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An HPLC assay for the norditerpenoid alkaloid methyllycaconitine, a potent nicotinic acetylcholine receptor antagonist

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Abstract

The extremely potent and selective nicotinic acetylcholine receptor antagonist methyllycaconitine, MLA, and related norditerpene alkaloids are finding increasing use as neurochemical probes and as targets for structure-activity relationship studies. In this work, an assay procedure for MLA which utilises ion suppression reverse-phase HPLC with UV absorbance detection at 270 nm is described. The method detected 280 ng MLA on column.

Keywords: Reverse-phase chromatography; HPLC; Methyllycaconitine; Ion suppression; Nicotinic acetylcholine receptor antagonist

1. Introduction

Methyllycaconitine (MLA) (Fig. 1) is a norditerpenoid ester alkaloid found in many species of the plant genus *Delphinium* (Ranunculaceae) which is highly toxic both to insects and mammals [1–3]. This toxicity arises from its highly potent and selective antagonist activity at nicotinic acetylcholine receptors [4,5]. We are currently engaged in structure-activity relationship (SAR) studies of MLA and its analogues using other natural alkaloids and semisynthetic derivatives prepared from MLA itself [6–8]. However, the extremely high activity of MLA (inhibitor dissociation constant, $K_i = 2$ nM in a competition binding assay against α-bungarotoxin [4]) means that even low levels of contamination of other test alkaloids with this compound will lead to a significant distortion of bioassay results. Accordingly, we have developed a sensitive HPLC assay for MLA. This practical assay is used to monitor alkaloids and their derivatives prior to biological testing. As MLA and related alkaloids are increasingly finding a use as selective probes in neurochemical studies [9,10], it is important that standard methodologies are developed for the assessment of the chemical purity of these alkaloids.

In addition, the method has been designed to be readily adaptable to LC/MS applications and for scale-up for semi-preparative use. Majak et al. [11] have reported a reverse-phase, ion-pair HPLC system for MLA. However, the external standard method of quantitation they used is insufficiently precise for our purposes, whilst their use of a hexanesulphonate counterion precludes LC/MS

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and scale-up for semi-preparative use. A normal-phase system more recently reported by Manners and Pfister [12] avoids these problems, but their system does not fully resolve MLA from its parent norditerpenoid alcohol, lycoctonine.

Several HPLC methods for the (structurally) closely related *Aconitum* alkaloids have been reported including a reverse-phase ion-pair quantitative assay [13], a qualitative reverse-phase system [14], preparative HPLC on a normal-phase system [15] and LC/MS [16]. In this last report, several of the test alkaloids were poorly resolved chromatographically and identification was achieved by selected-ion monitoring [16].

2. Experimental

2.1. Materials

Seeds of garden hybrid *Delphinium*, a cultivar closely related to the American 'Pacific Giant' and derived predominantly from the species *D. elatum*, were kindly donated by Blackmore and Langdon Ltd., Pensford, Bristol.

An authentic sample of MLA citrate was supplied by Professor M. H. Benn, University of Calgary, Canada. Lappaconitine was purchased from Latoxan, Rosans, France. Reagent grade ammonium acetate and formic acid, and HPLC grade acetonitrile were used in the preparation of the mobile phase. All solvents used in the extraction and separation of the alkaloids were HPLC grade.

2.2. Extraction of alkaloids

Ground, defatted seeds of garden hybrid *Delphinium* were continuously extracted with chloroform in a soxhlet extractor. The extract was concentrated under reduced pressure and partitioned with aqueous sulphuric acid solution (0.1 M). The acid phase was washed with one aliquot of chloroform, then basified to pH 10 with saturated aqueous sodium carbonate solution, and extracted with diethyl ether. After washing with water and drying over anhydrous Na_2SO_4, the ether was removed in vacuo at 40 °C to leave a crude total alkaloid mixture as an off-white foam.

The alkaloid mixture was separated by vacuum liquid chromatography over basic alumina (TLC grade) eluted with a step gradient of hexane, diethyl ether and methanol mixtures of increasing polarity. Delpheline (Fig. 1) was the first major alkaloid eluted followed by MLA and then a mixture of more polar components. Delpheline was further purified by recrystallisation from ethanol:hexane (1:1, v/v).

Lycoctonine (Fig. 1) was prepared by hydrolysis of MLA with 0.1 M NaOH in ethanol for 16 h at 20 °C and purified by recrystallisation from ethanol. The alkaloids were authenticated by comparison of their ^{1}H NMR, ^{13}C NMR and mass spectra with literature values [1,17].

2.3. Apparatus

HPLC was carried out using a JASCO PU980 pump, a 100 µl Rheodyne injection loop and a JASCO UV975 variable UV detector at 270 nm. A 25 cm × 4.6 mm i.d. Hypersil ODS 5 µm column was used with a mobile phase of 0.2 M aqueous ammonium acetate adjusted to the required pH (3.0–5.0) with formic acid. Acetonitrile was used as organic modifier. All mobile phase mixtures were pumped at a flow rate of 1 ml min^{-1}.

Lappaconitine (Fig. 1), a commercially available norditerpenoid alkaloid with an aromatic chromophore, was used as an internal standard.

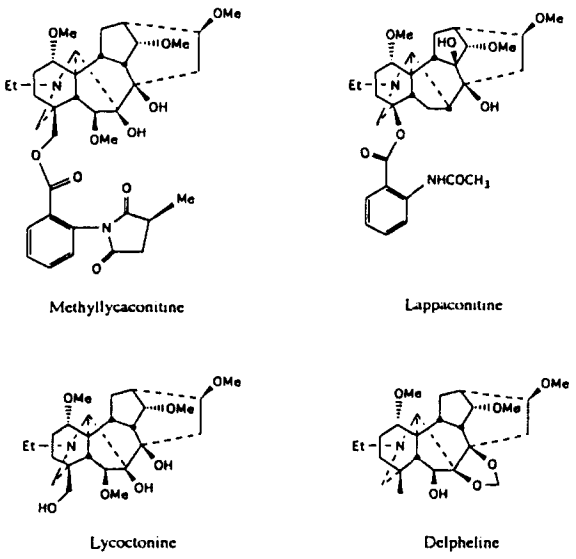

Fig. 1. Chemical structures of MLA, lycoctonine, delpheline and lappaconitine.

2.4. Calibration curves

A calibration curve was constructed by injecting, in duplicate, a series of mixtures consisting of 2.8–100 μg ml^{-1} of MLA with 25 μg ml^{-1} of lappaconitine in mobile phase. MLA peak height over lappaconitine peak height was plotted against MLA concentration and a line fitted by linear regression. Six replicates of solutions containing 5 μg ml^{-1} and 25 μg ml^{-1} of MLA were injected in order to assess the intra-day precision of the method.

2.5. Assays

For the assay of MLA in a crude alkaloid extract from cultivated *Delphinium* seed, approximately 2 mg of the extract, accurately weighed, was dissolved in mobile phase (10 ml). Aliquots (1 ml) were taken and mixed with 1 ml of a 0.05 mg ml^{-1} solution of lappaconitine in mobile phase. For the assay of MLA in purified alkaloid samples, approximately 10 mg of the alkaloid, accurately weighed, was dissolved in mobile phase (1 ml), and mixed with 1 ml of a 0.05 mg ml^{-1} lappaconitine solution.

3. Results and discussion

3.1. Optimisation of mobile phase

The aim was to develop a reverse-phase system which utilised a volatile mobile phase which would provide the flexibility for routine analytical use and also be adaptable for LC/MS and for semi-preparative HPLC. The use of a large organic anionic counterion, as employed in previous chromatographic methods for norditerpenoid alkaloids [11,13], was precluded by this requirement, but ionisation of these relatively weak bases was suppressed by buffering the mobile phase at relatively high pH values. Increasing the pH from 3.0 to 4.0 to 5.0 increased the capacity ratio, K', for MLA from 2.0 to 3.8 to 6.0 respectively when the acetonitrile content of the mobile phase was maintained at 35% (v/v). However, storage of samples in mobile phase at pH 3 showed progressive loss of MLA with more than 50% lost after 3 months and the concomitant appearance of several extra peaks in the chromatogram. At pH 5, MLA showed slight instability on prolonged storage, but no loss and no breakdown products were detected during the course of an intra-day precision experiment. Accordingly,

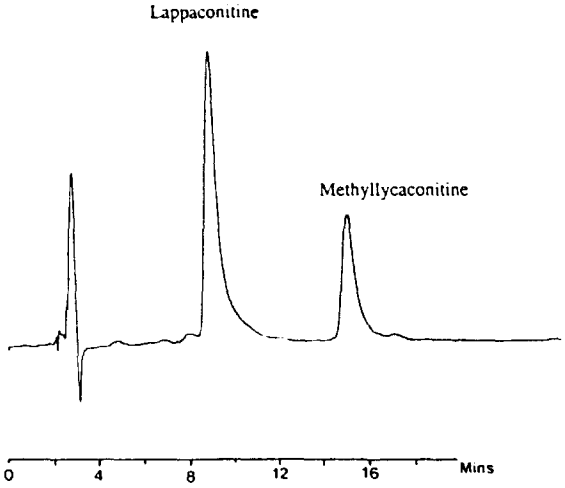

Fig. 2. A chromatogram obtained for 25 μg ml^{-1} of MLA and 25 μg ml^{-1} of lappaconitine using a 25 cm × 4.6 mm column of Hypersil ODS 5 μm and a mobile phase of 0.2 M ammonium acetate adjusted to pH 5 with formic acid:acetonitrile (70:30, v/v), 1 ml min^{-1} and detection by UV absorbance at 270 nm.

pH 5 was chosen for routine use, care being taken to prepare the injection solutions immediately prior to chromatography. Increasing the acetonitrile content from 25% (v/v) to 30% (v/v) to 35% (v/v) to 40% (v/v) at a constant pH of 4.0 led to a decrease in the K' value from 9.5 to 4.4 to 2.9 to 2.5 respectively. A mobile phase of 0.2 M ammonium acetate solution (pH 5) and acetonitrile 70:30 v/v) was eventually selected. Fig. 2 shows a chromatogram of a mixture of MLA and lappaconitine obtained using these conditions. MLA had a K' value of 6.5 and lappaconitine a K' value of 3.4, with a resolution of 2.4 even though the lappaconitine in particular showed tailing. The number of theoretical plates, N, for MLA was 3700 and 1700 for lappaconitine. The minor peak following MLA is nudicauline, which we have found as a minor contaminant in most of the MLA samples we have analysed.

3.2. Calibration

The calibration curve was linear from an MLA concentration of 2.8 to 100 μg ml^{-1} with a regression coefficient of 0.999. The line had a slope of 16.717 (SD = 0.136) and an intercept of 0.0268 (SD = 0.0055). The relative standard deviation (intra-day) at 5 μg ml^{-1} was 5% and 3.6% at 25 μg ml^{-1}. The lowest point on the calibration curve was equivalent to 280 ng of MLA on column. However, a peak for MLA was clearly detectable down to

approximately 50 ng on column, showing the potential for assaying down to this level by constructing appropriate calibration curves with a lower concentration of internal standard. Using a normal phase HPLC system with UV absorbance detection at 280 nm, Manners and Pfister [12] reported a detection limit for MLA of 300 ng on column. By using UV absorbance detection at 220 nm they were able to detect 30 ng on column, but this wavelength revealed that their chromatographic system did not fully resolve MLA from its parent alcohol, lycoctonine. This effectively prevented them from using this shorter wavelength for the quantitative analysis of MLA.

3.3. MLA content of alkaloids isolated from garden hybrid Delphinium seeds

Using UV detection at 270 nm, only those alkaloids possessing an aromatic acyl group were detected. However, LC/MS studies, to be reported elsewhere, showed that this system resolved MLA from all other alkaloids in the crude mixture. Using the assay described here the total crude alkaloid extracted from *Delphinium* seeds was shown to contain 56% (w/w) of MLA. The co-occurring alkaloid, delpheline, isolated from the crude mixture by vacuum liquid chromatography and recrystallisation, contained 0.2% (w/w) of MLA. Lycoctonine, prepared by base catalysed hydrolysis of MLA and purified by recrystallization contained no detectable MLA. The method can thus be applied both to the measurement of MLA in crude plant extracts and to isolated alkaloid samples in order to determine the precise levels of any trace contamination with this highly potent alkaloid prior to bioassay.

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