Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 1008–1013
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

# Development and validation of a rapid HPLC method for the determination of oseltamivir phosphate in Tamiflu® and generic versions

J. Joseph-Charles [a], C. Geneste [a], E. Laborde-Kummer [a], R. Gheyouche [b], H. Boudis [b], J.-P. Dubost [a,*]

[a] *Université Victor Segalen Bordeaux 2, UFR des Sciences Pharmaceutiques, Laboratoire de Chimie Analytique, 33076 Bordeaux Cedex, France*
[b] *Université Benyoucef Ben Khedda, Faculté de Médecine d'Alger, Département de Pharmacie, Laboratoire de Chimie Analytique, 16100 Alger Centre, Algeria*

Received 7 January 2007; received in revised form 1 April 2007; accepted 3 April 2007
Available online 7 April 2007

**Abstract**

Oseltamivir phosphate (OP) is an antiviral drug that is used in the treatment and prophylaxis of both influenza A and influenza B. It is effective against all known influenza viruses than can infect humans, including pandemic influenza viruses and may be the most appropriate antiviral option against avian influenza caused by H5N1 virus. Tamiflu®, the registered trademark used under exclusive license by Roche laboratories with OP as active pharmaceutical ingredient, is considered the best treatment for the bird flu disease.

A simple, selective, linear, accurate and precise HPLC method was developed and validated for rapid assay of OP aimed to the quality control of Tamiflu® capsules and generic versions. Isocratic elution at a flow rate of 1.2 mL/min was employed on a Zorbax CN column (150 mm × 4.6 mm; 5 μm) at ambient temperature. The mobile phase consisted of methanol and 0.04 M formic acid pH 3.0 (50:50, v/v). The UV detection wavelength was 226 nm and 20 μL of sample was injected. Sotalol hydrochloride was used as the internal standard (IS). The retention times for OP and IS were 3.40 and 2.25 min, respectively. The method was successfully applied to commercial pharmaceuticals, Tamiflu® and generic versions. The proposed method could be applicable for routine analysis of OP and monitoring of the quality of marketed drugs as possibly counterfeit Tamiflu®.


*Keywords:* Oseltamivir phosphate; Tamiflu®; Bird flu; RPLC–UV; Generic versions; Counterfeit

## 1. Introduction

Oseltamivir phosphate (OP) is the first orally available inhibitor of influenza virus neuraminidase, an enzyme involved in the release of new virus particles from infected cells. The structure of oseltamivir shows it possesses a hydrophobic moiety (Fig. 1). Oseltamivir's hydrophobic group is responsible for its poor oral absorption; thus, the phosphate salt has been developed that allows oral administration of this drug. OP is a prodrug that is rapidly and extensively metabolized via hepatic esterases to oseltamivir carboxylate (OC), the active form, a potent and selective inhibitor of influenza virus neuraminidase [1–4]. It was developed by Gilead Sciences and is currently marketed by Hoffmann-La Roche (Roche) under the trade name Tamiflu. Tamiflu® is the leading drug against avian flu. The manufacturer has permitted arrangements for a number of companies to produce Tamiflu under license to meet fast-growing world demand for this drug [5]. Tamiflu® is already in short supply throughout the world as countries gather stockpiles of the drug among fears of a possible avian flu pandemic [6,7]. Tamiflu® will be unaffordable for many poor countries, increasing the chance of a pandemic. Generic manufacturers would fill any potential market shortages of Tamiflu® in the event of an epidemic and sell generic versions at a considerably lower price than Tamiflu® in countries where the patent is not valid. Following the potential for counterfeit copies of Tamiflu, there is a risk that medicinal products supplied may be substandard or counterfeit versions. In late January 2006, the United States FDA issued

* Corresponding author.
*E-mail address:* jean-pierre.dubost@u-bordeaux2.fr (J.-P. Dubost).

0731-7085/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jpba.2007.04.002

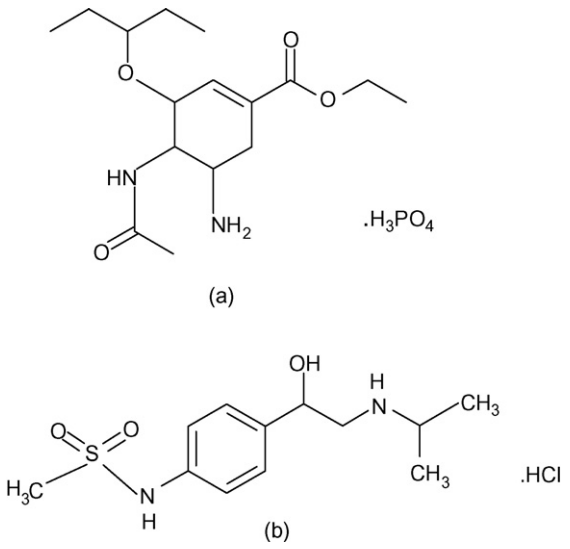


Fig. 1. Chemical structures of: (a) oseltamivir phosphate and (b) sotalol hydrochloride.

an alert about counterfeit prescription oseltamivir [8]. Problems with drug supply may increase the amount of counterfeit Tamiflu.

Hitherto there are few analytical methods reported for estimation of oseltamivir. An enzymatic assay based on neuraminidase inhibition was used for detection of oseltamivir carboxylate (OC) in rat plasma [9]. The determination of OC in pre-clinical plasma samples using HPLC with pre-column fluorescence derivatization was described [10]. OP and OC were determined by HPLC-Tandem MS in human and animal plasma and urine after a solid phase extraction [11]. These methods are complicated, costly and time consuming rather than a simple HPLC–UV method. So it is unsuitable to use these highly sensitive methods for the routine quantitative assay of OP capsules where the content of active pharmaceutical ingredient is high in the formulation. An article describes a liquid chromatographic assay for testing Tamiflu® purchased over the Internet and in local pharmacies in Thailand and Vietnam [12], however main drawbacks are time consuming sample preparation procedures including sonication of the capsule at 40 °C for 20 min, cooling to room temperature (about 30 min), centrifugation, run time of 7 min and lack of internal standard (IS).

The aim of the present work was to develop and validate a simple, fast and reliable isocratic RP-HPLC method with UV detection for the determination of OP in Tamiflu® and generic versions. The important features and novelty of the proposed method included simple sample treatment with sonication of small amount of powder sample at ambient temperature, centrifugation, dilution; short elution time (less than 5 min) with internal standard eluted prior to OP; short analysis time (less than 30 min); good precision (R.S.D. less than 5%) and high recovery (greater than 95%). Confirmation of the applicability of the developed method validated according to the International Conference on Harmonisation (ICH), to determination of OP in Tamiflu® capsules and generic Tamiflu capsules has been also performed.

## 2. Experimental

### 2.1. Chemicals and reagents

HPLC grade methanol (MeOH) and formic acid (A.R. grade) was purchased from Prolabo (VWR, Fontenay-sous-Bois, France), 1M sodium hydroxide (A.R. grade) from Panreac Quimica (Barcelona, Spain). High quality water, applied throughout the study, was obtained by means of a Jencons Autostill model 4000X apparatus (Fisher Bioblock Scientific, Illkirch, France). Oseltamivir phosphate standard sample was provided by Heterodrugs (Mumbai, India), an Indian pharmaceutical company that has been granted a sub-license by Roche to produce and manufacture oseltamivir and generic versions of Tamiflu. The internal standard, sotalol hydrochloride (Fig. 1), was obtained from Bristol-Myers Squibb (Epernon, France). All substances were used without any further purification.

Tamiflu® capsules, commercially available in France, were purchased through a local pharmacy. Generic versions of Tamiflu®, i.e. Saiflu and Flufly capsules were from the Saidal Group (Algerian Pharmaceutical Industry) and Julphar company (Gulf Pharmaceutical Industries), respectively. All of the commercial samples were supplied as hard capsules containing 75 mg oseltamivir base for oral use, in the form of oseltamivir phosphate. The molecular weight is 312.4 for oseltamivir free base and 410.4 for OP. In addition to the active ingredient, each capsule contains pre-gelatinized starch, talc, povidone K 30, croscarmellose sodium and sodium stearyl fumarate.

### 2.2. Instrumentation and analytical conditions

The HPLC system (Merck, Darmstadt, Germany) consisted of a pump (type L-6200 Intelligent Pump) equipped with a Rheodyne model 7161 injection valve with a 20 μL loop (Rheodyne Inc., Cotati, CA, USA), a UV-detector (type L-4000) set at 226 nm, an integrator (type D-2500). The analytical column, a Zorbax CN (150 mm × 4.6 mm i.d., 5 μm particle size) (Agilent Technologies, Massy, France) was operated at ambient temperature (20 ± 1 °C). Isocratic elution with methanol/0.04 M formic acid adjusted to pH 3.0 (portable pH/mV meter PHM201 connected to pHC3005-8 combined pH electrode) (Radiometer Analytical, Lyon, France) by 1 M sodium hydroxide (50:50, v/v) was used at a flow rate of 1.2 mL/min. The mobile phase was prepared freshly, filtered through a 0.20 μm polyamide-membrane filter (Sartorius, Goettingen, Germany) using a glass vacuum filtration apparatus Heto Master Jet type SUE 300Q (Heto Lab Equipment, Allerød, Denmark) and degassed by sonicating for 5 min before use (Ultrasonics type TS540, Fisher Bioblock Scientific, Illkirch, France). A BHG Hermle centrifuge type Z 360-0 (Berthold Hermle, Gosheim, Germany) was employed for the sample pre-treatment. The UV spectrum of OP for selecting the working wavelength of detection was taken using a Secomam S1000 PC UV–vis spectrophotometer (Secomam, Domont, France).

### 2.3. Stock and working standard solutions

Stock standard solutions of 1 mg/mL of OP were prepared freshly by accurately weighing approximately 20 mg of OP into a 20 mL volumetric flask and making up to volume with water. These solutions were further diluted with water in 20 mL volumetric flasks to obtain five working standards in the concentration range of 30, 40, 50, 60 and 70 μg/mL of OP covering 60–140% of the intended test concentration of 50 μg/mL for the pharmaceutical formulation. These calibration standards were added with 2.0 mL of freshly prepared 100 μg/mL aqueous solution of sotalol hydrochloride as internal standard (final concentration, 10 μg/mL) and made up to volume with water. All the solutions were prepared in triplicates. Before being subjected to analysis, all the working standard solutions were filtered through a 25 mm nylon membrane syringe filter (pore size 0.45 μm).

Before injecting solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. The calibration curve was plotted with the five concentrations of the 30–70 μg/mL working standard solutions. So chromatography was repeated thrice for each dilution. Calibration solutions were prepared daily and analysed immediately after preparation.

### 2.4. Assay sample preparation

The contents of five commercial capsules (labelled concentration 75 mg oseltamivir base corresponding to 98.5 mg OP) were each weighed and their mean mass was determined. After homogenizing the powder by grinding in a glass mortar, an accurately weighed portion of the pooled sample equivalent to 20 mg OP was quantitatively transferred into a 100 mL volumetric flask with about 95 mL of water. The solution was sonicated for 15 min, brought to volume with water, mixed well and centrifuged at 5000 rpm for 5 min. A 5.0 mL aliquot of the supernatant was transferred into a 20 mL volumetric flask, 2.0 mL of a 100 μg/mL solution of IS was added and diluted to volume using water. The theoretical OP concentration after dilution was 50 μg/mL (100% of OP). An aliquot of this solution was filtered through a 25 mm nylon membrane syringe filter (pore size 0.45 μm) prior to the injection into the HPLC system. Peak area ratios of OP to that of IS were then measured for the determinations.

### 2.5. Validation procedure

The objective of method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines [13]. The method was validated for linearity, precision (repeatability and intermediate precision), accuracy specificity, short-term stability and system suitability.

Standard plots were constructed with five concentrations in the range of 30–70 μg/mL prepared in triplicates to test linearity. The ratio of peak area signal of OP to that of IS was plotted against the corresponding concentration to obtain the calibration graph. The linearity was evaluated by linear regression analysis that was calculated by the least square regression method.

The precision of the assay was studied with respect to both repeatability and intermediate precision. Repeatability was calculated from six replicate injections of freshly prepared OP solution in the same equipment at a concentration of 100% (50 μg/mL) of the intended test concentration value on the same day. The experiment was repeated by assaying freshly prepared solution at the same concentration additionally on 2 consecutive days to determine intermediate precision. Peak area ratios of OP to that of IS were determined and precision was reported as %R.S.D.

Method accuracy was tested (% recovery and %R.S.D. of individual measurements) by analysing samples of OP at three different levels (60, 100 and 140%) in pure solutions using three preparations for each level. The results were expressed as the percentage of OP recovered in the samples.

Specificity was assessed by comparing the chromatograms obtained from sample of pharmaceutical preparation and standard solution with those obtained from excipients which take part in the commercial capsules and verifying the absence of interferences.

Sample solution short-term stability was tested at ambient temperature (20 ± 1 °C) for 3 days. In order to confirm the stability of both standard solutions at 100% level and capsule sample solutions, both solutions protected from light were reinjected after 24 and 48 h at ambient temperature and compared with freshly prepared solutions.

A system suitability test was performed by six replicate injections of the standard solution at a concentration of 50 μg/mL verifying IS/OP resolution >2; %R.S.D. of peak area ratios of OP to that of IS ±2%; %R.S.D. of each peak retention time ±2%.

## 3. Results and discussion

### 3.1. Optimization of the chromatographic conditions

Preliminary experiments have been performed on the basis of the HPLC method described by Wiltshire et al. [11]. CN stationary phase with polarity bonded-phase was selected to produce the interaction between nitrile group and OP analyte, resulting in short retention time. The effect of the composition of the mobile phase on the retention time of OP was thoroughly investigated. The concentration of the buffer (10–80 mM formic acid) as well as the amount of methanol (50–70%, v/v) were optimized to give symmetric peak with short retention time. An improvement was observed in the retention time and in the stability of peak symmetry when increasing the buffer concentration but it was important not to use as high concentration as 80 mM because of significant absorbance of formic acid at the detection wavelength of 226 nm. Acid formic concentration of 40 mM was found to be suitable.

Changes in methanol percentage from 50 to 65% resulted in decreasing the retention of OP but no significant change in peak symmetry was observed. On the other hand, the concentration of methanol of 70% involved modification of reversed-phase

system into normal-phase system based on the cyano stationary phase. Increasing the amount of methanol from 70% led to an increase of retention time. To allow internal standard elution prior to OP, the optimum methanol concentration was found to be 50%.

The effect of pH in the chromatographic elution of both compounds is related to the degree of ionisation. OP is a basic compound with p$K_a$ value 7.75 [14]. At the optimum pH value (pH 3.0) it is positively charged, which leads to its rapid elution with appropriate peak symmetry. The same low pH decreases also the retention time of the internal standard, sotalol hydrochloride, due to ionisation of its basic site (p$K_a$ = 9.8 and 8.3) [15]. There was no advantage in decreasing pH value to 2.5. A pH value of 3.0 was chosen for the optimum separation of the compounds, as at this pH the analyte peaks were well defined with complete baseline resolution.

### 3.2. Choice of internal standard

Several substances were tested as internal standards. Among these, sotalol hydrochloride has been chosen as the most appropriate in the present analysis because it is stable. In the present study, it did not interfere with the matrix of pharmaceutical samples and it was well separated from OP. Moreover, a significant advantage of this IS was its elution time that was shorter than that of OP resulting in short run time, less than 5 min. A typical chromatogram for OP and IS using the proposed method is shown in Fig. 2. A sharp and symmetrical peak was obtained with good baseline for each compound, thus facilitating the accurate measurement of the peak area. The average retention times for OP and IS were found to be 3.43 ± 0.03 and 2.27 ± 0.02 min, respectively. Under the described HPLC parameters, the respective compounds were clearly separated and their corresponding peaks were sharply developed at reasonable retention times.

Table 1
Statistical analysis of calibration curves in the HPLC determination of oseltamivir phosphate ($n$ = 15)

| Validation parameters | HPLC |
|---|---|
| Concentration range (μg/mL) | 30–70 |
| Number of concentration levels | 5 |
| Regression equation | |
| Slope ($b$) | 0.0397 |
| Standard deviation on slope ($S_b$) | 0.0007 |
| Intercept ($a$) | 0.0319 |
| Standard deviation on intercept ($S_a$) | 0.0393 |
| Determination coefficient ($r^2$) | 0.9954 |
| Residual sum of square | 0.02225 |
| $F$-value | 2807 |

### 3.3. Validation of methods

#### 3.3.1. Linearity

Five points calibration graphs were constructed covering a concentration range 30–70 μg/mL (see Section 2.3). Three independent determinations were performed at each concentration. Linear relationships between the ratio of the peak area signal of OP to that of IS versus the corresponding drug concentration were observed, as shown by the results presented in Table 1. The standard deviations of the slope and intercept were low. The determination coefficient ($r^2$) exceeded 0.99. The calculated $F$-value equal to 2807 is highly significant.

A Student's $t$-test was performed to determine whether the experimental intercept ($a$) of the regression equation was not

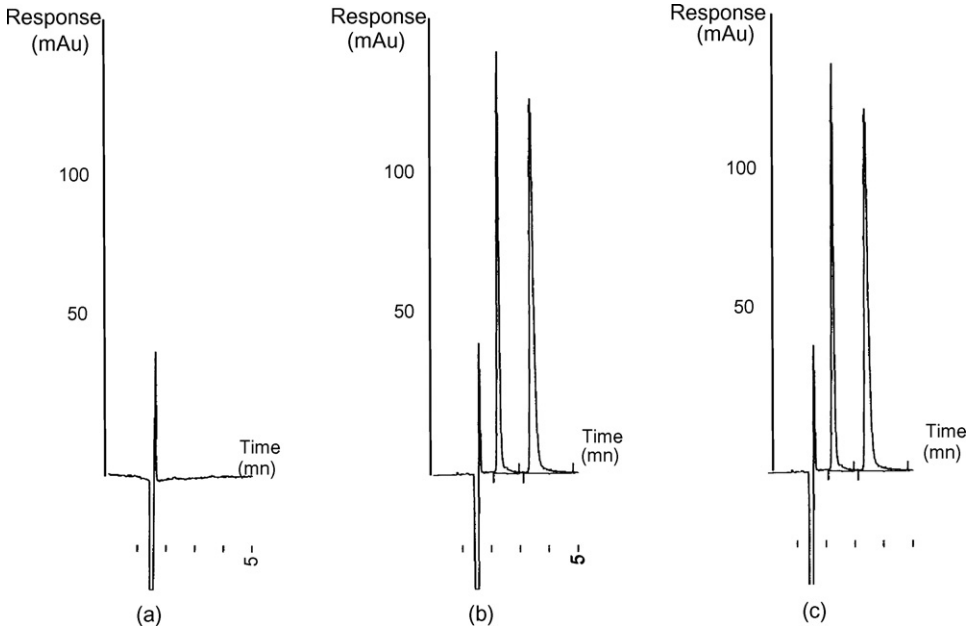


Fig. 2. Typical chromatograms obtained from the analysis of (a) excipients; (b) OP standard solution (50 μg/mL) and (c) OP extracted from capsules (50 μg/mL) containing 10 μg/mL of IS. Retention times for IS and OP were 2.2 and 3.4 min, respectively. The chromatographic conditions were as described in Section 2.

Table 2
Accuracy study for oseltamivir phosphate ($n = 9$)

| Concentration levels (μg/mL) | Mean recovery (%) | R.S.D. (%) |
|---|---|---|
| 30 | 100.01 | 0.81 |
| 50 | 101.74 | 1.67 |
| 70 | 102.28 | 0.65 |

Table 3
System suitability study

| | Retention time (min) | | Area ratio OP/IS |
|---|---|---|---|
| | OP (50 μg/mL) | IS (10 μg/mL) | |
| Mean ($n = 10$) | 3.43 | 2.27 | 2.00 |
| %R.S.D. | 0.91 | 1.02 | 1.10 |

significantly different from the theoretical zero value. It concerns the comparison of $t = a/S_a$, where $a$ is the intercept of the regression equation and $S_a$ is the standard deviation of $a$, with tabulated data of the $t$-distribution. As the calculated $t$-value ($t = 0.81$) does not exceed $t_0$ (0.05, 13) = 2.16, the intercept of regression equation is not significantly different from 0.

### 3.3.2. Precision

The repeatability study ($n = 6$) carried out showed a R.S.D. of 0.858% for the peak area ratios of OP to that of IS obtained, thus showing that the equipment used for the study worked correctly for the developed analytical method and being highly repetitive. For the intermediate precision, a study carried out by the same analyst working on 3 consecutive days ($n = 3$) indicated a R.S.D. of 0.744%. Both values were far below 5%, the limit percentage set for the precision and indicated a good method precision.

### 3.3.3. Accuracy

The data for accuracy were expressed in terms of percentage recoveries of OP in the real samples. These results are summarized in Table 2. The mean recovery data of OP in real sample were within the range of 100.01 and 102.28%, mean %R.S.D. was 1.04%, satisfying the acceptance criteria for the study.

### 3.3.4. Specificity

The HPLC chromatogram recorded for the mixture of the drug excipients revealed no peak within a retention time range of 5 min. The results showed that the developed method was specific as none of the excipients interfered with the analytes of interest (Fig. 2).

### 3.3.5. Stability

The stability of OP in standard and sample solutions containing IS was determined by storing the solutions at ambient temperature (20 ± 1 °C) protected from light. The solutions were checked in triplicate after 3 successive days of storage and the data were compared with freshly prepared samples. In each case, it could be noticed that solutions were stable for 72 h, as during this time the results did not decrease below 97%. This denotes that OP is stable in standard and sample solutions for at least 3 days at ambient temperature, protected from light and is compatible with IS.

### 3.3.6. System suitability

The resolution factor between IS and OP, in the developed method, was above 2. The %R.S.D. of peak area ratios of OP to that of IS and retention times for both drug and IS were within 2% indicating the suitability of the system (Table 3). These results indicate the applicability of this method to routine with no problems, its suitability being proved.

The statistical evaluation of the proposed method revealed its good linearity, reproducibility and its validation for different parameters and led us to the conclusion that it could be used for the rapid and reliable determination of OP in Tamiflu® capsules and generic versions.

## 3.4. Assay of capsules

The validated method was applied for the assay of three commercial capsules containing 98.5 mg of OP: Tamiflu®, Saiflu and Flufly. Each sample was analysed in triplicate after extracting the drug as mentioned in assay sample preparation of the experimental section (Section 2.4) and injections were carried out in triplicate. Fig. 2 shows an HPLC chromatogram of OP in pharmaceutical capsules. None of the capsule ingredients interfered with the analyte peak. The results presented in Table 4 are in good agreement with the labelled content. Assay results, expressed as the percentage of the label claim, were found to be 100.5% for Tamiflu®; 100.9% for Saiflu; 96.8% for Flufly showing that the content of OP in the capsule formulations conformed to the content requirements (95–105%) of the label claim. Low values of standard deviation denoted very good reproducibility of the measurement. The above results demonstrated that the developed HPLC method achieved rapid

Table 4
Results obtained for determination of oseltamivir phosphate in Tamiflu® and generic versions

| Sample | OP (theoretical value) (mg/capsule) | OP (determined value)[a] (mg/capsule) | Recovery[a] (%) | R.S.D.[a] (%) |
|---|---|---|---|---|
| Tamiflu® | 98.5 | 99.0 | 100.5 | 1.5 |
| Saiflu® | 98.5 | 99.4 | 100.9 | 1.2 |
| Flufly® | 98.5 | 95.4 | 96.8 | 1.9 |

[a] Mean of nine determinations.

and accurate determination of OP and could be used for the determination of OP in drug substance and pharmaceutical formulations.

## 4. Conclusion

A validated isocratic HPLC–UV method has been developed for the determination of OP in dosage forms: Tamiflu® and generic versions. The proposed method is simple, rapid, accurate, precise and specific. Its chromatographic run time of 5 min allows the analysis of a large number of samples in a short period of time. Therefore, it is suitable for the routine analysis of OP in pharmaceutical dosage forms. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC–MS or GC–MS that are complicated, costly and time consuming rather than a simple HPLC–UV method. Considering the possible worldwide development of counterfeit Tamiflu®, the proposed method could be useful for the national quality control laboratories in developing countries.

## References

[1] K. McClellan, C.M. Perry, Drugs 61 (2001) 263–283.
[2] W.B. Dreitlein, J. Maratos, J. Brocavich, Clin. Ther. 23 (2004) 327–355.
[3] F.G. Hayden, R.L. Atmar, M. Schilling, C. Johnson, D. Poretz, D. Paar, L. Huson, P. Ward, R.G. Mills, N. Engl. J. Med. 341 (1999) 1336–1343.
[4] K.G. Nicholson, F.Y. Aoki, A.D.M.E. Osterhaus, S. Trottier, O. Carewicz, C.H. Mercier, A. Rode, N. Kinnersley, P. Ward, Lancet 355 (2000) 1845–1850.
[5] M. Enserink, Science 312 (2006) 382–383.
[6] A. Abbott, H. Pearson, Nature 427 (2004) 472–473.
[7] J. Oxford, Expert Opin. Pharmacother. 6 (2005) 2493–2500.
[8] World Health Organization, WHO Media Centre, Counterfeit medicines, Fact sheet No. 275, Revised February 2006, Available from http://www.who.int/mediacentre/factsheets/fs275/en/.
[9] W. Li, P.A. Escarpe, E.J. Eisenberg, K.C. Cundy, C. Sweet, K.J. Jakeman, J. Merson, W. Lew, M. Williams, L. Zhang, C.U. Kim, N. Bischofberger, M.S. Chen, D.B. Mendel, Antimicrob. Agents Chemother. 42 (1998) 647–653.
[10] E.J. Eisenberg, K.C. Cundy, J Chromatogr. B 716 (1998) 267–273.
[11] H. Wiltshire, B. Wiltshire, A. Citron, T. Clarke, C. Serpe, D. Gray, W. Herron, J. Chromatogr. B 745 (2000) 373–388.
[12] N. Lindegardh, T.T. Hien, J. Farrar, P. Singhasivanon, N.J. White, N.P.J. Day, J. Pharm. Biomed. Anal. 42 (2006) 430–433.
[13] International Conference on Harmonisation, ICH Guidelines Q2(R1): Validation of Analytical Procedures, Text and Methodology Ref CPMP/ICH/381/95, Available from URL: http://www.ich.org/cache/compo/276-254-1.html.
[14] Product Monograph, Tamiflu, Hoffmann-La Roche Limited, Revised August 31, 2006, Missianga, Ontario, Canada, Available from: URL: http://www.rochecanada.com/pdf/tamifluSIE.pdf.
[15] R.T. Foster, R.A. Carr, Sotalol, in: H.G. Brittain, K. Florey (Eds.), Analytical Profiles of Drug Substances and Excipients, 21, Academic Press, Inc., San Diego, 1992, pp. 501–533.