Increasing the Sensitivity for Oseltamivir using ZIC®-HILIC Chromatography

A new analytical protocol for Oseltamivir (Tamiflu®), a drug used to counter influenza epidemics and its carboxylate derivative in plasma, urine and saliva that is approximately 500 times more sensitive than existing methods has been developed and validated. This new, validated protocol couples a ZIC®-HILIC column with MS-MS detection to allow for the use of smaller sample volumes and provides higher throughput than previously used methods.

Introduction

Oseltamivir (OP) (Tamiflu®) (I) is a leading anti-viral agent that is employed to counter a serious epidemic or pandemic of influenza. It is an ester prodrug that is rapidly hydrolyzed *in vivo* into its active metabolite, oseltamivir carboxylate (OC) (II), a potent and selective inhibitor of influenza virus neuroaminidase. Several years ago, an LC-MS method for OP and OC in plasma and urine using a CN column that can monitor the drug was reported; however, the low level of organic solvent (50 % MeOH) inhibits optimum electrospray ionization and leads to a relatively poor detection limit.

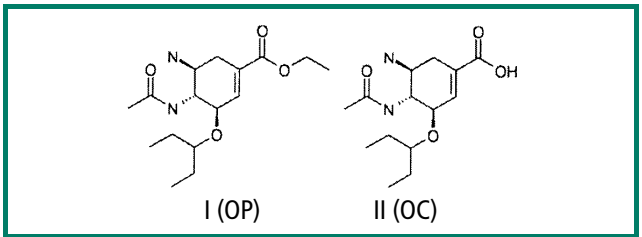

Figure 1: Structure of Oseltamivir (OP) (Tamiflu®) (I) and Oseltamivir Carboxylate (OC) (II).

A new LC-MS method for OP and OC has recently been reported by N. Lindegårdh and co-workers[1], which uses a ZIC®-HILIC column in gradient mode and a mobile phase with a considerably higher organic composition, thereby providing an assay with lower limit of detection for OP of 1 ng/mL, 1 ng/mL and 5 ng/mL in plasma, saliva and urine and 10 ng/mL, 10 ng/mL and 30 ng/mL of OC in plasma, saliva and urine. This method is approximately 500 times more sensitive than the existing method. It has been validated, matrix and recovery effects have been investigated and it was shown that potential co-administered drugs (zanamir and peramvir) do not interfere.

Experimental

Sample Preparation: 50 µL of internal standard solution (D-OP and D-OC) was added to 50 µL of sample in a 96 well plate and NH_4OAc (500 µL) was added. After mixing and centrifugation, the sample was loaded on a conditioned MPC-SD standard well SPE plate (the sample volume was added for plasma and saliva, 200 µL was used for urine). MeOH and NH_4OAc were used to condition the plate.

The plate was washed with water, methanol and MeOH/water and the column tips were then dried. A 96 well collection plate was inserted in the vacuum manifold, methanol/NH_4OAc, 50 mM (90:10) added to each well and the collection plate was covered with a seal mat.

Separation: The samples were separated with an Agilent 1200 system with a binary LC pump, vacuum degasser, temperature controlled micro-well plate autosampler and a thermostated column compartment. A ZIC®-HILIC column (5 µm, 50 x 2.1 mm; p/n 2712-052) column (Merck SeQuant, Umeå, Sweden) protected with a ZIC®-HILIC guard column (14 mm x 1.0 mm; p/n 2712-711) was employed using a 10 mM NH_4OAc in water + 1% HOAc/acetonitrile gradient (85% A - 60% A in 1.90 min) at 500 µL/min. In a separate experiment, OC and OP were separated with a ZIC®-HILIC column (3.5 µm, 20 x 2.1 mm; p/n 2701-022) column with acetonitrile/10 mM NH_4OAc in water + 1% HOAc (85:15).

Detection: Detection was performed with an API5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City CA) operated in the positive ion mode. Quantification was performed using selective reaction monitoring for the transitions m/z 313-225 and 316-228 for OP and D-OP and 285-197 and 288-200 for OC and D-OC.

Reference

1: This application note is condensed from the scientific paper *"Development and validation of a liquid chromatographic-tandem mass spectrometric method for determination of oseltamivir and its metabolite oseltamivir carboxylate in plasma, saliva and urine"* by N. Lindegårdh, et al., Journal of Chromatography B, 859 (2007) 74-83. Figure 2-3 are reprinted with permission of Elsevier Publishing.

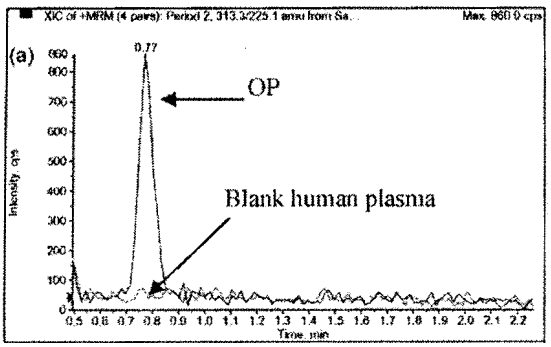

Figure 2: Chromatogram of OP in Plasma with a ZIC®-HILIC Column (5 µm, 50 mm x 2.1 mm) (reprinted with permission).

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Results

The chromatograms of OP in plasma (Figure 2) and OC in plasma (Figure 3) were collected at the lower limit of quantitation (LLOQ) [four times the limit of detection (LOD)]. Similar results were obtained for the drug and its metabolite in urine and plasma. The LOD and LLOQ for the compounds of interest in the three matrices are shown in Table 1.

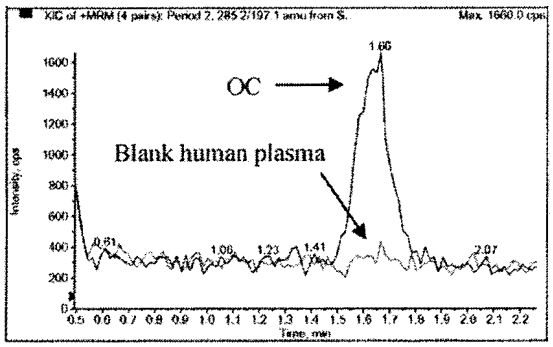

Figure 3: Chromatogram of OC in Plasma with a ZIC®-HILIC Column (5 µm, 50 mm x 2.1 mm). (reprinted with permission).

Table 1: LOD, LLOQ and Calibration Range of OP and OC in Plasma, Saliva and Urine

Matrix & Compound	LLOQ ng/mL	LOD ng/mL	Calibration Range ng/mL
Plasma			
OP	1	0.25	1-600
OC	10	2.5	10-10000
Saliva			
OP	1	0.25	1-300
OC	10	2.5	10-10000
Urine			
OP	1	1.25	5-1500
OC	10	7.5	30-30000

It is clear that the ZIC®-HILIC column provides a rapid separation with superb resolution in a short period of time. In addition, the separation was performed using a 20 x 2.1 mm column packed with 3.5 µm particles, which provided the separation shown in Figure 4.

Validation

Precision, accuracy linearity and calibration data were obtained from data collected over a four day period. Precision data for OP (OC) were collected for samples ranging from 15 ng/mL to 750 ng/mL; the CV for inter-assay measurements was 2.7% (2.2%) and the CV for intra-assay measurements was 2.0% (1.6%).

The analysis of blank samples indicated that the blank did not interfere with any of the peaks (see Figure 1). Potential neuraminidase inhibitors that could be combined with OP during clinical trials (zanamivir (RT=2.8 min) and permavir (RT=1.5 min)) do not interfere in the assay. Recovery studies were performed; as examples, the recovery of OC in urine was >90% and 80% for OP.

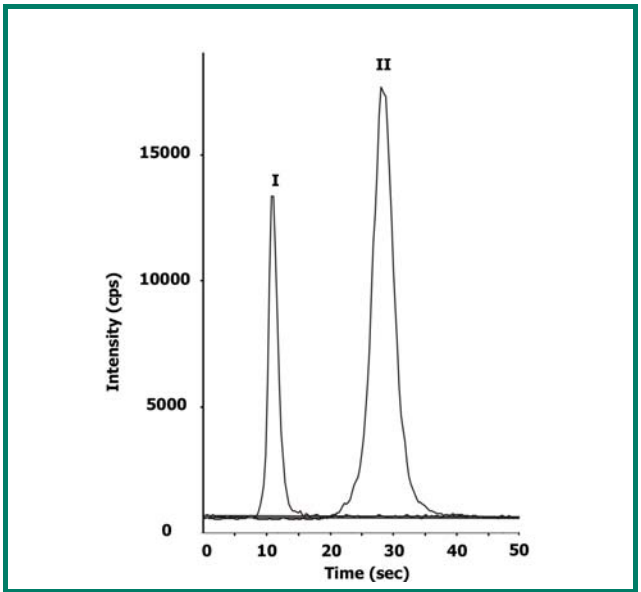

Figure 4: Separation of Oseltamavir (I) and OC (II) with a ZIC®-HILIC Column (3.5 µm, 20 mm x 2.1 mm).

Conclusions

The use of a ZIC©-HILIC column provides a separation that is approximately 500 times more sensitive than the existing assay for OP and can be used for detection of the drug and OC in plasma, saliva and urine (the existing assay was not applicable to saliva). This new assay requires a smaller sample volume and is more rapid than the existing assay.

About ZIC®-HILIC Chromatography

The ZIC®-HILIC stationary phase is based on the covalently bonded permanent zwitterionic sulfobetaine group indicated in Figure 5. It is available with a silica support in 3.5, 5 and 10 µm particle sizes in various column dimensions from capillary to semi-preparative (75 µm up to 20 mm ID). In addition, it is available with a polymeric support in 5 µm particles (ZIC®-*p*HILIC). Merck SeQuant also publishes the tutorial booklet A Practical Guide to HILIC, which is available free of charge.

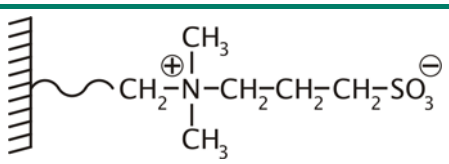

Figure 5: The Bonded Zwitterionic Sulfobetaine Group of ZIC®-HILIC.

About Merck SeQuant

Merck SeQuant is a Swedish company that develops a broad range of innovative products for separation and purification of complex samples via chromatography. The company was founded in 1987 by research workers from Umeå University. Products include ZIC®-HILIC columns for HPLC, Membrane Suppressors and Suppressor Regeneration Systems for Ion Chromatography, Proteomic Calibration Kits and Post Column Reaction Delay and Mixing Coils that are marketed and supported around the world by a series of distributors.

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