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Investigation of the metabolism of azaperone in the horse☆

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Abstract

Urine samples collected from a horse after intramuscular administration of 40 mg of azaperone were extracted at pH 10 before and after acid hydrolysis. The extracts were concentrated and analysed by LC–MS–MS. Two N-dealkylated metabolites, N-despyridinylazaperol and N-despyridinylazaperone, and a low concentration of azaperone were detected in the unhydrolysed urine. Six metabolites; hydroxyazaperol, two hydroxyazaperones, azaperol, N-despyridinylazaperol and N-despyridinylazaperone were detected in the hydrolysed urine extracts. Using XAD-2 resin extraction, three glucuronide conjugated azaperone metabolites (hydroxyazaperol glucuronide, hydroxyazaperone glucuronide and azaperol glucuronide) were detected in the urine. The mass spectra of these metabolites show the same characteristic daughter ions as the unconjugated metabolites. The glucuronide conjugated azaperone metabolites were partially hydrolysed in the heated nebulizer interface to the unconjugated metabolites.

1. Introduction

Azaperone (1-(4-fluorophenyl-4-[4-(2-pyridinyl)-1-piperazinyl]-1-butanone), a short-acting neuroleptic butyrophenone tranquiliser, is commonly administered to pigs shortly before transportation to reduce stress and aggressiveness. It has also reportedly been used to calm horses before racing.

After subcutaneous administration of radioactive azaperone to rats, approximately 22% and 78% of the radioactivity were excreted in the urine and faeces, respectively, within 96 h [1]. Azaperol, a hydrogenated metabolite of azaperone, was isolated and identified in pig tissues [2,3]. In man, following a suicide attempt involving the ingestion of approximately 2 g of azaperone, azaperone, azaperol and N-despyridinylazaperone were identified in the patient's urine [4]. Using GC–MS for the analysis of hydrolysed horse urine extracts, we have previously reported hydrogenation of the keto group and hydroxylation of the pyridinyl ring of azaperone to form azaperol and hydroxyazaperol [5]. Recently, Woods *et al.* [6] synthesized 5'-hydroxyazaperone and 5'-hydroxyazaperol and identified these metabolites in equine urine. No phase II (conjugation) metabolic study of azaperone has been reported yet in the literature.

Atmospheric pressure ionisation (API)-LC–

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tandem mass spectrometry (MS–MS) has recently been applied to drug metabolism studies [7]. Polar, thermally sensitive and non-volatile chemicals (*e.g.* conjugated drug metabolites and proteins) which can not be detected by GC–MS or need to be derivatized before GC–MS analysis can be analysed directly by LC–MS as intact molecules. However, atmospheric pressure ionisation is mild ionisation technique that does not produce many characteristic fragments for chemical identification and structure elucidation. To overcome the lack of fragmentation, collision-induced dissociation (CID)-MS–MS can be used to produce additional fragments from a mass-selected parent ion. Another advantage of using CID-MS–MS is to identify compounds in complex biological extracts. Equine urine extracts usually have a high chemical noise that interferes with the drug analysis, especially when the concentration of the analyte is low (sub-microgram/ml urine level). CID-mass spectrometry monitors the fragmentation of a selective ion with a specific m/z ratio. This can eliminate most of the background interference except the background noise with the same m/z ratio.

In the present study, the phase I and phase II metabolism of azaperone in horses was investigated using an atmospheric pressure ionisation LC–MS–MS. An analytical procedure for azaperone metabolites in equine urine was developed to be used in regulating the use of azaperone in the racing industry.

2. Experimental

2.1. Reagents and chemicals

Azaperone (Stresnil) was obtained from Pitman Moore (Don Mills, Ont., Canada). Azaperol was synthesized from azaperone by reduction with sodium borohydride as described by Rauws *et al.* [2]. XAD-2 resin was obtained from Rohm and Hass (West Hill, Ont., Canada) and was pre-conditioned as described by Frincke and Henderson [8]. Solvents (glass distilled OmniSolv grade) were obtained from BDH (Vancouver, Canada). All other chemicals were reagent grade, unless specified otherwise.

2.2. Drug administration and sample collection

Stresnil (40 mg) was administered intramuscularly to standard-bred mares. Urine samples were collected at various time intervals for a 5 day period. In our previous study [5], we demonstrated that the excretion of metabolites of azaperone in equine urine peaked between 1 to 4 h after drug administration. Hence, urine samples collected for the first 12 h post administration were used in this study.

2.3. Urine extraction

Urine samples (75 ml) were adjusted to pH 10 with 10 ml of borate buffer (saturated) and ammonium hydroxide (conc.) and extracted with 4% isopropanol in dichloromethane (100 ml). The organic layers were backwashed with 0.1 mol/l HCl (100 ml) and re-extracted at pH 10 with 4% isopropanol in dichloromethane (100 ml). The extracts were dried at 50°C with a stream of nitrogen, reconstituted in 0.5 ml of methanol and analysed by LC–MS–MS. The aqueous layers were acidified to pH 1 with 4 mol/l HCl and autoclaved at 120°C for 10 min. The autoclaved samples were extracted at pH 10 with 4% isopropanol in dichloromethane, backwashed and re-extracted as described previously.

For the extraction of conjugated metabolites, urine samples (50 ml) were adjusted to pH 9.5 with 5 ml of ammonium acetate buffer (5 mol/l) and concentrated ammonium hydroxide. The samples were passed through approximately 2 g of XAD-2 resins. The XAD-2 resin was washed with 20 ml of 50% ethyl acetate in dichloromethane, 10 ml of 10% acetic acid in water and 10 ml of dichloromethane. The resin was then eluted with 10 ml of 10% methanol in ethanol. The eluates were dried, reconstituted in methanol and analysed by LC–MS–MS.

2.4. LC–MS–MS

The chromatographic system consisted of an ABI (Foster City, CA, USA) 140A syringe

pump equipped with a 5-μm, 250 × 2.1 I.D. mm Suplex pKb-100 (Supelco, Oakville, Canada) column. The mobile phase was gradient programmed from 20% acetonitrile and 80% ammonium acetate buffer pH 10 (50 mmol/l) to 100% acetonitrile in 20 min and the flow-rate was 0.2 ml/min. Mass spectral analyses were performed with a Sciex (Thornhill, Ont., Canada) API III triple quadrupole LC–MS–MS system with a heated nebulizer interface. The orifice diameter was 125 μm. The nebulizer heater temperature was set at 400°C and the corona discharge current was set at 3 μA. The collision induced dissociation (CID) was achieved in the second quadrupole using argon as the collision gas. The argon curtain thickness was 500×10^{12} molecules/cm^2 and the collision energy was 50 eV. Positive ion mode was used for the study. The CID spectra were obtained by scanning from 10 a.m.u. up to 10 a.m.u. above the M + 1 ions in 1 a.m.u. steps with a dwell time of 5 ms. The full scan mass spectra were obtained under the same conditions as the CID spectra except that no collision gas was used and the scan range was 200 a.m.u. to 700 a.m.u. in 1 a.m.u. steps with a dwell time of 5 ms.

3. Results and discussion

A C_{18} LC column (Supelcosil, 3 μm 250 × 2.1 mm I.D.) was used initially for the separation of azaperone and its metabolites. Probably due to the interaction between the silanol groups in the silica-based column and amine groups in the analytes [9], we failed to achieve a good resolution and separation. Subsequently a deactivated reversed phase Suplex pKb-100 column was used and a good separation between azaperone and its metabolites was achieved. For the separation of conjugated metabolites, better resolution was achieved with a higher pH ammonium acetate buffer and, therefore, a pH 10 buffer was used as the mobile phase for this study. The reason for the better resolution at high pH may be due to the formation of uncharged amine groups in the analytes.

Full scan LC–MS, daughter-ion monitoring, parent-ion monitoring and neutral loss monitoring LC–MS–MS were used in our study. All the potential metabolites were detected using daughter-ion monitoring. No additional metabolites were detected by the other monitoring techniques which may be due to the high chemical noise of the equine urine matrix, especially in the low molecular mass region. Furthermore, the quality of the mass spectra from the full scan LC–MS, parent-ion monitoring and neutral loss monitoring LC–MS–MS were poor. Hence, only the daughter-ion monitoring CID mass spectra are presented in this study.

Fig. 1 shows the CID mass spectra and the proposed daughter-ion assignments of azaperone and azaperol analytical standards. Azaperone and azaperol standards (500 ng/injection) were separated by the LC column and analysed by CID-MS–MS. The daughter ions *m/z* 123 and 165 in the CID mass spectrum of azaperone corresponded to fragments derived from the butyrophenone moiety of the molecule. Daughter ion *m/z* 312 ($M + 1 - H_2O$) of azaperol appeared to be the dehydrated ions (from hydroxy group to corresponding alkene group) of protonated azaperol. Daughter ions *m/z* 149 and 192 in the CID mass spectrum of azaperol appeared to be derived from dehydrated azaperol.

There were two observations to support this hypothesis. The parent-ion mass spectra of *m/z* 149 and 192 of azaperol showed the parent ion at *m/z* 312 and 330. We have synthesized the dehydrated azaperol by incubating azaperol under strong acid conditions (4 mol/l HCl) at 100°C and the synthetic product (molecular mass 311) also produced the *m/z* 149 and 192 ions in the CID mass spectrum. Daughter ions 78, 121 and 147 corresponding to the fragments from the pyridinyl–piperazinyl moiety of the molecules were present in the mass spectra of azaperone and azaperol. Some small ions surrounding the parent ions of *m/z* 328 and 330 were observed in the CID mass spectra of azaperone and azaperol analytical standards (Fig. 1). They may be the result of the combined effects of the hydrogenation and dehydrogenation of the $[M + 1]^+$ ions and the poor resolutions of the mass spectrometer. For the LC–MS–MS analysis, the resolution of the mass spectrometer sometimes

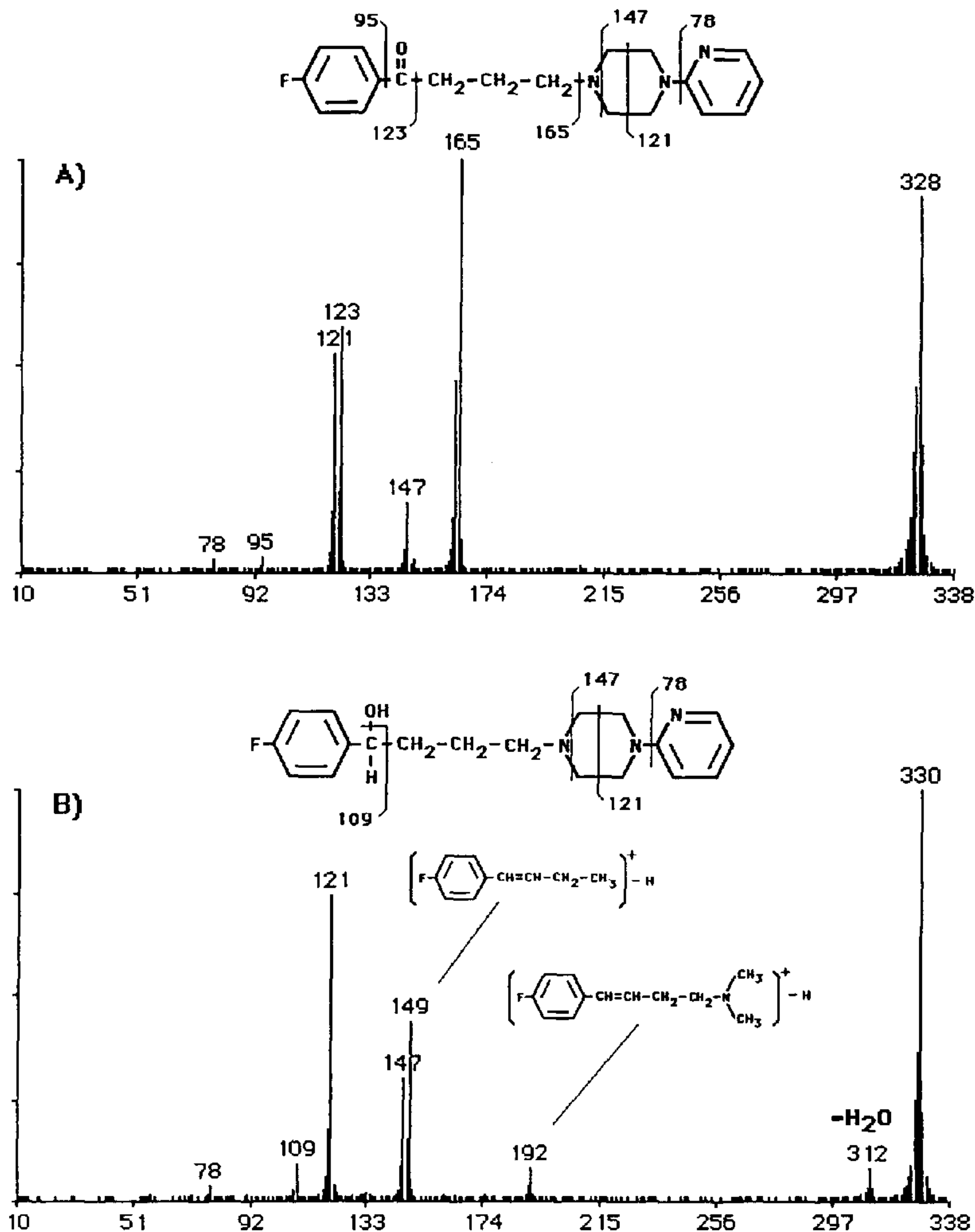

Fig. 1. Daughter-ion mass spectra of (A) azaperone, and (B) azaperol analytical standards.

had to be adjusted in order to increase the sensitivity.

Fig. 2 presents the LC chromatograms of the total daughter-ion monitoring (*m/z* 251, 253 and 328) of unhydrolysed urine extracts from the horse 3 h post i.m. administration of 40 mg of azaperone. A small peak (peak III) in the chromatogram of the daughter-ion monitoring of *m/z* 328 showed the same retention time (Table 1) and identical characteristic ions (Figs. 1A and 3C) as the azaperone analytical standard. This indicated that only trace amounts of unmetabolized azaperone were excreted in the equine urine. The daughter ions of the potential meta-

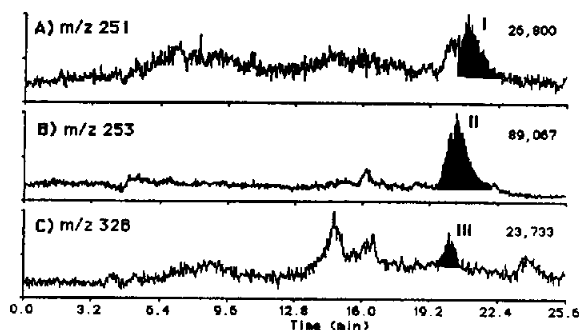

Fig. 2. LC chromatograms of the total daughter-ion monitoring of unhydrolysed urine extracts from the horse 3 h post i.m. administration of 40 mg of azaperone. (A) Daughter-ion scan of *m/z* 251 (M + 1 ion of N-despyridinylazaperone). (B) Daughter-ion scan of *m/z* 253 (M + 1 ion of N-despyridinylazaperol). (C) Daughter-ion scan of *m/z* 328 (M + 1 ion of azaperone).

bolites of azaperone were scanned by CID mass spectrometry. Two potential metabolites were detected in the unhydrolysed urine extract. The CID mass spectra of peaks I and II suggested the presence of N-dealkylated metabolites of azaperone and azaperol. These two peaks were not detected in the pre-administration urine extracts. The *m/z* 251 daughter-ion mass spectrum of peak I, which corresponded to the M + 1 molecular ions of N-despyridinylazaperone (Fig. 3A), showed major fragments at *m/z* 123 and 165 indicating that the metabolites retained the unchanged butyrophenone moiety of the azaperone molecule. The *m/z* 253 daughter-ion mass spectrum of peak II which corresponded to the M + 1 molecular ion of N-despyridinylazaperol (Fig. 3B), showed major fragments at

Table 1
LC retention times and major fragment ions from collision-induced dissociation of (M + 1) ions of azaperone and its proposed metabolites

Proposed compound	Retention time (min)	*m/z*
Azaperone standard	20.1	165, 328[a], 123, 121, 147, 95, 78
Azaperol standard	19.0	330[a], 121, 149, 147, 312, 192, 109, 78
N-Despyridinylazaperone (peak I)	20.9	251[a], 165, 123
N-Despyridinylazaperol (peak II)	20.4	253[a], 149, 109, 235
Azaperone (peak III)	20.1	165, 328[a], 123, 121, 95, 147
Hydroxyazaperol (peak IV)	17.5	346[a], 137, 149, 163, 192, 328, 109, 94
Hydroxyazaperone (peak V)	17.5	344[a], 165, 177, 137, 123
Hydroxyazaperone (peak VI)	18.2	165, 344[a], 137, 123, 95
Azaperol (peak VII)	19.0	330[a], 121, 149, 147, 312, 192, 109, 78
N-Despyridinylazaperol (peak VIII)	19.8	253[a], 149, 109, 235
N-Despyridinylazaperone (peak IX)	20.9	251[a], 165, 123
Hydroxyazaperol glucuronide (peak X)	13.9	522[a], 328, 346, 137, 192, 149, 504, 165, 163, 109
Decomposed[b] hydroxyazaperol glucuronide (peak XI)	13.9	346[a], 137, 149, 163, 328, 192, 109, 94
Hydroxyazaperone glucuronide (peak XII)	14.7	520[a], 165, 344, 177, 137, 123, 95
Decomposed[b] hydroxyazaperone glucuronide (peak XIII)	14.6	165, 344[a], 123, 137, 95
Azaperol glucuronide (peak XIV)	14.9	506[a], 330, 149, 121, 312, 192
Decomposed[b] azaperol glucuronide (peak XV)	14.9	330[a], 121, 149, 147, 312, 109

[a] $(M+1)^+$ ion.
[b] Decomposed in the heated nebulizer interface to form the unconjugated metabolites.

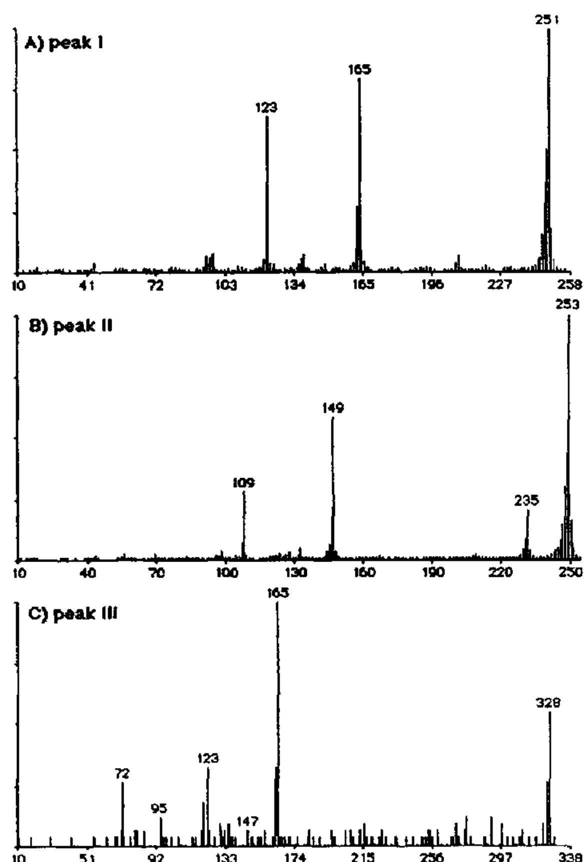

Fig. 3. Daughter-ion mass spectra of (A) peak I from Fig. 2A, and (B) peak II from Fig. 2B and peak III from Fig. 2C.

m/z 109 and 149 which corresponded to the dehydrated fragments as detected in the CID spectrum of azaperol. The detection of the dehydrated fragment (*m/z* 235) in peak II also indicated the presence of a hydroxy group in the metabolite. Furthermore, no ions at *m/z* 78, 121 and 147 were detected in the CID spectra of either metabolite indicating that the pyridinyl–piperazinyl moiety had been modified.

Fig. 4 presents the LC chromatograms of the total daughter-ion monitoring (*m/z* 346, 344, 330, 253 and 251) of hydrolysed urine extracts from the horse 3 h post i.m. administration of 40 mg of azaperone. Both enzyme hydrolysis and acid hydrolysis were evaluated in our preliminary study. Similar results were obtained by both methods. However, the acid hydrolysis required less time and therefore it was chosen for our study. The presence of azaperol in the hydrolysed urine extract was confirmed by comparing the retention times (Table 1) and the CID mass spectra of LC peak VII (Fig. 5D) with the azaperol analytical standard (Fig. 1B). The CID mass spectra of five other LC peaks showed fragmentation patterns consistent with metabolites of azaperone. Peaks VIII and IX had the same retention time and identical characteristic daughter ions (Fig. 5E and 5F) as peaks I and II in Fig. 2B and 2A indicating the presence of N-despyridinylazaperol and N-despyridinylazaperone. The LC peak at 17.5 min (peak IV, Fig. 4) may be the M + 1 molecular ions of hydroxyazaperol. The daughter-ion mass spectrum (Fig. 5A) of this peak showed the major fragments at *m/z* 109 and 149 which corresponded to the hydrated fragments as detected in the CID spectrum of azaperol. The presence of ions at *m/z* 94 and 137, which corresponded to the hydroxylated fragments *m/z* 78 and 121 which were detected in the pyridinyl–piperazinyl

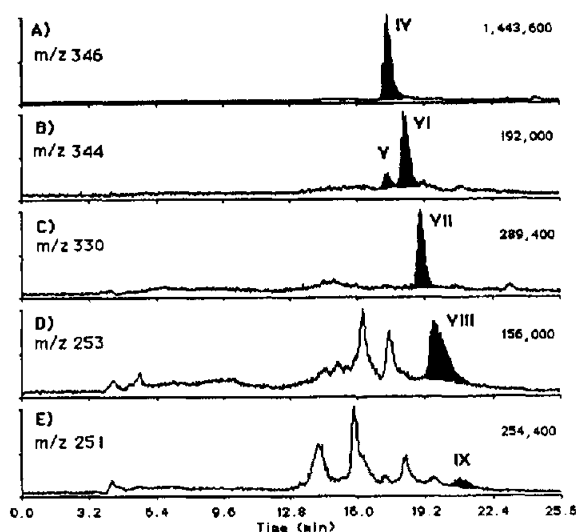

Fig. 4. LC chromatograms of the total daughter-ion monitoring of hydrolysed urine extracts from the horse 3 h post i.m. administration of 40 mg of azaperone. (A) Daughter-ion scan of *m/z* 346 (M + 1 ion of hydroxyazaperol). (B) Daughter-ion scan of *m/z* 344 (M + 1 ion of hydroxyazaperone). (C) Daughter-ion scan of *m/z* 330 (M + 1 ion of azaperol). (D) Daughter-ion scan of *m/z* 253 (M + 1 ion of N-despyridinylazaperol). (E) Daughter-ion scan of *m/z* 251 (M + 1 ion of N-despyridinylazaperone).

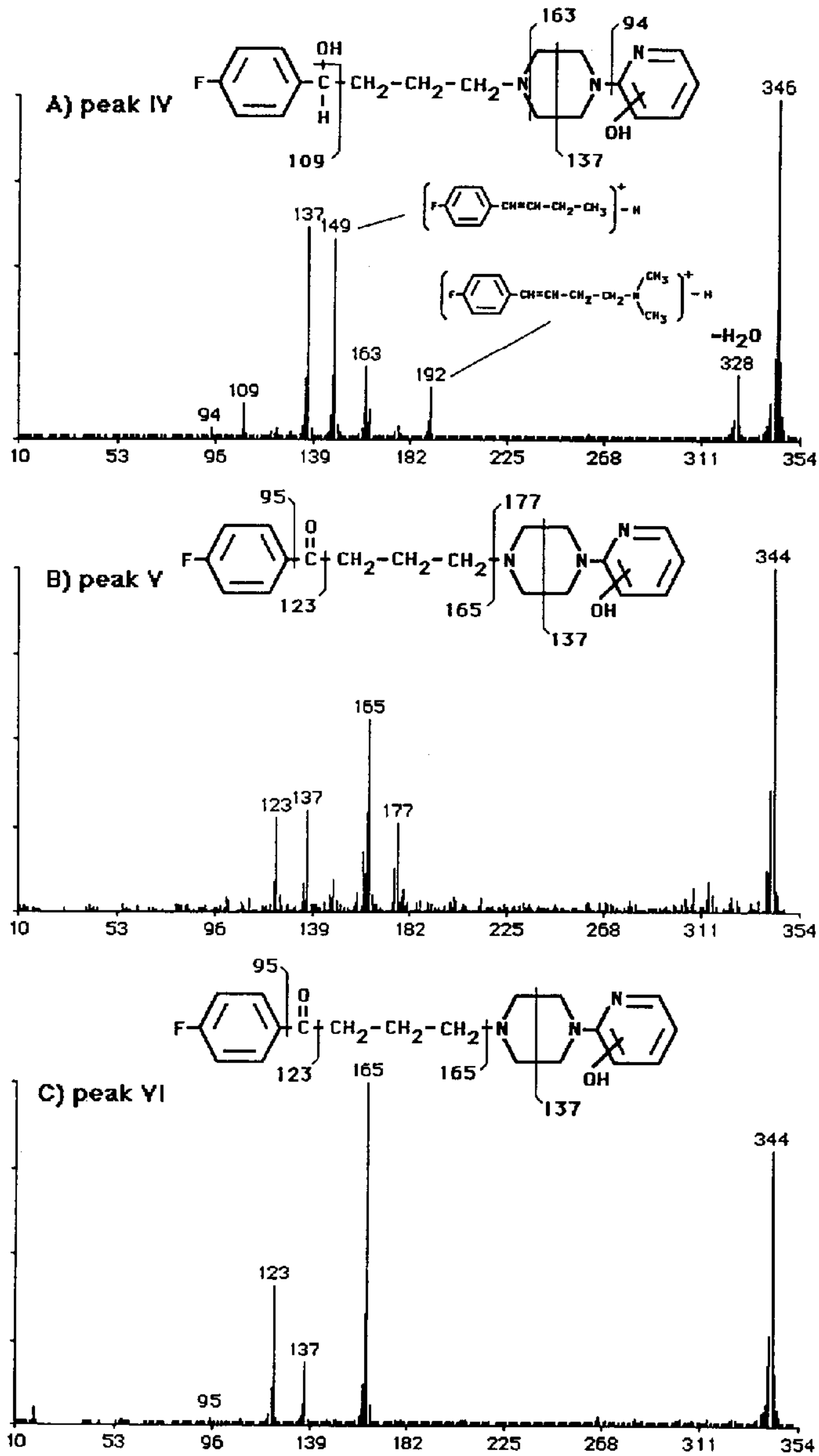

Fig. 5. Daughter-ion mass spectra of (A) peak IV in Fig. 4A, (B) peak V in Fig. 4B, (C) peak VI in Fig. 4B, (D) peak VII in Fig. 4C (E) peak VIII in Fig. 4D, and (F) peak IX in Fig. 4E. (contnd. on next page).

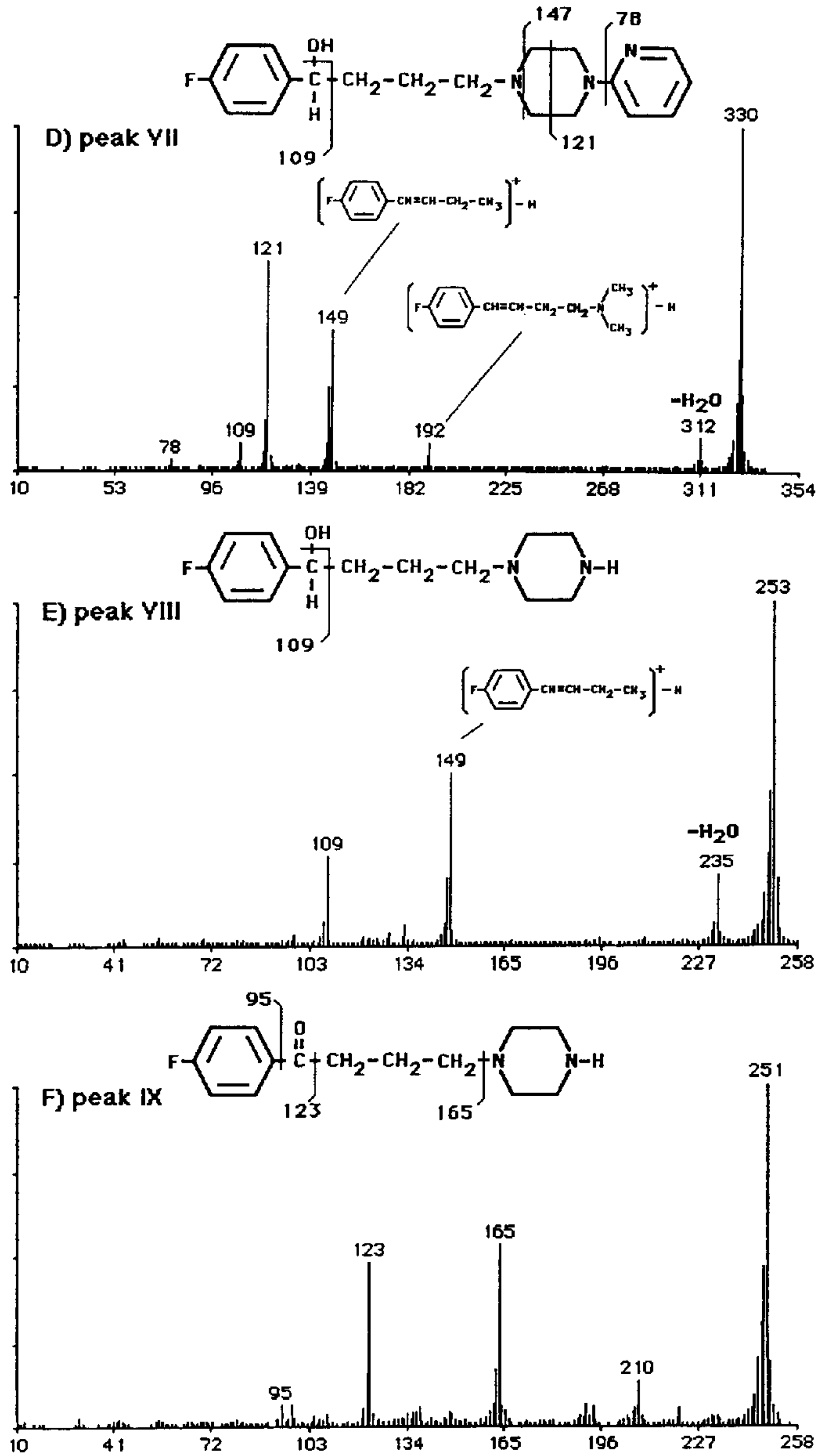

Fig. 5. continued.

moiety of azaperone and azaperol CID spectra, indicated that the hydroxylation may be on the pyridinyl ring. Peaks V and VI (Fig. 5B and 5C) may correspond to the isomeric forms of hydroxy-azaperone. The detection of fragments at *m/z* 123 and 165 in their mass spectra indicated the presence of the unchanged bytyrophenone moiety as in azaperone. The absence of ions at *m/z* 78, 121 and 147 and the presence of an ion at *m/z* 137 indicated the hydroxylation had occurred in the pyridinyl ring.

Fig. 6 presents the LC chromatograms of the total daughter-ion monitoring (*m/z* 522, 346, 520, 344, 506 and 330) of the XAD-2 extracted urine sample from the horse 3 h post i.m. administration of 40 mg of azaperone. Peak X

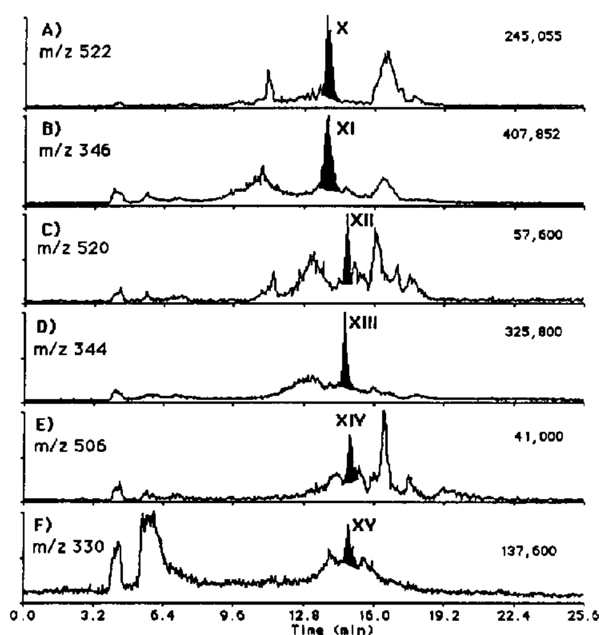

Fig. 6. LC chromatograms of the total daughter-ion monitoring of the XAD-2 extracted urine sample from the horse 3 h post i.m. administration of 40 mg of azaperone. (A) Daughter-ion scan of *m/z* 522 (M + 1 ion of hydroxyazaperol glucuronide). (B) Daughter-ion scan of *m/z* 346 (M + 1 ion of hydroxyazaperol). (C) Daughter-ion scan of *m/z* 520 (M + 1 ion of hydroxyazaperone glucuronide). (D) Daughter-ion scan of *m/z* 344 (M + 1 ion of hydroxyazaperone). (E) Daughter-ion scan of *m/z* 506 (M + 1 ion of azaperol glucuronide). (F) Daughter-ion scan of *m/z* 330 (M + 1 ion of azaperol).

(*m/z* 552) and peak XI (*m/z* 346) which might correspond to the M + 1 ions of glucuronide-conjugated hydroxyazaperol and hydroxy-azaperol had identical retention times. However, peak XI had an identical mass spectrum as that of peak IV. In reversed-phase LC chromatography, glucuronide conjugated metabolites should have shorter retention times when compared to that of the unconjugated metabolites. Indeed, we detected that the retention time of peak IV (probably, hydroxyazaperol) in the hydrolysed urine was 17.5 min, which was longer than that of 13.9 min for peak X. The possible explanation of the detection of *m/z* 346 ions at the same retention time as *m/z* 522 ions was that the glucuronide conjugated hydroxyazaperol was partially hydrolysed in the heated nebulizer interface to hydroxyazaperol. In a previous study of glucuronide and sulphate conjugated steroids [10], we also observed that conjugates were partially broken down in the heated nebulizer interface into the unconjugated steroid. The CID mass spectrum of peak X (parent ion *m/z* 522) showed the same characteristic daughter ions (Fig. 7A) as that of peak IV in Fig. 5A and a M + 1 ion corresponding to the molecular ion of peak IV plus a glucuronide, supporting the idea that peak X corresponding to hydroxyazaperol glucuronide. Decreasing the temperature of the heated nebulizer from 400°C to 350°C did not change the amount of *m/z* 522 ions *vs.* *m/z* 346 ions significantly but decreasing the orifice voltage increased the amount of *m/z* 522 ions *vs.* *m/z* 346 ions.

Peaks XII and XIV might correspond to the hydroxyazaperone glucuronide and azaperol glucuronide, respectively. Peaks XIII and XV showed identical retention times as peaks XII and XIV, respectively, but with identical characteristic fragments as the CID mass spectra of peaks VI and VII indicating that these peaks correspond to the hydrolysed products (hydrolysed in the LC–MS interface) of hydroxy-azaperone glucuronide and azaperol glucuronide, respectively. No corresponding glucuronide metabolites of peaks VII and IX were detected in the XAD-2 resin extracted urine samples which may be due to the low

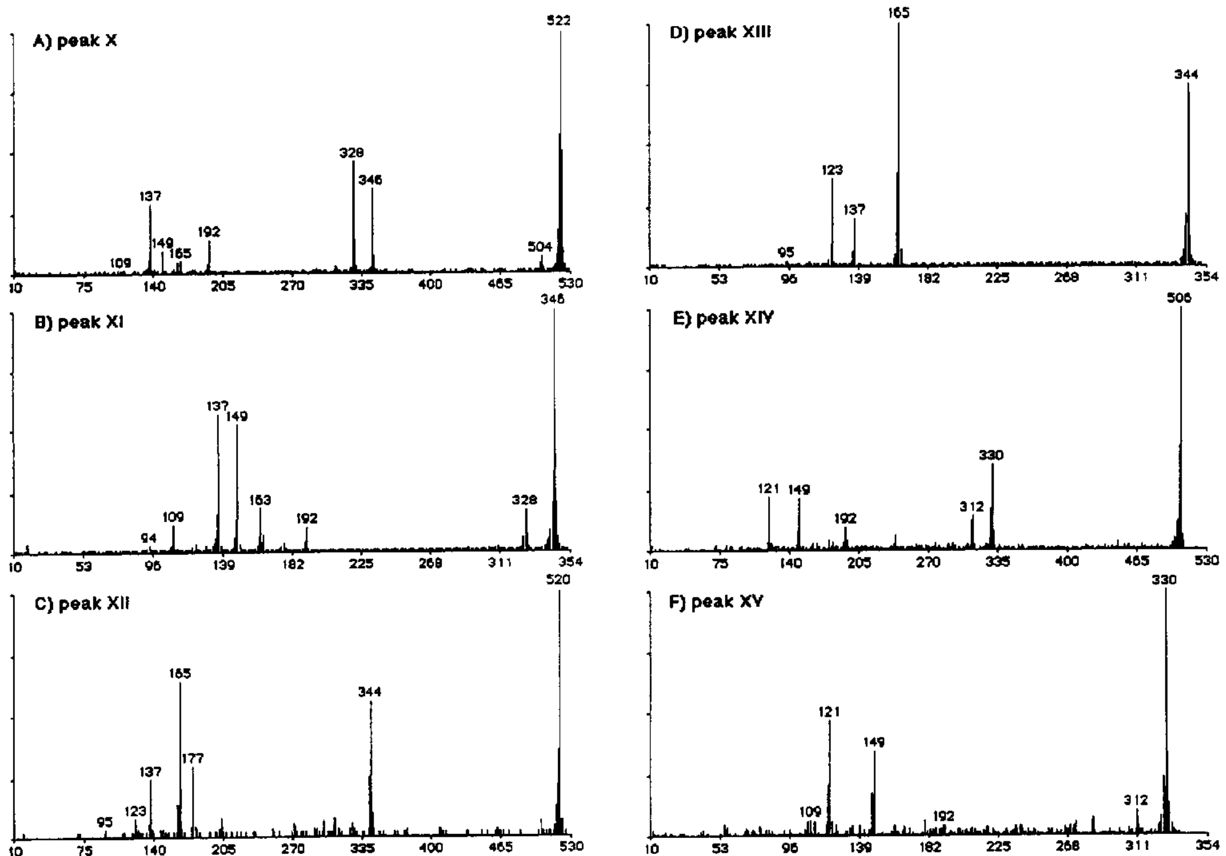

Fig. 7. Daughter-ion mass spectra of (A) peak X in Fig. 6A, (B) peak XI in Fig. 6B, (C) peak XIII in Fig. 6D, (E) peak XIV in Fig. 6E, and (F) peak XV in Fig. 6F.

concentration of these metabolites present in the urine.

4. Conclusion

Fig. 8 presents our proposed metabolic pathway of azaperone in the horse. Our results indicated that the majority of the metabolites were excreted in the urine in the conjugated form. No hydroxyazaperone, hydroxyazaperol or azaperol were detected in the unhydrolysed urine. N-Despyridinylazaperone and N-despyridinylazaperol may be partially conjugated, since both metabolites were detected in the unhydrolysed and hydrolysed urine extracts. The detection of azaperone, N-despyridinylazaperone and azaperol in the urine was consistent with the findings in man [4] and cattle [2]. N-despyridinylazaperol had not been previously reported. Moreover, the glucuronide conjugated azaperone metabolites have been successfully detected by LC–MS–MS in this study.

5. Acknowledgements

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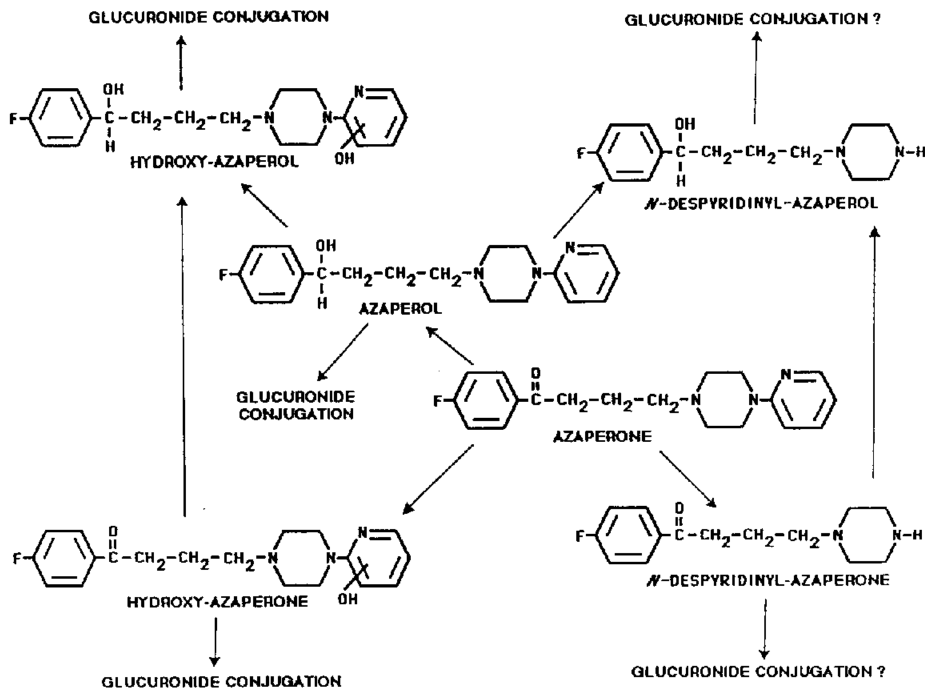

Fig. 8. Proposed metabolic pathway of azaperone in horses.

6. References

[1] J. Heykants, L. Pardoel and P.A.J. Janssen, *Arzeim. Forsch. (Drug Res.)*, 21 (1971) 982.

[2] A.G. Raus, M. Ollling, J. Freudenthal and M. ten Ham, *Toxicol. Appl. Pharmacol.*, 35 (1976) 333.

[3] N. Haagsma, E.R. Bathelt and J.W. Engelsma, *J. Chromatogr.*, 436 (1988) 73.

[4] M. van Boven and Daenens, *J. Anal. Toxicol.*, 16 (1992) 33.

[5] Y.C. Chui, B. Esaw, R. Zarate and L. Young, *Proceedings of 8th International Conference of Racing Analysts and Veterinarians, Harare, Zimbabwe, February, 1990, p. 71.*

[6] W.E. Woods, S. Stanley, J. Yang, D. Cook, D. Gerkin, R. Detra, R. Sams, D. Watt, H. Tai and T. Tobin, *Proceeding of 9th International Conference of Racing Analysts and Veterinarians, New Orleans, Louisiana, USA, March, 1992*, p. 187.

[7] A.L. Burlingame, T.A. Baillie and D.H. Russell, *Anal. Chem.*, 64 (1992) 467R.

[8] J.M. Frincke and G.L. Henderson, *Drug Metab. Dispos.*, 8 (1980) 425.

[9] C.F. Poole and S.K. Poole, *Chromatography Today*, Elsevier, Amsterdam, 1991, Ch. 4, p. 312.

[10] Y.C. Chui, G.K. Poon, B. Esaw and B. Laviolette, *Proceedings of The 40th ASMS Conference of Mass Spectrometry and Allied Topics, Washington, DC, USA, June, 1992*, p. 1889.