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Determination by high-performance liquid chromatography of phenylbutazone in samples of plasma from fighting bulls

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Abstract

The purpose of this study was to investigate the possible presence of phenylbutazone in plasma samples from fighting bulls killed in 2nd and 3rd category bullrings in the province of Salamanca (Spain) in 1998, 1999 and 2000. For quantitative and qualitative determination, a high-performance liquid chromatograph was used, equipped with a photodiode-array detector and setting wavelengths at 240, 254 and 284 nm. The mobile phase optimized for the simultaneous detection of dexamethasone, betamethasone, flunixin and phenylbutazone, was 0.01 *M* acetic acid pH 3 in methanol (35:65 v/v) at a flow rate of 1 ml/min. Plasma samples were deproteinized with 400 μl of acetonitrile and 20 μl of the supernatant were injected directly into the chromatographic system equipped with a Lichrospher 60 RP select B column and guard column. For the quantitative analysis, standard calibration curves were made in a concentration range between 0.25 and 30 μg/ml, using betamethasone as internal standard. The retention time of phenylbutazone was 8.7±0.2 min and recovery was 83%. The detection and quantification limits were 0.016 and 0.029, respectively for λ=240 nm. The study results show that 17 of the 74 samples analyzed in 1998, 18 of those from 1999 and 10 of those from 2000 were positive for phenylbutazone.

Keywords: Phenylbutazone

1. Introduction

Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) is a non-steroid anti-inflammatory agent (NSAID) from the pyrazolone group. From among its anti-inflammatory, antipyretic and analgesic effects, the first one is the most evident. Phenylbutazone (PBZ), a weakly acid with a pK_a of 4.5, is more readily and completely absorbed through the gastrointestinal tract than if it is injected, therapeutic blood levels being reached in 30 min. Ninety percent of the drug is bound to plasma proteins, and this may be why it is only slowly metabolized. Its

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main metabolite is oxyphenbutazone (OPB), which also has analgesic, antipyretic and above all anti-inflammatory effects [1]. The mechanism of phenylbutazone's anti-inflammatory action is the inhibition of endoperoxide isomerase in prostaglandin synthesis, whereas its analgesic property is thought to be regulated by a separate, centrally acting effect [2].

The drug has been widely used in horses to treat bone and joint inflammation, laminitis and inflammation of the soft tissues [3]. The half-life of PBZ is species, dose, and route dependent, but it is generally long. At a dosage of 5–6 mg/kg i.v., the $t_{1/2}$ in cattle ranged from approximately 30–82 h [4]. In view of the long elimination half-life of phenylbutazone in cattle, withholding periods have to be established before slaughter.

Several reports have been published describing methods for the detection of PBZ and OPB in horse urine samples using high-performance liquid chromatography (HPLC) [5–7] or gas chromatography–mass spectrometry (GC–MS) [8–10]. For the determination of phenylbutazone in plasma, many of the described methods include extraction followed by detection and quantification by HPLC–UV [5,11–22]. These methods, however, are tedious, less reproducible and facilitate the oxidation of drugs during analysis. Among the methods employing direct injection by HPLC to quantify phenylbutazone are those proposed by McKenzie [23] and Salvadori et al. [24]. A multi-residue HPLC method with photodiode-array detection has been reported for the determination of 12 NSAIDs in plasma, among them PBZ and FLU [25]. Although this study includes a wide range of matrices and other influencing factors to validate the method, the procedure involves a hydrolysis and solid-phase extraction (SPE) steps, which increases the analysis time. However, Salvadori et al. [24] describe a simple and rapid procedure for the detection and quantification of phenylbutazone in plasma based on the direct injection of deproteinized plasma into the chromatographic system, with final confirmation by GC–MS.

Here we studied the presence of phenylbutazone in samples of plasma from bulls killed in 2nd and 3rd category bullrings in the province of Salamanca (Spain) during 1998, 1999 and 2000 using direct injection of deproteinized plasma into an HPLC system, following a modified version of the procedure of Salvadori et al. [24].

2. Experimental

2.1. Reagents and apparatus

Standards of phenylbutazone [50-33-9] and betamethasone [987-24-6] were purchased from Sigma (Alcobendas, Madrid, Spain). The methanol used as mobile phase was of HPLC grade (Merck, Darmstadt, Germany). The acetonitrile (HPLC grade) and glacial acetic acid (reagent-grade) were obtained from Scharlau (Barcelona, Spain). The water used in the experiments was purified using an Elgastat UHQ system (Elga, High Wycombe, UK). Eppendorf tubes of 1.5 ml and 1% sodium heparin (Rovi, Madrid, Spain) as anticoagulant were used to prepare the samples. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma was used for the derivatization procedure.

2.2. Sample collection

After the bulls had been killed in the fight, blood samples were obtain by bleeding from cervical, jugular and carotid vessels converging with vena cava anterior and the aorta anterior. The samples were collected in 100-ml precipitating flasks containing 1 ml of previously diluted heparin (1 ml of 1% heparin in 5 ml). The blood was then centrifuged in 10 ml tubes at 3500 rpm (equivalent to an acceleration of 2195 *g* for the centrifuge used) for 10 min and the resulting plasma samples were stored at −20 °C until analysis.

2.3. Preparation of standards

Methanolic solutions of phenylbutazone and betamethasone (internal standard) were prepared at concentrations of 100 μg/ml and were stored at 4 °C. Then, the appropriate dilutions were prepared to obtain the calibration data and study the recovery and quantitative determinations.

2.4. Analytical method

In the preparation of plasma samples, 400-μl aliquots of plasma placed in Eppendorf tubes were deproteinized with 400 μl of acetonitrile, vortexed, and centrifuged at 3500 rpm (2195 *g*) for 5 min. For HPLC analysis, 20 μl of the resulting supernatant was injected into the chromatographic system.

The liquid chromatograph consisted of a Spectra System (ThermoQuest, San Jose, CA, USA) equipped with a P-4000 quaternary gradient pump (ThermoQuest), a 6000 LP UV photodiode-array detector (ThermoQuest), a 7125NS Rheodyne injector, a Dell Optiplex GX1 computer (Benton Harbor, MI, USA) with ChromQuest V.2.51 (ThermoQuest) chromatographic software and an HPLC column heater (Croco-Cil®, Sainte-Foy-La-Grande, France). The chromatographic column was a Lichrospher 60 RP select B (250×4 mm I.D., 5 μm) and a Lichrospher 60 RP select B guard column (4×4 mm I.D., 5 μm) from Merck (Darmstadt, Germany). The wavelengths monitored in the detector were 240, 254 and 284 nm.

The composition of the mobile phase was 0.01 *M* acetic acid pH 3 in methanol (35:65, v/v) at a flow rate of 1 ml/min, column temperature was maintained at 35 °C.

2.5. Validation procedure

2.5.1. Qualitative determination

Identification of positive samples was accomplished by comparison of the spectra of the presumably phenylbutazone-positive peaks with those recorded in the library of the chromatograph. For this purpose a library was compiled with spectra corresponding to plasma samples spiked with different anti-inflammatory drugs, among them phenylbutazone.

Final confirmation of the positive samples was achieved using the GC–MS system from Shimadzu (GC-17A and QP-5000, Duisburg, Germany) in scan mode (acquisition range 50–360 u.m.a.), using a DB-5 capillary column (30 m×0.25 mm I.D.) from J&W (Fulsom, CA, USA). The injection port was set at 250 °C and the interface at 300 °C. The oven temperature was programmed from 150 to 280 °C at 20 °C/min and injection was performed in splitless mode [11]. The positive samples were derivatised with BSTFA and 1 μl was injected into the GC–MS system. Fig. 1 shows the chromatogram of a sample of plasma positive for phenylbutazone and its mass spectrum and GC–MS chromatogram of bull plasma sample without phenylbutazone.

2.5.2. Quantitative determination

In the quantitative analysis, betamethasone was used as internal standard at a fixed concentration of 2 μg/ml. To obtain the standard calibration curves, aliquots of plasma were spiked with 0.25, 0.50, 1.0, 1.5, 2.0, 5.0, 10, 15, 20 and 30 μg/ml of phenylbutazone, applying the above method for each concentration in triplicate.

2.5.3. Recovery

The recovery was determined by comparing the slope of plasma standard curve to that of the methanol [26]. For this part of the study, aliquots ($n=3$) of plasma and methanol were spiked with the same concentrations of phenylbutazone (0.25–30 μg/ml) used to obtain the calibrations. Throughout the recovery study, betamethasone (2 μg/ml) was also used as internal standard. Recovery was considered as:

$$\%\ \text{Recovery} = \frac{\text{Slope of plasma standard curve}}{\text{Slope of methanol standard curve}} \times 100$$

2.5.4. Limit of detection and limit of quantification

To obtain the limit of detection and the limit of quantification, a series of blanks of plasma samples ($n=10$) were analyzed, calculating the mean and SD, and the following formula was applied:

$$C_L = (y_B + Ks_B)/m$$

with C_L, limit of detection or quantification; y_B, mean of response of ''n'' blanks; $K=3$, limit of detection and $K=10$, limit of quantification; s_B, SD of the response of 10 blanks; m, slope of plasma standard curve.

3. Results and discussion

Optimization of the conditions of liquid chromatography was carried out for the simultaneous analysis of the glucocorticoids dexamethasone (DEX) and

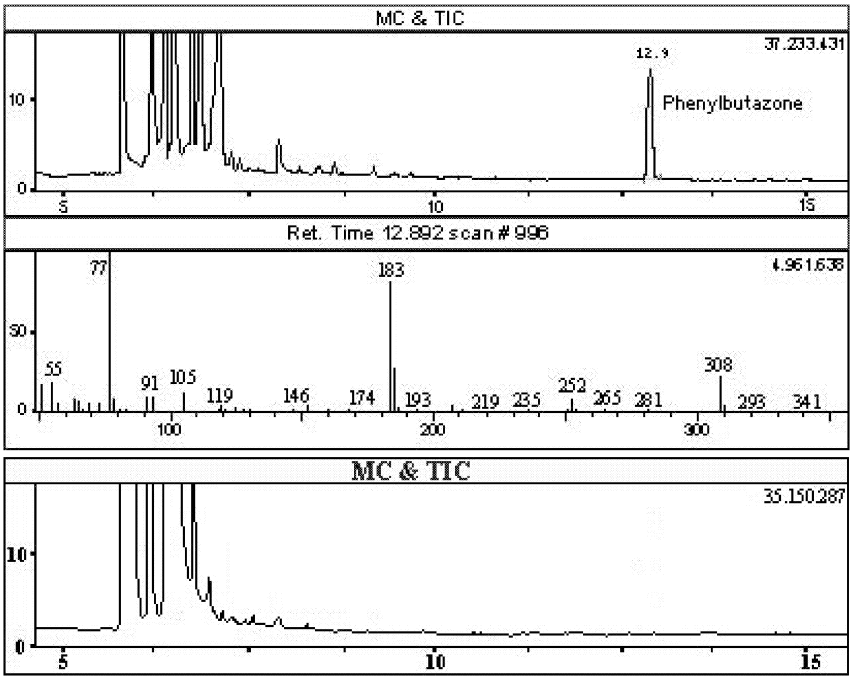
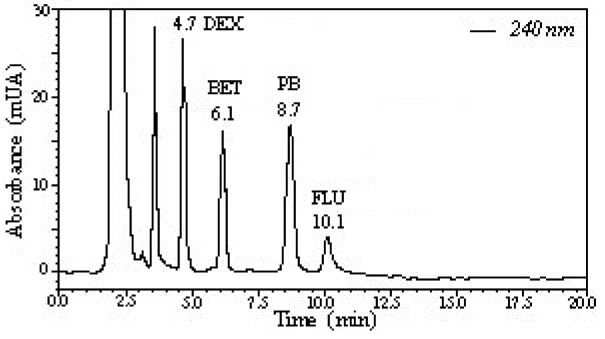


Fig. 1. GC–MS chromatogram and spectrum of a plasma sample with phenylbutazone and GC–MS chromatogram of bull plasma sample without phenylbutazone.

betamethasone (BET) and the anti-inflammatory agents phenylbutazone (PBZ) and flunixin (FLU), all drugs suspected to be present in this type of sample. The total time required for the analysis of these four components was 11 min, although upon analysing unknown samples the total time taken to obtain the chromatogram was prolonged to 20 min, the time necessary for the elution of other drugs possibly present in the samples (Fig. 2). After screening the samples taken during the first year of the study and

(a)



(b)

Drug	Retention time (min)
Dipirone	3.58
Prednisolone	4.03
Dexamethasone	4.57
Oxiphenbutazone	4.89
Methylprednisolone	4.95
Ketoprofen	5.83
Bethametasone	5.97
Sulindac	6.28
Naproxen	6.38
Suxibuzone	7.69
Phenylbutazone	8.74
Flurbiprofen	9.81
Flunixin	10.13
Indomethacin	10.88
Ibuprofen	12.32
Mefenaminc acid	19.33
Meclofenamic acid	19.86

Fig. 2. (a) Chromatogram of a mixture of standards of dexamethasone (DEX), betamethasone (BET), phenyllbutazone (PBZ) and flunixin (FLU). (b) Co-elution of other drugs in the determination of phenylbutazone by HPLC.

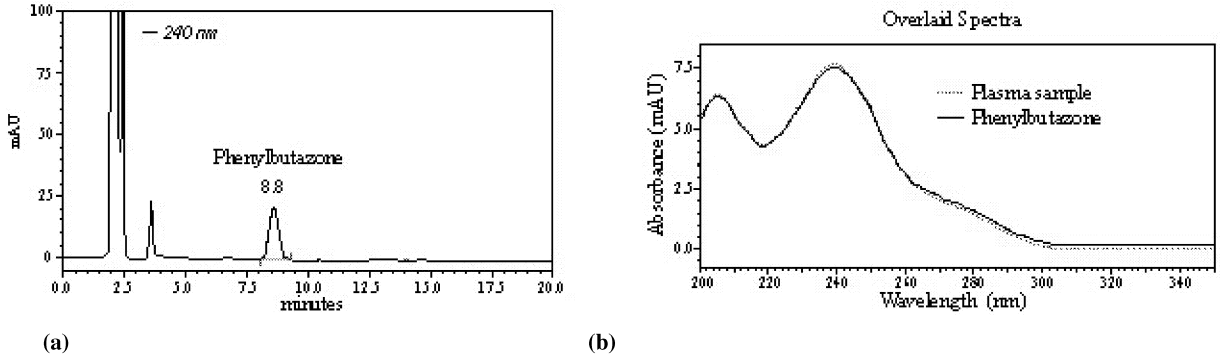


Fig. 3. (a) HPLC chromatogram of plasma sample containing 2 μg/ml of phenylbutazone. (b) UV spectrum of a phenylbutazone standard and the recorded spectrum of a plasma sample.

checking that betamethasone was not present in any of the samples, this substance was chosen as the internal standard because it has a retention time similar to that of phenylbutazone and because it shows a good peak height relationship with phenylbutazone for the two wavelengths selected ($\lambda = 240$ and 254 nm). The retention time of phenylbutazone under the chromatographic conditions applied was 8.7 ± 0.2 min. Fig. 3a shows the chromatograms obtained at a wavelength of 240 nm of a plasma containing 2 μg/ml of phenylbutazone. It may be seen that there are no interferences of endogenous plasma substances at the retention times of the peaks of interest.

The analytes detected using the HPLC-photodiode-array detector were identified and confirmed by assessing their retention times in connection with those of the spectra library, and the absorption maxima in the spectrum of the analyte should be at the same wavelengths as that of a standard of the same analyte. Also, there should be no visual differences above 220 nm for the parts of the spectrum with a relative absorbency $\geq 10\%$ at any point monitored [25,27]. Fig. 3b compares the spectra of a standard of phenylbutazone with that of an unknown plasma sample. The complete agreement between the two spectra confirms that the sample analyzed was positive for the substance. For greater security in the identifications, several samples were checked with GC–MS by comparing their mass spectra with that of a phenylbutazone standard, Fig. 4 shows the HPLC–UV chromatogram of bull plasma sample with and without phenylbutazone. The retention time of the methylated derivative of phenylbutazone was 12.9 min, with $m/z = 308$, 252, 183, 105, 91, 77, 55 (Fig. 1).

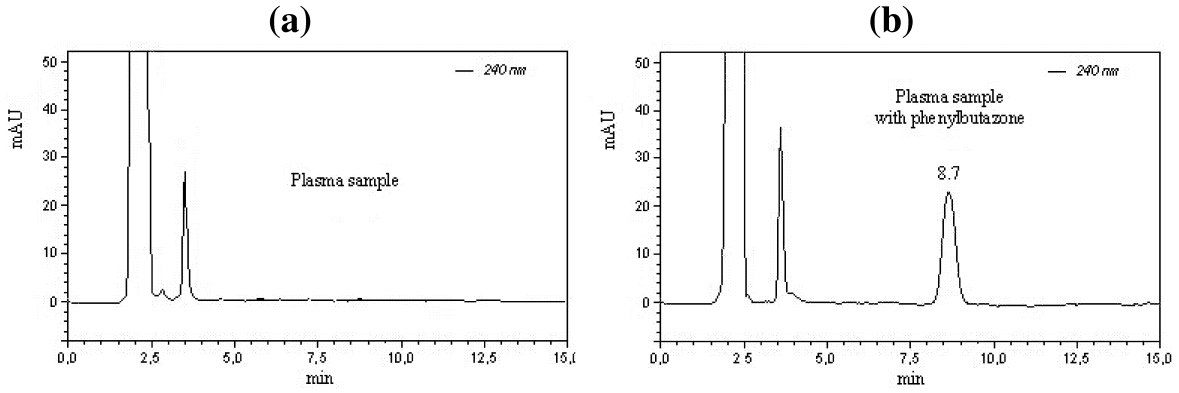


Fig. 4. (a) HPLC–UV chromatogram of bull plasma sample without phenylbutazone (2.27 μg/ml) and (b) with phenylbutazone.

The equations of the standard curves for 240 nm using 10 data points ($n=10$) are $y=0.4005x+0.0280$ ($r^2=0.9997$) and $y=0.4847x-0.0135$ ($r^2=0.9998$) for plasma and methanol, respectively, and for 254 nm are $y=0.3165x+0.0136$ ($r^2=0.9997$) and $y=0.3771x-0.0339$ ($r^2=0.9998$) for plasma and methanol, respectively. It may be seen that both calibration curves show good linearity, as deduced from their regression coefficients. The coefficients of variation obtained from the triplicate analysis of each of the calibration concentrations did not surpass 5% at the two wavelengths mentioned above, meaning that precision is acceptable across the concentration range studied. Regarding the reproducibility of linear standard curves, the assays performed inter-day with the plasma matrix (3 days) displayed coefficients of variation of 1.72 and 1.69% for wavelengths of 240 and 254 nm, respectively, while with methanol (2 days), these coefficients had values of 1.83 and 1.69% for the same wavelengths, respectively.

The recoveries calculated through the slope relationship in Section 2.5.3 (Table 1) are similar to those reported by Salvadori et al. [24], with the difference that betamethasone was used as the internal standard instead of naproxen. If naproxen is used as internal standard, typical correlation coefficient, slope and intercept for 240 nm were $y=0.2840x-0.0194$ ($r^2=0.9999$) and $y=0.3053x+0.0361$ ($r^2=0.9995$) for plasma and methanol, respectively, and for 254 nm were $y=0.8837x-0.0835$ ($r^2=0.9997$) and $y=0.9353x+0.1409$ ($r^2=0.9996$) for plasma and methanol, respectively. Recovery increases up to 93% for 240 nm and 94% for 254 nm. The sensitivity of the HPLC–UV method was greater than that reported by Salvadori et al. [24] for the determination of phenylbutazone by liquid chromatography. The limits of detection and quantification calculated using the formula described in Section 2.5.4 are shown in Table 1.

The results for the plasma samples in which phenylbutazone was detected are shown in Table 2, which gives the concentration, SD, and coefficient of variation of each of the samples. It may be considered that the precision in the quantification of the positive samples is acceptable. This may be deduced upon observing the coefficients of variation shown in Table 2, whose values do not surpass 8%, although most lie at 2–3%. Quantification was accomplished at 240 nm, because this wavelength shows the best limits of detection and quantification. The results obtained show that 17 of the 74 samples analyzed for 1998 and 18 of the 83 for 1999 contain the drug. In the case of the last year studied (2000), the number of positive samples decreased considerably, with 10 positive samples out of the 87 analysed. The concentrations of phenylbutazone found in the total number of positive samples were as follows: 28 samples had amounts lower than 2 μg/ml; 10 lay between 2 and 8 μg/ml and seven were above 8 μg/ml.

4. Conclusions

The procedure described allows the quantitative and qualitative detection of phenylbutazone in plasma samples from fighting bulls. The sensitivity of the method, with a detection limit at 0.016 μg/ml and a quantification limit of 0.029 μg/ml ($\lambda=240$ nm), is better than that of Salvadori et al. [24].

The presence of phenylbutazone was checked and quantified in unknown samples of plasma from bulls

Table 1
Results of validation procedure for phenylbutazone

λ	Sensitivity (μg/ml)		Precision				Recovery (%)
	Detection limit	Quantitation limit	Intra-assay C.V. (%)		Inter-assay C.V. (%)		
			Plasma[a]	Methanol[b]	Plasma[a]	Methanol[b]	
240 nm	0.016	0.029	2.04	1.73	1.72	1.83	83
254 nm	0.027	0.061	1.69	1.87	1.69	1.69	84

[a] $n=3$ determinations.
[b] $n=2$ determinations.

Table 2
Concentration, SD and coefficient of variation (C.V.) of plasma samples positive for phenylbutazone

1998			1999			2000		
C (μg/ml)	SD	C.V. (%)	*C* (μg/ml)	SD	C.V. (%)	*C* (μg/ml)	SD	C.V. (%)
23.37	0.62	2.67	17.08	0.40	2.36	13.11	0.41	3.14
18.03	0.80	4.41	8.48	0.34	3.98	8.66	0.65	7.48
8.67	0.13	1.48	6.67	0.33	5.96	1.86	0.03	1.52
3.56	0.08	2.23	6.43	0.12	1.86	1.51	0.05	3.62
2.87	0.06	2.07	4.65	0.13	2.81	1.16	0.07	6.18
2.76	0.09	3.32	4.12	0.07	1.66	0.82	$4\ E^{-4}$	0.05
1.99	0.06	3.05	2.67	0.03	1.19	0.52	0.01	2.31
1.74	0.04	2.52	2.27	0.10	4.59	0.20	0.01	5.10
1.59	0.06	3.54	2.04	0.07	3.19	0.08	0.001	1.45
1.34	0.05	3.38	1.96	0.11	5.52	0.06	$4\ E^{-4}$	0.64
1.16	0.04	3.36	0.92	0.01	1.40			
1.11	0.04	3.22	0.39	0.02	5.45			
0.70	0.01	0.74	0.39	0.02	5.64			
0.49	0.02	3.15	0.29	0.01	2.86			
0.47	0.02	3.85	0.28	0.02	6.44			
0.30	0.00	0.83	0.22	0.01	2.42			
0.29	0.02	6.16	0.18	0.004	2.22			
			0.16	0.003	1.87			

killed in 2nd and 3rd category bullrings in the province of Salamanca (Spain), and it is possible to use the method for the simultaneous analysis of dexamethasone, betamethasone, phenylbutazone and flunixin in plasma from fighting bulls.

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