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Rapid plasma-citrulline measurement by UPLC-MS/MS to determine enterocyte-mass reduction

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Introduction

Plasma citrulline is a biomarker of enterocyte dysfunction because it is almost exclusively synthesized in the intestine with only minor amounts being generated in the endothelium and by the urea cycle in the liver. Once released into the circulation, the amino acid is rapidly converted to arginine in the kidney (1).

Currently, free plasma citrulline usually is measured as part of an amino acid analysis requiring a multistep derivatization and minimal 16 minutes of chromatography (2). Liquid chromatography tandem mass spectrometry (LC-MS/MS) has been introduced for citrulline measurement but critical details about sample preparation conditions lacked (3). We wanted to develop a state of the art method for monitoring the intestinal function of patients on intensive chemotherapy (4). Accordingly, we evaluated ultra performance (UP)LC-MS/MS, and hydrophilic interaction (HILIC) chromatography which facilitates the use of acetonitrile as a solvent and thus promotes desolvation and ionization of the analytes with a 10-fold gain in the limit of detection (5). With acetonitrile (AcN) for extraction, an evaporation/reconstitution step was redundant to exchange the solvent. Also, interference by polar species from the matrix was eliminated because they elute after the analytes of interest (6).

Experimental

Materials and chemicals. L-citrulline (cat no 1.12117.0050) was obtained from Merck; the internal standard D_7-citrulline (L-citrulline-2,3,3,4,4,5,5-d_7, cat. No. D-6396) from CDN isotopes, Quebec. ULC/MS grade AcN, methanol, glacial acetic acid (no 489741) and formic acid (FA, no 589151) were supplied by Biosolve BV, Valkenswaard, The Netherlands. Stock solutions and working standard solutions were prepared in water, distributed in small aliquots and frozen at -80 °C.

Protein precipitation/extraction. We added 10 µL of plasma, or water as a blank, or standard solutions of L-citrulline at concentrations of: 10, 25, 50, 100 and up to 2000 µmol/L in distilled water to 50 µL of 0.1 mol/L HCl resulting in a final pH of 1.6. This was mixed by vortex with 1 mL AcN/H_2O 9:1 (v/v) in which the internal standard D_7-citrulline was dissolved at a concentration of 0.2 mg/L. After a further vortex mix the solution was centrifuged for 5 min at 16,000g and the supernatant was dispensed into autosampler vials.

UPLC-MS/MS system. Solvent delivery and sample introduction were performed using a Waters ACQUITY Ultra Performance LC system (Waters, Milford, MA, USA). Separation was performed on a HILIC column, 2.1 x 100 mm, packed with 1.7 µm particles designed to withstand 15,000 psi. Mobile phases A and B consisted of distilled water containing 0.1% (v/v) FA, and AcN with 0.1% FA, respectively. The gradient program was as follows: flow rate in all steps at 0.45 mL/min unless otherwise indicated. Initial: 90% B; 0 to 2.5 min: a gradient to 55% B; 2.5 to 3.5 min: 2% B; 3.5 to 4.9 min: reversion of the mobile phase to 90% B at a flow rate of 0.60 mL/min; 4.9 to 5 min; 90% phase B. We used full loop injection to introduce 10 µL of sample into the WATERS TQD system. L-citrulline and the deuterated IS under these conditions were eluted at about 1.55 min.

Fragments of L-citrulline and the IS were detected by selected reaction monitoring using mass-to-charge (*m/z*) transitions of 176→70 for quantitation and 176→113 for confirmation of L-citrulline and 180→74 for quantitation of the IS (figure 1 at the top shows the fragments), with a dwell time of 0.120 sec; cone gas was set at 50 L/h and cone voltage at 20.0 V; while collision energy was set at 22, 15 and 22 electron volt, respectively, for the above mentioned transitions at a delay time of 0.005 sec. All aspects of system operation and data acquisition were controlled using Masslynx v4.1 software with automated data processing using the Quanlynx Application Manager (Waters).

Method validation

In evaluating this new method, we first strived to reach the best possible precision with which it is possible to judge and eventually correct the accuracy of the

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Abbreviations: UPLC: ultra performance liquid chromatography; HILIC hydrophile interaction chromatography, MS/MS: tandem mass spectrometry; AcN: acetonitrile (methylcyanide); AP-EI: Atmospheric Pressure Electrospray Ionisation; IS: internal standard; AUC: area under the curve; FA: formic acid; Rt; retention time

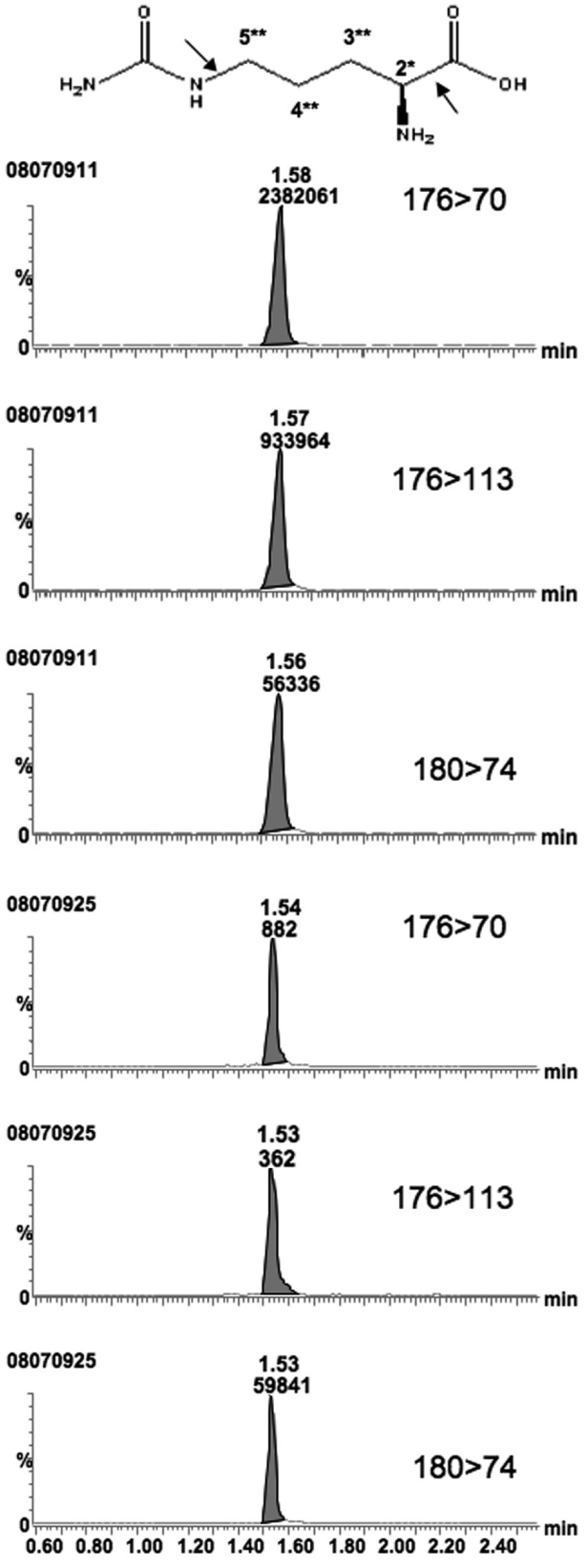

Figure 1. Top: molecular structure of L-citrulline (molecular weight 176) and breaking points to generate daughter fragments of *m/z*: 60; 72 and 113 (from left to right, respectively, the last fragment includes the middle). Below: registrations of peaks with the transitions: 176→70 for quantitation; 176→113 for recognition and reserve quantitation and 180→74 for quantitation of the IS. Samples had concentrations of 1958 µmol/L (AUC: 2382061) and 0.12 µmol/L (AUC: 882). No other peaks were present in the registration indicating excellent specificity. Ion suppression, if any, was between 0.65 and 1.31 min.

method. Experimentally we thus followed the NCCLS Approved Guidelines for Preliminary Evaluation of Clinical Laboratory Methods (EP10-A). Three samples with low, medium and high concentrations (as well as several standards) were analysed in triplicate (at specific order in the series) for 5 separate days. As an extension we spiked the basal sera L,M and H sera with 3 different amounts of the analyte to establish recovery. In this way, insight can be obtained into the within and between day imprecision, recoveries and accuracy, and a limited insight in linearity. The linearity study was extended to lower concentration to establish Limit of Quantitation (LOQ). For additional accuracy judgement we performed a correlation study with results obtained by the presently in house operating HPLC-fluorimetry. The limit of quantification (LOQ) was defined as the lowest concentration that could be measured with an imprecision <10%.

Results and discussion

Assay specific findings

The influence of acid. AP-ESI requires protonation; H^+-ions also favor retention to the HILIC columns at a phase of >80% AcN. We could demonstrate that with 0.1 N HCl (pH=1.6, thus < pK2 of citrulline), results were best with regard to the peak area and the response factor in comparison with several other acids.

Interferences: 1) Complete identity for standards prepared either in distilled water (x) or plasma with either low normal or normal citrulline concentrations (y) was observed after correcting for the initial basal concentration: y = 1.002 x+1.803 and y = 0.9986 x-1.9491, respectively. 2) During routine analysis the baseline did not fluctuate at all, demonstrating that any ion suppression (defined as a decrease of 25% in the base line between Rt 0.65 and 1.31 min) was absent (figure 1). This could be confirmed by an experiment in which a low amount of citrulline was infused at the time of sampling.

Efficiency. As shown in Figure 1, citrulline releases at about 1.55 min with a total run of 5 minutes. UPLC is thus minimally three times faster than the fastest HPLC variant with the additional advantage of a better peak separation

Method validation

Results on intra-day and interday precision, linearity, recoveries and method comparison are shown in table 1. The results with regard to the overall precision and accuracy stem with the promised quality of LC-MS/MS analysis: precision scores < 5 to 10%, perfect linearity over a 20,000 fold range; excellent recoveries and a perfect agreement with an in house HPLC-UV fluorescence method.

Sample material and stability

The use of serum, EDTA- or heparin plasma samples showed comparable results: whole blood samples with EDTA were stable for up to 72h at room temperature allowing overnight postage to the laboratory. Serum, EDTA- or heparin plasma was also stable for 72h at room temperature or for a week 4 ºC (5).

Reference values.
EDTA plasma of 20 adult males and 40 females (plasma creatinine values for both sexes <110 and 90 µmol/L, respectively and normal liver function) sent in by general practitioners gave plasma citrulline concentrations of 30.5 ± 8.8 µmol/L (mean and SD). Reference values derived from these data are: 13.0-48.1 µmol/L independent of gender and comparable with the literature where average values range from 31.1 to 40 with a SD of about 8 µmol/L (reviewed in 5).

Table 1. Summary of the method validation with regard to precision and accuracy

Precision

Concentration (µmol/L)	SD	CV (%)
Intra-day (n=20)		
5.6	0.2	4.3
34.9	0.8	2.3
64.3	1.3	2.0
Between-day (n=30)		
4.8	0.6	12.0
33.8	1.3	3.9
64.0	2.2	3.5

Recovery
Overall between 98.0 and 100.3%;
See example as a selection. Conc. in umol/L

Basal sample Conc.	Expected Conc.	Observed Conc. (mean ± SD)
5.7	74.0	72.0 ± 0.6
5.7	34.8	33.6 ± 0.8
5.7	15.2	15.3 ± 0.3

Linearity and LOQ
From 0.12 up to 1958 umol/L correlation coefficient >0.999
Completely identity of water- and plasma based calibrators
Limit of quantitation at a CV<10%: 0.3 umol/L with 10 ul sample.

Trueness
Citrulline content from the Sigma amino acid standard solution (product no 6407) after 10-fold dilution: 247.1 ± 7.1 ug/L (99.1 ± 2.8% of target value)

Method Comparison
Compared to HPLC-fluorescence in house method (x) with Passing and Bablok. 202 controls and patients on myeloblative therapy.
Slope: 1.028 (95% CI: 1.004-1.049)
Intercept: 0.301 (95% CI: 0.114-0.543)
Difference plot: an average difference of 0.39 umol/L (95% CI: 0.17-0.61 umol/L)

Citrulline measurement during myeloablative therapy
Following chemotherapy, plasma citrulline concentration gradually decreased until day 18 (= 7 days post stem cell transplantation) from average 25 to 5 µmol/L, followed by a gradual increase thereafter. Profound hypocitrullinemia appears to predict bacteraemia (7).

Conclusion
The UPLC-MS/MS method described here provides a robust means of rapidly measuring citrulline concentrations for use as a quantitative biomarker of functional enterocytes in different disease states in humans.

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