Application of Ultra-Fast LC-MS/MS to bioanalytical assays in a drug discovery environment

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Introduction

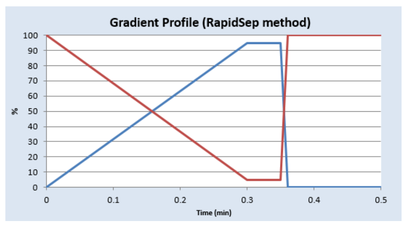
Within the past 20 years, small molecules bioanalysis using liquid chromatography/tandem mass spectrometry (LC-MS/MS) on a triple quadrupole-MS has become a common practice in support of drug metabolism and pharmacokinetics (DMPK) studies. The LC-MS/MS assay provides the specificity, flexibility and sensitivity that allows a quick and reliable drug quantification from a wide variety of biological matrices. However, there has always been an increasing demand in analytical capacity and faster sample turnaround time in order to identify potential lead compounds. For this reason, it has become necessary for our bioanalytical laboratories to develop a new analytical method to cope with this major challenge during the drug discovery stage.

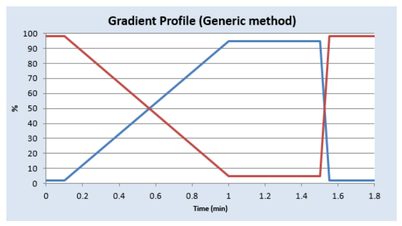
Recently, we have expanded our LC-MS/MS capabilities using Ultra-Fast Separation Chromatography (RapidSep) for bioanalytical analysis to improve overall efficiency of DMPK studies. With the new RapidSep set up, it has become possible to reduce the chromatographic run time from 108 s to 30 s per injection. To demonstrate the potential of the RapidSep over the traditional methods, we have applied the RapidSep set up to a real study sample; monitoring the PK profile following IV, PO administration to male (C57Bl/6J) mouse at 1,10 mg/kg. In this assessment, we have evaluated all essential PK parameters such as clearance, volume of distribution, AUC, half-life ($T_{1/2}$), C_{max}, T_{max}, bioavailability, blood: plasma ratio, and dose formulations for the potential of its applicability.

Method

RapidSep method vs Generic method

- Column: Halo 2.7 μm vs Kinetex XB-C18 2.6 μm
- Dimension: 10 x 2.1 mm **/** 50 x 2.1 mm
- Mobile phase A: 10 mM ammonium formate, 0.1% formic acid (aq)
- Mobile phase B: Methanol
- Temperature: N/A **/** 50ºC, Flow rate: 1.5 mL/min **/** 0.8 mL/min
- Detection: MRM





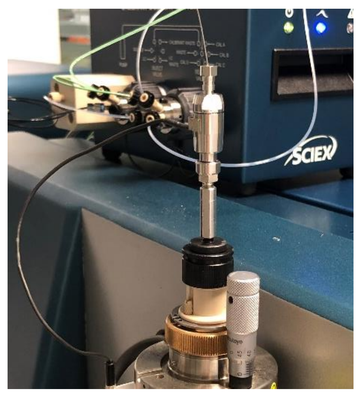
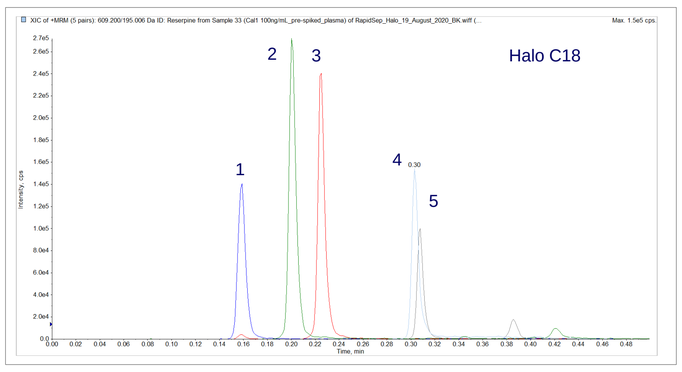


Figure 1: The column-source connection

For the RapidSep system/method, the column was attached directly to the MS source as shown in Figure 1. to minimise column dead volume.

Column performance



Test compound

Parameter	Atenolol	Nadolol	Metoprolol	Reserpine	Rosuvastatin
R	–	3.1	5.5	11.0	12.0
T	1.1	1.2	1.4	1.2	1.6
K'	7.0	9.0	10.5	14.0	14.5
α	1	1.3	1.5	2.1	2.1
t_R (sec)	9.6	12.0	13.8	18.0	18.6

R resolution factor, T tailing factor, K' capacity factor, α selectivity factor, t_R retention Time

Firstly, the column performance was evaluated by testing with five different standards. The analysis was conducted utilising the Agilent 1290 Infinity II dual needle μHPLC system coupled with the MRM detection in ESI positive mode on a ABSciex QTRAP 5500 mass spectrometer. Chromatographic separation was achieved using a reverse phase HALO C18 column cartridge with 10mM ammonium formate + 0.1% formic acid in water and methanol as the mobile phases. As a result, the peak of interest was well separated within a 30 second run time.

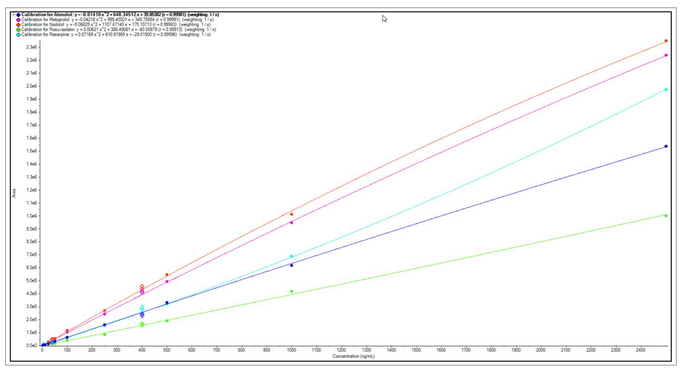


Figure 2. Calibration lines from the developed RapidSep method.

Initial assessment: The developed RapidSep method achieved consistent recoveries and the method revealed the calibration range (covered) from 1-5000 ng mL^{-1} for all five standards. The within- batch precision were within the ±20 %. acceptable limit. After qualification, the method was applied to the analysis of real study samples to examine the chromatographic quality and the risk of ion-suppression / enhancement in biological matrices.

Method

Sample Preparation: Biological samples were prepared by protein precipitation.

1 volume rat plasma /blood + 3 volumes MeOH containing IS → Centrifuge @ 4000RPM, 30min, 4ºC → 1 volume supernatant + 2 volumes water → Mix → LC-MS/MS analysis

The rat plasma samples were spiked with metoprolol (internal standard); extracted using protein precipitation.

Chromatography

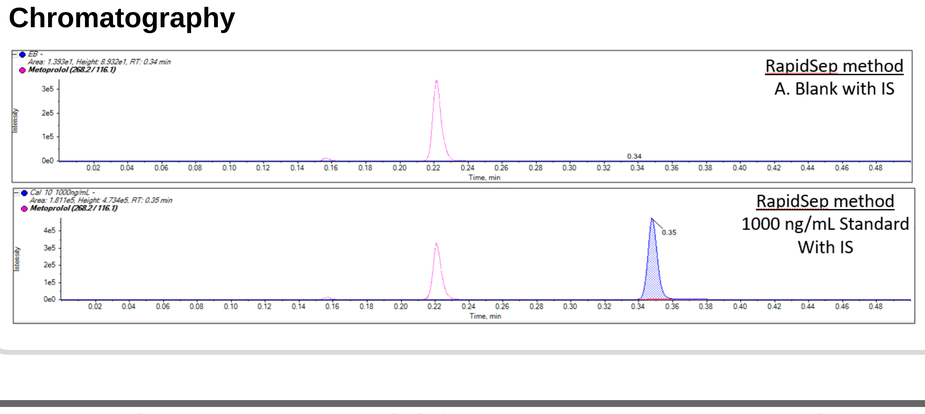


Figure 3. Representative LC-MS/MS chromatograms of A) blank rat plasma with IS, B) drug spike in rat plasma at 1000 ng/mL with IS. Both IS (Retention time (RT) = 0.23 minutes) and analyte peak (RT= 0.35 minutes).

Plasma matrix assessment

Linear range

Std. #	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	STD11	STD12
Nominal conc. (ng/mL)	1.00	2.50	5.00	10.0	25.0	50.0	100.0	250.0	500.0	1000.0	2500.0	5000.0
Determined conc. (ng/mL) 1	0.975	2.60	5.10	10.5	25.2	50.2	102	241	491	981	2704	4406
Accuracy (%)	97.6	104	102	105	101	100	102	96	98.1	98.1	108	88.1
Value >±20%	~~Value~~ Excluded from calculation of mean accuracy								NC: Not calculated			

Concentration of lowest acceptable calibration standard: **1** nmol/L STD1
Concentration of highest acceptable calibration standard: **5000** nmol/L STD12
N/A: No peak detected

Accuracy and Precision

A Generic method

Sample #	Analyte QC	Analyte QC	Analyte QC	Analyte QC
Nominal conc (ng/mL)	4.0	40	400	4000
Determine Cc 1	4.33	41.3	411	4071
2	3.70	40.5	429	4311
3	3.82	41.2	410	3916
Mean	3.95	41.0	417.0	4100
Stdev	0.338	0.483	10.3	199
Accuracy	**98.8**	**102.5**	**104.3**	**102.5**
Precision (%)	8.6	1.18	2.47	4.85

Linear range

Std. #	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	STD11	STD12
Nominal conc. (ng/mL)	1.00	2.50	5.00	10.0	25.0	50.0	100.0	250.0	500.0	1000.0	2500.0	5000.0
Determined conc. (ng/mL) 1	0.896	~~3.355~~	5	9	25	51	107	234	494	894	2425	5407
Accuracy (%)	89.6	-	94.7	93.6	99.8	101	107	93.7	98.8	89.4	97.0	108
Value >±20%	~~Value~~ Excluded from calculation of mean accuracy								NC: Not calculated			

Concentration of lowest acceptable calibration standard: **1** nmol/L STD1
Concentration of highest acceptable calibration standard: **5000** nmol/L STD12
N/A: No peak detected

Accuracy and Precision

B RapidSep method

Sample #	Analyte QC	Analyte QC	Analyte QC	Analyte QC
Nominal conc (ng/mL)	4.0	40	400	4000
Determine Cc 1	3.85	37.4	399	4892
2	4.53	40.5	379	5024
3	4.85	41.2	369	4418
Mean	4.41	39.7	382.0	4780
Stdev	0.511	2.06	15.3	319
Accuracy	**110.3**	**99.3**	**95.5**	**119.5**
Precision (%)	11.6	5.19	4.01	6.67

Figure 4. Comparison of Generic method against the RapidSep method. (A and B) Intra-assay linearity, accuracy and precision assessment using Generic vs RapidSep method.

Precision and Accuracy of spiked QCs

The spiked quality control sample precision, and accuracy were demonstrated at N=3 at the low (4ng/mL), and at medium 1 (40ng/mL), medium 2 (400ng/mL) and high (4000ng/mL) concentrations. The assay showed a linear calibration range / covered range from of 1-5000 ng/mL. The calibration curve was fitted with a quadratic regression $1/x^2$ whilst giving a r^2 value ≥ 0.99. This data shows that the RapidSep method provides similar data compared to the generic method from plasma matrix.

Blood matrix assessment

Linear range

Std. #	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	STD11	STD12
Nominal conc. (ng/mL)	1.00	2.50	5.00	10.0	25.0	50.0	100.0	250.0	500.0	1000.0	2500.0	5000.0
Determined c 1	0.997	2.40	~~2.68~~	11.5	26.6	53.7	104	254	482	901	2325	4534
Accuracy (%)	99.7	95.9	-	115	107	107	104	101	96.4	90.1	93.0	90.7
Value >±20%	~~Value~~ Excluded from calculation of mean accuracy								NC: Not calculated			

Concentration of lowest acceptable calibration standard: **1** nmol/L STD1
Concentration of highest acceptable calibration standard: **5000** nmol/L STD12
N/A: No peak detected

Accuracy and Precision

A Generic method

Sample #	Analyte QC	Analyte QC	Analyte QC	Analyte QC
Nominal conc (ng/mL)	4.0	40	400	4000
Determine Cc 1	4.39	40.6	399	4023
2	3.34	40.5	418	4070
3	4.23	41.2	321	3715
Mean	3.99	40.8	379.0	3940
Stdev	0.563	0.419	51.4	193
Accuracy	**99.8**	**102.0**	**94.8**	**98.5**
Precision (%)	14.1	1.03	13.56	4.90

Linear range

Std. #	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	STD11	STD12
Nominal conc. (ng/mL)	1.00	2.50	5.00	10.0	25.0	50.0	100.0	250.0	500.0	1000.0	2500.0	4000.0
Determined c 1	0.922	2.92	~~1.77~~	10.7	26.9	56.1	94.1	229	446	853	2265	4595
Accuracy (%)	92.2	117	-	107	108	112	94.1	91.7	89.3	85.3	90.6	115
Value >±20%	~~Value~~ Excluded from calculation of mean accuracy								NC: Not calculated			

Concentration of lowest acceptable calibration standard: **1** nmol/L STD1
Concentration of highest acceptable calibration standard: **4000** nmol/L STD12
N/A: No peak detected

Accuracy and Precision

B RapidSep method

Sample #	Analyte QC	Analyte QC	Analyte QC	Analyte QC
Nominal conc (ng/mL)	4.0	40	400	4000
Determine Cc 1	4.17	38.6	400	4731
2	3.39	40.5	379	5024
3	2.51	41.2	369	4595
Mean	3.36	40.1	383.0	4780
Stdev	0.833	1.37	15.8	219
Accuracy	**84.0**	**100**	**95.8**	**119.5**
Precision (%)	24.8	3.42	4.13	4.58

Figure 5. Comparison of Generic with RapidSep method. A and B) Intra-assay linearity, accuracy and precision assessment using Generic vs RapidSep method.

Precision and Accuracy of spiked QCs

Spiked quality control sample precision and accuracy were demonstrated at N=3 at the low (4ng/mL), and at medium 1 (40ng/mL), medium 2 (400ng/mL) and high (4000ng/mL) concentrations. The assay has shown to have a linear calibration range of 1-5000 ng/mL. A quadratic regression $1/x^2$ was used to fit the calibration curve, yielding a r^2 value ≥ 0.99. This data demonstrates that the RapidSep method produces similar results to different types of matrices.

ADME summary graph

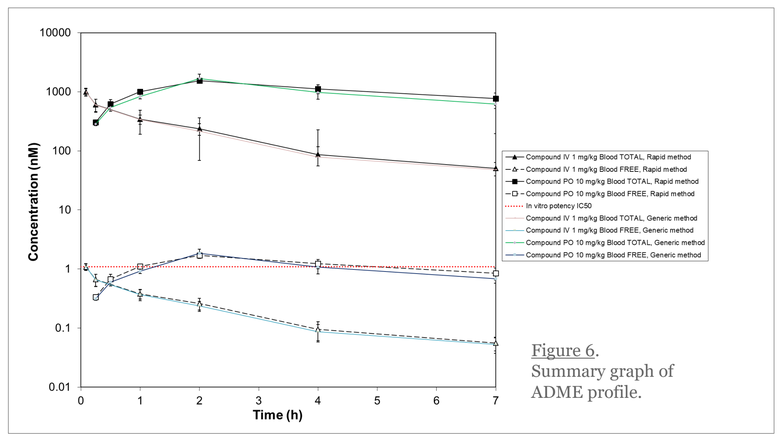


Figure 6. Summary graph of ADME profile.

A Summary graph showing the Mean TOTAL and FREE concentrations of the drug; following IV, PO administration to the male (C57Bl/6J) mouse at 1,10 mg/kg using the generic method vs the RapidSep method. These graphs are superimposed to each other suggesting the Rapidsep method is equally as effective as the generic method.

Pharmacokinetic parameters assessment

Generic method

PK Parameter	Compound IV 1 mg/kg Blood 1	2	3	Mean / Median	SD	Compound PO 10 mg/kg Blood 4	5	6	Mean / Median	SD
Dose (mg/kg)	1.00	1.00	1.00	**1.00**	-	10.0	10.0	10.0	**10.0**	-
C0 / Cmax (ng/mL)	561	626	581	**589**	**33.2**	793	622	896	**770**	**138**
C0 / Cmax (nM)	1222	1363	1267	**1284**	**72.3**	1727	1356	1953	**1679**	**302**
Clast (ng/mL)	15.4	22.1	28.3	**21.9**	**6.44**	235	323	292	**283**	**44.7**
tlast (h)	7.00	7.00	7.00	**7.00**	-	7.00	7.00	7.00	**7.00**	-
tmax (h)	-	-	-	-	-	2.00	2.00	2.00	**2.00**	-
t1/2 (h)	2.02	2.03	2.10	**2.05**	**0.0450**	-	-	-	-	-
MRT (h)	2.42	2.53	2.95	**2.63**	**0.277**	-	-	-	-	-
Vdss (L/kg)	4.7	3.3	4.2	**4.08**	**0.690**	-	-	-	-	-
CL / CL_F (mL/min/kg)	32.1	21.9	24.0	**26.0**	**5.42**	-	-	-	-	-
AUCinf (ng.hr/mL)	519	763	694	**658**	**126**	-	-	-	-	-
AUCinf (nM.hr)	1130	1662	1512	**1435**	**274**	-	-	-	-	-
AUC0-t (ng.hr/mL)	474	698	608	**593**	**113**	3268	2857	3025	**3050**	**207**
AUC0-t (nM.hr)	1032	1521	1325	**1293**	**246**	7121	6225	6591	**6646**	**450**
Fraction Absorbed	-	-	-	-	-	0.694	0.607	0.643	**0.648**	**0.0439**
Bioavailability (%) Using AUCinf	-	-	-	-	-	-	-	-	-	-
Bioavailability (%) Using AUC0-t	-	-	-	-	-	55.1	48.2	51.0	**51.4**	**3.48**
Number of Points used for Lambda z	4.00	4.00	5.00	**4.00**	-	-	-	-	-	-
AUC % Extrapolation to infinity	8.67	8.47	12.4	**9.84**	**2.19**	-	-	-	-	-
AUC % Back Extrapolation to C0	7.89	6.21	6.00	**6.70**	**1.03**	-	-	-	-	-

RapidSep method

PK Parameter	Compound IV 1 mg/kg Blood 1	2	3	Mean / Median	SD	Compound PO 10 mg/kg Blood 4	5	6	Mean / Median	SD
Dose (mg/kg)	1.00	1.00	1.00	**1.00**	**0.00**	10.0	10.0	10.0	**10.0**	-
C0 / Cmax (ng/mL)	518	651	547	**572**	**70.2**	662	714	747	**708**	**42.7**
C0 / Cmax (nM)	1128	1419	1193	**1246**	**153**	1443	1556	1627	**1542**	**93.1**
Clast (ng/mL)	17.0	26.5	29.6	**24.4**	**6.54**	292	446	318	**352**	**82.3**
tlast (h)	7.00	7.00	7.00	**7.00**		7.00	7.00	7.00	**7.00**	
tmax (h)	-	-	-	-		2.00	2.00	2.00	**2.00**	
t1/2 (h)	1.74	2.15	2.20	**2.03**	**0.253**	-	-	-	-	-
MRT (h)	2.34	2.74	3.09	**2.72**	**0.378**	-	-	-	-	-
Vdss (L/kg)	4.4	3.2	4.1	**3.9**	**0.633**	-	-	-	-	-
CL / CL_F (mL/min/kg)	31	19	22	**24**	**6.25**	-	-	-	-	-
AUCinf (ng.hr/mL)	534	865	755	**718**	**169**	-	-	-	-	-
AUCinf (nM.hr)	1163	1885	1644	**1564**	**368**	-	-	-	-	-
AUC0-t (ng.hr/mL)	491	783	661	**645**	**147**	3023	3735	3239	**3332**	**365**
AUC0-t (nM.hr)	1070	1706	1440	**1405**	**319**	6588	8138	7059	**7262**	**795**
Fraction Absorbed	-	-	-	-	-	0.580	0.717	0.622	**0.640**	**0.0700**
Bioavailability (%) Using AUCinf	-	-	-	-	-	-	-	-	-	-
Bioavailability (%) Using AUC0-t	-	-	-	-	-	46.9	57.9	50.2	**51.7**	**5.66**
Number of Points used for Lambda z	5.00	4.00	5.00	**5.00**	-	-	-	-	-	-
AUC % Extrapolation to infinity	8.01	9.51	12.5	**10.0**	**2.26**	-	-	-	-	-
AUC % Back Extrapolation to C0	7.17	5.72	5.22	**6.04**	**1.01**	-	-	-	-	-

All typical PK parameters such as clearance, volume of distribution, AUC, half-life ($T_{1/2}$), C_{max}, T_{max} and bioavailability are calculated, and the differences between the two methods are less than ±20 %.

Summary/conclusions

The data presented here demonstrates the success on Rapid Chromatography used to analyse discovery PK. It has shown to be reproducible and reliable whilst generating equivalent pharmacokinetic results to those obtained by the traditional generic method. To address throughput challenges, we have successfully reduced the total injection-to-injection cycle time to 60 s as opposed to 138 s with a direct RapidSep column setup. The introduction of this approach in our laboratory, in place of current analytical methods, will provide at least a 50% improvement in LCMS capacity without compromising chromatographically sensitivity and resolution.

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