Sensitive Quantitation of Glucagon in Rat Plasma with Trap-and-Elute MicroLC-MS

Using the SCIEX M3 MicroLC system for Pharmacokinetics Analysis in Small Sample Volumes

Remco van Soest and Lei Xiong
SCIEX, Redwood City, CA, USA

Pharmacokinetic (PK) studies in rats and mice are restricted in the number of time points that can be measured per animal, as the volume of blood that can be drawn from these small animals is limited. This often results in PK studies using multiple animals, which is not ideal from a scientific standpoint. Due to this limitation, and the ongoing efforts to Reduce, Refine and Replace test animals (the 3R's), there has been considerable interest in micro-sampling and other techniques that facilitate PK studies in smaller volumes of blood. In this technical note we describe how MicroLC is used to increase sensitivity for the quantitation of Glucagon in 25 µL rat plasma samples. Glucagon is a peptide hormone that increases glucose levels, and as such is used for e.g. the treatment of hypoglycemia in diabetes patients. Several glucagon analogues with better stability are currently in development.

MicroLC, using 0.3 mm ID columns at 5-20 µL/min flow rates, can improve LC-MS sensitivity by a factor of up to 10, compared to employing the more commonly adopted 2.1 - 3 mm ID columns at flow rates of 0.5-1 mL/min.[1,2] In a previously published technical note[3] we described how using Trap-Elute MicroLC-MS with 0.3 mm ID columns can achieve a 4x lower limit of quantitation (LLOQ) of a mAb in rat plasma. Here we used the same approach for the LC/MS analysis of Glucagon in plasma extracts using a simple off-line solid phase extraction (SPE) clean-up. 20 µL of SPE eluent was loaded onto a short trap column at 35 µL/min, followed by switching the trap column in-line with the separation column for analysis at 10 µL/min.

Key Benefits of using the M3 MicroLC system for Pharmacokinetics

- Allows more time points in small animal PK studies
- Peptide quantitation at levels up to 10 x lower than what can be measured with High Flow LC-MS
- High throughput by using a Trap-Elute workflow
- Increased column lifetime and reduced need for cleaning of the MS through removal of salts and other impurities using a Trap-Elute workflow

Materials and Methods

Sample Preparation: Glucagon was acquired from Sigma-Aldrich (St. Louis, MO, USA). ([$^{13}C_6$]Leu^{14})-Glucagon (1-29) (BACHEM, Torrance, CA, USA) was used as internal standard. Rat Plasma (Sprague Dawley, K2 EDTA) was acquired from BioreclamationIVT, (Westbury, NY, USA). Standard samples were prepared by spiking plasma with glucagon and internal standard. After spiking, 1% acetic acid was added to each sample. 25 µL of each standard was processed following a Solid Phase extraction (SPE) method that we developed earlier for the analysis of Glucagon in human plasma[4]. For SPE a mixed-mode Waters Oasis MAX µElution Plate (Waters, Milford, MA, USA) was used. Final extract volume was 110 µL after diluting with water to ensure low enough acetonitrile content for injection on the HPLC.

***HPLC Conditions – High Flow LC**:* A SCIEX ExionLC™ AD HPLC system was used for the high flow LC-MS analysis. The column used was a 100 x 2.1 mm Kinetex C18 2.6 µm 100 Å column from Phenomenex (Torrance, CA, USA), kept at 40°C. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. Wash solvent for the autosampler was 20/20/60 methanol/acetonitrile/IPA. The gradient was linear from 10 to 50% B in 5 min, with a 5 min wash at 90%B. Flow rate was 0.5 mL/min. Injection volume was 20 µL.

***HPLC conditions – MicroLC**:* A SCIEX M3 MicroLC-TE system, consisting of two MicroLC gradients and an integrated autosampler, was used in combination with a source mounted column oven. Mobile phase A in the analytical gradient pump was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid. For the separation a 100 x 0.3 mm HALO Peptide ES-C18 2.7 µm 160 Å column (MacMod, Chadds Ford, PA, USA) was used. The gradient used was the same as what was used for the high flow method, 10 - 50% B in 5 min, with a 10 min 50% B wash step. Flow rate was 10 µL/min. The column temperature was 40°C. Injection volume was 20 µL, and the autosampler needle and valve wash consisted of two cycles using mobile phase B, followed by one cycle using mobile phase A.

***Trapping Conditions**:* Mobile phase A in the loading gradient pump was water with 0.1% formic acid, Mobile phase B was acetonitrile with 0.1% formic acid. As the trap, a 10 x 0.5 mm 5 µm 120 Å ChromXP™ C18 CL trap column (SCIEX) was used. Sample was loaded from the injection loop onto the trap column using 100% A for one minute at 35 µL/min. The trap was washed with 90% B at 70 µL/min for 5 minutes after every analysis.

Table 1. Source and gas parameters

	High Flow LC	MicroLC
Electrode ID	100 µm	25 µm
Curtain Gas	30	30
Collision GAS	9	9
IonSpray Voltage	5000	5000
Temperature (°C)	500	350
Ion Source Gas 1	40	20
Ion Source Gas 2	40	20

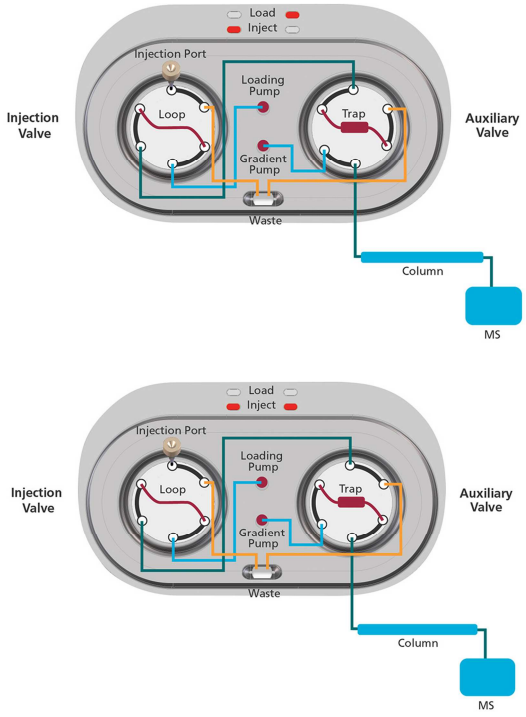

Figure 1. Valve positions for the trap loading part of the method (top) and the analysis part of the method (bottom).

***Mass Spectrometry**:* A SCIEX QTRAP® 6500+ LC-MS/MS system was used in MRM mode. The transitions used for quantitation were 697.6 to 813.4 for Glucagon, and 699.1 to 814.8 for the internal standard. Cycle time was 0.24 s. See reference 4 for further details on the method used. For the MicroLC experiments the standard electrode was replaced with a 25 µm ID electrode (SCIEX). Source and gas parameters are listed in Table 1. MultiQuant 3.0.2 software was used for data analysis.

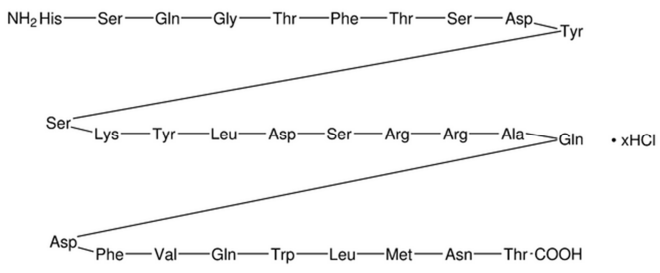

Figure 2. Amino acid sequence of Glucagon. Molecular weight is 3482.75

Sensitivity Improvement

Rat plasma samples were prepared by spiking with 0.1 to 100 ng/mL Glucagon, and 15 ng/mL of the internal standard. In order to prepare a sufficient amount of sample to accommodate 5 replicate injections of 20 µL with both high flow LC and microLC from the same extract, all standards were prepared in duplicate and pooled after SPE.

Figure 3 shows the XIC's for Glucagon with both methods at the 0.5 ng/mL level. Signal improved by a factor 10, and S/N improved by a factor of approximately 5.

Table 2 lists the calculated concentrations for the calibration curves with the CV % and accuracies. Using the requirements of precision < 20% and accuracy between 80 and 120% at the LLOQ, and at any higher concentration a precision <15% and accuracy between 85% and 115%, the LLOQ of glucagon quantitation in unstripped rat plasma for the high flow method was 0.5 ng/mL. The LLOQ for the microLC method improved by a factor of 5 to 0.1 ng/mL.

Both the High Flow and MicroLC methods showed good linearity with an r of 0.99 using a weighting of $1/x^2$ (Figure 4).

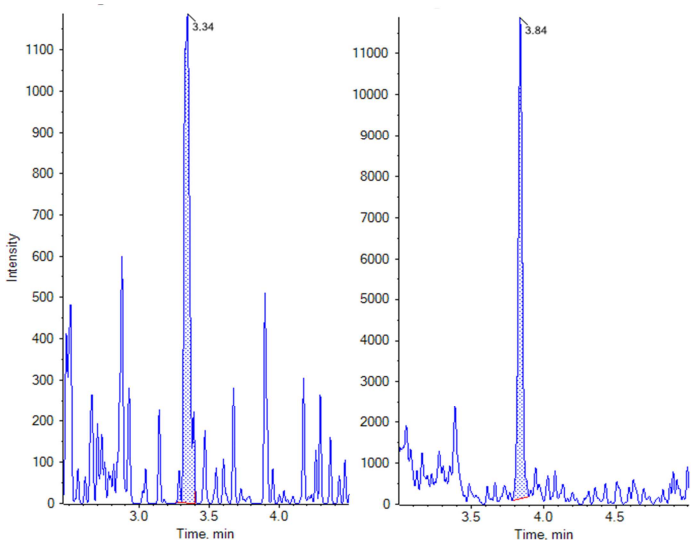

Figure 3. Sensitivity comparison between the High Flow LC-MS (left) and MicroLC-MS (right) methods at the 0.5 ng/mL level. S/N of the XIC for the Glucagon transition improved by a factor of 5

Carryover

Using High Flow LC-MS, no carryover was observed after injection of the 100 ng/mL sample. The Trap-Elute microLC method showed carryover of ca. 0.1%. The carryover was determined to come from the analytical column, so a longer column wash step can be applied for further reduction of the observed carryover. Alternatively the ULOQ for the microLC method could be reduced to fulfill the maximum allowable carryover requirement of 20% of the response at the LLOQ level after injecting a sample at the ULOQ level.

Table 2. Standard curve data for the high flow and MicroLC-MS methods

	High Flow LC-MS			Micro LC-MS		
Actual Concentration (ng/mL)	Mean calculated concentration (ng/mL)	Accuracy (%)	CV (%)	Mean calculated concentration (ng/mL)	Accuracy (%)	CV (%)
0.100	N/A	N/A	N/A	0.098	97.97	18.06
0.125	N/A	N/A	N/A	0.129	103.25	10.12
0.167	N/A	N/A	N/A	0.171	102.47	8.13
0.250	N/A	N/A	N/A	0.242	96.74	7.2
0.500	0.517	103.52	18.43	0.454	90.89	5.41
1.00	0.913	91.30	14.25	1.09	108.72	3.8
3.00	3.10	103.50	8.02	3.18	106.03	3.5
10.0	10.51	105.09	8.89	10.1	101.52	4.85
30.0	30.2	100.60	6.86	29.6	98.71	5.26
100	9.60	95.99	3.47	93.7	93.70	3.87

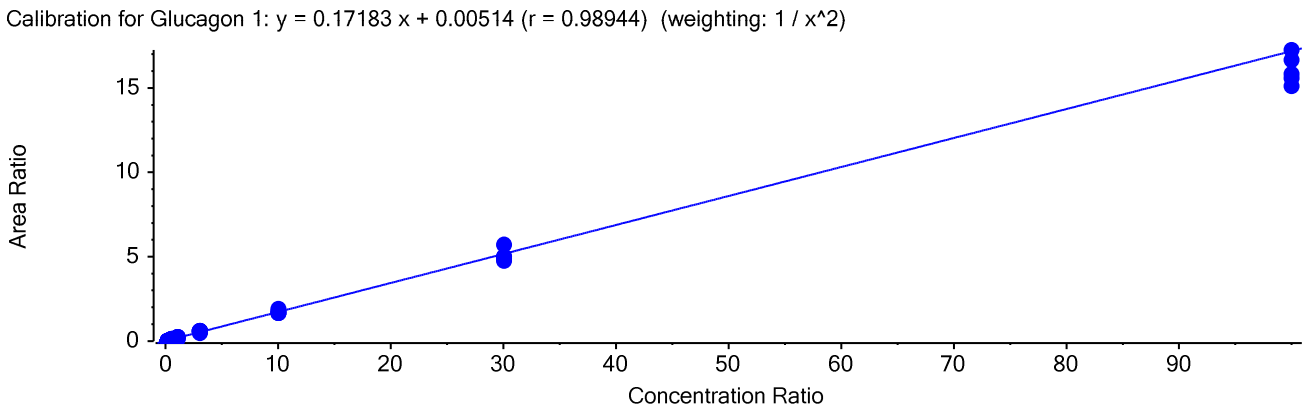

Figure 4. Calibration curve for the quantitation of Glucagon using micro LC-MS.

Conclusions

We have shown that quantitation of glucagon in 25 µL rat plasma samples using mixed-mode off-line SPE can be performed with a 5x lower LLOQ using a trap-elute MicroLC-MS method at 10 µL/min, as compared to using a direct inject High Flow LC-MS method at 500 µL/min. The trap-elute method ensures similar throughput while injecting the same 20 µL of sample, and protecting the MicroLC column and MS from salts and other contaminations.

This workflow offers a solution for the quantitation of Glucagon in small volume plasma samples, e.g. for preclinical pharmacokinetic studies in rats or mice.

References

1. Covey,T.R., Thomson B.A., Schneider, B.B. Atmospheric Pressure Ion Sources*; Mass Spec. Reviews*, 2009, 00, 1-29.
2. Covey T.R., Schneider B.B., Kovarik, Corr, Javahari et al.;The Central Analytical Figures of Merit of ESI, MALDI, and APCI.; *Cole RB, ed. Electrospray and MALDI mass spectrometry*. 2010, Chapter 13. Hoboken: John Wiley & Sons, Inc.
3. Improving Sensitivity for an Immunocapture LC-MS Assay of Infliximab in Rat Plasma Using Trap-and-Elute MicroLC-MS SCIEX Technical Note RUO-MKT-02-3413
4. iMethod Application for bioanalytical studies of Glucagon in human plasma using LC-MS http://sciex.com/products/ products/methods/imethod-application-for-bioanalytical-studies-of-glucagon-in-human-plasma-using-lc-ms

Document number: RUO-MKT-02-4268-A

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices