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Trace level identifications of fentanyl and eutylone in equine plasma, pharmacological significance and probable origins – a case report and analysis

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Summary: Consistent with recently increased street availability and recreational use of the potent synthetic opioid fentanyl, there has been a parallel increase in trace level plasma identifications of fentanyl in racing horses, at times in association with trace level amounts of other human recreational substances such as the synthetic cathinone eutylone, currently classified as a designer drug. Fentanyl has three basic effects in the horse, i.e., an analgesic effect, a locomotor stimulation response and a potential endurance effect. Fentanyl is considered relatively straightforward to synthesize following a four-step procedure. Eutylone is the most frequently identified cathinone-related substance identified in the US and is considered a synthetic stimulant. Eutylone is inexpensive to produce and mimics the effects of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine, commonly known as ecstasy, and is, like fentanyl, a street marketed human recreational substance. Both substances are listed in horseracing as Class 1 with Penalty Class A substances, therefore having the highest penalties for identifications in horses given their stimulant properties, according to the ARCI (*Association of Racing Commissioners International*). Given that racing authorities recognize the potential for substances of "human use and addiction" to inadvertently transfer to racing horses, we were asked to develop an Irrelevant Plasma Concentration (IPC) for fentanyl in horses. Additionally, the finding of a trace level of eutylone along with a trace level of fentanyl increases the likelihood that these paired trace level identifications were caused by inadvertent transfer from a human using a combination of recreational substances. With regard to fentanyl, review of the published pharmacology of fentanyl suggested that locomotor responses disappear below plasma concentrations of 5 ng/mL, with the locomotor response peaking at 50 ng/mL. Similarly, the antinociceptive effects of fentanyl require concentrations above 6.5 ng/mL. An effective plasma concentration (EPC) of 25 ng/mL was therefore decided on. Dividing this EPC by 500, the conservative *Toutain & Lassourd* safety factor (SF) gives a 50 pg/ml IPC for fentanyl in the horse. This value was not exceeded by any of the low fentanyl concentrations identified in 125,000 post-race samples in the 2018–2022 time range. Plasma concentrations of fentanyl in the sub-40 pg/mL range are therefore pharmacologically irrelevant with a significant likelihood of transfer from recreational users. This IPC value is consistent with a number of recent trace level plasma fentanyl identifications in equine samples and judgements in these matters by regulatory authorities that the likely source of these trace level plasma fentanyl identifications was inadvertent transfer from human recreational users to the horses in question. The first detections of fentanyl detections in racehorses occurred in the period 1978–1983 corresponding to the introduction of sensitive radioimmunoassay screening methods. This contrasts with the more recent uptick in 2018–2022, apparently due to exposure of horses to inadvertent trace level transfers from recreational users of fentanyl. Eutylone continues to be a concern, but more information on its equine pharmacokinetics is necessary before similar development of EPC and IPC can be determined.

Keywords: fentanyl, eutylone, environmental exposure, detection, pharmacological significance, racehorses

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Introduction

On September 17th, 2020, two winning horses at Charles Town, West Virginia (WV) were disqualified for post-race "positives" based on the detection of fentanyl and eutylone in their postrace samples (*King* 2020). Eutylone (*Krotulski* et al. 2021) is an illicit recreational substance/drug known colloquially as a "bath salt", chemically related to the naturally occurring plant substance cathinone (*Kind* et al. 2012), and apparently being used recreationally in combination with fentanyl. The regulatory rulings specifically noted that "the standard penalty for a first offense Class A medication violation of year suspension/$10,000.00 fine" was not imposed. As reported in BloodHorse magazine, (*King* 2020) the ruling noted both trainers' overall clean medication records and the fact that the substances were identified at "a trace level, which

lends credibility to the probability that the horse was inadvertently exposed to the drug in some manner."

These rulings also noted that an employee of one trainer who had access to both horses prerace had refused to take a drug test for fentanyl, and which employee was subsequently suspended. Overall, these events are consistent with these trace level postrace fentanyl and eutylone identifications being an outcome of their inadvertent transfer to the horses in question at trace levels from a human or humans using these substances recreationally. These likely events were considered by the regulatory authorities, who redistributed the purses but imposed no multiple medication violation points on the trainers involved, recognizing the substantial evidence supporting lack of trainer culpability with regard to these trace level "substances of human use and addiction" identification events.

In response to this specific West Virginia case and related regulatory events we were invited to review the pharmacology of fentanyl in horses and to identify an Irrelevant Plasma Concentration (IPC) for fentanyl as a guide for regulators with regard to the pharmacological significance or lack thereof of low concentration serum/plasma identifications of fentanyl, as we now report.

The substances in question, fentanyl and eutylone

Fentanyl, N-phenyl-N-[1- (2-phenylethyl)piperidin-4-yl]propenamide (Fig 1) , is a μ-opioid receptor agonist about 100 times more potent than morphine (*Tobin* 1978). Like all morphine related μ-opioid agonists fentanyl has three basic actions in the horse, an analgesic response, a clearcut locomotor stimulation response and a potential endurance effect. Regarding the latter, there are suggestions that pharmacologically effective concentrations of a μ-opioid agonist may prolong the racing endurance of a horse by suppressing the perception of exercise stress approaching the end of a race (*Tobin* et al. 1979). For these reasons horse racing has long been concerned about fentanyl and its improper use in racing horses, with the matter of fentanyl being an opiate and a DEA Schedule II substance making fentanyl identifications a particularly sensitive issue with regard to the social image of horse racing (*Tobin* 1981).

Fentanyl was first synthesized by Dr. Paul Janssen in Belgium (*Stanley* 2014) who synthesized a number of opiate related agonists including phenoperidine in 1957 and fentanyl in 1960. As well as being 100 or so times more potent than morphine, fentanyl is much more lipid soluble, giving it a remarkably rapid onset of action following IV administration and a significantly better therapeutic index (safety record) than morphine. Fentanyl was introduced into human medicine in Europe in 1963, in the US in 1968 and fentanyl is currently the opioid most often used intravenously for intraoperative analgesia in the United States and worldwide (*Stanley* 2014).

Fentanyl is marketed worldwide as an opioid analgesic. Approved formulations include transdermal patches, oral transmucosal lozenges, buccal tablets, buccal soluble film, sublingual tablets, nasal sprays and most recently a sublingual spray (*Stanley* 2014). Furthermore, given that its chemical synthesis is relatively straightforward as set forth in Figure 2, the street availability of illicitly synthesized fentanyl, hereinafter street fentanyl, has significantly increased, leading to increased recreational use of fentanyl, with the associated potential for trace level environmental transfers to horses from humans either prescribed or using fentanyl recreationally. A further point of interest is that recreational use often involves combi-

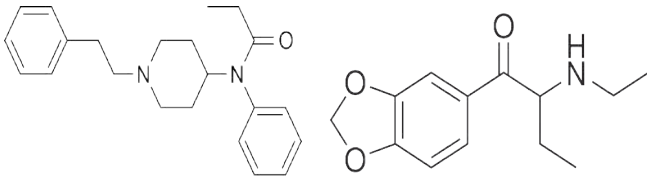

Fig. 1 a) Left, Fentanyl (N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl] propenamide); Molecular Formula $C_{22}H_{28}N_2O$, Molecular Weight, 336.471 g/mol. b) Right, Eutylone, ((±)-1-(1,3-benzodioxol-5-yl)-2-(ethylamino)butan-1-one); Molecular Formula $C_{13}H_{17}NO$, Molecular Weight, 235.283 g/mol. | *a) Links: Fentanyl (N-Phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]propenamid), Summenformel $C_{22}H_{28}N_2O$, Molekulargewicht 336,471 g/mol. b) Rechts, Eutylon, ((±)-1-(1,3-benzodioxol-5-yl)-2-(ethylamino)butan-1-on), Summenformel $C_{13}H_{17}NO$, Molekulargewicht, 235,283 g/mol.*

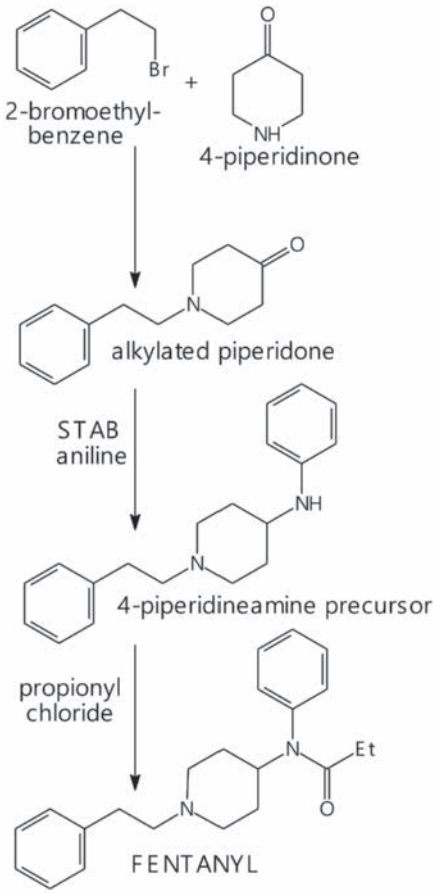

Fig. 2 Simple 4-step synthetic routes to fentanyl. The more recent *Valdez* et al. (2014) method has the advantage that all intermediate product yields exceed 90%, unlike the older *Suh* et al. (1998) method with yields in the 60–80% range. (STAB is sodium triacetoxyborohydride). | *Einfache 4-stufige Synthesewege zu Fentanyl. Das neuere Valdez et al. (2014) Methode hat den Vorteil, dass alle Zwischenproduktausbeuten über 90% liegen, im Gegensatz zur älteren Methode von Suh et al. (1998) mit Ausbeuten im Bereich von 60–80%. (STAB ist Natriumtriacetoxyborhydrid)*

nations of fentanyl with other psychoactive substances (*Wernau* 2023, *Daniulaityte* et al. 2019) such as eutylone, as in this West Virginia matter, and more recently with the veterinary sedative xylazine (US DEA, 2023).

Eutylone, Figure 1b, is a synthetic cathinone-related Novel Psychoactive Substance (NPS) and in 2021 it was the most frequently identified cathinone related substance by the US Drug Enforcement Administration (DEA) (*Krotulski* et al. 2021). Eutylone was first forensically identified in the US in September 2018 and by 2020 it was one of the most prevalent illicit synthetic stimulants reported in US forensic toxicology. *Krotulski* et al (2021) reported that by April 2020 eutylone had been confirmed in 83 human forensic cases, all but one case east of the Mississippi River, an east coast US distribution consistent with this current equine case being reported in West Virginia racing. This combined fentanyl and eutylone identification is of particular interest because the amounts identified are consistent with recent racing industry evaluations of the likely inadvertent transfer of fentanyl from human recreational users as the reason for the recent 2018 and thereafter uptick in trace level plasma identifications of fentanyl in racing horses.

Low concentration detections of fentanyl in racing horses

Concerns first arose in horse racing in the 1970s about the possible misuse of fentanyl in racing horses (*Combie* et al. 1979a). Administering fentanyl by rapid intravenous injection to horses we observed clearcut, rapid onset and highly reproducible dose related locomotor responses to fentanyl. We soon showed that this locomotor response is characteristic of opioid-related substances in horses, as we reported in the veterinary literature (*Combie* et al. 1979 a and b, *Kamerling* et al. 1985). Additionally, we developed a number of immunoassay-based screening tests for fentanyl in racing horses. Such tests had a Limit of Detection (LOD) for fentanyl of about 100 picograms/ml, which at that time provided a rapid, sensitive, and highly useful screening test for fentanyl (*Woods* et al. 1986, *McDonald* et al. 1987, *Tobin* et al. 1988).

With regard to the matter of equine drug testing, in the 40 or so years since fentanyl was first detected in horse racing the sensitivity of equine drug testing has increased at least 1,000-fold (*Fenger* et al. 2018). In a recent sequence of low plasma concentration fentanyl identifications the Limit of Detection (LOD) for fentanyl was 1 picogram/ml or less and the lowest concentration equine plasma fentanyl identifications that we are aware of were reported at 0.30 picograms/ml, and at 0.76 picograms/ml, both in Pennsylvania racing (*Mostollor* 2018). In all, eleven identifications were analyzed and detected in approximately 125,000 post-race equine samples analyzed between January 2018 and August 2022. Values ranged from 0.3–40 pg/mL. The question that has therefore arisen in the equine regulatory community is what if any is the pharmacological significance of these low picogram or sub-picogram/ml plasma concentration identifications of fentanyl.

A significant part of the reason for this question is the now widespread availability and use of street fentanyl, often in combination with other psychoactive substances and the associated increased incidence of human overdose deaths related to fentanyl abuse (*Wernau* 2023, *Daniulaityte* et al. 2019). This now widespread availability and use of street fentanyl has the potential to give rise to inadvertent transfer of trace level amounts of human use fentanyl to racing horses, as has long been the case for cocaine/benzoylecgonine (*Camargo* et al. 2006), morphine (*Camargo* et al. 2005), methamphetamine (*Brewer* et al. 2016), and gabapentin (*Brewer* et al. 2022) and other human prescription medications and recreational substances (*Washington Horse Racing Commission* 2022). As set forth in *Camargo* et al. (2006) in the case of cocaine, inadvertent transfer could arise from a cocaine abuser's hands to the mouth or muzzle of a horse and yield concentrations similar to those that are occasionally found in urine samples collected from show and racehorses. Spread of cocaine by casual contact is consistent with the fact that it is readily absorbed through human skin. Dermal and mucosal exposures of horses may result in the presence of cocaine metabolites in urine. Similar considerations likely relate to fentanyl, a drug known to be more lipophilic than morphine, as well as eutylone and related synthetic cathinones.

An irrelevant plasma concentration (IPC) for fentanyl

Review of the published scientific literature on fentanyl in horses shows that the locomotor response to fentanyl is lost when the plasma concentration of fentanyl falls below 5 ng/mL (*Combie* 1979). Similarly, *Echelmeyer* and coworkers (2019) have shown that fentanyl concentrations of greater than 6.5 ng/mL are required for an antinociceptive effect of fentanyl in the horse. These data are in good agreement with the results of *Combie* (1979) who reported the minimal plasma concentration of fentanyl associated with a locomotor response in horses as being 5 ng/mL and the peak locomotor response occurring at plasma concentrations of about 50 ng/mL. Assuming an Effective Plasma Concentration (EPC) for fentanyl in equines of about 25 ng/mL and dividing this concentration by 500, the highly conservative Effective Plasma Concentration (EPC) value divisor suggested by *Toutain & Lassourd* (2002) gives an Irrelevant Plasma Concentration (IPC) for fentanyl of 50 picograms/ml. We note that the eleven fentanyl concentrations determined post-race during 2018–2022 come in well below this conservatively calculated fentanyl Irrelevant Plasma Concentration (IPC). As such, it is highly unlikely that any of these $\leq$ 40 picogram/ml reported plasma concentrations of fentanyl were associated with any pharmacological effect of fentanyl at the time of the races in question. Eutylone identification was important in establishing that the source of fentanyl was likely from a human recreational user; however, more research is necessary before we can develop an IPC for eutylone.

History of fentanyl detections in racing horses

These environmental transfer interpretations for recently reported trace level identifications of fentanyl are consistent with the biphasic pattern of US fentanyl identifications as recorded by the *Association of Racing Commissioners International*

(ARCI) (*Holloway* 2020). As presented in Figure 3, fentanyl detections in racing horses were first reported in 1978 consistent with the then availability of a highly sensitive radioimmunoassay screening test for fentanyl. Fentanyl "positive" calls peaked at 29 in 1979 and then declined to zero in 1984/1985, consistent with recognition by potential users of the fact that fentanyl had become readily detectable by racing chemists. The following years from 1986 to 2017, a period of 31 years, presented a total of 10 fentanyl identifications, with zero identifications between 1995 and 2008, but 2018 presented 12 fentanyl identifications, zero in 2019 and a total of five in 2020.

The most likely explanation for this recent 2018 and thereafter increase in fentanyl identifications is an increase in the number of low concentration trace level fentanyl detections. Such an uptick in trace level identifications would be consistent with the presumably increased random exposure of horses to trace amounts of environmental fentanyl associated with the increased recreational use of street fentanyl (*Wernau* 2023, *Daniulaityte* et al. 2019). Given this environmental reality and the lack of pharmacological significance of trace level fentanyl, it may be appropriate for racing authorities to identify an analytical "cut-off" or Screening Limit of Detection (SLOD) for fentanyl in equine plasma, similar in principle to the environmental "cut-offs"/screening limits in place for cocaine/benzoylecgonine, methamphetamine and gabapentin and other human medications and recreational substances in many US racing jurisdictions (*Tobin* et al. 2012).

Based on the above analysis, there is no likelihood of a significant pharmacological effect associated with an identification of fentanyl at a plasma concentration of less than 50 picograms/ml. The second consideration is that given the now widespread availability of street fentanyl, the likelihood is that detections of trace level amounts of fentanyl at concentrations < 40 pg/mL are caused by inadvertent transfer of trace amounts of fentanyl from humans to horses. As such, the sub-40 pg/ml plasma identifications of fentanyl at concentrations are pharmacologically irrelevant with a significant likelihood of being the result of inadvertent transfer of fentanyl to the horses in question from a recreational user of street fentanyl or less likely from an individual prescribed an FDA approved product containing fentanyl (*Stanley* 2014).

Regulatory recognition of fentanyl as a substance of human use inadvertently transferring to racing horses

The final question is the identification of fentanyl sources in cases of possible environmental contamination. In the matter of the 300 femtogram/ml Pennsylvania identification the Stewards decided that "due to mitigating circumstances, there will be no further action on this drug positive" (*Fenger* et al. 2018). In the 760 femtogram/ml Pennsylvania matter the fentanyl identification was linked to the horse trailer driver being a user of fentanyl (*Mostollor* 2018), presumably non-prescription fentanyl. As such, the lowest concentration plasma identification was apparently dismissed, and the second lowest was considered caused by non-trainer related environmental transfer from a human user. Similarly, two of the 1–40 pg/ml plasma samples were associated with the employee in the West Virginia fentanyl/eutylone matter who had declined three requests to present for fentanyl testing, which was considered a mitigating circumstance for the trainers involved. Based on the prior good medication regulation record of the trainers involved, the Stewards adjudicating this matter considered these circumstances as indicative of inadvertent contamination being the source of this fentanyl identification and the trainers were found "not responsible for the medication violation" and no penalties were assessed against their licenses (*King* 2020). A point of particular interest is that the Pennsylvania Rules of Racing specifically recognize "substances of human use" as set forth in 403.16. "Environmental contaminants and substances of human use", in which section (b) notes that "Substances of human use and addiction may be found in the horse due to its close association with humans". Section (c) of this rule states that "If probative and substantial evidence is presented to the Bureau Directors prior to a hearing or presented to the Judges or Stewards during a hearing which indicates that a positive test may have been a result of environmental contamination, including inadvertent exposure due to human drug use, or dietary intake, or is endogenous to the horse, those factors may be considered in mitigation of any disciplinary action against the affected trainer" (*Legal Information* Institute, 2019) fully consistent with the above referenced fentanyl rulings.

The Pennsylvania Rules of Racing are also consistent with the current *Association of Racing Commissioners International* (ARCI) Model Rules which note that " (2) Substances of hu-

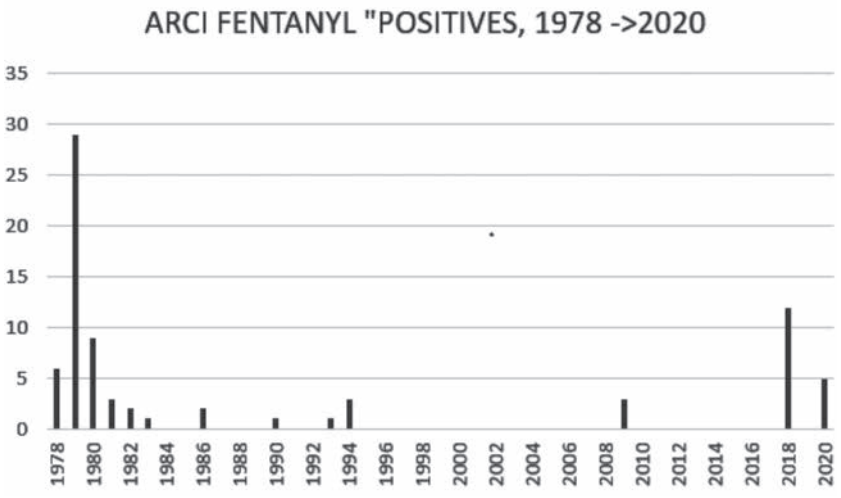

Fig. 3 Fentanyl identifications in US racing 1978–2020 as reported to the Association of Racing Commissioners International (ARCI). Shown are the number of positives versus year. | *Fentanyl-Identifizierungen im US-Rennsport 1978–2020, wie der Association of Racing Commissioners International (ARCI) gemeldet. Das Diagramm zeigt die Anzahl der positiven Ergebnisse auf der y-Achse und das Jahr auf der x-Achse.*

man use and addiction may be found in the horse due to its close association with humans. (3) If the preponderance of evidence presented in the hearing shows that a positive test is the result of environmental contamination, including inadvertent exposure due to human drug use, or dietary intake, or is endogenous to the horse, those factors should be considered in mitigation of any disciplinary action taken against the affected trainer. Disciplinary action shall only be taken if test sample results exceed the regulatory thresholds in the most recent version of the ARCI Endogenous, Dietary, or Environmental Substances Schedule." We also note, however, that the current ARCI Endogenous, Dietary, or Environmental Substances Schedule does not include fentanyl on its list of substances, so these West Virginia and Pennsylvania fentanyl rulings break new ground with respect to their recognition of fentanyl as a cause of trace level contamination of racing horse and their equine drug testing samples resulting from "inadvertent exposure due to human drug use" (ARCI, 2017, 2020).

Human drug testing "cut-offs" for fentanyl

Consistent with these moves by racing regulators away from zero tolerance testing for fentanyl, on October 24th, 2019, the Biochemical Testing Advisory Committee (BTAB) of the US Military Personnel Drug Abuse Testing Program "voted unanimously to add fentanyl and norfentanyl to the panel with a cutoff of one nanogram per milliliter." To the best of our knowledge, this "cut-off" is both an ELISA screening and a confirmatory testing "cut-off" (DOD Instruction 2020). This BTAB action is presumably a recognition of events similar to those set forth above in racing regulation, namely an increase in trace level fentanyl detections associated with inadvertent transfer from recreational users to "clean" individuals. It is also interesting that in a SAMHSA Drug Testing Advisory Board June 11–12, 2019 Minutes – Open Session, during the discussion, it was explained "that, although there is not a screening test specifically for fentanyl in the DoD panel, the presumption of a positive test will rely on an individual being positive for fentanyl and another drug – for example, cocaine and fentanyl, or an opioid and fentanyl ", formal recognition in human drug testing of a pattern of low concentration fentanyl detections occurring in association with detections of other recreational substances (*Dept Health & Human Services* 2019).

Conclusions

In summary, in the United States street fentanyl is now a widely available and used recreational substance, and is apparently being detected in racing horses at plasma concentrations of 40 picograms/ml or less, including one plasma identification at 0.30 picograms/ml. These plasma concentrations are well below the quite conservative 50 picograms/ml *Toutain & Lassourd* (2002) based Irrelevant Plasma Concentration (IPC) and at least two of these identifications, one at 0.76 picograms/mL and another at 0.30 picogram/mL, were considered by the regulatory authorities as being most likely caused by inadvertent transfer from a human user or users of presumably non-prescription fentanyl. Given these circumstances and the current widespread availability of street fentanyl, it is appropriate for equine regulatory authorities to carefully consider the lack of pharmacological relevance of plasma identifications of fentanyl at plasma concentrations of less than 50 picograms/ml and the significant probability that such irrelevant trace level identifications are associated with inadvertent transfer of fentanyl from human users, most likely recreational users of street fentanyl to racing horses.

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Conflict of interest statement

C. Fenger, K. Brewer, and T. Tobin are veterinarians and equine forensic scientists who have testified in equine forensic science matters and related areas. A. Morales-Briceño and C. Fenger are equine veterinarians who practice in the United States (C. Fenger) and South America and the Middle East (A. Morales-Briceño) in horse racing and sports-related areas. A. F. Lehner is an analytical toxicologist.

Author contributions

T. Tobin and K. Brewer performed the primary regional regulatory and forensic literature searching and analysis and assembled the forensic data, with A. Morales-Briceño focusing particularly on the European, Spanish, Middle East and South American veterinary and regulatory literature and experience. C. Fenger, Executive Director of the North American Association of Racetrack Veterinarians (NAARV), contributed to the writing and reviewed and approved the proposed Irrelevant Plasma Concentration for fentanyl. A. F. Lehner reviewed and

edited the manuscript and researched fentanyl syntheses. T. Tobin coordinated, organized, and drafted the various drafts of this manuscript with ongoing contributions from all authors, and all authors reviewed and approved the final manuscript submitted for publication.

Animal welfare statement

This research paper assembled, reviewed, and analyzed scientific, regulatory, and forensic data, and no animal experiments were performed.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Thomas *Tobin* https://orcid.org/0000-0001-8506-3147

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