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Development of a Mitra Tip Extraction Assay Coupled with LC-MS/MS for Quantitation of L-Citrulline, L-Arginine, and L-Argininosuccinic Acid: A Novel Approach for Biomarker Analysis

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PURPOSE

Biomarkers such as L-Citrulline, L-Arginine, and L-Argininosuccinic Acid play crucial roles in various physiological processes and pathological conditions. However, their accurate quantitation presents challenges due to sample complexity and low concentrations. Here, we present a novel approach utilizing Mitra tip extraction coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the simultaneous quantitation of these biomarkers. Mouse models are known to produce small blood volumes while Mitra tips offer advantages in sample collection, allowing for convenient and reproducible extraction from biological matrices. Whole Blood samples were collected using Mitra tips, followed by elution with a suitable solvent. Chromatographic separation was achieved on a high-performance liquid chromatography system, coupled with a triple quadrupole mass spectrometer operating in multiple reaction monitoring (MRM) mode. The method demonstrated excellent linearity (r > 0.99) over a wide concentration range for all biomarkers. Lower limits of quantitation (LLOQ) were determined to be within clinically relevant ranges. Precision and accuracy were assessed, meeting regulatory criteria. Furthermore, the method exhibited minimal matrix effects and satisfactory recovery rates. Application of this method to clinical samples demonstrated its utility in quantifying L-Citrulline, L-Arginine, and L-Argininosuccinic Acid with high sensitivity and specificity. This innovative approach offers a reliable and efficient method for biomarker analysis, facilitating research in various disease states and therapeutic intervention

OBJECTIVE

To present the development and validation of a novel Mitra Tip Extraction Assay coupled with LC-MS/MS for the quantitation of L-Citrulline, L-Arginine, and L-Argininosuccinic Acid, and to demonstrate its efficacy as a robust and reliable method for biomarker analysis in clinical and research settings. This poster aims to showcase the assay's advantages in terms of accuracy, sensitivity, and ease of use compared to traditional extraction and quantitation methods.

METHOD(S)

Special Precautions:

0.9% NaCl, 2% BSA in water is used as surrogate matrix for Calibrators, QCs, and Matrix Blank samples. Native matrix QCs are also included in each run, prepared in K2EDTA mouse whole blood.
Sample preparation requires collection onto Mitra tip and a drying time between 3 and 24 hours. Calibrators prepared and collected onto Mitra tip (Neoteryx by Trajan) the day prior to extraction are considered freshly prepared.
Extracted samples that fall outside the quantitation range will be diluted with extracted blank surrogate matrix with IS. Diluted samples will then be reassayed. Sufficient blank matrix IS samples should be included with each extraction to dilute unknown samples.

Extraction Summary:

Calibrators, QC's, Matrix Blanks, and Unknown samples are each aliquoted into corresponding well of 96-well plate. Use pipette tip to help dislodge sample tip from stem of Mitra collection apparatus. Aliquot 20µL of internal standard solution prepared at 25µM for each analyte in water. For carryover control samples, aliquot 20uL of water instead of internal standard. Aliquot 100uL of 10µM EDTA in water to all samples for Mitra tip rehydration. Centrifuge samples for approximately 1 minute at 1,000rpm.Vortex plate to mix samples for 30 minutes at room temperature. Precipitate samples with 400µL 0.1% FA in MeCN. Vortex plate to mix samples for 10 minutes at room temperature. Centrifuge samples at 4,000 rpm for 10 minutes. Aliquot 320µL of 100mM AmForm in water/MeCN (10/90 v/v) ph3.5 (MOBILE PHASE B) into a clean 96-well plate; this will be the final plate. Use nimbus to transfer 80uL from extraction plate to final plate. Vortex plate to mix final plate for 2 minutes at room temperature. Centrifuge final plate at 3,000 rpm for 2 minutes. Store at room temperature while waiting for extraction.

After initial quantitation, any samples that are outside the quantitation range should be diluted and rerun. To dilute samples; take extracted sample and dilute with extracted blank surrogate matrix with IS from the same extraction run, either 1:1 or 1:2 ratio, into a new well. Rerinject Curve, QC's, Matrix Blanks, and Diluted samples.

Calibration Curve Standard Data for L-Citrulline in Surrogate Matrix

	Run NuMber	5.00	10.0	25.0	50.0	100	300	450	500
	1	4.71	8.70	25.6	54.6	92.3	299	418	445
		5.44	9.91	27.0	53.9	119	301	429	474
	2	4.67	10.7	26.0	48.4	99.5	287	439	465
		*4.93	*9.93	*25.9	*54.3	*101	*303	*507	*519
		5.17	9.58	26.8	48.8	106	311	455	493
	3	5.32	10.1	24.5	48.3	111	281	471	501
		4.54	10.9	22.4	50.1	99.3	274	507	477
	4	4.90	9.40	28.2	51.1	85.6	285	445	489
		5.06	10.2	25.2	54.3	96.8	286	461	544
	*+/- 15% of nominal concentration								
Mean		4.98	9.94	25.7	51.2	101	291	453	486
S.D.		0.325	0.712	1.77	2.72	10.6	12.1	27.7	29.3
%CV		6.5	7.2	6.9	5.3	10.5	4.2	6.1	6.0
%Bias		-0.4	-0.6	2.8	2.4	1.0	-3.0	0.7	-2.8
n		8	8	8	8	8	8	8	8

Calibration Curve Standard Data for L-Argininosuccinic Acid in Surrogate Matrix

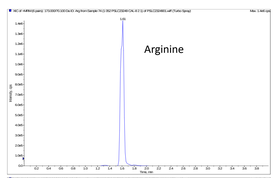
	Run NuMber	5.00	10.0	25.0	50.0	100	300	450	500
	1	5.05	9.09	23.5	52.5	94.1	294	410	458
		5.16	9.95	25.3	53.5	114	319	452	511
	2	4.40	9.48	25.4	49.4	97.3	281	445	480
		5.70	9.80	26.6	49.6	110	305	447	513
	3	5.14	10.1	24.3	47.2	109	287	470	489
		4.87	10.1	23.5	52.4	103	281	489	476
	4	4.83	9.26	27.2	50.3	85.5	290	428	504
		5.04	11.0	25.0	53.6	93.8	303	472	531
Mean		5.02	9.85	25.1	51.1	101	295	452	495
S.D.		0.367	0.600	1.34	2.29	9.79	13.2	25.4	23.7
%CV		7.3	6.1	5.3	4.5	9.7	4.5	5.6	4.8
%Bias		0.4	-1.5	0.4	2.2	1.0	-1.7	0.4	-1.0
n		8	8	8	8	8	8	8	8

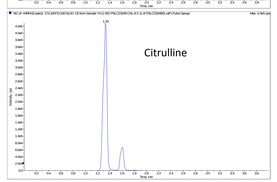
Calibration Curve Standard Data for L-Arginine in Surrogate Matrix

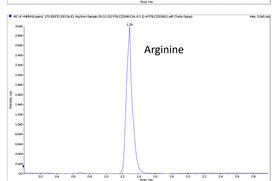
	Run NuMber	5.00	10.0	25.0	50.0	100	300	450	500
	1	5.23	9.64	24.9	50.8	96.6	318	438	475
		4.72	10.4	24.7	55.6	111	262	428	506
	2	5.16	10.2	25.0	56.5	110	298	508	479
		4.75	10.2	24.0	43.8	96.8	287	445	458
	3	5.05	11.1	25.7	47.6	114	288	509	482
		4.90	9.35	23.2	47.7	98.5	274	474	479
	4	5.09	*12.3	28.2	50.8	89.6	301	451	491
		4.59	10.9	25.0	50.8	90.1	289	440	544
	*Outlier								
Mean		4.94	10.3	25.1	50.5	101	290	462	489
S.D.		0.232	0.626	1.46	4.20	9.58	17.0	31.8	26.0
%CV		4.7	6.1	5.8	8.3	9.5	5.9	6.9	5.3
%Bias		-1.2	3.0	0.4	1.0	1.0	-3.3	2.7	-2.2
n		8	7	8	8	8	8	8	8

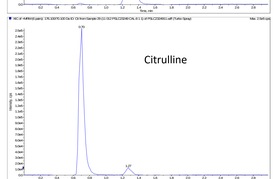
Extraction Recovery Data for L-Citrulline in Surrogate Matrix

	L-Citrulline			L-Arginine			L-Argininosuccinic Acid		
	LQC Level Control	MQC Level Control	HQC Level Control	LQC Level Control	MQC Level Control	HQC Level Control	LQC Level Control	MQC Level Control	HQC Level Control
	0.258	2.59	7.59	0.148	1.60	4.55	0.212	2.12	6.07
	0.308	2.86	8.32	0.176	1.86	4.44	0.226	2.27	6.44
	0.302	2.73	7.81	0.162	1.56	4.38	0.226	2.12	6.48
Mean*	0.289	2.73	7.91	0.162	1.67	4.46	0.221	2.17	6.33
S.D.*	0.0273	0.136	0.373	0.0136	0.165	0.0847	0.0079	0.0852	0.225
%CV**	9.4	5.0	4.7	8.4	9.9	1.9	3.6	3.9	3.6
n	3	3	3	3	3	3	3	3	3
	LQC Level Extracted	MQC Level Extracted	HQC Level Extracted	LQC Level Extracted	MQC Level Extracted	HQC Level Extracted	LQC Level Extracted	MQC Level Extracted	HQC Level Extracted
	0.177	1.73	5.18	0.098	0.97	2.98	0.144	1.37	4.37
	0.191	1.74	4.77	0.112	1.01	3.20	0.152	1.46	4.37
	0.219	1.82	4.87	0.124	1.09	2.78	0.157	1.46	3.93
Mean*	0.195	1.76	4.94	0.111000	1.02	2.99	0.151000	1.43	4.22
S.D.*	0.0213	0.0519	0.214	0.0128	0.064	0.206	0.00639	0.0495	0.254
%CV**	10.9	2.9	4.3	11.5	6.3	6.9	4.2	3.5	6.0
n	3	3	3	3	3	3	3	3	3
% Recovery**	67.5	64.5	62.5	68.5	61.1	67.0	68.3	65.9	66.7





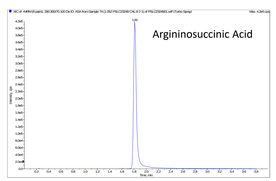




Moderna Chromatography of a Standard 8 Calibrator

Column	Agilent Poroshell 120 HILIC 1.9µM, 2.1x150
Mobile Phase A	10 mM Ammonium Formate in Water, pH 3
Mobile Phase B	100 mM Ammonium Formate in water pH 3/0.1% Formic Acid in Acetonitrile (10/90)

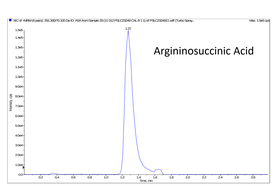
Time (min)	A %	B %	Flow (mL/min)
0.5	5	95	0.8
3.00	15	65	0.8
3.50	15	65	0.8
3.60	5	95	0.8
4.00			Stop



Aliri Chromatography of a Standard 8 Calibrator

Column	Agilent Poroshell 120 HILIC 1.9µM, 2.1x150
Mobile Phase A	10 mM Ammonium Formate in Water, pH 3.5
Mobile Phase B	100 mM Ammonium Formate in water pH 3.5/Acetonitrile (10/90)

Time (min)	A %	B%	Flow (mL/min)
0.1	10	90	0.2
3			Stop



Parallelism in Surrogate Matrix

	L-Citrulline			L-Arginine			L-Argininosuccinic Acid		
	HQC 51.0 µM DF=5	HQC 127 µM DF=2	HQC 254 µM DF=1	HQC 88.0 µM DF=5	HQC 220 µM DF=2	HQC 440 µM DF=1	HQC 56.0 µM DF=5	HQC 140 µM DF=2	HQC 280 µM DF=1
	54.9	142	254	76.0	216	434	63.5	143	288
	53.2	123	253	71.3	207	428	60.9	142	283
	55.1	122	269	82.0	205	452	59.4	135	284
Mean	54.4	129	259	76.4	209	438	61.3	140	285
S.D.	1.04	11.3	8.96	5.36	5.86	12.5	2.07	4.36	2.65
%CV	1.9	8.8	3.5	7.0	2.8	2.9	3.4	3.1	0.9
%Theoretical	106.7	101.6	102.0	86.8	95.0	99.5	109.5	100.0	101.8
%Bias	6.7	1.6	2.0	-13.2	-5.0	-0.5	9.5	0.0	1.8
n	3	3	3	3	3	3	3	3	3

Matrix Effect Data in Native or Surrogate Matrix

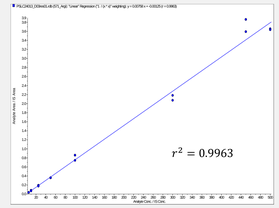
	L-Citrulline				L-Arginine				L-Argininosuccinic Acid			
	MQC 257 µM Lot 1 Native	MQC 276 µM Lot 2 Native	MQC 256 µM Lot 3 Native	MQC 150 µM Lot 4 Surrogate	MQC 478 µM Lot 1 Native	MQC 519 µM Lot 2 Native	MQC 453 µM Lot 3 Native	MQC 150 µM Lot 4 Surrogate	MQC 154 µM Lot 1 Native	MQC 155 µM Lot 2 Native	MQC 154 µM Lot 3 Native	MQC 150 µM Lot 4 Surrogate
	272	267	265	145	523	499	488	152	164	136	149	150
	272	280	250	148	497	525	440	137	156	144	133	138
	288	255	238	147	510	483	453	148	164	136	134	140
Mean	277	267	251	147	510	502	460	146	161	139	139	143
S.D.	9.24	12.5	13.5	1.53	13.0	21.2	24.8	7.77	4.62	4.62	8.96	6.43
%CV	3.3	4.7	5.4	1.0	2.5	4.2	5.4	5.3	2.9	3.3	6.4	4.5
%Theoretical	107.8	96.7	98.0	98.0	106.7	96.7	101.5	97.3	104.5	89.7	90.3	95.3
%Bias	7.8	-3.3	-2.0	-2.0	6.7	-3.3	1.5	-2.7	4.5	-10.3	-9.7	-4.7
n	3	3	3	3	3	3	3	3	3	3	3	3

CONCLUSION(S)

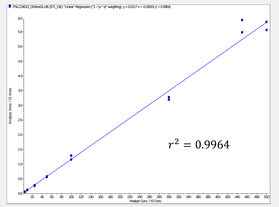
The implementation of a Mitra tip assay coupled with LC-MS/MS quantitation offers a robust and efficient method for the quantitation of citrulline, arginine, and argininosuccinic acid. This approach provides several advantages, including minimal sample preparation, reduced sample volume requirements, and improved sensitivity and specificity compared to conventional methods. Our results demonstrate the feasibility and reliability of this methodology in accurately measuring these important biomarkers in biological samples. Furthermore, the Mitra tip assay streamlines the analytical process, making it suitable for high-throughput applications in clinical and research settings.

The combination of Mitra tip sampling with LC-MS/MS analysis presents a promising avenue for the quantitation of citrulline, arginine, and argininosuccinic acid, offering researchers and clinicians a valuable tool for studying metabolic pathways and assessing physiological conditions. Further validation studies and application in diverse sample matrices will continue to refine and expand the utility of this approach in biomedical research and clinical diagnostics

Calibration Curve of Arginine



Calibration Curve of Citrulline



Calibration Curve of Argininosuccinic Acid