Metabolomic Profiling of Uremia With an LC-EC Array-MS Parallel Platform.

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Overview

Purpose: The objective of this project was to characterize the metabolomic profile of patients with uremia using a novel HPLC-electrochemical (EC) array—mass spectrometry (MS) parallel platform.

Methods: Plasma samples were obtained from 29 uremic hemodialysis patients and 30 healthy control subjects. Gradient coulometric array methods were developed for the global measurement of water-soluble analytes and for the targeted measurement of fat soluble vitamins and antioxidants. Our novel parallel HPLC-EC-MS metabolomics platform was used to simultaneously profile multiple known and unknown redox active and/or ionizable water soluble compounds in plasma. EC and MS data were subjected to principal component analyses (PCA), and results from uremic samples were compared to samples from healthy subjects.

Results: In all cases, uremic samples were readily differentiated from healthy controls by PCA. To our knowledge, this is the first metabolomics study specifically designed to assess the dysmetabolism that accompanies uremia. We have provisionally identified numerous redox active and/or ionizable metabolites whose concentrations are altered in this disease.

Introduction

Metabolites are key components of diverse biochemical processes, and changes in their concentrations can be regarded as the response of biological systems to environmental changes. The comprehensive metabolite profile, i.e., the 'metabolome', can be viewed as the final stage in a chain of events ranging from gene function to metabolism, and thus the metabolic phenotype may be the most direct reflection of the current state of a biologic system.

Kidney function plays a critical role in maintaining circulatory and organ system functional homeostasis, and loss of kidney function leads to dysregulation of many metabolic pathways leading to uremic syndrome. Since alterations in gene and protein expression are subject to complex homeostatic control and feedback mechanisms, it has been suggested that metabolomics may provide a higher level of integrated information than other "-omic" approaches and be more reflective of underlying pathology. Thus, uremia may be an ideal disease state applicable to metabolomic studies.

Increased oxidative stress is a metabolic alteration known to contribute to the pathogenesis of atherosclerosis, and uremia has now been identified as an increased oxidative stress state. To date, oxidative stress in uremia has primarily been evaluated by targeted analysis of the plasma concentration of individual oxidized solutes or antioxidants. Several studies have demonstrated an association between the plasma concentration of individual oxidized solutes or antioxidants and subsequent cardiovascular mortality in dialysis patients. However, a comprehensive biochemical assessment of uremia that incorporates simultaneous determination of multiple redox active and/or ionizable solutes has not been accomplished.

Recently, there has been considerable progress in developing an integrated analytical approach capable of measuring multiple redox active metabolites in biological matrices. Through the complementary application of EC array and MS techniques combined with high-performance liquid chromatography (LC), simultaneous detection of multiple known and unknown redox active and/or ionizable metabolites is now possible (1,2). We hypothesized that application of these technologies to the study of uremia would permit detection of multiple metabolites differing between uremic patients and healthy subjects. Accordingly, we sought to initially characterize a circulating uremic redox active and/or ionizable metabolome.

Methods

Study Subjects
Plasma samples were obtained from 10 patients with end-stage renal disease (ESRD) receiving chronic maintenance hemodialysis therapy (drawn immediately pre-dialysis) and from 10 healthy control subjects. The ESRD group was comprised of all Caucasian subjects (5 men) with a mean (±SD) age of 65 (±14) years. Since diabetes mellitus is the leading cause of ESRD, and diabetes may independently affect the circulating metabolome, we chose to study 5 ESRD subjects with and 5 ESRD subjects without diabetes mellitus. The healthy control group was comprised of all Caucasian subjects (5 men) who were 51 (±7) years of age.

All subjects were between the ages of 18 and 85. No subjects had evidence of active malignancy, severe gastrointestinal dysfunction, a history of a functional kidney transplant within six months prior to study entry, or recent cardiovascular procedures. Subjects were excluded from consideration if they were taking vitamin E supplements ≥ 60 IU per day, or ascorbic acid ≥ 500 mg per day. No subjects were routinely taking anti-inflammatory medications. Prisoners, patients with significant mental illness or cognitive deficits, and other vulnerable populations were excluded from the study. The study adhered to the Declaration of Helsinki and was approved by the Maine Medical Center Institutional Review Board.

Sample Preparation
Venous blood was drawn into Vacutainer® (Becton Dickinson, Franklin Lakes, NJ) tubes containing ethylenediamine tetra-acetic acid (ETDA), immediately placed on ice and centrifuged at 1700 g (4 °C) for 15 min. Plasma was removed and frozen within one hour of sample collection and stored at -70 °C until analysis.

Liquid Chromatography
The LC-EC array-MS platform consisted of an HPLC system, and an linear ion trap mass spectrometer with an electrospray ionization (ESI) source in parallel with a sixteen channel EC array detector (Thermo Scientific Dionex CoulArray Detector with Thermal Organizer).

Method 1: Water Soluble Metabolites
An LC method optimized for redox active water-soluble metabolites was used with EC-array detection alone. Mobile phase A consisted of 0.1 M monobasic sodium phosphate, 10 mg/L sodium dodecyl sulfate (SDS) and 50 nM nitrilotriacetic acid (NTA); pH 3.35. Mobile phase B consisted of 0.1 M monobasic sodium phosphate, 50 mg/L SDS, 50 nM NTA, 50% methanol (v/v); pH 3.45. The gradient timeline was 10% B for 10 min, a linear gradient to 40% B in 10 min, and a linear gradient to 90% B in 10 min, before returning to initial conditions. A C18 MCM column (150 mm x 4.6mm; 5 µm; Thermo Fisher Scientific) was used with a flow rate of 1.0 mL/min and column temperature of 37°C. EC array potentials were held at 60 mV increments from 0 mV to +900 mV (16 channels).

Method 2: Lipid Soluble Compounds
An LC method optimized for lipid-soluble redox-active metabolites was used with EC-array detection alone. Mobile phase A consisted of methanol:0.2 M ammonium acetate (90:10, v/v), pH 4.4. Mobile phase B consisted of methanol:1-propanol:1.0 M ammonium acetate (78:20:2, v/v/v), pH 4.4. The gradient timeline was a linear gradient from 0%-80% B in 10 min, a linear gradient from 80%-100% B in 10 min, and a 7 min hold at 100% B before returning to initial conditions. A C18 MD-150 column (150 mm x 3 mm; 3 µm; Thermo Fisher Scientific) was used with a flow rate of 0.8 mL/min and column temperature of 37°C. EC array potentials (mV vs. Pd) were: +200, +400, +500, +700, +800, -1000, -1000, +500 (8 channels).

Method 3: Parallel LC-EC array-MS Conditions
An LC method developed for the separation of a diverse range of analytes (e.g., metabolites soluble in aqueous and organic solvents) was used with parallel EC-array and MS detection. Mobile phase A consisted of 2% aqueous methanol (v/v) and mobile phase B consisted of 80% aqueous ACN (v/v) each containing 50 mM formic acid and 10 mM ammonium formate. A 15 min linear gradient from 0%-80% mobile phase B was used with a Shiseido® C18 MG column (4.6 mm x 75 mm; 3 mm Shiseido Co Ltd.), flow rate of 1.0 mL/min and column temperature of 35 °C. Flow was passively split post-column in the ratio of 4:1 EC:MS (Figure 1). MS data were acquired in positive ion enhanced MS full-scan mode. EC array potentials were held at 70 mV increments from +100 mV to +1150 mV (16 channels). Data acquisition from MS and EC array was synchronized with sample injection by using a simple contact closure from the autosampler, which allowed automated runs to be performed. A single sample injection led to the production of two separate and information-rich data files with this approach.

Data Analysis
EC data were analyzed using CoulArray™ for Windows® software 3.1. EC-array data was transferred to Pirouette® (Infometrix, Inc., Woodinville, WA) for chemometric analysis using a CoulArray version 2.0 Software Utility (Pattern Recognition Setup Wizard).

Results and Discussion

Water soluble metabolites in plasma samples were assessed using the LC-EC array platform. EC array chromatograms shown in Figures 1A and 1B are representative of the observed higher number and/or relative quantity of redox active peaks present in uremic subject plasma as compared to healthy subjects. Representative EC array chromatograms of lipid soluble metabolites are shown in Figures 2A and 2B, respectively. In contrast to the observations made from water soluble metabolite profiles, bidirectional differences in lipid soluble metabolite concentrations between uremic and healthy subjects are readily apparent.

FIGURE 1. Water soluble metabolites: Representative EC array chromatograms from plasma samples obtained from (A) patient with ESRD, and (B) healthy subject. Data were generated using an LC method optimized for polar (water-soluble) metabolites with EC-array detection alone (Method 1). Only channels 1-8 are shown for clarity

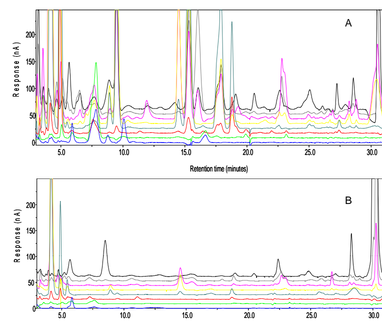
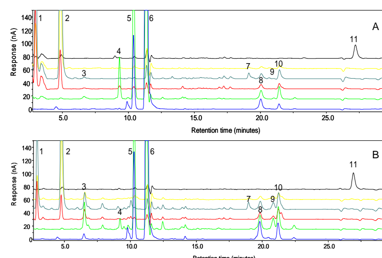
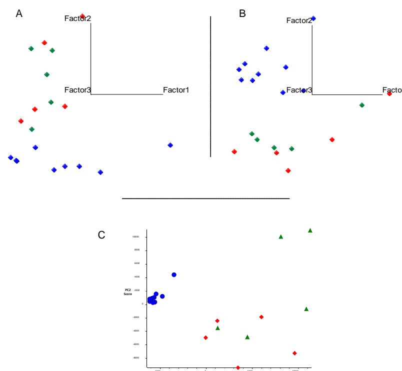


FIGURE 2. Fat soluble metabolites: Representative EC array chromatograms from plasma samples obtained from (A) patient with ESRD, and (B) healthy subject. Data (Table 1) were generated using an LC method optimized for lipid-soluble metabolites with EC-array detection alone (Method 2). Only channels 1-5 and 8 are shown for clarity. Peak identity: (1) retinol, (2) retinyl acetate, (3) lutein, (4) δ-tocopherol, (5) γ-tocopherol, (6) α-tocopherol, (7) retinyl palmitate, (8) lycopene, (9) α-carotene, (10) β-carotene, (11) CoQ10.



Water soluble metabolite data obtained using the parallel EC array-MS platform from all subjects were analyzed using principal components analysis (PCA). Figure 3A depicts a PCA scores plot of factors 1 and 2, which shows a single cluster of healthy subjects that is easily separated from the ESRD patients. Similarly, a PCA scores plot of lipid soluble redox active metabolite profiles, shows clear differentiation of uremic patients and healthy control subjects (Figure 3B). A PCA scores plot of MS data again demonstrates clear differentiation of uremic and healthy subjects based on the global plasma profiles of ionized species (Figure 3A). Although control and ESRD groupings could be easily differentiated by all three analytical approaches, there was considerable overlap between ESRD and the diabetic-ESRD groups. This suggest that the impact of ESRD on the plasma metabolome is so extensive that any differences due to diabetes are simply overwhelmed.

FIGURE 3. Representative PCA scores plot for (A) polar compound EC array data; (B) lipid-soluble compound EC array data; and (C) MS data. Individual green, red, and blue data points represent samples drawn from non-diabetic ESRD, diabetic ESRD, and healthy subjects, respectively (analytes measured using Method 3).



Quantitative comparison of selected lipid soluble redox active compounds in uremic patients versus healthy control subjects is presented in Table 1.

Table 1. Quantitative comparison of selected lipid soluble compounds*

	Healthy subjects	*ESRD patients*	*P*
α-tocopherol	11,861 ± 682	9,880 ± 713	0.03
α-carotene	86 ± 19	26 ± 4	0.002
β-carotene	267 ± 38	100 ± 19	0.0004
coenzyme-Q_{10}	695 ± 42	471 ± 24	< 0.0001
δ-tocopherol	56 ± 6	121 ± 15	0.0002
γ-tocopherol	1,310 ± 141	2,167 ± 194	0.0002
lutein	145 ± 13	75 ± 10	< 0.0001
lycopene	435 + 31	179 ± 19	< 0.0001
retinol	575 ± 36	1,126 ± 99	< 0.0001
retinyl palmitate	30 ± 7	30 ± 3	0.03

* Data presented as mean±SEM (ng/mL)

Conclusion

- The coupling of MS in parallel with the EC array is a powerful analytical approach providing additional, orthogonal data through the simultaneous measurement of redox active and ionizable metabolites.
- This pilot study showed that this platform has great potential for investigating the effects of disease on the human plasma metabolome.
- PCA readily differentiated controls from ESRD patients, but could not differentiate between ESRD and ESRD-diabetic patients.
- Future research will be directed at developing diagnostic profiles of plasma redox active metabolites that identify ESRD patients at higher risk of adverse cardiovascular outcomes, and at determining the utility of diagnostic metabolite profiles for assessing the response to therapies designed to alleviate the uremic syndrome (including dialytic therapies, kidney transplantation, and pharmacologic therapies).

References

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