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A simple and rapid liquid chromatographic assay for evaluation of potentially counterfeit Tamiflu®

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Abstract

A simple and rapid liquid chromatographic assay for the evaluation of potentially counterfeit oseltamivir (Tamiflu®) has been developed and assessed. The assay uses approximately 1 mg Tamiflu® powder when used for authentication and content estimate. The procedure was validated using 50 replicates analysed during five independent series with a total R.S.D. of 11.2%. The assay can also be used to monitor the exact content of oseltamivir in Tamiflu® capsules. One Tamiflu® capsule was transferred to a 250 mL volumetric flask and 150 mL water was added. The flask was placed in an ultrasonic bath at 40 °C for 20 min to dissolve the capsule. The solution was allowed to cool to room temperature before the flask was filled up to the mark (250 mL). A small aliquot was centrifuged and then directly injected into the LC-system for quantification. Oseltamivir was analysed by liquid chromatography with UV detection on a Hypersil Gold column (150 mm × 4.6 mm) using a mobile phase containing methanol–phosphate buffer (pH 2.5; 0.1 M) (50:50, v/v) at a flow rate of 1.0 mL/min. The assay was implemented for the analysis of Tamiflu® purchased over the Internet and at local pharmacies in Thailand and Vietnam.

Keywords: Avian influenza; Birdflu; Counterfeit; Oseltamivir; Tamiflu®; LC

1. Introduction

Oseltamivir (Tamiflu®) is an ester prodrug, which is rapidly and extensively hydrolysed in vivo to its active metabolite oseltamivir carboxylate, a potent and selective inhibitor of influenza virus neuraminidase [1]. Tamiflu® is considered the leading currently available antiviral to counter a serious epidemic or pandemic outbreak of influenza [2,3]. The current concerns over avian influenza A (H5N1) have created an increased demand for this drug. Pharmaceutical counterfeiting is a well-recognised global health problem with a particular impact in developing countries where drug-regulatory systems are weak or ineffective [4]. There have been many alarming reports lasting recent years of counterfeit antimalarials, antibiotics, hormones and steroids, analgesics and antipyretics, anti-asthma and anti-allergy drugs [5–11]. The threat from avian influenza has led to stockpiling leading to shortage of supply. Internet pharmacies have seized on this opportunity and are selling Tamiflu® at significantly inflated prices. Counterfeit Tamiflu® labelled as generic Tamiflu® has also appeared on the market, and was recently seized by US customs [12]. To date there are no published methods for determination of oseltamivir in Tamiflu® capsules and only one published method for the determination of oseltamivir in plasma using solid-phase extraction and LC-MS [13].

The aim of the work described in this paper was to develop a simple readily applicable rapid liquid chromatographic assay for quality control and authentication of Tamiflu® capsules. The assay was validated and applied to test Tamiflu® purchased over the Internet and in local pharmacies in Thailand and Vietnam.

2. Materials and methods

2.1. Chemicals

Oseltamivir and Tamiflu® were obtained from F. Hoffmann, La Roche Ltd. (Basel, Switzerland). The structure is shown in

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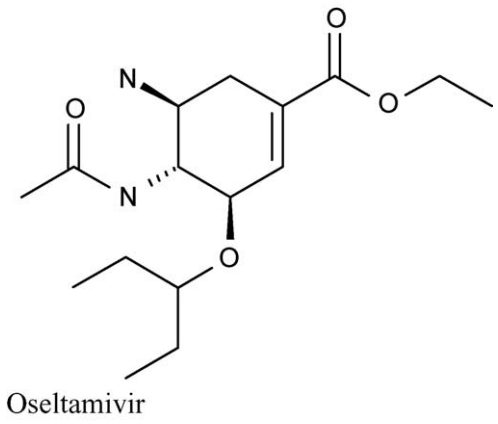

Fig. 1. Structure of oseltamivir.

Fig. 1. Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC water were obtained from JT Baker (Phillipsburg, USA). The phosphate buffer solutions were prepared by mixing appropriate amounts of sodium hydroxide and ortho-phosphoric acid, obtained from Merck (Darmstadt, Germany), with HPLC water.

2.2. Instrumentation

The LC system was a LaChrom Elite system consisting of a L2130 LC pump, a L2200 injector set at 25 °C, a L2300 column oven set at 25 °C and a L2450 DAD detector (Hitachi, Tokyo, Japan). The detector was set at 220 nm. Data acquisition was performed using LaChrom Elite software (VWR, Darmstadt, Germany). The compounds were analysed on a Hypersil Gold (150 mm × 4.6 mm) column (Thermo Electron Corporation, Waltham, USA) protected by a short guard column SecurityGuard C18 (4 mm × 3 mm, I.D) (Phenomenex Inc., Cheshire, UK) using a mobile phase containing methanol–phosphate buffer (pH 2.5; 0.1 M) (50:50, v/v) at a flow rate of 1.0 mL/min.

2.3. Preparation of standards

Stock solutions of oseltamivir 1 mg/mL were prepared freshly in deionised water. Calibration standards at 0.200, 0.300 and 0.400 mg/mL were prepared by dilution of the stock solution in water. A calibration curve was constructed from triplicates at each calibration level using peak-area against concentration and non-weighted linear regression for quantification. The calibration standards were prepared freshly on each day of analysis.

2.4. Analytical procedure—exact content

One capsule of oseltamivir (Tamiflu®) was transferred to a 250 mL volumetric flask and approximately 150 mL deionised water was added. The volumetric flask was put into an ultrasonic bath at a temperature of 40 °C for 20 min to dissolve the capsule completely. Approximately 90 mL of deionised water was added and the solution was left to cool to room temperature (about 30 min) before the flask was filled up to the mark to produce a theoretical concentration of 0.300 mg/mL oseltamivir. The flask was inverted a few times to mix the solution and 1 mL was then transferred to an Eppendorf® cup. The Eppendorf® cup was centrifuged at about 15,000 × *g* for 5 min on a micro centrifuge, three aliquots of 100 μL were transferred to glass inserts and 10 μL was injected into the LC-system.

2.5. Analytical procedure—authentication and content estimate

Initially the weight of five full Tamiflu® capsules and five empty Tamiflu® capsules were used to calculate the average amount of powder (i.e. active drug plus excipients) in a Tamiflu® capsule and the approximate ratio of Tamiflu® powder (mg)/amount oseltamivir (mg). Each capsule of Tamiflu® being checked was carefully opened by turning the upper part of the capsule until it detached. A spatula was used to transfer 0.600–1.600 mg Tamiflu® powder to a small homemade weighting boat (prepared using the bottoms of antistatic polystyrene hexagonal weighing boats, Heathrow Scientific, IL, USA). This was then transferred to an Eppendorf® cup and water (1000, 1500 or 2000 μL) was added to get a final theoretical concentration in the range of 0.200–0.400 mg/mL. The Eppendorf® cups were vortex mixed, 100 μL was transferred to glass inserts and 10 μL was injected into the LC-system.

2.6. Validation

The repeatability of the LC system was evaluated using five replicates of stock solution (1 mg/mL) and eight replicates of dissolved Tamiflu® powder (theoretical concentration 0.300 mg/mL). Linearity and regression model was evaluated using back-calculated values for the calibration curves. A typical chromatogram showing the three calibration standard levels is shown in Fig. 2. Accuracy and precision for the procedure described in Section 2.4 (exact content) was illustrated by analysis of six capsules of Tamiflu® (same batch but three different packages), with each sample analysed in triplicate. Accuracy and precision for the procedure described in Section 2.5 (authentication) was illustrated by 10 replicates of Tamiflu® powder (0.500–1.600 mg) analysed during five different analytical runs

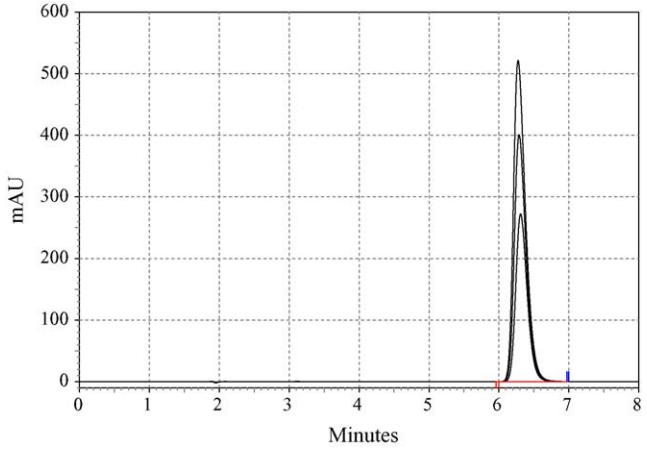

Fig. 2. Overlay of chromatograms from calibration standards 0.200, 0.300 and 0.400 mg/mL.

and 10 replicates of Tamiflu® powder (7–10 mg) in one analytical run using a freshly prepared calibration curve and stock solution each time. Stability of oseltamivir (pure standard and Tamiflu®) in water was evaluated at room temperature and at 4 °C for 24 h.

Selectivity was evaluated by analysis of different commonly available and cheap drugs or constituents, which could be used as substitutes for oseltamivir in counterfeit Tamiflu®. The following drugs or tablets were evaluated: Omeprazole, multivitamins, Paracetamol, Vitamin C, Ibuprofen, Multi B vitamin, Tropolidine/pseudoephedrine (Actifed®), Aspirin, Placebo (chloroquine), Amoxicillin.

The procedure described in Section 2.4 was applied for the analysis of Tamiflu® obtained over the Internet and at pharmacies in Thailand and Vietnam.

3. Results and discussion

Oseltamivir phosphate is freely soluble in water and methanol and has one basic pK_a estimated to about 7.75–8.80 [14,15]. The Tamiflu® capsule itself is also easily dissolved in water and complete dissolution is facilitated by heat and ultrasonic bath treatment.

3.1. Validation

The repeatability of the LC system was excellent with a R.S.D of 0.11% for injections of the stock solution ($n = 5$) and a R.S.D. of 0.30% for the injections of dissolved Tamiflu® ($n = 8$).

Back calculated values for the calibration standards validated the use of simple non-weighted linear regression for quantification. The assay was linear in the tested calibration range (0.2–0.4 mg/mL) and the accuracy and precision (R.S.D.) for the back-calculated calibration standards were less than 0.3% for all series. Accuracy and precision for the procedure described in Section 2.4 using six capsules of Tamiflu® (Batch B1138, MFD 06 2005) is shown in Table 1. The average weight ($n = 5$) of powder in a Tamiflu® capsule was 165.4 mg (S.D. 0.94), which gives an approximate amount of 2.21 mg Tamiflu® powder/mg oseltamivir. The amount of oseltamivir in a capsule was calculated as $c_m \times v_d \times 2.21/x_w \times 75$, where c_m is the measured concentration (μg/mL), v_d is the volume (mL) used to dissolve the Tamiflu® powder, 2.21 the amount (mg) of Tamiflu® powder/mg oseltamivir and x_w the amount (mg) of Tamiflu® powder weighed.

Table 1
Accuracy and precision—exact content procedure

	Found (mg/mL)	Oseltamivir/capsule (mg)	R.S.D. (%)
Capsule 1 pack 1	0.301	75.4	0.017
Capsule 2 pack 1	0.300	75.0	0.507
Capsule 3 pack 1	0.299	74.8	0.510
Capsule 4 pack 1	0.299	74.6	0.255
Capsule 1 pack 2	0.303	75.6	0.333
Capsule 1 pack 3	0.300	75.1	0.078
Average	0.300	75.1	0.536

Accuracy, intra-, inter- and total-assay precisions for the procedure described in Section 2.5 are summarised in Table 2. As can be seen the variation is quite large which is likely to be caused by the fact that the Tamiflu® powder is not completely homogenous with respect to excipients. In retrospect the actual performance of the balance over 6 months using a certified 1 mg weight ($n = 8$) showed an average weight of 0.9995 and a R.S.D. of 0.0926%. The use of a larger quantity powder (i.e. 7–10 mg) indeed improved the accuracy and precision (Table 2) but is less suitable if the capsules are to be used for treatment once demonstrated to be authentic. Oseltamivir was stable at room temperature and at 4 °C for at least 24 h. None of the evaluated drugs interfered with the oseltamivir peak. The content of oseltamivir in Tamiflu® obtained over the Internet and at some local pharmacies are reported in Table 3. All samples contained amounts within the ±5% tolerance limits specified by Roche

Table 2
Accuracy and precision—authentication and content estimate procedure

	Theoretical oseltamivir/capsule (mg)	Mean found	R.S.D. (%)	% Deviation (found vs. added)
Intra-assay 1 mg ($n = 50$)	75.0	85.7	8.8	14.2
Inter-assay 1 mg ($n = 5$)			7.0	
Total-assay 1 mg ($n = 50$)			11.2	
Intra-assay 10 mg ($n = 10$)	75.0	77.1	4.7	2.8

Table 3
Tamiflu® samples obtained over the Internet and at local pharmacies

	Found (mg/mL)	Oseltamivir/capsule (mg)	R.S.D. (%)	% Deviation (found vs. stated)
Internet supplier 1	0.300	75.1	0.315	0.1
Internet supplier 2	0.308	76.9	0.187	2.6
Local pharmacy pack 1, Thailand	0.299	74.7	0.340	−0.4
Local pharmacy pack 1, Vietnam	0.290	72.5	0.137	−3.4
Local pharmacy pack 2, Vietnam	0.301	75.2	0.282	0.2
Local pharmacy pack 3, Vietnam	0.299	74.7	0.057	−0.4

for release of finished product (F. Hoffmann, La Roche Ltd., personal communication).

4. Conclusion

A simple and rapid LC assay for quality control and authentication of Tamiflu® capsules has been developed and validated. The assay when used for authentication can use as little as 0.6 mg Tamiflu® powder thus enabling the remaining of the capsule to be used for treatment. The assay can also be used as a quality control tool to monitor the exact content in Tamiflu® capsules. The assay was successfully implemented for analysis of Tamiflu® purchased over the Internet and in local pharmacies in Thailand and Vietnam.

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