Universidad Nacional de San Luis, Facultad de Química, Bioquímica y Farmacia, Chacabuco y Pedernera, San Luis, Argentina

Determination and assay validation of the bioactive sesquiterpene lactone xanthatin isolated from *Xanthium cavanillesii* using capillary electrophoresis

L. S. Favier, G. Acosta, M. R. Gomez, A. O. María, C. E. Tonn

Received March 8, 2006, accepted April 10, 2006

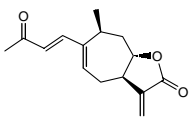
Dra. Laura Silvina Favier, Universidad Nacional de San Luis, Facultad de Química, Bioquímica y Farmacia, Chacabuco y Pedernera, San Luis, Argentina (5700)
lfavier@unsl.edu.ar

Pharmazie 61: 981–984 (2006)

This study describes the development and validation of a method for quantification of the antiulcer experimental drug xanthatin in tablets by capillary electrophoresis (CE). Solid oral dosage forms based on xanthatin were designed and assayed on rats. A CE methodology was developed; the parameters evaluated were: background electrolyte composition, concentration and pH, applied voltage and sample preparation. The method was validated in terms of range of linearity, limits of detection (LOD) and quantification (LOQ), accuracy, precision and selectivity and then applied to the pharmaceutical dosage forms. Xanthatin determination was carried out in less than 3 min with a 20 mM sodium tetraborate buffer, pH 9.20. Drug concentration per tablet found was 2.97 ± 0.2 mg. Calibration plots were linear over at least three orders of magnitude of analyte concentrations, LOD and LOQ were 7.6 and 26 μg mL^{-1} respectively. For accuracy evaluation a recovery test was performed, the values being better than 98.6%. With respect to precision, the results obtained were better than 1.02 RSD% (repeatability) and 1.54% (intermediate precision). After the manufacturing process the resulting tablets were biologically active. The methodology developed is useful, simple and rapid for xanthatin determination in tablets.

1. Introduction

Xanthatin is a natural product isolated from *Xanthium cavanillesii* Schouw (Asteraceae) that has shown high antiulcerogenic activity in rats and low toxicity in cell line tumors (Favier et al. 2005). Chemically, xanthatin is a sesquiterpene lactone (The Merck Index 1989) and the major component found in the plant after successive extractions with organic solvents. In the present work the purified drug was used as the active principle of tablets prepared by direct compression (Tabletting Specification Manual 1981). Given that not all drugs currently exhibit the appropriate rheology and compressibility required to be manufactured by direct compression, these studies were carried out on three formulations processed with different excipients.



Xanthatin

Natural xanthanolides have been reported to have antileishmanial, antimicrobial and antifungal activities (The Merck Index 1989; Lavault et al. 2005). Taking into account the promising results obtained in the bioassays reported and since xanthatin is not included in official compendia we sought an adequate analytical methodology which allowed us to quantify the active principle present in a pharmaceutical oral dosage form. Moreover, the literature does not offer any method for xanthatin determination.

Due to its low sample consumption, short analysis time and high separation efficiency, capillary electrophoresis (CE) has become an important analytical technique, particularly for the analysis and separation of herbal sample constituents (Hu and Dovichi 2002; Chen et al. 2002; Sun et al. 2003).

With the aim of carrying out quality control of the tablets designed in a reliable, fast and economical way, a CE method was developed for xanthatin quantification. The present methodology was validated in terms of range of linearity, limits of detection (LOD) and quantification (LOQ), accuracy, precision and selectivity as previously reported in the literature (Houzéet al. 2005; Fongea et al. 2004; Gomez et al. 2002).

2. Investigations and results

2.1. Evaluation of purity and physical properties

In order to obtain more detailed information of the drug compound various studies were carried out. A sample of xanthatin in fully deuterated chloroform was recorded and no impurity was detected. Structural elucidation was carried out in comparison with the genuine compound (Favier et al. 2005).

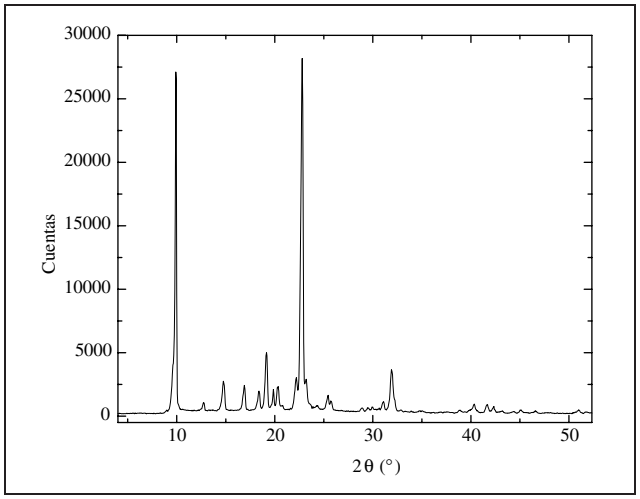


Fig. 1: X-ray diffraction pattern of xanthatin

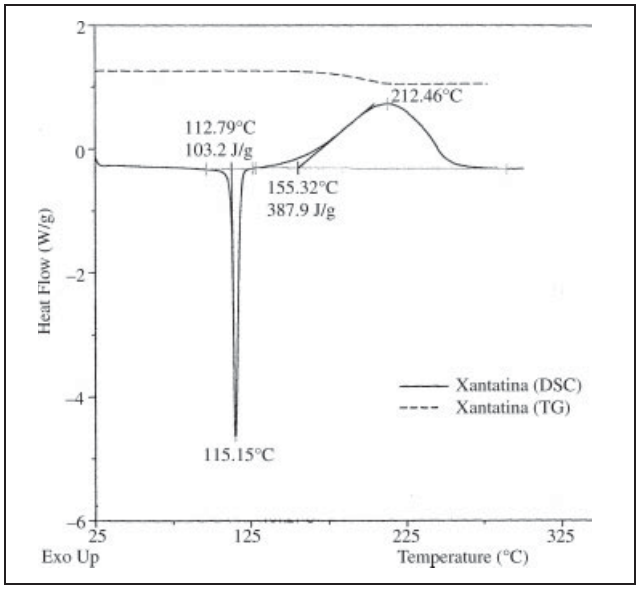


Fig. 2: Thermoanalysis plots: differential scanning calorimetry (DSC) and thermogravimetry (TG)

Approximate solubility properties were evaluated in descriptive terms according to the USP XXVIII; the drug showed to be sparingly soluble, 30 to 100 parts of solvent being required to dissolve 1 part of solute. This result agrees well with the absence of hydrophilic functionalities in the xanthatin molecule.

The X-ray powder diffractometry results indicate from its diffraction pattern that xanthatin posseses a crystal line structure (Fig. 1).

DSC thermograms of the sample are shown in Fig. 2. Xanthatin gave an endothermic peak at 115.15 °C due to melting and showed no weight loss in the TG curve. At 212.4 °C there is an exothermic peak in DSC with loss of weight observed in TG which would suggest decomposition.

2.2. Capillary electrophoresis

2.2.1. Method development

In the first place, the analytical method was optimized with the aim of achieving the best experimental conditions. The migration behavior of xanthatin was investigated by capillary zone electrophoresis (CZE). The influence of different parameters (nature and concentration of the running buffer, pH, applied voltage, capillary temperature, injection time and pressure and sample preparation) on migration time and efficiency was systematically investigated.

Different background electrolytes (BGEs) were tested; from the results it was found that a very efficient and stable electrophoretic system for determination of the analyte was achieved by using sodium tetraborate BGE.

Subsequently, the effect of buffer pH was investigated at a fixed buffer concentration (10 mM), within the range 6.0–10.0, adjusted with 0.1 mol · l^{-1} NaOH and 0.1 mol · l^{-1} HCl. At pH 9.20 analysis times were adequate for xanthatin assay.

Buffer concentration also has a significant effect on separation performance through its influence on electroosmotic flow (EOF) and the current produced in the capillary. Keeping other parameters constant (pH: 9.20, 30 kV, 25 °C) the buffer concentration was varied from 5–75 mM. Sodium tetraborate 20 mM was selected for further studies.

Electrophoretic parameters were then optimized consecutively; the best results with regard to selectivity, reproducibility, baseline behaviour and current performance were obtained with sodium tetraborate solution (20 mM), pH 9.20 as running buffer. The capillary temperature was maintained at 25 °C. Samples were maintained at 25 °C and pressure-injected on the anodic side at 3.5 kPa for 5 s; the applied voltage was 30 kV and detection was performed at 212 and 250 nm.

2.2.2. Quantitative xanthatin determination

The weight variation test would be a satisfactory method of determining the drug content uniformity of tablets if they comprised essentially all (90 to 95%) active ingredient. To assure uniform potency for tablets of low-dose drugs, a content uniformity test was used (United States Pharmacopeia 2003).

Once the conditions for separation were established, the CE method was applied to the determination of xanthatin in tablets (Fig. 3). The electropherogram shows no interference between active principle and tablet excipients under the optimized experimental conditions. Elution of xanthatin occurred before 3 min. The final concentration determined in each tablet was 2.97 ± 0.2 mg.

2.2.3. Method validation

Validation was carried out as recommended in the literature (United States Pharmacopeia 2003; ICH Harmonised Tripartite Guideline 1994; Fabre and Altria 2001; Breier et al. 2005). The linear relationship between the concentration of the analyte and the corresponding corrected peak area was studied. A calibration graph was constructed by plotting concentration (mg mL^{-1}) versus corrected peak area. The slopes and intercepts of regression equations, linearity range, correlation coefficient and limits of detection (LOD) and quantification (LOQ) are listed in the Table.

Table: Linear regression data for analysis of xanthatin

Concentration range (μg mL^{-1})	R^2 (n = 6)	Slope	Intercept	LOQ (μg mL^{-1})	LOD (μg mL^{-1})
10–100	0.999	70251.17	2368.33	26	7.6

The method was validated for precision with respect to the migration time and the peak area of the analyte. Precision was tested at two levels: repeatability and intermediate precision. Repeatability refers to the dispersion observed in a short period of time (0.17 RSD% for the time 1.02 RSD% for the peak area). Intermediate precision was measured by varying the capillary, the operator and the day of work. In all cases the values were better than 0.51 RSD% for the time and 1.54 RSD% for the peak area.
Accuracy was found by performing a recovery test. An analyte standard solution was added to different aliquots of xanthatin sample. The final concentration of xanthatin was measured and the recovery was calculated as: measured value – base value in the sample/added value. The recoveries were in all cases better than 98.6%.
The recovery results demonstrated the separation of xanthatin from related substances (potential impurities); thus selectivity of the present method was found to be adequate. In addition, the sample peaks were monitored in the UV range from 200–400 nm with a DAD-detector and assessment of peak purity showed peak homogeneity indicating the specificity of the method (ICH Harmonised Tripartite Guideline 1994; Fabre and Altria 2001; Mikus et al. 2004). It was shown that the major peak at approximately 3.0 min was only xanthatin without interference by any excipient from the formulation.

2.3. Determination of cytoprotective gastric activity of xanthatin tablets in rats

Previous studies have demonstrated that the α-methylene-γ-butyrolactone ring, presented in a series of sesquiterpene lactones and synthetic related compounds having a common Michael acceptor, plays a key role in antiulcerogenic bioactivity in rats (María et al. 2000). It has been reported that xanthatin showed significant protective activity against ulcer induced by absolute ethanol (Favier et al. 2005).

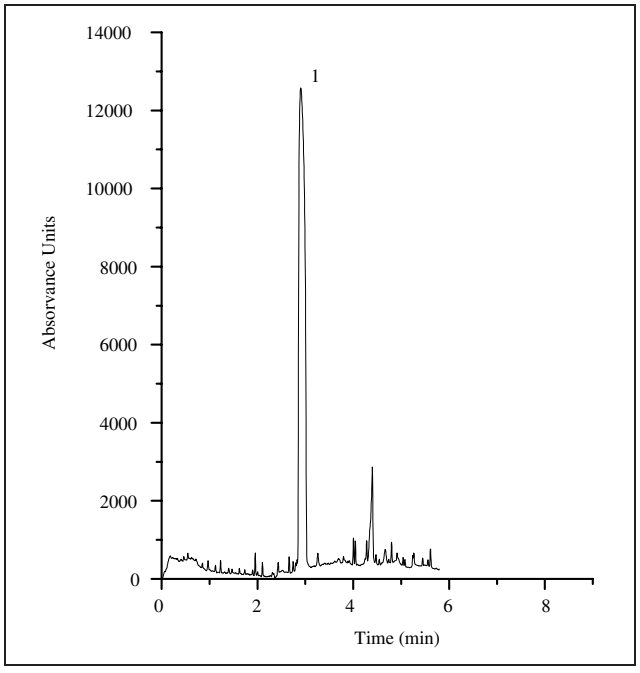


Fig. 3: Electropherogram of diluted sample of xanthatin tablet under optimized conditions. Conditions: 20 mM sodium tetraborate buffer, pH 9.20; capillary, 57 cm full lengh, 50 cm effective length, 75 μm ID, 375 μm OD; hydrodynamic injection at 3.5 kPa, 5 s; 30 kV constant voltage; 25 °C, detection by UV absorbance at 212 nm. Peak identification: 1, xanthatin

In order to evaluate the bioactivity of xanthatin when it is present in a complex mixture of excipients, a dosage of 9 mg/animal in tablet form was given to rats 60 min prior to the necrotizing agent. Absolute ethanol (1 mL) administered orally was employed as the necrotizing agent, and 1 h later the animals were decapitated. The stomachs were removed, opened along the greater curvature, and washed gently with saline solution. The degree of erosion in the glandular part of the stomach was assessed from a scoring system designed by Marazzi-Uberti and Turba (Giordano et al. 1992) from 0 (no erosions) to 5 (maximal damage). A value of 0.62 ± 0.37 was observed, corresponding to 87.2% ulcer inhibition.

3. Discussion

Xanthatin, a sesquiterpene lactone common in *Xanthium cavanillesii* Schouw (Asteraceae) was determined in less than 3 min. The results demonstrate that the developed CE method proposed here is a useful, simple and rapid technique for xanthatin determination. The CE methodology was successfully applied to tablets. It can be concluded that this method may be applicable to the routine quality control of formulations containing xanthatin. The results indicated that the CE assay could be a helpful tool for xanthatin quantification in common pharmaceutical analyses such as dissolution tests, drug determination in biological fluids or determination of the drug in *Xanthium cavanillesii* Schouw (Asteraceae) and its related medicinal preparations.

4. Experimental

4.1. Instrumentation

A Beckman P/ACE MDQ instrument (Beckman Instruments, Inc. Fullerton, CA) equipped with a diode array detector and a data handling system comprising an IBM personal computer and P/ACE System MDQ Software was utilized for the capillary electrophoresis analysis. The fused-silica capillaries were obtained from MicroSolv Technology Corporation and had the following dimensions: 57 cm total length, 50 cm effective length, 75 μm ID, 375 μm OD. CE solutions were degassed by ultrasonication (Testlab, Argentina), and running electrolytes and samples were filtered through 0.45 μm Titan Syringe filters (Sri Inc., Eaton Town, NJ. USA). The pH of the electrolytes was measured by an Orion 940 pHmeter equipped with a glass-combined electrode.

4.2. Materials and reagents

Pure xanthatin was obtained after successive extractions with organic solvents and purifications using chromatographic methods starting from *Xanthium cavanillessi* Schouw (Asteraceae) collected in Argentine (Favier et al. 2005). Although xanthatin is listed in the Merck Index, it is not included in any official monograph, thus additional studies were carried out. Identification and purity assays were performed by ^{1}H and ^{13}C NMR (Bruker AC 200). Some physical properties of the powder were also tested: solubility, X-ray analysis, differential scanning calorimetry (DSC) and thermogravimetric analysis (TG).
For tablet making, an inert precompacted mix (lactose monohydrate 67%, maize starch 28% and polyvinylpyrrolidone 5%) was used and was supplied by Lab. Puntanos S.E. Magnesium stearate was used as a lubricant and crosscarmelose sodium, microcrystalline cellulose and starch glycolate were used as disgregant agents. For tablet manufacture the traditional procedure for direct compression was followed (Lachman 1986).
Sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$) and sodium dihydrogenphosphate (NaH_2PO_4) were supplied by Mallinckrodt (Saint Louis, USA).
The water used in all studies was ultra-high-quality water (18 MΩ cm) obtained from a Barnstead Easy pure RF compact ultrapure water system. All other reagents and solvents were of analytical grade quality. All solutions were degassed by ultrasonication (Testlab, Argentina). Running electrolytes and samples were filtered through 0.45 μm Titan Syringe filters (Sri Inc., Eaton Town, NJ. USA).

4.3. Procedure

At the beginning of the day, capillary preconditioning consisted of a 5 min flush with water, 5 min with 0.1 M NaOH, 5 min with water and 10 min with the background electrolyte (BGE). Before starting a new run, the

capillary was flushed for 2 min with water, 2 min with 0.1 M NaOH and 1 min with water and equilibrated 2 min with the BGE. Flushing was performed at a pressure of approximately 140 kPa.

Stock standard solutions used in the construction of calibration curves were prepared by dissolving 10 mg of xanthatin in 100 mL of 20 mM sodium tetraborate buffer, pH 9.20 (0.1 mg mL^{-1}) and then diluting to obtain standard solutions within the concentration range: 10–100 mg l^{-1}.

Sample solutions were prepared as follows: ten tablets (n = 10) of each formulation were finely powdered, mixed and blended. A quantity equivalent to one tablet was accurately weighed and suitably diluted as required to reach the desired final concentration. All solutions were filtered through a 0.45 μm membrane prior to injection.

Acknowledgements: The authors thank the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional de San Luis (Argentina). The authors would like to thank Professor Dr Rubén H. Manzo for his valuable advice. We gratefully acknowledge the collaboration of Dr. Jorge Gonzalez and L. A. del Vitto. Part of this work has been previously included in the Doctoral Thesis of L. S. Favier.

References

Breier AR, Paim CS, Steppe M, Schapoval EES (2005) Development and validation of dissolution tests for fexofenadine hydrochloride capsules and coated tablets. J Pharm Pharm Sci 8: 289–298.

Chen G, Zhang L, Zhao J, Ye J (2002) Determination of hesperidin and synephrine in Pericarpium *Citri reticulatae* by capillary electrophoresis with electrochemical detection. Anal Bioanal Chem 373: 169–173.

Fabre H, Altria KD (2001) Key points in method validation in capillary electrophoresis, LCGC Europe May 2001: 302–310.

Favier LS, María AO, Wendel GH, Borkowski EJ, Giordano OS, Pelzer L, Tonn CE (2005) Anti-ulcerogenic activity of xanthanolide sesquiterpenes from *Xanthium cavanillesii* in rats. J Ethnopharmacol 100: 260–267.

Fongea H, Kaale E, Govaerts C, Desmet K, Van Schepdael A, Hoogmartens J (2004) Bioanalysis of tobramycin for therapeutic drug monitoring by solid-phase extraction and capillary zone electrophoresis. J Chromatogr B 810: 313–318.

Giordano O, Pestchanker M, Guerreiro E, Saad R, Enriz D, Rodríguez A, Jáuregui E, Guzmán J, María A, Wendel G (1992) Structure-activity relationship in the gastric cytoprotective effect of several sesquiterpene lactones. J Med Chem 35: 2452–2458.

Gomez MR, Olsina RA, Martínez LD, Silva MF (2002) Simultaneous determination of dextromethorphan, diphenhydramine and phenylephrine in expectorant and decongestant syrups by capillary electrophoresis. J Pharm Biomed Anal 30: 791–799.

Houzé P, Thabet H, Delfour A, Larrouy L, Le Bricon T, Baud FJ (2005) Quantification of pralidoxime methylsulfate (Contrathion) in human urine by capillary zone electrophoresis. J Chromatogr B 826: 63–68.

Hu S, Dovichi NJ (2002) Capillary electrophoresis for the analysis of biopolymers. Anal Chem 74: 2833–2850.

ICH Harmonised Tripartite Guideline. Text on validation of analytical procedures (1994).

Lachman L, Lieberman H, Kanig J (1986) The theory and Practice of Industrial Pharmacy. 3rd Ed. Lea & Febiger, Philadelphia pp. 318.

Lavault M, Landreau A, Larcher G, Bouchara J, Pagniez F, Le Pape P, Richomme P (2005) Antileishmanial and antifungal activities of xanthanolides isolated from *Xanthium macrocarpum*. Fitoterapia 76: 363–366.

María AO, Donadel O, Wendel G, Guzmán J, Guerreiro E, Giordano O (2000) Gastric anti-ulcer activity of several α, β-unsaturated carbonyl compounds in rats. Biol Pharm Bull 23: 555–557.

Mikus P, Kubacak P, Valáskova I, Havránek E (2004) Capillary zone electrophoresis determination of loratadine in tablets. Pharmazie 59: 260–262.

Sun Y, Guo T, Sui Y, Li F (2003) Fingerprint analysis of *Flos carthami* by capillary electrophoresis. J Chromatogr B 792: 147–152.

Tabletting Specification Manual, Industrial Pharmaceutical Technology Standard Specifications for Tabletting Tools (1981) revision. American Pharmaceutical Association, Washington, DC.

The Merck Index. 11th Ed. (1989) Merck & Co., Inc. Rahway, N.J. USA, pp. 1586.

United States Pharmacopeia, 26-National Formulary 21 (2003) The United States Pharmacopeia Convention, Rockville, MD.