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Metabolism and excretion of promazine by the horse

J. J. WEIR AND J. SANFORD

Department of Veterinary Pharmacology, Glasgow University Veterinary Hospital, Bearsden Road, Bearsden, Glasgow, Scotland

The urinary excretion of promazine and its metabolites has been examined in five horses, by ultraviolet spectrometry and thin-layer chromatography. In each horse, excretion continued for at least 96 h although the amount excreted was low, being about 11% of the dose. No correlation of excretion and pH or urinary volume was observed. Glucuronic acid conjugates predominated, their ratio to unconjugated metabolites being about 5:1. Promazine was found only in small amounts and traces of unconjugated sulphide metabolites were found in only one horse. At least nine metabolites of promazine were detected, the major one being the glucuronide of 3-hydroxypromazine.

The metabolism and excretion of the phenothiazine tranquillizers* is complex and shows marked variations from species to species (Goldenberg & Fishman, 1961; Goldenberg, Fishman & others, 1964).

Five common metabolic pathways of these drugs occur; namely, oxidation at the sulphur atom (Salzman & Brodie, 1956; Fishman & Goldenberg, 1960), oxidation at the terminal side-chain nitrogen atom (Fishman, Heaton & Goldenberg, 1962), demethylation at the terminal dimethyl amino-group (Ross, Young & Maass, 1958; Walkenstein & Seifter, 1959), and hydroxylation of the phenothiazine nucleus followed by conjugation with glucuronic acid (Lin, Reynolds & others, 1959; Huang, Sands & Kurland, 1961; Fishman & Goldenberg, 1963). A large number of metabolites are thus possible. With promazine some 30 metabolites have been detected in human urine (Goldenberg & others, 1964). Studies have also shown extensive localization of such compounds in the body (Salzman & Brodie, 1956; Fyodorov, 1958; Walkenstein & Seifter, 1959), low percentage urinary excretion (Fyodorov & Shnol, 1956; Nadeau & Sobolewski, 1959; Goldenberg & others, 1964), and prolonged excretion, sometimes lasting several months (Forrest, Forrest & Mason, 1961; Huang & others, 1961). (See review by Emmerson & Miya, 1963.)

These drugs have proved most useful in veterinary practice, both for the restraint of temperamental animals, and for pre-anaesthetic medication, particularly in the fields of equine medicine and surgery (Raker & English, 1959; Raker & Sayers, 1959).

Little information is available about their metabolism and excretion in the horse. Carey & Sanford (1963) detected the presence of at least seven metabolites of promazine in horse urine, and found that polar metabolites predominated and were excreted for a much longer period. Schubert (1967) suggested that urinary excretion in the horse continues for several days after a single intravenous injection of chlorpromazine, and that a small percentage of the dose is excreted. He detected

* Those phenothiazine derivatives possessing an amino-alkyl side-chain attached to the ring nitrogen atom.

four metabolites, the predominating one being chlorpromazine sulphoxide. The unchanged drug was found in only very small amounts.

It was decided to make a detailed analysis, both of metabolism and excretion, on one of this group of drugs. Promazine was chosen as a representative of the group because it may be given safely in a relatively large dose.

EXPERIMENTAL

Urine samples

The horses used were light hunters (3 geldings and 2 mares). Each horse was brought in from grass three days before the experiment and was fed the same diet of hay and concentrates before and during the experiment. A single intramuscular dose of promazine hydrochloride (5 mg/kg as a 5% solution), was administered at about 11 a.m. This dose invariably caused deep sedation lasting 12–18 h, but the horses remained standing. Urine was collected over successive periods of 8 h for 96 h after injection and the pH of each sample was measured. For quantitative analysis, 100 ml of each sample was used, and 300 ml for obtaining a qualitative excretion pattern. The residual urine was pooled for further analysis.

Urine collections were made from mares by passing a catheter at 2 hourly intervals. In geldings, collections were made by strapping a polythene container to the animal. All samples were stored in sterile bottles at −20° because in preliminary experiments storage at 4° allowed a build-up of impurities which tended to mask the phenothiazine peaks on the ultraviolet spectra of extracts.

Extraction procedures

The procedure for quantitative analysis was similar to that of Bolt (1965). However, owing to the large amounts of contaminants found in horse urine, and its tendency to form emulsions with organic solvents, several extra purification steps were included. Emulsions were usually broken at any stage by centrifuging at approximately 2,500 rev/min. If centrifugation failed, the addition of a little anhydrous ammonium sulphate succeeded.

Unconjugated metabolites (*Procedure* 1). Each 100 ml aliquot of urine was adjusted to pH 9·5–10 using N ammonia solution. The white precipitate which formed was removed by centrifuging, and decanting the supernatant urine. This precipitate contained no phenothiazines as shown by reaction with 50% sulphuric acid. The urine was then extracted with three 10 ml portions of 1,2-dichloroethane (previously washed with half its volume of 0·1N ammonia solution, 0·1N hydrochloric acid, and distilled water successively), and the organic extracts were pooled. The residual urine was stored for analysis of conjugated metabolites.

After removal of any emulsion, the organic extract was washed with 0·1N ammonia solution (2 × 50 ml), followed by distilled water (2 × 50 ml), and was finally extracted with 10 ml 0·1N hydrochloric acid. This acid extract was assayed for phenothiazine content by ultraviolet spectrometry.

Glucuronide conjugated metabolites (*Procedure* 2). As analysis of residual urine from Procedure 1 for glucuronide conjugates involved a prolonged incubation step to hydrolyse the glucuronides to their aglycones, this urine was heated for 10 min on a boiling water bath at three successive 24 h intervals before incubation, and was used thereafter under sterile conditions to avoid the possibility of formation of contaminants by microbial action at 37°.

The urine was adjusted to pH 4·5 using 5N hydrochloric acid and 5 ml β-glucuronidase (Ketodase 5,000 units/ml) were added. The sample was then incubated for 24 h at 37°. After incubation the pH was adjusted to 9·5–10 using ammonia solution, and the extraction carried out as for Procedure 1.

The concentration of sulphide, and of sulphoxide metabolites in each group was calculated using the background cancellation method of Flanagan, Lin & others (1959), as modified by Bolt (1965) for promazine assay.

The rates of excretion for four groups of metabolites—unconjugated sulphides, unconjugated sulphoxides, glucuronide conjugated sulphides, and glucuronide conjugated sulphoxides—were calculated for successive pooled 8 h samples. The methylated or hydroxylated derivatives and promazine *N*-oxide, which are common metabolites of promazine in other species, produce maxima on the ultraviolet spectrum at the same wavelength as the parent drug, and are included in the group of unconjugated sulphide derivatives.

The same procedures were used for qualitative analysis, using larger volumes of solvent. However, the extracts were not taken into acid solution, but dried over anhydrous ammonium sulphate, then evaporated under reduced pressure to approximately 1/300 of their original volume. The resultant solutions were used for thin-layer chromatography. In addition, the heating step was omitted from Procedure 2, as the interfering compounds gave no abnormal spots when chromatographed.

Chromatography

Activated silica thin-layer plates, after spotting, were developed to a height of 15 cm. For unconjugated metabolites the solvent system used was methanol–acetic acid–water (5:3:2), and for glucuronide conjugated metabolites the system was acetone–isopropanol–1% ammonia (9:7:4) (Goldenberg & others, 1964). Spots were visualized by spraying with a 50% sulphuric acid solution, and were identified by comparison with known standards.

RESULTS

Ninety-six h excretion patterns were plotted for six experiments. On examining the combined rates of excretion of the four groups of metabolites for each horse, it was noted that the patterns fell into two groups.

In the first group (A) a maximum rate of excretion was reached within 16 h after injection, but in the second group (B) this was not achieved until 24–32 h after injection. Each pattern of excretion was shown in 3 out of the 6 experiments and the average rates are shown in Fig. 1.

For each horse, excretion patterns of individual groups of metabolites proved to be varied and irregular. Excretion was also prolonged, continuing, in at least one of the four metabolic groups, up to 96 h. Typical examples from each group of horses are shown in Fig. 2A,B. Excretion of unconjugated sulphoxide metabolites did not exceed a rate of 2·5 mg/h for any horse, and the average rate of excretion remained at around 0·5 mg/h for the duration of the experiment. Unconjugated sulphide metabolites appeared in small quantity only in one case (Horse 1), from approximately 32 h after injection until the end of the experiment.

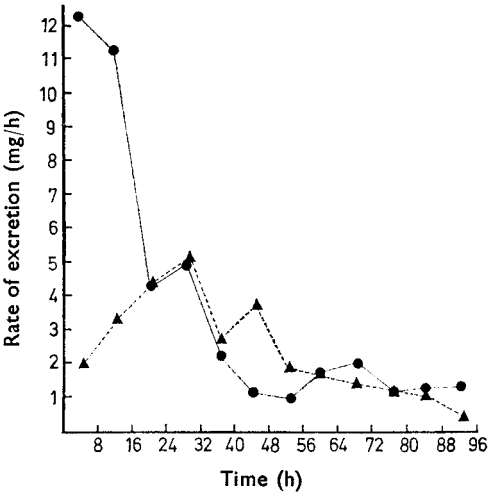

FIG. 1. Urinary excretion of total promazine metabolites by the horse. Rates of excretion were obtained from the total concentration of metabolites in pooled 8 h samples. Each point represents the average of three experiments. In excretion pattern (A), ——●——, the maximum rate was reached within 16 h, and in pattern (B), - - - ▲ - - -, within 24–32 h.

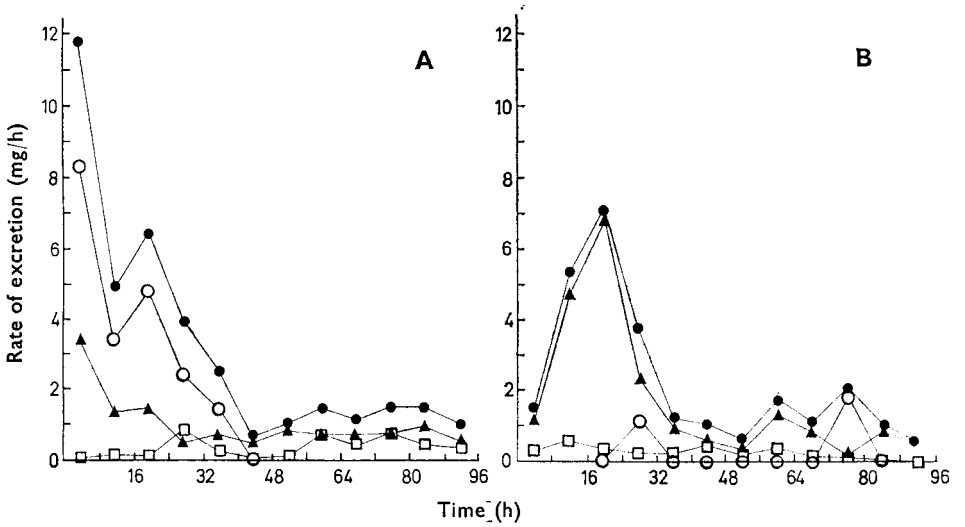

FIG. 2. Excretion patterns of individual groups of metabolities from (A) one horse representative of those shown in Fig. 1A and (B) one horse representative of those shown in Fig. 1B. □ Unconjugated sulphoxide metabolites. ▲ Glucuronide conjugated sulphoxide metabolites. ○ Glucuronide conjugated sulphide metabolites. ● Total metabolites.

Excretion of glucuronide-conjugated sulphoxide metabolites continued throughout the 96 h period in five horses. The remaining horse (animal No. 2) showed no trace of this type of metabolite. With one exception (No. 5b), the rate of excretion was less than 3·5 mg/h, and the average rate of excretion was less than 2 mg/h for the duration of the experiments.

Glucuronide-conjugated sulphide metabolites were in great evidence in four of the horses. The fifth horse, which was dosed on two occasions, showed no trace of this type of metabolite the first time, and, on the second occasion, it was only detected in

trace amounts in two samples. In the other four horses, excretion was very varied throughout each series of samples, but had ceased by 80 h after injection.

The pH of the urine samples varied between 7·0 and 8·5. No correlation was seen between differences in pH and the rate of excretion of metabolites, neither was this related to urinary volume.

Table 1. *The urinary excretion as % of dose after intramuscular administration of promazine hydrochloride to the horse*

Distribution	Animal No. 1	2	3	4	5a	5b	Average % of dose
% Unconjugated	3·6	1·8	0·2	1·3	3·2	1·0	1·8
% Glucuronides	18·1	7·8	7·5	9·6	5·9	6·1	9·2
Total	21·7	9·6	7·6	10·9	9·1	7·1	11·0
Glucuronides : unconjugated ..	~5 : 1	~4 : 1	~63 : 1	~7 : 1	~2 : 1	~6 : 1	~5 : 1

Table 1 shows the percentage of the dose excreted in the urine during each experiment, the average ratio of glucuronides to unconjugated metabolites being 5 : 1. The percentage of the dose excreted over the 96 h was low, the average value being approximately 11%.

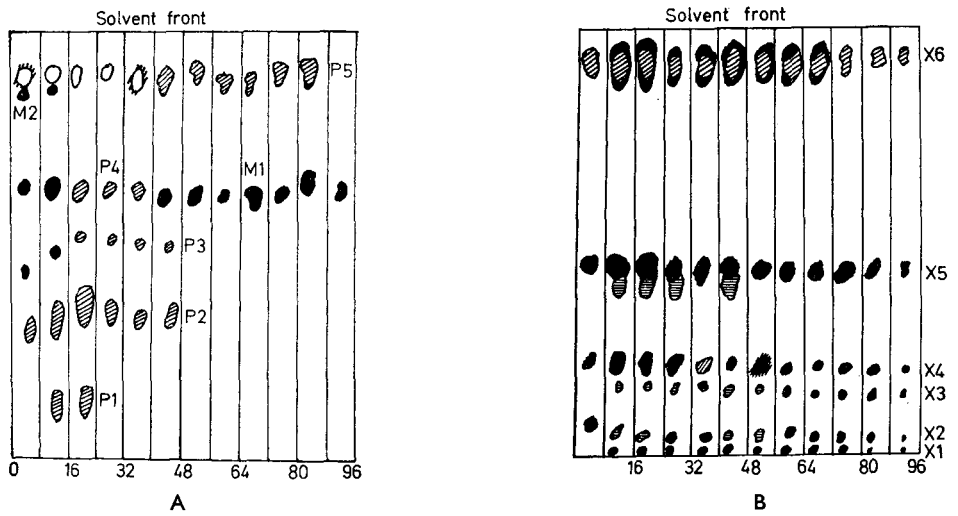

FIG. 3. Typical chromatograms of urinary extracts of (A) unconjugated and (B) glucuronide conjugated metabolites of promazine over 96 h. Each division represents the chromatogram of an 8 h pooled urine sample. The unconjugated metabolites were developed in the system methanol–acetic acid–water (5 : 3 : 2) and the conjugated metabolites were developed in the system acetone–isopropanol–1% ammonia (9 : 7 : 4). Chromatograms were visualized with a 50% sulphuric acid solution. Spot P2 was identified as promazine sulphoxide; P4 was identified as the parent drug and both M1 and X5 were identified as 3-hydroxypromazine. P1, P3, P5, M2, X1–4 and X6 were not identified. Colours: Black = purple, diagonal hatch = pink, cross hatch = bluish purple, white = yellow.

Fig. 3 shows typical chromatograms following the excretion of individual metabolites over 96 h. At least four unconjugated metabolites were detected in addition to the unchanged drug, which was also present in low concentration (P4). By comparison with standard metabolites two of these were tentatively identified as promazine sulphoxide (P2) and 3-hydroxypromazine (M1).

At least five glucuronide-conjugated metabolites were detected, the major one being identified as the glucuronide of 3-hydroxypromazine (X5), by comparison with the standard metabolite. The spot was also eluted from the plate using methanol, and identified conclusively by examination of its ultraviolet spectrum after reaction with 50% sulphuric acid as described by Beckett & Curry (1963).

DISCUSSION

The four groups of metabolites investigated in this series of experiments constitute the major metabolites known to be excreted in other species. The other common pathway of sulphate conjugation was investigated but only trace amounts were found.

Collection of urine over a 96 h period was chosen since work on other species had suggested prolonged excretion of these compounds (Forrest & others, 1961; Huang & others, 1961). Indeed, the present results indicate the need for more lengthy excretion studies on phenothiazine derivatives in the horse.

Masking of the ultraviolet spectra of urinary extracts of phenothiazine derivatives has previously been reported by Schubert (1967). The formation of further interfering compounds, by bacterial action, occurs at 4°, and is greatly increased by prolonged incubation at 37°. In view of this, thorough washing and a heating procedure to reduce growth of micro-organisms should be adhered to in the analysis of metabolites in horse urine.

So far we have eluted, and identified by ultraviolet spectroscopy, the major metabolite of promazine found in horse urine. It was not possible to elute the minor metabolites. The difficulty is thought to be due to the small concentrations of such metabolites found on thin-layer plates.

The varied and irregular excretion pattern noted in this series of experiments is in accordance with findings in other species. Eiduson, Geller & Wallace (1963) reported variable urinary excretion patterns of thioridazine after oral administration to man, and suggested a relation between the amount of drug excreted and urinary volume. No such relation was seen for promazine in the present experiments. Neither was there any obvious explanation for the two sets of metabolic patterns experienced in the present series of experiments. Carr (1962), reviewing the metabolism of this type of drug, mentioned "their unusual metabolic patterns and their individual excretion differences that vary with the different drugs and different patients".

The low percentage of dose recovered also agrees with research carried out in other species (Fyodorov & Shnol, 1956; Nadeau & Sobolewski, 1959), although reports varied considerably (Emmerson & Miya, 1963). Beckett, Beaven & Robinson (1963) recovered 7% of an oral dose of chlorpromazine from human urine, and attributed the low recovery to poor reabsorption of conjugated metabolites excreted in the bile, suggesting the possibility of a high concentration of metabolites in the faeces. Indeed, high levels of phenothiazine metabolites have been reported in the faeces of various species (Fyodorov & Shnol, 1956; Emmerson & Miya, 1963; Eiduson & Geller, 1963).

On the other hand, extensive localization of these drugs in various organs has been reported (Salzman & Brodie, 1956; Fyodorov, 1958; Walkenstein & Seifter, 1959). Slow release from sites of localization may explain the delayed excretion, and could also account for the low recoveries of metabolites.

The predominance of glucuronide-conjugated metabolites agrees with findings in other species. Beckett & others (1963) reported the ratio of glucuronides to

unconjugated to sulphate-conjugated metabolites recovered after an oral dose of chlorpromazine to man as approximately 12 : 5 : 4. Only trace amounts of sulphate-conjugated metabolites were found in our investigation, which may explain the larger ratio of glucuronides to unconjugated metabolites obtained.

In conclusion, the present series of experiments has borne out the varied and irregular excretion patterns experienced in other species, and the large number of metabolites associated with this group of drugs. Since at least one of the four groups of metabolites can be detected over a period of 96 h, this method of analysis should prove of value in cases of suspected doping by phenothiazine derivatives.

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