Quantification of phenylbutazone in equine plasma for doping control in horse racing using strong anion exchange solid phase extraction followed by liquid chromatography with UV detection

Frank Lin and Nicole Heiser

Faculty Mentor: Liguo Song

Department of Chemistry, Western Illinois University, Macomb IL

Abstract

A method using strong anion exchange solid phase extraction (SAX-SPE) followed by liquid chromatography ultraviolet detection (LC-UV) for the analysis of phenylbutazone (PBZ) and its metabolite oxyphenbutazone (OPBZ) in equine plasma for doping control in horse racing has been developed. By using SAX-SPE, commonly regulated non-steroidal anti-inflammatory drugs (NSAIDs) by the United States Equestrian Federation (USEF), i.e. PBZ, OPBZ, diclofenac, flunixin, ketoprofen, meclofenamic acid and naproxen, and an internal standard, i.e. tolfenamic acid, were first selectively extracted. Then, baseline separation of PBZ, OPBZ from other NSAIDs, internal standard, and residual components of equine plasma was achieved using LC-UV. Finally, PBZ and OPBZ in equine plasma were quantified after an internal calibration curve was created.

Introduction

Phenylbutazone (PBZ) is the second oldest non-steroidal anti-inflammatory drug (NSAID) after aspirin. It is relatively inexpensive, readily obtainable and especially effective in the management of acute and chronic musculoskeletal conditions in horses that produce mild pain due to inflammation of bone, joints, and soft tissues [1].

The winner of the 1968 Kentucky Derby, Dancer's Image, was disqualified after traces of PBZ were discovered in a post-race analysis. PBZ was legalized for use on most tracks around the United States in 1968, but it had not yet been approved by Churchill Downs. While PBZ has a great therapeutic value in the treatment of acute inflammatory conditions, which can enable the training of a sore racehorse to maintain fitness, it can also be misused to mask the lameness of a seriously injured racehorse. Therefore, a plasma threshold for PBZ in a racehorse has been established for the health and welfare of the racehorse and its rider. For example, in Illinois, as in many other states, 2.0 µg/mL of PBZ in equine plasma is not allowed in the racehorse in a post-race analysis. Plasma PBZ lower than the threshold is considered therapeutic dosage and does not violate the medication rules.

Post-race analysis of PBZ usually begins with a presumptive test using an enzyme-linked immunosorbent assay (ELISA). Positive samples are further submitted for confirmation and quantification by liquid chromatography (LC). Due to its specificity and sensitivity, most recently published LC methods employ mass spectrometric (MS) detection [2]. However, coupling LC with ultraviolet (UV) detection is much more accessible by equine drug testing laboratories than LC-MS. LC-UV can also achieve the required sensitivity in the analysis of PBZ. In this study, we wish to develop a LC-UV method for the analysis of PBZ in equine plasma. Because UV is less specific than MS, there are two major objectives: 1) to selectively extract NSAIDs from interfering components in equine plasma, , e.g. serum albumin, globulins, fibrinogen, regulatory proteins and clotting factors, and solutes such as electrolytes, amino acids, vitamins, organic acids, pigments, and enzymes; and 2) to achieve a baseline separation of PBZ from other commonly used NSAIDs for horses which include OPBZ, diclofenac, flunixin, ketoprofen, meclofenamic acid and naproxen.

Experimental

SAX-SPE procedure

1. **Sample pretreatment:** Dilute 200 µL equine plasma and 200 µL internal standard solution with 1.6 mL of 5% (V/V) ammonia in HPLC water.
2. **Column conditioning**: Use gravity flow to condition the SAX-SPE column with 1 mL methanol, followed by 1 ml water. Do not allow the column to go dry during conditioning. Recondition if the phase goes dry.
3. **Sample loading**: Use gravity flow to load the prepared 2 mL sample through the column.
4. **Column wash**: Use gravity flow to apply 2×500 µL HPLC water through the column. Then, use gravity flow to apply 2×500 µL HPLC methanol through the column. Finally, use a gentle vacuum through the column to remove residual moisture and ensure a completely dry column.
5. **Analyte elution**: Use gravity flow to apply 2×500 µL SPE eluting solution through the column.
6. **Eluate drying**: Use gentle nitrogen stream to dry the eluates completely at room temperature.
7. **Sample resuspension**: Add 200 µL HPLC mobile phase into each dried eluate and vortex for a couple of minutes.

LC Instrumental conditions

1. Column: Phenomenex Kinetex C18 (250 mm × 4.6 mm, 5 µm particle)
2. Eluent A: water with 0.1% acetic acid
3. Eluent B: methanol/acetonitrile 95:5 (v/v) with 0.1% acetic acid
4. Mobile phase composition: 68% B
5. Flow rate: 1 mL/min
6. Injection volume: 20 µL
7. Detection: UV at 254 nm

Results

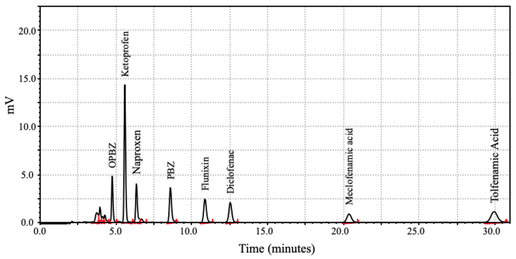
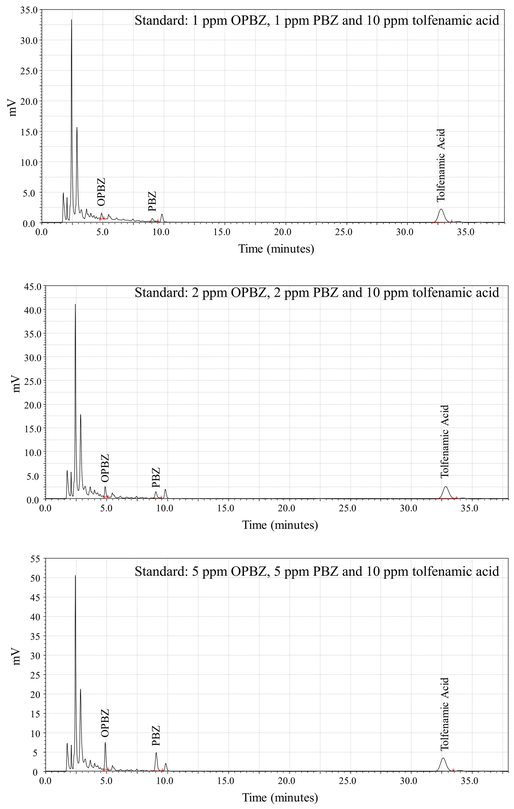
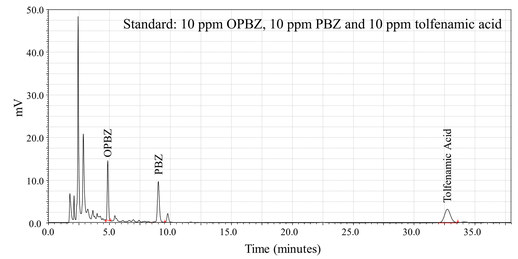
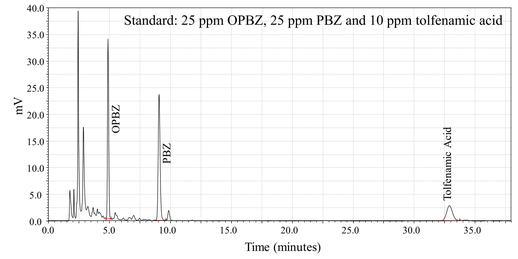


Figure 1. LC separation of 5 ppm commonly regulated NSAIDs and internal standard (tolfenamic acid) by USEF.







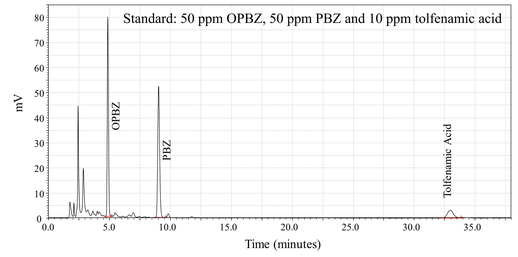


Figure 2. LC separation of OPBZ, PBZ and internal standard (tolfenamic acid) in equine plasma after SAX-SPE.

Table 1. OPBZ calibration data

Sample	C_{OPBZ} (ppm)	PA_{OPBZ}	C_{IS} (ppm)	PA_{IS}	C_{OPBZ}/C_{IS}	PA_{OPBZ}/PA_{IS}
std. 1	1.00	6801	10	74203	0.10	0.0917
std. 2	2.00	15455	10	91111	0.20	0.1696
std. 3	5.00	49197	10	116624	0.50	0.4218
std. 4	10.00	95732	10	108575	1.00	0.8817
std. 5	25.00	227705	10	95863	2.50	2.3753
std. 6	50.00	539724	10	108231	5.00	4.9868

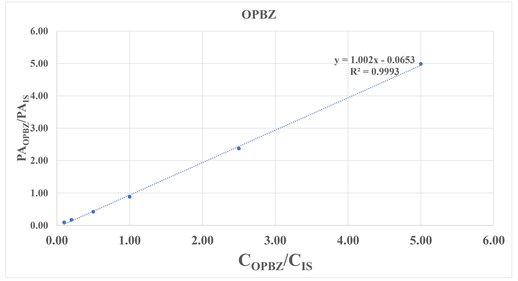


Figure 3. OPBZ internal calibration curve.

Table 2. PBZ calibration data

Sample	C_{PBZ} (ppm)	PA_{PBZ}	C_{IS} (ppm)	PA_{IS}	C_{PBZ}/C_{IS}	PA_{PBZ}/PA_{IS}
std. #1	1.00	6246	10	74203	0.10	0.0842
std. #2	2.00	15555	10	91111	0.20	0.1707
std. #3	5.00	53352	10	116624	0.50	0.4575
std. #4	10.00	106942	10	108575	1.00	0.9850
std. #5	25.00	253985	10	95863	2.50	2.6495
std. #6	50.00	562431	10	108231	5.00	5.1966

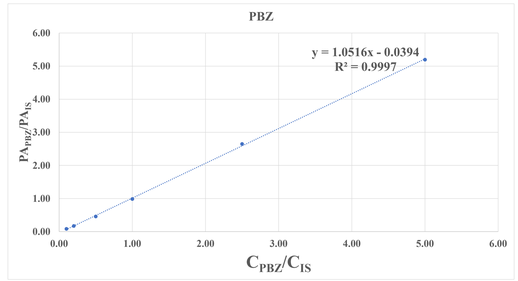


Figure 3. PBZ internal calibration curve.

$$C_{OPBZ\ or\ PBZ} = C_{OPBZ\ or\ PBZ/IS} \times C_{IS} = \frac{PA_{OPBZ\ or\ PBZ/IS} - \text{Intercept}}{\text{Slope}} \times 10\ ppm$$

Table 3. OPBZ analysis data

Sample	C_{OPBZ} (ppm)	PA_{OPBZ}	C_{IS} (ppm)	PA_{IS}	C_{OPBZ}/C_{IS}	PA_{OPBZ}/PA_{IS}
Plasma 1	5.03	14035	10	32024		0.4383
Plasma 2	4.81	42627	10	102272	0.48	0.4168
Plasma 3	5.49	34127	10	70449		0.4844
Average	5.11					
STD	0.34					
%STD	6.75					

Table 4. PBZ analysis data

Sample	C_{PBZ} (ppm)	PA_{PBZ}	C_{IS} (ppm)	PA_{IS}	C_{PBZ}/C_{IS}	PA_{PBZ}/PA_{IS}
Plasma #1	4.86	15105	10	32024		0.4717
Plasma #2	5.09	50665	10	102272	0.51	0.4954
Plasma #3	4.61	31359	10	70449		0.4451
Average	4.85					
STD	0.24					
%STD	4.93					

Conclusions

- A method using SAX-SPE followed by LC-UV for the analysis of PBZ and its metabolite, OPBZ, in equine plasma has been developed.
- Internal calibration of OPBZ and PBZ in equine plasma using linear regression have been obtained from 1 to 50 ppm.
- The lowest concentration in the linear range, i.e. 1 µg/mL, is below the plasma thresholds set by Illinois Racing Board, i.e. 2 µg/mL.
- A equine plasma with unknown concentration of OPBZ and PBZ has been analyzed in triplicate; and the %RSD of the analysis was 6.75% and 4.93% for OPBZ and PBZ, respectively.

References

1. L. R. Soma et al. J. Vet. Pharmacol. Ther., 35 (2012) 1-12.
2. B. Heffron et al. J. Anal. Toxicol., 37 (2013) 600-604.

Acknowledgement

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