Testing for therapeutic medications: analytical/pharmacological relationships and 'limitations' on the sensitivity of testing for certain agents

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Publication #244 from the Equine Pharmacology and Experimental Therapeutics Program at the Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky
Published as Kentucky Agricultural Experiment Station Article # 98–14–134 with the approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station

Tobin, T., Harkins, J. D., Sams, R. A. Testing for therapeutic medications: analytical/pharmacological relationships and 'limitations' on the sensitivity of testing for certain agents. *J. vet. Pharmacol. Therap.* **22,** 220–233.

Proper veterinary care of horses requires that horses in training have access to modern therapeutic medication. However, the sensitivity of equine drug testing now allows for detection of pharmacologically insignificant concentrations of many therapeutic medications. In 1995, the Association of Racing Commissioners International (ARCI) resolved that members 'address trace level detection so as not to lead to disciplinary action based on pharmacologically insignificant traces of these substances'. The rationale behind this approach is to prevent overly-sensitive testing from inhibiting the proper and appropriate veterinary care of performance horses. This review describes a scientific approach to implement this resolution using local anaesthetics as a model system and compares this approach with others currently in place.

For the purpose of this discussion, a 'trace' concentration is defined as a pharmacologically-insignificant concentration. Initially, the target pharmacological effect (e.g. local anaesthesia) was identified, and the dose response relationship was quantified. The 'Highest No Effect Dose' (HNED) was estimated and then administered to horses. Next, the target analyte was identified, synthesized, if necessary, and quantified in blood or urine; the concentrations observed after administration of the HNED are, by definition, true concentrations and hence are pharmacologically insignificant.

The key to this approach has been the synthesis of a unique series of authentic equine metabolite standards, which has allowed scientific identification of the concentration at which the pharmacological effect was indistinguishable from control values. Traces found at less than this concentration are, by definition, 'no effect limits', 'no effect traces' (NETs), 'no effect cut-offs', 'no effect limitations on the sensitivity of testing', or 'subtherapeutic residues'. Conversely, this approach will also identify potent medications for which the sensitivity of testing may need to be improved. Within the context of these experiments, the data create an analytical/pharmacological database that should assist industry professionals in interpreting the significance of trace concentrations of these medications or their metabolites in official samples. The most favourable outcome of this research is more medically appropriate use of therapeutic medications in performance horses, yielding substantial benefits to the health and welfare of these horses.

(Paper received 18 December 1998; accepted for publication 19 March 1999)

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INTRODUCTION

Modern analytical methods used by racing analysts are characterized by low limits of detection, resulting in identification of certain medications and/or their metabolites long after the pharmacological effects have dissipated. This review outlines both the basic regulatory mechanisms and the information bases being developed by the performance horse industry to cope with this problem.

This review will illustrate the need for limitations on the sensitivity of testing (limits) for certain medications that are widely recognized as therapeutic agents in horses. The need for

limits is already well established for regulation of environmental, dietary and endogenous substances, where the virtually unanimous international solution to this problem has been the development of specific 'thresholds' (Tobin, 1995). This review will present the case for similar limits for certain therapeutic medications. If the drug is a prohibited performance-altering agent, such as an ARCI Class 1 agent that has no generally accepted medical use in the racing horse, the question of when pharmacological effects cease is irrelevant. These drugs have no place in horse racing, and their detection at any concentration should be and is vigorously pursued. However, it is worth noting and will be detailed later that American racing is the only major racing area in the world that does not have an integrated, area-wide approach to the problem of ineffective trace residues of therapeutic medications.

For the purpose of this review, a therapeutic medication is an agent identified as such by the American Association of Equine Practitioners (AAEP; Table 1). These medications are commonly and appropriately used on horses in training by equine veterinarians, and their use is well established in the profession. It should be emphasized that the AAEP list includes a considerable range of agents, many of which have the ability to affect racing performance. This point is made clear by the listing of many of these as Class 2 and 3 agents in the ARCI Uniform Classification System for Foreign Substance (Association of Racing Commissioners International, 1998). In this classification system, the agents with the highest potential to affect performance have the lowest rating. There are no Class 1 agents approved for use in horses, and Class 5 agents include medications with low potential for abuse like cromolyl and DMSO (Table 1).

Table 1. American Association of Equine Practitioners' Therapeutic Medications List

Name	ARCI Class	Name	ARCI Class
Diazepam	2	Dipyrone	4
Fluphenazine	2	Flumethasone	4
Hydroxyzine	2	Flunixin	4
Ketamine	2	Guaifenesin	4
Lidocaine	2	Hydrocortisone	4
Mepivacaine	2	(Cortisol)	
Reserpine	2	Ibuprofen	4
Acepromazine	3	Isoflupredone	4
Albuterol	3	(Fluroprednisolone)	
Aminophylline	3	Isoxsuprine	4
Atropine	3	Ketoprofen	4
Butorphanol	3	Meclofenamic Acid	4
Clenbuterol	3	Methocarbamol	4
Detomidine	3	Methylergonovine	4
Glycopyrrolate	3	Methylprednisolone	4
Pentazocine	3	Nandrolone	4
Pentoxifylline	3	Naproxen	4
Procaine	3	Phenytoin	4
Promazine	3	Prednisolone	4
Pyrilamine	3	Prednisone	4
Terbutaline	3	Stanozolol	4
Xylazine	3	Testosterone	4
Acetysalicylic Acid	4	Thiosalicylate	4
Aminocaproic Acid	4	Triamcinolone	4
Betamethasone	4	Trichlormethiazide	4
Boldenone	4	Cimetidine	5
Dantrolene	4	Cromolyn	5
Dembroxol	4	Dimethylsulfoxide	5
(Dembrexine)		Dimethylsulphone	5
Dexamethasone	4	Ranitidine	5

This table was generated by circulating a list of several hundred medications to AAEP members and asking them to indicate which agents they routinely used in their practice. The data was collected and reviewed by the AAEP and presented for publication as Appendix G in the Proceedings of the 'Testing for Therapeutic Medications, Environmental and Dietary Substances in Racing Horses', pp. 191–192 1995, Lexington, KY.

The McKinsey Report (1991), an outside review of the medication control in the performance horse industry, assigned its highest priority to 'threshold levels for drugs permitted in animals on raceday', and also listed as a priority the development of 'trace' levels for therapeutic drugs commonly used in training. Echoing this theme, the European Horseracing Scientific Liaison Committee (EHSLC) in its recent 'Veterinary Drug Detection Times' booklet points out that the 'three central reasons for having rules to control the use of drugs in horse racing are (1) to ensure fair competition (2) to protect the welfare of horses, and (3) to protect the breed from becoming debased' (European Horserace Scientific Liaison Committee, 1997). The EHSLC booklet also notes that 'the rules of racing are not intended to discourage the proper veterinary care of racehorses if such treatment would not threaten any of these important objectives. Furthermore, modern forensic analysis can sometimes detect drugs (including metabolites) long after administration and, as such, can make it difficult for veterinary surgeons to give advice about how soon after treatment a horse may be raced.' Clearly, if horses are not to be deprived of proper veterinary care, suitable information on the time after administration that therapeutic agents or their metabolites may be detected in racing horses must be made available to the veterinary profession.

In 1995, the ARCI adopted a resolution whose final two paragraphs (National Conference, Oklahoma City, OK, April 1995) read as follows:

> 'The Association of Racing Commissioners International strongly recommends that its membership adopt a policy that all chemical findings in official test samples undergo a documented review process by the official veterinarian or appropriate veterinary consultant prior to the initiation of any regulatory action.
>
> And, further, the ARCI recommends that its members specifically implement procedures to have an official veterinarian or veterinary consultant review findings for ARCI class 4 and 5 substances to address 'trace' level detection so as not to lead to disciplinary action based on pharmacologically insignificant '*traces*' of these substances.' (Emphasis added)

The final phrase of these paragraphs 'pharmacologically insignificant traces of these substances' sets forth the scientific challenge to show, with reasonable scientific certainty, that

regulators can distinguish between pharmacologically significant and pharmacologically insignificant concentrations of these analytes. The goal of this review is to set forth the theoretical basis for such distinctions and to present some practical examples.

Definitions

For the purposes of this review, a 'limit', 'threshold', or 'cutoff' is any defined drug or metabolite concentration in a biological fluid that relates to a regulatory event. In racing, concentrations greater than the stipulated 'limit' initiate regulatory action, while concentrations below the 'limit' are of no regulatory interest. The terms 'limit', 'threshold', 'cut-off', and 'limitation on the sensitivity of testing' are equivalent in scientific and regulatory terms. In this review, the term 'limit' will be used as the standard descriptor for this concept.

This research is to identify 'no effect points', which are specific, pharmacologically defined concentrations in biological fluids at or below which the residues of the agents in question are 'pharmacologically insignificant', as set forth in the 1995 ARCI resolution. This 'no effect point' is also, by definition, the highest 'no effect limit' possible. Any concentration of the medication found in a biological sample that is below this 'no effect point' is also referred to as a 'no effect limit', a 'no effect threshold', a 'no effect trace', a 'no effect cut-off', a 'no effect limitation on the sensitivity of testing', or a 'subtherapeutic residue' and is a *pharmacologically insignificant trace,* as set forth in the ARCI resolution.

The use of these broadly equivalent definitions of what one author (T.T.) initially chose to call a 'no-effect threshold' may seem redundant. However, it will become clear that this problem has been approached by a number of different groups worldwide (Tobin, 1995), and each group has selected, for reasons clear to them, their own politically correct descriptors. To avoid offending any particular group and to make the close relationship between these descriptors apparent, their scientific equivalence will be repeatedly emphasized in this review. For the purposes of this review, the word 'limit' will be used to identify the 'no effect limitations', 'no effect cut-offs', 'no-effect thresholds', or 'subtherapeutic residues', all of which represent *pharmacologically insignificant traces*.

Historical background

At the beginning of this century, all foreign substances administered to a horse were, by definition, administered in contravention of the rules of racing. Furthermore, analytical methods were not highly developed, so it was unlikely that an analyst would unequivocally identify a large number of foreign substances (Tobin, 1981). Indeed, at that time the number of chemical substances known to exist was relatively small.

In contrast, analytical methods are now highly developed. Today, many therapeutic medications; dietary, environmental and endogenous substances; and their metabolites can be detected at very low concentrations long after their pharmacological or therapeutic effects have ceased. The lower limits of detection are due both to the advent of ELISA screening and to improved mass spectral confirmation techniques. However, it is not clearly understood by lay people and some industry professionals that *all drugs and medications are still present in biological samples collected from horses long after the pharmacological effects are over and long after most analytical methods cease to detect them* (Tobin *et al*., 1982).

A starting point; the number of drug molecules administered

A useful starting point for understanding the scope of this problem when presenting it to lay audiences is to consider the actual number of drug molecules injected as a clinically effective dose of a medication. The number of drug molecules injected in a single dose can be as high as about 10^{21} (Table 2). This extremely large number points to the primary reason why some medications can be retained at low but detectable concentrations in horses for relatively long periods.

Consider a typical dose of the common therapeutic medication, phenylbutazone. Using a half-life for phenylbutazone of 7.22 h, 90% of the remaining drug, or one log unit of the medication remaining in the horse, is eliminated each day (Fig. 1a). A typical dose of phenylbutazone in a horse contains about 10^{21} molecules, and it will take 21 days to eliminate the *entire* amount of drug. Furthermore, phenylbutazone or its metabolites can be detected in urine samples for up to 14 days after administration, while the pharmacological effect of phenylbutazone lasts only 24–36 h (Fig. 1b; Tobin, 1981).

A practical example of the duration for which 'trace' residues of drugs or metabolites can be detected is set forth by the experience of regulators in Hong Kong (D. Crone, personal communication), who at one time used a testing procedure that detected phenylbutazone for about 1 week after the last dose. A more sensitive test, which detected phenylbutazone for about 2 weeks, became available and was adopted. However, the Hong Kong authorities eventually concluded that the test with the lower (2 week post dosing) limit of detection served no useful purpose, and they chose to return to the original test. The Hong Kong authorities had, at least for this particular test, *chosen to limit the sensitivity of their testing method*. As the pharmacological activity of a single dose of phenylbutazone is generally considered to last no longer than about 24 h, both of these analytical cut-offs used in Hong Kong are very conservative 'no-effect limits'.

These theoretical predictions are broadly consistent with practical experience. For example, the Hong Kong Jockey Club

Table 2. Number of therapeutic medication molecules administered/dose

Drug	No. of molecules
Naproxen	10^{22}
Furosemide	10^{20}
Fentanyl	10^{18}
Etorphine	10^{16}
Hyaluronic Acid	10^{16}

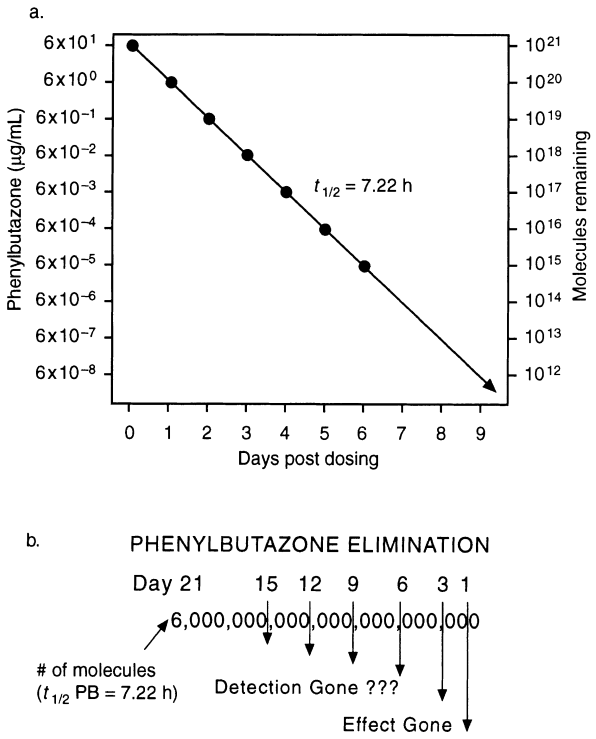

Fig. 1. (a) Elimination of phenylbutazone molecules following administration assuming a half-life of 7.22 h; (b) schematic showing the loss of pharmacological effects in relationship to the point where the agent is no longer detectable. According to these simple models, the last phenylbutazone molecules are being eliminated at ≈ 21 days post dosing.

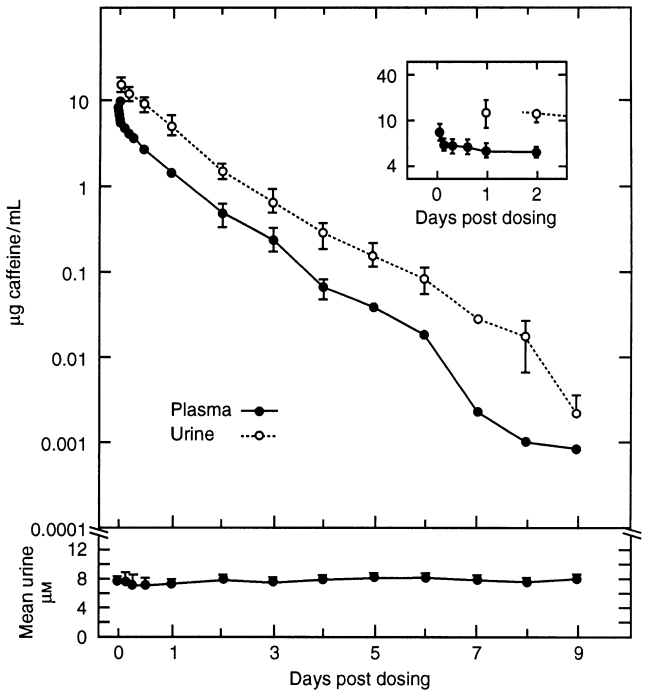

Fig. 2. Mean plasma and urinary concentrations in horses administered caffeine (4 mg/kg i.v.). Inset graph is an expanded plot of the plasma and urinary values in the first 2 h after dosing. Bottom panel: mean pH of urine at the indicated times after caffeine dosing. Reproduced with permission from Greene *et al.* (1983).

Laboratory has reported detection of phenylbutazone, presumably in urine as oxyphenbutazone, for up to 14 days after dosing. With reference to the Hong Kong example, it may be assumed that the horse with the longer detection time had an effective urinary half-life twice that of the horse of Fig. 1, and that oxyphenbutazone was concentrated about 100 fold by the urine pH effect. Furthermore, the number of phenylbutazone molecules in this horse drops from about 10^{21}–10^{14}, or by seven orders of magnitude, to reach the limit of detection. This scenario fits well with the reported 14 day detection time for urinary phenylbutazone in Hong Kong using their most sensitive methods.

Another specific example: caffeine

Caffeine provides another example of limited sensitivity testing. When caffeine is administered to a horse, it is eliminated with a plasma half-life of about 19 h and is detected for about 9 days after administration if immunoassay procedures are used for detection. Additionally, the urinary concentration of caffeine declines in parallel with the plasma concentration, and the urine concentration consistently averages about three times the corresponding plasma concentration (Fig. 2; Greene *et al.*, 1983).

The ELISA test for caffeine has a very low limit of detection. For example, the Neogen ELISA test (Neogen Inc, Lexington, KY) for caffeine has an I_{50} (the drug concentration that causes 50% inhibition of the test) of about 6 ng/mL. One way to reduce the number of low-concentration identifications is to select a reasonable 'limit' or 'cut-off' that is low enough to prevent horses from competing on a pharmacological effect of caffeine but high enough to avoid the reporting of insignificant traces of caffeine that result from environmental contamination.

Hong Kong scientists and racing officials have provided leadership in this area. Hong Kong has an administrative 'limit' or 'cutoff' for caffeine in plasma of 10 ng/mL and in urine of 30 ng/mL. If caffeine is identified at less than these concentrations, no administrative action is taken. Therefore, Hong Kong authorities have established a limit on their regulatory calls, with the result that *even though caffeine may be present in a chemical sense, it is not considered present in an administrative or regulatory sense*. Based on the fact that the pharmacological activity of a single dose of caffeine is unlikely to last longer than about 72 h, both of these analytical 'limits' are again very conservative 'no-effect' concentrations.

'Detection times' and 'withdrawal times'

The 'detection time' is the time period after administration during which an agent (or its metabolite) has been detected in a plasma or urine sample. However, detection time experiments are generally performed in only a small number of horses, and a vigorous disclaimer for protection against liability usually accompanies the information (Canadian Pari-Mutuel Agency, 1994; Australian Equine Veterinary Association, 1992; European Horserace Scientific Liaison Committee, 1997). The veterinarian then considers the reported 'detection time' and

other information available and advises clients on an appropriate period prior to the event to 'withdraw' or withhold further administration of the agent. This period, which is based on knowledge and experience of the vagaries of individual animals, is almost always longer than the 'detection time' and is called a 'withdrawal time'. Ideally, the practicing veterinarian needs a compendium of 'detection times' and/or estimated 'withdrawal times' for each of the therapeutic medications used to treat racehorses (Kellon & Tobin, 1995).

Bringing the 'withdrawal times' problem down to manageable size

It has been suggested that the problem of withdrawal times is too difficult to tackle successfully as there are over 87 000 widely used chemicals and 4000 common prescription agents. It has also been argued that it would be impossible to develop the necessary data on this number of chemicals. However, at a 1994 workshop in Lexington, KY, entitled 'Testing for Therapeutic Medications, Environmental and Dietary Substances in Racing Horses', it was pointed out that data needed to be developed for only a small fraction of these agents to make a significant impact on this problem.

As limitations or withdrawal time data would be developed only for agents commonly used in equine medicine, the list of candidates is immediately reduced to the medications currently listed by the AAEP as therapeutic agents (Norwood, 1995). Comparison of this list with the chemical identifications made on racehorses shows that over 50% of the reported identifications are caused by only nine agents: procaine, isoxsuprine, methocarbamol, dexamethasone, flunixin, prednisolone, acepromazine, promazine, and pyrilamine. The scientific challenge is to identify the plasma or urinary concentrations of these agents or their major metabolites at which their pharmacological effects disappear, as set forth in the ARCI resolution.

Identification of the 'critical pharmacological effect'

Before a database on analytical/pharmacological relationships can be developed, the specific pharmacological effect of concern to the racing industry must be identified. For some agents, this is a straightforward process. For example, loss of pain sensation is clearly the pharmacological effect of concern for local anaesthetics. With other agents, it is sometimes not so direct; for example, after oral administration of isoxsuprine to horses, no pharmacological responses have yet been identified (Harkins *et al.*, 1998b). However, identification of the critical pharmacological effect of concern to racing is a central part of this process and one on which there are currently active research programmes.

The 'Highest No Effect Dose'

Once the critical pharmacological effect for an agent has been identified, the highest no effect dose (HNED) is then determined. For example, using the heat lamp/local anaesthesia/abaxial sesamoid block model, the hoof withdrawal latency, dose and time response curves for procaine were developed. The data show that the HNED of procaine is about 5.0 mg/site and that the duration of action of procaine as a local anaesthetic is brief (Fig. 3). In this way, a family of dose response curves (Fig. 4) and HNEDs have been identified for bupivacaine (Lehner *et al.*, 1998), ropivacaine (Harkins *et al.*, 1999a), mepivacaine (Harkins *et al.*, 1999c; Woods *et al.*, 1998), lidocaine (Harkins *et al.*, 1998a), procaine, cocaine and benzocaine (Harkins *et al.*, 1996a), Sarapin® (Harkins *et al.*, 1997a), and fentanyl (Harkins & Tobin, 1999). Some of these agents are highly potent, some are of intermediate potency, and some are pharmacologically inactive in this model. In other work, 'behaviour chambers' and other laboratory models have been used to determine the HNED of other medications (Harkins *et al.*, 1997b).

Critical metabolites

A complication in this process is that the residue found in horse urine after administration of some of these agents, and on which the chemical identification is made, is often not the parent drug but a metabolite that is generally unavailable to regulators. To solve this problem, several of these metabolites including 3-hydroxymepivacaine, 3-hydroxylidocaine, 3-hydroxybupivacaine, 2-(1-hydroxyethyl) promazine sulfoxide, O-desmethylpyrilamine, 3-hydroxypromazine (Table 3) have been synthesized, purified, characterized and authenticated. These metabolites are used as standards for identification and quantification, as calibrators in routine screening, as reference standards, and for the preparation of specific qualitative and quantitative samples for proficiency or double blind testing and quality assurance work. Also, the data and materials developed in this research can form the basis of new and more effective tests for these agents if such tests are needed (Harkins *et al.*, 1998a, 1999c).

Surprisingly, as this research developed it became clear that the structures of the local anaesthetic metabolites found in horse urine had not been definitively identified. This structural uncertainty likely holds for other equine metabolites as well and has required that forensic reports be worded in a sufficiently broad manner to accommodate these uncertainties. The synthesis of authentic equine metabolites and isomers in our laboratory has answered these structural questions for several local anaesthetics, thereby improving the quality of the identification information available for metabolites of this group of agents (Lehner *et al.*, 1998).

Assembling the data

Once the HNED and the critical metabolite are identified, the relevant 'no effect point' can be determined in plasma or urine. For example, the HNED for procaine in the abaxial model was determined to be about 5 mg/site SQ. That dose was administered to horses, and the free procaine or free procaine plus its glucuronide metabolite ('total procaine') were quantified in urine. The peak concentration of free procaine recovered from these urine samples was about 28 ng/mL, thereby establishing a basis for a 'no effect point' for procaine in equine urine. Similarly, the peak concentration recovered after enzyme hydrolysis

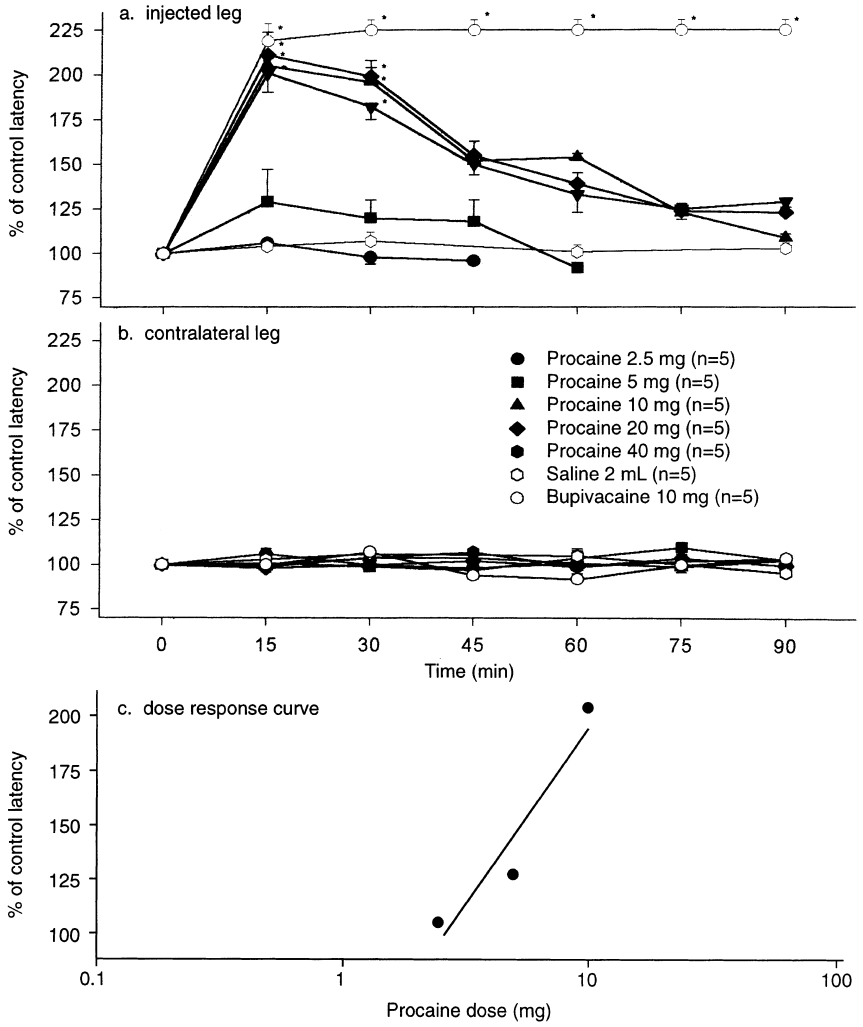

Fig. 3. (a) Change in hoof withdrawal reflex latency (HWRL) following injection of procaine; (b) change in HWRL in contralateral leg; (c) dose response curve. *Significantly different from control values ($P < 0.05$). Reproduced with permission from Harkins *et al.* (1996a).

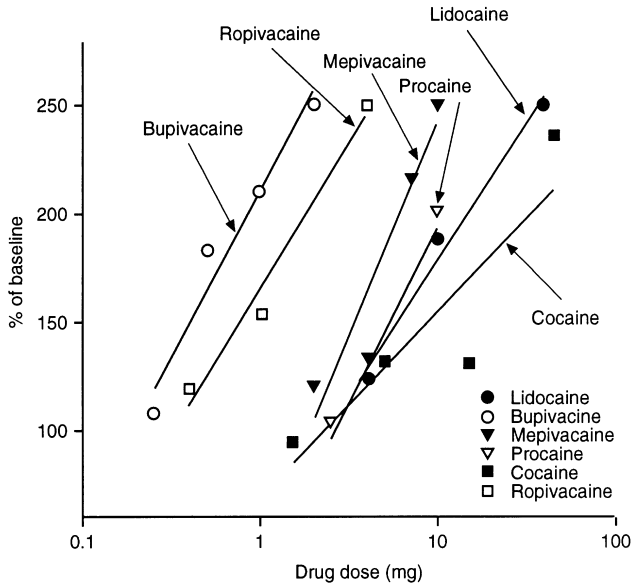

Fig. 4. Dose response curves determined by the heat lamp/local anaesthesia/abaxial sesamoid block model.

Table 3. Critical therapeutic medication metabolites synthesized

Parent Drug	Metabolite/Analogue
1 Lidocaine	3-OH-Lidocaine
2 Bupivacaine	3-OH-Bupivacaine
3 Mepivacaine	3-OH-Mepivacaine
4 Pyrilamine	O-Desmethylpyrilamine
5 Acepromazine	2-(1-Hydroxyethyl)promazine sulfoxide
6 Acepromazine	2-(1-Hydroxyethyl)promazine
7 Promazine	3-OH-Promazine
8 Phenylbutazone	Phenylbutazone-d^9

(conjugated plus nonconjugated) was 45 ng/mL (Fig. 5), thereby establishing a basis for a 'no effect point' for total procaine (free plus conjugated) in equine urine.

As procaine is a basic drug ($pK_a = 8.98$) and these horses were producing alkaline (pH = 8.5) urine, a 30 ng/mL cut-off for parent procaine must be regarded as very conservative. Urinary concentrations of free procaine are likely to be substantially increased if the urine pH is more acidic, the usual condition in postrace urine samples. Therefore, it is expected that the concentration of free procaine in acidic postrace urine will be

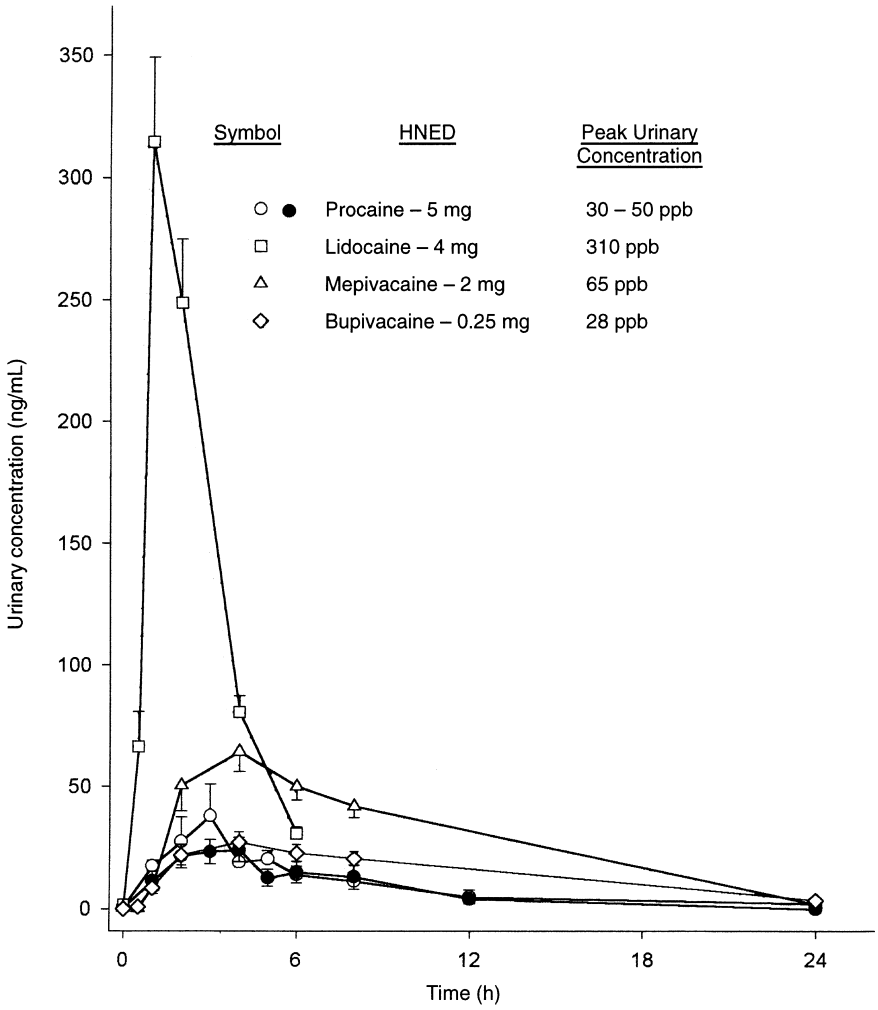

Fig. 5. The symbols indicate urine concentrations of recovered 3-hydroxylidocaine (□), recovered 3-hydroxymepivacaine (△), recovered 3-hydroxybupivacaine (◇), recovered free procaine (●), and procaine following enzymatic hydrolysis (○) after subcutaneous injection of the HNEDs of each agent.

higher than this concentration, as has been shown for free lidocaine (Sams, 1997).

Further analytical/pharmacological relationships

Other recently completed work has determined an HNED of lidocaine (5 mg/site) and a relatively high urine concentration of 3-hydroxylidocaine of ≈ 310 ng/mL in horses administered this dose (Harkins *et al.*, 1998a). Similarly, the HNED of mepivacaine was determined to be 2 mg/site, and the urinary concentrations of 3-hydroxymepivacaine was ≈ 65 ng/mL from horses administered that dose (Harkins *et al.*, 1999c). The HNED of bupivacaine was determined to be 0.25 mg/site, and the concentration of the major urinary metabolite of bupivacaine (3-hydroxybupivacaine) was ≈ 28 ng/mL. Preliminary work with ropivacaine suggests that this agent is similar to bupivacaine regarding local anaesthetic potency (Harkins *et al.*, 1998a, 1999a). Table 4 summarizes the progress to date for developing 'cut-offs' or 'no effect limitations'.

Analytical/pharmacological relationships: plasma or urinary cut-offs?

It is generally recognized that plasma concentrations of medications are often more predictable and easier to relate to pharmacological effects than are urinary concentrations, as urinary concentrations are subject to the influences of urine volume, flow rate, specific gravity and pH. However, there can be analytical limitations with respect to plasma 'cut-offs' that make their application unattainable. The classic example is procaine. Based on research by our group, the estimated plasma 'cut-off' for procaine would be ≈ 1 ng/mL (Harkins *et al.*, 1996a), well below the limit of quantitation of current routine analytical methods. A second problem with procaine is that it is hydrolyzed very rapidly by plasma esterases. Therefore, a plasma 'cut-off' for procaine requires the use of specially prepared collection tubes containing esterase inhibitors. Finally, and of particular significance in Kentucky, is the fact that Kentucky racing authorities do not collect blood samples in their postrace testing programme, which makes the concept of a plasma 'cut-off' for procaine moot.

Because of the potency of procaine and other local anaesthetics, their concentration in plasma is too low for current routine testing techniques to detect. Therefore, plasma thresholds for local anaesthetics are irrelevant, and urinary thresholds were developed by default. However, a plasma threshold, when practical, remains scientifically more acceptable than a urinary threshold.

Table 4. Current status of 'limits' project

Agent*	Metabolite**	HNED Dose***	Limit****
No Measurable Pharmacology			
1 Benzocaine	Not Required	No Effect	Ineffective (Harkins *et al*., 1996a)
2 Sarapin	Not Required	No Effect	Ineffective (Harkins *et al*., 1997a)
3 Isoxsuprine	Not Required	No activity orally	(Harkins *et al*., 1996b; 1998b)
4 Fentanyl	Not Required	No Effect	No nerve blocking effect on pain perception (Harkins & Tobin, 1999)
'Limits' on testing sensitivity			
5 Procaine	Not Required	5 mg/site	30–50 ng/mL (Harkins *et al*., 1996a)
6 Cocaine	Available commercially	> 5 mg/site	Various approaches in place (Harkins *et al*., 1996a)
7 Lidocaine	Synthesized (3- & 4-OH-lidocaine)	4 mg/site	310 p.p.b. (Harkins *et al*., 1998a; Tobin *et al*., 1998)
8 Mepivacaine	Synthesized (3- & 4-OH-mepivacaine)	2 mg/site	65 p.p.b. (Harkins *et al*., 1999c)
9 Bupivacaine	Synthesized (3- & 4-OH-bupivacaine)	0.25 mg/site	~ 28 p.p.b. (Harkins *et al*., 1999b; Lehner *et al*., 1998)
New test development			
10 Ropivacaine	Synthesized (3- & 4-OH-ropivacaine)	0.4 mg/site	(Harkins *et al*., 1998b)
'Limits' work in progress			
11 Acepromazine	Synthesized (HEPS)	1 mg/1000lb	In Progress
12 Detomidine	Available	< 0.2 mg/kg	(Harkins *et al*., 1997b)
13 Pyrilamine	Synthesized (Desmethylpyrilamine)	In progress	In progress
14 Promazine	Synthesized (3-OH-promazine)	In progress	In progress

*Agent for which a 'limit' is being determined; **Status for metabolite synthesis; ***Status of the Highest No-Effect Dose (HNED) determinations; ****Status of the final phase, 'limit' determination, references. Notes on metabolites: (1) Many drugs are excreted in horse urine linked to glucuronic acid, which makes them highly water-soluble and accelerates their elimination in urine. (2) To recover a linked (conjugated) drug from urine, the chemist first splits (hydrolyzes) the glucuronide link by incubating the urine with b-glucuronidase, an enzyme which breaks the drug-glucuronide bond. (3) If the glucuronide molecule is directly linked to the drug, the chemist recovers unchanged drug, which is the case for procaine and isoxsuprine. Therefore, synthesis of specific metabolites is not required. (4) However, most drugs are metabolized before they are linked to glucuronic acid. As the metabolites are not available commercially, they were synthesized in our laboratory for the 'limits' programme. (5) The 'limit' range for procaine (30–50 ng/mL) represents both the free drug concentration (30 ng/mL) and the total concentration of free and conjugated drug (50 ng/mL). (6) The presence of conjugated drug in the urine makes it more likely that the drug actually went through a horse, whereas the absence of conjugated drug creates suspicion that the drug did not pass through a horse.

Limitations of this database

The database reported in this communication applies primarily within the context of these experiments or closely related circumstances. These data are sensitive to dose and route of administration of the agent in question. If the agent is administered by a different route, as repeated doses, as a different formulation, or with another therapeutic rationale, then the analytical-pharmacological database reported here may not necessarily be applicable to the specific regulatory circumstances.

Furthermore, the database is method-dependent. When this work began, *no validated quantitative analytical methods* were available for any of these therapeutic medications or their urinary metabolites. In fact, few, if any, of these urinary metabolites were available until they were synthesized for this programme. Therefore, the methodologies on which these data were developed are specific to this work; any variation, adaptation, or substitution of these methods may affect the interpretation of the database. The analytical methodologies used, however, are based on or adapted from those currently used in racing chemistry. The enzymatic hydrolysis method was adapted from the original reports (Combie *et al*., 1982) and is based on that used by Truesdail Laboratories, Tustin, CA which performs postrace testing for Kentucky, California, and other states. Development of a standardized validated enzyme hydrolysis method for glucuronidated agents and their metabolites would significantly improve the reproducibility of quantitative analytical data involving a glucuronide hydrolysis step.

Similarly, no validated quantitative analytical methods were available for any of the hydroxylated metabolites that form the basis of this experimental work. Progress in this field would be facilitated by the development of validated quantitative methods for these drugs and metabolites to increase the level of confidence in quantitative analytical data. Finally, it must be remembered that these data were developed in a research setting. Therefore, only experienced regulators, who can determine the utility of a research database, should interpret and apply these data in a regulatory setting.

Related worldwide approaches to this question

The need to establish 'limitations' on the sensitivity of testing for therapeutic medications is not simply an American problem, but is rather a worldwide problem. The universal nature of this problem was highlighted at an international workshop on this subject at the University of Kentucky in 1994 entitled 'Testing

for Therapeutic Medications, Environmental and Dietary Substances in Racing Horses. This workshop reviewed the concept of limitations on the sensitivity of testing and endorsed the Canadian approach to this question (Tobin, 1995).

The Canadian approach

The approach used by the Canadian authorities is described as the 'deliberate nonselection of unnecessarily sensitive testing methods for specific substances' (Stevenson, 1995). Once the Canadians develop what they consider to be a satisfactory method for a therapeutic agent, they do not modify the method to alter the limit of detection (Weber, 1995). In lay terms, they 'freeze' or 'fix' the sensitivity of the method, and the sensitivity of the test is not allowed to change over time. Because the limit of detection of the method is fixed, Canadian scientists can administer specified clinical doses of the agents and develop specific 'detection times'. These detection times are published in a booklet by The Canadian Pari-Mutuel Agency which is the most comprehensive effort yet in this area, covering about 70 different medications and formulations (Canadian Pari-Mutuel Agency, 1994). However, it should be remembered that these methods are only applied to therapeutic substances and dietary or environmental contaminants. Other performance-altering substances are actively pursued by the Canadian authorities using the most sensitive testing available.

Furthermore, a deliberate effort has been made to relate the sensitivity of testing or testing 'limits' to the point at which therapeutic efficacy is lost (M. Weber, personal communication). Therefore, the underlying philosophy and goals of the Canadian programme are similar to those of a 'no effect limit' programme. Finally, the Canadian system includes outreach programmes to educate veterinarians and horsemen on detection time data, and *Canadian regulators announce changes in testing methods prior to the introduction of any new tests for therapeutic medications*. These policies and outreach efforts promote higher trainer/veterinarian confidence in the reported detection times for each agent.

The Australian approach

The Australian Equine Veterinary Association, along with the Conference of Principal Clubs (Racing Clubs) and the four official Australian Racing Chemistry Laboratories, has reported detection times for about 50 different therapeutic formulations used by Australian veterinarians (Australian Equine Veterinary Association, 1992). A booklet entitled 'Detection of Therapeutic Substances in Racing Horses' listing the Australian 'detection times' of all the medications tested is available. Review of the circumstances associated with the Australian data suggests that its development was less centralized than the Canadian data. Like the Canadians, the Australians have also 'frozen' their analytical methodology so the 'detection time' data provided to equine veterinarians remains consistent between laboratories and over time. Unfortunately, the National Equine Integrity and Welfare Advisory Board, the Australian Racing Board committee from which the AEVA obtain their detection time data, declined to allow incorporation of any data from this booklet in Table 5.

The European Horse Racing Scientific Liaison Committee Approach

In 1997, the British Jockey Club and several other European racing authorities have endorsed the development of 'detection times' for use in jurisdictions overseen by the EHSLC (European Horserace Scientific Liaison Committee, 1997). These are the most recently developed detection times, and the current list includes only eight or nine agents, although the list is expected to expand as time and resources permit.

The International Federation of Horse Racing Authorities (IFHRA)

In the most recent draft of its guidelines on prohibited substances, the International Federation of Horse Racing Authorities stipulates that 'Thresholds can only be adopted for substances endogenous to the horse; substances arising from plants traditionally grazed or harvested in equine feed; and substances in equine feed arising from contamination during cultivation, processing or treatment, storage or transportation' (Table 6). On the other hand, the document states that 'With the objective of preventing infringements, horse racing authorities may, at their discretion make available "**detection times**"; give forewarning of new or modified tests; and provide an analytical service to establish whether a horse entered to race contains a prohibited substance the trainer specifies'

As alluded to in the definitions section, the IFHRA avoids the term 'threshold' in reference to therapeutic medications. However, it should be understood that a 'detection time' can only be established after the sensitivity of the analytical method is fixed. The position of the IHFRA that detection times may be made available is therefore *an implicit recommendation* that 'limits', 'analytical cut-offs', 'decision-levels', 'limitations on the sensitivity of testing', or 'thresholds' are appropriate approaches to the regulatory problems created by the persistence of ineffective trace residues of therapeutic medications.

Other 'approaches': The United States

As outlined above, the one factor common to all of these approaches is control over the sensitivity of laboratory methods. For this approach to work, the laboratories must use stable and well-characterized methods, for if the limits of detection of the methods change, then the detection times also change. However, in the United States, there is no such body as the Canadian Pari-Mutuel Agency, the Australian Conference of Principal Clubs, or the European EHSLC to monitor the sensitivity of the testing methods.

The approach in the US has been much less integrated. The first limitation established was the National Association of State Racing Commissions (NASRC) plasma threshold for phenylbutazone, initially 2 μg/mL and later raised to 5 μg/mL. Somewhat later, California introduced plasma thresholds for flunixin, naproxen and meclofenamic acid, and Pennsylvania also introduced a plasma threshold for flunixin. In 1995 California introduced a series of urinary 'decision levels' for six therapeutic medications, and the use of plasma thresholds for furosemide of 50–100 ng/mL is becoming more widespread. These thresholds, in conjunction with the internationally accepted thresholds for

Table 5. Worldwide* List of 'Thresholds', 'Decision levels', and 'Detention times' for therapeutic medications in racehorses collected from various sources as of October 1998. Detection times vary with different jurisdictions/areas. The shortest and longest detection times are presented. Some of the variability is due to different doses/preparations. No attempt was made to adjust for those differences

'Thresholds'

Medication	Threshold	Fluid	Jurisdiction/Area
Phenylbutazone	5 μg/mL (5000 ng/mL)	plasma	North America* (ARCI)
	700 ng/mL	plasma	Jockey Club of Brasileiro
Oxyphenbutazone	5 μg/mL (5000 ng/mL)	plasma	North America* (ARCI)
Furosemide	50–100 ng/mL	plasma	Oklahoma & Others
	100 ng/mL	plasma	Jockey Club of Brasileiro
Flunixin	1 μg/mL (1000 ng/mL)	plasma	California
Flunixin	0.1 μg/mL (100 ng/mL)	plasma	Pennsylvania
Meclofenamic Acid	1 μg/mL (1000 ng/mL)	plasma	California
Procaine	25 ng/mL	plasma	Canada
	100 ng/mL	plasma	Rio de Janiero
Caffeine	10 ng/mL	plasma	Hong Kong, Rio de Janeiro
	30 ng/mL	urine	Hong Kong
Dipyrone	1000 ng/mL	plasma	Rio de Janiero
Imipramine	20 ng/mL	plasma	Rio de Janiero
Indomethacin	50 ng/mL	plasma	Rio de Janiero
Lidocaine	25 ng/mL	plasma	Rio de Janiero
Mephenesin	200 ng/mL	plasma	Rio de Janiero
Pyrilamine	5 ng/mL	plasma	Rio de Janiero
Promazine	20 ng/mL	plasma	Rio de Janiero
Tetramisole	80 ng/mL	plasma	Rio de Janiero

'Decision levels'

Medication	Decision level	Fluid	Jurisdiction/Area
Acepromazine	25 ng/mL	urine	California
Mepivacaine	10 ng/mL	urine	California
Promazine	25 ng/mL	urine	California
Albuterol	1.0 ng/mL	urine	California
Atropine	10 ng/mL	urine	California
Benzocaine	50 ng/mL	urine	California
Procaine	10 ng/mL	urine	California
Salicylates	750 μg/mL	urine	California

'Detection times'

Medication	'Detection times'	Fluid	Jurisdiction/Area
Acepromazine	24–96 h	urine	Canada, Europe
Acetaminophen	96 h	urine	Canada
Azaperone	36 h	urine	Canada
Betamethasone	24 h	urine	Canada
Bupivacaine	24 h	urine	Canada
Butorphanol	72 h	urine	Canada, Europe
Chloropheniramine	72 h	urine	Canada
Chlorpromazine	96 h	urine	Canada
Cimetidine	48 h	urine	Canada
Clenbuterol	72 h	urine	Canada
Cromoglycate	24–36 h	urine	Canada
Dantrolene	36 h	urine	Canada
Dembrexine	72 h	urine	Canada
Dexamethasone	24–36 h	urine	Canada, Europe
Dextromethorphan	96 h	urine	Canada
Diclofenac	36 h	urine	Canada
Diflunisal	96 h	urine	Canada
Dimethylsulfoxide	36 h	urine	Canada
Diphenhydramine	48 h	urine	Canada
Dipyrone	36–120 h	urine	Canada, Europe
Dyphylline	96 h	urine	Canada
Ephedrine	36 h	urine	Canada

Table 5. Continued

'Detection times'			
Drug	'Detection times'	Fluid	Jurisdiction/Area
Ergonovine	36–60 h	urine	Canada
Ethacrynic Acid	36–48 h	urine	Canada
Floctafenine	96 h	urine	Canada
Flumethasone	24 h	urine	Canada
Flunixin	48–72 h	urine	Canada
Flurbiprofen	60 h	urine	Canada
Furosemide	24–72 h	urine	Canada, Europe
Glycopyrrolate	36–48 h	urine	Canada
Guaifenesin	24 h	urine	Canada
Ibuprofen	48 h	urine	Canada
Indomethacin	48 h	urine	Canada
Isoflupredone	48 h	urine	Canada
Isoxsuprine	36 h	urine	Canada
Ketamine	96 h	urine	Canada
Ketoprofen	120 h	urine	Europe
Lidocaine	24–36 h	urine	Canada
Meclofenamic Acid	48 h	urine	Canada
Mefenamic Acid	48 h	urine	Canada
Mepivacaine	48–108 h	urine	Canada, Europe
Methocarbamol	24 h	urine	Canada
Methylprednisolone	96 h–> 44 days	urine	Canada, Europe
Methyl Salicylate	6 h	urine	Canada
Naproxen	96–120 h	urine	Canada
Oxyphenbutazone	48 h	urine	Canada
Penicillin Procaine G	48–425 h	urine	Canada
Pentazocine	72 h	urine	Canada
Pentoxifylline	48 h	urine	Canada
Pethidine	72 h	urine	Europe
Phenothiazine	96 h	urine	Canada
Phenylbutazone	96–144 h	urine	Canada, Europe
Piroxicam	72 h	urine	Canada
Prednisolone	24 h	urine	Canada
Prednisone	24 h	urine	Canada
Procaine HCl	48 h	urine	Canada
Promazine	96 h	urine	Canada
Pyrilamine	36 h	urine	Canada
Romifidine	72 h	urine	Europe
Sulindac	96 h	urine	Canada
Theophylline	96 h	urine	Canada
Thiosalicylic Acid	30 h	urine	Canada
Tiaprofenic Acid	120 h	urine	Canada
Triamcinolone Acetonide	24 h	urine	Canada
Trichlormethiazide	24–36 h	urine	Canada
Tripelennamine	36 h	urine	Canada
Xylazine	24–72 h	urine	Canada, Europe
Zomepiractc	96 h	urine	Canada

*Does not include Australian 'detection time' data.

dietary and environmental contaminants, now total more than 20 thresholds currently in place throughout the world. Additionally, if one includes the detection time data developed in Canada, Australia and by the EHSLC groups (Table 5), the number of 'cut-offs'/detection times now available for therapeutic medications, dietary and endogenous substances comes to a total of approximately 100 agents.

The advantage of specified quantitative 'limits' over the regional 'detection time' systems described for Canada, Western Europe and Australia is that they are truly international. A quantitative 'limit' is the same everywhere provided the methods are properly validated and appropriate standards are available. Conversely, a 'detection time' reported in Canada is often not the same as a detection time reported in Australia or the UK,

Table 6. 'Thresholds' approved by the International Federation of Horse Racing Association for endogenous substances, environmental contaminants, and dietary supplements

Agent	Threshold
Arsenic	0.3 μg/mL in urine
Carbon dioxide	37 mmol/L in plasma
Dimethyl sulphoxide	15 μg/mL in urine 1 μg/mL in plasma
Hydrocortisone	1 μg/mL in urine
Nandrolone	Free and conjugated 5 α-estrane-3β, 17α-diol to free and conjugated 5(10)-estrene-3β, 17α-diol in urine at a ratio of 1
Salicylic acid	750 μg/mL in urine 6.5 μg/mL in plasma
Testosterone	0.02 μg free and conjugated testosterone/mL urine (geldings) Free and conjugated testosterone to free and conjugated epitestosterone in urine at a ration of 12 (mares and fillies)
Theobromine	2 μg/mL in urine

presumably because the detection methods differ (Table 5). In this regard, the Canadians have made a deliberate effort to limit the sensitivity of their detection methods, based on the time of loss of pharmacological effect, while the basis for the limits in the Australian and the EHSLC testing procedures are unknown.

Quantitative 'cut-offs' or 'fixed' analytical methods?

It is clear from this overview that the regulatory approaches to dietary and environmental substances have been fundamentally different from those for therapeutic medications. Universally, the approach to dietary and environmental substances has been the development of *defined quantitative 'thresholds'*, which are accepted across the world. On the other hand, the approach to therapeutic medications has been the development of three independent and different detection time schedules in Australia, Canada and the USA. Beyond this, the regulation of therapeutic medications in the USA is a mixture of a number of thresholds/decision levels and a large number of locally developed and poorly defined 'detection times'. The reason for these two fundamentally different approaches lies largely in the limitations of current analytical procedures for therapeutic medications.

The reason the threshold approach has been applied to dietary and environmental substances is the relative ease with which such data could be developed, as it was a natural evolution of the analysts' skills. As the analysts were routinely identifying dietary and environmental contaminants in equine urine, they quantified the contaminants and established quantitative thresholds. The entire process was under the analysts' control and well within the range of their technical skills and the scope of their routine analytical work, and these results have been reported in the scientific literature.

This then raises the question of why has the approach to therapeutic medications been so different and so fragmented? The answer is that, for one reason or another, the industry failed to acquire the tools, namely authentic *equine medication metabolite standards*, needed to establish quantitative 'limits' for therapeutic medications. Lacking such standards, it was forced to take a series of less scientific, *ad hoc*, and largely local approaches, which have presented the racing world with three different sets of detection times. Similar *ad hoc* approaches in the USA have given this country an unknown number of individual and potentially variable 'detection times' for virtually each USA racing jurisdiction.

Racing is in this regulatory predicament because, lacking authentic metabolite standards, analysts have been unable to specifically identify and quantify metabolites of therapeutic medications in equine urine. Without metabolite standards, all the industry can do is *match* the mass spectrum obtained in a test sample with the mass spectrum obtained from a drug administration sample. Lacking authentic standards, the chemist is at times restricted to speculating as to the chemical structure of the materials identified in horse urine and has even less idea as to their possible urinary concentrations.

Given these restrictions, all the industry could do when asked by the racing community for medication guidelines was to dose horses with a typical clinical dose and identify the time for which the medication or its partially characterized metabolite was detectable. Because of the limitations set forth above, if the material identified in urine was a metabolite, then little useful information about the material identified, its chemical identity, and particularly the concentration identified, could be developed.

A further consideration is that 'detection times' generally appear to have been developed in the absence of pharmacological information. Therefore, as pointed out by Houghton (1995), a detection time may or may not be related to loss or absence of pharmacological effect. Again, lacking the tools to quantify the metabolites found in equine urine, the industry has been unable to approach a fundamentally important question.

In contrast, using the approach outlined in this review, the critical pharmacological effect is identified and defined experimentally, and the dose response relationship for the effect is described. The HNED is identified, and where appropriate, the critical metabolite is synthesized, unequivocally identified, and quantified. After these steps have been completed, a 'no effect point' is identified, and regulatory 'cut-offs', ''limitations', or 'subtherapeutic residues' for the identified substances can be established.

Application of this 'no effect point' concept does not lead simply to limitations on the sensitivity of testing. In addition to setting pharmacologically relevant 'no effect traces', 'analytical cut-offs', 'decision-levels', or 'limitations on the sensitivity of testing', the approach outlined in this review also identifies highly potent agents for which the sensitivity of analytical methods needs to be increased. For example, research presented here (Harkins *et al*., 1999b) and elsewhere points to the high local anaesthetic potency of bupivacaine. While the data identify a clear 'no effect point' for this agent, these same data also show that the sensitivity of routine screening methods for bupivacaine might well bear improvement.

Any limitation, 'cut-off', or analytical method that permits only the presence of a pharmacologically insignificant concentration of a substance is, by definition, a 'no effect limit'. The use

of pharmacologically based no effect traces enables these 'limits' to be established on a scientific basis, and realistic 'withdrawal times' can be determined. Furthermore, as pointed out by Houghton (1995), 'the integrity of the industry will be improved, and this concept will enhance the reputation of the forensic services'. Non-trivial collateral advantages include unequivocal forensic identification of the metabolites and the availability of authentic metabolites as world-wide standards and for quality assurance work. The only disadvantages noted are the problems associated with using quantitative methods, the possibility of secondary or unrelated pharmacological effects, and cost. If the only significant problem is cost, then the relatively modest cost of scientifically establishing 'cut-offs' for a small number of important therapeutic agents must be balanced against the significant benefits that such 'no effect limits' or 'no effect cut-offs' bring to the health and welfare of performance horses worldwide.

CONCLUSIONS

Proper veterinary care and humane considerations require that horses in training have access to modern therapeutic medications. On the other hand, the sensitivity of current analytical testing inhibits the use of such therapeutic medication, through fear that ineffective residual traces will result in disciplinary action.

Previous approaches to this problem have been hampered by the unavailability of suitable authentic standards for equine metabolites. Lacking such standards, the industry has been limited to dosing horses and measuring simple 'detection times' for each agent. These 'detection times' are unique to each laboratory unless the methods are rigorously standardized between laboratories. Three different organized sets of detection time data based on standardized methods are available from Canada, Australia and Western Europe. No such nationally recognized standardized methods exist in the USA; all USA detection times are, in principle, unique to each laboratory and infinitely variable. More fundamentally, with the exception of the Canadian data, no effort has been made to link detection times to the absence of pharmacological effect.

The approach presented here is new and soundly based on the development of authentic equine metabolite standards, which allow unequivocal identification and accurate quantification of unique metabolites in equine urine. Based on the availability of these standards, the metabolite traces are related to defined pharmacological effects to establish 'no pharmacological effect limits', 'cut-offs', or 'limitations on the sensitivity of testing'. Application of these standards to equine therapeutic medication testing will minimize the interference of testing with the proper veterinary care of horses, thereby significantly improving the health and welfare of these animals.

ACKNOWLEDGMENTS

Supported by grants entitled 'Development of a test for procaine in horses' and 'Thresholds and clearance times for therapeutic medications in horses' funded by The Equine Drug Council and The Kentucky Racing Commission, Lexington, KY and by research support from the National, Florida, and Nebraska Horsemen's Benevolent and Protective Associations, and Mrs. John Hay Whitney.

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