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Research Article

Orotic acid quantification in dried blood spots and biological fluids by hydrophilic interaction liquid chromatography tandem mass spectrometry

Orotic acid (ORA) is an intermediate metabolite in the pathway of pyrimidine nucleotides; its urinary excretion is useful to diagnose the hereditary orotic aciduria and some hyperammonemic inherited defects of urea cycle enzymes and amino acid transporters. ORA analysis is based on stable isotope dilution by GC-MS or LC-MS/MS methods. We developed a fast assay that measures the ORA in dried blood spots (DBS), plasma and urine using hydrophilic interaction LC-MS/MS. Within- and between-day analytical imprecision (CV%) of three quality control levels, in plasma, DBS and urine, ranged from 0.8 to 14.1%, while the inaccuracy ranged from −13.5 to 9.4%. In healthy children ($n = 20$), ORA concentrations were less than 0.69 μM in plasma, less than 0.82 μM in DBS and from 0.2 to 1.4 mmol/mol of creatinine in urine. A patient with citrullinemia showed ORA levels of 133 μM in plasma and 39 μM in DBS. A patient with hyperammonemia-hyperornithinemia-homocitrullinemia (HHH) syndrome presented a urinary ORA level of 9.1 mmol/mol of creatinine. The method is potentially able to discriminate affected patients from reference subjects; the clinical validation should be expanded on a higher number of patients.

Keywords: Hydrophilic interaction LC / Inherited metabolic diseases / MS/MS / Orotic acid
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1 Introduction

Orotic acid (ORA; 1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine-carboxylic acid; uracil-6-carboxylic acid) is an intermediate metabolite in the *de novo* synthetic pathway of pyrimidine nucleotides. The hereditary orotic aciduria (http://www.ncbi.nlm.nih.gov/omim/) [1, 2], an autosomal recessive disorder, results from a deficiency of uridine-5′-monophosphate (UMP) synthase, a bifunctional protein that catalyzes the transformation of ORA into UMP. ORA is efficiently cleared from blood by the kidney, both by filtration and tubular secretion. ORA accumulates in liver, enters into plasma and is excreted in the urine, giving rise to orotic aciduria, in many different situations in addition to the UMP synthase deficiency. In fact, ORA excretion also increases in several urea cycle disorders [3] due to the accumulation of carbamoyl phosphate (CP) within the mitochondrial matrix when there is a mismatch between the fluxes through CP synthetase and the urea cycle steps. Thereafter, the spill over beyond the control step of CP in the cytoplasm, where it can enter into *de novo* pyrimidine nucleotide synthesis, causes an overloading of the pathway. In particular, the ornithine transcarbamoylase defect is the disorder more characterized by orotic aciduria and hyperammonemia. In addition, patients affected by other disorders due to defect of ornithine transporter 1 (or HHH syndrome), of argininosuccinate synthetase, of argininosuccinate lyase and of lysine transporter (*i.e.* lysinuric protein intolerance) also exhibit mild orotic aciduria and hyperammonemia [3].

Determination of ORA is helpful for the differential diagnosis of hyperammonemia disorders, which cannot be readily diagnosed by amino acid chromatography, thus reducing the need for enzyme determination in tissue biopsies. Elevated blood ammonia concentrations can have detrimental effects, including brain damage, coma and death, then a fast diagnosis is, in most cases, determinant to ameliorate the prognosis of patient life. Altogether, these disorders show a total incidence of 1:94 000. Moreover, there

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Abbreviations: CP, carbamoyl phosphate; **DBS,** dried blood spot; **HILIC,** hydrophilic interaction LC; **IS,** internal standard; **MRM,** multiple reaction monitoring; **ORA,** orotic acid; **QC,** quality control; **UMP,** uridine-5′-monophosphate

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is a drug-induced orotic aciduria; in fact, allopurinol and 6-azauridine cause increased ORA excretion [3–5]. To date, until when Ito *et al.* [6] described a LC-ESI-MS/MS method for a large number of urinary metabolites, including ORA, the widely accepted approaches to the determination of urinary ORA were stable-isotope-dilution GC-MS [7–9] and ion-exchange chromatography methods with ultraviolet detection [10], each of which has difficulties and limitations. Other LC-MS/MS methods have been also developed to determinate ORA and intermediate metabolites in urine specimens and/or filter paper urine strips [11, 12]. Rashed *et al.* [13] proposed a more specific, high-throughput, sensitive stable-isotope-dilution LC-ESI-MS/MS method for the determination of urinary ORA. la Marca *et al.* [14] also described a fast LC-MS/MS method for the determination of ORA in urine with external calibration, easy sample preparation and no derivatization.

Small polar analytes are not well retained during a conventional RPLC even with high aqueous mobile phases, whereas a classic normal-phase LC, using apolar solvents, is not generally suitable for routine applications because of poor reproducibility and difficulty in interfacing with MS detection. Hydrophilic interaction LC (HILIC) is a method where polar stationary phase (bare silica or derivatized silica) is used in conjunction with a low aqueous/high organic mobile phase [15]. Polar compounds as ORA are well retained during HILIC and this chromatographic technique interfaced with ESI-MS works very well with the typical HILIC eluent, such as ACN/H_2O, whereas using normal-phase solvents, the efficiency of ionization is not easily achieved [16]. In addition, LC columns packed with sub-2 μm particles sorbents has improved significantly the peak capacity of several polar analytes, such as peptides, oligonucleotides and other compounds [17, 18]. Recently, the application of these columns for HILIC mode offers similar improvements for the separation of polar compounds and oligosaccarides [19, 20].

In this study, we present a HILIC-MS/MS method, using a column packed with sub-2 μm particles, by a stable isotope dilution procedure for the quantitative determination of ORA in dried blood spots (DBS), plasma and urine. The method requires only minimal sample preparation and provides a short run time; finally, the analysis from DBS is advantageous because the sample card is easy to ship, can be long-term archived and also used for retrospective studies [21].

2 Materials and methods

2.1 Materials and reagents

The analytical solvents of HPLC grade ACN and ammonium acetate were obtained from J.T. Baker (Deventer, The Netherlands). Purified water was generated by a Milli-Q reagent water system (Millipore, Bedford, MA, USA). ORA standard was purchased from Sigma-Aldrich (Steinheim, Germany), and [1,3-$^{15}N_2$]ORA used as internal standard (IS) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Filter paper for DBS preparation was of grade 903 and was purchased from Whatman GmbH (Dassel, Germany).

2.2 LC-MS/MS apparatus

The HPLC system with autosampler (Alliance 2695, Waters, USA), equipped with an analytical column (Zorbax RX-SIL, 1.8 μm, 50 × 2.1 mm id, Agilent Technologies, USA) and a security guard column (Silica guard column, 4 × 2 mm id, Phenomenex, USA), was coupled with an electrospray triple quadrupole mass spectrometer (Micromass Quattromicro, Waters), operating in negative mode. A post-column split valve was used to divert two-thirds of the flow into waste. An isocratic HILIC was performed by an aqueous mobile phase constituted of ACN 90% and ammonium acetate 4 mM. The flow rate was maintained at 0.15 mL/min and the column temperature at 30°C. The HPLC autosampler temperature was set at 25°C.

2.3 Standard solutions, HILIC and MS settings

Stock standard solutions were prepared in water to provide solutions of 1 mg/mL for ORA (6.4 mM) and IS (6.3 mM). Stock solutions were stored at −40°C until use. MS tuning was performed by flow injection analysis of standard working solutions prepared freshly diluting stock solution to provide a concentration of 50 μM (ACN/H_2O, 50:50). ESI negative mode was performed using capillary voltage and cone voltage at 2.9 kV and 15 V, respectively; source temperature was set at 120°C and desolvation temperature at 300°C. Collision energy was 10 eV. Under collision-induced dissociation conditions deprotonated ORA (*m/z* 155) and its IS (*m/z* 157) gave one major fragment of *m/z* 111 for ORA and *m/z* 113 for IS, both deriving from the neutral loss of carbon dioxide [M–H^-–CO_2]. The quantitative analysis was performed on selected ion chromatograms acquired by a multiple reaction monitoring (MRM) mode of the following transitions *m/z* 155 > 111 and *m/z* 157 > 113. The injection volume was 2 μL and the total analysis run time was 5 min.

The effect of different ACN/H_2O ratios has been investigated on retention time and signal intensity during HILIC-MS/MS method development, keeping the ammonium acetate constant at 4 mM. Two microliter of a working mixture solution at 5 μM was injected using a flow rate of 0.15 mL/min.

2.4 Samples, calibrators and quality controls preparation

2.4.1 Samples

DBS, plasma and urine samples, from apparently healthy children ($n = 20$) aged 0.1–10 years and from two patients

affected by urea cycle disorders (two samples from a patient affected by citrullinemia and six samples from a patient affected by HHH), were collected from those already analyzed in the Clinical Biochemistry Laboratory of University Hospital of Foggia and in the laboratory of Pediatrics Department of University of Naples. All the samples analyzed in this study were reserve materials that were not needed for further diagnostic investigations avoiding to take extra materials or more volume of sample from patients. Plasma and urine samples were stored at −20°C until analysis. The amount of blood spotted was at least 30 μL and the cards were stored at 4°C until analysis.

To evaluate plasma ORA extraction procedure in order to obtain the best peak shape and signal intensity, aqueous solutions with different percentages of ACN (from 40 to 80%), with a final dilution of sample from 1:5 to 1:20 and with fixed amount of ammonium acetate (4 mM), were used. The best extraction procedure was obtained mixing 20 μL of plasma with 20 μL of an aqueous solution of IS (5 μM) and 260 μL of ACN with ammonium acetate (4.6 mM). After mixing for 5 min, the sample was centrifuged at 6000 rpm for 3 min, and then 2 μL of supernatant were injected into LC-MS/MS.

The best DBS extraction procedure consists of adding 10 μL of an aqueous solution of IS (5 μM), 20 μL of water and 120 μL of ACN with ammonium acetate (5 mM) to a spot of 6 mm (Ø). The solution, after mixing, was incubated for 20 min at 30°C, and then 2 μL of supernatant were injected.

The urine samples were filtered to remove any particulate material through membrane filter (0.22 μm; Millex-GV, Millipore, France) and then diluted 1:10 with water before the analysis. Twenty microliter of diluted urine were mixed with 20 μL of a solution of IS (5 μM) and diluted with 260 μL of ACN with ammonium acetate (4.6 mM), mixed and allowed to stand for 5 min at room temperature, of which 2 μL were injected. The urinary level of ORA was normalized to the creatinine concentration.

2.4.2 Calibration curves

Calibrators were prepared by spiking human blood/plasma pools, from apparently healthy subjects, with 80 μL of ORA standard solution (500 μM) in a final volume of 800 μL; this solution was diluted with plasma or blood to obtain the concentrations of 50, 25, 12.5, 5, 1, 0.5 and 0.25 μM. Blood calibration points were then spotted on filter paper. The ORA concentration in blank DBS/plasma pools was calculated using the intercept/slope ratio value of a first calibration curve based on concentrations measured in DBS/plasma with standard solutions. Then, the calibration curves were re-calculated using the true values of calibrators obtained by applying the following formula: $(Ci \cdot Vi) + (Cs \cdot Vs) = (Cf \cdot Vf)$, where Ci, Cs and Cf are the initial, spiked and final concentrations, respectively, and Vi, Vs and Vf are the initial, spiked and final volumes, respectively. DBS samples with ORA concentration greater than 50 μM were opportunely re-analyzed punching a smaller spot of 3 mm Ø (3.1 μL). Plasma samples with ORA concentration greater than 50 μM were opportunely diluted and re-analyzed.

The urinary concentrations were calculated using a calibration curve prepared with aqueous standards at the following concentrations 50, 25, 12.5, 6.25, 3.12 and 1.56 μM.

All the DBS/plasma/urine calibrators were prepared freshly for each assay and analyzed along with patient samples and quality control (QC) samples.

2.4.3 QCs

DBS/plasma samples used in the validation study were prepared as follows. Pooled blank blood was divided into two different aliquots. The first one was spiked with ORA standard solution to obtain the final concentration of 15 and 2.5 μM and then spotted on filter paper to obtain DBS QCs. The second aliquot was centrifuged to obtain plasma which was spiked with ORA standard solution at concentration of 15 and 2.5 μM. QCs for urine were obtained spiking a pool of normal urine samples with the ORA standard solution to obtain the final concentration of 150 and 25 μM. The blank pools of blood, plasma and urine were used as low QC levels only for imprecision evaluation.

The QC aliquots and the clinical samples were stored at −20°C until use. The three levels of QC samples were analyzed to evaluate the within-day and between-day imprecision and inaccuracy. The within-day parameters were evaluated on 15 replicate on each level on 1 day. The between-day parameters were evaluated on each level once a day for 15 days along a period of 4 weeks.

2.5 Recovery and LOQ

Recovery was calculated on medium and high level of QC samples using the following formula: $RA = [QA(O+S) - QA(O)]/QA(S)$, in which RA is the recovery, QA(S) the amount of analyte A added (spiked value), QA(O) the quantity of analyte A in the original sample, and QA(O+S) the amount of analyte A recovered from the spiked sample.

Samples of unaffected subjects were diluted to obtain ORA concentrations from 0.1 to 0.3 μM for plasma and DBS, and from 0.6 to 2.0 μM for urine. These samples were used to calculate the LOQ in plasma, DBS and urine (five replicates of each level). The LOQ was the concentration that produced a signal-to-noise ratio higher than 10, imprecision of less than 20% and accuracy from 80 to 120%.

2.6 Matrix effect

The matrix effect was evaluated by a continuous infusion of IS (0.33 μM) introduced at a flow rate of 20 μL/min into the effluent from the HILIC column before introduction into mass spectrometer. The ion suppression/enhancement was evaluated on plasma, DBS and urine matrices, prepared as

described above, by injecting 2 μL into the HILIC-MS/MS system and recording the MRM signal of IS (m/z 157 > 113).

2.7 Statistical analysis

The acquired data were processed using the QuanLynx software (v. MassLynx 4.0, Waters) to calculate calibration curves and sample concentrations. The data presented as average, SD and CV% were processed by Microsoft Excel software for personal computer (v. 11.0, Microsoft).

3 Results and discussion

3.1 Mobile-phase selection: Effect of different ACN/H_2O ratio on HILIC

Generally, HILIC is performed using a mobile phase of 5–40% of water in ACN. By increasing the percentage of ACN, both signal intensity (area) and ORA retention were improved. Significant differences in signal intensity and retention time were obtained using ACN percentage from 85 to 95%. Less ACN (< 85%) leads to poor retention and worse peak shape. For these reasons, a mobile phase

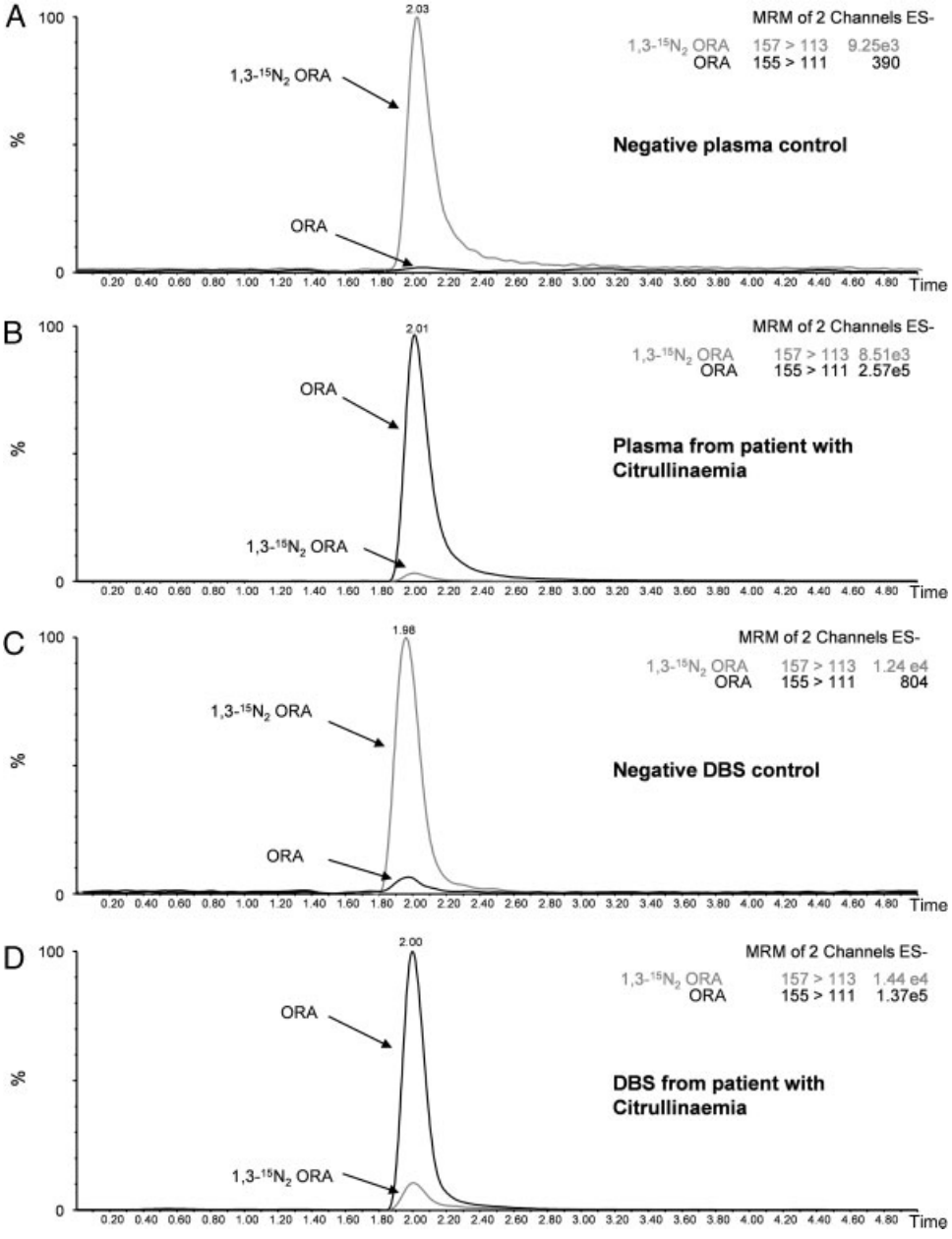

Figure 1. Ion chromatograms obtained monitoring the MRM transitions of ORA and IS. (A) Negative plasma, (B) plasma sample from patient affected by citrullinemia, (C) negative DBS, (D) DBS sample from patient affected by citrullinemia. In both plasma and DBS samples of patient affected by citrullinemia, the ORA was more than 100 times higher than negative samples.

constituted by ACN/H_2O of 90:10 (v/v), which provided a good compromise among retention, peak shape and signal intensity, has been chosen. The retention time of ORA and IS ranged from 1.98 to 2.03 min using a flow rate of 0.15 mL/min, the flow rate was not optimized in terms of yielding minimal theoretical plate height. In Fig. 1, the MRM chromatograms of ORA in negative and positive plasma and DBS samples are depicted. In Fig 2, the MRM chromatograms of ORA obtained from negative and positive urine samples are shown.

3.2 Validation study

Validation study was performed evaluating calibration curves, QCs, real samples, recoveries, LOQ and matrix effect.

3.2.1 Calibration curves, within-day and between-day variabilities

The linearity of calibration curves, prepared in plasma, DBS and water was estimated by evaluation of coefficient of correlation (*r*), intercept and slope as summarized in Table 1. Five different curves for each matrix were analyzed in different days by injection of two replicates for each concentration level. Plasma with high level of ORA (133 μM), over the upper level of calibration, was accurately measured after dilution of sample (1:3), the concentration calculated was 138 μM and the recovery was 104%. Urine with ORA concentration of 10 mM, diluted 10, 20, 50 and 100 times, provides accurate results from 94.3 to 101.7%, each dilution was performed three times.

The three levels of QC samples were analyzed during a sequence of unknown samples. The within-day imprecision (CV%) and inaccuracy (%) were calculated by analyzing, in the same analytical run, each level of QC samples 15 times. The ORA imprecision (CV%) on three QC samples ranged from 2.2 to 14.1 for plasma, from 1.4 to 9.4 for DBS and from 6.3 to 8.8 for urine, while the inaccuracy on two QC samples (medium and high levels) ranged from −13.5 to −13.3% in plasma, from 1.6 to 6.6% in DBS and from 8.6 to 9.0% in urine (Table 2).

The between-day imprecision and inaccuracy were calculated by analyzing each level of QC samples once a day for 15 days along 4 wk. The imprecision (CV%) was esti-

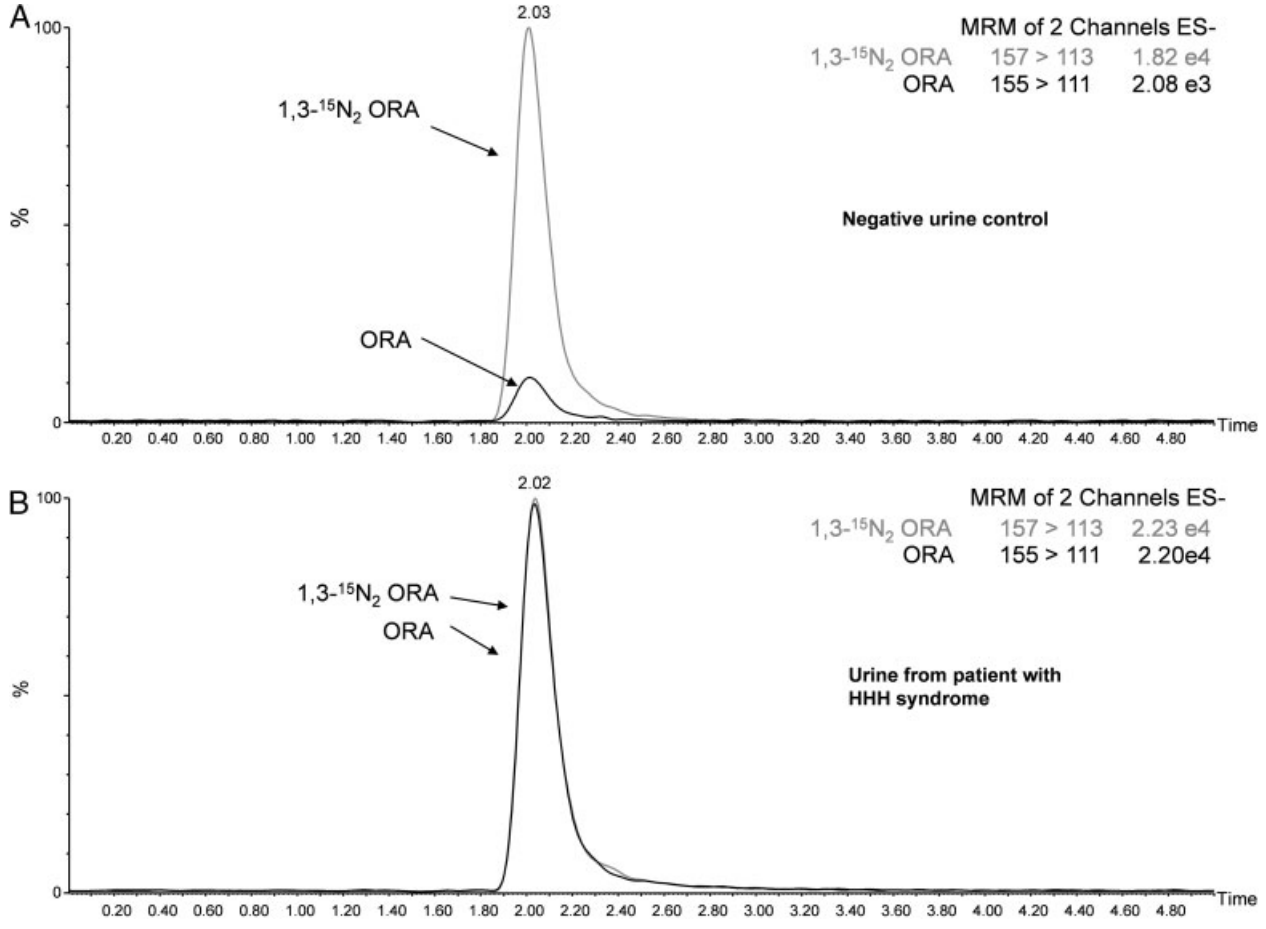

Figure 2. Ion chromatograms obtained monitoring the MRM transition of ORA and IS from urine samples. (A) Negative urine, (B) urine sample from patient affected by HHH syndrome. The signal of ORA obtained in HHH sample is ten times higher than negative sample.

Table 1. Parameter ranges of calibration curves of ORA (μM) obtained from different matrices

Matrices	*n*	Concentration (μM)	Slope from/to	Intercept from/to	*r* from/to
Water	5	1.56–50	0.2232/0.2294	0.0118/0.0348	0.9998/0.9999
Plasma	5	0.25–50	0.2220/0.2336	−0.0002/−0.0001	0.9995/0.9998
DBS	5	0.25–50	0.2320/0.2375	−0.0004/−0.0003	0.9993/0.9997

Table 2. Within-day and between-day imprecision and inaccuracy study of ORA in plasma, DBS and urine QCs[a)]

Concentrations (μM)	Within-day ($n = 15$)		Between-day ($n = 15$)	
	Imprecision (CV%)	Inaccuracy (%)	Imprecision (CV%)	Inaccuracy (%)
Plasma				
Blank pool	14.1	–	13.8	–
2.5	2.2	−13.5	9.2	−6.2
15	3.5	−13.3	7.3	−6.9
DBS				
Blank pool	9.4	–	3.0	–
2.5	2.1	1.6	0.8	1.9
15	1.4	6.6	3.7	9.4
Urine				
Blank pool	8.8	–	10.5	–
25	6.3	8.6	6.0	6.3
150	8.6	9.0	7.3	7.8

a) Blank pool concentrations were estimated at 0.2 μM in plasma, at 0.40 μM in DBS and at 5 μM in urine.

mated from 7.3 to 13.8 in plasma, from 0.8 to 3.7 in DBS and from 6.0 to 10.5 in urine. The inaccuracy ranged from −6.9 to −6.2% in plasma, from 1.9 to 9.4% in DBS and from 6.3 to 7.8% in urine (Table 2).

Urine and plasma QC samples showed good stability both overnight at 25°C on the autosampler and after a freeze-thaw cycle.

3.2.2 Recovery and LOQ

Between-day recovery was calculated on medium and high level of QC samples on a single analysis on each of 15 days. Recovery from medium and high level of plasma QCs (2.5 and 15 μM) ranged from 82 to 104% and from 82 and 103%, respectively. Recovery from medium and high level of DBS QCs (2.5 and 15 μM) ranged from 99 to 103% and from 97 and 116%, respectively. Recovery from medium and high level of urine QCs (25 and 150 μM) ranged from 101 to 117% and from 97 and 116%, respectively.

In plasma, the LOQ of ORA was 0.18 μM, the imprecision was 5.5% and the accuracy was 116%. In DBS, the LOQ was 0.23 μM, the imprecision was 4.5% and the accuracy was 112%. In urine, the LOQ was 1.8 μM, the imprecision was 3.7% and the accuracy was 103%.

3.2.3 Matrix effect

The matrix effect of plasma, DBS and urine was investigated on signal of ORA IS (m/z 157 > 113) to evaluate the ion suppression/enhancement due to potential co-eluting endogenous salts/compounds. Ion chromatograms of extracted plasma, DBS and urine in which the MRM responses of IS were continuously monitored are shown in Fig. 3. In all the investigated matrices, the ion suppression of the MRM response was observed from 0.9 to 1.75 min, whereas the ORA and IS peaks were recorded at a retention time of 2.0 min (Figs. 1 and 2).

3.2.4 Real samples

This HILIC-MS/MS method was used to analyze plasma, DBS and urine samples from 20 apparently healthy subjects. The ORA concentrations were up to 0.69 μM in plasma, up to 0.82 μM in DBS and from 0.2 to 1.4 mmol/mol of creatinine in urine samples (Table 3). Reference values obtained in plasma and DBS do not differ statistically. In this study, even if ORA levels measured in only 20 samples are reported, the data obtained are in agreement with those previously reported in the literature for negative controls, aged from 0–10 years, which showed that ORA levels were less than 1 μM in plasma [22] and in urine ranged from (mmol/mol of creatinine) 0.05 to 5.98 [13] and from 0.47 to 4.10 [3]. The comparison of reference values in DBS was not performed because this is the first work that shows the ORA levels in DBS. The analysis of ORA in paper DBS has significant advantages for laboratory purposes; in fact, the cards for neonatal screening should be also useful to detect, even retrospectively, the metabolic disorders of ORA. Whole blood from a patient affected by citrullinemia (patient O. P.) was spotted on filter paper to obtain DBS, while the remaining whole blood was centrifuged to obtain plasma. Plasma level of ORA from O. P. patient was of 133 μM, while in DBS was of 39 μM (Table 3). The discrepancy observed between the two values could be due to a lower concentration of ORA inside the red blood cells, this aspect should be studied more accurately in a higher number of affected patients. Previously, in a patient affected by citrullinemia has been reported a wide range of plasma ORA, from less than 1 μM to more than 60 μM [22]. Until now, only few plasma data are reported in the literature, and our study provides just preliminary results for ORA levels in plasma and DBS; then reference values

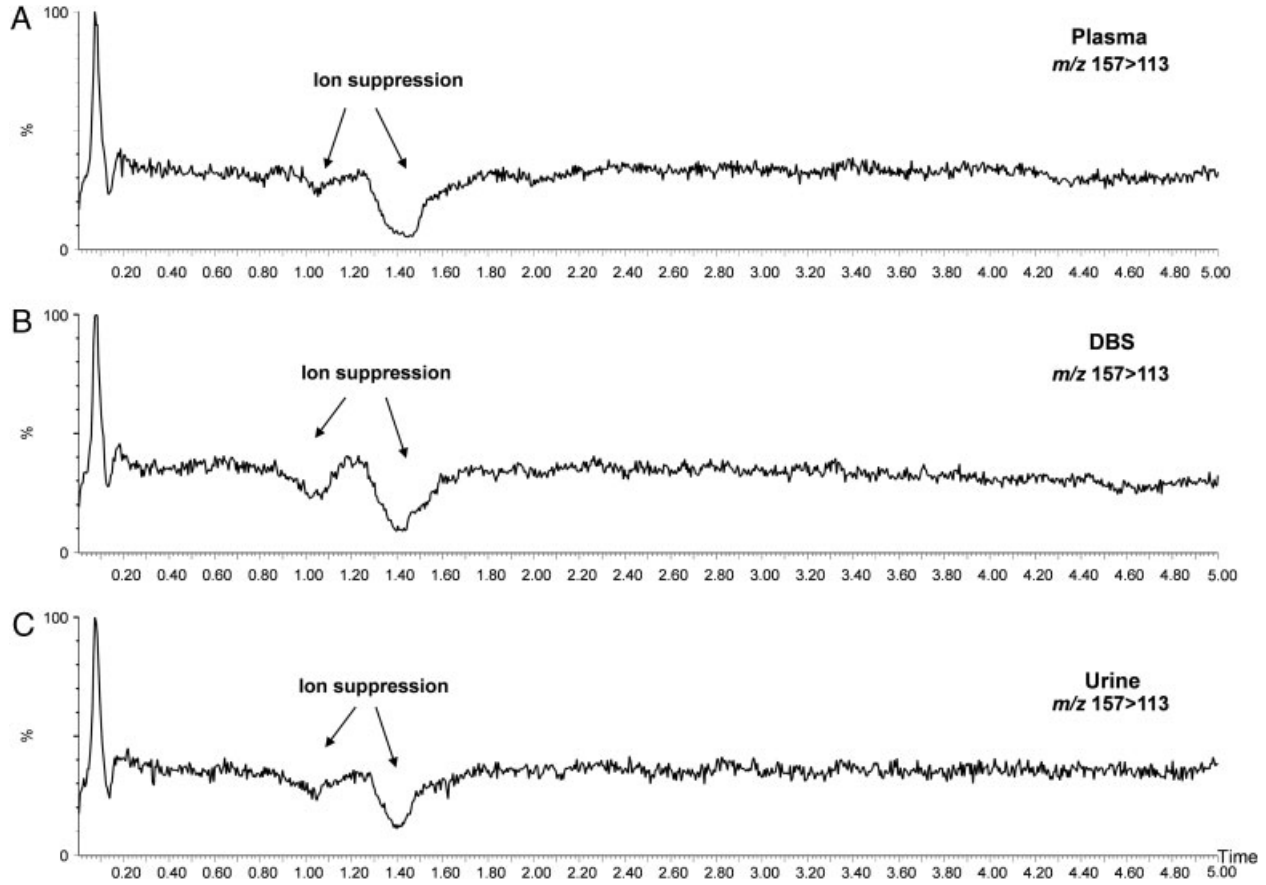

Figure 3. Ion suppression of 1,3-$^{15}N_2$ ORA (*m/z* 157 > 113) ion current by injection of (A) extracted blank plasma pool, (B) extracted blank DBS and (C) extracted blank urine pool.

Table 3. Plasma, DBS and urine reference values of ORA from negative control samples compared with affected patient samples

Samples	Negative control samples			Affected patient samples	
	n	Mean (SD)	Range	Patient O. P.[a)]	Patient O. V.[b)]
Plasma (μM)	20	0.23 (0.18)	0.0–0.69	133	<0.18[c)]
					<0.18[c)]
DBS (μM)	20	0.27 (0.23)	0.0–0.82	39	–
Urine (mmol/mol of creatinine)	20	0.56 (0.42)	0.2–1.4	–	9.1[d)]
					2.5[c)]
					0.9[c)]
					0.2[c)]

a) The patient was affected by citrullinemia type I.
b) The patient is affected by HHH syndrome.
c) The samples have been collected during the follow-up of therapeutic treatment.
d) The sample has been collected on early days of diagnosis.

should be determined in a higher number of control subjects and patients affected by orotic acidurias to define the cut-off levels, in plasma and in DBS, over which the diagnosis must be confirmed in urine sample.

Urine samples from the patient affected by HHH syndrome (patient O. V.), collected at different times during the follow-up of therapeutic treatment, were also analyzed (Table 3). The urine samples collected on early days after the diagnosis presented an ORA value of 9.1 mmol/mol of creatinine, while during the follow-up, the values ranged from 0.2 to 2.5 mmol/mol of creatinine. These data are in agreement with those reported in the literature [23].

4 Concluding remarks

This is the first report of a HILIC-MS/MS method for the determination of ORA that could be potentially used to screen hereditary orotic aciduria, urea cycle disorders and for a differential diagnosis of disorders with hyperammonemia using DBS. The method requires small sample volume and minimal sample preparation and a total time of analysis of 5 min. The analysis of ORA in DBS is advantageous for the screening of some metabolic diseases, because the sample card can be long-term archived, is easy to ship and can be used for retrospective study. This method is potentially able to discriminate affected from unaffected patients, even if the clinical validation should be expanded on a higher number of affected subjects.

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