Electrospray LC/MS Analysis of Glycopyrrolate in Equine Sport

Authors: P. Teale1 and S. Lowes2

1. Horseracing Forensic Laboratory Ltd, Newmarket, Suffolk, CB8 7DT, UK

2. Micromass UK Limited, Altrincham, Cheshire, WA14 5RZ

Introduction

Glycopyrrolate (glycopyrronium bromide) (Figure 1) is a quaternary ammonium anticholinergic drug administered to horses to assist breathing. Concerns about the abuse of this drug have been expressed by horseracing authorities worldwide and several positive cases have been detected. The on-line LC/MS[(1)] method currently in use at the Horseracing Forensic Laboratory for the specific detection of glycopyrrolate is described in this application note[(2)].

Glycopyrrolate is usually administered by intravenous or intramuscular routes at low levels (approximately 4µg per kg). Excretion in urine is rapid, taking place in less than eight hours, and predominantly in the form of the parent drug. ELISA kits are commercially available for the detection of glycopyrrolate in equine urine (Bioman Products Inc, Mississauga, Ontario, Canada) and provide an effective preliminary screening procedure for drug control. However in drug detection in sport, it is mandatory to identify the drug present in the body fluid under test, and ELISA technology is insufficiently selective for this purpose. Mass spectrometry offers high selectivity, and when coupled with the appropriate ionisation method for the sample under scrutiny, also high sensitivity. Initial mass spectrometric methods developed for glycopyrrolate involved base hydrolysis of the drug followed by methylation of the cyclopentylmandelic acid thus produced, and subsequent GC/MS detection of this methyl ester[(3)].

Various methodologies based on this approach have been developed and used to confirm the administration to horses of glycopyrrolate in drug control programmes in equine sports.

The advent of electrospray ionisation (ES) mass spectrometry presented the opportunity for developing an on-line LC/MS method capable of identifying the intact parent drug, and thus by-passing laborious and time-consuming derivatisation procedures. Electrospray ionisation is renowned in the biopolymer field[(4)], but its reliability and capacity to analyse thermally labile lower molecular weight compounds has ensured its increasing application in the detection and identification of drugs and their metabolites[(5)].

The method described herein is shown to be a rapid and reliable one, appropriate for glycopyrrolate qualification at the low ng/ml level using the closely related mepenzolate bromide (Figure 1) as internal standard.

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Experimental

Glycopyrrolate (1.2mg) was administered via an intravenous route to a thoroughbred mare (450kg). Urine samples were collected at controlled intervals via a catheter which was removed after 12 hours.

The urine samples were extracted for LC/MS analysis employing a modification of the method of Matassa et al[(1)], which is outlined in Figure 2, and briefly consists of adding the internal standard at the specified level of 20ng/ml to the urine sample and applying the whole to a carboxylic acid phase cartridge (International Sorbent Technology Ltd., Hengoed, UK) at neutral pH. The cartridge was then washed with buffer, methanol and chloroform, before being eluted with 5% acetic acid in methanol. Removal of this latter solvent and redissolving the sample in the mobile phase completed the clean-up procedure.

Chromatography was performed with a Hewlett Packard 1050 quaternary gradient HPLC pump in conjunction with a vacuum degasser. Sample injection (20µl) was effected with a Hewlett Packard 1050 series autosampler. Separations were performed with a Chromsep 5 C8 HPLC column (Chrompack, UK) of dimensions 100mm length x 3mm i.d, using an isocratic solvent system of 80:20 (volume:volume) acetonitrile: 20% aqueous ammonium acetate (50mM, pH 3.0) at a flow rate of 300µl/minute. The column eluent was interfaced via a splitter (1:10) to a Micromass Platform benchtop single quadrupole mass spectrometer (Micromass UK Limited, Altrincham, Cheshire, UK) operated in conjunction with MassLynx™ software and equipped with an electrospray ionisation source. Positive ionisation MS data were recorded over the range m/z 100-360 at 2 seconds/scan for full scan spectra or by selected ion recording (SIR) mode monitoring the M^+ ions of the glycopyrronium and

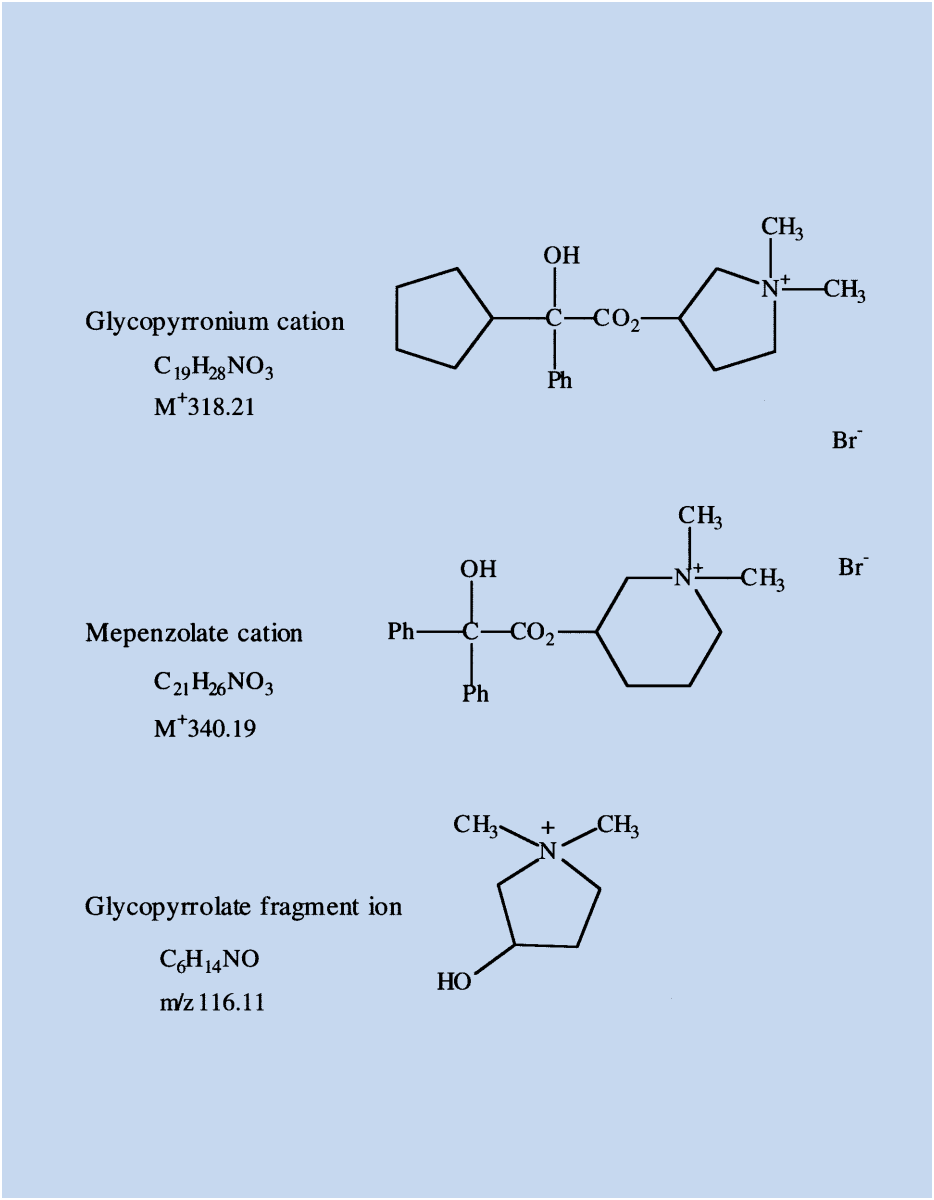

Figure 1. Chemical structures and formulae for glycopyrronium and mepenzolate cation and the assumed glycopyrrolate fragment ion.

mepenzolate cations at m/z 318 and 340 respectively for quantitative analysis. Responses were measured from the area under the molecular or fragment ion chromatograms of interest. The source temperature was maintained at 60°C and the capillary voltage operated at 3.6kV. The sampling cone voltage was varied through the range 20 to 45V, depending on whether the molecular ion was being optimised or in-source collisionally induced dissociation (CID) was being used to generate fragment ions.

Results and Discussion

ES Analysis of Glycopyrrolate

The electrospray ionisation method occurs as a consequence of a strong electric field acting on the surface of the solution eluting from the capillary of the ES interface, which results in dispersion of the solute and solvent into an aerosol of charged droplets. The solvent evaporates, aided by the drying gas, the droplets diminish in size, and eventually sample ions are expelled to be analysed by the mass spectrometer. Further solvent stripping takes place in the intermediate pressure region (ca. 1mbar) between the source and the MS analyser (Figure 3).

The presence of the polar solvent which frequently contains an electrolyte assures the "soft" nature of ES ionisation. Thus for organic compounds of less than ca. 1000 daltons molecular weight, ES generally yields singly charged, protonated molecular ions $(M+H)^+$, or deprotonated molecular ions $(M-H)^-$, in the positive and negative ionisation modes respectively so confirming the molecular weight. In addition, increasing the sampling cone voltage to generate in-source CID generates fragment ions from which structural information can be deduced[6].

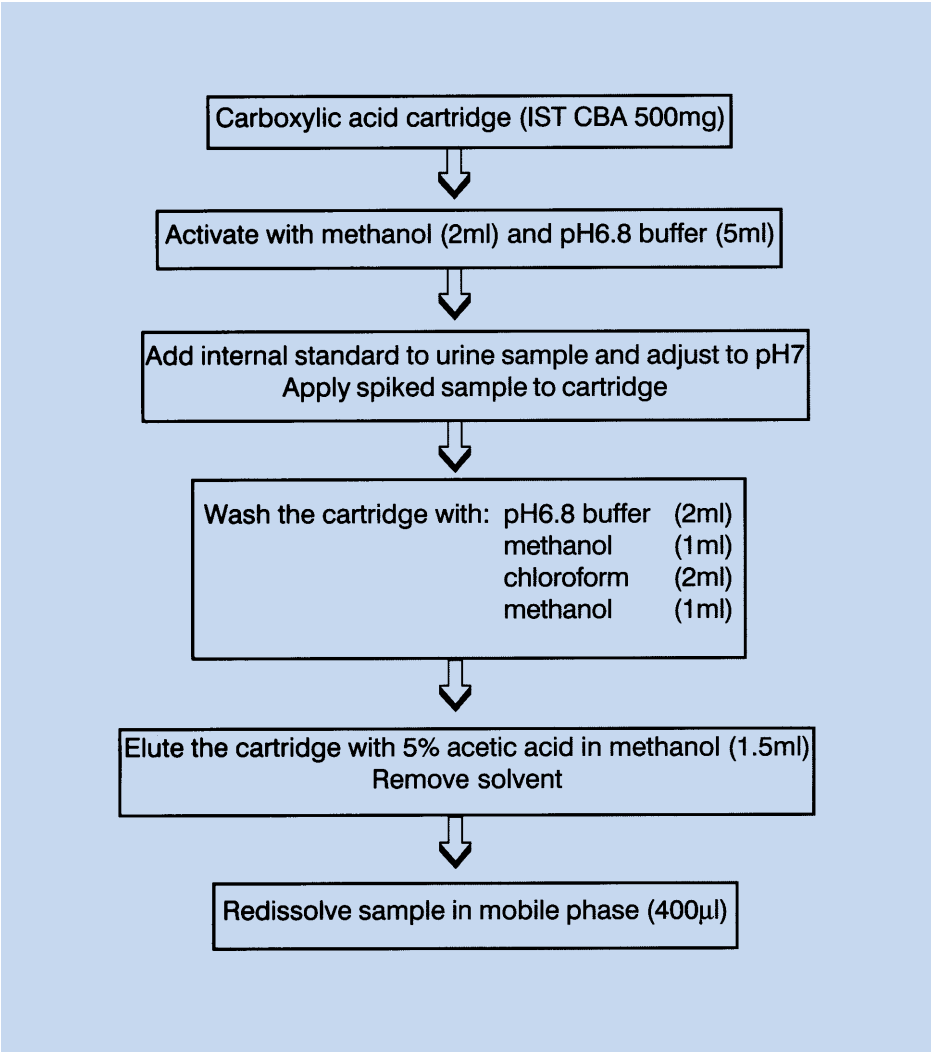

Figure 2. Extraction procedure for horse urine samples.

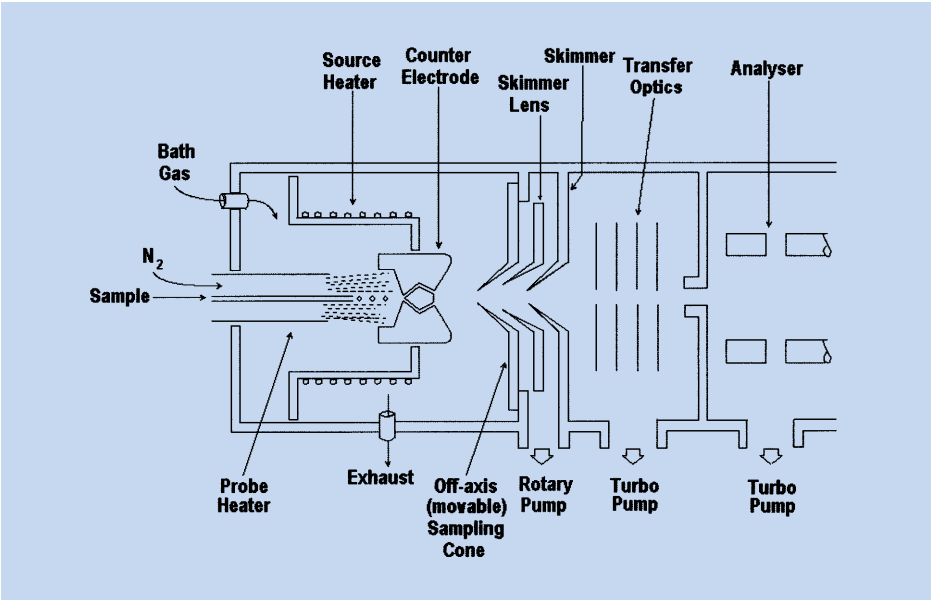

Figure 3. Schematic diagram of the Electrospray Interface.

In this study positive ionisation ES of glycopyrrolate produced a base peak ion at m/z 318 corresponding to the glycopyrronium cation, M^+ (Figure 4a). This ionisation mechanism, whereby proton addition is not observed due to the fact that the molecule is already charged, is common to ionic compounds analysed by ES[7].

Whilst the molecular ion itself is often diagnostic of an analyte of interest, confirmatory fragment ions can be valuable when looking at unknowns and complex mixtures such as those existing in biological matrices. In source CID is simple to perform on the Platform electrospray system, and can be incorporated readily into the MassLynx™ scanning function so that alternative scans can be programmed to be acquired with different sampling cone voltages throughout the LC/MS run. The CID spectrum (Figure 4b), obtained by employing a higher sampling cone voltage, shows a diagnostic fragment ion of glycopyrrolate at m/z 116 with the proposed structure (Figure 1) whereby the charge is retained on the quaternary ammonium site, and the ester functionality has been cleaved. Another ionic compound mepenzolate bromide was chosen as internal standard, due to its similar chemical structure and ES behaviour.

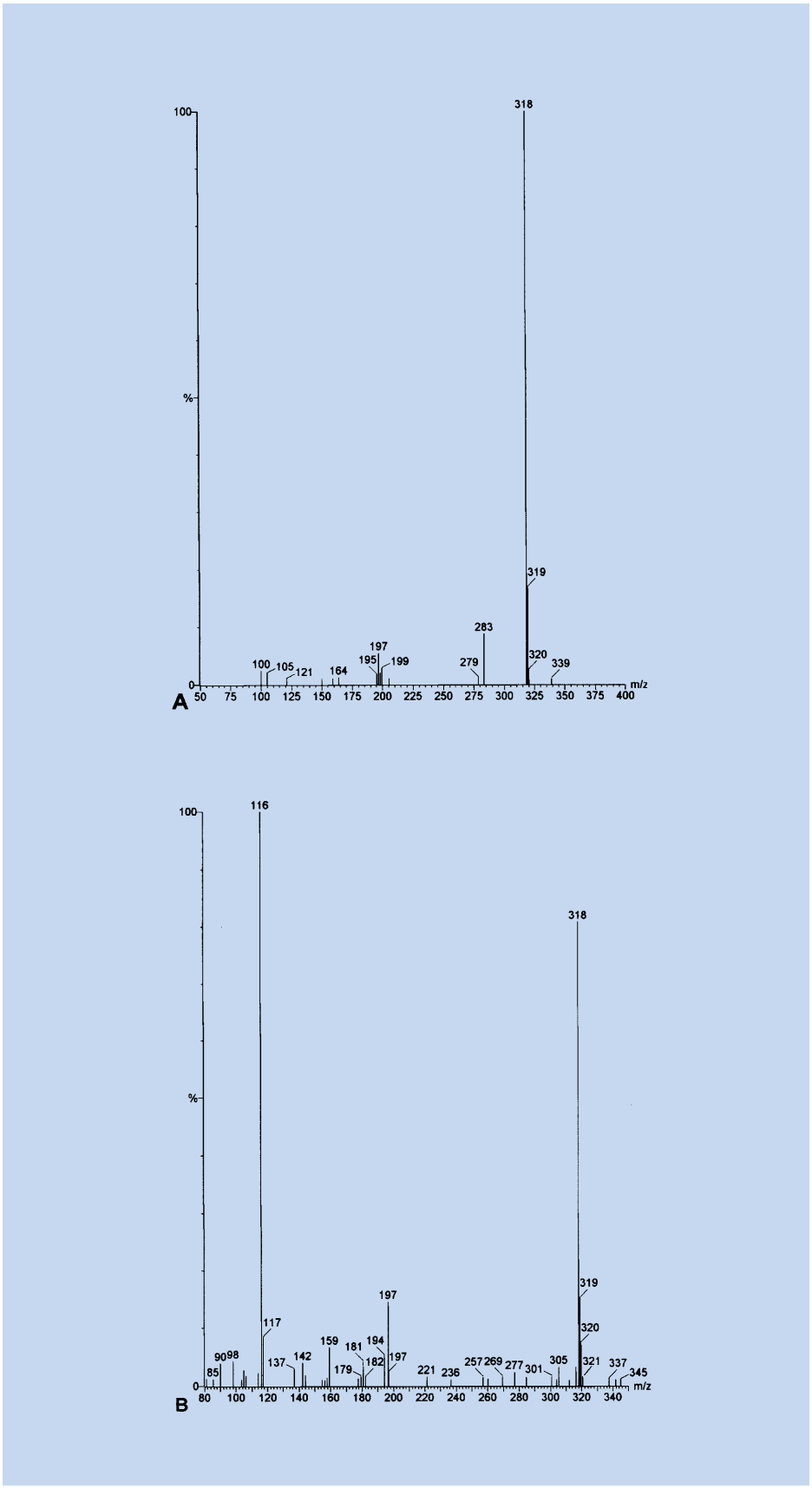

Figure 4. Positive ion ES mass spectrum of glycopyrrolate, (a) optimised on the M^+ ion, (b) using in-source Collision Induced Decomposition to generate structual information.

Detection of Glycopyrrolate in Horse Urine

Having ascertained the characteristic behaviour of glycopyrrolate to ES ionisation, detection of the drug in horse urine samples was investigated. The urine samples were spiked with the internal standard mepenzolate bromide, extracted as described previously and analysed by positive ionisation ES using selected ion recording (SIR) of the M^+ ions of glycopyrrolate and mepenzolate bromide, m/z 318 and 340 respectively. A low sampling cone voltage was employed to optimise these ions and minimise fragmentation.

The selected ion chromatograms of m/z 318 and 340 for a 5 hour post-administration urine sample (1.2mg intravenous dose) (Figure 5a) show the internal standard (retention time 3.41 minutes) well separated from glycopyrrolate (retention time 4.56 minutes). The retention times are in close accordance with those obtained when the pure samples were analysed under identical conditions. The corresponding responses from a “blank” urine sample, where no drug had been administered but the sample had been spiked with the internal standard, show a similar profile for the internal standard, but no evidence for glycopyrrolate, as expected (Figure 5b). The relatively short time taken for elution under these isocratic conditions makes possible a fast throughput of samples.

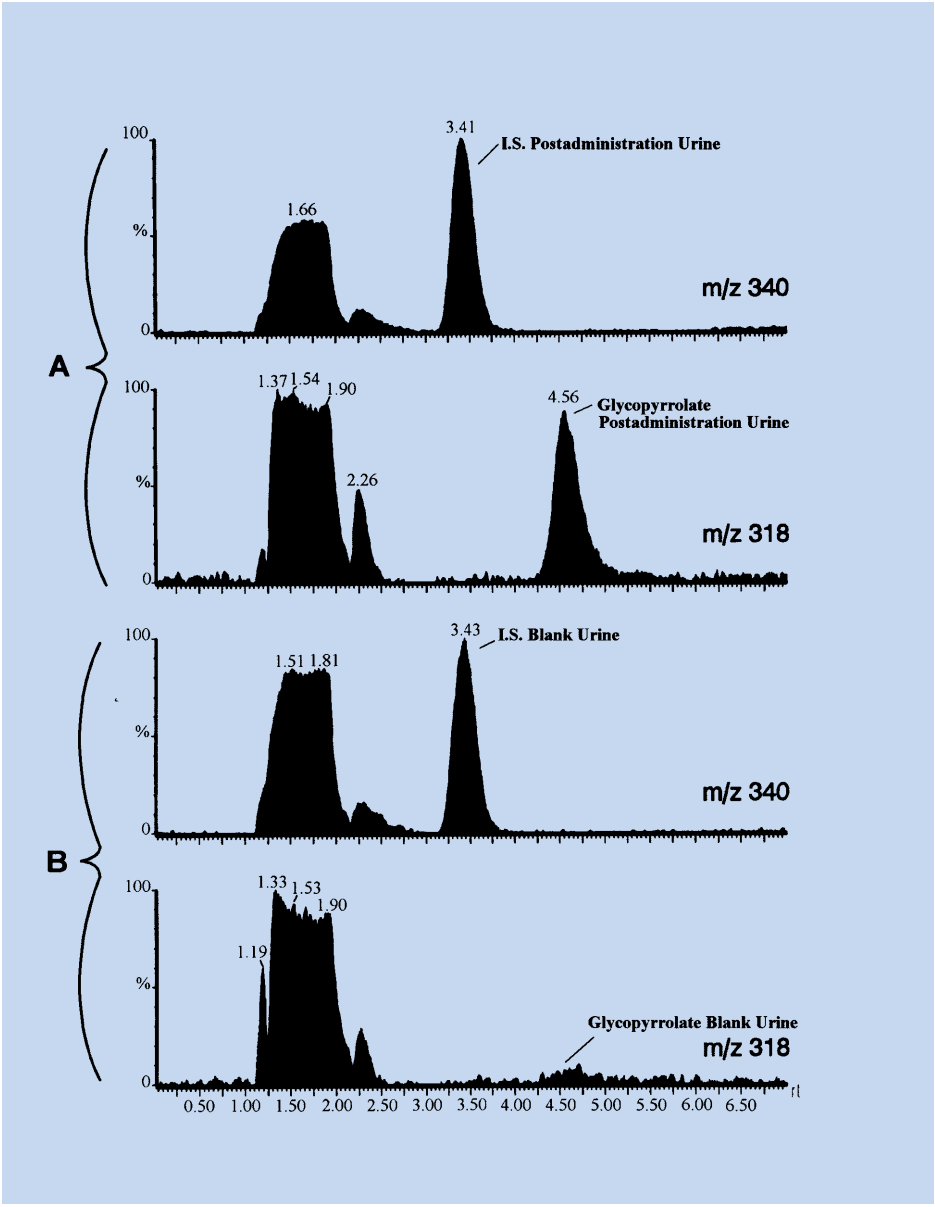

Figure 5. Quantification of glycopyrrolate on a C8 column at low cone voltage using mepenzolate bromide as internal standard (a) a 5 hour post-administration urine sample, (b) a blank urine sample.

Method Calibration Curve

A calibration curve was calculated from the analysis of standard concentrations of glycopyrrolate ranging from 1ng/ml to 50ng/ml in horse urine, (Figure 6). In each case the horse urine was spiked with the internal standard at 20ng/ml concentration prior to extraction. The calibration curve shows the concentration of glycopyrrolate (expressed in ng/ml) plotted against the ratio of glycopyrrolate to mepenzolate bromide. As each glycopyrrolate response has been referenced against the response from a fixed concentration of the internal standard, the highest degree of accuracy and reliability is ensured. The calibration curve demonstrates a high degree of linearity (correlation coefficient r=0.9995), with the lowest concentration producing a chromatographic peak of signal:noise = 15:1. The analysis was repeated several times and each time the correlation coefficient exceeded 0.998.

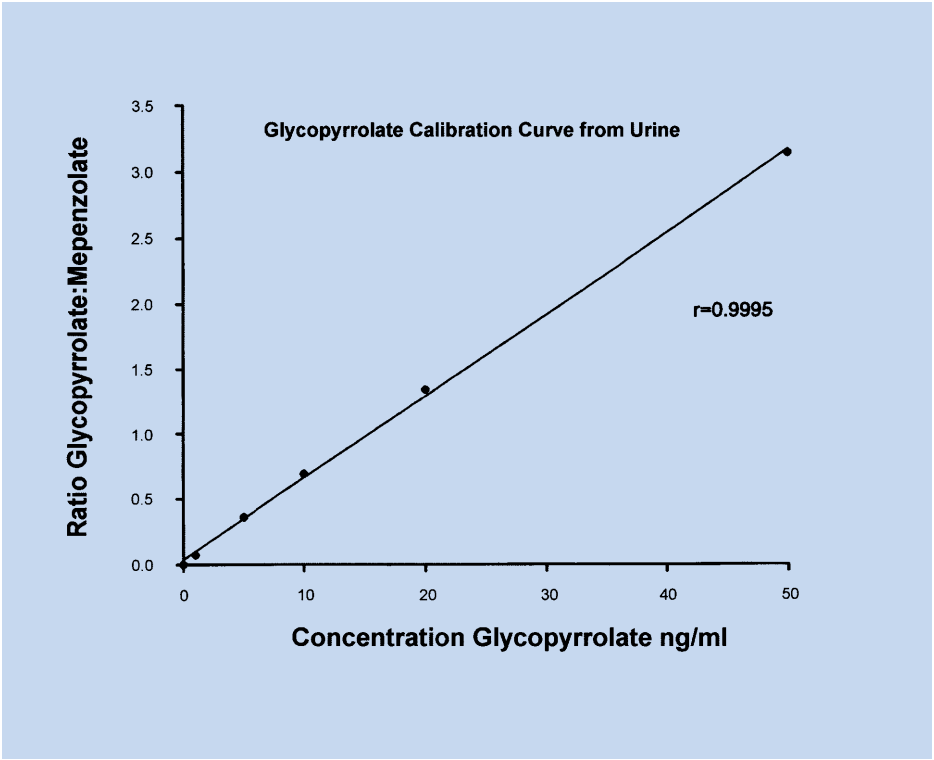

Figure 6. Glycopyrrolate calibration curve from horse urine.

Excretion Profile

The developed LC/MS method was used to conduct a time course study for the urinary excretion of glycopyrrolate from a 1.2mg intravenous administration to a horse. An excretion curve of time post-dose against concentration of glycopyrrolate in ng/ml of urine (Figure 7) shows that the drug is excreted rapidly in less than eight hours. The peak level detected, after 0.5 hours, was 444ng/ml. Excretion of the drug is therefore rapid, with levels falling below the limit of detection by 10 hours post-dose method such as this one, with reliable detection limits in the low ng/ml level, is required for effective control.

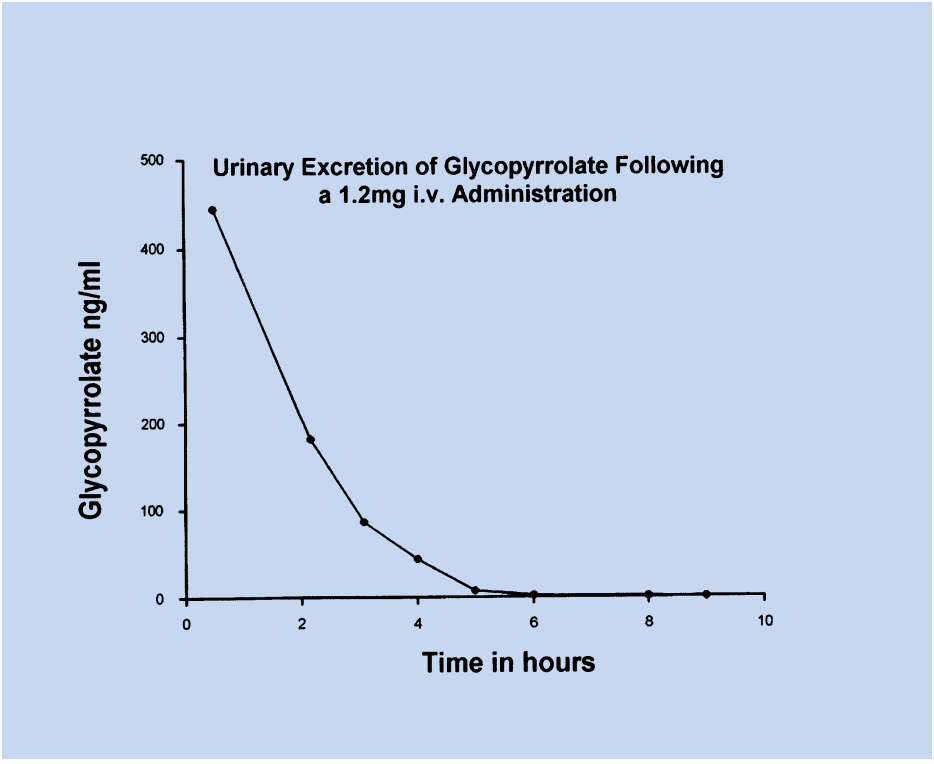

Figure 7. Equine urinary excretion of glycopyrrolate following a 1.2 mg intravenous administration.

Summary

Glycopyrrolate is an anticholinergic drug prohibited in horseracing due to its respiratory enhancing properties. This application note outlines the development of a selective and sensitive method for detection of this drug, in which the use of an internal standard provides additional reliability.

The amenability of glycopyrrolate to ES analysis has been illustrated and diagnostic spectra produced exhibiting both the molecular weight of the cation and structural information, the latter afforded by in-source collision induced decomposition.

High quality quantification of glycopyrrolate in equine urine has been achieved. On-line LC/MS of horse urine samples, extracted and spiked with an internal standard, showed glycopyrrolate SIR detection limits at the low ng/ml level with a chromatographic cycle time of less than 10 minutes.

The robustness of the extraction technique together with the high degree of reliability and ease-of-use of the analytical instrumentation has led to the introduction of this methodology into drug screening programmes at the Horseracing Forensic Laboratory.

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MICROMASS UK Limited
Floats Road
Wythenshawe
Manchester M23 9LZ
Tel: + 44 (0) 161 945 4170
Fax: + 44 (0) 161 998 8915
http://www.micromass.co.uk

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