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Simultaneous Analysis of Tryptophan, Kynurenines and Several Amino Acids Using GC/Q-TOF in Negative Chemical Ionization Mode

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Introduction

Tryptophan metabolism occurs via two primary pathways. One is initiated by the enzyme tryptophan hydroxylase and synthesizes serotonin and melatonin and the second pathway, commonly referred to as the kynurenine pathway, is initiated by indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase and produces nueroprotective kynurenine, neurotoxic quinolinic acid and ultimately NAD+. The kynurenine pathway is up-regulated in many inflammatory disease states including Huntington's disease, ALS, schizophrenia, Alzheimer's and dementia. A novel GC/MS/MS method using negative chemical ionization (NCI) which illustrated the first *in vivo* simultaneous measurement of extracellular tryptophan, kynurenine, 3-hydroxy kynurenine (3-HK) and quinolinic acid in rat brain has been previously reported [1]. A GC/NCI-Q-TOF used for both targeted and non-targeted analysis of tryptophan metabolites of the kynurenine pathway is described.

Using GC/Q-TOF in NCI and global screening mode of rat brain samples collected by striatal microdialysis *in vivo*, six kynurenines and several amino acids had LODs of 1.0 fmol with RSDs less than 10% over five replicate injections. Raw TOF data was mined for non-targeted compounds such as 5-hydroxy-tryptophan (5-HT) which has potential anti-depressive properties. 5-HT was easily identified as well as other amino acids and endogenous compounds.

Background

Tryptophan metabolic pathway is up-regulated in dozens of disease states including HIV, Huntingdon's and schizophrenia

Biogenic amines and amino acids represent multiple neurochemical pathways

Current need for multiplex targeted assay and ability to identify new bio-markers

Current assay via GC/MS/MS [1]

Experimental

The analytical method was developed on the Agilent 7890A/7200 GC/Q-TOF in electron capture negative chemical ionization mode using methane as the reagent gas. A mid column backflush configuration was developed using two 15 m HP-5MS columns connected at the mid-point by a helium purged union. The oven program was 60 °C for 1 min then 13 °C/min to 230 °C for 0 min for a run time of 14.077 minutes. All of the analytes were monitored as their corresponding poly-fluorinated derivatives by reacting with pentafluorpropanol and pentafluoropropionic acid anhydride. The_derivatized samples were analyzed by injecting 1 µL into the 250°C inlet of the GC/Q-TOF system in splitless mode.

Lesioned and non-lesioned rat brain samples were collected by striatal microdialysis *in vivo* by perfusion with Ringer solution at a rate of 1 µl/min. Samples were collected in 1 h-fractions and naïve rats were sampled for a consecutive 5 hours. The microdialysates from rats that received intrastriatal injections one week earlier were collected for 1-2 h to obtain baseline values. The rats then received an intra-peritoneal injection of kynurenine and dialysis continued [1].

For the derivatization [1]: standards were dissolved in 0.1 M NaOH containing 0.1% (w/v) ascorbic acid and diluted to the final concentration with 1% (v/v) aqueous formic acid. Fifty micoliters of a solution containing internal standards (500 nM $^{[2H5]}$L-tryptophan, 10 µM $^{[2H6]}$L-kynurenine and 50 nM $^{[2H3]}$quinolinic acid) were added to 50 µL of the biological sample and dried. The samples were reconstituted using 120 µL of pentafluorpropanol and 130 µL of pentafluoroacetic anhydride and heated at 75°C for 30 min. The samples were dried down again, and taken up in 50 µL of ethyl acetate for injection. One µL was injected into the GC.

Analytes

Name	Acrony	Type	Empirical Forr	Mono m	Type	Derivative Monc
Quinolinic Acid	Quin	KYN	C7 H5 N O4	167	Di-carboxylic acid	431.0211
3-hydroxy-kynur	3HK	KYN	C10 H12 N2 C	224.1	Carboxylic acid / Phenol / di-amine	794.0164, 218.9
Kynurenine	Kyn	KYN	C10 H12 N2 C	208.1	Carboxylic acid / Primary & Second	632.0423, 612.0
Kynurenic acid	Kyna	KYN	C10 H7 N O3	189	Carboxylic acid / Phenol	453.0418, 320.0
Tryptophan	Try	Amino Acid	C11 H12 N2 C	204.1	Carboxylic acid / Primary & Second	628.0474, 608.0
Anthranilic Acid	AA	Amino Acid	C7 H7 N O2	137	Carboxylic acid / Primary Amine	415.0261
Glutamate	Glu	Amino Acid	C5 H9 N O4	147.1	di-Carboxylic Acid / Amine	557.0315
Dopamine	DA	Neurotransm	C8 H11 N O2	153.1	Catecholamine	591.0157, 375.9
Serotonine	Ser	Neurotransm	C10 H12 N2 C	176.1	Phenol / di-amine	614.0317, 594.0

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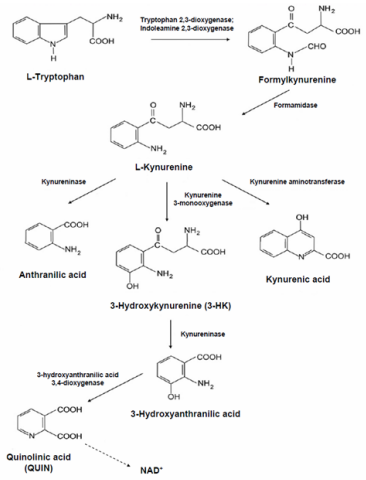
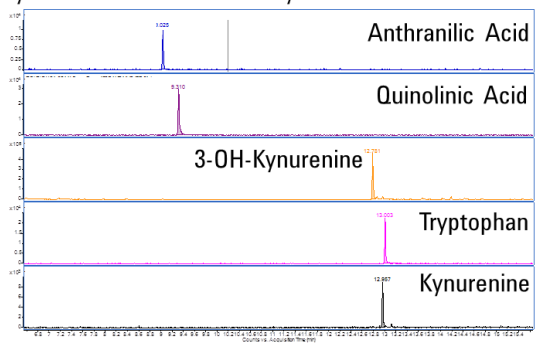
Results and Discussion

The kynurenine pathway

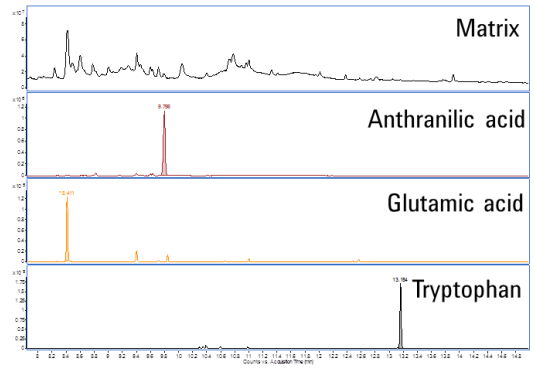


Exact mass Extracted Ion Chromatograms (EIC)

Kynurenines in rat brain dialysate

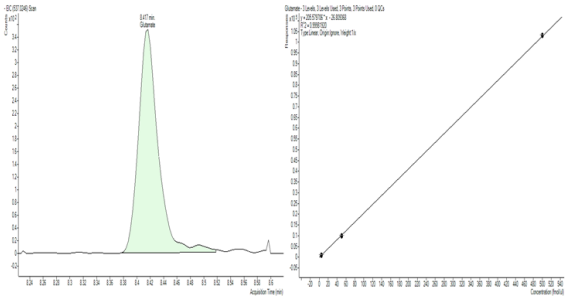


Amino Acids in mouse brain extract

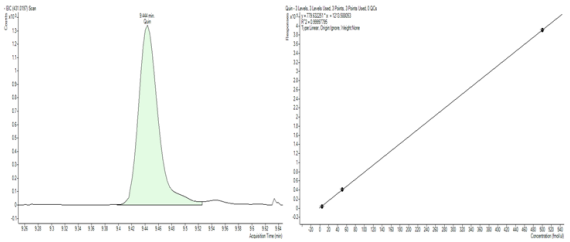


Select examples and results

Glutamate



Quinolinic Acid



Uncorrected mass accuracy & resolution

Name	Acronym	Derivative Mono m/z	Observed	Uncorrected d ppm	Resolution (FWHM)	comment
Quinolinic Acid	Quin	431.0211	431.0179	-7.4242	15393	
3-hydroxy-kynurenine	3HK	794.0164, 218.9950	218.9949	-0.4566	7821	EIC = 218.9950
Kynurenine	Kyn	632.0423, 612.0361	612.0349	-1.9607	22668	EIC = 612.0361
Kynurenic acid	Kyna	453.0418, 320.0320	320.0335	4.6870	11036	EIC = 320.0320
Tryptophan	Try	628.0474, 608.0412	608.0405	-1.1512	22520	EIC = 608.0405
Anthranilic Acid	AA	415.0261	415.0241	-4.8190	15371	
Glutamate	Glu	557.0315, 537.0258	537.0228	-0.0006	19179	EIC = 537.0258
Dopamine	DA	591.0157, 375.9964	375.9973	2.3936	14461	EIC = 375.9964
Serotonine	Ser	614.0317, 594.0197	594.0242	7.5755	23761	EIC = 594.0197

Quantitative results

Sample			Glutamate Results		Anthranilic Acid Results		Quin Results	
Data File	Type	Level	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy
STD1_02.d	Cal	3	501.82	100.36	499.61	99.92	499.88	99.98
STD2_02.d	Cal	2	48.00	96.00	54.32	108.65	51.34	102.68
STD3_02.d	Cal	1	5.18	103.63	1.07	21.37	3.78	75.60
New Mouse Brain_01.d	Sample		26799.16		89.67		17.39	

Sample			Dopamine Results		Qualifier (571.0087) Results		KYNA Results	
Data File	Type	Level	Final Conc.	Accuracy	Area	Ratio	Final Conc.	Accuracy
STD1_02.d	Cal	3	499.65	99.93	5657.22	1.86	505.97	101.19
STD2_02.d	Cal	2	53.80	107.60	543.40	1.62	43.43	86.87
STD3_02.d	Cal	1	1.55	30.95	37.09	1.92	5.60	111.94
New Mouse Brain_01.d	Sample		58.01		2668.21	7.37	338.00	

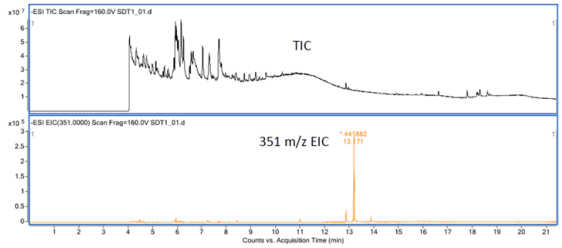
Sample			3-HK Results		Qualifier (630.0258) Results		Kyn Results	
Data File	Type	Level	Final Conc.	Accuracy	Area	Ratio	Final Conc.	Accuracy
STD1_02.d	Cal	3	497.33	99.47	9121.44	0.32	500.92	100.18
STD2_02.d	Cal	2	52.93	105.87	582.80	0.21	39.86	79.73
STD3_02.d	Cal	1	4.73	94.67	50.52	0.48	14.22	284.30
New Mouse Brain_01.d	Sample						47.91	

Sample			Tryp Results		Serotonine Results	
Data File	Type	Level	Final Conc.	Accuracy	Final Conc.	Accuracy
STD1_02.d	Cal	3	501.09	100.22	500.02	100.00
STD2_02.d	Cal	2	38.03	76.07	49.81	99.63
STD3_02.d	Cal	1	15.88	317.58	5.17	103.39
New Mouse Brain_01.d	Sample		171.58		24.31	

Results and Discussion

Using this hybrid GC/Q-TOF method, detection limits of one femtomol were achieved for the targeted analytes with RSDs less than 10% over five replicate injections. The data was mined for other compounds not previously observed in the targeted GC/MS/MS method such as 5-hydroxy-tryptophan (5-HT) which has potential anti-depressive properties. 5-HT was easily identified demonstrating the viability of this hybrid methodology for the simultaneous measurement of the entire kynurenine pathway and potentially all pathways of tryptophan metabolism in a single analysis.

5-hydroxytryptophan



The kynurenine pathway (KP) is the major catabolic route of dietary tryptophan in mammals and plays a significant role in biology. Interest in the role of KP in the central nervous system is mostly related to the neuroprotective properties of kynurenic acid and the excitotoxic quinolinic acid as well as the ability of 3-hydroxykynurenine (3-HK) and to scavenge reactive free radicals. These metabolites are involved in a number of important neurophysiological processes and may causally participate in neurological and psychiatric diseases [1]. The table below illustrates quantitative results from two technical replicates of rat brain dialysate.

Rat Brain Sample:
Average of 2 injections (fmol/µl)

Glutamate	AA	Quin	Dopamine	KYNA	3-HK	Kyn	Tryp	Ser
Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.
26857.07	96.85	17.69	69.11	382.84	0.00	66.76	248.20	49.63

Conclusions

High resolution mass spectrometry (HRMS) and accurate mass are the inherent analytical features of a time-of-flight (TOF) mass spectrometer. These features allow differentiation of nearly identical ions in heavy matrices through extraction of narrow window (10 ppm or less) ion chromatograms. The addition of a quadrupole on the TOF instrument allows the isolation of single precursor ions and an exact mass MS/MS spectrum for structural elucidation. In the method defined herein, tryptophan, kynurenine, 3-hydroxy-kynurenine, kynurenic acid and quinolinic acid along with anthranilic acid, glutamate, dopamine and serotonin were targeted. Raw TOF data was mined for compounds not observed in the targeted GC/MS/MS method reported earlier. The ability to develop this type of targeted and non-targeted hybrid HRMS methodology is a powerful tool for global screening of bio-analytical samples for known and unknown bio-markers.

References

[1] F.M. Notarangelo, H.-Q. Wu, A. Macherone, D.R. Graham and R. Schwarcz: GC/MS/MS detection of extracellular kynurenine and related metabolites in normal and lesioned rat brain. Anal. Biochem., 421, 573-581 (2012).

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