Unifying Processes to Measure Entecavir in Human Matrices in Two Geographically Distinct Laboratories

Q² Solutions
a Quintiles Quest Joint Venture

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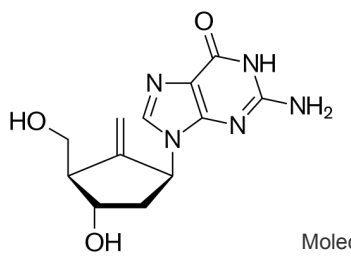
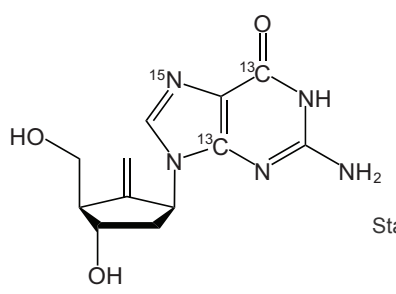
Introduction

- We often receive requests for one-off studies requiring analysis of compounds that are not in the Sponsor's pipeline. These may be in support of drug interaction, comparator, or co-administration studies. In this case, sensitive assays were required for the measurement of entecavir in human plasma and human urine.
- Although the clinical study was to be supported in North America, there was capacity for assay development activities in Europe. This was used as an opportunity to test how transferrable an assay could be between the two facilities. The development and validation were performed in Oss with a partial validation and subsequent sample analysis in Ithaca, New York.



Entecavir
$C_{12}H_{15}N_5O_3$
Molecular Weight: 277.28 Da



Entecavir-[$^{13}C_2$, ^{15}N]
Stable-isotope Labeled Internal Standard (IS)
$C_{10}{}^{13}C_2H_{15}N_4{}^{15}NO_3$
Molecular Weight: 280.26 Da

Entecavir Plasma Assay

Sample Preparation

- All samples were prepared at room temperature.
- Calibration standards and quality control (QC) samples were prepared in human plasma with dipotassium ethylenediaminetetraacetic acid (K_2EDTA) anti-coagulant.
- Calibration curve range was from 25.0 pg/mL to 20,000 pg/mL.
- QC samples were prepared at 75.0, 500, 5000, and 18,000 pg/mL with a dilution QC at 180,000 pg/mL (analyzed with a 10-fold dilution).
- 200 µL of sample was extracted by protein precipitation (PP) using acetonitrile on a Phenomenex 96-well Strata PP plate.
- Following evaporation, samples were reconstituted in 0.1% formic acid in water.

Liquid Chromatography with Tandem Mass Spectrometry (LC/MS/MS) Conditions

Chromatographic Conditions

LC System:	Waters Acquity
LC Column:	Waters BEH C_{18} (2.1 x 50 mm, 1.7 µm particle size)
LC Column Temperature:	30 °C
Mobile Phase A:	0.1% Formic Acid in Water
Mobile Phase B:	Methanol
Initial LC Conditions:	300 µL/min at 1% Mobile Phase B

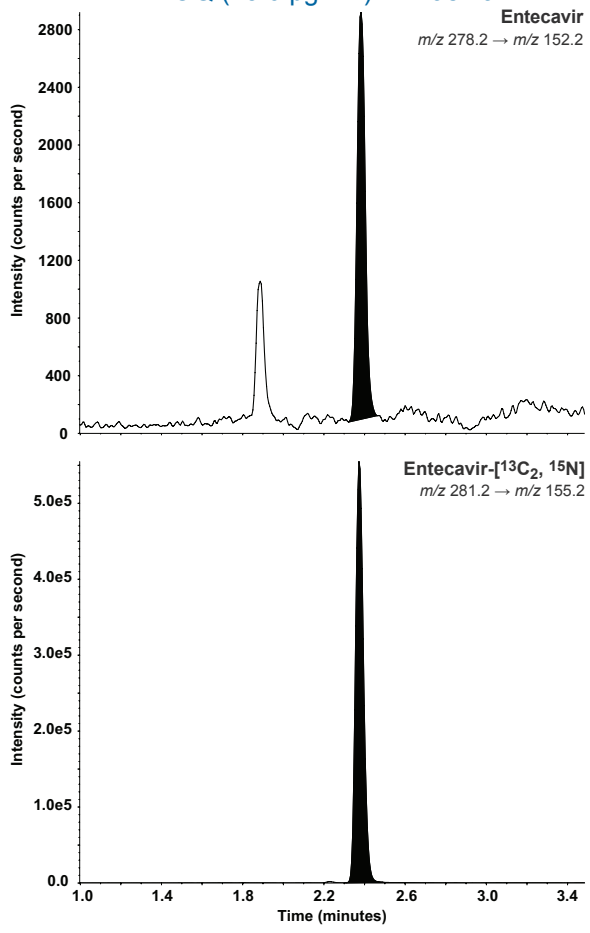
Gradient		
Time (min)	Function	Value
0.0	B Conc. (%)	1
3.0	B Conc. (%)	20
3.1	B Conc. (%)	99
3.5	B Conc. (%)	99
3.6	B Conc. (%)	1
4.0	Stop	

Mass Spectrometry Conditions

Mass Spectrometer:	AB SCIEX API 5000
Ionization:	Positive Ion, Turbo Ion Spray
Desolvation Temperature:	550 °C
Ion Spray Voltage:	4500 V
Dwell Time:	150 ms

Analyte and IS	Transitions Monitored (±0.2 for each mass)	Retention Time
Entecavir	*m/z* 278.2 → *m/z* 152.2	~2.4 minutes
Entecavir-[$^{13}C_2$,^{15}N]	*m/z* 281.2 → *m/z* 155.2	~2.4 minutes

LLOQ (25.0 pg/mL) in Plasma



Urine Assay Development

- Direct adaptation of the plasma LC/MS/MS conditions for analysis of urine extracts proved impossible.
- Using the acidic mobile phase, significant chromatographic interferences were observed so an alternative approach was developed using a basic mobile phase.
- Sensitivity under basic conditions was reduced so larger sample aliquots were necessary to meet the required lower limit of quantitation (LLOQ).

Entecavir Urine Assay

Sample Preparation

- All samples were prepared at room temperature.
- Calibration standards and QC samples were prepared in human urine.
- Calibration curve range was from 25.0 pg/mL to 15,000 pg/mL.
- QC samples were prepared at 75.0, 500, 3000, and 12,000 pg/mL with a dilution QC at 75,000 pg/mL (analyzed with a 10-fold dilution).
- 1000 µL of sample was extracted by solid-phase extraction using a Waters 96-well Oasis MCX LP plate.
- Following evaporation, samples were reconstituted in 100:0.5:0.6 water/1 M ammonium bicarbonate/ammonium hydroxide.

LC/MS/MS Conditions

Chromatographic Conditions same as for plasma except:

LC Column Temperature:	40 °C
Mobile Phase A:	100:0.5:0.6 Water/1 M Ammonium Bicarbonate/ Ammonium Hydroxide

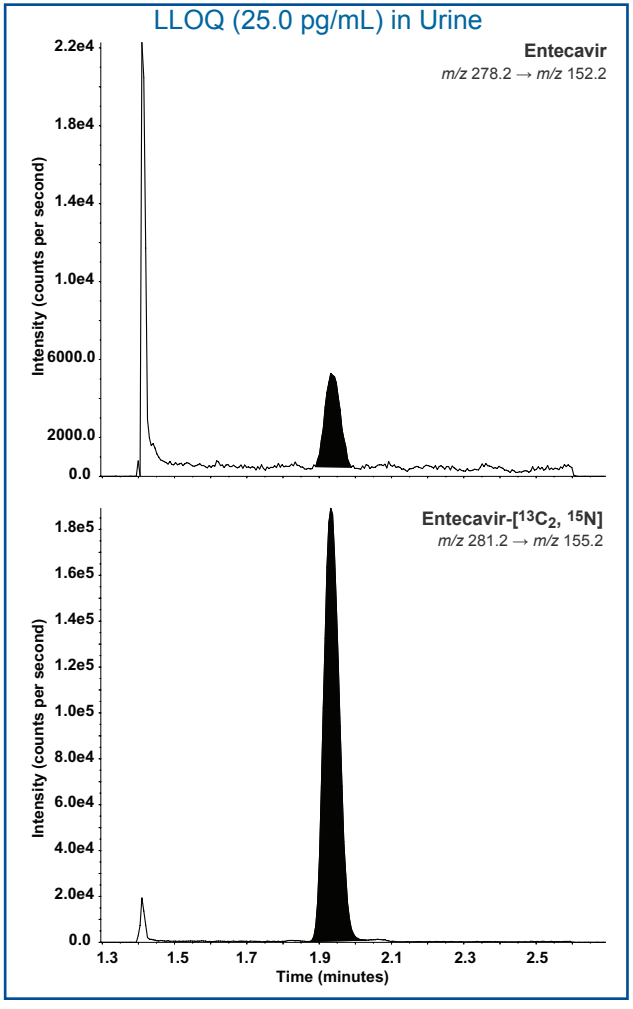
Gradient		
Time (min)	Function	Value
0.0	B Conc. (%)	1
0.5	B Conc. (%)	1
3.0	B Conc. (%)	8
3.5	B Conc. (%)	8
3.6	B Conc. (%)	99
4.0	B Conc. (%)	99
4.1	B Conc. (%)	1
4.5	Stop	

Mass Spectrometry Conditions same as for plasma except:

Ion Spray Voltage:	5500 V

Analyte and IS	Retention Time
Entecavir	~2.0 minutes
Entecavir-[$^{13}C_2$,^{15}N]	~2.0 minutes

LLOQ (25.0 pg/mL) in Urine



Assay Transfer to Ithaca

- Following validation of the entecavir human plasma and urine assays in Oss, the plan was to directly transfer each assay for use in Ithaca. The assays were followed exactly as written except that a Phenomenex Impact PP plate was used in place of the Strata PP plate. Loss of chromatographic retention was observed for both matrices. Troubleshooting the assay isolated the issue to the change in Acquity model (fixed loop versus flow through injection) and the mode of autosampler wash. Altering the weak autosampler wash to match the starting LC conditions fixed the analyte retention issue for both assays.
- A single accuracy and precision run was then performed in each matrix to demonstrate suitability for sample analysis.

Intra-Run Accuracy and Precision in Plasma

Entecavir in Human Plasma

QC (pg/mL)	LLOQQC 25.0	QC1 75.0	QC2 500	QC3 5000	QC4 18000	DilQC (10-fold dilution) 180000
	24.4	71.2	490	4940	17100	179000
	25.6	90.2[a]	497	4930	17400	180000
	24.4	75.8	507	4980	17100	186000
	27.4	73.9	493	4920	17100	184000
	21.8	71.8	494	5020	17200	181000
	22.7	719[b]	491	5090	17600	182000
Mean	24.4	76.6	495	4980	17300	182000
SD	2.00	7.83	6.22	65.4	207	2610
CV (%)	8.2	10.2	1.3	1.3	1.2	1.4
RE (%)	-2.4	2.1	-1.0	-0.4	-3.9	1.1
n	6	5	6	6	6	6

Intra-Run Accuracy and Precision in Urine

Entecavir in Human Urine

QC (pg/mL)	LLOQQC 25.0	QC1 75.0	QC2 500	QC3 3000	QC4 12000	DilQC (10-fold dilution) 75000
	26.9	81.8	549	3240	12600	80600
	25.5	76.9	547	3190	12600	79700
	26.6	77.8	542	3150	12600	78700
	26.2	78.4	552	3220	12700	79500
	25.2	79.2	552	3170	12600	78800
	27.4	78.4	537	3280	12600	80500
Mean	26.3	78.8	547	3210	12600	79600
SD	0.839	1.68	5.96	47.9	40.8	809
CV (%)	3.2	2.1	1.1	1.5	0.3	1.0
RE (%)	5.2	5.1	9.4	7.0	5.0	6.1
n	6	6	6	6	6	6

Precision: CV (%) = (SD/Mean) × 100
Accuracy: RE (%) = [(Mean - Nominal)/Nominal] × 100
[a]Deviation from nominal >15.0%; value included in statistics.
[b]Replicate 6 was deactivated as a statistical outlier.

Conclusions

- Robust assays with adequate sensitivity and selectivity were developed and validated for the measurement of entecavir in human plasma and urine.
- The plasma assay utilized a simple protein precipitation procedure followed by LC/MS/MS under acidic conditions.
- Direct adaptation of the plasma LC/MS/MS conditions to urine analysis was not possible due to excessive chromatographic interference.
- Solid-phase extraction followed by LC/MS/MS using a basic mobile phase gave clean extracts for the urine assay.
- A change in Acquity platform between sites meant that a change in the wash composition was necessary but no other alterations were made to the assays.
- Transfer to a geographically distinct lab for sample analysis was successful for both matrices.
- Sample analysis has proven to be reproducible with a 100% passing rate for incurred sample reanalysis (data not shown).

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