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Chiral analysis of methorphan in opiate-overdose related deaths by using capillary electrophoresis

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ABSTRACT

An enantioselective CE-based determination of methorphan and its main metabolites in blood is described. Enantiomeric separations were carried out in 50 cm × 50 μm (ID) uncoated fused silica capillaries, using a background electrolyte composed of 150 mM sodium phosphate pH 4.4 added with 5 mM 2-(hydroxypropyl)-β-cyclodextrin and methanol 20% (v/v), at a constant voltage of 25 kV. Sample injections were performed under field amplified sample stacking conditions. Detection was by recording UV absorbance at the wavelength of 200 nm. Linearity of response was assessed within a concentration range from 25 to 500 ng/mL for dextrometorhan, levomethorphan and their main metabolites (namely dextrorphan and levorphanol, respectively). Folcodine was used as internal standard. Under these conditions, the limit of quantification resulted 25 ng/mL for each one of the analytes. The intra-day and inter-day precision, in terms of coefficient of variation (CV) were below 3.7% and 14.9 % for migration times and peak areas, respectively. The present method was successfully applied to the analysis of post-mortem blood samples from ten subjects died for heroin overdoses. Among the samples "positive" for methorphan ($n = 4$), the *d*-enantiomer was found in concentrations ranging from 214 to 1282 ng/mL. The concentration of its main metabolite dextrorphan in the same samples ranged from 49 to 389 ng/mL.

1. Introduction

Methorphan (3-methoxy-*N*-methylmorphinan) is a well-known synthetic analogue of codeine. Being a chiral compound, it exists in form of two enantiomers, strongly different in their activity. The *l*-enantiomer, levomethorphan, acts as an opiate agonist and consequently, through the binding with the μ opioid receptors, a narcotic analgesic, being its main metabolite levorphanol 4–5 times more potent than morphine, with a longer half-life [1]. Levomethorphan use is typically characterized by habituation, tolerance, physical dependence and withdrawal syndrome. Consequently, levomethorphan, as well as its metabolite levorphanol, is a Schedule II controlled substance.

On the contrary, the *d*-enantiomer, dextrometorphan shows no narcotic activity and therefore is not under international control. Dextromethorphan keeps an antitussive action, and for this reason it is present in the formulation of several anti-cough medications, in many countries available over-the-counter. Other data suggest that the main dextromethorphan metabolite, dextrorphan, is the active principle. Therefore, also this compound is not scheduled. Recently, however, dextromethorphan has been reported to display a high binding affinity for the *N*-methyl-d-aspartate (NMDA) receptors, which could explain a, still limited, but potentially expanding, recreational abuse as a "safe" alternative to ecstasy [2–8].

Since 2010, the National Early Warning System (N.E.W.S., Dept. of Antidrug Policy, Presidency of the Council of Ministers) has released numerous warnings related to "street heroin" adulterated with methorphan. Similar information is also available in the recent scientific literature [9–12].

It is important to point out that almost all the current analytical methods used for the analysis of clandestine heroin are not enantioselective, and therefore, do not distinguish between the two stereoisomers of methorphan possibly present as adulterants. It is also important to ascertain the stereochemical nature of the methorphan found in the blood collected from opiate overdoses, in order to better investigate the intoxication mechanism. Moreover, since the effects of both the enantiomers of methorphan are thought to be caused by their major metabolites [13], namely levorphanol and dextrorphan, from levomethorphan and dextromethorphan, respectively, also these two compounds should be determined for a proper interpretation of the results.

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On these grounds, it is important to develop a simple and straightforward analytical procedure possessing chiral selectivity and high sensitivity suitable for the specific analysis of the enantiomers of methorphan and its metabolites in biological samples.

The traditional methods applied to the analysis of chiral compounds are typically based on GC and HPLC, through the formation of diastereoisomers or using chiral columns [14–16].

In the latest decades, however, after a pioneering paper by Fanali [16], capillary electrophoresis (CE) has found numerous applications in the analysis of chiral compounds, also in the field of forensic toxicology [17–18]. As it is well known, the main advantages of CE are represented by the possibility of achieving enantioselectivity just by dissolving a chiral selector in the electrophoretic buffer, thus enabling resolution also in the presence of reduced selectivity factors, as it is usual in the chiral separations.

To the best of our knowledge, only two research groups reported the separation of methorphan stereoisomers with CE by using cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) [19–20]. However, both the methods had been applied to the determination of methorphan enantiomers in urine samples.

Moreover, the other chiral methods reported in the literature for methorphan analysis, based on the use of HPLC–DAD, HPLC–MS, and GC–MS, were not applied to the analysis of cadaveric blood [12–15].

On these grounds, the aim of the present work was to develop a simple analytical method based on chiral CZE, suitable for the analysis of the (+) and (−) isomers of methorphan, as well as their main metabolites, dextrorphan and levorphanol, in post-mortem human blood.

2. Materials and methods

2.1. Reagents

Levomethorphan, levorphanol, and dextrorphan were obtained from LGC Standard (Sesto San Giovanni, Milan, Italy). Dextromethorphan, β-cyclodextrin (β-CD), dimethyl-β-cyclodextrin (dimethyl-β-CD), 2-(hydroxypropyl)-β-cyclodextrin (HP-β-CD), potassium dihydrogen phosphate, sodium carbonate, sodium hydroxide, hydrochloric acid, *n*-propanol, acetonitrile, methanol, *n*-hexane, ethylacetate and folcodine (I.S.) were purchased from Sigma–Aldrich (St. Louis, MO, USA). A stock solution 1 mg/mL for each one of the standards, i.e. dextromethorphan, levomethorphan, dextrorphan and levorphanol, was prepared in methanol and stored at −20 °C. The ultra pure water used in the present study was obtained from an aqua MAX Ultra 370 Series water purification system (Young Lin Instruments, Anyang, Korea).

2.2. Instrumentation

Analyses were performed in uncoated fused-silica capillaries 60 cm long (50 cm effective length) with inner diameter (i.d) of 50 μm (Composite Metal Services, The Chase, Hallow, UK) using a P/ACE MDQ automated capillary electropherograph (Beckman Coulter, Fullerton, CA, USA). The instrument was equipped with a diode array detector, set to monitor the wavelength of 200 nm. The optimized running buffer was composed of 150 mM potassium dihydrogen phosphate at pH 4.4, added with 5 mM HP-β-CD and methanol 20% (v/v).

Sample injections were performed as follows:

i) the injection end of the capillary was dipped in water for 6 s,
ii) a water plug was hydrodinamically injected by applying 0.1 psi for 3 s,
iii) the sample was electrokinetically injected by application of 7 kV for 15 s.

Then, the separations was carried out by applying a constant voltage of 25 kV (resulting current: 60 μA). Both capillary temperature and sample holder were maintained at 15 °C. Before each run, the capillary was rinsed sequentially with NaOH 1 M, water and buffer electrolyte, for five minute each. In order to obtain reproducible separations, fresh separation buffer was used each day.

Data acquisition and data reporting were carried out by using the software "32 Karat" Version 8.0 (Beckman Coulter). Resolution (R) was calculated according to the equation $R = [2(t_2 - t_1)]/(W_1 + W_2)$, where t_1 and t_2 represent the migration times of two adjacent peaks, while W_1 and W_2 are the peak widths at half height.

2.3. Sample collection and preparation

Post-mortem blood samples were collected from the femoral veins of corpses undergoing judicial autopsies and frozen at −20 °C until analysis. In the present work, four cases of heroin-related fatal overdose were studied.

Before analysis, blood samples underwent a liquid/liquid extraction according to the procedure described by Lin et al. [21] with slight modifications. Briefly, 500 μL of blood fortified with folcodine as I.S. (final concentration: 500 ng/mL) was added in together with 250 μL of saturated sodium carbonate solution. The mixture was vortexed for 30 s. A 1.5 mL of *n*-hexane-ethylacetate (1:1, v/v) was added and the sample was again vortex-mixed for 5 min. The mixture was then centrifuged at 2000 g for 5 min. The organic layer was evaporated under a stream of nitrogen and then the dried residue was reconstituted in 100 μL of a mixture methanol/HCl 10 mM (90/10). Eventually, the solution was directly injected in CE. Whenever the sample concentration resulted above the upper limit of quantification, an appropriate dilution was performed with water containing IS and eventually extracted.

3. Method optimization and validation

3.1. Optimization of the separation conditions

In capillary electrophoresis, chiral separation is generally based on differences of the interactions of the enantiomers of the analyte with a chiral selector dissolved in the separation electrolyte. Several different chiral selectors have been used, including CDs, macrocyclic antibiotics, carbohydrates and chiral crown ethers [22]. Among these agents, cyclodextrins are by far the most frequently used for their good solubility in aqueous buffer, low toxicity and low UV absorption.

In the present study, due to the basic chemical characteristic of the analytes, a phosphate buffer at acidic pH (4.4) was initially tested. Among the available CDs, β-CD, dimethyl-β-CD and HP-β-CD were tested at different concentrations in the range of 1.25–50 mM. Already in the early experiments, native β-CD proved inefficient to produce enantioseparation of methorphan, whereas modified CDs were suitable for chiral analysis. However, using dimethyl-β-CD, migration times were longer than 22–25 min, whereas HP-β-CD produced faster and more efficient separations, and therefore, was chosen for further development.

In the process of optimization, the concentration of phosphate buffer electrolyte was tested between 10 and 300 mM, recording both peak resolution and analysis time. High buffer concentrations proved to improve the resolution, but above 150 mM, because of excessive currents, efficiency, and therefore, resolution dropped.