



Online solid phase extraction liquid chromatography using bonded zwitterionic stationary phases and tandem mass spectrometry for rapid environmental trace analysis of highly polar hydrophilic compounds – Application for the antiviral drug Zanamivir

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ABSTRACT

Zanamivir (Za) is a highly polar and hydrophilic antiviral drug used for the treatment of influenza A viruses. Za has been detected in rivers of Japan and its environmental occurrence has the risk of inducing antiviral resistant avian influenza viruses. In this study, a rapid automated online solid phase extraction liquid chromatography method using bonded zwitterionic stationary phases and tandem mass spectrometry (SPE/LC–MS/MS) for trace analysis of Za was developed. Furthermore, an internal standard (IS) calibration method capable of quantifying Za in Milli-Q, surface water, sewage effluent and sewage influent was evaluated. Optimum pre-extraction sample composition was found to be 95/5 v/v acetonitrile/water sample and 1% formic acid. The developed method showed acceptable linearities ($r^2 \geq 0.994$), filtration recovery ($\geq 91\%$), and intra-day precisions ($RSD \leq 16\%$), and acceptable and environmentally relevant LOQs ($\leq 20 \text{ ng L}^{-1}$). Storage tests showed no significant losses of Za during 20 days and $+4/-20 \text{ }^\circ\text{C}$ ($\leq 12\%$) with the exception of influent samples, which should be kept at $-20 \text{ }^\circ\text{C}$ to avoid significant Za losses. The applicability of the method was demonstrated in a study on phototransformation of Za in unfiltered and filtered surface water during 28 days of artificial UV irradiation exposure. No significant ($\leq 12\%$) phototransformation was found in surface water after 28 days suggesting a relatively high photostability of Za and that Za should be of environmental concern.

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1. Introduction

Antiviral drugs are widely used to treat influenza A viruses but their fate and effects in the environment are not well studied [1]. Water living birds are the natural hosts of influenza A virus [2] and all known human pandemic viruses have contained genetic elements from avian influenza strains [3,4]. If influenza-infected birds are exposed to the antiviral drugs neuraminidase inhibitors (NAIs) in their water environment, resistance can develop [5,6], and could eventually transmit to humans as a resistant pandemic, which

would be of major public health concern [7]. The NAI oseltamivir (Tamiflu[®], Roche) has been mostly studied as it is the predominantly used anti-influenza drug [8], and also stockpiled worldwide in case of a severe influenza pandemic [9,10]. The active metabolite oseltamivir carboxylate (OC) is largely excreted from treated humans, is not removed or degraded by traditional sewage treatment [11,12], nor by direct phototransformation [11,13], and has repeatedly been measured in sewage effluents and river water, see for instance [14–19]. Zanamivir (Za) (Relenza[®], GlaxoSmithKline), is the second most used NAI [8]. It is used primarily when there is resistance to oseltamivir [20], has been less stockpiled, and much less environmentally studied. Additionally, the high polarity and hydrophilicity of Za (predicted values: pK_{a1}

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3.25; pK_{a2} 11.93; water solubility 7.31 g L^{-1} ; and $\log P$ -2.3 and -5.8 [21]) have made reliable quantification in water a challenge [1,22,23]. At present, Za has been measured in rivers in Japan [14–16] and photodegradation after 18 days in surface water, with the formation of four photoproducts, has been reported [22]. However, much of its fate and effects in the environment needs to be studied, which is a highly relevant task especially in the light of increasing Za usage.

Since Za is highly polar and hydrophilic, low retention is obtained on conventional reversed phase liquid chromatography (RP-LC) columns such as the most commonly used octadecyl carbon chain (C18)-bonded silica column. This was also observed in this study. Hydrophilic interaction liquid chromatography (HILIC), in contrast to RP-LC unless ion-pairing is used, has however the ability to significantly retain highly polar and hydrophilic analytes (HPHAs) [24–26]. Today, there are many commercially available HILIC columns with different stationary phase chemistries. Most of the columns carry ionic charges therefore it is recommended to use buffer salts in the mobile phase to control the ionic interactions between the analytes and the stationary phase, in addition to keeping a controlled mobile phase environment. Polar partitioning is the main retention mechanism but the presence of ionic interactions can have a substantial impact on retention. A column with charged functional groups has much greater interaction with charged analytes than that of an uncharged stationary phase and these electrostatic interactions provide the possibility of changing retention time and controlling selectivity by altering pH and/or buffer strength. Truly zwitterionic HILIC stationary phases, such as the ZIC-HILIC, provide sites for weak electrostatic interactions due to the close proximity of ion and counter-ion in balanced proportions within their functional groups. A pleasant side-effect using HILIC is that analytes usually elute with a high content of organic solvent which in turn may improve ionization efficiency and detection limit with mass spectrometry detection [27].

In environmental trace analysis, the extraction of polar analytes (e.g. pharmaceuticals) from aqueous samples is usually made by offline solid phase extraction [28]. In terms of HPHAs in aqueous samples, a “wide selection” of ion-exchange SPE sorbents have been used, for instance: OASIS HLB [29]; OASIS WCX [30]; and Strata X-CV [31]. However, in order to improve sample throughput, and minimize manual labor, automated time effective online SPE has become increasingly popular [32]. Although most of the method development involving HILIC has been in the area of bioanalysis [33], online SPE of environmental samples in combination with HILIC has been reported [34]. For instance Roen et al. successfully quantified nerve agent degradation products in water by the use of a porous graphite carbon online SPE in combination with HILIC [35]; Zhang et al. developed a cation-exchange restricted access material online SPE-HILIC method to determine melamine and cyromazine in milk [36]; and van Nuijs et al. used OASIS HLB and OASIS MCX SPE in the analysis of drugs of abuse (including metabolites) in sewage waters [37].

The aim of this study was to develop and evaluate a rapid automated ZIC-HILIC based online solid phase extraction liquid chromatography tandem mass spectrometry (SPE/LC-MS/MS) method, to our knowledge the first method using ZIC-HILIC in both the enrichment of the analyte (online SPE) and in the analytical column, capable of analysing Za in Milli-Q, surface water, sewage effluent and sewage influent. As the environmental fate of Za has been rarely studied, the method applicability was tested in a phototransformation study where Za in filtered and unfiltered surface water was exposed to simulated sunlight irradiation for 28 days, where the percentage of transformation was determined using the developed method.

2. Experimental

2.1. Chemicals

Za and ^{13}C - $^{15}\text{N}_2$ -Za standards were classified as analytical grade ($>98\%$) and were obtained from GlaxoSmithKline (Stevenage, Hertfordshire, UK). The stock solutions of Za and ^{13}C - $^{15}\text{N}_2$ -Za, and dilutes, were prepared in Milli-Q water and stored at -18°C . Calibration standards of 10 ml were prepared in acetonitrile/Milli-Q water (95/5 v/v, 1% formic acid).

LC/MS grade quality of acetonitrile (ACN) was purchased from Merck Millipore (Lichrosolv-hypergrade, Darmstadt, Germany) and the purified water was prepared using a Milli-Q Advantage, including a UV radiation source, ultrapure water system (Millipore, Billerica, USA). Formic acid (FA) (Sigma-Aldrich, Steinheim, Germany) was used to acidify mobile phases.

2.2. Optimization of sample pre-treatment

Due to the nature of HILIC, aqueous samples need to be diluted with a high content of organic solvent in order for the analytes to be retained on the SPE and analytical columns. In this study, ACN was used to dilute the samples, and FA was used to make the Za molecules positively charged to better interact with the distal charge of the ZIC-HILIC stationary phase.

The Design of Experiment (DOE) method was used initially in the optimization of the amount of ACN and FA used in sample pre-treatment to obtain the highest utility of information. A DOE method is characterized by controlled (independent) input variables, and observed and measured output variables. The input variables in this study were the proportion of water sample/ACN and FA, respectively, used in the sample pre-treatment, and the output variable was the signal area of Za measured with the online SPE/LC-MS/MS analysis system and the method described in 2.3 and 2.4, respectively. The experiments were planned based on a mixed experiment plan where variables were tested at two and three diversity levels. The experimental plan and the results are shown in the Supplementary material (Table S1).

Milli-Q and sewage treatment influent/effluent waters were included in the optimization of the sample pre-treatment. Influent and effluent waters were collected from Umeå STP and Za was not present above its LOQ (20 ng L^{-1} and 15 ng L^{-1} , respectively) in these waters. In the DOE method, constant variables were injection volume and Za concentration at 1 mL and 1000 ng L^{-1} , respectively. After the initial optimization using DOE, 5 ml injection volume was used in order to improve the LOQ.

2.3. Analytical system

In this study an autosampler from PAL HTC autosampler was used and it was equipped with cooled sample trays (CTC Analytics AG, Zwingen, Switzerland). The LC pumps (Surveyor and Accela), mass analyzer (TSQ Quantum Ultra EMR, triple stage quadrupole MS/MS) and software (Xcalibur) were made by Thermo Fisher Scientific (San Jose, CA, USA). The online SPE is based on a column switching system using a 6- and a 10-port valve described in details by Khan et al. [38].

2.4. Online SPE/LC-HESI-MS/MS method

As presented in Section 3.1, the sample dilution in the pre-treatment was decided to be 95% ACN and 1% FA. In total, the sample pre-treatment in the evaluated on-line SPE/LC-MS/MS method included filtration of 0.5 mL aqueous sample ($0.45 \mu\text{m}$ Filtropur S polysulfone, Sarstedt, Germany), dilution by 9.5 mL ACN (5/95% v/v), addition of $100 \mu\text{L}$ FA (1% v/v) and addition of Za-IS at a concentration

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