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Pemoline, a central nervous system stimulant reportedly occurring naturally in equine samples in Europe and elsewhere – a review and analysis

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Summary: Pemoline, (*RS*)-2-amino-5-phenyl-1,3-oxazol-4(5*H*)-one, is a member of the 4-oxazolidinone group of substances and a central nervous system stimulant closely related structurally and pharmacologically to aminorex. With the increased sensitivity of equine drug testing, low concentration identifications of pemoline have increasingly been identified in horse racing. In 2009 two pemoline and one tetramisole identification in English racing were reported in horses administered levamisole, leading to suggestions that levamisole, known to metabolize to aminorex, could also metabolize to pemoline. In April 2016 the French, German, and South African horseracing laboratories reported frequent identifications of pemoline in equine urine samples, such that these laboratories had "*in-house*" reporting limits below which urinary pemoline identifications were not reported. In 2016 and again in 2018 the matter of potential pemoline identifications in Indiana horse racing was communicated by the Indiana Horse Racing Commission to Indiana horsemen, with requests that horsemen avoid use of levamisole. Soon thereafter, in late 2018, we became aware that plant barbarin was a potential source of equine urinary aminorex identifications. In Spring 2019 we therefore harvested flowering "Yellow Rocket" (*Barbarea vulgaris*), a barbarin-containing invasive plant widely distributed in North America and administered it orally to horses. Urine samples collected from these horses were found to contain aminorex. Aminorex, closely related chemically and pharmacologically to pemoline, is therefore a naturally occurring alkaloid that may be identified in equine urine, suggesting possible similar botanical origins for pemoline, now not infrequently being identified at low ng/mL concentrations in equine urine samples in Europe and elsewhere. Addressing the regulatory implications of these findings, we have therefore reviewed the pharmacological literature on pemoline in the horse and using the Toutain & Lassourd safety factor of 500 we now propose 2 ng/mL of pemoline as an Irrelevant Plasma Concentration (IPC) of pemoline in horses.

Keywords: Pemoline, race horse, doping, drug testing, urine sample

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Introduction

Pemoline and aminorex

Pemoline (*RS*)-2-amino-5-phenyl-1,3-oxazol-4(5*H*)-one, Figure 1, is a member of the 4-oxazolidinone group of substances and is closely related structurally to aminorex and 4-methylaminorex.[1] Like aminorex, pemoline is a central nervous system stimulant, first marketed for use in human medicine in Europe in the nineteen sixties. In 1975 it was approved in the United States for the treatment of Attention-Deficit/Hyperactivity Disorder (ADHD) in persons 6 years of age and older and it has also been used in the treatment of narcolepsy. In 1996 Abbot Laboratories alerted the medical community to cases of serious liver toxicity and deaths associated with its marketed pemoline formulation, Cylert®. This association with a significant incidence of liver toxicity[2] resulted in the withdrawal of pemoline from therapeutic use in the United Kingdom in 1997, Canada in 1999 and the United States in 2005.

In horse racing in the United States, pemoline is classified by the Association of Racing Commissioners International

(ARCI) as a Drug Class 1, Penalty Class A substance, reflecting its close pharmacological relationship to methylphenidate[3], also a Class 1, Penalty Class A substance. In the United States Horseracing Integrity and Safety Authority regulatory system, pemoline is classified as a SO or Banned Substance, with the proviso that *"Pemoline is a metabolite of aminorex, which is a metabolite of levamisole. If there is credible evidence that the detection of pemoline in a horse's sample is the consequence of exposure to levamisole, the classification of pemoline may be revised to S7 (B)"*, to our knowledge the penalty classification for an approved therapeutic medication "overage".[4]

We will now review current knowledge of the relationships between levamisole, aminorex and pemoline and the reported detection of pemoline in equine post-race samples world-wide, starting with a 2009 sequence of events in English racing.

Pemolines reported in British racing believed to be associated with levamisole adminstration

The first sequence of pemoline identifications that drew attention to a possible relationship between a levamisole administration, Figure 2, and post-race detection of pemoline was a circumstance in 2009 in British racing where a number of horses were treated on a veterinarian's advice with Levacide, a levamisole-containing product. Two horses tested "positive" for pemoline, with a third horse testing "positive" for tetramisole. Reviewing this situation, we noted the close structural relationship between aminorex, a well-recognized metabolite of levamisole, and pemoline, wherein pemoline would be a simple oxidation product of aminorex. These chemical facts were communicated to the British Horseracing Authority Disciplinary Department which held that *"the Panel was satisfied that the source of both pemoline and tetramisole was a five-day course of 120 ml of Levacide for [horse #1] and [horse #2] and [horse #3] and a four-day course for [horse #4], which commenced on August 18, 2009 and was given under veterinary advice."* [5]

The European and South African experience with pemoline

Based on extensive international forensic experience, it now appears that pemoline – like aminorex – may also be a naturally occurring substance in equine urine. We first became aware of this possibility in correspondence with analytical colleagues concerning a claimed pemoline identification in Canadian horse racing. Discussions concerning this matter led to a worldwide exchange of correspondence on the matter of low concentration urinary identifications of pemoline in European horses and the then and still unknown origins of these European pemoline identifications. The first communication was on April 8th, 2016, from Dr. *Yves Bonnaire*, Director of the French Laboratoire des Courses Hippiques, LCH in France. In this communication Dr. *Bonnaire* set forth as follows.[6]

"Dear colleagues,
We are frequently finding small amount of pemoline in our routine (French and overseas) samples as well as in our experimentation horses (having received no pemoline or tetramisole or any related antiparasitic drug). The apparent concentration is sub nanogram up to 5 ng/ml. The drug is fully characterized (SRM and HRMS). <u>These findings do suggest a natural occurring origin</u>. Is there any other lab (s) having similar experience?
Best regards
Yves"

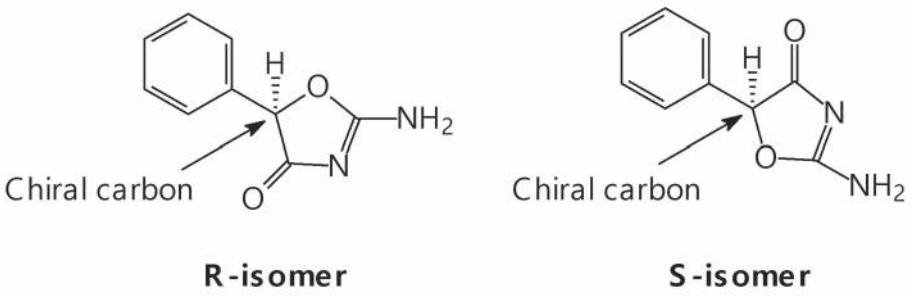

Fig. 1 Pemoline, (R and S)-2-amino-5-phenyl-1,3-oxazol-4(5H)-one, $C_9H_8N_2O_2$, molar mass, 176.175 g·mol^{-1} pKa 10.5. Chemically, pemoline exists as two enantiomers, the R-enantiomer left and the S-enantiomer, right. The pemoline previously approved for therapeutic use in the United States and elsewhere was racemic pemoline. | *Pemolin, (R und S)-2-Amino-5-phenyl-1,3-oxazol-4(5H)-on, $C_9H_8N_2O_2$, Molmasse 176,175 g·mol^{-1}, pKa 10,5. Chemisch gesehen existiert Pemolin als zwei Enantiomere, das R-Enantiomer links und das S-Enantiomer rechts. Das zuvor in den USA und anderswo für die therapeutische Verwendung zugelassene Pemolin war racemisches Pemolin.*

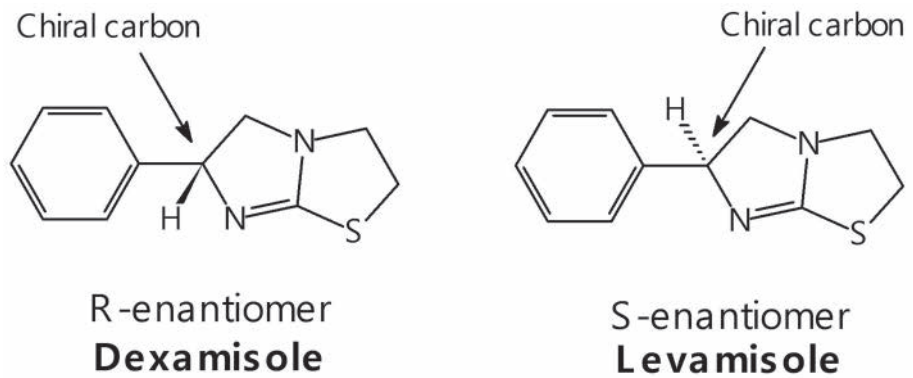

Fig. 2 Tetramisole, (R and S)-6-Phenyl-2,3,5,6-tetrahydroimidazo(2,1-b) thiazole, $C_{11}H_{12}N_2S$, molar mass 204.29 g.mol-1. Chemically, tetramisole exists as two enantiomers, the R-enantiomer dexamisole (left) and the S-enantiomer levamisole (right). The S-enantiomer, i.e., levamisole is the more biologically active enantiomer. | *Tetramisol, (R und S)-6-Phenyl-2,3,5,6-tetrahydroimidazo(2,1-b)thiazol, $C_{11}H_{12}N_2S$, Molmasse 204,29 g.mol-1. Chemisch gesehen existiert Tetramisol als zwei Enantiomere, das R-Enantiomer Dexamisol (links) und das S-Enantiomer Levamisol (rechts). Das S-Enantiomer, also Levamisol, ist das biologisch aktivere Enantiomer.*

(Our underlining of the *"natural occurring origin"* related wording)

On April 9th, 2016, Dr. *Terence Wan* of the Hong Kong Jockey Club Laboratory noted that his laboratory was the one that specifically identified pemoline at an estimated concentration of 2.5 ng/mL in a sample received from the French Laboratory concerning a pemoline unrelated matter.[7] On April 12th, 2016, Dr. *Bonnaire* expanded his response concerning pemoline in LCH samples as follows and noted that the French laboratory was using a 5 ng/mL urinary concentration of pemoline as an "in house" reporting limit.[6]

"Now we are screening by Q-exactive and as the sensitivity has increased almost all routine samples do contain pemoline at low level. This is why we do not consider pemoline at low level as potentially reportable as we have too many positive detection. This molecule is also found in our experimentation samples (as explained in my previous email), we have conducted at the time (long time ago) several analysis which were not conclusive and we decided at the time to adopt an in house reporting level (5 ng/ml). This is why several of our negative sample do contain pemoline or pemoline like substances."

Similarly, on April 12th, 2016, Dr. *Marc Machnik*[8] of the German Sport University reported that his laboratory also detected pemoline at low concentrations in equine urine and that, based on these findings, they have concluded that they should not report pemoline at concentrations below 10 ng/mL in urine samples. Likewise, Dr. *Magda Rosemann*, a scientist working in the South African equine drug testing laboratory also reported detecting pemoline in their South African samples when they screened these samples at high sensitivity.[9]

Pemoline concerns in Indiana racing

Consistent with these reports of urinary identifications of pemoline in European and South African racing horse samples, on May 23rd, 2016 the Indiana Horse Racing Commission (IHRC) issued an "*IHRC Advisory Notice to Horsemen*" entitled "*Horsemen Reminded to Avoid Products Containing Levamisole and Tetramisole*" based on the fact that there is "very *strong evidence in the scientific literature…that the administration of levamisole and potentially tetramisole, to horses results in production of aminorex and pemoline as metabolites*".[10]

Further consistent with the above referenced 2016 "*IHRC Advisory Notice to Horsemen*", on July 20th, 2018 one author (*Thomas Tobin*) was contacted by Dr. *Michael Mann*, DVM, the Indiana Director for the North American Association of Racetrack Veterinarians. He reported verbal warnings from Indiana Horse Racing Commission personnel to a number of trainers at Indiana Grand racetrack concerning the reported presence of trace amounts of pemoline in analytical samples and warning the horsemen about the use of levamisole.[11] The approach taken on this matter in Indiana racing was to not take regulatory action on these apparently low concentration pemoline screening identifications but to simply warn the involved horsemen about the risks of using levamisole, one possible innocent source of low concentration pemoline identifications in equine urine samples.

The problem with these Indiana notifications linking levamisole and pemoline was that the horsemen were generally unaware of any sources of levamisole in their barns which made their avoidance of levamisole use a moot issue. These regulatory notifications also left the horsemen aware and concerned that their horses were presenting potential analytical identifications of pemoline despite there being, to their knowledge, no known sources of pemoline in the diet or immediate environs of these horses.

Discussions with personnel at the Indiana Racing Commission testing laboratory established that these communications were based on screening identifications of pemoline and not on confirmed identifications. On July 23rd the regulatory significance of the reported lack of confirmation of these trace level screening pemoline identifications was communicated to Dr. *Mann*[12] and on July 25th the same was formally communicated to the Indiana Horse Racing Commission.[13] Thereafter the matter of these trace level screening identifications of pemoline in racing samples in Indiana has not been presented to Indiana horsemen as a regulatory concern.

In a related analysis of this Indiana pemoline situation on or about July 19th, 2018, one author (Thomas Tobin) sent a report to the Executive Director of the North American Association of Racetrack Veterinarians (NAARV), Dr. *Clara Fenger,* summarizing the facts and speculations presented above and setting forth that at least two European Laboratories had adopted "in-house" reporting limits for pemoline in post-race urine, one at 5 ng/mL and one at 10 ng/mL, as set forth above.[14] This communication also made clear that at that time, July, 2018, the source(s) of the pemoline findings in Europe was or were unknown, and that the existence of this long known, unidentified and possibly natural source of pemoline findings in European racing urine samples needed to be taken into account when evaluating the regulatory significance of low concentration pemoline findings in US racing samples or elsewhere.

Levamisole and pemoline identifications / "positives" in US racing

Given the above reported identifications of pemoline in British, French, German and South African post-race urine samples, we elected to review the number of pemoline identifications reported in US racing. Our first request to the Association of Racing Commissioners International (ARCI) was for a listing of pemoline identifications reported in US racing samples. Only one pemoline identification was reported and when we reviewed this identification it was clear that this identification was a levamisole-associated pemoline identification.

We therefore requested from ARCI a listing of all levamisole identifications, which data are presented in graphic form in Figure 3[15]. Levamisole identifications started in 1987 but remained low until 2002 when there were 11 identifications. The next significant number of levamisole identifications was in 2012, with 12 identifications in New York racing, and of these 12 identifications 11 were reported as containing pemoline. Then in 2013 there were 2 levamisole identifications, one of which was reported as containing pemoline, and 1 levamisole identification in 2014 this identification also report-

ed as containing pemoline. The year 2015 saw 9 levamisole identifications with just 1 pemoline identification while 2016 saw 15 levamisole identifications with 5 pemoline identifications. From 2017 on there were 40 plus levamisole identifications reported but no further reports of pemoline identifications. Overall, the ARCI data show a total of 129 levamisole identifications, and 21 of the respective samples also were reported to contain pemoline, with no ARCI reports of pemoline identifications in the absence of levamisole.

There are a number of possible interpretations of the above ARCI data. One interpretation is that pemoline is a downstream metabolite of levamisole, with the missing pemoline detections in Figure 3 relating to the fact that some regulatory analysts simply chose not to confirm its presence for purposes of reporting. This interpretation is consistent with the high percentage, i.e., 91% of "positive" samples reported from the 2012 New York samples contained both levamisole and pemoline and also from the 2016 spike in Illinois, which reported 5 identifications of samples containing both levamisole and pemoline, for a full 100% link between levamisole and pemoline identifications in Illinois. Similarly, the large spike in levamisole alone "positive" samples was largely driven by a large number of levamisole "positive" samples in Louisiana racing where the analytical focus appears to have been on detecting and quantifying levamisole in both the plasma and urine samples with no other analytes reported present in these Louisiana levamisole identifications.

A second interpretation of the ARCI data is that suggested by the European experience, namely that pemoline is a naturally occurring substance in at least some horse urines and that the pemoline is therefore randomly likely to be found in association with levamisole in post-race urine samples.

A third interpretation is that the detection of pemoline in a levamisole containing sample is due to pemoline being an analytical artefact associated with the urinalysis procedure, most likely related to the enzymatic or other hydrolysis steps prior to analysis.

A fourth but much less likely possibility is that a pemoline finding in any sample is due to the illicit administration of pemoline to the horse in question.

Aminorex, a levamisole metabolite found in horses urine following exposure to Brassicaceae plants

Shortly after the July 2018 communication was sent to Dr. *Fenger* and the North American Association of Racetrack Veterinarians (NAARV), we became aware of a report by the LGC laboratory in England of identifications of aminorex in sport horse samples in England with no known exposure of these horses to either levamisole or aminorex.[16] Additionally, the LGC report pointed to botanical barbarin, an alkaloidal substance found in members of the Brassicaceae plant family as likely being at least one natural source of these aminorex findings reported by LGC, which analysis was supported by discussions with a number of colleagues.

A compelling North American related consideration in this matter was that the pasture plant *Barbarea vulgaris*, colloquially "Yellow Rocket", which flowers in North American pastures from late April to June, depending on the latitude, is a member of this Brassicaceae plant family, thereby giving rise to the possibility that consumption of this plant by horses could give rise to aminorex findings. In Spring 2019, we therefore harvested pasture flowering yellow rocket plants and fed them to a number of horses, suitably disguising the aversive taste of these plants by mixing them with grass and sweet feed. As reported in the *Irish Veterinary Journal*, analysis of post-administration samples from these horses were "positive" for aminorex, revealing that consumption of a naturally occurring pasture plant could result in urinary aminorex findings in horses.[17]

These possible botanical, biochemical and human behavioral activities that may give rise to urinary identifications of levamisole and pemoline, alone or in combination, are shown in Figure 4.

Current knowledge of the relationships between levamisole, aminorex and pemoline

At this time levamisole is well known to metabolize to aminorex and a reported 26 other metabolites, but pemoline has not been reported detected in plasma or urine samples from post-levamisole administration experimental horses. Pemo-

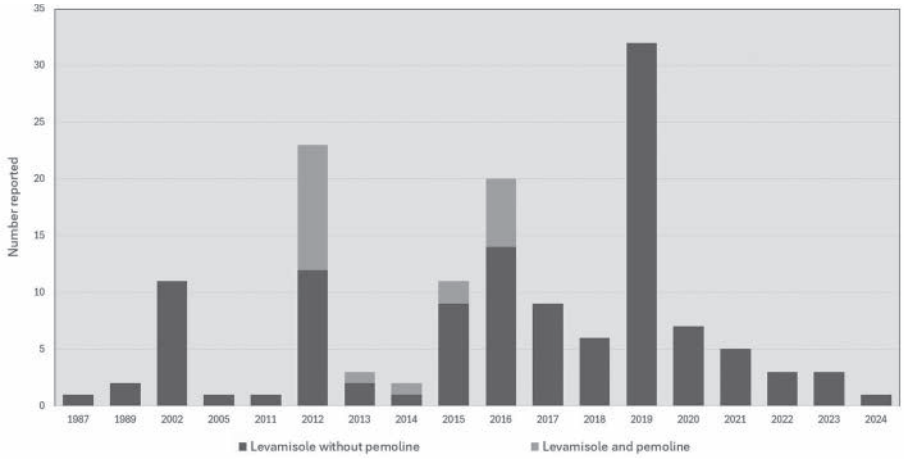

Fig. 3 Numbers of levamisole identifications reported in US racing 1987 to date. Levamisole identifications with no pemoline reported, dark columns, samples containing both levamisole and pemoline, grey columns. Note that in 2012 New York reported 11 of 12 post-race urine samples as "positive" for levamisole and also "positive" for pemoline. The ARCI has had no reports of pemoline identifications in the absence of levamisole. | *Anzahl der bei US-Rennen seit 1987 gemeldeten Levamisolnachweise. Levamisolnachweise ohne gemeldetes Pemolin, dunkle Säulen, Proben, die sowohl Levamisol als auch Pemolin enthalten, graue Säulen. Beachten Sie, dass New York 2012 11 von 12 Urinproben nach dem Rennen als „positiv" für Levamisol und auch „positiv" für Pemolin meldete. Dem ARCI liegen keine Berichte über Pemolinnachweise ohne Levamisol vor.*

line, however, has been identified as an "in vitro" metabolite of aminorex in equine liver microsome experiments by *Scarth* and colleagues, suggesting that an aminorex/pemoline transformation is at least a biochemical possibility in the horse.[18] *Scarth* et al. noted that "*No pemoline was detected after incubation with levamisole or tetramisole, but pemoline was detected after dosing with aminorex*". This is presumably a consequence of the low expected amounts of aminorex from levamisole, in turn pushing any generated pemoline below detectability." The microsomal method of *Scarth* may in fact not be optimal for mirroring levamisole metabolism, since levamisole is predicted to be a CYP450 1A2 & 2D6 inhibitor[19] and the latter two monooxygenases may very well be responsible for aminorex oxidation to pemoline. Pemoline is frequently detected in post-race equine urine samples in association with levamisole, to the point that it appears that the frequency of detection of pemoline in post-levamisole administration racing samples is, as a practical matter, determined principally by the interests/policies of the laboratory performing the post-race analyses. Additionally, aminorex has also been identified in equine urine samples following administration of the North American pasture plant *Barbarea vulgaris*, consistent with the at times identification of aminorex in equine samples in the absence of known levamisole exposure. These pasture sources of aminorex raise the possibility of similar pasture sources for the pemoline identifications of unknown origins reported in French, German and South African samples and also apparently at times in US samples. As a closely related substance, it appears possible that there is a similar botanical cause of the reported findings of low urinary concentrations of pemoline in urine samples from horses racing in France, Germany, and South Africa and also, apparently, in horses racing in Indiana. Given these circumstances, it is appropriate for regulatory authorities to follow the now long in place example of the French and German analytical laboratories and define an Irrelevant Plasma Concentration (IPC) reporting limit for pemoline in equine blood/plasma/serum samples.

An irrelevant plasma concentration (IPC) for pemoline

The determination of a reporting limit for blood/plasma/serum samples requires identification of an Irrelevant Plasma Concentration (IPC) for pemoline in equine blood/plasma/serum samples. Studies on the effects of pemoline in horses[20] have shown that administration of pemoline at doses of 2.5 and 5 mg/kg intravenously produces clear-cut dose-related locomotor responses in their experimental horses. While no pemoline blood concentration data were available for the 5 mg/kg IV dose, review of the available blood concentration data for the 2.5 mg/kg dose showed that the oral 2.5 mg/kg administration yielded peak plasma concentrations in the order of 1 µg/mL, while the 2.5 mg/kg IV dose was associated with somewhat higher peak plasma pemoline concentrations. Based on these data, a blood plasma serum concentration of 1,000 ng/ml is a conservative Effective Plasma Concentration (EPC) estimate for pemoline in a racing horse. Dividing this EPC by the conservative Toutain safety factor of 500 presents an Irrelevant Plasma Concentration for pemoline in horses of 2 ng/mL.[21] Given the fact that pemoline is likely to be found in equine urine at substantially higher urinary concentrations than the corresponding plasma (or serum) concentrations it seems likely that the urinary reporting limits used in France and Germany, as referenced above, are reasonable and appropriately conservative as Irrelevant Urinary Concentrations (IUCs).[22]

A further consideration is that review of the equine pharmacokinetic data for pemoline in the horse as reported by *Igwe* and colleagues[20] shows that the mean terminal plasma half-life for pemoline is unusually long at 39.4 hours. Therefore, horses exposed to ongoing dietary or environmental sources of pemoline will require five pemoline half-lives or about 8 days to reach steady state serum or urinary concentrations following exposure to a dietary source of pemoline. Furthermore, the relationship between plasma or serum and urinary

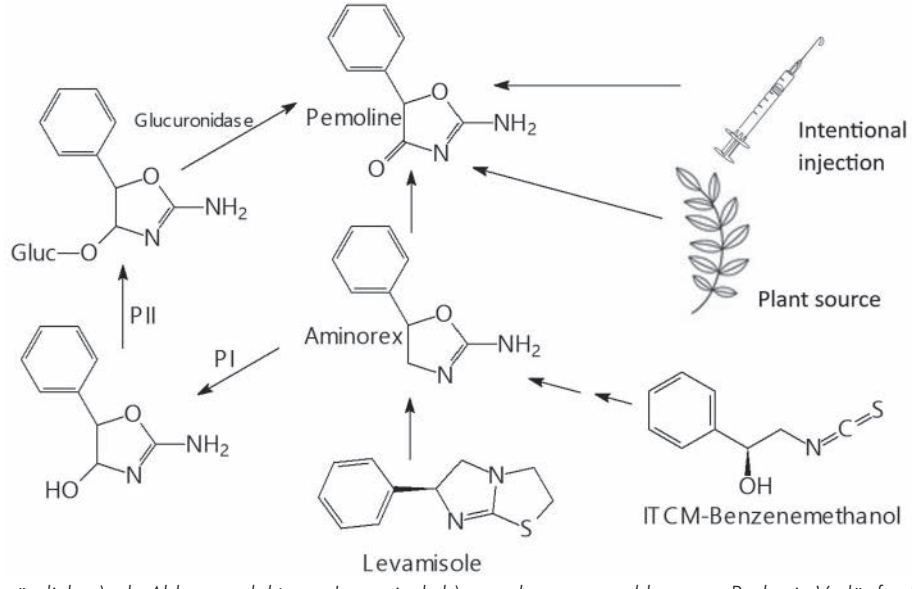

Fig. 4 Possible sources of pemoline in horse urine. Three sources involve aminorex as an intermediate, specifically a) as a breakdown product of levamisole; b) from the proposed barbarin precursor ITCM-benzenemethanol (α-(isothiocyanatomethyl)-benzenemethanol); c) as a glucuronidase-induced artifact from a hypothetical hydroxylated aminorex glucuronide (PI = Phase I metabolism; PII = Phase II metabolism; Gluc = glucuronic acid moiety). Note that the direct aminorex ➔ pemoline conversion has been demonstrated in vitro, by use of equine liver microsomes[18]. The plant image represents derivation from an unidentified plant source, while the syringe represents deliberate administration of pemoline. | *Mögliche Quellen von Pemolin im Pferdeurin. Drei Quellen beinhalten Aminorex als Zwischenprodukt, nämlich a) als Abbauprodukt von Levamisol; b) aus dem vorgeschlagenen Barbarin-Vorläufer ITCM-Benzolmethanol (α-(Isothiocyanatomethyl)-Benzolmethanol); c) als Glucuronidase-induziertes Artefakt aus einem hypothetischen hydroxylierten Aminorex-Glucuronid (PI = Phase-I-Stoffwechsel; PII = Phase-II-Stoffwechsel; Gluc = Glucuronsäure-Anteil). Beachten Sie, dass die direkte Umwandlung von Aminorex in Pemolin in vitro mithilfe von Pferdelebermikrosomen nachgewiesen wurde*[18]*. Das Pflanzenbild stellt die Ableitung aus einer nicht identifizierten Pflanzenquelle dar, während die Spritze die absichtliche Verabreichung von Pemolin darstellt.*

concentrations of pemoline is unknown but most likely highly variable due to urinary pH effects, so the most appropriate matrix for pemoline regulation in horse racing is blood/plasma/serum.[22] Overall, given the ongoing experience of our colleagues in Europe, introduction of a 2 ng/mL blood/plasma/serum screening limit for pemoline in US racing should effectively and conservatively address the matter of the detection of pharmacologically irrelevant concentrations of pemoline from botanical or other naturally occurring sources in US equine drug testing.

The derived IPC of 2 ng/mL can be validated by application of pharmacokinetic principles based on the studies of *Igwe* and *Blake*.[20] If we assume horses are exposed to background sources of pemoline, primarily plant sources, and consumption is about 1 mg/kg every 48 hours, then an EPC will range from 656–755 ng/mL applying bioavailability ranging 0.833–0.863 and clearances ranging 23.8–27.4 mL/kg/hr. Dividing the EPC by the Toutain safety factor gives values ranging from 1.27–1.51 ng/mL, just below the value calculated for the IPC above. Since a limited number of horses were used for the pharmacokinetic study, and no studies have been conducted on the pharmacokinetics of pemoline originating from plants and given the possibility of a wide variation in pharmacokinetic parameters of pemoline in larger groups of horses, the conservative IPC of 2 ng/mL is the best attainable value at the current time.

Pemoline is a basic compound given a reported pKa of 10.5. This bears on the manner with which it gets excreted in urine. Renal excretion completes the process of elimination that begins in the liver, and polar drugs such as protonated pemoline get filtered in the kidneys and are incapable of undergoing reabsorption. They thus get excreted in the urine. Urinary pH significantly impacts excretion, as drug ionization changes depending on the alkaline or acidic environment. Therefore, increased excretion occurs with weakly acidic drugs in basic urine and weakly basic drugs in acidic urine.[23]

Pemoline at a calculated IPC of 2 ng/mL is (2 × 10e-6 ug/L)/176 MW, or 0.011 μmole/L. At a pH as low as 5.0 following exercise, these data can be fit to the *Henderson-Hasselbalch* equation:[24]

$$pH = pKa + \log([A^-]/[HA])$$

where $A^{[-]}$ represents free pemoline and HA represents acid-conjugated pemoline, or pemoline +H. At pH 5.0, the ratio of $[A^{[-]}]/[HA]$ calculates as 0.0000032, or 0.0000032 moles of free pemoline relative to acidified pemoline, i.e. pemoline +H. The inverse yields 312500 moles pemoline +H relative to pemoline. In an animal at rest with a plasma pH of 7.4, the log ratio calculates as 0.00089, or 0.00089 moles of free pemoline relative to acidified pemoline. The inverse yields 1123 moles pemoline +H relative to pemoline. Thus, acidification of urine results in 278-fold higher amounts of pemoline + H on a molar basis, resulting in higher amounts in acidic urine[24].

The enantiomers of pemoline detected in racing horses

One classic significant scientific and potential regulatory concern is the matter of the specific enantiomers of pemoline (Figure 1) that are being detected in racehorse samples. To our knowledge the only form of pemoline commercially available in amounts suitable for equine administration is racemic pemoline, no longer available in the US and many countries given its propensity to cause liver toxicity.[25] Given this reality, it would be of considerable scientific and forensic interest to determine the enantiomeric composition of the pemolines that are being identified in equine urine samples and compare their urinary enantiomeric profiles with the enantiomeric profiles of pemoline recovered from horses administered pharmaceutical pemoline, and – if it can be definitively demonstrated as a source – from horses administered levamisole. This approach has previously been used for identification of aminorex[26] as arising from levamisole, and which approach is facilitated by the commercial availability of chemically pure reference standards of each pemoline enantiomer[27] and *Zhu* et al.[28] have provided a mechanism for separation of pemoline enantiomers by cyclodextrin-modified micellar electrokinetic chromatography. Additionally, given the fact that pemoline exists as two enantiomers it might also be of interest to review the pharmacology and pharmacodynamics of its enantiomers to determine whether one enantiomer is more pharmacologically active and/or less toxic than the other.

Potential botanical sources of pemoline

Figure 5 provides a summary of the proposed structural relationships between levamisole and its breakdown products aminorex and pemoline (top) as well as for comparison the glucobarbarin isothiocyanate metabolite and its breakdown products, aminorex and barbarin. The latter mechanism proposes involvement of an alkylamine from plant sources. In either mechanism, aminorex would be hypothesized to go on to produce pemoline by an oxidation reaction[5] (symbolized as [O]). However, the matter of finding support for this proposed oxidation is difficult. One could argue in favor of its possibility as follows. Molecular modeling of aminorex with Hyperchem software[29] demonstrates significant electronegativity on the phenyl carbons as well as carbon-4 of the 2-oxazoline ring, providing reasonable targets for electrophilic oxygenation events. *Henderson* et al. (1995)[30] studied a compound related to aminorex – 4-methylaminorex – as an analog of psychoactive phenethylamines, and their metabolism studies in the rat suggested that the 4-methyl group of 4-methylaminorex may inhibit metabolism in a manner similar to methyl substitution on the α-group of β-phenylethylamines, e.g. methamphetamine. Furthermore, aminorex ring opens on hydrolysis to a beta-hydroxyphenethylurea, a route unavailable to 4-methylaminorex.[31]

Philip et al.[31] suggested hydroxy-levamisole and aminorex as levamisole metabolites that could be assessed to indicate deliberate levamisole administration in horses; however, the former compound ranged to only 0.25 % relative abundance compared to the parent drug, and aminorex even less. *Igwe and Blake* (1983)[20] showed extensive tissue distribution and a particularly long half-life for pemoline in horses after oral administration, suggesting that – if present – it could be present at much lower levels, possibly below the limit of detection of *Phillip* et al. (2022).[31] The situation is different in humans where the reported half-life is on the order of 11

hours.[33] Owing to the current use of levamisole as an adulterant of illicit cocaine, *Hess* et al.[34] studied human metabolism of levamisole and claimed to find aminorex but not pemoline. However, the ion source of their ESI (+)-MS/MS detector was set at 400 °C; one could argue that this setting acted to limit pemoline detectability in favor of aminorex, since aminorex melts at 137 °C, while pemoline melts much higher at 256 °C with decomposition.[35] Such decomposition might render pemoline undetectable in sample extracts, where the limit of quantitation was at least 3-fold higher for pemoline than for aminorex. One may conclude that aminorex oxidation to pemoline is a possibility from a chemical perspective, but it is pushed below detectability in sedentary experimental mammals exposed to levamisole or aminorex. The situation may be different for fully oxygenated athletes like racehorses.

On the other hand, stronger arguments can be made against the transition from aminorex to pemoline. A scan of the literature via PubMed or SciFinder shows no reference to oxidation of the 2-oxazoline ring in related compounds to a 4-ketone. Ring oxidation may preferentially proceed to ring-opening type reactions, similar to ones described by *Galetto* et al.,[36] for 2-oxazolidinones. The 4-methyl group of 4-methylaminorex, in contrast, protects against any ring opening.[37] *Ho* et al.[38] studied levamisole administration to horses and found aminorex, its phenyl ring positional isomer rexamino, and yet a different $C_9H_{10}N_2O$ isomer 4-phenyl-2-imidazolidinone, yet no pemoline. Plasma and urine elimination rates showed a preponderance of 4-phenyl-2-imidazolidinone compared to the other discovered compounds, leading the authors to suggest that compound as a marker for likely levamisole administration, in contrast to situations where only aminorex may appear from other sources such as plant ingestion, e.g. from yellow rocket.[17] *Hundertmark* et al.[39] have taken this discussion further in studies of the levamisole constituent tetramisole used in adulterating illicit cocaine. In serum samples from 73 cocaine misusers, only p-hydroxy-tetramisole and 4-phenyl-2-imidazolidinone were identified as tetramisole metabolites when using an assay with high specificity for aminorex, which was not found. Their conclusion is that – despite a low limit of detection in their assay for aminorex – confusion between isomeric $C_9H_{10}N_2O$ compounds aminorex and 4-phenyl-2-imidazolidinone is a strong possibility, particularly if investigation is highly dependent on GC/MS assays and not LC/MS. If this idea is correct, the interpretations relevant to equine drug testing, therefore, are that perhaps aminorex in fact occurs only from plant sources and not necessarily from levamisole, aminorex is not necessarily a precursor to pemoline, and these European and elsewhere reported low concentration pemoline findings have most likely arisen from

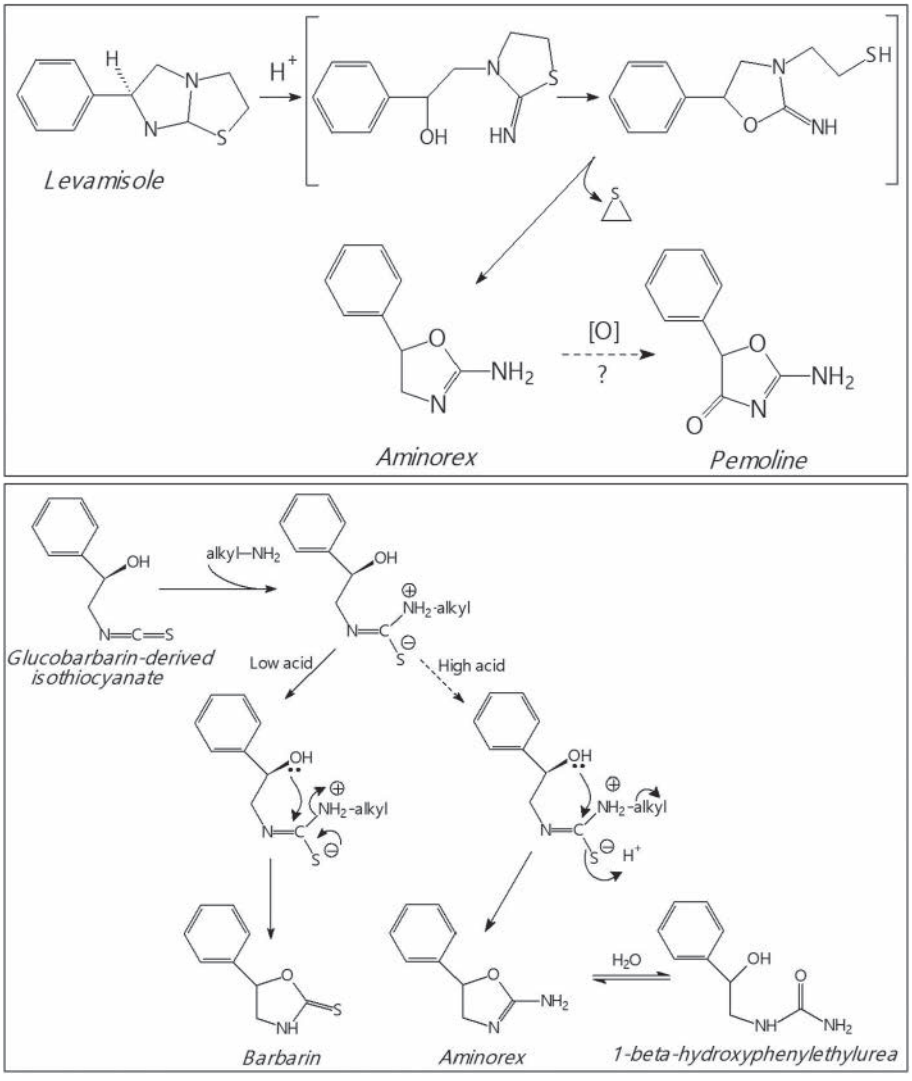

Fig. 5 Proposed mechanisms to account for the formation of aminorex and pemoline from levamisole (top, after *Gutierrez* et al.[5]) or barbarin and aminorex from the barbarin precursor glucobarbarin and its isothiocyanate breakdown product (bottom). Aminorex in the latter mechanism could hypothetically also undergo oxidation to pemoline, although it has been shown to ring open to 1-beta-hydroxyphenylethylurea.[32] | *Vorgeschlagene Mechanismen zur Erklärung der Bildung von Aminorex und Pemolin aus Levamisol (oben, nach Gutierrez et al.[5]) oder Barbarin und Aminorex aus dem Barbarin-Vorläufer Glucobarbarin und seinem Isothiocyanat-Abbauprodukt (unten). Aminorex könnte im letzteren Mechanismus hypothetisch auch zu Pemolin oxidiert werden, obwohl gezeigt wurde, dass es den Ring zu 1-beta-Hydroxyphenylethylharnstoff öffnet.*[32]

an unidentified botanical source in a manner similar to the barbarin and aminorex relationship (Figure 5).

Closing summary

This communication presents two clear patterns of pemoline identifications in equine urine samples. The pattern first reported in post-race English regulatory samples and confirmed in New York and Illinois samples suggests a potential for an at times high linkage rate between levamisole and pemoline identifications in post-race urine samples, although this relationship may simply be the result of higher sensitivity of testing for pemoline in those laboratories. The second and quite unexpected pattern of pemoline identifications is that first reported in France, Germany and South Africa where pemoline is identified in post-race urine samples and considered to be of natural and presumably botanical origins. Responding to these unexpected and to our knowledge unrelated patterns of pemoline identifications and following the lead of our European colleagues, we therefore propose in Irrelevant Plasma Concentration (IPC) Screening Limit/Regulatory Threshold of 2 ng/ml for pemoline in equine blood/plasma/serum.

Note added in proof

On or about December 18th, 2023, a Pennsylvania horseperson was notified of a pemoline "*violation*" in a horse racing on November 13th, 2023 at Penn National Racecourse [40]. The horseperson reached out to the Pennsylvania HBPA who contacted one author (*Thomas Tobin*) concerning this unusual substance identification in US racing [41]. The concentration claimed identified in the sample was 143 picograms/ml in blood, and the horseperson was completely unaware of any possible source for this identification. Responding to the PAHBPA request Thomas Tobin sent a detailed opinion setting forth the evidence that low concentrations of pemoline are routinely identified and considered to be "*of naturally occurring origin*" in European horses [42]. This analysis was communicated by the horseperson to HIWU with a request that it be reviewed by the Scientific Advisory Committee and his alleged violation rescinded [41]. On April 22nd, 2024 the horseperson was informed by the HIWU General Counsel that "*HIWU has determined that this finding will not be pursued as either an Adverse Analytical Finding or as an Atypical Finding*" and that "There will be no further disciplinary action taken against you" or the horse in connection with this matter [43], a result suggesting regulatory recognition by HIWU of the lack of forensic significance of low concentration pemoline identifications in US horse racing.

Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
ARCI	Association of Racing Commissioners International
DEA	Drug Enforcement Administration
EPC	Effective Plasma Concentration
FDA	Food and Drug Administration
GC/MS	Gas Chromatography/Mass Spectrometry
HFL	Horseracing Forensic Laboratory
HISA	Horseracing Integrity and Safety Authority.
HIWU	Horseracing Integrity and Welfare Unit.
HRMS	High Resolution Mass Spectrometry
IHRC	Indiana Horse Racing Commission
IPC	Irrelevant Plasma Concentration
IUC	Irrelevant Urinary Concentration
LCH	Laboratoire des Courses Hippiques
LC/MS	Liquid Chromatography/Mass Spectrometry
NAARV	North American Association of Racetrack Veterinarians
SRM	Selected Reaction Monitoring
SL	Screening Limit
US	United States

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Authors' contributions

TT conceived and directed the project and TT, CKF of the North American Association of Racetrack Veterinarians (NAARV), GAM, Director of the New York Drug Testing and Research Program, RLH of Holland Management Inc., AMB of Caracas, Venezuela and Dubai, United Arab Emirates and LD of Louisiana State University reviewed the data interpretation and analysis and approved the proposed regulatory guidelines from an equine practitioner, researcher, and regulatory scientist's perspective. KB and AFL performed the data searching, chemical structure evaluations and statistical analyses and TT coordinated and edited all drafts of this manuscript with ongoing contributions from all authors and all authors reviewed and approved the final manuscript submitted for publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available in the public domain as referenced in the manuscript or from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate are not applicable: As a review of the relevant scientific and regulatory literature, no ethics approval or consent to participate was necessary or required and all the authors have consented to publication of this case report and analysis.

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