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Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 1113–1119

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Determination of adrenergic agonists from extracts and herbal products of *Citrus aurantium* L. var. *amara* by LC☆

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Received 26 June 2001; received in revised form 31 July 2001; accepted 2 August 2001

Abstract

The purpose of this study was to set up a HPLC method to separate adrenergic amines (*dl*-octopamine, *dl*-synephrine and tyramine) and to determine their content in fruits, extracts and herbal products of *Citrus aurantium* L. var. *amara*. A rapid method for the quantitative analysis of these amines is described, based on their separation by RP-HPLC technique with UV detection. The analysis were conducted on a Lichrospher RP-18 column at room temperature, using a mobile phase consisting of 0.02 M citric acid–0.02 M NaH_2PO_4 (7:3 v/v) and adjusted to a final pH of 3. The detection was at 220 nm. Since some of these amines are chiral compounds and their enantiomers showed different pharmacological activity, the direct separation of synephrine enantiomers was carried out with HPLC on a β-cyclodextrin stationary phase. The mobile phase consisted of methanol–NaH_2PO_4 25 mM pH 3.5 (20:80 v/v) and tetrabutylammonium hydrogen sulfate 10 mM in ratio of 30:70 v/v in isocratic condition and the detection was at 220 nm. The two proposed methods were applied to the analysis of fruits, extracts and herbal products of *C. aurantium* L. var. *amara*. Taking into account that some authors have reported that *l*-synephrine may be converted into its *d*-form by high temperature, this optical isomerization was monitored by the same HPLC method used for the separation of enantiomers.

Keywords: *Citrus aurantium* L. var. *amara*; Adrenergic agonists; Synephrine; Reversed phase HPLC; Enantioseparation; β-cyclodextrins

1. Introduction

Citrus aurantium L. var. *amara* extracts have been recently introduced for antiobesity treatment in view of their composition in adrenergic amines (Fig. 1) [1] such as synephrine, octopamine and tyramine. However, the most important active constituent of the plant is synephrine and commercially available extracts are standardized for their content of this active compound. Synephrine

☆ This work was presented at 9th International Meeting on Recent Developments in Pharmaceutical Analysis, RDPA '01, Lipari, Aeolian Islands.

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00153-X

produces effects on human metabolism which could be useful for reducing fat mass in obese humans since it stimulates lipolysis, rises metabolic rate and oxidation of fat through increased thermogenesis [2,3]. It is also well known that synephrine and the other amines found in *C. aurantium* have effects on the cardiovascular system through adrenergic stimulation [4,5].

Some of these amines, such as synephrine, are chiral compounds and their enantiomers showed different pharmacological activity both in qualitative and in quantitative terms [6]. Furthermore their determination in crude drugs and in herbal products seems very important viewing the great commercial diffusion of these derivatives in the last years.

Several HPLC methods with electrochemical detection were described for the separation and quantification of biogenic amines in mouse central nervous system [7–9] and in *C. aurantium* crude drugs [10].

In the present study, the simultaneous analysis of *dl*-octopamine, *dl*-synephrine and tyramine was carried out with a RP-HPLC method with DAD-detection. This method was applied for the characterization of fresh and dried fruits, dried extracts and herbal products of *C. aurantium*.

Although the separation of synephrine and its metabolite had been accomplished by different methods such as capillary gas chromatography [11] and HPLC with electrochemical detection [12], the determination of synephrine enantiomers in complex matrices such as extracts and herbal products has not been properly investigated by RP-HPLC method with UV-detection.

Furthermore, the present paper describes a new simple method for the separation of synephrine enantiomers by means of HPLC-DAD technique based on β-cyclodextrin stationary phase.

Since some authors described that *l*-synephrine could be converted into its *d*-form at high temperature [13], this method was applied to the study of optical isomerization of synephrine induced by heat.

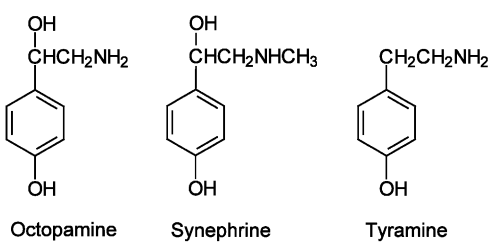

Fig. 1. Structures of adrenergic amines in *C. aurantium*.

2. Experimental

2.1. Chemicals and reagents

Octopamine, synephrine, tyramine, citric acid and sodium phosphate were purchased from Sigma (Milan, Italy). Tetrabutylammonium hydrogen sulfate was from Fluka (Buchs, Switzerland). Methanol HPLC grade was from Merck (Darmstadt, Germany).

C. aurantium L. var. *amara* fruits, kindly obtained by Dr Daniele Dallai of the Botanical Garden of the University of Modena and Reggio Emilia (Italy), came from Hanbury Botanical Institute of the University of Genova.

C. aurantium extracts and herbal products were obtained commercially from local markets.

2.2. Apparatus and HPLC conditions

2.2.1. Determination of octopamine, synephrine and tyramine

The HPLC system consisted of a HPLC 1100 Series (Hewlett-Packard, Waldbronn, Germany) with a diode array detector and a Lichrospher RP-18 column (125 × 4 mm, 5 μm, Hewlett-Packard), coupled with a Lichrospher RP-18 precolumn (4 × 4 mm, 5 μm, Hewlett-Packard).

The mobile phase was formed by 0.02 M citric acid–0.02 M NaH_2PO_4 (7:3 v/v) and adjusted to a final pH of 3. The mobile phase was pumped at a flow rate of 1 ml/min in isocratic condition. The detection was set at 220 nm.

The system was operated at room temperature.

2.2.2. Calibration curves

The standard solutions were prepared by dilution of the stock solutions with water to reach a concentration range of 1.8–35.9 ng/μl for octo-

pamine, 50.2–502.0 ng/μl for synephrine and 2.5–50.8 ng/μl for tyramine.

Duplicate 5 μl injections of each concentration were made and chromatographed under the conditions described above.

The peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

2.2.3. Separation of synephrine enantiomers

The HPLC system consisted of a HPLC 1100 Series (Hewlett-Packard) with a diode array detector and a Lichrocart Chiradex column (250 × 4 mm, 5 μm, Merck).

The mobile phase was formed by solvent A (methanol–NaH_2PO_4 25 mM pH 3.5 (20:80 v/v))–solvent B (tetrabutylammonium hydrogen sulfate 10 mM) in ratio of 30:70 v/v. The flow rate was 0.4 ml/min and the column was kept at 3 °C by means of a column thermostat HP 1100 Series (Hewlett-Packard). The detection was set at 220 nm.

2.2.4. Calibration curve

The stock standard solution was prepared by dissolving mg 5.049 of *dl*-synephrine in 10 ml of ultra pure water. The calibration curve was obtained from peak areas of the standard solutions over the concentration range 12.8–252.5 ng/μl for *l*-synephrine and 12.3–247.4 ng/μl for *d*-synephrine.

2.3. Sample preparation

A weighted amount (0.2–2.0 g) of pulverized sample (*C. aurantium* dried fruits, extracts and herbal products) was extracted with water (20 ml) at room temperature under magnetic stirrer for 30 min. After centrifugation for 15 min, the supernatant solution was filtered under vacuum into a volumetric flask. The residue was re-extracted in the same way. The final volume of the extracting solution was set at 50 ml. In the case of fresh fruits pulp, a weighted amount (18–20 g) was extracted in the way described above and the solution was brought to the final volume of 25 ml. In aqueous solution all the analytes were found to be stable.

A total of 5 μl of each solution were then injected and the analysis was repeated twice.

This procedure was followed both for the determination of amines and for synephrine enantiomers separation.

2.4. Heat stability of synephrine in C. aurantium fruits

In order to evaluate the eventual optical isomerization of synephrine induced by heat, a portion of homogenized fruits pulp was refluxed with water at 100 °C for 24 h. A sample of this solution was analyzed every hour according to the HPLC method already described.

3. Results and discussion

3.1. Validation of the method for octopamine, synephrine and tyramine separation

3.1.1. Linearity

The regression equation was $y = 11.11x - 2.68$ for octopamine (S.D. of the slope 0.23, S.D. of the intercept 4.19), $y = 11.87x - 8.26$ for synephrine (S.D. of the slope 0.14, S.D. of the intercept 40.76) and $y = 9.82x - 6.98$ for tyramine (S.D. of the slope 0.38, S.D. of the intercept 11.06). The calibration curves indicate a good linear relationship: the correlation coefficient was 0.998 for octopamine, 0.999 for synephrine and 0.995 for tyramine.

3.1.2. Limits of detection and quantification

The limit of detection (LOD) of the proposed method, calculated taking a signal-to-noise ratio of three as criteria, was 0.1 ng/μl for octopamine, 0.8 ng/μl for synephrine and 0.3 ng/μl for tyramine. The limit of quantification (LOQ), measured by preparing serial diluted solutions, was 0.9 ng/μl for octopamine, 2.3 ng/μl for synephrine and 1.2 ng/μl for tyramine.

3.1.3. Reproducibility

The reproducibility, tested by repeating the extraction procedure and analysis eight times, was 1.39% for synephrine, 2.47% for octopamine and 2.68% for tyramine.

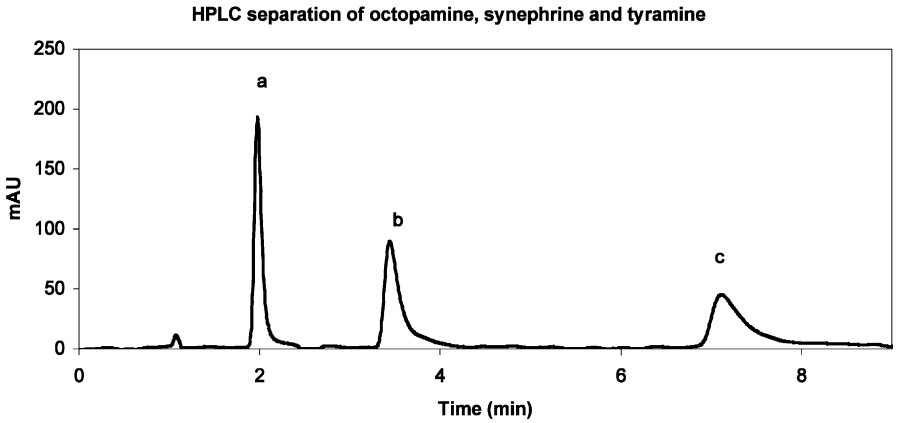

Fig. 2. Chromatogram of a standard mixture of octopamine, synephrine and tyramine. Peaks: (a) octopamine; (b) synephrine; (c) tyramine. HPLC conditions: Lichrospher RP-18 column (125 × 4 mm, 5 μm), coupled with a Lichrospher RP-18 precolumn (4 × 4 mm, 5 μm). Mobile phase: 0.02 M citric acid–0.02 M NaH_2PO_4 (7:3 v/v), pH 3. Flow rate: 1 ml/min. Detection at 220 nm.

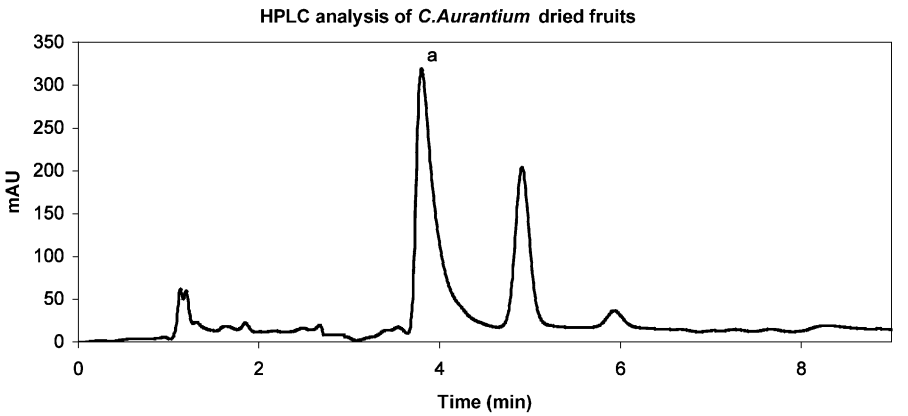

Fig. 3. Chromatogram of a sample solution obtained from dried fruits. Peaks: (a) synephrine. The chromatographic conditions are the same of Fig. 2.

3.1.4. Recovery

This study was performed by addition of known amount of standard to a known concentration of *C. aurantium* samples. The resulting mixtures were assayed and the recovery obtained was 99.7% for synephrine, 99.2% for octopamine and 99.3% for tyramine.

3.2. Determination of octopamine, synephrine and tyramine

Fig. 2 shows the chromatogram obtained from RP-HPLC separation of a mixture of octopamine, synephrine and tyramine standards.

The retention times of the three amines were 1.9 min for octopamine, 3.4 min for synephrine and 7.1 min for tyramine, respectively.

A typical chromatogram for the extracted sample solution is described in Fig. 3.

Table 1 summarizes the contents in adrenergic amines in all the analyzed samples.

In the *C. aurantium* products, we found that the *dl*-synephrine content was the main one between the three amines determined, although it was very low in fresh fruits (0.020%). In only one sample (herbal product n.2) we found a high content of octopamine (0.147%).

Table 1
Contents of *dl*-octopamine, *dl*-synephrine and tyramine in *Citrus aurantium* L. var. *amara* products by means of RP-HPLC method

Sample	Octopamine (%)	Synephrine (%)	Tyramine (%)
Fresh fruits	<LOQ	0.020	<LOQ
Dried fruits	<LOQ	0.352	<LOQ
Dried extract n.1	0.028	3.003	0.056
Dried extract n.2	0.023	3.079	0.055
Herbal product n.1	0.013	0.989	0.019
Herbal product n.2	0.147	0.664	0.022
Herbal product n.3	0.015	0.250	0.032

3.3. Validation of the method for synephrine enantiomers separation

3.3.1. Linearity

The regression equation was $y = 31.39x + 206.71$ for *l*-synephrine (S.D. of the slope 0.68, S.D. of the intercept 97.81), $y = 27.41x + 316.69$ for *d*-synephrine (S.D. of the slope 0.60, S.D. of the intercept 77.50). The correlation coefficient was 0.998 for each synephrine enantiomer.

3.3.2. Limits of detection and quantification

The LOD of the proposed method, calculated taking a signal-to-noise ratio of three as criteria, was 0.4 ng/μl for each enantiomer, while the LOQ, measured by preparing serial diluted solutions, was 1.1 ng/μl.

3.3.3. Reproducibility

The reproducibility, tested by repeating the extraction procedure and analysis eight times, was 1.39% for *l*-synephrine and 3.12% for *d*-synephrine.

3.3.4. Recovery

This study was performed by the addition of a known amount of standard to a known concentration of *C. aurantium* samples. The resulting mixtures were assayed and the recovery obtained was 99.8%.

3.4. Separation of synephrine enantiomers

Fig. 4 shows the chromatogram obtained for a standard solution of *dl*-synephrine.

The retention times were 21.4 min for *l*-synephrine and 22.5 min for *d*-synephrine. The separa-

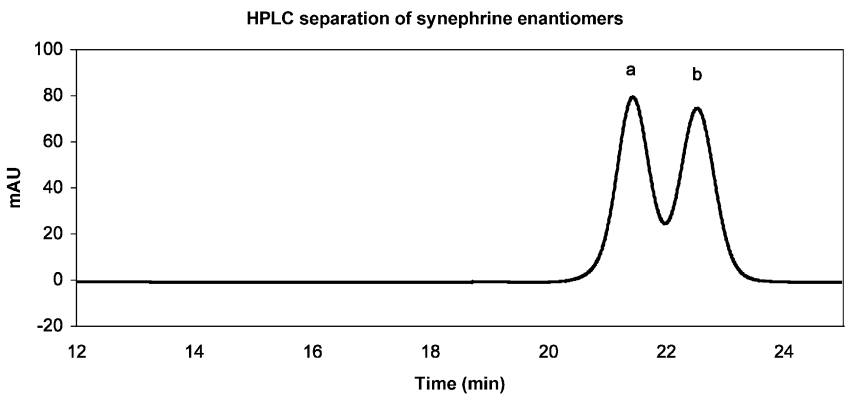

Fig. 4. Chromatogram of a standard solution of *dl*-synephrine. Peaks: (a) *l*-synephrine; (b) *d*-synephrine. HPLC conditions: Lichrocart Chiradex column (250 × 4 mm, 5 μm). Mobile phase: solvent A (methanol/NaH_2PO_4 25 mM pH 3.5 (20:80 v/v))–solvent B (tetrabutylammonium hydrogen sulfate 10 mM) in ratio of 30:70 v/v. Flow rate: 0.4 ml/min. Temperature: 3 °C. Detection at 220 nm.

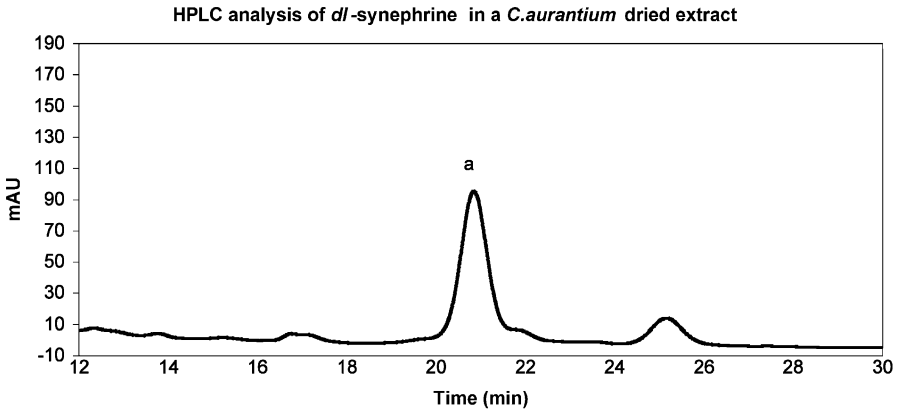

Fig. 5. Chromatogram of a sample solution obtained from a dried extract. HPLC conditions are the same of Fig. 4. Peaks: (a) *l*-synephrine.

Table 2
Contents of *dl*-synephrine enantiomers in *Citrus aurantium* L. var. *amara* products determined by HPLC technique on β-cyclodextrin stationary phase

Sample	*dl*-synephrine (%)	*l*-synephrine (%)	*d*-synephrine (%)	*l*-synephrine (relative %)	*d*-synephrine (relative %)
Fresh fruits	0.027	0.025	0.002	92.389	7.611
Dried fruits	0.380	0.380	<LOQ	100.000	0.000
Dried extract n.1	2.996	2.996	<LOQ	100.000	0.000
Dried extract n.2	2.847	2.847	<LOQ	100.000	0.000
Herbal product n.1	1.050	0.979	0.071	93.271	6.729
Herbal product n.2	0.616	0.584	0.031	94.912	5.088
Herbal product n.3	0.237	0.237	<LOQ	100.000	0.000

tion factor (α) was 1.05 and the resolution (R_s) was 0.73.

Fig. 5 shows a chromatogram of a sample extract of *C. aurantium*, while Table 2 describes the content of each enantiomer in all the analyzed sample.

L-synephrine was present in all the analyzed samples; when *d*-synephrine was determined, we observed that *l*-synephrine content was higher than *d*-synephrine. A significant amount of *d*-synephrine was found in fresh fruits (7.6%) and in some different commercial products.

3.5. Heat stability of synephrine

Fig. 6 shows the results obtained from the study of the heat stability of synephrine in *C. aurantium* fruits. We observed no significant changes of the relative % of synephrine enantiomers.

4. Conclusion

The RP-HPLC technique set up for the deter-

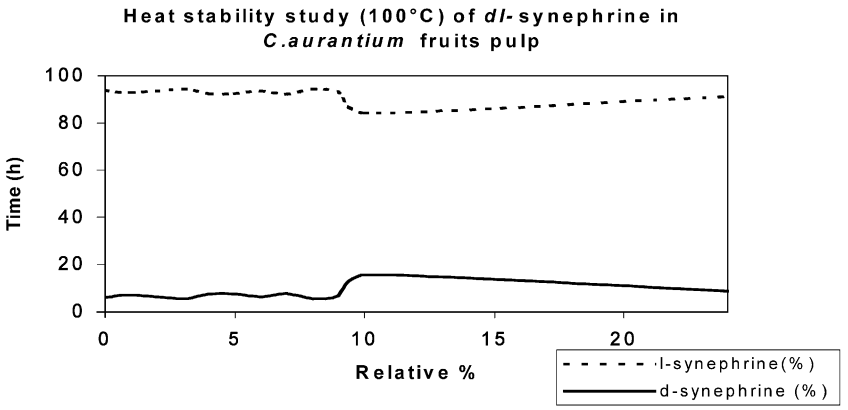

Fig. 6. Effects of temperature on synephrine enantiomers in *C. aurantium* fruits pulp.

mination of biogenic amines in *C. aurantium* L. var. *amara* was found to be rapid, sensible and selective. The analysis conditions were efficient to separate synephrine, octopamine and tyramine without interferences with the flavonoids and the other constituents of citrus extract.

The present chiral separation by means of Chiradex column in the condition described above proved to be useful and suitable to quality control of *C. aurantium* phytoderivatives, viewing the great diffusion of these extracts for the antiobesity treatment and the different and important activities of synephrine enantiomers.

Considerig the results obtained from the heat stability study, we observed that synephrine was not enantiomerized at the temperature usually adopted for the extraction procedures.

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