

Quantitation of Enantiomers Using Normal Phase Capillary LC/MS/MS with Post Column Add-On Flow and Electrospray Ionization

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Introduction

There is an increasing need for selective and sensitive LC/MS methods for quantifying stereoisomers in biological samples and in pharmaceutical materials. It has been shown that normal phase chiral separation using a microflow HPLC/UV system offers considerable benefits such as speed of analysis, solvent saving and lower column back pressure that allow for more rapid separation of enantiomers. In considering LC/MS applications, however, the compatibility between the chromatographic method and the mass spectrometry ionization conditions is of critical importance. Despite the effectiveness of immobilized polysaccharide coated columns for chiral separations, this approach has not been fully exploited for LC/MS applications due in part to the incompatibility between the solvents used in normal phase LC and electrospray ionization. Post-column addition of make-up solvent can be used to circumvent the incompatibility between normal phase HPLC and electrospray ionization. This approach is particularly well-suited for microflow HPLC because of its inherent low-flow rates. We show here that good LC/MS/MS sensitivity and quantitation can be achieved with post column addition of make-up solvent without compromising the chromatography performance or needing to divert the bulk flow. The separation of several pharmacologically significant enantiomers using polysaccharide chiral columns under normal phase conditions and with ESI/MS/MS detection will be

Experimental

Microflow HPLC Systems: The Eksigent ExpressLC-800™ System, an 8-channel parallel HPLC, was used for the chiral method screening and development. Eksigent ExpressLC-100™ System, a single-channel microflow HPLC, was used for the LC/MS/MS study. A zero-dead volume micro-tee union and a Harvard syringe pump were used for the post column add-on flow. The add-on solvent was water/0.1% formic acid with flow rates in the 1 – 20 μ L range. All post column transfer lines were of 30 or 40 μ m i.d. and kept short (total less than 50 cm) to minimize dispersion and back-pressure.

Mass Spectrometry: An API-4000 triple quadrupole from Applied Biosystems and a TSQ Quantum Access triple quadrupole from Thermo were employed for the LC/MS/MS studies. For both mass spectrometers, a 30 μ m i.d. fused-silica transfer line was inserted into the stainless steel electrospray needle to just past the emitter opening.

Separation Conditions: Polysaccharide coated type AD-H, AS-H, OD-H and OJ-H (150 x 0.3 mm) columns from Chiral Technologies were employed for the chiral separations. The mobile phases were A: Isopropanol or ethanol and B: hexane or heptane, with 0.1% TEA. The flow rate was 8 μ L/min.

Chemicals: Warfarin, Ketoprofen, Phenoprofen and 5-methyl-5-phenylhydantoin were obtained from Sigma-Aldrich. A proprietary compound of undisclosed structure was also used to demonstrate the applicability of chiral method screening and subsequent quantitation.

Results

Chiral Method Screening and Optimization

Chiral method screenings were carried out using the Eksigent ExpressLC-800™ parallel HPLC system with 2 channels each of the 4 polysaccharide columns (AD-H, AS-H, OD-H and OJ-H). Solvent A was EtOH (channels 1-4) and IPA (channels 5-8), and solvent B was hexane or heptane. A generic gradient of 5% to 60% A in 45 minutes was used for the initial screening with all 8 channels running in parallel. After the appropriate column and solvent were selected, further optimizations were carried out on a single channel instrument. For further information on chiral method screening see Sajonz et. al. *Chirality*, 2006 Nov, 18 (10) 803-13. An example of method optimization for the proprietary compound used in LC/MS/MS quantitation study is shown below.

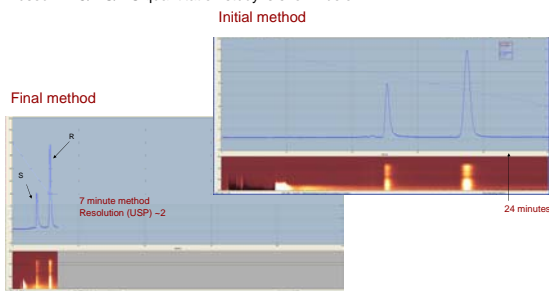


Figure 1. The development of a 7-min method using AD-H column and IPA in mobile phase A for the proprietary compound used in LC/MS/MS quantitation study. Optimizations were achieved by changing the mobile phase B additive concentration and by adjusting the gradient profile.

Chiral LC/MS Analysis of Racemic Mixtures

Normal phase chiral HPLC methods for the separation of racemic warfarin, ketoprofen, phenoprofen and 5-methyl-5-phenylhydantoin were developed using the Eksigent ExpressLC-800 system. The final optimized method for each compound was then used for the LC/MS experiments with the Eksigent ExpressLC-100 system. The ratio of the add-on flow to the HPLC outflow varied from compound to compound, as specified in the figure legend. The effect of the add-on flow on the mass spec signal response is discussed in the next section. The MRM method and mass spec conditions were optimized for each compound in 80/20 water/acetonitrile/0.1% formic acid at an add-on flow rate of 5 μ L/min with the 30 μ m i.d. fused silica transfer line inserted to just past the opening of the stainless steel spray needle. Results shown below were obtained with AD-H column, IPA/heptane/0.1% HOAC, 50 nL injection of 100 μ g/ml compound in methanol.

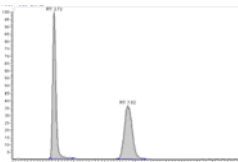


Figure 2. Warfarin, ESI(-) 307>161, 15/85 IPA/HEP, 8 μ L/min flow rate, 15 μ L/min add-on

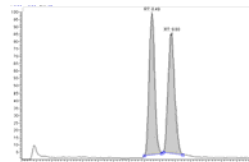


Figure 3. Ketoprofen, ESI(-) 253>209, 5/95 IPA/HEP, 8 μ L/min flow rate, 9 μ L/min add-on

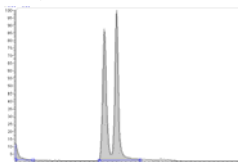


Figure 4. Phenoprofen, ESI(-) 241>197.3, 5/95 IPA/HEP, 8 μ L/min flow rate, 7 μ L/min add-on

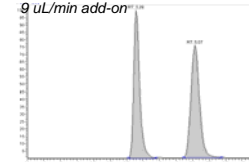


Figure 5. 5-methyl-5-phenylhydantoin, ESI(+) 191>120, 5/95 IPA/HEP, 8 μ L/min flow rate, 9 μ L/min add-on

Effect of Add-On Flow on Peak Width and Peak Height

The peak shape and width vary widely depend on the compound, the column chemistry and the separation condition. However, for the compounds investigated here, the peak width for each compound (in the range of 10 – 30 sec FWHM) was largely unaffected by the add-on flow from 1 to 15 μ L/min. This minimal dispersion is attributable to the use of ZDV union and small i.d. capillary transfer lines. As shown below by comparing the UV and MS chromatograms obtained in series, there is little difference between the normalized UV signal (at 8 μ L/min) and MS signal (8 μ L/min HPLC + 8 μ L/min add-on). Note that the Eksigent nano UV flow cell has a volume of only 45 nL.

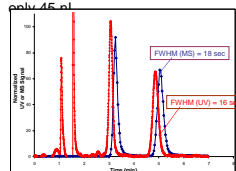


Figure 6. UV at 230 nm (red trace) and MRM at 253>209 (blue trace) obtained in series for 5-methyl-5-phenylhydantoin. Note the ~0.2 min delay of MS signal.

At low add-on flow rates, the MS response (peak height) increases with increasing add-on flow as the ESI becomes more efficient. At high add-on flow rates, the MS signal begins to decrease probably due to bulk dilution. This is also compound and separation condition dependent.

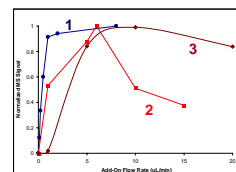


Figure 7. The effect of add-on flow on MS response (normalized) for (1) 5-methyl-5-phenylhydantoin, (2) phenoprofen and (3) a proprietary compound. The HPLC was at a constant flow rate of 8 μ L/min. In general, a ratio of 1:1 HPLC flow to add-on flow would give an optimal result.

Quantitation

The optimized 7-min HPLC method with AD-H column and normal phase separation condition (as shown in Figure 1) was used for LC/MS/MS quantitation of the proprietary compound. A representative MRM chromatogram for an asymmetric -R/-S mixture is shown below on the left. The calibration curve for the late eluting enantiomer from 1 ng/ml to 1 μ g/ml is shown below on the right. The lower limit of quantitation was 4 ng/ml with S/N ~ 10.

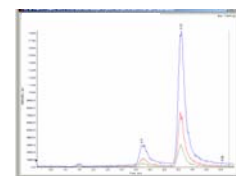


Figure 8. A typical MRM chromatogram of -R and -S mixture. Shown here are 3 MRMs taken with a dwell time of 350 msec. For subsequent experiments, a single MRM transition is used with the dwell time increased to 3 sec.

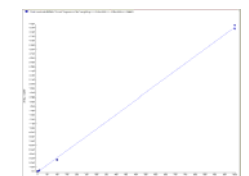


Figure 9. Calibration curve for the late eluting enantiomer, from 1 to 1000 ppb, at 2 points for each concentration. LLOQ ~ 4 ppb with S/N ~ 10. The %CV for 5 runs at LLOQ is ~ 5%.

Conclusion

We demonstrated that the coupling of normal phase microflow HPLC to conventional electrospray ionization mass spectrometry with post-column add-on flow can indeed provide an efficient and sensitive (at 1ng/ml level) LC/MS/MS method for the analysis of chiral drug molecules. This approach allows the use of chromatographic mobile phases that yield poor ionization or other undesirable effects for MS detection. Microflow HPLC instrument with its inherent low flow rates and rapid gradient mixing is particularly suited for this type of application. This approach significantly increases the scope of normal phase chromatography applications and provides additional analytical tools for LC/MS analysis of chiral molecules in biological samples.