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Solid-phase extraction techniques for the determination of glycopyrrolate from equine urine by liquid chromatography–tandem mass spectrometry and gas chromatography–mass spectrometry

L. C. Matassa*, D. Woodard, R. K. Leavitt, P. Firby and P. Beaumier

Mann Testing Laboratories Ltd., 5550 McAdam Road, Mississauga, Ontario L4Z 1P1 (Canada)

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ABSTRACT

Glycopyrrolate (Robinul) is a quaternary ammonium salt which serves as a respiratory enhancing drug. It is reportedly used in horse racing to improve breathing. Extraction of glycopyrrolate from equine urine employing unique solid-phase extraction techniques gave a residue suitable for liquid chromatography–tandem mass spectrometry (LC–MS–MS) and gas chromatography–mass spectrometry (GC–MS). LC–MS–MS analysis employed an extract derived from 5 ml of urine subjected to cation-exchange chromatography. The daughter ion of m/z 318 monitored in the positive-ion mode was m/z 116. Recovery of glycopyrrolate was 99.5% and the within-run coefficient of variation of two quality control samples (1.0 and 10 ng/ml) was less than 5%. The between-run coefficient of variation for the same two quality control samples was less than 6.5%. The minimal detectable concentration for the assay was 250 pg/ml. Due to the extremely low concentration of glycopyrrolate in urine, qualitative detection via full-scan GC–MS required XAD-2 extraction of 50 ml of urine, cation-exchange chromatography clean-up and a tandem hydrolysis–derivatization procedure. The target analyte for GC–MS qualitative analysis was the methyl ester of hydrolyzed glycopyrrolate. Glycopyrrolate could be detected in post-administration (1 mg intravenously) urine samples for up to 9 h by both LC–MS–MS and GC–MS. The success of the method was due to a combination of the extreme sensitivity of the LC–MS–MS method and the very selective extraction process for quaternary ammonium salts.

INTRODUCTION

Doping control in performance animals numbered over one million urine and blood samples around the world in 1989 [1]. The vast majority of samples were obtained from racing horses and dogs. More than 2000 drugs were prohibited. Among drug families most commonly abused were respiratory enhancing drugs, specifically bronchial dilators. Reputedly, the most commonly abused member of this family was glycopyrrolate. Prior to December 1990 no sample was ever found positive for this drug because suitable methods did not exist for its extraction and detection from equine samples.

Glycopyrrolate is a quaternary ammonium salt which completely antagonizes acetylcholine action on structures innervated by postganglionic nerves and on smooth muscles that respond to acetylcholine but lack cholinergic innervation [2]. In terms practical to the horse racing industry, glycopyrrolate assists in breathing.

Extracting glycopyrrolate from aqueous matrices such as equine urine presented a formidable challenge. The cationic nature of the drug does not lend itself well to liquid–liquid extraction. Previously published methods for analysis of glycopyrrolate in biological matrices have employed organic soluble ion pairs. Murray *et al.* [3] determined glycopyrrolate in human plasma by extracting an iodide–glycine drug complex into dichloromethane. Detection was achieved by gas chromatography (GC) with a nitrogen-sensitive detector following thermal dequaternisation in

the injection port. Attempts at repeating the process in this laboratory resulted in very dirty extracts and poor recoveries from equine urine, probably due to the extremely high concentration of carbonates inherently present in this matrix.

Sams [4] developed an ion-pairing extraction method utilizing heptanesulphonic acid and dichloromethane. However, we found unacceptable recoveries of the drug from equine urine. The method was also very time-consuming and labor-intensive.

Other methods of determining glycopyrrolate in biological matrices include a radioreceptor assay [5] and an enzyme-linked imunonosorbent assay [6]. These methods are inappropriate for positively identifying glycopyrrolate due to their poor specificity. Positive identification can be made only with mass spectrometry (MS).

Methods reported here use a new solid-phase extraction technique to selectively isolate glycopyrrolate from equine urine. Clean-up Carboxylic Acid-2 (CCX-2, Worldwide Monitoring) is a phase which possesses both cation-exchange and hydrophobic properties. Thus, glycopyrrolate was adsorbed onto the phase using the cationic nature of the molecule. Interfering matrix components were eluted by interrupting hydrophobic interactions. Final elution of the drug could be achieved by disrupting the ionic complex while ensuring that hydrophobic interactions between the phase and the target analyte did not occur. Detection and quantification was achieved by Ion Spray liquid chromatography–tandem mass spectrometry (LC–MS–MS). Qualitative full-scan mass spectra were obtained using GC and Ion Trap mass spectrometry (GC–MS) following XAD-2 extraction and CCX-2 clean-up.

EXPERIMENTAL

Reagents, drug standards and administration samples

Glass-distilled organic solvents were used throughout this work. Water used for buffers and wash solutions was deionized prior to use. Except as noted, all chemicals were reagent grade and purchased from BDH (Toronto, Canada). Tetrabutylammonium hydrogensulphate and methyl iodide were obtained from Aldrich (Milwaukee, WI, USA). Glycopyrrolate and mepenzolate were gifts from A. H. Robbins and Merrell Dow, respectively.

XAD-2 resin (100 μm particle size) was purchased from Rohm and Haas (Philadelphia, PA, USA). CCX-2 cation-exchange columns (40 μm particle size) were purchased from Worldwide Monitoring (Horsham, PA, USA). An Absorbex SPU solid-phase extraction apparatus was employed for cation-exchange extraction.

Glycopyrrolate and mepenzolate stock standard solutions were prepared at 1.0 mg/ml in methanol. Dilution to 1.0 μg/ml gave respective spiking standards.

Standard-bred mares were administered one of the following doses: 1 or 2 mg intravenously. Urine samples were collected by indwelling catheter on an hourly basis. Samples were frozen until the time of analysis.

Preparation of solid phases

XAD-2 resin (1 dm^3) was added to 20 l of tap water. Concentrated hydrochloric acid (75 ml) was carefully added and the mixture shaken. After standing for 30 min, the aqueous phase was drained. The resin was rinsed with tap water for 15 min and aspirated. Tap water (10 l), methanol (8 l) and 40% (w/v) sodium hydroxide (500 ml) were added. The mixture was shaken and allowed to stand for 24 h prior to exhaustive washing with tap water until neutral pH was obtained.

CCX-2 cation-exchange columns (500 mg, 14 ml) required activation immediately prior to use. This was achieved by washing with two aliquots of 2.5 ml methanol then two aliquots of 2.5 ml deionized water and finally two aliquots of 2.5 ml phosphate buffer (0.1 *M*, pH 7.00). Columns were not allowed to dry at any time during the activation process. Flow-rates were maintained at 1–2 ml/min.

Extraction for GC–MS

Equine urine (50 ml) was buffered to pH 9.5 with 10 ml carbonate buffer (0.1 *M*, pH 9.5). Wet XAD-2 resin (3 g) was added and the mixture rotated for 30 min. The aqueous phase was aspirated off and the resin washed with 10 ml of water. Following extensive aspiration the semi-dry resin was eluted with 20 ml of a solution of meth-

anol–0.5 *M* ammonium acetate buffer pH 3.00 (95.5). The eluent was concentrated to near dryness on a steam bath. The residue was reconstituted by adding 0.5 ml of methanol and 7 ml of 0.1 *M* phosphate buffer (pH 7.00). The resulting solution adsorbed onto a preconditioned cation-exchange column. Wash and elution steps were performed as outlined for LC–MS–MS analysis. Following evaporation of the elution solvent, the residue was dissolved in 2.0 ml of sodium hydroxide (0.2 *M*). Tetrabutylammonium hydrogensulphate (50 μl, 0.1 *M*) and 5 ml of methyliodide in dichloromethane (0.5 *M*) were added. The mixture was shaken, capped and heated to 65°C for 30 min with shaking and venting every 5 min. The organic layer was separated and passed through anhydrous sodium sulphate. Following evaporation at 40°C under nitrogen, the residue was reconstituted in 50 μl of toluene and subjected to GC–MS analysis.

Extraction for LC–MS–MS

Equine urine (5 ml) was buffered to pH 7.00 by addition of 3 ml of 0.1 *M* phosphate buffer (pH 7.00). Mepenzolate (12.5 ng) was added to give a final concentration of 2.5 ng/ml internal standard. Water (5 ml) was added and the sample centrifuged for 5 min at 800 *g*. The supernatant was applied to the CCX-2 column at a pressure of 3.5–7.0 kPa. After all urine had passed through, the column was washed sequentially with 5 ml of methanol and 5 ml of water. The column was allowed to dry at maximum vacuum for 5 min. Glycopyrrolate and mepenzolate was eluted by adding 4 ml of a solution of methanol–0.5 *M* ammonium acetate buffer pH 3.00 (95:5). Flow-rates were maintained at 1–2 ml/min during all wash and elution steps. The eluent was dried under nitrogen at 60°C and reconstituted in 0.1 ml of methanol.

Gas chromatograph–mass spectrometer

A 5890 gas chromatograph (Hewlett Packard, Toronto, Canada) was coupled to an Ion Trap 700 mass spectrometer (Finnigan MAT, San Jose, CA, USA). The gas chromatograph was equipped with a split–splitless injector and a 7673A autosampler (Hewlett Packard). The fused-silica capillary column was 30 m × 0.32 mm I.D. (0.25 μm) Restek 50/50 (Restek, Bellefonte, PA, USA). The injection port was operated at 275°C with a splitless time of 0.7 min. The oven temperature profile was 70 to 280°C at 15°C/min. Helium at 1 ml/min was the carrier gas. The following temperatures were maintained: transfer line at 275°C and trap analyzer at 230°C. The MS scan range was 80–250 a.m.u. at 1 scan/s. The electron multiplier voltage and filament current were set at 1900 eV and 20 μA respectively. The injection volume was 2 μl.

Liquid chromatograph–mass spectrometer–mass spectrometer

LC–MS–MS experiments were performed on a Sciex Model API III triple quadrupole mass spectrometer (Thornhill, Canada) equipped with an atmospheric pressure ionization (API) source and an Ion Spray interface. An Applied Biosystems 140A solvent delivery system (Mississauga, Canada) was coupled to a Spectra-Physics SP8880 autosampler (Mississauga, Canada). The LC column was a Hamilton PRP-1, 150 mm × 4.1 mm I.D. with LiChroma 10 μm packing (Chromatographic Specialties, Brockville, Canada). The mobile phase consisting of methanol–50 m*M* ammonium acetate pH 3.0 (80:20) was passed through the column at 0.8 ml/min and split 95:5 post column. The injection volume was 10 μl. High-purity air at 550 kPa was used as the nebulizing gas. Argon was the collision gas and nitrogen was the curtain gas. Dwell times were 90 and 600 ms for daughter scan (318, 111/121, 313/323; 340, 125/135, 335/345) and multiple reaction monitoring (318–116, 340–130) LC–MS–MS, respectively. All samples were analysed in the positive-ion mode.

RESULTS AND DISCUSSION

Cation-exchange extraction characteristics of glycopyrrolate were determined by Ion Spray LC–MS–MS. The target drug eluted at 2.3 min. The MS–MS spectrum consisted of ion clusters at *m/z* 318 and 116. Mepenzolate (internal standard) eluted at 2.1 min and gave an MS–MS spectrum of two ion clusters at *m/z* 340 and 130 (Fig. 1). Ion clusters obtained in the MS–MS mode arose as a result of broadening of the mass

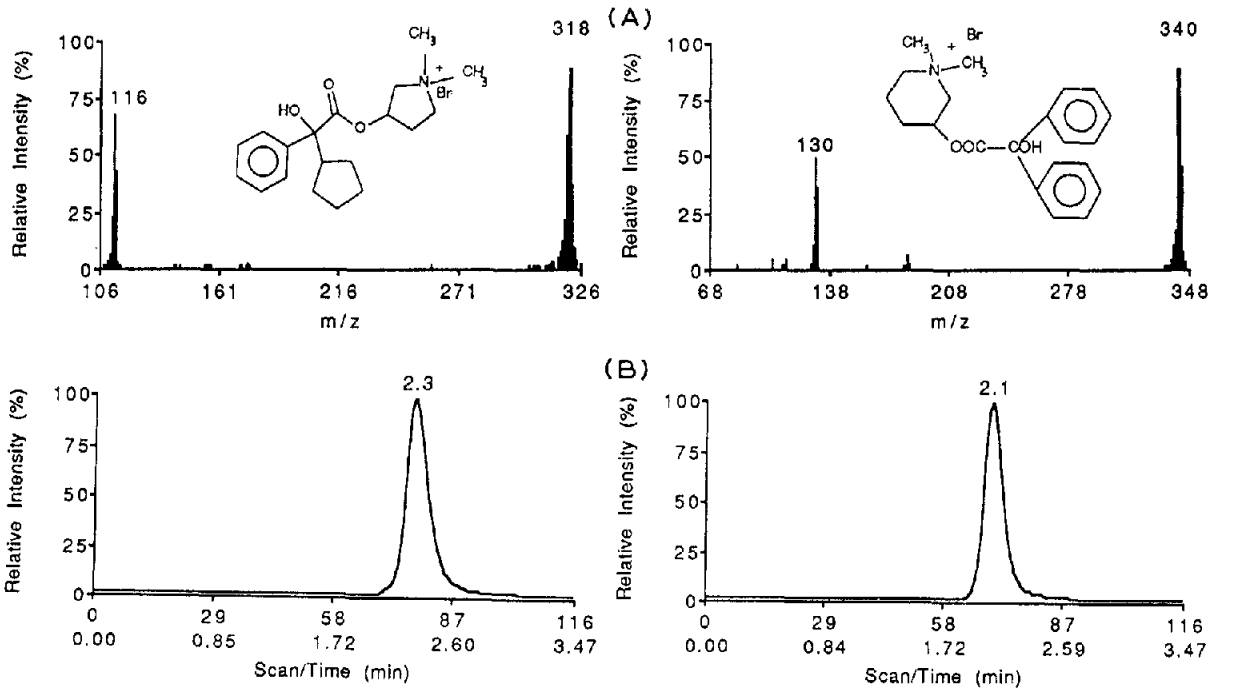

Fig. 1. LC–MS–MS chromatograms and spectra of (A) glycopyrrolate and (B) mepenzolate standards.

spectrometer resolution to achieve enhanced sensitivity.

Multiple reaction monitoring (MRM) chromatograms were used to determine glycopyrrolate and mepenzolate. MRM selectively filtered out all ions unrelated to the target analytes. Thus, chromatograms were obtained only for compounds with parent ions of 318 and 340 *and* which give daughter ions of 116 and 130, respectively. The result was extreme detection selectivity for glycopyrrolate ad mepenzolate (Fig. 2).

Quantification of glycopyrrolate was achieved by preparing standard curves of peak-height ratio (glycopyrrolate/mepenzolate) against concentration of spiked glycopyrrolate. A pool of drug free equine urine was used to prepare spikes at concentrations of 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 ng/ml. The resulting standard curve was linear in the concentration range tested. Linear regression analysis gave correlation coefficients consistently greater than 0.998.

Extraction recovery was calculated in quintuplicate at 1.0 and 10 ng/ml. Mean recovery (± S.D.) from all determinations was 99.5 ± 1.6%. The recovery of the 1.0 ng/ml spike was not different from the recovery of the 10 ng/ml spike, both being quantitative.

Within-run variability and accuracy were evaluated by analysing quality control samples (n = 5) at two different concentrations. Mean concentrations (±S.D.) and coefficients of variation (C.V.) were 1.00 ± 0.05 ng/ml and 5.0% for the 1.0 ng/ml sample and 9.84 ± 0.19 ng/ml and 1.9% for the 10.0 ng/ml sample.

Between-run variability and accuracy were evaluated over different days (n = 5). Mean (±S.D.) concentrations and C.V.s were 0.99 ± 0.06 ng/ml and 6.3% for the 1.0 ng/ml sample and 9.9 ± 0.22 ng/ml and 2.2% for the 10.0 ng/ml sample.

The method detection limit was defined as the minimum concentration of glycopyrrolate that could be identified, measured and reported with 99% confidence that the analyte concentration was greater than zero. As per Glaser *et al.* [7], this was determined to be 0.25 ng/ml using replicate analyses (n = 7, S_c = 0.08) of the sample matrix containing glycopyrrolate at 1 ng/ml. The excellent sensitivity of the method was no doubt enhanced by the selectivity afforded by MRM.

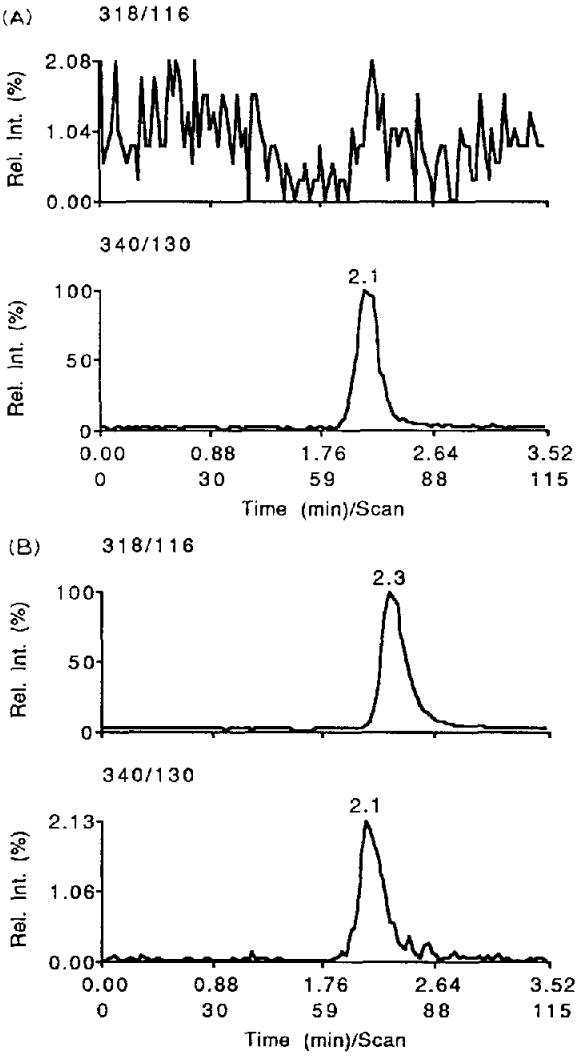

Fig. 2. Multiple reaction monitoring chromatograms of (A) blank urine containing 2.5 ng/ml mepenzolate (internal standard) and (B) 1 mg intravenous glycopyrrolate post-administration urine containing 2.5 ng/ml mepenzolate (internal standard).

The sorbent employed in CCX-2 columns had both ion-exchange and hydrophobic adsorption properties. Attention to buffer pH values was critical to the success of the cation-exchange extraction. The carboxylic acid moiety of the sorbent had a pK_a of 4.8 [8]. Thus, at the conditioning and application pH of 7.0 the exchanger existed as the carboxylate anion and extraction of cationic compounds was maximized. Methanol and water washes removed urine components which were hydrophobically bound without concomitant loss of cationic analytes. Decreasing pH of the conditioning or application buffers resulted in drastically reduced recovery because some ion-exchange sites on the sorbent were protonated.

Following adsorption, dissociation of carboxylate anion–cationic sorbent complexes was achieved by reprotonating the exchanger. Use of 100% ammonium acetate buffer (pH 3.00, 6 ml) resulted in no recovery of glycopyrrolate or internal standard. However, as the methanol component of the elution solvent was increased to a maximum of 98%, recovery was increased. It was postulated that in the absence of methanol, dissociated target analytes were adsorbed back onto the sorbent through hydrophobic interactions. Methanol in the elution solvent prevented such interactions from occurring. Thus, glycopyrrolate could be adsorbed to the phase using either ionic or hydrophobic interactions. Careful selection of wash and elution solvents allowed most matrix inteferences to be selectively separated from the target analyte.

Conditions under which the elution solvent was evaporated seemed less critical. No change in recovery was noted when the extract was dried at room temperature or on a steam bath. Enzyme hydrolysis of intravenous administration samples did not result in higher recoveries of glycopyrrolate. This supported previously reported findings [5]. Using a radioreceptor assay for analysis of human urine Kentala *et al.* [5] found that glucuronide or sulphate conjugation played only a minor role in the metabolism of glycopyrrolate in humans.

The GC–MS method was developed as a qualitative test. The urine volume had to be increased to 50 ml to achieve the sensitivity required to detect a 1-mg intravenous administration for 8 h post-dosing. The 5-ml LC–MS–MS extraction procedure did not afford an extract concentrated enough for GC–MS analysis. Further, XAD-2 pre-extraction was required because the cation-exchange columns would not allow more than 10 ml equine urine to be used. The very high protein content of equine urine plugged the CCX-2 column.

The quaternary ammonium structure of glycopyrrolate could not be chromatographed on the fused-silica capillary GC column. Thermal de-

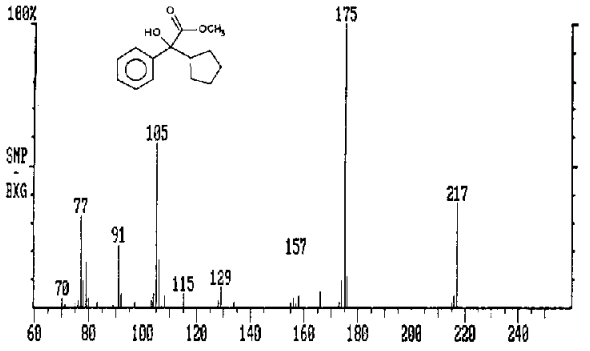

Fig. 3 Electron-impact GC–MS spectra of hydrolysed and methylated glycopyrrolate standard (MW = 234).

quaternisation as previously reported [3] led to very low response for the tertiary amine. It was postulated that hydrolysis of the ester function of the drug would give rise to a carboxylic acid which could be derivatised with methyl iodide. Thus, the unique feature of the GC–MS method was the one-step hydrolysis, derivatisation and extraction of a methyl ester derivative of the glycopyrrolate hydrolysis product. By comparison with a synthetically prepared standard, the hydrolysis step was quantitative. Representing full-scan mass spectra are shown in Fig. 3.

Analysis of post-administration urine samples showed the presence of glycopyrrolate by both LC–MS–MS and GC–MS for 12 and 9 h following intravenous doses of 2 and 1 mg, respectively. Preliminary investigation of mepenzolate administrations to standard-bred mares (10 mg intravenously) indicated detection up to 6 h post-administration was possible using the methods outlined above. Further, it is conceivable that other members of this class of quaternary ammonium bronchial dialators (ipratropium bromide, methscopolamine bromide, propantheline bromide) could be analysed in the same fashion and will be the topic of future work.

CONCLUSION

A quantitative LC–MS–MS method for analysis of glycopyrrolate in equine urine has been developed. The method is very sensitive owing to excellent recovery and selectivity achieved by the extraction process and the inherent selectivity of the MS–MS. The method is simple to perform and many samples can be analysed simultaneously. A qualitative GC–MS technique has also been developed. It features a unique one-step hydrolysis, derivatisation and extraction which allows detection of a glycopyrrolate hydrolysis product. As a result of the development of these methods, laboratories involved in doping control are now in a posistion to detect and confirm glycopyrrolate.

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