Variability Analysis of Human Plasma and Cerebral Spinal Fluid Reveals Statistical Significance of Changes in Mass Spectrometry-Based Metabolomics Data

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Analytical and biological variability are issues of central importance to human metabolomics studies. Here both types of variation are examined in human plasma and cerebrospinal fluid (CSF) using a global liquid chromatography/mass spectrometry (LC/MS) metabolomics strategy. The platform shows small analytical variation with a median coefficient of variation (CV) of 15−16% for both plasma and CSF sample matrixes when the integrated area of each peak in the mass spectra is considered. Analysis of biological variation shows that human CSF has a median CV of 35% and plasma has a median CV of 46%. To understand the difference in CV between the biofluids, we compared plasma and CSF independently obtained from different healthy humans. Additionally, we analyzed another group of patients from whom we compared matched CSF and plasma (plasma and CSF obtained from the same human subject). A similar number of features was observed in both biofluids, although the majority of features appeared with greater intensity in plasma. More than a dozen metabolites shared between the human CSF and plasma metabolomes were identified based on accurate mass measurements, retention times, and MS/MS spectra. The fold change in these metabolites was consistent with the median biological CV determined for all peaks. The measured median biological CV together with analysis of intragroup variation of healthy individuals suggests that fold changes above 2 in metabolomics studies investigating plasma or CSF are statistically relevant with respect to the inherent variability of a healthy control group. These data demonstrate the reproducibility of the global metabolomics platform using LC/MS and reveal the robustness of the approach for biomarker discovery.

The application of mass spectrometry to metabolomics is rapidly increasing,[1−6] primarily because mass spectrometry makes it possible to detect and measure a variety of small biological molecules over a wide dynamic range.[7−10] Targeted mass spectrometry-based metabolite assays are currently widespread in clinical diagnoses, including screening neonates for metabolic disorders, diagnosing over 200 organic acidemias, and detecting over 30 other disorders of metabolism resulting in small molecule deregulation.[11] Global (untargeted) metabolomics, which focuses on the identification of detectable metabolite changes between different phenotypes using comparative analyses, may be particularly useful for the discovery of new disease biomarkers.[12−16]

The strength of global metabolomics lies in the discovery phase: that is, the potential to detect and identify unanticipated changes in metabolite profiles which are characteristic of, for example, a disease state. For diseases that are difficult to diagnose or for which the biochemical mechanism is unknown, this information can provide new diagnostic biomarkers or therapeutic targets. Metabolomic profiles measured by using global approaches have recently uncovered potential urinary markers for

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prostate cancer progression[17] and have led to the generation of specific, testable biochemical hypotheses.[18,19] Early diagnostic biomarkers may be especially useful for neurodegenerative diseases, in which damage to the tissue of the central nervous system (CNS) may occur long before noticeable symptoms develop. Diagnosis and treatment of diseases of the CNS can be difficult and are complicated by the fact that many diseases lack specific diagnostic tests.[20,21] Although blood is the most commonly used biofluid for clinical chemistry diagnoses due to the minimally invasive nature of its collection, its rich metabolome, and its reflection of the metabolic state of the entire organism, cerebrospinal fluid (CSF) may represent a better source of metabolites for diseases of the CNS since it is closer in proximity to the tissue of disease origin and may better represent its neurochemical state.

We present a global metabolomics method applicable to biofluid analysis and validate its reproducibility for disease biomarker discovery using human plasma and human CSF. This approach combines liquid chromatography/mass spectrometry (LC/MS) with XCMS, an open-source software program designed for nonlinear retention-time alignment and integration of untargeted metabolomics data. These experiments establish the analytical variation of a global metabolomics strategy based on mass spectrometry for two unique and complex matrixes: human plasma and CSF. Key difficulties of untargeted metabolomics approaches are the variability within sample groups and the identification of metabolites. More than a dozen metabolites were identified using the METLIN mass spectral metabolite database in combination with accurate mass measurements and MS/MS. The variation of these metabolites between individuals was quantified in both human plasma and CSF, and the coefficient of variation (CV) analysis for all features reported by XCMS shows that human CSF has less variation compared to human plasma in healthy individuals.

EXPERIMENTAL SECTION

Sample Preparation and Metabolite Extraction. Independent sets of healthy human plasma and CSF were received from the Department of Neurobiology, the Scripps Research Institute. The samples were stored at −80 °C until extraction. A procedure for metabolite extraction from human plasma using a methanol protein precipitation protocol was described previously[22] and was adapted for CSF sample preparation. For each individual sample, four volumes of ice-cold methanol were added to one volume of plasma or CSF, immediately vortexed for 45 s, and left to precipitate at −20 °C for 1 h. Samples were then centrifuged at 13 000 rpm for 15 min at 4 °C, and the supernatants were transferred to fresh vials. Centrifugation was repeated to ensure complete removal of precipitated protein. The supernatant was dried in a SpeedVac without heat and subsequently resuspended to one-half of the original CSF volume in an aqueous solution of

5% acetonitrile. Samples were stored at 80 °C until LC/MS analysis. Immediately prior to the experiments, samples were thawed and vortexed for 30 s. During analysis, samples were maintained at 6 °C in a thermostatted autosampler.

LC/MS. Experiments were performed with an electrospray-ionization time-of-flight mass spectrometer (ESI-TOF; Agilent 1100 LC, TOF 6210) operating in positive mode. Chromatographic separation was performed using a reverse phase C18 column (Zorbax SB-300 C18 Capillary 5 μm, 0.5 mm × 150 mm; Agilent Technologies). The capillary pump flow rate was 9 μL/min. Mobile-phase A was water with 0.1% formic acid, and mobile-phase B was acetonitrile with 0.1% formic acid. An injection volume of 8 μL of extracted sample was used for each run with the following method: 5 min isocratic hold at 5% B; 45 min linear gradient from 5% to 95% B; 5 min isocratic hold at 95% B; 10 min isocratic hold at 5% B. Between runs a wash step was used to minimize carry over consisting of a 12 min linear gradient from 5% to 95% B; 3 min isocratic hold at 95% B; 2 min return to 5% B; 8 min isocratic hold at 5% B.

Data Analysis. LC/MS data were processed using XCMS, open-source software developed in our lab, which can be freely downloaded as an R package (http://masspec.scripps.edu/xcms/xcms.php).[23] XCMS applies a nonlinear retention time correction, performs peak-picking, and matches peaks across runs. It reports integrated areas of each detected peak in individual samples and calculates the Welch's t test for two sample groups. Features are listed in a file containing their integrated intensities (extracted ion chromatographic peak areas), observed fold changes across the two sample groups, and t test p-values for each sample. XCMS parameters were specified as steps = 2, step = 0.01, method = binlinbase, and fwhm = 50, unless otherwise specified. Multivariate PCA analysis was performed with SIMCA-P (version 11, Umetrics) using the complete metabolomics data sets (.tsv files), plotting PCA dimensions 1 and 2.

Metabolite Identification. Metabolites were identified based on accurate mass, retention time, and matching the MS/MS spectra of unknowns to standard model compounds. Some glycerophosphocholines and acylcarnitines were identified in the absence of available standards. In these cases, glycerophosphocholines were identified through accurate mass and MS/MS product ions m/z 104 and m/z 184. Acylcarnitines were identified through accurate mass, a MS/MS product ion of m/z 85, and a neutral loss of m/z 59. MS/MS spectra of all other standards are present in the METLIN database (http://metlin.scripps.edu/).[24] MS/MS experiments were performed on a quadrupole time-of-flight mass spectrometer (Q-TOF; Agilent 6510) with the collision energy set to 20 eV.

RESULTS AND DISCUSSION

Design of the Experiment. The study was designed to determine the reproducibility of the LC/MS platform and the data analysis scheme for metabolomic profiling of human biofluids for disease biomarker detection. Figure 1 illustrates the experimental workflow of the global metabolomics strategy. After LC/MS

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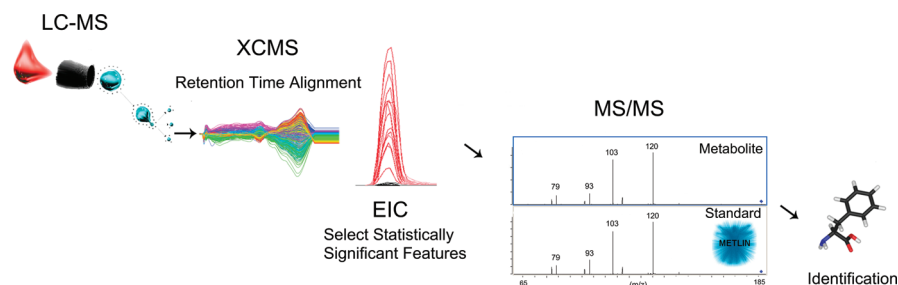
Figure 1. Workflow of the global metabolomics strategy. Briefly, plasma and CSF samples are extracted with methanol and applied to a capillary-flow HPLC column interfaced with a TOF mass spectrometer equipped with an electrospray source. Each sample results in a three-dimensional data set consisting of m/z, retention time, and ion signal intensity. XCMS software is then used to automatically find peaks, nonlinearly align the data sets in the time domain, integrate the peaks, and generate extracted ion chromatograms (EICs) for each peak. Compounds (peaks) of interest were then identified using a QTOF mass spectrometer to fragment the ions of interest, and this fragmentation pattern was compared with a library of authentic model compounds. Compounds are identified using a minimum of the accurate mass and MS/MS spectral matching with the QTOF.

analysis of biofluid extracts, XCMS performs nonlinear retention time alignment and integrates the peak areas of all detected features. The peak areas are used to statistically analyze metabolite changes between two or more phenotypic groups. Metabolites of interest are then identified using a MS/MS strategy based on comparison of the MS/MS spectra from the unknown compound to the MS/MS spectra of standard model compounds, the data for which are freely available in the METLIN metabolite database. Positive identification is based on accurate mass, matching retention times between unknown and the standard, and a match between the unknown MS/MS spectrum and that of the standard compound.

The discovery and identification of disease biomarkers based on metabolomics is a growing field.[25,26] The success of these studies depends on the selection of a suitable sample group and on the reproducibility of measurements. Here we examine the analytical reproducibility of the global LC/MS-based metabolomics approach and use this approach to compare the metabolite profiles of human plasma and CSF for several groups of individuals.

Analytical Variation (LC/MS + XCMS). The importance of statistical strategies in metabolomics has been the subject of recent reviews[27] discussing several data-analysis tools, including XCMS.[23,28−33] Pooled human plasma and human CSF extracts were used to assess the analytical variability of the LC/MS platform and XCMS analysis procedure. Plasma samples from individual patients were pooled together to create representative average samples. The CSF samples were similarly pooled. Analytical replicates of a pooled sample of plasma or CSF were then run in a series of injections, each separated by a shorter run serving

Table 1. Measured Variation in Human Plasma and CSF[a]

| biofluid	replicate type	no. of replicates	no. of features	mean CV (%)	median CV (%)
plasma	analytical	14	5 274	25	16
plasma	biological	14	3 641	56	46
CSF	analytical	12	4 889	26	15
CSF	biological	12	4 794	49	35

[a] Variation in human plasma and CSF features for four data sets. Analytical and biological replicates were run for both plasma (14 individuals) and CSF (12 individuals). CVs (%) were calculated from the integrated intensities of features reported by XCMS.

to wash and re-equilibrate the column, to evaluate analytical variability. The first five samples were injected to condition the system prior to the series of sample injections, and the data from the conditioning injections were not included in the analysis. A principle component analysis showed that conditioning of the system greatly improved the reproducibility of the leading sample injections. This trend has been previously reported, and Zelena et al. have shown that the number of injections required to condition the system may vary for different biofluids.[34] The data were processed with XCMS independently for each biofluid, which detected approximately 5 300 features in the human plasma replicates and 4 900 features in the human CSF replicates in the mass range of m/z 100 to m/z 1000. We estimate the number of distinct metabolites to be substantially less than the number of detected features, since a single metabolite may give rise to multiple mass spectral peaks, including the molecular ion, in-source fragments, adducts, and dimers, and each of these is reported in XCMS as a separate peak or "feature". Additionally, there will be some spurious peaks arising from background and noise that are classified as signals.

Table 1 contains the summary of measured analytical variation in the replicate runs. The median coefficient of variation (CV) was calculated by using the peak areas of all features reported by XCMS. Peak areas showed high analytical precision, with a median CV of 16% or less for features across replicate analyses, for both types of biofluids.

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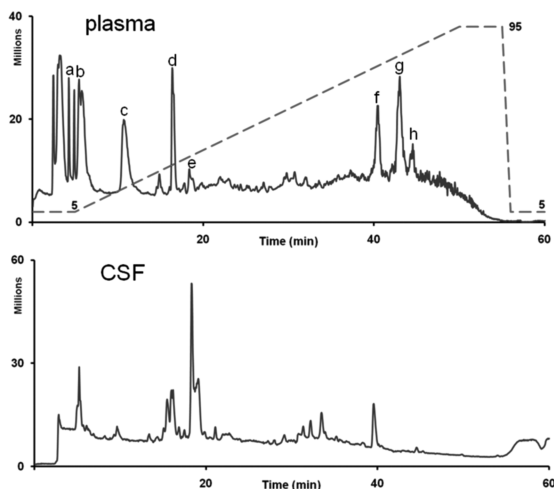
Figure 2. Total ion chromatogram (TIC) of human plasma (top) and CSF (bottom). The following metabolites appear in the plasma chromatogram: a, methionine; b, leucine/isoleucine; c, phenylalanine; d, tryptophan; e, caffeine; f–h, lysophospholipids. The dashed line shows the mobile phase condition during the run; 95% corresponds to a condition of 5% mobile phase A and 95% mobile phase B.

Biological Variation. Several reports suggest the importance of considering biological variability in human biofluid analysis since metabolic profiles may be sensitive to genetic, dietary, lifestyle, or environmental factors.[35,36] Variation in sample handling during the collection and postcollection procedures can also appear as interindividual variation.

A challenge in the application of metabolomics for disease biomarker discovery is to determine the effect of human variation on metabolite profiles. The success of metabolomics in nonhuman model systems is partially due to the controlled nature of such variation in the subjects, environmentally, behaviorally, and genetically. Typically, animal studies may have several specimens and contain several subjects per group or category.[37] In human studies, the inability to control the genetics, living conditions, diet, and other factors leads to the question of the number of human patients required to form statistically meaningful data sets.[38]

Typical chromatograms for human plasma and human CSF are shown in Figure 2; plasma is dominated by peaks arising from lysophospholipids. XCMS was used to compare the data from the individual samples which were pooled in the analytical replicate analysis. The results are summarized in Table 1. XCMS detected approximately 3 600 features in the human plasma biological replicates and 4 800 features in the human CSF biological replicates in the mass range of m/z 100 to m/z 1000. The total number of detected features decreased for the plasma biological replicates relative to the pooled plasma analytical replicates, but this was not observed for the CSF replicates. The majority of features showed low interindividual variation, suggesting that there are characteristic plasma and CSF metabolite profiles including a core

set of relatively low-variance metabolites in healthy individuals. The median CV of the features detected in human plasma biological replicates was 46%. Human CSF biological replicates showed less variation than plasma replicates with a median CV of 35%.

Biomarker discovery typically employs a comparison of peak areas to statistically analyze metabolite changes between two or more phenotypic groups. On the basis of integrated peak areas, XCMS reports variations between two groups, including the average fold change difference and the t test p-values. To determine the magnitude of significant changes which would be detected by XCMS in a within-group comparison of healthy individuals, 12 biological replicates of human CSF were compared by dividing the biological replicate samples into two groups of 6 and running XCMS to detect changes between the two groups. For the within-group comparison of CSF samples, 11 of the 4 800 features detected were reported to change by 2-fold or greater within a 95% confidence interval (p-value <0.05), and only 3 of these features were reported within a 99% confidence interval (p-value <0.01). The number of potential "false positives" was slightly higher for human plasma. This test was repeated for 14 human plasma biological replicates, which were split into two groups of 7. Of the 3 600 features detected, 48 features were reported to change by 2-fold or greater within a 95% confidence interval; however, only 5 of these were within a 99% confidence interval. For both human plasma and CSF, only one feature was reported to change by 3-fold or greater within a 99% confidence interval. On the basis of these calculations, applying a filter to include detected changes which are only 2-fold or greater should minimize the effect of biological variability which is inherent in a healthy control group.

When matched plasma and CSF from the same individual was compared (for a sample group consisting of 10 male individuals), less variation in the basal metabolite profiles was observed in CSF relative to plasma. Figure 3 illustrates a comparison of integrated intensities and CV for plasma and CSF sampled from these 10 individuals. To understand the nature of the differences, we compared the composition of each. A total of 70% of the shared features have a greater intensity in plasma, which is dominated by lysophospholipids. A study on the characterization of the human CSF metabolome has previously been performed by Wishart et al., showing that lysophospholipids are present at very low concentrations in CSF.[39] Because of ion suppression, the relative intensities of features in this study cannot serve as a measure of concentration in such different biofluids, but it provides an idea of the large scale differences between plasma and CSF. Ion suppression results from the limited charge available to all metabolites eluting from the column at a particular time and the larger number of readily ionizable phospholipids in plasma.

A graph illustrating the relationship of the CV of plasma and CSF (Figure 3, right) further supports the higher variation of plasma compared to CSF. A large number of features demonstrate high variation in plasma, with a CV near 80–90%, whereas in CSF these same features have a CV of 50% or less. These results show that there is a correlation between metabolite intensities in plasma

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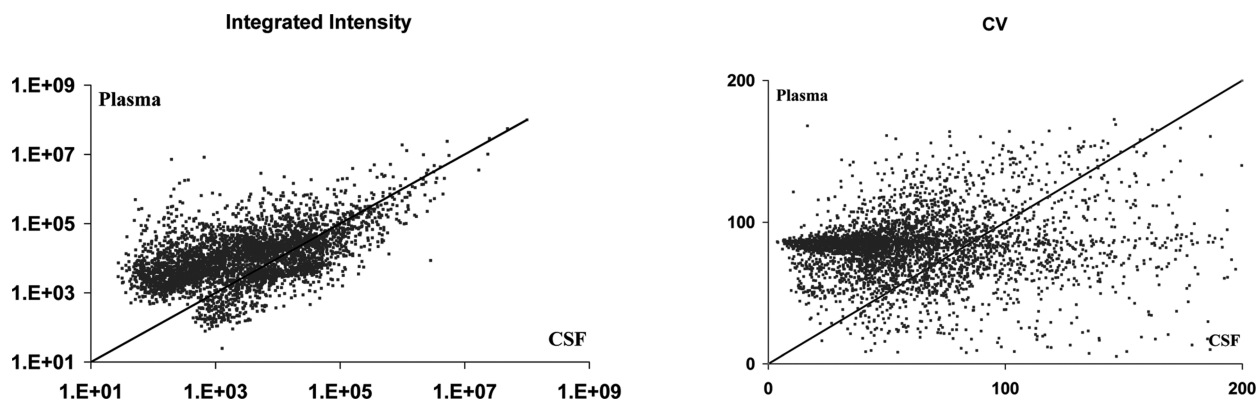
Figure 3. Comparison of matched human plasma and CSF from 10 individuals: (left) integrated intensity of features in human plasma versus CSF, and 70% of shared features have a greater intensity in plasma and (right) coefficient of variation in human plasma versus CSF.
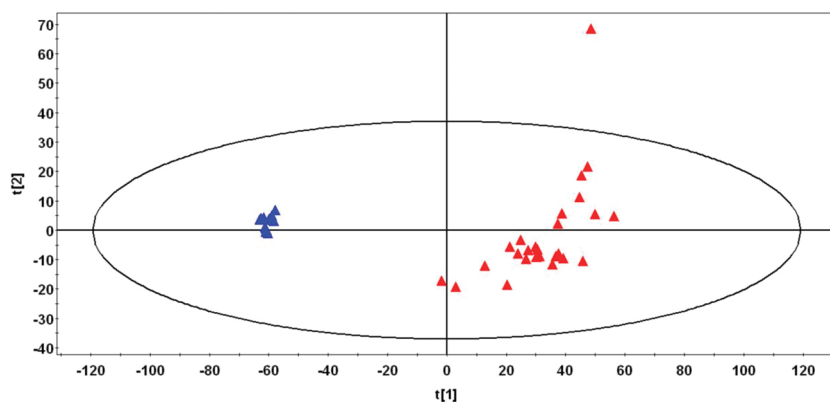
Figure 4. PCA analysis of human plasma (red triangles) and human CSF (blue triangles) metabolomics data sets. Note the greater spread in the plasma compared with CSF.

and CSF but that the interindividual differences in metabolite profiles are more conserved in CSF relative to plasma.

A principal component analysis of biological replicates of plasma and CSF shows more variability in individual plasma metabolite profiles compared to CSF metabolite profiles. The PCA plot in Figure 4 illustrates tight clustering of the CSF samples, whereas much greater spread is observed among the plasma samples which contain an outlier.

Scatter plots, shown in Figure 5, illustrate the relationship between intensity and CV, for both analytical and biological replicates of each biofluid. In the biological replicates of human CSF (Figure 5b), a high-frequency cluster appears of medium intensity and low CV (<20%) data. A similar cluster also appears in the biological replicates of human plasma (Figure 5d). Selection of low-variation clusters might aid in choosing candidate biomarkers that benefit from having efficient ionization, reproducible detection, and low variation among healthy individuals.

Displayed on the left-hand side of Figure 5 are histograms depicting the frequency of features binned according to their CV. In both plasma and CSF, the histograms of analytical replicates are similar, with a narrow distribution and a maximum number of features having a CV of approximately 10%. For the biological replicates, the envelope of the histogram is wider compared to analytical replicates, corresponding to a greater number of features having a higher CV. There is also a shift in the histogram to the right, the result of the biological variability on the distribution. In the histogram of biological plasma replicates, there is a cluster of features which seem to retain conservation and low variation

among individuals. This cluster is not evident in the histogram of biological CSF replicates, indicating that there is much less variation in CSF metabolite profiles overall.

Finally, we compared the biological variation of ions of unique m/z in each biofluid. The average CV was calculated for groups of features binned according to their m/z values (Figure 6). While no direct correlation between CV and m/z was observed for either biofluid, the average CV was more consistent over different m/z ranges for CSF compared to plasma. The relative consistency in CV versus m/z for both plasma and CSF suggests that many features of each biofluid have comparable biological variability. To further investigate the variability of unique features in each biofluid and to compare the variation of specific features in plasma to those in CSF, we identified several metabolites present in both biofluids. These metabolites are listed in Table 2, with their experimentally measured m/z values and their product ions, which were used for identification against authentic model compounds in the METLIN database. A model match is shown in Supplemental Figure S1 in the Supporting Information for the metabolite tyrosine, including a box plot depicting the variation in tyrosine intensities in plasma and CSF. Retention times of compounds are also taken into account to accurately identify metabolites and are listed in Table 2. The variation and intensity of identified metabolites were compared across matched plasma and CSF from 10 male individuals. Table 3 lists the variation and intensity observed for each metabolite in plasma versus CSF, and a detailed summary is in Supplementary Table S1 in the Supporting Information. The relative change in the identified metabolites was
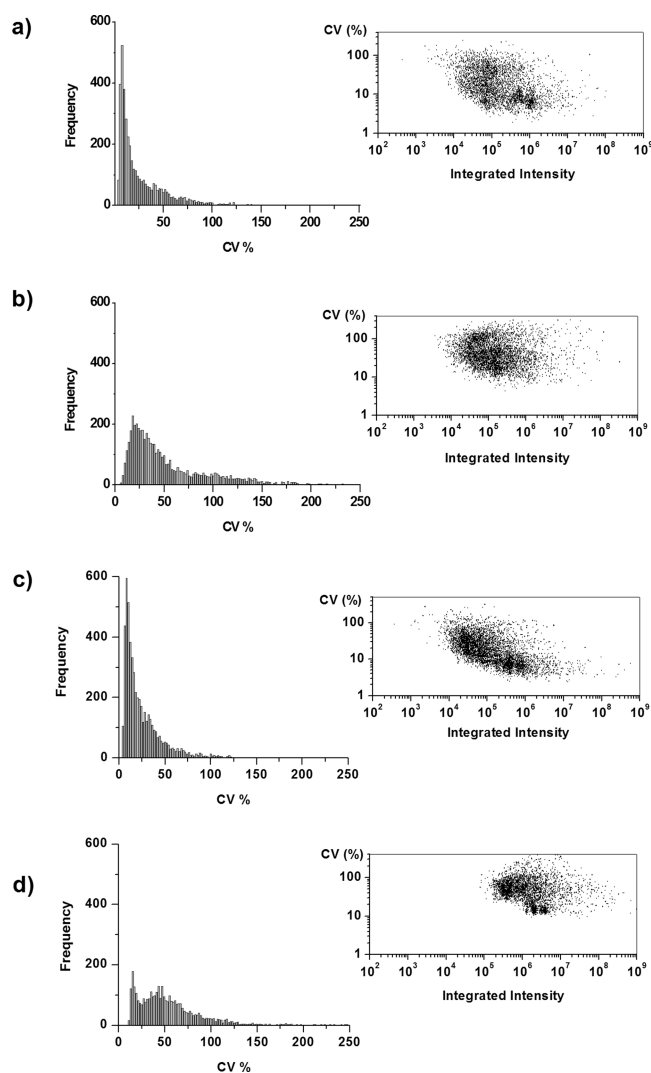
Figure 5. (Left) Histogram showing the frequency distribution of the coefficient of variation (CV) and (right) plot of integrated intensity versus the CV: (a) analytical replicates of human CSF, (b) biological replicates of human CSF, (c) analytical replicates of human plasma, and (d) biological replicates of human plasma.
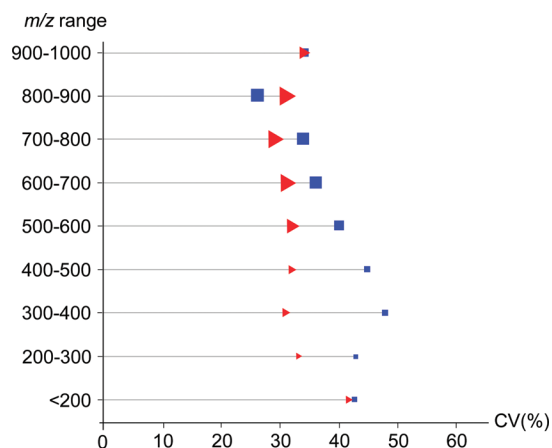
Figure 6. Plot of the average CV over a range of *m/z* values for biological replicates of plasma (blue squares) and CSF (red triangles). The total number of features over each range of *m/z* values is represented by the relative size of the corresponding icon (i.e., larger icons represent *m/z* ranges for which more features were detected).

Table 2. MS/MS Parent Ions, Fragment Ions, and Chromatographic Retention Times of Identified Metabolites in Human Plasma and CSF[a]

metabolite ID	retention time (min)	*m/z*	product ions (*m/z*)
phenylalanine	7	166.086	120, 103, 93, 79
carnitine	2	162.112	103, 85, 60
valine	3	118.090	78, 55
methionine	3	150.059	133, 104, 61, 56
tyrosine	4	182.081	147, 136, 123, 91
citrulline	4	176.064	113, 70
tryptophan	13	205.097	188, 159, 146, 118
cortisol	23	363.221	327, 309, 267, 121
decanoyl carnitine	32	316.241	257, 85, 60
glycerolphosphocholine (16:0)	39	496.341	184, 104
lauric acid	43	201.185	89, 69, 57, 43
palmitic acid	49	257.247	71, 57, 43

[a] Major product ions observed in MS/MS spectra of listed metabolites with the collision energy set to 20 eV.

Table 3. Variation of Individual Metabolites in Matched Human Plasma and CSF from 10 Male Individuals[a]

metabolite ID	CV (%) CSF	CV (%) plasma	integrated intensity (thousands) CSF	integrated intensity (thousands) plasma
phenylalanine	15	19	4 225	13 230
carnitine	19	24	229	878
valine	27	17	480	1 482
methionine	65	40	43	130
tyrosine	14	31	141	394
citrulline	29	19	79	230
tryptophan	19	21	2 416	7 772
cortisol	14	30	49	154
decanoyl carnitine	32	130	105	530
glycerolphosphocholine (16:0)	100	19	915	2 979
lauric acid	25	60	31	83
palmitic acid	7	114	25	120
average	31	43		

[a] Variation of individual metabolites in matched human plasma and CSF from 10 male individuals.

consistent with the median biological CV determined for all peaks. These observations, together with analysis of intragroup variation of healthy individuals, indicates that fold changes above 2 in metabolomics studies investigating plasma or CSF are statistically relevant with respect to the inherent variability of a healthy control group.

CONCLUSIONS

This study demonstrates the reproducibility and robustness of a global LC/MS metabolomics platform applicable to quantitation and identification of metabolites in biofluids. In replicate analyses of human plasma and human CSF, we observed low analytical variation (median CV of 16%). Measurements on biological replicates of human plasma and CSF showed that the CV for CSF was 11% lower than the CV of plasma and that similar numbers of features were observed in both biofluids. Both of these characteristics, low variability and a large number of detected features, are important for the efficacy of biomarker studies. Because of the difficulty of characterizing variability for each population and of obtaining large numbers of samples for human

studies, particularly CSF, filters can be applied when interpreting the statistical significance of fold changes between sample groups. Estimates based on these experiments suggest that for metabolomics studies using human plasma or CSF, a fold-change cutoff of 2 will minimize the effects of biological variation inherent in a healthy control group. Similar fold-change cutoffs are routinely applied in gene chip experiments.[40]

(40) Butte, A. J.; Ye, J.; Niederfellner, G.; Rett, K.; Haring, H. U.; White, M. F.; Kohane, I. S. *Pac. Symp. Biocomput.* **2001**, *6*, 6–17.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.