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Determination of levamisole and tetramisole in seized cocaine samples by enantioselective high-performance liquid chromatography and circular dichroism detection

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This article is dedicated to the memory of Prof. Gian Piero Spada (1956–2013).

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ABSTRACT

Levamisole, an anthelmintic drug, has been increasingly employed as an adulterant of illicit street cocaine over the last decade; recently, the use of tetramisole, the racemic mixture of levamisole and its enantiomer dexamisole, was also occasionally observed. A new enantioselective high-performance liquid chromatography (HPLC) method, performed on cellulose *tris*(3,5-dimethylphenylcarbamate) chiral stationary phases in normal-phase mode, was validated to determine the enantiomeric composition of tetramisole enantiomers in seized cocaine samples. Furthermore, the hyphenation of the validated HPLC method with a circular dichroism (CD) detection system allowed the direct determination of elution order and a selective monitoring of levamisole and dexamisole in the presence of possible interferences. The method was applied to the identification and quantitation of the two enantiomers of tetramisole in seized street cocaine samples.

1. Introduction

Levamisole (Fig. 1) is a veterinary anthelmintic drug which was employed as a therapeutic agent for humans until the end of the 20th century, when it was withdrawn due to adverse side effects such as agranulocytosis, cutaneous vasculopathy and leukoencephalopathy [1]. Levamisole gained forensic interest after the increase of its use as an adulterant in illicit cocaine samples; as a result, levamisole is now found in the majority of cocaine seized worldwide, linked to debilitating and eventually fatal immunologic effects in cocaine abusers [2]. The use of levamisole as adulterant is likely due to its physicochemical properties, which are quite similar to those of cocaine, although being a much cheaper and more easily accessible substance [3]. Moreover, the presence of levamisole should hypothetically enhance the effects of cocaine, since a possible synergism for their interaction has been proposed [4]. Therefore, the health risks associated to the use of levamisole as adulterant are worth highlighting, due to possible high contents in some illicit street cocaine samples which can eventually exceed the content in pure cocaine [3]. The presence of levamisole determines an additional health threat due to aminorex, one of its main metabolites [5,6]. Analytical assays for the determination of levamisole have been reported by gas chromatography–mass spectrometry (GC–MS) in human urine samples [5] and by high-performance liquid chromatography–mass spectrometry (HPLC–MS) in cocaine samples [6].

Quite recently, the analysis on seized illicit cocaine samples showed that some manufacturing laboratories have begun using tetramisole, a racemic mixture of levamisole and its enantiomer dexamisole (Fig. 1), as a substitute for levamisole as adulterant. Therefore, the identification and determination of both enantiomers is essential for a better evaluation of the health risks arising from the presence of these adulterants, in view of the different biological activity of the two enantiomers [7] and the stronger adverse effects of dexamisole in humans. Furthermore, the enantiomeric composition of adulterants in seized samples of cocaine may help determining the origin of the illicit drug [7]. Enantioselective

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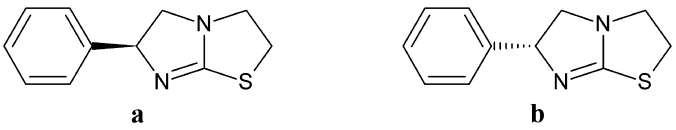

Fig. 1. Chemical structures of tetramisole enantiomers; a: levamisole; b: dexamisole.

analyses on tetramisole have already been developed and performed by capillary GC with flame ionization detection (FID) using fused silica columns coated with β-cyclodextrin [8], by HPLC [9] and capillary electrophoresis (CE) [10] using β-cyclodextrins as chiral selectors, and by HPLC on polysaccharide-based chiral stationary phases (CSPs) [10]; the advantages of enantioselective separation methods over polarimetric methods for the determination of tetramisole enantiomers were also experimentally demonstrated [10].

In the following study, an enantioselective HPLC method has been validated and applied for the detection and determination of levamisole and tetramisole in cocaine samples in the presence of some of the more frequently used adulterants; a cellulose *tris*(3,5-dimethylphenylcarbamate) CSP was used in the normal-phase mode. To enhance the selectivity of the HPLC assay, an online HPLC–circular dichroism (CD) method was developed and used for the identification and determination of the elution order levamisole and dexamisole in seized cocaine samples, in the presence of several adulterants. Indeed, the well established hyphenation of enantioselective HPLC and CD detection [11–14] allows to determine the stereochemistry of the eluted fractions with high detection selectivity; this feature is particularly important when dealing with complex matrices [15], as in the case of street samples of cocaine.

2. Materials and methods

2.1. Materials

Compounds *rac*-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]-[1,3]thiazole hydrochloride (tetramisole), (−)-(*S*)-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*][1,3]thiazole hydrochloride (levamisole), *N*-(4-ethoxyphenyl)acetamide (phenacetin), 2-(diethylamino)-*N*-(2,6-dimethylphenyl)acetamide (lidocaine), 2-(diethylamino)ethyl 4-aminobenzoate hydrochloride (procaine), ethyl 4-aminobenzoate (benzocaine) and 3,7-dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione (caffeine) were purchased from Sigma–Aldrich (Milan, Italy). Analytical-grade solvents (*n*-hexane, 2-propanol, ethanol 99.7% and diethylamine) were purchased from Sigma–Aldrich (Milan, Italy). Stock solutions of analytes were prepared in ethanol at a 1.00 mg mL^{-1} concentration. The standard solutions used for method validation were prepared by dilution of stock solutions with mobile phase.

2.2. Enantioselective HPLC analysis

2.2.1. Instrumentation

The enantioselective HPLC analysis was carried out on a chromatographic system consisting of a Jasco (Tōkyō, Japan) PU-980 HPLC pump, a MD-910 diode array detector, a LG-2080-02 ternary gradient unit, a DG-2080-53 degasser, a Jones (Lakewood, CO, USA) model 7955 HPLC column chiller, a Rheodyne 7725i syringe loading injector and a 20 μL sample loop; a Chiralcel® OD-H column (CSP: cellulose *tris*(3,5-dimethylphenylcarbamate) coated on silica gel; 250 × 4.6 mm I.D., 5 μm particle size; Daicel, Illkirch, France) and a Lux™ Cellulose-1 column (CSP: cellulose *tris*(3,5-dimethylphenylcarbamate) coated on silica gel; 250 × 4.6 mm I.D., 5 μm particle size; Phenomenex, Castel Maggiore, Italy) were used. CD detection was implemented by connecting the system to a Jasco J-810 spectropolarimeter equipped with a 10 mm pathlength HPLC flow cell and a Rheodyne 7010 injector set up as a three-way valve for stopped-flow measurements. Single-wavelength chromatograms were recorded at 230 nm in both diode array and CD detection modes; CD detection was carried out using a 2 s data pitch, a 5 nm spectral bandwidth and a 2 s time constant.

2.2.2. Chromatographic conditions

The enantioselective HPLC analysis was performed using a mobile phase consisting of a *n*-hexane–2-propanol mixture (80:20, v/v) in isocratic conditions; 0.1% (v/v) diethylamine was added to the mobile phase to neutralize the cationic forms of analytes and improve the chromatographic resolution. Constant flow rates (0.7 mL min^{-1} on Chiralcel OD-H column, 1.4 mL min^{-1} on Lux Cellulose-1) and column temperature (15 °C) were used.

2.2.3. Method validation

The enantioselective HPLC method was validated using the Lux Cellulose-1 column and diode array detection.

Selectivity was assessed by analysis of a 150 μg mL^{-1} standard solution of tetramisole hydrochloride; the relevant chromatographic parameters (capacity factor k, number of plates N, asymmetry factor A_S, selectivity α, and resolution R_S) were determined according to the half-height method using the Jasco ChromNav software. Interference from other analytes was investigated by analysis of a mix of frequent adulterants found in street cocaine samples (lidocaine, phenacetin, procaine, benzocaine and caffeine) with tetramisole; different concentrations were used depending on the spectroscopic response of analytes at 230 nm. Capacity factors for cocaine and its metabolite norcocaine were derived from the chromatograms of analyzed street cocaine samples; their identity was confirmed by GC–MS analysis (Figs. S1–S3 in the Supporting Information) [16].

Linearity (n = 3) was evaluated by analyzing six standard samples of levamisole hydrochloride (6.25, 9.375, 18.75, 37.5, 75 and 150 μg mL^{-1}); triplicate injections were performed for each solution. A calibration curve was obtained by plotting the peak areas (in mAU min) against the correspondent concentrations of neutral analyte (μg mL^{-1}) and applying a linear regression model ($y = a + bx$), for which the correlation coefficient (r^2) and the quality coefficient (QC_{mean}) were determined [17,18].

Sensitivity was assessed by calculating the limit of detection (LOD) and limit of quantitation (LOQ) for levamisole from the calibration curve using the standard deviation of the y-intercept (s_a) and the slope (b) obtained by linear regression (LOQ = 10s_a/b; LOD = LOQ/3).

Intra-assay and inter-assay precision (n = 6) was determined by repeated injections of a 75 μg mL^{-1} standard solution of levamisole hydrochloride. The average capacity factors and peak areas, with the corresponding relative standard deviations (%RSD), were determined for both assays.

Accuracy (n = 3) was evaluated by spiking a 0.5 mg mL^{-1} solution of seized street cocaine powder (see next subsection for sample preparation) with three different concentrations (60, 75 and 90 μg mL^{-1}) of levamisole hydrochloride; duplicate injections were performed for each sample. The average recoveries and relative standard deviation (%RSD) were determined for each concentration.

2.2.4. Street drug samples analysis

The validated HPLC method with diode array detection was applied to the analysis on samples of street cocaine powders (X_1–X_4) seized by Italian law enforcement agencies. Aliquots of 100 mg of finely ground samples were dissolved in ethanol by sonication to obtain a concentration of 10 mg mL^{-1}, diluted to