Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 212–214

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Quantification of 4′-geranyloxyferulic acid, a new natural colon cancer chemopreventive agent, by HPLC-DAD in grapefruit skin extract

S. Genovese[a,*], F. Epifano[a], G. Carlucci[a], M.C. Marcotullio[b], M. Curini[b], M. Locatelli[a]

[a] Dipartimento di Scienze del Farmaco, Università degli Studi "G. D'Annunzio" Chieti-Pescara, Via dei Vestini 31, 66100 Chieti Scalo (CH), Italy
[b] Dipartimento di Chimica e Tecnologia del Farmaco, Sez. Chim. Org., Università di Perugia, Via del Liceo, 06123 Perugia (PG), Italy

ARTICLE INFO

Article history:
Received 14 October 2009
Received in revised form 19 January 2010
Accepted 22 January 2010
Available online 1 February 2010

Keywords:
3-(4′-Geranyloxy-3′-methoxyphenyl)-2-trans-propenoic
Grapefruit
HPLC-DAD method
Prenyloxyphenylpropanoids
Rutaceae

ABSTRACT

Oxyprenylated natural products (isopentenyloxy-, geranyloxy- and the less spread farnesyloxy-compounds and their biosynthetic derivatives) represent a family of secondary metabolites that have been consider for years merely as biosynthetic intermediates of the most abundant *C*-prenylated derivatives. Many of the isolated oxyprenylated natural products were shown to exert *in vitro* and *in vivo* remarkable anti-cancer and anti-inflammatory effects. 4′-Geranyloxyferulic acid [3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic] has been discovered as a valuable chemopreventive agent of several types of cancer. After development of a high yield and "eco-friendly" synthetic scheme of this secondary metabolite, starting from cheap and non-toxic reagents and substrates, we developed a new HPLC-DAD method for its quantification in grapefruit skin extract. A preliminary study on C18 column showed the separation between GOFA and boropinic acid (having the same core but with an isopentenyloxy side chain), used as internal standard. The tested column were thermostated at 28 ± 1 °C and the separation was achieved in gradient condition at a flow rate of 1 mL/min with a starting mobile phase of H_2O:methanol (40:60, v/v, 1% formic acid). The limit of detection (LOD, S/N = 3) was 0.5 μg/mL and the limit of quantification (LOQ, S/N = 10) was 1 μg/mL. Matrix-matched standard curves showed linearity up to 75 μg/mL. In the analytical range the precision (RSD%) values were ≤2% and the accuracy (bias%) between ±12%. This method was used to evaluate for the first time the presence of this analyte in natural extract of grapefruit. In conclusion, this method showed LOQ values able to selective quantification of this analyte in grapefruit skin extract.

1. Introduction

Secondary metabolites of phenylpropanoid acid biosynthetic origin containing sesquiterpenyl, monoterpenyl and isopentenyl chains attached to a phenol group represent quite a rare class of natural products. Only in the last decade were these compounds studied extensively from a chemical and pharmacological point of view [1–4]. This group of natural products are mainly found in plants belonging to the Rutaceae, Apiaceae, and Compositae families, for which prenylation of phenolics could be claimed as a chemotaxonomic marker [5]. In particular prenyloxycinnamic acids were shown to exert the most valuable and promising biological effects [6].

In the last years we focused our interest on studying the chemical and biological properties of prenyloxyphenylpropanoids commonly found in edible fruits and vegetables in order to get further insights in their dietary feeding chemopreventive properties of severe acute and chronic diseases, like cancer and neurological disorders [7,8]. Although several phytochemical and pharmacological reports were reported in the literature during the last decade, studies aimed to build up analytical methodologies to reveal the presence of prenyloxyphenylpropanoids in plant extract are not numerous, so this is a field of research of current interest.

3-(4′-Geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid, named in the text as 4′-geranyloxyferulic acid (Fig. 1B) (GOFA), is a secondary metabolite biosynthetically related to ferulic acid in which a geranyl chain is attached to the phenolic group.

It was isolated in 1966 for the first time from the bark of *Acronychia baueri* Schott, an Australian small tree belonging to the family of Rutaceae [9]. Although known for more than four decades, only in the last ten years some of the pharmacological properties of this natural product began to be characterized. In particular GOFA showed a series of interesting biological effects such cancer chemoprevention by dietary feeding in rats and other effects closely

Abbreviations: GOFA, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic; SPE, solid phase extraction; HPLC-UV/vis, high performance liquid chromatography-ultraviolet/visible detector; LOD, limit of detection; LOQ, limit of quantification; S/N, signal to noise ratio.

* Corresponding author. Tel.: +39 08713554653; fax: +39 08713554912.
E-mail address: s.genovese@unich.it (S. Genovese).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.01.041

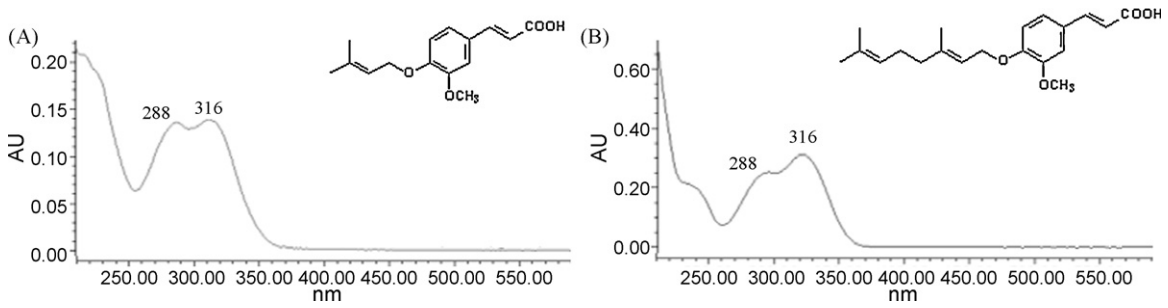

Fig. 1. Chemical structures of internal standard (boropinic acid (A) and analyte (3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic (B)) and its UV/vis spectra.

related to cancer growth and development, that metabolite were recently reviewed [10]. In continuation on our studies on this class of natural products, we wish to report herein a novel HPLC-DAD methods for the quantification of GOFA in complex matrices deriving from edible fruits (e.g. grapefruits).

2. Experimental

2.1. Materials and reagents

3-(4′-Geranyloxy-3′-methoxyphenyl)-2-trans-propenoic and boropinic acid (used as internal standard) were synthesized as already reported and their purity was assessed by GC/MS [10]. Methanol (HPLC-grade) from Carlo Erba (Milan, Italy) was employed for the analyses. Double distilled water was obtained by a Millipore Milli-Q Plus waters treatment system (Millipore, Bedford, MA, USA). For solid phase extractions were used Sep-Pak Vac 1cc (100 mg) C18 cartridges (Waters, Milford, MA, USA).

2.2. Chromatographic condition

HPLC analyses were performed using a Waters liquid chromatograph equipped with a model 600 solvent pump while a 2996 photodiode array detector and Empower v.2 Software (Waters, Milford, MA, USA) was used for data acquisition. A C18 reversed-phase packing column (GraceSmart RP18, 4.6 × 150 mm, 5 μm; Grace, Deerfield, IL, USA) was used for the separation and the column was thermostated at 28 ± 1 °C using a Jetstream2 Plus column oven. The UV/vis acquisition wavelength was set in the range of 210–600 nm. Analog output channel A was set at wavelength 316 nm with a bandwidth of 9.6 nm. The qualitative analyses were achieved at a wavelength of 288 nm. The injection volume was 20 μL. The mobile phase was directly on-line degassed by using Degassex, mod. DG-4400 (Phenomenex, Torrance, CA, USA). Mobile phase composition was water (solvent A) and methanol (solvent B), both with 1% formic acid. Separation was achieved under isocratic elution conditions (1 min at 60% B) followed by linear gradient (in 2 min to 90% B), isocratic condition (2 min at 90% B) and column re-equilibration (9 min at 60% B) at 1 mL/min flow rate. All the prepared sample solutions were centrifuged and the supernatant was directly injected into HPLC-UV/vis system.

2.3. Calibration standards and quality control samples

Stock solutions of 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid and boropinic acid were prepared separately by dissolving the crystalline pure powders in methanol in order to achieve a primary concentration of 1 mg/mL, and stored in aliquots at −20 °C. Working standard solutions were prepared by appropriate dilutions of the 1 mg/mL stock solutions to obtain solutions at concentration levels in the 10–750 μg/mL range and stored at −20 °C for a period of time not longer than 4 weeks. Working solution of internal standard was obtained by diluting boropinic acid stock solution to 400 μg/mL. All stock and working standard solutions were stored in glass tubes. Separate solutions were used to prepare calibration standards and QC samples. Volume of working standard solution spiked was less than 15% of total samples volume.

3. Result and discussion

3.1. Optimization of LC separation

Several HPLC method variables influencing separation of 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid and boropinic acid were investigated. The best result in terms of peak response (area and shape), chromatographic resolution and overall run time was obtained with GraceSmart RP18 (4.6 mm × 150 mm, 5 μm) thermostated at 28 ± 1 °C. In the optimized analytical conditions, mean retention times were as follows: 6.71 ± 0.01 min (R.S.D. = 0.46%) for the internal standard and 9.12 ± 0.09 min (R.S.D. = 2.0%) for analyte. Conditions set for column purge and re-equilibration ensured column pressure and chromatogram background stability. The calculated capacity factor (k') were 2.67 for internal standard and 3.96 for 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid. The dead retention time, calculated with uracile, was 1.83 min.

3.2. Limit of detection

Limits of detection (LODs) were estimated by measuring S/N values obtained in mobile phase spiked at 1 μg/mL level and extrapolating the corresponding values to S/N = 3. LOD value was 0.5 μg/mL for the analyte.

3.3. Linearity and limit of quantification

The calibration curves showed a good linearity in the concentration range between 1 and 75 μg/mL ($R^2 \geq 0.9880$). The back-calculated calibration standard points showed R.S.D.% values ranging from 1.7% to 6.5%. The percent difference between the standard concentrations calculated from the calibration curve and theoretical ones for both tested analytes ranged from −14% to 10%. Limit of quantification (LOQ) was evaluated according to the guidance for industry on the validation of bioanalytical methods, i.e. as the lowest analyte concentration corresponding to a response at least 10 times higher than blank response and which can be determined with 80–120% accuracy and 20% precision [11]. The back-calculated concentration data obtained from calibration curves allowed to assess 1 μg/mL as the validated LOQ of the analytical method for analyte. This method is selective for the quantitative analyses because two wavelengths were used for a correct identification and quantification of the analyte. Wavelength at 316 nm was used for the quantitative analyses and wavelength at 288 nm

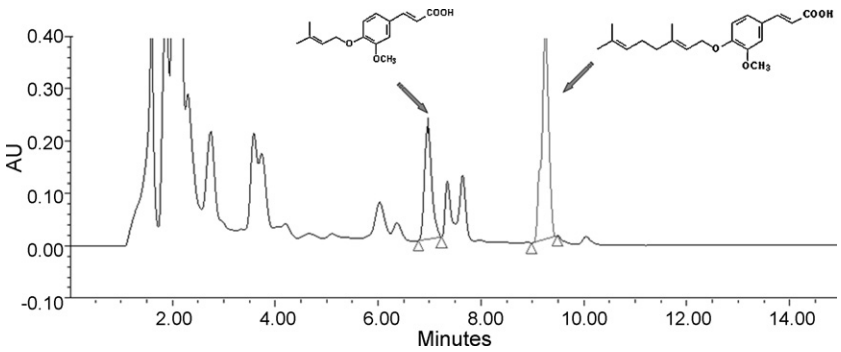

Fig. 2. HPLC-DAD profile for the analyses of grapefruit skin extract.

for the qualitative ones. The assay was also sensitive reasonably well for the direct quantitative determination of this compound in extracts of real matrices (Fig. 2).

3.4. Precision and accuracy

The accuracy and precision results for 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid, obtained analyzing QC samples prepared at three different concentration levels of 7.5, 30 and 60 μg/mL. For precisions, R.S.D.% values calculated for all the tested levels ($n = 6$ each) did not exceed 8% and 10%. For accuracies, bias values ranged from −3.6% to 7.9%.

3.5. Preliminary results in grapefruit skin extract

The assay was applied in a preliminary study for the direct qualitative determination of the title compound in grapefruit skin. No relevant effects were observed in extraction method both with Strata® C18 (Phenomenex) and Sep-Pak® C18 (Waters) SPE cartridges. The experimental conditions are the following: 1 mL of organic modifier (methanol) for the activation followed by 1 mL of Milli-Q water for the equilibration.

2 mL of extracts were loaded afterwards, then 2 mL of Milli-Q water was used for washing and the elution of analyte was accomplished with 2 mL of methanol. The elutes were dried under vacuum and reconstructed with 0.5 mL of mobile phase. With these two SPE columns it is possible to obtain a similar recovery values for internal standard and for 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid. This step is necessary to eliminate any possible matrix interferences and to obtain the sample in the same HPLC starting conditions. In this way it is possible to optimize the chromatographic separation and resolution. In the skin of grapefruit it was observed a total recovery strictly more than 100% while in the pulp no significant signals were found with this assay. It is interesting to note that this high recovery value may be probably associated to an endogenous presence of the analyte in this source, for this reason more detailed studies to quantify the analytes are necessary and will be performed in the next future. The optimized grapefruit skin extractions were exaustively achived using methanol (1:3, p/v) as extraction solvent and treated with ultrasound for 40 min and overnight maceration. Then the extracts were filtered on 0.45 μm polyamide filter and stored at −20 °C until analysis.

4. Conclusion

In this short communication we have shown, for the first time, that our methodology could be effective for the qualitative determination of a colon cancer chemopreventive agent from natural sources like the title molecule.

Our study represents the first report about the presence of 4′-geranyloxyferulic acid in white grapefruits. Studies to investigate if GOFA is also contained in other varieties of grapefruits like the “red” one, as well as in other edible agrumes are now in course in our laboratories.

In view of its well-documented pharmacological effects so far determined both *in vitro* and *in vivo*, in the next future we will validate our set-up analytical methodology in matrices to quantify the presence of 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid in edible plants belonging to the family of Rutaceae like lemons, oranges, grapefruits, carrots, celery and several others. This could be also the stimulus to correlate the presence of this anti-tumor secondary metabolite with a potential dietary cancer chemoprevention strategy, based on feeding with fruits and vegetables commonly consumed as food all over the world.

References

[1] M. Curini, G. Cravotto, F. Epifano, G. Giannone, Chemistry and biological activity of natural and synthetic prenyloxycoumarins, Curr. Med. Chem. 13 (2006) 199–222.
[2] P. Pattanaprateeb, N. Ruangrungsi, G.A. Cordel, Cytotoxic constituents from *Cratoxylum arborescens*, Planta Med. 71 (2005) 181–183.
[3] A.R. Bilia, A.W. Yusuf, A. Braca, A. Keita, I. Morelli, New prenylated anthraquinones and xanthones from *Vismia guinensis*, J. Nat. Prod. 63 (2000) 16–21.
[4] A.F. Magalhães, A. Tozzi, E.G. Magalhães, I.S. Blanco, M.C. Soriano, Flavonoids from *Lonchocarpus muehlbergianus*, Ann. Acad. Bras. Cienc. 76 (2004) 651–661.
[5] F. Epifano, S. Genovese, L. Menghini, M. Curini, Chemistry and pharmacology of oxyprenylated secondary plant metabolites, Phytochemistry 68 (2007) 939–953.
[6] M. Curini, F. Epifano, S. Genovese, Synthesis of a novel prodrug of 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans propenoic acid, Bioorg. Med. Chem. Lett. 15 (2005) 5049–5052.
[7] T. Tanaka, M.B. de Azevedo, N. Duran, J.B. Alderete, F. Epifano, S. Genovese, M. Tanaka, T. Tanaka, M. Curini, Colorectal cancer chemoprevention by two beta-cyclodextrin inclusion compounds of auraptene and 4′-geranyloxyferulic acid, Int. J. Cancer 126 (2010) 830–840.
[8] S. Genovese, F. Epifano, M. Curini, M. Dudra-Jastrzebska, J.J. Luszczki, Prenyloxyphenylpropanoids as a novel class of anticonvulsive agents, Bioorg. Med. Chem. Lett. 19 (2009) 5419–5422.
[9] R.H. Prager, H.M. Thregold, Some neutral costituents of *Acronychia baueri*, Aust. J. Chem. 5 (1966) 451–453.
[10] M. Curini, F. Epifano, S. Genovese, M.C. Marcotullio, L. Menghini, 3-(4′-Geranyloxy-3′-methoxyphenyl)-2-trans propenoic acid: a novel promising cancer chemopreventive agent, Anticancer Agents Med. Chem. 6 (2006) 571–577.
[11] CDER and CVM Guidance for Industry, Bioanalytical Method Validation. Food and Drug Administration, May 2001. http://www.fda.gov/cder/guidance/4252fnl.pdf.