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Simultaneous determination of morphine, codeine and 6-acetyl morphine in human urine and blood samples using direct aqueous derivatisation: Validation and application to real cases

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ABSTRACT

Opiates play a relevant role in forensic toxicology and their assay in urine or blood is usually performed for example in workplace drug-testing or toxicological investigation of drug impaired driving. The present work describes two new methods for detecting morphine, codeine and 6-monoacetyl morphine in human urine or blood using a single step derivatisation in aqueous phase. Propyl chloroformate is used as the dramatizing agent followed by liquid–liquid extraction and gas-chromatography–mass spectroscopy to detect the derivatives. The methods have been validated both for hydrolysed and unhydrolysed urine.

For hydrolysed urine, the LOD and LOQ were 2.5 ng/ml and 8.5 ng/ml for codeine, and 5.2 ng/ml and 15.1 ng/ml for morphine, respectively. For unhydrolysed urine, the LOD and LOQ were 3.0 ng/ml and 10.1 ng/ml for codeine, 2.7 ng/ml and 8.1 ng/ml for morphine, 0.8 ng/ml and 1.5 ng/ml for 6-monoacetyl morphine, respectively. In blood, the LOD and LOQ were 0.44 ng/ml and 1.46 ng/ml for codeine, 0.29 ng/ml and 0.98 ng/ml for morphine, 0.15 ng/ml and 0.51 ng/ml for 6-monoacetyl morphine, respectively.

The validated methods have been applied to 50 urine samples and 40 blood samples (both positive and negative) and they can be used in routine analyses.

1. Introduction

Blood and urine are very useful biological matrices in clinical and forensic toxicology due to their importance for assessing short-term and recent exposure to drugs, respectively. Blood concentration can be directly related to the observed symptoms or behaviour [1,2] and its analysis is essential to verify, for example, if a subject is driving under the influence of drugs. Urine analyses are performed worldwide to evaluate recent intake of drugs and they can be easily collected by a non-invasive procedure. Furthermore, drug concentrations are much higher than those detected in other matrices and non-chromatographic methods can be used easily.

Even though the prevalence of opiates in workplaces or in cases of driving impairment is low if compared to that of other drugs of abuse [3] (such as cannabinoids and cocaine), morphine, codeine and 6-monoacetyl morphine (6-MAM, the specific indicator for heroin intake) remain the selected drugs in testing panels because of their importance.

According to the 2009 annual report of the European Monitoring Centre for Drugs and Drug Addiction [4], heroin intake in Europe remains a serious public health issue and still accounts for a large proportion of the overall health and social costs associated with drug use [5]. Moreover, morphine is used as an analgesic in the treatment of severe pain and codeine is widely used for the treatment of cough and moderate pain too, so their presence in biological fluids must be often assessed and may not be correlated to heroin abuse, particularly in the case of codeine.

Usually, the first approach for the detection of these substances is a preliminary immunoassay test (mainly for urine) but, due to its low specificity, positive results must necessarily be confirmed by either gas or liquid chromatography, both coupled with a spectrometric detector (GC–MS or LC–MS).

Using GC–MS as the detection method, the sample must be submitted to preliminary treatment before analysis. This procedure usually involves different steps, such as hydrolysis of glucuronides for urine or deproteinisation for blood, organs, saliva, extraction of the analytes from the biological matrix and, as the final step, their derivatisation.

Morphine is excreted in the urine mainly as 3- and 6- glucuronides, so urine analysis for total morphine detection usually begins with hydrolysis of the conjugates to liberate free morphine [6] before extraction and chromatography. Hydrolysis can be performed either enzymatically [7–9] or by addition of strong acids [10,11], which results a higher yield [7].

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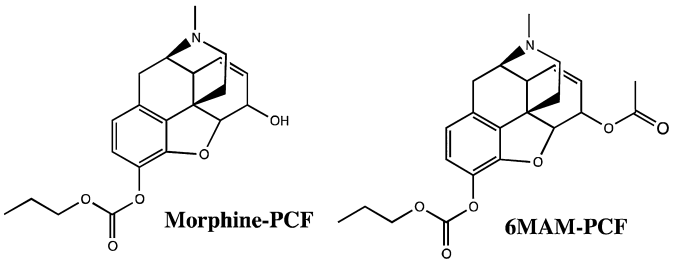

Fig. 1. Chemical structures of morphine and 6-MAM PCF derivatives.

Due to the amphoteric nature of morphine and 6-MAM, the co-extraction of codeine can be problematic [12] and the poor chromatographic characteristics of underivatised morphine necessitate the production of stable derivatives. According to Chen et al. [13], the acetyl derivative showed the greatest stability among those examined.

In this paper, we report for the first time the use of propyl chloroformate (PCF) for direct derivatisation of morphine and 6-MAM (Fig. 1) in human urine samples after acidic hydrolysis and in human whole blood after deproteinisation. Codeine does not react with propyl chloroformate but it can be extracted, together with morphine and 6-MAM derivatives, as an underivatised substance.

Unhydrolysed urine samples were analysed by simultaneous DLLME (dispersive liquid–liquid micro extraction) and derivatisation have been carried out. In this method, the appropriate mixture of extraction solvent and disperser solvent are injected into the aqueous sample by syringe. A cloudy solution consisting of fine particles of extraction solvent, which is entirely dispersed into aqueous phase, is formed. The extraction solvent is then recovered from the bottom of the conical test tube after centrifugation. Finally, the organic phase is collected and injected directly for the analysis [14].

The methods developed, both for the urine that for the blood, have been validated for daily use and they were applied to 50 forensic samples of human urine and 40 samples of human blood.

2. Materials and methods

2.1. Chemicals and reagents

Morphine, codeine, 6-MAM and their deuterated analogues were obtained from LGC Standards S.r.l. (Milan, Italy) at concentrations of 1 mg/ml or 100 μg/ml. Acetonitrile (AcCN), PCF, chlorobutane and Triethylamine (TEA) were purchased from Sigma-Aldrich (Milan, Italy). All solvents and other reagents were of analytical or HPLC grade and were purchased from Carlo Erba Analyticals (Milan, Italy).

2.2. Calibrators and controls

Using the standard solutions, two working solutions (1 and 10 μg/ml) for each analyte were prepared in methanol. The working solutions were then used to prepare five calibrators as follow: 20–50–100–200–500 ng/ml for codeine and morphine in both hydrolysed and unhydrolysed urine; 2–5–10–20–50 ng/ml for 6-AM only in unhydrolysed urine; 2–5–10–20–50 ng/ml for all analytes in whole blood. Each calibrator contained the analytes spiked into drug-free human blood or urine. The working solutions were also used to prepare three levels of control samples: 50, 100 and 150 ng/ml for codeine and morphine in hydrolysed and unhydrolysed urine; 5, 10 and 15 ng/ml for 6-MAM in unhydrolysed urine; 5, 10 and 15 ng/ml for all analytes in whole blood. For each analyte the relative deuterate was used as internal standard and was directly added to each sample at the following concentration: 100 ng/ml of codeine and morphine in hydrolysed urine, 100 ng/ml of codeine and morphine, 10 ng/ml of 6-MAM in unhydrolysed urine; 10 ng/ml for codeine, morphine and 6-MAM in whole blood.

2.3. Sample preparation

Hydrolysed urine – 5 μl of a methanolic solution (10 μg/ml) of morphine-D_3 and codeine-D_3 (internal standards, IS) were first added to 500 μl of urine followed by 100 μl of concentrated HCl; the solution was then incubated for 30 min in an autoclave. After cooling, the sample was centrifuged at 5470 × *g* for 5 min and the supernatant was transferred into a clean glass tube. Subsequently, 1 ml of water, 100 μl of acetonitrile, 100 μl of 12 N NaOH and 1 ml of saturated aqueous solution of sodium carbonate, were added to the hydrolysed urine under vortex agitation. Finally, the sample was derivatised by the addition of 30 μl of TEA and 30 μl of PCF. The extraction of analytes was performed by liquid–liquid extraction with diethyl ether. The organic phase was then evaporated under a gentle stream of nitrogen, and the residue reconstituted with 50 μl of ethyl acetate and submitted to GC–MS analysis.

Unhydrolysed urine – 5 μl of methanolic solution (10 μg/ml) of morphine-D_3 and codeine-D_3 and 10 μl of methanolic solution (1 μg/ml) of 6-MAM-D_3 were added, as internal standards, to 500 μl of urine together with 1 ml of distilled water. The solution was basified by the addition of 1 ml of pH 10 carbonate buffer and subsequently 500 μl of AcCN and 30 μl of TEA were added. Derivatisation and extraction were performed in a single step, using the 'dispersive liquid–liquid microextraction' (DLLME) technique. A solution consisting of methanol (disperser solvent: 240 μl), chloroform (extraction solvent: 80 μl), and PCF (derivatisation reagent: 30 μl) was added rapidly to the sample and the mixture was vortexed. A cloudy solution formed in the vial. The mixture was then centrifuged for 5 min at 7150 × *g* and after centrifugation an aliquot (50 μl) of the bottom phase was taken with a 50 μl micro syringe and transferred to a clean vial and submitted to GC–MS analysis.

Whole blood – 10 μl of methanolic solution of internal standards (1 μg/ml; morphine-D_3, codeine-D_3 and 6-MAM-D_3) were added to 1 ml of whole blood. Deproteinisation was obtained by the addition of 1.5 ml of AcCN followed by centrifugation for 5 min to 5470 × *g*. The supernatant, after being collected and transferred to a clean tube, was diluted with 3 ml of water. Subsequently, 1 ml of saturated aqueous solution of sodium carbonate was added to the sample. Finally, morphine and 6-MAM were derivatised by the addition of 30 μl of TEA and 30 μl of PCF. The extraction of analytes was performed by liquid–liquid extraction with chlorobutane. The organic phase was then evaporated under a gentle stream of nitrogen, and the residue reconstituted with 50 μl of ethyl acetate and analysed by GC–MS.

2.4. GC/MS instrumentation and conditions

All samples were analysed using a Shimadzu GC/MS QP2010 Ultra coupled to an AOC-20i autosampler (Shimadzu, Milan, Italy). Analyte separation was achieved using a 15 m Rtx® −5MS column (Crossbond® 5% diphenyl/95% polysiloxane, 0.25 mm internal diameter and 0.25 μm film thickness) from Restek (Pennsylvania, USA).

The injection port was set at 270 °C (injection volume 1 μl in splitless mode); the initial oven temperature was 100 °C held for 90 s and then increased by 17 °C/min as far as 320 °C, which was then held for 1 min. The transfer line was set at 250 °C and helium at 1.5 ml/min was used as the carrier gas.