Extremely Low LC-MS/MS Lithium Adduct Detection of Rapamycin in Animal Blood and Tissues

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1 Overview

- Study design required very low Rapamycin quantitation limit in animal blood and tissues
- Utilizing an analyte lithium adduct improved sensitivity of Rapamycin MS/MS detection
- Combination of lithium adduct detection with HILIC chromatography and µElution extraction significantly reduced the analyte lower quantitation limit and ensured a validatable method at Rapamycin range 10 – 10,000 pg/mL in animal whole blood
- Method was successfully applied to Rapamycin analysis in animal tissue homogenate

2 Introduction

Rapamycin, also known as sirolimus (Figure 1), is a natural microlide immunosupressant, extracted from Streptomyces Hydroscopicus as a mixture of main and minor isomers. In the human body Rapamycin inhibits T lymphocytes activation and proliferation, as well as antibody production. In whole blood Rapamycin is highly distributed in red blood cells (RBC), where it's bound to intracellular proteins. In vivo Rapamycin undergoes lactone ring degradation to its linear form (seco-Rapamycin), and enzyme dependant oxidation and demethylation.

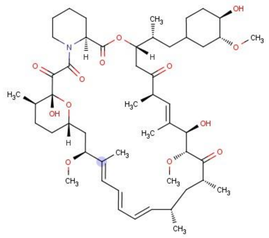

Figure 1. Rapamycin structure

3 Methods

Chromatography

Shimadzu Nexera UPLC LC-30AD equipped with Sielc Primesep B2 3 µm 4.6x100 mm column at 50ºC
Isocratic separation with 92 % MeCN in 0.1 mM lithium acetate aqueous at flow rate 1.2 mL/min

Mass Spectrometry

AB Sciex QTRAP 6500 operated in positive ion spray mode

	Mass Transition	Q2 Collision Energy, eV
Rapamycin	920 > 393	68
Rapamycin-$^{13}Cd_3$	924 > 393	68

Extraction Procedure

- Blood is treated with 1M zinc sulfate solution to destroy red blood cells
- Acetonitrile is added to precipitate proteins and release drug into solution
- Samples are centrifuged, supernatant is diluted with water and loaded onto Oasis HLB 96-well µElution plate
- After washing with 25% acetonitrile, samples are eluted with 50 µL of acetonitrile and injected directly into LC-MS/MS system

4 Results

Rapamycin easily forms ammonia and alkali metal adducts in gas phase (Figure 2). These molecular ions compete against each other, whereas the regular hydrogen parent ion isn't detected. However, in the presence of lithium acetate in mobile phase the distribution balance moves completely to the formation of Rapamycin lithium adduct, resulting in 4-5 times ion counts gain in Q1 scan (Figure 3).

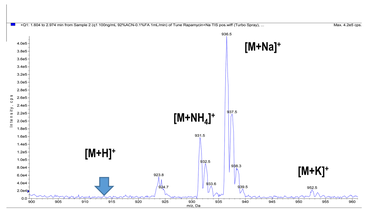

Figure 2. Ammonia, sodium and potassium adducts of Rapamycin in Q1 scan. The most abundant one is sodium adduct m/z ion. There is no peak at m/z of hydrogen adduct

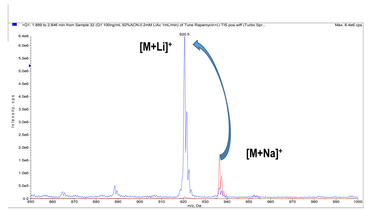

Figure 3. Rapamycin forms strong Q1 lithium adduct if lithium acetate introduced in mobile phase

After dissociation the molecular ions continue to keep appropriate metal atom within the fragment body. Both lithium and sodium Rapamycin adducts produce fragments with similar pattern, differing by a shift of 16 amu, just with slightly altered relative ions abundance (Figure 4). This shift corresponds to the difference in the atomic mass of sodium and lithium metals. Lithium MRM transition of Rapamycin parent/daughter ions [920-393 m/z] corresponds to sodium MRM transition [936-409 m/z].

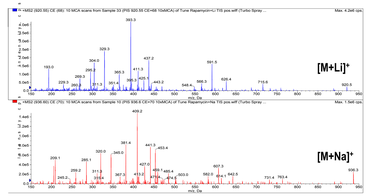

Figure 4. Product ion spectrum of Rapamycin with and without lithium acetate in infusion mobile phase

In solution Rapamycin presents as a mixture of major isomer B (pyran ring for carbon 14) and minor isomer C (oxepane ring for carbon 14). The isomers are easily separated in reversed phase chromatography, with isomer C eluting later and sitting on the tail of the main isomer B peak (Figure 5). However, attempting to completely resolve these peaks to baseline by reducing the gradient slope fails - there is always a noticeable bridge in between the peaks, presumably due to the presence of minor amounts of other isobaric impurities, significantly complicating consistent Rapamycin major isomer C peak integration.

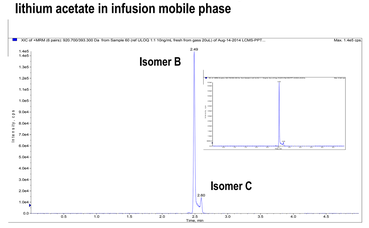

Figure 5. Typical gradient reversed phase chromatography of Rapamycin

HILIC chromatography, performed at high organic content, is usually a good alternative when compound partition is undesirable and isomers separation should be revoked. Rapamycin was separated as a single peak on Primesep B2 3 µm 4.6x100 mm column at 50ºC under isocratic conditions: 92% acetonitrile in 0.1 mM lithium acetate aqueous at 1.2 mL/min flow rate (Figure 6). Combination of lithium adduct detection with high organic HILIC chromatography allowed to attain an LLOQ of 10 pg/mL in rat whole blood (Figure 7).

Method was successfully pre-validated over the range 10 – 10,000 pg/mL in rat whole blood. Pre-validation batch met all the acceptance criteria for selectivity, precision and accuracy (Table 1). Rapamycin fit linear regression with $1/x^2$ weighing (Figure 8). Rapamycin extraction recovery was about 75%. Rapamycin has been proven to be stable in rat whole blood for 4 hours at room temperature, and after three freeze-thaw cycles. Additionally, the method has been successfully pre-validated in dog whole blood, and also applied to Rapamycin determination in animal lung and brain homogenates.

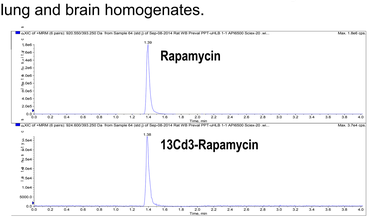

Figure 6. Rapamycin ULOQ 10 ng/mL, extracted from rat whole blood, and separated by HILIC chromatography

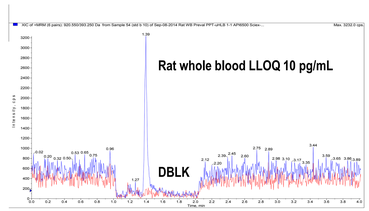

Figure 7. Rapamycin LLOQ 10 pg/mL, extracted from rat whole blood, and separated by HILIC chromatography

Theoretical Concentration (pg/mL)	Calculated Concentration (pg/mL)	% Accuracy (Mean)	% CV
10.0	9.3 11.5 10.8 11.3 10.1 9.4	104.0	9.1
25.0	26.0 24.1 25.0 24.3 25.3 25.8	100.4	3.0
2500	2366.7 2319.3 2425.5 2431.6 2610.2 2433.3	97.2	4.1
8000	7947.0 7851.7 7889.1 7452.5 7229.7 7597.9	97.8	3.7

Table 1. QC back calculated concentrations, precision and accuracy from 6 different donors at four concentration levels for Rapamycin, extracted from rat whole blood

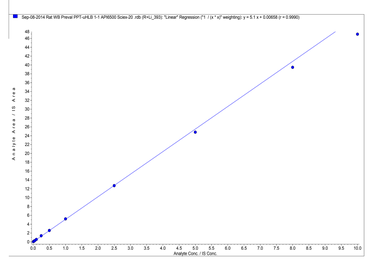

Figure 8. Rapamycin calibration curve from 10 pg/mL to 10,000 pg/mL in rat whole blood

5 Conclusion

A highly sensitive, accurate, precise and robust LC-MS/MS method has been developed for the quantitative determination of Rapamycin in animal whole blood and tissues. Utilizing a lithium adduct for Rapamycin detection, combined with non-evaporative µElution extraction with direct injection on HILIC chromatography allowed to significantly reduce the analyte lower quantitation limit in animal matrix.