Troubleshooting to Prevent Possible Oxidation of a Phenolic Compound during the Sample processing

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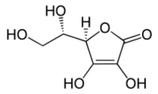
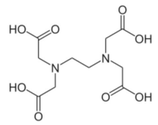
Introduction

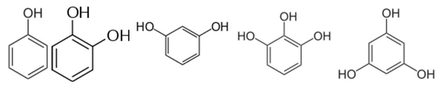


Ascorbic acid



Ethylenediaminetetraacetic acid (EDTA)

Phenolic compounds such as those found among anesthetics agents, anti-inflammatory drugs or neurotransmitters are very sensitive to oxidation in solution or in biological matrix. The use of an antioxidant like ascorbic acid is an effective way to prevent the oxidation mechanism of phenolic compounds. Unfortunately, in this case study, the ascorbic acid in the presence of EDTA in biological matrix promotes the aromatic hydroxylation of the parent drug, overestimating the concentration of the phenolic metabolite. Different antioxidants were unsuccessfully tested to replace ascorbic acid. Sample processing was therefore modified by elimination of the evaporation step of the protein precipitation extraction and removal of stabilization with ascorbic acid.



Phenolic compound structure

Method

The new extraction method was developed using 50 µL of human NaF/$K_2C_2O_4$ stabilized plasma alkalinized with potassium carbonate and acetonitrile has precipitation solvent. A part of the supernatant was transferred using a liquid handling system and diluted using a solution composed of water, acetonitrile and ammonium formate to reach the same composition as the mobile phase for HILIC chromatography. The biological matrix was aliquoted in different type of tubes to evaluate the compatibility of the metabolite with each type of tube.

Extraction Procedure

Add 50 µL of human NaF/$K_2C_2O_4$ plasma
↓
Add 200 µL of Internal Standard
↓
Mix for 60 seconds
↓
Add 1000 µL of Acetonitrile
↓
Mix for 60 seconds
↓
Centrifuge for 5 minutes at 3000 rpm at 4°C
↓
Transfer 125 µL of the mixture using a liquid handling system into a 96-well collection plate
↓
Add 50 µL of dilution solution

LC-MS/MS Analysis

Chromatographic mode:	Hilic Mode
Analytical Column:	Atlantis HILIC Silica, 50 x 4.6 mm, 3 um
Column temperature:	25°C
Elution mode:	Isocratic
Mobile Phase:	Water/Acetonitrile and Ammonium Formate 10 mM
Flow Rate:	1.000 mL/minute
Injection volume:	10 µL
Retention Times:	1.80 minutes for Parent drug
	2.11 minutes for Phenolic metabolite
	2.05 minutes for the Internal Standard
Acquisition time:	3.50 minutes
Autosampler temperature:	4°C
Detector:	AB Sciex API 4000
Source:	TurboIonSpray, Positive mode

Results

The parent drug and the phenolic metabolite were validated over the dynamic range of 0.5 to 500 ng/mL in human NaF/$K_2C_2O_4$ plasma using the evaporation free method. Matrix effect, hemolysis effect, lipemic effect and potentially interfering drugs were evaluated and showed no impact on the quantification. The recovery of the new method was evaluated and was of 90% for the parent drug (80% for the phenolic metabolite) with a good reproducibility **(Table 3)**. When different plastic tubes were compared, it was observed that 2 mL amber polypropylene microtubes introduced a bias of -24 to -29% on the metabolite concentration compared to other plastic tubes such as 12 x 75 mm amber polypropylene tubes or 15 mL plastic tubes **(Tables 4 and 5)**.

Table 1. Within-run Accuracy and Precision in Human NaF/$K_2C_2O_4$ Plasma for old Method with Evaporation Step

	LLQC 0.50 ng/mL		QC1 1.50 ng/mL		QC2 250.00 ng/mL		QC3 375.00 ng/mL		ULQC 500.00 ng/mL	
	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias
	0.50	0.00	1.34	-10.67	245.72	-1.71	343.04	-8.52	535.74	7.15
	0.50	0.00	1.29	-14.00	260.86	4.34	365.14	-2.63	486.33	-2.73
	0.54	8.00	1.42	-5.33	267.25	6.90	375.48	0.13	497.12	-0.58
	0.54	8.00	1.35	-10.00	273.71	9.48	372.22	-0.74	509.66	1.93
	0.61	22.00	1.51	0.67	276.17	10.47	401.35	7.03	513.15	2.63
	0.53	6.00	1.36	-9.33	273.59	9.44	390.77	4.21	515.44	3.09
N	6	6	6	6	6	6	6	6	6	6
Mean	0.537	7.33	1.378	-8.11	266.217	6.49	374.667	-0.09	509.573	1.92
SD(±)	0.0403		0.0768		11.4844		20.3419		16.8953	
CV(%)	7.50		5.57		4.31		5.43		3.32	

Table 2. Within-run Accuracy and Precision in Human NaF/$K_2C_2O_4$ Plasma for new Method with no Evaporation Step

	LLQC 0.50 ng/mL		QC1 1.50 ng/mL		QC2 250.00 ng/mL		QC3 375.00 ng/mL		ULQC 500.00 ng/mL	
	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias	Mesured Conc. (ng/mL)	% Bias
	0.56	12.00	1.55	3.33	255.43	2.17	395.91	5.58	521.93	4.39
	0.52	4.00	1.53	2.00	254.54	1.82	379.94	1.32	521.05	4.21
	0.52	4.00	1.52	1.33	256.80	2.72	382.78	2.07	512.77	2.55
	0.47	-6.00	1.50	0.00	241.22	-3.51	377.31	0.62	520.57	4.11
	0.51	2.00	1.48	-1.33	249.66	-0.14	367.67	-1.95	496.64	-0.67
	0.48	-4.00	1.45	-3.33	243.01	-2.80	353.73	-5.67	486.61	-2.68
N	6	6	6	6	6	6	6	6	6	6
Mean	0.510	2.00	1.505	0.33	250.110	0.04	376.223	0.33	509.928	1.99
SD(±)	0.0322		0.0362		6.6683		14.3154		14.8955	
CV(%)	6.31		2.41		2.67		3.81		2.92	

Table 3. Recovery of Phenolic Metabolite in Human NaF/$K_2C_2O_4$ Plasma for new Method with no Evaporation Step

	Untreated Standard	Extracted Sample
	Analyte Response Ratios	Analyte Response Ratios
	0.116030	0.089810
	0.115348	0.101203
	0.114105	0.097632
	0.122507	0.088983
	0.111445	0.086956
	0.111696	0.094049
Mean	0.1151885	0.0931055
SD(±)	0.00404177	0.00552486
CV(%)	3.51	5.93
Concentration	0.04	1.50
Concentration Factor	0.029	
Mean Recovery (%)	74.33	

	Untreated Standard	Extracted Sample
	Analyte Response Ratios	Analyte Response Ratios
	18.8118877	16.866461
	20.060012	17.267375
	20.283188	17.418744
	20.193516	16.054151
	19.639764	16.397066
	19.717843	16.100650
Mean	19.7843667	16.6874078
SD(±)	0.54097647	0.58998882
CV(%)	2.73	3.54
Concentration	7.25	250.00
Concentration Factor	0.029	
Mean Recovery (%)	84.35	

	Untreated Standard	Extracted Sample
	Analyte Response Ratios	Analyte Response Ratios
	31.277228	25.448477
	31.345396	26.466278
	30.205700	25.807202
	30.260544	24.921523
	30.443542	24.735638
	30.254955	24.185992
Mean	30.6312275	25.2608517
SD(±)	0.53341167	0.81646448
CV(%)	1.74	3.23
Concentration	10.87	375.00
Concentration Factor	0.029	
Mean Recovery (%)	82.43	

Table 4. Impact of Type of Polypropylene Tubes tested at Low, Medium and High Concentration Levels on the Phenolic Metabolite

Tube Format	QC1 (1.5 ng/mL)		QC2 (250 ng/mL)		QC3 (375 ng/mL)	
	(ng/mL)	% Bias	(ng/mL)	% Bias	(ng/mL)	% Bias
Amber polypropylene tube (2 mL)	1.38	-8.00	171.47	-31.41	283.77	-24.33
	1.45	-3.33	168.76	-32.50	278.99	-25.60
Amber polypropylene tube (12 X 75 mm)	1.44	-4.00	234.83	-6.07	351.53	-6.31
	1.50	0.00	235.79	-5.68	354.46	-5.48
Clear polypropylene tube (12 X 75 mm)	1.38	-8.00	233.35	-6.66	352.18	-6.09
	1.44	-4.00	231.03	-7.59	354.83	-5.38
Clear polypropylene tube (15 mL)	1.52	1.33	243.35	-2.66	371.93	-0.82
	1.53	2.00	241.22	-3.51	369.95	-1.35

Table 5. Evaluation of Two Polypropylene Tube Formats for Clinical Sampling at Low and High Concentration Levels in Four Different Matrices

Matrix lot	QC1 (1.50 ng/mL)			QC3 (375 ng/mL)		
	Amber PP 12X75 mm	Clear PP 12X75 mm	% Diff	Amber PP 12X75 mm	Clear PP 12X75 mm	% Diff
100037049/F	1.43	1.39	2.80	380.03	367.50	3.30
100037050/F	1.43	1.44	0.69	365.58	372.74	1.92
100037059/M	1.28	1.29	0.78	359.12	361.09	0.55
100037060/M	1.25	1.28	2.34	359.93	367.62	2.09

Results

Table 6. Matrix Effect at Low Quality Control Level (1.50 ng/mL)

Matrix type	Untreated Standard (MFQC1)		Reference Solution (RSQC1)		Calculated Matrix Factor (Analyte)	Calculated Matrix Factor (IS)	IS-Normalized Matrix Factor
	Analyte Responses	Internal Standard Responses	Analyte Responses	Internal Standard Responses			
Normal	13125	109573	11290	96399	1.028790	1.012902	1.015686
Normal	13228	113686	13679	113823	1.036864	1.050923	0.986622
Normal	13907	112934	12822	108766	1.090087	1.043971	1.044174
Normal	12048	104596	12758	111790	0.944371	0.966894	0.976706
Hyperlipemic	13061	114631	12830	107471	1.023774	1.059659	0.966135
5% Hemolyzed	12613	112095	13167	110815	0.988658	1.036216	0.954104
Mean			**12757.7**	**108177.3**			**0.9905712**
SD(±)							**0.03358225**
CV(%)							3.39

Table 7. Matrix Effect at High Quality Control Level (500 ng/mL)

Matrix type	Untreated Standard (MFULOQ)		Reference Solution (RSULOQ)		Calculated Matrix Factor (Analyte)	Calculated Matrix Factor (IS)	IS-Normalized Matrix Factor
	Analyte Responses	Internal Standard Responses	Analyte Responses	Internal Standard Responses			
Normal	4375935	110263	4250760	107307	0.995723	0.995652	1.000071
Normal	4501264	112518	4512889	113690	1.024241	1.016014	1.008097
Normal	4506294	112520	4344556	111605	1.025386	1.016032	1.009206
Normal	4526886	112750	4494593	112714	1.030072	1.018109	1.011750
Hyperlipemic	4436177	111548	4462409	111701	1.009431	1.007255	1.002160
5% Hemolyzed	4442055	113844	4303170	107450	1.010769	1.027988	0.983250
Mean			**4394729.5**	**110744.5**			**1.0024223**
SD(±)							**0.01037691**
CV(%)							**1.04**

Conclusion

The evaporation free extraction was developed to prevent the possible oxidation of the phenolic compound and give more stable quantification of its metabolite. Also, the tube experiment revealed the incompatibility of the phenolic metabolite with some of plastic types.

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