Journal of Chromatography B, 871 (2008) 164–173

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

The human cerebrospinal fluid metabolome☆

David S. Wishart[a,d,e,*], Michael J. Lewis[a], Joshua A. Morrissey[a], Mitchel D. Flegel[a], Kevin Jeroncic[a], Yeping Xiong[b], Dean Cheng[a], Roman Eisner[a], Bijaya Gautam[a], Dan Tzur[a], Summit Sawhney[d], Fiona Bamforth[c], Russ Greiner[a], Liang Li[b]

[a] Department of Computing Science, University of Alberta, Edmonton, AB, Canada T6G 2E8
[b] Department of Chemistry, University of Alberta, Edmonton, AB, T6G 2E8, Canada T6G 2E8
[c] Department of Clinical Laboratory Medicine, University of Alberta, Edmonton, AB, Canada T6G 2E8
[d] Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada T6G 2E8
[e] National Institute for Nanotechnology, 11421 Saskatchewan Drive, Edmonton, AB, Canada T6G 2E8

ARTICLE INFO

Article history:
Received 11 February 2008
Accepted 2 May 2008
Available online 8 May 2008

Keywords:
Cerebrospinal fluid
CSF
Metabolome
Metabolomics
Nuclear magnetic resonance
Mass spectrometry

ABSTRACT

With continuing improvements in analytical technology and an increased interest in comprehensive metabolic profiling of biofluids and tissues, there is a growing need to develop comprehensive reference resources for certain clinically important biofluids, such as blood, urine and cerebrospinal fluid (CSF). As part of our effort to systematically characterize the human metabolome we have chosen to characterize CSF as the first biofluid to be intensively scrutinized. In doing so, we combined comprehensive NMR, gas chromatography–mass spectrometry (GC–MS) and liquid chromatography (LC) Fourier transform–mass spectrometry (FTMS) methods with computer-aided literature mining to identify and quantify essentially all of the metabolites that can be commonly detected (with today's technology) in the human CSF metabolome. Tables containing the compounds, concentrations, spectra, protocols and links to disease associations that we have found for the human CSF metabolome are freely available at http://www.csfmetabolome.ca.

1. Introduction

Metabolomics is an emerging area of "omics" research that involves the global or near global analysis of the small molecule metabolites (<1500 Da) found in living organisms (i.e. the metabolome). While still in its infancy we are already beginning to see applications of metabolomics in many fields, including disease diagnostics [1], pharmaceutical research and development [2], and agriculture and food safety [3]. These applications are leading to the discovery of many useful biomarkers and the development of a number of improved screening assays. Continued advances in detection and separation technologies certainly suggest that the potential range of metabolomics applications will continue to grow. However, a common criticism about this field is the fact that in any given metabolomics study, relatively few metabolites are identified or quantified. In other words, metabolomics is not as quantitative as the other "omics" sciences. With the release of the first draft of the Human Metabolome [4], we believe an important step has been taken to make metabolomics studies much more quantitative. In an effort to lay an even more solid foundation to quantitative metabolomics we have started to systematically determine the *detectable* metabolic composition of clinically important biofluids and tissue types. Based on its relative metabolic simplicity and its potential importance to central nervous system (CNS) diseases, we have selected cerebrospinal fluid (CSF) as our first biofluid to be comprehensively characterized. Presented herein is the most complete catalogue of the human CSF metabolome to date.

CSF is the secretion product of the central nervous system that fills the ventricles and the subarachnoid space of the brain and spinal column [5,6]. Apart from it's role in protecting the brain from physical shock, CSF also has a function in circulating nutrients and chemicals filtered from the blood along with waste management by removing organic acids either by active transport or bulk flow from the extracellular fluid in the brain to the subarachnoid compartment, and ultimately into the venous blood stream and the lymphatic system [5,6,7]. Since the composition of CSF is directly dependent upon metabolite production rates in the brain [7], analysis of the CSF metabolome can offer biochemical insights into central nervous system disorders, such as brain injury [8], Parkinson's disease [9] and multiple sclerosis [10].

☆ This paper is part of a special volume entitled "Hyphenated Techniques for Global Metabolite Profiling", guest edited by Georgios Theodoridis and Ian D. Wilson.

* Corresponding author. Tel.: +1 780 492 0383; fax: +1 780 492 1071.
E-mail address: david.wishart@ualberta.ca (D.S. Wishart).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.05.001

Over the past 50 years several different routes have been pursued to characterize the CSF metabolome including: (1) modern metabolomic or metabolic profiling approaches; (2) referential clinical chemistry studies and (3) targeted metabolite identification studies. In terms of metabolite profiling methods, several different groups have applied ^{1}H NMR [11–14], gas chromatography–mass spectrometry (GC–MS) [7,13,14] and amino acid analysis [15,16] to characterize a significant portion of the CSF metabolome. Large numbers of referential clinical chemistry studies, largely focusing on a single metabolite at a time, were also conducted on CSF in the 1960s and 1970s [17,18]. The intent of these studies was to determine reference concentrations for many easily detected compounds. Information on these compounds and their concentration ranges has been compiled in a number of well known clinical chemistry texts [19]. With improvements to instrumentation sensitivity and separation capacity, dozens of other targeted metabolite studies have been conducted on CSF that have led to the identification and quantification of many previously undetectable CSF metabolites. Unfortunately, this information is not located in any central repository and is rather scattered across numerous journals and periodicals [4].

In order to facilitate future CSF research, it is important to establish a comprehensive, electronically accessible database of the detectable metabolites in human CSF. In this report we present a catalogue of detectable metabolites (including their concentrations and disease associations) that can be found in human cerebral spinal fluid. This catalogue was assembled using a combination of both experimental and literature-based research. Experimentally, we used nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry, Fourier transform–mass spectrometry (FTMS) and liquid chromatography (LC) to separate, identify, quantify and validate CSF metabolites. To compliment these "global" metabolic profiling efforts, our team also surveyed and extracted metabolite and disease-association data from more than 2000 books and journal articles that had been identified through computer-aided literature mining. In undertaking this effort we wished to address four key questions: (1) what compounds can be or have ever been identified in CSF? (2) What are the concentration ranges for these metabolites? (3) What portion of the CSF metabolome can be routinely identified and/or quantified in CSF using conventional, untargeted metabolomics methods? (4) What analytical methods (NMR, GC–MS, LC–MS) are best suited for comprehensively characterizing the CSF metabolome? Comprehensive tables containing the compounds, concentrations, spectra, protocols and links to disease associations that were uncovered or identified from this work are freely available at http://www.csfmetabolome.ca.

2. Experimental

2.1. CSF collection

Lumbar CSF samples were collected from 50 patients screened for meningitis in accordance with guidelines established by the University of Alberta Health Research Ethics Board. As part of the disease screening procedure, CSF samples were required to be stored at 4 °C for 2 days, after which they were placed in a freezer for long-term storage at −80 °C. Studies with CSF and other biofluids indicate that these fluids are quite stable at low (<5 °C) temperatures [16,20,21]. Samples that were colored due to blood contamination (erythrochromic and xanthochromic) [5] were eliminated from further analyses, leaving a total of 35 usable CSF samples. The typical volume of each CSF sample was 0.5–1.0 mL. Since degradation of CSF metabolites has been seen in CSF left at room temperature for >2 h [16,20], NMR samples were prepared and spectra collected almost immediately after thawing.

2.2. NMR spectroscopy

Fresh CSF samples were prepared by transferring a 300 μL aliquot of CSF fluid to a 1.5 mL Eppendorf tube followed by the addition of 35 μL D_2O and 15 μL of a standard solution (3.73 mM DSS (disodium-2,2-dimethyl-2-silapentane-5-sulphonate), 233 mM imidazole, and 0.47% NaN_3 in H_2O, Sigma–Aldrich, Mississauga, ON). The CSF sample (350 μL) was then transferred to a standard SHIGEMI microcell NMR tube. In total, 35 CSF samples were prepared in this manner, each containing 0.16 mM DSS, 10 mM imidazole, and 0.02% NaN_3 at a pH of 7.3–7.7. The samples were not filtered prior to data collection as CSF contains very little protein [5]. Furthermore, previous studies have found that using ultracentrifugation to remove high molecular weight metabolites does not improve the quantitative or qualitative analysis of low molecular weight metabolites [20].

All ^{1}H NMR spectra were collected on a 500 MHz Inova (Varian Inc., Palo Alto, CA) spectrometer equipped with either a 5 mm HCN Z-gradient pulsed-field gradient (PFG) room-temperature probe or a Z-gradient PFG Varian cold-probe. ^{1}H NMR spectra were acquired at 25 °C using the first transient of the tnnoesy-presaturation pulse sequence, which was chosen for its high degree of high quantitative accuracy [21]. Spectra were collected with 64 transients using a 4 s acquisition time and a 1 s recycle delay. For certain confirmatory experiments, higher field (800 MHz Varian Inova) instruments and larger numbers of transients (256) were used.

2.3. NMR compound identification and quantification

Prior to spectral analysis, all FIDs were zero-filled to 64k data points, and a line broadening of 0.5 Hz was applied. The methyl singlet of the buffer constituent DSS served as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All ^{1}H NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 4.6 (Chenomx Inc., Edmonton, AB). The Chenomx NMR Suite software allows for qualitative and quantitative analysis of an NMR spectrum by "fitting" spectral signatures from an internal database of reference spectra to the full NMR spectrum [22]. Specifically, the spectral fitting for each metabolite was done using the standard Chenomx 500 MHz (pH 6–8) metabolite library, with a set of additional compound signatures (1,5-anhydrosorbitol, dimethyl sulfone, 2-oxoisovalerate, 3-hydroxyisobutyrate) that were added in-house. It has been previously shown that this fitting procedure provides absolute concentration accuracies of 90% or better [23]. Concentration data was corrected for bandpass filter attenuation as previously described [21]. Each spectrum was processed and analyzed by multiple NMR spectroscopists to minimize compound misidentification and misquantification. Concentrations were averaged over 35 patient samples. As a further check of sample identification and quantification accuracy, "outlier" concentrations were identified (using a *Q*-test with a 99% confidence interval) and further analyzed to check or correct the compound identification. Extreme outlier concentrations, which may have represented an underlying or undetected disease condition, were removed in calculating the final metabolite concentration averages and standard deviations. In addition to these statistical checks, we also used sample spiking to confirm the identity of every spectral signature seen in our NMR spectra. This was done by adding 20–200 μM of the presumptive compound to selected CSF samples and checking to see if the corresponding ^{1}H NMR signals changed as expected.

2.4. GC–MS compound identification and quantification

Because of the need for relatively larger volumes for GC–MS and LC–MS work (particularly for quantification as well as for separation and derivatization trials), a pooled CSF sample was prepared from the un-used portion of the 35 samples used in the NMR studies. This ~10 mL sample was divided equally between the LC–MS and GC–MS studies. The CSF extraction and derivatization protocol for our GC–MS work was adapted from previously described methods [24]. Fresh GC–MS samples were prepared by transferring a 200 μL aliquot of CSF fluid to a 1 mL Eppendorf tube followed by the addition of 800 μL of 8:1 HPLC grade methanol:de-ionized water. The sample was then vortexed for 2 min followed by centrifugation at 5000 rpm for 5 min to remove any precipitate from the supernatant. After centrifugation, 200 μL of the supernatant was transferred into a 2 mL glass vial with a 400 μL insert and evaporated to dryness using a Speedvac concentrator. To the residue was added 40 μL of methoxyamine hydrochloride (Sigma–Aldrich), and was incubated at 30 °C for 90 min using a hotplate. After methoximation, 40 μL of MSTFA derivitization agent (Sigma–Aldrich) and 20 μL of proline internal standard solution were added to the residue followed by incubation at 30 °C for 45 min. GC–MS samples were then vortexed twice throughout incubation to ensure complete dissolution. Samples were analyzed immediately after derivitization.

Derivatized extracts were analyzed using an HP 6890/5975 quadrupole GC/MS operating in an electron impact (EI) mode. For analysis, 1 μL of extract was injected splitless onto a DB-5 column (J&W Scientific, Folsom, CA) with helium carrier gas set to a flow rate of 1 mL/min and initial oven temperature of 70 °C. The temperature was increased at 1 °C/min to 76 °C, and then increased at 6.1 °C/min, for a final run time of 45 min (and a final temperature of 310 °C). Samples were run using full scan at a mass range of 50–500 m/z, with a solvent delay of 6 min. Retention indices were calculated using an internal alkane standard [24].

Trimethylsilated metabolites were identified using AMDIS GC/MS processing software in one of two ways. Those peaks matching to known retention indices had their MS data (EI fragment spectra) compared to AMDIS's NIST/EPA/NIH library to confirm the compound's identity. This identity was double-checked using additional published retention index libraries [25]. Those peaks having no match to published retention indices and/or no match to the AMDIS GC/MS spectral library, were identified using pure standards obtained from the Human Metabolite Library [4] and quantified using external five-point calibration curves. Where peak baseline resolution was not observed, peak deconvolution software was used to separate peaks based on Gaussian shape recognition. Using the mass spectral information obtained in this manner, peaks were successfully identified and peak areas successfully quantified. As a final check, pure standards of all 41 compounds initially identified by this process were derivitized and run through the GC–MS (using the same protocol described above) to confirm their identity, retention indices and EI spectra.

2.5. FT ICR–MS compound identification

CSF samples from the previously prepared pooled CSF sample were analyzed using a Bruker Daltonics 9.4T Apex-Qe FT-ICR mass spectrometer operating in positive and negative ion mode equipped with a Waters UPLC system. For a typical analysis, 8 μL of CSF was injected onto a Waters BEH C18 column (Waters Corp., Milford, MA, 1.0 internal diameter (i.d.) × 150 mm). The flow rate was 50 μL/min using a mobile phase of 0.1% acetic acid solution in water (solution A) and acetonitrile (solution B). The column was equilibrated in a 5% B solution. After an initial equilibration time of 5 min, the solvent was ramped to 98% B over a period of 60 min. Further separation was achieved using a HILIC UPLC column (Waters Corp., Milford, MA, 1.0 i.d. × 150 mm). The mobile phase constituents for this separation were 8.5 μm ammonium acetate in 95% acetonitrile (solution A), and 8.5 mM ammonium acetate in 55% acetonitrile (solution B). After injection, a mobile phase concentration of 5% B was held for 10 min, then ramped at 1.3% B/min for 30 min. The mobile phase concentration was ramped to 100% B to a final time of 60 min.

Metabolites were identified and confirmed by high-resolution FT mass spectrometry (FTMS) by comparing their parent ion and fragment ion masses to known masses or fragment ion spectra with the Human Metabolome Database [4], DrugBank [26] and known literature. Unknown peaks needing further confirmation were identified by detailed analysis of MS/MS spectral patterns or comparison of LC retention time and/or MS/MS data obtained with authentic chemical standards obtained from the Human Metabolome Library.

2.6. Literature survey of CSF metabolites

In addition to the experimental analysis of the CSF metabolome mentioned above, a complete literature review of known metabolites and metabolite concentrations in CSF was also conducted. This literature survey was also facilitated by several computational tools developed for the Human Metabolome Database [4]. One of the more useful programs was an in-house text-mining tool called PolySearch (http://wishart.biology.ualberta.ca/polysearch/). This program was used to generate a hyperlinked list of abstracts and papers from PubMed containing relevant information about CSF metabolites and CSF concentration data. Specifically, PolySearch compiled a ranked list of metabolites based on the frequency of word co-occurrence with the terms "CSF", "cerebrospinal fluid" or "cerebral spinal fluid" in conjunction with words such as "concentration", "identification", "quantification", "mM", or "micromol". PolySearch also extracted key sentences from the abstracts, then labeled and hyperlinked the metabolites mentioned in the text. From the resulting papers and abstracts, our annotators extracted metabolite information (metabolite identities, concentrations, disease states, etc.) and entered the data into our database system. The resulting list of literature-derived CSF metabolites helped confirm metabolites found in our experimental analyses. The literature-derived concentration values also simplified some of the searches for putative metabolite matches.

3. Results and discussion

In this study, we have attempted to perform a quantitative, "base-line" characterization of the human CSF metabolome using a combination of both experimental and literature-based approaches. The literature-based data proved to be critical to the identification of a number of previously unidentified or misidentified peaks in our experimental data sets. Likewise, the experimental data allowed correction or confirmation of a number of questionable literature-derived values. The combination of both methods allowed us to assemble a very complete picture of the *detectable* CSF metabolome. It also allowed us the opportunity to address four key questions: (1) what compounds can be or have ever been identified in CSF? (2) What are the concentration ranges for these metabolites? (3) What portion of the CSF metabolome can be routinely identified and/or quantified in CSF using conventional, untargeted metabolomics methods? (4) What analytical methods (NMR, GC–MS, LC–MS) are best suited for comprehensively characterizing the CSF metabolome?

3.1. The content of the CSF metabolome—the CSFmetabolome database

A complete listing of the type and quantity of endogenous metabolites that can be detected in human CSF is given at http://www.csfmetabolome.ca. This freely available, web-enabled database provides a list of the metabolite names, concentration ranges (normal and disease associated), diseases and references for all human CSF metabolites that have ever been detected or described in the literature. It also contains the concentration data compiled from the experimental studies described here. Each CSF metabolite entry in this database is linked to a MetaboCard button which, in turn, is hyperlinked to the Human Metabolome Database (HMDB). The HMDB data provides up to 90 data fields covering details on the nomenclature, chemistry, biology and biochemistry of the metabolite of interest [4]. The CSFmetabolome database itself is searchable by compound name and concentration ranges. It can also be re-sorted to display metabolites on the basis of their name, concentration or disease association. Users may also use the ChemQuery search tool to search the database via the chemical structure, SMILES string or mass of the compound of interest. The CSFmetabolome database also supports searches on the basis of NMR chemical shifts (NMRSearch), mass spectra (MS-Search) and GC–MS data (GCMS-Search).

Currently the human CSFmetabolome database contains 308 *detectable* metabolites, as defined in the present literature. This is not a number that will remain unchanged. Rather it reflects the total number of endogenous metabolites (organic and inorganic) that have ever been detected and quantified by ourselves and others. Certainly as technology improves, it is likely that this number will increase as other, lower abundance metabolites are detected and added to future versions of the CSFmetabolome database. Likewise, if the list was expanded to include intermittent, exogenous compounds such as drugs, food additives and drug metabolites, it could be substantially larger. Inspection of our on-line tables generally shows that CSF contains very few detectable hydrophobic or lipid-like molecules. This is further emphasized in Table 1, which provides a listing of the metabolite categories in CSF and the number of representative compounds that can be found in this biofluid. Certainly lipids and steroids do exist in CSF, but they are at very low abundance and therefore, not easily detectable. Overall, the composition of CSF is dominated by amino acids, metal ions or salts, steroids and steroid derivatives, short chain fatty acids, hydroxy acids, short chain fatty acids, alcohols, dicarboxylic acids and carbohydrates.

Not unexpectedly, a significant number of the metabolites in CSF are neurotransmitters or metabolites of neurotransmitters. These include the catecholamines, excitatory amino acids (GABA, NAA, glutamate), acetylcholine and choline. Their presence obviously reflects the neurotransmitter activity and metabolism in the brain and central nervous system. Based on the existing literature data, the majority of metabolites in CSF are relatively small molecules (<400 Da) with a maximum molecular weight of 1500 Da (for sphingolipids). This restricted size limit may be an artifact of the experimental methodology employed, or it may reflect on the upper limit of small molecules that can easily pass through the blood brain barrier without the need for active transport.

3.2. Metabolite concentrations in CSF—literature survey

The CSFmetabolome database provides both concentration averages and concentration ranges for 308 endogenous metabolites corresponding to both normal and diseased conditions. These concentrations were derived from both literature reviews and from our own experimental efforts (*vide infra*). In many cases, multiple concentration values are given for "normal" conditions. This is done to provide users/readers a better estimate of the potential concentration variations that different technologies or laboratories may measure. As a general rule, there is good agreement between most laboratories and methods. However, the general consensus in this table does not reflect the true state of the raw literature. A number of literature-derived concentration values were eliminated through the curation process after being deemed mistaken, disproven (by subsequent published studies), mis-typed or physiologically impossible. Much of the curation process involved carefully reading and re-reading the primary literature to check for unit type, unit conversion and typographical inconsistencies.

Other than the inorganic ions such as sodium (145 mM), bicarbonate (10 mM), potassium (3 mM), calcium and magnesium (~1 mM), the 12 most abundant organic metabolites found in CSF are glucose (5 mM), urea (4 mM), lactic acid (2 mM), gluatmine (500 μM), citrate (400 μM), acetic acid (300 μM), fructose (200 μM), myo-inositol (170 μM), galactose (170 μM), ascorbic acid (160 μM), pyruvic acid (150 μM) and acetic acid (120 μM). The 12 least abundant metabolites in CSF are estradiol (1 pM), serotonin (10 pM), 8-isoprostane (20 pM), cyanocobalamin (25 pM), norepinephrine (100 pM), 3,4-dihydroxybenzeneacetic acid (100 pM), pregnenalone (130 pM), allopregnanolone (160 pM), epinephrine (220 pM), dihydropbiopterin (400 pM), homovanillic acid (400 pM) and 5-hydroxyindoleacetic acid (400 pM). Despite the high abun-

Table 1
Type and abundance of 37 different compound classes in human CSF

Category #	Compound class	Number of compounds found in CSF
1	Amino acids	56
2	Minerals and elements	27
3	Fatty acids	18
4	Steroids and steroid derivatives	18
5	Hydroxy acids	16
6	Alcohols and polyols	16
7	Dicarboxylic acids	13
8	Peptides	12
9	Carbohydrates	11
10	Nucleosides	10
11	Inorganic ions and gases	10
12	Cyclic amines	10
13	Catecholamines and derivatives	9
14	Pterins	9
15	Nucleotides	9
16	Keto-acids	7
17	Indoles and indole derivatives	6
18	Purines and purine derivatives	5
19	Carnitines	5
20	Miscellaneous	5
21	Prostanoids	5
22	Aromatic acids	4
23	Pyrimidines and pyrimidine derivatives	4
24	Amino ketones	4
25	Aldehydes	4
26	Leukotrienes	2
27	Tricarboxylic acids	2
28	Ketones	2
29	Biotin and derivatives	1
30	Porphyrins	1
31	Glycolipids	1
32	Amino acids phosphates	1
33	Cobalamin derivatives	1
34	Quinolines	1
35	Acyl phosphates	1
36	Amino alcohols	1
37	Lipids	1

dance of acids and acidic amino acids, CSF is heavily buffered by bicarbonate ions, allowing it to maintain a constant pH of 7.3 [7]. The relatively high levels of glucose and urea in CSF also reflect the primary activity of CSF (waste disposal and nutrient circulation). Other metabolites, such as trimethylamine, methanol, acetone, $DMSO_2$, etc. are primarily metabolic waste products that need to be removed from the brain.

One point that is particularly interesting is the fact that the concentration of the average metabolite in normal CSF varies by about ±50%, with some metabolites varying by as much as ±100% (such as acetoacetic acid, acetone and glutamic acid). Likewise, drawing conclusions about potential disease biomarkers without properly taking into account this variation would be ill-advised. Since CSF is like the "brain's urine" we believe this variation may be due to a number of factors, including age, gender, diurnal variation, health status, activity and diet [27]. Some entries in the CSFmetabolome database show these variations explicitly with information about the populations (age, gender) from which these metabolite concentrations were derived. Clearly more study on the contributions to the observed variations in CSF is warranted, although given the difficulty with which CSF is normally acquired, these studies will be difficult.

3.3. Experimental quantification and identification—NMR

Fig. 1 illustrates a typical high-resolution NMR spectrum of CSF. As can be seen in this figure, most of the visible peaks are annotated with a compound name. In processing and analyzing the 35 CSF spectra we were generally able to assign >95% of all visible peaks and account for >99% of the spectral area for each CSF spectrum. In other words the level of assignment is essentially complete. On average, a single CSF sample will yield about 45–50 identifiable metabolites by NMR. Analysis of the entire collection of CSF samples yielded a total of 53 metabolites of which 47 could be precisely quantified (Tables 2 and 3). Of the 53 compounds identified, three metabolites are exogenous or potentially exogenous, including acetaminophen (a drug), propylene glycol (a possible container contaminant) and isopropanol (from needles used to perform the lumbar punctures). However, propylene glycol and isopropanol are also known to be produced in the human body [28].

As seen in Table 3 some compounds are found in only a few CSF samples, while about 45 compounds (with >90% occurrence) seem universal. Efforts certainly were made to identify these "rare" compounds in a larger fraction of CSF samples. However, we found that collection of NMR spectra for longer periods of time or at higher fields only improved quantification accuracy but did not lead to an increase in the number of signals detected. Inspection of Table 3 also reveals the generally good agreement between the NMR-measured concentrations and those reported in the literature (often obtained by other analytical means). However, not all of the NMR-derived CSF concentrations agree with the literature derived values. Compounds exhibiting the greatest discrepancy between NMR measured values and literature derived values include: 3-hydroxyisobutyrate, acetone, glycerol, dimethylsulfone, mannose, oxalacetate, succinate and propylene glycol. Some of these may be explained by the inherent volatility or chemical instability of certain compounds (dimethylsulfone, acetone and propylene glycol). Other discrepancies may be due to sample collection/preservation effects or possibly sample size effects (2 patients versus 35 patients). A third source that might account for some of the observed variation may be technical problems with the analytical methods themselves or the calibration standards used in the original analyses. We have carefully re-investigated our concentration measurements by NMR for these nine "problem" compounds and are convinced that they are accurate.

Table 2
List of CSF metabolites identified by ^{1}H NMR, GC–MS and LC–FTMS

#	NMR	GC–MS	LC–FTMS
1	1,5-Anhydrosorbitol	**2-Hydroxybutyrate**	*Acetaminophen*
2	2-Aminobutyrate	4-Aminobutyrate (GABA)	Benzoic acid
3	**2-Hydroxybutyrate**	**Acetate**	Citrate
4	2-Hydroxyisovalerate	**Acetoacetate**	*Creatinine*
5	2-Oxoglutarate	Adenosine	Fructose
6	2-Oxoisovalerate	**Alanine**	Galactose
7	3-Hydoxybutyrate	Ascorbate	Glucose
8	3-Hydroxyisobutyrate	Asparagine	Inositol
9	3-Hydroxyisovalerate	Aspartate	Isoleucine
10	*Acetaminophen*	**Citrate**	Leucine
11	**Acetate**	Cysteine	*Mannose*
12	**Acetoacetate**	Ethanolamine	Paraxanthine
13	Acetone	**Fructose**	Phenylalanine
14	**Alanine**	**Galactitol**	Succinyladenine
15	Arginine	Galactose	Tryptophan
16	Choline	**Glucose**	Tyrosine
17	**Citrate**	**Glutamine**	*Xanthine*
18	Creatine	**Glycerol**	
19	*Creatinine*	**Glycine**	
20	Dimethyl sulfone	**Isoleucine**	
21	Dimethylamine	**Lactate**	
22	Formate	**Leucine**	
23	**Fructose**	**Lysine**	
24	**Galactitol**	Mannitol	
25	**Glucose**	**Methionine**	
26	Glutamate	**Myo-Inositol**	
27	**Gluatmine**	**Oxalacetate**	
28	**Glycerol**	**Phenylalanine**	
29	**Glycine**	Phosphate	
30	Histidine	**Pyroglutamate**	
31	**Isoleucine**	**Pyruvate**	
32	Isopropanol	Ribotol	
33	**Lactate**	**Serine**	
34	**Leucine**	**Succinate**	
35	**Lysine**	**Threonine**	
36	*Mannose*	Thymine	
37	Methanol	**Tryptophan**	
38	**Methionine**	**Tyrosine**	
39	**Myo-Inositol**	**Urea**	
40	**Oxalacetate**	Uric acid	
41	**Phenylalanine**	**Valine**	
42	Propylene glycol		
43	**Pyroglutamate**		
44	**Pyruvate**		
45	**Serine**		
46	**Succinate**		
47	**Threonine**		
48	Trimethylamine		
49	**Tryptophan**		
50	**Tyrosine**		
51	**Urea**		
52	**Valine**		
53	*Xanthine*		

It should be noted that given the invasive nature of CSF collection as well as the ethical and legal issues associated with obtaining CSF, it is almost impossible to collect this fluid from truly healthy individuals. Many CSF samples are collected "with cause" or as a precautionary measure due to some underlying condition. Consequently defining a normal or healthy concentration range for a given set of CSF metabolites is always going to be somewhat challenging. Nevertheless, we would estimate that the list of 53 metabolites given in Table 2 and the concentrations give in Table 3 essentially defines the "normal NMR-detectable CSF metabolome". Furthermore, we believe that this set of 53 should make NMR characterization of unprocessed CSF essentially automatic.

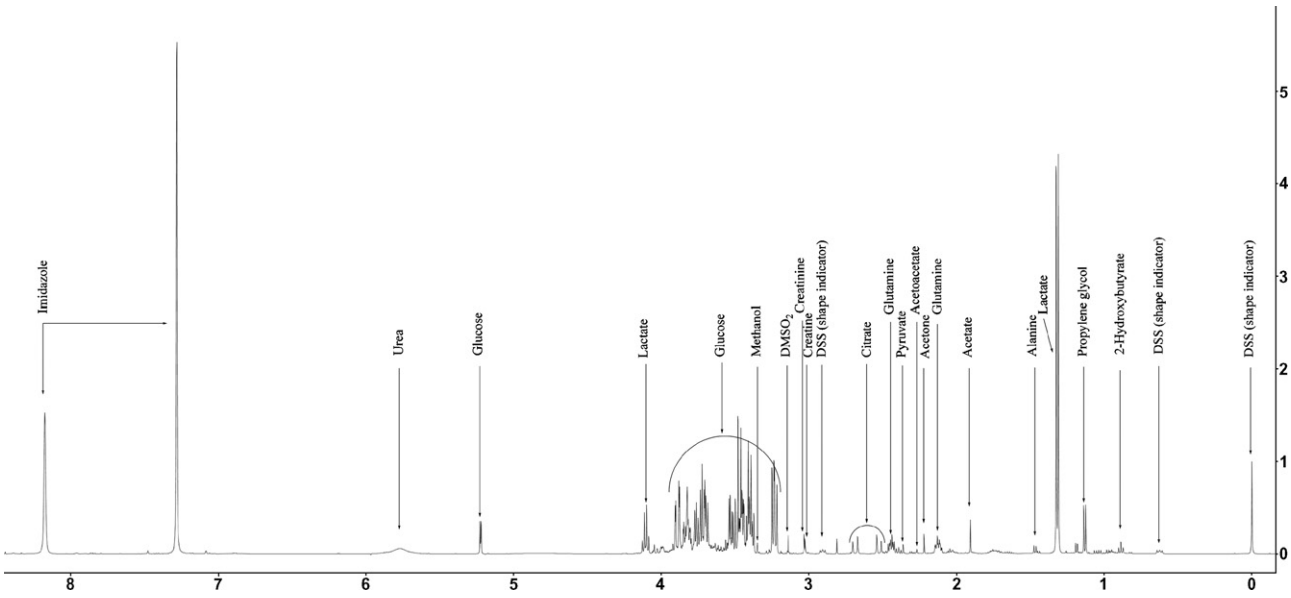

Fig. 1. Varian 500 MHz ^{1}H NMR spectrum of human CSF. Resonances are labeled with their corresponding metabolite names.

3.4. Experimental quantification and identification—GC–MS

Fig. 2 illustrates a high-resolution GC–MS spectrum of our pooled sample of CSF. As can be seen in this figure, most of the visible peaks are annotated with a compound name. Approximately 95% of peaks in the total ion chromatogram were identified. All chemical peaks for identified metabolites were verified by pure standards and correlated to literature values. In total we were able to identify 41 metabolites via GC–MS (Table 2). An additional 15 metabolites that could not be detected/quantified by NMR were quantified by GC–MS using external calibration (Table 4). Compound concentrations that were below the 1 μM limit could not be accurately quantified, however these compounds were identified based on identification methods previously described. Given that there are slightly over 75 compounds in the CSFmetabolome database that have normal concentrations >1 μM, one might have expected that the number of compounds detectable by GC–MS would be much higher than 41. One possible reason for this lower-than-expected number is our use of a relatively slow scanning quadrupole instrument that may not have allowed sufficient sampling across the GC peaks to permit full spectral deconvolution. The use of faster scanning quadrupole or TOF instruments with greater sensitivity may have improved compound detection or identification. Indeed, a recent report by Pears et al. [14] has shown that up to 80 metabolite signals could be detected in the CSF of domestic sheep using a higher quality GC–TOF instrument. However, only 45 of the 80 metabolites could be unambiguously identified (versus 41 reported here).

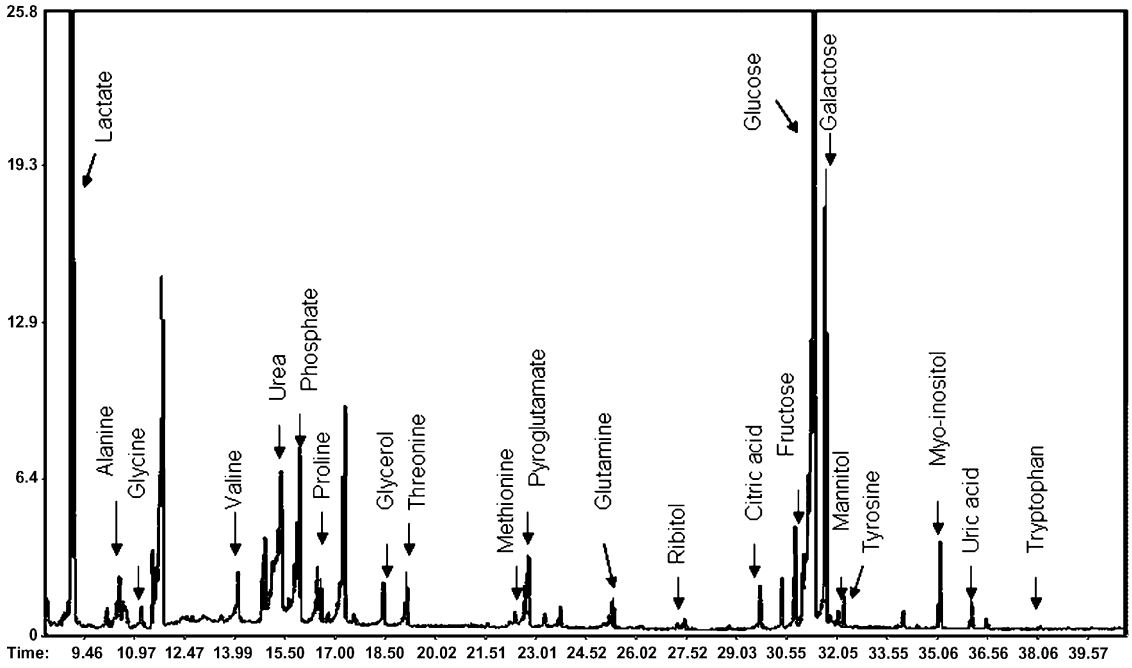

Fig. 2. HP 6890/5975 GC/MS spectrum of human CSF. Peaks are labeled with their corresponding metabolite names.

Table 3
Concentrations of metabolites in 35 CSF samples as measured by NMR

#	Compound name	Average (μM)	Standard deviation (μM)	% Occurrence	Literature value
1	1,5-Anhydrosorbitol	25	13	100	18 ± 5
2	2-Hydroxybutyrate	40	24	100	35 ± 24
3	2-Hydroxyisovalerate	8	6	91	7 ± 7
4	2-Oxoglutarate	5	4	91	9 ± 3
5	2-Oxoisovalerate	6	3	89	8 ± 7
6	3-Hydroxybutyrate	34	31	100	46 ± 24
7	3-Hydroxyisobutyrate	6	3	100	18 ± 18
8	3-Hydroxyisovalerate	4	2	100	N/A
9	Acetaminophen	11	6	21	N/A
10	Acetate	58	27	100	100 ± 30
11	Acetoacetate	12	14	94	6 ± 6
12	Acetone	20	21	97	67 ± 24
13	Alanine	46	27	100	37 ± 7
14	Choline	3	1	97	8 ± 5
15	Citrate	225	96	100	176 ± 50
16	Creatine	44	13	100	N/A
17	Creatinine	43	12	100	65 ± 25
18	Dimethylsulfone	2	1	97	11 ± 6
19	Dimethylamine	2	1	91	N/A
20	Formate	32	16	94	N/A
21	Fructose	160	91	100	240 ± 20
22	Glucose	2960	1110	100	5390 ± 1650
23	Glutamate	40	52	26	33 ± 7
24	Glutamine	432	204	100	444 ± 80
25	Glycerol	39	14	100	14 ± 3
26	Histidine	14	8	86	12 ± 2
27	Isoleucine	7	5	94	8 ± 3
28	Isopropanol	22	56	97	N/A
29	Lactate	1651	626	100	1590 ± 330
30	Leucine	16	9	100	19 ± 4
31	Lysine	29	13	100	28 ± 8
32	Mannose	24	13	97	64 ± 8
33	Methanol	44	36	100	N/A
34	Methionine	5	4	86	6 ± 3
35	myo-Inositol	84	40	100	133 ± 20
36	Oxalacetate	27	15	97	7 ± 2
37	Phenylalanine	15	13	91	18 ± 7
38	Propylene glycol	33	50	85	1 ± 0.5
39	Pyroglutamate	47	30	100	41 ± 30
40	Pyruvate	53	42	97	71 ± 30
41	Serine	42	18	100	42 ± 15
42	Succinate	3	2	82	29 ± 5
43	Threonine	30	12	68	28 ± 5
44	Tryptophan	5	3	46	2 ± 1
45	Tyrosine	12	9	91	10 ± 4
46	Valine	19	13	97	24 ± 7
47	Xanthine	13	7	21	5 ± 1

Table 4
Concentrations of metabolites in CSF by GC–MS

#	Compound name	Concentration (μM)	Literature value (μM)
1	2-Hydroxybutyrate	85.8	35 ± 24
2	4-Aminobutyrate	<1	0.3 ± 0.1
3	Adenosine	<1	0.01 ± 0.01
4	Ascorbate	178	164 ± 20
5	Asparagine	4.43	5.4 ± 1.4
6	Aspartate	2.30	2.8 ± 1.2
7	Galactose	107	166 ± 99
8	Glycerol	17.5	13.5 ± 3
9	Glycine	<1	8 ± 4
10	Mannitol	4.49	4.6 ± 1.2
11	Ribitol	17.8	3.7 ± 1.2
12	Succinate	47.2	29 ± 5
13	Thymine	<1	N/A
14	Urea	3820	4160 ± 1800
15	Uric acid	15.5	16 ± 12

These differences in metabolite numbers may also reflect inherent species differences (sheep versus human) or they may reflect intrinsic differences in the GC–MS deconvolution software and protocols.

It is also of some interest to compare the results of our GC–MS studies with the NMR studies. As seen in Table 2 and Fig. 3, NMR and GC–MS methods identify a common set of 28 compounds, while NMR detects 25 compounds that GC–MS methods cannot detect. Additionally GC–MS detects 13 compounds that NMR cannot routinely detect. Curiously, NMR is not able to detect 2 very high abundance compounds (ascorbic acid and galactose) that could be easily detected by GC–MS. The reasons for these differences could be manifold. For those compounds found in NMR but not GC–MS, it may be that the metabolites of interest were either too volatile for GC–MS detection, lost in sample preparation or eluted during the solvent delay. For those compounds found in GC–MS but not in NMR, the compounds may not have proton signals for NMR detection (uric acid, phosphate) or the concentrations were below detectable limits (adenosine, thymine). In all cases, the existence of NMR detectable metabolites was explicitly checked in our GC–MS analyses and vice versa. Collectively, GC–MS and NMR when combined together can identify 66 compounds.

As previously noted, approximately 5% of the peaks remain unidentified in our GC–MS analyses. These unidentified peaks in the total ion chromatogram were all of uniformly low intensity.

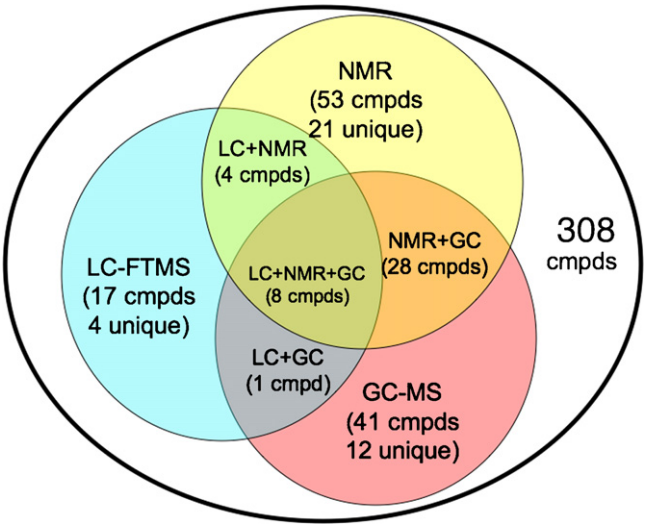

Fig. 3. Venn diagram showing the overlap of CSF metabolites detected by global NMR, GC–MS and LC–FTMS methods compared to the detectable CSF metabolome.

As such, electron impact ionization fails to produce enough fragmentation information for a positive identification. Nevertheless, numerous standards were run to confirm retention times and mass spectral information, likewise, other GC–MS metabolome libraries were also queried but with no success. Overall, GC–MS and NMR appear to be very complimentary techniques for the identification and quantification of small molecules in CSF.

3.5. Experimental quantification and identification—LC–FTMS

Fig. 4 illustrates some of the FTMS ion chromatograms collected using reverse phase C18 UPLC and HILIC columns attached to an electro-spray interface. Both positive and negative electrospray ionization modes and a variety of UPLC columns were used in order to maximize the number of peaks detected. A shallow gradient was purposely used for metabolite detection using an acquisition rate of approximately 3 s per scan. As seen in Fig. 4A, more than 30 peaks were detected in a single run using a C18 UPLC chromatogram in the positive ion mode (Fig. 4A). Furthermore, many metabolites co-eluted resulting in multiple metabolites being detected within a single chromatographic peak. Compared to the positive ion mode, the negative ion mode resulted in far fewer peaks (Fig. 4B). This may be due to the lower sensitivity of the FTMS in negative ion mode. This lack of sensitivity also biased our LC–MS method against the detection of organic acids, which are among the most abundant molecules in CSF. In order to reduce ion suppression and potentially improve the resolution of polar metabolites, a HILIC (hydrophilic interaction chromatography) column was used to resolve those metabolites not retained on the C18 column. Fig. 4C shows an example of a HILIC ion chromatogram (–ion mode). Data from the HILIC chromatography runs were generally more successful in matching known CSF compounds.

From the pooled CSF sample and using eluents from both C18-RP and HILIC HPLC columns a total of more than 200 unique features were detected using a combination of positive and negative mode detection. Metabolites were identified and confirmed using the accurate mass of the parent ion (to 4 decimal places), LC-retention data from authentic standards and MS/MS spectral patterns, also obtained from authentic standards. As seen in Table 2, a total of 17 metabolites were formally identified (but not quantified) via LC–FTMS. The remaining 200 features appear not to match any known compounds (via parent ion mass comparison) in CSF. It is also likely that many of these "features" are breakdown or ionization byproducts of well-known metabolites. It is somewhat surprising that our LC–MS methods were unable to detect any of the most abundant molecules in CSF.

The lack of success in identifying CSF metabolites by LC–MS underlines at least three of the weaknesses of this particular approach to global metabolic profiling. The first weakness lies in the fact that liquid chromatography, relative to gas chromatography, is an inherently poor method for achieving high-resolution and reproducible separations of polar compounds. LC methods typically achieve their best resolution when separating hydrophobic molecules. The second weakness lies in the fact that MS methods, while incredibly sensitive, are only sensitive to molecules that ionize well and which "fly" easily in a spectrometer. Those ions that fly well, do not necessarily correspond to the most abundant or even the most biologically important ions. This makes the detection of routine compounds difficult by LC–MS. The third weakness of LC–MS methods in global metabolomic profiling lies in the lack of referential MS or MS/MS databases. Being able to compare a mass list or a set of MS/MS peaks to a set of standard spectra would greatly facilitate compound identification. NMR and GC–MS are much more developed in this area than LC–MS. Indeed many GC–MS and NMR specific databases now publicly available [4,29,30]. Reference LC–MS and MS/MS spectra for large numbers of metabolites collected in appropriate biological matrices (rich in sodium, potassium, phosphate, and other adduct-forming components) are simply not available. This seriously limits the capacity to identify "obvious" metabolites in biological fluids. As a result, one is often forced to spike authentic standards into the biofluid of interest to make a positive ID.

Had we analyzed a biofluid containing a larger portion of non-polar metabolites (such as blood or serum), it is likely that the LC–FTMS approach would have achieved a much higher level of success. Likewise, the use of chemo-selective derivitization, prior to LC-separation [31,32] would have no doubt substantially improved the overall performance of our LC–FTMS approach. Despite these shortcomings, it is still clear that LC–FTMS or LC–MS/MS can be used as a complimentary technique to GC–MS and NMR for the analysis of small molecules in CSF.

3.6. Method comparison

To summarize, we used three different global metabolic profiling methods: (1) NMR, (2) GC–MS and (3) LC–MS to characterize as much of the known CSF metabolome as possible. We were able to positively identify a total of 70 unique metabolites including 1 drug and 2 potentially exogenous compounds. NMR spectroscopy was able to identify 53 compounds, GC–MS was able to identify 41 compounds and LC–FTMS was able to identify 17 compounds. Together, the three methods were able to identify a common set of just 8 metabolites (citrate, fructose, glucose, isoleucine, leucine, phenylalanine, tryptophan and tyrosine: underlined in Table 2). GC–MS and LC–MS were only able to positively identify just one shared metabolite (galactose: boxed in Table 2), GC–MS and NMR were able to identify a common set of 28 metabolites (bold in Table 2) while NMR and LC–MS were able to identify a common set of 4 metabolites (creatinine, acetaminophen, mannose and xanthine: italics in Table 2).

In terms of the portion of the *detectable* human CSF metabolome that these methods can sample, NMR is able to access ~17% (52/308) of the endogenous CSF metabolome, GC–MS is able to access 13% (41/308) while LC–MS is able to access 5% (16/308). When combined the three methods are able to obtain data on 22% of the endogenous CSF metabolome (69/308). It is important to emphasize that the approaches used here were "global" in

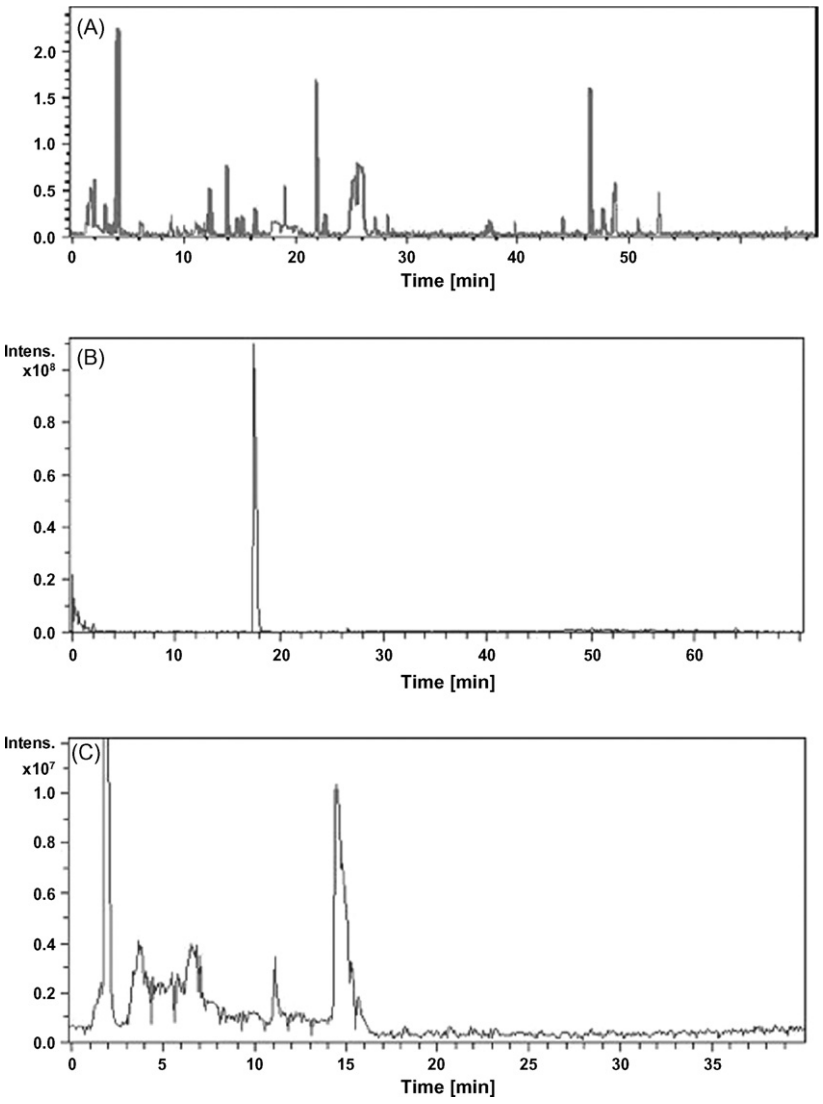

Fig. 4. CSF ion chromatograms collected from C18 and HILIC UPLC runs collected on a 9.4 Tesla Bruker FTMS equipped with an electro-spray interface. (A) C18 UPLC–FTMS positive ion mode base peak ion chromatogram of CSF. (B) C18 UPLC–FTMS negative ion mode base peak ion chromatogram of CSF. (C) HILIC UPLC–FTMS negative ion mode base peak ion chromatogram.

their intent, meaning that the detection and quantification of these metabolites was not targeted. The use of more sophisticated or targeted detection and separation protocols (immunodetection, solid phase extraction, chemical derivitization, etc.) along with the use of a higher-end GC–MS instrument (GC–TOF) would likely have led to the experimental detection of more compounds. However, for this study, we wanted to address the question of how well high throughput, global metabolomic methods could perform in identifying and quantifying metabolites in CSF.

While NMR may appear to be the most suitable method for CSF characterization—both in terms of its breadth of coverage and its amenability for quantification, it appears that NMR is already near at its practical limit of detection and quantification. It also appears that GC–MS is hovering near its limit of detection as well. Certainly the use of compound-selective isolation and concentration techniques could lead to some improvements in what could be detected or quantified by NMR and GC–MS. Indeed, over the past 20 years GC–MS techniques have been used to detect approximately 1/3 of the CSF metabolome. On the other hand, the modest performance seen for LC–FTMS in detecting CSF metabolites suggests that there is considerable room for methodological improvement in this area. In particular, it is very clear that the high abundance of polar compounds in CSF seriously limited the chromatographic separation achievable with standard RP and HILIC columns. The use of hydrophobic enrichment tags (similar in concept to trimethylsilation in GC–MS) using *p*-chlorophenylalanine mediated chemical labeling [31] or dimethyl isotopic labeling [32] has been shown to confer enhanced LC retention and resolution of small metabolites. Intelligent use of UV or fluorescent chemo-selective tags can also be employed as a way of improving the detection or quantification of many analytes. Preliminary data (Liang Li, personal communication) suggests this chemo-selective tagging approach could lead to detection and relative quantification by LC–FTMS of perhaps 150 metabolites in the CSF metabolome.

4. Conclusion

We began this study in an effort to address four key questions: (1) what compounds can be or have ever been identified in CSF? (2) What are the concentration ranges for these metabolites? (3) What portion of the CSF metabolome can be routinely identified and/or quantified in CSF using conventional, untar-

geted metabolomics methods? (4) What analytical methods (NMR, GC–MS, LC–MS) are best suited for comprehensively characterizing the CSF metabolome? Our computer-aided literature survey allowed us to identify 308 metabolites that constitute the detectable human CSF metabolome. In assessing this collection we found that CSF is a metabolically diverse biofluid, with representative metabolites spanning 33 different compound categories. More specifically we found that CSF, not unexpectedly, is rich in amino acids, inorganic salts, organic acids, and sugars. A large variety of catecholamines and steroids are also present, but at very low abundance. CSF metabolites range in concentration from 1 picomolar (for estradiol) to 145 mM (for sodium), although many metabolites have "normal" concentrations can vary by more than 50%. Approximately 75 metabolites in CSF have normal concentrations above 1 μM.

Our experimental efforts revealed that global metabolic profiling methods can (and should) routinely detect 70 different compounds in CSF, or about 22% of the detectable metabolome. NMR methods (alone) can detect and quantify 53 compounds, GC–MS methods (alone) can detect and quantify 41 compounds, and LC–FTMS methods (alone) can detect 17 different compounds. The fact that these three methods could only detect 8 common metabolites underlines the fact that global metabolic profiling methods must use more than one detection technology to obtain a complete picture of any given biofluid metabolome. While clear differences do exist in the number and type of compounds detected by the three technologies employed in this study, the intent was not to negatively bias any technology, but simply to characterize the human CSF metabolome with the tools we had at hand. In our hands, NMR appears to be the best method for performing global or non-targeted metabolic profiling of CSF. However, its general lack of sensitivity (>1 μM) suggests that NMR will tend to miss a number of metabolites (i.e. inflammatory or oxidation-status markers) of clinical interest. GC–MS appears to have similar or slightly better sensitivity to NMR and the two methods, when combined, can detect and/or quantify 66 metabolites. Potentially, the use of GC–TOF instrument or a fast scanning quadrupole instrument would have yielded more favorable results for the GC–MS studies. Interestingly, we found that LC–FTMS methods did not perform particularly well in characterizing the CSF metabolome. However, recent developments and continuing advances in LC–MS and LC–MS/MS technologies [31,32] suggest that this technology should soon match and eventually surpass the performance of NMR and GC–MS methods.

Our primary objective for undertaking these studies and compiling this data was to advance the fields of quantitative metabolomics and global metabolic profiling. Experimentally, our data should serve as a useful benchmark from which to compare other technologies or assess coming methodological improvements in CSF characterization. From a clinical standpoint, we think the information contained in the CSFMetabolome database should provide clinicians, clinical chemists and neuroscientists a convenient, centralized resource from which to learn more about CSF and to better appreciate the window it can provide on brain function and the biochemical activity in the brain.

Acknowledgements

This research was supported by Genome Alberta, Genome Canada and the University of Alberta. The authors would like to thank Connie Sobsey, Jun Peng and Ryan Fradette for their help in preparing the figures and Dr. Xingye Su for help with implementing the HILIC technique.

References

[1] D.I. Ellis, R. Goodacre, Analyst 131 (2006) 875.
[2] J.C. Lindon, E. Holmes, J.K. Nicholson, FEBS J. 274 (2007) 1140.
[3] R.A. Dixon, D.R. Gang, A.J. Charlton, O. Fiehn, H.A. Kuiper, T.L. Reynolds, R.S. Tjeerdema, E.H. Jeffery, J.B. German, W.P. Ridely, J.N. Seiber, J. Agric. Food Chem. 54 (2006) 8984.
[4] D.S. Wishart, D. Tzur, C. Knox, R. Eisner, A.C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, C. Fung, L. Nikolai, M. Lewis, M.-A. Coutouly, I. Forsythe, P. Tang, S. Shrivastava, K. Jeroncic, P. Stothard, G. Amegbey, D. Block, D. Hau, J. Wagner, J. Miniaci, M. Clements, M. Gebremedhin, N. Guo, Y. Zhang, G.E. Duggan, G.D. MacInnis, A.M. Weljie, R. Dowlatabadi, F. Bamforth, D. Clive, R. Greiner, L. Li, T. Marrie, B.D. Sykes, H.J. Vogel, L. Querengesser, Nucleic Acids Res. 35 (2007) D521.
[5] C. Lentner, A. Wink, Geigy Scientific Tables, 8th rev. ed., CIBA-Geigy, Basel, Switzerland, 1981.
[6] M.B. Segal, J. Inherit. Metab. Dis. 16 (1993) 617.
[7] G. Hoffmann, W. Meier-Augenstein, S. Stockler, R. Surtees, D. Rating, W. Nyhan, J. Inherit. Metab. Dis. 16 (1993) 648.
[8] B. Toczylowska, M. Chalimoniuk, M. Wodowska, E. Mayzner-Zawadzka, Brain Res. 1104 (2006) 183.
[9] F.J. Jiménez-Jiménez, J.C. Rubio, J.A. Molina, M.A. Martín, Y. Campos, J. Benito-León, M. Ortí-Pareja, T. Gasalla, J. Arenas, J. Neurol. Sci. 145 (1997) 183.
[10] I.L. Simone, F. Federico, M. Trojano, C. Tortorella, M. Liguori, P. Giannini, E. Piccola, G. Natile, P. Livrea, J. Neurol. Sci. 144 (1996) 182.
[11] E. Holmes, T.M. Tsang, J.T. Huang, F.M. Leweke, D. Koethe, C.W. Gerth, B.M. Nolden, S. Gross, D. Schreiber, J.K. Nicholson, S. Bahn, PLoS Med. 3 (2006) e327.
[12] B.C. Sweatman, R.D. Farrant, E. Holmes, F.Y. Ghauri, J.K. Nicholson, J.C. Lindon, J. Pharm. Biomed. Anal. 11 (1993) 651.
[13] R.A. Wevers, U. Engelke, U. Wendel, J.G. de Jong, F.J. Gabreels, A. Heerschap, Clin. Chem. 41 (1995) 744.
[14] M.R. Pears, R.M. Salek, D.N. Palmer, G.W. Kay, R.J. Mortishire-Smith, J.L. Griffin, J. Neurosci. Res. 85 (2007) 3494.
[15] G.P. Gerrits, F.J. Trijbels, L.A. Monnens, F.J. Gabreels, R.A.D. Abreu, A.G. Theeuwes, B. van Rayy-Selten, Clin. Chim. Acta 182 (1989) 271.
[16] T.N. Ferraro, T.A. Hare, Anal. Biochem. 143 (1984) 82.
[17] L. Sweetman, Fed. Proc. 27 (1968) 1055.
[18] M.B. Bowers Jr., Neuropharmacology 11 (1972) 101.
[19] C.A. Burtis, E.R. Ashwood, Tietz Textbook of Clinical Chemistry, third ed., WB Saunders, Philadelphia, PA, 1998.
[20] S. Maillet, J. Vion-Dury, S. Confort-Gouny, F. Nicoli, N.W. Lutz, P. Viout, P.J. Cozzone, Brain Res. Brain Res. Protoc. 3 (1998) 123.
[21] E.J. Saude, C.M. Slupsky, B.D. Sykes, Metabolomics 2 (2006) 113.
[22] A.M. Weljie, J. Newton, P. Mercier, E. Carlson, C.M. Slupsky, Anal. Chem. 78 (2006) 4430.
[23] E.J. Saude, B.D. Sykes, Metabolomics 3 (2007) 19.
[24] J. A, J. Trygg, J. Gullberg, A.I. Johansson, P. Jonsson, H. Antti, S.L. Marklund, T. Maritz, Anal. Chem. 77 (2005) 8086.
[25] N. Schauer, D. Steinhauser, S. Strelkov, D. Schomburg, G. Allison, T. Moritz, K. Lundren, U. Roessner-Tunali, M. Forbes, L. Willmitzer, FEBS Lett. 579 (2005) 1332.
[26] D.S. Wishart, C. Knox, A.C. Guo, S. Shrivastava, M. Hassanali, P. Stothard, Z. Chang, J. Woolsey, Nucleic Acids Res. 34 (2006) D668.
[27] C.M. Slupsky, K.N. Rankin, J. Wagner, H. Fu, D. Chang, A.M. Weljie, E.J. Saude, B. Lix, D.J. Adamko, S. Shah, R. Greiner, B.D. Sykes, T.J. Marrie, Anal. Chem. 79 (2007) 6995.
[28] A.E. Jones, R.L. Summers, J. Emerg. Med. 19 (2000) 165.
[29] C.A. Smith, G. O'Maille, E.J. Want, C. Qin, S.A. Trauger, T.R. Brandon, D.E. Custodio, R. Abagyan, G. Siuzdak, Ther. Drug Monit. 27 (2005) 747.
[30] J. Kopka, N. Schauer, S. Krueger, C. Birkemeyer, B. Usadel, E. Bergmüller, P. Dörmann, W. Weckwerth, Y. Gibon, M. Stitt, L. Willmitzer, A.R. Fernie, D. Steinhauser, Bioinformatics 15 (2005) 1635.
[31] E.E. Carlson, B.F. Cravatt, J. Am. Chem. Soc. 129 (2007) 15780.
[32] K. Guo, C. Ji, L. Li, Anal. Chem. 79 (2007) 8631.