Evaluating the integration of CE, ESI and Mass Spectrometry for the Quantitative Analysis of Underivatized Amino Acids in the Cationic Metabolome

John C. Hudson

SCIEX, Regina, SK, Canada

Metabolomics can be defined as a comprehensive analytical approach for the study of all low-molecular-weight species present in a given biological system of interest. The main objective of global metabolomics or untargeted metabolite profiling studies is to successfully analyze as many low-molecular-weight species (typically defined as <1000 Da or <1500 Da) as possible in a single experiment.[1]

The term metabolomics, in the modern sense, encompasses broad objectives of analyzing all the endogenous metabolites in a biological system both qualitatively and quantitatively, and also of determining the levels of those metabolites in, for example, a disease state indicated by changes in biomarker levels in bio-fluids.[2-4]

There are many approaches in the design of experiments. Metabolomic profiling (fingerprinting), in which changes in relative levels of compounds in a bio-fluid such as urine, may be up or down-regulated, is common. Profiling can be targeted or untargeted in which specific compounds are of interest, or any compounds in an untargeted approach are under scrutiny.

All these approaches are influenced by the fact that the number of compounds and their physico-chemical attributes are so diverse that no single analytical technique can cover them all. Techniques such as GC/MS, LC/MS, NMR, capillary electrophoresis (CE) and mass spectrometry (MS), all have advantages and limitations.[5] Comprehensive analysis by even a combination of techniques is simply impossible due to the diversity of analyte types. Then, the most important factor to consider is the subset of the metabolome to be investigated and which technique is best applied to that group of small molecules. Since the compounds, as metabolites of biological processes, are mostly polar and charged species (easily ionized), they lend themselves to electro-migration techniques, i.e., CE.[6] These compounds include organic acids, inorganic ions, amino acids, vitamins, carbohydrates, peptides, nucleotides and nucleosides, etc.[5] This diverse group of compounds, when investigated by CE, can provide a better understanding of compound inter-dependency, particularly when analysis is done by the same technique. Capillary electrophoresis, being the analytical technique of choice for polar molecule analysis, makes a perfect hyphenation partner with electrospray ionization mass spectrometry (CE-ESI-MS).[6-7]

The development in recent years of an integrated interface for CE-MS composed of a capillary and emitter to form a seamless integrated process with electro-spray ionization, CESI, holds the promise of orders of magnitude in improvement of detection levels.[2] Ramautar et al.[9] concluded that this approach was superior to using the sheath-liquid sprayers, which have been in use since the advent of CE-MS. Of particular interest is the low sample requirement (less than 10 nL injections). This feature permits analysis of specimens from animal studies where specimen size can be on the order of a few microliters or less.[10-11] Other studies have focused on small molecules from the cationic metabolome and the development of methods of analysis using CESI-MS.[8]

The objectives of this study include: 1) the selection and assessment of a performance standard (PS), including internal standards, used to evaluate daily instrument performance; 2) the development of sampling protocols for bio-fluids, including urine, plasma, serum and oral fluid (saliva) and 3) quantitative analysis of amino acids in bio-fluids using simple sample preparation protocols.

Experimental standards

Internal standards stock solutions

The following 2 internal standards based on the work of Soga et al.[8] were prepared at 1 mg/mL in DDI water and stored at 4 °C prior to use:

Internal standard 1 (IS-1) was 3-Aminopyrrolidine (3-AP) dihydrochloride Sigma 404624-5G

Internal standard 2 (IS-2) was L-Methionine Sulfone (L-MS) Sigma M0876-1G

Mixed internal standards spiking solution preparation

A mixed solution of 0.1 mM IS-1 and 0.02 mM IS-2 was prepared by adding 86 µL of IS-1 (3-AP) and 36 µL of IS-2 (L-MS) internal standards stock solutions to a 10 mL volumetric flask, which was brought to volume with DDI water and stored at 4 °C prior to use. This solution was used to spike bio-fluids as described in the protocols described later in this work.

Amino acids standards stock solution

The Amino Acid Standard H (prod. #20088) from Thermo Fisher Scientific (San Jose, CA) contained 2.5 µmol/mL of each amino acid (except for cystine, which was 1.25 µmol/mL) and was stored at -20 °C prior to use.

The amino acids in the mixture included: L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine·HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine.

Performance standard (PS)

The system performance standard was prepared diluting the amino acids standards stock solution, 1/100, and adding the 2 internal standards (IS-1 and IS-2) at 1 ppm each.

Amino acids quantitative standard

NIST Human Plasma SRM 1950 (NIST, Gaithersburg, MD) was used in the quantitative studies and was stored at -20 °C prior to use.

Bio-fluids: plasma/serum, urine and oral fluids (negative matrix controls)

Bio-fluid synthetic substitutes used in this study included Srine (synthetic urine), OraFlx (synthetic oral fluid) and SeraFlx (synthetic serum/plasma substitutes), obtained from DYNA-TEK, Inc. (Lenexa, KS, www.dtitesting.com) and ordered through Cerilliant Corporation (Round Rock, TX).

Reagents

Distilled and deionized (DDI) water was obtained from a Barnstead Nanopure Infinity ultrapure water system (Thermo Fisher Scientific). Methanol (0.1 N), and 1 Nsodium hydroxide , concentrated ammonium hydroxide, 0.1NHCl and glacial acetic acid were all reagent grade and were obtained from VWR Scientific (Bridgeport, NJ). The background electrolyte (BGE) and conductive liquid was 10% acetic acid in DDI water.

Mass spectrometer instrument

A Waters Xevo TQ mass spectrometer was controlled with MassLynx Software 4.1 and an applied ESI voltage set at 1.3 kV.

Mass spectral data was acquired using multiple reaction monitoring (MRM) mode. See Table 1 for the MRMs of the amino acid and internal standards.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Compound name	Parent	Daughter	Cone(V)	Collision (V)
Glycine (Gly)	76.0	30.0	19	6
3-Aminopyrrolidine (IS-1)	87.1	70.0	25	12
Alanine (Ala)	90.1	44.1	18	10
Serine (Ser)	106.1	60.1	19	9
Proline (Pro)	116.1	70.1	17	13
Valine (Val)	118.1	72.1	20	11
Threonine (Thr)	120.1	74.1	21	10
Isoleucine (Iso) or Leucine (Leu)	132.1	86.1	19	10
Aspartic acid (Asp)	134.0	74.0	21	12
Lysine (Ly)	147.1	84.1	21	15
Glutamic acid (Glu)	148.1	84.0	20	14
Methionine (Met)	150.1	104.1	17	10
Histidine (His)	156.	110.1	21	13
Phenylalanine (Phe)	166.1	120.1	20	14
Arginine (Arg)	175.1	70.1	19	14
Tyrosine (Tyr)	182.1	136.1	21	13
L-methionine sulfone (IS-2)	182.2	136.4	25	11
Cystine (Cys)	214.1	152.0	20	14

Table 1. Performance Standard - MRM Transitions

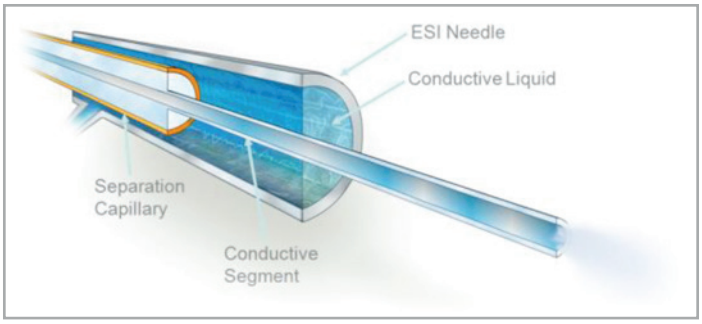

Figure 1. The CESI emitter.

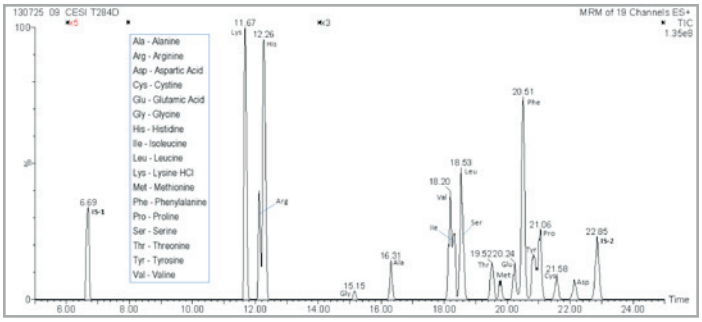

Figure 2. Performance standard, 0.025 µmol/mL.

Capillary electrophoresis instrument

The CESI 8000 Plus High Performance Separation-ESI Module (PN A98089) from SCIEX (Brea, CA) was controlled by 32 Karat Software V10.1. The OptiMS Silica Surface Cartridge (PN B07367) contained a 30 µm ID x 90 cm total length capillary. The cartridge and sample storage temperatures were set at 25 °C and 10 °C, respectively. These settings were used for all methods used in this work, including equilibration, separation and shutdown. See Figure 1 for a diagram of the emitter.

Capillary Conditioning

The OptiMS Silica Surface cartridge was conditioned by rinsing the capillary sequentially with methanol, water, 1 N sodium hydroxide, hydrochloric acid, water and BGE.

Injection

Pressure injection was used in most separation methods in this work. Electrokinetic (voltage) injection can also be used to increase the loading and resolution of the components. For both injection modes, the use of internal standards is necessary to compensate for any bias resulting from differences in the mobilities of the analytes and for changes in injection times at higher versus lower concentrations.

Separation Method

The BGE consisted of 10% aqueous acetic acid. Separation runs of 25 min were performed at 30 kV.

Daily Capillary Conditioning

Prior to daily system performance evaluation or sample analysis, the capillary was conditioned by manually rinsing with BGE for 20 min at 50 psi followed by replenishment of the conductive liquid, which is also BGE, by pressure rinsing at 50 psi for 0.5 min.

Evaluating system performance

An instrument performance standard (PS) was prepared at 0.025 µmol/mL and included 2 internal standards, 3-AP and L-MS at 1 ppm, as described in the internal standards stock solutions section. Separation of the PS components is shown in the TIC of Figure 2.

Shutdown Methods

Daily shutdown consisted of a BGE rinse with the ESI voltage off. Prior to long-term storage, the capillary was rinsed with BGE, water and methanol, followed by air drying using a vacuum.

Capillary Storage

The OptiMS capillary was stored between +18 °C and +26 °C.

Sample Preparation

General

The sample preparation protocols used and described in this work are based on recommended approaches for analysis of subsets of compounds in the human metabolome.[1,2,11] No single approach or analytical technique can cover the breadth of analyte types encountered, resulting in the necessity for compromise and selectivity in the design of experiments in metabolomics studies.

Of great importance in sample preparation is the availability and suitability of negative bio-fluids, including urine, plasma, serum and oral fluid controls. These matrix controls must be evaluated for interfering components in both qualitative and quantitative procedures.

Protocols

Urine Analysis Protocol

1. Filter each sample with a 3kD NanoSep filter by centrifuging at 14,000 rpm (Beckman Microfuge18) for 30 min. Analyze 50 µL of each urine sample, including calibrators, blanks and unknowns,.
2. For calibrators, to 50 µL of Srine, add 50 µL of mixed internal standards solution, add 50 µL of each calibrator solution (substitute water for blanks), add 350 µL of water and vortex.
3. For unknowns, to 50 µL of each urine sample, add 50 µL of the mixed internal standards solution, add 400 µL of water and vortex.
4. Filter each sample with a 3kD NanoSep filter by centrifuging at 14,000 rpm for 30 min.
5. Transfer 150 µL of each calibrator or unknown sample to 200 µL micro vials for CESI-MS analysis.
6. Inject sample using pressure at 2 psi for 5 to 10 s.

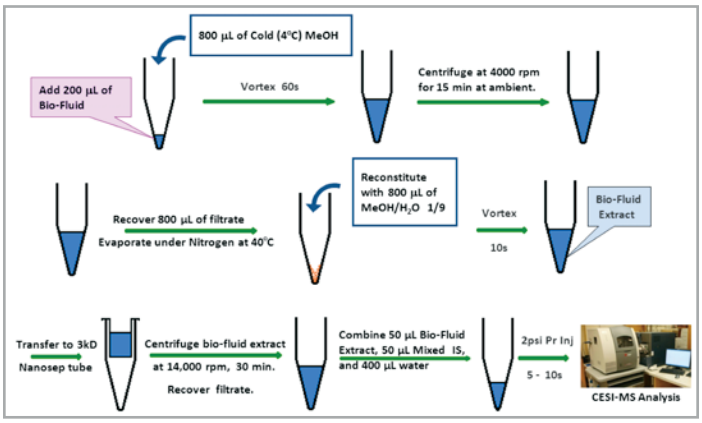

Figure 3. Sample preparation, plasma or oral fluid.

Plasma, Serum and Oral Fluid Analysis Protocol

1. Analyze 200 µL of each unknown bio-fluid specimen or blank synthetic bio-fluid. See Figure 3.
2. Add 800 µL of cold (4 °C) methanol to a 200 µL bio-fluid aliquot in a 1.5 mL centrifuge tube.
3. Vortex for 60 s and centrifuge at 14,000 rpm for 15 min at room temperature.
4. Pipette 800 µL aliquots of each filtrate into 12 x 75 mm culture tubes.
5. Evaporate under nitrogen carefully just to dryness (or in a centrifugal evaporator) at 40 °C.
6. Reconstitute each residue in 800 µL of 1/9 MeOH/Water.
7. Vortex each tube for 10 s.
8. Filter each sample with a 3kD NanoSep filter by centrifuging at 14,000 rpm for 30 min.
9. Prepare calibrators with 50 µL of blank synthetic bio-fluid solution from step 8, 50 µL of mixed IS, 50 µL of the calibrator solution and 350 µL of water (total 500 µL).
10. Prepare unknowns and blanks with 50 µL of unknown or synthetic bio-fluid from step 8, 50 µL of mixed IS and 400 µL of water (total 500 µL).
11. Transfer 150 µL of each calibrator unknown sample to 200 µL micro vials for CESI-MS analysis.
12. Inject samples with pressure injections at 2 psi for 5 or 10 s

Results and Discussion

CESI-MS Performance Standard

A commercially available amino acid mixture (see the experimental standards section) was assessed as a suitable instrument performance standard for CESI-MS. The standard solution covered the expected concentration range for bio-fluid levels (infants to adults, 2,500 to 1 µmol/L).

Migration time reproducibility was assessed using a 1/100 dilution of the amino acid standard mixture and electrokinetic injection, 16 s/10 kV, followed by data collection on CESI-MS. Migration times with %RSD of <0.18 were observed for all amino acids and both internal standards (n = 6).

To further assess the usefulness of the amino acid standard mixture in quantitative analysis, dilutions were prepared and used as spiking solutions for plasma, oral fluid or urine separate calibrations.

Bio-fluids analysis

A number of different bio-fluids are used for metabolomics analysis. These include plasma, oral fluid, serum, urine and tissue samples, such as liver (which require more involved extraction procedures).

Urine is a specimen of choice for untargeted and profiling studies in the metabolomics field. It is readily available in the least intrusive collection means and in sufficient quantity. However, in order to do quantitative analysis of metabolites in urine, it is necessary to use either a standard addition method or a blank matrix urine sample in the calibration protocol.

Correction for variable void volume and electrolyte concentration is also necessary for quantitative comparison.

Plasma, serum and oral fluid all contain proteins that must be removed before CESI-MS analysis. Proteins can adsorb on the inner surface of the capillary and change the capillary characteristics, such as electroosmotic flow and subsequently analyte migration parameters. As described above, the sample preparation protocol requires a deproteinization step, usually by means of cold organic solvent protein precipitation followed by centrifugation and a final filtration step.

After the proteins have been removed, the remaining supernatant can be analyzed by simple dilution (dilute and shoot) techniques. Dilution is typically 1/10 resulting in minimal matrix effects (ion suppression or enhancement).

Quantitative determination of amino acids

Urine analysis

In this work, calibrations were prepared for the amino acids in the performance standard. The 2 internal standards, bracketing compounds of interest, provided increased precision of injection and better reproducibility by using relative migration times coupled with an MRM transition for each targeted compound. Internal standards were also very important in these calibration methods, allowing the use of peak area ratios instead of response factors in regression analysis. Synthetic urine (Surine) was used in the preparation of the calibrators and negative controls. A urine sample from a volunteer was used as an unknown in the protocol.

A number of calibrations were prepared and run as part of method development in order to adjust injection parameters and the amount of internal standards added to the urine samples. Calibrations were linear over 3 or 4 orders of magnitude with r2 values of 0.990 or greater. See Figure 4 for valine as an example calibration in urine.

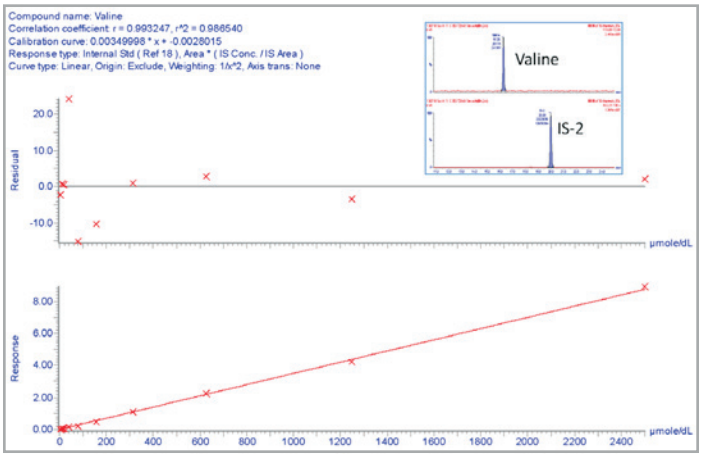

Figure 4. Linear regression analysis, valine (2500 to 5 µmol/L in urine).

Carryover in urine analysis

The protocol was examined for carryover by running blank urine samples following the highest concentration standard, 2500 µmol/L.

No significant carryover was detected in blank urine extracts. In the preserved Serine negative control, 2 artifacts were present. The artifact peaks differed in migration times from those of the expected amino acids based on their MRM transitions.

Plasma or Serum Analysis

Plasma is the bio-fluid commonly selected for quantitative analysis. It is more consistent in component concentration than oral fluid or urine but has the most intrusive sample collection protocol. Plasma and serum both require removal of proteins prior to dilution and analysis. The extraction protocol of Figure 3 again provides clean extracts that are then diluted 1/10

(50 µL to 0.5 mL). A dilution factor of 5 times must also be applied in the final plasma calculations to correct for 200 µL of unknowns analyzed. Synthetic plasma/serum (SeraFlx) was used in the preparation of the calibrators and negative controls. A traceable quantitative human plasma sample (NIST SRM 1950) was used in this evaluation.

Calibrations were linear over 4 orders of magnitude with r2 values of 0.990 or greater. See Figure 5 for proline as an example calibration in plasma.

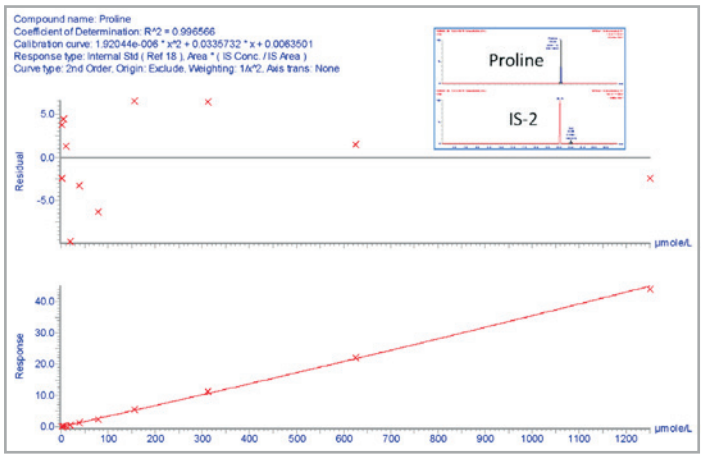

Figure 5. Linear regression analysis, proline (1250 to 1 µmole/L in plasma).

LOD/LOQ Determination

The limit of detection and quantification was 1.2 µmol/L (the concentration of the lowest calibrator). Peak-to-peak (PtP) signal to noise was calculated from the low calibrator and is shown in Figure 6 for arginine, LOD SN >3 and LOQ SN >10.

NIST Human Plasma Standard Reference Material (SRM)

Samples of NIST Standard Reference Material (SRM) 1950 were analyzed using the plasma protocol (Figure 3). Table 2 shows the results for the NIST standard determined experimentally compared to the documentation for the SRM. Most of the experimental values were within accepted tolerance limits. It should be noted that the NIST reference values were determined using techniques other than CESI-MS and were averaged results from multiple technologies (e.g., LC/MS, GC/MS, NMR).

Amino acid	NIST	±	Expt.
Alanine (Ala)	300	26	314.5
Arginine (Arg)	81.4	26	70.7
Aspartic acid (Asp)	N/A		N/A
Cystine (Cys)	7.8	0.4	11.9
Glutamic acid (Glu)	67	18	N/A
Glycine (Gly)	245	16	277.6
Histidine (His)	72.6	3.6	63.5
Isoleucine (Iso)	55.5	3.4	68.9
Leucine (Leu)	100.4	6.3	115.6
Lysine HCl (Ly)	140	14	113.5
Methionine (Met)	22.3	1.8	33.6
Phenylalanine (Phe)	51	7	47.1
Proline (Pro)	177	9	161.9
Serine (Ser)	95.9	4.3	91.5
Threonine (Thr)	119.5	6.1	122.5
Tyrosine (Tyr)	57.3	3	59.7
Valine (Val)	182.2	10.4	201.1

Table 2. NIST concentrations versus experimental data.

Conclusions

1. CESI-MS, the integration of CE and ESI into a single dynamic process and interfaced to mass spectrometry, provides the resolution and sensitivity required to analyze the smallest of polar compounds encountered in the many research fields of metabolomics.
2. This important advancement in separation and ionization technology permits replicate quantitative analysis on less than 100 µL of bio-fluid and detection of many challenging metabolites at diagnostic levels in small samples from infants. In fact, the protocols described in this work can be scaled down to bio-fluid sample requirements of 10 µL or even less.
3. The small sample volumes injected, less than 5 nL, create a significant advantage to metabolomics researchers, who often work with bio-fluids obtained by the most intrusive of collection methods. As a result, the small specimen volumes needed will minimize the effect of those intrusive methods and require smaller specimens overall.
4. The bio-fluid sample preparation protocols described in this work resulted in LOD/LOQ values for representative low molecular weight analytes, amino acids of less than 408 amol. Linear regression analysis over 4 orders of magnitude (2.5 to 2500 µmol/L) resulted in r2 values of 0.990 or greater.

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