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Determination of oseltamivir carboxylic acid in human serum by solid phase extraction and high performance liquid chromatography with UV detection

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Abstract

This study was aimed at developing a fast and sensitive method for determination of oseltamivir carboxylic acid (OCA), the active moiety of anti-influenza agent, oseltamivir phosphate, in human serum by high performance liquid chromatography and UV detection. The analyte and an internal standard (vanillin) were extracted from human serum by a solid phase extraction (SPE) procedure. Chromatographic separation was achieved using a reverse phase C18 column with a mobile phase consisting of 0.05 M phosphate buffer containing triethylamine (1 mL/L; pH 3.0) and acetonitrile (70:30, v/v). The detection wavelength was set at 215 nm. The average recoveries of the drug and internal standard were 98 and 85%, respectively. The calibration curve was linear over a concentration range of 15–6400 ng/mL of OCA in human serum. The lower limits of detection and quantification were 5 and 15 ng/mL, respectively. The coefficient variation values of both inter- and intra-day analysis were less than 12% whereas the percentage error was less than 4.5. The stability of the drug at the serum samples maintained at −40 °C for 60 days was found to be 100% from the initial value and no interferences were found from either endogenous components in serum or commonly co-administrated antiviral drugs. The validated method was applied to a randomized cross-over bioequivalence study of two different oseltamivir phosphate preparations in 24 healthy volunteers.

Keywords: HPLC; Oseltamivir; Influenza; Bioequivalence study

1. Introduction

Oseltamivir, [ethyl(3*R*,4*R*,5*S*)-4-deacetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate] (Fig. 1A), the ethyl ester pro-drug of oseltamivir carboxylate (OCA; Fig. 1B), [(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid] is the first orally active neuroaminidase inhibitor available for the prophylaxis and treatment of influenza A and B. The inactive parent drug is rapidly absorbed and extensively metabolized by esterases in the gastrointestinal tract and liver to its active carboxylate form. Consequently, only low levels (3–5% of that of the metabolite) of the intact drug are found in the systemic circulation [1]. Oseltamivir phosphate was firstly supplied by the Roche pharmaceutical company however, due to serious health problem of influenza and to meet fast-growing world demand for the drug, a number of pharmaceutical companies in different countries are currently marketing oseltamivir phosphate under license of the Roche pharmaceutical company. Thus a fast and accurate method is needed to support human single dose studies of oseltamivir phosphate. A few analytical methods are available for the analysis of drug or its active metabolite in the biological fluids. An analytical method using high performance liquid chromatography (HPLC) with UV detection has been reported for the analysis of pro-drug in pharmaceutical preparations including bulk samples, raw materials and finish products [2]. An enzymatic assay based on neuraminidase inhibition was reported for analysis of OCA [3]. A sensitive assay using HPLC–MS–MS has been described by Wiltshire et al. [4] for simultaneous analysis of the ethyl ester and active carboxylate metabolite of oseltamivir in biological fluids. In their method

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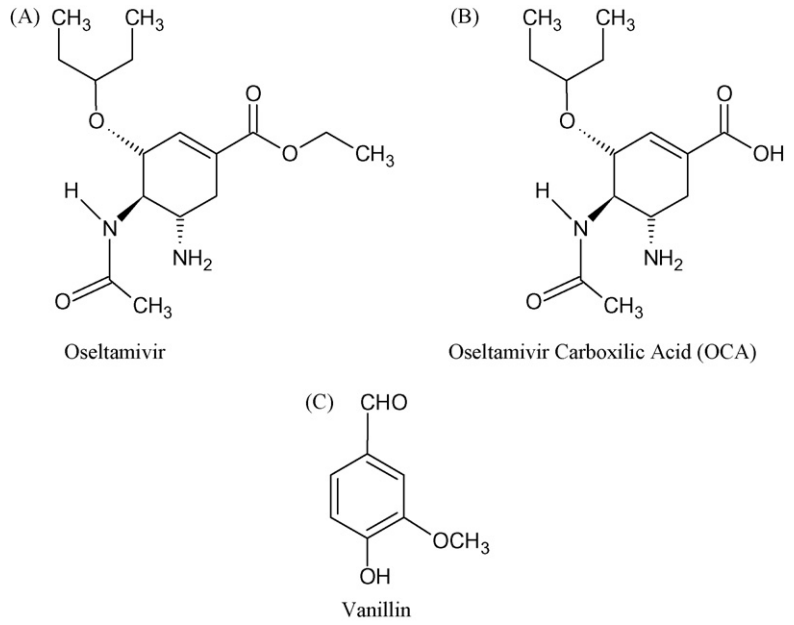

Oseltamivir

Oseltamivir Carboxilic Acid (OCA)

Fig. 1. Chemical structures of (A) oseltamivir phosphate, (B) oseltamivir carboxylic acid and (C) vanillin.

which has not been evaluated in single dose studies of the drug in healthy volunteers, limits of quantification (LOQ) of 1 and 10 ng/mL were reported for the pro-drug and the active neuroaminidase inhibitor, respectively. However, this technique needs an expensive instrument which is not available in many laboratories especially in under-developing countries. A derivatization technique using naphthalenedialdehyde as labeling agent and HPLC coupled with fluorescence detection with LOQ of 50 ng/mL has been published for determination of OCA in rat plasma [5]. In this method however, a toxic reagent (KCN) and long incubation time (45 min) are required for derivatization of the analyte. Also their method is not sensitive enough for use in single dose pharmacokinetic studies. The present paper is the first report of a simple reverse phase HPLC–UV method to determine the active carboxylic metabolite of oseltamivir phosphate in human serum. This method was applied and validated in a single dose bioequivalence study, following oral administration of two different oseltamivir preparations in 24 healthy volunteers.

2. Experimental

2.1. Chemicals

OCA (purity 96.0%) was synthesized by Cipla Ltd. Company (Mumbai, India) and kindly provided by Bakhtar Bioshimi pharmaceutical company (Kermanshah, Iran). Vanillin (I.S.; Fig. 1C) was from Merck (Darmstadt, Germany). All reagents used were of analytical grade except acetonitrile which was HPLC grade. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Preparation of standard solutions

A stock solution of OCA (1000 μg/mL) was prepared in distilled water. For working standards, the appropriate volumes of this solution were diluted with acetonitrile to obtain different solutions ranging from 0.15 to 64 μg/mL. Working standard solution of the I.S. (2.5 μg/mL) was prepared in methanol. A 1.0 M solution of citric acid was prepared in water. A 0.1 M phosphate-buffered saline (PBS) prepared in de-ionized water contained sodium chloride (154 mM), potassium phosphate monobasic (1.5 mM) and sodium phosphate dibasic (8.5 mM). The pH of the buffer was adjusted to 7.4 using either sodium hydroxide or phosphoric acid. An elution solvent consisting of acetonitrile and water (3:1, v/v) and a washing solvent consisting of 0.1 M HCl in water–acetonitrile (5:95, v/v) were prepared. All solutions were stored at 4 °C and were stable for at least 3 weeks.

2.3. Sample preparation

Solid phase extraction cartridges (C18, 500 mg, 6 mL, Cronus, Maisemore Gloucester, UK) were conditioned with the PBS buffer (5 mL) followed by distilled water (5 mL), acetonitrile (5 mL) and 1 mL of the washing solvent. To 1 mL of serum sample (blank, calibration or unknown) in a test tube, 100 μL of the I.S. and 100 μL of the citric acid solution were added. Following brief mixing on vortex mixer, the sample was loaded onto the cartridge. The loaded cartridge was washed with the washing solvent (1 mL) and the analytes were selectively eluted with the elution solvent (500 μL) into a glass tube. After evaporation of the organic solvent using a Speed-Vac concentrator (Eppendorf, Hamburg, Germany), a volume of 20 μl was injected into the HPLC system.

2.4. Instrumentation and chromatographic conditions

The HPLC system consisted of two pumps (LC-10AD), a column oven (CTO-10A), a UV–vis spectrophotometer detector (SPD-10AD) operated at wavelength of 215 nm, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu,

Kyoto, Japan. The analytical column was a Shimpack CLC-ODS (Shimadzu, Kyoto, Japan), 150 mm × 4.6 mm I.D., 5 μm particle size which was protected by a Shim-pack G-ODS guard column (10 mm × 4.0 mm I.D., 5 μm particle size). A mixture of 0.05 M phosphate buffer containing triethylamine (1 mL/L; pH 3.0) and acetonitrile (70:30, v/v) was used as the mobile phase. The column oven temperature was set at 58 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 1.6 mL/min.

2.5. Validation of the method

2.5.1. Calibration curve and linearity

Calibrators were prepared with standards of 15, 30, 60, 120, 240, 480, 960, 1600, 3200 and 6400 ng/mL using pooled human blank serum obtained from the normal subjects. In disposable glass tubes (100 mm × 16 mm), after evaporation of 100 μL from each working solution of the analyte, under a gentle stream of nitrogen at 50 °C, the residues were reconstituted in 1 mL of drug-free human serum, mixed for 10 s on a vortex mixer and subjected to extraction and analysis as described above. The linearity of the method was checked on the same day ($n = 6$) and in 6 consecutive days. Calibration curves were obtained using a weighted regression with a weighting factor of $1/(\text{concentration})^2$ by linear least-squares regression analysis of plots of peak-area ratio of OCA to I.S. versus drug concentrations.

2.5.2. Accuracy, precision and sensitivity

Quality control samples used in method validation were prepared with the drug working solutions to make low (15 ng/mL), medium (480 ng/mL) and high (3200 ng/mL) concentrations of the analyte. Intra- and inter-day variations were calculated by repeated analysis ($n = 6$) of different concentrations of OCA in a single analytical run and in 10 analytical runs performed on different days, respectively. The limit of detection was defined as the concentration of drug giving a signal-to-noise ratio of 3:1. The lower limit of quantification was defined as the lowest serum concentration of the drug quantified with a coefficient of variation of less than 20% (range recommended by the Conference Report on Bioanalytical Methods Validation [6]).

2.5.3. Specificity, selectivity, recovery and stability

The specificity of the method was examined by presence of disturbing endogenous peaks in 24 human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except for the addition of the I.S. Selectivity of the assay was examined by analysis of several potentially co-administrated drugs with oseltamivir. The absolute recoveries of OCA at the above mentioned concentrations (15, 480 and 3200 ng/mL) as well as the I.S. at applied concentration were calculated in replicates ($n = 5$) by comparing the respective peak areas obtained by extraction of the samples from serum, with those obtained from the same amounts of unextracted solutions in acetonitrile. Stability of the quality control serum samples (15, 480 and 3200 ng/mL) was subjected to short-term (12 h, 25 °C) room temperature, three freeze–thaw (−40 to 25 °C) cycles and long-term (60 days, −40 °C) stability tests. Subsequently, the concentrations were measured in comparison to freshly prepared samples. Stability of the solutions of OCA and the I.S. were studied over a period of 3 weeks by comparing of the peak areas at different times.

2.6. Application of the method

The present method was applied to a randomized cross-over bioequivalence study of two different oseltamivir preparations in 24 male healthy volunteers aged 26.5 ± 4.2 years and weighing 72.3 ± 5.1 kg with normal biochemical parameters. Written informed consent was obtained from the subjects and the study protocol was approved by Ethics Committee of Kermanshah University of Medical Sciences. After an overnight fasting, all the volunteers received a single oral dose of 150 mg oseltamivir phosphate from either Bakhtar Bioshimi (Kermanshah, Iran) or F. Hoffmann-La Roche Ltd. (Switzerland) pharmaceutical companies on 2 working days separated by a wash-out period of 3 weeks. Blood sampling were carried out at suitable intervals up to 24 h using disposable glass tubes (100 mm × 16 mm) and the samples were stored at −40 °C until analysis. Pharmacokinetic parameters including maximum concentration (C_{max}), area under the concentration time curve from zero to the time of last sampling (AUC_{0-t}) and area under the concentration time curve from zero to infinity ($AUC_{0-\infty}$) were compared. Student's *t*-test was used for statistical analysis of the data and statistical significance was defined at the level of $p < 0.05$.

3. Results

3.1. Specificity and selectivity

Representative chromatograms of human blank serum spiked with the I.S. and human blank serum spiked with the I.S. and OCA at the concentration of 660 ng/mL are shown in Fig. 2A and B, respectively. Under the chromatographic conditions described, the I.S. and OCA were eluted with retention times of 8.0 and 9.1 min, respectively. No endogenous components from serum were found to interfere with the elution of the drug or I.S. Fig. 2C and D shows the chromatograms of serum samples obtained at 6 and 24 h after a single oral dose of 150 mg oseltamivir phosphate from a healthy volunteer, respectively.

The results of the selectivity study showed that using the same analytical conditions, acyclovir, lamivudine, zidovudine, nelfinavir, ribavirin, methyldopa, theophylline, cimetidine and acetaminophen were eluted before the solvent front while, amantadine, idoxuridine, rimantadine, cinnarizine, glybenclamide, hydrocortisone, omeprazole, naproxen, indomethacin, celecoxib, ibuprofen, diazepam, chlordiazopoxide and oxazepam were not eluted within 10 min. All the drugs were tested at concentrations range of 10–1000 ng/mL.

3.2. Sensitivity and linearity

The LOD and LOQ were found to be 5 and 15 ng/mL, respectively. The calibration curves were linear over the concen-

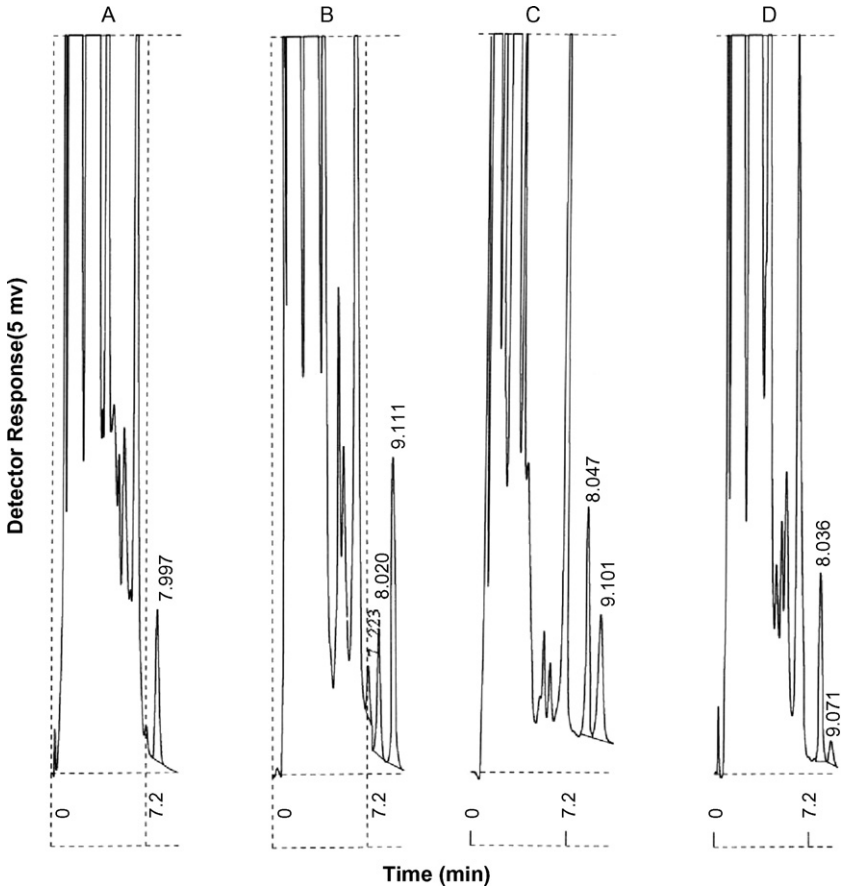

Fig. 2. Typical chromatograms obtained from an extract of (A) human blank serum spiked with vanillin as the (B) human blank serum spiked with 660 ng/ml OCA and vanillin as the I.S. and (C and D) serum samples obtained at 6 and 24 h after a single oral dose of 150 mg of the drug from a healthy volunteer containing 140 and 28 ng/mL of the drug, respectively. Peaks eluted at 8.1 and 9.1 min correspond to the I.S. and OCA, respectively.

tration ranges of 15–6400 ng/mL. The correlation coefficients for calibration curves were equal to or better than 0.9975. Intra- and inter-day reproducibility for calibration curves were determined on the same day in replicate ($n = 6$) and on different days ($n = 6$), respectively, using the same pooled serum sample. The intra-day average slope of the fitted straight lines was 0.3552 ± 0.02 ng/mL (C.V. = 5.6%) and the mean intercept of the calibration curves was 2.88 ± 0.194 (C.V. = 6.7%). The corresponding mean (±S.D.) coefficient of the linear regression analysis was 0.9975 ± 0.011 (C.V. = 0.1%). For calibration curves prepared on different days ($n = 10$), the mean ± S.D. of results were as follows: slope 0.3676 ± 0.013 ng/mL (C.V. = 3.5%), coefficient of the linear regression analysis = 0.9980 ± 0.011 (C.V. = 0.1%) and intercept = 2.28 ± 0.172 (C.V. = 7.5%).

3.3. Recovery, accuracy, precision and stability

The inter- and intra-day accuracy and precision values of the assay method are presented in Table 1. The coefficient variation values of both inter- and intra-day analysis were less than 12% whereas the percentage error was less than 4.5. The mean

Table 1
Precision and accuracy results of the validation

Known concentration (ng/mL)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (% mean deviation)
Inter-day ($n = 10$)			
15	14.3 ± 1.70	11.90	−4.4
480	485.2 ± 8.1	1.67	1.1
3200	3241 ± 49.7	1.53	1.3
Intra-day ($n = 6$)			
15	15.5 ± 1.78	11.54	3.3
480	488.7 ± 9.1	1.87	1.8
3200	3267 ± 67.5	2.07	2.1

Accuracy has been calculated as a percentage of the nominal concentration.

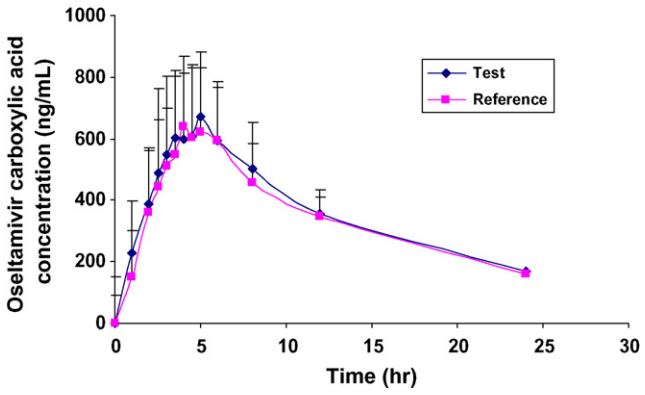

Fig. 3. Mean ± S.D. serum concentrations vs. time profiles of OCA for two oseltamivir preparations in 24 human volunteers after administration of a single 150 mg oral dose.

Table 2
Mean (S.D.) pharmacokinetic parameters of OCA for two preparations in 24 healthy volunteers after administration of a single 150 mg oral dose

Parameter\prep.	Test	Reference	P value[a]
T_{max} (h)	4.44 (1.0)	4.06 (1.0)	NS
C_{max} (ng/mL)	748.6 (240)	745.7 (259)	NS
AUC_{0-24} (ng h/mL)	8424.5 (3070)	8019.6 (2637)	NS
$AUC_{0-\infty}$ (ng h/mL)	10989.2 (4801)	10640.4 (3732)	NS
$T_{1/2}$ (h)	10.00 (3.1)	10.5 (3.0)	NS

T_{max} = time to maximum concentration, C_{max} = maximum concentration, AUC = area under the concentration time curve and $T_{1/2}$ = elimination half life.
[a] NS = no significant difference ($P < 0.05$).

extraction efficiencies of OCA and I.S. from serum were found to be 98 ± 3% and 85 ± 5%, respectively. The stock solutions of OCA and the I.S. were stable for at least 21 days when stored at 4 °C. The stability of the drug was found to be 100% from the initial value, after 60 days maintenance of the serum at −40 °C and following three thaw–freeze cycles.

4. Application of the method

This method has been successfully used for the determination of serum concentrations of OCA in a randomized cross-over bioequivalence study following single oral administration of two different oseltamivir phosphate preparations in 24 healthy volunteers. Typical serum concentration–time profiles of OCA have been shown in Fig. 3 and resulted pharmacokinetic parameters have been summarized in Table 2.

5. Discussion

OCA does not contain any strong UV light absorbing groups and it has only a weak UV absorbance in the low wavelength range. Furthermore, the drug is highly polar in nature ($\log p = -2.1$) and is therefore extremely difficult to extract from biological fluids such as serum by commonly used organic solvents. Also low blood levels are obtained after single dose administration of the drug in human pharmacokinetic studies. Thus, for assay of the analyte using conventional HPLC–ultraviolet method, high efficient extraction procedure is required and the drug should be chromatographed without any interfering or co eluting peak. All our attempts failed to extract OCA from the serum samples with a good recovery (>50%) either using liquid–liquid extraction by different solvents, or with protein precipitation methods. Due to presence of an ionizable carboxylic acid moiety in OCA molecule (Fig. 1B), the drug can be extracted by solid phase extraction using its pH dependent retention on C18 cartridge. As it has been reported by Eisenberg and Cundy [5], lowering pH of the washing solution below 3 protonates the carboxylic acid group and retains the drug onto the cartridge. Thus, un-wanted compounds can be washed with acidified solvents and the analyte is selectively eluted using non-acidified medium such as mixture of acetonitrile and water. In our method however, recovery of extraction and the shape of chromatograms were significantly improved using the PBS buffer for conditioning of the cartridges. Oseltamivir phosphate is rapidly converted to the active carboxylate form and although it has been reported that low blood levels of the intact drug can be detected in plasma [1], our method failed to detect peak of the intact drug in plasma. The mobile phase composition was investigated and we found that pH of the buffer solution plays an important role in the resolution of the chromatograms also, sufficient amounts of triethylamine is essential to obtain symmetric peaks. A number of chemical agents were tested to choose I.S. and vanillin was selected considering its UV spectrum, retention time, recovery and resolution from the drug and endogenous peaks.

6. Conclusion

In conclusion, a new, sensitive and specific method has been validated for the determination of oseltamivir carboxylic acid (OCA), the active moiety of anti-influenza agent, oseltamivir phosphate, in human serum using HPLC method and UV detection. This method which has demonstrated to be suitable for its use in pharmacokinetic studies of oseltamivir phosphate, is rapid with run time of 10 min and sensitive with LOQ of 15 ng/mL.

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