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

Tiwalola Ogunleye and Madison Chao

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 naproxen 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 naproxen from other NSAIDs, internal standard, and residual components of equine plasma was achieved using LC-UV. Finally, naproxen in equine plasma was quantified after an internal calibration curve was created.

Introduction

Naproxen is a non-steroidal anti-inflammatory drug (NSAID). It is possibly the most effective NSAID for the treatment of musculoskeletal pain from soft tissue injury, muscle soreness, and bone and joint problems [1]. In racehorses, however, naproxen has the capacity to affect racing performance, thus its usage is subject to doping control regulations. In order to discriminate between doping and therapeutic use of naproxen, a threshold of 40 ppm in equine plasma has been adapted in samples collected immediately after the race by the United States Equestrian Federation (USEF).

Post-race analysis of naproxen 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 naproxen. In this study, we wish to develop a LC-UV method for the analysis of naproxen 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 naproxen from other commonly used NSAIDs for horses, i.e. phenbuazone, oxyphenbutazone, diclofenac, flunixin, ketoprofen, and meclofenamic acid.

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 SPE column with 1 mL methanol, followed by 1 ml HPLC 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 totally 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 totally at room temperature.
7. **Sample resuspension**: Add 200 µL HPLC mobile phase into each dried eluate and vertex 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

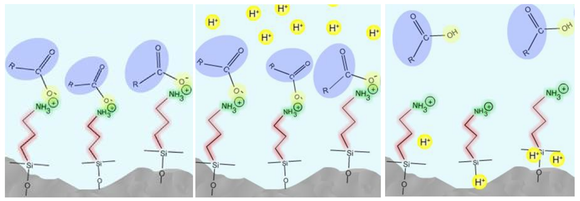


Figure 1. SAX-SPE mechanism: analyte elution through analyte neutralization (www.chromacademy.com).

Results

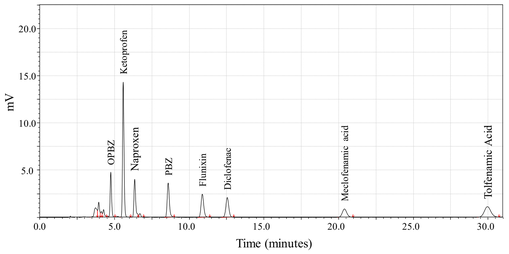
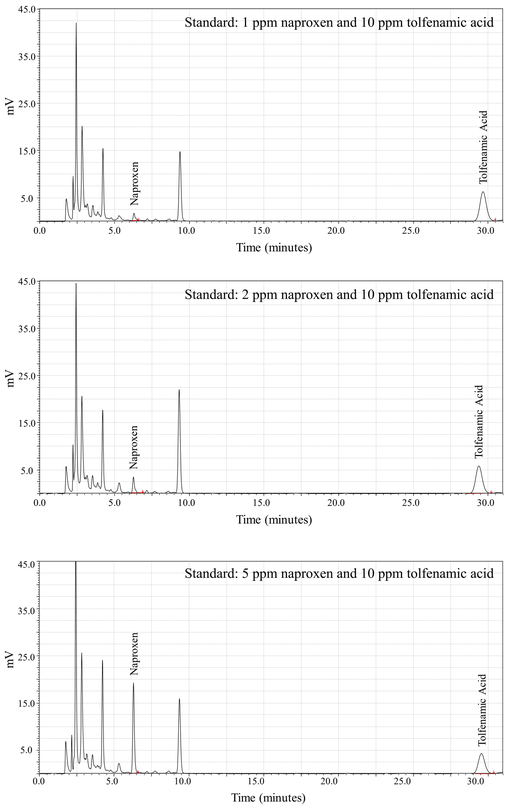


Figure 2. LC separation of 10 ppm commonly regulated NSAIDs and internal standard (tolfenamic acid) by USEF.



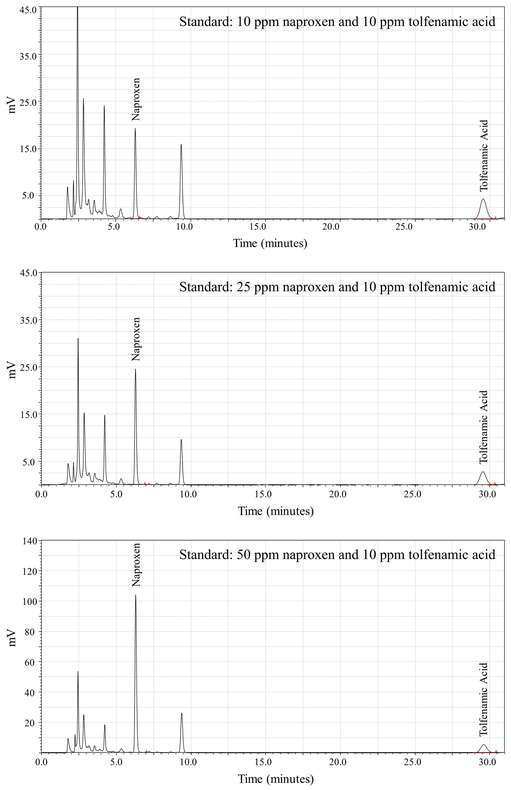


Figure 3. LC separation of standard samples in equine plasma after SAX-SPE.

Table 1. Naproxen calibration data

Sample	C_{NAP} (ppm)	PA_{NAP}	C_{IS} (ppm)	PA_{IS}	C_{NAP}/C_{IS}	PA_{NAP}/PA_{IS}
std. #1	1.00	14340	10	189837	0.10	0.0755
std. #2	2.00	29530	10	177072	0.20	0.1668
std. #3	5.00	29606	10	86841	0.50	0.3409
std. #4	10.00	146913	10	132888	1.00	1.1055
std. #5	25.00	189457	10	85922	2.50	2.2050
std. #6	50.00	795243	10	165362	5.00	4.8091

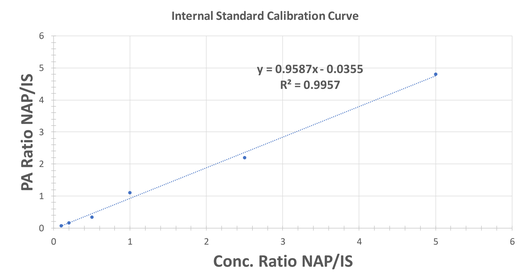


Figure 4. Naproxen internal standard calibration curve.

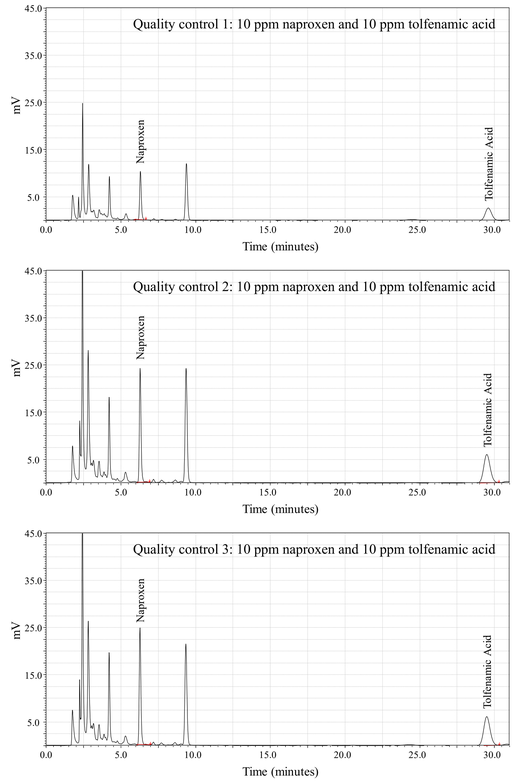


Figure 5. LC separation of quality control samples in equine plasma after SAX-SPE.

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

Table 2. Naproxen analysis data

Sample	C_{NAP} (ppm)	PA_{NAP}	C_{IS} (ppm)	PA_{IS}	C_{NAP}/C_{IS}	PA_{NAP}/PA_{IS}
Plasma 1	9.25	61969	10	72685		0.8517
Plasma 2	9.18	94472	10	111826	0.92	0.8448
Plasma 3	9.43	104749	10	120546		0.8690
Average	9.29					
STD	0.13					
%STD	1.40					

Conclusions

- A method using SAX-SPE followed by LC-UV for the analysis of naproxen in equine plasma has been developed.
- Internal calibrations of naproxen in equine plasma using linear regression have been obtained from 1 to 50 ppm.
- The lowest concentration in the linear range, i.e. 1 ppm, is well below the plasma threshold set by USEF, i.e. 40 ppm.
- A equine plasma with unknown concentration of naproxen has been analyzed; and the %RSD of four analysis was 1.40%.

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

The authors appreciate the partial financial support by NSF (CHE-1827209) and WIU CAS Undergraduate Research Grant.