

**METHODOLOGY FOR THE SCREENING AND CONFIRMATION OF  
PICROTOXIN IN EQUINE URINE BY EXTRACTION AND GAS  
CHROMATOGRAPHY/ MASS SPECTROMETRY**

**Abstract**

Picrotoxin, a chemical mixture of the compounds picrotin and picrotoxinin (1:1), is obtained from the seeds of the plant *Anamirta cocculus*. Picrotoxin-related material can be extracted from equine urine by a standard BU (base urine; base/neutral extract) procedure. Since picrotoxin has no functionality to infer base or acid character it is proposed that it is isolated as a neutral species. Using GC/MS as the screening technique, a metabolite/ thermal decomposition product of picrotoxin can be detected in equine urine as a function of time following administration. Confirmation is based on the formation of this same product from a picrotoxin standard obtained from a chemical supply house.

This procedure has been used to detect and confirm use of picrotoxin, an RCI category 1 substance, in quarter horses racing in the State of Louisiana in six cases. This material is usually sold on the back-side in an alcohol-based solution (benzyl alcohol/water) under the name "Pickle Juice".

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**List of Standard Reference Materials**

Picrotoxin, a mixture (1:1) of picrotin and picrotoxinin

## **SUMMARY OF RESULTS OF METHOD VERIFICATION STUDY**

Four laboratories participated in the interlaboratory verification study. All laboratories reported the ability to detect and confirm the presence of picrotoxin-related compound(s) in urine samples obtained from a picrotoxin administration set. The administration set was generated by dosing four horses with 200 mg picrotoxin/horse (Acros, Inc; Lot# A010957101) by the intravenous (IV) route. Samples from each horse were collected at 0,1,2,4,6,8,24,48 and 72 hours post dose, pooled and aliquoted for submission.

Following the SOP provided, one of the four laboratories reported the detection and confirmation of the picrotoxin decomposition product in the 1,2,4 and 6 hour urine samples; one laboratory reported detection and confirmation in samples up to and including the 8 hour sample and one laboratory was able to detect and confirm this material in samples up to and including the 24 hour post administration sample. The variability in detection is not known but most likely relates to individual instrument sensitivity and conditions. The fourth laboratory deviated from the SOP in having used an on-column injection system for the GC/MS analysis. However, the use of this system indicated that the thermal decomposition product/metabolite identification established in the original SOP is most likely derived from a process involving injection port thermal rearrangement of the molecule and that this product is not a metabolite of picrotoxin. This was suggested by the ability to detect both picrotin (1-8 hours) and picrotoxinin (1-24 hours) individually in the respective samples using on-column injection. These results imply an alternative approach that affords direct detection and analysis of the components of picrotoxin. However, these results are derived from an SOP deviation and will, therefore, require further confirmation by other laboratories.

Laboratories involved in this study were Truesdail Laboratories, Industrial Laboratories, The Equine Medication Surveillance Laboratory of Louisiana State University and The Pennsylvania Equine Toxicology and Research Laboratory.

## **Safety Precautions**

The pharmacology and toxicology of picrotoxin have been studied in both humans and animals. It is a powerful stimulant that affects all parts of the CNS and at sufficiently high doses is a potent convulsant. Depending on dose, it can affect movement, memory, anxiety level and learning as well as mood. At high doses picrotoxin produces seizures, which are characterized by confusion, followed by convulsions that are intermittent and coordinated. Accompanying seizure activity are salivation, increased blood pressure, respiration and vomiting.

Picrotoxin has been marketed as an antidote to barbiturate and opiate overdose. Conversely, picrotoxin exposure and toxicity can be reversed with sedatives but is most effectively accomplished by benzodiazepines, such as diazepam.

Picrotoxin is sold as an off-white to yellow powder. Precautions to avoid exposure to drug powder or dust should include working with the drug under a hood, wearing a particle mask and eye protection, gloves and a disposable lab coat or protective suit. Labware coming into contact with the drug should be wiped clean of particles, the wipes properly disposed, and the labware rinsed with alcohol or acetone into a proper solvent/waste disposal receptacle. Solutions of the drug should be handled with gloves. Any exposed area should be washed with soap and water immediately.

## **SCOPE**

Picrotoxin is not detectable by currently defined tlc procedures or by any available immunoassay kits. This procedure is intended for application to the detection and confirmation of picrotoxin administration through the base-urine extraction and analysis of the equine urine extract by GC/MS. The methods described have not been applied to other body fluids or urine from other species. The methods are designed to accomplish qualitative detection and confirmation of the administration of picrotoxin to the horse using the formation of a picrotoxin-related product that is extractable from urine and is also formed from stock solutions of picrotoxin, which is used as reference material.

## **APPLICABLE CONCENTRATION RANGE**

Absolute limits of detection and confirmation have not been established. However, following the administration of picrotoxin (IV, 0.28 mg/kg) the drug was detectable by GC/MS (full scan) in urine for up to six (6) hours post administration. Urine samples from a horse administered the drug IV at a dose of 0.1 mg/kg contained no detectable level of the drug at any time point. This dose and route of administration of the drug also had no obvious affect on the horse. Analyses of urines from horses administered the drug have not routinely been conducted using selected ion monitoring, which could extend the ability to detect and confirm picrotoxin administration beyond the six hour period described or to detect administration of lower doses. As indicated in the results of the interlaboratory studies, detection and confirmation beyond the six hour time period

(1-24 hours) is feasible. Laboratory results suggest that limits of detection and confirmation in the low tens-of-ngs/ml are required, particularly for the later (24 hours) time period.

### LIMITATIONS

These procedures have not been examined for use in blood or other body fluids other than urine from the horse. Detection has only been demonstrated for doses of 0.28 mg/kg and 200 mg/horse IV. The method described is for qualitative analysis only.

### REFERENCES

Early attempts to develop methods of analysis for picrotoxin administrations were based on its use as an antidote for barbiturate overdose. Such methods were often indirect, insensitive and nonspecific. For example, Duff and Dille (J. Pharmacol. Exp. Therap., 67, 353-357, 1939) injected extracts from blood obtained from animals receiving picrotoxin into frogs and counted the number of frogs undergoing seizure activity and their relative severity. A search of the literature failed to reveal a standard instrumental analysis method for screening and/or confirmation of picrotoxin in biological fluids.

### DEFINITIONS

GC/MS	gas chromatography/mass spectrometry
BU	base-urine
RCI	Racing Commissioners International
IV	intravenous
SOP	standard operating procedure
CNS	central nervous system
MW	molecular weight
DCM	dichloromethane
ISO	isopropanol

### PRINCIPLE

Picrotoxin consists of two molecules in a 1:1 ratio (compound weight of 602 amu); picrotin (MW 310 amu) and picrotoxinin (MW 292), as shown in Figure 1. Picrotoxinin is pharmacologically active whereas picrotin is not. Picrotoxin is water soluble and neutral to litmus. GC/MS analysis, using a heated (200-250C) injection port, of organic solvent (ethyl acetate) solutions or organic extracts (base/neutral or acid/neutral) of picrotoxin produce a major peak whose mass spectrum does not directly reflect the structure of either picrotin or picrotoxinin. However, small satellite peaks are observed, especially as the injection port temperature is lowered (200C or less), that are identifiable as these compounds. This is an indication that picrotoxin undergoes thermal

decomposition in the injection port producing one major product with a mass of 266 amu (Figure 2 a and b). The degree of conversion of picrotoxin to this compound (% yield) is not known but the peak obtained from a standard increases with the age of the standard. Thus, the 266 amu compound is a marker for picrotoxin administration only and cannot, at this time, be used for quantitative purposes. GC analysis of picrotoxin using on-column injection may obviate this process of analysis for this product and permit more direct and efficient detection. This possibility is supported by the results obtained in the interlaboratory study discussed earlier.

Thus, base/neutral extracts of urine spiked with picrotoxin and assayed by GC using an injection port temperature of 200-250C provide a peak that can be identified by retention time and mass spectrum as being identical to the product obtained from analysis of picrotoxin standards by the same methods.

Administration of picrotoxin to a horse produces profound stimulation and collection, extraction and GC/MS analysis of the urine affords detection of this same compound at 1, 2, 4 and 6 hours or longer post administration using full scan. It is not known whether the compound extracted and injected is picrotoxin or one of its components or whether the compound detected is actually a metabolite in this instance. Current data support the idea that the material is a product of thermal decomposition/rearrangement.

A procedure for screening, detecting and confirming the use of picrotoxin can be established by monitoring this product.

## **STANDARDS**

Picrotoxin; Sigma Chemical Co., St. Louis, MO (picrotin and picrotoxinin also individually available). Off-white-yellow powder/crystals. Stable at room temperature, protect from light. Working stock solutions of picrotoxin are prepared in ethyl acetate at a concentration of 10 ug/ml and stored at -20C. These stock solutions have proven to have an indefinite storage life; indeed, the yield of the 266 amu analyte appears to increase with age, implying that it is itself a decomposition product of picrotoxin and also arises from a decomposition/rearrangement upon injection.

## **REAGENTS AND SUPPLIES**

Supplies and reagents for extractions should follow those given for a base-urine extraction procedure.

Bromthymol blue

Sodium carbonate

Dichloromethane:isopropanol (DCM:ISO) 75:25

HPLC grade water

Ethyl acetate

Carbonate buffer with bromthymol blue (pH 10-11, 1 M);

L Dissolve 106 g of sodium carbonate and 200 mg of bromthymol blue in 1 of HPLC water in a 2 L beaker. Place magnetic stirring bar in beaker and stir on stirrer until all solids are completely dissolved. Store in a closed glass bottle and label appropriately.

Picrotoxin working stock standard;

Add 1 mg of picrotoxin standard to 100 mls of ethyl acetate. Stir until dissolved. Label appropriately.

Tank of high purity, dry nitrogen

15 ml threaded glass conical centrifuge tubes and teflon lined screw caps

disposable glass serological pipettes

disposable glass pipettes and rubber bulbs

### **APPARATI**

Nitrogen evaporator; N\_EVAP, Model 115, Organomation Associates, Inc., or equivalent

GC/MS system; Hewlett Packard 5970 MSD, 5890 GC with autosampler or equivalent

Vortex mixer

Rotating sample mixer

Stirring bar

Magnetic stirrer

Vacuum aspiration device (side-arm vacuum flask connected to house vacuum; one-hole rubber stopper fitted with glass insert, Tygon vacuum tubing and a glass disposable pipette)

Centrifuge suitable for 15 ml conical tubes, 2000 rpm speed

### **SAMPLING**

No special handling or sampling conditions have been identified.

### **SAMPLE PREPARATION**

No special sample preparation conditions have been identified. Samples are normally stored at -20C prior to thawing for analysis. No decomposition of the picrotoxin product has been noted with repeated freeze-thaw cycling (n = 4 cycles).

### **CONTROLS PREPARATION**

Negative control urine should be screened by extraction and GC/MS analysis against pure reference standard for picrotoxin prior to its use in this procedure to assure

the absence of picrotoxin and or other interfering substances. Positive control(s) is prepared by spiking the negative control tube with 500 ul of working stock (10 ug/ml), evaporating to dryness and adding 8.0 ml of negative control urine. Method blanks are generated using HPLC grade water in place of urine. Extraction and analysis are conducted as described below.

## PROCEDURE

Extractions are performed as defined in the SOP for base-urines (BU).

1. Pour 8 mls of blank urine into a tube labeled urine blank and 8 mls of blank urine into a tube labeled positive control. Pour 8 mls of HPLC grade water into a tube labeled method blank.
2. Pour 8 mls of urine from each sample into appropriately labeled tubes.
3. Spike the positive control sample with picrotoxin standard (500 ul of 10 ppm ethyl acetate working stock).
4. Add 1.8 ml of 1 M carbonate/bromthymol blue solution to each tube using disposable serological pipette(s) or equivalent.
5. Cap each tube and place on a rotating mixer (20 revolutions/minute) for ten minutes. Emulsion formation becomes considerable if rotated significantly longer.
6. Centrifuge samples for 15 minutes at 2000 rpm.
7. Uncap samples and aspirate aqueous (top) layer. Allow tubes to set for approximately 10 minutes and aspirate any remaining aqueous (blue) layer.
8. Transfer solvent layer to clean 15 ml glass conical centrifuge tube using disposable glass pipette and rubber bulb.
9. Evaporate to dryness under a gentle stream of dry nitrogen in a water bath set to 35C. The nitrogen stream should form a "dimple" on the surface of the sample.
10. The residue is dissolved in 200 ul of ethyl acetate and submitted for GC/MS analysis.

Following GC/MS tuning, QC analyses and an instrument bakeout, samples are injected (2 ul each) in the sequence; pure standard (500 ul of 10 ppm ethyl acetate working stock), zero injection bakeout, method blank, urine blank, suspect sample(s), spiked sample and pure standard.

The GC (HP 5870 equipped with autosampler) is operated using the following conditions; injection port 250C, purge function activated at 0.75 minutes post injection, splitless. Column of 30 M DB-5, 0.25 mm ID, 0.1 mm coating, transfer zone of 300C and a head pressure of 15 psi. Temperature program; 50C for 1 minute, increasing 30C/minute to 300C with a 5 minute hold.

The MS (HP 5970 Mass Selective Detector, MSD) is operated in the electron impact, positive ion, full scan mode, eV of 70 and emission current of 30 ma.

Samples are run and data reduction is performed reviewing the retention time and mass spectrum of the 266 amu standard, screening the extracted ion current profiles for the major ions at the correct retention time. Ion ratios are reviewed and criteria for

determining the confirmation of the presence of the drug are assessed (See appropriate SOP). Following a determination that the sample meets all criteria for a suspect positive the original sample is then re-accessed and this SOP repeated. If both analyses meet necessary criteria for the determination of a positive the appropriate paperwork is completed and submitted to the Quality Assurance Officer (See appropriate SOP).

### **CALCULATIONS**

Calculations in this SOP consist of proper math applied to determination of weight-to-volume dilutions made to obtain stated concentrations.

### **TEST RESULTS REPORT**

Refer to appropriate SOP.

### **QUALITY CONTROL**

Refer to appropriate SOP.

### **FOOTNOTES AND COMMENTS**

A selected ion monitoring method has also been applied to the screening of samples for picrotoxin. Up to 5 major ions can be readily obtained from the spectrum and used to lower the limit of detection of picrotoxin in equine urine samples.