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Development and validation of a pre-column reversed phase liquid chromatographic method with fluorescence detection for the determination of primary phenethylamines in dietary supplements and phytoextracts

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ABSTRACT

A sensitive and selective reversed-phase liquid chromatographic (RP-LC) method was developed and validated to determine octopamine, tyramine and Tyrosine (Tyr) in complex matrices as formulations and phytoextracts (*Citrus aurantium*), after pre-column derivatization with *o*-phthaldialdehyde (OPA) reagent. The chromatographic separations were performed at room temperature on a Phenomenex Luna C18 column using methanol and sodium acetate buffer (pH 5.5) by varying composition gradient elution as mobile phase and detected flurometrically at λ_{em} = 455 nm with λ_{ex} = 340 nm. The results obtained by the proposed method were compared with those achieved by a validated direct RP-LC method with fluorescence detection at λ_{em} = 310 nm with λ_{ex} = 275 nm, as reference method, using a Phenomenex Gemini C18 column under isocratic elution conditions with acetonitrile and sodium 1-heptanesulphonate (pH 3), as mobile phase. The higher sensitivity of the derivatization method (detection limit about 0.06 pmol) allowed the sure determination of octopamine present in traces in the examined samples. The repeatability of method (RSD) was ≤1.90% and there was no significant difference between repeatability and intermediate precision data. Recovery studies showed good results 99.5–101.3% with RSD ranging from 0.8 to 1.2%. All analyses were performed by mild conditions in absence of preliminary difficult extraction methodologies or laborious step of sample pre-treatment.

1. Introduction

Recently, dietary supplements formulated with fruits of *Citrus aurantium* have been marketed as an appetite suppressant to support weight loss. The unripe fruits of *C. aurantium* L. var. *amara* (Rutaceae; vernacular name: bitter orange) contain high levels of flavonoids (flavanones, flavones and flavonols) [1] and adrenergic amines which differ in the number and position of hydroxyl substituents and include synephrine, octopamine, tyramine, N-methyltyramine and hordenine. The most active components in *C. aurantium* are synephrine and octopamine. Synephrine is structurally similar to epinephrine, while octopamine is similar in structure to norepinephrine. Synephrine is the primary alkaloid found in the immature fruits, whereas the other alkaloids are present at significantly lower levels. *C. aurantium* alkaloids increase the metabolic rate and promote the oxidation of fat through increased thermogenesis. Synephrine and octopamine activate selectively the β-3 adrenoreceptors and seem to inhibit cAMP production [2–6]. After the ban in April 2004 of *Ephedra sinica* in the US many products for weight loss and bodybuilding now contain *C. aurantium* extracts instead. Although no direct adverse events have been associated with their ingestion thus far, they increase blood pressure and has the potential to rise the risk of cardiovascular events. In addition, *C. aurantium* should be used with caution because of suspected possible interferences with CYP 450 enzymes. Thus, it can cause an alarming increase in the blood levels of many drugs [4,7]. Octopamine is known as a "false neurotransmitter" in humans because it alters the normal function of the brain and is believed to stimulate the production of growth hormone [8]. Therefore, it was included since 2006 in the "The Prohibited List International Standard" by WADA (World Anti-Doping Agency) into stimulant category prohibited in sport competitions [9]. The seriousness of effects above given show the necessity of reliable analytical methods applicable to quality control of commercial dietary supplements, which are able to establish the octopamine content to avoid possible adulterations.

A variety of approaches have been used for the determination of phenethylamine alkaloids in plant materials and extracts, juice and in dietary supplements. Traditionally, the methods involve reversed-phase liquid chromatography (RP-LC) with UV, electrochemical and mass spectrometry detection or capillary

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electrophoresis (CE) [10]. RP-LC with UV detection is the preferred technique in phytochemical analysis for its versatility and the used mobile phases contain in several cases an ion-pair reagent to increase the peak resolution and symmetry [5,11–13]. On other hand, RP-LC with UV detection in absence of an ion-pair reagent was reported in other papers [14–17]. So far, the LC analysis of these compounds using fluorescence or chemiluminescence detection offers great sensitivity and selectivity [5,18,19]. In particular, LC with fluorescence detection was used for the determination of bioactive primary amines in orange juice and drinks by post-column derivatization with *o*-phthaldialdehyde (OPA) reagent [11,20,21].

Typical fluorogenic derivatization reagents for amino function reported in literature are dansyl chloride (Dns-Cl), 9-fluorenylmethyl chloroformate (FMOC), *o*-phthaldialdehyde (OPA), fluorescamine, nynhydrin, phenylisocyanate, phenylisothiocyanate (PITC), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [22,23], but some of them can give a variety of drawbacks. For example, PITC derivatization is long and involves several stages of drying under vacuum before the injection into chromatographic system; on the other hand, with FMOC, the excess of the derivatization reagent can give rise to problems if it is not removed by extraction with pentane or it is not derivatized with an amine whose derivative does not interfere. In addition, Dns-Cl requires a long time of reaction and a low concentration, otherwise the increase of the peak area background decrease the sensitivity of the determination [24,25]. In general, OPA is used as post-column fluorogenic reagent owing to the instability of its adducts, but several papers show that it can be employed in pre-column derivatization with reliable results [26–30]. In general, to improve the sensitivity pre-column derivatization procedures are preferred because they can be carried out without further instrumentation, in short time of derivatization reaction and without limitation of reaction solvent or derivatization reaction rate. Besides, the pre-column labeling offers the advantage to decrease the polarity of the compounds so that they can be retained on the reversed stationary phase. OPA is a compound devoid of significant native fluorescence which react in short times (1 min) and in mild conditions (room temperature) selectively with the primary amino function to give highly fluorescent adducts. OPA pre-column derivatization does not show the presence of excess reagent interfering with the analytes resolution. However, as far as we know OPA was not previously studied as pre-column reagent for phenethylamine alkaloid analysis in plant materials, extracts, juice and dietary supplements.

The aim of the present work is to verify the applicability of OPA as pre-column derivatization reagent for the RP-LC fluorescent determination (λ_{em} = 455 nm with λ_{ex} = 340 nm) of octopamine and other primary phenethylamines in dietary supplements and phytoextracts having a complex matrix. In addition, results obtained by a reference direct RP-LC method using fluorescence detection (λ_{em} = 310 nm with λ_{ex} = 275) were included and evaluated critically. The performance of both methods was investigated with respect to linearity, detection and quantification limits, precision and accuracy.

2. Experimental

2.1. Materials

(±) Octopamine hydrochloride >95%, tyramine hydrochloride 99% (±) synephrine 98%, quinine 98% (used as internal standard, IS), methanol and acetonitrile were purchased from Sigma–Aldrich (Milan, Italy), whereas hordenine and N-methyltyramine from Carbone Scientific Co. Ltd. (London, United Kingdom). *o*-Phthaldialdehyde (OPA) and L-Tyrosine (Tyr) 99% were obtained from Fluka (Buchs, Switzerland). Purified water by a Milli-RX (Millipore, Milford, MA, USA) apparatus was used for the preparation of all solutions and mobile phases. All the other chemicals were of analytical reagent grade.

2.2. Solutions

Standard solutions of Tyr, octopamine and tyramine were prepared in water and were subjected to derivatization reaction (method A), whereas the standard solutions of all phenethylamines were prepared in a mixture A:B (60:40, v/v), where A is water and B is the appropriate mobile phase for the direct method (method B). Phenethylamine concentrations are reported in Table 1. The mass of the standard compound hydrochloride was converted to the corresponding free base form using a molecular mass conversion. The reagent OPA solution was prepared by dissolving 100 mg in 1.5 mL of methanol; then, to the obtained solution 100 μL of 2-mercaptoethanol and 11.2 mL of sodium borate buffer (pH 9.5; 0.4 M) were added. The mixture was stored in the dark at 4 °C and was allowed to stand for 24 h before use. An aliquot of 10 μL of mercaptoethanol was added every 2 days to help to maintain the reagent strength. The solution remains stable for about 2 weeks [31]. IS solution corresponding to 250 and 6.25 μg/mL for the analysis of formulations (capsules) and phytoextracts was prepared in water, respectively. Borate buffer (pH 9.5, 0.4 M) solution was prepared by adjusting the pH of a boric acid solution to 9.5 with 1 M sodium hydroxide [32]. Sodium acetate buffer (pH 5.5, 0.05 M) was prepared by dissolving sodium acetate in water and adjusting to pH 5.5 with glacial acetic acid [33]. Sodium 1-heptanesulphonate (SHS) (pH 3, 12 mM) was prepared by dissolving SHS in water and adjusting to pH 3 with phosphoric acid.

2.3. Equipment

The liquid chromatograph consisted of a Jasco Model LG-980-02S ternary gradient unit, a Jasco PU-1580 pump and a Jasco FP-920 fluorescence detector (Jasco Corporation, Tokyo, Japan). All separations were performed at ambient temperature (25 °C). The data were collected on a PC equipped with the integration program Borwin and ChromNav. The solvents were degassed on line with a degasser model Gastorr 153 S.A.S Corporation (Tokyo, Japan). Manual injections were carried out using a Rheodyne model 7125 injector with 20 μL sample loop. A column inlet filter (0.5 μm × 3 mm i.d.) model 7335 Rheodyne was used. The centrifugation was performed both by an ALC 4235A and an eppendorf Centric 150 Tehtnica (Opto-Lab, Concordia, Modena, Italy) centrifuge. Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with thermostatically controlled heating (30–80 °C) was used for ultrasonication.

2.4. Derivatization procedure

A 50 μL aliquot of the phenethylamines solution was treated with 50 μL of sodium borate buffer (pH 9.5, 0.4 M) and 40 μL of the reagent OPA solution were added. The reaction was carried out at ambient temperature in a tube (1.0 mL) for 1 min covering 15 s ultrasonication in presence of the appropriate IS solution. Then, 100 μL of a mixture A:B, where A is methanol and B is sodium acetate buffer (pH 5.5, 0.05 M) in the ratio 50:50, v/v, were added and 20 μL aliquot of the resulting clear solution was injected into the chromatograph.