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Short Communication

High-performance liquid chromatographic assay for laudanosine in biological fluids and tissue for neurotoxic studies in rats

IQBAL RAMZAN

Pharmacy Department, The University of Sydney, Sydney, N.S.W. 2006 (Australia)

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ABSTRACT

A procedure for the determination of laudanosine, the central nervous system active metabolite of the neuromuscular blocking drug atracurium, in serum, cerebrospinal fluid and brain is described. The method uses a readily available internal standard, ethavrine, and a single-step protein precipitation with acetonitrile followed by high-performance liquid chromatographic separation with ultraviolet detection. Norlaudanosine, the major metabolite of laudanosine, can also be quantified. Linearity of detector response was obtained between 1 and 25 μg/ml or μg/g and the method is suitable for determining neurotoxic concentrations of laudanosine in experimental animals.

INTRODUCTION

Laudanosine (Fig. 1a), a naturally occurring opium alkaloid [1], is a major metabolite of atracurium, a newly introduced bisquaternary non-depolarizing neuromuscular blocking drug [2]. Laudanosine itself is metabolized *in vivo* to at least six different compounds, the most prominent being norlaudanosine (or tetrahydropapaverine, Fig. 1b) [3].

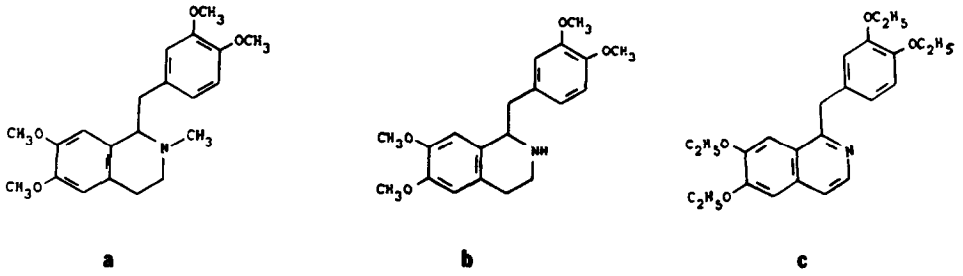

Fig. 1. Structures of laudanosine (a), norlaudanosine (b) and the internal standard, ethavrine (c).

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Laudanosine is of interest to anaesthetists since it has central nervous system (CNS)-stimulant properties in animals; it causes seizures in rabbits [4], dogs [5,6] and rodents [7]. Laudanosine also increases the minimum anaesthetic concentration (MAC) of halothane [8]. While laudanosine-induced seizures, following atracurium dosing, have not been reported in humans, it is, however, detected in human plasma following atracurium administration especially during prolonged use and in patients with renal or hepatic desease; concentrations of laudanosine may reach convulsant levels [9,10].

To assess the risk factors associated with laudanosine neurotoxicity a high-performance liquid chromatographic (HPLC) assay has been developed for its quantitation in serum and CNS fluid and tissue. Compared to the currently available assay for laudanosine [6,11], this method is simple, does not require prior extraction of the drug from the biological matrix and utilizes a readily available internal standard. Norlaudanosine can also be detected. The method has been used to measure neurotoxic concentrations in rats and has general applicability for similar studies in other animals.

EXPERIMENTAL

Drugs, chemicals and reagents

DL-Laudanosine, norlaudanosine (tetrahydropapaverine) hydrochloride and internal standard, ethavrine hydrochloride (Fig. 1c) were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was from American Burdick and Jackson (Muskegon, MI, U.S.A.), anhydrous sodium sulphate from Sigma, zinc sulphate ($+7H_2O$) from Fisher Scientific (Pittsburgh, PA, U.S.A.) and sodium hydroxide and hydrochloric acid from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Chromatography

The HPLC system consisted of a Waters U6K variable-volume loop injector (Water Assoc., Milford, MA, U.S.A), a Waters M45 pump, a 2 cm × 4.6 mm I.D. C_{18} DB (deactivated for basic compounds) guard column and a 25 cm × 4.6 mm I.D., 5-μm C_{18} DB analytical column (Supelco, Bellefonte, PA, U.S.A.); a Waters 440 fixed-wavelength detector was connected to a Waters extended-wavelength module set at 229 nm (cadmium lamp), and a Hitachi-EM Science D2000 chromato-integrator (EM Science, Cherry Hill, NJ, U.S.A.) was set at attenuation 5 (32 mV full scale). The column temperature was ambient and the mobile phase, water–acetonitrile (40:60, v/v) containing 0.006 *M* sodium sulphate, was filtered and degassed prior to use. The pH was not adjusted and varied between 6 and 6.5. The flow-rate was 1.5 ml/min and the usual operating pressure was between 10.3 and 13.8 MPa.

Biological sample processing

Rat serum (50 μl) was placed in a 1.5-ml Eppendorf tube (Brinkmann In-

struments, Westbury, NY, U.S.A.) followed by addition of 5 μl of ethavrine (0.05 mg/ml in 0.01 *M* hydrochloric acid, pH 2–2.5) as internal standard and 100 μl of acetonitrile for protein precipitation. The mixture was vortex-mixed for 15 s and centrifuged for 2 min in a high-speed centrifuge. A 50-μl aliquot of the supernatant was injected into the column. Protein precipitation could also be carried out using 50 μl of an equal mixture of 10% (w/v) zinc sulphate and 0.5 *M* sodium hydroxide but the amount of internal standard needed to be increased (ten-fold) since this precipitant co-precipitates ethavrine with the serum proteins. For the laudanosine assay from cerebrospinal fluid (CSF), 10–50 μl samples were injected directly into the column without prior protein precipitation.

Laudanosine concentrations in rat brain were determined in cerebral tissue from one hemisphere. The tissue (~0.5 g) was accurately weighed; 50 μl of the internal standard solution and 1 ml of acetonitrile were then added, and the mixture was homogenized in a motor-driven tissue homogenizer (Wheaton Instruments, Millville, NJ, U.S.A.). Following centrifugation, 50 μl of the supernatant were analyzed as for serum samples. Zinc sulphate could again be used as the protein precipitant but it co-precipitates the internal standard with brain proteins.

For routine analyses, calibration curves for laudanosine between 1 and 25 μg/ml (or μg/g) were constructed using either drug-free rat serum, CSF or brain and standard solutions of laudanosine made in 0.01 *M* hydrochloric acid (pH 1–1.5). If necessary this curve could be extended to a higher (25–100 μg/ml) concentration range. For the norlaudanosine assay, standard solutions were prepared in water without the aid of acid.

RESULTS

Laudanosine showed three UV absorption maxima in the mobile phase at 205, 229 and 280 nm (Fig. 2a), hence its detection was carried out at 229 nm, the wavelength available with the extended wavelength module in this laboratory.

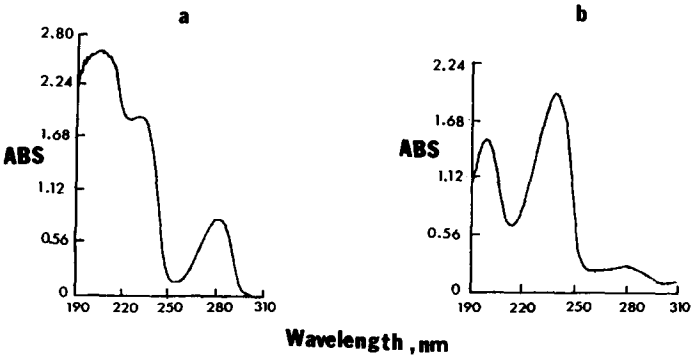

Fig. 2. UV spectra of (a) laudanosine and (b) the internal standard, ethavrine.

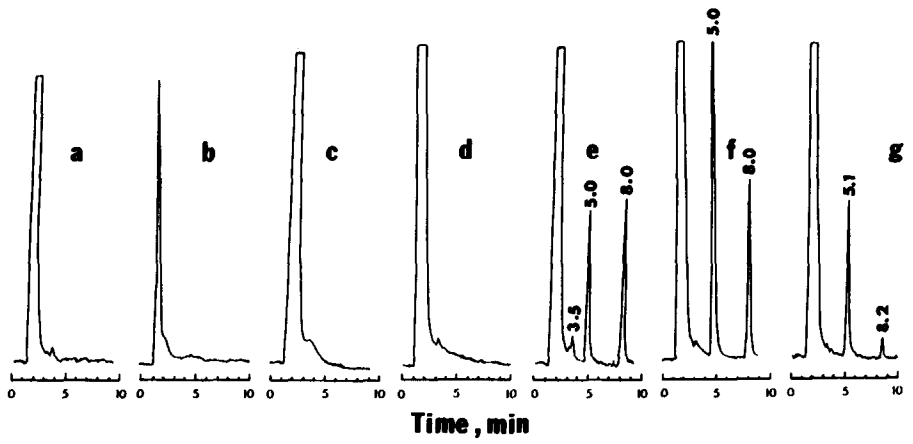

Fig. 3. Chromatograms of (a) blank drug-free rat serum, (b) CSF, (c) brain and (d) human serum. These chromatograms were generated after acetonitrile protein precipitation except for CSF (b), which was injected directly. Acetonitrile-treated rat serum spiked with 10 μg/ml each of norlaudanosine and laudanosine (and the internal standard) are shown in (e) while (f) illustrates a chromatogram from a serum sample containing 24.3 μg/ml laudanosine from a rat infused with laudanosine to a maximal seizure (norlaudanosine was not detected). The last chromatogram (g) shows that zinc sulphate protein precipitation of serum co-precipitates the internal standard, ethavrine.

Norlaudanosine exhibited a similar UV spectrum but ethavrine, the internal standard has a maximum at 240 nm (Fig. 2b).

Using the described conditions, laudanosine metabolite, norlaudanosine, laudanosine and the internal standard (ethavrine) had retention times of 3.5, 5.0 and 8.0 min, respectively. There were no interfering peaks from blank (drug-free) rat serum (Fig. 3a), CSF (b), brain (c) or human serum (d). Fig. 3e shows a chromatogram of rat serum spiked (and protein precipitated with acetonitrile) with 10 μg/ml norlaudanosine and laudanosine together with the internal standard while Fig. 3f shows a similar chromatogram from a rat infused with laudanosine to a maximal seizure. Fig. 3g illustrates that protein precipitation of serum samples with zinc sulphate co-precipitates the internal standard, ethavrine.

Laudanosine-to-ethavrine peak-area ratios were linear with laudanosine concentrations from 1 to 25 μg/ml and linear regression yielded correlation coefficients in excess of 0.995. The detection limit was 1 μg/ml using 50 μl of sample and the coefficient of variation of the assay varied between 2.3 and 7.6% (for CSF and brain, $n = 3$, respectively). Norlaudanosine could only be quantified between 2.5 and 10 μg/ml and detection limit was $\sim$ 2 μg/ml. There were no interferences from other drugs used in anaesthesia (*e.g.* barbiturates or narcotic analgesics). The neuromuscular blocker atracurium which generates laudanosine on metabolism elutes with the solvent front when injected into the column.

A rat (323 g body weight) infused intravenously with laudanosine at 0.68 mg/min exhibited a maximal seizure (as evidenced by forelimb flexion and tonic

hindlimb extension) at a dose of 10 mg or 32 mg/kg. At onset of the seizure, serum, CSF and brain concentrations of laudanosine were 24.3, 5.2 μg/ml and 7 μg/g respectively. When the serum and brain samples were re-analyzed in duplicate at another time the concentrations did not differ by greater than 6.8% (CSF assays not repeated due to limited sample volume).

DISCUSSION

Previously utilized HPLC assays for laudanosine in biological fluids (mainly in serum or plasma and sometimes in CSF) have involved solid-phase extractions followed by ion-exchange chromatography and fluorescence detection [6,11]. These assays are very sensitive and as such are suitable for low concentrations encountered in clinical practice. A synthetic quaternary derivative of laudanosine (N-methyllaudanosine), which is not available commercially, is used as the internal standard but the assay can be used to quantify atracurium, the neuromuscular blocking drug which on metabolism yields laudanosine. The currently developed HPLC method is simpler in that no extraction of the drug from the biological matrix is required before chromatography; it uses a cheap and commercially available internal standard and is the first technique where the drug can be analysed from brain tissue, also by direct protein precipitation without prior extraction. In addition, the claimed primary metabolite of laudanosine, norlaudanosine, can be detected as well. The full range of possible metabolite(s) of laudanosine have been quantified previously but only using a gas chromatographic (and mass spectrometric) technique [3]. However, the present method is not as sensitive and thus may be useful only for neurotoxic studies in animals where concentrations in excess of that in patients are likely to be encountered. However, it is possible to increase sensitivity by fluorescence (rather than UV) detection presumably as in previous assays [6,11].

Atracurium is used commonly and thus caution about the possible CNS excitatory effects from its metabolite laudanosine is needed. This is especially so, for example, in anephric patients who are likely to have impaired elimination of laudanosine itself and who may in addition be at an increased risk (or show greater sensitivity) to laudanosine. Also of importance is the relationship between laudanosine in the systemic circulation and its concentration in the CNS where it presumably produces its neurotoxicity. The current assay procedure for laudanosine is useful for this purpose since it allows quantitation of laudanosine in serum as well as CSF and brain tissue so that risk factors associated with laudanosine can be identified without pharmacokinetic complications related to laudanosine distribution to the CNS.

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