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Multi-Functional Derivatization of Amine, Hydroxyl, and Carboxylate Groups for Metabolomic Investigations of Human Tissue by Electrospray Ionization Mass Spectrometry

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Abstract

Metabolomics, the study of small molecules involved in cellular processes, offers the potential to reveal insights into the pathophysiology of disease states. Analysis of metabolites by electrospray mass spectrometry is complicated by their structural diversity. Amine, hydroxyl, and carboxylate groups all affect signal responses differently based on their polarity and proton affinity. This heterogeneity of signal response, sensitivity, and resistance to competing ionization complicates metabolite quantitation. Such limitations can be mitigated by a dual derivatization scheme. In this work, primary amine and hydroxyl groups are tagged with a linear acyl chloride head containing a tertiary amine tail, followed by carboxylate groups coupled to a linear amine tag with a tertiary amine tail. This tagging scheme increases analyte proton affinity and hydrophobicity. In the case of carboxylate groups, the inherent anionic charge is inverted to a cationic charge. This dual tagging is completed within 2.5 hours, diminishes adduct formation, and improves sensitivity by >75-fold. The average limit of detection for 23 metabolites was 38 nM and the R^2 was 0.97. This process was used to investigate metabolite changes from human tissue. Examination of diabetic and non-diabetic human tissue showed marked changes in both energy metabolites and amino acids. Further examination of the tissue showed that HbA1C value is inversely correlated with fumarate levels. This technique potentially allows for the analysis of virtually all metabolites in a single analytical run. Thus, it may lead to a more complete picture of metabolic dysfunction in human disease.

Abstract

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Novel two step-derivatization of hydroxyl, amine, and carboxylate groups for expanding the metabolomics toolbox.

1. Introduction

Investigations into metabolism provide a snapshot of the bioactivity in the cell or tissue and offer insights into the pathophysiology of disease states. Metabolomics, or broad scale metabolite studies, have shed light on a variety of disease states and potential therapeutic targets^{1–6}. Metabolism driven diseases, such as diabetes, are a particularly well-suited application to metabolomic studies^{7–13}. While MS based metabolomics has been able to uncover novel biomarkers and pathway interactions, analytical challenges exist which prevent the field from achieving its full potential. Heterogeneity in analyte sensitivity which arises from proton affinity and polarity as well as signal splitting from adduct formation and neutral losses all complicate analyses¹⁴ and cause difficulties in quantitation. Issues like peak splitting from adduct formation may diminish signal and prevent the detection of molecules present in low abundance in complex samples.

Current analytical methods overcome some of these deficiencies. A prominent tool with this regard is chemical tagging of specific functional groups, so called semi-targeted analyses, which often increases sensitivity and in certain cases diminishes signal splitting^{15–17}. Targeting metabolite amine groups using DiART, Dansyl Cl, and Benzoyl Cl have shown signal enhancement and the ability to multiplex samples using isotope analysis^{18–20}. Hydroxyl groups have also been targeted for analyses though mainly in the form of phenols.^{21, 22, 19} Carboxylic acids have been investigated using amide reactions to a high degree of success.^{23–26} Despite these advances in detection of metabolites, a method that analyzes multiple classes in a single chromatographic run and minimizing in-source fragmentation and sodium adduct formation is absent.

In this work, a sequential two stage tagging scheme is developed to label amine, hydroxyl, and carboxylate groups in a single analysis. This approach enhances signal response by increasing both proton affinity and hydrophobicity to each group. The LC separation is performed using a mixed mode weak cation exchanger-reverse phase material to take advantage of these properties. This technique is applied to diabetic and non-diabetic human tissue for the targeted analysis of amino acids and energy metabolites. The goal of this research is to develop a tagging scheme to enhance the sensitivity, diminish analyte degeneracy, and improve chromatographic performance for structurally diverse metabolites.

2. Material and methods:

2.1 Reagents and materials:

All chemical standards, ammonium formate, formic acid, dimethylaminoacetyl chloride hydrochloride, triethylamine, thionyl chloride, N, N-Diethylethylenediamine, HATU, HOAt, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were purchased from Sigma Aldrich (St. Louis) and Fisher Scientific (Pittsburgh, PA). DL-Methionine-d₃ (S-methyl-d₃) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). HPLC-MS grade solvents were purchased from Honeywell Burdick & Jackson (Muskegon, MI).

2.2 Derivatization with Dimethylaminoacetyl chloride and N, N-Diethylethylenediamine:

2.2.1 Generation and regeneration of dimethylaminoacetyl

chloride: Dimethylaminoacetyl chloride was chlorinated using thionyl chloride to ensure chlorination after initial use of the reagent. 100 mM Dimethylaminoacetyl chloride hydrochloride and thionyl chloride were added to a round bottom flask at mole ratio of 1:3 in DMF. Triethylamine was added to neutralize the solution and temperature was raised to 90–100 °C for 45 minutes to remove excess thionyl chloride.

2.2.2 Analyte Derivatization: Dimethylaminoacetyl chloride (40 µL) was added 100µL of analytes in DMSO. The reactions were conducted at room temperature for 30 minutes followed by quenched with 1.0 µL of H₂O. Carboxylate-tag amide reaction conditions were optimized with a well-known peptide coupling reagent HATU²⁷ and HOAt²⁸. N, N-Diethylethylenediamine (42.0 µL), 60 µL of 500 mM HATU and 60 µL of 500 mM HOAt were added to the analyte solution and incubated at room temperature for 2 hours. Then the microcentrifuge tube was placed in Thermo vacuum centrifuge under vacuum until dry and reconstituted in water with 0.1% formic acid buffer (pH 4.0).

2.3 Metabolite extraction:

Fresh human muscles were obtained from human cardiac operations. All samples were procured in accordance of Saint Louis University Internal Review Board (IRB) with Protocol ID 24160 and 27077. During sternotomy, a small portion of the sternothyroideus muscle is divided to expose the left innominate vein. Muscle was removed and immediately flash frozen in liquid nitrogen in the operating theater. Muscle was freeze-dried using lyophilization (LABCONCO FreeZone 1 Liter Benchtop Freeze Dry System, Kansas City, MO) for 48 hours to maintain its metabolic integrity. Tissue was ground to powder by using liquid nitrogen cooled mortar and pestle.²⁹ 1.0 mg of powder muscle tissue was added in 500.0 µL ice cold 80/20 acetonitrile/H₂O (v/v) with 1 µM internal standard DL-methionine-d₃ (C/D/N Isotopes Inc., Canada), followed by sonication (Mixonix XL-2000, Qsonica, CT). Samples were then centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was collected for tagging/derivatization.

2.4 Liquid chromatography (LC)/mass spectrometry (MS):

LC-MS analyses were performed in positive ion mode with a Thermo Scientific LTQ ion trap mass spectrometer (Waltham, MA) equipped with ESI interface coupled with Thermo Scientific Dionex UltiMate 3000 series (Waltham, MA). The flow rate was 0.30 mL/min.

Separations were performed on a Mixed-mode WCX-RP column (150×2.1 mm, 3 μm) purchased from Dionex Corporation (Sunnyvale, CA). The sample loop on the injection valve was 5 μL. Mobile phase A was 5 mM ammonium formate at pH of 5.0, mobile phase B was acetonitrile, mobile phase C was 40 mM ammonium formate at pH of 3.5. The separation was initiated with 100% mobile phase A at t= 0 min with %B and %C at 0%. For t=5min B=5% and C=1%; t=6min B=10%, C=5%; t=7min B=70% C=30%; t=10min B=70%, C=30%, t=45min B=0%, C=0% with a 15 minute re-equilibration hold. For the parameters of positive ion mode, the spray voltage was +3.0 kV. The temperature of heated capillary was 200 °C. The collision energy for collision-induced dissociation (CID) was 35%. Full scan MS spectra were acquired in the ion trap with full scan range from 50–500 m/z, scan time: 3 microscans and maximum injection time was 10 ms.

3. Results and discussion:

3.1 Reaction optimization and labeling strategy

The goal of this study is to develop a reaction sequence which increases proton affinity and hydrophobicity for broad classes of metabolites. Figure 1A shows the reaction scheme for tagging amine, hydroxyl, and carboxylate moieties. Linear acyl chloride tags will react with both amines and hydroxyls to yield amide bonds and esters, both of which are resistant to hydrolysis. The tertiary amine of the tag provides high proton affinity to increase electrospray ionization efficiency/signal response. By performing the reaction in polar aprotic solvent, hydroxyl groups are able to be tagged. The reaction between acyl chloride and nucleophile is rapid and complete within 20 minutes (Supplemental S1). Investigations into analytes with secondary amines (Pro and His), showed no detectable reactivity with the acyl chloride tag. Under these conditions, unstable anhydrides are formed between the tag and carboxylates. Water is thus added to restore the carboxylates and quench the reaction. The second reaction in the sequence couples metabolite carboxylates and the second tag, a linear amine with an ethyl based tertiary amine. The reaction was optimized to HATU/HOAt as the reagent because it yielded no observable analyte side reactions (Supplemental S4). The reaction kinetics show completion in under 100 min (Supplemental S2). EDC as an amide coupling reagent in our hands had a significant number of side reactions with standard analytes and was thus abandoned³⁰. Figure 1B shows the combination of these two reactions for the analyte glycine.

3.2 LC-MS Figures of Merit

Separation of derivatized metabolites was optimized using a mixed-mode weak cation exchanger, C18 reverse phase chromatography. Reverse phase LC-MS alone yielded unacceptable resolution. Figure 2 shows the separation of 23 derivatized metabolite standards consisting mainly of amino acids and organic acids. Number of tags on each analytes are shown in Supplemental Table S2. This separation system was designed to separate cationic species with varying levels of hydrophobicities. Samples were introduced at pH 5 followed by increasing organic and salt solvent. This allowed for deprotonation of the stationary phase and for the sample solvent to pass through the column unretained to waste. Despite this rinse, sample solvent showed some retention and remained an interferent for two analytes, fumarate and succinate (Supplemental S3). Analytes showed good linearity

with the exception of succinate and asparagine, whose labelled m/z overlapped with solvent peaks. Critical to obtaining reproducible chromatography is the re-equilibration of at least 15 column volumes between injections. This was due to the WCX component of the analysis.

Tagging of the metabolites provided a unique mode for metabolite separation and enhanced signal due likely to the increase in hydrophobicity and proton affinity. The tertiary amine moieties in the tags allow analysis of carboxylates to be performed in positive mode. Figure 3 shows the signal improvement of tagged vs. untagged analytes. It is noteworthy that ESI-MS using positive mode is substantially easier to accomplish than negative mode. This method produces a charge inversion of anionic carboxylates to cationic tertiary amines via amide bond formation. Typically, amino acids and organic acids are analyzed using separate methods^{31, 32}. This technique allows for a single system to analyze both classes. Figure 3A shows an improvement of greater than 75-fold for derivatized malate vs. underivatized malate. Underivatized malate a dicarboxylic acid showed both low signal response as well as peak splitting from degeneracy of a sodium adduct. The sodium adducts is 40% of the malate signal response. With tagged malate, the sodium adducts is not observed above baseline in the spectra. The derivatization of the carboxylates in malate prevent sodium adduct formation. Signal degeneracies often complicate spectra and reduce signal intensity.

Analytical figures of merit are shown in Table 1. Limits of detection ranged between 1 to 159 nM (average 38 nM) for full scan mode. Responses were linear between 0.01 to 25 μ M with an average correlation coefficient of 0.965. Good reproducibility was also observed with average %RSD (n=3) of 17. Taken together, this two-step derivatization and mixed-mode separation system showed good sensitivity and reproducibility for targeted metabolite quantitation.

3.3 MS/MS Fragmentation

Analytes of interest were characterized by MS/MS using collision-induced dissociation (CID). Figure 4 shows four examples of fragmentation and yield two types of fragmentation pattern. Fragments coming from the tag alone are found in Figure 4A and B. Fragments coming from both the tag and the analyte are found in Figure 4C and D. The product ions (Figure 4, A and B) show fragments of tags cleaved next to the amide bond with loss of the tertiary amine and loss of the entire tag. In Figure 4C and D, product ions are observed from both backbone cleavage of target analyte and tag. This suggests that expansion of this system may be well adapted to multiple reaction monitoring on a triple quadrupole MS system.

3.4 Analysis of human heart tissue

Based on the promising results seen from standard metabolites, this method was applied to quantitate metabolites from human disease tissue. As shown in Figure 5A, metabolite levels were evaluated from human tissue with and without diabetes. Samples were quenched in liquid nitrogen immediately after harvesting to minimize possible metabolite degradation effects. Variance in metabolite levels is likely due inherent patient variation. Six samples were acquired in total. Diabetic tissue showed significant reduction in diabetic (n=3) tyrosine and fumarate compared to non-diabetic (n=3). The most pronounced change was in

succinate levels (5-fold), which were substantially elevated in diabetic tissue. Notably, fumarate and succinate are metabolically linked through the enzyme succinate dehydrogenase (Complex II in the electron transport chain) which uses succinate as its substrate and fumarate is the product. Damage or reduction of activity to succinate dehydrogenase is one hypothesis that could explain these results. Further studies are needed to investigate the role of this enzyme in diabetes.

Long term (3 month) blood glucose levels are characterized by the level of glycosylated haemoglobin (HbA1c). HbA1c of 6.5 was considered the cut-off between non-diabetic and diabetic. Figure 5B investigated the role of HbA1C levels in metabolism. Fumarate decreased with increasing level of HbA1c with $R^2 = 0.79$ correlation. Despite low sample numbers, this degree of correlation is strong considering the complex nature of true biomedical samples. Previous reports investigating diabetic animal models found that fumarate levels were altered in diabetes.³³

Conclusions

This work shows the utility of two distinct tags to label multiple functional groups in a variety of metabolites. Significant changes and inversely linear relationship was found in fumarate levels of human tissue. These results show simultaneous detection of diverse groups of metabolites which suggest larger metabolomic studies are possible which could lead to a more complete picture of metabolic changes.

Previous work in tagging metabolites has focused on combining multiple separation and tagging methods to expand the categories of metabolites detected. The initial system proposed here provides evidence that such goals can be achieved in a single platform. Future work will broaden the number of metabolites analyzed by using high resolution/peak capacity chromatography and high-resolution MS. Overall, this work enabled enhanced sensitivity of different metabolite classes through two step derivatization and mixed-mode separation, which not only circumvented limitations to ES-MS, but expanded the coverage of a single run of positive mode MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure. 1.

A) General reaction scheme of two tags with 3 different class of compounds; B) Reaction scheme of glycine

Figure. 2.

Reconstructed ion chromatograms of tagged organic acids and amino acids standards using Mixed-mode WCX column (150×2.1 mm, 3 μ m). Flow: 0.3 mL/min injection volume: 5 μ L.

* designates corresponding derivatized analyte. Isomers (Leucine/Isoleucine) are unresolved. The concentration of standards is 1 μ M.

Figure 3.

(A) Comparison of tagged malate (black) and untagged malate (blue) in different detection mode; (B) untagged malate (100 μ M) chromatogram in negative mode; malate (100 μ M) chromatogram with sodium adducts; (C) tagged malate (5 μ M) chromatogram in positive mode; tagged malate (5 μ M) chromatogram (black) with sodium adducts (red).

Figure 4.
MS/MS fragmentation of (A) tagged threonine, m/z 194.65 (B) tagged proline m/z 214.19
(C) tagged phenylalanine m/z 175.13 (D) tagged fumarate m/z 313.26

Figure 5.

(a) Fold changes in organic acids and amino acids of Diabetic vs. non-diabetic heart tissue; Error bars are SEM; * denotes $p < 0.05$; Fold changes are represented on a log10 scale. (b) Plot of fumarate's signal response/D-Met to the HbA1c value

Table 1.

Sensitivity, reproducibility, dynamic range of LC/MS results of derivatized standard metabolites (^a based on peak intensity; ^b based on limit of quantification and limit of linearity)

	LOD (nM) ^a	R ²	%RSD(n=3)	Linear dynamic range (μM) ^b	m/zs
Alpha-keto-glutarate	7	0.924	15	0.02–25	172.14
Pyruvate	51	0.947	14	0.17–25	187.14
Succinate	39	0.878	8	0.13–5	315.28
Fumarate	70	0.950	10	0.23–5	313.26
Malate	3	0.991	25	0.01–5	208.67
Asparagine	35	0.913	19	0.35–10	158.62
Aspartate	12	0.929	13	0.12–25	208.18
Glutamate	1	0.963	22	0.01–10	143.79
Glycine	36	0.998	6	0.36–25	130.11
Proline	1	0.996	19	0.01–5	214.19
Leucine/Isoleucine	28	0.989	8	0.28–5	315.28
Methionine	61	0.995	10	0.61–5	167.12
Phenylalanine	1	0.994	18	0.01–5	175.13
Serine	7	0.975	25	0.07–10	194.18
Threonine	79	0.996	5	0.79–10	194.65
Valine	1	0.999	23	0.01–5	151.13
Glutamine	159	0.937	14	1.60–5	165.63
Histidine	59	0.997	22	0.59–5	170.13
Arginine	22	0.977	26	0.22–10	179.65
Alanine	27	0.967	22	0.27–5	273.24
Tryptophan	70	0.962	4	0.70–10	194.64
Tyrosine	86	0.994	24	0.86–10	225.66
Lysine	16	0.913	29	0.16–5	208.17
Average	38	0.965	17	0.30–10	