Journal of Chromatography B, 906 (2012) 58–62

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Aqueous normal phase liquid chromatography coupled with tandem time-of-flight quadrupole mass spectrometry for determination of zanamivir in human serum

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ARTICLE INFO

Article history:
Received 17 May 2012
Accepted 14 August 2012
Available online 21 August 2012

Keywords:
Aqueous normal-phase
Zanamivir
Human serum

ABSTRACT

An aqueous normal phase (ANP) liquid chromatography coupled with a hybrid quadrupole time-of-flight mass spectrometry (ANP-LC-micrOTOFQ) method was used for the determination of zanamivir in human serum. Zanamivir was extracted with methanol from protein-precipitated human serum samples and further purified with SCX solid-phase extraction cartridges. Scherzo SM-C18, Agilent Zorbax SB-Aq, Cogent Diamond Hydride, Cogent Bidentate and Luna HILIC columns were compared and optimized for the retention and separation of zanamivir and the Luna HILIC and Diamond Hydride columns exhibited the best retention of zanamivir. The former provided a shorter retention time, a sharper peak and relatively high sensitivity, whereas the latter exhibited a longer retention time and less matrix interference. The analytical range of the calibration curve was between 5 and 1000 ng/mL.

1. Introduction

Pandemic influenza is a global outbreak of the disease that spreads easily from person to person and causes serious illness. Three major influenza pandemics swept the globe in the 20th century causing millions of deaths [1]. Zanamivir was the first commercially developed neuraminidase inhibitor and it is used in the treatment and prophylaxis of influenza caused by influenza A and B viruses. It is effective in preventing, controlling, or rapidly reducing illness with fever [2] and it is currently marketed by GlaxoSmithKline under the trade name Relenza® as a powder for inhaled delivery [3].

Determination of zanamivir or related drugs with a very low octanol–water partition coefficient (log *P*) remains a challenge because reverse phase C_{18} columns are limited in their capability to retain hydrophilic compounds, resulting in ion suppression in mass spectrometry (MS). Maintaining an appropriate chromatographic retention of the analytes is highly desired in most liquid chromatography (LC)–MS assays to avoid possible ion suppression and mass spectrometric interference. Hydrophilic interaction chromatography (HILIC) with low-aqueous/high-organic mobile phase is valuable in LC–MS systems for the analysis of polar analytes [4]. HILIC-MS/MS methods have been reported for the determination of zanamivir [3,5]. However, these methods did not significantly improve the retention behavior of zanamivir. Additionally, the retention time of zanamivir is shortened on HILIC columns after repetitive injections due to addition of acids in the mobile phase. Finally, matrix ionization suppression is also problematic in performing HILIC-ESI-MS/MS.

Aqueous normal phase (ANP) chromatography represents an important new technology for the separation of endogenous metabolites in biological matrices. It enables high resolution of hydrophilic compounds which are not adequately retained and separated on conventional stationary phases in reversed-phase operation mode [6,7]. HILIC is also known as an effective way to retain hydrophilic compounds, which has gained increased attention in recent years. The mechanism of separation on HILIC is based on partition of solutes between a water-enriched layer at the surface of hydrophilic bed and the bulk of the aqueous/organic mobile phase [8]. However, HILIC analyses provide only a partial solution to the total number of analytes that must be separated [9]. Aqueous normal phase chromatography is typically used when partition is not the main retention mechanism of hydrophilic compounds, but where interactions may involve adsorption and ion and/or ligand exchange. The ANP is silica hydride-based HPLC stationary phases, which are compatible with a wide range of mobile phase compositions ranging from 100% aqueous to 100% nonpolar organic solvents [7]. Silica hydride-based columns can be used in traditional reverse

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http://dx.doi.org/10.1016/j.jchromb.2012.08.020

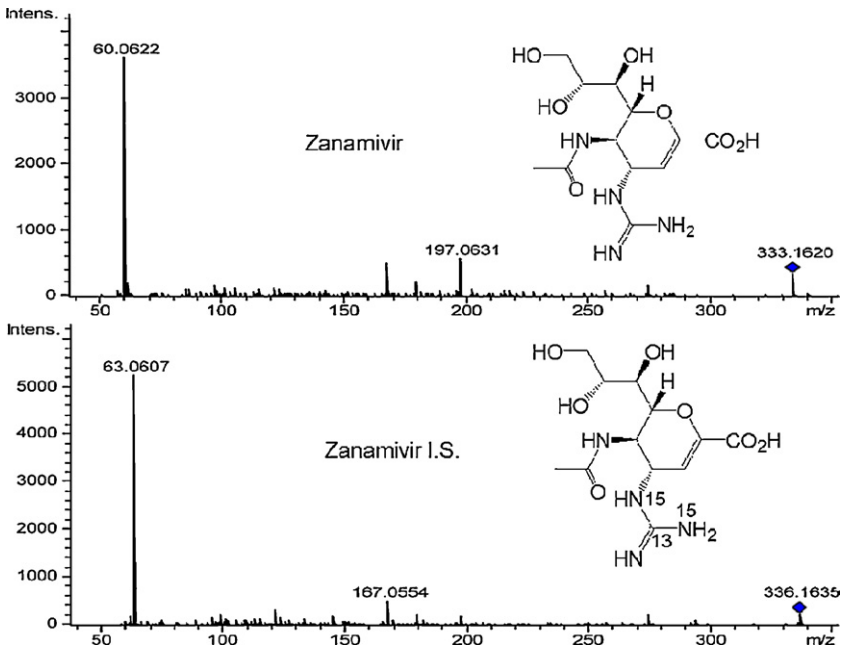

Fig. 1. MRM mass spectra of zanamivir and the isotopic labeled zanamivir internal standard (zanamivir I.S.), showing fragmentations of m/z 333 > 60 and 336 > 63, respectively.

phase (RP), ANP and organic normal phase (ONP) chromatography [10]. Silica hydride phases can make the transition from ANP to RP by addition of water to the mobile phase, whereas HILIC phases do not have such transition capability and thus can only retain polar compounds [11].

The present study focused on the retention of zanamivir on five different columns with unique properties to retain polar compounds. Influences of mobile phase constituent on the retention time of zanamivir were examined. The results demonstrated that a silica hydride column offered significantly more retention capabilities with zanamivir than the other columns.

2. Experimental

2.1. Chemicals and materials

Zanamivir (Fig. 1) was obtained from Beijing Apifocus Co. (Beijing, China) and its internal standard (I.S.), a ^{13}C, $^{15}N_2$-labeled analog was from Toronto Research Chemical Inc. (Ontario, Canada). HPLC/MS grade acetonitrile and methanol, HPLC grade water and acetic acid were purchased from Fisher Scientific (Morris Plains, NJ, USA). Ammonium acetate was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Human serum samples were collected according an approved protocol at the University of Hawaii at Manoa.

2.2. Preparation of standard solutions

The zanamivir stock solution (400 mg/L) was prepared in water. The working stock solution was prepared in acetonitrile at a concentration of 50 mg/L. The standards were stored at −20 °C until use. A set of calibration standards was prepared in mobile phase and blank matrix (human serum extracts) by appropriate dilutions of the working stock solution to yield final concentrations of zanamivir at 0.005, 0.01, 0.05, 0.2, 0.5 and 1.0 mg/L. The calibration range of zanamivir was 5–1000 ng/mL. Calibration standards and quality control samples were prepared on the day of the analysis as described above.

An internal standard of zanamivir was dissolved in deionized water to yield a stock solution at a concentration of 1 mg/mL. A working standard solution was prepared in deionized water by appropriate dilution of the stock solution to yield a final concentration of internal standard at 5 μg/mL.

2.3. Sample preparation

An aliquot of human serum (50 μL) was spiked with internal standard (5 μg/mL, 80 μL) and extracted via protein precipitation with 150 μL of methanol at room temperature for 5 min after vortex mixing. The tubes were then centrifuged at 11,950 × *g* for 5 min. The supernatant was loaded onto a Discovery DSC-SCX cartridge (50 mg, 1 mL, Supelco, Bellfonte, PA, USA), which had been primed with methanol (1 mL) and 10% aqueous acetic acid (1 mL). The cartridge was then washed with 2 × 0.5 mL of acetonitrile-10% aqueous acetic acid (7:3, v:v) followed by 2 × 0.5 mL of water. Zanamivir and zanamivir I.S. were eluted from the cartridge using 1 mL of methanol:water:triethylamine (45:45:10, v:v:v) into a clean tube. After evaporation of the solvent to dryness under a gentle stream of nitrogen, the residues were dissolved in mobile phase (200 μL) by vortexing for about 10 s. The extracts were filtered through 0.22-μm PTFE filters with syringes into autosampler vials for LC-micrOTOFQ analysis.

2.4. Columns and LC-micrOTOFQ-MS system

Scherzo SM-C18 (3 μm, 150 mm × 2.0 mm i.d.), Agilent Zorbax SB-Aq (1.8 μm, 100 mm × 2.1 mm i.d.), Cogent Diamond Hydride (4 μm, 100 Å, 150 mm × 2.1 mm i.d.), Cogent Bidentate (4 μm, 100 Å, 150 mm × 2.1 mm i.d.) and Luna HILIC columns (3 μm, 200 Å, 100 mm × 2.0 mm i.d.) were used for these studies.

Liquid chromatography was equipped with an Agilent 1200 system consisting of a capillary LC pump, a vacuum degasser, an autosampler and a thermostatted column compartment set at 28 °C (Agilent Technologies, Santa Clara, USA). The LC was interfaced with a micrOTOFQ quadrupole time-of-flight mass spectrometer from Bruker Daltonics (Bremen, Germany).

2.5. LC-micrOTOFQ-MS conditions and optimizations

Five columns were used for LC-micrOTOFQ-MS analysis, under isocratic or gradient conditions, with a mobile phase composed of

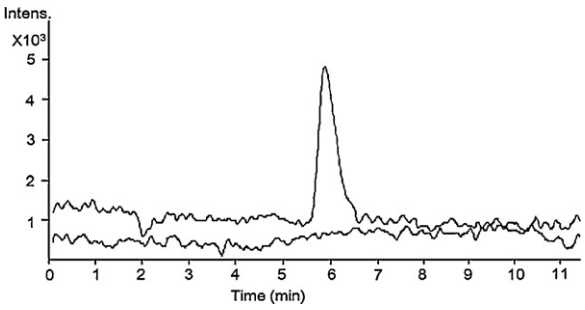

Fig. 2. MRM chromatograms (MRM 333→60) of human serum sample fortified with zanamivir at 0.05 mg/L and the blank human serum sample. The mobile phase was a mixture of acetonitrile and water (6:4, v:v), containing 0.2% acetic acid. The flow rate was 0.2 mL/min. The column was the Diamond Hydride column.

acetonitrile–water containing 0.2% acetic acid. The injection volume was 5 μL. The flow rate was 0.2 mL/min. The total run time was 15 min. The Diamond Hydride column and the conditions (acetonitrile:water = 6:4, with 0.2% acetic acid) that produced a retention time of 6 min were selected for detecting zanamivir in human serum samples.

The mass spectrometer was operated under positive electrospray ionization (ESI+) mode. The ion source parameters were as follows: voltage of 4.2 kV, nebulization with nitrogen at 2 bar, dry gas flow of 8.0 L/min and at a temperature of 195 °C.

The ion optics was optimized to the highest intensity in the mass range *m/z* 50–350 by direct infusion of ESI Tuning Mix (Agilent). The same solution and flow rate were also used for the mass calibration of the micrOTOFQ-MS, which was performed daily. The parameters of detection were optimized by direct infusion of 0.2 mg/L of zanamivir standard. During the initial phase of method development, it was shown that ESI+ mode gave a higher signal than ESI-mode. Zanamivir standard showed a protonated molecular ion $[M+H]^+$ at *m/z* 333 with high signal intensity. Strong signals of product ions fragmented from zanamivir (*m/z* 333 > 60) and zanamivir I.S. (*m/z* 336 > 63) were observed in MS/MS experiments with the multiple reaction monitoring (MRM) mode at a collision energy of 25 eV (Fig. 1). The product ions are protonated guanidine ions. Data acquisition and quantification were performed using Hystar software (Bruker).

3. Results and discussion

3.1. Method evaluation

3.1.1. Recovery, linearity, LOQ and LOD

The LC-micrOTOFQ method was evaluated for the analysis of zanamivir in human serum samples. The calibration lines were constructed by plotting the peak area ratios of zanamivir to the internal standard against the actual concentrations. The linear range was between 15 and 1000 ng/mL with a correlation coefficient of 0.99. The limit of detection and lower limit of quantification was 5 and 15 ng/mL, respectively, based on 3 and 10 times of the S/N ratio (Table 1). The average recoveries were between 72 and 75% of zanamivir at concentrations of 50 and 500 ng/mL in human serum samples. The relative standard deviations (RSD) ranged from 2% to 4% for these samples. Matrix effects were evaluated using blank human serum samples. The matrix effects were quantitatively estimated by comparing the peak area between the standard spiked in elution solution and the extracted blank matrix at the same concentration of the analyte. The results demonstrate that the low spike level (50 ng/mL) of zanamivir suffers from slight ion suppression (Table 1). Fig. 2 shows typical MRM chromatograms of the blank human serum and zanamivir fortified in human serum samples.

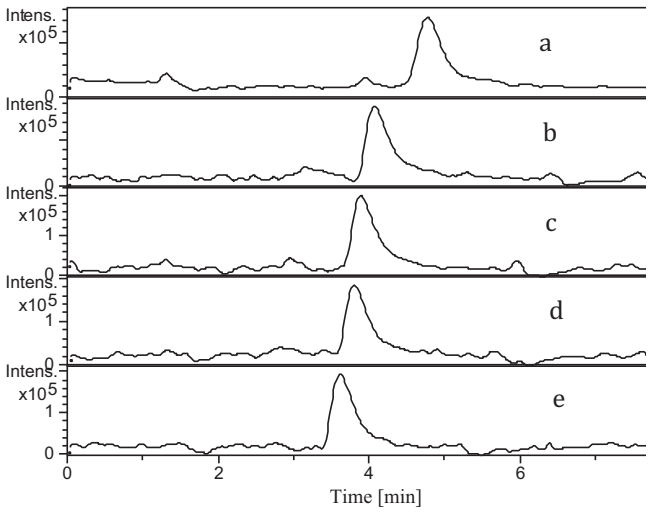

Fig. 3. LC–MS chromatograms showing retention time decrease as repetitive injections (from a to e) of zanamivir on the Luna HILIC column when acetic acid was added in the mobile phase (acetonitrile:water = 7:3, containing 0.2% acetic acid).

3.1.2. Accuracy and precision

Table 2 shows acceptable intra- and inter-day precisions and accuracies that were determined from five replicate analyses of zanamivir at the concentrations of 5, 10, 50, 200, 500 and 1000 ng/mL. The accuracy was determined by calculating percent relative errors (RE%), while the precision was determined by calculating RSD. In human serum, the intra-day and inter-day precisions ranged from 2% to 14% and 2% to 13%, respectively. The intra-day and inter-day accuracies ranged from −3.1% to 1.9% and −1.4% to 0.7%, respectively.

3.2. Comparison of different columns for zanamivir retention

Zanamivir is water soluble with a log *P* of −4.1. Zanamivir has a basic pK_a of 11.3 and an acidic pK_a of 3.8 [3]. Zanamivir showed no retention and poor peak shape on conventional C_{18} columns. HILIC separates compounds by passing a hydrophobic mobile phase across a neutral hydrophilic stationary phase, causing solutes to elute in order of increasing hydrophilicity – the opposite of RP chromatography [12]. HILIC is a supplement to RP-HPLC. Hydrophilic compounds such as zanamivir have been often analyzed with HILIC-MS/MS. HILIC columns, however, are limited in their compatibility to acid additives in mobile phase, whereas ion suppression is a significant challenge to the MS system. In the present work, the ESI+ mode of micrOTOFQ with acetic acid as an additive gave much higher signal of zanamivir than the negative mode. Acetic acid employed in the mobile phase caused serious loss of retention on the Luna HILIC column over repetitive injections (Fig. 3). It is noteworthy that a Waters Atlantis HILIC column (3 μm, 200 A, 100 mm × 2.0 mm i.d.) was preliminarily tested for the retention of zanamivir. However, the result was not as good as the Luna HILIC. Therefore, the Luna HILIC column was used in the present study. The Diamond Hydride stationary phase is essentially a silica hydride surface with less than 2% amount of bonded carbon on the surface. The Diamond Hydride column can be used for the separation of metabolites such as amino acids, carbohydrates and small organic acids in ANP mode [7]. Zanamivir contains amide, carboxyl, guanidino and hydroxyl groups and shares similar polarities with those metabolites that can be separated on the Diamond Hydride column. Thus, it is a good choice for determination of zanamivir.

We surveyed five different columns with unique properties to retain polar compounds. (a) The Scherzo SM-C18 column contains cation and anion ligands that allow for reverse phase separation in

Table 1
Average recoveries of zanamivir in human serum samples ($n = 5$), matrix effects, lower limit of quantification (LLOQ) and limit of detection (LOD).

Conc. (ng/mL)	Average recovery (% ± SD)	Matrix effect (%)	LLOQ (ng/mL)	LOD (ng/mL)
50	72 ± 1.6	84	15	5
500	75 ± 3.5	94		

Table 2
Inter- and intra-day validation statistics for zanamivir in human sera ($n = 5$).

Concentrations of zanamivir (ng/mL)	Intraday		Interday	
	Accuracy (RE%)	Precision (RSD%)	Accuracy (RE%)	Precision (RSD%)
5	−3.1	14	0.7	13
10	1.9	4	0.5	5
50	0.3	8	1.0	6
200	−1.5	5	−1.4	4
500	1.4	4	0.6	4
1000	−0.9	2	−0.4	2

addition to anion and cation exchange. (b) The Zorbax SB-Aq is an alkyl reversed-phase bonded phase designed to retain hydrophilic compounds when using aqueous mobile phases, including 100% water. (c) The Luna HILIC column retains a water-enriched layer on the surface of silica, which facilitates the transfer of polar compounds onto the stationary phase for increased retention. (d) The Cogent Bidentate column is bonded directly to the Type-C silica surface with two separate points of attachment, which can be operated in RP, NP and ANP. (e) The Diamond Hydride column is characterized to retain very polar compounds in ANP. All these analyses are performed with the same isocratic program and mobile phase (acetonitrile:water = 7:3, v/v, with 0.2% acetic acid). Under such a condition, the retention time of zanamivir was approximately 11 min on the Diamond Hydride column compared to 5 min on the HILIC and less than 3 min on the other three columns. Fig. 4 includes a comparison of the chromatograms of zanamivir on the five columns. Both Diamond Hydride and HILIC columns improved the retention of zanamivir, however, HILIC had serious matrix effects when matrices existed. The capacity factor (k') of zanamivir on these columns was calculated according to the equation of $k' = (V_R - V_M)/V_M$. The retention volume (V_R) is equal to retention time (t_R) multiplied by the volumetric flow rate (F, mL/min) of the mobile phase through the column, $V_R = t_R F$. The total volume of the

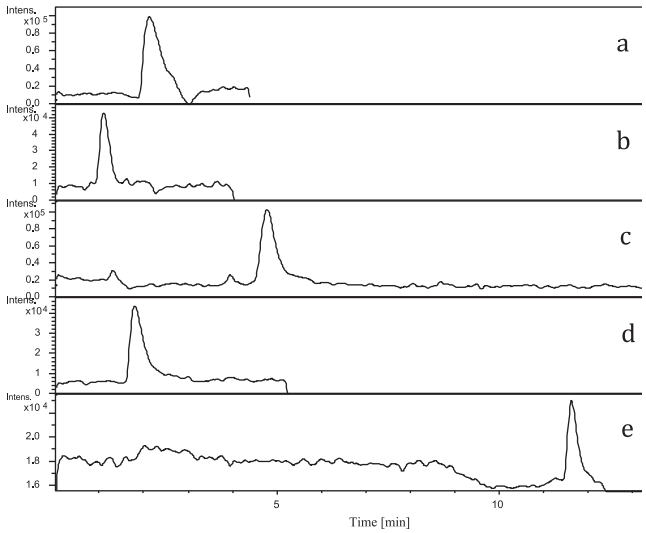

Fig. 4. Comparison of zanamivir retention times on five columns under the same LC–MS conditions (acetonitrile:water = 7:3, containing 0.2% acetic acid). (a) Scherzo SM-C18 column; (b) Agilent Zorbax SB-Aq column; (c) Luna HILIC column; (d) Cogent Bidentate column; (e) Cogent Diamond Hydride column.

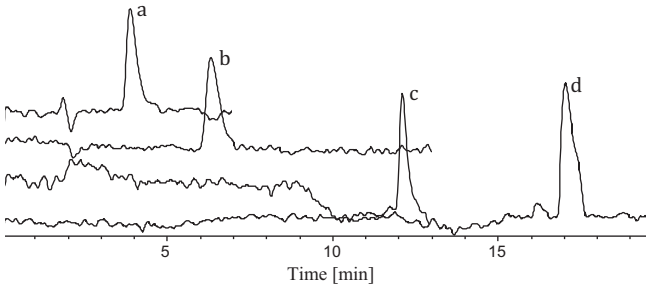

Fig. 5. Effects of percentage of acetonitrile in the mobile phase on zanamivir retention time on the Diamond Hydride column. Isocratic elution with (a) 50%, (b) 60% and (c) 70% aqueous acetonitrile (all contained 0.2% acetic acid); and (d) gradient elution – A: 0.2% acetic acid water, B: 0.2% acetic acid acetonitrile. 100% B for 4 min then changed to 100% A in 15 min, held 100% A for 3 min. The flow rate for both isocratic and gradient elution was 0.2 mL/min.

solvent within the column $V_M = (\pi d^2 L V_P)/4$. D is the diameter of column in mm. L is the length of the column in mm. An average pore volume (V_P) is assumed to be 0.70. The capacity factors are 0.35, 0.004, 3.08, 0.08 and 5.27 for Scherzo SM-C18, Zorbax SB-Aq, Luna HILIC, Cogent Bidentate and Diamond Hydride columns, respectively. The results show that the Diamond Hydride column retained zanamivir the best, followed by the HILIC column. However, the intensity of zanamivir on the Diamond Hydride column was lower than that on the HILIC column.

Varying ratios of organic solvents in mobile phase were also surveyed with the Diamond Hydride column (Fig. 5). The retention increased as the percentage of acetonitrile in the mobile phase increased and gradient elution resulted in longer retention. The results verified that the Diamond Hydride has sufficient capability to retain zanamivir. The ANP-LC-micrOTOFQ with the Diamond Hydride column was used for the analysis of zanamivir fortified in human serum samples. The method offers an alternative protocol for the analysis of zanamivir as well as other extremely polar drugs.

4. Conclusion

Since zanamivir is an effective drug against influenza A and B virus studies are underway to improve its bioavailability. The present study is the initial demonstration of the effective utilization of ANP-LC-micrOTOFQ to analyze zanamivir in human serum samples. Specifically, we also reveal that the retention capability of the Diamond Hydride column is particularly effective for zanamivir to avoid matrix effects. The method will likely have broad application in the analysis of zanamivir in biological samples including those from pharmacokinetic, toxicokinetic, and efficacy studies.

Acknowledgements

The authors would like to acknowledge Dr. Sandra Chang in John A. Burns School of Medicine (JABSOM), University of Hawaii at Manoa for the human serum samples. JG received a scholarship from the Chinese Scholarship Council. EHH is supported in part by NIH grant P20 RR16467/P20 GM103466. The analyses were done in the JABSOM Proteomics Core facility funded by NIH RCMI 2G12RR003061-26.

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