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Metabolic Investigation in Psychiatric Disorders

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Abstract

A multiplicity of theories have been proposed over the years that aim to conceptualize the pathophysiology of neuropsychiatric disorders, including impaired neurotransmission, viral infections, genetic mutation, energy metabolism deficiency, excitotoxicity, oxidative stress, and others. It is likely that complex disorders such as schizophrenia, bipolar disorder, and major depression are associated with multiple etiologies and pathogenetic mechanisms. In light of the interwoven biochemistry of human organs, identifying a network of multiple interacting biochemical pathways that account for the constellation of clinical and biological features would advance our understanding of these disorders. One such approach is to evaluate simultaneously the multiple metabolites in order to uncover the dynamic relations in the relevant biochemical systems. These metabolites are a group of low-molecular-weight, redox-active compounds, such as antioxidants, amino acids, catecholamines vitamins, lipids, and nucleotides, which reflect the metabolic processes, including anabolism and catabolism as well as other related cellular processes (e.g., signal transduction, regulation, detoxification, etc.). Such an analytic approach has the potential to yield valuable insights into the likely complex pathophysiological mechanisms that affect multiple metabolic pathways and thereby offer multiple windows of therapeutic opportunities.

Index Entries: High-pressure liquid chromatography; coulometric multielectrode array system; metabolomics; psychiatric disorders; oxidative stress.

Introduction

Research into the pathophysiology of major psychiatric disorders such as schizophrenia, depression, and bipolar disorder has historically been on neurotransmitter systems, generally with an emphasis on investigating a single neurotransmitter system. Although the pathophysiological role of dopaminergic, serotoninergic, and glutamatergic systems remains pre-eminent, it is increasingly apparent that these disorders are better conceptualized as having

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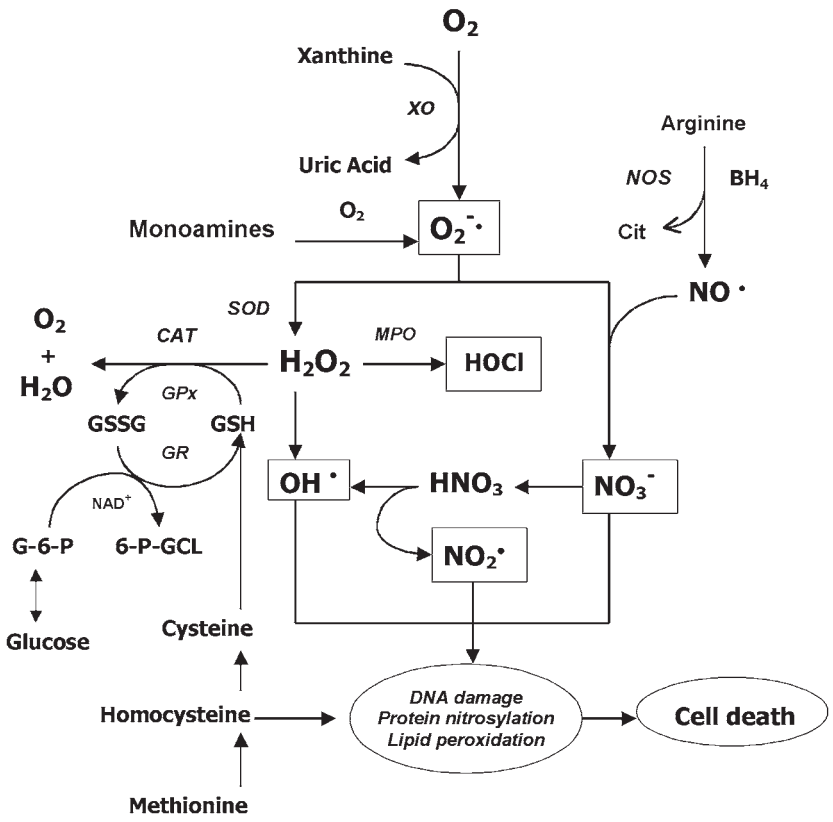

Fig. 1. Production and removal of oxygen and nitrogen free radicals in mammalian cells. XO, xanthine oxidase; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; G-6-P, glucose-6-phosphate; 6-P-GCL, 6-phosphogluconolactone; HOCl, hypochlorous acid; MPO, myeloperoxidase; NOS, nitric oxide synthase; BH4, 5,6,7,8-tetrahydro-L-bioterin; Cit, citrulline.

"multineurotransmitter" pathologies. This stems, in part, from an increasing appreciation of how neurotransmitter systems are, in fact, dynamically linked with each other. For example, investigating alterations in dopamine levels as a consequence of dopamine-blocking antipsychotic agents will fail to provide a true picture of the effects of antipsychotic agents on all of the systems that are affected by the antipsychotic agents. In addition to direct effects on neurotransmission, increased dopamine in the synaptic cleft can produce increase in free radicals, which, in turn, can lead to lipid peroxidation and interrupt signal transduction, and have other downstream effects. Measuring dopamine levels alone will fail to provide a broader picture of the complex interactions. Further, whether the multineurotransmitter pathologies are primary or secondary to other pathological processes such as oxidative stress *(1–3)* and membrane dysfunction *(4–6)* are yet to be determined. There is abundant evidence that alterations in key neurotransmitters can both be modified by and contribute to oxidative stress and membrane dysfunction. Therefore, to identify candidate pathological process(es) that account for the complex constellation of clinical and biological features in schizophrenia, as an example, it is advantageous to evaluate simultaneously the network of interacting biochemical pathways (Fig. 1) that produce and remove oxygen and nitrogen free radicals in mammalian cells.

The introduction of powerful and rapid multidimensional separation and characterization methods into psychiatric research can revolutionize investigation at the molecular level. The resolving power of these methods is superior to individual one-dimensional approaches, enabling comprehensive metabolic analyses. A novel approach is to simultaneously examine metabolites derived from these pathways (Fig. 1). These metabolites are a group of low-molecular-weight compounds, such as antioxidants, amino acids, catecholamines, vitamins, lipids, and nucleotides, which reflect the metabolic processes, including anabolism and catabolism as well as other related cellular processes (e.g., signal transduction, regulation, detoxification, etc.). Such an analytic approach has the potential to yield valuable insights into the likely complex pathophysiological mechanisms that affect multiple metabolic pathways and thereby offer multiple windows of therapeutic opportunities. Parallel with these developments has been advances in informatics capable of rigorous qualitative and quantitative analysis of the separation profiles *(7),* allowing the development of exploratory models that will propel biological psychiatric research.

Metabolomic Technologies

Classical Spectroscopic and Chromatographic Techniques

Metabolomics, the profiles of global metabolite in biological tissues, are typically generated with high-throughput nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) *(8).* These profiles are represented by analytical spectra that are compared by statistical approaches (e.g., pattern recognition). However, each of these approaches has limitations that preclude it from becoming a universal metabolomics platform *(9).* A major drawback of NMR is that certain sample preparation steps are needed because the individual resonances are sensitive to the chemical and physical environment of a molecule. In addition, it is difficult to measure high-abundance and very low-abundance metabolites simultaneously. On the other hand, MS is highly beneficial for wide screening of nondestructive metabolites in coupling with gas chromatography (GC) or high-pressure liquid chromatography (HPLC). However, lack of accurate quantification is a major liability for metabolomics assessments. In regard to the classical chromatographic techniques, both HPLC and GC are often too slow to become high-throughput platforms. It is possible, however, that a combined, parallel approach with HPLC, GC, and MS might achieve the best results.

Recent advances in MS technology, particularly high-throughput and high-resolution matrix-assisted laser desorption ionization–time of flight–MS (MALDI-TOF-MS), offer additional means to more rapidly and accurately identify and quantify the metabolome. These advances have been primarily applied to proteomics research, but recent technological breakthroughs, including "matrix free," now allow adaptation of these rapid and sensitive analyses to be used in the low-mass (*m/z* 100–1000) range *(10).* Coupled with recent advances in low-flow (e.g., nL/min) HPLC and liquid handling robotics systems that ensure reproducibility in fraction collection *(11),* a new era of metabolite identification and quantitation has begun.

HPLC With a Coulometric Multi-Electrode Array System

On the other hand, studies of a series of related metabolites are often complicated by cumbersome and different analytical methods, which require separate and multistep extraction and chemical reaction procedures. Thus, measurements of multiple parameters are always restricted by the limited sample size. HPLC-CMEAS (coulemetric multi-electrode array system provides high specificity and sensitivity for the determination of multiple redox-active low-molecular-weight compounds based on differences in oxidation-reduction properties of analytes *(12–15).* The basic concepts of HPLC-

CMEAS technology have been extensively elucidated *(16,17).* In brief, the CMEAS equips four cell packs in series, each consisting of four coulometric electrodes, and set to increasing specified potentials between 0 and 1000 mV, which allows resolution of coeluting 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 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 *(12–15).* More than 1000 electrochemically oxidizable compounds such as antioxidants, oxidative stress markers, and monoamine metabolites can be analyzed simultaneously in biological samples *(14,18).*

Modifications of Current Published HPLC-CMEAS Procedure for Measurements of Multiple Redox-Active Compounds

The previously reported HPLC-CMEAS protocols *(12,14,18)* are somewhat tedious, requiring dual columns and a complex gradient elution profile with variable flow rates. However, the major concern involves gradient profile with 100% mobil phase B (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. These spurious peaks 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 *(12,14).* The mixed gradient was then delivered from the PS/GM to a PEEK-lined pulse damper prior to flowing through the autosampler injector and onto the columns.

Without the use of previously reported two columns in series as well as of a complex gradient elution profile, we *(19)* have recently developed an automated procedure to simultaneously measure multiple redox-active low-molecular weight compounds in a single column with a simplified binary gradient profile (Fig. 2A). Our modified method does not require a PS/GM for suppression of spurious peaks, which is a useful simplification of the plumbing. No other chemical reactions are necessary for sample preparation. In order to reduce the running time and yet achieve a reproducible retention time by the autosampler injection, our gradient elution profile was also modified to produce a shorter equilibration time, stable retention time, and a reduced cost per test. This procedure can be applied to a variety of biological samples such as plasma and cerebrospinal fluid.

Quantitative analysis of peak identity and the peaks relative to absolute or reference standards are achieved by the ESA Coul Array for Windows 32 package. This software automatically subtracts backgrounds resulting from the gradient drift. The coulometric array is used to generate databases of all redox-active molecules with redox potentials from 0 to 900 mV (Fig. 2B). Peak identity is verified by the retention time, dominant channel, and the ratio of reactivity on the dominant channel to reactivity on the subdominant channels *(12,18).* The detector potentials are configured to allow compounds responding at three consecutive electrodes *(20).* 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 *(21).* 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.

Application of HPLC-CMEAS

Evaluation of Brain Metabolites

Recently, we have applied HPLC-CMEAS to study the glutathione redox state in postmortem brain from patients with schizophrenia

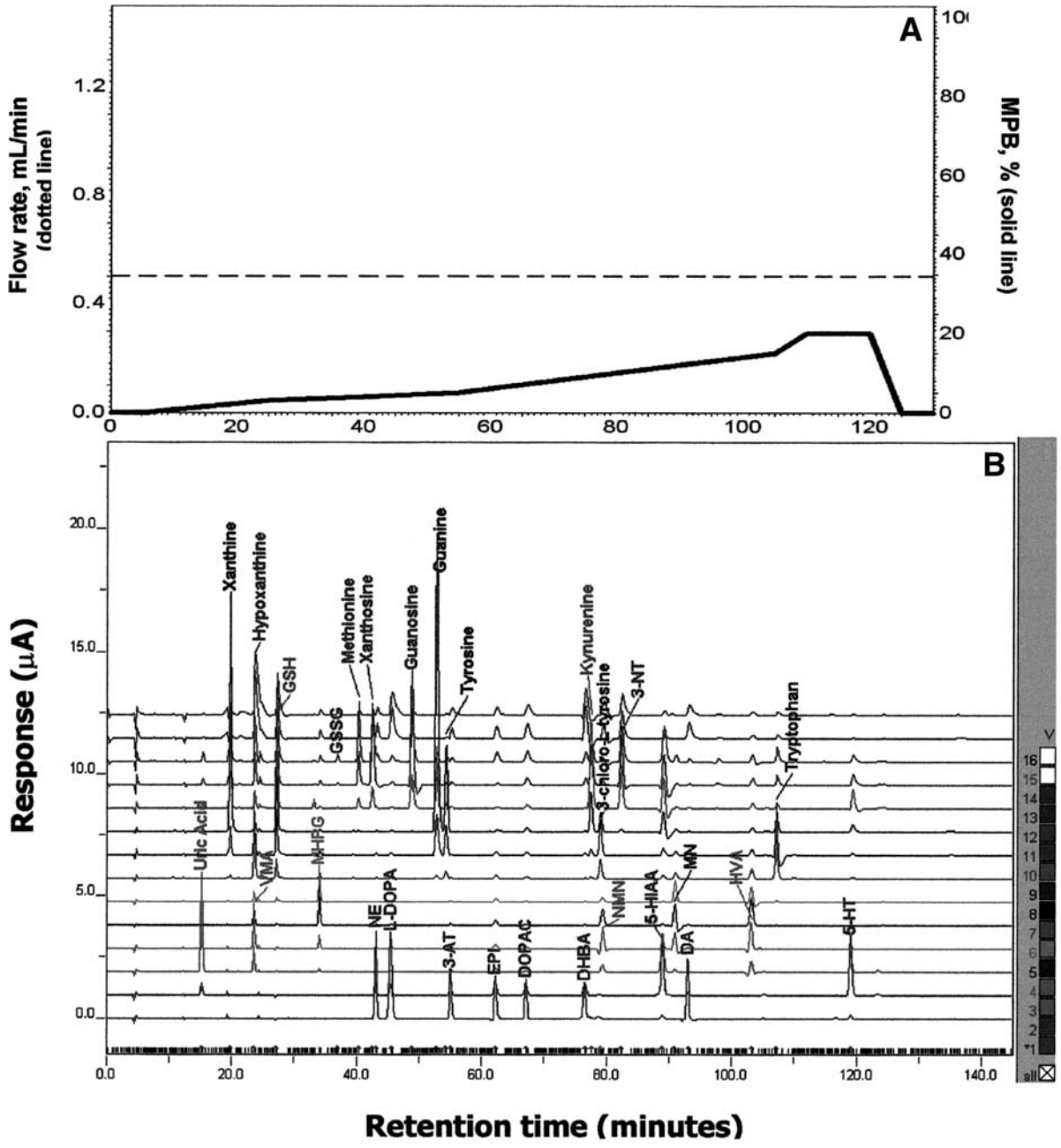

Fig. 2. Separation of low-molecular-weight, redox-active compounds by HPLC coupled with a CMEAS. **(A)** Flow rate and mobile-phase gradient profile; **(B)** 16-channel chromatograms obtained by separation of standard mixture in a single column (ESA Meta-250, 5 μm ODS, 250 × 4.6 mm inner diameter) under a 150-min gradient elution that ranged from 0% to 20% MPB with a fixed flow rate of 0.5 mL/min (A). The temperature of both cells and column was maintained at 25°C. The CMEAS was set to have increments from 0 to 900 mV in 60-mV steps. VMA, 4-hydroxy-3-methoxy-mandelic acid; GSH, glutathione; MHPG, 4-hydroxy-3-methoxyphenyl-glycol; GSSG, oxidized glutathione; NE, norepinephrine; L-DOPA, L-3-hydroxytyrosine; 3-AT, 3-amino-tyrosine; EPI, epinephrine; DOPAC, 3,4-dihydroxy-phenylacetic acid; DHBA, dihydroxybenzylamine (internal standard); NMN, normetanephrine; 3-NT, 3-nitro-tyrosine; 5-HIAA, 5-hydroxyindole-3-acetic acid; MN, metanephrine; DA, dopamine; HVA, homovanillic acid; 5-HT, serotonin.

(22). Figure 3 illustrates an example of metabolic profile of low-molecular-weight, redox-active compounds found in the caudate region from a control subject (Fig. 3A) and a patient with schizophrenia (Fig. 3B). The major metabolites include (1) low-molecular-weight antioxidants (ascorbic acid, uric acid, and glutathione), (2) purine catabolites (e.g., hypoxanthine, xanthine, xanthosine, guanosine, and uric acid), (3) tyrosine metabolites (tyrosine and homovanillic acid), and (4) tryptophan metabolites (tryptophan and 5-hydroxy-indoleacetic acid). As illustrated in Fig. 1, these compounds can serve as biological indices underlying the mechanisms of antioxidant defense system.

Another advantage of the HPLC-CMEAS method is to provide the dynamic range of analytes by ranges from picograms to micrograms on a column in five or more orders of magnitude *(18).* Therefore, in addition to the above-mentioned metabolites in the brain caudate, several minor metabolites reflecting oxidative damage (glutathione disulfide and 3-nitrotyrosine), tyrosine pathway (dopamine, norepinephrine, epinephrine, and 3-methoxy-4-hydroxy-phenylglycol), and tryptophan pathway (kynurenic acid and serotonin) are also identified. These can be exemplified by altering the response scale from microampere (μA) to nanoampere (nA) in channels 1 to 4 of Fig. 3 (Fig. 4). Consequently, increased levels of HVA and 5-HIAA become noticeable in the brain caudate region from this patient with schizophrenia.

Evaluation 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 study *(23–25).* Following validation of the HPLC-CMEAS methodology based on 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), approx 250 compounds in serum were identified as potential components of serum metabolome *(23).* Subsequent studies by Shi et al. further revealed different metabolite datasets as baseline profiles for male and female rats *(25).* Taken together, these studies suggest that quantitative analysis of selected serum metabolites can provide important information for identifying biomarkers chromatographically and for setting the stage for pattern-recognition-based approaches in establishing metabolome-based categorical separations.

Therapeutic Monitoring

One of the powerful applications of HPLC-CMEAS will be to examine prospectively the effects to current and novel treatments for psychiatric disorders. This will help in the investigation of predictors of therapeutic outcome. A major problem currently exists in the treatment of the psychoses with second-generation antipsychotic drugs. Despite having multiple advantages over earlier antipsychotics, second-generation antipsychotic drugs, sadly, have greater adverse metabolic consequences, as described in a recent review of extant research and clinical consensus *(26).* Weight gain, diabetes mellitus, and dyslipidemia risks have been identified, but more research is needed, in part, (1) to clarify the relative risks of these adverse outcomes among the various second-generation antipsychotic drugs and (2) to identify pretreatment or early treatment factors that can predict the occurrence of such outcomes for a given second-generation antipsychotic drug *(26).* Blood samples collected from well-characterized patients at appropriate times in relation to treatment could provide a sequence of metabolomic data containing clues to items (1) and (2). Figure 5 exemplifies changes in tryptophan pathways observed from plasma metabolites in first-episode neuroleptic-naive patient individual with schizophrenia following treatment with risperidone (second-generation antipsychotic drug) up to 2 yr.

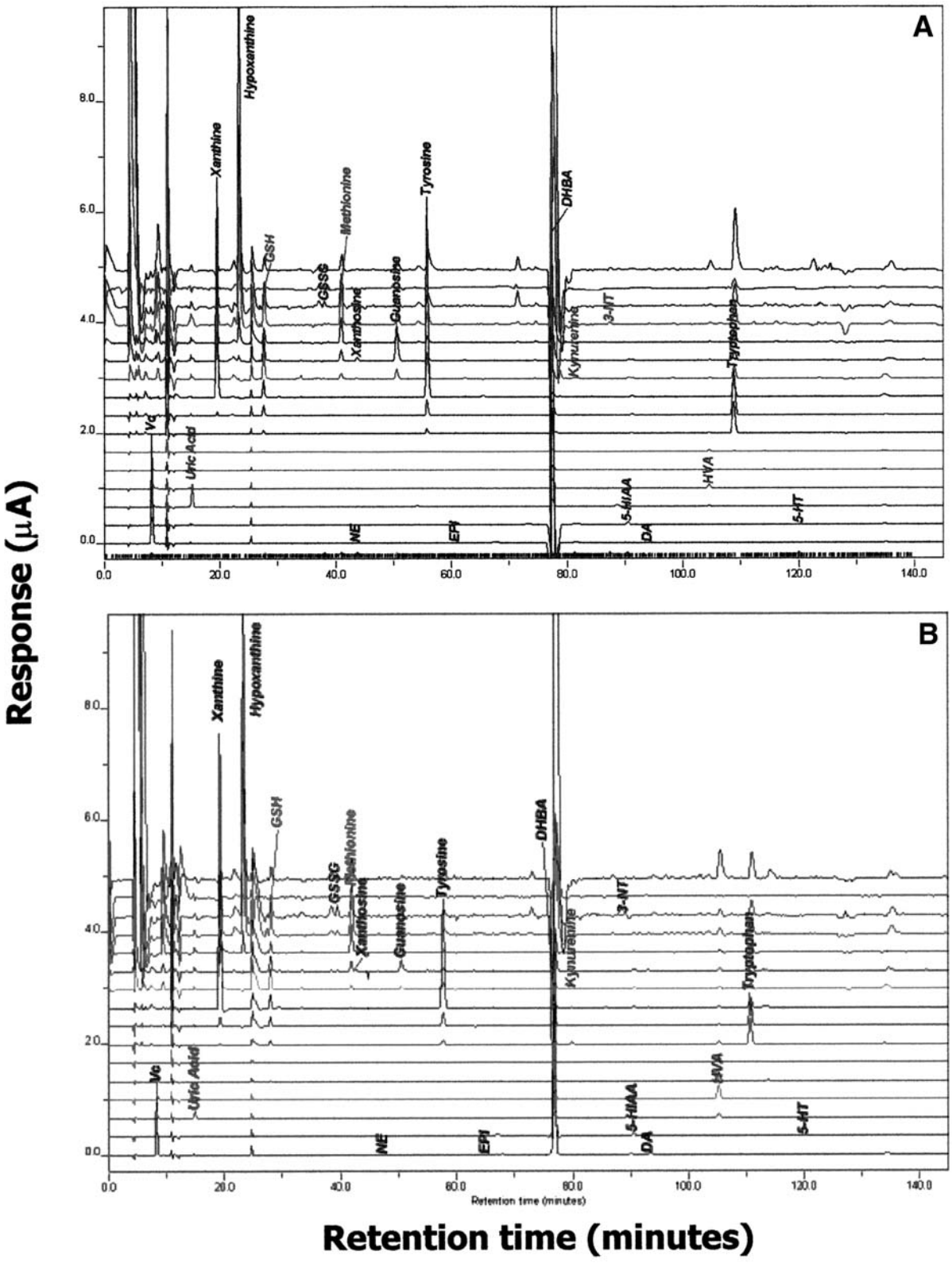

Fig. 3. Metabolic profile of low-molecular-weight, redox-active compounds in caudate region of postmortem brain by HPLC coupled with a CMEAS: **(A)** control subject without mental disorder; **(B)** patient with schizophrenia. The HPLC operating procedure is the same as described in Fig. 2.

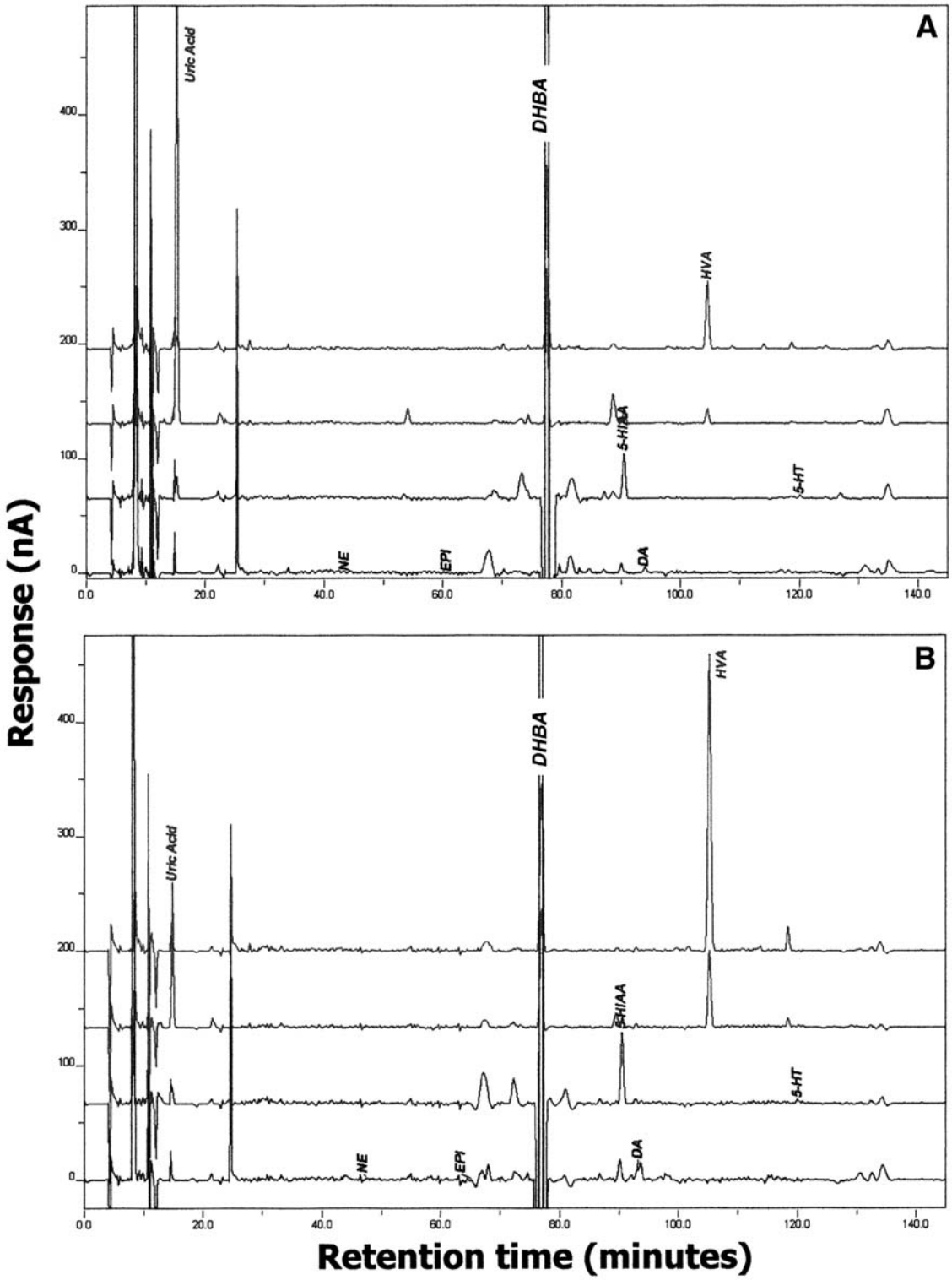

Fig. 4. Comparison of minor redox-active compounds in the postmortem caudate between a control subject without a mental disorder **(A)** and a patient with schizophrenia **(B)** in subchannels (channels 1–4 from Fig. 3) by HPLC coupled with a CMEAS. 5-HIAA, 5-hydroxyindole-3-acetic acid; HVA, homovanillic acid.

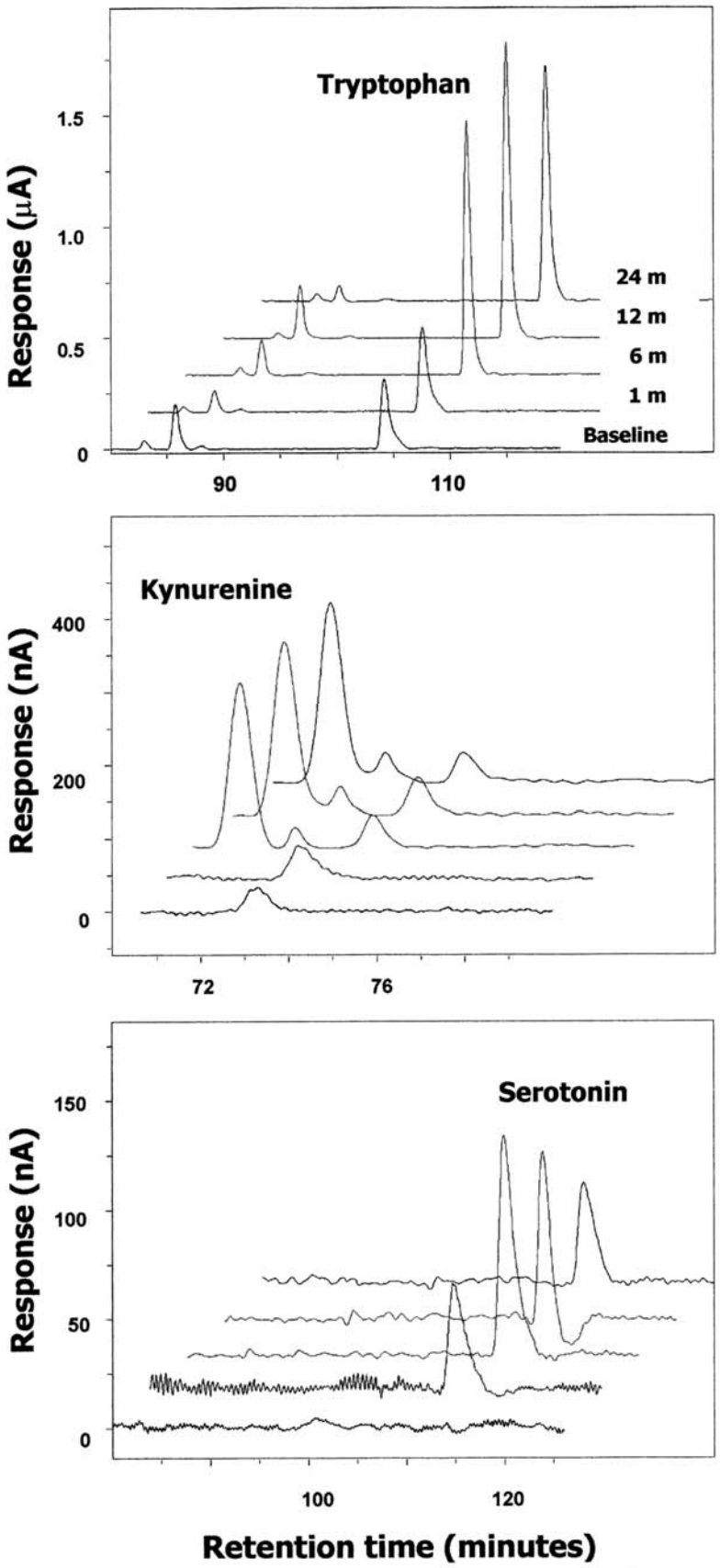

Fig. 5. Changes in tryptophan pathways observed from plasma metabolome in first-episode neuroleptic-naive patient individual with schizophrenia following treatment with risperidone up to 2 yr.

Conclusions

What has been lacking to date has been the ability to examine the complexity of systems simultaneously to understand their dynamic relations, which might offer clues to disease mechanisms. Metabolic investigations offer a novel opportunity to examine the heretofore discrete biochemical systems simultaneously, one that more accurately reflects in vivo relations between systems. Such a paradigm shift in the field can propel our understanding of the pathophysiology of psychiatric disorders to the next level, allowing theoretically derived treatments.

Biological research in the aforementioned disorders has yielded a vast amount of information about individual biochemical systems. For example, research into a small number of neurotransmitter systems—dopaminergic, serotonergic, glutamatergic, adrenergic—has identified a large number of alterations, putatively associated with the disorders in question. However, the ultimate usefulness of this data in understanding these disorders will remain limited because the neurotransmitter systems in the brain are linked to each other, to signal transduction systems, and to a broad variety of membrane-bound and cellular systems. Metabolic studies aim to assay the levels, activities, and interactions of all metabolites in a biological system and how these change in response to environmental stimuli. Changes in metabolites are directly influenced by environmental factors and genetic alterations. Thus, metabolic profiles can be a useful tool in examining individual and combined effects of environmental and genetic factors on disease processes.

Recently, differential analysis of normal subjects and patients with amyotrophic lateral sclerosis revealed numerous differences in metabolic profiles that could be easily measured and monitored by CMEAS *(7).* In addition, metabolic analysis of samples from autistic and control subjects could be separated by cluster analysis using 65 plasma biomarkers *(7).* In short, the data collected from HPLC-CMEAS allow multiple rather than single metabolites to be used in markers for a group, which will greatly improve the predictive diagnostics for phenotypes. More significantly, those comprehensive analyses that generate metabolic profiles represent not only biomarkers for disease but also metabolic maps that can be used to identify specific genes responsible for disease *(27).*

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