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Chemical stability of oseltamivir in oral solutions

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The stability of oseltamivir in oral aqueous solutions containing the preservative sodium benzoate was studied by a stability indicating HPLC-method. The separation was achieved on a RP-18 ec column using a gradient of mobile phase A (aqueous solution of 50 mM ammonium acetate) and mobile phase B (60% (v/v) acetonitrile/40% (v/v) mobile phase A). The assay was subsequently validated according to the ICH guideline Q2(R1). The extemporaneously prepared "Oseltamivir Oral Solution 15 mg/ml for Adults or for Children" (NRF 31.2.) according to the German National Formulary ("Neues Rezeptur-Formularium") was stable for 84 days if stored under refrigeration. After storage at 25 °C the content of oseltamivir decreased to 98.4%. Considering the toxicological limit of 0.5% of the 5-acetylamino derivative (the so-called isomer I) the solution is stable for 46 days. Oseltamivir was less stable in a solution prepared with potable water instead of purified water. Due to an increasing pH the stability of this solution decreased to 14 days. Furthermore a white precipitate of mainly calcium phosphate was observed. The addition of 0.1% anhydrous citric acid avoided these problems and improved the stability of the solution prepared with potable water to 63 days. Sodium benzoate was stable in all oral solutions tested.

1. Introduction

Oseltamivir [(3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-carboxylic acid ethyl ester phosphate, Scheme] is used to treat or prevent influenza in adults and children over the age of one (European Medicines Agency 2005). Oseltamivir is considered the leading currently available antiviral agent to counter a serious epidemic or pandemic outbreak of influenza alongside other public-health measures (Lindegårdh et al. 2006). In Germany public and hospital pharmacies are obliged to supply the population with antiviral oseltamivir oral solutions in case of an influenza pandemia (Robert Koch Institut 2006). The German Formulary lists for this purpose the formulation "Oseltamivir Oral Solution 15 mg/ml for Adults or for Children" (NRF 31.2.) (Neues Rezeptur-Formularium 2006). In case of an officially assessed pandemia such solutions have to be prepared in German public and hospital pharmacies and will be stored for a certain period of time. Thus, the solutions contain sodium benzoate as preservative.

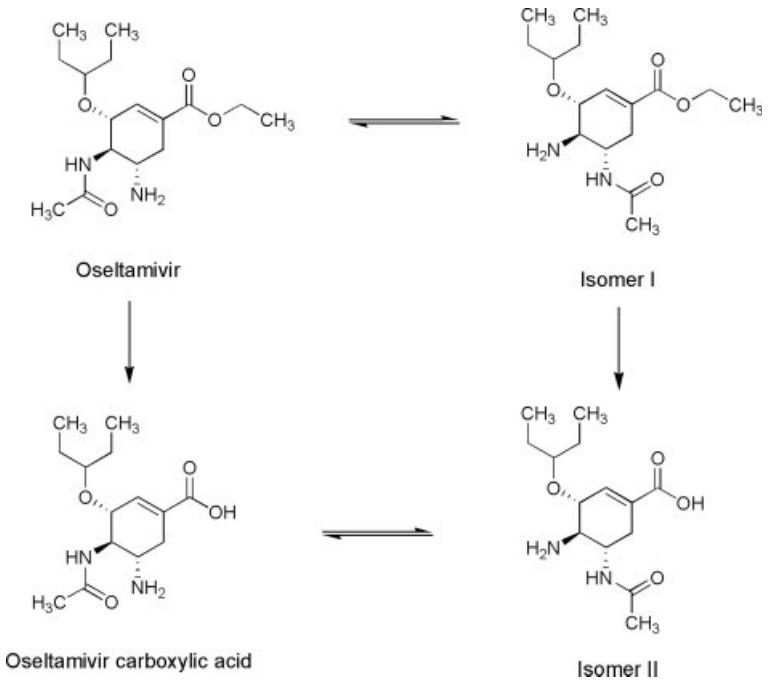
The stability of oseltamivir in aqueous solution at 70 °C has been investigated by HPLC and three degradation products have been identified (Oliyai et al. 1998). The ester group can be hydrolyzed to yield oseltamivir carboxylic acid. Moreover, *N*,*N*-acyl migration can occur in oseltamivir and in oseltamivir carboxylic acid, resulting in the respective isomers I and II (Scheme). For oseltamivir capsules a simple and rapid assay for the evaluation of potentially counterfeit products has been developed (Lindegårdh et al. 2006). However, a validated HPLC-method for the determination of oseltamivir in oral solution has not been published to date. Thus, the aim of the present study was to develop a validated HPLC-method for the simultaneous analysis of oseltamivir, its degradation products and the preservative sodium benzoate. Furthermore the stability of oseltamivir in the recommended German formulation and in a second formulation, prepared with potable water instead of "Purified Water" Ph. Eur., should be tested. This unusual way of preparation may be necessary, if in case of a pandemic influenza large volumes of oseltamivir oral solution are needed very quickly.

2. Investigations, results and discussion

2.1. HPLC method development

The HPLC-method used was based on a published stability indicating method using gradient elution with minor modifications concerning the gradient (Oliyai et al. 1998). Thus, the separations were performed at 22.0 ± 0.2 °C using a gradient of mobile phase A consisting of 50 mM aqueous ammonium acetate and mobile phase B consisting of 60% acetonitrile/40% 50 mM aqueous ammonium acetate (v/v). The mobile phase was initially set at 90% mobile phase A/10% mobile phase B, followed by a 20 min linear gradient to 100% mobile phase B, a 5 min linear gradient back to 90% mobile phaseA/10% mobile phase B and 5 min re-equilibration. The flow-rate was 1.0 ml/min and the detector was set at 230 nm. A typical chromatogram is shown in the Fig.

Scheme



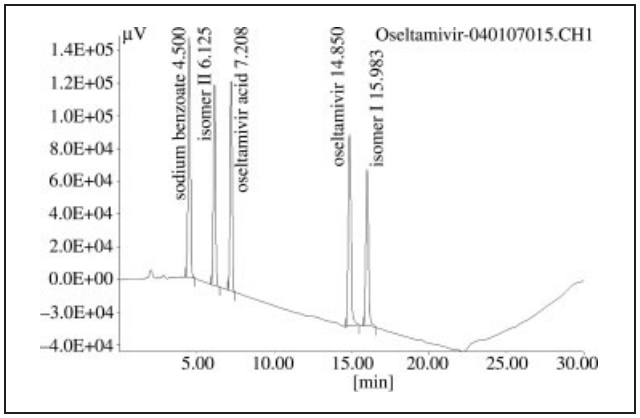


Fig.: Typical chromatogram of sodium benzoate (Retention time (RT) = 4.5 min), isomer II (RT = 6.1 min, Resolution (R_S) = 7.5), oseltamivir carboxylic acid (RT = 7.2 min, R_S = 5.1), oseltamivir (RT = 14.9, R_S = 30.9) and isomer I (RT = 16.0 min, R_S = 4.0). For experimental conditions see text

2.2. Method validation

The HPLC was valuated according to the ICH guideline Q2(R1) with respect to specificity, linearity, range, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and robustness (International Conference on Harmonisation 2005).

2.2.1. Specifity

As shown in the Fig. all analytes are well separated. The retention times were as follwos: sodium benzoate 4.5 min, isomer II 6.1 min, oseltamivir carboxylic acid 7.2 min, oseltamivir 14.9 min and isomer I 16.0 min. The HPLC assay is specific for each analyte, since the resolution of all components exceeds 1.5.

2.2.2. Linearity and range

Oseltamivir was calibrated in the concentration range 50–1000 µg/ml, sodium benzoate between 2.5–50 µg/ml and the degradation products, oseltamivir carboxylic acid, isomer I and isomer II in the concentration range 10–500 µg/ml using six different concentrations for each compound spread out evenly throughout the respective concentration range. Each solution was injected twice. The calibration data are summarized in Table 1. Correlation coefficients of at least 0.9992 were observed.

2.2.3. Limit of detection and limit of quantitation

Based on a signal-to-noise ratio of 3 : 1 the LODs for all analytes investigated varied between 2 and 10 µg/ml (Table 1). Considering that the LODs for the degradation products are at least 10 µg/ml the method is sensitive enough to detect the compounds in the undiluted oral so-

Table 1: Calibration data of oseltamivir phosphate, sodium benzoate and degradation products of oseltamivir

Parameter	Oseltamivir phosphate	Sodium benzoate	Oseltamivir carboxylic acid	Isomer I	Isomer II
Range (µg/ml)	50–1,000	2.5–50	10–500	10–500	10–500
Correlation	0.9992	0.9999	0.9994	0.9999	0.9999
LOD (µg/ml)	7	2	10	6	10
LOQ (µg/ml)	16	8	35	20	33

lution at least at the 0.05% level relative to oseltamivir phosphate. This is the reporting threshold defined by ICH guideline Q3A (International Conference on Harmonisation 2002).
Considering a signal-to-noise ratio of 10 : 1, the LOQs ranged between 8 and 35 μg/ml (Table 1). This corresponds to a relative concentration between 0.04% and 0.18%.

2.2.4. Precision

The repeatability of the method was determined by analysis of a standard solution, injecting the solution six times. Relative standard deviations (RSDs) for oseltamivir and sodium benzoate were 0.52% and 0.66%, respectively. Based on these results and performing three injections for each assay the confidence interval (P = 0.95) for oseltamivir is 394 ± 2.9 μg/ml and for sodium benzoate 20 ± 0.19 μg/ml.
The intermediate precision was evaluated by measuring the content of eight standard solutions prepared on eight different days. Each solution was injected four times. The RSDs for oseltamivir and sodium benzoate ranged from 0.28–0.65% and 0.08–0.56%, respectively.
To determine the repeatability of the assays of the degradation products, standard solutions containing 90 μg/ml of each analyte were injected six times. The following results were obtained: oseltamivir carboxylic acid, RSD 0.40%; isomer I, RSD 0.48%; isomerII RSD 0.34%.

2.2.5. Accuracy

Since the dosage forms tested were without exception compounded aqueous solutions with known components, the accuracy of the method was inferred from the proven specificity, linearity and precision (International Conference on Harmonisation 2005). Furthermore the accuracy is shown by the fact that the sums of the contents of oseltamivir and its degradation products are between 98.7 and 101.6% during the whole study (Table 2).

2.2.6. Robustness

The robustness of the assay was investigated using a test solution containing isomer I. This degradation product has the most critical separation from the principal peak. Varying the amount of acetonitrile by ±2% in mobile phase B and variations of the first gradient step (±2 min) had no significant influence on the separation.

2.3. Stability of oseltamivir and sodium benzoate in oral liquids

The HPLC method was applied to the analysis of the oseltamivir oral solutions A, B, C and D, stored at 25 °C in a thermostated chamber (solutions A, B and C) and at 6 °C in a refrigerator (solution D) for 84 days (Table 3). Assays and determinations of pH-value of the solutions were performed at the beginning of the study (T_0) and after 7, 14, 21, 28, 42, 56 and 84 days (Table 2).

2.3.1. pH-value

At T_0, the pH of the solutions A and D were 5.1. After 84 days storage at 25 °C oral solution A showed a pH of 4.9, solution D stored at 6 °C had an unchanged pH of 5.1. Oral solution B displayed a higher value at T_0, pH 5.7, due to the use of potable water (pH = 7.4), which slightly decreased to pH 5.6 after 84 days. Oral solution C prepared with pota-

Table 2: Content of oseltamivir and degradation products after storage

	Content (% ± SD of three determinations) after storage (d)							
	T_0	T_7	T_{14}	T_{21}	T_{28}	T_{42}	T_{56}	T_{84}
Oral solution A								
Oseltamivir	100.7 ± 0.6	101.6 ± 0.3	100.1 ± 0.5	99.4 ± 0.5	99.5 ± 0.1	99.3 ± 0.1	98.8 ± 0.1	98.4 ± 0.2
Oseltamivir carboxylate	n. d.	n. d.	0.11 ± 0.00	0.15 ± 0.00	0.20 ± 0.01	0.29 ± 0.01	0.39 ± 0.00	0.56 ± 0.00
Isomer I	n. d.	n. d.	0.24 ± 0.00	0.25 ± 0.01	0.33 ± 0.01	0.46 ± 0.01	0.61 ± 0.00	0.86 ± 0.00
Sum	100.7 ± 0.6	101.6 ± 0.3	100.5 ± 0.5	99.8 ± 0.5	100.0 ± 0.1	100.0 ± 0.1	99.8 ± 0.1	99.8 ± 0.2
Oral solution B								
Oseltamivir	101.1 ± 0.1	101.3 ± 0.1	99.6 ± 0.2	99.2 ± 0.4	98.8 ± 0.1	97.6 ± 0.4	97.7 ± 0.1	95.3 ± 0.3
Oseltamivir carboxylate	n. d.	0.15 ± 0.01	0.30 ± 0.01	0.41 ± 0.03	0.56 ± 0.01	0.80 ± 0.01	1.03 ± 0.01	1.47 ± 0.00
Isomer I	n. d.	0.17 ± 0.01	0.50 ± 0.02	0.59 ± 0.02	0.80 ± 0.02	1.12 ± 0.01	1.40 ± 0.00	1.91 ± 0.02
Sum	101.1 ± 0.1	101.6 ± 0.1	100.4 ± 0.2	100.2 ± 0.4	100.2 ± 0.1	99.5 ± 0.4	100.1 ± 0.1	98.7 ± 0.3
Oral solution C								
Oseltamivir	100.4 ± 0.6	101.6 ± 0.5	100.0 ± 0.5	99.6 ± 0.2	99.8 ± 0.9	99.4 ± 0.4	99.0 ± 0.3	99.2 ± 0.4
Oseltamivir carboxylate	n. d.	n. d.	n. d.	0.07 ± 0.01	0.10 ± 0.00	0.14 ± 0.00	0.21 ± 0.00	0.29 ± 0.00
Isomer I	n. d.	n. d.	0.17 ± 0.01	0.19 ± 0.01	0.25 ± 0.01	0.33 ± 0.01	0.45 ± 0.00	0.66 ± 0.00
Sum	100.4 ± 0.6	101.6 ± 0.5	100.2 ± 0.5	99.9 ± 0.2	100.2 ± 0.9	99.9 ± 0.4	99.7 ± 0.3	100.2 ± 0.4
Oral solution D								
Oseltamivir	100.7 ± 0.6	101.1 ± 0.3	100.1 ± 0.1	100.2 ± 0.1	100.6 ± 0.4	100.5 ± 0.1	101.2 ± 0.4	100.3 ± 0.4
Oseltamivir carboxylate	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	0.07 ± 0.00	0.09 ± 0.00
Isomer I	n. d.	n. d.	0.09 ± 0.01	0.05 ± 0.01	n. d.	0.12 ± 0.01	0.05 ± 0.00	0.07 ± 0.00
Sum	100.7 ± 0.6	101.1 ± 0.3	100.2 ± 0.1	100.3 ± 0.1	100.6 ± 0.4	100.6 ± 0.1	101.3 ± 0.4	100.5 ± 0.4

Isomer II was not detected during the study, n. d. = not detectable

ble water adding also citric acid showed a constant pH of 4.2 during the whole study. It can be summarized that the pH values of the oral solutions remained essentially constant for 84 days of storage independent of the actual conditions. The slight decrease in pH observed for solutions A and B by 0.1–0.2 pH units may be due to the formation of oseltamivir carboxylic acid (see discussion below).

2.3.2. Stability of oseltamivir

The results of the HPLC analysis of oseltamivir and its degradation products are summarized in Table 2. Isomer II could not be detected during the period of time investigated.

The concentration of oseltamivir in the oral solutions A and D at T_0 was 100.7% (see Table 2). After storage at 6 °C for 84 days solution D had a content of 100.3%. Thus, the formulation of the German Formulary is stable under refrigeration. In oral solution A stored at 25 °C for 84 days the concentration of oseltamivir decreased to 98.4%. Because the decomposition of oseltamivir is accelerated at higher pH (Oliyai et al. 1998), oral solution B, which was prepared with potable water, displayed a distinct decrease in concentration from 101.1% to 95.3% after 84 days at room temperature. Furthermore, a white precipitate was observed which was identified by atomic absorption spectroscopy and by color reaction with ammonium molybdate as a mixture of calcium phosphate and traces of magnesium phosphate. The degradation of oseltamivir in potable water can essentially be suppressed by the addition of citric acid and adjusting the pH to 4.2. Oseltamivir has a maximal stability at pH 4.0 (Oliyai et al. 1998). In oral solution C, also prepared with potable water but at pH = 4.2, only a small decrease of the oseltamivir content from 100.4% to 99.2% (T_{84}) was noted. No precipitation was observed in this solution.

The formation of degradation products was also studied (Table 2). In all solutions the content of oseltamivir carboxylic acid and isomer I at T_0 was below the reporting threshold. In the original formulation NRF 31.2., stored for 84 days under refrigeration (oral solution D), only a minor increase of the degradation products oseltamivir carboxylic acid (0.09%) and isomer I (0.07%) was observed. As expected, the content of the degradation products increased to 0.56% and 0.86% respectively, if this solution is stored for 84 days at 25 °C (oral solution A). The maximum decomposition was observed in oral solution B where 1.47% oseltamivir carboxylic acid and 1.91% isomer I were found. The results for oral solution C demonstrate that citric acid is an effective stabilizer (oseltamivir carboxylic acid: 0.29%, isomer I: 0.66%).

Due to toxicological reasons the content of isomer I in liquid oseltamivir preparations is limited at 0.5% (Sattelkau 2007). Considering this value and interpolating the measured content of isomer I in the oral solutions A, B, C and D, the stability of the preparations tested can be estimated as follows: oral solution A: 46 days, oral solution B: 14 days, oral solution C: 63 days and oral solution D: at least 84 days.

2.3.3. Stability of sodium benzoate

Sodium benzoate was determined simultaneously to oseltamivir. The concentration of sodium benzoate after 84 days of storage in each solution tested was found to be between 100.3 ± 0.3% and 101.1 ± 0.1% of the initial values. Thus, sodium benzoate is stable under the conditions studied.

Table 3: Composition of oseltamivir oral solutions tested

Sample	Ingredient	Quantity
Oral solution A (NRF 31.2)	Oseltamivir phosphate	1.971 g
	Sodium benzoate	0.1 g
	Purified water	to 100.0 ml
Oral solution B	Oseltamivir phosphate	1.971 g
	Sodium benzoate	0.1 g
	Potable water	to 100.0 ml
Oral solution C	Oseltamivir phosphate	1.971 g
	Sodium benzoate	0.1 g
	Anhydrous citric acid	0.1 g
	Potable water	to 100.0 ml

3. Experimental

3.1. Materials

Oseltamivir phosphate (Roche Standard no. RO-64-0796/002, Lot no. BS03071016), oseltamivir carboxylic acid [(3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-carboxylic acid] (Roche Standard no. RO-64-0802/002, Lot no. 504B243803), isomer I [(3*R*,4*R*,5*S*)-4-amino-5-acetylamino-3-(1-ethylpropoxy)-cyclohex-1-ene-carboxylic acid, ethyl ester] (Roche Standard no. RO-64-0952/000, Lot no. 19450B063) and isomer II [(3*R*,4*R*,5*S*)-4-amino-5-acetylamino-3-(1-ethylpropoxy)-cyclohex-1-ene-carboxylic acid] (Roche Standard no. RO-64-0951/000, Lot no. 1706-03) were supplied by Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals and reagents were European Pharmacopeia grade and were used without further purification. Buffers were prepared in deionized, distilled water. The solutions were stored in Aponorm®-bottles (WEPA GmbH, Hillscheid, Germany).

3.2. Instrumentation

The HPLC system used consisted of a Jasco PU-2089 Plus Quaternary Gradient Pump (Jasco GmbH, Groß-Umstadt, Germany) equipped with a 20 µl injector (Model 7725-I, Rheodyne) and a Jasco UV-2070 Plus UV/VIS-Detector. Jasco-Borwin-DV Chromatography-Software was used to collect and analyse the data. Separation of the analytes was performed on a Nucleodur 100-5 C18 ec column, 250 × 4 mm, 5 µm (Macherey-Nagel GmbH, Düren, Germany) thermostated with a Jasco Column Peltier-Thermostat. All pH values were measured using a Knick pH-meter 646 (Knick GmbH, Berlin Germany), calibrated with CertiPur® Buffer Solution pH 4.00 (Merck KgaA, Darmstadt, Germany). Atomic absorption analysis was performed with the Atomic Absorption Spectrophotometer 1100 (Perkin-Elmer, Überlingen, Germany).

The oseltamivir solutions were stored in a Thermo® Tec Climate Chamber at 25 ± 2 °C and 60 ± 5% relative humidity. Storage under refrigeration was performed at 6 ± 2 °C in a normal refrigerator.

3.3. Standard and sample solutions

Three batches of "Oseltamivir Oral Solution 15 mg/ml for Adults or for Children" (NRF 31.2.) were prepared according to the German Formulary as summarized in Table 3 (Neues Rezeptur-Formularium 2006). Solution C was prepared with potable water as supplied by the city of Eschborn, Germany (excerpt from official data: pH = 7.34, water hardness 20.5 °dH, calcium 115 mg/l, magnesium 19 mg/l). The oral solutions A, B and C were filled in 250-ml-amber glass bottles with PP-closures and stored at 25 °C. Additionally, a portion of oral solution A was stored a glass bottle under refrigeration at 6 °C (oral solution D).

Preparation of test solutions: 10.0 ml of the oral solution were diluted with 50 mM ammonium acetate buffer, previously adjusted with acetic acid 30% to pH 5.0, to 100.0 ml. 10.0 ml of this solution were diluted with ammonium acetate buffer, pH 5.0, to 50.0 ml (theoretical concentrations of oseltamivir phosphate and sodium benzoate were 394 µg/ml and 20 µg/ml, respectively). 20 µl of this test solution were injected three times. For the determination of degradation products 20 µl of the undiluted oral solution were injected.

Preparation of standard solutions: 0.1971 g oseltamivir phosphate and 0.0100 g sodium benzoate were dissolved in ammonium acetate buffer, pH 5.0, to 100.0 ml. 10.0 ml of this solution were diluted with ammonium acetate buffer, pH 5.0, to 50.0 ml. 20 µl of this test solution were injected four or six times.

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