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Metabolomic Analysis of Mammalian Cells and Human Tissue through One-Pot Two Stage Derivatizations using Sheathless Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry

Tianjiao Huang^{||}, Michael Armbruster^{||}, Richard Lee[‡], Dawn S. Hui[‡], and James L. Edwards^{*,||}

^{||} Department of Chemistry and Biochemistry, Saint Louis University, 3501 Laclede Ave, Saint Louis, MO 63102

[‡] Cardiovascular Comprehensive Care Center, Saint Louis University, 3635 Vista Ave, St louis MO USA

Abstract

Analysis of metabolites is often performed using separations coupled to mass spectrometry which is challenging due to their vast structural heterogeneity and variable charge states. Metabolites are often separated based on their class/functional group which in large part determine their acidity or basicity. This charge state dictates the ionization mode and efficiency of the molecule. To improve the sensitivity and expand the coverage of the mammalian metabolome, multifunctional derivatization with sheathless CE-ESI-MS was undertaken. In this work, amines, hydroxyls and carboxylates were labeled with tertiary amines tags. This derivatization was performed in under 100 minutes and resulted in high positive charge states for all analytes investigated. Amino acids and organic acids showed average limits of detection of 76 nM with good linearity of 0.96 and 10% RSD for peak area. Applying this metabolomic profiling system to bovine aortic endothelial cells showed changes in 15 metabolites after treatment with high glucose. The sample injection volume on-capillary was <300 cells for quantitative analyses. Targeted metabolites were found in human tissue, which indicates possible application of the system complex metabolome quantitation.

1. Introduction:

Metabolomics, the study of endogenous small polar molecules offers insight into the bioactivity of cells, serum or plasma[1]. Specific metabolite groups or pathways have been analyzed using a wide variety of detectors with varying levels of sensitivity and selectivity[2, 3]. Mass Spectrometry (MS) is a common detector choice for metabolomic studies but the

*Address Correspondence to: Dr. James L. Edwards, Department of Chemistry and Biochemistry, Saint Louis University, 3501 Laclede Ave, Monsanto Hall, St Louis, MO 63102, Ph 314-977-3624, jedward5@slu.edu.

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efficacy of the analysis is largely dependent on analyte ionization efficiency, separation method, and flowrate[4].

Each metabolite has a unique structure and different separation based MS methods are often optimized according to the compound functional groups. Derivatization schemes for specific functional groups boost analyte ionization efficiency either through increasing hydrophobicity or proton affinity[5–10]. Such techniques target only one specific functional group/class of compounds. A method capable of tagging broad classes of metabolites with a single rapid separation-MS technique is needed.

Separation of metabolites has often proven difficult due to the high degree of structural heterogeneity and varying charge states. In particular, common metabolites such as those related to glucose metabolism and the amino acids are often analyzed using two unique separation platforms[11–14]. Capillary Electrophoresis (CE) offers efficient separation with short equilibration times between sample runs[15, 16].

In recent years, CE-MS has been applied with great success to metabolomic studies. Of particular interest is the use of CE coupled to high resolution MS which has been applied to metabolite analysis of genetically modified maize[17–19]. In many cases, the vast structure diversity of the metabolome requires strategies such as chemical derivatization to simplify the data set and enhance analyte classes of interest. For instance, Hao et al. analyzed amine-containing metabolites by using isobaric tag with CE-ESI-MS/MS and LC-ESI-MS/MS[20]. This work was able to quantify 30 metabolites from four different samples. Yang et al. developed a new approach for carboxylic acid profiling in rat urine by CE-ESI-MS/MS with isotope coded derivatization[21]. This method profiled 32 metabolites and was able to detect a total of 59 potential carboxylic acids.

Analysis of diverse classes of metabolites by CE-MS is challenging because migration of acidic groups often requires pressure assistance and different ionization polarities. This work analyzes both organic acids and amino acids with a porous junction sheathless CE-ESI-MS system in a single run by derivatizing multiple functional groups in a rapid one pot, two stage schemes. Thus, different classes of metabolites share common tags that improve the ionization efficiency and in the case of carboxylates, inverts the charge. The major benefit in using CE-MS is the ability to separate and detect analytes of interest without the interferences from the reaction matrix.

2. Materials and methods

2.1 Reagents and materials

All chemical reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA) unless otherwise noted. N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b] pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU), 1-Chloro-N, N,2-trimethyl-1-propenylamine, 3-(Diethylamino) propionic acid, dimethylformamide, pyridine, N, N-Diethylethylenediamine, endothelial growth medium (EGM) and fetal bovine serum were purchased from Sigma-Aldrich. Ammonium formate, organic acids and amino acids

standards, D-glucose anhydrous, Phosphate Buffered Saline (PBS), liquid chromatography mass spectrometry (LCMS) grade methanol and water, formic acid and culture dishes were purchased from Fisher Scientific. The amino acids are noted according to their one letter code. Fused silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2 Tag synthesis and derivatization

Ghosez chlorinating reagent (1-Chloro-N, N,2-trimethyl-1-propenylamine. 300 mM) and 3-(Diethylamino) propionic acid (300 mM) were mixed in 1.5 mL Eppendorf Safelock Biopur tube that contained 1 mL anhydrous dimethylformamide (DMF) and left to react for 2 hours under room temperature. This resulted in the amine/hydroxyl tag, 3-(Diethylamino) propionyl chloride.

Anhydrous pyridine (4 μ L) and 3-(Diethylamino) propinoyl chloride (100 μ L) was added to dried analyte standards (50 μ L) at room temperature. After 30 minutes of reaction, the first derivatization step was quenched by adding 7 μ L of *N, N*-Diethylethylenediamine, followed by addition of 50 μ L HATU (1M) and left to react for 70 minutes at room temperature. After the dual-step derivatization, vials were diluted with 290 μ L 5 mM ammonium formate buffer.

2.3 Mammalian Sample Collection and Preparation

Bovine aortic endothelial cells were purchased from Lonza (Walkersville, MD) and maintained in endothelial growth medium (EGM) with 10% fetal bovine serum. Confluent cells of 5 cm dishes (2×10^6 cells) were incubated with either EGM media or EGM media plus 20 mM glucose for 15 minutes and then lysed. Lysing and extractions of metabolites followed previously published methods[22] with slight modifications. In brief, cells were quickly rinsed with room temperature Phosphate Buffered Saline (PBS) and rapidly quenched with 600 μ L ice-cold (0°C) 80/20 methanol/H₂O spiked with 5 μ M d3-methionine as the internal standard. Plates were scraped on dry ice-ethanol and cell suspensions were sonicated (Mixonix XL-2000, Qsonica, CT) with ten, 1s bursts. Centrifugation of cell lysates were at 14,000 rpm (20 $\times g$) to precipitate protein and cell membranes. Supernatants were collected and 20 μ L were transferred into vials and dried down for derivatization and sheathless CE-ESI-MS analysis.

Analysis of human tissue was obtained from sternotomy as previously described[23]. Muscles were flash frozen in liquid nitrogen at the operating room. Tissue was lyophilized, and ground in liquid nitrogen with mortar and pestle. Tissue was then ground to powder. Metabolites from 2 mg of the tissue powder were extracted using 600 μ L ice-cold 80/20 methanol/H₂O spiked with 5 μ M d3-methionine as described above.

2.4 Fabrication of sheathless CE-ESI-MS interface and instrumentation

The fabrication was performed using previously published methods with slight modifications[24–26]. Capillary dimensions were 50 μ m i.d., 365 μ m o.d. $\times$ 80 cm. The exterior of the capillary is coated with polyimide and the interior was left uncoated. Nanospray tip was pulled and etched with HF prior to porous junction formation with an emitter tip diameter of $\sim$ 15 μ m. After etching, the capillary was flushed and filled with run

buffer, 5 mM ammonium formate 10% MeOH (pH=2.5, adjusted with formic acid). Sample was introduced by hydrodynamic injection ($h = 32$ cm higher than the interface, $t = 20$ s). +20 kV was applied to the inlet reservoir using CZE1000R (Spellman, Hauppauge, NY, USA) and +2 kV was applied at the porous junction reservoir through nano-ESI interface on the MS.

Quadrupole ion trap LTQ XL mass spectrometer was used throughout the experiments (Thermo Scientific Waltham, WA) in positive mode for all analytes. Quantitative data was acquired in full scan mode, ranging from 50.0 to 500.0 m/z. For MS/MS experiments, the collision energy for Collision-Induced Dissociation (CID) was 35%. Scan time was 1 microscans and the maximum injection time was set to 10.00 ms. The capillary temperature was 150°C. The capillary voltage was 8.0 V and the voltage of tube lens was 85 V.

3. Results and Discussion

3.1 Optimizing Tagging Reactions

The structural diversity of organic acids and amino acids result in poor ionization efficiency and often requires multiple analytical platforms for analysis.[9, 22, 23] To remedy this problem, Figure 1 shows a dual tagging step within “one-pot” that adds the same high proton affinity (tertiary amine) moiety to all groups of metabolites. Polar aprotic solvents are used to ensure these reactions achieve quantitative yields. This design enables 1) tagged carboxylates to now migrate to the detector without pressure assistance, 2) Improvement in signal/ ionization efficiency for all metabolites 3) very low electroosmotic flow rates to optimize separations, and 4) elimination of neutral polar aprotic solvents and reagents as interferent due to complete separation from the analytes.

Primary amines and hydroxyl groups were tagged using the classic acyl chloride reaction which results in primary amide bond[27] and ester bond formation. Acyl chlorides as tagging reagents are prone to hydrolysis over time, which diminish their long-term reactivity. To ensure optimal tagging performance, tags were chlorinated daily as described above. Conventional chlorination reactions (e.g. thionyl chloride, oxalyl chloride and phosphorus trichloride) result in HCl formation which hinders reactivity with the analytes. *Ghosez's* reagent allowed 97.1% ($n=3$) conversion efficiency without the formation of an acid[28]. To quench this reaction, the second tag, *N,N*-Diethylethylenediamine was added. This results in two interfering peaks in our system, the second tag itself and the product of reaction between tag 1 and tag 2. To fully react analyte carboxylates with the amine tag, a coupling reagent HATU is added. The dual tagging scheme results in amide bond formation (from reaction #1 and #2) and ester formation both of which are stable and resistant to hydrolysis. Comparison of step 1 reaction and step 1 & 2 reactions were undertaken in supplemental figure 3. As shown in figure 2, one-pot two step derivatization not only expanded the detection of metabolites to include metabolites with only organic acid functional groups, but also improved the sensitivity.

Supplemental figure 2 shows the kinetic studies of each derivatization step. In supplemental figure 2A, the acyl chloride reaction was evaluated and found to be complete by 30 minutes. This reaction tagged both primary amines and hydroxyl groups. The organic acids

(excluding lactate) therefore showed no signal as they lack those functional groups. Compounds which contained two or more amines/hydroxyls were evaluated based on the presence of two tags and absence of detectable single tag in these cases (S, T, Y, and K). Figure 2B shows the reaction rate of reaction #1 and 2 was performed using analyte standards. Addition of the second tag followed by HATU showed reaction optimization at 70 mins. Reaction completion was evaluated based on the detection of maximum possible tag numbers for each analyte and the absence of signal from incomplete tagging of the same analyte.

3.2 Buffer optimization

In addition to reaction optimization, investigation of the buffer composition with regards to sensitivity and ionization efficiency is shown supplemental figure 1[3]. Investigations into the effect of background electrolyte (BGE) concentration (5–15 mM ammonium formate, 10% MeOH, pH=2.5) on S/N and peak area were investigated. Ammonium formate was chosen as the BGE in favor of ammonium acetate due to wide variations in migration times (data not shown). 10% MeOH was needed to achieve consistent and stable electrospray[29, 30]. BGE concentration was varied between 5–15 mM and investigated for signal response. For 16 of the 21 standard analytes, 5 mM BGE yielded the highest S/N. M, V, H, and R showed slight improvements at 10 mM BGE over 5 mM. When examining peak area, 5 mM BGE was superior in 18 of the 21 analytes. The improvement in signal response for low BGE is expected as it minimizes competing ionization.

3.3 Sheathless CE-ESI-MS Separation

Figure 3 shows the electropherogram and migration times for each tagged metabolite using 10% MeOH, 5 mM ammonium formate pH = 2.5. At pH=2.5, the neutral solvent migrated to the detector at $t_m \sim 50$ min ($\mu_{EOF} 1 \times 10^{-4}$ cm²/V*s). Tagged metabolites thus migrate almost completely based on their electrophoretic mobility and allows complete separation from neutrals (e.g. HATU/HOAt and DMF). This was critical to detection of analytes as these two components are at high concentration and diminish signal response when co-migrating with analytes. The separation window for cationic charged analytes was between 14–19 minutes. The tertiary amine derivatization resulted in a +1 charge for each tag on the analyte. This generally caused the structures of each metabolite to become more homogeneous. In many cases the, electrophoretic mobility became close, such as the succinate (14.44 min) and fumarate (14.36 min). In other cases, where analyte structures are very similar, such as lactate (2 tags) and pyruvate (1 tag), the tagging allowed a separation of 3.7min. Traditionally these two metabolites are difficult to properly separate by LC. One-pot two step derivatization with sheathless CE-ESI-MS also improves the peak profile and separation of the metabolites. As shown in figure 2, metabolites labeled with only step 1, organic acids such as fumarate, alpha-keto-glutarate and succinate were not detectable. When comparing metabolites which are tagged in both reaction 1 and reaction 2, the peak width is larger for reaction 1 compared to dual labeling of the metabolites.. As shown in table 1, the average plate numbers with two step derivatizations resulted in average of 137641 and step 1 labeling only yielded 13408.

As shown in Table 1, limits of detection were between 9 to 225 nM (average detection limit is 76 nM) for full scan mode. Responses were linear between 0.24 to 25 μ M with average linearity 0.956. Good reproducibility was also observed with peak area RSDs of 10% (n=3). Taken together, the derivatization and sheathless CE-ESI-MS system showed good sensitivity and reproducibility for targeted metabolite quantitation.

3.4 Fragmentation

After determining the reproducibility of the derivatization scheme and CE-ESI-MS system, fragmentation patterns by MS/MS of tagged analytes were investigated. Figure 4 shows the fragmentation of tagged α ketoglutarate (5A, organic acid) and asparagine (5B, amino acid). The typical cleavage occurred at the backbone between the tag and the analytes. Major fragments found in both spectra are the cleavage of tags with loss of tertiary amine groups. Because the analyte has a +2 charge the MS/MS yields product ions on both sides of the cleavage location. The strong product ion from the tag (+86 m/z) suggests that this system may be amenable to quantitation by using a triple quadrupole MS.

Examination of the amino acid MS/MS experiments showed intriguing fragmentation patterns. Typically, CID yields b/y ions from cleavage of amide bond. In this tagging scheme, amide bonds are formed from both reactions. In figure 4B, asparagine shows a distinct product ion at 143m/z which corresponds to the mass of the tag and the relevant analyte functional group. This indicates either an x or z product ion and not the typical b/y ion. This is likely due to the resonance and rigidity of the amide bond and the lack of a mobile proton. Because the two types of tags are structurally similar, it is not possible at this time to determine which tag is fragmented and therefore whether the product ion of interest is x, z, or both. For this system, bond dissociation tends to occur near the vicinity of amide bonds.

3.5 Analysis of short-term high glucose treatment with BECs and human cardiac tissue

Based on the strong performance of this system using commercially available standards, human tissue were analyzed[23]. Sample composition and small variations from the in-house manufactured capillary interface can influence migration times. Migration times were corrected to the deuterated internal standard, d3-methionine. Figure 5 shows the electropherogram of metabolites extracted from human tissue after derivatization. 10 metabolites were clearly detectable from human tissue. The other 15 metabolites were present though interferences from co-eluting compounds made unequivocal identification difficult. This work shows that there is significant potential in using this system to examine even the most complicated biological matrices.

Figure 6A shows a general workflow of lysing, one-pot two step derivatization and sheathless CE-ESI-MS. Of each set (n=3) of 5 cm culture plates, cells were lysed and 1/30 of the lysate were derivatized and 1/10 of that sample (<300 cells, calculated cell numbers are in supplemental equation 1) was injected on-capillary analyzed by the system. Figure 6B shows metabolite level changes from endothelial cells in response to short term high glucose exposure[9]. As shown in figure 6B, compared to control groups, 15 out of 21 metabolites, showed significant difference ($p < 0.05$) and 6 of them had $p < 0.001$ (p values are included in

table 1, table 1 included signal to noise ratios of +15 mM glucose treated endothelial cells versus controls.) Organic acids from the TCA cycles showed significant increased as expected. Pyruvate and lactate are directly linked metabolically and showed opposite changes which suggest pyruvate is being converted into lactate. Pyruvate levels decreased and lactate increased which suggests that the excess glucose is being diverted to lactate. As for amino acids, most showed an increase after high glucose treatment compared to controls. Both essential and non-essential amino acids showed increases upon high glucose which therefore likely resulted from an influx of amino acids from the media[31].

4. Conclusion

Described here is a novel sheathless CE-MS detection scheme for structurally diverse metabolites using a one pot-two-step derivatization. Conventional LC/MS of such metabolites, requires two distinct platforms with microliter sample volumes. Using a CE-MS system, injection of mammalian cell lysates was quantified. These volumes correspond to <300 cell lysates injected on-capillary. As only nanoliters samples are required for cellular analysis which achieved an average S/N of 19, this derivatization and sheathless CE-MS system could in theory be utilized for single islet or very large single cell metabolite profiling. There is no foreseen limit to the expansion of metabolite number or classes able to be investigated by this method. Expanding the reach of this technique using both high resolution MS and targeted triple quadrupole MS is currently underway.

Equation 1. Calculated cell numbers:

$$\underbrace{2E6}_A \times \underbrace{\frac{20\mu L}{600\mu L}}_B \times \underbrace{\frac{200 \times 10E-3\mu L}{50\mu L}}_C = 267 \text{ cells}$$

A: Full confluent cell numbers based on hemocytometer results

B: Sample preparation factor: 600 μ L is total lysate volume; 20 μ L is actual taken volume for derivatization and analysis

C: Injection volume factor: $200 \times 10E-3$ μ L is calculated injection volume; 50 μ L is reconstitution volume of lysate

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Novel sequential derivatization method that expands the detection of diverse classes of metabolites
- Hydroxyl, amine and carboxylate groups are derivatized with high proton affinity tags and separated by sheathless CE-ESI-MS
- Application of derivatization and sheathless CE-ESI-MS to human tissue and bovine aortic endothelial cell metabolome profiling
- Ability to detect 21 metabolites from <300 cells injected on-capillary

Figure 1. Two-step derivatization method for identification and quantitation of organic acids and amino acids. (A) Schematic of the workflow for one-pot two stage derivatization. (B) Reactions for alanine undergoing a one-pot two stage derivatization.

Figure 2.

Comparison of reaction 1 with reaction 1 and 2. Each standard concentration was 5 μ M. Reaction 1 targets amine and hydroxyl group labelling only. Reaction 1 and 2 labels amine, hydroxyl and carboxylate moieties.

Figure 3. Sheathless CE-ESI-MS selected ion electropherograms for a standard mixture of derivatized organic acids and amino acids at 1 μ M.

Figure 4. Fragmentation and MS/MS analysis of (A) tagged alpha-ketoglutarate (B) tagged asparagine. Structures of labeled alpha-ketoglutarate and asparagine are presented within the spectra. Red indicates acyl chloride tag. Blue indicates amine tag.

Figure 5. Selected ion electropherograms of two-step derivatized metabolites from extraction of human tissue using sheathless CE-ESI-MS.

* indicates the analyte of interest to distinguish from interfering peaks.

Figure 6.

(A) Schematic of two-step derivatization method in endothelial cells. Overview workflow for 21 metabolites two-step labelling and sheathless CE-ESI-MS analysis. (B) Organic acids and amino acids from bovine aortic endothelial cells after 15 mins of media +15 mM glucose treatment versus control. * denotes $p < 0.05$, ** denotes $p < 0.01$. Error bars are SEM. $n=3$.

Table 1.

Analytical Figures of Merit: Reproducibility, Linearity, and Sensitivity of standards. 1 denotes plate numbers from one-pot two step derivatization. 2 denotes plate numbers from step 1 reaction. a denotes t-test results from comparison of two groups: from bovine aortic endothelial cells after 15 mins of media +15 mM glucose treatment versus control. b denotes average signal to noise ratio from standards at concentration of 1 μ M. c denotes average signal to noise ratio of bovine aortic endothelial cells controls. d denotes average signal to noise ratio of bovine aortic endothelial cells after 15 mins of media +15 mM glucose treatment. Data analysis was Thermo Xcalibur Qual Browser with Boxcar 7 points smoothing.

	LOD (nM)	R ²	Peak Area %RSD (n=3)	Linear Dynamic Range (μ M)	m/z	Two-step derivatization N ¹	Step 1 derivatization N ²	P value ^a	Standards S/N ^b	Control S/N ^c	HG treatment S/N ^d
Alpha-keto-glutarate	38	0.961	8	0.12–25	172.14	71449	N/A	0	131	32	53
Pyruvate	17	0.927	10	0.06–25	187.14	47961	N/A	0.003	458	73	211
Succinate	26	0.993	9	0.08–25	158.14	270234	N/A	0.004	91	87	120
Fumarate	62	0.952	9	0.20–25	157.13	66812	N/A	0	115	54	99
Lactate	19	0.926	6	0.06–25	158.63	68403	2172	0	84	37	53
A	95	0.98	7	0.31–25	158.14	30695	21044	0.045	100	20	64
G	75	0.95	11	0.25–25	151.13	269859	22420	0	325	20	79
N	56	0.916	10	0.18–25	179.64	33885	12792	0.041	90	32	46
F	90	0.925	2	0.29–25	196.16	293937	15450	0.002	70	28	37
D	30	0.914	12	0.09–25	229.20	50033	1062	0.05	64	13	19
E	30	0.973	12	0.09–25	236.20	102080	15775	0.411	94	12	60
L/I	9	0.997	15	0.03–25	179.16	72900	2421	0.002	71	21	67
M	92	0.996	17	0.30–25	188.14	31159	15145	0.018	184	14	33
S	187	0.96	5	0.62–25	229.69	90252	6109	0.004	82	73	101
T	29	0.993	15	0.09–25	236.70	90360	21943	0.122	87	25	31
V	12	0.982	13	0.03–25	172.16	365348	8957	0	132	34	43
H	225	0.94	17	0.74–25	191.15	90469	34003	0.039	305	97	131
R	133	0.927	13	0.43–25	200.67	371027	1152	0.37	100	15	79
W	57	0.941	8	0.18–25	215.66	23713	22102	0.022	104	13	34
Y	80	0.962	6	0.26–25	267.70	362308	24294	0.164	225	16	42
K	139	0.954	13	0.45–25	250.22	87569	1092	0.546	83	16	53
Average	71	0.956	10			137641	13408		143	35	69