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Urinary Amino Acid Analysis: A Comparison of iTRAQ®-LC-MS/MS, GC-MS, and Amino Acid Analyzer

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Abstract

Urinary amino acid analysis is typically done by cation-exchange chromatography followed by post-column derivatization with ninhydrin and UV detection. This method lacks throughput and specificity. Two recently introduced stable isotope ratio mass spectrometric methods promise to overcome those shortcomings. Using two blinded sets of urine replicates and a certified amino acid standard, we compared the precision and accuracy of gas chromatography/mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) of propyl chloroformate and iTRAQ® derivatized amino acids, respectively, to conventional amino acid analysis. The GC-MS method builds on the direct derivatization of amino acids in diluted urine with propyl chloroformate, GC separation and mass spectrometric quantification of derivatives using stable isotope labeled standards. The LC-MS/MS method requires prior urinary protein precipitation followed by labeling of urinary and standard amino acids with iTRAQ® tags containing different cleavable reporter ions distinguishable by MS/MS fragmentation. Means and standard deviations of percent technical error (% TE) computed for 20 amino acids determined by amino acid analyzer, GC-MS, and iTRAQ®-LC-MS/MS analyses of 33 duplicate and triplicate urine specimens were 7.27 ± 5.22, 21.18 ± 10.94, and 18.34 ± 14.67, respectively. Corresponding values for 13 amino acids determined in a second batch of 144 urine specimens measured in duplicate or triplicate were 8.39 ± 5.35, 6.23 ± 3.84, and 35.37 ± 29.42. Both GC-MS and iTRAQ®-LC-MS/MS are suited for high-throughput amino acid analysis, with the former offering at present higher reproducibility and completely automated sample pretreatment, while the latter covers more amino acids and related amines.

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Keywords

amino acids; urine; gas chromatography-mass spectrometry; liquid chromatography-tandem mass spectrometry; amino acid analyzer; precolumn derivatization; stable isotope dilution; iTRAQ; propyl chloroformate

1. Introduction

Amino acids are the basic building blocks of proteins and play essential roles in energy metabolism, neurotransmission, and lipid transport. Their quantitative analysis is important in disease diagnostics [1-3] and, increasingly, in elucidating nutritional influences on physiology [4,5].

The prevailing method of amino acid analysis is cation-exchange chromatography followed by post-column derivatization with ninhydrin and UV detection [6,7]. Low throughput and specificity of detection have spurred development of novel methods for quantification of free amino acids in physiological fluids; these offer more specific detection, decreased lower limits of quantification (LLOQ), and higher throughput [8]. Methods described recently for urinary amino acid analysis include capillary electrophoresis time-of-flight mass spectrometry [9], enhanced ^{13}C NMR spectroscopy [10], gas chromatography-mass spectrometry (GC-MS) of pentafluorobenzylated amino acids [11], and isotope dilution reversed phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) of propyl chloroformate derivatized [12] and underivatized amino acids [13]. Lately, we described an automated isotope dilution GC-MS method for the analysis of amino acids derivatized with propyl chloroformate in native urine [14].

The goal of the present study was to compare sample preparation, runtime, number of analytes amenable to quantification, LLOQ, reproducibility, and validity of three methods in the analysis of urinary amino acids: a conventional amino acid analyzer, GC-MS of propyl chloroformate derivatives [14], and iTRAQ®-LC-MS/MS [15]. The amine-reactive isobaric tagging reagent iTRAQ® has been widely used in the tagging of peptides for multiplexed protein quantitation [15]. To the best of our knowledge, this is the first peer-reviewed report on the application of iTRAQ®-LC-MS/MS to the analysis of physiological amino acids, in particular, those found in human urine; it is based on differential derivatization of standard and sample amino acids with isobaric tags that show identical chromatographic retention, but can be distinguished by MS/MS upon dissociation of reporter ions that differ by one mass unit. The advantage of iTRAQ®-LC-MS/MS is the availability of 42 internal standards of physiological amino acids and amines for absolute quantification by isotope ratio analysis.

For the comparison of the three methods, blinded sets of 98 and 341 urine specimens, respectively, were analyzed. The urine specimens were aliquots from the timed 24-hour urine collections of the INTERMAP (INTERNational collaborative study of MACronutrients and blood Pressure) Study on relation between diet and blood pressure among 4,680 men and women ages 40–59 years in Japan, Peoples Republic of China (PRC), UK, and USA [16].

2. Experimental

2.1. Urine specimens

The urine samples were aliquots taken from the 24-hour urine specimens collected by the INTERMAP study from 1997–1999. Boric acid had been added as a preservative to the urine samples upon collection. Before preparation of aliquots for this study, samples had been stored

in liquid nitrogen. Aliquots were prepared in parallel in London, shipped on dry ice to the analytical study sites, and stored at -20°C until analysis.

The first test set comprised 30 triplicates and 4 duplicates from 34 INTERMAP urine specimens randomly selected from five (of 17) population samples surveyed by the INTERMAP Study: Sapporo (Japan), Aito Town (Japan), Guangxi (PRC), Chicago (US), and Minneapolis (US). The second set comprised 341 aliquots (91 duplicates, 53 triplicates) from 144 INTERMAP urine specimens from the same population samples, but different from those in batch 1.

2.2. Chemicals

A standard of 17 amino acids (Cat. No. 09428), phenol, isooctane, and thiodiglycol were purchased from Sigma-Aldrich (Taufkirchen, Germany). A certified amino acid solution (Standard Reference Material 2389) was obtained from NIST (National Institute of Standards and Technology, Gaithersburg, MD). Propanol (LC-MS grade) and chloroform (HPLC grade) were from Fisher Scientific GmbH (Ulm, Germany). The $[\text{U-}^{13}\text{C}, \text{U-}^{15}\text{N}]$ cell free amino acid mix was from Euriso-top (Saint-Aubin Cedex, France); $[2, 5, 5\text{-}^2\text{H}_3]$ -2-amino adipic acid was from C/D/N Isotopes Inc. (Quebec, Canada). The Phenomenex (Torrence, CA) EZ:faast kit for GC-MS and the Applied Biosystems (Foster City, CA) Amino Acid Analysis for Physiological Samples Kit were used for the derivatization of amino acids with propyl chloroformate and iTRAQ®, respectively.

2.3. GC-MS analysis

GC-MS amino acid analysis (14) was done on an Agilent (Palo Alto, CA) 6890/5975 GC-MS equipped with a programmable temperature vaporizing (PTV) injector and an MPS-2 Preperation (both from Gerstel, Muehlheim, Germany). Fifty μL of urine were transferred together with 20 μL of stabilization reagent containing 10 % isopropanol, 0.1% phenol, and 2% thiodiglycol, to a 2-mL autosampler vial (Gerstel). The amino acids were directly derivatized in diluted urine. Derivatives were extracted by isooctane and 2.5 μL of the organic extract were injected at a split ratio of 1:15. Internal standards were norvaline (Nval) and a mixture of uniformly ^{13}C , ^{15}N -labeled alanine, glycine, valine, leucine, isoleucine, proline, asparagine, aspartic acid, methionine, glutamic acid, phenylalanine, glutamine, lysine, histidine, tyrosine, and tryptophan; additional standards used for the second batch were $[2,5,5\text{-}^2\text{H}_3]$ α -amino adipic acid and $[2,3,4,5,6\text{-}^2\text{H}_5]$ hippuric acid. For GC separation, a Phenomenex ZB-AAA column was used, 15 m $\times$ 0.25 mm ID, 0.1- μm film thickness. The oven temperature was initially held at 70°C for 1 min, raised at $30^{\circ}\text{C}/\text{min}$ to 300°C , and held for 5 min. Column flow was 1.1 mL He/min. The temperature of the PTV injector, which held a SILTEC (Gerstel) liner, was set at 50°C for 0.5 min and ramped at $12^{\circ}\text{C}/\text{s}$ to 320°C . The transfer line to the MS was kept at 310°C . The MS was operated simultaneously in scan (50–420 m/z) and SIM (selected ion monitoring) mode.

For the first batch of urine specimens, quantification was performed by $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ isotope ratio analysis. The nearest eluting stable isotope-labeled amino acid was used for quantification of amino acids with no stable isotope labeled standard available. For the second batch, calibration curves were generated over a range of 0.3–2000 μM using unlabeled amino acid standards and a fixed concentration of stable isotope labeled amino acids for normalization of peak areas.

2.4. iTRAQ®-LC-MS/MS

Derivatization of urinary amino acids with iTRAQ® was performed semi-automated using the Apricot Designs TPS-24 Total Pipetting Solution™. Forty μL of urine were pipetted into 96-well plates and 10 μL of 10% sulfosalicylic acid containing 4 nmol of norleucine (Nle) (to calculate extraction efficiency) were added to precipitate proteins. After mixing for 30 s, the

plates were centrifuged in an Eppendorf Centrifuge 5810R for 5 min at 2,000 RPM (700×g). Ten μL of supernatant were mixed with 40 μL labeling buffer (0.45 M borate buffer, pH 8.5, containing 20 pmol/ μL norvaline to calculate derivatization efficiency). Ten μL of the diluted supernatant were mixed with 5 μL of iTRAQ® reagent 115 solution (1 tube mixed with 70 μL of isopropanol), and incubated at room temperature for 30 min. Then 5 μL of 1.2% hydroxylamine solution were added. Samples were allowed to evaporate overnight and were reconstituted with 32 μL of iTRAQ® reagent 114-labeled standard mix (5 pmol of each amino acid/ μL except for L-cystine, present at 2.5 pmol/ μL). Chromatographic separation of isobaric amino acids was achieved at 50°C using an Agilent 1100 HPLC system and an Applied Biosystems C18 5 μm column, 4.6 i.d. $\times$ 150 mm. The mobile phase consisted of 0.1% formic acid and 0.01% heptafluorobutyric acid in water (solvent A), respectively acetonitrile (solvent B). The column was equilibrated in 98% A and the gradient was 98%-72% A over 10 min, 72%-0% A over 0.1 min, hold at 100% B for 5.9 min. A flow rate of 800 $\mu\text{L min}^{-1}$ was used; injection volume was 2 μL . Tandem mass spectrometry was performed on an API 3200 (Applied Biosystems) with turbo ion spray in positive mode using the following parameters: Ion spray voltage (IS) 1500 V; auxiliary gas temperature (TEM) 700°C; curtain gas (CUR), nebulizer gas (GS1), and auxiliary gas (GS2) 20, 70, and 70 arbitrary units, respectively; collision gas medium; entrance potential (EP) 10V; declustering potential (DP) 20V; collision energy (CE) 30V; collision cell exit potential (CXP) 5V. Quantitative determination was performed by multiple reaction-monitoring (MRM) using one transition each for the analyte and the internal standard, according to manufacturer instructions. Chromatograms were processed with a beta version of Cliquant® software.

2.5. Amino acid analyzer

The amino acid analyzer Biochrom 30 was used (Laborservice Onken, Gründau, Germany) for analysis of the first batch of urine specimens. Sample preparation and analysis were performed using manufacturer standard protocols. Data were also available on urinary levels of 21 selected amino acids for the second batch of specimens, measured previously at the INTERMAP central laboratory in Leuven, Belgium, using a Biochrom 20 (Biochrom Ltd, Cambridge, UK) amino acid analyzer.

2.6. Statistics

Intra-specimen reliability of amino acid measurements was tested separately on batch 1 and 2 urine specimens, by calculating the technical error (TE) of measurements (18). It was computed as the square root of the sum of variance between corresponding measurements divided by the number of urine specimens analyzed. This is a modification of the original method for calculating the TE to allow inclusion of the variance of triplicate measurements. For calculation of percent TE (%TE), TE was divided by the mean of all split sample values and multiplied by 100. Pearson's correlation analysis was used to calculate the association between the three techniques for each urinary amino acid. The Bland–Altman test (19) was employed to determine the 95% limits of agreement between the techniques. Urinary amino acid concentrations are reported as micromolar ($\mu\text{mol/L}$) ranges both uncorrected and corrected for urinary creatinine, which was measured by the Jaffe method (16). The uncorrected values are given for direct comparison of urinary amino acid concentrations with the respective LLOQs, defined as the lowest point of the calibration curve that can be determined with 80–120% accuracy (20), for GC-MS and iTRAQ®-LC-MS/MS. Data were analyzed using SAS/STAT 9.1 software (SAS Institute Inc., Cary, NC) and descriptive statistical functions implemented in Excel® 2004 for Mac (version 11.4.1, Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. Reproducibility

First a blinded set of 98 urine samples was analyzed by GC-MS, iTRAQ®-LC-MS/MS, and the amino acid analyzer. The set comprised 34 different urine specimens, from which 2 or 3 replicate aliquots had been prepared. All 34 urine specimens were analyzed by the amino acid analyzer, while only 33 and 31 specimens could be subjected to GC-MS and iTRAQ®-LC-MS/MS, respectively, due to specimen volume limitations. Representative GC-MS and iTRAQ®-LC-MS/MS chromatograms of human urine are shown in Figures 1 and 2, respectively. Not all amino acids were amenable to analysis by all three methods (Table 1). Number of analytes covered ranged from 26 analytes for GC-MS to 40 and 42 for the amino acid analyzer and iTRAQ®-LC-MS/MS, respectively. Urinary serine, threonine, hydroxylysine, and hydroxyproline could not be measured reliably by GC-MS because of secondary interactions of their underivatized hydroxyl group with the liner (14). In addition, anserine, arginine, argininosuccinic acid, carnosine, citrulline, ethanolamine, γ -aminobutyric acid, homocitrulline, phosphoethanolamine, phosphoserine, taurine, and the methylhistidines were not amenable to GC-MS because of either thermal instability (*e.g.*, arginine) or low vapor pressure (*e.g.*, phosphoethanolamine). Quantification of β -alanine by iTRAQ® was impeded by coeluting matrix components, hence it was excluded. Urinary levels of some amino acids (*e.g.*, phosphoserine and cystathionine) were low, consequently all urine specimens did not yield concentration values above LLOQs, listed in Table 2 together with ranges of urinary amino acid levels, both uncorrected and corrected for urinary creatinine, observed for both batches of urine specimens. Adjustment for urinary creatinine takes into account inter-individual differences in glomerular filtration rate and facilitates comparison with urinary amino acid levels reported in the medical literature. For amino acids limited in quantification, data include actual number of specimens, given in brackets next to %TE value in Table 3.

Average percent technical error (%TE) over all sample replicates was calculated for each amino acid (Table 3). For 20 urinary amino acids, quantitative data were available from all three methods; reproducibility for those analytes was: mean $\pm$ SD of %TE (range) 7.27 ± 5.22 (2.13–19.03), 21.18 ± 10.94 (10.14–56.54), and 18.34 ± 14.67 (6.60–64.26), respectively, for amino acid analyzer, GC-MS, and iTRAQ®-LC-MS/MS. For α -aminoadipic acid (Aad), β -aminobutyric acid (Abu), β -aminoisobutyric acid (bAib), cystathionine (Cth), and cystine (Cys-Cys), no stable isotope-labeled standards were available for GC-MS analysis. Their concentrations had to be calculated by using the nearest eluting stable isotope standard as a reference. This fails to account fully for any variation of ionization; hence, technical error is expected to be greater. Excluding Aad, Abu, bAib, and Cys-Cys, %TE for GC-MS improved from 21.60 ± 11.07 (mean $\pm$ SD) to 16.93 ± 4.15 , range 10.14–23.11. For iTRAQ®-LCMS/MS, the corresponding values also improved from 18.85 ± 14.89 to 16.38 ± 11.19 , range 6.60–52.15, upon omission of the high %TE associated with bAib.

GC-MS and iTRAQ®-LC-MS/MS were further evaluated with a second batch of 341 split samples from 144 INTERMAP urine specimens. For 101 of these urine specimens, urinary levels of 21 selected amino acids had been analyzed previously in duplicate using a Biochrom 20 amino acid analyzer. For 13/21 amino acids with urinary levels determined successfully by amino acid analyzer, GC-MS, and iTRAQ®-LC-MS/MS, mean $\pm$ SD (range) of %TE was 8.39 ± 5.35 (2.66–19.01), 6.23 ± 3.84 (3.38–14.02), and 35.37 ± 29.42 (16.30–115.64), respectively. Excluding methionine, with urinary levels least reproducible for amino acid analyzer and iTRAQ®-LC-MS/MS (%TEs of 19.01 and 115.64, respectively) reduced average %TEs to 7.51 ± 4.48 , 5.89 ± 3.80 , and 28.68 ± 17.59 . Expanding the comparison to all 21 amino acids amenable to both amino acid analyzer and iTRAQ®-LC-MS/MS yielded average %TEs of 7.59 ± 4.96 and 30.90 ± 23.88 , respectively.

Overall, including %TEs of all amino acids with urinary levels amenable to analysis (Table 3), the amino acid analyzer yielded the best results with average %TEs of 7.43 ± 5.43 and 7.59 ± 4.96 (mean $\pm$ SD) for batches 1 and 2, respectively. GC-MS matched the reliability of the amino acid analyzer for the second batch of urine specimens, with average %TE of 8.28 ± 6.64 ; average %TE for the first batch of urine specimen was 21.69 ± 10.67 . There is no obvious reason for the improvement in precision for the second batch other than gain in experience by the operator of GCMS. Reproducibility of GC-MS measurements depends on the availability of stable isotope labeled amino acid standards that account for variation of ionization due to matrix effects. This is obvious from comparing the average %TE of 5.87 ± 3.59 for the 17 amino acids with stable isotope-labeled internal standards available compared to average %TE of 13.03 ± 8.31 for the 8 amino acids with concentrations determined using the nearest eluting stable isotope-labeled standard as reference. Hence, improvements in GC-MS performance depend on availability of additional stable isotope-labeled amino acids. This will not help the comparatively small number of 26 urinary amino acids and amines amenable to GC-MS analysis versus 34 and 40 for amino acid analyzer and iTRAQ®-LC-MS/MS, respectively. The latter method also carries the advantage of having stable isotope labeled standards available for 42 physiological amino acids and amines. One would expect iTRAQ®-LC-MS/MS to be highly reproducible. But for reasons discussed below, iTRAQ®-LC-MS/MS yielded the highest average %TE of 30.38 ± 19.16 for the second batch of urine specimens.

3.2. Correlation between methods

Data obtained by Biochrom 20, GC-MS and iTRAQ®-LC-MS/MS for the second batch of urine specimens were correlated with each other; Pearson r-values and 95% confidence intervals are listed in Table 4. The Pearson correlation coefficients for the 12 amino acids measured by both GC-MS and the amino acid analyzer ranged from 0.800 (Trp) to 0.980 (Gly). GC-MS and iTRAQ®-LC-MS/MS had 19 amino acids in common (cystathionine was excluded because of its low urinary levels) and showed generally good correlation. The single exception was cystine ($r=0.822$). The correlation coefficients for the remaining 18 analytes ranged between 0.934 (Glu) and 0.988 (Tyr). Urinary levels of 20 amino acids were available for the comparison of iTRAQ®-LC-MS/MS with the amino acid analyzer. Correlation coefficients for arginine (0.561), carnosine (0.801), cystine (0.811), isoleucine (0.802), taurine (0.885) tryptophan (0.764), and tyrosine (0.780) were poor; for the remaining 13 amino acids, they ranged from 0.899 (Phe, Val) to 0.951 (Lys).

3.3. Bland-Altman plots

Bland-Altman plots assess agreement between two different analytical methods: this graphical method plots concentration difference between the two techniques for each specimen against average of the two concentrations [17]. In addition, mean difference ($\bar{d}$), and lower and upper limits of agreement are shown as horizontal lines, with limits of agreement defined as mean difference ± 1.96 times standard deviation ($\bar{d} \pm 1.96$ SD). Table 5 gives data on mean difference, limits of agreement, and type of plot; each Bland-Altman plot was categorized into one of six types based on graphical appearance:

Type A—Type A represents ideal agreement between two methods, *i.e.*, absolute mean difference is almost zero and individual differences scatter randomly with no apparent systematic error; mean of the difference is $\leq 15\%$ of mean concentration for all measurements with two methods. An example comparing GC-MS with iTRAQ®-LC-MS/MS is shown in Figure 3a for glycine.

Type B—Absolute mean difference has a positive value and is $> 15\%$ of mean concentration. In this case, systematic error is detected, represented by the negative value for the mean

difference with individual differences scattering randomly. A representative type B plot for amino acid analyzer vs. iTRAQ®-LC-MS/MS of arginine is shown in Figure 3b.

Type C—Type C resembles type B, but mean difference has a negative value. An example is shown in Figure 3c for the analysis of glutamic acid by GC-MS and iTRAQ®-LC-MS/MS.

Type D—Type D plots represent proportional error in agreement between two methods. In this case, individual mean differences become proportionately more positive the higher the concentration of the analyte. This is exemplified in Figure 3d for the analysis of lysine by amino acid analyzer and GC-MS.

Type E—In type E plots, individual mean differences become proportionately more negative the higher the concentration of the analyte. An example for a type E plot is shown in Figure 3e for the analysis of cystine by GC-MS and iTRAQ®-LC-MS/MS.

Type F—In type F plots, individual mean differences show a ‘V-shaped’ distribution as standard deviation increases with concentration as shown for valine (amino acid analyzer vs. iTRAQ®-LC-MS/MS) in Figure 3f.

Only 19 of 51 (37.3%) Bland-Altman plots revealed excellent type A agreement. Glycine and tyrosine were the only quantitated amino acids that agreed well across all three methods. Absolute systematic errors were observed in 8 (15.7%) instances; proportional errors of type D and type E in 8 (15.7%) and 6 (11.8%) cases, respectively; and multiplicative errors of type F in 10 (19.6%) cases.

Since only 7 of 19 (36.8%) comparisons between GC-MS and iTRAQ showed excellent agreement on urinary amino acid concentrations, we validated the accuracy of these methods using a NIST certified amino acid standard.

3.4. Validity assessment with a certified standard

The certified NIST standard with 17 amino acids was analyzed to assess validity of GC-MS and iTRAQ®-LC-MS/MS concentrations. We quantitated 16 amino acids with the GC-MS method. Arginine could not be determined due to thermal instability of its propylformate derivative. Excellent correspondence with the NIST certified values was obtained for all amino acids measured by GC-MS and iTRAQ®-LC-MS/MS (Figure 4). The recoveries for GC-MS varied from 98–111% and for iTRAQ®-LCMS/MS from 91–106%. Overall, GC-MS tended to overestimate the NIST certified values by $5.33 \pm 3.70\%$ (mean $\pm$ standard deviation), whereas iTRAQ®-LC-MS/MS on average matched the certified values well ($-0.04 \pm 4.18\%$). The reproducibility of the GC-MS data was excellent with relative standard deviations (RSDs) of about 1% for most amino acids. The iTRAQ®-LC-MS/MS data showed RSDs of 3–6%.

Both GC-MS and iTRAQ®-LC-MS/MS quantitated accurately cystine in the NIST standard. In urine, however, iTRAQ®-LC-MS/MS consistently yielded higher levels of cystine with the difference from GC-MS and amino acid analyzer becoming proportionately greater with higher urinary cystine levels (Table 5). Cysteine may oxidize under non-acidic conditions to cystine [18]. Although the urine specimens were alkalized for the labeling of amino acids with iTRAQ®, the excess in urinary cystine by iTRAQ®-LC-MS/MS far exceeded the reported levels of urinary cysteine, typically present at about 10% of cystine [18]. Therefore, reasons other than oxidation of cysteine account for the apparent overquantitation of urinary cystine.

3.5. Comparison of methods

The three methods were compared with regard to sample preparation, amount of sample needed for analysis, runtime, number of analytes amenable to quantification, cost and limit of quantification (LOQ). A summary of the comparison is given in Table 6. Both amino acid analyzer and iTRAQ®-LC-MS/MS require protein precipitation. GC-MS allows the direct derivatization of amino acids with propyl chloroformate in native urine and, therefore, automation of the entire analytical procedure. The urine volumes needed for GC-MS and iTRAQ®-LC-MS/MS analysis are 40–50 µL, while 200 µL are required for the amino acid analyzer. Given that urine is typically available in large quantities, these differences in sample volume are negligible.

A drawback of the amino acid analyzer is the typical runtime of 130 min. In contrast, total runtimes for GC-MS and iTRAQ®-LC-MS/MS are 20 and 25 min, respectively. The LLOQs for the amino acid analyzer (2–3 µmol/L) are also on average higher than those for GC-MS (0.3–30 µmol/L) and iTRAQ®-LC-MS/MS (0.5–10 µmol/L). A disadvantage of GC-MS is the smaller number of amino acids amenable to analysis (14). In principle, 33 urinary amino acids can be detected by GC-MS [14], but only 22 amino acids were measurable above the LLOQ in ≥ 80% of urine specimens of the second batch. In contrast, it was possible to quantify 34 analytes in at least 80% of the urine specimens by iTRAQ®-LC-MS/MS.

The higher TEs of iTRAQ®-LC-MS/MS appear to be mainly due to excess of multiple reaction-monitoring transitions acquired in the third of the four predefined time windows. In the first, second, and fourth period, 3 (PSer, PEtN, Tau), 7 (Asn, Ser, Hyp, Gly, Gln, EtN, Asp), and 10 (Val, Nval, Met, Tyr, Hcys, Ile, Leu, Nle, Phe, Trp) amino acids are monitored, respectively. In contrast, in the third period 24 amino acids (Cit, Sar, bAla, Ala, Thr, Glu, His, 3MHis, 1MHis, Hcit, Asa, GABA, bAib, Abu, Aad, Ans, Car, Pro, Arg, Hyl, Orn, Cth, Cys-Cys, Lys) are monitored, with only half as many data points recorded. This has a significant influence on the reproducibility of peak areas. For the second batch of urine specimens, mean ± SD of %TE (range) was 33.09±14.60 (18.21–73.31) for period 3, while it was (excluding methionine) 21.16±7.39 (13.88–40.64) for periods 1, 2, and 4. This shortcoming may be alleviated by recent implementation of scheduled MRMs that allow definition of as many overlapping periods as there are amino acids, with each amino acid monitored only for the time period of its expected elution from the column. For maximum precision, chromatographic resolution of amino acids will have to be improved to limit number of overlapping periods. This will be the more true if iTRAQ®-LC-MS/MS analysis is expanded to other urinary analytes possessing one or more free amino groups, which will also require the synthesis of the corresponding iTRAQ® reagent 114-labeled standards.

In conclusion, GC-MS and LC-MS/MS are attractive alternatives to the amino acid analyzer. The advantages of GC-MS are its complete automation, short runtime, and higher precision; its one limitation is the smaller number of amino acids amenable to analysis. In comparison, iTRAQ®-LC-MS/MS excels in greater number of amino acids amenable to analysis and current availability of 42 stable isotope labeled standards. Incorporation of scheduled MRMs, improved chromatographic resolution, and an advanced integration algorithm may improve reproducibility of the iTRAQ® method.

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Nonstandard abbreviations

iTRAQ®, isobaric tagging for relative and absolute quantification; LLOQ, lower limit of quantitation; MRM, multiple reaction-monitoring; MSTFA, N-methyl-N-(trimethylsilyl) trifluoroacetamide; SIM, selected ion monitoring; TEM, technical error of measurement.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1

Names and abbreviations of 45 amino acids and related amines, and their amenability to analysis by each of the three methods.

Amino Acid	Abbreviation	iTRAQ®	GC-MS	Biochrom30
α -Aminoadipic Acid *	Aad	X	X	X
α -Aminobutyric acid	Abu	X	X	X
Alanine *	Ala	X	X	X
Anserine	Ans	X		X
Arginine *	Arg	X		X
Argininosuccinic Acid	Asa	X		
Asparagine *	Asn	X	X	X
Aspartic Acid *	Asp	X	X	X
β -Alanine	bAla	X	X	X
β -Aminoisobutyric acid	bAib	X	X	X
Carnosine	Car	X		X
Citrulline	Cit	X		X
Cystathionine	Cth	X	X	X
Cystine	Cys-Cys	X	X	X
Ethanolamine	EtN	X		X
γ -Aminobutyric Acid	GABA	X		X
Glutamine *	Gln	X	X	X
Glutamic acid *	Glu	X	X	X
Glycine *	Gly	X	X	X
Glycine-Proline	Gpr		X	
Homocitrulline	Hcit	X		
Homocystine	Hcys	X		X
Hippuric acid	Hip		X	
Histidine *	His	X	X	X
Hydroxylysine	Hyl	X	X	X
Hydroxyproline	Hyp	X	X	X
allo-Isoleucine	allo-Ile		X	
Isoleucine *	Ile	X	X	X
Leucine *	Leu	X	X	X
Lysine *	Lys	X	X	X
Methionine *	Met	X	X	X
1-Methylhistidine	M1His	X		X
3-Methylhistidine	M3His	X		X
Ornithine	Orn	X	X	X
Phosphoethanolamine	PEtN	X		X
Phenylalanine *	Phe	X	X	X
Proline *	Pro	X	X	X
Phosphoserine	Pser	X		X
Sarcosine	Sar	X	X	X

Amino Acid	Abbreviation	iTRAQ®	GC-MS	Biochrom30
Serine *	Ser	X		X
Taurine	Tau	X		X
Threonine *	Thr	X		X
Tryptophan *	Trp	X	X	X
Tyrosine *	Tyr	X	X	X
Valine *	Val	X	X	X

* For these amino acids, [U-¹³C, U-¹⁵N]- and deuterium-labeled (Aad) standards were available

Table 2

Range of urinary amino acid concentrations [$\mu\text{mol/L}$] uncorrected and corrected for urinary creatinine [$\mu\text{mol}/\text{mmol}$ creatinine] in batches 1 and 2 (434 and 433 urine aliquots, respectively), and lower limits of quantitation (LLOQs) [$\mu\text{mol/L}$] for GC-MS and iTRAQ®-LC-MS/MS.

Amino Acid	GC-MS (N=434) $\mu\text{mol/L}$	iTRAQ® (N=433) $\mu\text{mol/L}$	GC-MS $\mu\text{mol}/\text{mmol}$ creatinine	iTRAQ® $\mu\text{mol}/\text{mmol}$ creatinine	GC-MS LLOQ $\mu\text{mol/L}$	iTRAQ® LLOQ $\mu\text{mol/L}$
Asd	<3.00 – 99.28	3.58 – 153.12	0.05 – 0.83	0.07 – 1.22	3	0.5
Abu	0.38 – 35.80	<0.5 – 40.40	0.01 – 0.23	0.02 – 0.27	0.3	0.5
Ala	19.56 – 1072.70	22.19 – 1376.46	0.4 – 7.82	0.39 – 10.9	0.3	1
Ans	UD	<1.00 – 806.71	-	0.01 – 9.12	-	1
Arg	UD	<5.00 – 128.58	-	0.06 – 1.73	-	5
Asa	UD	<5 – 37.83	-	0.03 – 0.49	-	5
Asn	10.62 – 550.48	17.31 – 713.97	0.22 – 5.0	0.36 – 6.39	12	5
Asp	<3.00 – 65.44	0.66 – 49.67	0.02 – 0.48	0.01 – 0.35	3	0.5
bAlb	6.50 – 2299.96	4.64 – 2523.59	0.09 – 27.3	0.06 – 23.05	0.9	0.5
Car	UD	1.43 – 260.80	-	0.02 – 5.87	-	1
Cit	UD	<0.50 – 30.80	-	0.09 – 0.4	-	0.5
Cys-Cys	<12.00 – 355.24	<10 – 1491.36	0.15 – 2.43	0.21 – 15.62	12	10
EN	UD	60.45 – 803.76	-	0.67 – 10.53	-	0.5
GABA	UD	<1 – 23.96	-	0.01 – 0.49	-	1
Gln	32.06 – 1753.00	37.15 – 1867.69	0.66 – 21.51	0.59 – 31.49	30	0.5
Glu	1.60 – 38.76	2.18 – 36.19	0.06 – 0.72	0.05 – 0.65	3	0.5
Gly	70.60 – 5175.28	124.50 – 6524.52	1.44 – 69.44	1.89 – 121.8	3	0.5
Gpr	<3.00 – 35.36	UD	0.02 – 0.45	-	3	-
Hcit	UD	<5.00 – 163.69	-	0.07 – 1.13	-	5
Hip	42.08 – 5148.88	UD	0.34 – 111.3	-	30	-
His	54.58 – 2444.74	55.27 – 2865.53	1.16 – 19.25	0.95 – 34.4	12	0.5
Hyl	UD	<1.00 – 76.31	-	0.02 – 0.94	12	1
Hyp	UD	<0.5 – 65.15	-	0.003 – 0.41	3	0.5
Allo-Ile	<0.9 – 10.3	UD	0.004 – 0.06	-	0.9	-
Ile	1.44 – 40.72	1.47 – 51.24	0.03 – 0.25	0.03 – 0.32	0.9	0.5
Leu	3.42 – 96.56	3.63 – 103.33	0.07 – 0.63	0.07 – 2.45	0.3	0.5

Amino Acid	GC-MS (N=434) $\mu\text{mol/L}$	iTRAQ® (N=433) $\mu\text{mol/L}$	GC-MS $\mu\text{mol/mmol creatinine}^*$	iTRAQ® $\mu\text{mol/mmol creatinine}^*$	GC-MS LLOQ $\mu\text{mol/L}$	iTRAQ® LLOQ $\mu\text{mol/L}$
Lys	7.06 – 1862.82	8.6 – 2206.81	0.14 – 25.0	0.15 – 29.61	0.9	0.5
Met	<3.00 – 18.90	<0.5 – 18.81	0.01 – 0.13	0.004 – 0.24	3	0.5
M1His	UD	7.98 – 5614.71	-	0.1 – 63.44	-	1
M3His	UD	10.15 – 2966.78	-	0.14 – 40.04	-	0.5
Orn	1.66 – 75.78	<5.00 – 110.66	0.03 – 1.02	0.04 – 1.49	0.9	5
PEtN	UD	2.40 – 106.43	-	0.05 – 0.9	-	0.5
Phe	6.62 – 192.74	6.50 – 220.00	0.12 – 1.14	0.12 – 1.2	0.9	0.5
Pro	0.94 – 24.60	<5 – 24.19	0.02 – 0.32	0.03 – 0.25	0.3	5
Sar	0.92 – 7.94	0.6 – 11.01	0.01 – 0.14	0.01 – 0.14	0.9	0.5
Ser	UD	48.99 – 1092.64	-	0.72 – 9.77	-	0.5
Tau	UD	11.88 – 5238.65	-	0.27 – 71.84	-	1
Thr	UD	10.33 – 498.27	-	0.2 – 6.11	-	1
Trp	5.88 – 242.08	7.17 – 269.72	0.12 – 1.3	0.1 – 1.45	0.3	0.5
Tyr	8.76 – 350.36	8.8 – 363.43	0.18 – 2.03	0.14 – 2.4	0.9	1
Val	4.98 – 136.10	4.21 – 146.67	0.1 – 0.82	0.1 – 0.92	0.3	1

* Ranges are only given for amino acid concentrations above the LLOQ. UD, undeterminable

Table 3

Percent technical errors computed from duplicate and triplicate measurements of urinary amino acids for batches #1 and #2 of urine specimens. Number of duplicates or triplicates used for computing percent technical error is given in brackets. Urine specimens with amino acid levels below the lower limit of quantitation were excluded.

Amino acid	First batch			Second batch		
	iTRAQ (N=31)	GC-MS (N=33)	Biochrom30 (N=34)	iTRAQ (N=143)	GC-MS (N=144)	Biochrom20 (N=101)
Aad	11.08	34.84 (30)	6.72	22.73	4.08	ND
Abu	22.15 (30)	56.54	5.26	20.37	6.63	ND
Ala	9.90	16.33	2.20	23.54	3.38	4.02
bAla	UD	ND	5.65 (10)	UD	ND	ND
Ans	46.81 (22)	UD	5.24 (18)	50.53 (132)	UD	ND
Arg	17.67 (28)	UD	7.45	22.25 (140)	UD	15.60 (84)
Asa	<LLOQ	UD	<LLOQ	43.15 (94)	UD	ND
Asn	13.40	16.21	5.00	18.86	4.16	5.86
Asp	21.43	12.80 (16)	12.00	25.55	15.02 (138)	ND
bAlb	64.26	33.49	10.95 (30)	63.99	11.02	ND
Car	18.59	UD	9.36 (3)	29.32	UD	8.23 (100)
Cit	22.45	UD	6.60	30.01 (141)	UD	ND
Cth	8.72 (9)	13.18 (6)	17.62 (26)	25.81 (6)	9.98 (18)	ND
Cys-Cys	14.91	31.65	3.29	73.31* (142)	14.02 (139)	5.84
EtN	7.30	UD	5.27	13.88	UD	7.53
GABA	26.01 (22)	UD	25.42	26.57	UD	ND
Gln	25.11	22.70	3.98	22.27	13.95	3.84
Glu	11.99	19.92	19.03 (32)	22.03	3.93	ND
Gly	13.91* (30)	19.22	2.98	40.64	4.47	2.66
Gpr	UD	36.25 (17)	ND	UD	28.69 (121)	ND
Hcit	21.50* (26)	UD	ND	30.24 (138)	UD	ND
Hip	UD	ND	UD	UD	25.08	UD
His	18.26	10.14	2.13	27.15	4.39	3.30
Hyl	33.72 (28)	UD	11.72 (24)	43.01 (133)	UD	ND
Hyp	36.93 (31)	UD	<LLOQ	23.05 (37)	UD	ND
allo-Ile	UD	<LLOQ	ND	UD	5.23 (30)	ND
Ile	6.60	15.24	16.05 (28)	18.32	5.22	16.86 (60)

Amino acid	First batch			Second batch		
	iTRAQ (N=31)	GC-MS (N=33)	Biochrom30 (N=34)	iTRAQ (N=143)	GC-MS (N=144)	Biochrom20 (N=101)
Leu	52.15	14.29	9.06 (30)	16.59	4.13	ND
Lys	18.96	20.73	6.27	50.60	4.53	5.72
Met	16.12 (27)	20.16 (8)	<LLOQ	115.64 (102)	10.30 (79)	19.01 (95)
M1His	14.89	UD	6.76	35.78	UD	3.30
M3His	17.01	UD	2.92	21.17	UD	4.80
Om	15.40 (25)	23.11	4.00	33.76 (121)	9.13	ND
PEtN	6.90	UD	5.58* (33)	17.56	UD	ND
Phe	11.92	16.15	4.07	16.45	4.10	10.60 (99)
Pro	7.51 (7)	18.76	<LLOQ	18.21 (89)	5.65	ND
Pser	13.11 (2)	UD	<LLOQ	23.05 (37)	UD	ND
Sar	22.20	ND	11.40 (32)	23.74	7.49 (104)	ND
Ser	19.28	UD	2.39	15.38	UD	3.56 (100)
Tau	15.75	UD	4.53	20.84	UD	3.01
Thr	13.33	UD	2.56	23.75	UD	4.18
Trp	9.49	12.80	5.04	18.22	4.29	12.69 (82)
Tyr	9.51	22.10	2.57	16.30	4.37	6.63
Val	7.74	12.15	7.15	18.54	3.85	12.07 (98)

* One outlier exceeding 8 SDs of the mean excluded; ND, not determined; UD, undeterminable.

Table 4

Pearson correlation coefficients (R), 95 % confidence intervals (CI) and slopes computed from the mean concentrations of duplicate and triplicate measurements of 144 urine specimens using the amino acid analyzer Biochrom 20 and stable isotope ratio mass spectrometry of propyl chloroformate and iTRAQ® derivatized amino acids.

Amino Acid	GC-MS vs. Biochrom 20		iTRAQ®-LC-MS/MS vs. GC-MS		iTRAQ®-LC-MS/MS vs. Biochrom 20	
	R (95% CI)	slope	R (95% CI)	slope	R (95% CI)	slope
Aad	-	-	0.968 (0.955 – 0.977)	1.258	-	-
Abu	-	-	0.953 (0.935 – 0.966)	0.974	-	-
bAlb	-	-	0.967 (0.954 – 0.976)	0.722	-	-
Ala	0.970 (0.959 – 0.978)	0.928	0.979 (0.971 – 0.985)	1.175	0.944 (0.923 – 0.96)	0.823
Arg	-	-	-	-	0.561 (0.437 – 0.663)	0.900
Asn	0.953 (0.935 – 0.966)	0.719	0.986 (0.980 – 0.989)	1.050	0.940 (0.918 – 0.957)	1.170
Asp	-	-	0.929 (0.901 – 0.948)	0.618	-	-
Car	-	-	-	-	0.801 (0.733 – 0.852)	1.462
Cys-Cys	0.944 (0.922 – 0.959)	0.684	0.822 (0.759 – 0.868)	1.49	0.811 (0.746 – 0.860)	0.616
EtN	-	-	-	-	0.917 (0.886 – 0.939)	0.873
Glu	-	-	0.934 (0.908 – 0.951)	0.752	-	-
Gln	0.956 (0.94 – 0.968)	1.111	0.958 (0.942 – 0.970)	0.628	0.938 (0.915 – 0.955)	1.231
Gly	0.980 (0.973 – 0.986)	0.968	0.937 (0.913 – 0.954)	1.198	0.921 (0.891 – 0.942)	0.730
His	0.969 (0.957 – 0.977)	1.056	0.965 (0.952 – 0.975)	1.042	0.940 (0.918 – 0.957)	0.799
Ile	0.812 (0.747 – 0.861)	0.812	0.976 (0.966 – 0.982)	1.059	0.802 (0.736 – 0.854)	0.737
Leu	-	-	0.984 (0.969 – 0.984)	0.997	-	-
Lys	0.969 (0.957 – 0.978)	0.966	0.977 (0.969 – 0.984)	0.963	0.951 (0.932 – 0.964)	0.968
M1His	-	-	-	-	0.934 (0.909 – 0.952)	0.799
M3His	-	-	-	-	0.906 (0.871 – 0.931)	0.753
Om	-	-	0.963 (0.949 – 0.973)	1.310	-	-
Phe	0.909 (0.875 – 0.933)	0.778	0.986 (0.980 – 0.990)	1.018	0.899 (0.862 – 0.926)	1.015
Ser	-	-	-	-	0.939 (0.915 – 0.955)	0.856
Tau	-	-	-	-	0.885 (0.843 – 0.916)	0.694
Thr	-	-	-	-	0.946 (0.925 – 0.961)	1.071
Trp	0.800 (0.733 – 0.851)	0.782	0.981 (0.974 – 0.986)	0.907	0.760 (0.680 – 0.821)	0.841
Tyr	0.844 (0.788 – 0.885)	0.525	0.988 (0.983 – 0.991)	0.974	0.807 (0.740 – 0.857)	1.318
Val	0.912 (0.879 – 0.936)	0.995	0.983 (0.976 – 0.988)	0.952	0.899 (0.862 – 0.926)	0.851

Table 5
Mean differences ($\bar{d}$) and limits of agreement ($\bar{d} \pm 1.96$ SD) between methods in μM and types of Bland-Altman plots (TP*).

AA	Biochrom vs. GC-MS			GC-MS vs. iTRAQ			BIOCHROM vs. iTRAQ		
	$\bar{d}$	± 1.96 SD	TP	$\bar{d}$	± 1.96 SD	TP	$\bar{d}$	± 1.96 SD	TP
Aad				-7.45	-24.95 – 10.04	E			
Abu				-0.89	-4.96 – 3.18	A			
bAib				98.96	-320.6 – 518.6	D			
Ala	23.2	-55.7 – 102.0	A	-11.2	-134.1 – 111.7	F	11.9	-135.9 – 159.8	A
Arg							-4.76	-42.1 – 32.5	C
Asn	31.57	-39.2 – 102.4	D	-7.96	-49.1 – 33.1	F	23.7	-54.0 – 101.4	F
Asp				4.54	-2.1 – 11.1	D			
Car							70.8	1.1 – 140.5	D
Cys-Cys	18.0	-14.8 – 50.8	D	-26.29	-139.31 – 86.72	E	-8.27	-117.83 – 101.28	E
EtN							-15.1	-127.6 – 97.5	A
Gln	-59.3	-219.9 – 101.3	C	141.7	-83.0 – 366.3	D	82.4	-84.7 – 249.4	D
Glu				2.95	-3.3 – 9.2	B			
Gly	2.2	-292.1 – 296.5	A	-44.9	-927.0 – 837.2	A	-42.6	-954.8 – 869.6	A
His	-44.0	-254.4 – 166.3	E	-2.53	-340.7 – 335.7	F	-46.6	-440.3 – 347.1	F
Ile	-1.9	-6.2 – 2.4	C	-0.75	-4.5 – 3.0	A	-2.7	-8.5 – 3.1	C
Leu				-0.12	-8.2 – 8.0	A			
Lys	68.5	-67.3 – 204.3	D	1.8	-192.6 – 196.1	F	70.4	-158.0 – 298.8	F
M1His							28.9	-524.0 – 581.7	A
M3His							-8.3	-107.5 – 90.9	A
Orn				-2.8	-14.3 – 8.7	E			
Phe	6.4	-16.6 – 29.3	B	-2.7	-15.6 – 10.1	A	3.7	-22.0 – 29.3	A
Ser							-3.0	-128.6 – 122.7	A
Tau							-121.5	-993.1 – 750.2	E
Thr							23.2	-48.3 – 94.8	B
Trp	-9.03	-48.5 – 30.4	C	5.1	-11.0 – 21.3	A	-4.7	-33.9 – 24.4	A
Tyr	5.49	-82.2 – 93.2	A	2.7	-20.0 – 25.4	A	4.86	-38.5 – 48.2	A
Val	-2.35	-16.3 – 11.6	F	1.4	-8.0 – 10.8	F	-0.94	-17.0 – 15.1	F

* A, methods are interchangeable; B, absolute mean difference between two methods has a positive value exceeding 15% of mean concentration for all measurements; C, absolute mean difference between two methods has a negative value exceeding 15% of mean concentration for all measurements; D, absolute mean difference becomes proportionately more positive the higher the analyte concentration; E, absolute mean difference becomes proportionately more negative the higher the analyte concentration; F, absolute mean difference increases with analyte concentration.

Table 6

Method comparison

Method	GC-MS	LC-MS/MS	Amino acid analyzer
Protein precipitation	No	Yes	Yes
Sample volume	50 µL	40 µL	200 µL
Runtime	20 min	25 min	130 min
Number of amenable analytes	33	42	42
Cost per analysis	6 €	14 €	10 €
LOQ [µM]	0.3–30	0.5–50	2–3