Screening by immunoassay and confirmation & quantitation by GC-MS of buprenorphine and norbuprenorphine in urine, whole blood and serum

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INTRODUCTION

The purpose of these studies was to modify immunoassay methods for screening of buprenorphine (BU) and to develop GC-MS method for determination of buprenorphine and norbuprenorphine (NBU) in serum, whole blood and urine samples. The special focus during the development work of GC-MS method was concentrated on the derivatization of drugs and the urine hydrolysis.

Buprenorphine is a synthetic opioid having both analgesic and opioid antagonist properties. It is used in the treatment of severe pain and it is 25 - 40 times more potent than morphine. The analgesic effect with buprenorphine is gained at low doses (0.2 - 0.6 mg every 6 - 8 h). Buprenorphine is also used in the treatment of opiate abuse because of its prolonged duration of action. In the withdrawal treatment doses are several milligrams. Because the pain relieving effect is achieved at low doses there is a need for a sensitive assay to find out therapeutic doses in whole blood or serum. Buprenorphine is metabolized to the norbuprenorphine by N-dealkylation and conjugation. Norbuprenorphine is the active metabolite of buprenorphine. In the urine buprenorphine and its metabolite is found combined to the glucuronides.

METHODS

CEDIA-Screening

The urine samples were screened by the cloned enzyme donor immunoassay (CEDIA). The Buprenorphine CEDIA kit was obtained from Microgenics and the Dade Behring VIVA automated immunoassay screening device was used to modify the method. The kinetic measurement mode was selected and the measurement wavelength was set at 570 nm. The delay and minimum times were set at 156 and 80 s. The different ratios of sample and reagents volume were tested to attain the optimum cut off level and sensitivity. The cut off level 1.5 ng/ml was achieved with 30 µl sample, 150 µl reagent A and 150 µl reagent B volumes. The calibrator was prepared in drug-free urine. One GC-MS confirmed positive clinical sample and one negative clinical sample were used as control samples.
ELISA Screening

The whole blood samples were screened by the enzyme-linked immunosorbent assay (ELISA, Cozart Microplate Elisa). At first the used method was modified from the screening method for urine. In the course of time were noticed that method needs further development. When the manufacturer of the screening kit released a new kit designed for whole blood samples then the new kit was decided to evaluate. Samples, controls and calibrators were all whole blood and Microplate EIA Kit were made for whole blood samples. Calibrators were prepared in drug-free whole blood. A positive clinical sample was used as a control sample. The immunoassay was employed according to the instructions of the manufacturer.

GC-MS confirmation

Hydrolysis. The urine sample has to be hydrolyzed to release buprenorphine and norbuprenorphine from their glucuronide conjugates. The optimal incubation conditions of the enzymatic hydrolysis of 1 ml urine samples were found to be: 50 μl β-glucuronidase of *Helix Pomatia* and 200 μl Na-acetate as a buffer were added to the samples and incubated overnight (18h) at 37°C.

Extraction. The whole blood or serum samples (2 ml) and/or the hydrolyzed urine samples (1 ml) were extracted by liquid-liquid extraction with toluene containing deuterated buprenorphine (buprenorphine-d₄) as an internal standard (Table 1). Before the extraction the pH of samples were adjusted to the basic region (about 9) with Na₂HPO₄. After vortex mixing and centrifuging the solvent was transferred to a clean tube and evaporated in vacuum.

<table>
<thead>
<tr>
<th>Sample volume</th>
<th>Buffer</th>
<th>Solvent</th>
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<tbody>
<tr>
<td>Whole blood, serum</td>
<td>2 ml 100 mg Na₂HPO₄ powder</td>
<td>7 ml toluene *</td>
</tr>
<tr>
<td>Urine</td>
<td>1 ml 1,0 ml 0,5 M Na₂HPO₄ solution + 50 mg Na₂HPO₄ powder</td>
<td>7 ml toluene *</td>
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</table>

Table 1: The extraction of samples (* contains BU-d₄)*

Derivatization. The extraction residue was derivatized by silylation. 80 μl acetonitrile and 20 μl MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) were added to the extraction residue to create silyl derivatives of amine and hydroxyl groups.

GC-MS conditions. The samples were analysed with Agilent Network gas chromatograph-mass spectrometer, 6890/5973, instrument. The separation was carried out on a DB-35MS (30 m/0,32 i.d./0,25 μm film) fused silica capillary column by using helium as a carrier gas. The oven temperature program was: 180°C/1min→15°C/min→340°C/4 min. Selected ion monitoring (SIM) was used in the electron impact mode. The selected ions of analytes were as follows:
Buprenorphine 450, 482  
Norbuprenorphine 468, 500, 510, 542  
Buprenorphine-d4 454  

The chromatogram of a standard solution is shown in figure 1.

Figure 1: 1. Buprenorphine, 2. Norbuprenorphine.

RESULTS

CEDIA-Screening

The cut-off level was 1.5 ng/ml. True positive, true negative, false positive and false negative results were calculated after comparison between the CEDIA and GC-MS results. From screened samples there were 8 false positive out of 29 positive samples. These false positive samples were also analysed for opiates with GC-MS method. It was observed that buprenorphine CEDIA screening assay had cross reactivity with other opiates such as morphine, codeine, pholcodine and tramadol at the high levels. All screened negative samples were true negative.

ELISA Screening

The ELISA screening method was validated in the whole blood. The limit of detection was 1.0 ng/ml. The dose-response curve were determined for buprenorphine concentrations ranging from 0 to 20 ng/ml. When the concentration of buprenorphine increased the optical density decreased. The relative standard deviation (RSD) of the intra-day assay was 9.6 % and 7.25 % when 10 whole blood samples were spiked with buprenorphine at the concentration 5 ng/ml and 1,0 ng/ml. The drug-free whole blood samples spiked with other opioids (morphine, codeine, MAM, methadone, tramadol, dihydrocodeine) or norbuprenorphine were not given positive result even if spiked concentrations were high (up to 10mg/ml). The clinical samples and the samples from the drunken driver (total 91 samples) were screened by this method. The results were calculated after
comparison between the ELISA and the GC-MS results. From screened samples there were one false positive out of 8 positive samples. All screened negative samples were true negative.

**GC-MS confirmation**

The evaluation of reliability of the developed GC-MS method included validation and comparing matrices. The limits of quantitation (LOQ) were 0.5 ng/ml for both buprenorphine and norbuprenorphine. The relative standard deviation of accuracy and precision of the method at the concentration level 0.5 ng/ml were: 9.4 % (BU) / 4.6 % (NBU) for accuracy; 5.3 (BU) / 7.9 (NBU) intra-day assay; 13.0 (BU) / 27.7 (NBU) inter-day assay.

The linearity range for both analytes was 0.5 - 15 ng/ml. The GC-MS method was validated in the serum. In the comparing matrices were found that the urine or whole blood standard curve of norbuprenorphine are not reliable because norbuprenorphine does not remain in the spiked drug-free urine or whole blood.

**DISCUSSION & CONCLUSION**

The manufacturers ELISA method found to be suitable for screening of buprenorphine in the whole blood and it has been decided to the screen whole blood samples from drunken drivers or clinical whole blood samples with this method. It is also found to be suitable for screening of buprenorphine in the serum samples which were not possible earlier. However the screening of serum samples needs more examinations.

The development work of GC-MS method: The optimal time of urine hydrolysis were found. In the urgent cases four hours (4h) incubation time releases sufficiently drugs for detect them. The composition of silyl derivative of buprenorphine and norbuprenorphine needs only one derivatization reagent (MSTFA) and does not need heating after adding derivatization reagent. The method was found to be sensitive. The validation results were mainly good and acceptable. Norbuprenorphine does not remain spiked in the drug-free urine or the whole blood specimens, and therefore the standard curve of norbuprenorphine is not reliable. However, norbuprenorphine finding from the sample proves the drug intake. Buprenorphine remains spiked in the urine and in the whole blood and therefore the results of buprenorphine in those specimens are reliable. In drug-free serum both buprenorphine and norbuprenorphine remains spiked and the standard curves are reliable.

The GC-MS method and immunological screening by CEDIA has been accredited by the Finnish Accreditation Service. The accreditation of CEDIA screening method includes Dade Behring automated immunoassay screening device V-TWIN. All described methods have been used in routine analysis for serum, whole blood and urine in clinical samples and in samples from drunken driver for several years.

**References**

