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Determination of butorphanol in horse race urine by immunoassay and gas chromatography–mass spectrometry

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Abstract

An analytical procedure to screen butorphanol in horse race urine using ELISA kits and its confirmation by GC–MS is described. Urine samples (5 ml) were subjected to enzymatic hydrolysis and extracted by solid-phase extraction. The residues were then evaporated, derivatized and injected into the GC–MS system. The ELISA test (20 μl of sample) was able to detect butorphanol up to 104 h after the intramuscular administration of 8 mg of Torbugesic, and the GC–MS method detected the drug up to 24 h in FULL SCAN or 31 h in the SIM mode. Validation of the GC–MS method in the SIM mode using nalbuphine as internal standard included linearity studies (10–250 ng/ml), recovery (±100%), intra-assay (4.1–14.9%) and inter-assay (9.3–45.1%) precision, stability (10 days), limit of detection (10 ng/ml) and limit of quantitation (20 ng/ml). © 1997 Elsevier Science B.V.

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1. Introduction

Butorphanol [17-(cyclobutylmethyl)morphinan-3,14-diol] is a synthetic opioid, mixed agonist–antagonist, analgesic drug. It belongs to a group of compounds known as morphinans and was developed as a potent analgesic, which could be used as an alternative to morphine (Fig. 1) with less addictive characteristics.

Opioid drugs have been known since the dawn of history as the most effective medication available for the treatment of pain. In man, they produce sedation and respiratory depression. In horses, they also depress pain and respiration; but, at low doses, they stimulate the animals to move at a pace between a brisk trot and a gallop. This stimulant effect in horses is common to all the narcotic analgesics [1].

Among the many publications reporting methods for the determination of morphine and related compounds in biological samples, only a few deal with butorphanol [2–6].

Extraction of urine samples should yield a much higher positive rate than blood samples, as most opiates and opiate metabolites are concentrated and excreted preferentially in the urine [2,7]. Gas chromatography–mass spectrometry (GC–MS) is undoubtedly one of the most specific and sensitive methods of analysis, especially when coupled with a

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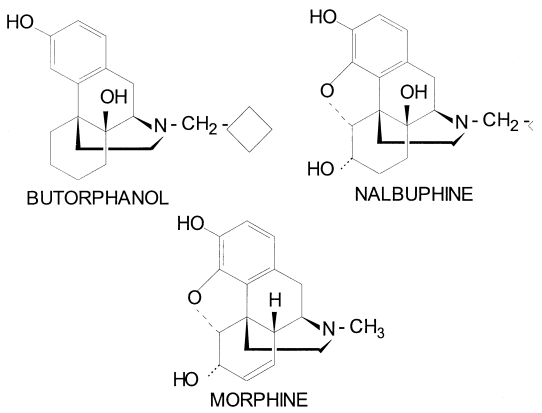

Fig. 1. Molecular structure of butorphanol, nalbuphine and morphine.

selective extraction technique such as solid-phase extraction (SPE). According to a widely accepted analytical strategy, GC–MS is a very useful and versatile tool for confirmation of many classes of drugs in urine after a preliminary screening using the enzyme linked immunosorbent assay (ELISA) technique.

This paper describes a sensitive method for isolation and chemical identification of butorphanol in horse urine using enzymatic hydrolysis, SPE and electron impact GC–MS analysis after a preliminary ELISA screening. A complete validation of the method including limit of detection (LOD) and quantitation (LOQ), recovery, precision, linearity and stability was carried out and is reported and discussed.

2. Experimental

2.1. Chemicals and reagents

Butorphanol tartrate and nalbuphine hydrochloride (used as internal standard) were purchased from Bristol-Myers Squibb (Princeton, NJ, USA) and Rhodia-Farma (São Paulo, SP, Brazil), respectively. Torbugesic from Fort Dodge Labs. (Fort Dodge, IA, USA) containing 10 mg/ml of butorphanol free-base was administered intramuscularly to a horse (0.8 ml). Stock standard solutions (1 mg/ml free-base) and working solutions (100 and 10 μg/ml) of drugs were prepared in methanol and stored at −16°C. Methanol (HPLC-grade), toluene, ethyl acetate, *n*-hexane, triethylamine and acetic acid were purchased from Merck (Darmstadt, Germany). Deionized water was obtained in-laboratory by a Milli-Q plus system (Millipore, Mulheim, France). 0.1 *M* phosphate buffer pH 6.0 (the phosphate buffer was prepared as follows: dissolve 1.70 g sodium phosphate dibasic anhydrous Na_2HPO_4 and 12.14 g of sodium phosphate monobasic, monohydrate NaH_2PO_4 in 800 ml of water; dilute to 1000 ml with water; mix and adjust pH to 6.0±0.1 with 100 m*M* Na_2HPO_4 or 100 m*M* NaH_2PO_4, as necessary) was used for the extraction procedure. N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and β-glucuronidase from *Patella vulgata* were obtained from Sigma (St. Louis, MO, USA). SPE columns XRDAF506 (6 ml), from United Chemical Technologies (Bristol, PA, USA), vacuum manifold from J.T. Baker (Phillipsburg, NJ, USA), Reacti-Therm III and Reacti-Vap III from Pierce (Rockford, IL, USA), were used for sample preparation. ELISA kits for butorphanol were from Neogen Corporation (Elisa Technologies Division, Lexington, KY, USA).

2.2. GC–MS equipment and conditions

The GC–MS analyses were performed on a Model 5890 series II capillary gas chromatograph directly interfaced to a Model 5971A mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). A Model 7673 automatic sampler (Hewlett-Packard) was used to introduce the sample onto a 25 m×0.2 mm I.D., 0.33 μm HP 1 column (Hewlett-Packard) in the splitless mode. The injector temperature was 270°C, interface oven temperature 300°C. The oven temperature program was as follows: initial temperature: 90°C for 0.5 min; program rate A: 45°C/min up to 180°C; rate B: 35°C/min up to 260°C; hold time: 1 min; rate C: 30°C/min up to 290°C; hold time: 11 min. Total run time: 17.79 min. The data were acquired after 10 min solvent delay in FULL SCAN (50–550 u) and selected ion monitoring (SIM) modes for butorphanol (*m*/*z* 344, 345, 346, 347, 326, 327, 299, 271, 399, 400) and nalbuphine (*m*/*z* 446, 447, 448, 501, 502, 206, 215, 358, 315, 428) at a total dwell time of 100 ms. Helium was used as the

carrier gas at a gas flow of 0.9 ml/min (linear gas velocity 35 cm/s).

2.3. Urine samples

Urine samples were collected from a horse administered 8 mg of Torbugesic by intramuscular injection after 2, 4, 6, 8, 10, 12, 14, 16, 24, 28, 32, 48, 52, 56, 76, 79, 96, 99, 104, 132, 152 and 155 h. Blank horse urine samples were collected before the administration. All samples were kept at 4°C until analysis.

2.4. ELISA screening

The samples, blanks and controls (20 μl), were pipetted into a multiwell microplate activated with an antibody specific to butorphanol. The test was used according to the manufacturer's instructions.

According to manufacturer's instructions, the I-50 (a term used to indicate sensitivity of the test; it means the drug concentration that shows 50% less color activity than the zero standard) of the kit was 0.33 ng/ml to equine urine, with a intra- and inter-assay precision of 6.52% and 4.81%, respectively. The studied linearity to equine urine was from 5 to 300 ng/ml. There is a cross-reactivity with nalbuphine (37%) and with naloxone, nalorphine and pentazocine (below 0.5%).

2.5. Sample processing

Five ml of urine samples were spiked with 500 ng of internal standard (nalbuphine) and acidified to pH 5.0 with acetic acid. One ml of β-glucuronidase (about 5.000 units of β-glucuronidase per ml of urine) was added and the samples mixed and heated at 65°C for 3 h in a water bath. After cooling to room temperature, the mixture was centrifuged at 900 *g* for 15 min and the supernatants were extracted by SPE. The XRDAF506 disposable columns were conditioned with 5 ml of methanol, 5 ml of deionized water and 3 ml of 0.1 *M* phosphate buffer (pH 6.0) sequentially without allowing the sorbent to dry (if it happens, start again using 5 ml of methanol). The supernatants of centrifuged samples were poured into each column reservoir and drawn slowly through the column. After washing the columns with 3 ml of 0.1 *M* phosphate buffer (pH 6.0) and 2 ml of 0.1 *M* acetic acid, the columns were allowed to dry for 5 min at full vacuum and again washed with 2 ml of *n*-hexane and 5 ml of methanol. After another drying period at full vacuum, the analyte was eluted with 5 ml of a mixture of ethyl acetate–triethylamine (9:1, v/v). The entire extraction was performed under suction at a flow-rate ≤3 ml/min, except in the elution step, which was at gravity flow (no suction). The eluates were collected in screw-cap tubes, evaporated to dryness at 55°C under a N_2 stream, and reconstituted with 100 μl of a mixture of MSTFA–toluene (3:7, v/v) prepared at the time of the use. The vials were capped and heated to 80°C for 15 min. After evaporation to dryness at 55°C under a N_2 stream and reconstitution with 50 μl of toluene, a 1-μl aliquot was injected onto the GC–MS system.

2.6. Identification and acceptance criteria

The chemical identification of butorphanol was performed using the ion ratio among the qualifier ions in FULL SCAN and SIM modes and the retention time criteria.

After injection of butorphanol standard (1–10 ng) onto the GC–MS system, the area ratio among the 12 more abundant and structurally definite ions for all injections was studied both in SIM and FULL SCAN modes: 344 (base peak), 345, 346, 347, 384, 399 (molecular ion), 400, 165, 185, 271, 326 and 327. The criteria used was the coefficient of variation (C.V.) for all ratios at the studied range concentration and three of them, with C.V.s below or equal to 20%, were chosen.

For butorphanol, the ratios obtained, on the same day and conditions in the range of 1–10 ng of standard and 10–250 ng/ml of spiked sample were: FULL SCAN: 28.49 for *m/z* 344/326 with a C.V. of 20.03%, 3.67 for *m/z* 344/345 with a C.V. of 2.00% and 0.14 for *m/z* 326/345 with a C.V. of 19.13%; for SIM conditions the area ratios were 23.88 for *m/z* 344/326 with a C.V. of 14.31%, 3.92 for *m/z* 344/345 with a C.V. of 2.81% and 0.17 for *m/z* 326/345 with a C.V. of 15.19%.

A 1% variation in the retention times was deemed acceptable.

To quantify butorphanol for the validation step,

we used the SIM mode and the ratio between the area of the base peak of butorphanol and the base peak of nalbuphine (344/446) was chosen.

2.7. Validation

2.7.1. Linearity

The calibration curve for butorphanol was performed in replicates ($n=5$) and determined by adding 50, 100, 200, 250, 350, 500 and 1250 ng butorphanol and 500 ng of nalbuphine (internal standard) to 5 ml of drug-free horse urine samples. These solutions were extracted, derivatized and analyzed according to the procedure described above.

2.7.2. LOD for GC–MS [8]

The LOD was determined to be the smallest quantity of the six concentrations of butorphanol studied, and it gave a C.V. below or equal to 20% for the base peak area (m/z 344) for which acceptance criteria (area ratio and retention time) were reached. 0.5, 1.0, 2.0, 4.0, 5.0 and 10 ng of butorphanol with 5 ng of nalbuphine ($n=5$) were injected.

2.7.3. LOD and LOQ of the method [8]

To determine the LOD and the LOQ of the method, the same injections performed to obtain the calibration curve were used. The LOD of the method was determined in the same way as the LOD of GC–MS.

The LOQ was defined as the concentration at which the acceptance criteria to butorphanol were met and the ratio between the area of the base peak of butorphanol and nalbuphine (344/446) showed a C.V. below 20%.

2.7.4. Intra- and inter-assay precision

The intra-assay precision was determined by analyzing five aliquots in replicate ($n=5$) of three different spiked samples (20, 50 and 200 ng/ml of butorphanol added with 100 ng/ml of nalbuphine as internal standard) and three samples collected at 14, 24 and 28 h after the administration of butorphanol. The concentrations of these collected samples were close to those of the spiked ones. The inter-assay precision was determined using the same spiked samples, prepared and analyzed on three different days.

2.7.5. Stability

This parameter was determined by the analysis of urine samples obtained from the same collection which were used in the intra-assay precision study ($n=5$). The samples were kept at 4°C and were analyzed (extracted and derivatized) five and ten days after the first determination. The variation in concentrations measured on different days was evaluated.

2.7.6. Recovery [9,10]

The recovery was evaluated by comparison between the mean concentration obtained after extraction and the theoretical added concentration.

For this determination, we used the following: (a) four urine samples, named internal calibration samples (ICSs) containing 20, 50, 100 and 200 ng/ml of butorphanol with 100 ng/ml of nalbuphine, in which butorphanol and nalbuphine were added before the enzymatic hydrolysis, extraction and derivatization and (b) four samples, called external calibration samples (ECSs), in which only the internal standard was added before the hydrolysis, extraction and derivatization, and butorphanol was added just before the derivatization step.

The % recovery was calculated for each concentration in replicates ($n=5$) using the area ratio between the base peak of butorphanol and nalbuphine (344/446), according to the following formula:

$$\%\ \text{Recovery} = \frac{\text{ICS } 344/446 \times 100}{\text{ECS } 344/446}$$

3. Results and discussion

The ELISA and GC–MS (in SIM mode) procedures allowed the detection of butorphanol up to 104 and 32 h post-administration to a horse, respectively. Despite the difference in detection ‘windows‘ between the two techniques, this does not present a problem, because stimulants are normally administered a few hours before the race [7,11].

There is a cross-reactivity of the ELISA kit to butorphanol with nalbuphine and pentazocine. None

of them interfere in the butorphanol analysis; besides the different retention times (pentazocine 5.62 min, butorphanol 9.39 min and nalbuphine 11.42 min), they have a completely different spectra. Further studies should be conducted to determine the period of the pharmacological effect of butorphanol in the horse.

It is well known that sensitivity and specificity are inversely correlated as in any other analytical technique. Capillary columns offer inertness, high ef-

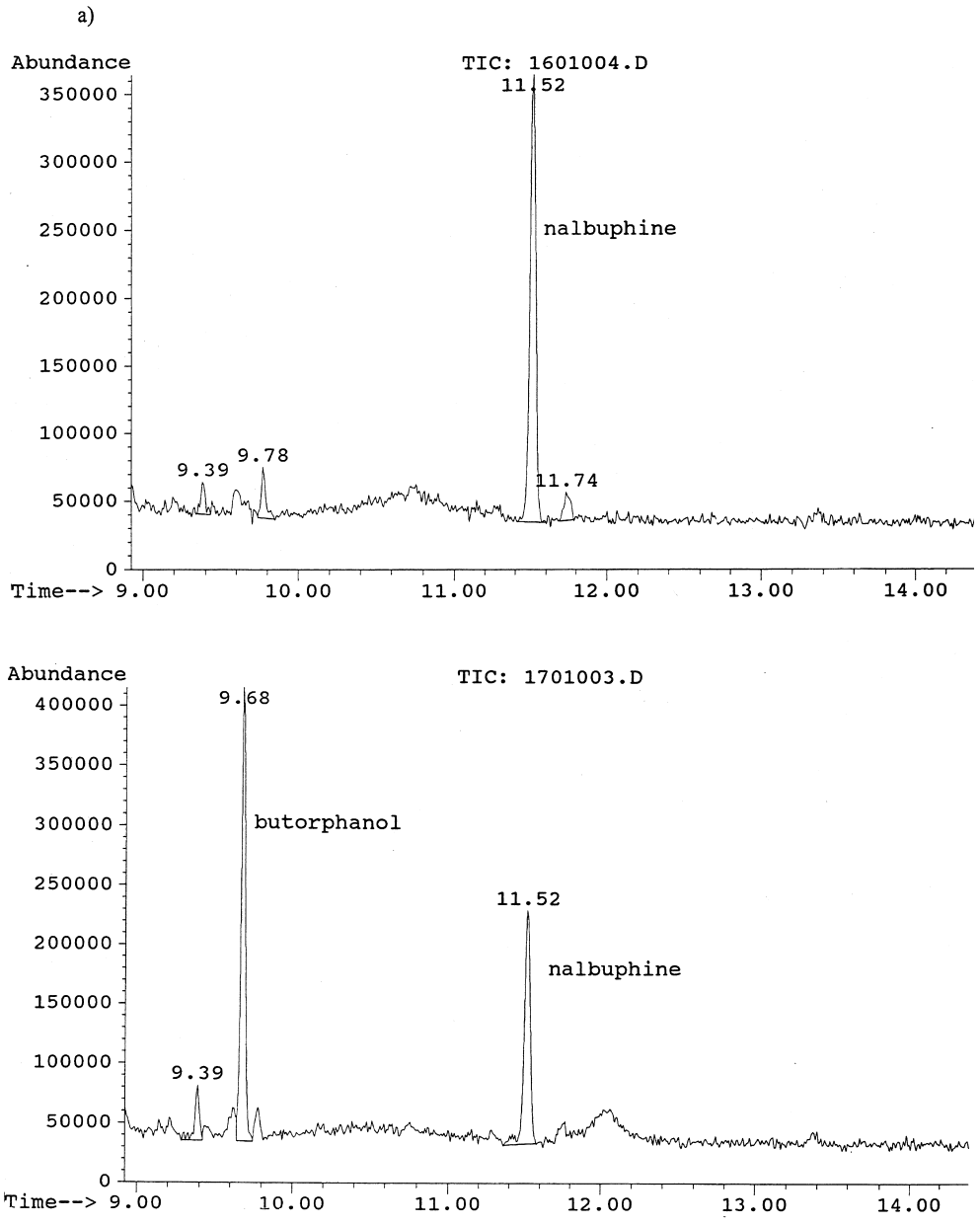

Fig. 2. (a) Total ion chromatogram of a blank and a positive sample of butorphanol with internal standard – nalbuphine (100 ng/ml, each); (b) spectrum of derivatized butorphanol; (c) spectrum of derivatized nalbuphine.

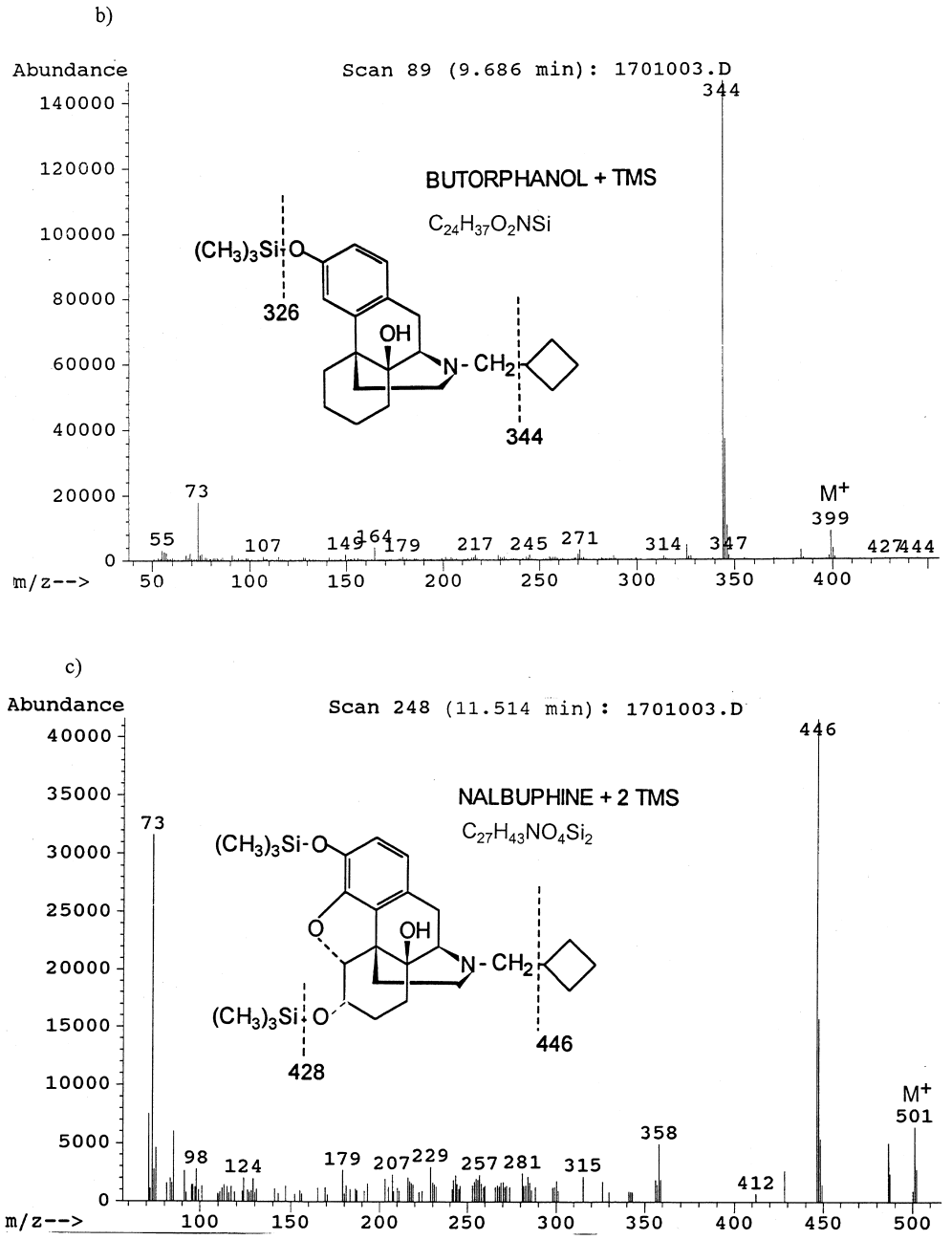

Fig. 2. (*continued*)

ficiency at low flow-rates, high speed of analysis and chemical and thermal stability. Even using these devices, when increasing sensitivity is needed, it is necessary to disregard the peaks which contribute to specificity and to collect data from a small amount of ions for a longer period of time, in the same manner as in done by using the SIM mode. According to Armbruster et al. [8], the number of ions that should be monitored in a run will depend on the specificity of the ions being monitored, but three is usually

regarded as a minimum. In addition, the intensity (area or abundance) of the ions must be reproducible and employed as part of the validation criteria. In the present work the chosen ions for the determination of the identity of butorphanol, analyzed with the FULL SCAN or SIM techniques were m/z 344, 345 and 326.

The ratios for that specific range were established; the C.V. value for two of the ratios was very near to the limit (20%). This is probably due to the large range of concentrations evaluated. For this reason, when confirming a positive sample, one should have a standard with a concentration similar to that of the sample. In other words, a positive sample should have ratios within 20% of the standard values.

Fig. 2 shows a total ion chromatogram of an authentic sample (100 ng/ml of butorphanol) and a blank and the spectra of derivatized butorphanol and nalbuphine.

Even though in doping control it is not necessary to quantify butorphanol, all the validation parameters were studied in SIM mode.

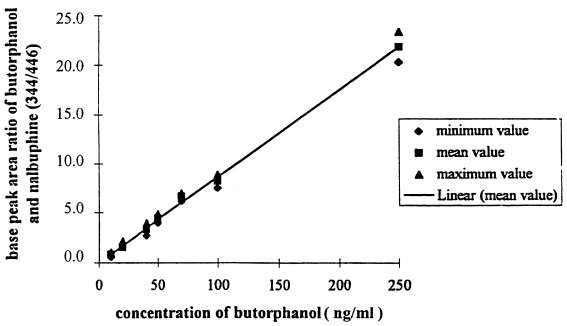

Fig. 3. Butorphanol calibration curve.

For quantitative analysis in GC–MS, just one ion of analyte and internal standard is normally chosen [12–21].

The main parameters essential to validate the performance of an analytical method are stability, precision, recovery and limits of quantitation and detection [22]. The validation results are listed in Table 1 and a calibration curve is shown in Fig. 3.

To be reliable, recovery must be checked for each

Table 1
Summary of the validation results for the butorphanol analysis by SPE and GC–MS detection

Parameter	Result
Linearity ($n=5$)	10–250 ng/ml ($y=0.0878x-0.0797$; $r^2=0.9979$)
LOD of GC–MS ($n=5$)	1 ng (SIM) or 5 ng (FULL SCAN)
LOD of the method ($n=5$)	10 ng/ml (SIM) or 40 ng/ml (FULL SCAN)
Intra-assay precision ($n=5$)	
Spiked samples	20 ng/ml, 6.3% 50 ng/ml, 4.5% 200 ng/ml, 14.9%
Real samples	28 h after administration, 5.0% 24 h after administration, 4.1% 14 h after administration, 5.4%
Inter-assay precision ($n=5$)	20 ng/ml, 9.3% 50 ng/ml, 45.1% 200 ng/ml, 22.8%
Relative recovery ($n=5$)	20 ng/ml, 100% 50 ng/ml, 107.3% 100 ng/ml, 106.3% 200 ng/ml, 100.2%
System stability ($n=5$)	10 days

matrix. The concentrations used should be at the same level as LOQ and as the maximum value of the calibration curve ($n=5$) [9,17].

The relative recovery value over 100% showed a different extraction rate between the analyte and the internal standard; on the other hand the recovery calculated without the internal standard, named absolute recovery, (absolute area of the m/z 344) was 86%.

The system stability has been defined in order to determine the stability of the analytes being tested in a sample solution. This is a measure of the bias in assay results generated during a preselected time interval, using a single solution. System stability should be determined by replicate analysis of the sample solution and is considered to be appropriate if the coefficient of variation calculated on the assay results obtained at different time intervals from the collections does not exceed more than 20% of the corresponding value of the system precision [23]. In dope testing analysis, the system stability must be of, at least, one week which is the accepted period between the sample collection and the release of results.

During one year of butorphanol analysis by ELISA screening at the Antidoping Laboratory, eight positive samples were found and three of them were confirmed by the GC–MS using FULL SCAN mode, with a comparison of the standard ratios.

It is well know that when a drug is found in horse race urine, this drug is either suspended or substituted for another one. This is common in an antidoping laboratory.

In conclusion, this method combines the power of the ELISA screening test and SPE with the selectivity of GC–MS in order to achieve a confirmation of butorphanol in urine samples. The results of our evaluation indicate that the procedure described above is able to detect and identify butorphanol in urine within acceptable limits of detection, linearity, recovery, stability and precision and acceptable period of time after administration.

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