Thin-Layer Chromatographic Detection of Etorphine in Equine Urine

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Abstract

Etorphine, a morphine congener, is metabolized in the horse to a glucuronide conjugate. The parent drug can be recovered after treatment with β-glucuronidase. Thin-layer chromatography, using high-performance silica gel plates, can detect the drug for several hours. The minimum amount detectable by the method (20 ng) appears to be governed by the colorimetric spray sequence used.

Introduction

Etorphine [6,7,8,14-tetrahydro-7α-(1-hydroxy-1-methylbutyl)-6,14-*endo*-ethenooripavine] is a synthetic opiate derivative. The drug is a derivative of the opium alkaloid thebaine. Thebaine has practically no analgesic action and can produce seizures at low doses.[1] Etorphine is valuable for capturing large wild animals such as elephants and rhinoceros because of its high potency. Among horseracing enthusiasts in the United States it is known as 'elephant juice' and is widely reported to be administered to racehorses in synergistic combination with diprenorphine as an analgesic and stimulant.

Etorphine is an extremely potent opioid. Low doses (0.009–0.100 mg/horse) stimulate locomotor activity.[2–3] These very low doses make the drug difficult to detect and confirm. A commercially available radioimmunoassay (RIA) kit has been successfully used to detect the drug in urines of racing animals.[4]

Because of its phenolic moiety, etorphine is metabolized to a glucuronide, similar to most morphine congeners. Treatment of the metabolite with β-glucuronidase, followed by thin-layer chromatography (TLC) of the freed parent drug, is the subject of this study.

Experimental

Reagents

Organic solvents were pesticide-analysis or spectroscopic quality (Fisher Scientific). Reagents were certified ACS quality (Fisher Scientific). β-Glucuronidase was from *Patella vulgata* (Sigma Chemical). Acetate buffer (pH 5; 1.0 M) was made by diluting sodium acetate (164.0 g) and glacial acetic acid (77.0 ml) to 2000 ml with distilled water. Ludy Tenger's reagent was prepared by mixing bismuth subcarbonate (0.5 g) dissolved in concentrated hydrochloric acid (3 ml) with sodium iodide (3 g) dissolved in distilled water (50 ml). *N,O*-Bis(trimethylsilyl)trifluoroacetimide (Pierce Chemical) was used to derivatize the etorphine for mass-spectral analysis.

Apparatus

High-performance silica gel thin-layer plates (Whatman HP-KF; 10 × 20 cm; 200 μm layer thickness) were used. Borosilicate-glass culture tubes with polypropylene screw-caps (150 × 16 mm and 125 × 16 mm; Fisher Scientific) served as extraction and evaporation vessels. A water bath, equipped for nitrogen flow, was used to evaporate solvents before chromatography (Organomation Associates, model III). Capillary spotting pipettes were prepared by drawing Pasteur glass pipettes (9 in) into capillary tips.

Procedure

Urine (5 ml) was mixed with acetate buffer (pH 5; 2 ml) and β-glucuronidase (1 ml; 5000 units) in a culture tube (150 mm), and the tube capped. The urine was incubated (3 h; 63°C). After incubation the urine was made pH 9.5 by adding concentrated ammonia solution dropwise and using a 'pencil' electrode and pH meter. Isopropanol (0.8 ml) and dichloromethane (6.0 ml) were added to the urine, the tube capped, and the mixture rotoracked (5 min) to partition the drug into the organic phase. The mixture was then centrifuged, and the dichloromethane phase transferred to a clean culture tube (125 mm). Sulfuric acid (0.1 M; 2 ml) was added to the dichloromethane phase, the tube capped, and the contents rotoracked (5 min) and centrifuged. The dichloromethane phase was discarded and dilute ammonia solution (0.6 M; 2.0 ml) was added to the sulfuric acid, thereby changing the pH to 9.3. Petroleum ether–dichloromethane (2:1; 6 ml) was added to the aqueous phase and the tube capped. The mixture was rotoracked (5 min), the tube centrifuged, and the upper (organic) phase transferred to a clean culture tube (125 mm). The organic phase was carefully evaporated under nitrogen in a water bath (35°C).

Dichloromethane (40 μl) was added to the residue in the tube and the entire contents carefully spotted under a hot air stream onto the origin of a TLC plate. The plate was developed for 3 cm with ethyl acetate–methanol–acetic acid (7:2:1) and afterwards dried under a stream of hot air. The plate was exposed to formaldehyde fumes for 1 minute, then carefully and lightly oversprayed with Ludy Tenger's reagent. This was followed by spraying with aqueous sodium nitrite (5%) until the plate background 'bleached' leaving drug spots a light brown [Figure 1]. The spots were best observed by viewing the plate with a combination of transmitted and reflected light. (The glass plate and thin silica gel layer permitted ready passage of light through the plate.)

Drug addition studies

To determine the minimum detectable quantity of etorphine using this procedure etorphine aliquots (400, 100, 50, 30, 20, 10 ng) were added to control urine samples (5.0 ml) which were then processed. A minimum of six urine samples at each concentration were analyzed. Visual observation of drug spots on the TLC plate after the spray sequence indicated that 20 ng of etorphine per 5.0 ml of urine was the minimum detectable by this procedure.

Figure 1 *TLC of urine extracts from a horse given 0.1 mg etorphine. The urines are a control (0-hour) sample and 2-, 4-, and 8-hour post-dose samples. Authentic etorphine standards are also on the plate.*

Identification and confirmation

The identification and confirmation of etorphine were accomplished by mass spectroscopy using electron-impact ionization. The drug can be isolated by preparative TLC and was introduced to the mass spectrometer as its TMS derivative. Characteristic fragments appeared at *m/z* 483, 396, 354, 272, 250, 232, 162, and 121.[5]

Results and Discussion

Etorphine can be detected in the urine of a horse by TLC analysis. Figure 1 is the photograph of a TLC plate with urine extracts from an etorphine-dosed horse. The animal received 0.1 mg of etorphine (IM). The photograph shows etorphine standard spots, a lane from a control urine (0 h), and lanes from urines

obtained 2, 4, and 8 hours after dosing. Note the drug spots from the 2-, 4-, and 8-hour urines and the absence of any spot in the control. Similar results were obtained from horses dosed with 0.03 mg and 0.009 mg (IM) but the drug could only be observed for 5 hours. These results are in good agreement with an RIA study.[6] For the 0.03-mg- and 0.009-mg-dosed horses RIA analysis showed urine concentrations of etorphine equivalents of 4 to 5 ng/ml (the minimum detectable by TLC) 5 hours after dosing.

The procedure gives well-defined spots with a clear, clean background. Below 20 ng in urine the drug could not be detected with the spray sequence used, though presumably there was some on the plate. A spray (or spray sequence) for detecting the drug in lower amounts would be desirable. Using an instrumental plate scanner might give a lower detection limit even with the present spray sequence but would be costly and would increase both analysis time and complexity. Using silanized glassware — particularly in the final solvent evaporation step — should be investigated. These and other aspects will be studied as the method is further refined.

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