

Plasma and urine pharmacokinetics of two formulations of dexamethasone in greyhound dogs

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Abstract

Dexamethasone, formulated as sodium phosphate and as phenylpropionate combined with sodium phosphate, was administered subcutaneously to six greyhounds. Plasma and urine were collected for up to 240 h and analysed with a limit of quantification (LOQ) of at least 100 pg/ml for dexamethasone. Dexamethasone, formulated as sodium phosphate, terminal half-life was 10.4 h in plasma and approximately 16 h in urine, and at 96 h, plasma hydrocortisone concentrations returned to background with dexamethasone levels around the LOQ. Dexamethasone, formulated as phenylpropionate combined with sodium phosphate, terminal half-life, was 25.6 h in plasma and approximately 26 h in urine, and at 96 h, plasma hydrocortisone concentrations returned to background with dexamethasone levels in three of the six greyhounds around the LOQ. Critical assessment of the pharmacokinetic and pharmacodynamic data indicated how it might be utilized for medication control in racing greyhounds.

KEYWORDS

dexamethasone, greyhound, pharmacodynamics, pharmacokinetics, plasma, urine

1 | INTRODUCTION

Dexamethasone, a synthetic corticosteroid, is a commonly used veterinary medicine. Injectable preparations require formulation, often as esters, to take account of the very limited solubility of glucocorticoids in water and to provide a range of formulations that extend duration of action (Bishop, 2000). For example, dexamethasone sodium phosphate is resorbed rapidly from the injection site, thus ensuring a rapid onset of activity, whereas dexamethasone phenylpropionate is absorbed more slowly from the injection site, thus ensuring a more prolonged duration of activity (Australian Pesticides & Veterinary Medicines Authority, 2002).

There are limited data on the pharmacokinetics of dexamethasone in dogs. 1 mg/kg of dexamethasone in alcohol or as isonicotinate was given intravenously or intramuscularly, respectively, to five mixed breed dogs (Toutain et al., 1983). Dexamethasone in plasma was measured for 10 h after administration, and the limit of quantification (LOQ) was 2 ng/ml. 1 mg of an undefined formulation of dexamethasone was injected intramuscularly into 25 greyhounds, and urine samples collected for up to 96 h (Hill et al., 1997) with dexamethasone being detected for 24 h.

For medication control of drugs for animals used in sport, urine is the sample matrix of choice (Morris, 2014). To inform risk assessment for medication control, information on contemporaneous plasma (as plasma levels drive effects) and urine levels, after clinical

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doses of the formulations used by clinicians, that define the terminal elimination phases, and with limits of quantification that are appropriate for medication control rather than therapeutics, is required.

The primary objective of this study was to describe extended plasma and urine concentrations and the pharmacokinetics of dexamethasone in greyhound dogs following administration of two commonly available commercial injectable preparations of dexamethasone, using a highly sensitive analytical assay. A secondary objective was to describe the pharmacodynamic effects, specifically the suppression of endogenous hydrocortisone. This data could then inform discussions of medication control in racing greyhounds.

2 | MATERIALS AND METHODS

Dexamethasone was administered as a single dose in two separate formulations. 2 mg/ml of dexamethasone sodium phosphate, equivalent to 1.52 mg/ml dexamethasone, (Dexadreson[®] Injection, MSD Animal Health Australia, Macquarie Park, NSW [DXD]) was given subcutaneously at a dose of 1.52 mg/dog. 1 mg/ml of dexamethasone sodium phosphate and 2 mg/ml as dexamethasone phenylpropionate, equivalent to 2.26 mg/ml dexamethasone, (Dexafort[®] Injection, MSD Animal Health Australia, Macquarie Park, NSW [DXF]) was given subcutaneously at a dose of 1.13 mg/dog. The two formulations were given at separate times; DXD administration was separated by 36 days from the day of DXF administration. Three neutered female and three entire male greyhounds were studied with a mean bodyweight of 32 kg (range 27.8–37.4 kg) and mean age of 3.8 years (range 3–5 years). Blood and urine were collected before drug administration. Blood samples were collected after 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h for both formulations. Urine samples were collected after 2, 4, 6, 8, 10, 12, 24, 48, 72, 96, 120 and 144 h for both formulations, and also at 168, 192, 216 and 240 h for the DXF formulation. The blood samples were heparinized and plasma collected by centrifugation at 4000 rpm (for 10 min). All samples were stored at –20°C. The frozen samples were transported on dry ice to the analytical laboratory.

Dogs were fed a commercial dry dog food (Dogpro PLUS Working Dog, Hypro Petcare P/L) with an additional portion of fresh meat, with the daily feed ration as two meals and had access to water at all times. 100–200 g of meat was given on the treatment day prior to drug administration. The study was conducted in accordance with the principles of the VICH GCP guidelines (International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, Good Clinical Practice, June 2000, effective July 2001). Ethics approval (TRIM 15/699(156)) was collected from the Secretary's Animal Care and Ethics Committee of the NSW Department of Primary Industry.

Concentrations of dexamethasone and hydrocortisone were measured in the pre- and post-administration urine and plasma. Urine samples (1 ml) were diluted with ammonium acetate buffer (0.5 M, pH 5.5, 6 ml), pH adjusted to 5.5–6, and then, enzyme hydrolysed with beta

glucuronidase. Samples were then extracted on mixed-mode C8-SCX columns (Bond Elut-Certify, 130 mg, 3 ml, Agilent, CA, USA) previously conditioned with methanol (2 ml) and water (2 ml). After urine loading, the column was washed with water (4 ml) then acetic acid (1 M, 2 ml) for pH adjustment and dried with nitrogen at 200 ml/sec for 10 min. The acid/neutral fraction was eluted with dichloromethane/ethyl acetate (4:1, 2 ml) and then washed with sodium hydroxide/sodium chloride (1 M/0.15 M, 0.5 ml). The organic layer was removed and evaporated under nitrogen at 50°C and reconstituted in formic acid/ammonium formate (0.1%) and methanol (50:50) and submitted for LCMS analysis.

Plasma samples (500 µl) were diluted with methanol (150 µl) followed by 2% formic acid (3.5 ml) before being transferred to solid-phase extraction cartridges (Bond Elut Plexa PCX, 30 mg, 3 ml, Agilent, CA, USA) previously conditioned with methanol (2 ml) followed by formic acid (2%, 2 ml). Cartridges were then washed with formic acid (2%, 2 ml) and dried with nitrogen at 200 ml/sec for 10 min. The acid/neutral fraction was eluted with ethyl acetate/methanol (9:1, 2 ml), evaporated under nitrogen at 50°C and then reconstituted in formic acid/ammonium formate (0.1%/0.1%) and methanol (50:50) and submitted for LCMS analysis.

Urine and plasma extracts were analysed by liquid chromatography-mass spectrometry using a Shimadzu 8060 triple quadrupole mass spectrometer (Shimadzu Corp., Kyoto, Japan) coupled to a Nexera LC-30AD (Shimadzu Corp., Kyoto, Japan) liquid chromatography. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Electrospray ionization was carried out with heater block, interface and DL temperatures of 300°C, 300°C and 275°C, respectively. The nebulizer, heating and drying gas flow rates were 3, 10 and 5 L/min, respectively.

For urine, dexamethasone was monitored in positive ion mode using the de-fluorinated in-source fragment of 373.1 as the precursor ion and daughter ions *m/z* 147.1 (for quantification) and *m/z* 171.1 (for identification). The internal standard, dexamethasone-d₄, was monitored using the transition *m/z* 377.1 >*m/z* 149.1. Hydrocortisone was monitored in positive ion mode using the precursor ion *m/z* 363.1 and daughter ions *m/z* 121.1 (for quantification) and *m/z* 309.1 (for identification). The internal standard, hydrocortisone-d₄, was monitored using the transition *m/z* 364.1 >*m/z* 121.1.

For plasma, dexamethasone was monitored in negative ion mode using the formate adduct of 437.2 as the precursor ion and daughter ion of *m/z* 361.3. The internal standard, dexamethasone-d₄, was monitored using the transition *m/z* 441.2 >*m/z* 363.2. Hydrocortisone was monitored using the same transitions as the urine method.

Chromatographic separation was achieved using a Poroshell 120 EC-C18 column (3 mm × 50 mm, 2.7 µm particle size) (Agilent Technologies, CA, USA). The mobile phase consisted of formic acid (0.1%) and ammonium formate (0.1%) (A) and methanol (B). The initial composition was 50% B, which was held for 0.3 min and then ramped to reach 98% B at 2.5 min. This was held for 1.5 min before being returned to 50% B and equilibrated for 1.5 min.

TABLE 1 Calibration ranges, limits of quantification (LOQ), limits of detection (LOD), correlation coefficients (R^2), inter-batch variability of precision and accuracy for the analysis of dexamethasone and hydrocortisone in plasma and urine

Plasma	Range	LOQ	LOD	R^2	Precision (% CV)	Accuracy
Dexamethasone	0–10 ng/ml	0.01 ng/ml	0.01 ng/ml	>.99	8.1%	5.4%
Hydrocortisone	0–10 ng/ml	0.5 ng/ml	0.1 ng/ml	>.99	0.9%	6.0%
Urine	Range	LOQ	LOD	R^2	Precision (% CV)	Accuracy
Dexamethasone	0–500 ng/ml	0.1 ng/ml	0.1 ng/ml	>.99	12.8%	8.2%
Hydrocortisone	0–200 ng/ml	0.5 ng/ml	0.25 ng/ml	>.99	11.7%	12.7%

Calibration ranges, correlation coefficients, limits of quantification (LOQ), and detection (LOD) and inter-batch variability of precision and accuracy for all methods are listed in Table 1.

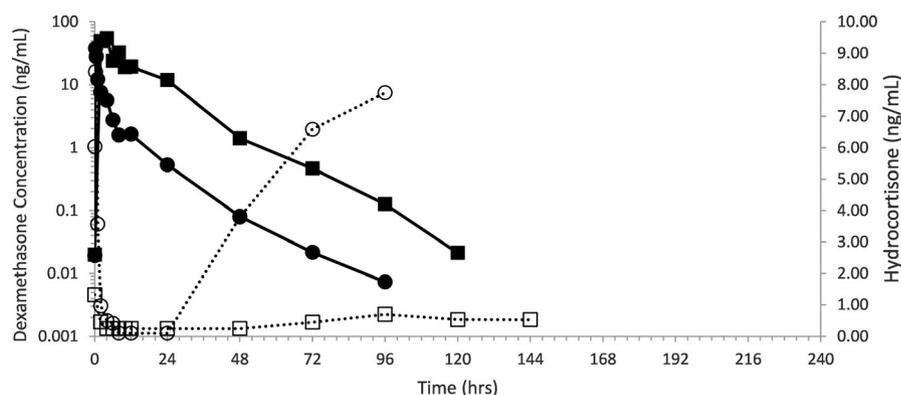
The PK profiles were analysed with a non-compartmental analysis using model 200–202 of Phoenix[®] WinNonlin[®] version 8.3 (Certara USA, Inc., Princeton, NJ) with logarithmic trapezoidal rule. Furthermore, the slopes and resulting half-lives for decay phases were determined from a linear regression fit to natural log concentration data as the two dexamethasone formulations showed complex decay phases in the plasma data.

Statistical analyses were performed using commercially available software (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA) to assess significant differences in hydrocortisone concentrations between

base line ($t = 0$) and each time point following dexamethasone administration. Data were analysed using mixed-effects analysis of variance, with the dog as the random effect and with time as the fixed effect. Post hoc comparisons were performed with a Dunnett's multiple-comparison test to preserve a nominal significance of 0.05.

3 | RESULTS

The elimination profile in six greyhounds after dexamethasone sodium phosphate administration (DXD—Dexadreson[®] formulation) in plasma and urine are shown in Figure 1 (first 24 h shown in Appendix S1) and the pharmacokinetic parameters are in Table 2.

FIGURE 1 Mean dexamethasone (filled symbols) and hydrocortisone (open symbols) concentration in plasma (circle symbols) and urine (square symbols) after administration of dexamethasone sodium phosphate (DXD) to six greyhounds**TABLE 2** Non-compartmental parameters for dexamethasone after administration of dexamethasone sodium phosphate (DXD) to six greyhounds

Animal	Weight (Kg)	C _{max} (ng/ml)	CL/F (mL/min/Kg)	HL1 (hrs)	HL2 (hrs)	HL3 (hrs)
1	34.7	37.2	8.75	0.427	2.23	10.7
2	29.8	39.7	9.81	0.61	2.55	9.28
3	36.8	46.7	8.14	0.379	2.23	11.0
4	33.5	32.9	9.44	0.43	2.36	5.96
5	29.4	29.2	11.5	0.489	2.68	13.8
6	27.8	43.3	7.98	0.453	2.79	14.2
Mean	32.0	38.2	9.27	0.455 ^a	2.45 ^a	9.95 ^a
Median	31.7	38.4	9.09	0.442	2.46	10.8

Note: C_{max} (peak plasma concentration), CL/F (mean apparent clearance adjusted for bioavailability), HL (half-life for each successive decay phase).

^aHarmonic mean.

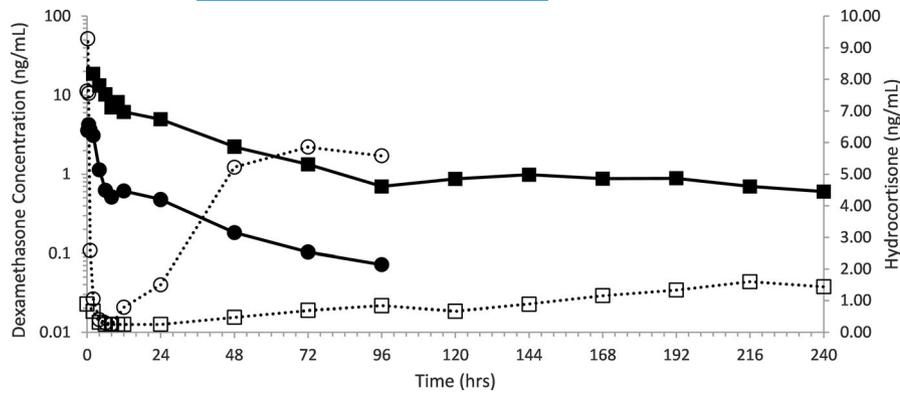


FIGURE 2 Mean dexamethasone (filled symbols) and hydrocortisone (open symbols) concentration in plasma (circle symbols) and urine (square symbols) after administration of dexamethasone sodium phosphate and dexamethasone phenylpropionate (DXF) to six greyhounds

Dexamethasone concentrations in plasma reach their peak by 15 min post-administration before declining rapidly, with a harmonic mean half-life of 0.46 h. At approximately 2 h, there appears to be a second slower decay phase which flattens between 6 and 8 h, with a geometric mean half-life of 2.45 h and then further decays into an even slower third phase, with a harmonic mean half-life of 9.95 h. The mean apparent clearance adjusted for subcutaneous bioavailability was 9.27 ml/min/Kg. Dexamethasone concentrations in urine increase rapidly over the first 2–4 h before declining and then flatten between 6 and 12 h. This is then followed by a slower second decay phase with an approximate half-life of 16 h, a similar order to the half-life for the third-decay phase observed in plasma. Figure 1 also shows how hydrocortisone concentrations decrease rapidly after the administration of DXD and then stay below the LOD until 24 h. After this time, hydrocortisone concentrations start to rise and all 6 dogs appear to return to background levels by 96 h; however, statistically significant differences from baseline using the Dunnett's post hoc test are only observed between 2 and 24 h.

The elimination profile in six greyhounds after dexamethasone sodium phosphate and dexamethasone phenylpropionate, administration (DXF—Dexafort[®] formulation) in plasma and urine are shown in Figure 2 (first 24 h shown in Appendix S1) and the pharmacokinetic parameters are in Table 3. Dexamethasone plasma concentrations increase rapidly over the first 2 h before declining rapidly with a harmonic mean half-life of 1.95 h. At approximately 8 h, there appears to be a second phase of absorption with dexamethasone concentrations increasing until 12 h followed by a slower decline, with a harmonic mean half-life of 25.3 h. The mean apparent clearance adjusted for subcutaneous bioavailability was 16.0 ml/min/Kg. Dexamethasone urine concentrations increase rapidly over the first 2 h before declining, and at approximately 8 h, there appears to be a second phase of absorption although less pronounced than in plasma. At 12 h, urine concentrations decline with a half-life of approximately 25 h, which is very similar to the second phase plasma half-life. At 100 h post-administration, the urine pharmacokinetics enters a very long phase with a half-life of approximately 200 h. Figure 2 also shows how hydrocortisone concentrations decrease rapidly after the administration of DXF and then stay below the LOD until 8 h. After this time, hydrocortisone concentrations start to rise and 3 out of the 6 dogs appear to return to background by 96 h;

however, statistically significant differences from baseline using the Dunnett's post hoc test are only observed between 2 and 24 h.

4 | DISCUSSION

The data produced in this study were compatible with the much more limited existing data that have informed clinical usage for many years (Hill et al., 1997; Toutain et al., 1983): For example, Toutain et al., 1984 determined a clearance of 6.4 ml/min/kg for IV administration of dexamethasone to dogs. This study herein usefully extends the duration of plasma data, adds extended urine data, as well as an indication of one component of the pharmacodynamic effect by measuring the reduction in endogenous hydrocortisone. As such, it addressed the primary and secondary objectives set out for this study and makes this information available for wider scientific and clinical use. Moreover, the data from this study can be combined with existing and future data and analysed using a population approach such as Non-Linear Mixed Effects (NLME) methodology (Schoemaker & Cohen, 1996). NLME is appropriate for analysing unbalanced data sets collected with analytical techniques of different sensitivity, but having generated similar data above the LOQ. Such an aggregation of data will solve the question of low statistical power of individual data sets and will facilitate harmonization between regulatory jurisdictions.

The data from this study also provide scientific information for the first stage, risk assessment (Toutain, 2010), of medication control. There are two established methodologies to derive medication control parameters from such administration studies (Toutain, 2013). The first methodology used in most horse and greyhound regulatory jurisdictions is to use the data to estimate the effective plasma concentration (EPC), irrelevant plasma concentration (IPC), irrelevant urine concentration (IUC) and so derive a screening limit (SL) and detection time (DT) (Toutain & Lassourd, 2002). The second methodology used in US horseracing is similar to that used for determining drug residues in meat for human safety such that, with a risk of $\leq 5\%$, that at least 95% of the animal population is under the specified drug level as for example used for dexamethasone (European Agency for the Evaluation of Medicinal Products & Committee for Veterinary Medicinal Products, 1997). It is important to be clear on the methodology used, for example, the latter approach can tend be

TABLE 3 Non-compartmental parameters for dexamethasone after administration of dexamethasone sodium phosphate and dexamethasone phenylpropionate (DXF) to six greyhounds

Animal	Weight (Kg)	Cmax (ng/ml)	CL/F (ml/min/Kg)	HL1 (hrs)	HL2 (hrs)
1	34.7	4.27	17.4	2.35	27.5
2	29.8	5.89	15.9	2	21.3
3	36.8	4.09	12.6	2.39	33.1
4	33.5	4.34	21.7	1.74	20.3
5	29.4	4.36	16.5	1.76	26.3
6	27.8	4.60	12.0	1.69	27.5
Mean	32	4.59	16.0	1.95 ^a	25.3 ^a
Median	31.65	4.35	16.2	1.88	26.9

Note: Cmax (peak plasma concentration), CL/F (mean apparent clearance adjusted for bioavailability), HL (half-life for each successive decay phase).

^aHarmonic mean.

more permissive in terms of drug levels, which will be an important factor in the second stage of medication control; risk management by regulators informed by scientific advice (Toutain, 2013).

A considerable body of published medication control parameters using the former methodology (EPC, IPC, IUC, SL, DT) is being utilized by regulators in horse racing and now in greyhound racing. However, using this approach can be questioned in some situations. With drugs that act both locally and systemically, such as glucocorticoids, there is no single concentration value which covers every site of action and the use of an IPC/IUC may be challenged (Toutain, 2010), although it has already been used in respect of dexamethasone isonicotinate in racehorses (Ekstrand et al., 2015).

Most administration studies for medication control focus on drug pharmacokinetics and do not include pharmacodynamic measurements. Glucocorticoids have both rapid and slower pharmacodynamic effects and the Toutain and Lassourd (2002) methodology also may not be appropriate if there is significant hysteresis. Rapid, non-genomic glucocorticoid actions are mediated through physiochemical interactions with cytosolic glucocorticoid receptor (GR) or membrane-bound GR. Non-genomic effects of glucocorticoids do not require protein synthesis and occur within seconds to minutes of GR activation. For example, when released from the inactive GR protein complex, the non-receptor tyrosine kinase protein c-Src activates signalling cascades that inhibit phospholipase A2 activity, phosphorylate annexin 1 and impair the release of arachidonic acid. Slower genomic effects of glucocorticoids involve binding to GR followed by translocation to the nucleus. Once inside the nucleus, GR binds directly to glucocorticoid-responsive elements (GREs) and stimulates target gene expression followed by protein synthesis, and therefore, there can be a delay in some effects. However, most of the anti-inflammatory effects of glucocorticoids appear to result from an important negative regulatory mechanism called transrepression, in which GR directly interferes with the transcriptional activation of key inflammatory proteins.

Therefore, if pharmacodynamic parameters are considered rather than pharmacokinetic alone, this can significantly affect a

determination of medication control information (Knych et al., 2020). However, with corticoids having a complex array of PD effects, there is a question with regard to which effects should be considered. For instance, in the herein study it is clear that inhibition of hydrocortisone in plasma occurs rapidly (statistically significant at 2 h). This has also been shown in horses where significant inhibition of hydrocortisone was observed in a few hours (Ekstrand et al., 2015; Knych et al., 2020). Initial suppression of hydrocortisone is rapid because dexamethasone causes incremental inhibition for as long as dexamethasone plasma concentrations are greater than the IC₅₀. However, the maximum observed suppression of hydrocortisone occurs much later than the time of occurrence of maximum dexamethasone plasma concentrations (time =0) because after hydrocortisone suppression reaches a maximum the return to baseline is then a function of both the rate of hydrocortisone production and dexamethasone elimination. Therefore, the response lasts beyond the presence of effective dexamethasone concentrations because of the time needed for the system to equilibrate (Sharma & Jusko, 1998).

While statistically significant differences from baseline were only observed between 2 and 24 h, six dogs may not be statistically powerful enough, given the number of comparisons prepared in the post hoc Dunnett's test, to reject the null hypothesis for later time points. Moreover, caution must be applied as 'not statistically different' is insufficient to conclude equivalence to baseline. The limited statistical power of this trial (6 dogs) prevents the conclusion that the hydrocortisone plasma concentrations at ≥48 h are significantly lower than baseline values. On the other hand, a conclusion that the difference is 'statistically significant' (eg 24 h) means there is strong evidence that the difference is not zero, but it will not be known whether the difference is large enough to be clinically or scientifically indistinguishable. With a limitation of 6 dogs, a simple a priori contrast approach could be applied to hydrocortisone levels using a *t* test comparison at a specified day (e.g. day 3 or 4) relative to pre-dose control. However, *t* tests between control and day 3 or 4 were not statistically significant but were significant for day 2. More complex linear contrast approaches where data are weighted by sets of contrasts could be applied and offer more power against Type II errors, especially if several control values of hydrocortisone were collected prior to administration. Therefore, caution must be applied to the herein results when interpreting the duration of hydrocortisone suppression.

The reported IC₅₀ values for inhibition of hydrocortisone production were very different between the Ekstrand et al., 2015 study (0.06 ng/ml) and the Knych et al., 2020 study (0.007 ng/ml). An estimate of the IC₅₀ for the herein Greyhound study is approximately 0.1 ng/ml using an indirect response model for the average data and occurs at 48 h post-administration.

Furthermore, the Knych et al., 2020 investigation examined the effect of dexamethasone on inflammatory stimulated whole blood and showed that key bio-markers of inflammation such as prostaglandins and metabolites of arachidonic acid were significantly reduced within 2–4 h of administration. This is consistent with the known rapid transrepression anti-inflammatory effects of glucocorticoids discussed above.

Given that the major anti-inflammatory effects and inhibition of hydrocortisone production by dexamethasone are rapid processes and that clinical indications in racing greyhounds relate to the administration of either DXD or DXF to greyhounds for their systemic anti-inflammatory effects, for example, its use for medial tibial periostitis, there is a strong case that in these circumstances the Toutain method has validity for medication control in racing greyhounds as it is in racing horses (Ekstrand et al., 2015).

In conclusion, this study makes available for dexamethasone, in widely available commercial formulations, extended plasma data, adds extended urine data, as well as providing an indication of one component of the pharmacodynamic effect by measuring the reduction in endogenous hydrocortisone. Greyhound racing regulators also now have the option of using this risk assessment information for risk management to decide an analytical cut-off value for screening of medications (Toutain, 2010). Further information on risk management using the methodology described by Toutain and Lassourd (2002) is provided in the supplementary online information.

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None.

CONFLICT OF INTEREST

Greyhound Racing Victoria funded this study; the administration study was performed at SCEC; the chemical analysis was performed at Racing Analytical Services Limited and the pharmacokinetic analysis was performed at the University of Nottingham. Tim Morris is Independent Scientific Adviser to the Greyhound Board of Great Britain and receives fees for this activity and holds an unpaid appointment the University of Nottingham. Steven Karamatic is Chief Veterinarian at Greyhound Racing Victoria and receives payment for this activity. Paul Zahra is, and Eric Li was, an employee of Racing Analytical Services Limited. Stuart Paine is an employee of the University of Nottingham and has received fees for advice from the Greyhound Racing Victoria. Sally Colgan was an employee of Eurofins SCEC at the time of the study.

AUTHOR CONTRIBUTION

Tim Morris coordinated the interpretation and reporting of this study. Stuart Paine performed pharmacokinetic analysis and interpretation. Paul Zahra and Eric Li developed methodology and provided chemical analysis. Steven Karamatic designed the study and contributed to regulatory interpretation. Sally Colgan was the principal investigator for the in-vivo study. All authors contributed to and reviewed the manuscript and are accountable for its contents.

ANIMAL WELFARE AND ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have adhered to international standards for the protection of animals used for scientific

purposes. Ethics approval (TRIM 15/699(156)) was collected from the Secretary's Animal Care and Ethics Committee of the NSW Department of Primary Industry.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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