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THE URINARY EXCRETION OF PHENOBARBITONE AND PENTOBARBITONE IN THE HORSE

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Abstract—The urinary excretion of phenobarbitone and pentobarbitone in the horse has been investigated. About 11% phenobarbitone was recovered in the 72 hr following oral administration, and the only metabolite detected was the *p*-hydroxyphenobarbitone glucuronide conjugate. Pentobarbitone was not detected in horse urine, but was excreted as the 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acids and 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid. The alcohol metabolites, which were not conjugated with glucuronic acid, were completely excreted within 21 hr following oral dosage and constituted 33 per cent of the administered pentobarbitone.

ALTHOUGH urine provides a ready sample source for drug detection in the horse, the knowledge available regarding the form of the urinary excretion of specific drugs is limited and the assumption must be made, at the present time, that the urinary excretion of drugs in the horse follows a pattern similar to that in other species. With this view in mind it was considered of importance to extend the observations on the fate of phenobarbitone and pentobarbitone in horse blood and saliva[1] to establish the form of the urinary excretion of these drugs for comparison with other species.

Much qualitative information is available in the literature on the urinary excretion of barbiturates in various species. Pentobarbitone is excreted unchanged only to the extent of less than 0·5 per cent of the administered dose in mice[2] and dogs.[3] The principal metabolites of pentobarbitone in dog,[3–5] man,[6–8] rabbit[9] and rat[8,9] are the diastereoisomeric alcohols 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid. Other metabolites reported[3] include the glucuronide of the dextrorotatory alcohol, 5-ethyl-5-(1-methyl-3-carboxypropyl)barbituric acid, urea and four unidentified metabolites.

Phenobarbitone is excreted unchanged and as *p*-hydroxyphenobarbitone in the urine of dog[10,11] and man.[11–15] The metabolite is conjugated with glucuronic acid almost wholly in dog urine[10,11] but only partially in the urine of man.[11–13] Using a ^{14}C label Benakis and Glasson[16] have detected four other metabolites which have not been identified.

As quantitative data on the urinary excretion rates of both barbiturates in any species are limited[3,15,17–23] it was desired to establish over what period the barbiturates or their metabolites were excreted in detectable quantities in horse urine, and to what extent of the original dose these constituted.

METHODS

The animals used have been described previously.[1]

Sodium pentobarbitone was administered by stomach-tube at a dose rate of 5 mg/kg in excretion rate determinations. In metabolite isolation experiments a stomach-tube with a bulbous end, which retained the stomach-tube in position for as long as required when the free end was tied to a head stall, was used and an initial dose of 4·4 mg/kg followed by hourly supplementary doses of 2·2 mg/kg administered. Sodium phenobarbitone was given in all cases by normal stomach-tube. A rate of 6 mg/kg was used in clearance rate experiments but for attempts at metabolite isolation higher doses were given.

Urine collection was by the method of Warwick[24] which had been modified to record the time at which each urine sample was voided. The modification consisted of a paddle inserted through the tube at the base of the funnel. When urine flowed down the funnel into the tube the paddle was depressed and closed an electrical circuit containing a relay which operated a pointer touching a smoked drum rotating once every 24 hr. In the phenobarbitone experiments urine samples were collected over a period of 72 hr during which time the ponies were exercised, usually just after the passage of a urine sample. With pentobarbitone a period of 24 hr was found to be sufficient.

Before analysis all urine samples, which contained no preservative, were subjected to the purification procedure advocated by Green *et al.*[25] which removed much of the interfering natural colouring material. Phenobarbitone was extracted from the purified urine with benzene. Metabolic material, which was insoluble in benzene,[11] was then extracted with ether. Pentobarbitone and its metabolites were extracted with ether after first saturating the urine with ammonium sulphate which acted as a 'salting-out' agent. As all extracts were eventually obtained in ethereal solution this was dried over a mixture of sodium sulphate and solid sodium bicarbonate (2:1 by bulk approximately). The latter reagent was found to be superior to the conventional buffer wash for removal of acidic natural urine components. It avoided loss of metabolic material due to its high water solubility and overcame the risk of pH changes in the commonly used bicarbonate buffer.[26] One of the pentobarbitone metabolites was removed from ethereal solution by the reagent and when this was required the mixed drying agent was dissolved in dilute acid and the solution extracted with ether.

Paper chromatography of extracts was carried out on Whatman 3 MM paper using a butanol/ammonia solvent,[27] an amyl alcohol/ammonia solvent[28] or the *iso*-propyl alcohol/ammonia solvent[29] originally described for phenolic acids but which was found to be suitable for metabolic material. Barbiturates were detected on chromatograms by u.v. light,[27] the cobalt chloride/ammonia reagent[27] or by eluting the suspected areas with 0·45 N sodium hydroxide and subsequently recording the u.v. spectra of the eluted material at different pH values.

Gas-liquid chromatography of pentobarbitone and its metabolites was carried out as described by Jain *et al.*[30] although temperatures and gas flow rates were altered to suitable values. Use was made of electron capture and flame ionisation detectors.

Infrared spectra of metabolites and parent barbiturates were recorded from potassium bromide discs on a Unicam SP.200 spectrophotometer.

β-glucuronidase hydrolyses were carried out essentially as described by Butler.[11] With the phenobarbitone metabolite, extraction of the urine with ether before

hydrolysis gave a much cleaner final extract. It was assumed that there was sufficient natural phosphate in the urine* to inactivate any sulphatase in the β-glucuronidase.

Phenobarbitone was determined using the complexometric reaction described by Lubran.[31] 10 ml of purified urine were saturated with ammonium sulphate and extracted with 50 ml of benzene which in turn was extracted with 10 ml of 0·45 N sodium hydroxide. After acidification of the alkaline extract the resulting solution was extracted with two 25 ml portions of ether which were then combined and dried over sodium sulphate/sodium bicarbonate. After evaporation of the ether the residue was dissolved in 3 ml of chloroform and the complexometric reaction described by Lubran[31] carried out on the solution.

The pentobarbitone alcohol metabolites were determined in a similar fashion. 10 ml of purified urine were extracted with two 25 ml portions of ether after saturation with ammonium sulphate. The combined ether extracts were dried over sodium sulphate/sodium bicarbonate before evaporation to dryness. The residue thus obtained was dissolved in 3 ml of ethyl acetate, because of the relative insolubility of the metabolites in chloroform,[5] and filtered through a cotton wool plug. To 1 ml of the filtrate was added 2 ml of chloroform and the complexometric reaction completed as for phenobarbitone.

RESULTS

Urinary excretion of phenobarbitone. Paper chromatographic examination of extracts of urine from ponies dosed with phenobarbitone did not reveal the presence of phenobarbitone although the complexometric determination indicated a concentration of about 10 μg/ml in the samples used. It was concluded that the presence of natural coloured urinary constituents masked the presence of phenobarbitone to any of the locating agents described. A large volume of urine was extracted and the extract subjected to paper chromatography in the butanol/ammonia solvent system. That region on the paper, at which phenobarbitone was expected to occur, was eluted with dilute sodium hydroxide and the acidified alkaline extract extracted with ether. The ether extract obtained was subjected to further paper chromatography in the *iso*propyl alcohol/ammonia solvent and the resultant chromatogram treated as was the first. The extract so obtained was chromatographed a final time with the amyl alcohol/ ammonia solvent. The appropriate region of this chromatogram, after elution, gave a strong positive reaction to the complexometric reaction of Lubran.[31] The differential u.v. spectrum[32] of the extract between solutions at pH 13 and 10·4 matched almost exactly the corresponding spectrum of pure phenobarbitone. It was concluded that the urine contained a barbiturate material which possessed the same R_f values as did phenobarbitone in three paper chromatography systems. Using the described quantitative determination the partition coefficient of the barbiturate material between benzene and a pH 7 phosphate buffer was determined and a value of 0·59 obtained. This was similar to the value of 0·53 obtained for phenobarbitone when small amounts of interfering chromogens in the urine were considered. When taken in conjunction with the knowledge that phenobarbitone is excreted in the urine of man,[15,18,19] rat[17,33] and dog[20] the above evidence was considered sufficient for it to be assumed that phenobarbitone was excreted to some extent unchanged in the urine of horses.

* F. Alexander, unpublished results.

Under no circumstances was there any indication of the presence of phenobarbitone metabolites on paper chromatograms of urine extracts. However after acid hydrolysis of the urine[10,11] followed by ether extraction, and purification of the extract, a metabolite was detected with an R_f value of 0·29 in the butanol/ammonia paper chromatography system. This metabolite was isolated from the 24 hr urine of a pony after dosage with 1·3 g of sodium phenobarbitone. The urine, after acid hydrolysis, was extracted with ether and the ether extract, after several purification stages, subjected to large scale paper chromatography. Alkaline elution of the appropriate regions of the chromatograms was followed by ether extraction of the acidified eluate. Several recrystallisations of the ether extractable material, by dissolving it in a few ml of acetone, diluting with a large volume of benzene and allowing to stand for several days, gave needle shaped crystals melting at 223–225°. The u.v. spectra of this material at pH 13, 10·4 and 1 were identical to the unusual characteristic spectra of *p*-hydroxyphenobarbitone[13] and the infrared spectrum was also in accordance with the isolated material being *p*-hydroxyphenobarbitone. The distribution coefficient of the isolated material between ether and water added further evidence in favour of this compound being *p*-hydroxyphenobarbitone and this assumption was therefore made.

Analysis of urine after β-glucuronidase hydrolysis revealed that a large fraction of the metabolite was conjugated with glucuronic acid. Omission of the β-glucuronidase resulted in no detection of *p*-hydroxyphenobarbitone.

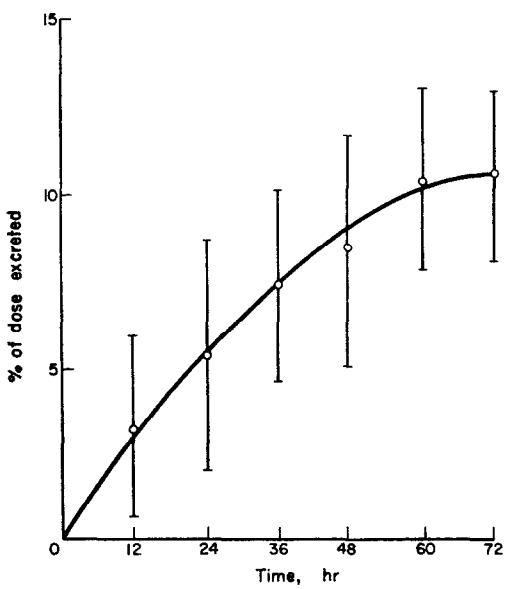

FIG. 1. Mean rate (±S.D.) of urinary excretion of phenobarbitone obtained from five experiments on five ponies following an oral dose of 6 mg/kg of sodium phenobarbitone.

The rate of the urinary excretion of phenobarbitone in five ponies was followed over a period of 72 hr and from the five excretion curves obtained a composite curve was constructed and is shown in Fig. 1. As can be seen about 11 per cent of the administered dose was excreted within 72 hr after oral administration.

The analytical method, when carried out on urine containing known amounts of phenobarbitone, gave a recovery of 70 ± 4 per cent in seven determinations with a concentration of from 4·49 to 4·83 μg/ml. The blank value for ten normal random urine samples was 0·9 ± 0·3 μg/ml and this mean value was subtracted from all quantitative determinations. Although the reagents used in the determination, with or without the presence of phenobarbitone, gave a colour similar in stability to that reported by Lubran,[31] it was found that with normal or phenobarbitone containing urine extracts appreciable rapid fading of the colour occurred and it was possible for the O.D. to fall below that of the reagent blank in the case of the former. This error was minimised by carrying out each determination on a standardised time schedule.

Urinary excretion of pentobarbitone. Pentobarbitone was not detected in horse urine extracts by the use of paper chromatography or by G.L.C. As the latter technique could detect the equivalent of less than 1 per cent of the administered dose used it was concluded that pentobarbitone was not excreted in significant quantities in horse urine.

On paper chromatograms, using the butanol/ammonia solvent, barbiturate reacting regions were detected at R_f 0·50 (metabolite A) and at R_f 0·08 (metabolite B). Isolation of the metabolites was attempted using the 48 hr urine obtained from a pony which had been given 7 g of sodium pentobarbitone over a period of 32 hr. Using the previously described extraction, followed by paper chromatography first using the butanol/ammonia solvent and then the *iso*propyl alcohol/ammonia solvent, A was obtained in reasonably pure condition. After several recrystallisations, as described for *p*-hydroxyphenobarbitone, A was obtained as white needle shaped crystals melting at 130°. This material was compared with a synthetic sample of 5-ethyl-5-(3-hydroxy-1-methylbutyl)-barbituric acid (m.p. 187–188°).* The two substances had similar infrared spectra and if the substances were considered identical the small differences could be considered due to differences in the relative amounts of the two diastereoisomers in each sample. Maynert and Dawson[5] have shown that the spectra of the two isomers do differ slightly. A and the synthetic material both had an R_f value of 0·45 in the *n*-butanol/ammonia paper chromatography system of Algeri and Walker.[34] Their partition coefficients between ether and water, and ethyl acetate and water were almost identical. Gas-liquid chromatography of each resulted in identical chromatograms having two distinct peaks separate from the solvent peak. It was therefore concluded that A was 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid.

The sodium sulphate/sodium bicarbonate residue from the original extraction was taken up in dilute hydrochloric acid and the solution extracted with ether. Continuous ether extraction of the butanol/ammonia chromatogram of the ether extract removed much of the non-barbiturate material. Alkaline elution of the appropriate portions of the chromatograms followed by ether extraction of the acidified alkaline elutate gave a small amount of material which was rechromatographed in the *iso*propyl alcohol/ammonia solvent. The developed chromatogram was extracted as before and a small amount of crude B obtained. After recrystallisation as described for *p*-hydroxyphenobarbitone and including a recrystallisation from concentrated hydrochloric acid pure B was obtained melting at 193°. This was acidic in nature and had an R_f value of 0·08 in the *n*-butanol/ammonia paper chromatography system of Algeri and

* Kindly provided by Y. J. Dickert of the Dow Chemical Co.

Walker.[34] The found partition coefficients of B between ether and a pH 4·56 buffer[3] and between the two phases formed on mixing equal volumes of water, ether, methanol and chloroform[3] added support to the postulation that B was 5-ethyl-5-(1-methyl-3-carboxypropyl) barbituric acid and this was therefore considered proved.

The amount and rate of urinary excretion of the alcohol metabolites were determined. From seven experiments on five ponies the mean amount excreted was 33 ± 7 per cent of the administered pentobarbitone (see Table 1). Only two ponies voided a

TABLE 1. TOTAL AMOUNT OF PENTOBARBITONE ALCOHOL METABOLITES EXCRETED BY PONIES AFTER AN ORAL DOSE OF 5 MG/KG OF SODIUM PENTOBARBITONE

Pony	Original dose excreted as metabolite A (%)
II	38
III	20
V	39
VI	40
VI	30
VII	35
VII	37

sufficient number of samples, during the period of metabolite excretion, for an excretion rate to be determined. The results of two experiments on each pony were collected and are presented in Fig. 2 from which it can be seen that excretion was complete

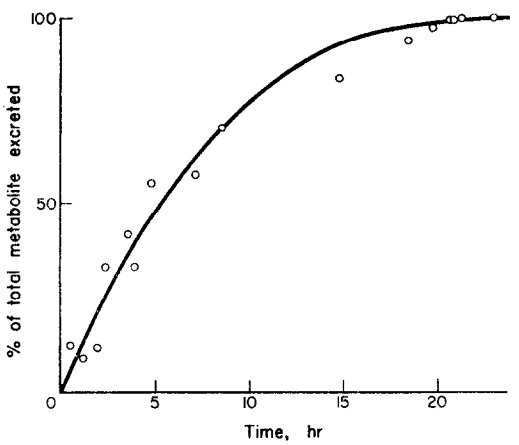

FIG. 2. Rate of urinary excretion of pentobarbitone alcohol metabolites. Results from four experiments on two ponies following oral doses of 5 kg/mg of sodium pentobarbitone.

after about 21 hr. From the analysis of metabolite containing urine before and after β-glucuronidase hydrolysis it was concluded that the alcohol metabolites were not conjugated with glucuronic acid to any appreciable extent.

It was found that the presence of ethyl acetate decreased the intensity of the colour produced in the complexometric method by about 50 per cent for the alcohols.

However the relatively high concentrations overcame any decrease in sensitivity. The recovery of known amounts of isolated metabolite from urine was 63 ± 3·5 per cent (n = 8, concentration, 115 μg/ml). From 17 random normal urine samples a blank value of 30 ± 10 μg/ml was obtained. The variation was sufficiently small, in view of the high concentrations involved, to be of no great significance and the mean blank value was subtracted from all quantitative results. No appreciable fading of the colour was noted during the period of analysis.

DISCUSSION

The metabolites isolated from horse urine show that phenobarbitone and pentobarbitone are metabolised in the horse in a similar manner to other species qualitatively but differ quantitatively.

The amount of free alcoholic metabolites of pentobarbitone excreted was approximately half of that excreted in the dog[3] and two-thirds of that excreted in man.[23] That these metabolites were not conjugated with glucuronic acid conflicts with the results of Titus and Weiss[3] who found that 13·6 per cent of the original dose was excreted as the glucuronate of the dextrorotatory alcohol in the dog. Although interfering chromogens prevented determination of the carboxylic acid metabolite it was estimated, from the intensity of the appropriate spot on paper chromatograms of urine extracts produced by the cobalt chloride/ammonia reagent, that a larger amount was present than in dog urine (4·5 per cent of the administered dose[3]). This was considered not improbable since *in vitro* experiments of Cooper and Brodie[9] have shown that rabbit and rat enzymes produce about equal amounts of mixed alcohols and acid. That only 33 per cent of the administered drug was accounted for as the mixed alcohols showed that a large amount was not accounted for. Other possible forms of urinary excretion, not investigated, were sulphate conjugation of one or both alcohol metabolites or the formation of greater relative amounts of the unidentified metabolites detected by Titus and Weiss.[3] A further possibility was the formation of metabolites not found in carnivores, on which most metabolic studies have been based, but produced by different mechanisms in herbivores.

Insufficient data exist in the literature to allow a comparison of the urinary excretion rates of phenobarbitone in the horse with those for other species. The only conclusion that may be drawn is that the total amount of phenobarbitone excreted in the horse was of similar magnitude to that in man,[15,18,19] dog[20] and rat.[17] The apparent complete conjugation of *p*-hydroxyphenobarbitone to at least glucuronic acid shows that in this respect the horse resembles the dog.[10,11] This conjugation makes the routine screening of horse urine in the detection of phenobarbitone suspect since unless hydrolysis is carried out the metabolite will not be detected. The concentration of phenobarbitone itself is too low (maximum about 10 μg/ml) relative to natural interfering chromogens to allow of its detection by paper chromatography, although the technique of Lubran[31] provides a useful means of detection. If this latter method is used to quantitatively determine phenobarbitone, or barbiturates in general, in horse urine attention will have to be paid to the fading of the colour produced. This point was not recorded in the original paper and may be peculiar to horse urine.

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