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Validation of a dual LC-HRMS platform for clinical metabolic diagnosis in serum, bridging quantitative analysis and untargeted metabolomics

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Abstract

Mass spectrometry-based metabolomics is a rapidly growing field in both research and diagnosis. Generally, the methodologies and types of instruments used for clinical and other absolute quantification experiments are different from those used for biomarkers discovery and untargeted analysis, as the former requires optimal sensitivity and dynamic range, while the latter requires high resolution and high mass accuracy. We used a Q-TOF mass spectrometer with two different types of pentafluorophenyl (PFP) stationary phases, employing both positive and negative ionization, to develop and validate a hybrid quantification and discovery platform using LC-HRMS. This dual-PFP LC-MS platform quantifies over 50 clinically relevant metabolites in serum (using both MS and MS/MS acquisitions) while simultaneously collecting high resolution and high mass accuracy full scans to monitor all other co-eluting non-targeted analytes. We demonstrate that the linearity, accuracy, and precision results for the quantification of a number of metabolites, including amino acids, organic acids, acylcarnitines and purines/pyrimidines, meets or exceeds normal bioanalytical standards over their respective physiological ranges. The chromatography resolved highly polar as well as hydrophobic analytes under reverse-phase conditions, enabling analysis of a wide range of chemicals, necessary for untargeted metabolomics experiments. Though previous LC-HRMS methods have demonstrated quantification capabilities for various drug and small molecule compounds, the present study provides an HRMS quant/qual platform tailored to metabolic disease; and covers a multitude of different metabolites including compounds normally quantified by a combination of separate instrumentation.

Keywords

untargeted metabolomics; targeted metabolomics; bioanalytical validation; mass spectrometry; Q-TOF; LC-HRMS; comprehensive metabolite profiling

Introduction

Untargeted metabolomics has become a popular tool in metabolite biomarker discovery and in the evaluation of metabolic pathway changes associated with disease (Dunn et al. 2011;

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Kell 2007; Mamas et al. 2011). Such methods are effective in identifying metabolites that have statistically significant changes among sample cohorts. Recent instrument and chromatographic advances have allowed investigators to measure hundreds to thousands of unique metabolites that are representative of diverse chemical classes, all within one or several runs (Crews et al. 2009; Yanes et al. 2011).

A disadvantage of pure untargeted metabolomic studies is that metabolites are only quantified relative to one another and an actual estimate of concentration often cannot be determined, an aspect that makes the technique better suited for research than actual clinical diagnosis. Certain metabolites have wide reference ranges, and a 5-fold change in relative intensity for example, may in fact have no phenotypic consequence, whereas the same relative change in a metabolite that is tightly regulated may be very clinically relevant. On the other hand, targeted metabolomics methods enable absolute quantification of metabolites of interest using stable isotope dilution, and quality control analysis. In the realm of clinical testing, different regulatory bodies (CAP, CLIA, GLP) require standard procedures for assurance of specificity, precision, accuracy, linearity, sensitivity, recovery and stability in the presence of potentially interfering compounds, and verification of calibration with at least a minimum or zero value, a low, middle, and a high value near the upper limit of the analytical range (Bansal et al. 2007; FDA 2001). Unfortunately, triple-quadrupole mass spectrometers, best suited for accurate quantification, lack the resolution and mass accuracy necessary to identify the other thousands of co-extracted analytes.

In LC-MS/MS targeted platforms, the compounds are generally quantified by selected reaction monitoring analysis (SRM), focusing just on the transition of the precursor to a product ion of a particular analyte (Honour 2011). SRM experiments reduce the background signal of unspecified compounds that have similar mass and elution times, and increase the duty cycle of the instrument, resulting in greater sensitivity and a linear dynamic range of quantification. A new generation of Q-TOF instruments combine the sensitivity and dynamic range of triple-quadrupole instruments with time of flight technology, allowing for high resolution and mass accuracy (Fung et al. 2011). We have used such an instrument to develop assay methods which allow for absolute quantification of a range of metabolites present in serum and plasma, while simultaneously collecting high resolution, accurate mass data for comprehensive untargeted metabolomics, as shown in the workflow in Figure 1. Several LC-HRMS have been recently published that have successfully quantified different compounds using related mass spec techniques, but the previous publications have focused on drug compounds or peptides, which are known to behave well in standard reverse phase conditions using C18 columns (Dillen et al. 2012; Henry et al. 2012; Kaufmann et al. 2011; Zhang et al. 2009). We have developed and validated methods that enable quantification of small polar compounds such as amino acids and organic acids in addition to the more hydrophobic chemical classes, enabling a hybrid quantification/discovery platform with more comprehensive capabilities and a clinically oriented focus.

Typically, clinical biochemical genetic tests include separate analyses of organic acids (Duez et al. 1996; Hoffmann et al. 1989; Kumps et al. 1999; Sweetman 1991), amino acids (Shapira 1989), and acylcarnitines (Millington et al. 2011; Vreken et al. 1999), using various instruments, but are measured here with a single system. For our purposes, we selected 55

compounds that are measured in the diagnosis of metabolic dysfunction; commonly associated with, but not limited to inherited metabolic disorders (Chace et al. 2005; Wilcken et al. 2008). These include all 20 proteinogenic amino acids, a selection of non-proteinogenic amino acids, organic acids, purines, pyrimidines, and acylcarnitines (Table 1).

Methods and Materials

Strategy

The concentration ranges used for the analytical validation of standards were selected to span normal reference ranges previously reported in plasma, but also encompassed some of the reported abnormal range seen in related diseases (Blau 2003; Hoffmann et al. 1993; Psychogios et al. 2011). These ranges extend from one to over two orders of magnitude in concentration, depending on the metabolite (Table 1). To further verify these ranges, we have processed 15 human serum samples and analyzed them along with the validation experiments. The 15 patients included 3 cohorts, representing (respectively) controls (4 patients), hepatocellular carcinoma (HCC) patients (5 patients), and patients with cirrhosis (6 patients). Though many of the compounds measured are key metabolites in inherited metabolic disorders (i.e. MCADD, MSUD, PKU, Tyrosinemia, etc.), it was not necessary to include such samples in the validation. The respective pathological concentrations are more readily detectable than the minor deviations seen in patients without Mendelian metabolic disease, and the analytical ranges we present here demonstrate that we can distinguish a diseased range for those various key metabolites. We instead confirm that our method enables quantification of these metabolites in normal and non-inherited metabolic disease samples, while demonstrating the versatility of the untargeted aspects of the platform. Previous untargeted studies of HCC and liver disease with a much larger sampling provided a good comparison for evaluating this platform, as these studies reported changes in potential biomarkers spanning a wide range of the physiochemical spectrum, including amino acids, organic acids, acylcarnitines, and a variety of bile and fatty acids (Chen et al. 2011; Zhou et al. 2012).

Sample preparation

We used a final concentration of cold 80% methanol for all sample extractions (Bruce et al. 2008). For calibration curve and quality control materials, 90 μ L of analyte-stripped serum (Biocell, Rancho Dominguez, CA), was supplemented with 10 μ L of a non-labeled standards mixture, followed by 400 μ L of methanol containing 10 μ L of a stable isotope standard mixture. Sera from patients were collected as part of a separate study approved by the UCSD IRB. Those serum samples were collected within 12 months of each other and processed within 4 hours of collection, as reported previously (Cooper et al. 2001), followed by aliquoting and immediate storage in a -80°C freezer. Samples were extracted with cold methanol as described above within several months after all sera were collected.

Stable isotopes used as internal standards were purchased from CDN Isotopes (Quebec, Canada) and Cambridge Isotopes Laboratory (Andover, MA). Several of the isotopes are ^{13}C and ^{15}N labeled. Although the majority are deuterated, all are labeled at carbon atoms positions that are not readily exchangeable with protons in solution. The specific

atoms that are labeled for each compound are noted in a table in the supplementary methods section. Two separate stocks of isotopic standard mixes were formulated based on chemical compatibility. These were dissolved in either 0.02N HCl (final concentration) or methanol. Separate concentrations of calibrator mixes were formulated to satisfy the unique physiological concentration range of each metabolite (Suppl. Methods). A calibration curve (blank, zero, and 8 concentration levels) and four different QC levels were evaluated. On each day of validation, each QC level was extracted in triplicate and run together with a calibration curve. All standard curves had coefficient of correlation values greater than 0.99, most of which contained all 8 calibrator points (Suppl. Data). To satisfy linearity, however, several compounds had either 1 or 2 outliers removed.

HPLC and MS methodologies

All quantification was performed with two different types of pentafluorophenyl columns (C18-PFP; 2.1mm × 150mm, 3μm particle, by Mac-Mode Analytical [Chadds Ford, PA], and Epic-PFP; 2.1mm × 150mm, 3μm particle, by ES Industries [West Berlin, NJ]) (Godat et al. 2010; Yang et al. 2010). Material from a single extraction was used for both column types. Amino acids and most of the acylcarnitines were quantified in positive mode on the Epic-PFP, while organic acids were quantified in negative ionization mode on the C18-PFP. The Epic-PFP provided better results for polar compounds in positive ion mode (data not shown). Eight compounds exhibited better performance with the C18-PFP in positive ionization mode (tryptophan, creatine, creatinine, cytidine, uridine, uracil, as well as isovalerylcarnitine and palmitoylcarnitine). As described for each compound table 1, three runs were used in total to quantify all 54 compounds, though most of these could be quantified with just two: positive mode on the EPIC-PFP and negative mode on the C18-PFP. Both columns were kept at 30° C for all runs. The flow rate was 300 μL/min (Shimadzu Prominence UFLC) for all runs, using similar reverse phase gradients (ACN/ 0.1%FA) for both columns, and optimized for comprehensive metabolite profiling.

All mass spec analysis was performed on an AB-Sciex model 5600 Triple-TOF using the Turbo ESI source. Instrument parameters are described in the supplementary methods. Nearly 70% of the samples were quantified in TOF-MS mode (Table 1). This was enabled by sufficient chromatographic selectivity of these compounds as well the ability to extract for narrow mass ranges during peak integration, enabled by the high resolution (>30,000) of the Q-TOF instrument. In all runs, each scan included a 250 millisecond TOF-MS scan followed by 50-100 millisecond individual product ion scans for specific compounds analyzed by MS/MS. Scan times were adjusted so that the total cycle was less than 1 second. This cycle time enabled at least 8-10 scans for each compound, based on their elution profiles. Several of the compounds were quantified in both MS and MS/MS mode for comparison (Table 2). For MS/MS identification of potential biomarkers, we combined the 15 patients' sera into 3 separate pools and analyzed these on the Q-TOF with a method that maximizes for data dependent acquisitions, acquiring MS/MS scans on up to 12 non-redundant precursor ions within a 1 second total scan cycle. All compounds were quantified using MultiQuant software (AB Sciex), while MarkerView software (AB Sciex) was used for untargeted analysis. Background subtraction, peak identification, and alignment were all

performed in MarkerView, as well as t-test and principal component analysis for the identification of statistically significant variations between patient cohorts.

Comparisons to standard clinical methods

Five of the patients' sera were also analyzed by standard clinical methods. The majority of the compounds measured in the above Q-TOF methods were also measured using GC-MS analysis for TMS-derivatives of organic acids (Agilent 5973), Amino acid analyzer for amino acids (Biochrom 30), and triple-quadrupole LC-MS/MS for butyryl-esters of acylcarnitines (API 2000). These methods were performed as previously described (Duez et al. 1996; Hoffmann et al. 1989; Kumps et al. 1999; Millington et al. 2011; Shapira 1989; Sweetman 1991).

Results

HPLC column performance

Chromatographic runs showed good repeatability in both intra-day and inter-day assays as shown in Figure 2. Figure 2 also demonstrates the versatility in retention of organic acids and amino acids, while maintaining efficient retention and peak shape of more hydrophobic compounds such as octanoic acid, as well as octanoyl- and palmitoylcarnitine. The ability of both PFP columns to separate isomers including 2- and 3-hydroxybutyrate, isoleucine and leucine, as well as 2-methylbutyryl-, isovaleryl- and valerylcarnitine, is shown in Figure 2B-G. Isobutyryl- and butyrylcarnitine were separated on the C18-PFP but not the Epic-PFP (data not shown). Nearly all the compounds maintained relatively sharp Gaussian profiles, though a few showed poorer peak shape. For example, arginine and lysine (Figure 2E) start to elute shortly at 1 minute in a broad peak that appears non-Gaussian, due in part to ionic suppression occurring near the void volume of the chromatographic runs (0.9min). Nevertheless, using stable isotopes of these compounds experiencing the same elution profiles and suppression effects, the quantification statistics were sufficient, as seen in Table 1. Most of the smaller organic acids eluted between 1.5 minutes and 4 minutes while the larger ones eluted later in the gradient. Nearly all of the amino acids also eluted within the first several (Figure 2). The unique feature of the PFP columns is that polar compounds such as organic and amino acids can be analyzed at low physiological levels under reverse phase conditions, while still showing sharp elution profiles of hydrophobic compounds. Figure 2H-I shows the chromatographic extraction from a serum sample run of 6 different hydrophobic compounds (2 bile acids and 4 long chain fatty acids) previously identified as potential biomarkers in a previous metabolomics study of HCC (Zhou et al. 2012). Though the changes in their abundances were not shown to be significant among our small cohorts, we show these traces to demonstrate the comprehensive capabilities of the Epic-PFP and C18-PFP columns in measuring both polar and non-polar/hydrophobic metabolites.

Quantification and bioanalytical validation

Table 1 demonstrates the high accuracy and precision results obtained in the experiments. The results for 54 of the 55 compounds tested are shown, excluding glycine, which could not be detected in the lower half of the standard curve samples. 34 of the remaining 54 compounds satisfied the following standard criteria (Bansal et al. 2007; FDA 2001): (a)

back-calculated accuracy deviations of 20% (80%-120%) for each day's replicate measurements of LOQ QC samples and a maximum of a 20% inter-day coefficient of variation (%CV), (b) a maximum of 15% accuracy deviation (85%-115%) of intra-day replicates of low, middle, and high QC samples, and a maximum %CV of 15. Four of the compounds included in this list of 34 only missed this criterion by 2 percentage points for just one of the QC level measurements and were therefore included with the other 30. Many of the compounds that met these requirements included amino and organic acids, several acylcarnitines, and several purines/pyrimidines. An additional 5 compounds including lysine, hypoxanthine, citrulline, propionyl- and octanoylcarnitine missed the criteria for the LOQ by several percentage points. Another 15 compounds had accuracy and CV deviations that did not satisfy the requirements for LOQ (indicated in Table 1), but did however meet the guidelines for the other three levels of quality control replicates, enabling their validation for an adjusted range still sufficient for quantification in human samples, as seen in the reported values found in the patient samples in table 3. In those 15 serum samples, only propionylcarnitine and octanoic acid were seen at concentrations lower than the low QC, while all the other measurements fell within the adjusted range for the compounds listed with asterisks in Table 1. Six compounds (uridine, cytidine, thymine, suberic acid, azelaic acid, and octanoic acid) did not have measurable values in the patient samples. For these compounds, nearly all measurements in the patient serums fell below the lower limit of quantification, and therefore their presence will likely only be quantified in patient samples with abnormal levels. Two compounds, citrate and creatinine, had very linear standard curves and had very good signal in the measurements, but had poor accuracy and precision results at the lower levels due to high residual endogenous concentrations of these compounds in the analyte-stripped serum, causing background levels which obscure measurements at the lower analytical ranges. For future quantification of creatinine and citrate in this platform, standard curves and QC's will be done by varying the isotopic standards of these compounds, while keeping the endogenous form constant. The results show that most of the compounds are accurately and reproducibly quantified in MS mode, though some of the compounds required MS/MS based quantification, due either to overlap with co-eluting analytes including isobaric compounds, or just due to increased sensitivity obtained in product ion scanning mode (Table 2). For example, the isotopic standard for taurine had nearly the identical mass as 5-oxoproline, necessitating specific product scans and quantitation on compound specific product ions. Similarly, an isobaric compound with a similar elution time and precursor mass as valine necessitated product ion quantification.

We used stable isotopes of each respective compound for quantification, except for a few cases. Asparagine was quantified with a glutamine standard and threonine was quantified with the stable isotope of methionine, as their respective isotopes were not available to us at the time of the study. Nonetheless the accuracy and precision results show these are adequate substitutes. Additionally, hypoxanthine was quantified using deuterated tyrosine. A hypoxanthine isotope was included but surprisingly eluted several minutes from the non-labeled standard (Figure 2), which we verified by MS/MS. This could be due to the tautomerization of hypoxanthine (Hernandez et al. 1996).

Comparisons to existing clinical methodologies (Cross-validation)

We have done preliminary comparisons between our current methodology with standard clinical methodologies currently used for amino acid, organic acid, and acylcarnitine analysis, as described in the methods. Five patients' sera samples were used for the comparison of many of the metabolites verified in the Q-TOF methods (Suppl. Table 1). Most of the amino acids and acylcarnitines showed accuracy measurements between 75 and 125% with standard deviations below 20%, while some of the organic acids showed greater discrepancy. This is not surprising considering that the GC-MS method did not use stable isotopes, and several of the organic acids were too low in concentration to be accurately reported by the older method. The preliminary comparisons showed that the Q-TOF methods exhibited increased sensitivity for a number of compounds, especially organic acids. Another advantage of the Q-TOF platform was the ability to better resolve isomers such as isovaleryl-, 2-methylbutyryl- and valerylcarnitine, which co-elute in the original method (Forni et al. 2010).

Patient Samples and Untargeted Analysis

We used untargeted methods to process the control, HCC, and cirrhotic samples from the same runs from which the quantitative data was extracted. Using marker view software, peaks were aligned and integrated followed by both multivariate and ANOVA statistical processing. From t-test analysis of all peak groups, we identified over 30 unique analytes of interest. One such peak was identified by MS/MS database matching (Tautenhahn et al. 2012) as inosine (box plot in Figure 1D), a compound determined in the previous HCC metabolomics study as being the most differentiated biomarker (Chen et al. 2011). Biological conclusions cannot be formed from this data though, due to the small sample number of each cohort, intended primarily for analytical validation.

Discussion

Using the high resolution and mass accuracy of a new Q-TOF instrument, we demonstrate that quantification of clinically relevant metabolites can be done in MS mode instead of MS/MS mode in many cases, allowing each cycle to collect data on all ionized precursor ions, which could later be evaluated by untargeted analysis. Quantifying in MS mode has been considered a challenge because of background ions from the matrix as well as lower signal intensity. We have been able to eliminate the background for most of the targeted analytes by employing recent advances in chromatographic stationary phases, specifically PFP-based columns. For several compounds, including taurine, ornithine, 5-oxoproline acid and valine, background signals from the serum matrix and/or spiked compounds obscured signals in MS mode, necessitating specific product scans (MS/MS) for accurate quantification. In other cases, as demonstrated in Table 2, product ion quantification improved accuracy and precision results, specifically at the lower levels of concentration. The fast scanning rate of the instrument (up to 100Hz) enabled us to collect short MS/MS scans within the same scanning cycle as the precursor ion data collection (TOF-MS scan). Triple-quadrupole instruments, which lack a time-of-flight tube but include an additional quadrupole for daughter ion selection, generally allow for even faster scanning with less ion loss, and could therefore quantify a greater number of compounds by MS/MS. However, a

triple-quadrupole instrument lacks the ability to profile co-eluting ions at high resolution and mass accuracy. The results show that the majority of analytes quantified on the Q-TOF, including most of the amino acids, many organic acids, acylcarnitines and purines/pyrimidines, met or exceeded accuracy and precision criteria common to analytical validation of triple-quadrupole methods. Though Q-TOF instruments were previously thought to lack the dynamic range for quantification of such compounds at physiological ranges, improvements in this new generation Q-TOF instrument, in conjunction with the aforementioned columns, allowed this goal to be achieved. The sensitivity levels also met or exceeded those measured by standard clinical methods routinely used for measuring many of these compounds. Organic acids were quantifiable at concentrations as low as 0.3 μ M (malic) and 5nM for several acylcarnitines. We used fewer replicates per day in the current validation than prescribed in other validation protocols, which often use 6 replicates to test intra-day characteristics (Honour 2011). This was due to length of our runs (40min. each), which limited the number of QC replicates we could analyze so that each batch did not greatly exceed 1 full day of collection, potentially compromising post-preparative stability. Further validation experiments will be performed to further determine long-term reproducibility and ruggedness of the method.

Though most of the compounds that were quantified were polar in nature, we have shown by extracting signals for a variety of fatty acids, bile acids, and other lipid forms from the patient serum samples, that this new Q-TOF/LC-MS strategy is effective in analyzing many classes of non-polar compounds with untargeted analysis, some of which we plan to quantitate in future iterations of this platform. Arguably, polar metabolite profiling (especially of organic acids) has been under-represented in traditional reverse-phase LC-MS methods. The PFP-columns have significantly advanced the ability to quantitate polar metabolites relevant to clinical applications, without the use of ion-pairing agents (which limit analysis to a single ionization mode) or HILIC columns, which may compromise analysis of hydrophobic compounds (Buescher et al. 2010; Ikegami et al. 2008).

A hybrid targeted/untargeted method held to the conventional rigorous validation standards used with triple-quadrupole methodology, permits for comprehensive metabolic diagnosis, without sacrificing all of the other information potentially useful for future untargeted studies. Specifically, such a platform would be effective as both a clinical and research tool for the diagnosis and follow-up of Mendelian metabolic disorders (i.e. where pathognomonic compounds are elevated, such as tyrosine in tyrosinemia, phenylalanine in PKU, and branched chain amino acids in MSUD), additionally enabling discovery of unforeseen metabolic changes, or the effects of drug therapies and consequent drug metabolism. These methods can also be used in a more traditional research approach for the study of non-Mendelian disease states such as liver disease and cancer, where known changes in major metabolic pathways (i.e. amino acid metabolism and cellular/energy pathways (Dejong et al. 2007; Moreno-Sanchez et al. 2007)) can be quantified, while still enabling the search for potential biomarkers. The current methods help to reduce the need for untargeted sample processing to occur within very short time frames, usually done to limit instrument drift effects. Having many internal standards and absolute quantification results of different classes of metabolites can improve the normalization of samples processed that are run many weeks or months apart, something currently presented as a

challenge in untargeted studies. Also, collecting full scan, accurate mass data with good chemometric properties allows one to revisit the original dataset if a new, relevant metabolite is later identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CLIA	Clinical laboratory improvement act
CAP	College for American pathologists
GLP	Good laboratory practices
HCC	Hepatocellular carcinoma
Cirr	cirrhotic patient
ND	not determined
TOF	Time of flight
LOQ	(lower) limit of quantification
QC	quality control
CV	coefficient of variation
HRMS	high resolution mass spectrometry
MSUD	Maple syrup urine disease
MCADD	Medium chain acyl-CoA dehydrogenase deficiency
PKU	Phenylketonuria

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Figure 1. Targeted/Untargeted Workflow

The figure describes the different analyses that can be done from a Q-TOF run. (A) Total intensity scan of a single MS run. Each run includes multiple experiments within each 0.9 second scan cycle, including a 0.25sec TOF-MS scan (B) from which specific masses can be extracted using narrow m/z windows. Statistical analyses such as PCA plots (C) and T-tests help distinguish cohorts and implicate ions of interest. (D) A box plot of one such analyte from the current study, identified as inosine by MS/MS data (>5 -fold difference between disease [HCC/cirrhotic] and control groups from our t-test analysis with p -values <0.05). From the same TOF-MS scan (50-1000 m/z) used in untargeted processing, narrow m/z ranges can be extracted for absolute quantification of labeled and non-labeled standards (E), using standard curve (shown on right) and QC analysis. Within the same cycle, short MS/MS scans of specific masses (F) permit quantification of product ions, shown here for lysine.

Figure 2. Resolution of quantified analytes on the PFP columns

(A) 6 overlaid QC traces are shown from 3 different days on the C18-pfp in negative mode. (B+C) Organic acids resolved on C18-pfp. Note the resolution between isomers 2- and 3-hydroxybutyric acid. (D) 6 overlaid QC traces from 3 different days on Epic-PFP in positive mode. (E) Polar and neutral amino acids resolved on Epic-PFP. (F) A Trace of non-polar amino acids as well as (iso)butyrylcarnitine and hypoxanthine (tautomer described in text) are shown resolved on the Epic-PFP. Note resolution between leucine and isoleucine. (G) Resolution of isovaleryl carnitine from 2-methylbutyryl- and valeryl-carnitine standards are shown. Only isovaleryl and 2-methylbutyryl carnitines were significantly present in the patient sera. (H-I) Peak extractions of various fatty acids and bile acids are shown, compounds that were reported in previous studies as potential biomarkers of HCC.

Table 1
Accuracy and Precision results of Clinical Validation

Metabolite	Range (uM)	Stable Iso-tope	Actual conc. of QC's in μM LOQ, low, medium, and high	Accuracy LOQ QC: <i>Inter-day avg. (Intra-day averages)</i>	Accuracy Low QC: <i>Inter-day avg. (Intra-day averages)</i>	Accuracy Medium QC: <i>Inter-day avg. (Intra-day averages)</i>	Accuracy High QC: <i>Inter-day avg. (Intra-day averages)</i>	Inter-day %CV: QC's LOQ, low, medium, high
amino acids (proteinogenic)								
alanine ^A	70-700	d4	70.170.313, 578	102(117.89.100)	83(86.81.82)	94 (94.96.93)	104(95.103,112)	17, 9, 8, 11
leucine ^A	30-250	d3	30.70.113.206	104(107.105.99)	109(107.114.107)	102(101.101.103)	101(104.101.97)	5, 4, 3, 5
isoleucine ^A	10-125	d10	10.25, 56,103	93 (91.96.91)	101(101.103.99)	101(105.98.98)	99(105.95.96)	3, 3, 4, 6
valine ^{#A}	40-350	d8	40.95.158.289	96(108.92.89)	99(101.97.93)	97(102.96.94)	98(99.97.98)	10, 6, 5, 5
proline ^A	40-500	d7	40, 100.215.413	91 (91.93.89)	102(101.105.101)	100(100.102.98)	98(104.94.95)	3, 3, 3, 5
phenylalanine ^A	20-200	d5	20.50.90.165	99(100.98.100)	111(108.115.109)	106(104.107.106)	96 (99.96.93)	3, 4, 3, 6
tryptophan ^B	1-120	d5	1, 6, 45, 99	ND	104(122.98.91)	103(102.103.103)	95(94.97.96)	14, 2, 3
tyrosine ^A	20-150	d4	20, 35, 68, 124	100(100.99.99)	96(95.98.95)	101 (100.103.101)	98(101.95.98)	1, 3, 2, 3
cystine ^A	30-200	d4	30.50.90,165	115(116,114,113)	99(96.100.100)	100(106.93.101)	107(110.101.112)	4, 8, 12, 10
methionine ^A	5-50	1- ¹³ C, d3	5, 13, 23, 41	100(103.100.96)	103(101.107.102)	100(102.99.100)	100(101.98.100)	4, 3, 2, 2
threonine ^A	25-300	1- ¹³ C, d3met	25, 75, 135, 248	93 (84.95.101)	101(110.97.97)	96(89.103.96)	89(88.87.91)	18, 15, 8, 4
serine ^{#A}	30-300	d3	30, 75, 135, 248	109(116,117.93)	103(95.120.95)	96(98.95.94)	103(104.101.105)	8, 20, 8, 8
asparagine ^{#A}	20-150	d5 gln	20, 38, 68, 124	109(111.118.99)	100(93.96.111)	104(106.107.98)	97(97.100.94)	12, 9, 7, 6
glutamine ^A	100-800	d5	100, 188, 350, 660	99(102.103.92)	98(92.102.99)	96(101.96.90)	100(102.102.96)	8, 8, 7, 6
aspartate ^{#A}	0.6-50	d3	0.6, 2.1, 18, 42	ND	100(111.99.91)	98(99.98.98)	106(99.112.106)	19, 9, 9
glutamate ^A	0.5-150	d3	0.5, 3, 53, 124	ND	89(83.81.105)	92(85.95.94)	97(98.96.97)	33, 13, 8
lysine ^{#A}	21-300	d4	21, 59, 130, 248	127*(120,142,120)	98(93.96.105)	91 (90.88.95)	98(106.90.98)	13, 8, 4, 7
histidine ^{#A}	40-250	d3	40, 70, 113, 206	100(113.100.87)	96(93.96.98)	95(102.87.96)	101(108.97.99)	7, 3, 7, 7
arginine ^A	20-150	d4	10, 38, 68, 124	96(100.97.90)	98 (97.103.93)	99(100.96.101)	101 (108.99.95)	5, 5, 3, 7
non-proteinogenic amino acids/other								
ornithine ^{#A}	10-200	d2	10, 35, 88, 165	99(103.94.99)	95(96.87.102)	100(103.98.97)	97(93.99,100)	14, 9, 11, 6
homocystine ^A	5-75	d8	5, 15, 34, 62	99(98.110.89)	97(102.104.86)	96(95.96.96)	98(95, 100, 98)	13, 12, 7, 4
creatine ^B	5-150	d3	5, 18, 63, 124	93 (97.91.92)	99(103.98.97)	95(96.91.99)	93 (97.90.93)	5, 3, 3, 3
creatinine ^B	2-100	d3	0.5, 1.5, 20, 41	N/A	114*(172.96.74)	103(108.92.108)	94(100.90.92)	53, 10, 8
pipecolic ^B	1-30	d9	1, 3.5, 13, 25	87(91.88.81)	98(103.97.94)	99(101.96.101)	96(101.94.95)	6, 5, 3, 3

Metabolite	Range (uM)	Stable Iso- tope	Actual conc. of QC's in μM LOQ, low, medium, and high	Accuracy LOQ QC: Inter-day avg. (Intra-day averages)	Accuracy Low QC: Inter-day avg. (Intra-day averages)	Accuracy Medium QC: Inter-day avg. (Intra-day averages)	Accuracy High QC: Inter-day avg. (Intra-day averages)	Inter-day %CV: QC's LOQ, low, medium, high
5-oxoproline ^{#C}	10-120	d5	10, 25, 53, 99	110(111.119,102)	103(112.99,97)	101 (113.95,96)	99(106.96,95)	10. 8. 9. 6
taurine ^{#C}	10-100	d4	10, 25, 45, 83	99(88.112,97)	95 (98.95,92)	94 (98.94,91)	102(110.99,97)	11, 4. 6. 9
citrulline ^A	2-100	d2	2, 7, 40, 83	101 (93.112,96)	100(111,95,93)	97(115.76,101)	99(110,95,93)	26, 15, 18, 9
purines/pyrimidines								
hypoxanthine ^A	0.5-50	d4tyr	0.5, 3, 20, 41	79(76,74,871)	113(109.116,115)	103(112,94,103)	102(113.94,99)	6, 7, 8. 10
uracil ^{#B}	0.3-3	2- ¹⁵ N	0.3, 0.7, 1.3, 2.5	93(106.98,90)	83(85.73,91)	93(89.90,101)	88(84.80,100)	19, 18, 13, 11
thymine ^{#A}	0.05-15	d4	0.05, 0.3, 5.3, 12.4	98(129.88,73)	94(98.101,83)	97(104.92,94)	95(99.95,89)	31. 11. 6. 7
cytidine ^{#B}	0.05-15	d2	0.05, 0.3, 5.3, 12.4	92(81,100,95)	96(95.97,96)	97(96.92,103)	95(98.89,96)	12, 4. 7. 5
uridine ^{#B}	0.05-15	d2	0.05, 0.3, 5.3, 12.4	ND	97(117,97,77)	103(101.100,109)	96(98,90,100)	18, 6, 6
orotic acid ^{#C}	0.2-7	2- ¹⁵ N	0.2, 0.7, 2.8, 5.8	100(99,112,90)	94(100,94,89)	101 (102,100,100)	100(97,100,103)	12, 7, 3. 6
guanosine ^A	0.1-3	d5	0.1, 0.35, 1.25, 2.48	91 (83,77,113)	85(92,74,90)	90(92,89,88)	96(105.77,107)	4. 12, 7, 17
acylcarnitines								
carnitine ^A	2-125	d3	2.9, 46, 103	79(82. 78,77)	116(115.116,117)	104(105.103,104)	101 (105.100,99)	6. 5. 2. 4
acetyl-carnitine ^A	0.5-50	d3	0.5, 3, 18, 41	ND	118(116.118,121)	110(110,108,111)	100(100,101,99)	3, 3, 4
propionyl-carnitine ^A	0, 05-5	d3	0.05, 0.3, 1.8, 4.1	130 [*] (127,159,106)	89(92.92,85)	85(86.88,81)	96(96.99,93)	23, 4. 4. 4
isobutyryl-carnitine ^A	0.005-0.5	d7	0.005, 0.03, 0.18, 0.41	111 (120.104,108)	91 (91,93,88)	87(89.94,79)	94(93.99,90)	10. 9. 8, 11
isovaleryl-carnitine ^B	0, 005-0.5	d3	0.005, 0.03, 0.18, 0.41	ND	102(105.102,100)	105(106.104,106)	98(100.100,95)	3, 3, 3
octanoyl-carnitine ^A	0.005-0.5	d3	0.005, 0.03, 0.18, 0.41	ND	99(96.101,99)	103(104.100,104)	100(103.98,98)	4. 3. 3
palmitoyl-carnitine ^B	0.005-0.5	d3	0.005, 0.03, 0.18, 0.41	113 [*] (110,119,NA)	87(95,76,91)	88(97,83,84)	102(103,101,103)	9, 11, 8, 1
organic acids								
2-OH butyric acid ^C	2-100	d3	2, 7, 40, 83	105(104.120,91)	103(109.103,98)	96(101,94,92)	96(102.92,93)	15, 5. 5. 6
3-OH-butyric acid ^C	6-300	2- ¹³ C	6.26, 125. 248	104(117.102,93)	99(105.96,95)	98(98.103,93)	95(102.92,89)	11. 6. 5, 7
citric ^C	21-300	d4c	21, 59, 125, 248	N/A	N/A	108(112.111,102)	100(122.90,87)	10, 18
fumaric ^{#C}	0.1-5	d2	0.1, 0.35, 1.8, 4.1	ND	84(92,69,90)	100(105,101,94)	104(121.96,94)	15, 7, 14
lactic ^C	510-3600	3- ¹³ C	510, 875, 1600, 2970	97(100.101,92)	103(109.100,101)	98(101.98,95)	95(104.91,91)	5, 5, 5, 9
malic ^{#C}	0.3-25	d3	0.3, 1.1, 8.8, 21	101 (99.114,91)	99(112.97,88)	95(100.92,94)	97(105,93,93)	13, 11, 4, 7

Metabolite	Range (uM)	Stable Iso-tope	Actual conc. of QC's in μ M LOQ, low, medium, and high	Accuracy LOQ QC: Inter-day avg. (Intra-day averages)	Accuracy Low QC: Inter-day avg. (Intra-day averages)	Accuracy Medium QC: Inter-day avg. (Intra-day averages)	Accuracy High QC: Inter-day avg. (Intra-day averages)	Inter-day %CV: QC's LOQ. low, medium, high
Pyruvic ^{#C}	6-300	2- ¹³ C	6,21,115, 248	ND	83(91,85,72)	109(117,109,102)	98(105,94,97)	10, 8,6
succinic ^C	1-50	4- ¹³ C	1.3.5,18,41	108(108.119.97)	99(104.101.92)	96(101,94,92)	97(104.96,91)	11, 7. 4. 6
octanoic ^C	5-300	d15	5,18,113, 248	ND	96(118.90,80)	85(98.79,79)	107(119.103.100)	17,10.9
suberic ^C	0.5-15	d4	0.5,1.5,6.3,12.4	84(91,86.76)	101 (107.95.99)	104(108.103.100)	98(99,98,97)	12, 6. 4. 3
azelaic ^C	0.5-70	d14	0.5. 2.7.5.17	ND	114(120.114.108)	108(114.106.103)	98(105.92.98)	7, 5.6
Alphaketo-glutaric ^{#C}	0.5-30	4- ¹³ C	0.5, 3,13,25	104(119.104,88)	99(101,95,101)	99(105,97,95)	100(104.93,104)	22, 6, 5. 7
ketoisovaleric ^C	0.5-40	5- ¹³ C	0.5, 3.15, 33	ND	94(100.93,89)	99(104.98.96)	99(105.96,96)	21, 5. 4. 5
2-oxoisocaproic ^C	1-60	d3	1,3.5,23,50	70* (59.81.70)	98(104.92,98)	102(107.98.100)	100(107.94.98)	11, 5. 4. 6

Note, all compounds used their own stable isotopic versions for quantification, except for where noted.

ND, not determined because the concentration was below the limit of quantification.

N/A, not available, due to a high endogenous level of analyte in the serum that prohibits accurate measurement at a quality control level.

LOQ, denotes lower limit of quantitation

^A denotes positive mode on EPIC-PFP column

^B denotes positive mode on C18-PFP column

^C denotes negative mode on C18-PFP column

[#] denotes quantification in MS/MS mode

* denotes that the measurement did not meet validation requirements for particular QC level.

Table 2
MS vs MS/MS quantification

metabolite	QC accuracies and %CV MS-mode <i>Inter-day %Acc (Inter-day %CV) for QC LOQ, low, med, and high</i>	QC accuracies and %CV MS/MS-mode <i>Inter-day %Acc (Inter-day %CV) for QC LOQ, low, med, and high</i>
malic	103(14%), 95(11%), 95(2%), 94(4%)	101(13%), 99(11%), 95(4%), 97(7%)
fumaric	103(53%), 63(11%), 99(10%), 97(10%)	150(38%), 84(15%), 100(7%), 104(14%)
pyruvic	73(3%), 90(31%), 99(21%)	83(10%), 109(8%), 102(6%)
alphaketoglutaric	88(34%), 102(9%), 102(8%), 98(6%)	104(22%), 99(6%), 99(5%), 100(7%)
orotic	74(16%), 95(11%), 101(5%), 96(7%)	100(12%), 94(7%), 101(3%), 100(6%)
uracil	88(26%), 86(14%), 82(14%), 84(14%)	98(19%), 83(18%), 93(13%), 88(11%)
thymine	96(11), 101(5%), 100(6%)	97(31%), 94(11%), 97(6%), 95(7%)
aspartate	110(28%), 111(14%), 96(12%)	100(19%), 98(9%), 106(9%)
guanosine	91(4%), 85(12%), 90(7%), 96(17%)	82(39%), 84(10%), 92(9%), 85(11%)

Comparing accuracies and %CV's of 9 compounds that were quantified in both MS mode and MS/MS using the 5600 Q-TOF. Bolded values indicate the better mode for quantification.

Table 3

Metabolite concentrations in patient samples

	Ala 70-700	Leu 30-250	Ile 10-125	Val 40-350	Pro 40-500	Phe 20-200	Try 6-120	Tyr 20-150	Cystine 30-200	Met 5-50	Thr 25-300	Ser 30-300	Asn 20-150	Gln 100-800	Lys 21-300	His 40-250	Arg 20-150	Asp 2-50	Glu 3-150
Control	180 (78)	115 (57)	60 (25)	176 (65)	235 (65)	64 (8.6)	45 (17)	80 (20)	37 (11)	25 (10)	185 (36)	81 (20)	47 (15)	547 (48)	220 (10)	59 (11)	96 (4.0)	6.8 (3.0)	40 (22)
HOC	241 (126)	96 (44)	48 (22)	140 (68)	201 (88)	108 (43)	57 (27)	142 (76)	43 (13)	83 (92)	268 (128)	153 (118)	72 (46)	606 (211)	226 (121)	60 (46)	96 (30)	16 (10)	41 (25)
Cirr.	198 (33)	79 (32)	39 (9.0)	125 (48)	167 (38)	90 (26)	47 (13)	106 (31)	51 (23)	36 (12)	180 (70)	108 (23)	50 (12)	545 (110)	204 (36)	52 (10)	95 (35)	15 (8.6)	46 (32)

	Ornithine 10-200	Homo- cystine 5-75	Creatine 5-150	Pipecolic 1-30	5-oxo- proline 10-120	Citrulline 2-100	Hypoxan- thine 0.5-50	Uracil 0.3-3.0	Thymine 0.05-15	Cytidine 0.05-15	Uridine 0.3-15	Orotic 0.2-7	Guanosine 0.1-3	Carnitine 2-125	Acetyl- carnitine 3-50	Propionyl- carnitine 0.005-0.5
Control	97 (18)	7.9 (3.8)	24 (15)	4.3 (1.8)	81 (5.9)	36 (21)	5.9 (2.6)	0.07 (0.03)	NA	0.03 (0.02)	4.3 (2.1)	0.20 (0.28)	0.76 (0.76)	39 (8.6)	8.9 (1.9)	0.09 (0.02)
HCC	108 (72)	4.8 (4.2)	20 (14)	6.3 (3.4)	86 (28)	34 (8)	7.1 (3.1)	0.07 (0.02)	NA	0.03 (0.02)	3.5 (0.94)	0.11 (0.04)	0.15 (0.03)	45 (20)	9.2 (3.8)	0.10 (0.03)
Cirr.	101 (24)	4.1 (2.8)	14 (4.9)	10 (9.1)	81 (16)	39 (11)	9.0 (4.1)	0.08 (0.02)	NA	0.06 (0.05)	4.3 (0.80)	0.14 (0.12)	0.16 (0.03)	33 (7.5)	10 (5.2)	0.06 (0.02)

	Isobutyryl- carnitine. 0.005-0.5	Isovaleryl- carnitine 0.03-0.5	Octanoyl- carnitine 0.03-0.5	Palmitoyl- carnitine 0.005-0.5	2-OH- butyrate 2-100	3-OH- butyrate 6-300	Fumarate 0.3-5	Lactate 510-3600	Malate 0.3-25	Pyruvate 21-300	Succinate 1-50	Octanoic 18-300	Suberic 0.5-15	Azelale 2-20	Alphaketo- glutric. 0.5-30	Ketoiso- valeric 3-40	2-oxoiso- caproic. 1-60
Control	323 (151)	131 (65)	108 (47)	81 (23)	22 (10)	52 (31)	3.1 (1.6)	1256 (287)	10 (11)	163 (49)	4.2 (1.8)	NA	NA	NA	14 (8.6)	10 (3.8)	20 (9.5)
HCC	176 (107)	64 (33)	81 (14)	94 (10)	42 (27)	18 (20)	3.3 (2.7)	2717 (858)	6.6 (14)	175 (95)	3.5 (1.0)	NA	NA	NA	18 (12)	13 (1.9)	18 (5.9)
Cirr.	141 (45)	68 (37)	125 (39)	121 (19)	37 (18)	62 (47)	3.9 (3.4)	2368 (890)	6.7 (1.6)	168 (60)	4.2 (13)	NA	NA	NA	19 (8.0)	10 (1.9)	16 (5.7)

All Concentrations are in μM. Standard deviations of average concentrations are shown in parentheses.

Ranges of concentrations measured are shown below each respective metabolite name. Those compounds that did not qualify at the LOQ target concentration (listed in 31) show an adjusted range based on the low QC.

N/A indicates that the concentration of the metabolite was too low to accurately measure in patient sera.