The United States Public Health Service Substance Abuse and Mental Health Services Administration is alerting medical professionals that a substantial percentage of cocaine imported into the United States is adulterated with levamisole, a veterinary pharmaceutical that can cause blood cell disorders such as severe neutropenia and agranulocytosis.

Levamisole HCl is the active ingredient in a number of veterinary drugs approved to treat worm infestations in animals. Levamisole HCl was also the active ingredient in a human drug for oral administration approved on June 18, 1990, as adjuvant treatment in combination with fluorouracil after surgical resection in patients with Duke's stage C colon cancer. This drug was withdrawn from the U.S. market around 2000, and it has not been marketed in the U.S. since then. The objective of this study was to develop a method to determine the amount of levamisole in urine samples. The procedure will be provided to state health laboratories as needed to be used in the evaluation of patients that have developed neutropenia or agranulocytosis in the setting of recent cocaine use. A gas chromatography–mass spectrometry method was validated and tested at two different laboratories, and the method limit of detection for levamisole is 1 ng/mL in urine when using a 5-mL sample.

Confirmation of the stereoisomer of levamisole was done by high-performance liquid chromatography using a chiral column.

The United States Public Health Service Substance Abuse and Mental Health Services Administration issued an alert on September 21, 2009, warning of numerous cases of agranulocytosis, an acute blood disorder consisting of a substantial reduction in certain types of white blood cells. These cases appeared among illicit drug users in the setting of recent cocaine use adulterated with levamisole (Figure 1), a veterinary pharmaceutical (1). As of July 2009, according to the U.S. Drug Enforcement Administration 69% of seized cocaine lots entering the U.S. contained levamisole (2). Levamisole is added at approximately 6% by weight to the cocaine hydrochloride bricks (3) with the intent to enhance the effects of cocaine (4).

Levamisole had been used historically in humans for such conditions as cancer and rheumatoid arthritis; hence, its adverse effects on blood cells are known. A study of 60 patients receiving levamisole for the treatment of rheumatoid arthritis found that 35% of the patients suffered a persistent decrease in neutrophil counts (5). Ingestion of cocaine containing levamisole can also cause agranulocytosis (6,7). Snorting, smoking, or injecting cocaine containing levamisole has been
associated with rapidly developing, life threatening infections due to the development of neutropenia and agranulocytosis (6,7).

Pharmacokinetic studies for levamisole have shown that levamisole is eliminated from plasma with a half-life of approximately 5.6 h in one study (8) and 241 min in a second study (9). Only approximately 3.2% of the oral dose was recovered as unchanged drug in the urine (8).

An analytical profile for levamisole that summarizes the physical and spectrometric properties for the determination of levamisole in illegal drug samples has been prepared (10). Gas chromatographic (GC) methods have been developed for levamisole in plasma and in animal tissues using a nitrogen-selective thermionic specific detector (8,11) with detection limits as low as 2 ng/mL in urine.

This gas chromatographic–mass spectrometric (GC–MS) method was developed and validated to provide a method for health officials to measure levamisole in urine when unexplained occurrences of agranulocytosis occur. Patients will likely be unaware that they have been exposed to levamisole.

Experimental

Chemicals

Levamisole hydrochloride, S-(-)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole hydrochloride, was purchased from United States Pharmacopeia lot #F2C122. Tetramisole hydrochloride, (+)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole hydrochloride, was purchased from Acros. Cyheptamide and isoamyl alcohol (3-methyl-1-butanol) Reagent-Plus® were purchased from Sigma-Aldrich. EMD OminiSolv grade hexane was purchased from Fisher Scientific.

GC–MS conditions

An Agilent 6890 GC/5975B MS was used in the method validation with Enhanced ChemStation software version MSD ChemStation D.03.00.552. A Varian 450-GC/Varian 320-MS TQ MS with Varian MS workstation system control (version 6.9.1) was used in the analysis of urine samples collected in New Mexico. A 1-µL injection volume was used with splitless injection for 1 min. An Agilent DB-5MS UI column (30 m × 0.25 mm, 0.25-µm film thickness) was installed, and a constant helium carrier gas flow of 1 mL/min was used in the validation testing with fortified urine samples. In the analysis of urine samples from New Mexico, two additional columns were employed. An HP-5MS (15 m × 0.25-mm i.d., 0.25-µm film thickness) column and a DB-17MS (30 m × 0.25-mm i.d., 0.25-µm film thickness) column were used. The column oven program initial temperature was 70°C with a 1-min hold, then increased to 270°C at 20°C/min, and held for 4 min. The transfer line was set at 280°C, injection port at 250°C, and MS quad at 150°C. The electron multiplier was set at 200 volts above autotune value. The source was operated in electron impact mode and set at 230°C. Selected ion monitoring is used to increase sensitivity. In the method validation using the DB-5MS UI 30-m column, data acquisition began at 9.5 min monitoring ions m/z 148 and 204 both with dwell time of 100 ms then switched at 11.6 min to ion m/z 193 with dwell time of 100 ms. Using alternative columns requires determining the actual retention time of the analytes and adjusting the times required for monitoring the analytes of interest. Levamisole chromatography is sensitive both to the column type and condition of the column. Peak tailing significantly increases with column use. After a year of use in analyzing a variety of samples, removal of the first meter of the column was required to restore the DB-5MS UI column to performance similar to that when it was first installed.

High-performance liquid chromatography (HPLC) conditions for chiral separation of tetramisole

Levamisole is one of the two stereo isomers of tetramisole. One of the samples obtained from the New Mexico Department of Health contained 1.2 µg/mL levamisole based on the GC–MS results. The GC–MS method does not differentiate between the two possible stereo isomers. In order to verify that levamisole was being used and not tetramisole a chiral separation was employed on this sample. The separation of the stereoisomers of tetramisole can be carried by HPLC (12,13). A Phenomenex Lux 5u Amylose-2 column (250 mm × 4.6 mm) was used for the separation of the isomers. An Agilent 1290 HPLC system was used for the analysis with a flow rate of 1 mL/min with an eluent composed of 95% acetonitrile/5% IPA/0.1% DEA and an injection volume of 10 µL. The same sample extract used for GC–MS analysis was used for the HPLC chiral analysis.

Preparation of extraction solution and internal standard solutions

Five-hundred milliliters of extraction solution was prepared by transferring 25 mL of isoamyl alcohol to a 500-mL volumetric and diluting to volume with hexane. A stock internal standard solution was prepared by transferring approximately 5 mg of cyheptamide accurately weighed to a 50-mL volumetric and diluting to volume with methanol. A working internal standard solution was prepared by transferring 2 mL of stock internal standard solution to a 100-mL volumetric and diluting to volume with methanol containing 1 mL of concentrated ammonium hydroxide per liter of methanol in order to convert the levamisole HCl to its neutral form.

Preparation of standard solutions

Stock levamisole (mw = 204.29) standard solution was prepared by transferring approximately 1.5 mg accurately weighed levamisole HCl (mw = 240.75) to a 10-mL volumetric and diluting to volume with working internal standard solution. A series of standard solutions was prepared by serial dilution with working internal standard solution to the analytical range of interest including the limit of detection (LOD) of 25 ng levamisole per milliliter of solvent. A 25 ng/mL standard is equivalent to a urine sample at 1 ng/mL when extracting a 5-mL sample with 0.2 mL internal standard solution. The 1 M sodium hydroxide solution was prepared by transferring 4 g of sodium hydroxide to a 100-mL volumetric, adding deionized water to volume, and mixing until dissolved.
Preparation of urine samples for analysis

A 5-mL sample of urine was transferred to a screw-top test tube when sufficient sample was available and 0.2 mL of working internal standard solution was added to the test tube. Two milliliters of 1 M sodium hydroxide and 3 mL of 95:5 hexane/isoamyl alcohol extraction solution were added. The mixture was capped, placed on an oscillating shaker for 30 min, and then centrifuged using a bench-top centrifuge to break the emulsion. The upper layer (hexane/isoamyl alcohol) was transferred to a 5-mL Reacti-vial with a transfer pipette. One milliliter of hexane was added to the test tube without shaking and then transferred to the 5-mL Reacti-vial while taking care to avoid transferring water to the Reacti-vial. The Reacti-vial was placed in a heating block at 55ºC ± 5ºC and evaporated to approximately 0.05 mL with a gentle stream of nitrogen. After cooling to room temperature, 0.2 mL of methanol was added to the Reacti-vial, and it was capped and mixed on vortex mixer for 30 s. The sample extract was transferred to an autosampler vial containing 0.25 mL insert for analysis.

Calculation of results

Areas for selected ions were determined at the retention time for levamisole, fragment ion m/z 148 and molecular ion m/z 204, and for cyheptamide, fragment ion m/z 193. The molecular ion 204 is used for quantitation of levamisole. The ratio of the area for ions m/z 204 to 193 is calculated for standards and samples. Linear regression analysis is carried out comparing the ratio of the area for ion 204 to ion 193 versus the levamisole concentration in the standards. The slope and intercept obtained from the linear regression analysis are used to calculate the concentration of levamisole present in the urine sample extract. The concentration of levamisole in the urine is determined by multiplying the concentration determined in the extract by the concentration factor used. A concentration factor of 25 was used in this work (5 mL/0.2 mL = 25).

Confirmation of identity

Ion m/z 204 is the levamisole ion used for quantitation, and ion m/z 148 is a qualifying fragment ion for levamisole. The area ratio for ions m/z 204 to 148 obtained with unknown samples should be within ±20% of the ratio calculated for the levamisole standards for confirmation of identity.

<table>
<thead>
<tr>
<th>Concentration ng levamisole/mL urine</th>
<th>Average Recovery</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.80</td>
<td>90.0%</td>
<td>11.4%</td>
</tr>
<tr>
<td>2.68</td>
<td>84.1%</td>
<td>2.8%</td>
</tr>
<tr>
<td>8.92</td>
<td>98.9%</td>
<td>4.5%</td>
</tr>
<tr>
<td>29.7</td>
<td>92.6%</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

Table I. Spiked Recoveries for Levamisole and Standard Deviation
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61 ng/mL and obtained blank corrected recoveries of 122.2%, 116.4%, and 113.9% with an average of 117.5% and a percent relative standard (%RSD) deviation of 3.6%. Precision was evaluated by determining the %RSD for an approximately 274 ng/mL standard. Analyst 1 had a %RSD of 2.4% with a tailing factor of 2.9, and analyst 2 obtained a %RSD of 1.6% and a tailing factor of 1.38.

Stability of the Agilent DB-5MS (30 m × 0.25 mm, 0.25-µm film thickness) UI column over a 15-month period was evaluated. The column, when initially installed, had a signal-to-noise peak-to-peak (S/N) for a 0.223 µg/mL sample of 27 with a tailing factor of 1.50. After 15 months of use running a variety of samples, the column performance had degraded significantly and had an S/N of 9 with a tailing factor of 8.25 with a 0.274 µg/mL sample. After removal of the first meter of column the column performance was restored and had an S/N of 36 and a tailing factor of 1.50 with the 0.274 µg/mL sample.

Analysis of specimens

Samples of urine have been analyzed by health agencies to determine if the patient's illness can be linked to levamisole. As part of the method validation samples previously analyzed by the New Mexico Health Department were stored frozen and shipped to the St. Louis, MO Food and Drug laboratory for comparative analysis. Results are tabulated in Table II.

The St. Louis, MO Food and Drug Administration laboratory analyzed reserve samples that had been stored at the New Mexico laboratory. The two methods’ results for samples 2010203487 and 800900598 were similar but differed from samples 2010203510 and 2010202911. Using the FDA-selected ion monitoring method, all the patients were found to have at least trace levels of levamisole present. These patients had tested positive for cocaine or opiates, and levamisole is now commonly found as a cutting agent with co-

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**Table II. Comparison of Results for the Determination of Levamisole in Full Scan GC–MS at the NM SLD with the Selected Ion Monitoring Method Results Obtained at the FDA Laboratory**

<table>
<thead>
<tr>
<th>Specimen #</th>
<th>NM SLD Result</th>
<th>FDA Result (ng/mL)</th>
<th>Time Frame*</th>
<th>Case Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010203487</td>
<td>Negative</td>
<td>1.1 &lt; LOQ</td>
<td>Unknown</td>
<td>Tested positive for cocaine but arrived at the hospital unresponsive and later died.</td>
</tr>
<tr>
<td>800900598</td>
<td>Positive</td>
<td>1212</td>
<td>9 h</td>
<td>Cocaine from crack pipe also tested positive (tested by law enforcement out of state, not SLD).</td>
</tr>
<tr>
<td>2010203510</td>
<td>Positive</td>
<td>1.0 &lt; LOQ</td>
<td>Unknown</td>
<td>Tested negative for cocaine. Tested positive for opiates but could not determine if it was heroin. Unable to contact patient for interview; still trying. Reoccurrence.</td>
</tr>
<tr>
<td>2010202911</td>
<td>Negative</td>
<td>9.3</td>
<td>Unknown</td>
<td>This patient was not interviewed because it was a reoccurrence, and he had already been interviewed. He tested positive for cocaine, but reported last use was 2 months prior.</td>
</tr>
</tbody>
</table>

* Approximate time elapsed between last cocaine exposure and specimen collection.
caine (2). A possible explanation for the difference in the results is that selected ion monitoring is more sensitive than full scan MS and that levamisole is not stable in urine even when refrigerated.

An additional urine sample from a patient with unexplained agranulocytosis was sent to the FDA for analysis. This patient had used cocaine within 5 days of the urine analysis but the exact timing is uncertain. Approximately 2.7 mL of sample was available for analysis rather than 5 mL. The sample had been refrigerated after collection and shipped to St. Louis for analysis. Extraction was done 14 days after original collection. Sample chromatogram is shown in Figure 2 and was found to contain 0.075 µg/mL of levamisole. Given the half-life of approximately 6 h in-vivo for levamisole, it was remarkable to find levamisole still detectable at easily detectable levels.

Confirmation of stereoisomer

The GC–MS method does not differentiate between the two stereoisomers of tetramisole. The high concentration of levamisole found in the urine of New Mexico patient 800900598 provided an opportunity to determine if the patient was exposed to a racemic mixture, (±)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole, or to levamisole alone. This chiral separation has been reported using chiral HPLC columns (13,14).

The concentration of levamisole in the extract of this patient would be approximately 30 µg/mL, which allowed for detection by HPLC–UV detection. This same approach should be applicable to HPLC–MS or HPLC–MS–MS with possibly even lower LODs. Figure 3 shows the HPLC chromatograms for the mixed stereoisomer tetramisole standard, for the levamisole standard, and for the urine extract sample using the Phenomenex Lux 5µm Amylose-2 column. Based on the area percent obtained for the two isomers, the isomer distribution in the urine extract was approximately 87% levamisole.

Conclusions

A method for levamisole was developed and validated to provide health officials and analysts a means to determine if patients with signs and symptoms of severe neutropenia or agranulocytosis have been exposed to levamisole. The method has an LOD of 1 ng/mL in a 5-mL sample. Recoveries were 99% at 8 ng/mL and 117.5% at 61 ng/mL. Method applicability to patient samples was demonstrated.

Acknowledgments

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References

1. United States Substance Abuse and Mental Health Services Administra- 
tion. Nationwide public health alert issued concerning life-threatening risk posed by cocaine laced with veterinary anti-

2. Centers for Disease Control. Agranulocytosis associated with co-
caine use—four states, March 2008–November 2009. MMWR 

3. J.F. Casale, E.M. Corbeil, and P.A. Hays. Identification of lev-
amisole impurities found in illicit cocaine exhibits. Microgram J. 

4. L.P. Raymon and D.S. Isenschmid. The possible role of levamisole 
in illicit cocaine preparations. J. Anal. Toxicol. 33: 620–622 
(2009).

5. G.T. Williams, S.A.N. Johnson, P.A. Dieppe, and E.C. Huskisson, 
Neutropenia during treatment of rheumatoid arthritis with lev-

6. N.Y. Zhu, D.F. Legatt, and A.R. Turner. Agranulocytosis after con-

7. D.R. Czuchlewski, M. Brackney, C. Ewers, J. Mann, 
M.H. Fekrazad, A. Martinez, K.B. Nolte, B. Hjelle, I. Rabinowitz, 
B.R. Curtis, J.G. McFarland, J. Baumbach, and K. Foucar. Clin-
copathologic features of agranulocytosis in the setting of lev-

assay and pharmacokinetics of levamisole and p-hydroxyle-
vamisole in human plasma and urine. Biopharm. Drug Dispos. 7: 
71–89 (1986).

9. J.M. Reid, J.S. Kovach, M.J. O’Connell, P.G. Bagniewski, and 
C.G. Moertel. Clinical and pharmacokinetic studies of high-dose 
levamisole in combination with 5-fluorouracil in patients with ad-

10. A.M.M. Valentino and K. Fuentecilla. Levamisole: an analytical 

11. R. Woestenbohrs, L. Michielsen, and J. Heykants. Determination 
of levamisole in plasma and animal tissues by gas chromatog-
raphy with thermionic specific detection. J. Chromatogr. 224: 

12. International Conference on Harmonization (ICH). Validation of 
analytical methods: methodology. ICH Q2 B, 1996 
http://www.fda.gov/downloads/Drugs/GuidanceComplianceReg- 
ulatoryInformation/Guidances/ucm073384.pdf.

ration of chiral antifungal drugs tetramisole, miconazole, and 
paclitaxel on two chiral stationary phases. Anal. Lett. 38:

14. L. Peng, S. Jaypal, R. Chankvetadze, and T. Farkas. Reversed-
phase chiral HPLC and LC/MS analysis with tris(chloromethyl-
phenylcarbamate) derivatives of cellulose and amyllose as chiral 

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