Detection and structure elucidation of alkaloid metabolites from *Papaver dubium* in equine urine

Paul Wynne[1] and John Vine[2]
[1]SGE Analytical Science, Ringwood, Victoria, Australia
[2]Racing Analytical Services Limited, Flemington, Victoria, Australia

Introduction

Routine analysis of an equine urine sample collected in a Tasmanian jurisdiction showed the presence of an unusual compound. Detected during routine screening of an acetylated basic extract by GCMS, the compound had mass spectral characteristics very similar to apomorphine (Figure 1). Although other abundant alkaloid metabolites were also detected, further investigation was undertaken to determine if the compound was a prohibited substance, a component of a herbal supplement or an analytical curiosity.

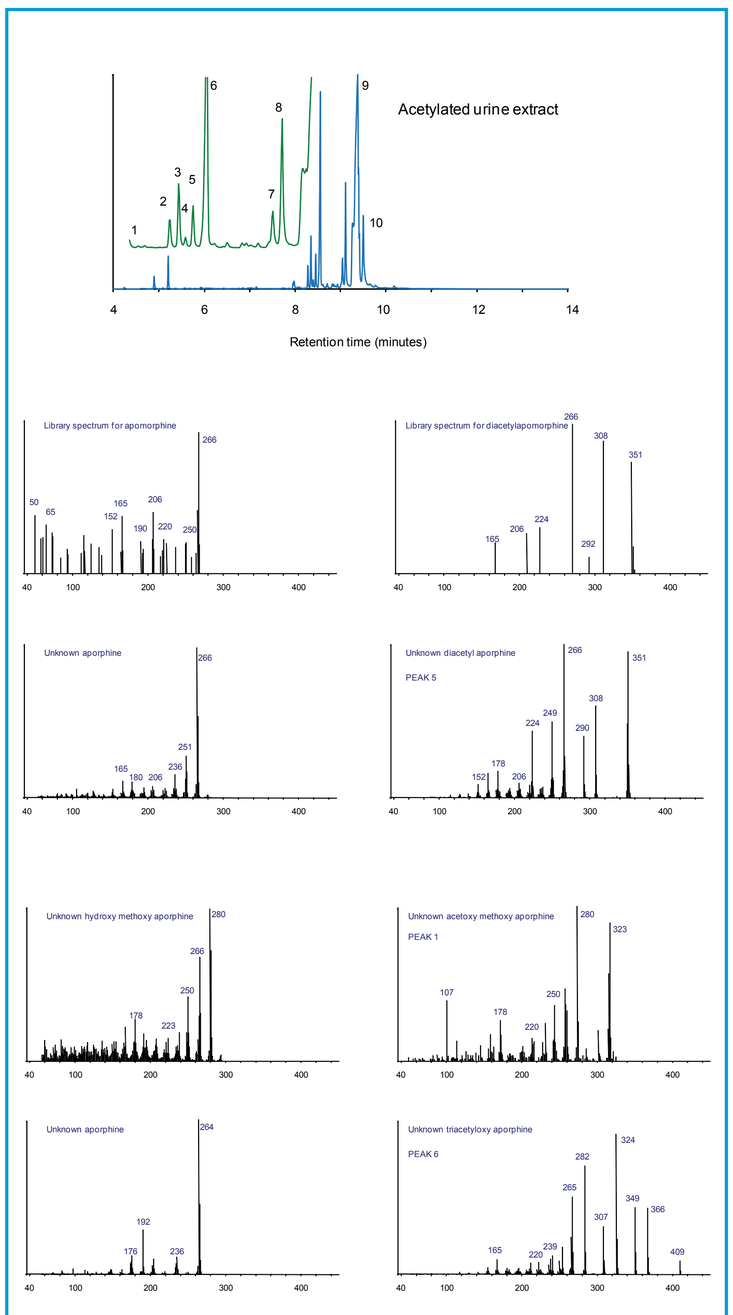

Figure 1. The GCMS chromatogram from the acetylated extract of a urine sample containing unknown aporphines and other alkaloid metabolites and the corresponding EI mass spectra before and after acetylation. Peaks 1,4 and 5 are identified as aporphines. Other peaks are identified as metabolites of other alkaloid classes.

Results and Discussion

Urinalysis showed the presence of several compounds that were tentatively identified as aporphines on the basis of their mass spectral data (Figure 1). Small differences in retention times and EI mass spectra showed that none were identical to apomorphine. Attempts to recover samples of feed from the stable returned a small quantity of remnant chaff that showed the presence of a badly degraded poppy capsule fragments. Taxonomic characteristics were similar to *Papaver dubium*, a wild species known to affect Tasmanian agriculture.

The fragment was matched with a capsule grown from the laboratory's reference collection (Figure 2). *Papaver dubium* is known to contain the aporphine alkaloid aporeine (1,2-methylenedioxyaporphine) which is also known as (+)-roemerine (Figure 3)[2]. As metabolic pathways for methylenedioxyphenyl compounds are known to proceed via the corresponding catechol to a methoxyphenol, 1,2-dihydroxyaporphine and 1-methoxy-2-hydroxyaporphine were predicted as aporeine metabolites along with possible N-desmethylation to noraporeine (Figure 3).

It is uncertain without a controlled administration of *Papaver dubium* whether or not the hydroxyl methoxyaporphine (Peak 1) and trihydroxyaporphine (Peak 6) are the result of secondary equine metabolism or derived directly from the plant material.

Figure 2. Papaver dubium *pod collected from a plant growing in Victoria (bottom) and similarly shaped fragments of degraded poppy pod collected from a feed sample.*

Targeted analysis of the urine and plant extract by LCMS[2] showed the presence of aporphines consistent with aporeine in the plant and the catechol metabolite in the urine (Figure 4). The catechol was also present in the plant as a minor metabolite. Fragmentation of the aporphines is proposed to proceed via sequential neutral loss of the nitrogen and the oxygen substituents and tend towards highly stable species that are resistant to further fragmentation (Figure 5). The m/z 219 ion arising from apomorphine is formed more readily than from the 1,2-substituted aporphines and its relative abundance may be of diagnostic value.

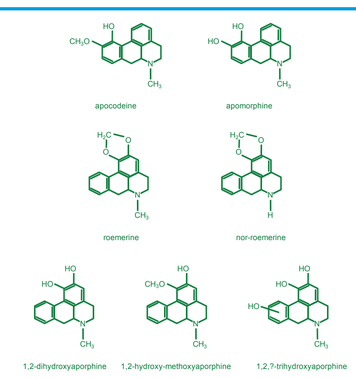

Figure 3. The structures of apocodeine, apomorphine, aporeine (roemerine) and possible urinary metabolites of aporeine.

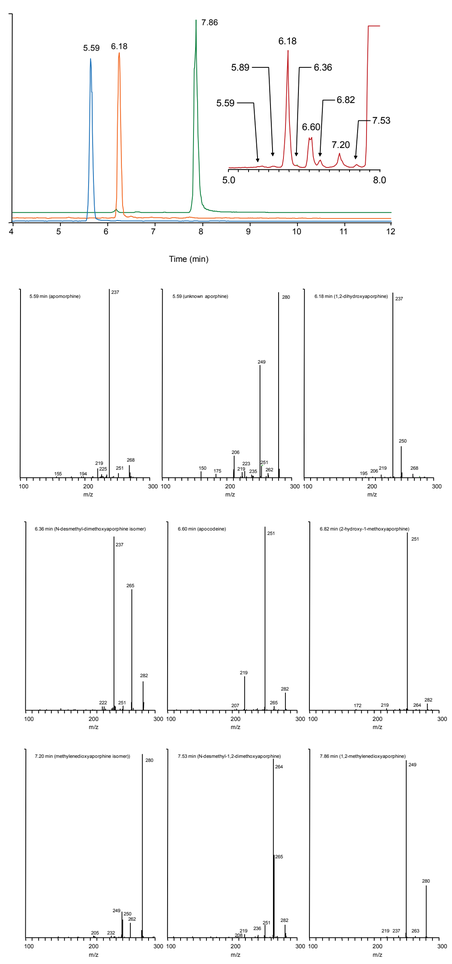

Figure 4. LC-ESI-MS[2] chromatograms for apomorphine spiked in urine (blue), unknown compound in urine (orange) and extract of Papaver dubium *(green) and the corresponding ESI mass spectra.*

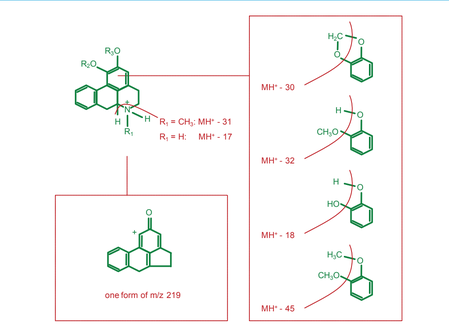

Figure 5. Characteristic neutral losses for substituted aporphines by ESI-MS and MS^n used for the identification of the major alkaloid in urine. Fragmentation produces stable conjugated product ions with m/z 219 that resist further losses.

Conclusion

Using ESI-MS[2], EI-GCMS and chemotaxonomic methods, the structure of a urinary metabolite of aporeine was deduced to be the catechol metabolite 1,2-dihydroxyaporphine. Both the catechol and aporeine are alkaloids of *Papaver dubium*. The catechol is isomeric with apomorphine but there is no evidence to suggest that it is a prohibited substance and in this case appears to have been administered inadvertently as a feed contaminant.

Experimental

A sample of urine (3 mL) was diluted with 0.1 M potassium phosphate buffer (pH 6.0, 4.5 mL) and the pH adjusted to 6.2-6.3. Each sample was then enzyme hydrolysed with ß-glucuronidase (2500 IU, E. coli, Sigma, St Louis, USA) for 2 hours at 50 °C and extracted on a Bond-Elut Certify column (120 mg, 3 mL, Varian, Harbor City, USA) as described previously[1]. The basic eluate was evaporated to dryness under a stream of nitrogen at 20 °C and a flow rate of 1 mL/min and the residues treated by one of two alternative routes. For GCMS analysis, the residue was acetylated with pyridine - acetic anhydride (2:1, 100 µL) in a screw-capped culture tube at 80 °C for 20 minutes. The excess reagents were evaporated under a stream of nitrogen at 60 °C and a flow rate of 1 mL/min and the residues reconstituted in 100 µL of ethyl acetate prior to analysis.

Alternatively, the basic eluate was evaporated to dryness under a stream of nitrogen at 20 °C and a flow rate of 1 mL/min. The residues were reconstituted in 100 µL of a 95:5 mixture of 1 % v/v aqueous acetic acid and methanol prior to analysis by LCMS.

Gas Chromatography Mass Spectrometry (GCMS)

EI-GCMS was performed on a Hewlett Packard 6890 GC - 5973 MSD equipped with a 7683 Autoinjector (Palo Alto, USA). The GC was equipped with a BPX5 column (12 m x 0.22 mm x 0.25 µm, SGE, Melbourne, Australia) and used helium as the carrier gas with a constant flow of 1.0 mL/min. The oven temperature was held at 75 °C for 2 minutes then heated at 30 °C/min to 300 °C with a final holding time of 9.0 minutes. Injections of 2 µL were pulsed splitless with a nominal head pressure of 60 kPa pulsed to 170 kPa prior to injection and returning to 60 kPa after 60 seconds. A scan range of 50-650 Da at 2.48 scan/sec was used.

Liquid Chromatography Mass Spectrometry (LCMS)

Electrospray Ionisation (ESI) - LCMS experiments were performed on a LCQ Classic mass spectrometer (Thermo Finnigan, San Jose, USA) equipped with a AS3000 Variable Loop Autoinjector, P4000 pump and SCM1000 solvent sparging unit (Thermo Separation Products (TSP), San Jose, USA). The column was a Zorbax SB-C18 (3.0 mmID x 150 mm x 5 µm, Agilent Technologies, Wilmington, USA) maintained at 55 °C and the mobile phase was a mixture of 1 % v/v aqueous acetic acid (solvent A) and methanol (solvent B) at a flowrate of 500 µL/min. A solvent gradient of A:B of 95:5 (0 min) to 10:90 (10 - 14 min) to 95:5 (16 min) was used. The injection volume was 10 µL. The electrospray ion (ESI) source operated with a capillary temperature of 220 °C, capillary voltage of 32 V, ion spray voltage of 4.5 kV, sheath gas at 65 units and auxiliary gas at 5 units. An isolation peak width of 1.5 Da was used for MS[2] precursor ions m/z 268, 280 and 282 at 30 % relative collision energy. Product ions were acquired over the range 100 to 300 Da.

References

[1]Batty, D.C., Wynne, P.M. and Vine, J.H. (1996) Comprehensive drug screening using a single solid-phase extraction cartridge. *Proc. 11th int. Conf. racing. Anal. Vet.* Eds: E.Houghton and D. Auer. R&W Publications, Newmarket, pp197-202.
[2]Barton, D.H.R., Bhakuni, D.S., Chapman, G.M. and Kirby, G.W. (1967) Phenol oxidation and biosynthesis. Part XV. The biosynthesis of roemerine, anonaine, and mecambrine. J. Chem. Soc. C, 1967, 2134 – 2140.

RACING ANALYTICAL SERVICES LTD

SGE Analytical Science
www.sge.com

AUSTRALIA & PACIFIC REGION CHINA EUROPE INDIA JAPAN MIDDLE EAST USA