IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES
IX. DETECTION OF DETOMIDINE IN EQUINE BLOOD AND URINE
BY RADIOIMMUNOASSAY

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SUMMARY

Detomidine is a potent non-narcotic sedative agent which is currently in the process of being approved for veterinary clinical use in the United States. Since no effective screening method in horses is available for detomidine, we have developed an $^{125}$I radioimmunoassay for detomidine in equine blood and urine as part of a panel of tests for illegal drugs in performance horses.

Our $^{125}$I radioimmunoassay has an I-50 for detomidine of approximately 2 ng/ml. Our assay shows limited cross-reactivity with the pharmacodynamically similar xylazine, but does not cross-react with acepromazine, epinephrine, haloperidol or promazine. The plasma kinetic data from clinical ($\geq$ 5mg/horse) as well as sub-clinical doses indicate first-order elimination in a dose-dependent manner. Within the first 30 minutes after intravenous (IV) administration of 30 mg/horse, plasma levels peak at approximately 20 ng/ml and then decline with an apparent plasma half-life of 25 minutes. Diuresis can occur with administration of clinical doses of detomidine and this effect was accounted for in the analysis of urine samples. Using this method, administration of 30 mg/horse can be readily detected in equine urine for up to 8 hours after IV injection. Additionally, doses as low as 0.5 mg/horse can be detected for short periods of time in blood and urine with use of this assay.

Utilization of this assay by research scientists and forensic analysts will allow for the establishment of proper guidelines and controls regarding detomidine administration to performance horses and assurance of compliance with these guidelines.
INTRODUCTION

Detomidine, 4(5)-(2,3-dimethylbenzyl)imidazole hydrochloride, is a new non-narcotic drug for which the manufacturers (Farmos Group Ltd., Turku, Finland) are seeking entry into the United States veterinary pharmaceutical market. Detomidine is pharmacologically related to the widely used sedative xylazine. Both are alpha-2 adrenoreceptor agonists causing sedation by decreasing the overall vigilance of the animal (Ruskoaho, 1986). However, detomidine is much more potent than xylazine in that a therapeutic dose is about 10 mg/horse for detomidine as compared with 500 mg/horse for xylazine (Jochle and Hamm, 1986). In addition, detomidine appears to be more specific for alpha-2 receptors in the central nervous system (Ruskoaho, 1986). It is currently being used in veterinary medicine in Europe and is suspected of being illegally administered to performance horses in both Europe and the United States.

Detomidine is classified as a sedative analgesic and muscle relaxant for clinical use (Clarke and Taylor, 1986). It is rapidly absorbed after administration by any route and is distributed throughout the body with a high affinity for the CNS (Jochle and Hamm, 1986). The principal physiological responses include bradycardia, vascular hypotension, and bradypnea. These effects are a transient response to detomidine administration with peak effects seen within the first 5 minutes post injection. Hypoxia and cyanosis can parallel the respiratory depression with this period apparently corresponding to the deeper analgesic and sedative effects (Kamerling et al., 1988).

Initial signs of analgesia occur as soon as 2 to 4 minutes after
intravenous (IV) injection. Additionally, with its ability to reduce gut motility, detomidine has been proposed as a drug of choice for controlling some forms of visceral pain or colic (Szeligowski et al., 1986).

Substantial diuresis can occur at one to two hours after drug treatment with other effects such as muscle tremors and sweating also occurring in animals exposed to high doses of detomidine (Jochle and Hamm, 1986).

The pharmacological characteristics of detomidine suggest its potential usefulness and potency for minor surgery, examination or manipulation of fractious animals, sedation of animals prior to shipping or management of some types of colic. In racing or showing horses it is sometimes advantageous to calm fractious animals prior to an event and the use of pharmaceutical agents for this purpose is expressly forbidden by most equine governing bodies. Sub-clinical doses of detomidine may cause subtle sedative effects and these effects may be observed after doses of 1 mg/horse or less. Such doses are currently not reliably detectable by any forensic method, and control of illegally administered detomidine is therefore a problem. For these reasons, we decided to develop a $^{125}$I radioimmunoassay (RIA) for detomidine that would be sufficiently sensitive for detection of this drug in equine blood and urine.

**MATERIALS AND METHODS**

**Horses**

A total of seven Thoroughbred or Standardbred mares (400-450 kg) were used throughout the study. The horses were kept at pasture and allowed free access to food and water. The horses were placed in standard box stalls (17 sq M) approximately 12 hours prior to dosing for
acclimatization.

**Dosing and Sampling**

Authentic detomidine standard, injectable detomidine (Domosedan®) detomidine metabolites and the newly developed analog medetomidine were obtained from Farmos Group Ltd., (Turku, Finland). Dose levels administered were 0.5, 1, 2.5, 5, 15, and 30 mg detomidine/horse. All injections were administered by rapid IV bolus into the jugular vein. All urine samples were collected by bladder catheterization and stored frozen until assayed. Blood samples were collected into vacuum tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ) containing potassium oxalate and sodium fluoride and immediately centrifuged. The plasma fraction was aliquoted and frozen until assayed.

**Whole Blood Distribution**

$^{125}\text{I}$ detomidine was added to equine whole blood samples. The samples were allowed to incubate at room temperature for 10 minutes, one hour or two hours. After incubation, the samples were centrifuged for 10 minutes at 3000 x g. The plasma and cell fractions were then analyzed for radioactivity (Beckman 5500 Gamma Counter, Beckman Instruments, Arlington Heights, IL).

**Radioimmunoassay Method**

The RIA method developed for detomidine is similar to methods previously reported (Woods *et al*, 1986, Weckman *et al*, 1988). Standard curves were run with each assay, including the cross-reactivity assays, to assure consistency. 50 µl of standards or samples were mixed with 100 µl of anti-detomidine antibody, 100 µl of $^{125}\text{I}$ detomidine and 150 µl of RIA buffer (50 mM tris(hydroxymethyl)aminomethane, pH 7.5 containing 0.1% gelatin). The tubes were allowed to incubate for one hour at room temperature. After the first incubation, 1.5 µl of normal rab-
bit serum plus 50 μl of goat antibody to rabbit gamma-globulin (Behring Diagnostics, La Jolla, CA) were added and the tubes were again allowed to incubate for one hour at room temperature. The second anti-body and the normal rabbit serum were added to separate the bound anti-
detomidine antibody from the unbound. After the second incubation, 1 ml of 50 mM tris buffer was added and the tubes were placed in an ice water bath for 10 minutes. The samples were then centrifuged (3000 x g) at 4 C for 10 minutes. After centrifugation, the top layer was aspirated leaving the cold antibody-ligand complex at the bottom of the tube. The tubes were then analyzed for activity (Beckman 5500 Gamma Counter, Beckman Instruments, Arlington Heights, IL). The data was then analyzed by use of a computer software package (RIA-AID, Robert Maciel Associates, Inc., Arlington, MA).

Plasma and urine samples were subjected to an extraction procedure prior to RIA analysis. Samples (1 ml) were pipeted into 1.5 ml micro-
centrifuge tubes and 3 drops of NaHCO3/NaOH (pH 10-12) were added along
with 400 μl of chloroform/isopropanol (3:1). The samples were then cen-
trifuged in a microcentrifuge (Eppendorf Microcentrifuge, Brinkman
Instruments Co., Westbury, NY) for 30 seconds at 8,800 x g and the top
layer discarded. The bottom layer was then transferred to clean glass
tubes and dried under a stream of nitrogen. Prior to immunoassay analy-
sis the samples were reconstituted with 25 μl of methanol and 150 μl of
50 mM Tris RIA buffer. The urine data resulting from clinical doses of
detomidine was corrected for its diuretic effect by analysis of creatine
nine content. Creatinine content was determined commercially by Veteri-
nary Laboratory, Inc. (Lexington, KY) and the data expressed as ng
immunoreactive detomidine/mg creatinine.
RESULTS

Figures 1 and 2 show typical standard curves illustrating the competitive binding of $^{125}$I detomidine and drug standards for our antidentomidine antibody. The assay was quite sensitive to detomidine with concentrations of detomidine as little as 0.5 ng/ml producing detectable displacement of $^{125}$I detomidine from the antibody. Our antibody showed little cross-reactivity with other parent compounds that we tested (Fig 1). The standard curve shows that the antibody does not cross-react with acepromazine, haloperidol, promazine or epinephrine. Xylazine at concentrations greater than 500 ng/ml shows minimal cross-reactivity with our antibody. Additionally, medetomidine, a newly developed analog of detomidine, and the two proposed metabolites of detomidine are recognized to a limited extent by our antibody (Fig 2).

![Graph showing detomidine antibody specificity by RIA analysis](image)

Figure 1. RIA standard curve for detomidine and cross-reactivity with acepromazine, epinephrine, haloperidol, promazine and xylazine. Only xylazine at concentrations above 500 ng/ml shows any cross-reactivity in our assay.
Figure 2. RIA standard curve for detomidine showing cross reactivity with medetomidine, (OH)-detomidine and (COOH)-detomidine. The carboxyl metabolite shows the least amount of cross reactivity with the detomidine antibody.

Prior to in vivo studies, the distribution of $^{125}$I detomidine in equine whole blood was determined. Figure 3 shows the results of incubating radiolabelled detomidine with whole blood. The solid bars demonstrate that approximately 90% of the detomidine is recovered in the plasma fraction regardless of incubation time.
Figure 3. Distribution of $^{125}$I detomidine in whole blood samples when incubated for 10 minutes, 1 hour and 2 hours. The solid bars show the percentage of the total radioactivity found in the plasma and the open bars show the percentage found in the cell fraction. Approximately 90% of the radiolabelled detomidine is recovered in the plasma.

We administered clinical and sub-clinical doses of detomidine to horses at dose levels ranging from 30 mg/horse to 0.5 mg/horse. After IV injection of 30 mg, plasma levels peaked at about 20 ng/ml and then decayed with an apparent plasma half-life of twenty five minutes to become undetectable after eight hours post administration (Fig 4). The peak plasma level attained, the time course of detection and the apparent plasma half-life declined in a dose-dependent manner with the lowest dose being detectable for less than one hour (Fig 5).
Figure 4. Plasma concentrations of immunoreactive detomidine following IV administration of 30, 15, 5 and 2.5 mg/horse.

Figure 5. Plasma concentrations of immunoreactive detomidine following IV administration of 5, 1 and 0.5 mg/horse.
The detection of detomidine or its metabolites in equine urine is complicated by the diuretic effect of detomidine (Fig 6). Clinical doses of detomidine can produce substantial diuresis, with the effects peaking at about 1.5 hours after drug administration and declining thereafter. Since this diuretic effect is likely to dilute out detomidine or detomidine metabolites in the urine, we chose to express the results of our urinary assays for clinical doses of detomidine in terms of ng immunoreactive detomidine per mg of creatinine.

![Diuretic Effect of Detomidine](image)

**Figure 6.** Diuresis produced by IV administration of 15, 5 and 2.5 mg/horse of detomidine.
Administration of 30 mg detomidine/horse was detected in urine by RIA for up to eight hours post administration (Fig 7). More importantly, sub-clinical doses are detectable in equine urine with use of this assay, although for only the first two hours post injection (Fig 8). Analysis for creatinine content is not required with sub-clinical doses since there is little or no diuretic effect seen at these dose levels.

Figure 7. Immunoreactive detomidine/mg creatinine found in equine urine following IV administration of 30 and 5 mg detomidine/horse.
Figure 8. Immunoreactive detomidine levels found in equine urine following IV administration of 1 and 0.5 mg detomidine/horse.

**DISCUSSION**

The $^{125}$I RIA developed for detomidine is similar to the assays we have developed for etorphine and fentanyl (Woods et al., 1988, Weckman et al., 1988). This assay is sensitive and specific for detomidine or detomidine metabolites. It has an I-50 for detomidine of about 2 ng/ml and does not cross-react with acepromazine, epinephrine, haloperidol, or promazine. It does cross-react with xylazine, but only at xylazine concentrations that are well above pharmacological significance. Additionally, it recognizes the hydroxylated and carboxylated metabolites of detomidine as well as the newly developed analog of detomidine, medetomidine. Using this assay, one can readily monitor the decline in plasma levels as well as the elimination phase associated with detomidine administration. The ability to detect sub-clinical doses of detomidine is most important since it is these dose levels that are suspected of being illegally administered to performance horses.
Sub-clinical doses of detomidine administered to a horse within a few hours of an equine event may have subtle pharmacological effects that would be advantageous in an overly fractious or nervous animal. Additionally, clinical doses may have residual sedative effects for as long as 24 hours (Wood et al., 1988). The use of pharmaceutical agents in this manner is forbidden by equine governing bodies and this creates the need for sensitive analytical methodologies for potentially abused drugs. Preliminary experiments suggest that the use of standard thin layer chromatographic techniques would not be sufficient to control the use of detomidine in performance horses and we therefore elected to develop an immunoassay for detomidine.

In our approach to this problem we synthesized several derivatives of detomidine and injected them into rabbits. Antibodies to these haptenes were harvested from these rabbits and evaluated in our assay. The hapten produced by derivatization of the imidazole moiety of detomidine elicited the most useful antibody.

We developed our RIA using this antibody and $^{125}$I detomidine. Our standard curves show that half-maximal inhibition of labeled detomidine binding occurs with the addition about 2 ng/ml of detomidine standard. In addition, our studies show that our antibody is specific for detomidine or detomidine metabolites and that interference from other compounds of similar pharmacological properties does not appear to pose a problem. Only the pharmacologically similar xylazine shows any cross-reactivity in our assay and then only at concentrations that are well above any plasma levels that one might expect to find after xylazine administration (Dyer et al., 1987).

The plasma kinetic data presented in figures 4 and 5 show that the assay readily detects the administration of detomidine in the horse,
although sub-clinical doses are not detectable for longer than one hour. Examination of Figure 4 reveals a peak plasma concentration of approximately 20 ng/ml after administration of 30 mg/horse and an apparent plasma half-life of 25 minutes. While the drug was detectable for eight hours after a 30 mg dose, it was only detectable in plasma for about thirty minutes after a 0.5 mg dose. The detection of low doses appears necessary to control the potential abuse of this medication in performance horses.

Our immunoassay will detect the administration of 1 mg of detomidine, and to lesser extent 0.5 mg, in equine plasma. The 1 mg dose is detectable for about two hours depending on the background levels seen in populations of horses. After the 0.5 mg dose the levels of detomidine that are detectable are much lower and are indistinguishable from background as soon as thirty minutes after injection. Therefore, adequate detection of this very low dose in equine plasma presents an analytical problem. However, the development of sensitive immunoassays would appear to be the practical course of action for the control of detomidine administration at the sub-clinical dose level.

The situation with respect to urine analysis is somewhat more promising. If the urine sample is extracted and analyzed for detomidine equivalents, levels that are substantially above background are found for up to two hours after the 1 mg/horse dose (Fig 8). With the 0.5 mg dose, the urinary levels are lower, but are still detectable at two hours after administration. Based on the data it would appear that sufficient levels of detomidine or detomidine metabolites are excreted in the urine in the first two hours to allow for the detection of subclinical doses.

Clinical doses are sufficiently detected in urine samples with the
use of this RIA analysis. As shown in Figure 7, detomidine or detomidine metabolites are effectively quantitated in equine urine after clinical doses when the immunoreactive material is expressed as ng detomidine/mg creatinine. When sub-clinical doses are used, it is not necessary to analyze for creatinine content, since there is little or no diuretic effect at these lower doses. The data indicates that the administration of this drug can be adequately detected by use of this assay in equine urine.

In other experiments we have incorporated this antibody into the enzyme-linked immunosorbent assay (ELISA) test format and evaluated its ability to detect detomidine in equine blood and urine. Based on our preliminary data, the ELISA test format does not appear to offer any major advantages in sensitivity or specificity.

In summary, detomidine's potency indicates that its potential abuse in the horse would involve very small doses and control of the use of this drug would require an assay with the ability to detect subclinical as well as clinical doses. While our assay shows the ability to detect subclinical doses of detomidine for short periods of time, there is the added problem of adequate confirmation. At this time there is no useful mass spectral method of confirmation and we are therefore concentrating our analytical approach to this drug into two areas. Firstly, we are developing other detomidine haptenes in the hopes of obtaining antibodies that will readily recognize the major metabolites of detomidine in the horse. Secondly, we are developing a mass spectral method for detomidine to allow for confirmation of any detomidine positive that may be suggested by immunoassay analysis.
REFERENCES


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