Trace Enrichment of *In Vitro* Drug Metabolites Using Turbulent Flow Chromatography

Introduction

A crucial step in drug development is to predict the metabolic fate of a new drug candidate in animals and humans. Typical *in vitro* metabolism studies involve incubating the drug with sub-cellular fractions, such as rat-liver microsomes (RLM). Each metabolite generated by the microsomes must be adequately isolated, detected, and identified, most-often using liquid chromatography (LC) with mass spectrometry (MS). In order to characterize trace amounts of metabolites that are difficult to detect, milliliter volumes of microsomal preparations are concentrated down to microliter injection volumes by evaporation, which is extremely time consuming (4 to 5 hours). This study demonstrates how turbulent flow chromatography (TurboFlow®) was successfully used to automate trace enrichment and separation of loxapine metabolites from an RLM preparation, while reducing the total analysis time down to 50 minutes.

System Information

Instrumentation:	Cohesive Aria TX-2 system
Detector:	Triple Quadrupole Mass Spectrometer
Columns:	TurboFlow Column: Cyclone® 0.5 x 50 mm
	Analytical Column: Epic Polar 4.6 x 50 mm

Experimental Design

An initial study determined the loading capacity of a 0.5 x 50 mm Cyclone column at a turbulent flow rate of 1.5 mL/min. Results were compared to those from experiments using a 1.0 x 50 mm Cyclone column, which required a flow rate to 5.0 mL/min to achieve turbulent flow. When compared to the 1.0 mm i.d. version, the 0.5 mm i.d. column provided adequate capacity for 200 µL injections of aqueous samples while reducing solvent consumption by a factor of four.

Using the Aria operating system, an initial method was developed to permit injecting 200 µL of an aqueous sample several times to enrich trace components on an HPLC column. The method used transferred analytes to an HPLC column via isocratic focusing, but did not initiate a gradient elution step for the final separation. The following steps summarize this "Load, Transfer, Enrich" approach:

1. Loaded 200 µL of an aqueous sample, such as loxapine (incubated in a RLM preparation) into a 0.5 x 50 mm Cyclone column at 1.5 mL/min to retain analytes and rinse away unwanted sample matrix. During this step, the HPLC column was equilibrated with 0.1% formic acid in water at 0.1 mL/min.
2. Eluted analytes from the Cyclone column with 30% acetonitrile in water — contained in a 200 µL transfer loop — at 0.2 mL/min, while merging this stream with a stream of 0.1% formic acid in water at 0.8 mL/min. This transfer step diluted the acetonitrile content of the analyte fraction to permit isocratic focusing at the head of the HPLC column.
3. While back flushing the TurboFlow column, the transfer loop was filled with 30% acetonitrile in water — in preparation for the next injection — and the HPLC column was equilibrated with 0.1% formic acid in water.
4. Steps 1-3 were repeated three more times to enrich analytes from 800 µL of sample at the head of the HPLC column.

A final 200 uL sample injection was made using a method, which loaded the sample under turbulent flow conditions, transferred, and enriched the analyte fraction into the HPLC column, and finally, eluted and separated the enriched analytes (representing all five injections) with a shallow gradient.

Results and Discussion

Compared to a single 50 µL injection of the loxapine-RLM incubate, the TurboFlow trace enrichment technique was used to process 1.0 mL of the sample, which resulted in a 20-fold increase in sensitivity (Figure 1) while maintaining good chromatographic peak shape. Peak identification by extracted ion chromatograms (IEC) confirmed the elution order of loxapine and its metabolites: 1.Hydroxy Loxapine; 2. Hydroxy Loxapine-N-oxide; 3. N-desmethyl Loxapine; 4. Loxapine; 5. Loxapine N-oxide. The trace enrichment technique clearly revealed additional sample components that could be characterized by MS/MS techniques.

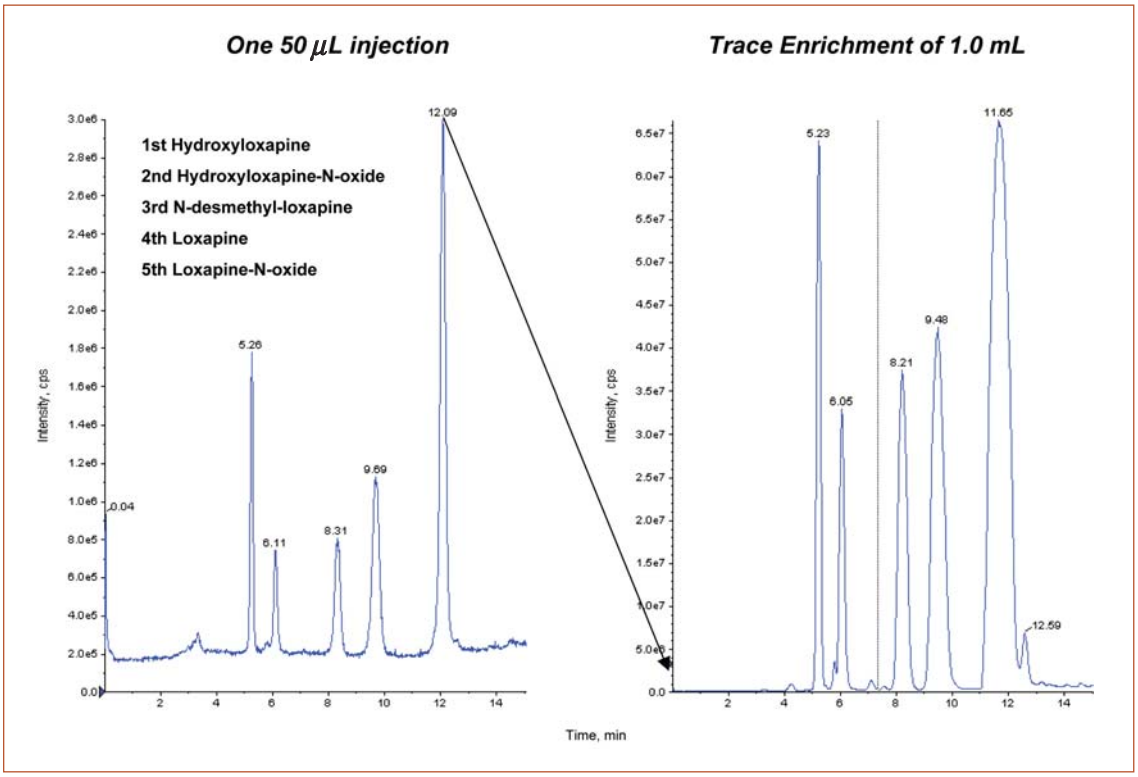

Figure 1. TurboFlow trace enrichment of loxapine-RLM incubate.

The TurboFlow column permitted an orthogonal two-dimensional separation that dramatically improved selectivity and sample capacity while minimizing sample loss due to adsorption processes frequently associated with offline sample preparation. Additionally, this approach virtually eliminated the problem of concentrating unwanted sample components, another major issue with offline techniques. The TurboFlow process initially excluded larger sample components while capturing the analytes of interest. This, in itself, dramatically improved both the column capacity for drug metabolites as well as the signal/noise (sensitivity). The resulting chromatogram shown in Figure 1 demonstrates that the peak shapes are maintained without increasing baseline noise due to concentrating matrix interferences along with the analytes of interest, resulting in a 20-fold increase in sensitivity.

Conclusion

Trace enrichment of metabolites from aqueous solutions was successfully accomplished by using turbulent flow chromatography. Multiple injections, using an Aria method to extract, transfer, and focus, enabled analyte enrichment from 1 mL of an aqueous mirosomal preparation in the analytical column, prior to separation. By using TurboFlow, enough separation of the substrate drug and its metabolites was achieved to permit sufficient detection and structure elucidation, while also bringing total analysis time down to 50 minutes.

References

1. Oberhauser, Charles J., et al, Turbulent Flow LC for LC-MS and LC-MS-MS Bioanalysis, *LC-GC 2000*; 18:716-724.
2. Herman, Joseph L., Generic Method for On-Line Extraction of Drug Substances in the Presence of Biological Matrices using Turbulent Flow Chromatography, *Rapid Communications in Mass Spectrometry 2002*; 16:421-426.

COHESIVE® TECHNOLOGIES

Cohesive Technologies Inc.
101 Constitution Boulevard
Franklin, MA 02038
Tel 508.520.5500
Toll-free 800.246.4550
Fax 508.528.8699
www.cohesivetech.com

Cohesive Technologies Ltd.
17 Cochran Close
Crownhill
Milton Keynes MK8 0AJ
United Kingdom
Tel +44 (0) 1908 267355
Fax +44 (0) 1908 267366

Application Note 11.0-0505