

*Bull Vet Inst Pulawy 49, 223-226, 2005*

# DETERMINATION OF PHENYLBUTAZONE AND OXYPHENBUTAZONE IN BOVINE PLASMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION

PIOTR JEDZINIAK, TERESA SZPRENGIER-JUSZKIEWICZ  
AND ANDRZEJ GIERAK<sup>1</sup>

Department of Pharmacology and Toxicology,  
National Veterinary Research Institute, 24-100 Puławy, Poland  
e-mail: jedzi@piwet.pulawy.pl

<sup>1</sup>Department of Chromatographic Methods, Faculty of Chemistry,  
Maria Skłodowska-Curie University, 20-031, Lublin, Poland

*Received for publication April 14, 2005.*

## Abstract

A simple and reliable method for the determination of phenylbutazone (PB) and its active metabolite oxyphenbutazone (OPB) in bovine plasma is described. After solvent extraction of plasma samples with acetonitrile and clean-up by solid phase extraction using C18 cartridges, analytes were determined by liquid chromatography with UV detection. Mean recovery values of PB and OPB from plasma samples fortified at the levels of 30–480 ng/ml were  $52.0 \pm 7.0\%$  and  $69.0 \pm 7.8\%$ , respectively. Repeatability, expressed as coefficient of variation, was below 15%. Limit of detection for PB was 18 ng/ml and for OPB - 12 ng/ml. Limit of quantitation of PB and OPB was 60 ng/ml each. Validation parameters and calculated performance criteria: decision limit ( $CC\alpha$ ) and detection capability ( $CC\beta$ ) indicate that the method is suitable for screening plasma samples for phenylbutazone and oxyphenbutazone.

**Key words:** phenylbutazone, oxyphenbutazone, residues, HPLC.

Phenylbutazone is an effective non-steroidal anti-inflammatory drug (NSAID) with antipyretic and analgesic activity, used in veterinary medicine for more than 50 years to treat bone and joint inflammation, laminitis and inflammation of soft tissues (1). It is widely used in dogs and horses but, because of toxicity and the lack of established maximum residue limit (MRL), is not approved for use in food producing animals. The most serious adverse reactions of phenylbutazone observed in humans and animals are: gastric and intestinal ulceration and bleeding, disturbances in platelet function, the prolongation of gestation or spontaneous labour and changes in renal function (12).

In spite of lack of approval there is some evidence of extra-label use of phenylbutazone in food producing animals, for the treatment of various inflammatory conditions (for example mastitis in lactating dairy cows) (3). This can result in residues of phenylbutazone in food, and because of its good oral availability and high toxicity, can pose health risk to the consumers. For this reason it is necessary to monitor the possible misuse of phenylbutazone in animal production. As the matrix to be controlled animal plasma is useful.

There are several methods described for the determination of phenylbutazone in plasma. Some of them use liquid-liquid and solid-phase extraction (SPE) for sample preparation and liquid chromatography with UV (4, 13) or diode array (5, 8, 9, 14, 17) for detection. Other methods use GC-MS technique (6, 15, 16). Some of the authors addressed the problems of the decomposition of PB and OPB during sample preparation (9 - 11).

In this paper we present simple and reliable method for the determination of phenylbutazone and oxyphenbutazone in animal plasma, with relative good coefficient of variation of the results and free of some disadvantages typical for plasma analysis such as clogging of SPE columns in the extract purification step.

## Material and Methods

**Reagents.** All reagents used for sample preparation were of analytical grade, and all reagents used for chromatography were HPLC grade. Acetonitrile, methanol, hexane, diethyl ether, acetic acid, and C18 SPE columns (6 ml, 500 mg) were purchased from J.T. Baker. Phenylbutazone, oxyphenbutazone, and ascorbic acid were purchased

from Sigma. Water used for analysis was obtained from Millipore Q Plus Station and had resistance above 18 m $\Omega$ .

**Standard solutions.** All standard solutions were prepared in methanol. Stock solutions of PB and OPB (1 mg/ml) were stored in  $-20^{\circ}\text{C}$  until used (stable for at least 6 months). Working standard solutions (100  $\mu\text{g/ml}$ ) and mixed standard solution (10  $\mu\text{g}$  of each PB and OPB/ml) were prepared on the same day of analysis.

**HPLC conditions.** Instrumental analysis was performed on HP 1050 liquid chromatograph (Hewlett Packard) equipped with quaternary pump, degasser, Rheodyne valve with 20  $\mu\text{l}$  loop, Inertsil ODS-2 column (150 mm x 4.6 mm, 5  $\mu\text{m}$  particle size, Supelco) and UV-Vis detector (240 nm), controlled by Chemstation software. Mobile phase was pumped in gradient (A - acetonitrile, B - 0.1 M acetic acid; 0-2 min: 20% A, 18 min: 60% A) with 1.2 ml/min flow. Column temperature was not controlled.

**Samples.** Drug free (blank) bovine plasma was prepared from heparinised blood collected from cows in the slaughter house. Plasma was checked for interferences during analysis and stored in 50 ml polypropylene screw-capped tubes in the freezer (below  $-18^{\circ}\text{C}$ ). Before using sub-samples were thawed overnight in the refrigerator ( $4^{\circ}\text{C}$ ).

**Procedure.** A 5 ml plasma sample was transferred to glass centrifuge tube. Control (fortified) sample was spiked with specified volume of mix standard solution, vortex-mixed during 1 min, and let to rest for 10 min. After that 5 ml of acetonitrile was added. Tube was vortex-mixed for 1 min and centrifuged (3 000 x g, 10 min). Supernatant was transferred to another tube, diluted with 20 ml of 0.01 M ascorbic acid water solution and applied to the C18 cartridge (previously preconditioned with 3 ml of acetonitrile, 3 ml of water and 3 ml of the mixture of acetonitrile and 0.01 M ascorbic acid water solution, 1+4). After application of the sample 2 ml of 0.01 M ascorbic acid and 2 ml of water were passed through the column to elute interferences. After this step column bed was dried under vacuum (approximately 300 mbar) for 45 min. Phenylbutazone and oxyphenbutazone were eluted with a 2.5 ml mixture of hexane and diethyl ether (1+1). The eluate was accurately evaporated to dryness

under stream of nitrogen at  $40^{\circ}\text{C}$ . Dry residue was reconstituted in 300  $\mu\text{l}$  of mobile phase (acetonitrile and 0.1 M acetic acid, 1+1) and 20  $\mu\text{l}$  was injected on the HPLC column.

**Validation.** Linearity of the method and recovery of PB and OPB from plasma were determined by analysis of calibration curves which were generated for both analytes running through HPLC analysis mixed calibration standard solution in the concentration levels over the working range (30-480 ng/ml), and plotting the recorded peak heights versus concentrations. The matrix calibration curves were prepared from the results of analysis of blank plasma samples fortified at the corresponded levels of PB and OPB carried out through the whole procedure.

Validation parameters: repeatability, reproducibility, decision limit and detection capability were calculated from the results of analysis of 3 series (analyses in different occasions) of the control samples. Control samples of bovine plasma were spiked with phenylbutazone and oxyphenbutazone on three concentration levels (30, 60, 120 ng/ml) and analysed in six replicates. The concentration of each drug was determined and intra- and inter-assay coefficients of variations (CV) were calculated.

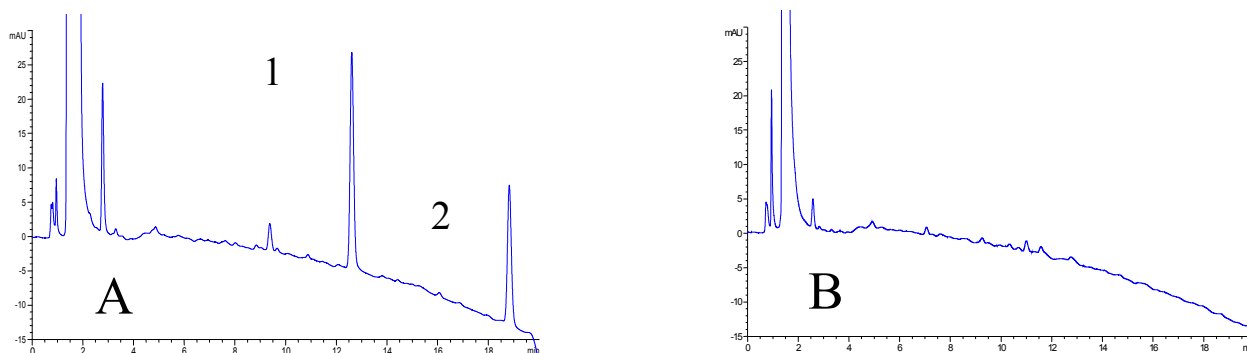
Decision limits ( $CC_{\alpha}$ ) and detection capabilities ( $CC_{\beta}$ ) were calculated according to requirements of Commission Decision 2002/657/EC (2), using results of reproducibility analysis.

The detection limits were calculated as the concentration with a signal to noise ratio equal 3 to 1.

The limits of quantitation corresponded to the lowest concentration that could be determined with a coefficient of variation (CV) less than 15%.

## Results

No interference peaks were observed in bovine plasma samples, taken randomly in the slaughter house and extracted using the described procedure. Chromatograms of plasma samples fortified with PB and OPB and blank plasma are shown in Fig 1.



**Fig. 1.** Chromatograms of extracts: A - bovine plasma spiked with 120 ng/ml oxyphenbutazone (1) and phenylbutazone (2); B - blank plasma sample.

Calculated validation parameters of the method are reported in Table 1. The linearity was determined over the concentration range of 30 to 480 ng/ml. Linear regression analysis of the results demonstrated good relationship of HPLC response to analyte concentrations (correlation coefficients for both PB and OPB curves were above 0.99).

Mean recoveries of PB and OPB from the bovine plasma, calculated by comparing results of

standard calibration curve representing 100% recovery, with matrix calibration curve, were 52.0% and 69.0%, respectively. Repeatability determined at three concentration levels, expressed as coefficient of variation, was below 15% for both analytes. Intra-laboratory reproducibility determined at three concentration levels analysed in 3 series of sample, expressed as coefficient of variation, varied between 8.5 and 28.7%.

**Table 1**  
Results of validation procedure for PB and OPB

Validation parameters	Phenylbutazone	Oxyphenbutazone
Limit of detection, ng/ml	18	12
Limit of quantitation, ng/ml	60	60
Standard calibration curve	$y = 0.0782x - 0.3436$ $r^2 = 0.999$	$y = 0.0878x - 0.3318$ $r^2 = 0.999$
Matrix calibration curve	$y = 0.041x - 0.1539$ $r^2 = 0.996$	$y = 0.061x - 0.1496$ $r^2 = 0.997$
Linearity, ng/ml	30-480	30-480
Repeatability (n=6) CV, %	30 ng/ml	13.9
	60 ng/ml	10.9
	120 ng/ml	8.5
Reproducibility, (n=6) CV, %	30 ng/ml	28.7
	60 ng/ml	13.5
	120 ng/ml	10.2
Mean recovery	52.0±7.0	69.0±7.8
CC $\alpha$ , ng/ml	69.5	69.9
CC $\beta$ , ng/ml	80.0	81.7

## Discussion

A simple method for the determination of phenylbutazone, and its active metabolite oxyphenbutazone in bovine plasma has been described. Solvent extraction of plasma samples with acetonitrile (7, 13) was preferred over other methods of extraction (6, 9, 17), ultrafiltration (4) or acid hydrolysis (8). It allowed precipitation of plasma proteins during extraction and avoided clogging the SPE column in the purification step and appeared to be less time consuming than other purification step methods.

Many authors (8, 9, 17) observed degradation of phenylbutazone and oxyphenbutazone during analysis, if samples were exposed to acidic conditions, left dry and open to the atmosphere, or when containing oxygen diethyl ether was used as elution solvent in solid-phase extraction. Our experiences indicate that addition of ascorbic acid solution as stabiliser to the extract before SPE cleaning-up, according to the procedure described by Gowik (8), makes the degradation slower but does not stop it completely. This was probably the reason why recoveries of the method

were rather low (52% and 69% for PB and OPB), however, similar to those observed by other authors: 63% and 55% (8), 51.5% and 37.9% (9), 53.5% and 43.3% (17).

Indices of precision of the method, expressed as CV of repeatability were on the accepted level (below 15%). Coefficients of variation of reproducibility for oxyphenbutazone were also below 15%, but for the lowest spiking level were higher (28.7% for PB and 16.3% for OPB). For this reason limits of quantification for both compounds were established on the level 60 ng/ml.

We have found that bed drying is critical point of the procedure during SPE step. As the mixture for elution of analytes (hexane-diethyl ether) is not miscible with water, any traces of water, resting on the column from previously used ascorbic acid water solution, have to be completely removed. Therefore long drying (minimum 45 min) is necessary.

Liquid chromatography conditions and gradient elution described in the procedure permit to obtain good separation of target analytes and matrix interferences. Validation parameters and calculated performance criteria: decision limit (CC $\alpha$ ) and detection capability

(CCβ) indicate that the method is suitable for screening plasma samples for phenylbutazone and oxyphenbutazone.

**Acknowledgments:** This study was supported by State Committee for Scientific Research (KBN) Grant PCZ 014-36, Warsaw, Poland.

### References

1. Boothe D.M.: The analgesic-antipyretic- anti-inflammatory drugs, In: *Veterinary Pharmacology and Therapeutics*, edited by H.R Adams, Iowa State University Press, Ames, Iowa 1995, pp. 432-446.
2. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. O J L 2002, **221**, 8-36.
3. Dascanio J.H.J., Mechor G.D., Gron Y.T., Kenny D.G., Broker C.A., Thompson P., Chiffelle C.L., Musser J.M.M., Warnic L.D.: Effect of phenylbutazone and flunixin meglumine on acute tonic mastitis in dairy cows. *Am J Vet Research* 1995, **56**, 1213-1218.
4. De Veau E.I.: Determination of non-protein bound phenylbutazone in bovine plasma using ultrafiltration and liquid chromatography with ultraviolet detection. *J Chromatogr B* 1999, **721**, 141-145.
5. Fiori M., Farne M., Civitareale C., Nasi A., Serpe L., Gallo P.: The use of bovine plasma serum albumin as ligand in affinity chromatographic clean-up of non-steroidal anti-inflammatory drugs from bovine plasma. *Chromatographia* 2004, **60**, 253-257.
6. Gonzalez G., Ventura R., Smith A.K., Torre R., Segura J.: Detection of non steroidal antiinflammatory drugs in equine plasma and urine by gas chromatography – mass spectrometry. *J Chromatogr A* 1996, **719**, 251-264.
7. Gonzalez Martin M.I., Sanchez Gonzalez C.I., Jimenez Hernandez A., Garcia Cachan M.D., Castro de Cabo M.J., Garzon Cuadrado A.L.: Determination by high-performance liquid chromatography of phenylbutazone in samples of plasma from fighting bulls. *J Chromatogr B* 2002, **769**, 119-126.
8. Gowik P., Jülicher B., Uchlig S.: Multi-residue method for non-steroidal anti-inflammatory drugs in plasma using high performance liquid chromatography – photodiode array detection. Method description and comprehensive in-house validation. *J Chromatogr B* 1998, **716**, 221-232.
9. Grippa E., Santini L., Castellano G., Gatto M.T., Leone M.G., Saso L.: Simultaneous determination of hydrocortisone, dexamethasone, indometacin, phenylbutazone and oxyphenbutazone in equine serum by high performance liquid chromatography. *J Chromatogr B* 2000, **738**, 17-25.
10. Gupta R.N.: Improved stability of phenylbutazone for its determination by liquid chromatography. *J Chromatogr* 1990, **550**, 160-163.
11. Hyde W., Ambrisco K.: Sample degradation consideration in phenylbutazone quantitation. In: *Proceedings*, 9th International Symposium of Racing Analysts and Veterinarians, 1992, New Orleans.
12. Lewicki J. Toksyczność i stosowanie fenylobutazonu u zwierząt. *Magazyn Weterynaryjny* 1995, **3**, 253-259.
13. Neto L.M.R., Andraus M.H., Salvadori M.C.: Determination of phenylbutazone and oxyphenbutazone in plasma and urine samples of horses by high-performance liquid chromatography and gas chromatography – mass spectrometry. *J Chromatogr* 1996, **678**, 211-218.
14. Peck K.E., Ray A.C., Manuel G., Rao M.M., Foos J.: Quantification of phenylbutazone in equine sera by use of high performance liquid chromatography with non-evaporative extraction technique. *Am J Vet Res* 1996, **57**, 25-27.
15. Singh A.K., Jang Y., Mishera U., Granley K.: Simultaneous analysis of flunixin, naproxen, ethacrynic acid, indomethacin, phenylbutazone, mefenamic acid and thiosalicylic acid in plasma and urine by high performance liquid and gas chromatography – mass spectrometry. *J Chromatogr* 1991, **568**, 351-361.
16. Takeda A., Tanaka H., Shinorhara T., Ohtake I.: Systematic analysis of acid, neutral and basic drugs in horse plasma by combination of solid-phase extraction, non-aquous partitioning and gas chromatography – mass spectrometry. *J Chromatogr B* 2001, **758**, 235-248.
17. Taylor M.R., Westwood S.S.: Quantitation of phenylbutazone and oxyphenbutazone in equine plasma by high performance liquid chromatography with solid-phase extraction. *J Chromatogr A* 1995, **697**, 389-396.