

Detection and Confirmation of Romifidine

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Abstract

Traditional liquid/liquid extraction methods as well as solid phase extraction were explored to find the best practical method for detection and confirmation of romifidine. As immunoassay kits are not yet available for this drug, thin-layer chromatography (TLC) is the method of choice for initial detection. The solid phase extraction employed was found to give less recovery than the thin-layer chromatography extraction methods. Complete procedures for detection and confirmation are included as well as a brief outline of some of the more significant TLC detection indicators.

Scope

The following SOP is proposed for TLC detection and GC/MS confirmation of Romifidine in equine urine.

The thin-layer chromatography (TLC) limit of detection for romifidine in equine urine is approximately 100 ng/ml using enzyme hydrolysis (EH), basic urine (BU) or ion pair urine (IPU) extraction's. The IPU method is our method of choice as the TLC visibility for romifidine is most pronounced in this extract. Romifidine was faintly detectable at a lower concentration (50ng/ml) in the EH extraction, but the EH extraction is less desirable due to romifidine's alignment with a common background spot. During routine screening, romifidine would most likely be detected in the IPU extract. Davidow is the solvent development system of choice regardless of extract.

The limit of detection by gas chromatography / mass spectroscopy (GC/MS) is 25 ng/ml using the IPU extraction method (blind scrape; 27 mls of urine was used).

Solid phase extraction (SPE) was also investigated. The limit of detection for GC/MS using the SPE method is 50 ng/ml (20 mls of urine was used).

References

Thin-layer chromatography (TLC) methods:

NASRC Quality Assurance Program (1982-1988)

ARCI Quality Assurance Program (1988-1995)

The TLC methods are modification (or verbatim) acquired from the above two programs.

Solid Phase Extraction (SPE) Method:

The solid phase extraction method used in this method development was derived from the application manual provided by United Chemical Technologies, Inc. for extraction of drugs using their Clean Screen® solid phase extraction columns.

Definitions

GC/MS: Gas Chromatography / Mass Spectroscopy

SOP: Standard Operating Procedure

TMS: Trimethylsilyl

BSTFA: bis(trimethylsilyl)trifluoroacetamide

EH: Enzyme Hydrolysis

IPU: Ion Pair Urine extraction

BU: Base Urine extraction

NU: Neutral Urine extraction

Prop: Propionic acid development solvent

Dav: Davidow development solvent

T-1: T-1 development solvent

Drag (D): Dragendorff's overspray reagent

Nitrite (N): Sodium nitrite overspray reagent

Cupric (C): Cupric chloride overspray reagent

FES: FES overspray reagent

Mand: Mandeline's overspray reagent

Nin: Ninhydrin overspray reagent

Fearons: Fearons overspray reagent

HCl: Hydrochloric acid overspray

Principle

Romifidine is an alkaline drug and was extractable in all of the alkaline extracts examined. Of the extracts examined the ion pair extract is the extraction of choice probably due to the potential ability of romifidine to form a zwitterion on its imidazoline ring.

Ion Pair Extraction (IPU): The first step in isolating a drug from a urine sample is to extract the drug from the urine into a solvent (organic phase). In order to move a chemical (drug) from an aqueous phase (urine) to an organic phase, the chemical (drug) must become neutrally charged (i.e. not charged). Some drug molecules have both a positively charged site as well as a negatively charged site in which both sites can not both be neutralized by pH adjustment (i.e. the usual way). To neutralize this type of species (a zwitterion species) one must use an ion pairing agent which can cap both the positive and negative sites and bring the drug into the organic phase.

Standards

Romifidine, 2-(2-Bromo-6-fluoroanilino)-2-imidazoline, an analgesic sedative, was obtained from Dr. Frans Delbeke, University of Gent, Belgium.

A 1.00 mg/ml stock solution was prepared by weighing out approximately 0.0100 grams of the romifidine powder and dissolving it in the appropriate amount of methanol (approximately 10.0 mls, depending on actual weight of romifidine) to yield a 1.00 mg/ml solution. A portion of this solution was diluted by a factor of ten (10) and appropriate amounts of this dilute solution were used to spike blank urine to yield urine concentrations of 0, 10, 50, 100, 200, and 500 ng/ml.

TLC Detection

Romifidine spiked urine samples of 0, 10, 50, 100, 200, 500 ng/ml were prepared. Each one was extracted by IPU, NU, BU, and EH methods, the table below summarizes the results.

	0	10	50	100	200	500	comment
IPU (Davidow)	-	-	-	DC+	DC+	DC+	
IPU (T-1)	-	-	-	-	-	-	Covered by dye
NU (Davidow)	-	-	-	-	-	Trace	Fearons
NU (Davidow)	-	-	-	-	-	-	3A:H2SO4
BU (Davidow)	-	-	-	DN+	DN+	DN+	
BU (T-1)	-	-	-	-	-	-	Modified Ehrlich's/HCl
EH (Davidow)	-	-	DN?	DN+	DN+	DN+	50 faint
EH (Propionic)	-	-	-	DN+	DN+	DN+	
EH (Propionic)	-	-	Trace	+	+	+	Fearons

Note: While spraying with the Drag Nitrite sequence on EH Davidow appears the best, the romifidine aligns with a common background spot that would make TLC detection difficult. Also, regarding the EH plate over-sprayed with Fearon's, Fearon's is not routinely sprayed on EH plates, this was done as an experiment to check Fearons sensitivity verses the Drag Nitrite Cupric sequence. Fearon's data is for information purposes, and to be used at your discretion. Furthermore, the GC/MS recovery was evaluated for three extracts with the following results: IPU > SPE > EH.

Note: A tremendous amount of variability was observed in determining the best extraction procedure for this drug. All of the alkaline extraction procedures that were evaluated (BU, IPU, SPE, EH) recovered romifidine with adequate success (EH was most often the least successful).

Note: The following over-sprays were also evaluated, but found to be ineffective: Ninhydrin, Fluram, FES-phenothiazine, Mandelin's and 3A:H₂SO₄.

Immunoassay Detection

The following kits were evaluated using a blank and a 200 ng/ml spiked sample: detomidine, promazine group, pyrilamine, tricyclic group, bronchodilator group, opiate group, etorphine, oxymorphone, anileridine, pentazocine, and lidocaine. None of these kits showed any cross reactivity. After testing the kits mentioned, mostly chosen at random, Neogen corporation was consulted regarding which ELISA kits might react with romifidine. As far as they could tell none of their kits would react with romifidine with any reliability (Thank you to D. Schroedter and S. Magsig of Neogen).

Procedure

Ion-Pair Extraction for Thin layer Chromatography

Reagents

- 1 Ion-pair buffer
- 2 3:1 dichloromethane/isopropyl alcohol
- 3 2N sodium hydroxide
- 4 1N hydrochloric acid
- 5 9:1 dichloromethane/isopropyl alcohol
- 6 Davidow solvent
- 7 Dragendorff's reagent
- 8 Sodium nitrite solution
- 9 Cupric chloride solution

Apparatus

- 1 E.M. Science, silica gel, Fluorescent, Thin-layer Chromatography Plates
- 2 Capillary plate spotter
- 3 Hot plate with small fan (for spotting TLC plates)
- 4 16 x 125 mm screw top test tubes with caps
- 5 10 ml graduated pipettes
- 6 Rotorack mixer for test tubes
- 7 Centrifuge
- 8 Vacuum aspiration apparatus
- 9 Disposable 15 x 85 mm tubes
- 10 Light box with 254 nm and 365 nm UV light
- 11 Water bath
- 12 Hot plate
- 13 Drying oven
- 14 Heating Block

Sample prep

- 1 Add 9.0 ml urine to a 16 x 125 mm screw top tube.
- 2 Add 2.0 ml ion-pair buffer and 4.0 ml 3:1 DCM:IPA.
- 3 Cap tube and place on rotorack. Adjust pH with 2N NaOH and/or 1N HCl until the aqueous (upper) layer is blue-green in color and the organic (lower) layer is pale yellow in color. The color of the aqueous layer is largely determined by the color of the urine and will vary from sample to sample. Resultant pH should be approximately 7.8. Rotorack after the pH has been adjusted for 15 minutes.
- 4 Centrifuge for 5 minutes. If necessary, invert the tube to break the pellet and the emulsion and re-centrifuge.
- 5 Aspirate the aqueous layer and transfer the solvent layer to a clean 15 x 85 mm tube. Concentrate to dryness in a 65°C water bath
- 6 Spot the residue on TLC plate using 9:1 DCM:IPA. Cool plate prior to development.

TLC Plate - Davidow

- 1 Develop in Davidow (use 40-50 ml/tank) for 5 cm. Dry plate well.
- 2 Observe with 365 nm UV light. Indicate fluorescence with =. Record Rf's.
- 3 Observe with 254 nm UV light. Indicate quenching with | |. Record Rf's.
- 4 Spray with Dragendorff's. Record colors and Rf's.
- 5 Spray with cupric chloride. Wait for three minutes. Record colors and Rf's.

Confirmation Of Romifidine by GC/MS:

Detection:

Romifidine was confirmable by GC/MS down to 25 ng/ml using the above IPU (ion-pair urine) extraction method. 27 mls of urine was used in preparation for GC/MS confirmation. All urine residue was spotted on a TLC plate (approximately 6 to 8 spots), developed in Davidow for five centimeters and the area scraped in the region of the romifidine standard, also spotted on the plate. Scrapings were eluted with 50:50 ethyl acetate:methanol (~200 µl), dried in a 60°C water bath under a gentle stream of nitrogen. Final residue was reconstituted in 10 µl ethyl acetate plus 10 µl BSTFA and heated for 20 minutes at 70°C in a sealed vial.

GC/MS Conditions:

Splitless for 0.8 min. at a Head Pressure of 15 psi
Column: 25 meter HP-5, 0.33 um film, 0.2 mm ID
Initial Temp: 80°C 1.51 min. hold
Program Rate: 20°C/min.
Final Temp: 280°C 13.74 min. hold

GC/MS was performed in full scan EI mode over the mass range of 40-550 amu.
Some significant ions for Romifidine monoTMS in order of abundance: 250,142,
331, 329, 99, 77
Retention time: ~11 minutes

Quality Assurance

TUNING Proper tuning of the GC/MS instrument is required before running standards controls and samples for the confirmation of any drug by GC/MS. Tuning to EPA 625 DFTPP tuning requirements was used in the development of this method. Other tuning compounds and requirements may be used depending on the standard operating procedures of your facility.

CONTROLS When performing a confirmation on any drug at least one positive control should be used to assure that the method is working and that a reasonable recovery is achieved. 100 ng/ml romifidine in urine, extracted by this method, should be easily detectable (as well as meeting criteria for calling a positive; see below). A positive control is a screened negative urine that is spiked with the drug under analysis (romifidine).

A negative control should also be used to assure that the drug under analysis is not being picked up as a contamination during handling and extraction of samples and controls. A negative control is a urine that has been screened negative for the drug under analysis. The control negative should show no signs of the drug under analysis via extracted ion profiles. Extracted ion profiles for the control negative should be done the same way as is done for reagent blanks (see explanation of how this is done under "Reagent Blanks").

REAGENT BLANKS A reagent blank is the same derivatizing reagent and/or solvent which is used to dissolve/ derivatize the residue from the sample and controls. Where these blanks are used is shown below (under: "Suggested Run order for Analysis"). Reagent blanks should show no sign of the drug under analysis. This is done by using extracted ion profiles of three of the major ions of the drug under analysis and scaling the view of these ions to the same scale as the extracted ion profiles of the sample, control positive or standard. The reagent blank ion profiles should be scaled to the smaller of the sample , control positive or standard which it is adjacent to according to the run order (see "Suggested Run order for Analysis", below).

CRITERIA FOR CALLING A POSITIVE

GC/MS analysis of drugs and their metabolites gives several different kinds of information which are used in the determination of a "positive". The two most important are the mass spectrum and the chromatographic retention time. Final determinations on positive samples are made based on the weight of all evidence and not solely on one or two parameters.

A. Mass Spectral Criteria

FIT: The mass spectrum of the sample is compared to the standard spectra in the computer library. The computer calculates how well the sample mass spectrum

matches the library and displays this in terms of a FIT number. The instruments used in this method development were an ion trap system (ITS40) and a quadrapole system (HP 5890/5971). A FIT of >899 is the goal to call a positive when using the ITS40. A fit quality of >89 is the goal to call a positive when using the HP data system (i.e. 90% or better for either system).

B. Chromatographic Criteria

Retention Time: A comparison is made between the retention time of the sample and the retention time(s) of the standard or the control positive. The goal for percent difference between the retention times is $\pm 1\%$.

C. Supporting information

While the retention time and FIT are the primary considerations in calling a positive, a number of criteria should be examined to support the initial call. Other factors that should be examined include:

Peak ratios: Generally, the peak ratios (percent relative abundance) of the largest 5 to 6 peaks in the mass spectrum of the drug under analysis are compared to the corresponding peaks in the mass spectrum of the standard or control positive. A goal of $\pm 30\%$ is set for corresponding peaks.

Suggested Run Order for Analysis

DFTPP instrument must pass tuning requirement

Standard the standard or standards for the drug or drugs being confirmed.

Blank a reagent blank of the same matrix as used for sample, standards, and controls.

CNTL(-) a blank urine extracted the same way and at the same time as the sample(s).

Sample extract of urine containing suspect(s).

Blank a reagent blank of the same matrix as used for samples, standards and controls.

Sample other samples with same suspect(s) may be run here, each followed by a reagent blank.

Blank a reagent blank of the same matrix as the samples, standards and controls.

CNTL(+) a blank urine which has been spiked with the drug(s) which is being confirmed, or a urine which has been previously confirmed to have that drug(s) present.

Blank a reagent blank of the same matrix as the samples standards and controls.

Standard the standard or standards for the drug or drugs being analyzed.

In confirmations performed at this facility the run order will include (after the control positive) another reagent blank followed by another injection of the standard. The second injection of the standard is to assure that the performance of the instrument has not changed significantly.

NOTE: The run order to be used as well as other criteria for calling a positive will depend on the standard operating procedures of your facility or other officiating bodies.

Reagents for Romifidine SOP

TLC Analysis

Extraction Solutions

Ion-Pair Buffer (IPU)

Heat 3.8L deionized water for one hour in 60°C water bath. Add 300 g sodium phosphate dibasic. Shake well. Add 75 ml 1N sodium hydroxide and 600 mg bromothymol blue dissolved in a minimum amount of methanol. Shake well. Adjust pH to 8.0 using concentrated H₃PO₄ and 2N NaOH (if needed). Filter.

Developing Solvents

Davidow: Ethyl acetate 3400 ml
Methanol 400 ml
Ammonium hydroxide 200 ml

Spray Reagents

- **Dragendorff's Spray**

Mix equal amounts of Solution A and Solution B.

Solution A: 9.4 g Bismuth Subnitrate dissolved in approximately 600 ml DI-H₂O. Add 306 ml Glacial Acetic Acid. Bring to 1 liter with DI-H₂O. Mix for several minutes and filter.

Solution B: 112.1 g Potassium Iodide in 1 liter DI-H₂O.

- **Cupric Chloride**

200 g cupric chloride in 600 ml DI-H₂O. Add 200 ml methanol.