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ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF OSELTAMIVIR

M. Espinosa Bosch[a], C. Bosch Ojeda[b], A.J. Ruiz Sánchez[c], F. Sánchez Rojas[b1]

[a] Department of Pharmacy, General Hospital, University Hospital "Virgen del Rocío", Manuel Siurot s/n, 41013, Sevilla, Spain
[b] Department of Analytical Chemistry, Faculty of Sciences, University of Málaga, Campus Teatinos s/n, 29071 Málaga, Spain
[c] Department of Organic Chemistry, Faculty of Sciences, University of Málaga, Campus Teatinos s/n, 29071, Málaga, Spain

ABSTRACT

Oseltamivir phosphate is an antiviral drug used in the treatment and prophylaxis of both influenza virus A and influenza virus B. Oseltamivir phosphate is an ethyl ester pro-drug that is rapidly and extensively metabolized by esterases in the gastrointestinal tract and liver to its active form, oseltamivir carboxylate. There are some analytical methods in the literature for the analysis of both compounds in the biological fluids and pharmaceutical preparations. In this work, we have recompiled these methods with the aim of to present the different options for the oseltamivir determination.

Keywords: Oseltamivir; pharmaceuticals; analytical determinations; biological samples; review.

[1] Corresponding author: *E-mail address:* fsanchezr@uma.es

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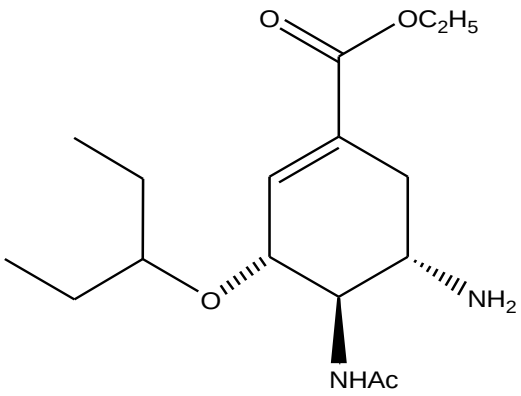
INTRODUCTION

Influenza is a highly contagious, acute febrile respiratory infection caused by the influenza virus. Cases typically occur in a seasonal pattern, with localised epidemics during the winter months. Influenza is an *orthomyxovirus*, comprising a lipid membrane surrounding a matrix protein shell and a core consisting of seven or eight ribonucleic acid (RNA)–nucleoprotein complexes. There are three serotypes of influenza virus – influenza A, B and C – which differ in their core proteins. Influenza A and B are responsible for nearly all influenza-associated clinical illnesses. The influenza virus contains two surface glycoproteins, which act as powerful antigens: haemagglutinin (HA) and neuraminidase (NA). Haemagglutinin facilitates the entry of the virus into cells of the respiratory epithelium, while neuraminidase facilitates the release of newly produced viral particles from infected cells [1].

Figure 1. Chemical structure of oseltamavir.



Two classes of antiviral medications are currently available for treatment or prophylaxis of influenza infections: neuraminidase inhibitors (NAIs) (oseltamivir and zanamivir) and the adamantanes (amantadine and rimantadine). The NAIs block the action of influenza neuraminidase, an enzyme present on the viral envelope that provides for the efficient release of progeny virion particles from the surface of an infected cell. The target of the adamantanes is the viral M2 matrix protein, an ion-channel protein that spans the viral envelope's lipid bilayer and is required for viral uncoating. Partly as a result of the emergence of amantadine resistant influenza viruses, alternative antiviral strategies have been developed. The sialidase activity in the virus neuramindase (NA) protein plays a critical role in the influenza virus replication cycle, and the design of NA protein inhibitors is currently one of the most common approaches in the development of anti-influenza virus drugs. The NA protein was the first virus glycoprotein for which a high resolution molecular structure was obtained. Subsequent functional and structural studies of the NA protein have allowed several pharmaceutical companies to produce NA inhibitors using a structure-based inhibitor design. Zanamivir and oseltamivir are the two NAIs that are currently available for the prevention of virus infection. These drugs target the active site of the NA protein, thus inhibiting its sialidase activity that is essential for virus release [2]. Zanamivir was the first NAI available, and it is currently marketed by GlaxoSmithKline under the market name of Relenza®. Oseltamivir, initially developed by Gilead Sciences, is currently produced by Hoffman-La Roche under the market name of Tamiflu®. These drugs, in particular

oseltamivir, are currently the primary drugs available for the prevention of influenza virus infection. Although zanamivir and oseltamivir are similar in their mode of action, the drugs have different biochemical properties, which influence how they are administered. Due to the poor bioavailability of zanamivir it must be administered by inhalation, and there have been several reports of respiratory complications following its inhalation. In contrast, oseltamivir is administered orally as a pro-drug ester, usually as oseltamivir phosphate (OP). Once administered, the pro-drug is processed by hepatic esterases into its active form (oseltamivir carboxylate (OC)). It is currently the drug of choice for the prevention of influenza virus infection, and as a consequence it is being stockpiled by organisations in many countries in anticipation of a pandemic

Oseltamivir is indicated for [3]:

- The post-exposure prophylaxis of influenza in patients aged 1 year and above who have had contact with a clinically diagnosed influenza index case when influenza is circulating in the community.

- The treatment of influenza in patients aged 1 year and above who present with influenza symptoms when influenza is circulating in the community. Treatment is effective when initiated within 48 hours of onset of the first symptoms.

There is a constant need to determine the concentrations of OP and OC in human and animal samples during post-marketing monitoring and research and in pharmaceutical samples. Hitherto there are few analytical methods reported for estimation of oseltamivir. Literature survey for oseltamivir analysis revealed several methods based on different techniques, such as bioassay, capillary electrophoresis, liquid chromatographic with fluorescence, mass spectrometric and UV detection, among others. The purpose of this paper is to review these analytical procedures developed in the last years for the determination of oseltamivir, the most important of the few antiviral drugs available for treatment of seasonal flu and a cornerstone in the defence against an influenza pandemic.

ANALYTICAL METHODS

Colorimetric methods

Simple and affordable colorimetric assays provide a practical means to rapidly monitor drug quality. Colorimetric tests are rapid and easy to perform, the reagents and equipment for colorimetric tests are inexpensive, relatively non-toxic, and are ideal for use in field situations. In this sense, OP possesses amine groups and the protonated form may act as a cationic site for anionic dyes such as Congo red and bromochlorophenol blue to produce coloured ion-pairing complexes extractable in ethyl acetate [4]. After complete phase separation, the top organic layer was transferred to a glass tube for absorbance measurements at 520 nm or 590 nm, respectively. Greater linearity and lesser variability are observed from the Congo red assay. Evaluation of the colorimetric assay for OC has not been performed by the authors. Ion-pairing with acidic dyes under the described conditions is less likely because the carboxy metabolite is a zwitterion.

Spectrofluorimetric methods

The previous reported colorimetric assay [4] is not sensitive enough with the initial determined concentration of analyte. In addition, the method can not be applied in analysis of the drug in biological fluids, and requires extraction process. Spectrofluorometry, because of its high sensitivity and selectivity, low cost, and wide availability in most quality control laboratories, is a powerful analytical techniques for the determination of drugs. Aydoğmuş [5] describes a simple, sensitive and economical spectrofluorometric method for the analysis of OP in its capsules with satisfactory results. The method was based on its reaction with fluorescamine in borate buffer solution (pH=8.5), to form fluorescent product which was measured fluorometrically at λ_{em} = 483 nm (λ_{exc} = 381 nm). This method may not be applicable for the analysis of OP in biological fluids since the other chemicals with primary or secondary amines group in the maintained matrix will interfere with the assay.

Enzymatic assay

An enzymatic assay based on neuraminidase inhibition was used for detection of OC in rat plasma [6]. However, the assay was time-consuming, suffered from a lack of specificity and was difficult to use in routine analysis.

Mass spectrometry methods (MS)

Nyadong et al. [7] present a method based on the selective recognition of oseltamivir by crown ethers added to desorption electrospray ionization (DESI) spray solvent. The authors exploit crown ether host–guest chemistry in a reactive DESI MS platform as an alternative approach for the rapid, sensitive and selective detection of oseltamivir in pharmaceutical preparations. The formation of a specific non-covalent complex, coupled with consecutive reaction monitoring (CRM) mode; where an oseltamivir fragment generated by an MS^3 experiment is monitored, ensures the highest degree of selectivity. Molecular recognition *via* host–guest complexation reactions in DESI MS has been shown to be a rapid, highly selective and sensitive method for screening the quality of Tamiflu® samples.

HPLC methods

The stability of oseltamivir in oral aqueous solutions containing the preservative sodium benzoate was studied by a stability indicating HPLC-method [8]. The separation was achieved on a RP-18 column using a gradient of mobile phase A (aqueous solution of 50 mM ammonium acetate) and mobile phase B (60% acetonitrile/40% mobile phase A).

A HPLC assay for quality control and authentication of Tamiflu® capsules has been developed and validated by Lindegardh et al. [9]. Oseltamivir was analysed by liquid chromatography with UV detection at 220 nm on a Hypersil Gold column using a mobile phase containing methanol-phosphate buffer (pH 2.5; 0.1 M) (50:50) at a flow rate of 1.0 mL/min.

Later, diverse methods using HPLC with UV detection are proposed:

- Isocratic HPLC method has been developed for the determination of OP in dosage forms, Tamiflu® and generic versions [10]. The conditions are as follow: Isocratic elution, at a flow rate of 1.2 mL/min, on a Zorbax CN column at ambient temperature. The mobile phase consisted of methanol and 0.04M formic acid pH 3.0 (50:50). The detection wavelength was 226 nm and 20µL 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 important features and novelty of this 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 IS 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%).
- Reverse phase HPLC (RP-HPLC) method for the analysis of oseltamivir active pharmaceutical ingredient (API) with gradient elution [11]. The method utilizes Kromasil C18, 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. Based on peak purity results, obtained from the analysis of force degraded samples using described method, it can be concluded that the absence of coeluting 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.
- Fuke et al. [12] describe the HPLC-UV detection at 230 nm of OC in biological materials, using floropipamide as IS, following mixed mode cation exchange extraction and application of this method to a case of death from falling after ingestion of a Tamiflu® capsule. Chromatographic separation was performed using a XTerra® MS C18 analytical column, column temperature maintained at 40 ºC, mobile phase consisted of acetonitrile–20 mmol/L potassium dihydrogen phosphate buffer (pH 3.0) (8:92) and flow rate was maintained at 0.2 mL/min. This is the first report on the distribution of OC in a human body.
- Finally, Bahrami et al. [13] present a simple RP-HPLC method to determine OC in human serum. The analyte and an IS (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 0.05M phosphate buffer containing triethylamine (1 mL/L; pH 3.0) and acetonitrile (70:30). The detection wavelength was set at 215 nm. This method which has demonstrated to be suitable for its use in pharmacokinetic studies of OP, is rapid with run time of 10 min and sensitive with LOQ of 15 ng/mL.

Chromatographic method with fluorescence detection is proposed by Eisenberg and Cundy [14]. Plasma samples were subjected to SPE on C18 extraction columns. After extraction, OC was derivatized with naphthalenedialdehyde in the presence of potassium cyanide to produce highly fluorescent cyano[*f*]benzoisoindole derivatives. Derivatized samples were stable for >24 h at 4ºC. The analytical column used was a Prodigy (ODS-2), column temperature maintained at 40 ºC, mobile phase consisted of 50 mmol/L sodium acetate in acetonitrile–water (27:73), flow- rate was maintained at 2 mL/min and the total run time was 10 min. The retention times were 4.3 min for IS and 5.2 min for OC. The samples were analyzed by an isocratic HPLC method using fluorescence detection at 420 nm excitation and 470 nm emission wavelengths.

HPLC with tandem mass spectrometric detection was the most attractive option for the simultaneous assay of OP and OC in biological samples. In this way, Wiltshire et al. [15] developed an HPLC–MS/MS assay for both compounds in plasma and urine. After SPE procedure, the drug and pro-drug were separated by RP-HPLC using a Nova-Pak CN HP cartridge housed in a radial compression module. The mobile phase was 50% methanol–50% 80 mM aqueous formic acid, pH 3. The flow-rate was 0.5 ml/min and the retention times for the two compounds were approximately 3.5 (OC) and 5 min (OP). The analytes were introduced into the mass spectrometer via the appropriate interface and the protonated molecular ions fragmented by collision with argon (nitrogen for PE-Sciex API 365). The resultant daughter ions were then detected.

Later, a method for the analysis of OP and OC in human plasma, saliva and urine using off-line SPE and liquid chromatography coupled to positive tandem mass spectroscopy has been developed by Lindegardh et al. [16]. OP and OC were analysed on a ZIC-HILIC column using a mobile phase gradient containing acetonitrile–ammonium acetate buffer (pH 3.5; 10 mM) at a flow rate of 500 µL/min. The ZIC-HILIC column has a sulfobetaine type zwitterionic stationary phase covalently attached to silica and is especially suitable for polar and hydrophilic analytes. The mobile phase should contain a high concentration of water miscible organic solvent (e.g. acetonitrile) to promote hydrophilic and weak electrostatic interactions between the analytes and the hydrophilic phase. Neutral and lipophilic compounds in general show poor retention on the column. An triple quadrupole mass spectrometer with a TurboV^{TM} ionisation source interface operated in the positive ion mode, was used for the multiple reaction monitoring LC–MS/MS analysis. This highly sensitive robust LC–MS assay should facilitate urgently needed pharmacokinetic studies on this important antiviral drug.

Heinig and Bucheli carried out the determination of OP and OC in rat plasma, cerebrospinal fluid and brain as well as in human plasma and urine by LC–MS/MS [17]. On-line SPE using an in-house assembled column-switching system provided good selectivity and robustness, removing the need for elaborate off-line sample preparation. Superior sensitivity and short analysis time were obtained by the authors. The method was successfully validated according to regulatory requirements and employed for analysis of study samples. Threefold deuterated OP and OC served as ISs. Protein precipitation with perchloric acid was followed by on-line SPE and gradient separation on a reversed-phase column. After electrospray ionization, the compounds were detected in positive ion selected reaction monitoring mode with run time 3.6 min.

Capillary electrophoresis methods

In general, the main advantages of capillary electrophoresis (CE) methods are speed, resolution, efficiency, analyte solubility and stability, minimal reagent and solvent consumption, compatibility with mass spectrometry, and the availability of several modes which have all made CE a popular analytical technique in the field of pharmaceutical and biomedical analysis.

The purposes of an investigation developed by Jabbaribar et al. [18] are to present the micellar electrokinetic chromatography (MEKC) and MEKC-sweeping methods for the

analysis of oseltamivir and its hydrolyzed product in a pharmaceutical formulation. The LOD and LOQ of MEKC method were improved using the sweeping technique. Separation column was an uncoated fused-silica capillary with total length of 90 cm and effective length of 72 cm. For MEKC, sample injections were hydrodynamically conducted at 100 mbar for 18 s. In the MEKC-sweeping mode, the samples were injected at 250 mbar for 1 min. A voltage of +30 kV was applied and the temperature was 25 °C. Background electrolyte (BGE) was prepared using boric acid and NaOH 2 M containing various concentrations of sodium dodecyl sulphate and then filtered through a membrane filter. Before each run, the capillary was rinsed with NaOH 0.1 M, water, and running buffer for 3.5 min each. The new capillary was conditioned with NaOH 1 M, water, and BGE for 20 min each. The detection was carried out at 214 nm.

Very recently, Laborde-Kummer et al. [19] carry out a rapid determination of OP in capsules by capillary zone electrophoresis (CZE). Electrophoresis was carried out on a system equipped with a diode array detector. An uncoated fused silica capillary with a total length of 60.2cm×75µm i.d. was used. Samples were hydrodynamically injected into the capillary for 5 s at 0.5 psi at the detector side. Consequently, the effective length of the capillary was 10 cm. The capillary was pre-conditioned prior to its first use by successively conducting a 5 min rinse with 0.1M HCl, a 2 min rinse with water, a 10 min rinse with 0.1M NaOH, a 2 min rinse with water and finally a 20 min rinse with electrolyte buffer. Separations were carried out at −15 kV at 25 ºC using a 50mM sodium phosphate buffer, pH 6.3. UV detection was carried out at 226 nm. Conditioning between runs consisted of a 2 min rinse with phosphate buffer. A short electrophoretic analysis time (less than 1.5 min) was obtained using the short end injection mode. The results obtained attest to the precision and the accuracy of the method. Furthermore, low cost and simple sample pre-treatment strengthen its potential applicability for routine analysis in the quality control of capsules with an advantage of no organic solvent consumption compared to HPLC method.

ENVIRONMENTAL STUDIES

Numerous studies have documented that a wide number of pharmaceuticals used in human and veterinary medicine have the potential to enter the aquatic ecosystem. After administration, a variety of pharmaceuticals are discharged as parent compounds or metabolites into wastewater treatment plants via excretion with urine and faeces. Research has shown that many pharmaceuticals are not completely removed during wastewater treatments, and as a result, pharmaceuticals have been found in a wide range of environmental samples including surface and groundwater. The occurrence of pharmaceuticals in the aquatic environment has become a subject of scientific and public concern. With respect to oseltamivir, a recent study conducted in Sweden has demonstrated that OC is not removed during conventional sewage water treatments which include mechanical, chemical and biological processes [20]. In this study conducted with water of an irrigation canal, the authors showed that 65% of the initial amount of the active antiviral OC was removed during a 36-day incubation period at 20 ºC. The study also showed that degradation of OC was driven by microbial processes.

Consequently, in case of urban areas with a large number of patients receiving Tamiflu, the potential risk of OC to contaminate the aquatic ecosystem should be

considered [20-22]. The publications show that OC is quite persistent in aquatic environments and is only removed by microbial degradation associated with sediment [21,23]. A hypothesis has been presented that OC residues in the environment, either after usage during a pandemic or for treatment of seasonal influenza, could expose the natural reservoir of influenza virus, dabbling ducks, to low levels of this antiviral which could promote resistance development [20,22].

The country where oseltamivir is used most is Japan, where it is used to treat seasonal flu. Söderström et al. [24] measured the levels of OC in water samples from the Yodo River system in the Kyoto and Osaka prefectures, Japan, taken before and during the flu-season 2007/8. No OC was detected before the flu-season but 2–58 ng/L was detected in the samples taken during the flu season.

Bartels and von Tümpling summarized the results of three different daylight exposure experiments with OC in different waters under sterile and non-sterile conditions simulating shallow water processes at the latitude of approximately 52°N [25]. Using a river water solution containing 50 µg/L OC under non-sterile conditions, a half-life time of 17.8 days was observed. Direct photolysis plays no or only a negligible role for the decomposition of OC. Degradation of OC seems to occur as a combination of microbial metabolism and indirect photolysis.

A recent environmental risk assessment for oseltamivir, based on those very high predicted environmental concentrations and on predicted no-effect concentrations derived from chronic ecotoxicity tests, concluded in no evident risk to sewage works and surface waters from the use of oseltamivir, even under pandemic conditions [26]. The same investigation group presents other contribution that is an extension to the marine environment [27]. The authors conclude that there are no grounds for environmental concerns from the medical use of Tamiflu, even when used in an inordinately widespread manner during an influenza pandemic.

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