Development, Validation and Application of a High Sensitivity On-Line Extraction LC-MS/MS Method for the Determination of a Parent Molecule and its Metabolites M1 and M2 in Human Plasma

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INTRODUCTION

The objective of this study was to validate the analytical method for the determination of a parent compound and its main metabolites (M1 and M2) in human plasma, over a concentration range of 0.1 to 20 pg/mL (parent) and 0.25 to 20 pg/mL (M1 and M2). A sample aliquot volume of 500 µL was used. This assay concentration range was required due to the high potency and hence low therapeutic doses of the parent compound administered. The typical Cmax was expected to be in the low pg/mL levels

The analytes are small molecules (~400 amu) that are very polar. The main functional groups are tertiary amine and sulphonamide.

Key characteristics of the method:

Analytical technique: LC-MS/MS

Sample preparation: SPE and On-Line Extraction

Matrix: Human Plasma (Lith Hep)

LLOQ (pg/mL):0.100 (Parent), 0.250 (M1 & M2)

ULOQ (pg/mL): 20.0 (Parent), 20.0 (M1 & M2)

Total analysis time: 7.0 minutes

EXPERIMENTAL

Sample Preparation

Sample volume: 500 µL

Internal standard: Parent-d10

SPE: Oasis MCX 30 mg

Off-line extract volume: 250 µL

On-Line extraction: Symbiosis Pharma

Extraction cartridge:Hysphere-C2-SE (10 x 2 mm)

On-line SPE

Instrument: Spark Holland Symbiosis

Sample load: 100 µL of off-line extract

Sample wash: 1000 µL H_2O:MeOH: ammonium acetate (1M): ammonia 90:10:1:0.2

Sample backward wash: 500 µL H_2O:MeOH: ammonium acetate (1M): ammonia 90:10:1:0.2

Sample elution: 200 µL H_2O:MeOH:FA (formic acid) 70:30:0.5

Chromatography

Column: Agilent, Poroshell 120 EC 2.7 µm, 50 x 3 mm

Column Temperature: 50°C

Mobile phase A:: FA (0.2%) in MeOH: H_2O:ammonium formate (1M) 10:90:0.2

Mobile Phase B: FA (0.2%) in MeOH: H_2O:ammonium formate (1M) 90:10:0.2

Flow rate: 600 µL/min

Run time: 7.0 minutes

Retention time: Parent 3.9 min

M1 3.1 min

M2 2.7 min

Parent-d10 3.9 min

Mass Spectrometry

Instrument: AB SCIEX API 5000

Instrument Parameters:

Q1 Resolution: Unit

Q3 Resolution: Unit

Interface: Turbo IonSpray (+ve)

Dwell time (each): 75 ms

IS: 1800 V

Temperature: 400°C

Figure 1: Spark Holland Symbiosis, interfaced with an AB Sciex API 5000

RESULTS

The validation comprised of three different runs to assess the precision and accuracy of the method. Additional experiments include selectivity, matrix effects, modification of ionisation and freeze/thaw, short-term, long term, whole blood and extract stabilities, effect of haemolysed plasma, recovery and carry-over.

Lower limit of quantitation

The lower limit of quantitation (LLOQ), as defined by the lowest QC at which inter-batch accuracy and the inter-batch precision was ≤ 20%. The signal to noise ratio at the LLOQ was greater than ten.

The LLOQ was 0.1 pg/mL for the parent and 0.25 pg/mL for M1 and M2 (Figure 2).

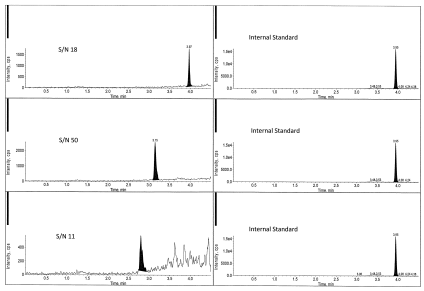

Figure 2: LLOQ at 0.1 pg/mL chromatogram for the parent, 0.25 pg/mL chromatogram for M1 and M2

Linearity

Linearity was demonstrated from the LLOQ to the highest evaluated concentration (ULOQ) for each analyte, using a linear regression of peak area ratio with a 1/x weighting.

All coefficients of determination (r^2) of the calibration lines for all analytes during the validation were better than or equal to 0.9953.

Precision and Accuracy

The overall intra-run and inter-run precision and accuracy data were within acceptable limits.

Intra-run and Inter-run data for parent

Concentration (pg/mL)	Mean intra-run precision and accuracy		Inter-run precision and accuracy	
	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)
0.100	9.9	89.6	9.8	90.2
0.300	3.7	96.2	5.4	96.3
4.00	2.1	96.8	2.5	96.8
16.0	1.5	97.3	1.5	97.5
100[a]	3.5	100	3.9	99.9

Intra-run and inter-run data for M1

Concentration (pg/mL)	Mean intra-run precision and accuracy		Inter-run precision and accuracy	
	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)
0.250	4.2	101	4.3	101
0.750	6.4	99.3	7.4	99.3
4.00	4.5	97.3	4.7	97.3
16.0	3.3	98.8	4.0	98.8
100a	4.9	99.3	5.1	99.5

Intra-run and inter-run data for M2

Concentration (pg/mL)	Mean intra-run precision and accuracy		Inter-run precision and accuracy	
	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)
0.250	9.0	90.3	11.3	90.8
0.750	6.6	98.6	6.6	98.7
4.00	5.0	95.8	5.6	95.8
16.0	3.4	95.4	4.2	95.6
100a	5.7	97.9	5.9	98.0

Stability

The parent molecule, M1 and M2 were found to be stable under the following conditions:

Condition	Minimum stability
Stability in Plasma at-80°C	3 months
Short Term in Plasma at RT	24 Hours
Maximum Freeze/Thaw cycles	3 Cycles
Extract Stability at 10°C	120 hours
Whole Blood Stability at 4°C	2 Hours
Whole Blood Stability at RT	2 Hours

Matrix Effects and Modification of Ionisation

The parent, M1 and M2 were found to have no significant matrix effects in six different individual human plasmas at low and high calibration standard levels.

Matrix factors and the IS normalised matrix factors approximated to 1.0 for all individuals and compounds. Therefore the method was not significantly affected by inter-individual variability.

Effect of Haemolysed Plasma

There was no effect on the quantification of the parent, M1 and M2 at the low calibration standard level prepared in 2% haemolysed plasma.

Carry over

No significant carry over was observed (<20% of the LLOQ) for the parent, M1 and M2.

Recovery

The mean recovery of the parent, M1 and M2 were found to be 84.1%, 81.3% and 81.2%, respectively.

Selectivity

There were no significant interfering peaks in the regions of the MRM chromatograms at the retention times of the parent, M1, M2 and parent-d10, in six different individual human plasmas (Figure 3).

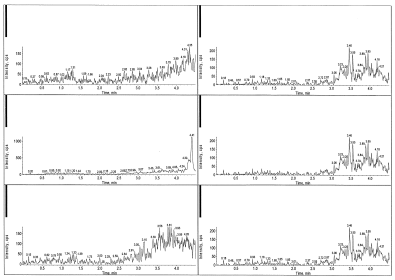

Figure 3: Selectivity sample chromatogram for parent, M1, M2 and parent-d10.

Application of the method

The methodology has subsequently been applied to clinical sample analysis. The typical parent profile presented in Figure 4 demonstrates the need for sub-pg/mL quantitation (as low as 0.1 pg/mL) in order to adequately describe the absorption and elimination phases. These data would not have been available if the sub pg/mL method was not applied.

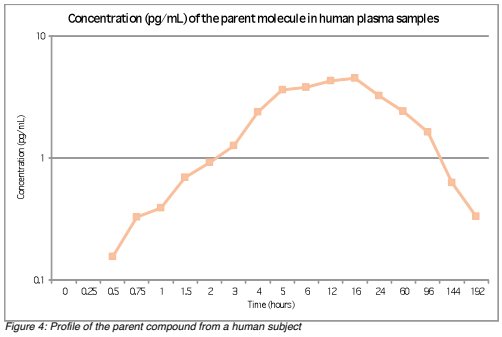

Figure 4: Profile of the parent compound from a human subject

CONCLUSION

The method utilises both off-line and on-line solid phase extraction to enable the detection limits and demonstrates the use of an orthogonal multidimensional approach. The method described for the determination of the parent and its main metabolites M1 and M2 in human plasma has been validated successfully over the concentration range 0.1 to 20 pg/mL for the parent and 0.25 to 20 pg/mL for M1 and M2 using an aliquot volume of 500 µL.

The method was demonstrated to be sufficiently accurate and precise, and to have sufficient selectivity, to reliably allow the determination of the parent, M1 and M2 in human plasma samples over the examined range. The sub-pg/mL LLOQ has provided high quality PK data from early clinical studies, enabling the rapid progression of the clinical programme.

A similar methodology has also been validated and applied to preclinical plasma samples achieving a parent LLOQ of 0.1 pg/mL, using a smaller plasma aliquot of 250 µL.

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