Ultra-Selective and Fast Analysis of Phenylbutazone in Ground Meat using Strata™-X-A Solid Phase Extraction Cartridges and Kinetex® 2.6 µm Core-Shell Technology

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Abstract

This technical note describes a simple yet effective solid phase extraction and cleanup method for phenylbutazone from meat with recovery values > 90%. Highly specific LC/MS/MS data is generated using a Kinetex 2.6 µm XB-C18 core-shell column enabling rapid run times under 5 minutes with excellent precision and accuracy.

This analytical method provides a limit of detection (LOD) of 1 ppb (µg/Kg) and a limit of quantitation (LOQ) of 2 ppb, which is well below 5 ppb the recommended concentration proposed by the EU reference laboratory for NSAIDs.[1]

Introduction

Communities worldwide were outraged when it was discovered that horse meat had been illegally incorporated into food products. As scientific laboratories raced to identify all sources of contamination, concern was also raised about the potential for veterinary drugs such as phenylbutazone (bute) to have entered the food chain. Many veterinary drugs that are safe and approved for use in animals can pose significant health risk to humans, especially children.

In Europe, horses can be classified as food producing animals and maximum residue limits have been set for pharmacologically active substances in foodstuffs of animal origin.

The concern in Europe was that non-food producing horses, which may have been treated with phenylbutazone at some point in their life, had entered the food chain.[2]

Phenylbutazone is a non-steroidal anti-inflammatory drug (NSAIDs) commonly prescribed for horses. Phenylbutazone can cause blood dyscrasias and is a carcinogen.[3] Due to its toxicity, phenylbutazone is not approved for human use. In order to establish the compliance with the legislation, a robust analytical method for phenylbutazone is required. The meat matrix is a sample cleanup challenge due to its high complexity. Several most anticipated matrix interferences from meat are lipids, proteins, and carbohydrates. A highly selective extraction/cleanup method is necessary in order to eliminate matrix interferences which might generate false-positive or false-negative results.

Previously published analytical procedures require liquid extractions with a variety of solvents such as acetonitrile and methanol, followed by a multi-step solid phase extraction method using different retention modes and LC/MS/MS analysis with long run times.[4-7] In this study, we demonstrate an ultra-selective single step solid phase extraction of phenylbutazone and a rapid, high resolution LC/MS/MS analysis using a Kinetex core-shell HPLC column.

Reagents and Chemicals

Phenylbutazone and Phenylbutazone-D10 was purchased from Sigma and C/D/N Isotopes, respectively. Methanol, acetonitrile, ethyl acetate were obtained from JT Baker. An Omni TH hand homogenizer (Omni International, Kennesaw, GA, USA) was employed for homogenization purposes. A SecurityGuard™ ULTRA cartridge was used in line with the LC column to extend lifetime of the column.

Experimental Conditions

Sample Pretreatment

1. Add 2 mL 0.1 N NaOH and 50 µL of 4 µg/mL Int Std (phenylbutazone-D10) to 2 ±0.1 g homogenized ground beef sample in an Erlenmeyer flask (or similar).
2. Cap and shake vigorously for at least 30 seconds using a vortex.
3. Homozenize the meat sample with the Omni TH hand homogenizer machine until the sample appears as a very thick slurry, then add 8 mL of methanol to the mixture.
4. Cap and place on a mechanical shaker for 1 hour at medium high to high setting. Make sure the vessels are properly secured.
5. Transfer the mixture to a 15 mL centrifuge tube and centrifuge for 10 minutes at room temperature (18-22 °C) and 3000-3200 rpm.
6. Add 2 mL of the resulting supernatant to 2 mL of deionized (D.I.) water for solid phase extraction.

Solid Phase Extraction (SPE)

The supernatant from extracted ground meat is further cleaned up using SPE. For best results, maintain a 1-2 mL/min flow through the SPE bed.

SPE Protocol	
Cartridge:	Strata-X-A, 100 mg/6 mL
Part No.:	8B-S123-ECH
Condition:	3 mL Methanol
Equilibrate:	3 mL DI Water
Load:	4 mL of Pretreated sample
Wash 1:	2 mL D.I. Water
Wash 2:	2 mL Acetonitrile
Wash 3:	2 mL Ethyl Acetate
Dry:	5 minutes under full vacuum
Elute:	2x 1.5 mL 1 % Formic Acid in Methanol
Dry Down:	Evaporate under a stream of nitrogen gas at 50 °C to dryness
Reconstitute:	Resuspend the residue with 500 µL of 50:50 Methanol/ 0.1% Formic Acid

Chromatographic Conditions

LC/MS/MS was performed using a Kinetex 2.6 µm XB-C18 50 x 2.1 mm HPLC column (p/n 00B-4496-AN) on an Agilent® 1200SL LC system (Agilent Technologies, Palo Alto, CA, USA) with an upper pressure limit of 600 bar, equipped with a binary pump, column oven, autosampler and interfaced with an API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA). The LC/MS/MS system was setup to scan in multiple reactions monitoring (MRM) mode. An electrospray ionization source operating in positive polarity was used for this analysis. Refer to **Table 1** and **2** for the compound dependent parameters and ionization source conditions.

Column: Kinetex® 2.6 µm XB-C18
Dimensions: 50 x 2.1 mm
Part No.: 00B-4496-AN
Mobile Phase: A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Methanol

Gradient:

Time (min)	B (%)
0.00	50
0.50	50
2.00	95
3.50	95
3.51	50
5.00	50

Flow Rate: 450 µL/min
Column Temp: 45 °C
Pressure: 3670 psi (253 bar)

Table 1.
MRM Transitions and Compound Dependent Parameters

ID	Q1	Q1	Dwell	DP	CE	CXP
Phenylbutazone 1	309.2	120	75	50	27	10
Phenylbutazone 2	309.2	160	75	50	29	13
Phenylbutazone 3	309.2	188	75	50	23	18

Table 2.
Electrospray Ionization Source Conditions

Nebulizing Gas (Gas1)	50
Drying Gas (Gas2)	50
Collision Gas (CAD)	6
Curtain Gas (CUR)	20
Drying Gas Temp (Tem)	600
IonSpray Voltage (IS)	5500

Results and Discussion

For prohibited and unauthorized analytes, the limit of detection (LOD) must be at or less than the Minimum Required Performance Limit (MRPL) as defined in Commission Decision 2002/657/EC.[8]

The determination of phenylbutazone from meat requires a LOD below 5 ppb. A highly sensitive detector, supported by a selective sample preparation are required in order to accurately analyze phenylbutazone at such low levels.

Conventional sample cleanup consists of an enzymatic hydrolysis followed by liquid extraction and solid phase extraction cleanup using a normal phase cartridge for lipid removal and a reversed phase cartridge for further clean up and analyte concentration. In this study, we employed a polymeric strong anion exchange method using Strata™-X-A to replace the two-step SPE cleanup method. Phenylbutazone has a strongly acidic α-hydrogen that is ionized above pH ~5 (**Figure 1**). In its ionized form, phenylbutazone can retain very strongly via ion-exchange mechanism on the Strata-X-A sorbent, which is surface-modified with quaternary amine groups. The powerful ion-exchange interactions allow for two aggressive washes— acetonitrile followed by ethyl acetate to furnish an extremely thorough cleanup of the meat matrix. The recoveries of phenylbutazone extracted by the Strata-X-A method are shown in **Table 3**. Ground beef spiked at 5 and 75 ppb were extracted and analyzed against a matrix matched calibration curve (**Figure 2**). The matrix-matched calibration curve of 1- 200 ppb (µg/Kg) yielded a linear fit (r=0.9991) with 1/x weighting factor, where x is the concentration. The LOD of this method is 1 ppb (µg/Kg). The 2 ppb (µg/Kg) spiked meat extract produced an average accuracy of 108.2% (n=2) against the calibration curve and can be used as the LOQ. In this study, a minimal injection volume of 5 µL was used. A slightly larger injection volume can further improve both LOD & LOQ levels.

Figure 1.
Phenylbutazone and Phenylbutazone-D10 Chemical Structures

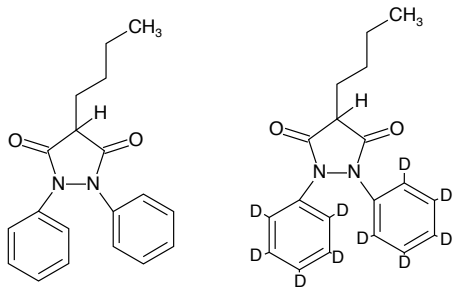


Figure 2.
Linearity Curve of Phenylbutazone from Meat extract (1-200 ppb µg/Kg) Linear with 1/x weighting factor, CC=0.9991

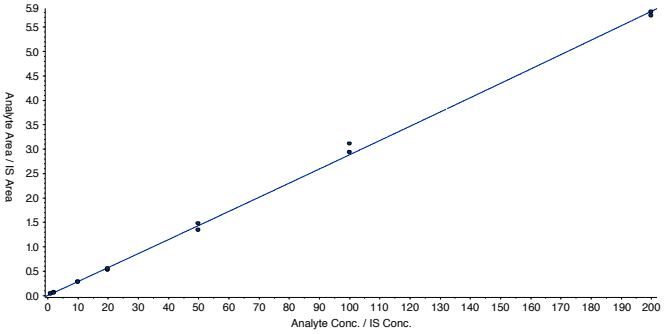


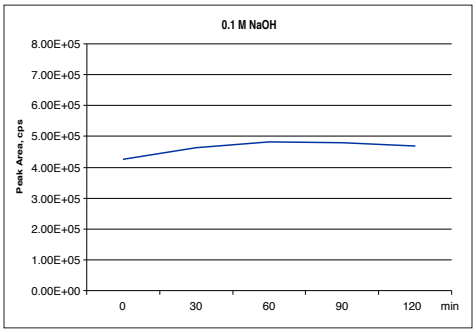
Table 3.
% Recovery of Phenylbutazone from Beef Extract at 5 ppb and 75 ppb (µg/Kg) n=4

Spiked Conc.	%CV	Accuracy
5	8.02	100.7
75	5.0	90.3

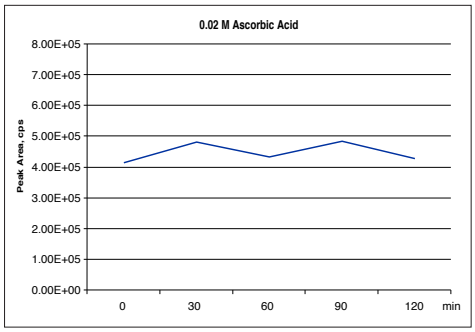
It has been reported that phenylbutazone suffers decomposition during analysis and ascorbic acid has been used as a preservative.[9] In order to ensure no degradation of phenylbutazone would occur during the analysis, a two-hour stability study in two different medium, alkaline (high pH) and acidic containing ascorbic acid was performed. Results showed no significant variation or degradation in phenylbutazone response in all tested solutions. (**Figure 3**)

Figure 3.
Stability study data of phenylbutazone in **(a)** an alkaline solution without preservative; **(b)** an acidic solution containing 0.02 M ascorbic acid as preservative

(a)



(b)



For LC/MS/MS analysis, a Kinetex® 2.6 µm XB-C18 core-shell HPLC column was chosen. The innovative core-shell technology can provide sub-2 µm performance at backpressure compatible with standard HPLC instruments. The 2.6 µm particle gives backpressure similar to a 3 µm particle, but the improved mass transfer kinetics of the core-shell particle significantly enhance resolving power, giving sub-2 µm performance. In the current analysis, the backpressure generated was 253 bar. The chromatogram of 10 ppb (µg/Kg) spiked ground beef extract is shown in **Figure 4**.

It is interesting to mention that during LC/MS/MS method development, the MRM transition 309.2→ 160.0 displayed a persistent broad peak eluting at a later retention time than phenylbutazone (**Figure 5**). This may have been a mobile phase related impurity that was introduced onto the LC column during the column equilibration period and did not come from the sample or extraction procedure. In ground beef extract, the same channel also displayed a sharp peak almost co-eluting with the other impurity peak observed earlier. However, both of these peaks are well separated from phenylbutazone on the Kinetex 2.6 µm core-shell particle combined with unique XB-C18 chemistry. The resolution of these impurities further allows the suitability of three MRM channels for low level determination (both screening and confirmation) of this toxic anti-inflammatory residue in meat.

Figure 4.
Chromatogram of 10 ppb (µg/Kg) Phenylbutazone and its internal standard (Phenylbutazone-D10) on Kinetex 2.6 µm XB-C18 50 x 2.1 mm. Peak assignment: 1) Phenylbutazone-D10 (I.S.), 2) Phenylbutazone, 3) Solvent/MP impurity

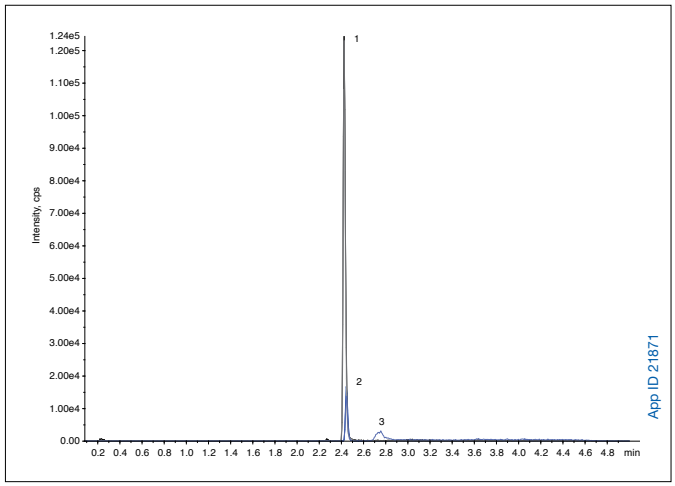
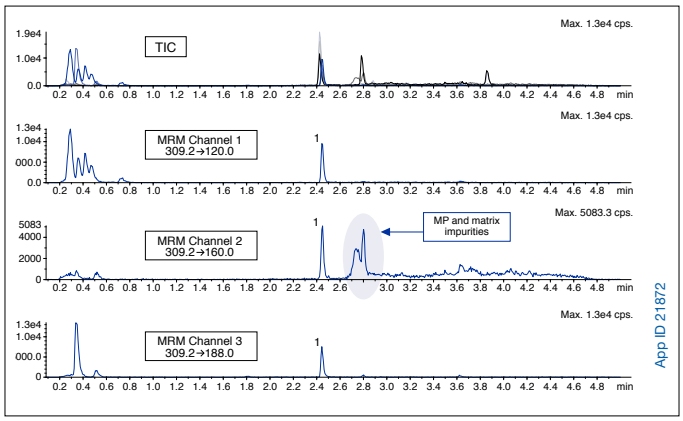


Figure 5.
A representative chromatogram of phenylbutazone extract from ground beef. Total ion chromatogram (TIC) and each individual MRM channels are displayed for clarity. Peak assignment: 1) Phenylbutazone



Conclusion

The current technical note describes a rapid and robust method to quantify and unequivocally identify phenylbutazone in ground meat samples. The single step strong anion exchange SPE method eliminates problematic lipids, proteins, and other matrix interferences prior to a high efficiency HPLC method using a Kinetex 2.6 µm XB-C18 column. This method generated a matrix-matched calibration with excellent linearity, which provided high precision and accuracy. The estimated detection and quantification limits are well below 5 ppb, the method performance requirement proposed by the EU reference laboratory for NSAIDs.

The sample pretreatment procedure is flexible to incorporate an enzymatic hydrolysis step if needed, as the highly selective strong anion exchange extraction and cleanup method is able to specifically extract and concentrate phenylbutazone amidst any intricate matrices.

References

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APPLICATIONS

Ordering Information

Kinetex® 2.6 µm Analytical Columns (mm)

	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard™ ULTRA Cartridges /3pk
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AJ0-8770
PFP	00A-4477-E0	00B-4477-E0	00C-4477-E0	00D-4477-E0	00F-4477-E0	AJ0-8773
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	AJ0-8774

SecurityGuard ULTRA cartridges require holder, Part No.: AJ0-9000. for 4.6 mm ID

2.6 µm Minibore Columns (mm)

	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard™ ULTRA Cartridges /3pk
XB-C18	00A-4496-AN	00B-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
PFP	00A-4477-AN	00B-4477-AN	00D-4477-AN	00F-4477-AN	AJ0-8787
HILIC	00A-4461-AN	00B-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788

for 2.1 mm ID

Strata™-X-A

Format	Sorbent Mass	Part Number	Unit
Tube			
	30 mg	8B-S123-TAK**	1 mL (100/box)
	30 mg	8B-S123-TBJ	3 mL (50/box)
	60 mg	8B-S123-UBJ	3 mL (50/box)
	100 mg	8B-S123-EBJ	3 mL (50/box)
	100 mg	8B-S123-ECH	6 mL (30/box)
	200 mg	8B-S123-FBJ	3 mL (50/box)
	200 mg	8B-S123-FCH	6 mL (30/box)
	500 mg	8B-S123-HBJ	3 mL (50/box)
	500 mg	8B-S123-HCH	6 mL (30/box)
Giga™ Tube			
	500 mg	8B-S123-HDG	12 mL (20/box)
	1 g	8B-S123-JDG	12 mL (20/box)
	1 g	8B-S123-JEG	20 mL (20/box)
	2 g	8B-S123-KEG	20 mL (20/box)
	5 g	8B-S123-LFF	60 mL (16/box)
96-Well Plate			
	10 mg	8E-S123-AGB	2 Plates/Box
	30 mg	8E-S123-TGB	2 Plates/Box
	60 mg	8E-S123-UGB	2 Plates/Box

**Tab-less tubes available. Contact Phenomenex for details.

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