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Short communication

Determination of ornithine in human plasma by hydrophilic interaction chromatography–tandem mass spectrometry

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Abstract

The amino acid ornithine (Orn) acts as a vital part in the physiologically fundamental urea cycle. As such, it is a main intermediate in the catabolic breakdown as well as in the synthesis of arginine and is involved in many other metabolic pathways with potential clinical implications. We here describe a LC–MS-MS method for the detection of Orn in human plasma which is fast, easy and precise. The sample preparation comprises only protein precipitation and the addition of the isotopic labeled I.S. The analytes are separated by hydrophilic interaction chromatography (HILIC) in less than 4 min on a silica column with an isocratic mobile phase consisting of 0.1% trifluoroacetic acid in water and acetonitrile in the ratio of 25:75. Orn and its I.S. are detected and quantified by APCI tandem mass spectrometry. The calibration function is linear from 7.5 to 205 μmol/l and covers the range of concentrations found in patients undergoing different clinical interventions. The quantification results are independent with regard to the biological matrix analyzed. The intra-day and inter-day relative standard deviations are 1.1% and 3.5%, respectively. As an application of the described method in clinical investigations, we report arginine and ornithine plasma concentration results from an arginine supplementation study enrolling healthy volunteers and patients suffering from hypercholesterolemia. After oral dosing of 110 mg/kg arginine, ornithine plasma concentrations rose from 54 to 148 μmol/l after 2 h and were back to baseline after 24 h. However, arginine to ornithine ratios kept constant during the complete observation time.

Keywords: Ornithine; Arginine; HPLC; APCI; MS-MS; HILIC

1. Introduction

The basic amino acid ornithine (Orn) (for molecular structure see Fig. 1) acts as a vital part in the physiologically fundamental urea cycle. It is synthesized from the catabolic breakdown of arginine (Arg) by the action of the enzyme arginase [1]. On the other hand, Arg can be synthesized from Orn via citrulline, closing the urea cycle. The homeostasis of the urea cycle is of vital importance for the organism [1] and is in the focus of current scientific research [2,3]. Since Arg is the source for the endothelial relaxing factor nitric oxide, Orn is capable to influence the production rate of NO in an indirect way. Orn is also a source for the amino acids proline and glutamate and is therefore involved in multiple aspects of the production and elimination of biochemical intermediates [4]. To elucidate the role of Orn in its many biochemical pathways, a fast, precise and cheap method for the determination of Orn in biological fluids would be of great value.

Currently, semi-quantitative measurements of Orn in human plasma are routinely performed by tandem mass spectrometry without chromatographic separation in the course of newborn screening [5] to detect inborn enzymatic defects like argininosuccinate lysase deficiency [6]. Methods providing quantitative results for Orn have been published also [7–10]. These methods feature ion exchange chromatography or reversed phase chromatography after derivatization with *o*-phthalaldehyde. Because the detection of the analytes in these methods is unspecific with regard to amino acids, Orn has to be separated from the other amino acids by chromatographic means, leading to very long chromatographic run times of about 35–120 min, depending on the method applied. A more sophisticated LC–MS-MS based method for the quantification

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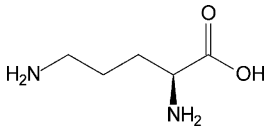

Fig. 1. Molecular structure of ornithine.

of Orn besides 75 other amino acids from biological fluids was described by Piraud et al. [11]. To retain and separate the underivatized analytes, they applied ion-pairing chromatography and a mobile phase gradient, leading to cycling times of more than 30 min. All of these methods suffer from the fact that they attempt to quantify Orn together with many other substances, leading to complex and inefficient protocols.

Here we describe a novel HPLC–MS-MS method especially optimized for the detection of Orn in human plasma, which leads to fast, precise and specific quantification. The method requires only very little sample preparation work and is, due to its short chromatographic run time, suitable for high-throughput analysis.

2. Experimental

2.1. Instrumentation

The HPLC part of the analytical apparatus consisted of an Agilent 1100 system (Waldbronn, Germany) comprising a binary pump, an autosampler and a thermostatted column compartment. The chromatographic separation took place on an Atlantis™ HILIC silica 5 μm 150 mm × 2.1 mm column (Waters, Eschborn, Germany), protected by a SecurityGuard system (Phenomenex, Aschaffenburg, Germany) equipped with a 4 mm × 2 mm silica filter insert. The analytes were detected by a Thermo Electron TSQ Discovery Max triple quadrupole mass spectrometer equipped with a APCI ion source. System control and data handling were carried out by the Thermo Electron Xcalibur software, version 1.2.

2.2. Chemicals

L-Ornithine hydrochloride was purchased from Sigma–Aldrich (Seelze, Germany) and its internal standard 3,3,4,4,5,5-D_6-L-ornithine hydrochloride was purchased from Cambridge Isotope Laboratories (Andover, Ma, USA). Acetonitrile was supplied from Riedel-de Haën (Seelze, Germany) in the quality grade "for LC–MS". Ultra pure water was produced in our laboratory by a Barnstead EASYpure UV system (Werner, Leverkusen, Germany). All other chemicals were in analytical grade or better.

2.3. Sample collection

Blood samples of about 5 ml were drawn into sampling tubes (BD Vacutainer Systems, Plymouth, UK) containing EDTA as anticoagulant. Blood cells were separated by centrifugation at 2440 × *g* for 10 min. The resulting plasma was stored at −80 °C until analysis.

2.4. Calibration and quality control samples

An Orn stock solution was made up by dissolving 25.3 mg L-ornithine hydrochloride (i.e. 150 μmol) in 100 ml water. Calibration samples were prepared by spiking pooled human plasma samples or water samples with 7.5–150 μmol/l Orn. Quality control samples were prepared in a similar manner from pooled human plasma at the basal concentration (i.e. without spiking) and at a spiking concentration of 150 μmol/l. The quality control samples were portioned and frozen at −80 °C until analysis.

2.5. Sample preparation

To 100 μl EDTA plasma, 20 μl of the I.S. solution (500 μmol/l D_6-L-ornithine in water) and 125 μl of a buffer solution (69 μmol/l formic acid and 22 μmol/l ammonium formate in water) were added. The protein precipitation was achieved by the addition of 1 ml acetonitrile to this mixture. After centrifugation at 10,000 × *g* for 5 min, the clear supernatants were forwarded to the HPLC system.

2.6. Chromatographic conditions and MS-detector settings

From the prepared samples, 5 μl were injected into the HPLC system. The analytes were separated by isocratic elution with a mobile phase consisting of 25% of 0.1% trifluoroacetic acid in water and 75% acetonitrile, at a flow rate of 0.4 ml/min. The column temperature was held constant at 30 °C. Under these condition, Orn and its I.S. eluted synchronously at 2.1 min. An entire chromatographic cycle took about 4.5 min. The mass spectrometric detection took place in the MS-MS mode with ionization of the analytes in an APCI ion source working in the positive mode. The temperatures of the APCI vaporizer and the ion source capillary were set to 450 °C and 206 °C, respectively. Under these conditions, Orn and its I.S. were ionized to the $[M+H]^+$ molecular ions, with mass to charge ratios of *m/z* 133 and *m/z* 139, respectively. Ion fragmentation took place at a collision energy of 15 eV with argon at a pressure of 1.4 mTorr serving as collision gas. The most abundant fragment ions of *m/z* 70 and *m/z* 76 were observed for Orn and its I.S., respectively.

2.7. Study subjects

Seventeen subjects participated in an Arg supplementation study, divided into a group of 7 healthy volunteers (age: 23–49 years, median 36 years; weight 64–100 kg, median 81 kg; all male) and a group of 10 patients suffering from hypercholesterolemia (age:33–55 years, median 47 years; weight 82–118 kg, median 96 kg; all male). All participants were enrolled in accordance with the Helsinki declaration and gave informed consent prior to participation. All subjects received a single Arg dose of 110 mg/kg or placebo. EDTA-plasma samples were drawn at 0, 2, 4 and 24 h after dosing. Orn concentrations were measured according to the here described method and Arg concentrations were determined according to an LC–MS-MS method described recently [12].

3. Results and discussion

3.1. Sample preparation, chromatography and MS detector settings

A main feature of the here described method is the very simple sample preparation. It consists only of the addition of the I.S., buffering and protein precipitation. Since there is no cumbersome extraction of the analytes with, for example, solid phase ion exchange columns, the procedure is fast, inexpensive and robust. The utilization of acetonitrile as protein precipitation agent fulfills the additional purpose of yielding a sample composition very similar to the mobile phase of the HPLC system, thus avoiding peak distortion by injecting samples with a higher elution strength than the mobile phase.

The basic amino acid Orn exhibits very polar properties in chromatography, making it difficult to retain on reversed phase HPLC columns. Reasonable retention can only be achieved by ion pairing or derivatization of Orn [7,9–11]. On the other hand, the hydrophilic interaction chromatography (HILIC) separation mechanism has been proved to be very useful when working with small polar compounds [13]. HILIC separations are characterized by the use of a polar stationary phase such as silica and a mobile phase consisting of a high percentage of acetonitrile (typically >70%) together with an aqueous buffer. Applying this technology to the separation of underivatized Orn, sharp peaks and a retention factor k of about 2 resulted. Typical chromatograms obtained by this method are depicted in Fig. 2.

Ionization for the mass spectrometric detection of Orn and its I.S. takes place in an APCI ion source, which is known for its more robust and stable performance in comparison to an ESI ion source [14]. Under the described conditions, strong and stable signals of Orn and its I.S. in form of their $[M+H]^+$ molecular ions with mass to charge ratios of m/z 133 and m/z 139, respectively, can be observed. No adduct ions or dimers and no source fragmentation products are formed. Due to the high selectivity of the tandem MS detection and since Orn does not share its molecular mass with other amino acids, no interferences are found. The only exception is a small peak at about 1.9 min which appears in the ion trace of Orn in all chromatograms from plasma, but not from water samples (see Fig. 2). The peak relates to the small fraction of Arg in the samples which is transformed in the ion source to Orn, presumably by the heat induced abstraction of the molecular fragment [HN=C=NH]. Nevertheless, since Arg is chromatographically completely separated from Orn, no impairment of the quantification of Orn results. A very small peak of Orn appears in blank water samples, which amounts to about 0.1 μmol/l (Fig. 2a). It stems from an impurity of unlabeled Orn in the isotopic labeled I.S. The introduced systematically error in the quantification by this impurity is about 1% at the lowest calibration level and therefore negligible.

Matrix effects on the ionization efficiency in the APCI source are evaluated following the method described by Souverain et al. [14]. In short, the I.S. solution is infused via a tee-union to the HPLC effluent at a constant rate while samples prepared from plasma without the I.S. are injected into the HPLC. The ion trace relating to the I.S. should be detected by the mass spectrometer at a constant intensity, since the I.S. is infused at a constant rate. If any matrix effect attenuating or enhancing the ionization efficiency during the chromatographic runtime, the signal intensity fades or rises accordingly. Applying this approach to our method, strong episodes of signal suppression by the plasma matrix can be observed. Nevertheless, regardless of the individual plasma sample tested, such signal suppressions generally ceased after about 2 min. Since the peak of Orn elutes later in the chromatographic runtime, no severe impairment of the peak intensity of Orn should be expected. However, the quantification results are not dependent on any matrix effect, since Orn and its isotope labeled I.S. sustain always the same matrix effect, leaving the ratios constant regardless of any matrix effects.

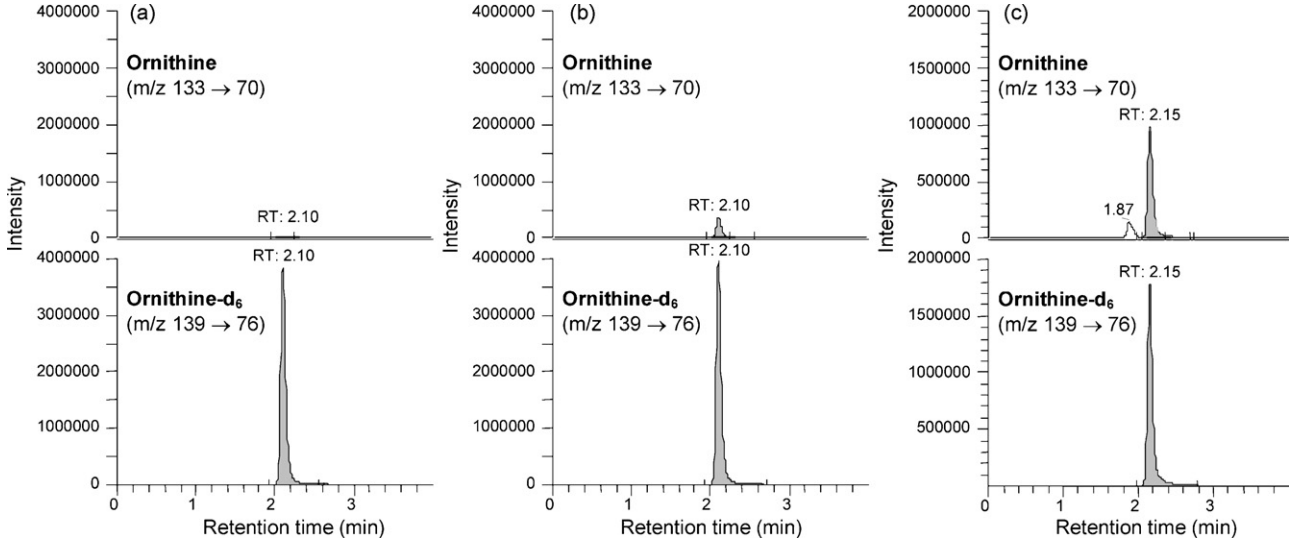

Fig. 2. Typical chromatograms obtained from (a) a blank water sample with I.S., (b) a calibration sample containing 7.5 μmol/l Orn in water and (c) an EDTA-plasma sample of a healthy volunteer (found Orn concentration: 50.0 μmol/l). Peaks at 2.1 min refer to Orn (upper ion trace) and its I.S. D_6-Orn, respectively. The peak at 1.87 min in the Orn ion trace of the EDTA plasma sample refers to the cross talking but chromatographically separated arginine.

3.2. Calibration

The calibration of endogenous substances such as Orn is always challenging to the analyst. Since no plasma matrix without an unknown concentration of Orn is available, the calibration has to be conduced by adding up known amounts of Orn to an unknown sample to produce a calibration function with an offset according to the endogenous concentration. Another method is to spike non-biological (i.e. water) samples without an endogenous concentration of Orn to produce a calibration function free of an offset. In this case it has to be proven that the calibration function from the non-biological matrix results in the same slope as the calibration function from authentic matrix. Otherwise, systematic errors in the quantification results are introduced if applying a calibration function yielded from non-biological samples on biological samples. In the here reported method, due to the application of an isotope labeled I.S., the slopes of the calibration functions are independent from the matrix, whether it is water or human plasma. The calibration function from water shows a slope of 0.0109 ± 0.0002 with an insignificant intercept of 0.0065 ± 0.0156. The corresponding data for a calibration from human plasma are 0.0105 ± 0.0002 with an intercept of 0.6195 ± 0.0158 corresponding to an endogenous Orn concentration of 55.9 μmol/l. The differences in the slopes of the two calibration functions are not significantly different ($p < 0.01$), and therefore, the quantification is independent of the matrix investigated. Hence, the unknown plasma samples can be quantified by applying the calibration obtained from water. The calibration function is linear in the range of 7.5–205 μmol/l. The lower limit of quantification is therefore 7.5 μmol/l (i.e. the lower end of the calibration range), whereas the limit of detection is 0.1 μmol/l, at a S/N ratio of 3.

3.3. Precision and accuracy

Intra-day precision measurements ($n = 10$) resulted in relative standard deviations of 1.1% at 55.8 μmol/l (i.e. basal level of pooled human plasma) and 1.3% at 201.2 μmol/l (basal level plus 150 μmol/l spiking), with an accuracy of −2.2%. The corresponding inter-day ($n = 6$) relative standard deviations were 3.5% at the basal level of 56.6 μmol/l and 2.5% at 204.9 μmol/l with an accuracy of −0.8%. These results satisfy the requirements for biological method development [15].

3.4. Stability of the samples and solutions

The long-term stability of the quality control samples was tested by comparison of the found Orn concentrations in fresh samples and after 5 month of storage at −80 °C. There were no differences greater than 5%, thus EDTA plasma samples can be regarded as stable for at least 5 month at −80 °C.

The stability of the Orn stock solution was tested in a similar way by comparison of a newly prepared stock solution with one after storage for 4 weeks at 5 °C. Again, no differences greater than 5% between the two solutions were detected, thus the Orn stock solution can be considered as stable for at least 4 weeks if stored at 5 °C.

4. Application of the method to clinical studies

First results with the here described method were obtained in the course of an Arg supplementation study enrolling seven healthy volunteers and nine patients suffering from hypercholesterolemia. The nutritional supplementation of Arg is believed to improve vascular function in patients with elevated cardiovascular risk factor such as hypercholesterolemia [16]. In the course of this study, Arg and Orn plasma concentrations were determined to shed light on the pharmacokinetic properties of Arg and its main metabolite Orn after the application of a single oral dose of 110 mg/kg Arg. The plasma concentration values of Arg and Orn obtained in this study are summarized in Table 1. Concentration levels before dosing were comparable to previously published data of 94.2 ± 25.8 μmol/l [17] for Arg and 55 ± 16 μmol/l for Orn [8]. Two hours after the oral dosing of 110 mg/kg Arg, plasma levels of Arg rose considerable above the concentrations observed after placebo. Synchronously, the Orn levels showed an analogous elevation. These elevated concentrations of Arg and Orn were declining after 4 h and were virtually back to baseline values after 24 h. The elevation of the plasma concentrations for Arg and Orn 2 h and 4 h after dosing was highly significant. On the other hand, the ratios between Arg and Orn remain approximately constant at a value of 1.59 ± 0.37, with no significant differences at the various sampling times. Furthermore, no significant differences between healthy volunteers and patients suffering from hypercholesterolemia could be observed. The fast absorption and elimination of Arg and the constant concentration ratio to Orn highlights the robust capac-

Table 1
Ornithine and arginine plasma concentration values

Subgroup	Treatment (single dose)	Analyte	Concentration (μmol/l) at different times after application			
			0 h	2 h	4 h	24 h
Healthy ($n = 7$)	Placebo	Orn	54.4 ± 16.1	59.3 ± 16.9	63.5 ± 18.8	46.8 ± 7.9
	110 mg/kg Arg	Orn	52.2 ± 11.9	134.4 ± 25.7*	96.4 ± 20.7*	54.3 ± 8.7
	Placebo	Arg	83.2 ± 11.2	89.4 ± 28.8	98.1 ± 20.8	80.9 ± 14.2
	110 mg/kg Arg	Arg	93.3 ± 17.0	233.6 ± 59.0*	151.4 ± 33.4*	100.0 ± 15.0
Hypercholesterolemia ($n = 10$)	Placebo	Orn	55.0 ± 14.2	54.8 ± 12.6	60.2 ± 12.2	53.4 ± 10.9
	110 mg/kg Arg	Orn	51.3 ± 9.5	161.3 ± 48.0*	115.9 ± 37.8*	55.3 ± 9.7
	Placebo	Arg	77.6 ± 7.9	82.3 ± 9.3	90.7 ± 14.4	86.4 ± 10.2
	110 mg/kg Arg	Arg	81.1 ± 8.8	211.2 ± 59.1*	145.0 ± 33.8*	86.6 ± 9.0

* Significant difference with respect to placebo treatment ($p < 0.005$).

ity of the urea cycle and related metabolic pathways to handle interventions and restore normal homeostatic conditions [4].

5. Conclusion

Here we present a new, fast and precise method for the determination of Orn in human plasma. This method is ideally suited to be used as a tool for the probing of the urea cycle under diverse clinical conditions and under different supplementation regimes of Arg and Orn. Furthermore, it is independent of the matrix and can therefore easily be adapted for the determination of Orn in other biological fluids than plasma, for example in cell culture supernatant.

References

[1] J.L. Deignan, J.C. Livesay, P.K. Yoo, S.I. Goodman, W.E. O'Brien, R.K. Iyer, S.D. Cederbaum, W.W. Grody, Mol. Genet. Metab. 89 (2006) 87.
[2] H. Murayama, M. Ikemoto, Y. Fukuda, S. Tsunekawa, A. Nagata, Clin. Chim. Acta. 375 (2007) 63.
[3] I.B. Debats, D. Booi, N.E. Deutz, W.A. Buurman, W.D. Boeckx, R.R. van der Hulst, J. Surg. Res. 134 (2006) 205.
[4] G. Wu, S.M. Morris Jr., Biochem. J. 336 (Part 1) (1998) 1.
[5] M.S. Rashed, P.T. Ozand, M.P. Bucknall, D. Little, Pediatr. Res. 38 (1995) 324.
[6] S. Stadler, K. Gempel, I. Bieger, B.F. Pontz, K.D. Gerbitz, M.F. Bauer, S. Hofmann, J. Inherit. Metab. Dis. 24 (2001) 370.
[7] D. Fekkes, A. van Dalen, M. Edelman, A. Voskuilen, J. Chromatogr. B 669 (1995) 177.
[8] J. Le Boucher, C. Charret, C. Coudray-Lucas, J. Giboudeau, L. Cynober, Clin. Chem. 43 (1997) 1421.
[9] M.L. Patchett, C.R. Monk, R.M. Daniel, H.W. Morgan, J. Chromatogr. 425 (1988) 269.
[10] W.Z. Zhang, D.M. Kaye, Anal. Biochem. 326 (2004) 87.
[11] M. Piraud, C. Vianey-Saban, K. Petritis, C. Elfakir, J.P. Steghens, D. Bouchu, Rapid Commun. Mass Spectrom. 19 (2005) 1587.
[12] J. Martens-Lobenhoffer, S.M. Bode-Böger, Clin. Chem. 52 (2006) 488.
[13] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[14] S. Souverain, S. Rudaz, J.L. Veuthey, J. Chromatogr. A 1058 (2004) 61.
[15] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[16] S.M. Bode-Böger, Eur. J. Clin. Pharmacol. 62 (2006) 91.
[17] T. Teerlink, R.J. Nijveldt, S. de Jong, P.A.M. van Leeuwen, Anal. Biochem. 303 (2002) 131.