

Non-Ionization Related Matrix Effects on Quantitative Determination of EC78 and its Metabolites in Human Plasma Using LC/MS/MS

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OBJECTIVES

To develop and validate a LC/MS/MS method for the quantitative determination of an anticancer agent (EC78) and its two metabolites, 5-hydroxyl-EC78 and 5-carboxyl-EC78, in human plasma.

METHODOLOGY

SAMPLE PREPARATION

1. Aliquot 50.0 μ L of each sample into 96-well plate.
2. Add 50.0 μ L working internal standard (500 ng/mL of EC78-d3 in 50:50 [v/v] MeOH: water).
3. Vortex mix for ~ 30 seconds.
4. To all samples add 50 μ L of freshly prepared 90:10 MeCN: ammonium hydroxide (~ 28% purity) in water (v/v).
5. Vortex mix for ~ 2 minutes.

PROTEIN PRECIPITATE EXTRACTION

1. Manually add 500 μ L of MeCN to all samples.
2. Cap the sample plate securely.
3. Vortex-mix the samples on a low to medium setting for 3 minutes.
4. Centrifuge the samples at ~ 3000 rpm for ~ 5 minutes to concentrate the precipitate.
5. Transfer the supernatant into a clean 96-well plate.
6. Evaporate the samples to dryness at ~ 40°C under nitrogen in a Turbovap (~ 40 minutes).
7. Reconstitute the samples with 200 μ L of 30:70(v/v) MeOH:H₂O.
8. Vortex-mix the samples using the multi-tube vortexer for ~ 2 minutes on a low setting.
9. Centrifuge at ~ 3000 rpm for ~ 5 minutes.

CHROMATOGRAPHIC CONDITIONS

Column:	Agilent Zorbax SP-Phenyl, 2x50 mm, Part# 860975-912
Mobile Phase:	A: 0.2% Formic acid in water B: Methanol C: 0.1% FA in 90:10 MeCN:water (back flush at 1.0 mL/min)
Gradient:	Gradient with column backflush (back flush column time: 3.1' to 5.0')
Injection Volume:	5– 20 µL
Column Temperature:	40°C
Flow Rate:	0.500 mL/min
Injector Temperature:	Room Temperature
Needle Wash 1:	50:50 DMF:water
Needle Wash 2:	0.1% Formic acid in water

MASS SPECTROMETER CONDITIONS

Instrument:	MDS Sciex API4000	
Ionization Mode:	Turboionspray, positive ion mode	
Source Temperature:	400°C	
SRM Transitions:	EC78 5-hydroxyl-EC78 5-carboxyl-EC78 EC78-d3	319 → 150 335 → 138 349 → 150 322 → 153

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

- Δ The method was originally developed using Varian Bond Elute 96 C2 100 mg SPE plates to extract 150 μ L of sample under neutral conditions. Very good accuracy and precision were obtained for all three analytes over a linear range of 1.00 to 500 ng/mL; however, when frozen dilution QCs (quality controls, 2000 ng/mL) were diluted 10 times with control matrix before extraction, the measured concentration of 5-carboxyl-EC78 was up to 150% of its target while the other two analytes were acceptable (Table 1).
- Δ This dilution QC bias of 5-carboxyl-EC78 was not affected by sample storage time and/or matrix lot difference, but was dependant upon the extraction method. The bias was not observed when the samples were extracted using a protein precipitate extraction (PPT) method under basic conditions (Table 2).
- Δ Matrix binding effects were proposed to cause this dilution QC bias for 5-carboxyl-EC78. At least two types of matrix-drug binding complexes, M1-D, and M2-D, were proposed to be involved in plasma samples (Figure 1):
 1. M1-D complex was relatively stable and it will not completely dissociate under the neutral SPE extraction conditions, but will fully dissociate under basic PPT extraction conditions;
 2. M2-D complex was relatively unstable. It can be disrupted completely in neutral SPE or basic PPT extraction conditions;
 3. M2-D complex cannot convert to the M1-D complex with additional matrix dilutions (at least during a short period of time);
 4. Within the linear range, the M1-D and M2-D complexes were present in the samples at similar ratios; however, when drug concentrations are above the linear range, the M1-D complex is saturated and M2-D is not. Therefore, the measured dilution QC concentrations will be higher when the samples are extracted in neutral SPE conditions, but not in basic PPT conditions.

Figure 1. Proposed drug-matrix binding complexes in plasma samples

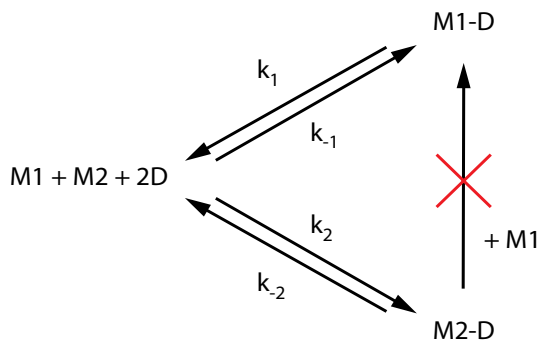


Table 1. Intra-assay accuracy and precision for EC78 and 5-carboxyl-EC78 (EC78-5COOH) extracted by SPE method: 150 μ L of samples was mixed with 50.0 μ L of IS working solution and 200 μ L of 100 mM ammonium acetate; samples were loaded onto a conditioned Varian Bond Elute C2 100 mg SPE plate. The sample plates were washed with 400 μ L of 100 mM ammonium acetate, then eluted with 400 μ L of 90:10 acetonitrile:water; the samples were evaporated and reconstituted in 200 μ L of 0.1% formic acid in 30:70 MeOH:water. All concentrations are in ng/mL.

	LLOQ		Low		Medium		High		Dilution	
	EC78	EC78-5COOH	EC78	EC78-5COOH	EC78	EC78-5COOH	EC78	EC78-5COOH	EC78	EC78-5COOH
Theor. C.	1.00	1.00	3.00	3.00	200	200	380	380	2000	2000
Found C. Mean	0.946	1.01	2.99	3.23	196	223	356	410	2040	2990
S.D.	0.0241	0.054	0.0655	0.128	4.51	12.6	3.66	9.65	17.6	45.8
%CV	2.5	5.3	2.2	4	2.3	5.7	1	2.4	0.9	1.5
%Theoretical	94.6	101	99.7	107.7	98	111.5	93.7	107.9	102	149.5
n	6	6	6	6	6	6	6	6	6	6

Table 2. Accuracy and precision comparison of dilution QCs extracted by SPE and PPT methods: fresh QCs and calibration curves were prepared on the day of extraction. Frozen QCs were pre-prepared and stored at -70°C for ~1 week; two identical batches were extracted using the same samples with either SPE or PPT as described in Table 1 or the methodology section, respectively. All concentrations are in ng/mL.

	Solid Phase Extraction (SPE)				Protein Precipitate Extraction (PPT)			
	Frozen Dilution QC		Fresh Dilution QC		Frozen Dilution QC		Fresh Dilution QC	
	EC78	EC78-5COOH	EC78	EC78-5COOH	EC78	EC78-5COOH	EC78	EC78-5COOH
Theor. C.	2000	2000	2000	2000	2000	2000	2000	2000
Found C. Mean	2050	2650	2010	2690	2070	2050	2020	1970
S.D.	21.9	56.9	49	78.3	78.1	91.3	36.1	50.8
%CV	1.1	2.1	2.4	2.9	3.8	4.5	1.8	2.6
%Theoretical	102.5	132.5	100.5	134.5	103.5	102.5	101	98.5
n	6	6	6	6	6	6	6	6

Figure 2. Plasma Blank for EC78

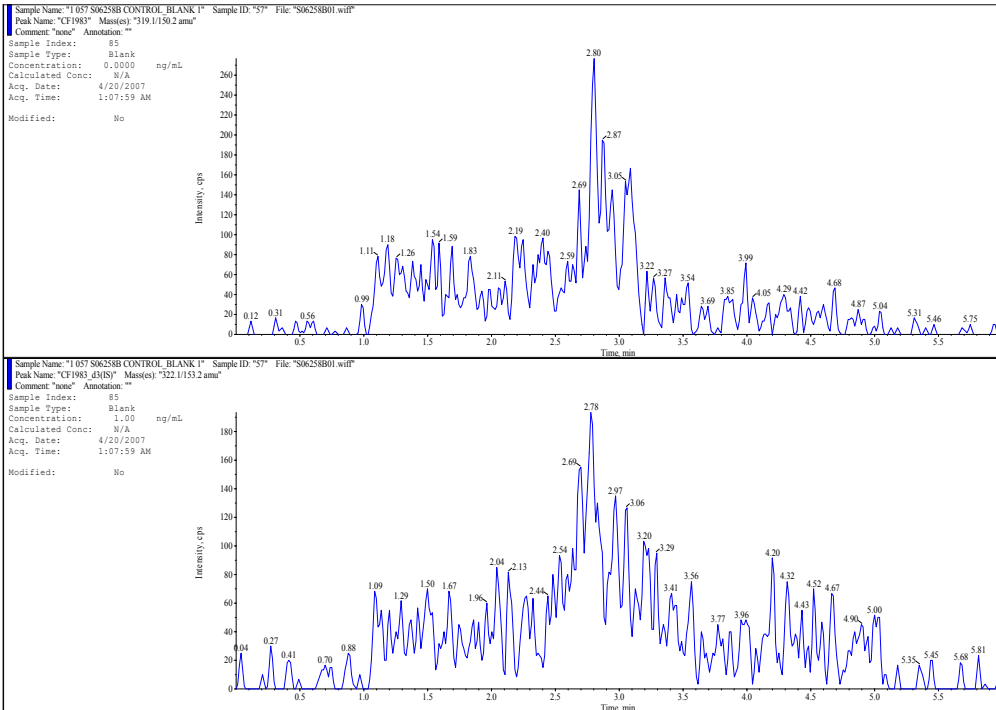


Figure 3. Low Standard (1.00 ng/mL) for EC78

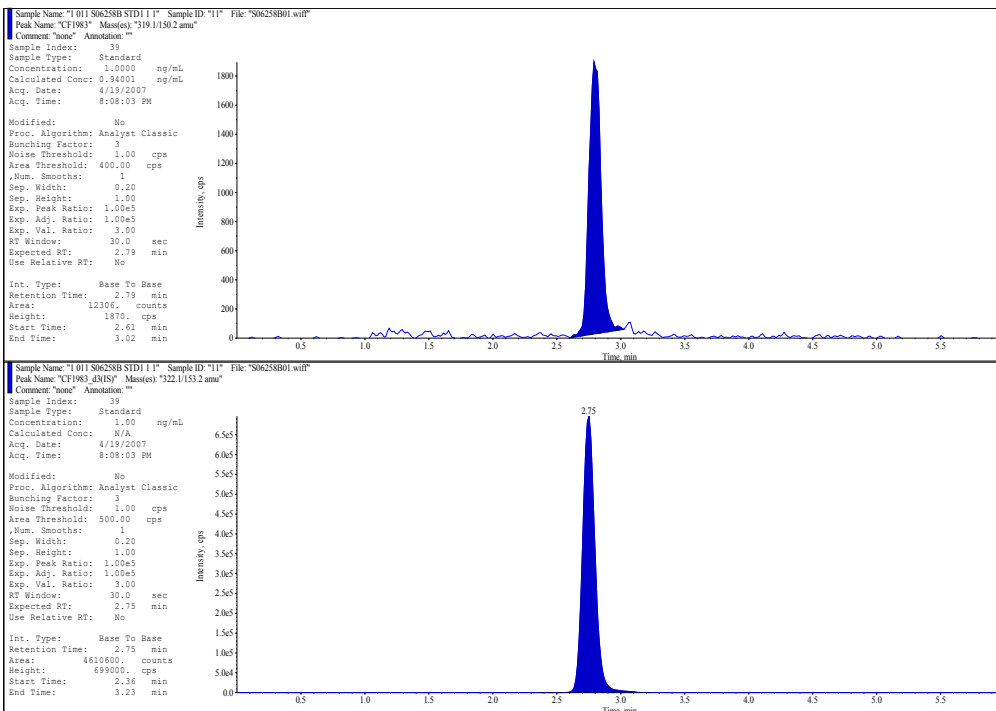


Figure 4. Plasma Blank for 5-hydroxyl-EC78

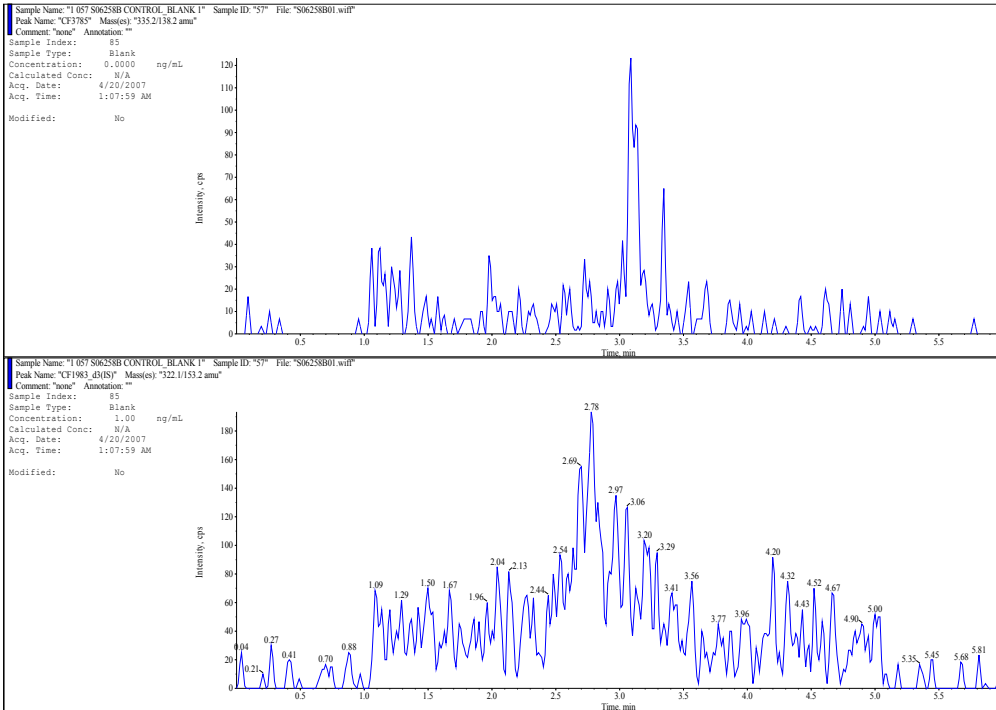


Figure 5. Low Standard (1.00 ng/mL) for 5-hydroxyl-EC78

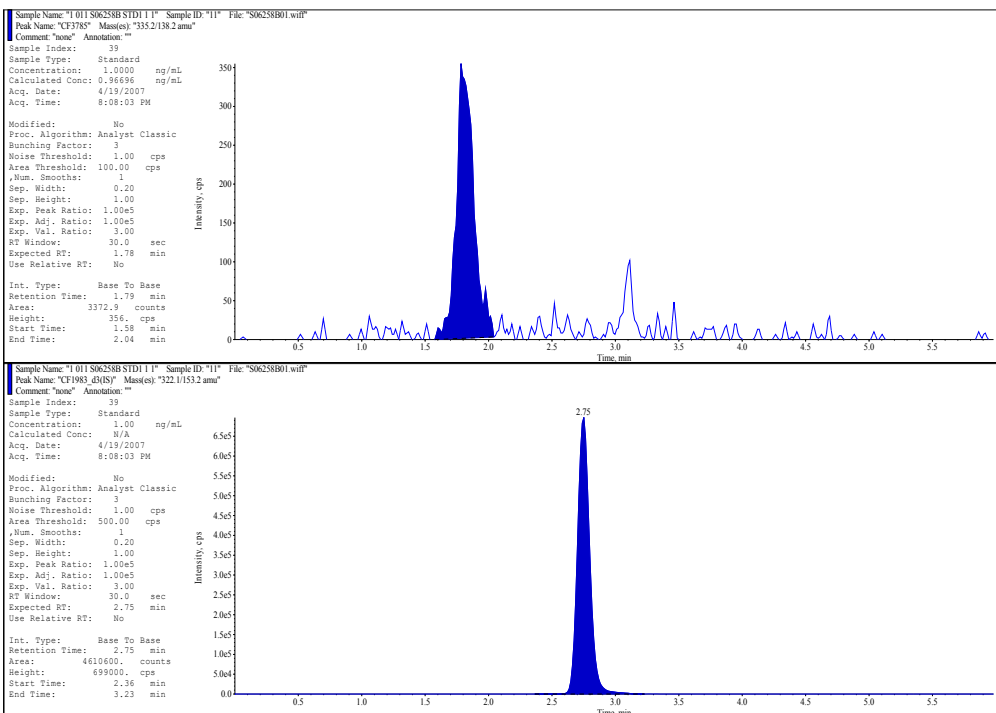


Figure 6. Plasma Blank for 5-carboxyl-EC78

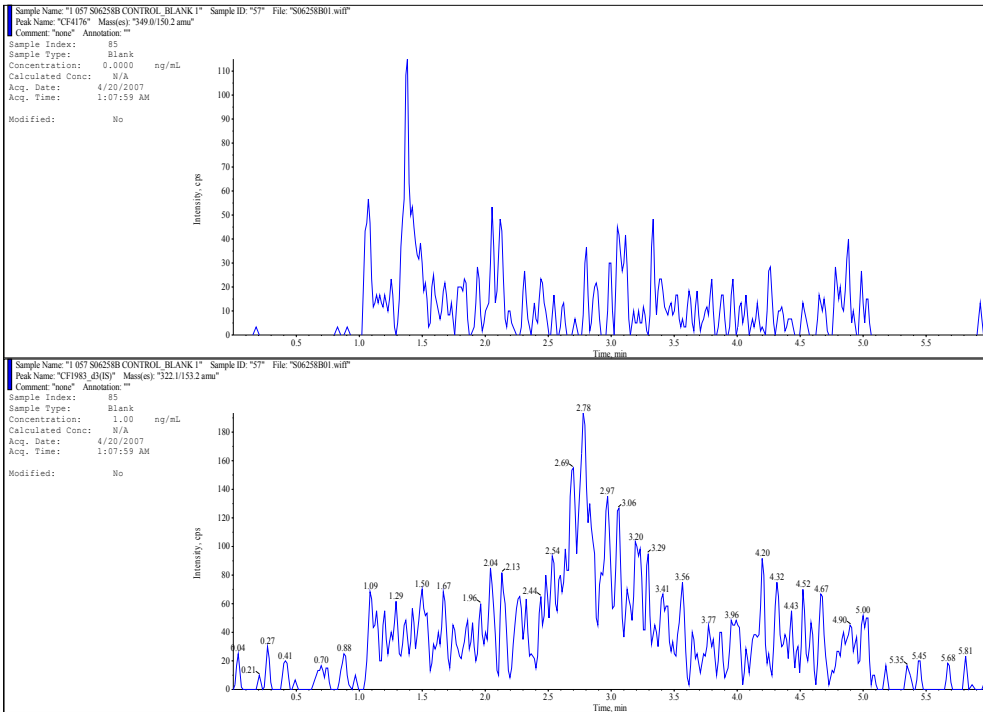
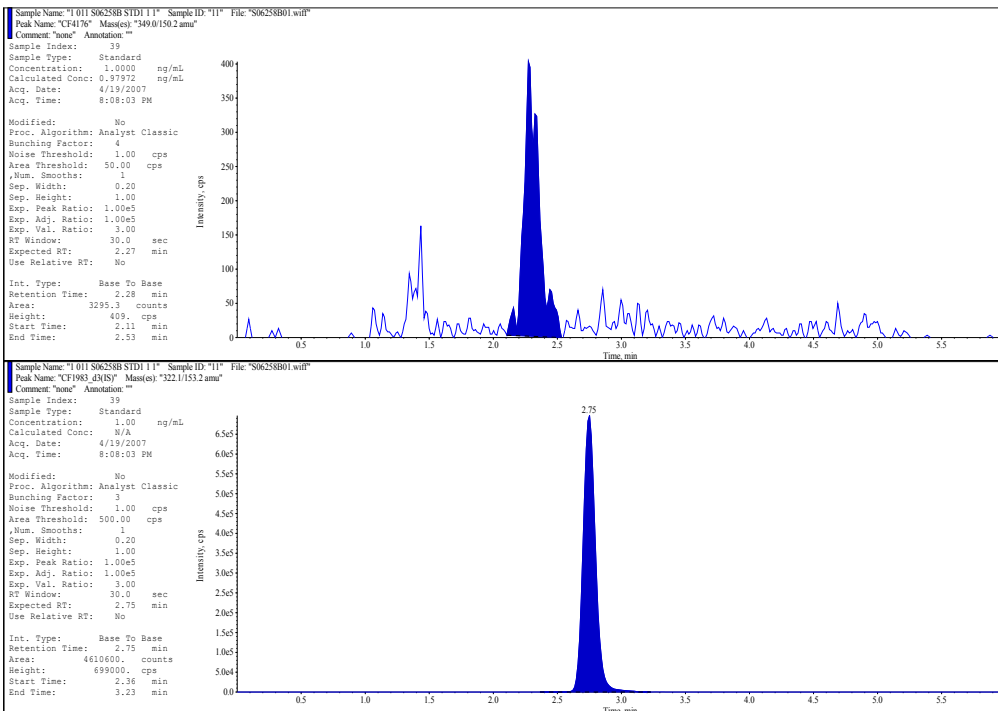


Figure 7. Low Standard (1.00 ng/mL) for 5-carboxyl-EC78



ASSAY VALIDATION

- Δ **Accuracy/precision:** Demonstrated at n=6 at LLOQ, Low, Medium, High concentrations over 3 validation runs. (Tables 5-7)
- Δ **Selectivity:** Demonstrated with blank and LLOQ concentrations in six sources of human plasma. (Table 8)
- Δ **Ability to dilute:** Demonstrated above ULOQ at DF=10. (Table 9)
- Δ **Extraction recovery:** Evaluated for analytes at Low, Medium, High concentrations (n=6). (Table 10)
- Δ **Carryover:** Evaluated in each run. No carryover present.

STABILITY

- Δ **Stock solution:** Room temperature for analytes and the I.S., 6 hrs 94 days at 1-8°C in DMF
- Δ **Stability in matrix:** Freezing/thawing, 3 cycles
Room temperature, 6 hrs
126 days at -70°C
- Δ **Extract:** Batch reinjection stability, 240 hours at RT

Table 3. Back-calculated concentrations of calibration standards for EC78; all concentrations are expressed as ng/mL

Theory	Mean	S.D.	%CV	%Bias	n
0.765	0.771	0.0240	3.1	0.8	8
1.54	1.52	0.0546	3.6	-1.3	8
3.07	3.07	0.0855	2.8	0.0	8
9.60	9.51	0.168	1.8	-0.9	8
38.3	39.2	0.812	2.1	2.3	8
192	191	4.14	2.2	-0.5	8
307	302	6.91	2.3	-1.6	8
383	390	11.5	2.9	1.8	8

Table 4. Back-calculated concentrations of calibration standards for 5-hydroxyl-EC78 and 5-carboxyl-EC78; all concentrations are expressed as ng/mL

5-hydroxyl-EC78					
Theory	Mean	S.D.	%CV	%Bias	n
1.00	1.03	0.0655	6.4	3.0	8
2.00	1.93	0.144	7.5	-3.5	8
4.00	3.94	0.297	7.5	-1.5	8
12.5	11.9	0.415	3.5	-4.8	7
50.0	49.7	1.72	3.5	-0.6	8
250	251	12.2	4.9	0.4	8
400	409	15.6	3.8	2.3	8
500	525	17.3	3.3	5.0	8
5-carboxyl-EC78					
Theory	Mean	S.D.	%CV	%Bias	n
1.00	1.02	0.0411	4.0	2.0	8
2.00	1.92	0.167	8.7	-4.0	8
4.00	3.99	0.154	3.9	-0.3	7
12.5	12.0	0.481	4.0	-4.0	7
50.0	49.1	2.19	4.5	-1.8	8
250	248	13.5	5.4	-0.8	8
400	409	19.0	4.6	2.3	8
500	527	23.0	4.4	5.4	8

Table 5. Intra- and inter-assay accuracy and precision for EC78 (ANOVA)

Nominal Concentration	LLOQ QC 0.765 ng/mL	Low QC 2.30 ng/mL	Medium QC 153 ng/mL	High QC 291 ng/mL
Mean Observed Concentration	0.737	2.35	152	285
%Bias	-3.7	2.2	-0.7	-2.1
Between Run Precision (%CV)	3.0	2.7	1.4	0.0
Within Run Precision (%CV)	3.7	3.7	2.2	2.4
Total Variation (%CV)	4.8	4.6	2.6	2.3
n	18	18	18	18
Number of Runs	3	3	3	3

Table 6. Intra- and inter-assay accuracy and precision for 5-hydroxyl-EC78 (ANOVA)

Nominal Concentration	LLOQ QC 1.00 ng/mL	Low QC 3.00 ng/mL	Medium QC 200 ng/mL	High QC 380 ng/mL
Mean Observed Concentration	0.996	3.07	199	377
%Bias	-0.4	2.3	-0.5	-0.8
Between Run Precision (%CV)	8.9	2.9	7.1	3.1
Within Run Precision (%CV)	6.0	5.1	7.6	4.0
Total Variation (%CV)	10.7	5.9	10.4	5.1
n	18	18	18	18
Number of Runs	3	3	3	3

Table 7. Intra- and inter-assay accuracy and precision for 5-carboxyl-EC78 (ANOVA)

Nominal Concentration	LLOQ QC 1.00 ng/mL	Low QC 3.00 ng/mL	Medium QC 200 ng/mL	High QC 380 ng/mL
Mean Observed Concentration	0.984	2.96	192	375
%Bias	-1.6	-1.3	-4.0	-1.3
Between Run Precision (%CV)	6.6	2.2	2.9	6.1
Within Run Precision (%CV)	8.6	7.3	10.5	4.0
Total Variation (%CV)	10.8	7.6	10.9	7.3
n	18	18	18	18
Number of Runs	3	3	3	3

Table 8. Selectivity in 6 lots of human plasma at the lower limit of quantification for EC78, 5-hydroxyl-EC78, and 5-carboxyl-EC78

	EC78 0.765 ng/mL	5-hydroxyl-EC78 1.00 ng/mL	5-carboxyl-EC78 1.00 ng/mL
Mean	0.797	0.922	0.996
S.D.	0.0241	0.140	0.0433
%CV	3.0	15.2	4.3
%Theoretical	104.2	92.2	99.6
%Bias	4.2	-7.8	-0.4
n	6	6	6

Table 9. Dilution quality control samples for EC78, 5-hydroxyl-EC78, and 5-carboxyl-EC78

	EC78 Dilution QC 1530 ng/mL DF=10	5-hydroxyl-EC78 Dilution QC 2000 ng/mL DF=10	5-carboxyl-EC78 Dilution QC 2000 ng/mL DF=10
Mean	1570	2070	1950
S.D.	42.6	62.8	85.4
%CV	2.7	3.0	4.4
%Theoretical	102.6	103.5	97.5
%Bias	2.6	3.5	-2.5
n	6	6	6

Table 10. Relative extraction recovery for EC78, 5-hydroxyl-EC78, and 5-carboxyl-EC78 at low, medium, and high QC concentration levels

Mean	Low (3.00 ng/mL)	Medium (200 ng/mL)	High (380 ng/mL)
Mean Recovery for EC78	67.9%	72.3%	76.7%
Mean Recovery for 5-hydroxyl-EC78	89.9%	89.3%	83.7%
Mean Recovery for 5-carboxyl-EC78	65.0%	62.5%	65.7%

CONCLUSION

A robust, simple and sensitive method was developed and validated to quantitatively analyze EC78 and its two metabolites in human plasma. Concentration-dependent extraction recoveries were observed in neutral SPE conditions due to matrix-drug binding effects in plasma samples. Basic protein precipitate extraction conditions demonstrated the elimination of these matrix effects.

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