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Determination of citrulline in human plasma, red blood cells and urine by electron impact (EI) ionization gas chromatography–mass spectrometry

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Abstract

A method was developed by using gas chromatography–mass spectrometry in the electron impact ionization mode to quantify citrulline in plasma, red blood cells (RBC) and urine. For all three fluids, citrulline was extracted on ion exchange resins, before derivatization to its propyl-heptaflorobutyryl-ester. Assay precision (coefficient of variation, CV) was <5%, recovery% was >90% and the within- and between-day CV were <10% on 200 μL of plasma and RBC, and 400 μL of urine. The current method allows for the detection of 20 pmol of natural citrulline in aqueous standards, and small volumes (<100 μL) of biological fluids.

Keywords: Nutrition; Protein; Amino acids; Stable isotopes

1. Introduction

Citrulline is a non-essential amino acid that is not incorporated into protein, and is present as a free amino acid in most biological fluids such as plasma, urine and cerebrospinal fluid [1–4]. Two main pathways account for citrulline biosynthesis [5,6]. First, citrulline is produced from glutamine in reactions that are generally considered part of the urea cycle by condensation of ornithine and carbamyl phosphate. Second, citrulline can also arise from the conversion of arginine to nitric oxide (NO) by nitric oxide synthetase (NOS) in most tissues. Conversion of arginine to citrulline accounts for ≈10% of circulating citrulline flux, implying that the rest, nearly 90%, must arise from glutamine [7–9]. In the liver, citrulline is quantitatively utilized by arginosuccinate synthase (ASS) for urea synthesis, so the liver does not release any free citrulline. In contrast, the citrulline formed in the gut is released as such into the bloodstream, and taken up by the kidney, where it can be converted to arginine by ASS. There is, therefore, no significant extra intestinal source of circulating citrulline.

Recent studies have suggested plasma citrulline concentration may be used as an index of small intestinal mass or function. Plasma citrulline indeed correlates with residual bowel length in patients with short bowel syndrome [8,9], and with small bowel function in patients with celiac disease [10], intestinal graft rejection [11,12], or radiation-induced intestinal damage [13]. Moreover, rodent studies suggest citrulline may have a protein anabolic effect in specific clinical conditions, as it enhanced nitrogen balance after extensive small intestinal resection [14], and muscle protein synthesis in old, undernourished rats [15].

Several methods have been used to determine citrulline concentrations in human plasma such as the diacetylmonoxime method [16], ion exchange chromatography [17], reversed phase high-pressure liquid chromatography (HPLC) [18–21],

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HPLC–mass spectrometry [2,22], HPLC–fluorescence [23–24] and gas chromatography–mass spectrometry [25,1]. HPLC has been used to measure citrulline in cerebrospinal fluid [26], and LC–MS for brain tissue citrulline assay [27]. Yet, fewer methods have been validated for quantifying citrulline in urine [28–29,2] and no data concerning citrulline concentrations in blood cells have been reported.

In this context, it is of interest to improve our understanding of citrulline metabolism in humans. The aim of the current study was to develop a robust and sensitive method to measure citrulline concentrations in human plasma, blood cells and urine, by using gas chromatography–mass spectrometry (GC/MS).

2. Experimental

2.1. Chemicals and standards

All chemicals (propan-1-ol, acetylchloride, hydrogen chloride, ammonium hydroxide, potassium hydroxide, 5-sulfosalicylic acid, ethylacetate and heptafluorobutyric anhydride), citrulline (L-2-amino-5-ureidovaleric acid) and ion exchange resins (Dowex 50WX8-200 and Dowex 1X8) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Homocitrulline (ε-carbamoyl lysine) was obtained from Advanced Asymetrics Inc. (Millstadt, USA) and L-[2,3, 3, 4, 4, 5, 5-$^2H_7$] citrulline (d_7-citrulline) from CDN Isotopes (Cil Cluzeau Info Labo, France).

2.2. Sample preparation

2.2.1. Calibration curves

On each day of analysis, two sets of standard curves were prepared to permit determination of citrulline concentration in plasma, red blood cells (RBC) and urine samples. Standards were prepared at concentrations similar to those found in biological samples. The first set of standards (for plasma and RBC citrulline determination) were prepared with 100 μL of 0.1 mol/L homocitrulline, as an internal standard and graded known volumes of a 0.1 mol/L citrulline solution (0–400 μL). As native urine contained a small amount of homocitrulline (data not shown), another internal standard, d_7-citrulline, was used for urine citrulline quantification. The second set of standards (for urine citrulline determination) was prepared with 100 μL of 0.1 mol/L d_7-citrulline and graded known volumes of a 0.1 mol/L natural citrulline solution (0–400 μL).

2.2.2. Plasma and RBC preparation

Blood samples (5 mL) were drawn into glass tubes containing EDTA. Blood cells and plasma were immediately separated by centrifugation at 3000 × *g* for 10 min at 4 °C, plasma was collected using a Pasteur pipette, and transferred into a new tube. Plasma and RBC were stored at −80 °C until analysis. Before biological samples were analyzed, a 10 nmol aliquot of 0.1 mol/L homocitrulline was added to a 200-μL aliquot of plasma or RBC. Blood cells were disrupted by adding 400 μL of distilled water. Plasma and blood cells were deproteinized with 100 or 200 μL of 10% (w/v) 5-sulfosalicylic acid (10% SSA), respectively. The same extraction and derivatization protocols were used for plasma and blood cell samples. Deproteinized plasma and blood cells were first acidified to pH < 2 with 400 μL of 0.1 mol/L HCl, and then poured on top of microcolumns containing 1 mL of Dowex 50 cation exchange resin. The cation-exchange columns were washed with 10 mL of deionized water, and citrulline was eluted with 2 mL of 6 mol/L NH_4OH to raise pH above 10. The eluate was dried under nitrogen, and derivatized to its propyl heptafluoro-butyryl derivative. Each dried fraction was first dissolved with 600 μL of a 1/5 (v/v) mixture of acetylchloride/ice-cold propanol, and incubated at 110 °C for 30 min. Each sample was then evaporated under a stream of nitrogen, spiked with 50 μL of heptafluorobutyric anhydride (HFBA), and incubated at 60 °C for 30 min. The derivatized citrulline was dried under nitrogen and dissolved in 100 μL of ethylacetate until analysis by GC/MS.

2.2.3. Urine preparation

Urine samples were aliquoted soon after collection and stored immediately at −80 °C until analysis. Even though the derivatization was the same as for plasma, a different extraction procedure had to be used for urine. A 10 nmol aliquot of d_7-citrulline was added to 400 μL of urine. Two milliliter of a 1 M KOH solution were first added to each 400 μL urine sample to reach a pH > 9, and samples were then poured on top of microcolumns containing 1 mL of Dowex 1X8 anion exchange resin. The eluate was collected, and columns were further washed with 3 mL of 1 mol/L KOH, to achieve more extensive citrulline elution. The citrulline-containing eluate was acidified to pH < 2 with 3.5 mL of 1 M HCl, and poured on top of microcolumns containing 1 mL of Dowex 50WX8 cation exchange resin. Columns were washed with 10 mL of deionized water, and citrulline was eluted with 2 mL of 6 mol/L NH_4OH to raise pH above 10. The eluate was dried under nitrogen, and derivatized to its propyl heptafluoro-butyryl derivative, as described for plasma and RBC. The derivatized citrulline was dried under nitrogen and dissolved in ethylacetate until analysis by GC/MS. A volume of 100 μL of ethylacetate was used for plasma and RBC samples, and 200 μL were used for urine samples, because the urine samples were subsequently filtered on a paper filter prior to injection into GC/MS. On each day of analysis, a derivatization blank was prepared with 200 μL of distilled water.

2.3. GC/MS analysis

Citrulline analysis was performed using an HP 5890 series II® gas chromatograph coupled with a 5971® mass selective detector (Hewlett-Packard, Palo Alto, CA, USA) using a DB-1® (30 m × 0.25 mm ID, 0.25 μm film thickness; J&W Scientific, Courtaboeuf, France) capillary column operated in the splitless mode. Injector and detector temperatures were 250 and 290 °C, respectively. The helium carrier flowed at a rate of 1.2 mL/min. The oven temperature was initially set at 80 °C, and then ramped from 80 to 170 °C at a rate of 15 °C/min, and raised to 220 °C at a rate of 8 °C/min, and then raised at 250 °C at a rate of 50 °C/min and maintained at 250 °C for 1 min. The mass spectrometer was operated in the electron impact (EI) ionization mode. The acqui-

sition time was 14 min. For each sample analyzed, a volume of 2 μL was injected into GC/MS system.

2.4. Human application

Blood and urine samples were obtained at 11 a.m., 3 h after breakfast, from five healthy adult male volunteers, according to procedures approved by the local ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale des Pays de la Loire). Urine samples were collected before breakfast from healthy adults who had been fasting overnight.

Biological samples were prepared after addition of 100 μL of 0.1 mol/L homocitrulline to 200 μL of plasma and red blood cells, and after addition of 100 μL, 0.1 mol/L d_7-citrulline to 400 μL of urine.

3. Results

3.1. Standards

The propyl-hepta-fluoro-butyryl ester derivatives of citrulline, homocitrulline and L-[2,3,3,4,4,5,5-$^2H_7$] citrulline (d_7-citrulline) were stable for several weeks when stored at 4 °C (data not shown). The complete mass spectra of these propyl-hepta-fluoro-butyryl ester derivatives obtained by GC/MS in the scan mode are shown Fig. 1. Citrulline and d_7-citrulline eluted at retention times of 8.09 and 8.07 min, respectively, and homocitrulline eluted at a retention time of 9.38 min. Chromatograms were free of interfering peaks. Monitoring of several ions (at m/z = 114, 309 for citrulline, 128 and 323 for homocitrulline, and 118 and 313 for d_7-citrulline) was used for confirmatory identification in biological samples. For quantitation, the monitoring of the prominent ions at m/z = 114, 128 and 118 for citrulline homocitrulline, and d_7-citrulline, respectively) afforded the best sensitivity, in the selected ion monitoring mode. Although these ions are in a low mass range, their high relative abundance was found to allow the detection of very small quantities of citrulline in biological samples.

3.2. Biological fluids

The chromatograms of citrulline, homocitrulline and d_7-citrulline obtained from the three biological fluids by GC/MS in the SIM mode were free of interfering peaks (Fig. 2).

3.3. Calibration curves

A typical standard curve obtained for each set of internal standards by plotting the 114/128 or 114/118 peak area ratios against citrulline/homocitrulline or citrulline/d_7-citrulline mole ratios is shown in Fig. 3. The linearity and slope were measured for each set of internal standards (Fig. 3a and b). High coefficients of correlation were consistently obtained for citrulline/homocitrulline ($y = 0.9443x + 0.013$, $r^2 = 0.9997$), as well as for citrulline/d_7-citrulline ($y = 2.2452x + 0.0324$, $r^2 = 0.9999$). The range covered by the calibration curves was between 0 and 40 nmol for a 200-μL plasma or RBC sample, corresponding to a range of blood citrulline concentrations between 0 and 200 μM, and 0–20 nmol in a 400 μL urine sample, corresponding to 0–50 μM citrulline concentration range in urine.

3.4. Assay validation

3.4.1. Assay precision of the GC/MS equipment

The assay precision (run-to-run variation) was determined by injecting the same biological sample (plasma, RBC, or urine: $n = 2$) five times consecutively into the GC/MS system (Table 1). Coefficients of variation (CV, %) calculated as: CV = 100 × SD/mean were consistently less than 5% for the biological samples.

3.4.2. Assay precision of the whole procedure

3.4.2.1. Assay accuracy. Accuracy was determined by measuring the recovery of known amounts of natural citrulline (10, 20 and 30 nmol) added to two aliquots of plasma, RBC and urine prior to sample processing. The recovery (%) calculated for each biological sample was 92 ± 7% for plasma, 95 ± 7% for RBC and 90 ± 1% for urine (Table 1). When the amounts of citrulline measured in biological samples were plotted against the spiked amounts, highly linear correlations were observed, with $r^2 = 0.9947$, 0.9978, and 0.9996 in plasma, red blood cells, and urine, respectively (Fig. 4a–c). Whereas the slope did not differ from 1 for red blood cells (Fig. 4b), it was, however, significantly below unity for plasma (Fig. 4a), and urine (Fig. 4c). The use of the "aqueous standard curve" would thus result in systematic quantification errors: for instance, 30 nmol/L spiked plasma would be measured as 33.07 nmol/L, whereas the expected value is 38.42 nmol/L (spiked plus endogenous amount), the difference is thus 14%. This finding underlines the difficulties of calibrating endogenous substances in biological matrices, and suggests that standard curves spiked with either plasma or urine should ideally be used for accurate quantification of citrulline in the corresponding biological matrix.

3.4.2.2. Within- and between-day reproducibility (Table 1). The precision of the GC–MS equipment, and that of the whole procedure, were assessed in separate experiments. The run-to-run reproductibility, reflecting solely the precision of the GC/MS equipment was determined by repeatedly injecting (3–5 times) the same sample (of either plasma, red blood cell, or urine) into the system. To determine the precision of the whole procedure, three separate aliquots of the same pool of plasma underwent separate extraction, derivatization, and analysis, either on the same day (to determine the within-day CV), or on days several weeks apart (to determine the between-day CV). For all the biological samples tested, the within-day and between-day coefficients of variation were consistently less than 10%, and within-day reproducibility was better than between-day (Table 1).

Table 1
Method validation

	Precision[a] of the GC/MS equipment (CV) (%)	Precision of the whole procedure (%)		
		Accuracy[b] (recovery)	Reproducibility within day[c] (CV)	Reproducibility between days[d] (CV)
Plasma	2.7	92 ± 7	4.2	4.4
RBC	3.8	95 ± 7	3.7	3.8
Urine	1.6	90 ± 1	6.2	9.1

[a] Precision (run-to-run variation) was measured by repeatedly injecting samples prepared from human plasma, red blood cell pellet and urine consecutively into the GC/MS system; coefficients of variation (CV, %) were calculated as: CV = 100 × SD/mean.
[b] Accuracy was determined by measuring recovery of known amounts of natural citrulline added to aliquots of plasma, red blood cell pellets and urine prior to sample processing.
[c] Within-day reproducibility was determined from the analysis by the GC/MS system, of three aliquots of a single plasma, red blood cell or urine sample that had been extracted, derivatized, in parallel, and were analyzed by GC/MS on the same day ($n = 5$).
[d] Between days reproducibility was determined by injecting into the GC/MS system, three aliquots of one plasma, RBC or urine sample that had been extracted, derivatized and analyzed on 2 different days.

3.4.2.3. Assay sensitivity. The assay sensitivity was determined on aqueous solutions of natural citrulline. The current assay allowed for the detection of 20 pmol of natural citrulline in aqueous solutions, and citrulline could be detected in very small volumes of biological fluids, down to 40, 30, 100 μL of plasma, RBC and urine, respectively, which contain approximately 1.6, 0.9, and 0.6 nmol citrulline, respectively. As derivatized samples were dissolved in a final volume of 100 μL ethylacetate (for plasma and RBC) or 200 μL ethylacetate for urine, and as 2 μL were injected, the amount of citrulline injected into the GC/MS was 31, 19, and 6 pmol citrulline for plasma, RBC, and urine, respectively.

3.5. Human application

The method developed in the current study was used for quantifying citrulline in plasma, RBC and urine samples from five healthy volunteers (Table 2). As the slope of the line describing the relationship between added amounts of citrulline and measured amounts is below 1 for plasma and urine (Fig. 4b and c), the true concentration of citrulline $[CIT]_{true}$ in plasma or urine was calculated as:

$$[CIT]_{true} = \frac{[CIT]_{apparent}}{k},$$

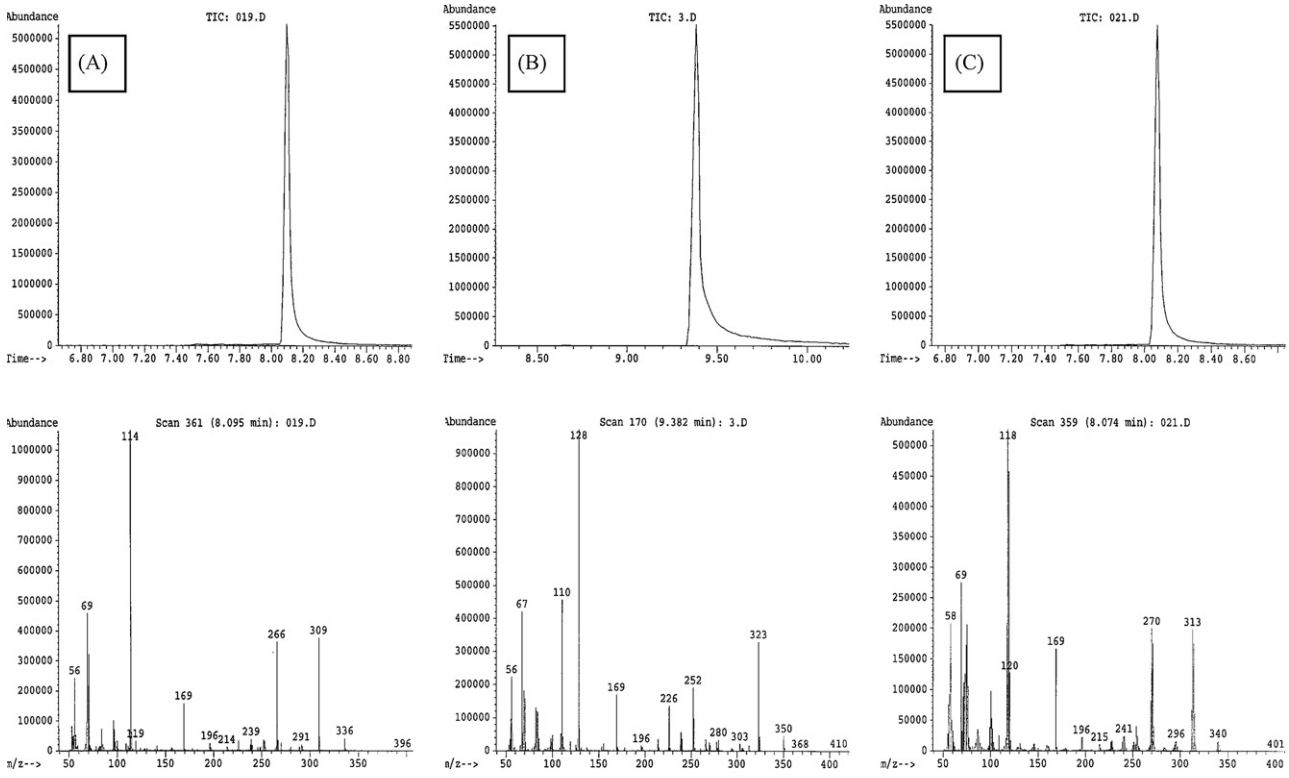

Fig. 1. Complete mass spectrum of propyl-hepta-fluoro-butyryl L-citrulline (A), propyl-hepta-fluoro-butyryl homocitrulline (B) and propyl-hepta-fluoro-butyryl d_7-citrulline (C), in aqueous solution (EI mode).

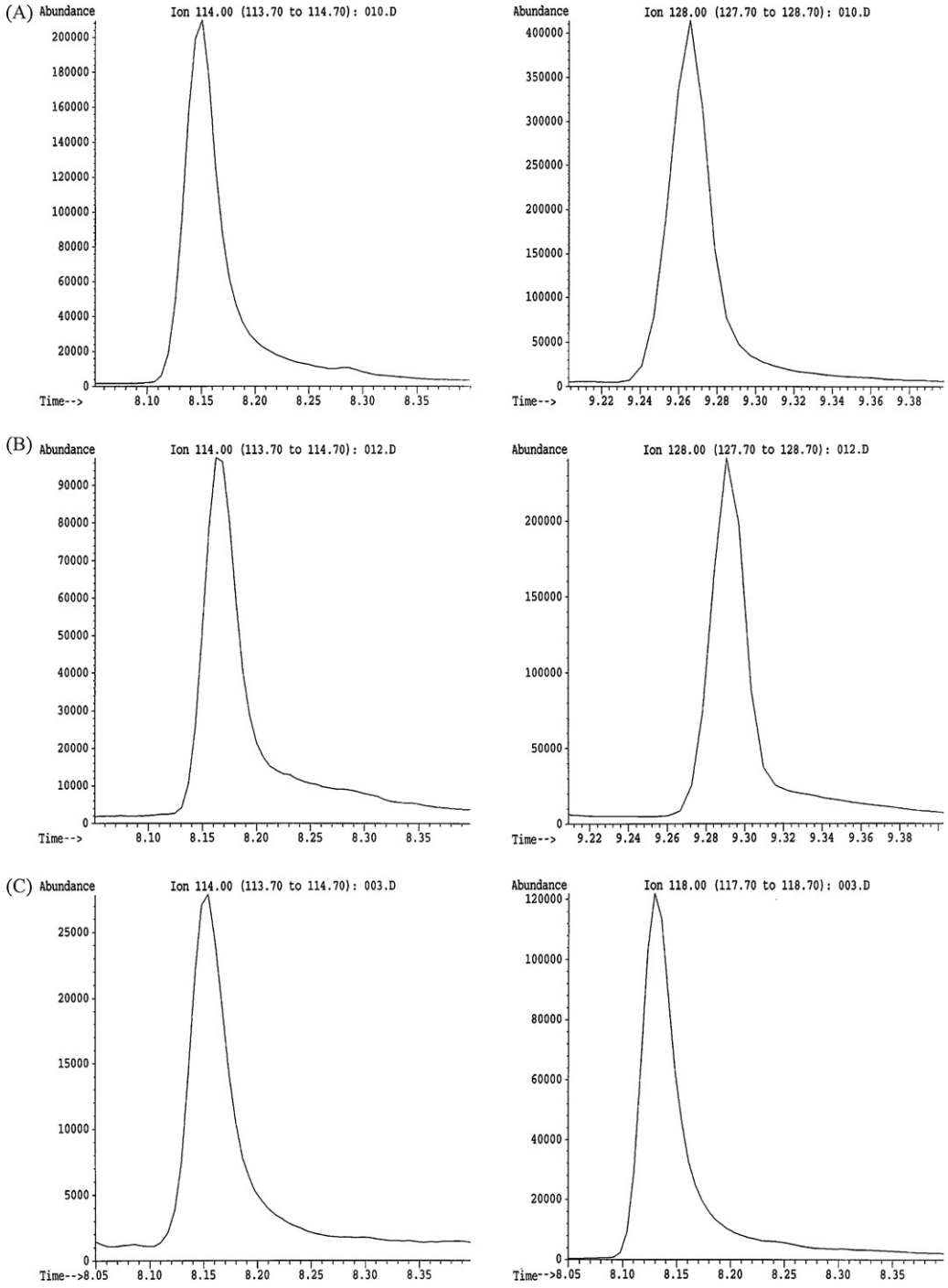

Fig. 2. GC/MS chromatogram of plasma citrulline (A), red blood cell citrulline (B) and urine citrulline (C), acquired in the selected ion monitoring mode.

where $[CIT]_{apparent}$ is the amount of citrulline calculated using the calibration curve constructed using aqueous standards (Fig. 3a and b for plasma and urine, respectively), and k, a correction factor, is the slope of the of the line describing the relationship between added amounts of citrulline and measured amounts in the corresponding matrix (e.g., 0.8218 and 0.8678 for plasma and urine, respectively; see Fig. 4a and c).

Plasma citrulline levels ($n = 5$) were $47 \pm 12\,\mu M$, and were slightly, but not significantly higher than RBC citrulline levels ($31 \pm 13\,\mu M$). Compared to plasma and RBC, urine citrulline levels were $7 \pm 3\,\mu M$, and thus considerably lower than those measured in plasma and RBC ($p < 0.001$ and $p < 0.01$, respectively, as tested using Student's t-test).

4. Discussion

To our knowledge, the current study is first to demonstrate that citrulline quantification can be performed by GC/MS operated in the electron impact ionization mode in very small volumes of

Table 2
Plasma, RBC and urine citrulline levels in five healthy adults, in fed state

Subject	Plasma citrulline (μM)	RBC citrulline (μM)	Urine citrulline (μM)
A	26.3	26.3	3.9
B	50.5	38.5	10.8
C	53.3	44.3	8.4
D	49.6	12.0	6.3
E	55.4	36.4	4.8
Mean	47.0	31.5	6.9[a,b]
SD	11.8	12.7	2.8

True citrulline concentrations were calculated as $[CIT]_{true} = [CIT]_{apparent}/k$, where $[CIT]_{apparent}$ is the amount of citrulline calculated using the calibration curve constructed using aqueous standards (Fig. 3a and b for plasma and urine, respectively), and k, a correction factor, is the slope of the line describing the relationship between added amounts of citrulline and measured amounts in the corresponding matrix (see Fig. 4a and c).

[a] Plasma versus urine ($p < 0.001$).

[b] RBC versus urine ($p < 0.01$).

three different biological matrices, such as human plasma, RBC and urine.

Although the extraction procedure for plasma and erythrocyte citrulline was adapted from Adams [30], the latter extraction procedure failed to yield a significant recovery of urinary citrulline. A different extraction scheme, using two sets of ion exchange resins, and adapted from that used for the separation of glutamine [31], was therefore used for urine citrulline.

The derivatization procedure leading to the formation of propyl-hepta-fluoro-butyryl ester derivatives has been used for many other amino acids, e.g., glutamine [32]. Although we tried

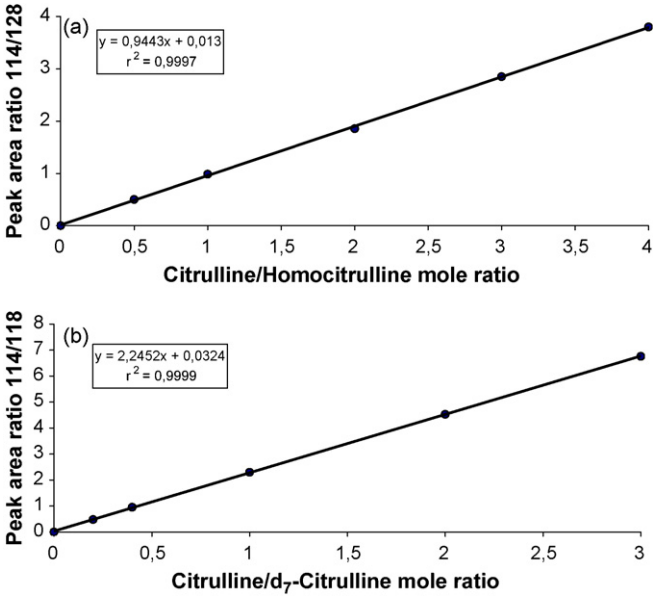

Fig. 3. (a) Standard curve for the GC/MS assay of citrulline, using homocitrulline as an internal standard. Relationship between 114/128 peak area ratio, and the citrulline/homocitrulline mole ratio in a set of standard aqueous solutions. (b) Standard curve for the GC/MS assay of citrulline, using L-[2,3, 3, 4, 4, 5, 5-$^2H_7$] citrulline as an internal standard. Relationship between 114/118 peak area ratio, and the citrulline/d_7-citrulline mole in a set of standard aqueous ratio solutions.

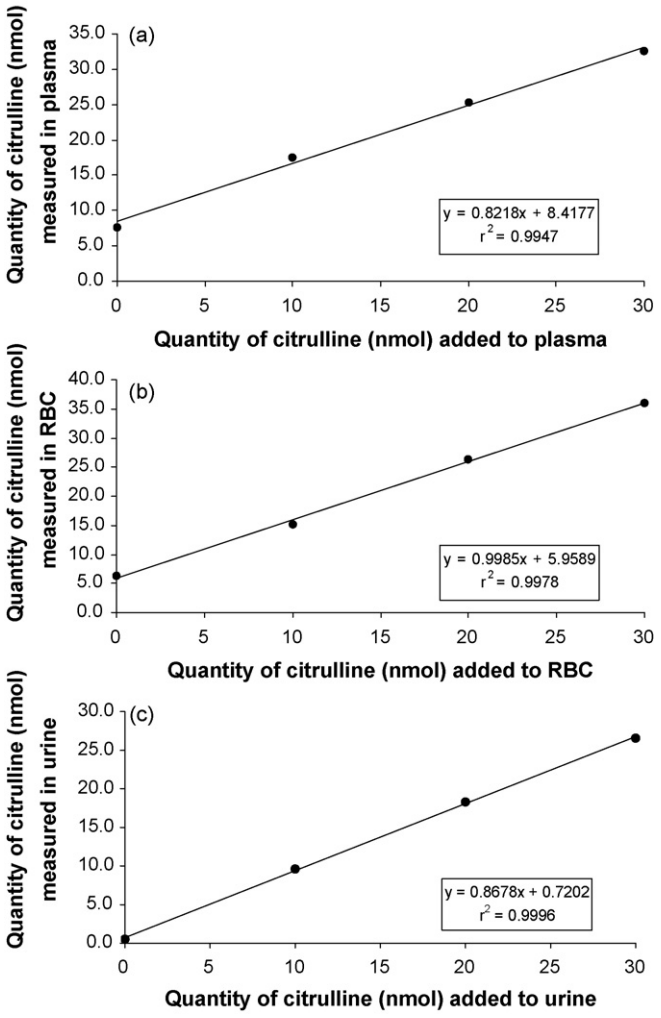

Fig. 4. (a) Relationship between the quantity of citrulline (nmol) measured with the current method in a biological sample, and the quantity of natural citrulline (nmol) spiked into that sample prior to extraction and derivatization: relationship is shown in plasma (a), RBC (b), and urine (c).

several derivatization procedures such as the tertbutyl, dimethylsilyl esters or methyl, heptafluorobutyl esters used by other workers [33,7], in our hands the use of propyl-hepta-fluoro-butyryl ester derivatives was found to produce better results in terms of peak shape, and stability of samples (data not shown).

Similarly, several internal standards such as thiocitrulline, $^{15}N_2$-citrulline and ^{13}C-citrulline were tried in the current study (data not shown): the best results were obtained for homocitrulline and L-[2,3, 3, 4, 4, 5, 5-$^2H_7$] citrulline. Indeed, calibration curves obtained for each set of internal standard were highly linear, with r^2 consistently between 0.999 and 1.000.

The current method proved sensitive, as attested by its low limit of detection (20 pmol of citrulline, i.e., less than 50 μL of plasma), and has satisfactory assay precision, reproducibility, and accuracy in the various biological fluids tested, as attested by the within-assay and day-to-day CV < 5%, and the >90% recovery. It is quite robust as derivatized samples can be stored for weeks prior to analysis, and assay can be performed using the electron impact mode of operation, a 'workhorse' instrument which

involves a lower cost of maintenance than chemical ionization GC/MS or liquid chromatography–mass spectrometry [34–35].

The plasma and urine citrulline levels obtained using the current method are consistent with those reported using other methods in similar in healthy adults [16,17,21,2,29]. We are not, however, aware of previous reports regarding RBC citrulline levels. The current method may thus prove useful for the measurement of the citrulline content in the intracellular compartment.

As many methods have been published for the determination of citrulline (e.g., [16–27]), the pros and cons of the present method deserve discussion. Like all techniques involving GC/MS [1,25], the current method involves a rather tedious extraction and derivatization procedure, a clear disadvantage compared to ion exchange chromatography [17], HPLC [23,24], or LC–MS [2,22]. On the other hand, like all methods using mass spectrometry as a technique of detection [1,25], the method opens the way to the performance of kinetic studies using stable isotope-labeled citrulline. Compared to previously reported GC/MS assays [1,25], the current method furthermore relies on electron impact GC/MS, a 'workhorse' instrument that is more widely available than LC–MS systems in analytical laboratories, and is a relatively low cost, low maintenance equipment. Finally, to the best of our knowledge, the current method is the only one tested in three biological matrices, i.e., plasma, red blood cells, and urine.

As the current method is robust and sensitive and can be used on fluids other than plasma, it appears suitable for use in clinical studies in populations of frail subjects or patients (e.g., preterm infants) in whom limiting the volume of blood draws is critical.

For instance, if citrulline were to be used as a therapeutic agent [14,15], it is important to determine how much citrulline is lost in urine. Similarly, when blood sampling is limited, measuring citrulline concentration in red blood cells may be helpful: as a matter fact, when blood is sampled in critically ill patients (e.g., premature infants), plasma is primarily used for the clinical monitoring of the patient's condition (measurement of glucose, electrolytes) whereas red cell pellets are usually disposed of as "waste": these waste samples could be used, provided red blood cell citrulline reflects plasma citrulline level. As such, the described method, should help in expanding the scope of clinical investigation of citrulline metabolism.

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