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Development and Validation of a Chromatographic Method for the Determination of Biflorin obtained from *Capraria biflora* Roots

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SUMMARY. A LC-method was developed and validated for quantification of biflorin obtained from *Capraria biflora* roots. The HPLC analysis was performed using a C18 column in isocratic conditions using methanol-water and UV detection at 510 nm. A high coefficient of determination was achieved for biflorin (0.9993). The method showed good repeatability (R.S.D. = 1.68 %), reproducibility (R.S.D. = 2.13 %) and good accuracy for biflorin peak (99.13 %, R.S.D. = 2.14 %).

RESUMEN. "Desarrollo y validación de un método de cromatografía líquida para la determinación de biflorina obtenida de las raíces de *Capraria biflora"*. Se desarrolló y validó un método de Cromatografía Líquida para la cuantificación de biflorina obtenida de las raíces de *Capraria biflora*. El análisis fue realizado utilizando una columna C18 en condiciones isocráticas utilizando metanol-agua como solvente, con detección UV a 510 nm. Se obtuvo un coeficiente de determinación alto para biflorina (0,9993). El método presentó buena repetibilidad (R.S.D. = 1,68 %), reproducibilidad (R.S.D. = 2,13 %) y exactitud para el pico de biflorina (99,13 %, R.S.D. = 2,14 %).

INTRODUCTION

Capraria biflora (Scrophulariaceae) is a small perennial shrub distributed in North and South America. In Brazil, the specie is widely cultivated in coastal region, where it is widely known as "chá-da-terra", "chá-da-américa" or "chá-dorio" [1-7]. From the roots of *C. biflora* was extracted a compound known as biflorin, a naphthoquinone, which structure corresponds to 6,9-dimethyl-3-(4-methyl-3-pentenyl)naphtho[1,8-bc]-pyran-7,8-dione (Fig. 1) [8-10].

Concerning the biological activity, the biflorin showed anti-bacterial and anti-fungal properties [9-11]. Thus, the development of semi-solid formulations containing this substance has a special interest. Actually, the absence of analytical methods for quantification of biflorin is an obstacle for the technological development.

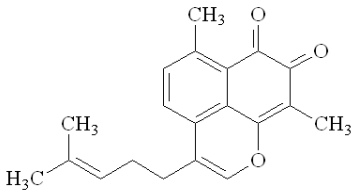

Figure 1. Chemical structure of biflorin.

The LC methodologies with UV detection have been largely used by the pharmaceutical industry for quality analysis of raw, intermediary and final products. The successful of the LC technique is due its versatility, precision, and relatively low cost [12].

The aim of this work was to develop and validate a simple and fast LC-method for quantification of biflorin isolated from C*apraria biflora*.

KEY WORDS: Biflorin, *Capraria biflora,* LC-method, Validation.
PALABRAS CLAVE: Biflorina, *Capraria biflora,* Cromatografía Liquida, Validación.

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EXPERIMENTAL

Plant material

Roots from *Capraria biflora* L. were collected in Itamaracá (Pernambuco/Brazil) (April/1997, January/1998 and January/1999) and taxonomically identified. Voucher specimen was deposited at herbarium of the Empresa Pernambucana de Pesquisa Agropecuária - IPA (Pernambuco/Brazil) under the registration number 57902.

Extraction and purification of biflorin

Roots from *C. biflora* (105.75 g) were extracted by maceration during 120 h with 2.5 L of ethanol 70o GL. The ethanolic extract was then concentrated at 50 °C under low pressure, yielding 16.71 g of dried material. The dried residue was fractionated by column chromatography using silica as stationary phase and mixtures of toluene-ethyl acetate (8:2; V/V) as elution medium [8,9]. The fraction containing biflorin was selected and re-crystallized in di-isopropyl ether, yielding 373.8 mg of biflorin.

NMR study

^{1}H NMR and ^{13}C NMR spectra from biflorin crystals were recorded at 300 MHz and 75 MHz, respectively (NMR Spectrometer, Varian Unit), using TMS as internal standard. The samples were dissolved in $CDCl_3$ and the 1H and ^{13}C chemical shifts (δ) are given in *ppm* and in Hertz (Hz) for couplings *(J)*.

GC-MS

The mass spectrum of biflorin was obtained by GC-MS analysis using a Finnigan GCQ mass spectrometer system equipped with a DB-5 (30 m x 0.25 mm i.d. x 0.5 µm) column. The injector and GC/MS interface were kept at 280 °C and helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The analysis was carried out in the splitless mode.

Chemicals and solvents

The mobile phase for HPLC analysis was prepared with methanol LC grade (Merck, Germany) and water (Milli-Q system, Millipore, USA).

LC system

The analysis was carried out in a Hewlett Packard HP 1100 liquid chromatograph equipped with a pump, an UV/VIS-detector (Hewlett Packard, USA) and a Spherisorb ODS C18 RP-column (250 mm x 45 mm i.d.) (Waters, USA). The peaks were detected at 510 nm. After filtration (0.44 (m, Millipore, USA), an isocratic elution was performed. Mobile phase was composite by methanol-water (85:15, V/V) at a flow rate of 1.0 ml/min.

Method development

Calibration curves of biflorin

Methanolic solutions of biflorin were prepared in concentrations from 1 to 10 µg/ml. The solutions were filtered through a 0.45 µm membrane (Millipore-HVHP, USA). The calibration curves were made by linear regression and the results represented the averaged of three curves performed by three injections of each concentration.

Linearity, precision, accuracy, detection and quantification limits

The method linearity, recovery, precision (repeatability and intermediary precision), detection and quantification limits were evaluated according to the ICH guidelines specifications [13]. The linearity of the curves was estimated by regression using the last square method. The slope, intercept (with respective confidence intervals) and coefficient of determination (R^2) were calculated and evaluated [14]. Thereby, three samples of the biflorin solution at three different concentrations (2, 5 and 10 µg/ml) were injected three times and the amount recovered was calculated.

For the repeatability assay, six diluted solutions at 5 µg/ml were prepared. Each diluted solution was injected in triplicate and the repeatability was evaluated for peak areas of biflorin through the relative standard deviation (RSD %). The intermediary precision was calculated from three analysts, at two different days. At each day a new solution was prepared and injected in triplicate. The data were expressed as relative standard deviation (RSD %), and tested by two-way ANOVA [14].

Statistical analysis

The individual data were grouped following each experiment. The mean with the respective deviation was used as a measurement of the central tendency and dispersion (standard deviation and relative standard deviation) [14].

RESULTS AND DISCUSSION

Extraction and identification of biflorin

After extraction and purification, the use of NMR experiments and mass spectrometry tech-

^{1}H - RMN (300 MHz, $CDCl_3$)
1.58 (s, 3H-15); 1.72 (s, *J* = 1.5Hz, 3H-14); 1.98 (s, 3H-16); 2.71 (s, 3H-17); 2.28 (td, *J* = 7.8Hz and 7.2Hz, 2H-11); 2.53 (t, *J* = 7.8Hz, 2H-10); 5.16 (tq, *J* = 7.2Hz and 1.5Hz, H-12); 7.07 (s, H-2); 7.40 (d, *J* = 8.4Hz, H-5); 7.52 (d, *J* = 8.4 Hz, H-4).

^{13}C - RMN (75.4 MHz, $CDCl_3$)
140.594 (C-2); 115.891 (C-3); 128.750 (C-3a); 128.189 (C-4); 136.301 (C-5); 146.356 (C-6); 126.173 (C-6a); 181.641 (C-7); 177.595 (C-8); 113.093 (C-9); 161.512 (C-9a); 123.729 (C-9b); 27.080 (C-10); 26.946 (C-11); 122.387 (C-12); 133.323 (C-13); 25.571 (C-14); 17.739 (C-15); 23.013 (C-16); 7.564 (C-17).

MS (70 eV)
m/z 308 (28) $[M]^+$, 281 (17.62%), 280 (100) [M - CO]+, 212 (27), 211 (92) $[M - CO - C_5H_9]^+$, 183 (18), 153 (19).

Table 1. Structural confirmation of biflorin.

C number	Experimental δ (ppm)	Literature δ (ppm)[15]
2	140.594	140.400
3	115.891	112.100
3a	128.750	129.500
4	128.189	128.400
5	136.301	136.400
6	146.356	146.600
6a	126.173	126.300
7	181.641	182.000
8	177.595	178.000
9	113.093	113.400
9a	161.512	161.700
9b	123.729	124.000
10	27.080	27.500
11	26.946	25.700
12	122.387	124.900
13	133.323	131.100
14	25.571	25.700
15	17.739	17.700
16	23.013	23.200
17	7.564	7.700

Table 2. Spectral data of ^{13}C NMR. Comparation between experimental data and related values [16-18].
* The spectrum was performed in $CDCl_3$ at 75.5 MHz.

niques led to identification of the compound 6,9-dimethyl-3-(4-methyl-3-pentenyl)naphtho[1,8-bc]-pyran-7,8-dione (biflorin) (Table 1). After comparison between the δ observed experimentally by ^{13}C NMR and spectrum of davidianones and mansonon, the o-naftoquinon structure of biflorin was confirmed [15,16] (Table 2).

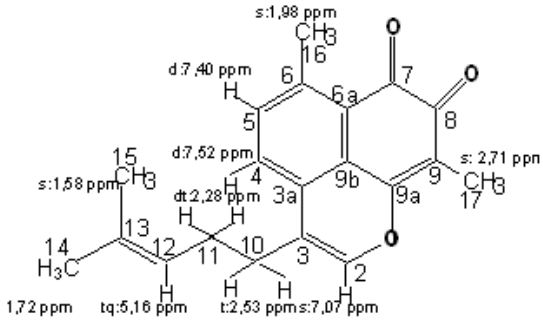

Figure 2. Proton identification by ^{1}H NMR.

The mass spectrum showed a molecular ion peak at m/z 308 (28.4%), the base peak at m/z= 280 (100.00%) was due carbon monoxide elimination. The fragment ion at m/z 211 (91.8%) corresponding to the lost of C_5H_9. The fragment ion at m/z 183 (17.8%) corresponding to C_7H_{13}. Another fragments ions were observed at m/z = 281 (17.62%), m/z = 212 (27.34%) and m/z = 153 (19.35%) (Fig. 2).

LC-Method Development

For the preliminary evaluation of the mobile phase, different combination of methanol:water (v/v) were tested. The data are summarized in Table 3.

All systems containing methanol:water (v/v) showed RSD lower than 3% for the retention time of the biflorin. Concerning the pH, no difference was observed for mobile phases, except

Elution systems (v/v)	pH	Retention Time (min)
Methanol:water (80:20)	6.90 ± 0.10 (1.44)	10.50 ± 0.50 (2.34)
Methanol:water (85:15)	6.33 ± 0.15 (2.41)	7.95 ± 0.06 (0.78)
Methanol:water (90:10)	6.00 ± 0.10 (1.67)	5.52 ± 0.01 (0.17)
Methanol:water (85:24:1)	4.10 ± 0.20 (4.90)	7.31 ± 0.09 (1.19)

Table 3. Preliminary evaluation of mobile phase. R.S.D. values are given in parentheses.

when acetic acid was added. However, the pH showed no decisive important influence on biflorin retention time or peak resolution. The mixture from methanol:water 85:15 (v/v) was selected for the LC analysis due its separation power in the experimental conditions. The system showed 17,469.20 theoretical plates and capacity factor of 2.10 for biflorin peak.

The linearity was evaluated for biflorin thought the construction of a calibration curve, which was obtained by plotting peak areas upon concentrations using ten different solutions. The calibration curve was evaluated statistically and the regression parameters showed linearity in the range from 1 to 10 µg/ml. The linear model was Y = 0.614 + 117.50X and the coefficient of determination for biflorin curve was higher than 0.99 (R^2 = 0.9993). Thus, the calculated straight line could explain more than 99% of the experimental data. The confidence intervals for intercept point included zero (-41.65 to 42.88). Therefore, the result confirms the absence of constant systematic errors.

The detection limit (LOD) and quantification limit (LOQ) are defined as the amount of analyte in standard solutions that yields an instrumental signal significantly different from the blank or background signal which equals to 3 and 10, respectively. For biflorin the LOD calculated was 0.32 µg/mL and the LOQ was 0.98 µg/mL.

The assay of recovery or accuracy was performed to evaluate any interference on the method response. The differences between the expected and observed concentrations of biflorin are presented in Table 4.

According to ICH, the repeatability of an analytical method is described as the standard deviation (or relative standard deviation), calculated from six repetitions of determinations from concentration of 100% from the standard [13]. For biflorin, the concentration of 5 µg/mL was choosing as central point from the calibration curve. The result was 4.87 ± 0.0816. The method was found to be precise with lower value for R.S.D. (1.68 %).

Tested concentrations (µg/mL)	Biflorin recovery (%)
2	101.5 ± 1.35
5	97.4 ± 2.60
10	98.5 ± 1.45
Mean (R.S.D.)	99.13 (2.14)

Table 4. Recovery results (%) for biflorin (n = 3).

	Analyst 1	Analyst 2	Analyst 3
Day 1	2,02 ± 0,06	2,08 ± 0,08	1,97 ± 0,04
Day 2	1,98 ± 0,06	1,97 ± 0,06	2,02 ± 0,05
Mean (R.S.D.)	2.04 ± 0.04 (2.13)		
$F_{ANALYSTS}$	0.518		
F_{DAYS}	0.161		

Table 5. Intermediary precision test for biflorin (n = 3).

The intermediary precision is performed to evaluate the accumulation of the random errors. In this work the variations between different days and analysts were studied. Thus, samples of 2 µg/mL from biflorin were injected three times by three different analysts at six different days. The data for intermediary precision are presented in Table 5.

The intermediary precision data were evaluated statistically by ANOVA, and the results showed that the variations introduced by both source of error (analysts and days) were very low, demonstrating the high reproducibility of the method.

CONCLUSION

The validation study performed for the LC-method of quantification of biflorin obtained from *Capraria biflora,* showed its suitability. High stability was observed for the method that suffered no significantly influence from source of variations like different analysts on different days. In conclusion, the proposed RP-LC method is simple, fast and precise, and can be used satisfactory for biflorin quantification.

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