DEVELOPMENT AND VALIDATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PINOCEMBRIN IN LEAVES OF PIPER ECUADORENSE SODIRO

Santiago Ojeda-Riascos[1]*, Mónica Valdivieso[1], James Calva[1], Vladimir Morocho[1], Gianluca Gilardoni[1], Eduardo Valarezo[1], and Omar Malagon[1]

[1]Universidad Técnica Particular de Loja, Departamento de Química y Ciencias Exactas Loja , Ecuador.

*Autor para correspondencia: esojeda@utpl.edu.ec

Recibido: 2019/06/28 Aprobado: 2019/11/30

DOI: https://doi.org/10.26621/XV21.2019.12.A05.PUCESI.2550.6684

RESUMEN

El pinocembrin es una flavanona que muestra actividades farmacológicas y biológicas tales como: antifúngicas, anticancerosas, antimicrobianas, antiinflamatorias y antioxidantes. Este metabolito secundario ha sido identificado en varias plantas pertenecientes a la familia Piperaceae. La planta Piper ecuadorense es un arbusto nativo utilizado en medicina tradicional por curanderos de la comunidad indígena Saraguro de Ecuador. Por lo tanto, debido a su importante actividad, es necesario desarrollar y validar un método empleando el HPLC-DAD con la finalidad de determinar el pinocembrin en los extractos de las hojas de Piper ecuadorense. El método utilizó una columna BDS Hipersil C-18 (5 µm, 4,6x250 mm i.d.) con la mezcla de acetato de amonio (0.01 M) y metanol (35:65 %, v/v) como fase móvil a una velocidad de flujo de 1 mL / min y la detección fue por UV a 290 nm. Los parámetros de linealidad, exactitud, precisión, recuperación, límite de detección y límite de cuantificación del método en HPLC fueron logrados. El método mostró una excelente linealidad (r2 = 0.9998) y la recuperación fue de 100.70-101.33%. También se alcanzó un alto grado de exactitud y precisión (RSD inferior al 3% para intraday e interday). Así, se desarrolló y validó con éxito un método simple, preciso, seguro y reproducible en el HPLC para analizar el pinocembrin en extractos de las hojas de Piper ecuadorense.

Palabras clave: HPLC-DAD; medicina tradicional ecuatoriana; Piperaceae; pinocembrin; *Piper ecuadorense*

ABSTRACT

Pinocembrin is a flavanon e that shows pharmacological and biological activities such as antifungal, anticancer, antimicrobial, anti-inflammatory, and antioxidants. This secondary metabolite has been identified in several plants belonging to the Piperaceae family. Piper ecuadorense is a native shrub used in traditional medicine by healers from the Saraguro indigenous community of Ecuador. Therefore, due his important activity, it is necessary develop and validate a method by using HPLC-DAD in order to determinate the pinocembrin in Piper ecuadorense leaf extracts. The method utilized a BDS Hipersil C-18 column (5 µm, 4.6x250 mm i.d.) with the mixture of ammonium acetate (0.01 M) and methanol (35:65 %, v/v) as the mobile phase at a flow-rate of 1 mL/min, and detection was by UV at 290 nm. The parameters of linearity, accuracy, precision, recovery, detection limit, and quantitation limit of the HPLC method were achieved. The method showed excellent linearity (r2 = 0.9998), and the recovery was 100.70-101.33%. A high degree of accuracy and precision (RSD less than 3% for intraday and interday) were likewise reached. In this way, a simple, precise, accurate and reproducible HPLC method was successfully developed and validated to analyze pinocembrin in Piper ecuadorense leaf extracts.

Keywords: HPLC-DAD; traditional Ecuadorian medicine; Piperaceae; pinocembrin; *Piper ecuadorense*

OPEN ACCESS
CC BY NC ND

INTRODUCTION

Piper ecuadorense Sodiro is a native shrub belonging to the family Piperaceae. The plant is popularly known as "matico de monte" (Ramírez et al., 2013). In Ecuador, Piper ecuadorense leaves have traditionally been used in aqueous infusion for the treatment of hangover, as a disinfectant or in wound healing (Tene et al., 2007). Moreover, the herbal healers from the Saraguro indigenous community, use the aerial parts in mixture with others plants for the treatment of "mal del aire" (Andrade et al., 2009). Actually, Few studies and no phytochemical works have been reported Piper ecuadorense Sodiro for until now (Ramírez et al., 2013). Only two flavanones, pinostrobin and pinocembrin have been reported as the active constituents, exhibiting antifungal activity (Ramírez et al., 2013). In addition, pinocembrin exhibited anticancer (WANGKANGWAN et al., 2009), antimicrobial (Guo et al., 2015), anti-inflammatory (Rasul et al., 2013)7-dihydroxyflavanone, and antioxidant activities (Sala et al., 2003).

Actually, the validation of an HPLC method is required for quantitative determination of drugs and their metabolites in biological samples and ensures that this methodology is selective, accurate and reproducible (Merken and Beecher, 2000; U.S. Department of Health and Human Services Food and Drug Administration et al., 2018)when finalized, will represent the Food and Drug Administration's (FDA's. It is necessary to validate analytical methods to yield reliable results which can be satisfactorily interpreted (Shah et al., 2000)Bioequiva- lence and Pharmacokinetic Studies (Conference held in Ar- lington, VA, December 3–5, 1990 and the report published in Pharmaceutical Research, 9: 588-592, 1992.

In this study, the quantitative determination of pinocembrin by HPLC-DAD was developed. The parameters linearity, accuracy, precision, recovery, limit of detection and limit of quantitation of the HPLC method were achieved. Thereby, the developed method is expected to be applicable in the analysis of pinocembrin in extracts of plant material and to determinate the degradation percentage of this compound in pharmaceutical forms.

MATERIALS AND METHODS

Chemicals and reagents

Analytical standards like pinocembrin (purity >95%) (Figure 1a) and chrysin (purity >97%, internal standard) (Figure 1b), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate (purity ≥ 99.0%) (Sigma-Aldrich, St. Louis, MO, USA) and methanol (J.T. Baker, USA) were of analytical grade.

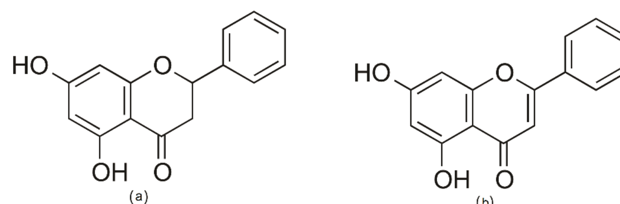

Figure 1. Structures of Pinocembrin (a) and chrysin (b)

Plant material

Piper ecuadorense leaves were collected in Zamora Chinchipe Province, Ecuador, in October 2009 (4° 7´53.99´´ S, 78° 56´28.30´´ W). The collection was authorized by the Ministerio del Ambiente de Ecuador (MAE) (N°001-IC-FLO-DBAP-VS-DRLZCH-MA). The plant material was identified by Bolívar Merino, "Reinaldo Espinosa" Herbarium, Universidad Nacional de Loja, Ecuador. A voucher specimen was deposited (herbarium specimen number PPN-pi-007) at the herbarium of the Universidad Técnica Particular de Loja, Loja, Ecuador.

Preparation of stock solutions and standards

Stock solutions of pinocembrin and chrysin (10 mg/50 mL each) were prepared by dissolving an appropriate amount in methanol and stored at 4°C in glass bottles until use. Working internal standard (I.S.) solution was 100 µg/mL. Individual working solutions were prepared in methanol and diluted to provide a series of the standard solutions of 0.5, 1, 10, 25, 50, 75, 100 µg/mL. These concentration ranges covered the concentrations expected in our experimental study. Calibration curves were constructed for the target analyte.

Sample preparation

Piper ecuadorense leaf extract (100 mg) was obtained as previously described by Ramirez et al., 2013. The extract was obtained from dried leaves of P. ecuadorense using ethanol. The extract was filtered and then evaporated under a 50 mbar absolute pressure at 37 °C. The dried extract was processed for the removal of sugars. The sugar free extract was directly separated by a preparative column chromatography, eluting with a n-Hex:EtOAc mixture increasing polarity gradient system. The fraction eluted with n-Hex:EtOAc 85:15 was pinocembrin.

HPLC analysis

HPLC analysis was carried out using an Alliance Waters 2695 series equipped with a 996 photodiode-array detector (PDA) and autosampler (Waters, Milford, MA, USA). Data

were acquired by the software Masslynx 4.1 (Waters). PDA spectra (200–600 nm scan range) were compared for pinocembrin and chrysin identification. Separation was achieved at 20°C on a BDS Hipersil C-18 column (5 µm, 4.6x250 mm i.d.).Mobile phase was composed of a mixture of 0.01 M aqueous ammonium acetate, pH 4.0, and methanol in a 35:65 % (v/v) ratio with the flow rate of 1 mL/min. The HPLC run time was 10 min. Sample injection volumes were 10 µL and detection was done by ultraviolet (UV) at wavelength 290 nm (Yang et al., 2009).

Validation of method

The guidelines for bioanalytical method validation in industry of the Food and Drug Administration (FDA) were followed (U.S. Department of Health and Human Services Food and Drug Administration et al., 2018)when finalized, will represent the Food and Drug Administration's (FDA's. Linearity, accuracy, intraday and interday precision, specificity, limit of detection (LOD) and limit of quantification (LOQ) were established.

Linearity validation

Calibration curves were plotted on three consecutive days, analyzing mixtures of each reference standard at seven different concentrations and plotting peak areas against concentration. The linearity of the detector response was determined by means of linear regression.

Accuracy and precision validation

The precision was established from the Piper ecuadorense leaf extract samples through replicate analyses using three concentration of pinocembrin (1, 25 and 50 µg/mL). Intraday accuracy and precision were established by analyses of the group of standards in five replicates. Interday accuracy and precision were conducted in five replicates for three consecutive days. A calibration curve was prepared for each assay to determinate the concentration of each sample.

Specificity validation

Peak identification was carried out by comparison with authentic standards, recording the UV spectrum of each peak. The UV spectra were taken at various points of the peaks to check peak homogeneity.

LOD and LOQ

Serial dilutions of reference standards were prepared with methanol and were then analyzed with the method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

Recovery

Percent recovery of pinocembrin was calculated by relating the analyte and internal standard peak area ratios obtained from samples and the standard solutions at the same concentration.

Analysis of pinocembrin in Piper ecuadorense extract

For HPLC, 10 mg of the extract were dissolved with 50 mL of methanol, followed by the addition of 0.5 ml of the I.S. The obtained solutions were filtered through a hydrophilic PVDF syringe filter (33 mm, 0.22 µm, Millipore, USA) and analyzed immediately in order to avoid possible chemical degradation. All sample assays were performed in triplicate.

RESULTS AND DISCUSSION

Method development

Appropriate conditions for quantitative determination of pinocembrin were determined as isocratic elution on C18 reversed-phase. The compound has a high UV absorption at 290 nm. This wavelength was used for quantification. Mixtures of aqueous ammonium acetate with methanol were tested as the mobile phase. The elution system was adequately set to separate the peaks at the baseline in 15 min (Figure 2a). HPLC method was similar to the previously reported method described by Yang et al., 2009 and is capable to quantify pinocembrin. The method is simple, selective and sensitive. The use of this method easily allowed the identification of pinocembrin in Piper ecuadorense leaf extract presented in Figure 2b. Fitting chromatographic peak shapes and reliable retention times (tR) for pinocembrin and chrysin (9.2 and 11.2 min, respectively) were achieved because doesn´t exist other compounds interfering in the assay.

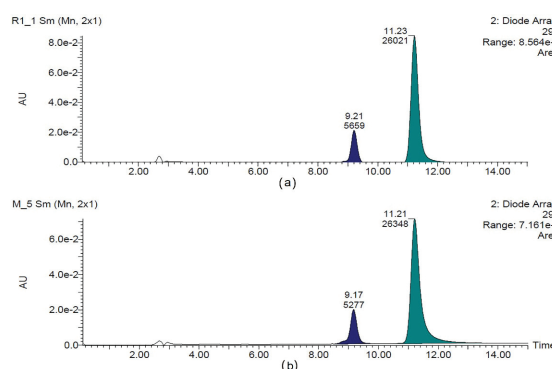

Figure 2. Representative HPLC-DAD (at 290nm) chromatograms of (a) pinocembrin (1 µg/ml) and chrysin (50 µg/ml); (b) real sample of Piper ecuadorense leaf extract (50 µg/ml) and chrysin (50 µg/ml)

Validation of the analytical method

Linearity

The linear calibration curves were obtained by plotting the peak areas of pinocembrin versus the concentration at seven levels (0.5, 1, 10, 25, 50, 75 and 100 µg/mL). The correlation coefficient (r2) and the equation for the calibration curve were calculated to be y = 741x + 222.48 (r2 = 0.9998, n = 7). Excellent linearity was observed between peak areas and concentrations (Table 1). The results of LOD and LOQ analysis for pinocembrin indicate that the established HPLC method is sufficiently sensitive for the determination of this compound in Piper ecuadorense leaf extracts.

Table 1. HPLC calibration data for pinocembrin

Compound	Linear range (µg/mL)	Linearity (r^2)	Equation	t_R (min)	LOD/LOQ (µg/mL)
Pinocembrin	0.5–100	0.9998	y = 740.999x+222.478	9.2	0.5/1.6

Accuracy and precision

The precision of this method was tested, performing intra- and inter-day multiple injections of a standard solution of pinocembrin. The accuracy of the analytical method was studied by spiking solutions of pinocembrin at concentrations of 1, 25 and 50 µg/mL into Piper ecuadorense leaf extracts, to evaluate recoveries of pinocembrin for this method.

The precision for concentrations of 1, 25 and 50 µg/mL of pinocembrin were 2.87, 1.14 and 1.46%, respectively. The accuracy of the pinocembrin concentration to three levels at 1, 25, and 50 µg/mL from the calibration curve was determined in five replicates. Table 2 illustrates the precision and accuracy range data from 1.14% to 3.08% and from 96.55% to 101.56%, respectively throughout the three concentrations.

Results suggest that this method is capable to quantify pinocembrin with high precision since the relative standard deviations (% RSD) were low. Furthermore, the low coefficient of variation (CV) values demonstrate precision of the method (Table 2). The specificity of the method was evaluated using UV absorption spectra at three points of each peak. Comparison of these spectra with those of authentic samples revealed that each peak was homogeneous and not overlapping with any impurity.

Table 2. HPLC Validation data: accuracy, precision, and recovery of pinocembrin

	Spiked concentration (µg/mL)		
	1	25	50
Accuracy and precision			
Intra-day precision			
Measured concentration (µg/mL)	0.82±0.21	24.92±0.36	50.02±0.83
Accuracy (%)	97.53±2.79	99.75±1.14	100.03±1.46
CV[a] (%)	2.87	1.14	1.46
Inter-day precision			
Measured concentration (µg/mL)	0.73±0.23	25.50±0.54	50.29±0.65
Accuracy (%)	96.55±2.97	101.56±1.69	100.50±1.14
CV (%)	3.08	1.66	1.14
Recovery			
Measured concentration (µg/mL)	1.05±0.10	26.17±0.31	50.75±0.81
Recovery	100.70 (±1.38)	103.69 (±0.98)	101.33 (±1.42)

[a] Coefficient of variation (CV, %) = 100 × (standard deviation/pinocembrin found).

LOD and LOQ

The LOD and LOQ were found to be 0.5 and 1.6 µg/mL, respectively, indicating the adequate sensitivity of the method (Table 1).

Recovery

Percent recovery values for the pinocembrin were from 100.70% to 101.33% and are illustrated in Table 2. The recoveries near to 100% indicate a good accuracy of this method.

Estimation of pinocembrin in Piper ecuadorense extract

The total pinocembrin content in the extract was found to be 6.64 (± 0.17) µg/mL. Additional peaks of some extract components did not appear in the chromatogram (Figure 2b), finding that pinocembrin and chrysin were the only compounds present in the chromatogram at the selected wavelength. Hence, there is not interference in the analysis with other components, showing the specificity of the method.

CONCLUSIONS

A simple, specific, precise, accurate and reproducible HPLC method was successfully developed and validated to analyze pinocembrin in Piper ecuadorense leaf extract.

The present work contributes to the scientific investigation on herbal drugs applied to traditional Ecuadorian medicine, as well as to quality control of the crude drug Piper ecuadorense. The proposed HPLC method showed good linearity, accuracy, and precision. In comparison to an earlier published method by Yang et al., 2009, the presented system focused on the analysis of pinocembrin in the Piper ecuadorense extract and achieved a better separation. This is the first report of a HPLC method to determinate pinocembrin in this plant.

Acknowledgements

This work was supported by the Universidad Técnica Particular de Loja project (PROY/IQA/0027).

REFERENCES

Andrade, J.M., Armijos, C., Malagon, O., Lucero, H., 2009. Plantas Silvestres empleadas por la etnia Saraguro en la Parroquia San Lucas, Provincia de Loja, Ecuador, 1ra ed. Ediciones UTPL, Loja, Ecuador.

Guo, W., Jiang, Y., Chen, X., Yu, P., Wang, M., Wu, X., Zhang, D., 2015. Identification and quantitation of major phenolic compounds from penthorum chinense pursh. by HPLC with tandem mass spectrometry and HPLC with diode array detection. J. Sep. Sci. 38, 2789–2796. https://doi.org/10.1002/jssc.201500303

Merken, H.M., Beecher, G.R., 2000. Measurement of food flavonoids by high-performance liquid chromatography: A review. J. Agric. Food Chem. 48, 577–599.

Ramírez, J., Cartuche, L., Morocho, V., Aguilar, S., Malagon, O., 2013. Antifungal activity of raw extract and flavanons isolated from Piper ecuadorense from Ecuador. Brazilian J. Pharmacogn. 23, 370–373. https://doi.org/10.1590/S0102-695X2013005000012

Rasul, A., Millimouno, F.M., Ali Eltayb, W., Ali, M., Li, J., Li, X., 2013. Pinocembrin: A Novel Natural Compound with Versatile Pharmacological and Biological Activities. Biomed Res. Int. 2013, 9. https://doi.org/10.1155/2013/379850

Sala, A., Recio, M.C., Schinella, G., Máñez, S., Giner, R., Cerdá-Nicolás, M., Rı́os, J.-L., 2003. Assessment of the anti-inflammatory activity and free radical scavenger activity of tiliroside. Eur. J. Pharmacol. 461, 53–61. https://doi.org/http://dx.doi.org/10.1016/S0014-2999(02)02953-9

Shah, V., Midha, K., Findlay, J., Hill, H., Hulse, J., McGilveray, I., McKay, G., Miller, K., Patnaik, R., Powell, M., Tonelli, A., Viswanathan, C., Yacobi, A., 2000. Bioanalytical Method Validation—A Revisit with a Decade of Progress. Pharm. Res. 17, 1551–1557. https://doi.org/10.1023/a:1007669411738

Tene, V., Malagón, O., Finzi, P.V., Vidari, G., Armijos, C., Zaragoza, T., 2007. An ethnobotanical survey of medicinal plants used in Loja and Zamora-Chinchipe, Ecuador. J. Ethnopharmacol. 111, 63–81. https://doi.org/http://dx.doi.org/10.1016/j.jep.2006.10.032

U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), 2018. Bioanalytical Method Validation Guidance for Industry.

Wangkangwan, W., Boonkerd, S., Chavasiri, W., Sukapirom, K., Pattanapanyasat, K., Kongkathip, N., Miyakawa, T., Yompakdee, C., 2009. Pinostrobin from Boesenbergia pandurata Is an Inhibitor of Ca 2+ -Signal-Mediated Cell-Cycle Regulation in the Yeast Saccharomyces cerevisiae. Biosci. Biotechnol. Biochem. 73, 1679–1682. https://doi.org/10.1271/bbb.90114

Yang, Z., Liu, R., Li, X., Tian, S., Liu, Q., Du, G., 2009. Development and validation of a high-performance liquid chromatographic method for determination of pinocembrin in rat plasma: Application to pharmacokinetic study. J. Pharm. Biomed. Anal. 49, 1277–1281. https://doi.org/http://dx.doi.org/10.1016/j.jpba.2009.02.030