Research Article

HPLC enantioseparation of alkaloid malacitanine using fluorimetric/polarimetric detection

This work reports two methods developed for the separation and determination of the enantiomers of the new alkaloid malacitanine (MLC) and the determination of the enantiomeric purity in mixtures. First, the isomers were separated using a Chirex 3020 (250 mm × 4.6 mm, 5 μm) chiral column with a mobile phase of cyclohexane–1,2-dichloroethane–ethanol–trifluoroacetic acid (64:30:6:0.6, v/v) at a flow rate of 1 mL/min and fluorimetric detection. Obtained retention times were 12.4 and 15.9 min (+ and −) with a resolution Rs of 1.13. Relative standard deviations (RSDs) were 2.5 and 2.4% at the 0.5-μg level (four determinations). Second, a nonenantioselective procedure for the determination of enantiomeric purity of MLC using a Lichrospher® Si-60 (250 mm × 5 mm, 5 μm) normal phase with a mobile phase of 100% ethanol at a flow rate of 0.9 mL/min coupled to two detectors in series, fluorimetric and polarimetric. RSD of 3.3% was obtained. Calculated enantiomeric purity by chiral chromatography gave 48.6% (−)-MLC in the near racemic product. Using polarimetric signal of the nonseparated enantiomers and comparing the slopes of the calibration curves (enantiomers) from the racemic product gave 47.8% (−)-MLC content. A study of accuracy of (−)-MLC gave recoveries from 98.3 to 100.7%.

Keywords: Alkaloids / Ceratocapnos heterocarpa / Chiral separation / Fluorimetric / Malacitaine / Polarimetric detection

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1 Introduction

Botanicals represent a formidable source of potential pharmaceutical and nutraceutical compounds because of the molecular diversity found in nature. Two main challenges can be faced in the analysis of phytochemicals: the lack of suitable standards, and the fact that many bioactive compounds are found at low levels in complex mixtures or plant extracts making detection and quantification very difficult. Frequently, natural products analysis suffers an additional difficulty; due to its inherent optical activity and since it is well known that a pair of enantiomers can display quite different activity and therapeutic and toxicological profiles, the analytical chemistry of these compounds is a promising research field strongly demanded by pharmaceutical companies.

Protoberberines represent a structural class of organic cations that have been mainly distributed in several plants and widely used in western natural medicine and traditional Chinese herbal medicine [1, 2]. Protoberberine alkaloids are reported to have anticancer [3–5], anti-infective [6, 7], antidiabetic [8], immunomodulatory [9, 10], and antimalarial activities [11, 12]. Most published papers have focused on the separation of chiral pharmaceutical products, obtained by organic synthesis, because the production and commercialization of enantiomerically pure drugs is a warranty of good pharmaceutical practices. Although, in several cases, significant differences between the pharmacology and toxicology of the individual enantiomers and the racemate have not been clearly demonstrated; in other instances, serious differences between them have been found. For example, it has been reported that l-tetrahydropalmatine has analgesic activity with a more potent tranquilizing effect than the d-isomer [13]. Recently, the antimalarial activity and structure–activity relationship of 22 protoberberines has been studied and discussed [7, 12]. No references to malacitanine (MLC) have been found until now.

(−)-MLC is an isoquinolinic alkaloid that can be extracted from Ceratocapnos heterocarpa, endemic plant found in south of Spain and north of Africa [14]. Together with heterocarpine is the unique tetrahydroprotoberberine containing a hydroxymethyl group at C-8.

MLC molecule has two chiral centers, C-8 and C-14, thus presenting two enantiomeric pairs. In this work, we used pure 8S,14S(−)-MLC (Fig. 1) and a nonracemic mixture of both enantiomers. The other enantiomeric pair (epimalach tinane) has not been used. Total synthesis has been performed by proton and C13 NMR. 8S,14S(−)-MLC has a specific rotation of [α]D = −87.3° (c = 0.05, methanol). MLC is
soluble in methanol and slightly soluble in ethanol. The ethanolic solution of MLC shows absorption spectrum maxima at λ<sub>max</sub> (log ε) 205 (4.75) and 286 (3.56) nm and a shoulder at 228 (406) nm. This ethanolic solution shows intense fluorescence emission centered at 318 nm when excited at 285 nm. Figure 2 shows absorption (A), excitation (B), and emission (C) spectra of MLC.

The enantiomeric form found in the nature is (−)-MLC in several papaveraceas such as <i>C. heterocarpa</i>. Biological/pharmacological effects have not been studied until now, however, it is probable that these effects are similar as in other protoberberines (antioxidant, anticolinergic, antisecrent, astringent, and antimicrobial agents).

In the present paper, we proposed a chromatographic method for the separation and determination of the enantiomers of MLC based on the use of a chiral column Chirex 3020 (250 mm × 4.6 mm, 5 μm) from Phenomenex (Torrance, CA, USA). Mobile phase was cyclohexane–1,2-dichloroethane–ethanol–trifluoroacetic acid (TFA) (64:30:6:0.6, v/v) at a flow rate of 1 mL/min. No previous analytical methods have been found for the determination of MLC and its enantiomers.

In addition, a procedure for the determination of enantiomeric purity using a conventional chromatographic column Si-60 (250 mm × 4 mm, 5 μm) from Merck (Darmstadt, Germany) in normal phase (100% ethanol) at a flow rate of 0.9 mL/min coupled to a polarimetric detector (Applied Chromatography Systems Limited) placed in series with the fluorimetric detector gives additional information to determine enantiomeric purity, because the absorbance or fluorescence signal is proportional to the total amount of stereoisomer present, while the polarimetric detector signal is dependent on the ratio of enantiomers present [15–17]. This methodology, based on the use of a combined signal from a generic detector (UV) and a chiroptic detector (O.R.) has been used previously in the enantiomeric ratio determination of several compounds [18–21].

2 Experimental

2.1 Chemicals and reagents

(S)-(−)-MLC and racemic (±)-MLC were isolated from the plant (<i>C. heterocarpa</i>) by extraction with methanol and subsequently separated by liquid chromatography column and preparative thin-layer chromatography [14]. The racemic was synthesized [22] (joined with (±)-epimalacitanine) of which was separated by preparative thin-layer chromatography reacting glycolaldehyde norcrasilofoline with 2.5 M hydrochloric acid medium.

2.2 Instrumentation

Measurements were performed with a Merck-Hitachi (Darmstadt, Germany) liquid chromatograph consisting of an L-6200 pump, an AS-4000 autosampler, an L-4250 UV-visible detector, an F-1080 fluorescence detector, and a D-6000 interface. Instrument parameters were controlled by Hitachi-Merck HM software.

A ChiraMonitor 2000 optical rotation (OR) detector (Applied Chromatography Systems Limited) placed in series after above-mentioned detectors. This detector was equipped with a collimated laser diode providing up to 30 mW of light at 830 nm, and a flow cell of 0.48 dm path length, 73 μL volume.

Data acquisition and transformation were accomplished by the Pico ADC-142 (Picotechnology Ltd., Cambridge, UK), which is an analog-to-digital converter with two input ranges. The instrumental parameters were controlled by Picolog software and the calculation of the areas (negative and positive peaks) with Microsoft Origin 7.5.

2.3 Chromatographic conditions

Enantiomeric separation was accomplished using a chiral chromatographic column (Chirex 3020, Phenomenex) consisting of (S)-tert-leucine and R-1-(o-naphthyl)ethylamine in normal-phase conditions. Several percentages of tertiary mixture were assayed and Rs values calculated from chromatographic data. The obtained data show that a percentage of 64% n-hexane, 30% dichloroethane, 6% ethanol, and 0.6% TFA gives the best resolution at a flow rate of 1 mL/min, with 1-μL injection.

Nonenantioselective chromatographic conditions were selected to obtain a good separation between injection and analyte peaks. A Si-60 (250 mm × 4 mm, 5 μm) column was used as stationary phase, 100% ethanol as mobile phase, and 0.9 mL/min flow rate.
Figure 2. Absorption (A), excitation (B), and emission (C) spectra of (−)-MLC. [MLC] = 36 μg/mL in ethanol.

2.4 Standard solutions

Stock standard solutions of (S)-(−)-MLC (1600 mg/L) and (±)-MLC (1600 mg/L) were prepared by dissolving the compounds in ethanol. Dilutions were in the appropriate mobile phase. The solvents used as mobile phase were gradient grade from Lichrosolv Merck (methanol, ethanol, 1,2-dichloroethane, TFA, and cyclohexane). All these solvents were filtered through 0.2-μm nylon membrane and then degassed for 1 h in ultrasonic bath before using.

3 Results and discussion

3.1 Enantioselective HPLC

3.1.1 Mobile-phase effect on retention and stereoselectivity

As expected, large contents on hexane promote higher retention time and polar alcohols give short retention time. However, the solubility of the analyte in cyclohexane is higher and this solvent was preferred as a mobile phase in spite of their high viscosity. The use of ethanol as a modifier appeared to be better to obtain short retention times than 2-propanol. To improve selectivity, the addition of 1,2-dichloroethane has proved to be effective, and TFA gives best chromatographic profiles. Finally, the mobile phase giving best separation of both enantiomers was cyclohexane-1,2-dichloroethane-ethanol-TFA (32:15:3:0.3; v/v) at a flow rate of 1 mL/min.

Preliminary experiences show that neither chiral stationary phase Chiradex, Merck (β-cyclodextrine inclusion mechanism) nor Chiraspher, Merck (Pirkle type) gave good separation of MLC enantiomers.

Using a chiral column Chirex 3020 and the mobile phase above cited, separation at baseline of both enantiomers can be obtained in a short total time. Figure 3A shows the fluorimetric chromatogram of the near racemic mixture of both enantiomers and Fig. 3B shows the chromatographic profile of the enantiomer (−)-MLC. As can be seen, retention time of 12.4 and 15.9 min was obtained for (+)-MLC and (−)-MLC, respectively. From these data and using the equation $R_s = \frac{2t_1}{w_1 + w_2}$, a resolution $R_s$ value of 1.13 and a value of $\alpha = 1.4$ were obtained. Once separated the enantiomers, the method validation was established. Linearity was assessed with four series at six concentrations levels and the correlation coefficients were calculated in order to prove the linearity of the calibration curves. To evaluate the intraday precision, three control samples at the 500 ng/mL level (fluorescence detector) (150 g/mL in O.R.) were injected. Statistical tests were performed at a level of confidence of 95% ($P = 0.05$). The limits of detection and quantitation were considered to be the concentrations that produced signal-to-noise ratios of 3 and 10, respectively. In Table 1, the obtained results are ordered. The enantiomeric purity determination was carried out using the expression:

$$\%S(−)−MLC = \left[ \frac{m_S}{m_S + m_R} \right]100$$

where $m_S$ and $m_R$ are the values of the slopes of the calibration curve for each enantiomer [23, 24]. From these data, the enantiomeric purity of the sample used was found to be 48.60% (−)-MLC.
methodology or appropriate chiral column that separates the enantiomers, and in these cases the use of chiral detection by circular dichroism (CD) or OR, whose response is specific for compounds exhibiting optical activity, can solve the problem. In this case, we used a column Si-60 (250 mm × 5 mm, 5 μm) and 100% ethanol mobile phase at a flow rate of 0.9 mL/min. The choice of the mobile phase was advised by the better solubility in ethanol. The Si-60 column gives results with a narrow elution peak than with a reverse-phase column.

Several samples containing 160 μg in an injection volume of 100 μL were injected in the chromatograph. Samples were prepared from standard R-(−)-MLC (1600 mg/L) and standard racemic (±)-MLC (1600 mg/L). Each sample was prepared by mixing different aliquots in the volume of both solutions. The result is a mixture containing a fix concentration of 1600 mg/L of MLC with different enantiomeric proportion. All measurements were performed by triplicate. Figure 4 shows the polarimetric profile of a blank, 100, 93.3, 87.1, 81.3, and 72.8% (−)-MLC (from upper to lower). As can be seen, a false-positive signal appears before the true polarimetric signal (negative) corresponding to the excess of (−)-MLC. This false-positive signal is due to the so-called

3.2 Nonenantioselective HPLC: polarimetric detection

The best option to establish a method for the determination of the enantiomers of a mixture, or proportion in the mixture (enantionic purity), is the enantioseparation by a suitable chromatographic procedure and comparison with standard pure enantiomers. However, sometimes there is no

![Figure 3](link-to-figure)

**Figure 3.** (A) Mixture of (+)-MLC and (−)-MLC chromatogram (flow rate 1 mL/min, fluorimetric detection, and sample concentration 16.00 mg/L, therefore, injection mass 0.160 g). Retention times 12.4 and 15.9 min for (+)-MLC and (−)-MLC, respectively. (B) (−)-MLC chromatogram (injection mass 0.40 g).

**Table 1.** Analytical performances

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![Figure 4](link-to-figure)

**Figure 4.** Polarimetric chromatograms. Polarimetric profile of several mixtures of MLC containing a fix total concentration and different percentages of enantiomers: blank, 100, 93.3, 87.1, 81.3, and 72.8% (−)-MLC (from upper to lower). Injected volume 100 μL, total injected mass 160 μg.
refractive index artefact (RIA) [25], because no separation was associated with the polarimetric signal. Obtained polarimetric signals are noisiest because sensitivity of polarimetry, as dispersive phenomena, is reduced. However, this method can be useful when no separation can be obtained and an indicative report is needed.

From these data, the plot of polarimetric signal/fluorimetric signal (peak area) against % (−)-MLC [26] gives a linear fit of $P = 1.4 - 0.03x$ ($x = \% (−)$-MLC) with a correlation coefficient of $−0.998$. Figure 5 shows that the crossing zero abscissa axis occurs at 47.8% (−)-MLC, in good concordance with that obtained by the fluorimetric/chromatographic method. If a true racemic mixture of MLC was used as a standard, crossing of fittest curve with abscissa axis would be at 0.0%.

4 Recovery assay

To determine the accuracy of the developed methods, a recovery assay at 80, 85, and 90% levels of (−)-MLC was performed.

For the analytical recovery assay study, three different quantities (μg) of (−)-MLC and (±)-MLC were injected in the chromatograph and submitted to the chiral method. The sum of (−)-MLC and (±)-MLC was 160 μg in a total volume of 100 mL. Each sample was prepared by mixing different aliquots of both solutions to obtain a final percentage of 80, 90, and 95% of (−)-MLC. The spiked samples were analyzed in triplicate and the obtained results are ordered in Table 2. Recovery assay shows that the accuracy of the method is excellent because recoveries are within 109–92% with standard deviation below 5%.

5 Conclusions

The new isoquinolinic alkaloid MLC was analyzed, previous chiral HPLC separation with a resolution of 1.13, and enantiomeric excess of mixtures of both enantiomers determined. A second nonenantioselective method with polarimetric detection, for the determination of enantiomeric excess of mixtures, was proposed.

The enantioselective chromatographic method of this new alkaloid is fast (10 min), selective ($R_s$ 1.13), precise (RSD 2.5%), and exact (recovery values between 98 and 101%). Thus, it is advisable for phytochemical enantiomeric analysis and to assess enantiomeric excess in enantioselective synthesis. The nonenantioselective method shows good analytical specifications and in addition is very simple, therefore, can be useful as first analysis results in organic synthesis.

Results obtained show that the methods developed in this study might be applied for the wide application in separation and determination of enantiomeric excess of the components of mixtures of enantiomers.

The authors have declared no conflict of interest.

6 References


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