New approach for the diagnosis of histamine intolerance based on the determination of histamine and methylhistamine in urine


a Department of Nutrition, Food Sciences and Gastronomy, XaRTA, INSA, School of Pharmacy and Food Sciences, University of Barcelona, Avinguda Prat de la Riba 171; 08921 Santa Coloma de Gramenet, Spain.

b Laboratory of Commodity Science, Department of Management, Sapienza University of Rome, Via del Castro Laurenziano 9; 00161 Rome, Italy.

Received 14 March 2017/ Accepted 8 June 2017

*Corresponding author: mcvidal@ub.edu
Abstract

Histamine intolerance is a disorder in the homeostasis of histamine due to a reduced intestinal degradation of this amine, mainly caused by diamine oxidase (DAO) enzyme deficiency, which provokes its accumulation in plasma and the appearance of adverse health affects. A new approach for the diagnosis of this intolerance could be the determination of histamine and its metabolites in urine. The aim of this work was to develop and validate a rapid method to determine histamine and methylhistamine in human urine by Ultra High Performance Liquid Chromatography and Fluorimetric detection (UHPLC-FL). The proposed method is a consistent procedure to determine histamine and methylhistamine in less than 11 minutes with adequate linearity and sensitivity. Relative standard deviation was always lower than 5.5%, ensuring method precision; and mean recovery was greater than 99% for both analytes. The structure of histamine and methylhistamine conjugated with OPA were confirmed by UHPLC-ITD-FTMS which enabled to unequivocally identify both analytes in standards and also in urine samples. The analysis of histamine and methylhistamine in urine samples could be a potential new approach for the routine diagnosis of histamine intolerance, more patient-friendly and with clear advantages in terms of equipment and personnel demand for sample collection in comparison with current plasmatic DAO activity determination.

Keywords

Histamine; Methylhistamine; Histamine intolerance; Diamine oxidase; Solid phase extraction; Ultra high pressure liquid chromatography.
1. Introduction

Histamine (2-[4-imidazolyl]ethylamine) is a bioactive amine discovered in 1910 by Dale and Laidlaw [1] which is synthesized by decarboxylation of the amino acid histidine, using pyridoxal phosphate (vitamin B6) as cofactor. It is mainly produced in mast cells, basophils, platelets, histaminergic neurons and enterochromaffin cells; where it is stored intracellularly in vesicles until its release upon stimulation. Histamine (HA) is involved in the regulation of different physiological functions such as the secretion of gastric juice, cell growth and cellular differentiation, the day-night rhythm, neurotransmission and immunomodulation [2,3].

Two metabolic pathways for HA are known in humans. Histamine-N-methyltransferase (HNMT) is the enzyme responsible for the ring methylation of HA and is mainly located in the liver and kidney and it carries out the conversion of HA to 1-methylhistamine (MHA), which will be finally converted to N-methylimidazoleacetic acid. As a cytosolic protein, HNMT metabolizes HA only in the intracellular space of cells [4-6]. On the other hand, diamine oxidase (DAO) is an enzyme of mainly intestinal location that performs the oxidative deamination of HA producing imidazole acetaldehyde, which will later be converted to imidazoleacetic acid and finally combined with ribose for its urinary excretion. As a secretory protein, DAO is responsible for scavenging extracellular HA after mediator release, being the main enzyme for the metabolism of intestinal HA [4-6].

Histamine intolerance (HIT) is a disorder in the homeostasis of HA due to a reduced intestinal degradation of this amine resulting in its accumulation in plasma and the appearance of multi-faced clinical symptoms, mainly headaches, flatulence, diarrhea,
abdominal pain, sneezing, rhinorrhea, hypotonia, arrhythmias, idiopathic urticaria and pruritus [2,3,7]. An enzymatic deficiency of DAO, key enzyme in the intestinal degradation of histamine, can occur based on genetic predisposition, in inflammatory and degenerative intestinal disorders or by pharmacological blockade [3,8,9]. The incidence of HIT has been estimated to be 1% of the population although this percentage may increase as a consequence of a better knowledge and diagnostic of this enzymatic deficiency. Current therapy for HIT is the limitation of foods containing HA, which may be complemented with encapsulated DAO enzyme to contribute to the degradation of intestinal HA [3,10].

Currently, the identification of individuals with HIT is based on plasmatic DAO activity through a biochemical assay that measures the amount of HA that can be degraded by this enzyme [11]. An alternative for the diagnosis of HIT by DAO deficiency could be the determination of HA and its metabolites in urine, considering that individuals with insufficient DAO activity would have a distribution profile of these compounds significantly different from healthy individuals. In fact, individuals with symptoms associated with HIT would show a higher urinary content of HA and its major metabolite produced by the HNMT metabolic pathway (MHA) than healthy population.

The analytical approach for the simultaneous determination of HA and its metabolites is complex. There are commercial kits that allow the determination of HA through ELISA and of its metabolites through radioimmunoassay (RIA) techniques. However, these immunological-based techniques do not allow simultaneous determination of HA and its metabolites, while, in the case of RIA, they also involve complications related to the use of radioactive material [12]. Alternatively, chromatographic methods, mainly based
on high performance liquid chromatography (HPLC) coupled with various detection systems, appear to be the most appropriate for the simultaneous separation and quantification of these compounds [13]. Few HPLC methods are available in the literature for the simultaneous determination of HA and MHA, mainly focused on the quantification of both compounds in laboratory animal plasma and other biological specimens, such as brain or intestinal tissues [14-19]. Although UV detection has been widely used, the high sensitivity and specificity necessary to detect these compounds in samples such as blood makes fluorescence (FL) or mass spectrometry (MS) detection systems more suitable for this purpose. More recently, ultra high performance liquid chromatography (UHPLC) has been proposed for the simultaneous determination of HA and MHA in mice hair and cerebrospinal fluid [20-22]. A fast chromatographic procedure (UHPLC) coupled with FL detection could be an advantageous approach for the routine determination of these analytes in human urine.

In order to have a new approach for the diagnosis of histamine intolerance, the aim of this work was to develop and validate a rapid and reliable method to quantify HA and MHA in urine. An UHPLC procedure coupled with an on-line o-phthalaldehyde (OPA) post-column derivatization and FL detection has been validated in terms of linearity, sensitivity, precision and recovery. Structural analysis of HA and MHA OPA derivatives using UHPLC-ITD-FTMS was carried out to unequivocally identify these compounds in urine samples.

2. Material and methods

2.1. Reagents and chemicals
Histamine dihydrochloride and 1-methylhistamine dihydrochloride were purchased from Sigma (St. Louis, MO, USA). Ultra pure water (18.2 MΩcm) was produced using a LaboStar System from Evoqua Water Technologies (Warrendale, PA, USA). Methanol and acetonitrile (both HPLC grade) were obtained from Fisher Scientific (Loughborough, UK). The other reagent-grade chemicals used were: acetic acid, formic acid, sodium acetate anhydrous, OPA, 2-mercaptoethanol and Brij 35 from Merck (Darmstadt, Germany); hydrochloric acid 0.1M (HCl), potassium hydroxide, boric acid and ammonium 30% from Panreac (Barcelona, Spain); and sodium octanesulphonate from Romil Chemicals (Cambridge, UK).

2.2. Standard solutions

Stock solutions of HA and MHA were prepared in 0.1 M HCl for a given concentration of 20 mg/L. Pooled working standard solutions (ranging from 0.05 to 10 mg/L) were prepared diluting and mixing aliquot of each compound stock solution with 0.1 M HCl. Standard solutions were protected from light and stored at 4°C until use and filtered through a 0.22 μm membrane filter (GHP, Waters Corp., Milford, MA, USA).

2.3. Samples preparation and MCX SPE procedure

Urine samples from twelve subjects aged 22-40 years were collected for 24 hours without the addition of preservatives. All volunteers received an information kit, including an informed consent form and a questionnaire to record any symptomatology associated with HIT. Samples were stored in a freezer (-20 °C) until analysis. Prior to experiments, samples were thawed at room temperature and completely homogenized.
Sample preparation consisted of an acidic hydrolysis by adding 750 μL of 0.1 M HCl to 10 mL of urine sample. The solution was placed in a heater at 90 °C for 30 minutes and subsequently cooled at room temperature. Later, a purification and concentration procedure was performed by solid phase extraction (SPE) using mixed cation exchange (MCX, 3 mL, 60 mg) cartridges acquired from Waters Corporation (Milford, MA, USA). The cartridges were conditioned with 2 mL of methanol, followed by 2 mL of distilled water. After adsorption of the sample, the cartridge was washed with 10 mL of 0.1 M HCl and dried for 1 minute under vacuum to remove the excess of water. The elution of HA and MHA was performed with 2 mL of 5% NH₄OH in methanol (v/v). The eluate was evaporated to dryness with a centrifugal vacuum concentrator (30 °C, 1465 rpm) and later redissolved in 200 μL of 0.1 M HCl and filtered through a 0.22 μm filter (GHP, Waters Corp., Milford, MA, USA) before UHPLC injection.

2.4. UHPLC-FL determination of HA and MHA

2.4.1. Equipment

Chromatographic separation was performed using an UHPLC-FL system consisting of a Waters Acquity™ Ultra Performance Liquid Chromatography equipment, which involved a quaternary pump, an auto-sampler and a FL detector; accomplished with a post-column reagent manager (Waters 510). The post-column pump was connected to a zero dead volume installed between the column outlet and the detector. An Acquity UPLC™ BEH C₁₈ column (1.7 μm, 2.1 mm x 50 mm) (Waters Corp., Milford, MA, USA) was used as the analytic column. Data acquisition was managed with the Empower version 3 system.
2.4.2. Chromatographic conditions

Mobile phase consisted of the eluent A as a solution of 0.1 M sodium acetate and 10 mM sodium octanesulphonate adjusted to pH 4.8 with acetic acid, and the eluent B as a mixture of solvent B-acetonitrile (6.6:3.4), where solvent B was a solution of 0.2 M sodium acetate and 10 mM sodium octanesulphonate adjusted to pH 4.5 with acetic acid. The mobile phase was filtered through a 0.22 μm filter. The flow rate of the mobile phase was 0.8 mL/min. A linear gradient was applied: 0 min, 80% A; 6 min, 70% A; 6.5 min, 0% A; 8.5 min 0% A; 9 min, 80% A. Finally, the system was re-equilibrated for 2 min at the initial conditions before the next injection. Derivatization reagent was prepared by mixing and aqueous solution of 31 g of boric acid and 26.2 g of potassium hydroxide with 0.2 g of OPA dissolved in 5 mL of methanol. To the above solution, 3 mL of 30% Brij and 3 mL of 2-mercaptoethanol as a reducing agent were added and final volume was brought to 1 L with water. The daily prepared post-column derivatization reagent was filtered through a 0.22 μm membrane filter and protected from light. The flow rate of the derivatization reagent was 0.4 mL/min. Automatic injection of 1 μL of the standard solution and samples was carried out. Vials filled with standard solutions or samples were cooled to 4 °C in the auto sampler, the column was kept at 42 °C and post-column reaction equipment was mantained at room temperature. Fluorescence detection at 340 nm for excitation and 445 nm for emission was applied.

2.5. LC-MS system
An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in positive mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a setting of 30,000 resolution at m/z 400. Operation parameters were as follows: source voltage, 3.5kV; sheath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 375 °C. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target 5 · 10^5 for MS mode and 5 · 10^4 for MS^n mode). Samples were analysed in full scan mode at a resolving power of 30,000 at m/z 400 and MS^2 events acquired at a resolving power of 15,000. An isolation width of 1 amu was used and precursors were fragmented by collision-induced dissociation C-trap (CID) with normalised collision energy of 35 V and an activation time of 10 ms. The mass range in FTMS mode was from m/z 100 to 1000. Data analysis was achieved using XCalibur software v2.0.7 (Thermo Fisher Scientific). An external calibration for mass was carried out before the analysis.

Liquid chromatography analysis was performed using an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump and a thermostated autosampler. Chromatographic separation was accomplished with an Acquity UPLC™ BEH C\textsubscript{18} column (1.7 μm, 2.1 mm x 50 mm) (Waters Corp., Milford, MA, USA) kept at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), and was delivered at a constant flow rate of 0.8 ml/min following a gradient elution: 0 min, 100% A; 3 min, 80% A and 20% B; 3.5 min, 80% A and 20% B; 3.6 min 40% A and 60% B; 4.6 min, 40% A and 60% B; 5 min, 100% A. Finally, the system was re-equilibrated for 2 min at the initial conditions before
the next injection. The autosampler plate was held at 10 °C and the injection volume
was 1 µL.

For the pre-column derivatization, 2% acid formic in water was used for the preparation of standard solutions and to resuspended urine samples. To 100 µL of standard solution, or redissolved urine sample, 400 µL of the derivatization reagent were added and thoroughly mixed using a vortex. UHPLC-MS analysis was performed immediately after the derivatization reaction due to the low stability of OPA derivatives.

2.6. Statistical analysis

The statistical analysis of data was performed with Statistical Software Package for Windows SPSS, version 22 (SPSS, Chicago, IL, USA). Analysis of the variance for linear regression was used to test the reliability of the method. The Student’s t-test was used to compare between data sets and the homogeneity of variances was assessed through Cochran’s C test.

3. Results and discussion

To properly determine total content of HA and MHA in urine, sample preparation consisted in the acid hydrolysis (0.1M HCl) combined with heat treatment. As reported in previous works, higher levels of these compounds were obtained in hydrolysed urine than in non-hydrolysed samples [17]. In order to set final sample hydrolysis parameters, several time and temperature conditions were assayed. Total conversion of conjugated
analytes to free HA and MHA was achieved after submitting the sample at 90 °C for 30 minutes.

Purification and concentration procedure through MCX SPE cartridges was implemented attending the low concentration of HA and MHA in urine and the need to minimize potential interferences of this matrix. When loading urine sample onto the SPE cartridge, acid pH values achieved by previous acidic hydrolysis facilitated the strong interaction of positively charged amine groups of HA and MHA with the sulfonic anion of MCX sorbent. A proper cleanup step washing with 0.1M HCl allowed the reduction of matrix interferences. Later elution with a basic 5% NH₄OH in methanol (v/v) solution ensured cleavage of the electrostatic interactions between ammonium ion of HA and MHA and sulfonic anion of the sorbent. The use of MCX SPE cartridges allowed five-fold pre-concentration of analytes and greater sample concentration was achieved by eluate evaporation to dryness with a centrifugal vacuum concentrator. Overall analytical procedure achieved a final concentration of the analytes of fifty-fold in relation to initial urine content.

Due to the low natural fluorescence of the analytes, the use of a derivatizing reagent in order to detect HA and MHA and increase the sensitivity of the method was required. The presence of amino groups in the structures of HA and MHA makes both compounds suitable for the derivatization with a large number of fluorogenic reagents, being OPA, fluorescamina and DBD-F \((4-(N,N\text{-dimethylamino sulfonyl})-7\text{-fluoro-2,1,3-}
benzoxadiazole)\) the most commonly used \([12,13,20]\). In this method, an online post-column OPA derivatization procedure was used, which ensured a high reproducibility by minimizing sample manipulation prior to the injection. Moreover, OPA reacts rapidly
with amines in the presence of a reducing agent, improving detection sensitivity, reducing the polarity of original amino compounds and increasing method selectivity [23]. The current method provides a significant improvement in comparison with some previous methods that mostly used pre-column derivatization techniques, which could face problems related to the low stability of OPA-amine derivatives [12,13].

According to previous methods, several mobile phase compositions and gradient programmes were assayed to get the best resolved peaks for HA and MHA [12,13,24]. Considering the slightly structural differences of the analytes, a mobile phase consisting in two eluents of different polarity and pH permitted to establish the necessary gradient in order to properly separate HA and MHA. This was achieved by a gradual and linear slight increase of eluent B, the less polar eluent of the mobile phase, during the first minutes of the chromatographic run. Just after the separation of both analytes, the proportion of acetonitrile was markedly incremented aiming to completely elute less polar urinary compounds, which consequently incremented the chromatographic run up to the final 11 minutes. Moreover, sodium octanesulphonate as ion-pairing reagent was added to the mobile phase in order to improve chromatographic separation of these hydrophilic and polar compounds. Fig. 1 shows the chromatograms of the standard solutions and of urine samples. The proposed method accomplished an acceptable separation between HA and MHA with a chromatographic resolution (R) of 1.5. HA and MHA were identified on the basis of the retention time by comparison with the standard.

The present UHPLC method reduces considerably the time required for urinary determination of HA and MHA in comparison with previously published HPLC-FL
methods, resulting in turn in decreased reagent costs and reduced environmental impact [14,15,17]. The reliability of this UHPLC method for routine analysis of urine samples was assessed in terms of linearity, sensitivity, precision and recovery.

3.1. Linearity

Linearity was tested at twelve different concentrations between 0.05 and 10 mg/L, performing seven measurements at each level. Analysis of the variance of the regression allowed assessing the linearity of the UHPLC method. Least-squares analysis resulted in a correlation coefficient of $r \geq 0.9999$ for both analytes ($p<0.001$). Calibrations data fit a linear regression model with determination coefficients ($r^2$) higher than 99.99% for all standard curves.

3.2. Sensitivity

The chromatographic limit of detection (LOD) and the limit of quantification (LOQ) were obtained following the IUPAC guidelines and using low-concentration HA and MHA standard regression curves ranging from 0.05 to 0.8 mg/L [25]. A blank consisting in 0.1 M HCl was used to determine baseline noise. LODs were 0.025 mg/L for HA and 0.028 mg/L for MHA. LOQs were 0.035 mg/L and 0.045 mg/L for HA and MHA, respectively. To confirm the established LODs and LOQs a standard solution at those level concentrations was analysed (Table 1).

3.3. Precision
Method precision was evaluated through repeatability by carrying out seven independent determinations of a urine sample from a volunteer using the same analytical conditions (Table 1). Urine and spiked urine with known amounts of HA and MHA (0.01, 0.04 and 0.15 mg/L) were studied in septuplicate in order to test the precision at different levels. The relative standard deviation (RSD) for HA and MHA at all concentration levels was lower than 5.5%, representing a satisfactory level of precision. The Horwitz equation for intra-laboratory studies confirmed the acceptability of these precision results.

3.4. Recovery

Method recovery was determined via accuracy evaluation by the standard addition procedure using urine samples spiked with three addition levels (0.01, 0.04 and 0.15 mg/L of HA and MHA). Seven determinations were performed for each addition level (Table 1). The mean recovery of HA was 99.25% (SD=1.86), which was not statistically different from the theoretical value of 100% (p>0.005 according to the Student’s t-test). For MHA, mean recovery was 99.8% (SD=0.21), which neither was statistically different from the theoretical value of 100% (p>0.005). The assumption of homogeneity of variances among the three spiking levels was tested using Cochran’s C test. Experimental values for both analytes remained under the Cochran’s test tabled value, confirming that the variance of recovery values was not dependent on the analyte content (p>0.005).

3.5. Structural analysis of HA and MHA OPA derivatives using UHPLC-HRMS
The structure of HA and MHA conjugated with OPA were confirmed by UHPLC-ITD-FTMS which enabled specific detection of both compounds in a complex matrix such as urine. The spectra showed the protonated molecule with a mass error of less than 2 ppm.

The mass spectra shown in Fig. 3 and Fig. 4 confirmed that the OPA complex for HA was formed with a molecular ion [M+H]+ at m/z = 288.1162 (-1.2 ppm error) in the standard and a molecular ion [M+H]+ at m/z = 288.1165 (0.0 ppm error) in the urine sample. For MHA, OPA conjugated complex was observed with a molecular ion [M+H]+ at m/z = 302.1319 (-1.1 ppm error) and at m/z = 302.1318 (-0.8 ppm error) in the standard and urine sample, respectively.

Moreover, injection in MS² mode was performed. The MS² spectra gave as result an ion with m/z 228.1132 for HA and an ion with m/z 242.1290 for MHA. Confirmation in samples was accomplished by injection of urine samples in the same conditions. Results are shown in Fig. 3 and Fig. 4.

3.6. UHPLC-FL sample analysis

Urine samples from twelve volunteers were analysed using the proposed method (Fig. 2). No significant differences were found in HA urinary content from different volunteers with a mean value of 0.143 ± 0.08 μmol/day, which is in good agreement with the values obtained by other works [17,26]. On the contrary, greater differences in MHA content were found in analysed urine samples. Concretely, two individuals who reported symptomatology related to HIT showed a significantly higher urinary content of MHA. Moreover, the increased MHA contents exceeded the threshold established as normal level of this analyte in urine [27]. These results support the initial hypothesis according
to which the decrease in DAO activity would cause an accumulation of MHA, the metabolite produced by HNMT. Therefore, this method could be an advantageous and more patient-friendly alternative to current plasmatic DAO activity determination used to diagnose histamine intolerance, being less invasive and avoiding the need for specific equipment and qualified personnel required for plasma sample extraction. Additionally, the distribution profile of histamine and its main metabolite in urine could provide a complementary evaluation of DAO activity, specifically considering that some authors have reported a wide variability in DAO activity both in healthy volunteers and patients diagnosed with HIT according to their symptomatology [28,29].

4. Conclusion

The proposed UHPLC method allows the satisfactory determination of urinary HA and MHA in less than 11 minutes. The use of MCX SPE cartridges was effective for the selective purification and concentration of HA and MHA in human urine. Overall sample treatment procedure achieved a final concentration of the analytes of fifty-fold in relation to initial urine content. Online post-column derivatization of the analytes with OPA permitted the sensitive detection of the analytes while minimizing sample manipulation prior to UHPLC injection. Unequivocal identification of HA and MHA OPA derivatives in standard and in urine samples has been accomplished through UHPLC-ITD-FTMS. To our knowledge, this is the first UHPLC-FL method with OPA post-column derivatization used to determine HA and MHA in human urine; thus becoming a potential new approach for the routine diagnosis of histamine intolerant individuals.
Further studies involving more volunteers are needed to validate MHA as a biomarker for the diagnosis of HIT.

Funding

This work was supported by Direcció General de Recerca of the Generalitat de Catalunya (2014-1438 SGR) and the Interministerial Commission for Science and Technology (CICYT) of the Ministerio de Educación, Cultura y Deporte (AGL 2012-39995). Oriol Comas-Basté is a recipient of a doctoral fellowship from the University of Barcelona (APIF2015).
References


Figure captions
Fig 1. Chromatograms of HA and MHA standard solution of 10 mg/L (A) and a urine sample (B).

Fig 2. Urinary content of HA and MHA (μmol/day) in twelve volunteers. Dashed lines indicate normal levels of HA and MHA reported by literature.

Fig 3. Representative chromatogram, FTMS spectra and MS² spectra of OPA derivative HA (A) and MHA (B) standards.

Fig 4. Representative chromatogram, FTMS spectra and MS² spectra of OPA derivative HA (A) and MHA (B) in a urine sample.
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<th>Sensitivity</th>
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<td>Addition level I 0.01 mg/L of HA and MHA</td>
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<sup>a</sup> Relative standard deviation (RSD) for seven determinations.

<sup>b</sup> Acceptable range for relative standard deviations according to Horwitz’s formula for intra-laboratory studies (1/2 - 2/3 of the interlaboratory study calculated by the formula).

<sup>c</sup> Mean recovery percentages and standard deviation in parentheses.

<sup>d</sup> Variance outlier test Cochran Cₗₑₐₜ (6,2,0.005) = 0.8534.
Urinary content (μmol/day)

Histamine

Methylhistamine

Methylhistamine <1.5 μmol/day

Histamine <0.8 μmol/day

0.00 0.40 0.80 1.20 1.60

1 2 3 4 5 6 7 8 9 10 11 12

Histamine

Methylhistamine