The development and validation of a novel LC-MS/MS method for the quantification of Cenicriviroc in human plasma and cerebrospinal fluid

Journal:	*Biomedical Chromatography*
Manuscript ID	BMC-19-0212.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	05-Jul-2019

Complete List of Authors:	Dilly Penchala, Sujan ; University of Liverpool, Department of Molecular and Clinical Pharmacology Alagaratnam, Jasmini ; Imperial College London, Division of Infectious Diseases, Department of Medicine Challenger, Elizabeth ; University of Liverpool, Department of Molecular and Clinical Pharmacology AMARA, Alieu; University of Liverpool, Department of Molecular and Clinical Pharmacology Else, Laura ; University of Liverpool, Department of Molecular and Clinical Pharmacology Winston, Alan; Imperial College London, Division of Infectious Diseases, Department of Medicine Khoo, Saye ; University of Liverpool, Department of Molecular and Clinical Pharmacology; Royal Liverpool University Hospital

Keywords:	Cenicriviroc, LC-MS/MS, Plasma, CSF, Human serum albumin

SCHOLARONE™
Manuscripts

The development and validation of a novel LC-MS/MS method for the quantification of Cenicriviroc in human plasma and cerebrospinal fluid.

Authors:

Sujan Dilly Penchala[1], Jasmini Alagaratnam[2], Elizabeth Challenger[1], Alieu Amara[1], Laura Else[1], Alan Winston[2] and Saye Khoo[1,3]

1. Department of Pharmacology, University of Liverpool, Liverpool L69 7SX, UK
2. Division of Infectious Diseases, Department of Medicine, Imperial College London, London W2 1PG
3. Royal Liverpool University Hospital, Prescot Road, Liverpool L7 8XP

Corresponding Authors:

Sujan Dilly Penchala

Department of Pharmacology, University of Liverpool, Liverpool L69 7SX, UK

P: +44 151 706 4045

E: sujan@liverpool.ac.uk

Word count:

Abstract	135
Manuscript	2469

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

28 # Abstract

29 A high performance liquid chromatography tandem mass spectrometric method was developed
30 and validated cenicriviroc quantification in human plasma and cerebrospinal fluid. The method
31 involved precipitation with acetonitrile and injecting supernatants onto the column. Separation
32 was achieved on an XBridge C_{18} column with a gradient elution of 0.1% formic acid in water
33 and acetonitrile. Analyte detection was conducted in positive ion mode using SRM. The m/z
34 transitions were: CVC (697.3→574.3) and CVC-d7 (704.4→574.3). Calibration curve ranged
35 from 5-1000 ng/ml for plasma and 0.241-15.0 ng/ml for CSF. The intra and inter day precision
36 and accuracy were <15% for both plasma and CSF across four different concentrations.
37 Cenicriviroc recovery from plasma and artificial CSF was >90%. The method was utilised for
38 the measurement of patients' plasma and CSF samples taking a dose of 50, 150 and 300mg qd.

39
40
41
42
43
44
45

46 **Keywords: Cenicriviroc, LC-MS/MS, Plasma, CSF, human serum albumin**

47
48
49
50
51
52
53
54
55
56

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

57 **Introduction**

58 Cenicriviroc (CVC) is a novel potent dual antagonist of the chemokine co-receptors 5 and 2
59 (CCR5/CCR2) and blocks HIV-1 entry (Kramer, Hassounah, Colby-Germinario, Oliveira,
60 Lefebvre, Mesplede and Wainberg 2015). CVC has been tested in Phase IIb trials, for the
61 treatment of non-alcoholic steatohepatitis in adult subjects with liver fibrosis and consistently
62 demonstrated an improvement in liver fibrosis (Tacke 2018). Cenicriviroc is also expected to
63 have potent anti-inflammatory activity (Lalezari, Gathe, Brinson, Thompson, Cohen, Dejesus,
64 Galindez, Ernst, Martin and Palleja 2011). Furthermore, CVC presents a long half-life (~40hrs)
65 supporting once-daily dosing (Marier, Trinh, Pheng, Palleja and Martin 2011, S. Palleja 2009).
66 The replication of HIV RNA in the central nervous system (CNS) creates the potential for HIV-
67 associated cognitive impairment. It is postulated that CCR5 antagonists could target HIV RNA
68 replication in sanctuary sites a method previously shown to have beneficial effects in the CNS
69 (Garvey, Nelson, Latch, Erlwein, Allsop, Mitchell, Kaye, Watson, Back, Taylor-Robinson and
70 Winston 2012). This method was developed and validated to assess Cenicriviroc in in plasma
71 and cerebrospinal fluid (CSF) as part of a Phase II feasibility study, (EudraCT 2015-002955-
72 85),

73

74 Plasma levels of Cenicriviroc have previously been quantified using liquid chromatography/
75 tandem mass spectrometry (LC-MS/MS) (Lefebvre, Gottwald, Lasseter, Chang, Willett, Smith,
76 Somasunderam and Utay 2016), but the methods have not been published. Also, to our
77 knowledge, there are no CSF methods published either. We present here a simple tandem mass
78 spectrometric method for quantification of Cenicriviroc in plasma and in CSF using protein
79 precipitation extraction and validated in accordance with EMA and FDA guidelines (2012,
80 2018).

81

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

82 ## Materials and Methods

83

84 ### Chemicals

85 CVC and CVC-d7 were obtained from Allergan Inc, Irvine, California; methanol and
86 acetonitrile (LC-MS grade) were obtained from Sigma–Aldrich. Deionized water (HPLC
87 grade) was obtained from an Elga Option 4 water purifier (Elga LabWater, High Wycombe,
88 United Kingdom). Further purification to 18.2 MΩ was carried out using a Purelab Classic
89 UVF (Elga LabWater). Whole blood, collected in K_2EDTA, was obtained from healthy drug-
90 free volunteers with Ethics approval from the NHS Health Research Authority. Artificial CSF
91 was obtained from Harvard Apparatus and Human Serum Albumin from Sigma–Aldrich, UK.

92

93 ### Equipment

94 The chromatographic system was made up of a variable loop Accela autosampler (temperature,
95 6°C) and an Accela LC pump (Thermo Fisher Scientific, Hemel Hempstead, United Kingdom).
96 The analyte and internal standard (IS) were eluted using a reverse-phase C_{18} XBridge column
97 (3.5 µm:50 mm × 2.1 mm; Waters UK) with a guard column (at an oven temperature of 40°C.
98 The mass spectrometer was a triple–quadrupole TSQ Quantum Access (Thermo Fisher
99 Scientific, UK) with a heated-electrospray ionization source. Tuning, data acquisition and
100 processing were carried out using TSQ Tune and LC Quan Software (Thermo Fisher Scientific)
101 respectively.

102

103 ### Stock solution preparation

104 Methanolic stock solutions (1 mg/mL) were prepared, with working solutions containing (100
105 µg/mL and 10 µg/mL of CVC) prepared by diluting the stock solution with more methanol.
106 The internal standard working solutions for plasma (100 ng/mL of CVC-d7) and CSF (10
107 ng/mL of CVC-d7) were also prepared in methanol.

108

109 For plasma, working calibration sub-stock solutions were prepared from 100µg/mL by diluting
110 in the appropriate volume of methanol to a final concentration of 50, 12.5 and 0.625 µg/ml.
111 Working calibration standards were prepared by spiking blank human plasma to yield 12.5,
112 250, and 1000 ng/mL CVC calibration standard solutions. The working calibration standard
113 solutions were serially diluted to yield final plasma concentrations of 5.0, 12.5, 50.0, 125.0,

For Peer Review

250.0, 500.0, 800.0, and 1000.0 ng/mL of CVC. All standards were prepared using calibrated air-displacement pipettes.

For CSF, working calibration sub-stock solutions were prepared from 10 µg/mL by diluting in the appropriate volume of methanol to a final concentration of 0.012, 0.019, 0.049, 0.097, 0.195, 0.433, 0.623 and 0.750 µg/mL. Calibration curves were prepared by spiking the working calibration sub-stock solution into artificial CSF containing 0.5% human serum albumin, yielding eight calibration standards (0.241, 0.389, 0.973, 1.947, 3.894, 8.653, 12.45, 15.0ng/mL). Quality Control (QC) samples were prepared from the CVC QC primary stock. These consisted of High QC (750 ng/mL), Medium QC (150 ng/mL), Low QC (11.3 ng/mL; 3x the lowest calibration standard concentration) and the lower limit of quantification (LLQ; 5.0 ng/mL) for plasma and for CSF (HQC; 12.50, MQC; 2.25, LQC; 0.720 and LLQ; 0.241 ng/mL).

Non-specific binding of Cenicriviroc in CSF

Hydrophobic compounds are often lost due to adsorption onto glass and plastic lab-ware when in low-protein biological matrices such as urine and CSF (Gu, Deng, Wang, Aubry and Arnold 2010, Ji, Jiang, Livson, Davis, Chu and Weng 2010, P.Nouri 2016). Minimising or resolving the adsorption loss is often achieved using a variety of methods including: addition of plasma, bovine serum albumin or surfactants such as Tween 20, sodium dodecylbenzenesulfonate or 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)(Chen, Bajpai, Mollova and Leung 2009, Silvester and Zang 2012, Xu, Du, Rose, Fu, Woolf and Musson 2005)

There was a significant loss of Cenicriviroc observed in aCSF, with increased variability from the nominal concentrations (%Bias) and within replicates. This loss was observed across the entire calibration range from 0.24 ng/mL to 15 ng/mL. This was due to non-specific binding of Cenicriviroc to the glass tubes when serial dilutions were performed in CSF. Separate preparation of individual calibrant levels in pure methanol, to eliminate the serial dilution step, followed by direct spiking into blank CSF did not reduce this effect.

The addition of human serum albumin at different concentrations (0.01-1%) was evaluated as a method of preventing the non-specific binding of CVC in CSF samples. Based on the results, artificial CSF was pre-treated with 0.5% human serum albumin before the preparation of calibration standards and quality control samples. (Table 1).

For Peer Review

The addition of 0.5% human serum albumin (w/v) greatly improved analyte response and decreased the variability.

TABLE 1

Sample Preparation

CVC was extracted from both plasma and CSF by protein precipitation using acetonitrile. Standards, QCs, blank and study samples (100µl) were aliquoted into 5mL glass test tubes, to each of which internal standard working solution (CVC-d7; 20µl) was added. 100µl of 0.1% formic acid solution in water was also added to the mixture followed by precipitation with 500µl of acetonitrile. Samples were vortexed for approximately 1 minute and then centrifuged (4000 rpm, 4°C, 5 minutes) and the supernatant transferred to autosampler vials. The vials were loaded onto autosampler trays and 5µL injected into the LC-MS/MS system for analysis.

LC-MS/MS Procedure

Chromatographic separation was achieved using a Waters C_{18} XBridge column (3.5µm: 50 mm × 2.1 mm) and 5mM ammonium acetate solution with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). Cenicriviroc was eluted from the column using a gradient method at a flow rate of 400µl/min. Mobile phase gradient started with 80% mobile phase A, which was held for 0.3 minutes then increasing in organic content to 85% mobile phase B in 0.8 minutes. This was maintained ~~over~~ up to 2.5 minutes followed by increasing the organic content to 100% ~~over~~ for 1~~3.5~~ minute~~s~~ and back to the initial conditions for reconditioning with a total run time of 5 minutes.

The electrospray ionisation (ESI) mass spectrometer (MS) was operated in positive ion mode using selective reaction monitoring (SRM). These were the MS settings:- electrospray voltage, 5.0kV; capillary temperature, 270°C; vaporiser temperature, 350°C; sheath and auxiliary gas pressures, 50 and 20 arbitrary units respectively. The collision gas Argon, was delivered at a pressure of 1.5mTorr with collision energy set to 20 and tube lens set to 50. The m/z transitions for CVC was 697.3→574.3 and CVC-d7, 704.4→574.3 and. The scan width was set at 0.01 and the scan time at 0.1 seconds. The peak width settings for Q1 and Q3 were set at unit resolution (0.7).

For Peer Review

Initial chromatographic optimisation was with a conventional C_{18} column using acetonitrile and formic acid as additives at low concentrations in mobile phase but this resulted in peak tailing. Altering the pH and buffers at to different concentrations did not improve the peak shape. Cenicriviroc ~~has~~ is a ~~strong~~ weak polyprotic bas~~ic~~e ~~pKa value~~ (pKa=6.39), making the molecule prone to secondary interaction with silanes of the column. Changing the column to a high purity silica type with end-capping was considered, in order improve the peak shape. The XBridge C_{18} column is known for its Ethylene Bridged Hybrid (BEH) technology and advanced end-capping. The previously used mobile phase was not sufficient to get a reasonable signal and a good peak shape using the XBridge column. However, addition of 5mM ammonium acetate to water containing 0.1% formic acid resulted in sharper peaks with excellent sensitivity.

Validation methodology

Selectivity

Six different lots of blank human plasma were used in this assessment. Selectivity was determined at the LLQ, where the precision should be ≤20% and accuracy within 20% of the nominal concentrations. (n = 6). Also, interference or noise response at the same retention time of internal standard were deemed acceptable if the % interference was less than 5% of the mean response of the internal standard areas in 6 LLQ samples.

Accuracy and Precision

Three separate accuracy and precision batches were run consisting of a calibration curve and LLQ, LQC, MQC and HQC samples in replicates of six. Data from the manufacturers' observed C_{max} following standard dosing (and adjusted dosing based on interaction with other ART agents that induce or inhibit hepatic iso-enzymes) served as a guide to setting the calibration range.

Carryover

The carryover was assessed by injecting blank samples, followed by assay LLQ (5 ng/mL) and upper limit of quantification (ULQ; 1000 ng/mL) standards, ~~were~~ run in duplicate. This was followed by 3 blank plasma samples. The % carryover (in the blank samples after ULQ) was

For Peer Review

calculated ~~after each subsequent run,~~ and expressed in relation to the assay LLQ; the % carryover should not exceed 20% of the LLQ concentration (EMA Bioanalytical method validation) .

Dilution integrity

CVC concentrations between 160-180% of the assay ULQ was spiked into plasma, which was subsequently diluted 1:2 and 1:4 with blank plasma. The samples were then analysed, with concentrations from the standard curve (including the appropriate dilution factor), and compared against the expected nominal concentration.

Recovery and Matrix effects

The % recovery and matrix effects were determined quantitatively using the methods of Matuszewski *et al* (Matuszewski, Constanzer and Chavez-Eng 2003). The % recovery (process efficiency) was obtained by comparing the peak-area of the analyte at LQC/MQC/HQC concentrations from extracted plasma samples, to the peak area of analyte spiked at an equivalent concentration in mobile phase. The % matrix effect compared the peak areas of CVC spiked into blank plasma extracts with the peak areas of CVC in mobile phase at an equivalent concentration.

Stability

The stability of Cenicriviroc in plasma and CSF under different conditions (QC samples; 6 per level) was assessed after:- heat inactivation at 58°C for 40 minutes; and over 3 freeze-thaw cycles spanning a period of 3 days. Bench-top stability was also assessed at room temperature over 6 hours (n=4, per level); and furthermore, by re-injecting an accepted precision and accuracy batch (6 QC per level) which had been left in the autosampler at 4°C for 48 hours, reinjection reproducibility was assessed.

Measurement of Cenicriviroc in human plasma and CSF

Blood samples for the pharmacokinetic analysis of CVC in a phase II open-label feasibility study in 7 HIV-positive volunteers were analysed using this method. Of the seven participants, only four completed all study procedures (EudraCT 2015-002955-85). The study was conducted at St Mary's Hospital, Imperial College Healthcare NHS Trust, London, UK, between March and December 2016. Human ethics approval was obtained (The Brighton and

For Peer Review

1
2
3 238 Sussex Research Ethics Committee, UK, reference: 15-LOC-1887). Cenicriviroc was
4
5 239 administered once daily in the morning with food, for 8 weeks. Dose was 150 mg daily when
6
7 240 administered with ART without significant effects on hepatic isoenzymes. This dose was
8
9 241 reduced to 50 mg daily or increased to 300 mg daily when administered alongside ART with
10
11 242 significant inhibition effects (HIV protease inhibitors) or with significant induction effects
12 243 (efavirenz) on hepatic isoenzymes, respectively. Blood was drawn at baseline and week 8.
13
14 244 Collected whole blood was immediately placed on ice until centrifugation to separate plasma
15
16 245 from the blood cells. Plasma was aliquoted and stored at −40 °C until analysis. The subjects
17 246 had their CSF collected by lumbar puncture. The CSF samples were aliquoted and immediately
18
19 247 placed at−40 °C freezer until analysis.
20
21
22 248
23
24
25 249
26
27
28 250 **Method Validation**

29
30
31 251 **Selectivity**

32
33
34 252 There was minimal background interference (<10% of the signal response at the LLQ for
35 253 CVC) and suitable selectivity in all six plasma batches chosen.
36
37
38 254 **Accuracy and Precision**

39
40
41 255 The accuracy and precision (both inter- and intra-assay) values at the assay LLQ fell within the
42 256 designated ±20% and were within ±15% of the nominal values for all QC levels. (**Table 2**).
43
44 257
45
46
47 258 **TABLE 2**
48
49
50 259 **Carryover**

51
52 260 The mean % carryover (n=3) observed in the first extracted blank sample following injection
53
54 261 of an ULQ sample (1000 ng/mL) was 0.13% for CVC. This represented 26.9% of the assay
55
56 262 LLQ that, upon injection of the second blank plasma sample, reduced to 0.03% for CVC which
57 263 is equivalent to 5.8% of the assay LLQ.
58
59
60 264 **Dilution integrity**

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

265 Samples diluted by a factor of 2 and 4 times showed calculated concentrations within ± 15%
266 of the nominal values. The %CV for the diluted samples was <10% for Cenicriviroc.

267

268 **Recovery and Matrix effects**

269 Matrix effects (ME), Recovery Efficiency (RE), and Process Efficiency (PE) were studied for
270 both plasma and CSF matrices each at three different concentrations. Overall recovery is
271 >90% in both plasma and CSF with negligible matrix effect; the data can be seen in **Table 3**.

272 **TABLE 3**

273

274 **Stability**

275 Stability data are presented in **Table 4**. Samples were stable for up to 48 hours following re-
276 injection within the LC-MS/MS autosampler (4°C) with concentrations within ±15% of the
277 respective nominal values. Cenicriviroc was also stable after heat treatment and through 3
278 freeze-thaw cycles (n=4). Furthermore, when left on the bench at room temperature, the
279 samples were stable for up to 6 hours.

280

281 **TABLE 4**

282

283 **Application of method to clinical pharmacokinetic study**

284 All subjects had plasma and cerebrospinal fluid cenicriviroc concentrations below the LLQ at
285 baseline. At week eight, peak plasma cenicriviroc concentrations was detectable in all four
286 subjects, but detectable in the cerebrospinal fluid in only two subjects (mean 0.82 and 0.40
287 ng/mL respectively), and below the LLQ in the other two subjects. Mean cerebrospinal fluid:
288 plasma cenicriviroc concentration ratio was no more than 0.18% (95% CI of the upper estimate
289 0.09% – 0.28%).(Alagaratnam, Dilly-Penchala, Challenger, Else, Legg, Petersen, Jones,
290 Kulasegaram, Seyedkazemi, Lefebvre, Khoo and Winston).

291

292 **TABLE 5**

293

294

For Peer Review

1
2
3 295
4
5 296
6
7 297
8

Conclusion

In this study, a sensitive, selective, accurate and robust LC–MS/MS method was developed and validated to quantify cenicriviroc in human plasma and CSF. This, to the best of our knowledge, is the first method quantifying cenicriviroc concentrations in CSF.

Furthermore, this assay will provide a greater understanding of CVC pharmacokinetics across different individuals under different treatment scenarios. The incurred sample analysis met the acceptance criteria and as previously mentioned, the method has been used to study the pharmacokinetics of cenicriviroc in plasma and CSF as part of a clinical trials.

Acknowledgments

The authors express their thanks to Allergan Pharmaceuticals for providing the compounds.

For Peer Review

Legends

Figure 1. Chromatograms of cenicriviroc and cenicriviroc-d7 in **CSF** in a) Blank aCSF sample b) Spiked with 0.24ng/ml (LOQ) c) Patient sample at week 8 d) Internal standard CVC-d7

Figure 2. Chromatograms of cenicriviroc and cenicriviroc-d7 in **Plasma** e) Chromatogram of Blank plasma sample f) Spiked with 5ng/ml (LOQ) g) Patient sample at week 8 h) Internal standard CVC-d7

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

322 **References**

323 FDA Guidance for Industry, Bioanalytical Method Validation. US Food and Drug Administration. 2018.

324 Alagaratnam J, Dilly-Penchala S, Challenger E, Else L, Legg K, Petersen C, Jones B, Kulasegaram R,
325 Seyedkazemi S, Lefebvre E, Khoo S and Winston A Cerebrospinal fluid exposure of cenicriviroc in HIV-
326 positive individuals with cognitive impairment. Br J Clin Pharmacol; **0** (0). DOI: 10.1111/bcp.13878.

327 Chen C, Bajpai L, Mollova N and Leung K Sensitive and cost-effective LC-MS/MS method for
328 quantitation of CVT-6883 in human urine using sodium dodecylbenzenesulfonate additive to eliminate
329 adsorptive losses. J Chromatogr B Analyt Technol Biomed Life Sci 2009; **877** (10): 943-7. DOI:
330 10.1016/j.jchromb.2009.02.045.

331 Garvey L, Nelson M, Latch N, Erlwein OW, Allsop JM, Mitchell A, Kaye S, Watson V, Back D, Taylor-
332 Robinson SD and Winston A CNS effects of a CCR5 inhibitor in HIV-infected subjects: a pharmacokinetic
333 and cerebral metabolite study. J Antimicrob Chemother 2012; **67** (1): 206-12. DOI:
334 10.1093/jac/dkr427.

335 Gu H, Deng Y, Wang J, Aubry AF and Arnold ME Development and validation of sensitive and selective
336 LC-MS/MS methods for the determination of BMS-708163, a gamma-secretase inhibitor, in plasma
337 and cerebrospinal fluid using deprotonated or formate adduct ions as precursor ions. J Chromatogr B
338 Analyt Technol Biomed Life Sci 2010; **878** (25): 2319-26. DOI: 10.1016/j.jchromb.2010.06.041.

339 Ji AJ, Jiang Z, Livson Y, Davis JA, Chu JX and Weng N Challenges in urine bioanalytical assays:
340 overcoming nonspecific binding. Bioanalysis 2010; **2** (9): 1573-86. DOI: 10.4155/bio.10.114.

341 Kramer VG, Hassounah S, Colby-Germinario SP, Oliveira M, Lefebvre E, Mesplede T and Wainberg MA
342 The dual CCR5 and CCR2 inhibitor cenicriviroc does not redistribute HIV into extracellular space:
343 implications for plasma viral load and intracellular DNA decline. J Antimicrob Chemother 2015; **70** (3):
344 750-6. DOI: 10.1093/jac/dku451.

345 Lalezari J, Gathe J, Brinson C, Thompson M, Cohen C, Dejesus E, Galindez J, Ernst JA, Martin DE and
346 Palleja SM Safety, efficacy, and pharmacokinetics of TBR-652, a CCR5/CCR2 antagonist, in HIV-1-
347 infected, treatment-experienced, CCR5 antagonist-naive subjects. J Acquir Immune Defic Syndr 2011;
348 **57** (2): 118-25. DOI: 10.1097/QAI.0b013e318213c2c0.

349 Lefebvre E, Gottwald M, Lasseter K, Chang W, Willett M, Smith PF, Somasunderam A and Utay NS
350 Pharmacokinetics, Safety, and CCR2/CCR5 Antagonist Activity of Cenicriviroc in Participants With Mild
351 or Moderate Hepatic Impairment. Clin Transl Sci 2016; **9** (3): 139-48. DOI: 10.1111/cts.12397.

352 Marier JF, Trinh M, Pheng LH, Palleja SM and Martin DE Pharmacokinetics and pharmacodynamics of
353 TBR-652, a novel CCR5 antagonist, in HIV-1-infected, antiretroviral treatment-experienced, CCR5
354 antagonist-naive patients. Antimicrob Agents Chemother 2011; **55** (6): 2768-74. DOI:
355 10.1128/AAC.00713-10.

For Peer Review

Matuszewski BK, Constanzer ML and Chavez-Eng CM Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 2003; **75** (13): 3019-30.

P.Nouri CHALLENGES IN CSF SAMPLE COLLECTION: NON-SPECIFIC BINDING AND BIOANALYSIS OF AD BIOMARKERS. Alzheimer's & Dementia 2016; **12** (7): 1140-P1141.

S. Palleja LW-S, R. Ogden, D. Martin, R. Driz, J. Sapirstein. *TBR-652, A CHEMOKINE RECEPTOR 5 (CCR5) ANTAGONIST, DEMONSTRATES GOOD ORAL BIOAVAILABILITY AND DESIRABLE PHARMACOKINETIC (PK) AND SAFETY PROFILES IN HEALTHY VOLUNTEERS* 49th ICAAC. San Francisco, CA, 2009.

Silvester S and Zang F Overcoming non-specific adsorption issues for AZD9164 in human urine samples: consideration of bioanalytical and metabolite identification procedures. J Chromatogr B Analyt Technol Biomed Life Sci 2012; **893-894**: 134-43. DOI: 10.1016/j.jchromb.2012.03.004.

Tacke F Cenicriviroc for the treatment of non-alcoholic steatohepatitis and liver fibrosis. Expert Opin Investig Drugs 2018; **27** (3): 301-311. DOI: 10.1080/13543784.2018.1442436.

Xu Y, Du L, Rose MJ, Fu I, Woolf EJ and Musson DG Concerns in the development of an assay for determination of a highly conjugated adsorption-prone compound in human urine. J Chromatogr B Analyt Technol Biomed Life Sci 2005; **818** (2): 241-8. DOI: 10.1016/j.jchromb.2005.01.004.

For Peer Review

LIST OF TABLES

Table 1. Variability from nominal concentrations (%Bias) observed before and after pre-treatment with human serum albumin (0.5% w/v)

Nominal Concentration (ng/mL)	Untreated artificial CSF		Artificial CSF pre-treated with 0.5% serum albumin human	
	Duplicate back calculated concentration (ng/mL)	%Bias	Duplicate back calculated concentration (ng/mL)	%Bias
0.241	0.309	28.2	0.200	-17.0
	0.277	14.9	0.280	16.0
0.389	0.336	-13.6	0.416	7.0
	0.347	-10.8	0.393	1.1
0.973	0.453	-53.4	0.898	-7.7
	0.548	-43.7	0.922	-5.2
1.947	1.292	-33.6	1.714	-12.0
	0.809	-58.4	1.821	-6.5
3.894	6.402	64.4	4.098	5.3
	4.681	20.2	3.699	-5.0
8.653	9.673	11.8	8.118	-6.2
	11.139	28.7	8.317	-3.9
12.450	14.520	16.6	15.043	20.8
	16.623	33.5	14.053	12.9
15.000	14.326	-4.5	16.535	10.2
	14.931	-0.5	15.884	5.9

For Peer Review

Table 2. Accuracy & Precision (inter- and intra-day) for CVC in Plasma & CSF

Plasma	LLQ (5 ng/mL)				LQC (11.3 ng/mL)				MQC (150 ng/mL)				HQC (750ng/mL)			
	Mean	SD	%CV	%Bias	Mean	SD	%CV	%Bias	Mean	SD	%CV	%Bias	Mean	SD	%CV	%Bias
Inter-day	5.23	0.54	10.26	4.59	11.90	0.82	6.90	5.33	159.52	12.96	8.12	6.35	792.53	54.68	6.90	5.67
Intra-day	5.46	0.305	5.59	9.21	12.38	0.51	4.15	9.58	165.01	4.66	2.83	10.00	789.03	33.90	4.30	5.20
CSF	LLQ (0.241 ng/mL)				LQC (0.720 ng/mL)				MQC (2.25 ng/mL)				HQC (12.5 ng/mL)			
	Mean	SD	%CV	%Bias	Mean	SD	%CV	%Bias	Mean	SD	%CV	%Bias	Mean	SD	%CV	%Bias
Inter-day	0.26	0.04	13.70	9.42	0.72	0.072	10.05	-0.59	2.36	0.32	13.51	3.62	12.48	1.78	14.25	-1.04
Intra-day	0.25	0.04	14.74	4.54	0.74	0.07	9.63	2.36	2.42	0.15	6.26	8.71	13.57	1.42	10.49	8.60

CV - Coefficient of Variation; SD – Standard Deviation; LQC – Low Quality Control samples; MQC – Medium Low Quality Control samples; HQC – High Low Quality Control samples

Table 3. Recovery & matrix effect of Cenicriviroc in plasma and CSF

Matrix	Nominal QC concentration in ng/mL (L, M, H)	Mean peak area A(n=6)	Mean peak area B(n=6)	Mean peak area C(n=6)	Mean peak Area ratio B2	Mean peak Area ratio C2	ME (%) B/A	Ext RE (%) C/B	PE (%) C/A	Analysis RE (%) C2/B2
Plasma	11.3	152234	155868	149635	0.39	0.41	102.4	96.0	98.3	105.1
	150	1896308	1842656	1891241	6.3	5.83	97.2	102.6	99.7	92.5
	750	8873971	8749945	8816279	31.47	28.41	98.6	100.8	99.3	90.3
CSF	0.72	6171	6684	5801	0.40	0.44	108.3	86.8	94.0	109.8
	2.25	27740	28987	31494	1.30	1.41	104.5	108.6	113.5	108.5
	12.5	163512	149438	143166	12.72	11.55	91.4	95.8	87.6	90.8

ME (%) - % Matrix effect; Ext RE (%) - % Extraction Recovery Efficiency; PE (%) - % Process Efficiency; Analysis RE (%) - % Analysis Recovery Efficiency; L – Low, M - Medium, H - High

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 4. Stability data for CVC under different storage conditions

Stability measured	QC	Precision (%CV)	Accuracy (%)
Benchtop (6h)	LQC	10.08	-9.69
	MQC	5.16	1.22
	HQC	10.81	-1.09
Heat inactivation	LQC	6.56	-3.47
	MQC	1.41	7.61
	HQC	4.24	0.679
Autosampler (48h)	LQC	1.79	-8.875
	MQC	5.57	1.016
	HQC	1.01	-1.186
Reinjection reproducibility (48h)	LQC	7.10	1.424
	MQC	4.94	4.614
	HQC	8.35	1.55

QC – Quality Control samples; LQC – Low Quality Control samples; MQC – Medium Low Quality Control samples; HQC – High Low Quality Control samples

For Peer Review

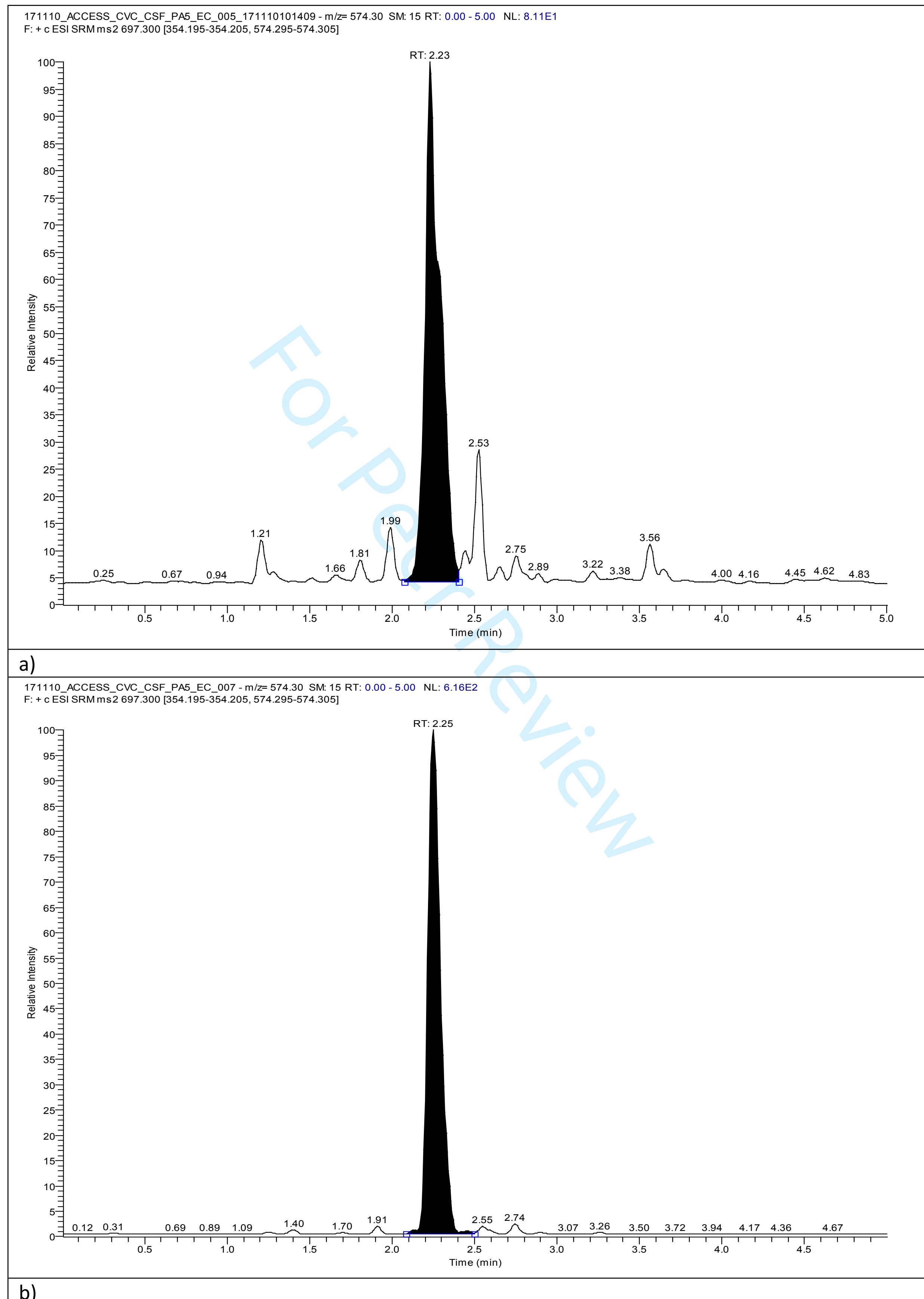
Table 5. Individual subject blood and cerebrospinal fluid concentration at week 8

Cenicriviroc concentration	Subject 1	Subject 2	Subject 3	Subject 4
CSF, ng/mL	0.82	0.40	(<LLQ)	(<LLQ)
Plasma, ng/mL	718.60	211.06	411.93	70.50
CSF: plasma cenicriviroc concentration (%)	0.11	0.19	---	---
Antiretroviral therapy	abacavir, lamivudine, raltegravir	lamivudine, atazanavir, ritonavir	tenofovir DF, emtricitabine, dolutegravir	tenofovir DF, emtricitabine, raltegravir
Cenicriviroc dose	150 mg	50 mg	150 mg	150 mg

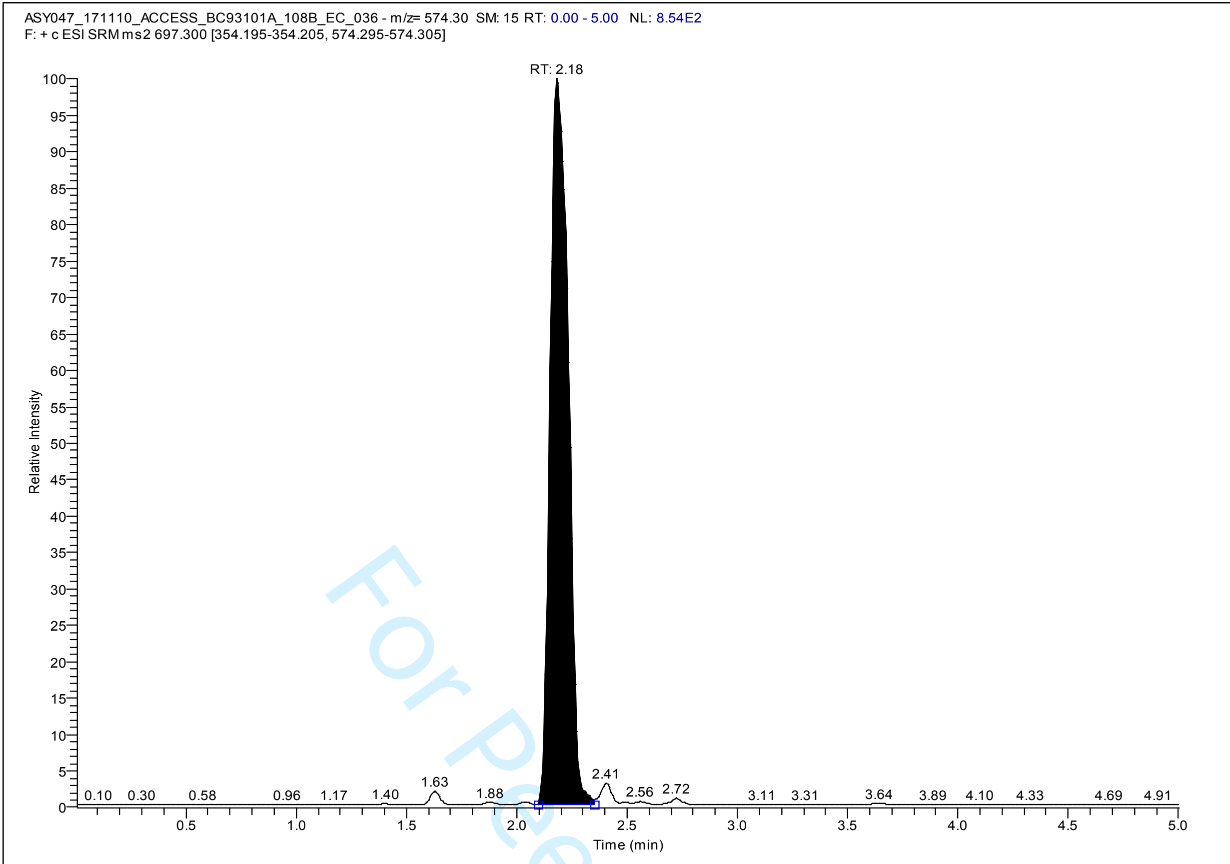
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

LIST OF FIGURES

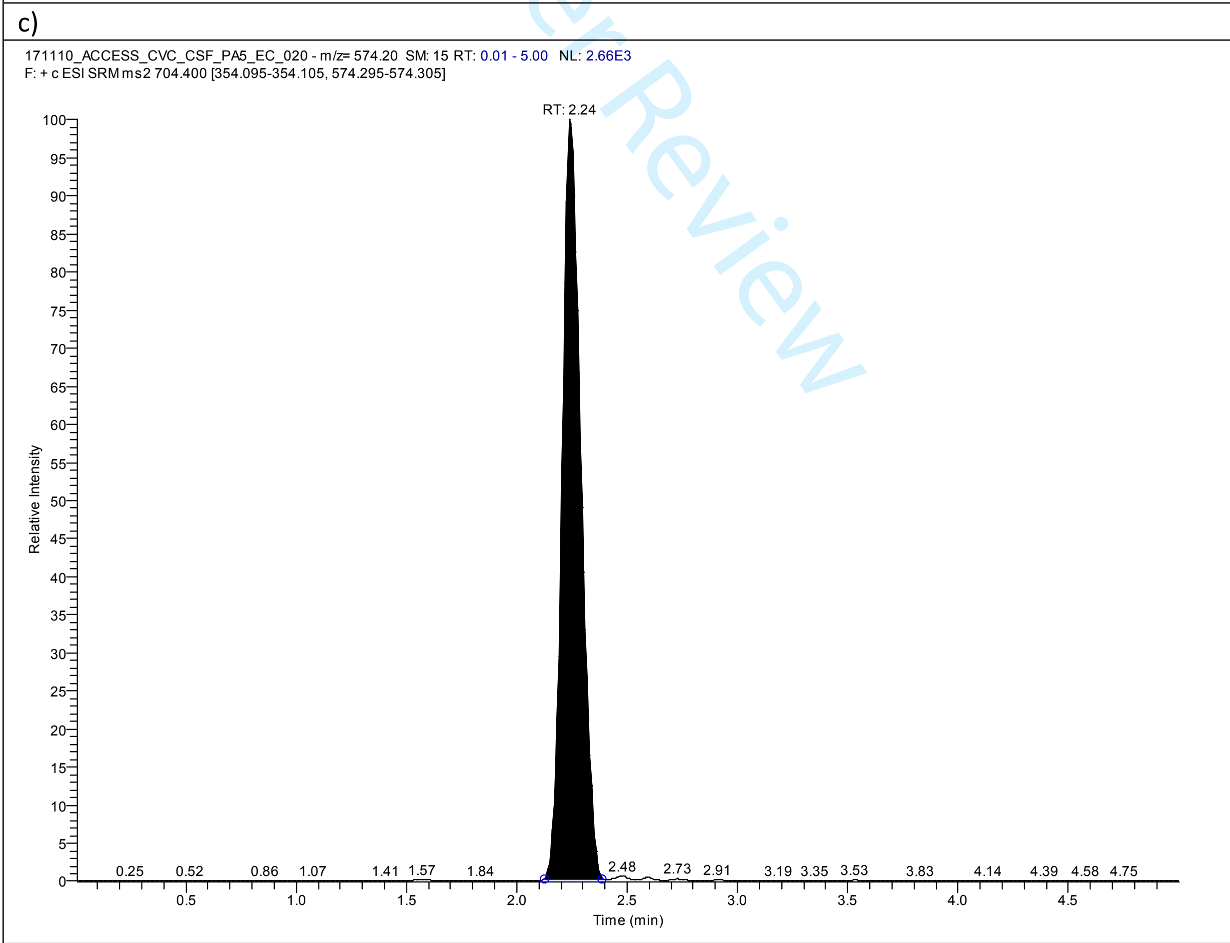
Figure 1. Chromatograms of cenicriviroc and cenicriviroc-d7 in **CSF**



For Peer Review



c)

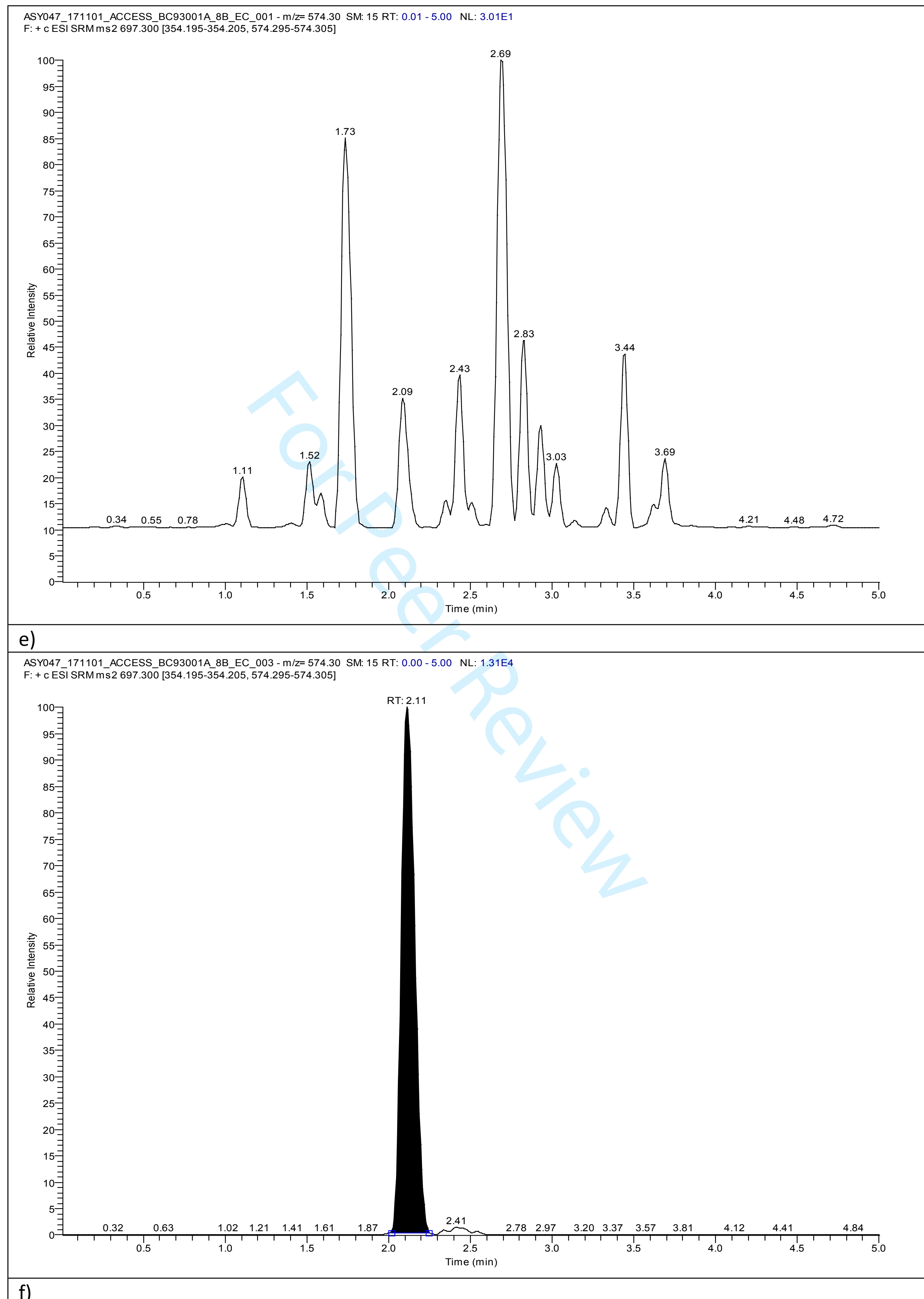


d)

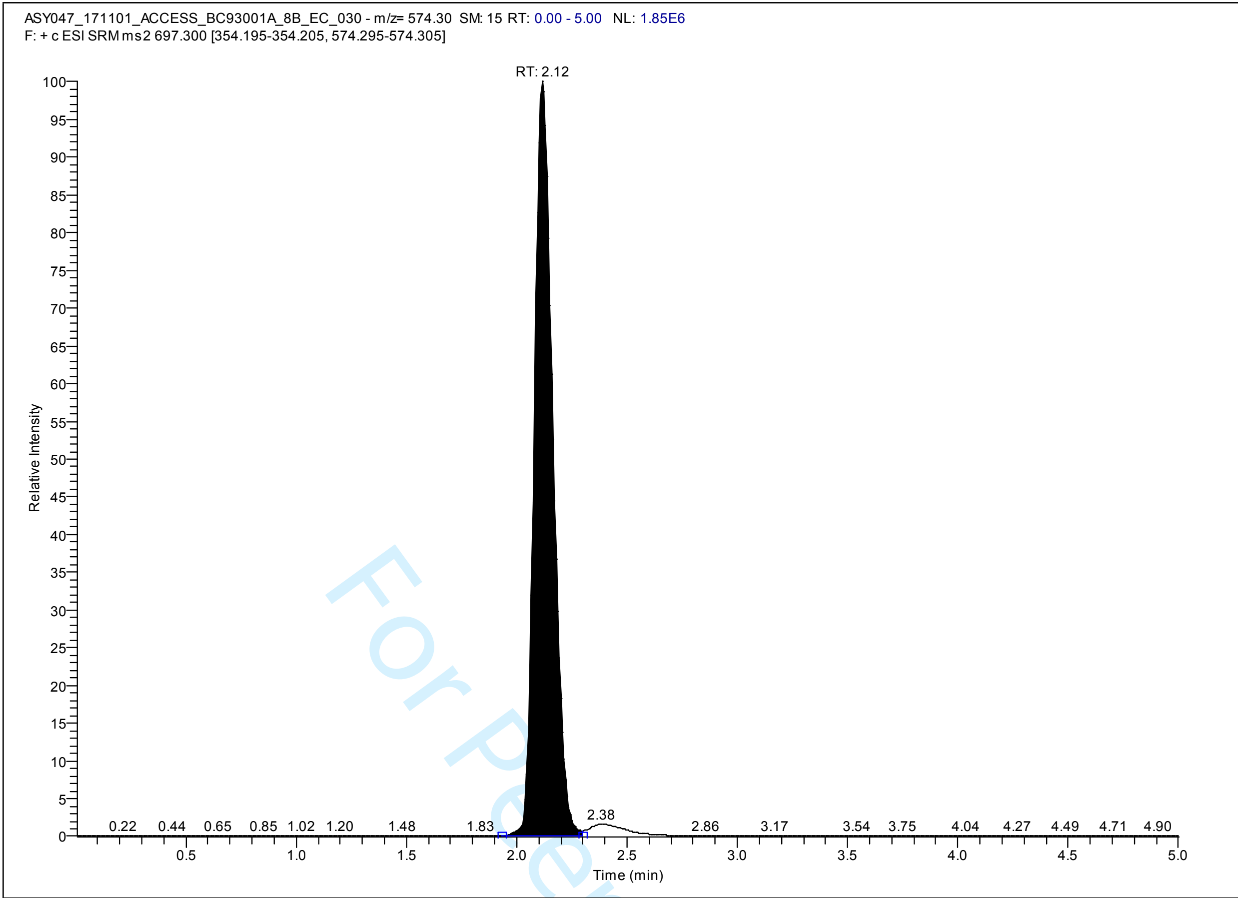
For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

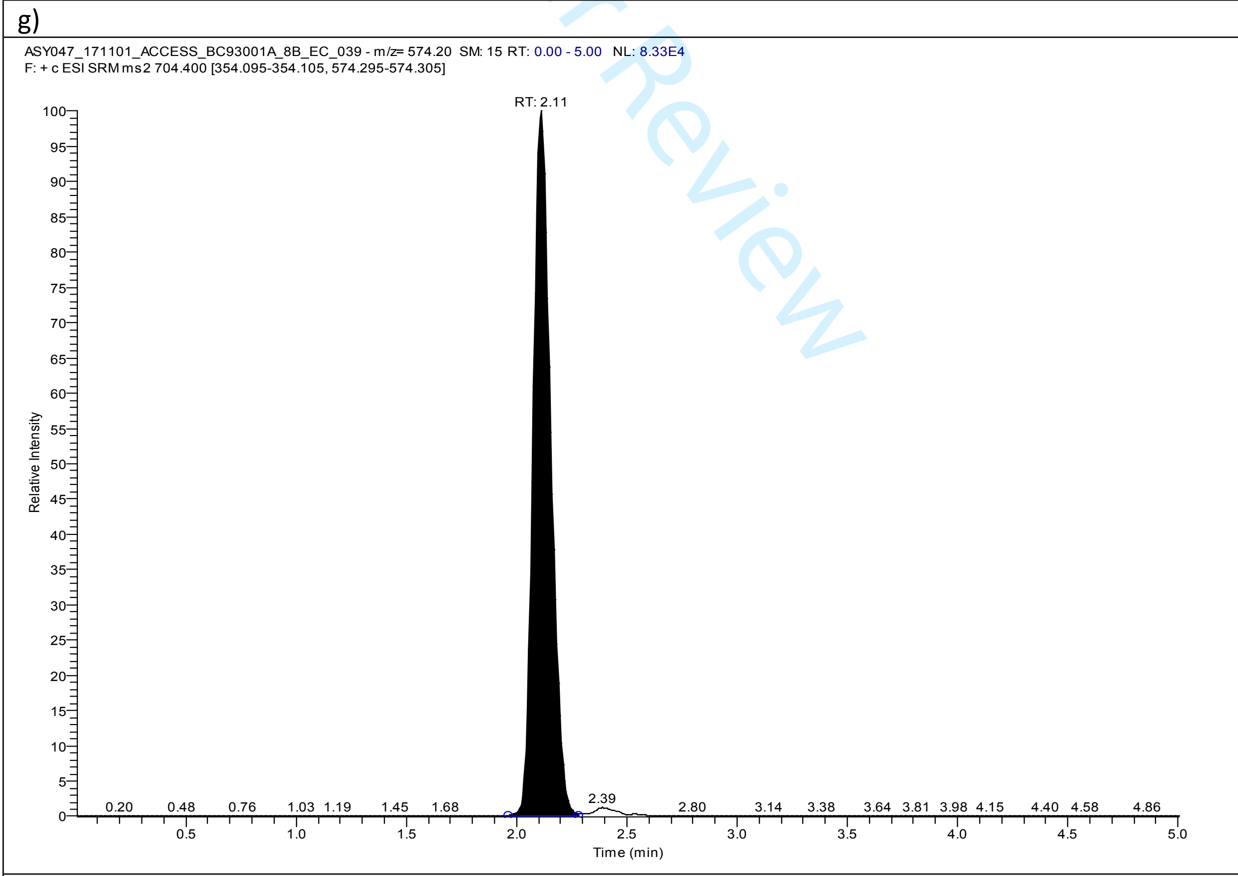
Figure 2. Chromatograms of cenicriviroc and cenicriviroc-d7 in **Plasma**



For Peer Review



g)



h)

For Peer Review