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Determination of phenylbutazone and oxyphenbutazone in plasma and urine samples of horses by high-performance liquid chromatography and gas chromatography–mass spectrometry

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Abstract

A method is described for the qualitative and quantitative determination of phenylbutazone and oxyphenbutazone in horse urine and plasma samples viewing antidoping control. A horse was administered intravenously with 3 g of phenylbutazone. For the qualitative determination, a screening by HPLC was performed after acidic extraction of the urine samples and the confirmation process was realized by GC–MS. Using the proposed method it was possible to detect phenylbutazone and oxyphenbutazone in urine for up to 48 and 120 h, respectively. For the quantitation of these drugs the plasma was deproteinized with acetonitrile and 20 μl were injected directly into the HPLC system equipped with a UV detector and LiChrospher RP-18 column. The mobile phase used was 0.01 *M* acetic acid in methanol (45:55, v/v). The limit of detection was 0.5 μg/ml for phenylbutazone and oxyphenbutazone and the limit of quantitation was 1.0 μg/ml for both drugs. Using the proposed method it was possible to quantify phenylbutazone up to 30 h and oxyphenbutazone up to 39 h after administration.

Keywords: Phenylbutazone; Oxyphenbutazone

1. Introduction

Phenylbutazone (PB) is a non-steroidal anti-inflammatory agent which has been used extensively in horses for the treatment of bone and joint inflammation, laminitis and soft tissue inflammation. Nevertheless, if the treatment is not followed by a suitable period of rest the animal will invariably become lame and that is the main cause for the premature withdrawal of horses from athletic life. For that reason and also to keep events free of drugs, phenylbutazone is considered a prohibited substance to horses in training by a large number of racing jurisdictions. In Brazil, phenylbutazone is prohibited in most thoroughbred racing tracks, but is allowed in showhorse events, at plasma levels of 2 to 8 μg/ml, depending on the jurisdiction.

Some methods have been described for the detection of PB and oxyphenbutazone (OPB) in horse urine using high-performance liquid chromatography (HPLC) [1–3] or gas chromatography–mass spectrometry (GC–MS) [4–6]. For the quantitation of these drugs in plasma many methods [1,7–18] have been described, all of them involving an extraction

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step followed by HPLC–UV detection and quantitation. These methods are time consuming, less reproducible and offer more opportunity for the likely oxidation of the drugs during analysis. MacKenzie [19] proposed a direct injection HPLC method to quantify PB in plasma and Hyde et al. [20] used a previous dialysis followed by direct injection into the system. This is an elegant procedure, totally automated but requires special devices for the dialysis.

This paper describes a procedure for the detection of PB and OPB in urine samples by HPLC and GC–MS and a simple and rapid procedure for the plasma quantitation of these drugs based on direct injection of deproteinized plasma into a HPLC system. This procedure is not deleterious to the analytical column and gives the enough sensitivity to be used in the antidoping control of racehorses.

2. Experimental

2.1. Reagents and materials

Analytical standards of phenylbutazone and diazepam (used as reference for retention times) were obtained from the pharmaceutical laboratory Farmasa S/A (São Paulo, SP, Brazil), oxyphenbutazone from Sintofarma S/A (São Paulo, SP, Brazil) and naproxen (used as internal standard for the quantitation procedure) from Sarsa S/A (São Paulo, SP, Brazil). Synthesis grade ethanol (Ferreira, São Paulo, SP, Brazil) was distilled before use. Methanol, acetonitrile and dichloromethane, all of chromatographic grade, glacial acetic acid (reagent grade) and all the other reagent grade solvents were purchased from Merck (Rio de Janeiro, RJ, Brazil). Liquemine (5000 UI of heparin/ml) was purchased from Roche S/A (São Paulo, SP, Brazil). The water used as mobile phase was freshly distilled, deionized and purified in Milli-Q equipment (Millipore, Bedford, MA, USA). Acetate buffer pH 3.5 (2 *M* sodium acetate–2 *M* acetic acid), saturated solution of sodium bicarbonate and qualitative filter paper (No. 10, 9 cm, Inlab, São Paulo, SP, Brazil) were used for the extraction procedure. For the purification by thin-layer chromatography (TLC), silica gel GF 254 0.25 mm, 20 × 20 cm plates were developed in chloroform–methanol–amonium hydroxide (90:10:0.25, v/v/v). Fluoropore membrane (Millipore, Bedford, MA, USA), 13 mm, 0.5 μm was used to avoid silica residues in the extract after TLC purification. Methelut (Pierce, IL, USA) was used for the derivatization procedure.

2.2. Standard solutions

Methanolic solutions of PB and OPB at 0.1% (m/v) and 0.01% (m/v) were prepared. Standard solutions 0.1% (m/v) of diazepam and naproxen were also prepared in methanol. All solutions were stored in the dark at 4°C.

2.3. Administration procedure

A 5 year-old thoroughbred mare with a mass of 410 kg received an intravenous dose of 3 g of phenylbutazone sodium (Butazolidina, Geigy, Brazil). Naturally voided urine samples were collected from 2–175 h after administration and stored at −20°C up to the time of analysis. Blood samples obtained from the jugular vein were collected up to 56 h after administration, placed into tubes containing 1 ml of Liquemine, centrifuged at 900 *g* for 5 min and the resulting plasma samples were stored at −20°C.

2.4. Sample processing

2.4.1. Urine samples

Urine samples were spiked with 0.5 μg/ml of diazepam. Aliquots of 20 ml were acidified with 5 ml of acetate buffer and extracted with 20 ml of dichloromethane–ethanol (95:5, v/v). After agitation (3 min) and centrifugation (900 *g*, 10 min) the organic layer was washed with 2 ml of a saturated solution of sodium bicarbonate, filtered through qualitative paper and evaporated at 40°C to dryness under nitrogen flow. For the HPLC analysis the residues were dissolved in 100 μl of methanol and 10 μl injected into the equipment. For GC–MS analysis the residues were previously purified by TLC using chloroform–methanol–amonium hydroxide (90:10:0.25, v/v/v) for 10 cm as the developing system. The silica corresponding to the UV quench was removed from the plate, the drugs eluted with circa 800 μl of methanol through a Fluoropore

membrane and the solvent evaporated at 40°C under nitrogen flow.

2.4.2. Plasma samples

Plasma samples were added to 2 μg/ml of internal standard at the time of analysis and aliquots of 400 μl were deproteinized with 400 μl of acetonitrile, vortexed for 1 min and centrifuged for 3 min. Aliquots of 20 μl were injected directly into the HPLC–UV system.

2.5. High-performance liquid chromatography

A liquid chromatograph CG-480C equipped with a UV detector CG-435 both from Instrumentos Científicos CG (São Paulo, SP, Brazil), an integrator HP-3396A from Hewlett-Packard (Palo Alto, CA, USA) and an AS-2000A autosampler from Merck and Hitachi (Tokyo, Japan) were used. The detector wavelength was fixed at 254 nm. The analytical column was a LiChrospher 100 RP-18 125 × 4 mm I.D., 5 μm and the guard-column was a LiChrospher 100 RP-18 4 × 4 mmI.D., 5 μm, both from Merck (Darmstadt, Germany). The column temperature was set at 35°C throughout the experiment. The isocratic mobile phase composition was 0.01 *M* acetic acid in methanol (45:55, v/v), at a flow-rate of 1 ml/min.

2.6. Gas chromatography–mass spectrometry

A gas chromatograph Model HP-5890 series II equipped with mass selective detector Model HP-5971A (Hewlett-Packard, Palo Alto, CA, USA) was used in the scan mode (acquisition range 50–360 a.m.u.) to obtain the full spectra of the derivatized drugs. The instrument was equipped with a splitless injection port and a methylsilicone capillary column, 12 m × 0.25 mm I.D., 0.33 μm, from Hewlett-Packard (Palo Alto, CA, USA). The oven temperature was programmed from 100 to 290°C at a rate of 10°C/min then keeping this temperature for 5 min. The injector port was set at 270°C and the interface at 300°C. Data handling was performed on a Hewlett-Packard MS Chemstation. The residue obtained after TLC purification was redissolved with 30 μl of methanol and 1 μl of this solution added of 0.5 μl of Methelut were injected into the GC–MS.

2.7. Validation procedures

2.7.1. Limit of detection and precision for the qualitative procedure

Blank urine samples spiked with six different concentrations in the range of 0.4 to 2.0 μg/ml were processed in replicates ($n=5$) as described above. The precision obtained from these samples was used to establish the limit of detection.

2.7.2. Precision, limit of detection and limit of quantitation

Plasma samples spiked with 0.25, 0.5, 1.0, 1.5, 2.0, 5.0, 10, 15, 20, 30 and 50 μg/ml were processed as described above and subjected to HPLC analysis. This process was done in replicates ($n=5$). The precision obtained from these samples was used to establish the limit of detection and limit of quantitation, according to the procedure described by Armbruster et al. [21].

2.7.3. Recovery

For the recovery study, water and plasma aliquots ($n=5$) spiked with eight different concentrations in the range of 0.5–20 μg/ml were processed as described above. The recovery was determined by comparing the slope of the plasma standard curve to that of the water standard curve and the result given in percentage [22].

$$\%\text{Recovery} = \frac{\text{slope of plasma standard curve}}{\text{slope of water standard curve}} \times 100$$

2.7.4. Selectivity

The selectivity of the method was determined by examining the separation of PB and OPB from endogenous plasma constituents in blank plasma. The effect of concomitant elution of other anti-inflammatory drugs and xanthines was also investigated. Methanolic solutions of these drugs were injected in the same conditions and their retention times determined.

2.7.5. Stability

Aliquots of samples collected after the administration of phenylbutazone containing high, medium and low concentrations of PB and OPB were stored at

−20°C for 1, 2 and 6 months. At the appropriate time, five aliquots of each concentration range were analysed for the stability study.

2.7.6. Quantitation

Quantitation was accomplished by generating internal standard calibration curves using control plasma fortified with appropriate dilutions of the 0.1% (m/v) solution of PB and OPB in methanol to obtain the desired concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, 5.0, 10, 15, 20, 30 and 50 μg/ml. Naproxen (2 μg/ml) was added to all samples and the procedure was done in replicates ($n=5$).

3. Results and discussion

The described procedure is used routinely in this laboratory for the detection of a wide range of acidic and neutral drugs. For HPLC analysis, the wavelength was fixed at 254 nm, which is not optimum for PB and OPB, but it was chosen because the method was intended to screen a wide range drugs.

As diazepam is not detected in horse urine samples once it undergoes extensive biotransformation, it was used as reference standard for retention times. The coefficient of variation for the relative retention times was <0.5% ($n=50$). The relative retention times were 1.21 (C.V.=0.2%) and 0.48 (C.V.=0.3%) to PB and OPB, respectively. The total time required from one sample analysis to the initiation of the next sample was 20 min.

The purification step for GC–MS analysis was performed by TLC. The main restriction to this technique is that it offers more opportunity for the likely oxidation of PB during analysis. Blowing down the air and filling the TLC tank with nitrogen avoid decomposition of the spots of PB and OPB during the run [23]. As it is a inexpensive technique, it was used as a clean-up procedure and for the GC–MS step, which was not intended for quantitation of the drugs. Urine samples ($n=3$) spiked with 0.6 μg/ml (which was the limit of detection for HPLC) were analysed by GC–MS showing that the oxidative decomposition was minimal and did not interfere for the purposes of the analysis.

The final confirmatory identification was performed by GC–MS. The retention times of the methylated derivatives of PB and OPB were 6.57 and 8.84 min, with $m/z=322$, 266, 183, 118, 105, 83, 77, 55 and 352, 296, 214, 213, 107, 92, 77, 55, respectively. Fig. 1 shows the total ion chromatogram and the fragmentation spectrum of an authentic positive urine sample, collected after a regular race. The limit of detection in urine samples was not studied as only positive samples in HPLC would be submitted to confirmation by GC–MS.

For the quantitative analysis, naproxen was chosen to be used as internal standard because of the difficulty in obtaining a close structural analogue of the analytes. Some available anti-inflamatory drugs were tested and the retention time of naproxen laid between those of PB and OPB with better peak height ratio precision than the others tested. Fig. 2 shows a HPLC chromatogram obtained from a plasma sample containing 5 μg/ml of PB and OPB. Acetonitrile and methanol were tested as deproteinizing agents. The deproteinization with acetonitrile required smaller volume of solvent than with methanol, leading to a smaller dilution of the sample.

Table 1 shows the results of validation procedures. The recovery showed some interference of matrix, probably due to the presence of proteins in the sample. To check that, a parallel recovery study was performed using the same formula described in Section 2.7.3 but, instead of using the slope of the water standard curve, the slope of a curve made of plasma which was first deproteinized and then spiked with different concentrations of the drugs was used. In this case it was noticed that the matrix did not interfere in the recovery values. Thus, one can conclude that the interference observed when recovery was calculated exactly according to the formula, was probably due to plasma protein.

A series of 20 plasma blank samples were tested and the chromatogram profile indicated no co-elution of endogenous plasma substances. None of drugs tested for co-elution interfered with the quantitation of PB and OPB (Table 2).

For the stability test, the frozen plasma samples containing PB and OPB were analysed on each of the established occasions and the results showed no apparent time-dependence decrease in the medium

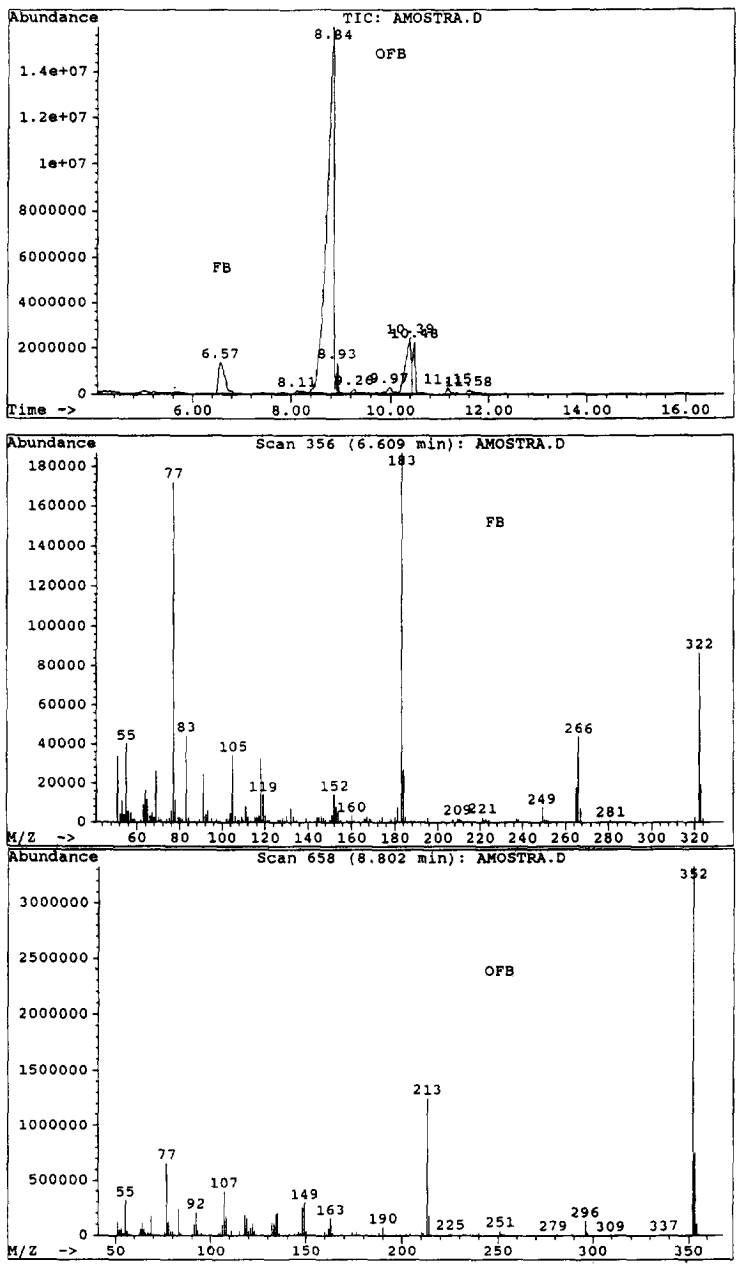

Fig. 1. Total ion chromatogram and the fragmentation spectrum of an authentic positive urine sample collected after a regular race.

and high concentrations, although there was a possible significant downward trend at the lower concentration.

Fig. 3 shows the plasma concentrations of PB and OPB. Plasma levels peaked at 31.1 μg/ml for PB and 3.9 μg/ml for OPB in 1 and 15 h, respectively. It was possible to detect PB and OPB up to 39 h (C.V.=14.1%) and 56 h (C.V.=3.4%), respectively,

Table 1
Results of validation procedures for PB and OPB

Parameter	PB	OPB
Concentration range (μg/ml)	0.5–30	0.5–50
C.V. (%)	7.8	4.7
Recovery (%)	83	105
LOD (μg/ml)[a]	0.5	0.5
C.V. (%)	5.9	11.8
LOQ (μg/ml)[b]	1	1
C.V. (%)	7.2	11.7
Curves	$y=0.364 + 0.445\,x$	$y=0.389 + 0.706\,x$
r^2	0.994	0.998

[a] LOD: limit of detection.
[b] LOQ: limit of quantitation.

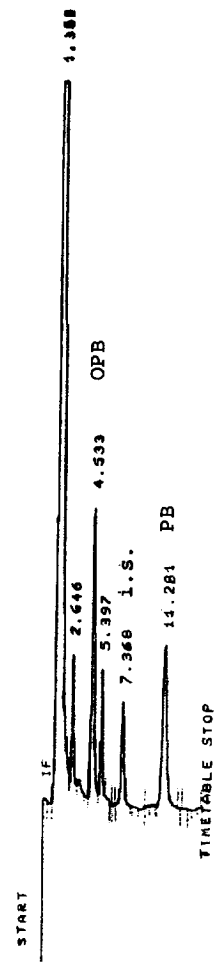

Fig. 2. HPLC chromatogram obtained from a plasma sample containing 5 μg/ml of PB and OPB.

Table 2
Study of the co-elution of other drugs in the HPLC detection of PB and OPB

Drug	Retention time (min)	Relative retention time
Oxyphenbutazone	**4.53**	**0.62**
Ketoprofen	5.82	0.79
Naproxen	7.37	1
Sulindac	8.33	1.13
Phenylbutazone	**11.28**	**1.53**
Flunixin	12.38	1.68
Suxibuzone	13.93	1.89
Flurbiprofen	16.29	2.21
Niflumic acid	17.26	2.34
Indomethacin	20.27	2.75
Diclofenac	20.56	2.79
Prednisone	2.73	0.37
Prednisolone	3.54	0.48
Betamethasone	4.86	0.66
Isoflupredone	5.01	0.07
Dexamethasone	5.02	0.68
Methylprednisolone	5.16	0.7
Theobromine	1.11	0.15
Caffeine	2.36	0.32
Theophylline	4.86	0.66

and to quantify them up to 30 h (C.V.=3.8%) and 39 h (C.V.=9.7%), respectively. According to Tobin [24], after this period the drug has no more pharmacological effect. The method detected PB and OPB in urine for up to 48 and 120 h, respectively.

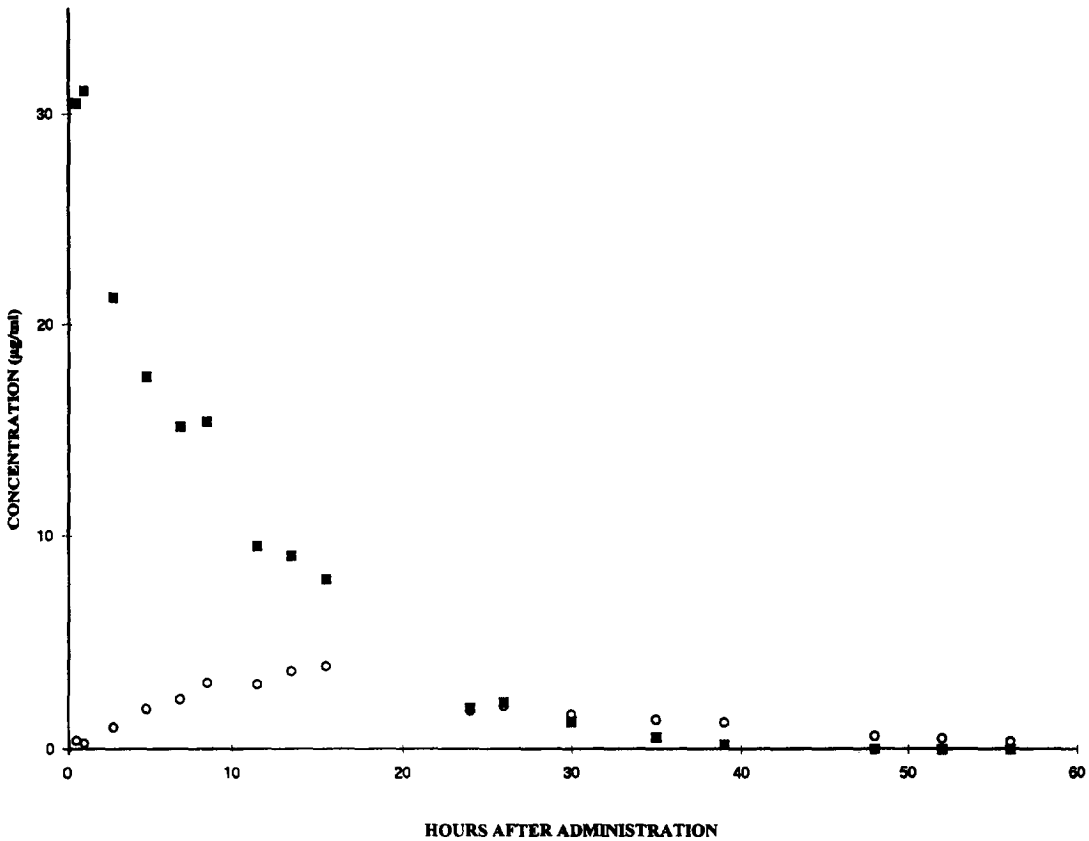

Fig. 3. Plasma levels of PB (■) and OPB (○) in the horse.

4. Conclusion

The procedure reported here for the quantitation of PB and OPB involves a simple deproteinization step followed by direct injection on the HPLC equipment, allowing the analysis of four samples per hour. It was applied to over 500 plasma samples without contamination of the analytical column, provided that a new guard-column is used every 80–100 samples. The method requires no extraction, no evaporation step and is sensitive enough to be used in the antidoping control of racehorses where controlled use of these drugs are permitted by the racing jurisdictions.

The qualitative procedure, although more laborious because it involves an extraction step, showed enough sensitivity and reliability to be used in antidoping analysis.

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