

Stability Indicating RP-HPLC Method Development and Validation for Oseltamivir API

Balasubramanian NARASIMHAN,*^a Khan ABIDA,^a and Kona SRINIVAS^b

^aDepartment of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology; Hisar–125001, Haryana, India; and ^bRanbaxy Research Laboratories; Gurgaon–122015, Haryana, India.

Received August 7, 2007; accepted January 14, 2008; published online January 22, 2008

The present study describes the development and subsequent validation of a stability indicating reverse-phase HPLC (RP-HPLC) method for the analysis of oseltamivir active pharmaceutical ingredient (API). The proposed RP-HPLC method utilizes Kromasil C₁₈, 5 μ m, 250 mm \times 4.6 mm i.d. column (at ambient temperature), gradient run (using acetonitrile and triethylamine as mobile phase), effluent flow rate (1.0 ml/min), and UV detection at 215 nm for analysis of oseltamivir. The described method was linear over the range of 70–130 μ g/ml ($r^2=0.999$). The precision, ruggedness and robustness values were also within the prescribed limits (<1% for system precision and <2% for other parameters). Oseltamivir was exposed to acidic, basic, oxidative and thermal stress conditions, and the stressed samples were analyzed by the proposed method. Chromatographic peak purity results indicated the absence of co-eluting peaks with the main peak of oseltamivir, which demonstrated the specificity of assay method for estimation of oseltamivir in presence of degradation products. The proposed method can be used for routine analysis of oseltamivir in quality control laboratories.

Key words reverse-phase-HPLC; oseltamivir; stability indicating assay; forced degradation

Oseltamivir is an antiviral drug used in the treatment and prophylaxis of influenza virus A and B infections. It was developed by Gilead Sciences and is currently marketed by Haffmann-La Roche (Roche) under the trade name Tamiflu. Oseltamivir is readily absorbed from the gastrointestinal tract after oral administration of oseltamivir phosphate. It is extensively converted to oseltamivir carboxylate by esterases located predominantly in the liver. Oseltamivir carboxylate is eliminated entirely by renal excretion. Less than 20% of an oral radiolabeled dose is eliminated in feces.¹⁾ Chemically it is (3R,4R,5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester.²⁾

Stability testing forms an important part of the process of drug product development. The purpose of stability testing is to provide evidence on how the quality of a drug substance varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and enables recommendation of storage conditions, retest periods, and shelf life to be established. The assay of oseltamivir API (Active Pharmaceutical Ingredient) in stability test sample needs to be determined using stability indicating method, as recommended by the International Conference on Harmonization (ICH) guidelines³⁾ and USP.⁴⁾

The present work was designed to develop a simple, precise and rapid analytical LC procedure, which would serve as stability indicating assay method for analysis of oseltamivir API. Literature survey for oseltamivir analysis revealed several methods based on different techniques, viz. LC-MS assay for its quantification in plasma and urine,⁵⁾ LC assay for evaluation of Tamiflu⁶⁾ and HPLC method for the determination of oseltamivir in Tamiflu.⁷⁾

The present study is the first time report on stability indicating assay of oseltamivir in presence of degradation products by HPLC. In this method gradient elution method is selected for the analysis of oseltamivir API because it gave better base line separation and peak width, which is suitable for the routine analysis of oseltamivir. Although the gradient elution method uses two-pump system, the gradient run pro-

vided a proper peak with baseline, which prompted us to select this method for the analysis of oseltamivir API.

In view of above in the present study we hereby report the development and validation of a stability indicating gradient reverse-phase HPLC (RP-HPLC) method for analysis of oseltamivir API in presence of degradation products as per ICH guidelines.

In order to establish the stability indicating nature of the method, force degradation of oseltamivir was performed under various stress conditions (basic, acidic and oxidative), and stressed samples were analyzed by the proposed method. The proposed LC method was able to separate the drug from degradation products generated during forced degradation studies. The developed method was validated as per ICH guidelines⁸⁾ and its updated international convention.⁹⁾ The linearity of response, precision, ruggedness and robustness of the described method has been checked.

Experimental

Chemical and Reagents Oseltamivir standard and API were provided by Ranbaxy Research Laboratories, Gurgaon, India. Acetonitrile and hydrogen peroxide were from Qualigens, Mumbai, water was from Milli Q (Millipore), Billerica, triethylamine and sodium hydroxide were from Merck Ltd.; Mumbai.

HPLC Instrumentation and Conditions The HPLC system—Alliance, Waters 2695 separation module having maximum pressure of 5000 psi, detector—Waters 2996, photodiode array detector. To develop a precise, linear, specific and suitable stability indicating RP-HPLC method for analysis of oseltamivir, different chromatographic conditions was applied and the results are presented in Table 1 and the following optimized conditions were applied for final analysis. The chromatographic separation was performed using Kromasil C₁₈, 5 μ m, 250 \times 4.6 mm i.d. column, at ambient temperature, eluted at the flow rate of 1.0 ml/min using gradient run. The mobile phase consisted of acetonitrile and 0.2% triethylamine buffer (v/v), apparent pH adjusted to 3 \pm 0.1 with 10% phosphoric acid, filtered through 0.45 μ m nylon filter and degassed in ultrasonic bath prior to use. Wavelength was selected by scanning standard solution of drug over 200 to 400 nm using Shimadzu model 2450 double beam UV–visible spectrophotometer. Measurements made with an injection volume of 20 μ l and ultraviolet (UV) detection at 215 nm, showed reasonably good response.

Standard and Sample Preparation The standard and sample stock solutions were prepared separately by dissolving standard and sample in a sol-

* To whom correspondence should be addressed. e-mail: naru2000us@yahoo.com

Table 1. Method Development for RP-HPLC Analysis of Oseltamivir API

Fig. No.	Column used	Mobile phase	Isocratic/ gradient	Inj. vol.	Observation	Result
1a	Gemini C ₁₈ , 5 μ (150 \times 4.6) mm	K ₂ HPO ₄ +KH ₂ PO ₄ (pH 7): ACN ^{a)}	Isocratic	20 μ l	Peak width was more	Method rejected
1b	Chemsil C ₁₈ , 5 μ (150 \times 4.6) mm	Triethylamine (pH 3): ACN	Isocratic	20 μ l	Absorbance was very less	Method rejected
1c	Kromasil C ₁₈ , 5 μ (150 \times 4.6) mm	Ammonium acetate (pH 6.7): ACN	Isocratic	20 μ l	Peak shape was not OK	Method rejected
1d	Kromasil C ₁₈ , 5 μ (250 \times 4.6) mm	Ammonium acetate (pH 6.7): ACN	Isocratic	20 μ l	Base line was not OK	Method rejected
1e	Kromasil C ₁₈ , 5 μ (250 \times 4.6) mm	Triethylamine (pH 3): ACN	Isocratic	20 μ l	Peak width was more	Method rejected
1f	Kromasil C ₁₈ , 5 μ (250 \times 4.6) mm	Triethylamine (pH 3): ACN	Isocratic	20 μ l	Peak width was more	Method rejected
1g	Kromasil C ₁₈ , 5 μ (250 \times 4.6) mm	Triethylamine (pH 3): ACN	Gradient	20 μ l	Base line was not OK	Method rejected
1h	Kromasil C ₁₈ , 5 μ (250 \times 4.6) mm	Triethylamine (pH 3): ACN	Gradient	20 μ l	Run time was more	Method rejected
1i	Kromasil C ₁₈ , 5 μ (250 \times 4.6) mm	Triethylamine (pH 3): ACN	Gradient	20 μ l	Both peak shape and absorbance were OK	Method accepted

From the results observed the following optimum chromatographic conditions were finalized for the analysis of oseltamivir API.

Gradient run:

Time	Buffer	ACN
0	80	20
5	80	20
8	60	40
12	30	70
18	80	20
22	80	20

 a) ACN: acetonitrile.

vent mixture of water: acetonitrile (70:30, v/v) and diluting with the same solvent.

Optimization of Chromatographic Conditions The chromatographic conditions were optimized by different means. (Using different column, different buffer and different mode of HPLC run, Table 1, Fig. 1.)

Forced Degradation Studies Oseltamivir was allowed to hydrolyze in different strengths of base (0.005 N, 0.05 N NaOH), acid (0.05 N, 0.5 N, 1 N HCl) and hydrogen peroxide (30%, 10%, 3%, 1%). Oseltamivir was also studied for its thermal degradation at 100 °C, 120 °C and 200 °C for 1 h. An accurately weighed 50 mg of oseltamivir API was dissolved in 1 ml of respective base (NaOH), acid (HCl) or hydrogen peroxide and kept for specified period after which the volume was made upto 50 ml with water: acetonitrile (70:30, v/v). Five milliliters of the above solution was diluted with water: acetonitrile (70:30, v/v) to get a concentration of 100 ppm. Blank was also treated in the same way. The results of stability studies are presented in Table 2, Fig. 2.

Validation Linearity was determined by injecting different concentration of sample solutions (70—130 μ g/ml, Fig. 3). For system precision, standard solution (100 μ g) was injected in six replicate injections to check % RSD (relative standard deviation) and for method precision six time samples were prepared and each of those was injected in duplicate. Mean of all of these values gives rise to assay value.

To establish the within-day (intra-assay) and between-day (inter-assay) accuracy and precision of the method, oseltamivir was assayed on one day and three separate days. Intra-assay and inter-assay were calculated and the data are presented in Tables 3 and 4 respectively.

Robustness of method was investigated by varying the chromatographic conditions such as change of flow rate (\pm 10%), organic content in mobile phase (\pm 2%), wavelength of detection (\pm 5%) and pH of buffer in mobile phase (\pm 0.2%). Robustness of the developed method was indicated by the overall % RSD between the data at each variable condition (Table 5).

Limit of Detection (LOD) The detection limit is determined by the analysis of samples with the known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

The LOD was calculated as follows:

$$\text{LOD} = 3.3(\sigma/S)$$

Limit of Quantitation (LOQ) The quantitation limit is determined by the analysis of sample of known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

The LOQ was calculated as follows:

$$\text{LOQ} = 10(\sigma/S)$$

Specificity The specificity of the proposed method was performed by

analyzing degraded sample solution of oseltamivir.

Stability in Analytical Solution For stability in analytical solution sample was analyzed initially and at different time intervals.

Results and Discussion

Chromatographic Conditions To develop a precise, linear, specific and suitable stability indicating RP-HPLC method for analysis of oseltamivir, different chromatographic conditions were applied and the results observed are presented in Table 1 and Figs. 1a—i. Isocratic elution is simple, requires only one pump and flat baseline separation for easy and reproducible results. X. F. Li *et al.*¹⁰ suggested that the time to achieve baseline separation for analysis of components is usually long under isocratic elution. The similar results have been observed with current study (Table 1) where in different isocratic runs the base line or peak width were not significant to carry out RP-HPLC analysis whereas the gradient run provided a proper peak with baseline (Fig. 1i) which was selected for the analysis of oseltamivir API. This is similar to the results observed by Sharma *et al.*¹¹ during their analysis of tea chemicals by RP-HPLC, where they observed gradient elution provided better base line separation and peak width in comparison to isocratic run.

In case of RP-HPLC various columns are available, but here Kromasil C₁₈, 5 μ m, 250 mm \times 4.6 mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good and acetonitrile was selected as mobile phase, because of its favorable UV transmittance. Among the different mobile phases employed the mobile phase consisted of acetonitrile and 0.2% triethylamine (v/v) with an apparent pH adjusted to 3 \pm 0.1 with 10% phosphoric acid was found to be suitable for the analysis of oseltamivir API. Further, a flow rate of 1.0 ml/min, an injection volume of 20 μ l and UV detection at 215 nm was found to be best for analysis.

Forced Degradation Studies Singh and Bakshi¹² suggested a target degradation of 20—80% for establishing stability indicating nature of the assay method, even as the

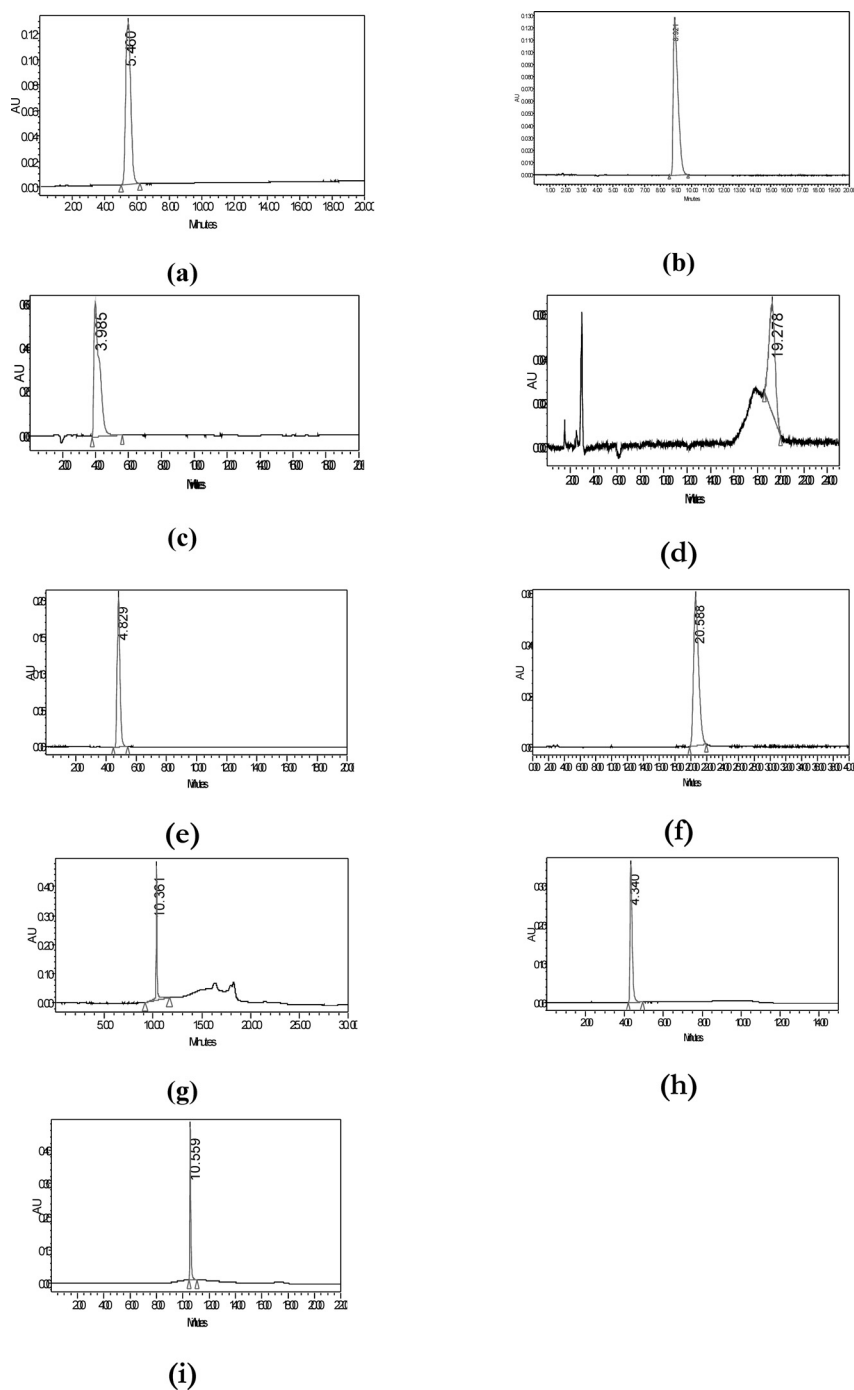


Fig. 1. The Chromatograms of Different Trials Shown in Table 1

intermediate degradation products should not interfere with any stage of drug analysis. In the present study even though the conditions used for forced degradation are in the range of producing 20–80% target degradation, degradation of oseltamivir could not be achieved even after prolonged duration.

During the study it was observed that upon treatment of oseltamivir with different strengths of base (0.005 N, 0.05 N NaOH), acid (0.05 N, 0.5 N, 1 N HCl) and hydrogen peroxide (30%, 10%, 3%, 1%) the degradation was observed only with the higher strengths of alkali (0.05 N NaOH), acid (1 N HCl) and hydrogen peroxide (30%) whereas with the lower strengths of alkali (0.005 N NaOH), acid (0.05 N, 0.5 N HCl)

and hydrogen peroxide (1%, 3%, 10%) no degradation (0%, Table 2) was observed. Table 2 indicates the extent of degradation of oseltamivir under various stress conditions. Figures 2a to e show the chromatograms of forced degraded samples. Further it is important to note that from the chromatograms (Figs. 2a to e), it is evident that although the degraded peaks are observed, the peak of oseltamivir is unaffected, which indicates that these degraded peaks are due to the presence of impurities (amine or polar groups) and the oseltamivir is stable under the applied stress conditions like heat, acid and alkaline hydrolysis and oxidative degradation states.

Linearity The calibration curve showed good linearity in the range of 70–130 µg/ml, for oseltamivir API with cor-

Table 2. Results of Force Degradation Studies of Oseltamivir API

Stress condition/duration/solution	% degradation
Alkaline degradation (0.05 N NaOH, 1.5 h)	10
Alkaline degradation (0.05 N NaOH, 2.5 h)	15
Alkaline degradation (0.05 N NaOH, 5 h)	25
Oxidative degradation (1% H ₂ O ₂)	0
Oxidative degradation (3% H ₂ O ₂)	0
Oxidative degradation (10% H ₂ O ₂)	0
Oxidative degradation (30% H ₂ O ₂ , 90 °C, 10 min)	39
Acidic degradation (0.05 N HCl)	0
Acidic degradation (0.5 N HCl)	0
Acidic degradation (1 N HCl, 85 °C, 25 min)	10
Thermal degradation (solid sample, 100 °C, 1 h)	11
Thermal degradation (solid sample, 120 °C, 1 h)	22

Table 3. Intra-assay Precision Data of Proposed RP-HPLC Method (Method Ruggedness)

	Mean (% w/w)	S.D.	% RSD
Assay-1	99.79	0.408	0.41
Assay-2	99.27	0.236	0.24
Intra assay	99.53	0.420	0.42

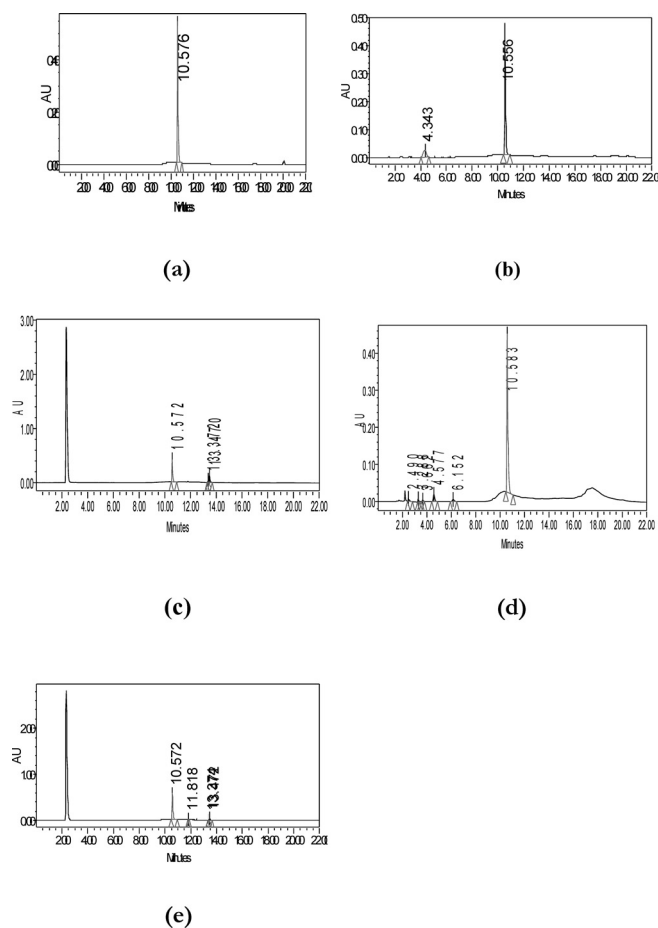


Fig. 2. Chromatograms of (a) Untreated Sample Solution, (b) Base Hydrolyzed-Degraded Sample, (c) Oxidative-Degraded Sample, (d) Acid Hydrolyzed Degraded Sample, (e) Thermal Degraded Sample

relation co-efficient (r) of 0.999 (Fig. 3). A typical calibration curve has the regression equation of $y=25358x+62100$ for oseltamivir.

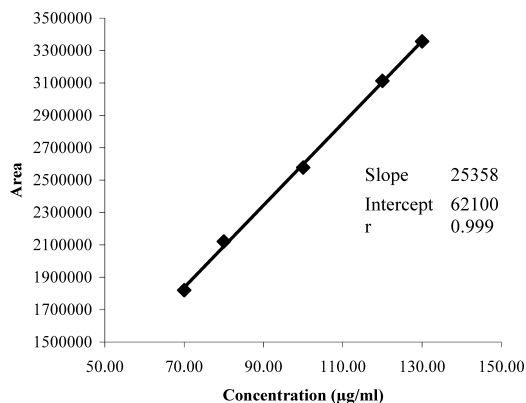


Fig. 3. Linearity Curve for Oseltamivir

Table 4. Inter-assay Precision Data of Proposed RP-HPLC Method

	Mean (% w/w)	S.D.	% RSD
Assay-1	99.26	0.081	0.08
Assay-2	99.41	0.248	0.25
Inter assay	99.33	0.192	0.19

Table 5. The Influence of Changes in Chromatographic Parameters on RP-HPLC Analysis of Oseltamivir API (Method Robustness)

Change in parameter	% RSD
Flow (0.9 ml/min)	0.64
Flow (1.1 ml/min)	0.94
Wave length (210 nm)	1.89
Wave length (220 nm)	0.33
pH (2.8)	0.03
pH (3.2)	0.83
Organic phase composition (-2%)	0.87
Organic phase composition (+2%)	0.90

Precision The results of system precision (% RSD=0.25) and method precision (% RSD=0.10) are found within the prescribed limit of ICH guidelines (% RSD<1%, and % RSD<2% respectively in case of system precision and method precision).

Intra-assay and Inter-assay The intra- and inter-day variation of the method was carried out and the high values of mean assay and low values of standard deviation and % RSD (% RSD<2%) within a day and day to day variations for oseltamivir revealed that the proposed method is precise (Tables 3, 4).

Method Robustness Influence of small changes in chromatographic conditions such as change in flow rate ($\pm 10\%$), organic content in mobile phase ($\pm 2\%$), wavelength of detection ($\pm 5\%$) and pH of buffer in mobile phase ($\pm 0.2\%$) studied to determine the robustness of the method are also in favor (Table 5, % RSD<2%) of the developed RP-HPLC method for the analysis of oseltamivir API.

LOD and LOQ The minimum concentration levels at which the analyte can be reliably detected (LOD) and quantified (LOQ) were found to be 0.05 and 0.3 $\mu\text{g/ml}$, respectively.

Specificity and Stability in Analytical Solution The results of specificity indicated that the peak was pure in pres-

ence of degraded sample. It is important to mention here that the oseltamivir API was stable in solution form upto 24 h at 25 °C. The appearance of broad peak at RRT 4.343 may be attributed to the presence of amine or polar groups in the degraded sample.

The results of linearity, precision, inter and intra-day assays, method robustness, LOD, LOQ and specificity and stability in analytical solution established the validation of the developed RP-HPLC assay for the analysis of oseltamivir.

Conclusion

In conclusion, a sensitive and selective stability indicating RP-HPLC method has been developed and validated for the analysis of oseltamivir API. Based on peak purity results, obtained from the analysis of force degraded samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of oseltamivir indicated that the developed method is specific for the estimation of oseltamivir in presence of degradation products. Further the proposed RP-HPLC method has excellent sensitivity, precision, and reproducibility. Even though no attempt was made to identify the degraded products, proposed method can be used as a stability indicating method for assay of oseltamivir.

References

- 1) Maryadell N. J. O., Hekelman K. J., "The Merck Index—An Encyclopedia of Chemicals, Drugs and Biologicals," 14th ed., Merck & Co. Inc., Whitehouse Station, NJ, 2006, pp. 1187—1188.
- 2) Oseltamivir-Wikipedia, <http://en.wikipedia.org/wiki/oseltamivir>.
- 3) ICH, "Stability Testing of New Drug Substances and Procedure," Proceeding of the International Conference on Harmonization, Geneva, 1993.
- 4) "The United States Pharmacopoeia," 26th ed., US Pharmacopoeia convention, MD, 2003, pp. 1151—1154.
- 5) Wiltshire H., Wiltshire B., Citron A., Serpe C., Gray D., Herron W., *J. Chromatogr. B*, **745**, 373—388 (2000).
- 6) Lindegardh N., Hien T., Farrar J., Singhasivanon P., White N. J., Day N. P. J., *J. Pharm. Biomed. Anal.*, **42**, 430—433 (2006).
- 7) Joseph-Charls J., Geneste C., Laborde-Kummer E., Gheyouché R., Boudis H., Dubost J. P., *J. Pharm. Biomed. Anal.*, **44**, 1008—1012 (2007).
- 8) ICH, "Validation of Analytical Procedures: Methodology," Proceeding of the International Conference on Harmonization, Geneva, 1996.
- 9) ICH, "Guideline on Analytical Method Validation," Proceeding of International Convention on Quality for the Pharmaceutical Industry, Toronto, Canada, 2002.
- 10) Li X., Ma M., Cheng A., Zheng J., Tam Y. K., *Anal. Chim. Acta*, **457**, 165—171 (2002).
- 11) Sharma V., Gulati A., Ravindranath S. D., *J. Food Compos. Anal.*, **18**, 583—594 (2005).
- 12) Singh S., Bakshi M., *Pharm. Tech.*, **24**, 1—14 (2000).