ThP 560

High-Sensitivity Analysis of Drugs in Ultra-Small Volumes Plasma Samples Using Micro-Flow LC-MS/MS

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1. Overview

We report an example of the implementation of high sensitivity microflow LC–MS/MS method allowing an effective application of micro-sampling techniques for the accurate quantification of antiarrhythmic drug Verapamil and its metabolite Nor-Verapamil.

2. Introduction

Drugs and other xenobiotics entering the body are generally subject to metabolism that facilitates their detoxification and elimination. The ability to predict the metabolic fate of compounds before the first doses are given to humans is highly desirable for reasons of both efficacy and safety [1].

However, the size of the animal models usually restricts the sample volume that can be safely taken during a pharmacokinetic time study for the drug and its metabolites. This quickly results in the need for many animals and a significant amount of drug material. Discovery-stage PK studies can be cost prohibitive, especially when using rare and expensive mouse strains.

Therefore, an analytical system capable of producing the maximum data from a small sample volume is highly desirable to maximize scientific information and reduce animal usage.

Reduction of animal usage in discovery-stage PK studies it is possible by using micro-sampling-based approaches (serial sampling for single mouse), but an increase in sensitivity it is often required when such small amount of sample are used.

The implementation of microflow LC–MS methods could result in sensitivity gain allowing an effective application of micro-sampling techniques.

3. Methods and Materials

3-1. Reagents

Analytical standards of Verapamil, Nor-Verapamil, and deuterated Verapamil (D6) were purchased from Wako Chemicals. Individual stock solutions at 100 mg/mL were prepared in Methanol and further diluted in blank plasma to make calibration standards (7 levels) and QC (2 levels). The calibration range was from 0.5 to 185 µg/L.

All other reagents were of analytical grade from Sigma-Aldrich. Solvents used were of LC-MS grade from Wako chemicals.

3-2. Sample Preparation

Spiked plasma samples (2 µL) were diluted 1:30 with Precipitant solution (Acetonitrile + Formic Acid 0.1%) containing labelled internal standards. After incubation at room temperature for 20 min samples were centrifugated and supernatant was transferred into vial, and 5 µL were injected on the trapping column (Shim-pack MCT LC8 5µm) then back-flushed to analytical column (Shim-pack PLONAS Biphenyl 2.7µm 100x0.2mm, Shimadzu Corp.) with a gradient of water and acetonitrile containing 0.1% formic acid. Verapamil and Nor-Verapamil were quantified using isotope dilution analysis in MRM mode.

3-3. Analytical Conditions

A Mikros LC system (Shimadzu Corp.) was used with a trap-and-elute configuration (Figure 1).

Figure 1 Instrument configuration

Table 1 Analytical conditions

System	: Nexera Mikros
Analytical column	: Shim-pack PLONAS Byphenyl (2.7µm , 0.2x100 mm)
Trap Column	: Shim-pack MCT LC8 (5 µm, 0.3x5 mm)
Temperature	: 40°C
Mobile Phases	: Load: Water:Acetonitrile 98:2 + 0.1% formic acid A: Water +0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow Rate	: 4 µL/min
Injection Volume	: 5 µL
Loading time	: 1 min (250 ul/min)
Gradient	: 26%B 1 min, 26% B -> 95% B 3 min, 95% B 1 min
Total Run Time	: 11 min

3-4. Detection Conditions

Detection was performed using high-sensitivity triple quadrupole LCMS-8060 (Shimadzu Corp.)

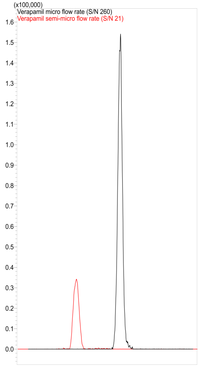
Table 2 Detection conditions

System	: LCMS-8060		
Ionization	: Micro ESI		
Probe Voltage	: +2.6 kV (positive ionization)		
Temperature	: Interface: no heating Desolvation Line: 250°C Heater Block: 400°C		
Gas Flow	: Nebulizing Gas: 1 L/min Heating Gas: -- Drying Gas: --		
Dwell Time / Pause time	: 10 ms / 1 ms		
MRM	: Compound	MRM Quant	MRM Qual
	Verapamil	455.0 > 150.25	455.0 > 303.3 (165.2)
	Nor-Verapamil	440.95 > 165	440.95 > 150
	Verapamil D6	461.3 > 309.3	461.3 > 165.25 (150.25)

4. Results

4-1. Evaluation of signal intensity using micro-flow

In order to assess the effectiveness of micro-flow rate analysis in increasing the signal response for the molecules of interests, Verapamil and its metabolite Nor-Verapamil were spiked in plasma sample at lowest concentration level (0.5 ug/L). After sample preparation, 5 ul of supernatant were firstly injected in the MIKROS system.



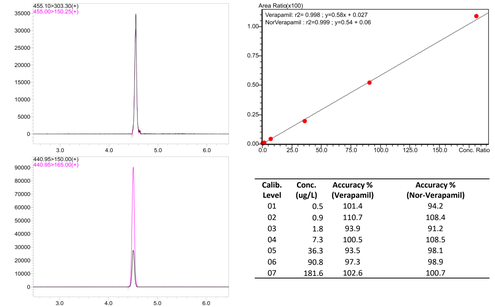
Subsequently the plug and play micro ESI ion source was substituted with ESI source and the Mikros Pump was used at semi micro flow rates for repeating the same analysis in semi-micro conditions (table 3, conditions comparison). Increase of signal intensity when using micro-flow rates system was >4.5 fold (>10 fold in S/N) for Verapamil and >3.5 fold for NorVerapamil (Figure 2).

Parameter	Micro LC-MS/MS method	Semi-Micro LC-MS/MS Method
Injection volume (ul)	4	4
Flow rate (ul/min)	4	441
Analytical column	0.2x100 mm 2.7 um Biphenyl	2.1x100 mm 2.7 um Biphenyl
Linear velocity (cm/sec)	4.145	4.145
Sample concentration (ng/L)	500	500

Figure 2 Comparison between Mikros and semi-micro LCMS/MS (spiked plasma sample at LLMI = 0.5 ug/L, TIC chromatogram)

4-2. Calibration

Calibration curves were calculated by internal standardization using a linear regression model with 1/x weighting. Acceptance criteria was an accuracy comprised between 85 to 115%.



Calib. Level	Conc. (ug/L)	Accuracy % (Verapamil)	Accuracy % (Nor-Verapamil)
01	0.5	101.4	94.2
02	0.9	110.7	108.4
03	1.8	93.9	91.2
04	7.3	100.5	108.5
05	36.3	93.5	98.1
06	90.8	97.3	98.9
07	181.6	102.6	100.7

Figure 3 Calibration curve , accuracy and LLMI chromatogram for Verapamil and Nor-verapamil

4-3. Recovery

Total recovery (i.e. combining extraction and matrix effect) was evaluated by comparing peak areas in lower range level QC in plasma to an equivalent prepared in solution. Each type of sample was prepared in triplicate. Results are shown in Table 3. The mean recoveries were >97% illustrating the good extraction rate and the low matrix effect.

Table 3 Recovery

Molecule	Peak Area (plasma)	Peak Area (neat std)	Overall Recovery (%)
Verapamil	179,517	182,805	98
NorVerapamil	150,453	146,364	97

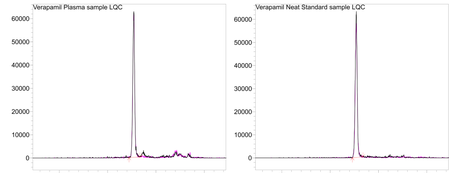


Figure 3 Chromatogram of Verapamil (MRM) in plasma spiked extracted sample and neat standard solution (LQC 1 ug/L)

5. Conclusions

- The use of micro-flow rates for the quantification of Verapamil and Nor-Verapamil allowed to obtain a substantial increase in sensitivity (signal intensity and signal to noise) without any limitation regarding the injection volume (using a trap and elute system). For that reason a reduction of initial plasma sample was possible without affecting the analytical performances of the method.
- The reported method is a proof of concept showing benefits in using micro-flow rates for drugs quantitation in biological fluids and could find application in discovery-stage PK studies.
- The use of ultra-small plasma quantities (2 ul) is furthermore compatible with micro sampling device, such as MSW2 (Microsampling Wing™ × Microsampling Windmill™), that are designed based on the principles of the 3Rs (Replacement, Reduction and Refinement) to minimize the burden to animals.

References

[1] Rapid Commun. Mass Spectrom. 2014, 28, 1293–1302