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Determination of multiple redox-active compounds by high-performance liquid chromatography with coulometric multi-electrode array system

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Abstract

Studies of the antioxidant defense system and the related metabolic pathways are often complicated by cumbersome analytical methods, which require separate and multi-step extraction and chemical reaction procedures. Further, assaying multiple parameters is limited because of the usual small sample amounts. High-performance liquid chromatography coupled with a coulometric multi-electrode array system provides us high specificity and sensitivity to measure electrochemically oxidizable compounds in biological samples. In contrast to previously reported methods with two columns in series and a complex gradient elution profile, we have developed an automated procedure to simultaneously measure multiple redox-active low-molecular weight compounds that utilizes a single column with a simplified binary gradient profile. No other chemical reactions are necessary. In order to reduce the running time and yet achieve a reproducible retention time by the auto sampler injection, our gradient elution profile was modified to produce a shorter equilibration time, stable retention time, and a reduced cost per test.

Keywords: Coulometric multi-electrode array system; Redox-active compounds

1. Introduction

There are many diseases with etiologic heterogeneity. To identify candidate pathological process(es) that account for the constellation of clinical and biological features in these diseases, it is necessary to evaluate a network of multiple interacting biochemical pathways simultaneously in biological samples. However, studies of a series of related metabolites are often complicated by cumbersome and different analytical methods, which require separate and multi-step extraction and chemical reaction procedures. Thus, measurements of multiple parameters are always restricted by the sample amounts, particularly the biopsied materials.

High-pressure liquid chromatography (HPLC) coupled with a coulometric multi-electrode array system (CMEAS) provides high specificity and sensitivity for determination of multiple redox-active low-molecular weight compounds based on differences in oxidation–reduction properties of analytes [1–4]. The CMEAS equips four cell packs in series, each consisting of four coulometric electrodes, and set to increasing specified potentials between −1000 and 2000 mV, which allows resolution of co-eluting compounds whose oxidation or reduction potentials differ by as little as 60 mV. Multisensor, coulometric detectors can oxidize and measure nearly 100% of the oxidizable compounds in a sample. The

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coulometric array detectors utilize a combination of retention time and ratio of response across adjacent detectors as a molecular fingerprint to enable identification of a specific peak in HPLC chromatograms [1–4].

Using the HPLC–CMEAS, more than 1000 electrochemically oxidizable compounds such as antioxidants, oxidative stress markers, and monoamine metabolites, can be analyzed simultaneously in the biological samples [2,5]. These previously reported protocols, however, are somewhat tedious, requiring dual columns and a complex gradient elution profile with variable flow rates. In addition, a special "passive gradient mixer or peak suppresser/ gradient mixer" was placed in the flow stream before the HPLC injector to reduce a mobile phase derived contaminant signal. The present study reports a simplified HPLC protocol to separate multiple redox-active low-molecular weight compounds in a single analysis.

2. Experimental

2.1. Chemicals

1-Pentane-sulfonic acid (PSA) was purchased from J.T. Baker (Phillipsburg, NJ). Lithium acetate, methanol, acetonitrile, isopropanol, and all the standard compounds were from Sigma (St. Louis, MO). Glacial acetic acid was obtained from Fisher (Pittsburgh, PA). Nylon filter membrane (0.2 μm) was obtained from Waters (Bedford, MA). All water was purified by Millipore Mill-Q Reagent Water System.

Most standard stock solutions consisting of 2 mg/mL each were prepared from HPLC-grade H_2O. Some standards that are not soluble in H_2O completely are pre-dissolved in 0.1 mL of 0.1N NaOH or 0.1 M $HClO_4$ before subsequent dilutions with H_2O. All stock solutions were stored at −70 °C. Before run, different sets of working standard mixtures consisting of five different concentration levels (50, 100, 500, 1000, and 5000 ng/mL each) were prepared by a series of dilutions from the concentrated stock solution. 3,4-Dihydroxybenzylamine (DHBA) was served as an internal standard.

2.2. Instrumentation

CoulArray Model 5600 HPLC system (ESA Inc., Chelmsford, MA) consists of two Model 582 pumps, one dynamic gradient mixer, two PEEK pulse dampers, a Model 542 refrigerated autosampler injector, a CoulArray organizer module, and a serial array of 16 coulometric electrodes. The system was controlled and chromatograms were analyzed by a Dell personal computer Model Dimension XPS R450 using the ESA CoulArray for Windows-32 software program (Version 1.04).

2.3. Preparation of the mobile phase

The solutions used for two mobile phases were essentially the same as described by Milbury [1]. The current procedure used to prepare these two mobile phases, however, was quite different from that described by Kristal et al. [5]. Mobile phase A (MPA) consists of 1.1% (w/v) of 1-pentane-sulfonic acid (pH, 3.0). At any given time, we prepare 2 L of MPA freshly by dissolving 22 g of 1-pentane-sulfonic acid in a mixture of 1994 mL of H_2O and 6 mL of glacial acetic acid (Fisher Scientific, reagent A.C.S., 17.4N). The final pH of MPA is 3.0 ± 0.1.

Mobile phase B (MPB) consists of 0.1 M lithium acetate in solvent mixture of methanol, acetonitrile, and isopropanol, 80/10/10 (v/v/v). In the present study, we prepare 1 L of MPB freshly for every 2 L of MPA. Lithium acetate (10.2 g) was dissolved in a solvent mixture consisting of 800 mL methanol, 100 mL acetonitrile, 100 mL isopropanol, and 16 mL glacial acetic acid. The final pH of MPB is 6.4 ± 0.1.

Both mobile phases were filtered through 0.2 μm nylon filter membrane and then degassed with helium for 20 min before applying to HPLC.

2.4. Sample preparation

Freshly drawn blood with anticoagulant citrate dextrose (ACD) is first centrifuged at 800 rpm for 15 min to remove red blood cells. Internal standard (50 μL), dihydroxybenzylamine (10 μg/mL), and 250 μL of saline solution were added to 50 μL of plasma sample. The mixture was then filtered through a disposable membrane (0.22 μm pore size) micropartition system (Millipore Ultrafree-MC) under centrifugation to remove any compounds above 10,000 nominal molecular weight limit. A similar procedure was also used to obtain protein-free cerebrospinal fluid (CSF) with exception that 50 μL of internal standard solution was added to 150 μL of CSF without addition of saline solution. Aliquot of plasma or CSF filtrate (deproteinized sample) was then separated by HPLC (see below).

2.5. HPLC operating procedure

Each sample (50 μL) was run on a single column (ESA Meta-250, 5 μm ODS, 250 mm × 4.6 mm i.d.) under a 150-min gradient elution that ranged from 0% to 20% MPB with a fixed flow rate of 0.5 mL/min (Fig. 1A). The temperature of both cells and column was maintained at 25 °C. Similar to the previously published method [1–4], the current gradient elution also apply 2 min of a high-potential cell-cleaning step and restore the column condition to the 100% MPA at the end of each run. The CMEAS was set to have increments from 0 to 900 mV in 60 mV steps. The properties of various antioxidant compounds or monoamine metabolites are often closely related to the same structural characteristics. The use of several coulometric sensors in series that are maintained at different potentials provides resolution and detection of co-eluting compounds having different electrochemical features.

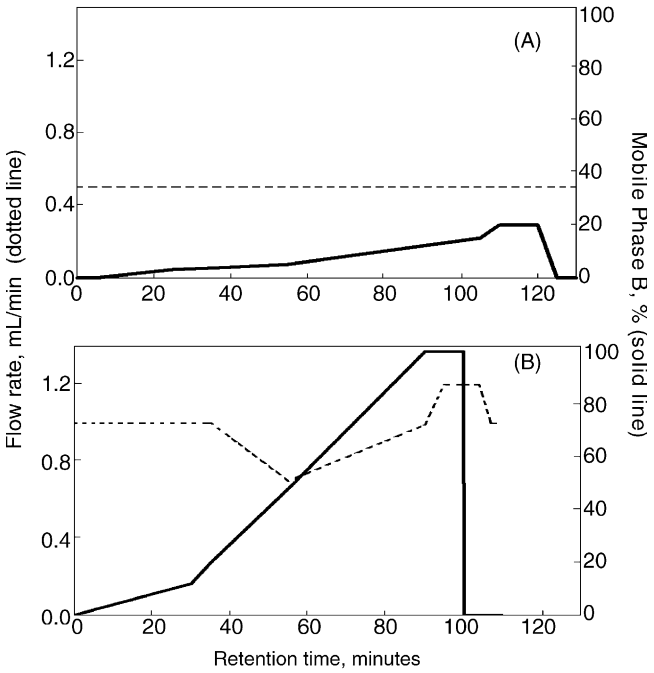

Fig. 1. Comparison of flow rate and mobile phase gradient profile between (A) the present modified procedure and (B) previously published method by Kristal et al. [5].

2.6. Data analysis

The ESA CoulArray for Windows 32 package was used for quantitative analysis of peak identity and the peaks relative to absolute or reference standards. This software is able to automatically subtract backgrounds resulting from the gradient drift. The coulometric array was used to generate databases of all redox-active molecules with redox potentials from 0 to 900 mV. Peak identity was verified by the retention time, dominant channel, and the ratio of reactivity on the dominant channel to reactivity on the subdominant channels [1,5,6]. The detector potentials are configured to allow compounds responding at three consecutive electrodes [7]. The analyzing software defines this set of three responses as a peak cluster and the sensor having the highest response within a cluster is called the dominant channel [8]. Both peak purity and peak identity can be verified by relative reactivity across multiple different potentials because the ratio of the dominant to the adjacent detector responses is characteristic of a given compound.

Ratio conformity is defined as [ratio of the adjacent channel to the dominant channel in unknown]/[ratio of the adjacent channel to the dominant one in the standard] × 100. Two ratio window (RW) settings have been used to quantitate compounds: RW 30 uses channels with <30% ratio variation, whereas RW 100 uses all channels. Recently, Shi et al. [8] have demonstrated that the narrow ratio window (RW 30) increases quantitative accuracy in coulometric electrode array analysis of the metabolites in biological samples. In the present study, the concentration of each peak was thus calculated according to the dominant channel which have >70% ratio conformity to contribute to the quantitation.

3. Results and discussion

To improve the previously reported procedures [1,2,5] and reduce the cost per test for monitoring multiple redox-active low-molecular weight compounds by HPLC–CMEAS, we have modified the column installation, mobile phase preparation, gradient elution profile, and column cleaning protocol as follows.

3.1. Column installation

In order to monitor multiple redox-active compounds in a single run, two serial columns were installed for sample separation in the previously described procedure [1,2,5]. Owing to the length of column and high flow rates (0.7–1.2 mL/min, Fig. 2A), the column pressure can easily reach above the maximal setting (340 bar) for the designated ESA Model 582 pump. Consequently, the stainless tubing was required to run the above HPLC procedure. To reduce column pressure as well as the aziotropic viscosity affects, the present procedure only uses one column with a fixed lower flow rate (0.5 mL/min) while maintaining the similar resolution and running time as previously established.

In addition to the META250 column [2], other types of HPLC column, such as C18 ESA HR80 [8] or TosoHaas TSK-GEL ODS-80 [5] columns, have also been used successfully in series. Although we have not tested other columns, it is likely that these previously tested two series columns can also be adapted to run the current HPLC condition.

3.2. Mobile phase

Kristal et al. [2,5] prepared their working mobile phases from dilution of the concentrated stock solution. However, the concentrated MPA stock solution (dissolving 399.98 g of pentane sulfonic acid in 1300 mL H_2O and 200 mL glacial acetic acid first, and then bring to 2 L with H_2O) is inherently contaminated and needs to be cleaned by electrolyzing the concentrated buffer over pyrolytic graphite at 1000 mV versus a palladium reference [Pd(H)] for 12–24 h. The electrochemical cleaning of the MPA concentrate also balances the oxidation potential of the buffer solution that adds to the long-term stability of coulometric electrode detectors. By contrast, we are able to dispense with the above cleaning procedure because we prepare our working MPA (2 L) freshly without dilution from the concentrated stock solution.

To reduce the acetic acid content in MPB, we have substantially modified the procedure of Kristal et al. [2,5] for MPB preparation. Instead of preparing the working MPA from the concentrated lithium acetate (4 M) solution, we made 0.1 M lithium acetate freshly from the solvent mixture that was pre-adjusted to pH 3.0 with acetic acid. Therefore, the final pH

Table 1
Reproducibility of retention time and quantitative channel ratio responses

Peak ID	Standards	Dominant channel	Retention time		Channel ratio responses	
			Mean ± S.D., n = 19	CV (%)	Mean ± S.D., n = 19	CV (%)
1	Uric acid	3	15.18 ± 0.60	3.95	0.90 ± 0.02	2.22
2	Xanthine	9	20.01 ± 0.62	3.10	0.72 ± 0.05	6.94
3	4-Hydroxy-3-methoxy-mandelic acid	4	23.6			
4	Hypoxanthine	13	23.7			
5	Glutathione (reduced form)	10	28.57 ± 0.98	3.43	0.46 ± 0.02	4.35
6	4-Hydroxy-3-methoxyphenyl-glycol	6	34.0			
7	Oxidized glutathione (GSSG)	12	40.12 ± 1.77	4.41	0.80 ± 0.06	7.50
8	Methionine	12	42.62 ± 1.49	3.50	0.43 ± 0.01	2.33
9	Xanthosine	11	43.78 ± 1.82	4.16	0.53 ± 0.01	1.89
10	Norepinephrine	1	45.42 ± 1.17	2.58	0.89 ± 0.03	3.37
11	L-3-Hydroxytyrosine (L-DOPA)	1	45.5			
12	Guanosine	11	51.79 ± 2.29	4.42	0.60 ± 0.03	5.00
13	Guanine	9	56.54 ± 2.46	4.35	0.89 ± 0.03	3.37
14	Tyrosine	9	58.86 ± 2.54	4.32	0.86 ± 0.02	2.33
15	3-Amino-tyrosine	1	55.0			
16	Epinephrine	1	62.82 ± 1.61	2.56	0.82 ± 0.04	4.88
17	3,4-Dihydroxyphenylacetic acid (DOPAC)	1	67.1			
18	Dihydroxybenzylamine (internal standard)	1	77.51 ± 1.70	2.19	0.81 ± 0.05	6.17
19	Kynurenine	10	81.10 ± 2.19	2.70	0.55 ± 0.05	9.09
20	3-Chloro-L-tyrosine	8	79.0			
21	Normetanephrine	4	79.3			
22	3-Nitro-tyrosine	11	85.96 ± 2.20	2.56	0.36 ± 0.02	5.56
23	5-Hydroxyindole-3-acetic acid (5-HIAA)	2	91.46 ± 2.46	2.69	0.73 ± 0.05	6.85
24	Metanephrine	5	90.8			
25	Dopamine	1	94.05 ± 1.76	1.87	0.96 ± 0.02	2.08
26	Homovanillic acid (HVA)	4	105.84 ± 2.33	2.20	0.55 ± 0.04	7.27
27	Tryptophan	8	111.12 ± 2.51	2.26	0.43 ± 0.03	6.98
28	Serotonin (5-HT)	2	120.37 ± 1.78	1.48	0.80 ± 0.03	3.75

Note: Peak ID and dominant channel are the same as those labeled in Fig. 1. Channel ratio response was determined by the ratio of peak area of the dominant channel to the sum of peak areas of pre-dominant, dominant, and post-dominant channels.

value (approximately 6.5) of our MPB is much higher than the previously reported (pH 3.0).

It should also be noted that 1-pentane-sulfonic acid and lithium acetate combination in Milbury's mobile phases [1] have several functions. First, PSA as an ion-pairing reagent equilibrates rapidly with a C18 column allowing stable repetitive gradients to 100% organic modifier. Thus, PSA in the MPA increases column stability by acting as a detergent for large peptide fragments, lipids, and lipoproteins that are present in any "deproteinized" biological sample. Secondly, lithium is bacteriostatic and prevents the occasional growth of bacterial/fungal cultures in elements of the fluidic system that affect stability over long time periods. Thirdly, the extension of the gradient to 100% B in each assay [1–5] removes lipids, glycoproteins, and lipoproteins resulting in good stability over a 1 to 2-year-period.

3.3. Gradient elution

The gradient profile of the current method (Fig. 1) is quite different from the previously published method by Kristal et al. [5]. Particularly, complete removal of 100% MPB at the end of run (Fig. 1B) requires much longer equilibration time with MPA than the present method. A quick equilibration was possible with much lower % of MPB (Fig. 1A) than those previously reported gradient elution. In any given batched analysis, a blank run is always included at the beginning of the sequence. Using the auto injector, we are able to maintain a stable retention time (Table 1) with the proposed gradient elution even after 150 injections.

To compensate for aziotropic viscosity effects, variable high flow rates (0.7–1.2 mL/min) were applied to the previously reported gradient elution profile (Fig. 1B). Such effects, however, were not present in the current condition using a single HPLC column. Thus, we reduce the flow rate to a fixed 0.5 mL/min (Fig. 1A) with an intention to decrease the total MPA volume during our gradient elution.

In addition, analytes with peak ID from 1 to 28 were eluted within the 70 min (Fig. 2B) during the 110 min of previously published gradient run [5]. On the other hand, the same set of compounds (peak ID 1–28, Fig. 2A) was separated evenly by the current procedure over the entire HPLC run. Thus, our modified gradient system may provide a better resolution for some closely eluted compounds, e.g., peaks 7 and 12 or 8 and 13 shown in Fig. 2B.

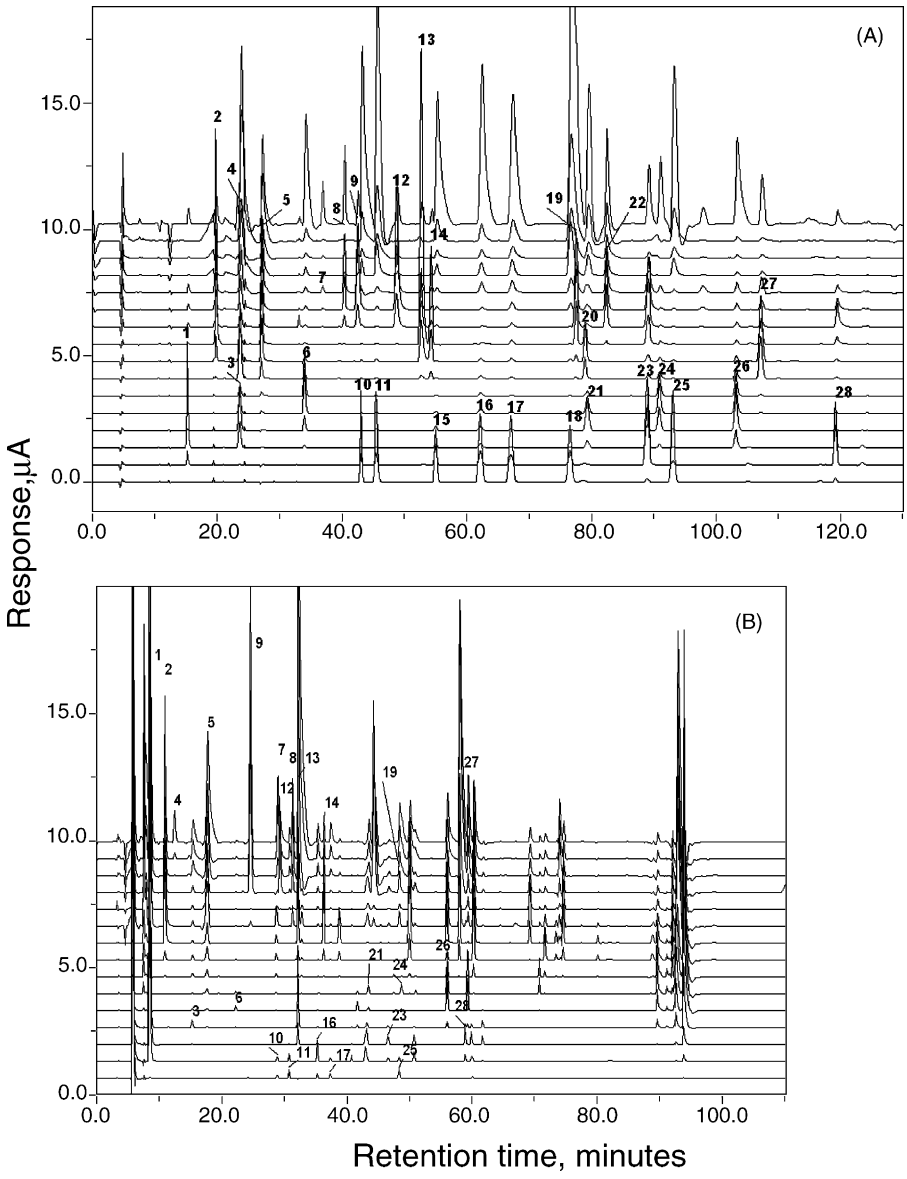

Fig. 2. Comparison of HPLC–CMEAS chromatograms of the standard mixture using between (A) the present modified gradient method and (B) the previously published [5] gradient method. Chromatogram (B) was provided by the ESA Laboratories Inc. The identification number of standards in each chromatogram is summarized in Table 2.

3.4. Column washing and equilibration

The previously reported gradient profile [1,2,5] contains 100 % MPB at the end of run, which acts as the organic modifier to wash out any residues (shown as the spurious peaks after 90 min) such as lipids and polysaccharides in the sample (Fig. 2B). These spurious peaks, however, were also present in the gradient of blank runs. In their experiments, a special "peak suppresser/gradient mixer" (PS/GM, not commercially available) was required to place in the flow stream before the HPLC injector to reduce the effect of mobile phase contaminants [1,2]. The mixed gradient was then delivered from the PS/GM to a PEEK lined pulse damper prior to flowing through the autosampler injector and on to the columns.

In the present study, however, less extensive column washing is applied, and thus the method does not require a mechanical device (e.g., PS/GM) for suppression of spurious peaks, which is a useful simplification of the plumbing. The maximal gradient of MPB was reduced drastically from 100% of prior methods to 20% in the present gradient system (Fig. 1A). The lower percentage of MPB at the end of gradient run substantially reduces these spurious peaks from the chromatogram (Fig. 2A) *without* applying peak suppresser/gradient mixer.

In order to obtain a reproducible retention time, it is necessary to remove the MPB completely from the column between the run. If not, the presence of MPB in the column will continuously shorten the retention time as the number of injection increase. Consequently, certain peaks such as

Table 2
Intra-assay reliabilities and recovery

Analytes	Plasma baseline (ng/mL)	Spiked amount (ng/mL)	Total (ng/mL, n = 10)	Recovery (n = 10)		Coefficients of variation (%)
				ng/mL	%	
Guanosine	303	3949	4323 ± 130	4020 ± 130	101.8 ± 3.3	3.23
Kynurenine	146	3643	3564 ± 170	3418 ± 170	93.8 ± 4.7	4.97
3-Nitro-tyrosine	38	5342	4853 ± 92	4815 ± 92	90.1 ± 1.7	1.91
5-Hydroxyindole-3-acetic acid	26	5384	5024 ± 197	4998 ± 197	92.8 ± 3.7	3.94
Homovanillic acid	56	5485	4990 ± 161	4934 ± 161	90.0 ± 2.9	3.26
Serotonin	47	1379	1435 ± 33	1388 ± 33	100.6 ± 2.4	2.38

guanine and tyrosine will become inseparable. Because Milbury's gradient contains 100% MPB, the column needs to be equilibrated with >25 mL of MPA (25 min with flow rate at 1.0–1.2 mL/min) to remove MPB between runs. This equilibration step will undoubtedly raise the cost per test since 1-pentane-sulfonic acid, which is the only chemical in the MPA, costs approximately $200 per 100 g. In contrast, the present gradient only contains 20% MPB maximally, which leads to a lesser volume (10 mL) of MPA for equilibration between runs.

The deteriorated resolution between guanine and tyrosine peaks can usually serve as an index to measure column efficiency. To regenerate column, it is recommended that the column is maintained at lower flow rate, e.g., 0.01 mL/min, with 100% MPA over the weekend. This same procedure is also applicable when column is in idle condition.

3.5. Chromatographic stability

Table 1 shows the retention time and dominant channel of 28 analytes. Nineteen of the 28 analytes were mixed as a standard mixture and tested for chromatographic stability. Following 19 runs over a period of 1 month, all 361 analytes (19 × 19) were within 4.5% of the mean retention time. Previously, Kristal et al [2] have demonstrated that all 225 analytes (from 25 runs of a mixture containing nine analytes) were within 2% of the mean retention time. However, in their study, samples were run within a period of 4 days. By contrast, in the present study, samples were run as a quality control in different batches over a period of 1 month.

In addition to retention time, coulometric array detectors also utilize the ratio of response across adjacent detectors (Table 1) as a molecular fingerprint to identify a specific peak in HPLC chromatograms [9,10]. All analytes measured in this study had quantitative channel ratio responses (R-values) within 10%. The R-values were determined without confounding analyses of co-eluting peaks on the subdominant channel.

3.6. Assay precisions

In a typical assay, the standard curve for all analytes in this study was established between 0.2 and 200 ng (on column) or from 4 ng/mL to 4 μg/mL. Kristal et al. [5] have shown that the dynamic range of analytes by HPLC–CMEAS method ranges from 1–10 pg to 1–3 μg on column (five or more orders of magnitude). In the present study, a variety of redox-active compounds with concentration ranging from 200 pg (on column) to 2 μg (on column) can be measured simultaneously in the plasma sample (Fig. 3B). In the absence of peak suppresser, we can reliably measure redox-active low-molecular compounds as low as 100 pg (on column), which is two times of the noise signal generated by the blank run.

To test the precision and recovery of HPLC–CMEAS method, aliquots of pooled plasma samples were compared before and after spiking with six known amounts of standards (5-hydroxyindole-3-acetic acid, dopamine, xanthine, methionine, tryptophan, and ascorbic acid). The whole analytical procedure including deproteinization was carried out for each sample. The intra-assay precisions (n = 10) of six spiked redox-active compounds in plasma (Table 2) ranged from 1.91% to 4.97% coefficients of variation (CV). The mean recovery of 10 within-run assays ranged from 90% to 102%. On the other hand, within the range of the standard curve, the CV for between-run assays (n = 9) were in the range of 1.91%–5.88% for these six spiked compounds (Table 3).

Table 3
Inter-assay reliabilities and recovery

Analytes	Plasma baseline (ng/mL)	Spiked amount (ng/mL)	Total (ng/mL, n = 9)	Recovery (n = 9)		Coefficients of variation (%)
				ng/mL	%	
Guanosine	789	3949	4678 ± 72	3889 ± 100	98.5 ± 2.5	2.57
Kynurenine	50	3643	3616 ± 118	3566 ± 128	97.9 ± 3.5	3.59
3-Nitro-tyrosine	35	5342	5095 ± 219	5060 ± 219	94.7 ± 4.1	4.33
5-Hydroxyindole-3-acetic acid	101	5384	5525 ± 235	5424 ± 237	100.7 ± 4.4	4.37
Homovanillic acid	32	5485	5283 ± 310	5251 ± 309	95.7 ± 5.6	5.88
Serotonin	55	1379	1418 ± 17	1363 ± 26	98.8 ± 1.9	1.91

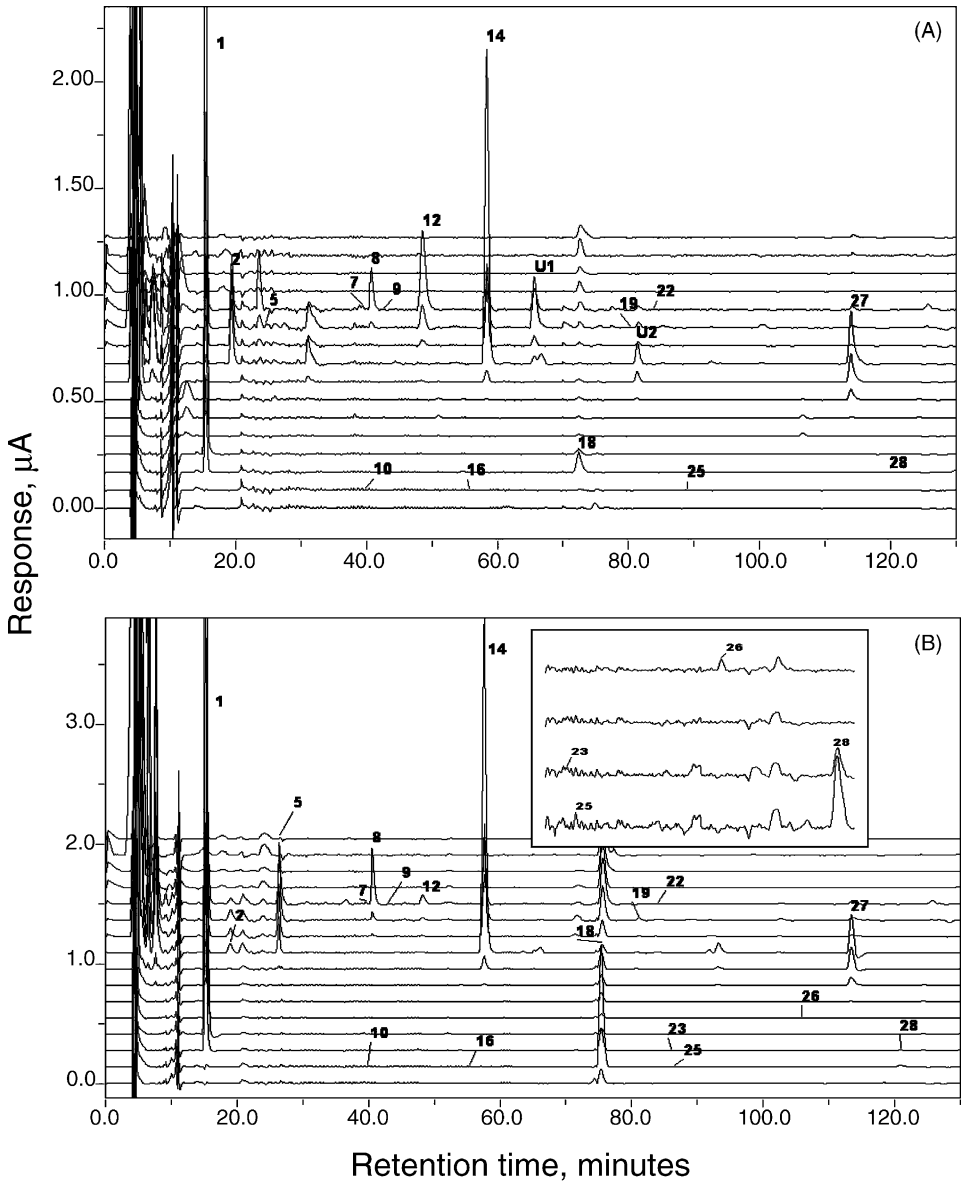

Fig. 3. Sample chromatograms of deproteinized cerebrospinal fluid (A) and plasma (B) using the present modified flow rate and mobile phase gradient profile. The identification number of standards in each chromatogram is summarized in Table 2. The detection range of analytes in plasma sample (B) ranges from 200 pg (peak 28) to 2 μg (peak 1) on column.

The mean recovery of between-run assays also ranged from 96% to 101%.

3.7. Stability of serum metabolome

Recently, Kristal's laboratory have conducted a series of investigations concerning the issues of maintaining stable patterns of response in rat serum samples in a dietary restriction (DR) study [11–13]. Nearly 1200 serum compounds can be examined simultaneously by using a HPLC–CMEAS method. Vigneau-Callahan et al. [11] further addressed the issues concerning the analytical validity (HPLC running condition, computer-automated peak identification, mathematical compensation for chromatographic drift, etc.) and biological variability (individual variability, cohort–cohort variability, outliers). Following validation of the HPLC–CMEAS methodology based upon the above criteria, approximately 250 compounds in serum have sufficient analytical reproducibility for potential use as components of serum metabolome [11].

Subsequent studies by Shi et al. distinguished 101 (female) and 112 (male) chromatographically identifiable compounds that were different between ad libitum (AL) consumption and DR 6-month-old rat [12]. Using independent cohorts of AL and DR 6-month-old rats, they further revealed 29- and 63-metabolite datasets as baseline profiles for male and female rats, respectively [13]. Taken together, these studies suggest that quantitative analysis of selected serum metabolites can

yield sufficient information to classify a group of rats by dietary intake, to identify biomarkers chromatographically, and to set the stage for pattern recognition-based approaches in establishing metabolome-based categorical separations.

4. Summary

In contrast to the two columns in series, required peak suppresser/gradient mixer and complex gradient elution profile, we have simplified the previously published HPLC method to separate multiple redox-active low-molecular weight compounds simultaneously in a single column (Fig. 2A). These compounds include, but are not limited to (1) low-molecular weight antioxidants (e.g., ascorbic acid, uric acid, and glutathione); (2) purine catabolites (e.g., hypoxanthine, xanthine, xanthosine, guanine, guanosine and uric acid); (3) oxidative damage products (glutathione disulfide, 3-nitrotyrosine, and 8-hydroxydeoxyguanosine); (4) tyrosine metabolites (tyrosine, dopamine, norepinephrine, epinephrine, 3-methoxy-4-hydroxyphenylglycol, and homovanillic acid); and (5) tryptophan metabolites (tryptophan, kynurenic acid, serotonin, and 5-hydroxy-indoleacetic acid). This procedure can be applied to a variety of biological samples such as plasma and cerebrospinal fluid (Fig. 3). No other chemical reactions are necessary. The current gradient elution was modified to provide a stable peak retention time with automated injection. Moreover, our gradient profile substantially reduces those spurious peaks occurring late in the blank runs when the final gradient was 100% B. The intra- and inter-assay precisions of six spiked redox-active compounds in plasma ranged from 1.91% to 5.88% CV. The mean recovery of within- and between-run assays also ranged from 90% to 102%. However, further investigations are needed to demonstrate whether analytical performance can be maintained in a long-term study.

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