INTER-LABORATORY COMPARISON OF PHENYLBUTAZONE QUANTIFICATION

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Most racing jurisdictions in the United States allow the presence of phenylbutazone (PBZ) in post race equine blood samples at pre-determined limits. Therefore, it is important that the quantitative determination of PBZ in equine blood samples be accurate and consistent. Jurisdictions utilising the ARCI 'Split Sample Model Rule' require analytical methods that minimise inter-laboratory variability. The objective of this study was to compare the variability of PBZ quantitative determinations using 2 different methods. Serum samples were obtained from horses (n=2) before and 4, 6, 8 and 12 h after iv administration of 2 g PBZ. Serum samples were pooled, mixed to homogeneity, divided into equal aliquots and frozen at -20°C. In addition, blank serum from the same horses, supplemented with a known quantity of PBZ prepared from a USP reference standard, was mixed, divided and frozen as above. Duplicate aliquots of each sample, including 2 identical supplemented samples, were sent frozen to all participating laboratories in a blinded fashion. The laboratories were requested to analyse the samples for PBZ by high performance liquid chromatography using one or both of the methods that had been validated previously through collaborative studies performed by the Testing Integrity Program.

Briefly, Method 'A' consisted of a liquid-liquid extraction and Method 'B' involved a salt extraction technique. The results for Samples 1 to 6 (mean ± sd) for Method 'A' were 9.1 ± 0.7, 6.1 ± 0.9, 5.0 ± 0.9, 2.4 ± 0.5, 5.7 ± 0.6 and 5.9 ± 0.7 µg/ml, respectively. The results for Samples 1 to 6 (mean ± sd) for Method 'B' were 9.7 ± 0.3, 6.1 ± 0.6, 5.0 ± 0.3, 2.2 ± 0.2, 5.9 ± 0.4 and 5.9 ± 0.6 µg/ml, respectively. The results as determined by Method 'A' and 'B' were not significantly different ($P>0.05$). In addition, no significant differences ($P>0.05$) were found in the mean sample determinations when individual laboratory results were compared.

AN OVERVIEW OF ANALYTICAL/PHARMACOLOGICAL RELATIONSHIPS AND THE NEED FOR LIMITATIONS ON THE SENSITIVITY OF TESTING FOR CERTAIN AGENTS

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ABSTRACT

In 1995, the Association of Racing Commissioners International (RCI) resolved that members "address trace level detection so as not to lead to disciplinary action based on pharmacologically insignificant traces of these substances". This review outlines one approach to the implementation of this resolution.

As set forth above, a 'trace' is a pharmacologically insignificant concentration. Our approach has been to identify the target pharmacological effect ('critical pharmacological effect') and the dose response relationship. We have then established the highest no-effect dose (HNED) and administered it to horses. In addition, we have identified a target analyte, synthesised it, when appropriate, and quantified it in blood and urine samples collected at various times after administration of the HNED. The concentrations observed after administration of the HNED are, by definition, 'pharmacologically insignificant'.

This approach allowed for rational determination of the concentration at which the pharmacological effect was statistically indistinguishable from control values. Quantitative thresholds below this concentration are, by definition, no-effect thresholds (NETs).

Within the context of these experiments, the data define an analytical/pharmacological database that should assist industry professionals in interpreting the significance of measured concentrations of these analytes in post race samples, with the most favorable outcome being substantial benefit to the health and welfare of the horse.

GENERAL BACKGROUND

For the purposes of this presentation, a therapeutic medication is defined as a drug on the list approved by the American Association of Equine Practitioners (AAEP; Table 1). Use of these medications is well established among equine veterinarians (Tobin 1981, 1995).

The European Horseracing Scientific Liaison Committee (Anon 1997) suggests that the 3 central reasons for having rules to control the use of drugs in horseracing are:

1) to ensure fair competition;
2) to protect the welfare of horses;
3) to protect the breed from becoming debased.

They also note 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".

On the other hand, if horses are not to be deprived of proper veterinary care, information on the times for which therapeutic agents may be detected in racing horses ***must*** be made available to veterinarians.

In 1995, the RCI adopted a resolution whose final 2 paragraphs (National Conference, Oklahoma City, Oklahoma, 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 RCI recommends that its members specifically implement procedures to have an official veterinarian or veterinary consultant review findings for RCI Class 4 and 5 substances to

TABLE 1: American Association of Equine Practitioners' therapeutic medication list

Name	ARCI class	Trade name	Name	ARCI class	Trade name
Diazepam	2	Valium	Dipyrone	4	Novin, Methampyrone
Fluphenazine	2	Prolixin, Permitil	Flumethasone	4	Flucort, etc
Hydroxyzine	2	Atarax	Flunixin	4	Banamine
Ketamine	2	Ketalar, Ketaset, Vetalar	Guaifenesin	4	Gecolate
Lidocaine	2	Xylocaine	Hydrocortisone (cortisol)	4	Cortef, etc
Mepivacaine	2	Carbocaine	Ibuprofen	4	Motrin, Advil, etc
Reserpine	2	Serpasil	Isoflupredone (fluroprednisolone)	4	Predef
Acepromazine	3	Atravet, Promace	Isoxsuprine	4	Vasodilan
Albuterol	3	Proventil, Ventolin	Ketoprofen	4	Orudis
Aminophylline	3	Aminophylline, etc	Meclofenamic acid	4	Arquel
Atropine	3		Methocarbamol	4	Robaxin
Butorphanol	3	Stadol, Torbugesic	Methylergonovine	4	Methergine
Clenbuterol	3		Methylprednisolone	4	Medrol
Detomidine	3	Demosedan	Nandrolone	4	Nandrolin, Laurabolin
Glycopyrrolate	3	Robinol	Naproxen	4	Equiproxen, Aleve, Naproysn
Pentazocine	3	Talwin	Phenytoin	4	Dilantin
Pentoxifylline	3	Trental, Vazofirin	Prednisolone	4	Delta-Cortef
Procaine	3	Novocaine	Prednisone	4	Meticorten
Promazine	3	Sparine	Stanozolol	4	Winstrol-V
Pyrilamine	3	Equihist	Testosterone	4	
Terbutaline	3	Brethine, Bricanyl	Thiosalicylate	4	
Xylazine	3	Rompun	Triamcinolone	4	Vetalog, etc
Acetysalicylic acid (aspirin)	4		Trichlormethiazide	4	Naqua
Aminocaproic acid	4	Amicar, Caprocid	Cimetidine	4	Tagamet
Betamethasone	4	Betasone, etc	Cromolyn	5	Intal
Boldenone	4	Equipoise	Dimethylsulphoxide (DMSO)	5	
Dantrolene	4	Dantrium	Dimethylsulphone (MSM)	5	
Dembroxol (dembrexine)	4	Sputolysin	Ranitidine	5	Zanta
Dexamethasone	4	Azium, etc			

address trace level detection so as not to lead to disciplinary action based on pharmacologically insignificant traces of these substances'.

The last phrase of these paragraphs "pharmacologically insignificant traces of these substances" puts forward the scientific challenge, which is to show, with reasonable scientific certainty, that we can identify what constitutes a pharmacologically insignificant concentration of these drugs. This review sets forth the theoretical and practical basis for such distinctions, both for ARCI Class 4 and 5 agents and also for selected AAEP therapeutic medications of higher ARCI class, as described in the EHSLC (Anon 1997), Canadian (Anon 1994) and Australian (Anon 1992) 'detection time' guidelines.

Problem of withdrawal times: the need for limitations on the sensitivity of testing for therapeutic medications

Many medications are detectable long after the pharmacological effects are over. If the drug is a prohibited performance-altering agent, 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 pursued vigorously.

However, if the detected substance is a legitimate therapeutic medication (or a metabolite thereof) that has been administered to promote the health and welfare of the horse, then the regulatory position should be considerably different. For such analytes, either the industry needs defined limitations on the sensitivity of testing so that 'detection times' can be determined and 'withdrawal time' guidelines can be developed, with the aim of safeguarding the health and welfare of the horse, or the industry needs sufficient information to interpret the findings based on a review of the data as described by the RCI.

Detection times and withdrawal times

A 'detection time' is the longest time after administration at which an analyte has been identified in plasma or urine. 'Detection time' experiments are generally performed on a small number of horses (<10) and vigorous disclaimers usually accompany detection time information (Anon 1992; Anon 1994; Anon 1997).

The veterinarian then considers this highly disclaimed information and advises clients as to an appropriate time period prior to the event to 'withdraw' or withhold administration of the drug. This period, which is based on knowledge and experience of the vagaries of testing and of individual animals, is generally longer than a 'detection time' and is called a 'withdrawal time'. Ideally, the practising veterinarian should have a

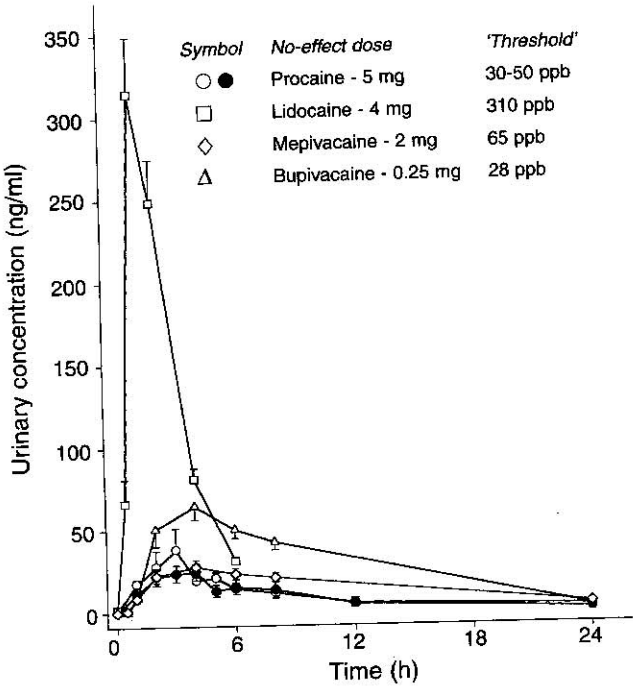

Fig 1: The symbols indicate urine concentrations of recovered 3-hydroxylidocaine (□), recovered 3-hydroxymepivacaine (Δ), recovered 3-hydroxybupivacaine (◇), recovered free procaine (●), and procaine following enzymatic hydrolysis (○) after subcutaneous injection of the HNEDs of each agent.

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compendium of 'detection times' and should estimate 'withdrawal times' for each of the therapeutic medications used to treat racehorses based on a thorough assessment of each situation (Kellon and Tobin 1995).

Reducing the 'withdrawal times' problem to a manageable size

Threshold or 'withdrawal time' data need to be developed only for therapeutic agents on the list of drugs identified by the AAEP (Table 1; Norwood, 1995) and included in RCI Class 4 or 5. Analysis of this list shows that about 9 of the agents result in more than 50% of the reported identifications. These 9 agents, listed in no particular order, are procaine, isoxsuprine, methocarbamol, dexamethasone, flunixin, prednisolone, acepromazine, promazine and pyrilamine. Nine is a manageable number of drugs and our research has focused on them, the challenge being to identify the concentrations in plasma or urine at which these agents become pharmacologically insignificant.

Various methods are available to identify the concentration at which the pharmacological activity disappears. Below that point, any remaining 'residue' is insignificant. However, to establish whether a pharmacological effect is relevant to racing, we must first determine which is 'critical' as far as racing is concerned (Harkins and Tobin 1994; Harkins *et al.* 1996).

Identification of the 'critical pharmacological effect'

The starting point for this work is to identify the pharmacological effect of concern to racing. For local anaesthetics, this is relatively straightforward as the loss of pain sensation is clearly the pharmacological effect of concern. For other drugs, it may be much less straightforward. For example, after oral administration of isoxsuprine to horses, no pharmacological responses were identified (Harkins *et al.* 1998b). However, identification of the critical pharmacological effect of concern to racing is a necessary first step in this process.

The highest no-effect dose

Once the critical pharmacological effect of a drug has been identified, the HNED is determined. For example, by using the heat lamp/local anaethesia/abaxial sesamoid block model, a family of dose response curves (Fig 2) and HNEDs have been identified for bupivacaine, ropivacaine, mepivacaine, lidocaine, procaine, cocaine, benzocaine, Sarapin and fentanyl (Harkins *et al.* 1995, 1996, 1997a, 1998a, 1999). Some of these agents are highly potent, some are of intermediate potency and the remainder are inactive. Other investigations have used 'behaviour chambers' and other laboratory models to determine the HNED of an agent (Harkins *et al.* 1997b).

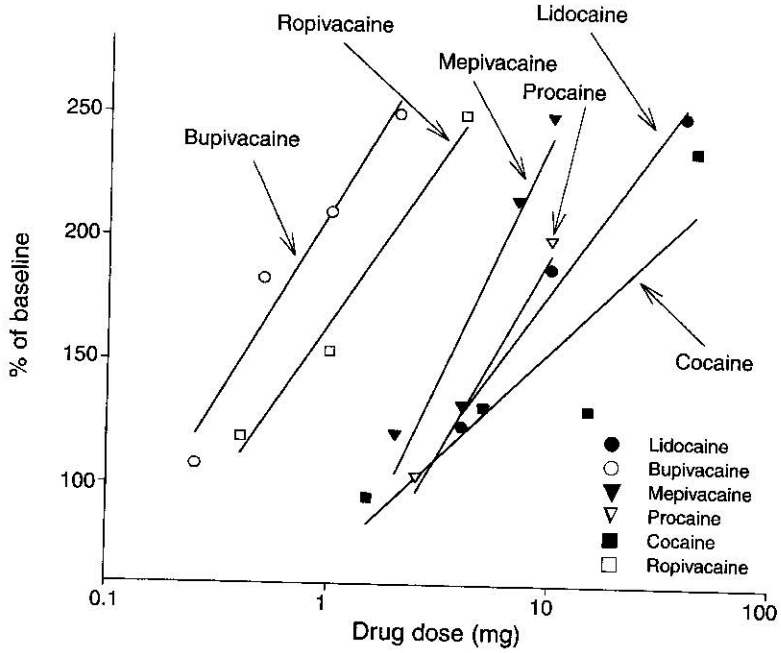

Fig 2: Dose response curves determined by the heat lamp/local anaesthesia/abaxial sesamoid block model.

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CRITICAL METABOLITES

A complication in this process, however, is that an analyte found in horse urine may not be the parent drug but a metabolite thereof. We have therefore synthesised, purified, characterised and authenticated a series of drug metabolites including, 3-hydroxymepivacaine, 3-hydroxylidocaine, 3-hydroxybupivacaine, 2-(1-hydroxyethyl) promazine sulphoxide, O-desmethylpyrilamine and 3-hydroxypromazine (Table 2). These metabolites are used as standards for identification and quantification, and as specific qualitative and quantitative supplements for quality assurance assessment (Harkins *et al.* 1998a, 1999).

ASSEMBLING THE DATA

Once the HNED and the critical metabolite have been identified, the relevant 'threshold' value can be determined. For example, the HNED for procaine in the abaxial sesamoid block model was about 5 mg/site subcutaneously. This dose was administered to horses and the free procaine, or free procaine plus its glucuronide metabolite ('total procaine'), were quantified subsequently in urine. The peak concentrations of free procaine recovered were about 28 ng/ml, while the peak concentration recovered after enzyme hydrolysis (conjugated plus unconjugated) was 45 ng/ml (Fig 1), thereby establishing a basis for thresholds for free or total (ie free plus conjugated) procaine in equine urine.

Because procaine is a basic drug and the horses were producing alkaline (pH = 8.5) urine, a 28 ng/ml threshold for free procaine must be regarded as very conservative. Urinary concentrations of free procaine are likely to be increased substantially if the urine pH is more acidic, which is normally the case for post race urine samples. Therefore, it may be expected that the concentration of free procaine will be higher than this threshold in acidic post race urine, as shown by Sams (1996) for free lidocaine.

FURTHER ANALYTICAL/PHARMACOLOGICAL RELATIONSHIPS

Other recently completed work has determined a HNED for lidocaine of 5 mg/site, which on administration to horses gave a relatively high urine concentration of 310 ng/ml 3-hydroxylidocaine (Harkins *et al.* 1998a). Similarly, the HNED for mepivacaine was established at 2 mg/site, and the concentration of 3-hydroxymepivacaine measured in horses administered this dose was about 65 ng/ml (Harkins *et al.* 1999). Bupivacaine was found to have a HNED of 0.25 mg/site which gave a urinary concentration of approximately 30 ng/ml for the major metabolite, 3-hydroxybupivacaine. Preliminary work with ropivacaine suggests that this agent is about half as potent as bupivacaine with regard to local anaesthetic potency (Table 3).

TABLE 2: Critical therapeutic medication metabolites synthesised

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 sulphoxide
6) Acepromazine	2-(1-hydroxyethyl) promazine
7) Promazine	3-OH-promazine
8) Phenylbutazone	Phenylbutazone D9

TABLE 3: The HNED and ED_{50} for the local anaesthetics tested

Local anaesthetic	HNED	ED_{50}
Bupivacaine	0.25 mg	0.75 mg
Ropivacaine	0.40 mg	1.80 mg
Mepivacaine	2.0 mg	5.20 mg
Procaine	2.5 mg	8.05 mg
Lidocaine	4.0 mg	13.65 mg
Cocaine	5.0 mg	25.18 mg

LIMITATIONS OF DATABASE

The database reported above applies primarily to the context of these experiments or closely related circumstances. If the drug is administered by a different route, as repeated doses, as a different formulation or with another therapeutic rationale, the analytical/pharmacological database may not be applicable to the specific regulatory circumstances. Further, the database is method sensitive, because there are no standardised analytical methods for any of the drugs for which we have developed thresholds. The methodologies on which the database was developed are specific to this work, and any variation, adaptation or substitution of these methods may affect interpretation of the database. However, the methodologies used were adapted from those frequently used in racing chemistry; for example, the enzymatic hydrolysis method was adapted from that of Combie *et al.* (1982) and is used by Truesdail Laboratories, which performs post race testing for Kentucky, California and other states. On the other hand, development of a standardised, validated enzyme hydrolysis method for conjugated metabolites would significantly improve the reproducibility of quantitative data involving a glucuronide hydrolysis step.

Similarly, no validated, quantitative, analytical methods were available for measuring the unique hydroxylated metabolites that form the basis of this experimental work. Progress in this field, therefore, would be facilitated by development of validated, quantitative methods for these drugs and metabolites.

Finally, it must be noted that our data were developed in a research setting. Only experienced regulators who can evaluate a research database and determine its applicability to field situations should apply these data to a regulatory setting.

ANALYTICAL/PHARMACOLOGICAL RELATIONSHIPS: PLASMA OR URINARY CUT-OFFS?

It is recognised that plasma concentrations of drugs are more predictable and easier to relate to pharmacological effect than urinary concentrations. Conversely, urinary concentrations are subject to the influences of urinary volume, flow rate and pH and other factors that can influence the concentration of medications and metabolites in the urine.

However, there can be technical difficulties with plasma thresholds that make their application impractical. The classic example is procaine. Based on research by Harkins *et al.* (1996), the estimated highest no-effect threshold for procaine in plasma is approximately 1 ng/ml, well below the limit of quantitation of current analytical methods. A second problem with procaine is that it is hydrolysed rapidly by plasma esterases. Thus, a plasma threshold for procaine requires the use of specially prepared collection tubes containing esterase inhibitors, which are expensive. Finally, and of some significance in Kentucky, is the fact that Kentucky racing authorities until recently did not collect blood samples in their post race testing programme, so rendering moot the concept of a plasma threshold for procaine.

RELATED APPROACHES TO THIS QUESTION: THE CANADIAN APPROACH

The Canadian approach is described as 'deliberate non-selection of unnecessarily sensitive testing methods for specific substances'. Thus, once a satisfactory test method for a therapeutic agent is developed, the limit of detection for that test is not altered (Weber 1995). Because the method is fixed, Canadian scientists can dose horses and develop specific 'detection times' which are published in a booklet (Anon 1994) and provide the most comprehensive collection of data in this area, covering some 70 medications and formulations.

THE AUSTRALIAN APPROACH

The Australian Equine Veterinary Association, along with the Conference of Principal Clubs (Racing Clubs) and the 4 official Australian Racing Chemistry Laboratories, have reported detection times for about 50 different therapeutic formulations used by Australian veterinarians (Anon 1992).

TABLE 4: Current status of 'thresholds' project

Agent*	RCI class	Metabolite†	HNED dose**	Threshold‡
1) Benzocaine	4	NR	No effect	Ineffective; published
2) Sarapin	NC	NR	No effect	Ineffective; published
3) Fentanyl	1	NR effect on pain perception	No effect	Tested for nerve blocking
4) Procaine	3	NR	5 mg/site	30–50 ng/ml; published
5) Cocaine	1	Available commercially	>5 mg/site	Various approaches in place
6) Lidocaine	2	Synthesised (3-OH-lidocaine)	4 mg/site	Submitted; 310 ppm
7) Mepivacaine	2	Synthesised (3-OH-mepivacaine)	2 mg/site	Submitted; 65 ppm
8) Bupivacaine	2	Synthesised (3-OH-bupivacaine)	0.25 mg/site	~40 ppm?
9) Ropivacaine	2	Not synthesised (3-OH-ropivacaine)	2 mg/site	N/A
10) Acepromazine	3	Synthesised (HEPS)	1 mg/1,000 lbs	In progress
11) Isoxsuprine	4	NR	No activity orally	2 papers in press
12) Detomidine	3	Available (?)	Determined	In progress
13) Pyrilamine	3	Synthesised (desmethylpyrilamine)	In progress	In progress
14) Promazine	3	Synthesised (3-OH-promazine)	In progress	In progress

*Agent for which a threshold is being determined; † Status for metabolite synthesis; ** Status of the highest no-effect dose (HNED) determinations; ‡ Status of the final phase, threshold determination; NC = Not classified; NR = Not required

THE EHSLC APPROACH

More recently, the English Jockey Club endorsed the development of detection times for use by those racing in jurisdictions overseen by the European Horseracing Scientific Liaison Committee. The most recently developed detection times (Anon 1997) currently amount to a list of 8 or 9 agents, although this list is likely to be expanded.

OTHER APPROACHES

The USA approach has been much less integrated. The first threshold, for phenylbutasone, was established by the National Association of State Racing Commission's, at 2 µg/ml and later raised to 5 µg/ml. California then introduced thresholds for flunixin, naproxen and meclofenamic acid; and Pennsylvania introduced a threshold for flunixin. More recently, California introduced 'decision levels' for 6 therapeutic medications. These, in conjunction with internationally accepted thresholds for dietary and environmental contaminants, now total more than 20 thresholds in place around the world.

An advantage of these thresholds over 'regional' systems described for Canada, Western Europe and Australia, is that they are international. A quantitative threshold is the same everywhere provided the methods are well characterised and properly validated. Conversely, detection times developed in Canada may or may not be the same as those developed in Australia or Western Europe.

THRESHOLDS AND/OR ANALYTICAL LIMITATIONS?

Practically, the only way researchers can communicate results to colleagues, regulators, veterinary practitioners and other industry professionals is by presenting them as concentrations in plasma (preferably) or urine. This tradition of expressing thresholds in terms of concentration is established in the area of dietary and environmental contaminants, which are uniformly regulated by means of plasma or urinary concentrations.

The reason threshold concentrations have not been applied to residues of therapeutic medicines may be the differing nature of the skills required to develop this information. The threshold approach to dietary and environmental substances, pioneered by Moss (1976), was a natural evolution of the analysts' skills. Analysts routinely identified contaminants in urine, quantified them and established quantitative thresholds. The entire process was under the analysts' control and well within their range of skills.

The establishment of detection times is also within the skills of an analyst. By treating a horse with a typical clinical dose, the time for which the drug or its metabolite is detectable can be determined. The detection time is then presented with the dosing information as well as a disclaimer. No information about the material identified, its chemical identity, or its concentration is presented. In many cases, the lack of quantitative information is likely to relate directly to a lack of authentic standards, as described in this overview.

Additionally, detection time data are generally 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.

On the other hand, with the approach outlined above, the critical pharmacological effect is identified and defined experimentally, and the dose response relationship described. The HNED is calculated and

the critical metabolite is identified, synthesised and quantified. Based on these data, a threshold concentration for the identified substances can be scientifically determined.

Thresholds as no-effect thresholds

Any regulation allowing the presence of a substance at pharmacologically insignificant concentrations is, by definition, a no-effect threshold. The use of pharmacologically-based thresholds enables them to be established on a scientific basis, and realistic withdrawal times can be determined. Further, as pointed out by Houghton (1995), the integrity of the industry will be improved and this will enhance the reputation of the forensic services. The only disadvantages are the problems associated with using quantitative methods, the possibility of secondary or unrelated pharmacological effects and expense.

Regarding expense, the relatively modest cost of scientifically establishing thresholds for a relatively small number of important therapeutic agents must be balanced against the benefits that workable no-effect thresholds would bring to the health and welfare of performance horses worldwide.

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