalative administration of an aerosol containing 500,000 IU (100 mg) of nystatin setrile powder. Between-day and within-day precision and accuracy were better than 15% of the respective mean and nominal values. Recovery ranged between 67 and 102%. The systemic exposure of nystatin after repeated inhalation was very low with maximum plasma levels up to 50 ng/ml.

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References

[1] E.L. Hazen, R. Brown, Science 112 (1950) 423.
[2] S.B. Zotchev, Curr. Med. Chem. 10 (2003) 211.
[3] A.H. Groll, S.C. Piscitelli, T.J. Walsh, Adv. Pharmacol. 44 (1998) 343.
[4] J.M. Hamilton-Miller, Bacteriol. Rev. 37 (1973) 166.
[5] The pharmacological basis of therapeutics, Macmillian Publishing Co. Inc., New York, 1975.
[6] A. Rios, M. Rosenblum, G. Crofoot, R. Lenk, J. Infect. Dis. 168 (1) (1993) 253.
[7] A.H. Groll, D. Mickiene, K. Werner, S.C. Piscitelli, T.J. Walsh, J. Chromatogr. B: Biomed. Sci. Appl. 735 (1999) 51.
[8] B. Sauer, R. Matusch, J. Chromatogr. A 672 (1994) 247.
[9] W. Mechlinski, C.P. Schaffner, J. Chromatogr. 99 (1974) 619.
[10] Center for Drug Evaluation and Research, 2001, http://www.fda.gov/cder/guidance/4252fnl.htm.
[11] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Int. J. Pharm. 82 (1992) 1.
[12] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[13] P. Wilson, A. Stewart, V. Flournoy, S.W. Zito, A. Vancura, J. AOAC Int. 84 (2001) 1050.
[14] A.M. Almeida, M.M. Castel-Branco, A.C. Falcao, J. Chromatogr. B Anal.: Technol. Biomed. Life Sci. 774 (2002) 215.