

DEVELOPMENT OF AN ELISA FOR THE DETECTION OF PHENYLBUTAZONE AND OXYPHENBUTAZONE

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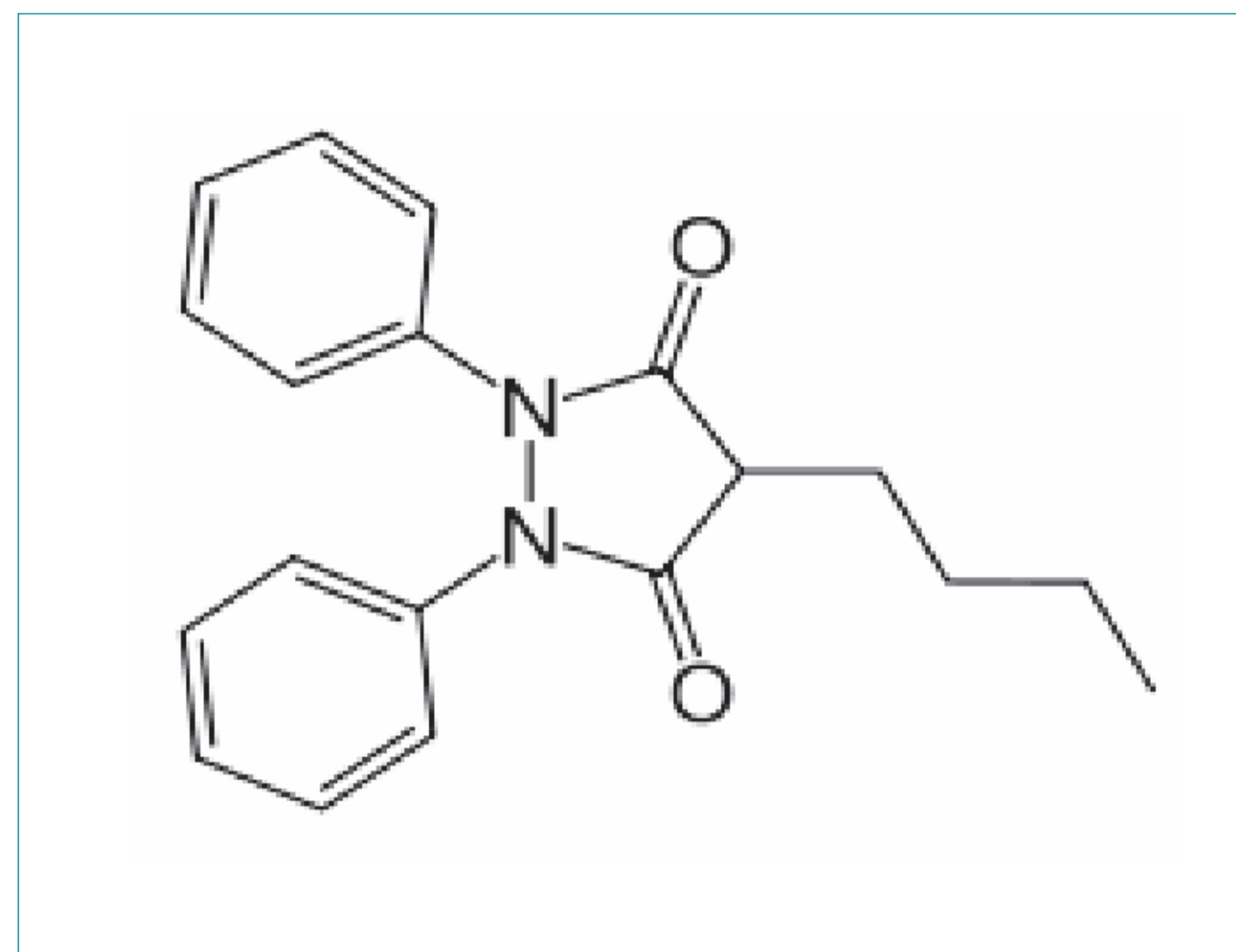
Introduction

Phenylbutazone was one of the first non-steroidal anti-inflammatory drugs (NSAIDs) approved for use in horses and dogs. High doses of phenylbutazone may be considered a rules violation under some equestrian organizations, as the drug may remain in the bloodstream four to five days

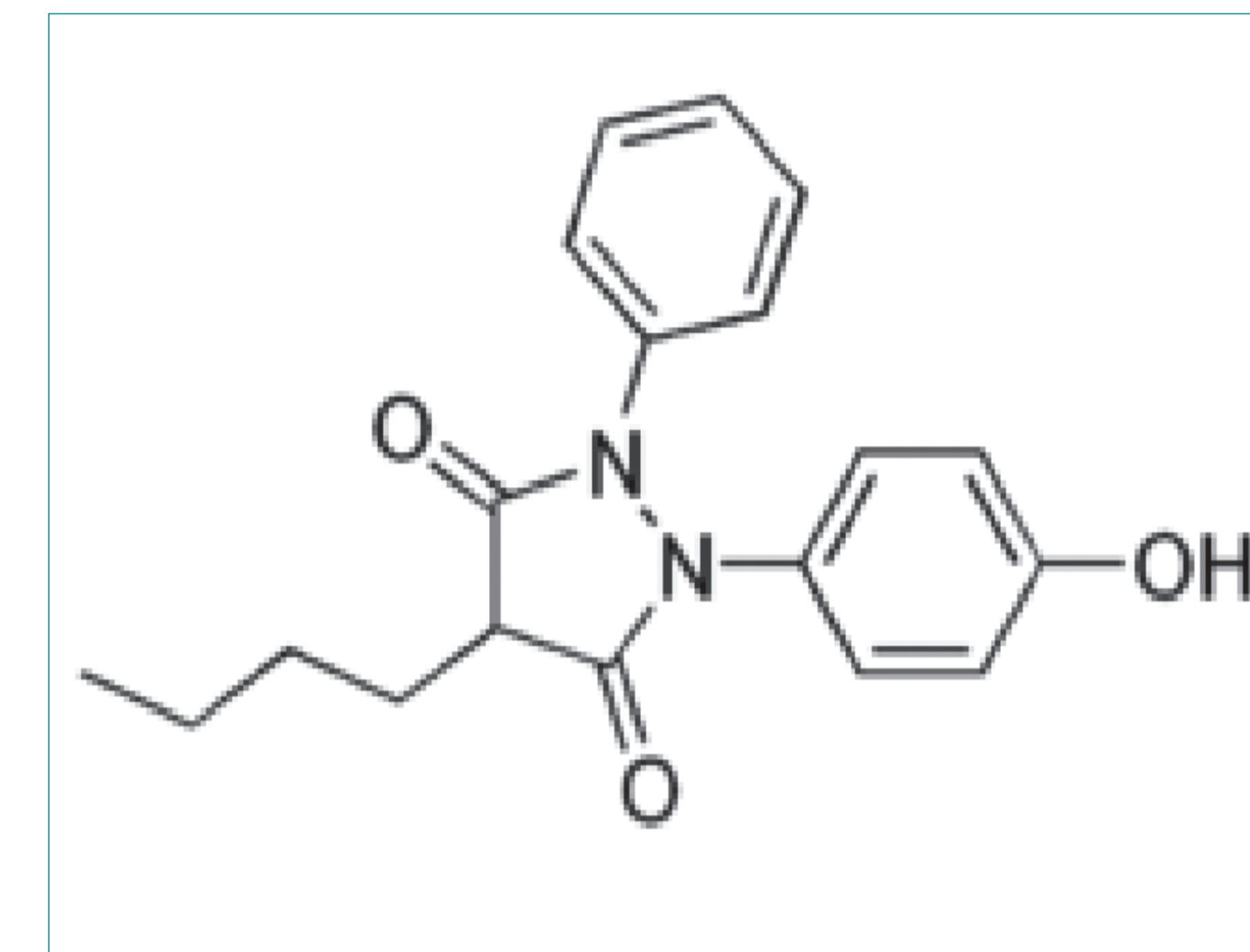
after administration. Currently, the use of phenylbutazone in food producing animals is prohibited in most countries, MRLs have not been set for phenylbutazone residues and any detection is considered a violation. We report the development of a competitive ELISA for

the detection of **phenylbutazone** and its **metabolite oxyphenbutazone** in serum/plasma samples, which is of interest for monitoring and regulatory purposes.

Chemical Structures



Phenylbutazone



Oxyphenbutazone

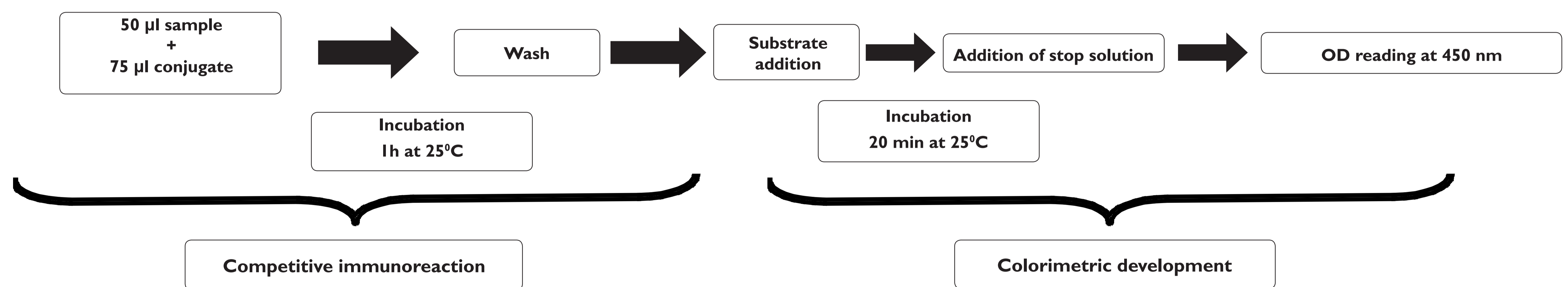
Methodology

Competitive ELISA

In-house made capture antibody was immobilised and stabilised on 96 well microtitre plates. The assay is based on a competitive reaction where any free analyte contained in the standards/samples competes for binding sites of the capture antibody with horseradish

peroxidase labelled conjugate. Following the incubation and washing steps, enzyme substrate is added. Measurement of the optical density is carried out at 450nm once the colour reaction is stopped producing a colour change from blue to yellow. Colour intensity is inversely proportional to the concentration of the analyte present.

Sample Preparation (serum/plasma):
Dilution 1/5 with diluted diluent/wash buffer.



The analytical performance of the developed ELISA kit (PB3456, Randox Laboratories, Crumlin, UK) was assessed.

Analytical parameters

Limit of Detection (LOD)
LOD was defined as mean concentration of negative samples + 3SD.

Specificity/Cross-reactivity
The specificity, expressed as % cross-reactivity (%CR) was calculated as follows:
 $\%CR = \frac{[IC_{50}(\text{Phenylbutazone})]}{[IC_{50}(\text{Cross-reactant})]} \times 100$
The IC₅₀ for each analyte was calculated by taking 50% of the optical density (OD) from the zero calibrator and reading this OD value from

the x-axis (concentration in ng/ml) of the respective calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Precision
Intra-assay precision (n=12) was determined from the mean results corresponding to six different concentration levels within the same run and was expressed as %CV.

Recovery
A negative sample was spiked at a concentration 5 ng/ml. 20 replicates were assessed. Results were expressed as % recovery.

Results

Limit of Detection (LOD) - Phenylbutazone

Limit of Detection* **2.01 ng/ml**
Calibration Range **0-90 ng/ml**

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*Includes include assay dilution factor (x5).

Specificity/Cross-reactivity (CR)

Analyte	% CR
Phenylbutazone	100
Oxyphenbutazone	156

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Precision

Analyte	Intra-assay precision (n=12)					
	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Phenylbutazone	%CV	%CV	%CV	%CV	%CV	%CV
	1.9	3.1	2.5	2.2	2.4	2.5

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Recovery

Analyte	% Recovery (n=20)
Phenylbutazone	92%

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Conclusion

- Data indicate that the developed competitive ELISA detects phenylbutazone and its metabolite oxyphenbutazone in serum/plasma samples.
- Simple sample preparation, only dilution required (1/5).
- LOD = 2.01 ng/ml for phenylbutazone. Intra precision values are typically %CV≤3% over a range of concentration levels and the %recovery was 92% for a concentration of 5 ng/ml.
- Once applied to the microtitre plate 40 samples can be screened in 90min.
- The developed ELISA represents a valuable and convenient analytical tool that can be used for the in vitro determination of phenylbutazone and oxyphenbutazone in serum/plasma samples.