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Plasma citrulline measurement using UPLC tandem mass-spectrometry to determine small intestinal enterocyte pathology

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ABSTRACT

Citrulline is a nonessential free amino acid, detectable in various biological fluids such as plasma, urine and cerebrospinal fluid. The plasma citrulline concentration is increasingly considered to be a reliable biomarker of enterocyte function. Current analysis usually involves lengthy HPLC separations as a part of classical amino acid profiling, or mass spectrometry usually in combination with derivatization. We employed UPLC-HILIC–tandem mass-spectrometry (MS/MS) of acetonitrile-derived supernatants from plasma samples of control subjects and of patients who had received myeloablative chemotherapy. Detection was achieved by the selected reaction monitoring of transitions: m/z 176 → 70 and 180 → 74 (for the deuterated standard), respectively. The method was precise and accurate with inter-day CV < 3.9% (n = 30), recoveries ranging from 98.0 to 100.3% and high linearity from 0.3 to at least 2000 μmol/L. The results for 202 plasma samples agreed well with those obtained by the classical HPLC-fluorescence method. By a simple protein precipitation/extraction step and the UPLC separation the result can be available within 30 min of receipt with a capacity of at least 12 assays per hour. Citrulline in blood and plasma or serum was stable for at least 2 days at room temperature which would permit postal transport to the laboratory. The UPLC–MS/MS method for measuring plasma citrulline concentrations is fast and robust and is therefore an ideal tool for monitoring the intestinal enterocyte capacity of patients with various pathological conditions.

1. Introduction

Recent studies in patients with short bowel syndrome, villous atrophy syndrome, Crohn's disease and acute mucosal enteropathy as well as those receiving anti-neoplastic treatments have highlighted the significance of citrulline as a biomarker of enterocyte dysfunction (reviewed in [1–3]). The utility of citrulline for this purpose is due to the fact that 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 taken up by the kidney where it is rapidly converted to arginine [1,2].

Currently, free plasma citrulline usually is measured as part of an amino acid analysis which is expensive. Some variant methodologies using columns with particle sizes of 2–3 μm could reduce the time needed for chromatography to about 16 min [4,5]. To enhance sensitivity and selectivity a multistep pre-analytical step including derivatization is also obligatory, with the drawback of suboptimal scores in quality control programs [6–10]. In a recent publication liquid chromatography tandem mass spectrometry (LC–MS/MS) was used on dried blood spot samples though critical details about sample preparation conditions were lacking [10]. Furthermore, citrulline concentrations from dried blood spot show large variations compared with results obtained with plasma [11]. Thus, to meet the anticipated increase in number of requests for plasma citrulline assays there is a need for a rapid and robust analytical method which covers a wide concentration range for normal levels in excess of 40 μmol/L to the very low concentrations encountered in patient samples following intensive chemotherapy [3]. Accordingly, we evaluated ultra performance (UP)LC, because we reasoned that the increased specificity of tandem mass measurements only needs separations of compounds suppressing ion response. We were also encouraged by our previous experience with the development of a HPLC-fluorescence method and the availability of suitable hydrophilic interaction (HILIC) chromatography columns that require mobile phases that are high in organic

Abbreviations: UPLC, ultra-performance liquid chromatography; HILIC, hydrophile interaction chromatography; MS/MS, tandem mass spectrometry; AcN, acetonitrile (methylcyanide); AP-EI, atmospheric pressure electrospray ionization; IS, internal standard; AUC, area under the curve; Rt, retention time.

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content. Due to their volatility, desolvation and ionization of the analytes is promoted resulting in increased electrospray ionization mass-sensitivity yielding a 10-fold lower limit of detection. With acetonitrile (AcN) for extraction, an evaporation/reconstitution step is not only redundant, but also eliminates interference by polar species from the matrix because they elute after the analytes of interest [12].

A rapid and robust method for measuring plasma citrulline concentrations would also allow the results to be used for monitoring the intestinal enterocyte capacity of patients with various pathological conditions.

2. Experimental

2.1. Materials and chemicals

L-Citrulline (cat. no. 1.12117.0050) and the various acids were obtained from Merck; the internal standard d_7-citrulline (L-citrulline-2,3,3,4,4,5,5-d_7, cat. no. D-6396) was purchased from CDN isotopes, Quebec and contained 65 atom% deuterated atoms, divided over 99.27% 2,5,5-d_3 and 39% 3,3,4,4-d_4. Water was prepared by USF-ELGA, Rossmark, Ede, The Netherlands and ULC/MS grade AcN, methanol, glacial acetic acid (No. 489741) and formic acid (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.

2.2. 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 16,000 × *g* after which the supernatant was dispensed into autosampler vials. This protein precipitation/extraction at a pH < p*K* of 2.4 occurs theoretically at maximal solubility [2,12].

2.3. UPLC–MS/MS system

Solvent delivery and sample introduction were performed using a Waters ACQUITY Ultra Performance LC system (Waters, Milford, MA, USA) equipped with a thermostat for both the sample and column compartments which were maintained at 4 and 55 °C, respectively. A Waters ACQUITY TQ tandem quadrupole mass spectometer, interfaced with an atmospheric pressure electrospray ionization (AP-ESI) source was used for the analysis. Separation was performed on a HILIC column, 2.1 mm × 100 mm, packed with 1.7 μm particles (ACQUITY UPLC BEH HILIC, part No. 186003461, from Waters) designed to withstand 15,000 psi. Mobile phases A and B consisted of distilled water containing 0.1% (v/v) formic acid, and AcN with 0.1% formic acid, respectively. The gradient program was as follows: flow rate in all steps at 0.45 mL/min unless otherwise indicated. Initial: 90% B; 0–2.5 min: a gradient to 55% B; 2.5–3.5 min: 2% B; 3.5–4.9 min: reversion of the mobile phase to 90% B at a flow rate of 0.60 mL/min; 4.9–5 min; 90% phase B. We used full loop injection to introduce 10 μL of sample into the system. L-Citrulline and the deuterated IS under these conditions were eluted at about 1.55 min.

The AP-ESI was operated in the positive ion mode. Nitrogen was used as the nebulizing and desolvation gas at a flow rate of 100 and 1000 L/h, respectively; argon at a pressure around 3×10^{-3} mbar was used as collision gas at a flow rate of 15 mL/h. The ion source and the desolvation temperature were maintained at 130 and 400 °C, respectively. 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 (Fig. 1 at the top shows the fragments), with a dwell time of 0.120 s; 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 eV, respectively, for the above mentioned transitions at a delay time of 0.005 s. 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).

3. Method validation

3.1. Testing ion suppression

Under continuous registration, we infused a constant flow of 0.5 μmol/L citrulline solution directly in the MS while we also started the autosampler to inject once 10 μL of a plasma extract at a concentration of about 40 μmol/L standard diluted with HCl and AcN as described above.

3.2. False positive interference by blanks

To select the best option for protein precipitation/extraction, we paid attention to the false positive signals at the selected transitions of the respective blanks. In addition, we tested whether the transition 176 > 113 which we usually used for recognition resulted in more accurate values when applied for quantitation, versus the transition 176 > 70. This is of special interest when analysing low concentrations of citrulline. However, throughout this study, the transition 176 > 70 could be used.

3.3. Linearity and limit of quantification

On different occasions, we measured calibration curves by analysing standards either in distilled water or plasma with low normal or normal citrulline concentrations. The least-squares linear regression curves were calculated by use of Excel software. We applied the CLSI-EP6 protocol to test the linearity of the method. The dilutional linearity and limit of quantification (LOQ) of the assay were performed by serial dilution of both the aqueous standard sample as well as the low value plasma sample with water. LOQ was defined as the lowest concentration that could be measured with an imprecision <10% on multiple occasions by different operators.

3.4. Imprecision

We determined intra- and inter-assay variation by use of 3 plasma samples with low, medium and high concentrations of citrulline and obtained intra-assay imprecision from 20 replicates measured in a single series and inter-assay imprecision from 30 assays over a 30 days period.

3.5. Recovery

We estimated mean relative recoveries by adding three different concentrations of citrulline to a plasma sample with low basal citrulline concentration and measured recoveries in six replicates of these samples.

3.6. Method comparison

202 plasma samples from normal individuals as well as from patients who had received myeloablative chemotherapy were used for method comparison. All samples had been stored at −80 °C.

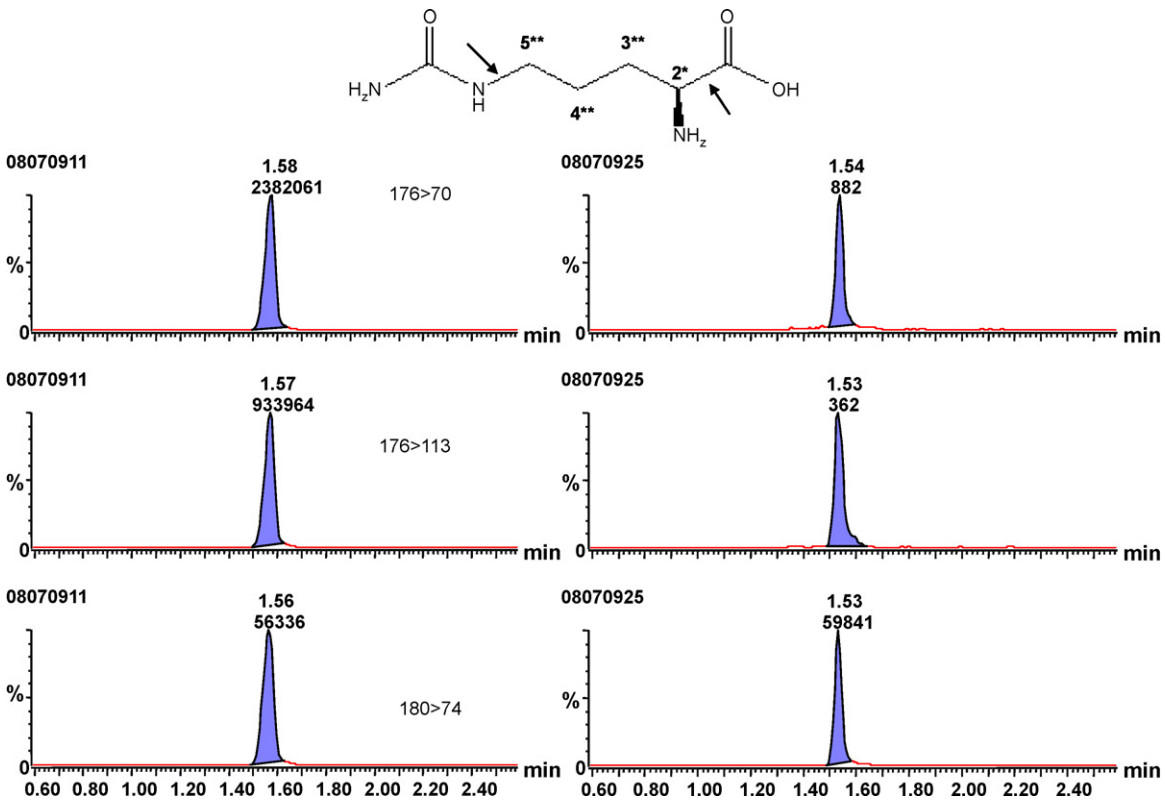

Fig. 1. (Top) Molecular structure of L-citrulline ($M+H^+ = 176$) and breaking points to generate daughter fragments of *m/z*: 60; 72 and 113 (from left to right, respectively, the last fragment includes the middle). (Below) Registrations of peaks with the transitions: 176 → 70 for quantitation; 176 → 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.

The UPLC–MS/MS method was compared to a HPLC-fluorescence method with on-line derivatizations with o-phtaldialdehyde [4,6]. We applied Passing–Bablok regression analysis [13] to compare methods.

3.7. Sample material and stability

We obtained both serum, EDTA- and heparin plasma from 20 subjects to compare citrulline concentrations in the various blood collection tubes. Stability in whole blood was tested in EDTA anti-coagulated blood of four subjects that had been stored for 4, 24, 48 or 72 h at room temperature or at 4 °C. Stability in serum, EDTA- or heparin plasma was tested in samples from three subjects with basal citrulline levels of 20, 30 and 95 μmol/L, respectively, that had been stored at room temperature or at 4 °C for 24, 48 h or 7 days, or frozen at −20 °C and were reanalyzed after one to three freeze-thaw cycles.

3.8. Biological variation and reference values

We determined biological intra- and inter-day variation by analyzing 11 plasma samples from five patients on the day of transplantation as well as 1 week later that have been obtained at regular intervals during the day between 9.00 a.m. and 9.00 p.m. To establish reference values, we selected 60 EDTA plasma samples of 20 adult males and 40 females sent in by general practitioners (plasma creatinine values for both sexes <110 and 90 μmol/L, respectively, and normal liver function). The studies were approved by the local Ethics Committee.

4. Results and discussion

4.1. Optimizing the protein precipitation/extraction step and analyte separation in the UPLC-HILIC–MS/MS method.

4.1.1. The influence of acid

AP-ESI requires protonation. Hence H^+-ions have to be added to the system, preferably in the mobile phase. In addition, these ions also stimulate retention to the HILIC columns when highly organic phases like >80% AcN are used. Like all amino acids, citrulline is a biacid; it has two p*K*s: p*K*1 at about 9.4 and p*K*2 about 2.3 [2,11]. At neutral pH the molecule behaves as a zwitterion favoring partition into the high organic phase as a function of the hydrophobicity of the mobile phase. Below p*K*2 a higher ionic strength contributes. We tested the magnitude of the signal as function of the pH of the sample, using various strong and weak acids at maximal organic phase composition of 90% AcN. Our results showed the assay in which 10 μL of sample was added to 50 μL 0.1 mol/L HCl, before mixing with AcN + IS, was superior to the other variants tested. The signals for the standards and IS were highest; moreover, the IS signal in the plasma or serum samples was >90% of that in the aqueous samples (or calibrators and blanks) when 10 μL (not 50 μL) of sample was protein precipitated/extracted. In all other acids and sample volumes tested, the IS signal in the plasma samples was ≤ 50% of that in the aqueous samples. Nevertheless, the response factors (area L-citrulline/area IS) with the use of different acids as proton donors were always comparable, resulting in similar end-concentrations and reflecting the validity of the IS to control the protein precipitation/extraction under all conditions tested.

Table 1
Intra- and inter-day imprecision for three plasma samples with low, intermediate and high (patho)physiological concentrations of citrulline.

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

SD, standard deviation and CV, variation coefficient.

4.1.2. Ion suppression

When present, ion suppression (i.e. a decrease of 25% in the base line between Rt 0.65 and 1.31 min) was outside the typical Rt for citrulline (at 1.55 min). In fact, the IS coelutes with L-citrulline which compensates for matrix effects if present. During routine analysis the baseline did not fluctuate at all demonstrating that ion suppression is not significant (Fig. 1).

4.2. Assay validation

4.2.1. Linearity and limit of quantification

Linearity was present up to at least 1958 μmol/L. Fig. 1 shows the lowest and highest concentration with excellent chromatographic characteristics at high levels. Throughout this study, correlation coefficients >0.999 were observed. 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: $y = 1.002x + 1.803$ and $y = 0.9986x - 1.9491$, respectively.

Linearity and absence of an intercept were found at high dilutions both for the aqueous standard sample as well as the low value plasma sample; and the latter was independent of the use of 176 > 113 or 176 > 70 as transition. An estimated quantification limit of 0.3 and 0.6 μmol/L, respectively, was established for both transitions when analysing 10 μL of sample.

4.2.2. Imprecision

The inter-day CV was about twice as high as the intra-day CV (Table 1). The low value control had a CV of 12% inherent in the 6–10-fold difference in concentrations. Nevertheless, the absolute SDs were the lowest (Table 1).

4.2.3. Recovery

As already apparent from the spiking experiment to plasma showing high linearity, recoveries were satisfactory with mean recoveries of between 98.0 and 100.3% as shown in Table 2.

4.2.4. Method comparison

Results for the 202 plasma samples showed excellent agreement between the results observed by the HPLC-fluorescence method (x) and the UPLC-HILIC–MS/MS method (Passing and Bablok analysis). The slope was 1.028 (95% CI: 1.004–1.049), while the intercept was 0.301 (95% CI: 0.114–0.543) (Fig. 2). The difference plot revealed an average difference of 0.39 μmol/L (acceptable range: 0.17–0.61 μmol/L). As shown in Fig. 1, citrulline releases at about 1.55 min, at a total run of 5 min; the latter value can even by shortened given the specificity of the method. In our present

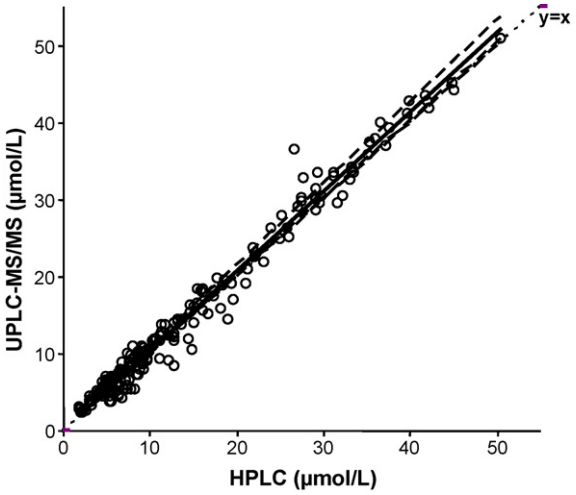

Fig. 2. Comparison of pooled results by the HPLC-fluorescence method and the UPLC–MS/MS method for 202 samples. Bracket lines: 95% CI; dashed line: line of identity ($y = x$).

HPLC-fluorimetry method citrulline releases at 18.5 min with a run duration of 25 min; while in a published rapid variant method citrulline releases at 7 min at a total run time of 12 min [5]. Thus colums with more theoretical plates have already decreased run time in HPLC. Nevertheless, the UPLC is still three times faster than the fastest variant with an additional advantage of a better peak separation. However, also the very simple extraction step contributes to the efficiency of the present method. In addition, a derivation step is redundant. Very recently, a total amino acid method with MS/MS was published [14] with a Rt for citrulline of 1.7 min, rather similar to our results, but, with a relatively high imprecision (CV of 17%) and a suboptimal accuracy (a slope of 0.68 with a comparison method and a recovery of 116%). In addition, the work-up looks extensive including an additional evaporation and a derivatization step.

4.2.5. Sample material and stability

Comparing serum, EDTA- or heparin plasma samples of 20 subjects showed that the citrulline concentrations did not differ significantly as the slopes and intercept were within the limits for agreement (results not shown). This underlies the specificity of our method at variance with HPLC-fluorescence methods requiring oxidative derivatizations. As discussed by Chuang et al. [15] EDTA and metabisulphite contaminating heparin from the anticoagulants interfere in various ways.

The samples of whole blood in EDTA of 4 subjects were stable for up to 72 h, irrespective whether they were stored at room temperature or at 4 °C (Fig. 3a). Serum, EDTA- or heparin plasma of 3 subjects with basal citrulline levels of 20, 30 and 95 μmol/L, respectively, appeared stable when stored at room temperature for up to 48 h, or for 7 days at 4 °C (Fig. 3b). If stored at ambient temperature for 7 days, citrulline averaged 4.3 ± 2.6 μmol/L (range 0.6–8.7 μmol/L) or 11 ± 6% (range 3–19%) higher, irrespective of whether serum, heparin- or EDTA plasma was tested (Fig. 3b). Finally, concentrations were stable in serum or plasma samples that

Table 2
Mean recoveries of the UPLC-MS/MS assay for plasma citrulline.

Basal sample concentration (μmol/L)	Expected sample concentration (basal + spiked) (μmol/L)	Observed sample concentration mean (±SD) (μmol/L)	Recovery mean (±SD) in % (n = 6)
5.7	74.0	72.1 ± 0.6	98.0 ± 0.8
5.7	34.8	33.6 ± 0.8	96.6 ± 2.2
5.7	15.2	15.3 ± 0.4	100.3 ± 2.2

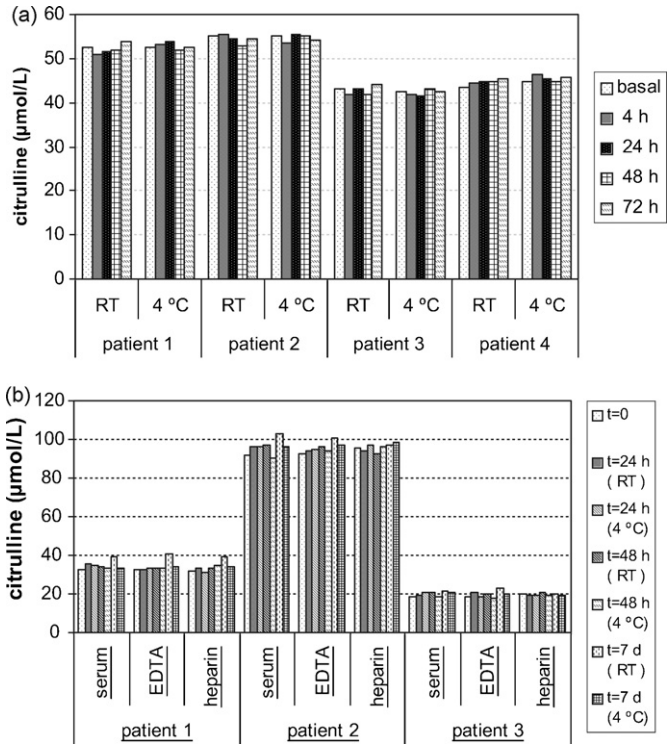

Fig. 3. Storage experiments of whole blood or serum and plasma. (a) Stability of whole blood of 4 subjects stored at room temperature or at 4 °C for up to 72 h. (b) Stability of serum, EDTA- or heparin plasma of three subjects stored for up to 7 days at room temperature or at 4 °C.

had been subjected to 1, 2 or 3 freeze-thaw cycles. The results support the conclusion that whole blood anticoagulated with EDTA can be send to the laboratory by overnight postage. For reasons of accuracy we would not choose for blood spots [11], but rather for small vials or capillaries. The other findings are more of value in research settings.

4.2.6. Reference values

Mean and SD plasma citrulline concentrations were 30.5 ± 8.8 μmol/L and reference values derived from these data were 13.0–48.1 μmol/L independent of gender. For comparison: reference values among Caucasians of 40 ± 10 μmol/L [16], 38 ± 8 μmol/L [17], 38.1 ± 6.4 μmol/L [18], 40 ± 10 μmol/L [19], and 32 ± 9 μmol/L [5] have been reported. The differences are minor and are likely ascribable to the selection of the study population. Furthermore, serious compromised glomerular filtration rate potentially increases circulating plasma citrulline levels (reviewed in [2]). It can be questioned whether these reference values are also suitable for neonates and children. Obviously, these subjects have less enterocyte surface, but any release of citrulline from it likely is diluted in a lower plasma volume and is metabolized by a smaller kidney. Alltogether, this equilibrium suggests a regulatory function of higher order. This equilibrium apparently explains that premature neonates [20] and virus-affected children aged 2 years [21], already have plasma citrulline concentrations typical for adults.

4.2.7. Biological variation during myeloablative therapy

Eleven plasma samples of five patients had been collected at regular intervals during the day of transplantation as well as 1 week later. The CV% within each subject on both days correlated highly (r^2 = 0.96; n = 5); over both days CV's averaged 10.0 ± 5.1% (range 4.8–18.2%). A once daily sampling therefore appears adequate to monitor enterocyte function. The magnitude of the biological variation permits following closely the effect of myeloablative chemotherapy by determining daily plasma citrulline concentrations. As illustrated in Fig. 4 there is a gradual decrease in plasma citrulline concentration from the first day of chemotherapy until nadir is reached on day 18 (=7 days post-stem cell transplantation) followed by a gradual increase thereafter.

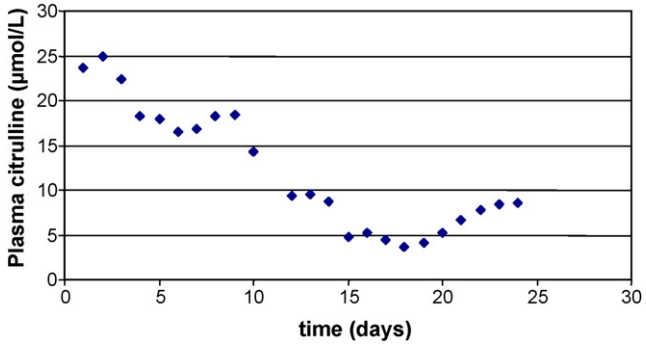

Fig. 4. Course of plasma citrulline levels in a single patient during myoablative therapy.

A decreased level of citrulline correlates with a reduced functional enterocyte mass indicating malfunction of the small intestines. This is independently of the inflammatory or nutritional status of patients with various conditions including short bowel syndrome, bowel ischaemia, Coeliac disease, as well as following intestinal transplantation, cytoreductive therapy and therapeutic or accidental irradiation [1–3,22–24]. A rapidly available result of citrulline in daily clinical practice would enable the clinician to precisely monitor the course of intestinal dysfunction and would offer him or her a tool to manage a patient with signs of intestinal failure which is also relevant in premature children [20]. In the setting of a stem cell transplantation after high-dose chemotherapy profound hypocitrullinema appears to predict bacteraemia [25]. In situations of intestinal failure monitoring citrulline might help direct the use of nutritional support.

5. 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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