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Title: **Chromatographic Detection of Trimetoquinol (Inolin®) and its Major Urinary Metabolites in the Horse: A Preliminary Report**

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Summary:

Trimetoquinol (TMQ) (1-(3',4',5'-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, m.w. 345) is the prototype tetrahydroisoquinoline pharmaceutical. TMQ is marketed as a bronchodilator in human medicine; in horse racing, TMQ is listed as an Association of Racing Commissioners International (ARCI) class 3 foreign substance. As such, TMQ is considered to have the potential to affect racing performance in horses and a validated qualitative confirmatory method is required to regulate its use in racing. In horses, TMQ is an extremely potent cardiac stimulant, with IV doses of 0.2 μg/kg (~100 μg/horse), or less, producing an acute stimulation of heart rate. Intratracheal (IT) administration of similar doses of TMQ yielded no observable cardiac responses, and no locomotor effects were observed after IV doses of up to 2 μg/kg. Based on these preliminary pharmacodynamic findings, 8 μg/kg of TMQ IV was selected as a safe and effective dose for studies on the metabolism and analytical detection of TMQ in horses. We developed a solid phase extraction method for recovery of TMQ and its metabolites from equine urine, identified suitable high performance liquid chromatographic conditions for these substances and our internal standard, papaverine, and developed a highly sensitive ESI(+)-LC-MS-MS method (estimated LOD, 0.1 ng/ml) for TMQ and its major metabolites in equine urine. Multiple Reaction Monitoring (MRM) analysis of unhydrolyzed post-administration urine showed small amounts of TMQ, along with glucuronide, methylated, and sulfated metabolites, with glucuronide metabolites predominating. Following glucuronidase hydrolysis, recovered parent TMQ peaked at relatively high concentrations (>300 ng/ml) within one hour of administration and thereafter declined. The methylated metabolites of TMQ peaked later and at comparable total concentrations, and thereafter declined more slowly. These data suggest that glucuronide hydrolysis of early post-administration urine samples will allow recovery of readily identifiable quantities of parent TMQ. These findings, combined with the highly sensitive LC-MS-MS detection of parent TMQ described herein suggest that glucuronide hydrolysis of post-administration urine, followed by LC-MS-MS or other analysis, will allow effective regulatory control of this agent in racing horses.

Introduction

Trimetoquinol (TMQ) (1-(3',4',5'-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, m.w. 345) is the prototype of the tetrahydroisoquinoline class of pharmaceuticals. TMQ (Inolin®) is marketed in Japan as a bronchodilatory agent for the treatment of asthma and other respiratory tract conditions in humans (1). It has one center of asymmetry, and the S(-)-isomer of TMQ has been shown to be biologically active on β-adrenoceptors (1). In contrast, the R(+)-isomer possesses thromboxane A2/prostaglandin H2 antagonist activity (2). The chemical structure of TMQ differs significantly from classical β-adrenoceptor agonists such as epinephrine (Fig. 1). It lacks the α-hydroxy group of catecholamines, and the amino nitrogen is contained within a tetrahydroisoquinoline ring. However, TMQ possesses a 3',4',5'-trimethoxybenzyl ring at the carbon-1 position of the tetrahydroisoquinoline ring that is required for biological activity (3). The structure of TMQ resembles that of papaverine, a phosphodiesterase inhibitor and vasodilator, chosen as an internal standard for our assays (Fig. 1).

TMQ is an agonist for the β2-adrenoceptors, which are cell membrane-bound G-protein coupled receptors that mediate physiological functions of the endogenous catecholamines, epinephrine and norepinephrine. These adrenoceptors were initially classified as β1- (heart, white adipose tissue) and β2- (lungs, vasculature) adrenoceptors (4) based on the rank order of sympathomimetic amine potencies.

A new receptor subtype with atypical characteristics resulted from studies on TMQ in rats. A cloned and expressed β-adrenoceptor demonstrated these atypical properties, i.e. non-β1 and non-β2, proving the existence of a third subtype of β-adrenoceptor, designated β3-adrenoceptor (5). Atypical β-adrenoceptors play an important role in lipid metabolism, and their selective agonists may have potential use for the treatment of obesity (6). Attempts have been made to develop a selective β3-adrenoceptor agonist to combat human obesity and many analogs of TMQ were synthesized for that purpose (7-10).

Stimulation of β1-adrenoceptors in the heart lead to a positive inotropic effect (increase in contractility) and a positive chronotropic effect (increase in heart rate). Hoffman (11) proposes that direct effects on the heart are determined largely by β1-adrenoceptors, although β2-adrenoceptors are also involved. Unless the agent is a selective β2-adrenoceptor agonist (e.g. albuterol and salmeterol), it will also directly interact with the β1-adrenoceptors in the heart. However, to a certain extent, selective β2-adrenoceptor agonists may interact with β1-adrenoceptors as well. β2-Adrenoceptors are present in smooth muscles such as the vasculature and bronchia. The stimulation of these receptors induces smooth muscle relaxation, which leads to hypotension and bronchodilation, leading to the usefulness of these agents for treatment of asthma.

The basic mechanism of action of TMQ and its cardiovascular and bronchodilatory properties suggest that it has the potential to improve the performance of racing horses. In horse racing, TMQ is listed as an ARCI class 3 foreign substances. As such, TMQ is generally considered to have the potential to affect the racing performance in horses and a validated qualitative confirmatory method is required to regulate its use in racing. We have therefore performed a preliminary characterization of the pharmacodynamics of TMQ in the horse and developed a solid phase extraction method and an LC-MS-MS analytical method to detect TMQ and its metabolites in horse urine with a view to developing an analytical method upon which the regulatory control of this agent in horses can be based.

Materials and Methods:

Horses:

Six mature Thoroughbred mares from the University of Kentucky herd, weighing 486-521 kg were fed twice a day with grass hay and feed (12%), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. The animals from the herd were vaccinated annually for tetanus and dewormed quarterly with ivermectin (MSD Agvet, Rahway, NJ). A routine clinical examination was performed before each experiment to assure that the animals were healthy and sound. During experimentation, horses were provided water and hay *ad libitum*. The mares served as their own controls. Animals used in our experiments are managed according to the rules and regulations of the Institutional Animal Care and Use Committee at the University of Kentucky, which also approved the experimental protocol.

Drug administration:

Initial steps in the research reported were made possible by the generous provision to us of small quantities of TMQ by our colleagues at Truesdail Laboratories, Tustin, CA. S(-)TMQ was later made available to us in more substantial quantities by Tanabe Seiyaku Co., Ltd. (Chuo-ku, Osaka, Japan), with a certificate of analysis indicating an estimated purity of 99.3 – 100.9%, which was confirmed by in-house GC/MS evaluation of a BSTFA + 1% TMCS (Pierce) – derivatized sample. Trimetoquinol hydrochloride (Inolin®) (8 μg/kg) was administered orally (PO), intravenously (IV) and intratracheally (IT) to horses after dissolving it in ethanol (0.5 ml), which was then diluted with saline (2.5 ml). Urine samples were collected immediately before (0h) and at 1, 2, 4, 6, 8 and 24h after administration using a Harris flush tube (24 Fr x 60 in; Seamless, Ocala, FL). Urine samples were divided into 50 ml aliquots stored at –20°C until assayed. For pharmacological responses, five horses were used to simultaneously record heart rates and locomotor activities following dosing with TMQ (2, 0.2, and 0.02 μg/kg), epinephrine (20 and 2 μg/kg), and norepinephrine (20 and 2 μg/kg).

Pharmacological Responses:

Heart rates (HR) were recorded at 1 min intervals during each experiment by an on-board heart rate computer (Polar CIC Inc, Port Washington, NY). An elastic strap with an attached transmitter and receiver was placed around the chest of the horse. The transmitter was placed at the level of the left elbow, over the heart. Electrode gel was used to insure proper conduction of the HR signal.

Locomotor chambers consisted of two 3.4 x 3.4 m box stalls equipped with Minibeam sensors (SM31E and SM2A31R, Banner Engineering, Minneapolis, MN) spaced equally around the stall 45 cm above the floor. An interruption was scored each time the horse disrupted the beam of light and this output was summed and recorded on a data logger (CR10, Campbell Scientific, Inc., Logan, UT).

All behavioral experiments followed a rigorous standard protocol to reduce variability from extraneous effects. A horse was placed in each behavior stall at 1200 h, and the HR strap was attached. The horse was allowed to acclimate to the stall for 3 h. Recording of locomotor and HR activities was begun at 1500 h. Baseline activity was recorded for 10 min, and then the control and experimental treatments were administered. Locomotor and HR data were recorded for 10 h until 0100 h the following morning. Total number of interruptions were summed for every 15 min interval. Controls and experimental trials were run simultaneously.

Statistical Analysis

Analysis of variance with repeated measures was used to compare heart rate values and locomotor activity values of control and treatment horses for the different doses of TMQ, epinephrine, and norepinephrine. Significance was set at $P<0.05$.

Analytical Procedures: Sample Preparation and Analysis:

For beta-glucuronidase hydrolysis, urine samples were treated for 3h at 65°C with *Patella vulgata* beta-glucuronidase (1000 units of Sigma Type L-II/mL urine brought to pH 5 with 1 M sodium acetate buffer) as previously described (12). The resultant hydrolysates were centrifuged to remove sediment, and the decanted liquid was subjected to solid phase extraction (SPE).

Calibration curves were generated by spiking different amounts of stock solutions of TMQ in 2 ml of blank urine, in order to obtain concentrations up to 600 ng/ml. 2 ml of 1M sodium acetate buffer (pH 5.0) were added to each sample to obtain pH 5.0. SPE was performed on a battery of Zymark RapidTrace SPE Workstation extraction modules (Hopkinton, MA) and also manually (Speedisk 48 Pressure Processor, J.T. Baker, Inc.). In either case SPE columns (United Chemical Technologies, Bristol, PA, type CSDAU Clean-screen) were conditioned by adding sequentially 3 ml dichloromethane/isopropanol/NH_4OH (78:20:2, v/v/v), 3 ml methanol, 3 ml deionized water, and 2 ml 1M sodium acetate buffer (pH 5.0). Samples brought to pH 5.0 with 2 ml 1M sodium acetate buffer were loaded, then the column was washed sequentially with 3 ml deionized water, 3 ml 1M acetic acid, 3 ml methanol. Then each column was eluted with 3 ml dichloromethane/isopropanol/NH_4OH (concentrated) (78:20:2, v/v/v) into glass tubes. The eluent was evaporated to dryness under a stream of nitrogen in a 40°C water bath in a Zymark TurboVap LV evaporator (Hopkinton, MA). The residue was dissolved directly in 100 µl acetonitrile:0.05% formic acid, 1:9, then transferred to a microinjection vial and sealed. Ten µl were injected into the Agilent (Atlanta, GA) 1050 HPLC–Micromass Quattro II ESI(+)MS/MS (Beverly, MA).

Extracts were also infused at 1.2 ml/h directly into the electrospray probe of the Quattro II MS/MS (Micromass, Beverly, MA) by means of a Harvard syringe pump equipped with a 500 µl Hamilton gas-tight syringe. Full scan electrospray ionization (ESI+) mass spectra were obtained on analytical trimetoquinol standards at 10 µg/ml in 1:1 acetonitrile: 0.05% formic acid (aq), pH~4, and

on urine extracts, both by direct infusion. All spectra were optimized by combination of 1-2 min of uniformly acquired data, background subtraction, and peak smoothing.

MS/MS tuning and HPLC:

The mass spectrometer was tuned for positive ion spectra by direct infusion of 10 μg/ml trimetoquinol in acetonitrile:0.05% formic acid (aq), 1:1. The peak shape and intensity of the monoprotonated TMQ m/z 346 ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens, and RF lens settings. Skimmer lens offset was left at 5 V. Collision gas (argon) and collision energy were adjusted for collisionally-induced dissociation (CID) in the central hexapole by optimizing settings as needed for the second quadrupole. Generally, the collision gas was set to 1-3 X 10^{-3} mbar. Increasing the photomultiplier setting 100-150 V above the regular 650 V sufficiently increased sensitivity. In general for positive mode, the source cone voltage was set at 23 V, the collision energy was set at –18 V, the capillary of the ESI probe was set at +3.2 kV, the skimmer was set at 1.1 V, and the HV lens was set at 0.53 kV. Source temperature was set at 120°C. Gradient chromatography with the HPLC-ESI(+)-MS/MS utilized settings for solvents A (acetonitrile + 0.05% formic acid) and B (deionized water + 5% acetonitrile + 0.05% formic acid) as described in Table I.

Results

Heart rate and locomotor activity

Figure 2 illustrates the pharmacological potency of TMQ and compares it with epinephrine and norepinephrine. After administration of 2 ug/kg of TMQ (~1 mg/horse), the heart rate (HR) was very rapidly elevated from a basal level of around 30 to 190 bpm and remained over 100 bpm for ~20 min (Figure 2a). This HR response was higher than that following a ten-fold greater dose of epinephrine (Figure 2b) or of norepinephrine (Figure 2c); following these administrations HR was

elevated to ~150 bpm and remained over 100 bpm for only ~ 3 min. Heart rate effect of lower doses of TMQ were also comparable to those obtained following ten-fold greater doses of epinephrine and norepinephrine, as set forth in Fig 2.

Figure 3 illustrates the considerable pharmacological potency of TMQ compared to other highly potent drugs such as etorphine and clenbuterol. On the other hand, in contrast with these clearcut and highly potent cardiac responses following IV administration of TMQ, intratracheal administration of TMQ (up to 0.1 mg) failed to produce any cardiac response. Similarly, preliminary evaluations of the potency of TMQ via oral route (10-100 mg) in the horse also suggest that its bioavailability and efficacy by this route is markedly reduced.

Ionization of TMQ

On dissolution in acetonitrile: 0.05% formic acid (aq), see Fig. 4 [left], TMQ readily produced an m/z 346 $[M+H]^+$ protonated pseudomolecular ion. This species produced one prominent daughter ion at m/z 164 on collision-induced dissociation in the tandem quadrupole instrument, see Fig. 4 [right]. This ion arises by cleavage of the C-C bond at the methylene bridge between trimethoxybenzyl and isoquinoline ring systems to release neutral 3,4,5-trimethoxytoluene, m.w. 182 with shift of a proton (Fig. 5 – right panel). The m/z 164 ion has an intensity dependence on cone voltage setting as shown in the inset of Fig. 4, with maximum intensity in the vicinity of 20 volts; collision energy variation for collision-induced dissociation is shown in Fig. 5 [left panel] and indicates optimal production of m/z 164 in the vicinity of 18 volts.

TMQ detection by LC-ESI(+)MS/MS

The production of the m/z 164 daughter ion enabled ready detection of TMQ with an MRM [multiple reaction monitoring] detection methodology on the Quattro II. This was performed in conjunction with similarly structured papaverine (cf. Fig. 1) as an internal standard. HPLC conditions are listed in Table 1. Fig. 6 shows an example of TMQ HPLC chromatography. Solid phase extraction was performed with papaverine as internal standard, and Fig. 7 indicates the linearity of the analytical method. Manual SPE, which employs gas pressure-driven elution, was moderately more efficient than robotic extraction by means of the Zymark RapidTrace, which employs piston-driven positive displacement, but either method provided more than adequate linearity and sensitivity (Fig. 7). We estimate the limit of detection (LOD) of this method at about 20 pg on column and about 100 pg/ml in equine urine.

TMQ metabolites in unhydrolyzed urine:

TMQ metabolism was investigated by prediction of possible metabolites based on literature reports, chemical intuition, and prior experience, as summarized in Table 2. We first investigated the metabolites identifiable in unhydrolyzed urine. A predominant metabolite discovered by application of this predictive approach was TMQ-glucuronide, which is, presumably, a mixture of the two possible TMQ-O-glucuronides formed at the isoquinoline hydroxy groups (m.w. 521 each). Fig. 8 shows the time course profile of apparent TMQ-glucuronide monitored as the transition m/z 522->164, with internal isoquinoline structure released as the m/z 164 fragment. One of these cleavages corresponds to one observed earlier for parent TMQ (Fig. 5). Evaluation of this fragmentation pattern indicates that the urinary concentrations of glucuronide metabolites of TMQ peak within 1 hour or less post-IV administration of TMQ.

Another metabolite identified in unhydrolyzed urine was a methylated TMQ-glucuronide, m.w. 535. Fig. 9 demonstrates, based on raw areas for the m/z 536-> 178 transformation, near equivalence in apparent raw peak area to that for the TMQ-glucuronide m/z 522->164, with the peak in relative abundance occurring around 2 hours post-administration, rather than within 1 hour, followed by a relatively prolonged elimination. The m/z 178 ion is 14 amu greater than the principal m/z 522 daughter ion, indicating the presence of the methyl group on the isoquinoline ring system (compare structures in Fig. 8 and 9).

Other evaluations identified an apparent TMQ-sulfate (m/z 426); however it is apparently a minor metabolite, as shown in Fig. 10.

TMQ metabolites in hydrolyzed urine

Fig. 11 shows the release of TMQ from TMQ-glucuronide following hydrolysis with beta-glucuronidase. Note the two y-axes, one for hydrolyzed urine [left] and one for unhydrolyzed urine [right], necessitated by the two orders of magnitude between amounts. Results indicate 100-fold increase in recovery of TMQ after glucuronidase treatment. Comparison of methylated and non-methylated TMQ post-hydrolysis is shown in Fig. 12 and reveals agreement with the patterns seen with the corresponding glucuronides (Fig. 9).

Fig. 13 illustrates the overall effect of enzyme hydrolysis on glucuronides, specifically that both methylated-TMQ-glucuronide (m/z 536 ->178) and TMQ-glucuronide (m/z 522->164 or 522->364) decrease post-hydrolysis although not with 100% cleavage efficiency; whereas the released methyl metabolite (m/z 360->178) increases post-hydrolysis.

Discussion:

The established abilities of TMQ to both stimulate heart and dilate bronchioles lend credence to suggestions that TMQ has the potential for improper use in racing horses. We therefore developed solid phase extraction (SPE) methods for TMQ in horse urine using both manual and robotic systems. Manual extraction provided a standard curve with a slightly higher slope, but both methods performed in a very satisfactory manner.

The pharmacological data presented here confirm that intravenously administered TMQ is an extremely potent cardiac stimulant in the horse. The increase in HR following intravenous injection of TMQ (2 μg/kg) was similar to that obtained from 10-fold higher doses of epinephrine and norepinephrine (20 μg/kg) [Fig. 2]. A particularly intriguing finding is that similar doses (0.05-0.2 μg/kg) of TMQ administered intratracheally (IT) and oral doses (0.05-0.2 mg/kg) up to 1,000 times greater had no effect on HR [Fig. 3]. The fact that IT administration had no effect on HR suggests that TMQ does not redistribute rapidly from the lung. This knowledge, combined with previous studies (13) showing that TMQ administered by inhalation has a significant bronchodilatory effect in humans, raises concerns that even smaller doses of TMQ administered IT could produce bronchodilating effects and that TMQ administered in this way would be correspondingly more difficult to detect in post-administration urine samples.

In response to a colleague's suggestion, we performed a preliminary evaluation of TMQ pharmacodynamics after oral administration. As shown in figure 3, orally administered TMQ produced no detectable effect on HR at doses of up to 0.2 mg/kg, although at this dose some of the animals sweat observably. Overall, these data show that the pharmacodynamics of TMQ in the horse is highly route-dependent. By the oral route, the threshold dose for a pharmacodynamic effect, namely clinically apparent sweating, is about 200 μg/kg or approximately 100 mg/horse. By the intravenous route the threshold dose for a pharmacodynamic effect on HR is extremely low, on the order of less than 0.005 μg/kg or approximately 2.5 μg/horse, a 40,000-fold reduction in the threshold dose, and on the order of

potency of etorphine, one of the most potent pharmacological agents reported in the horse. These results suggest that the dose/response relationship for TMQ can vary over a 40,000-fold range in the horse, depending on the route of administration. The range may be even greater when one consider apparent potency on IT administration. This is an exceptionally wide range in dose/response relationships, and is comparable with the range of dose/response relationships observed over the entire family of narcotic analgesics in the horse (Fig. 3).

With the goal of enabling regulatory control of this highly potent agent, we developed the ESI(+)-MS/MS method to characterize and detect TMQ and its metabolites in horse urine. Our extraction method allowed good recovery of analytes and preliminary evaluations suggest on the order of 100% extraction efficiency.

TMQ is readily detectable by ESI(+)-MS/MS, and its mass spectrum is shown in Fig. 4 [left]. Isotopic abundances for m/z 346, 347 and 348 match expectations (100%, 22% and 3.2%). Also in figure 4 we see the daughter ion spectrum indicating a pronounced m/z 164 from parent ion m/z 346. This ion is the catechol isoquinoline ring system, with the release of 3,4,5-trimethoxytoluene, as presented in Fig. 5 [right].

A typical chromatogram of a TMQ standard (20 ng/ml) and the internal standard papaverine is shown in Fig. 6. Both compounds have short retention times as might be expected on a 1mm diameter column, but the peaks separate dependably and with high quality chromatography. The method demonstrates good linearity with either manual or automated extraction (Fig. 7), and our current estimate for the limit of detection (LOD) is about 20 pg on column or 100 pg/ml in equine urine based solely on the m/z 346->164 MRM detection of parent TMQ. More extensive studies on method validation are in progress.

Parent unconjugated TMQ was observed in unhydrolyzed urine (Fig. 11) but only on the order of 1% of the amounts of TMQ glucuronide; the principal metabolite identifiable in unhydrolyzed urine

was TMQ-O-glucuronide (m/z 522). It seems probable that each of the two isoquinoline hydroxy groups are the principal targets of glucuronide conjugation. TMQ-glucuronide concentration, following 8 μg/kg IV dose, peaked within one hour at about 300 ng/ml (Fig. 8), returning to below the LOD between 8-24 hours post-administration. The time profile of TMQ-glucuronide raw area of the m/z 522->164 is shown in Fig. 8 [left], the m/z 164 fragment representing the isoquinoline ring, as shown in Fig.8 [right].

Review of the literature suggested the presence of methylated TMQ as another metabolite (14 and Table 2). We provisionally interpret these data as indicating the presence of a methylated TMQ-glucuronide, m/z 536, with methyl groups attached to one or both diol hydroxy groups of the isoquinoline ring, with the glucuronide attached to the remaining hydroxyl. Fig. 9 [right] presents a possible structure for m/z 536. Fig. 9 [left] shows the observed elimination pattern for these methylated TMQ-glucuronides. Note that relative abundance for 536->178 and 522->164 is close to equivalence, with the difference that 536->178 elimination peaks within 2 hours and not within 1 hour as seen for 522->164. We can infer that, since methylated TMQ-glucuronide is less polar than TMQ-glucuronide, it takes longer to be eliminated from the horse. It is also apparent that all the curves for m/z 536 (536->178, 536->181 and 536->355) correspond to one another indicating that m/z 178, 181 and 355 are daughter ions for one, the other or both methyl glucuronides. Additionally, the m/z 178 ion is 14 amu greater than the principal m/z 522 daughter ion (m/z 164), which indicates the presence of the methyl group in the isoquinoline ring (compare Fig. 8 and 9).

Other evaluations identified a possible sulfate metabolite (m/z 426) (Fig. 10). In the absence of authentic standards, we make the assumption that the raw HPLC area of these conjugates directly reflects their relative concentrations, which assumes in turn that the energetics of mass spectral fragmentations are similar. This is not unreasonable since glucuronide, methyl-glucuronide and sulfate species all undergo two cleavages to release isoquinoline ring systems in each case (cf. structures in Figs. 8 and 9, for example). Based on these rationalizations, we believe methyl-TMQ-glucuronide and TMQ-glucuronide are approximately equivalent in released urinary concentrations and that TMQ-

sulfate is apparently a minor metabolite. Note that the raw area of m/z 426->164 (TMQ-sulfate species) decreased after hydrolysis. This is shown in Fig. 10 along with the expected decrease of m/z 522->164 for glucuronide after hydrolysis. Corresponding decrease in m/z 426->164 is likely due to sulfatase activity in our glucuronidase preparation, or the instability of sulfate esters in the pH 5 65°C incubation, or both.

The data of Fig. 11 confirm that the major TMQ metabolite consists of TMQ-glucuronide species, since glucuronidase-released TMQ also peak one hour post-administration (compare Figs. 8 and 11). As shown previously, in unhydrolyzed urine the abundance of m/z 346 is againon the order of 1% that for hydrolyzed urine.

Since HPLC area can be provisionally considered directly proportional to concentration based on arguments presented above, Fig. 12 compares HPLC areas of glucuronidase-released methylated TMQ with those of nonmethylated TMQ. Nonmethylated-TMQ (m/z 346->164) abundance peaks within 1h post-administration at a higher raw MRM area. On the other hand, methylated-TMQ (m/z 360->178) abundance peaks 2h post-administration at a somewhat lower HPLC area. M/z 360 also presents a slower rate of elimination, confirming that it is a less polar and presumably more lipophilic species.

The identifications of TMQ species as parent TMQ, methylated TMQ, TMQ-glucuronide and methylated-TMQ-glucuronide are further supported by their relative behaviors on glucuronidase treatment. As shown in Fig. 13A, hydrolysis induces a reduction in the relative amounts of m/z 536->178 HPLC areas, as one would expect from a glucuronide. Corresponding increase in the free methylated species (m/z 360->178) post-hydrolysis is then shown in Fig. 13B. The same relationship holds for the non-methylated TMQ-glucuronide (m/z 522->164, Fig. 13C) relative to the hydrolysis-released TMQ (m/z 346->164, Fig. 13D).

In the absence of a deuterated TMQ standard we found that papaverine acted as a suitable internal standard for the following reasons. Papaverine is readily available at low cost and is unregulated. Its structural similarities to TMQ and its metabolites made it an acceptable candidate. Finally the m/z 340->202 transition for papaverine was sufficiently different from anticipated metabolites (Table 2) as to not mislead us into misidentifying TMQ metabolites in SPE extracts.

Other metabolites of TMQ exist and we are continuing to study and identify their structures and relative abundances, and we will report on these in a future report. Specifically, we are in the process of acquiring full scan ESI(+)-MS/MS daughter ion spectra on each of the metabolites and we will report these and their structural deconvolutions.

In summary these results suggest that a reliable and sensitive confirmatory method for TMQ would consist of glucuronidase hydrolysis, SPE extraction and LC-ESI(+)-MS/MS MRM analysis. We are currently evaluating qualifier ion transitions such as m/z 346->183, 346->329, 346->161 in comparison to m/z 346->164 as a quantifier transition. Optionally, an additional level of confirmation could involve determination of the presence of methylated TMQ released hydrolytically, but this approach requires availability of either 3- or 4-O-methyl-TMQ standard, or both.

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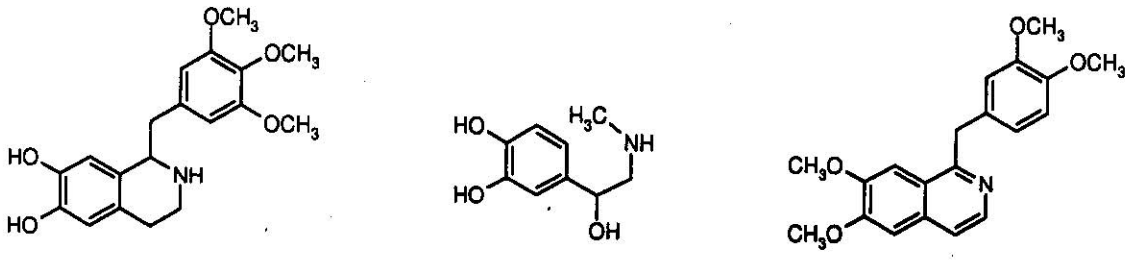


Fig. 1. TMQ [left], m.w. 345 in comparison with epinephrine [center], m.w. 183 and the internal standard used in our studies, papaverine [right], m.w. 339.

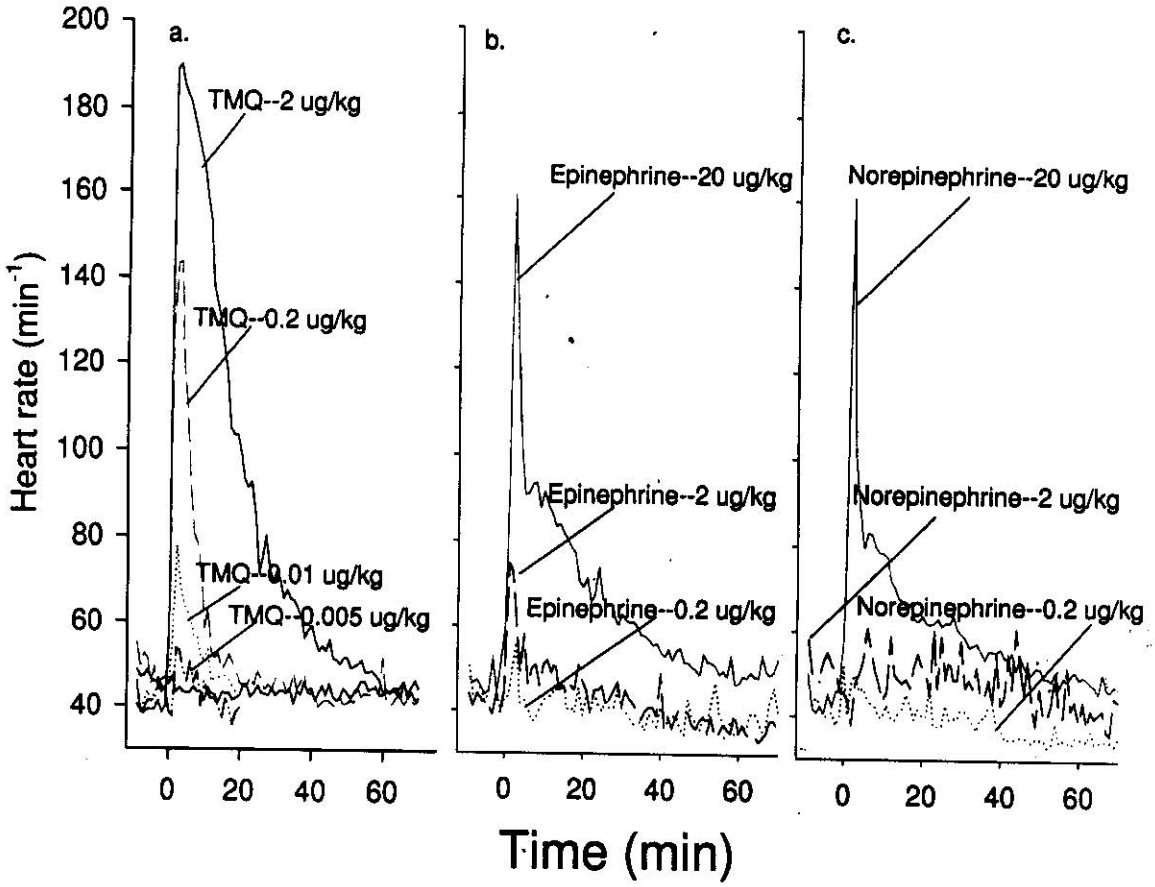


Fig. 2. Heart rate stimulation following intravenous injection of the indicated doses of a) TMQ, b) epinephrine, and c) norepinephrine in 5 horses.

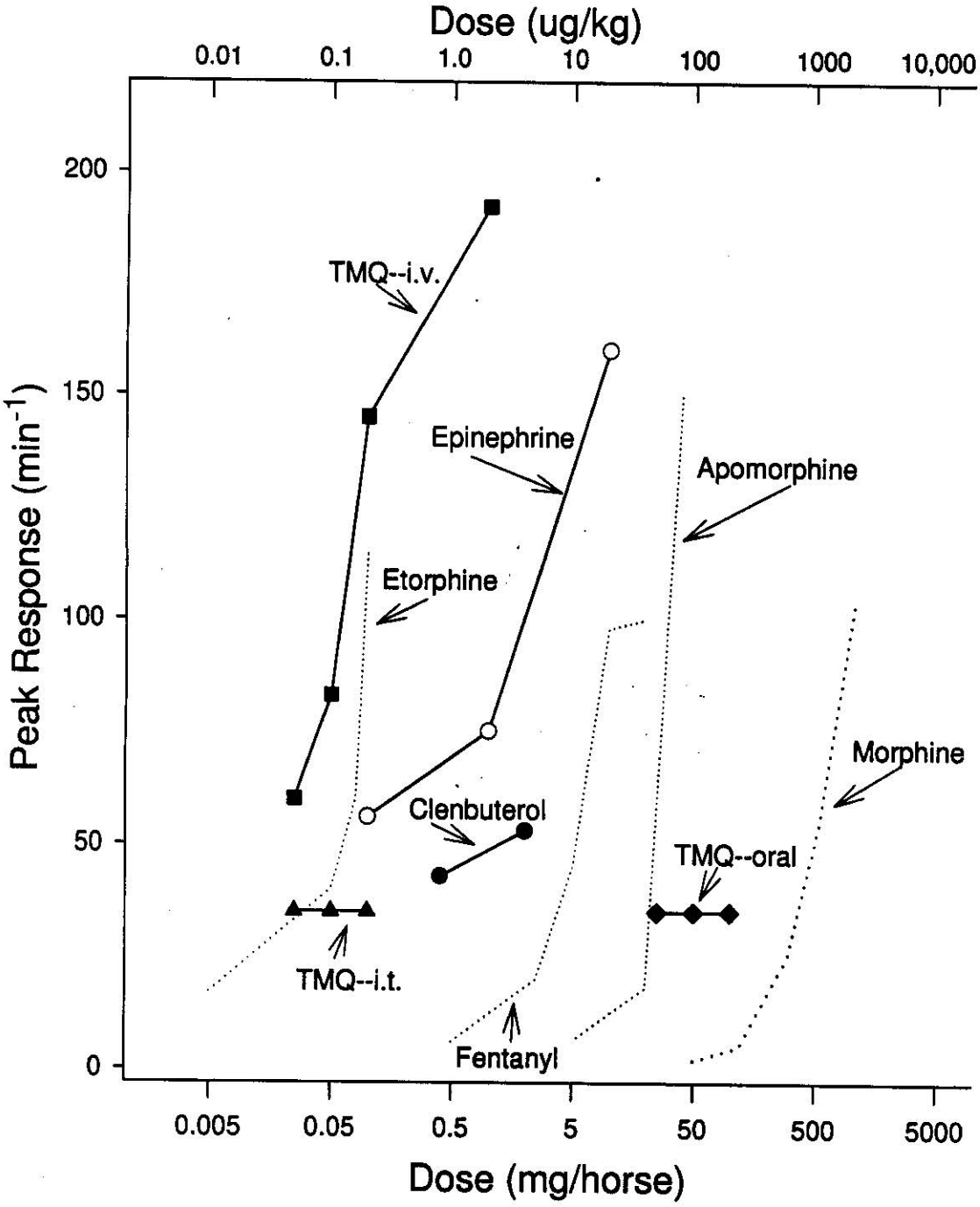


Fig. 3 Dose response curves for peak HR effects for TMQ following intravenous (■-■), intratracheal (▲-▲), and oral (♦-♦) administration, and intravenous (●-●) clenbuterol and (○-○) epinephrine. Norepinephrine data were essentially identical to epinephrine (data not shown). The dotted lines show locomotor response for a range of selected narcotic analgesics in the horse [modified from Tobin (15)], including the most potent pharmacology previously reported in the horse, the locomotor response to etorphine.

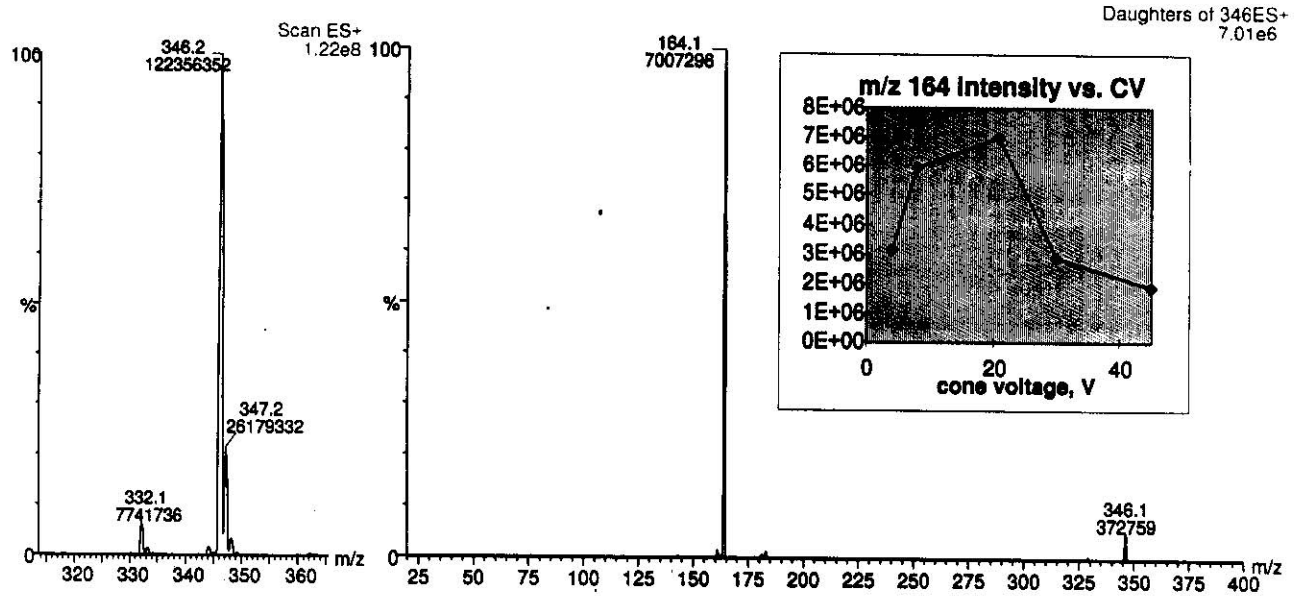
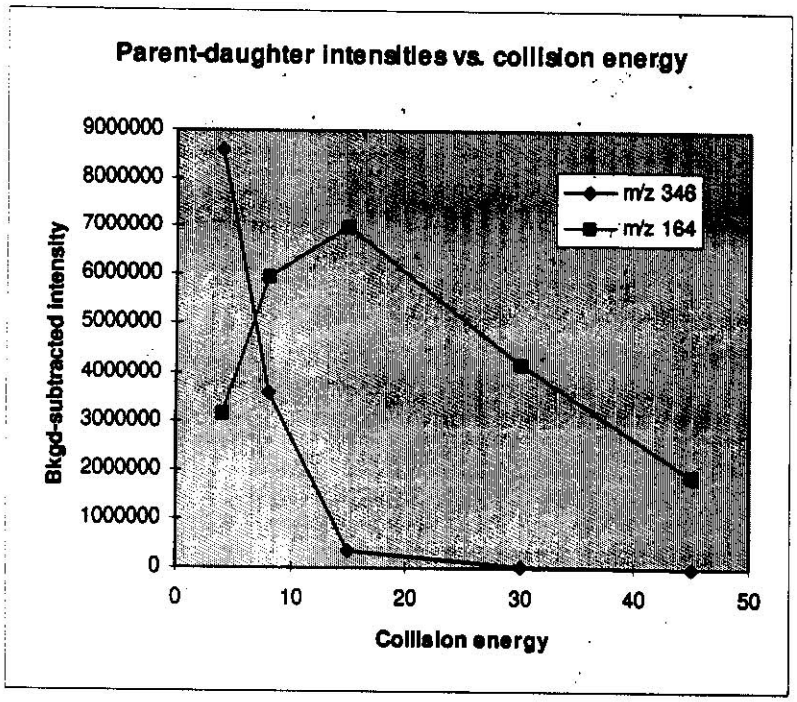


Fig. 4. TMQ ESI(+) mass spectra. The left hand panel shows full scan spectrum on 10 ug/ml TMQ in 0.05% formic acid:acetonitrile, 1:1; the right hand panel shows daughter ions of the m/z 346 $[M+H]^+$ parent at a cone voltage setting of 21. The inset in the right hand panel demonstrates dependence of m/z 164 intensity on the cone voltage setting.



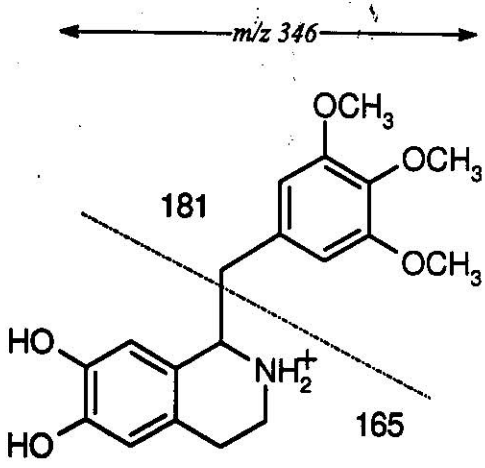


Fig. 5. [Left] Variation of parent and daughter ion intensities of TMQ on direct infusion-ESI(+)-MS/MS with changes in collision energy. [Right] Bond cleaved in protonated TMQ to release a 182 m.w. neutral molecule of 3,4,5-trimethoxytoluene and m/z 164 daughter ion fragment.

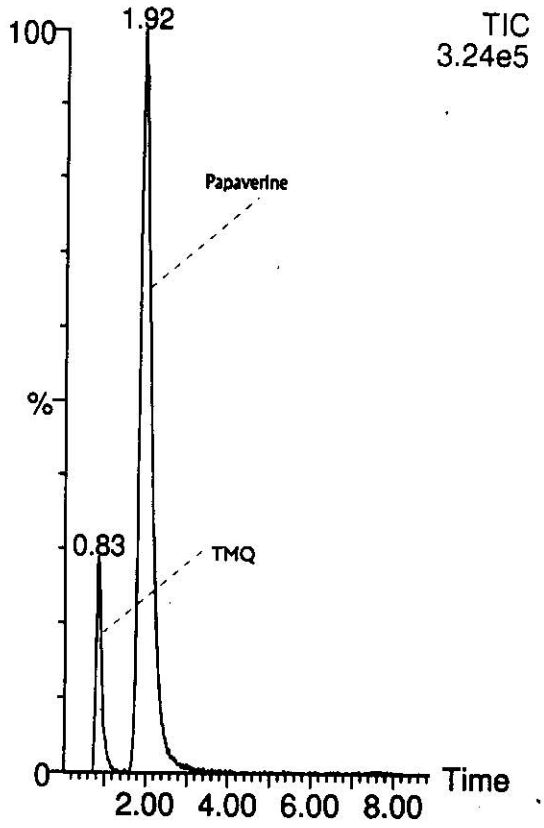


Fig. 6. TMQ standard (20ng/ml in urine extracted by SPE) run by LC-ESI(+)-MS/MS. Along with 20 ng/ml chromatogram, the internal standard, papaverine.

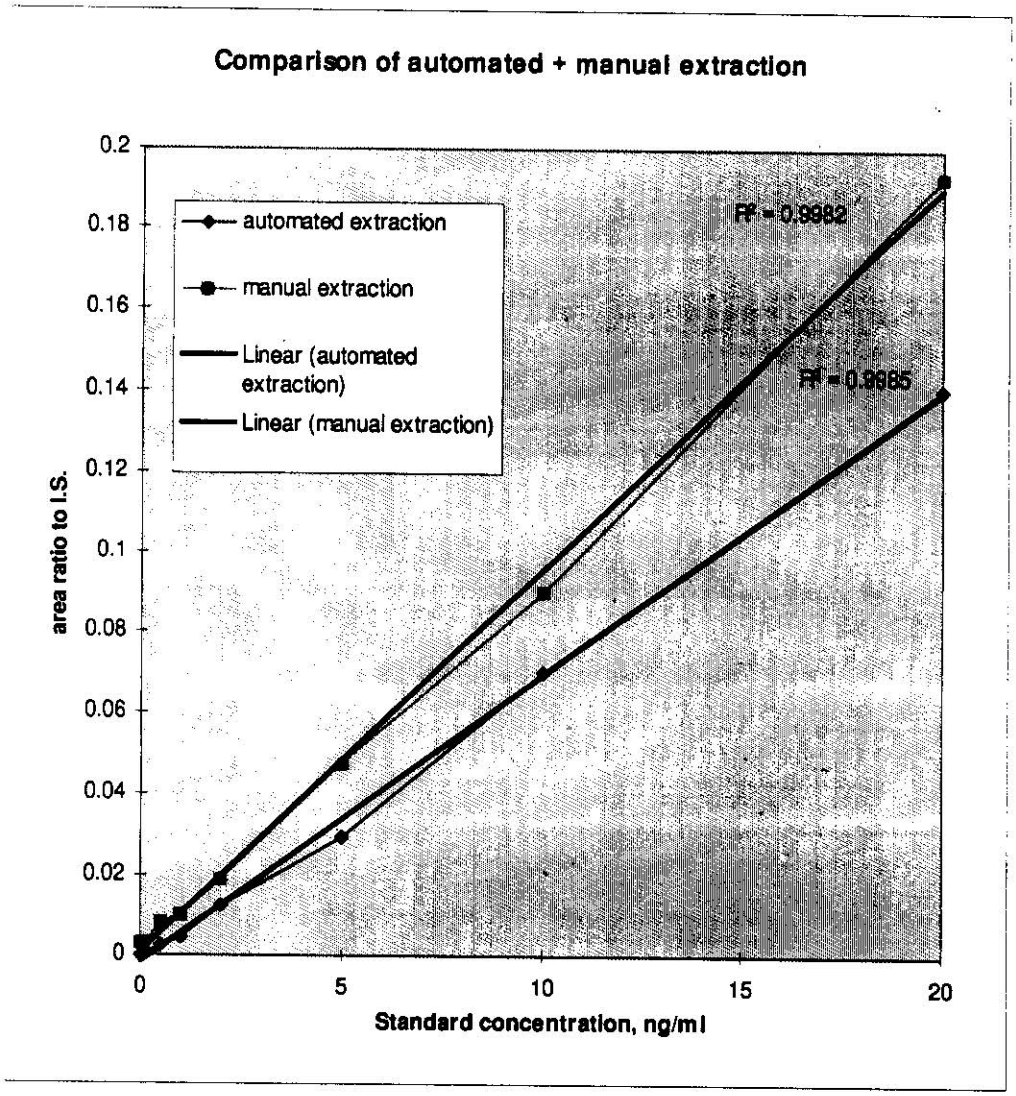


Fig. 7. Linearity of SPE extraction of TMQ in comparison to the papaverine internal standard. Although both methods were linear and sensitive, minor differences in extraction methodology resulted in slightly better recovery with the manual SPE method.

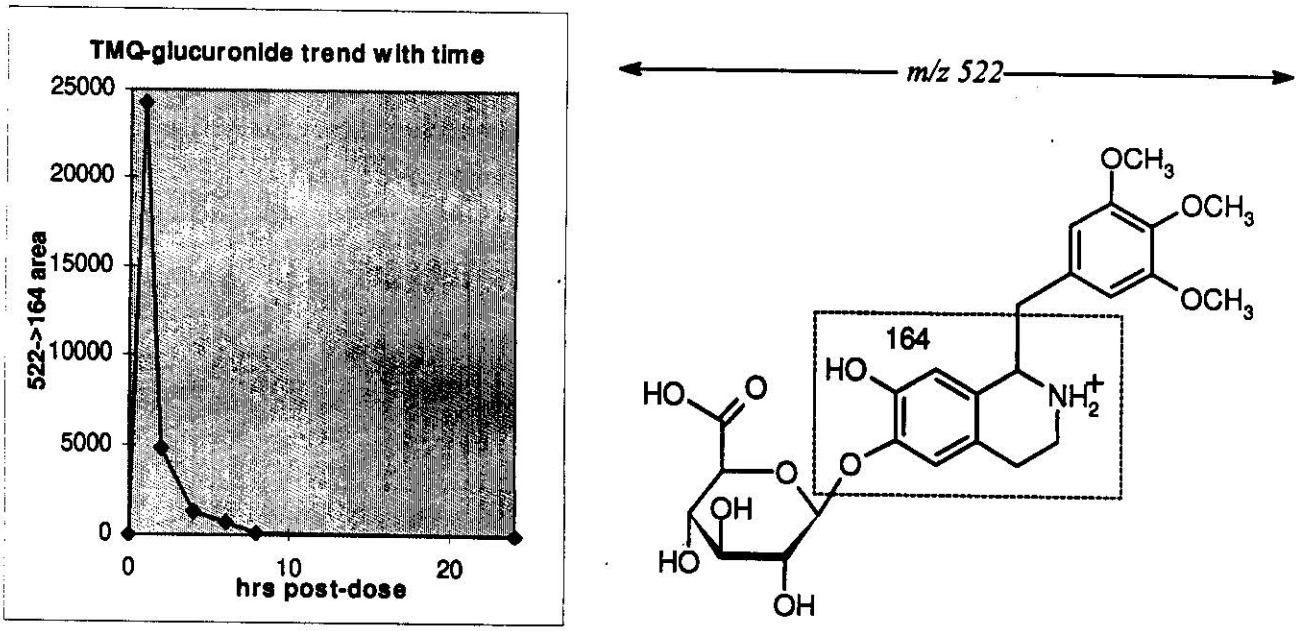


Fig. 8. Apparent time course of TMQ-glucuronide elimination: TMQ-glucuronide was detected as a m/z 522->164 fragmentation by LC/ESI(+)-MS/MS. The left panel indicates the time course of TMQ-glucuronide elimination as monitored by m/z 522->164 fragmentation. The right panel indicates the proposed structure for the glucuronide(s) and the source of the m/z 164 fragment as arising from cleavages of the m/z 522 ion.

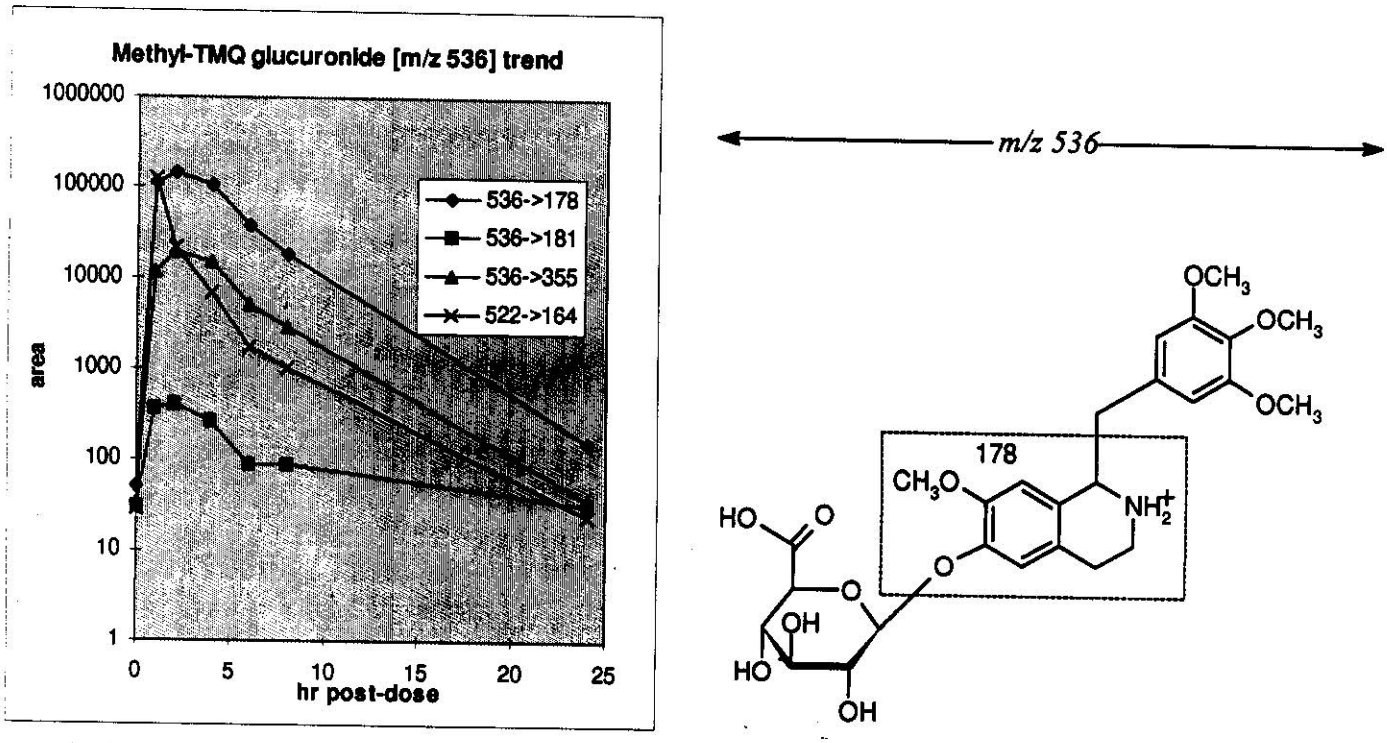


Fig. 9. Excretion profile of the methylated-TMQ-glucuronide (m.w. 535) and its daughter ions in contrast to that of TMQ-glucuronide (m.w. 521). Note logarithmic y-axis to enable comparison to the lower abundance 181 and 355 daughter ions. The right panel indicates one likely structure for the glucuronide and the source of the m/z 178 and other fragments as arising from the two cleavages.

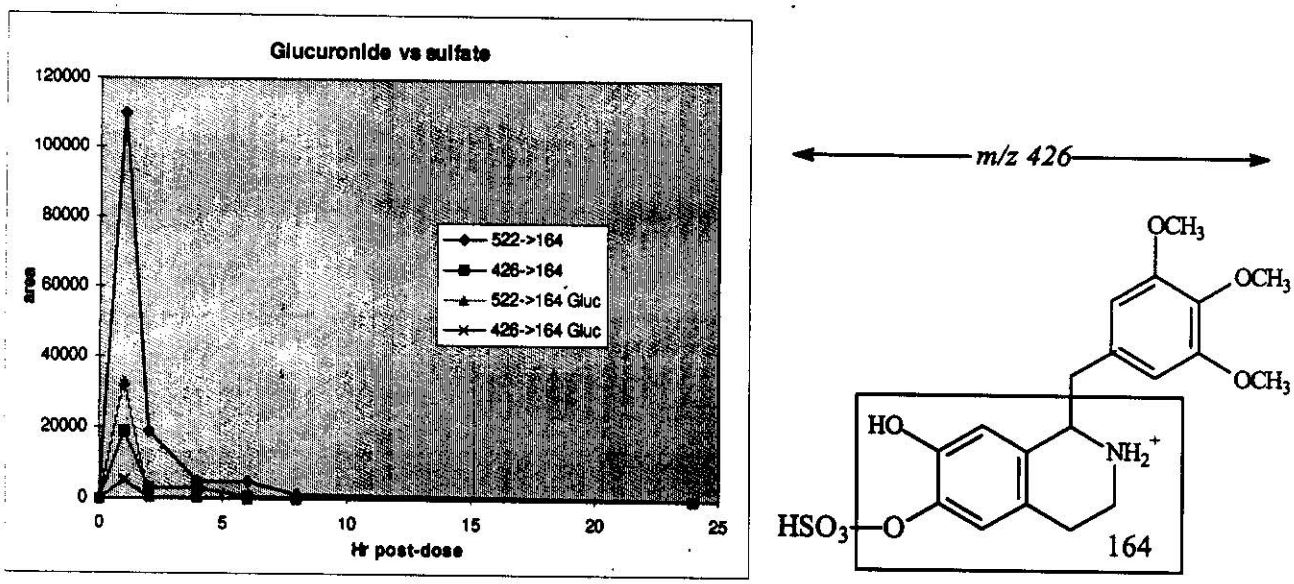


Fig. 10. Excretion profile of TMQ-sulfate, m.w. 425, in comparison to TMQ-glucuronide, m.w. 521. Profile of excretion post-dose is shown based on the raw areas of the corresponding fragmentations, both with ["Gluc"] and without beta-glucuronidase treatment [left]. Right-hand panel illustrates one structure for the sulfate ester of TMQ with the origin of the m/z 164 fragment from the m/z 426 $[M+H]^+$ parent.

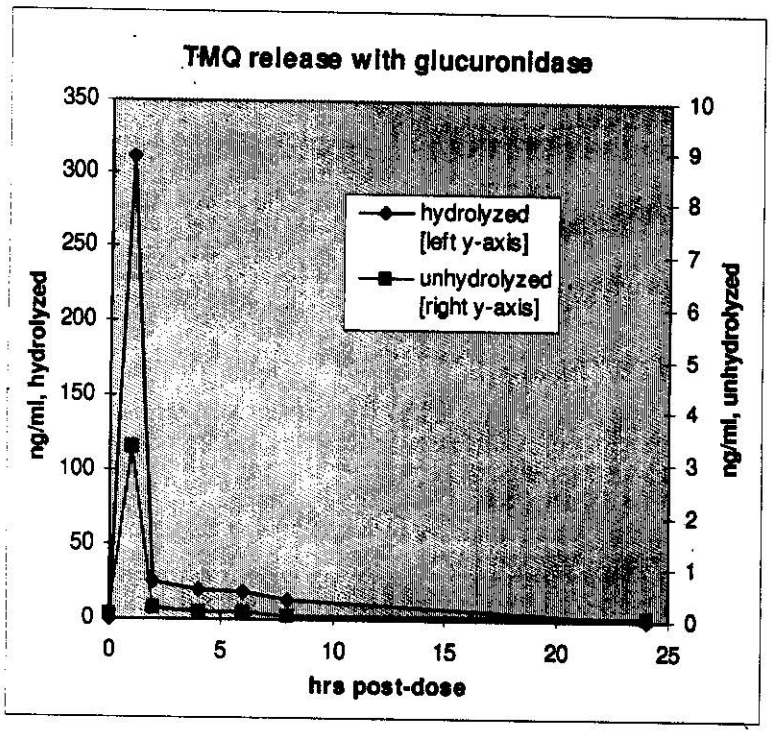
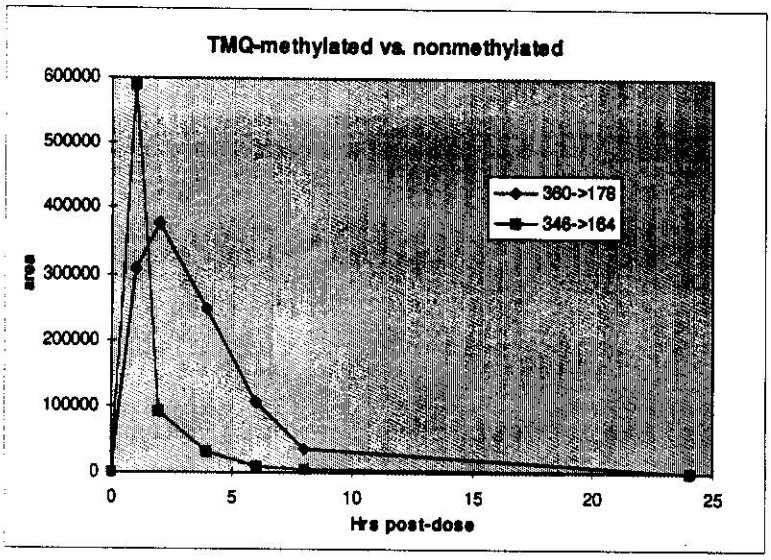


Fig. 11. TMQ in urine samples following 8 μg/kg dose. The urine was either unhydrolyzed (■—■), or hydrolyzed 3 hrs at 65°C with beta-glucuronidase (Patella vulgata) (u—u) as indicated. Note the left y-axis corresponds to hydrolyzed, the right y-axis corresponds to unhydrolyzed enabling display of the low TMQ concentrations in the unhydrolyzed samples.



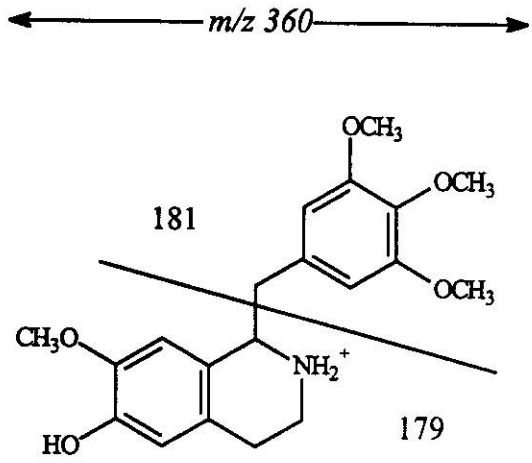


Fig. 12. Comparative time course and approximate relative concentrations of methylated (m.w. 359) and non-methylated (m.w. 345) TMQ species recovered from equine urine post-administration and glucuronidase hydrolyzed urine [left panel]. Right-hand panel shows one structure for methylated TMQ and the origin of the m/z 178 fragment.

Effect of glucuronidase on TMQ-glucuronides and methylated TMQ-glucuronides:

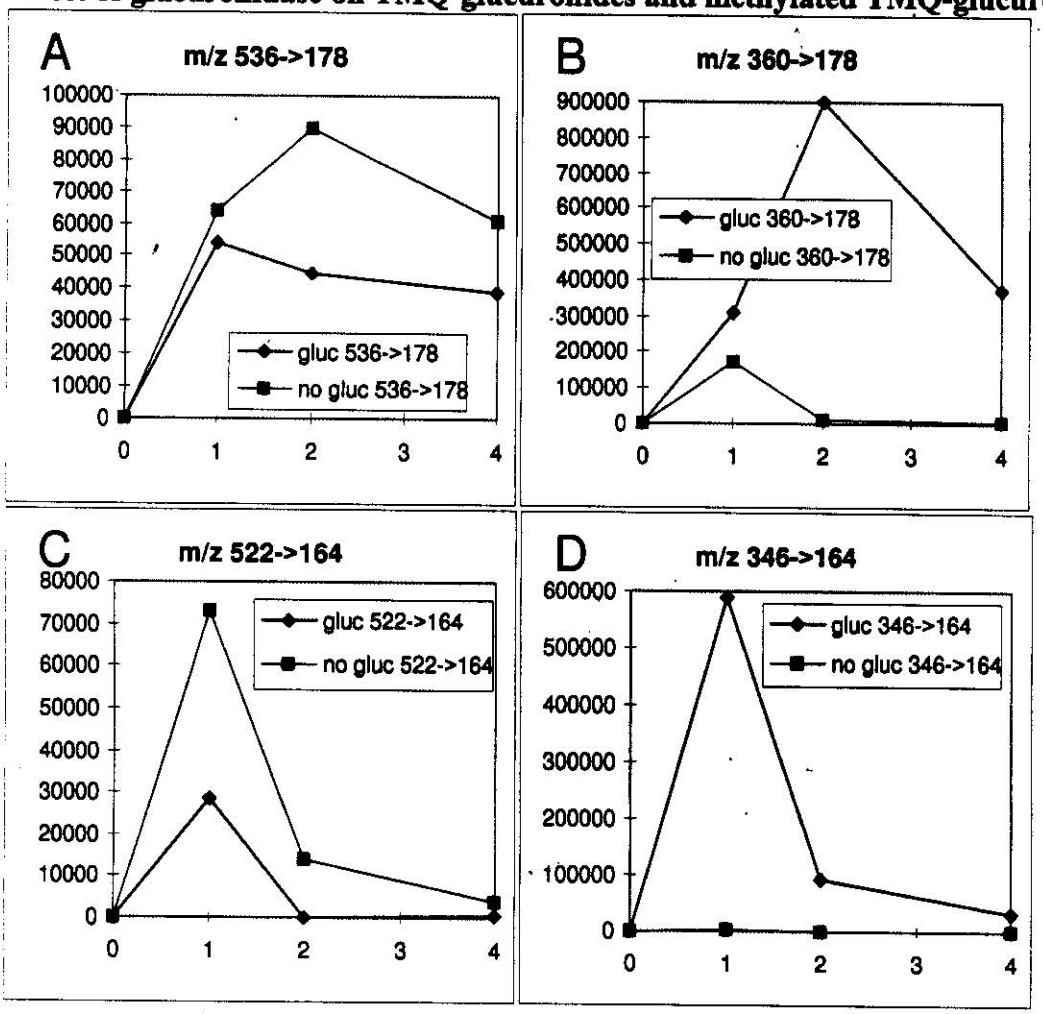


Fig. 13. Illustration of the relative decrease in TMQ- and methylated-TMQ-glucuronides (parent ions at m/z 522 and 536, respectively) following glucuronidase treatment with concurrent increase in free TMQ and methylated-TMQ (parent ions at m/z 346 and 360, respectively). Plots of raw HPLC areas as a function of time (hr) post-administration up to 4 hrs. "Gluc" (u—u) represents beta-glucuronidase treatment, "No gluc" (■—■) represents no such treatment in each case. **A**, m/z 536->178 (parent methylated-TMQ-glucuronide fragmentation to methylated catechol isoquinoline ring); **B**, m/z 360->178 (parent methylated-TMQ fragmentation to methylated catechol isoquinoline ring); **C**, m/z 522->164 (parent TMQ-glucuronide fragmentation to catechol isoquinoline ring); **D**, m/z 346->164 (parent TMQ fragmentation to catechol isoquinoline ring).

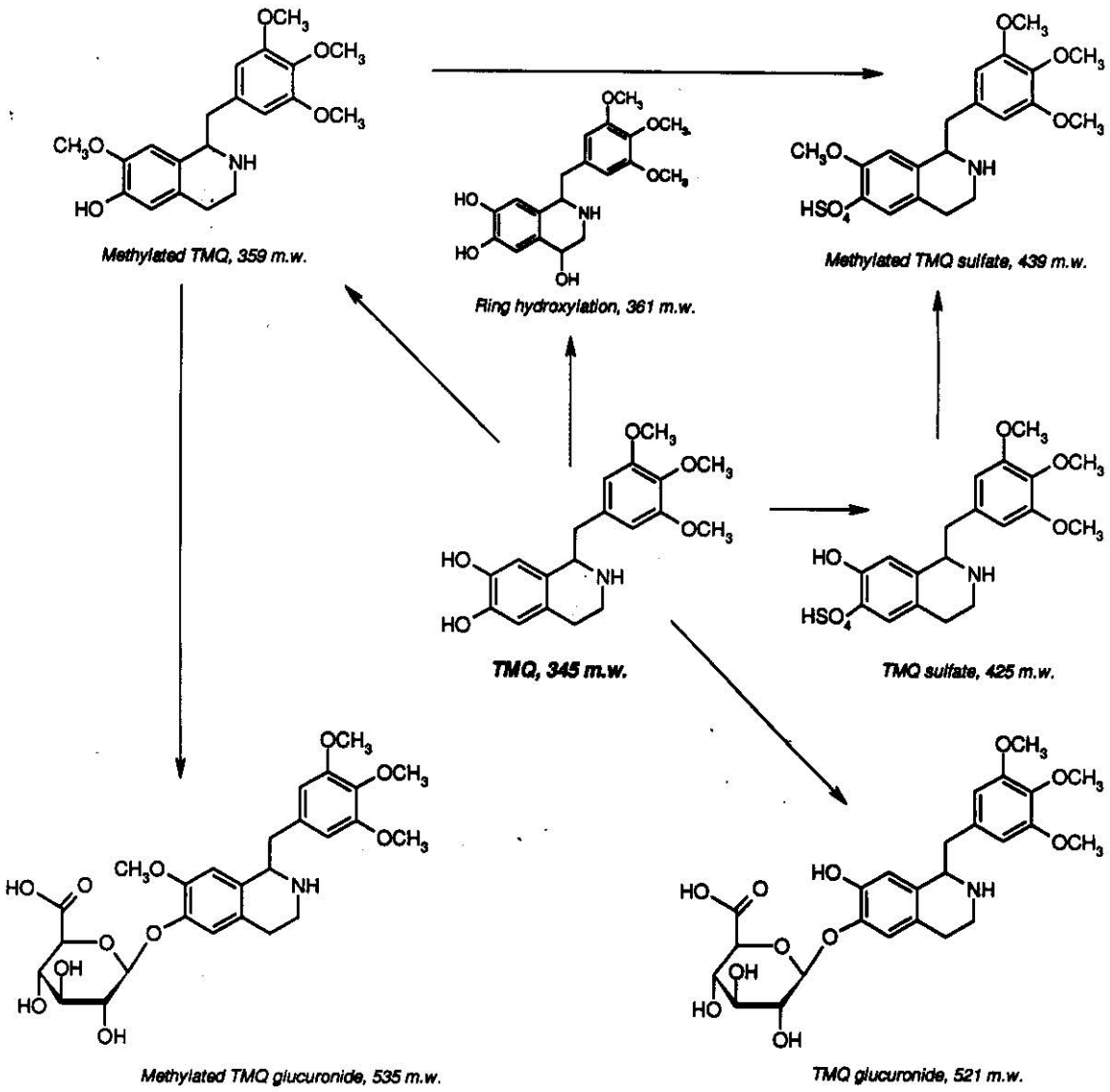


Fig. 14. Proposed pathways of TMQ metabolism. Substituent placements at the isoquinoline diol group are arbitrary, and it is likely that the possible alternative structural arrangements also occur *in vivo*. The imino ring hydroxylation product is observed to occur at about 0.4% or less in 0.05% formic acid: acetonitrile and has not yet been definitively demonstrated to occur in the horse.

Table 1. HPLC Gradient for TMQ and papaverine.
Initial Conditions:

A%	10.0
B%	90.0
Flow (ml/min)	0.150

HP1050 LC Gradient Timetable

Time	A%	B%	Flow
0.00	10.0	90.0	0.150
1.00	10.0	90.0	0.150
2.50	89.5	10.5	0.150
4.50	89.5	10.5	0.150
5.00	10.0	90.0	0.150
16.00	10.0	90.0	0.150

Table 2. Predicted metabolites of TMQ as a guide to discovering equine metabolism of this drug.

Compound description	M.W.	ESI+, [M+H+], m/z
TMQ	345	346
scission product w double bond formed in imino ring	161	162
scission product [trimethyoxytoluene] *	182	-----
CH3-->H [demethylation]	331	332
dehydration of hydroxylated imino ring	343	344
Methylation	359	360
imino ring hydroxylation	361	362
demethylated--> acetate	374	375
diol-->acetate	387	388
demethylated--> sulfate	411	412
Sulfate of dehydrated hydroxy-TMQ	423	424
diol-->sulfate	425	426
methylation-->sulfate	439	440
Sulfate of hydroxyl-TMQ	441	442
demethylated--> glucuronide	507	508
glucuronide of dehydrated hydroxy-TMQ	519	520
diol-->glucuronide	521	522
methylation-->glucuronide	535	536
glucuronide of hydroxyl-TMQ	537	538
glucuronide of TMQ sulfate	601	602
glucuronide of methylated TMQ sulfate	615	616

* Trimethoxytoluene not likely to be seen with ESI(+)MS/MS.