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THE BLOOD AND SALIVA CLEARANCES OF PHENOBARBITONE AND PENTOBARBITONE IN THE HORSE

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Abstract—The clearance of phenobarbitone and pentobarbitone from blood and saliva has been studied in the horse. Although the concentration of each barbiturate was lower in the saliva than in the blood the clearance rates in both fluids were parallel, the concentration in the saliva being directly dependant on the non-plasma protein bound barbiturate. Following oral administration, phenobarbitone showed a non-logarithmic decline in blood concentration with time over the period 0–6·5 hr. Pentobarbitone gave clearances of 46 per cent per hr and 49 per cent per hr from blood and saliva respectively following i.v. injection. Following oral administration of each drug the maximum concentration in the saliva was reached in from 10 to 20 min. Plasma protein binding was 44 per cent for pentobarbitone and 15 per cent for phenobarbitone; only the latter was bound to saliva protein, to the extent of 7 per cent.

ALTHOUGH saliva samples are frequently used in routine screening for drugs in racehorses comparatively little attention has been paid to the fate of specific drugs in this fluid. Moreover, as far as can be ascertained no work has been published on the fate of barbiturates in the horse so that it was considered of importance to study two common barbiturates, phenobarbitone and pentobarbitone.

Many investigators have described the fate of these barbiturates in various other species and extensive information is available on the blood clearance rates of both which has been reviewed by Richards and Taylor,[1] although little has been published on the salivary secretion of barbiturates. Rasmussen[2] studied the secretion of barbiturates in the saliva of cows and goats at an almost constant blood concentration. However, as ruminant saliva has an alkaline pH (pH 8·0–8·4[2]) it was considered that the more nearly neutral saliva of horses (pH 7·3–7·7[6]) might affect the salivary secretion of these drugs. As reports have been made of salivary flow inhibition by barbiturates[3–5] the effect on saliva flow by the two barbiturates was observed.

METHODS

Animals. Six ponies were used ranging in weight from 140 to 308 kg. Two were stallions, three were geldings and one was a mare. Two of the geldings, which had permanent parotid fistulae and have been described by Alexander[6] were used in saliva clearance experiments.

Drug administration. Sodium phenobarbitone was administered by stomach-tube at dosage rates of 8 mg/kg for blood clearance experiments and 3·7, 4·4, 6·2 and 6·6

mg/kg for saliva clearance experiments. These doses were insufficient to cause more than a temporary inco-ordination for a short time after dosage.

Sodium pentobarbitone was given by i.v. injection of an aqueous solution into a jugular vein. In blood clearance experiments a dose of 10 mg/kg was used which was sufficient to cause complete anaesthesia lasting from 1 or 2 min to over 15 min. Certain of the ponies showed excitement when regaining consciousness and were given light chloroform or halothane anaesthesia which was terminated when signs of excitement passed. In the saliva clearance experiments the dosage rate was reduced to 4 mg/kg which was the maximum amount that could be given without causing the ponies to refuse to eat and so produce saliva.[6] Some saliva clearance results were also obtained following oral administration.

Sample collection. 15 ml blood samples were collected, from a jugular vein, in evacuated tubes containing lithium oxalate as anticoagulant. Saliva samples were collected from the two ponies with parotid fistulae as described by Alexander.[6] 100 and 50 ml samples were collected for pentobarbitone and phenobarbitone analysis respectively and after acidification with dilute hydrochloric acid, set aside to await analysis. Samples of both blood and saliva were collected prior to drug administration to allow the value of apparent barbiturate in each to be established. These values were subtracted from the results for samples collected after drug administration. Blood samples were collected at 1, 2, 4 and 6·5 hr, and 15, 45, 75, 120, 180 and 240 min for phenobarbitone and pentobarbitone respectively. Since saliva flow was irregular sample collection was usually when convenient.

Analysis. Blood samples were analysed by the method of Goldbaum[7] as modified by Brackett and Finkle* to a differential spectrophotometric technique. 6 ml of blood were extracted with 50 ml of chloroform and 40 ml of the filtered chloroform extracted with 5 ml of 0·45 N sodium hydroxide. The O.D. of the alkaline solution was recorded at 260 mμ and this measurement repeated after a pH adjustment to 10·4; from the difference in the two readings, after correcting the latter for dilution by the buffer, the amount of barbiturate present was calculated from the appropriate calibration curves. Adjustment of the pH of the alkaline solution from 13 to 10·4 was achieved by adding to 3 ml of the alkali 0·5 ml of an ethylamine buffer prepared from 32·8 g ethylamine hydrochloride, 10·6 g ammonium chloride and distilled water to make 100 ml.

Saliva sample analysis differed from that of blood only in the method of extraction. The saliva was mechanically rolled, at a rate of approximately 1 rev/sec for 15 min, with an equal volume of ether in a stoppered bottle to prevent emulsion formation. After separation from the ether the saliva was extracted with a further equal volume of ether. The combined ether extracts were centrifuged to remove the small amount of emulsified saliva and extracted with 10 ml of 0·45 N sodium hydroxide. Measurement of the O.D. of the extract at pH 13 and 10·4, as described for blood analysis, allowed the saliva-barbiturate concentration to be established.

Protein binding. Protein binding determinations were carried out by the ultrafiltration method of Goldstein and Aronow[8] using 15 ml of plasma or saliva to which had been added 0·5 ml of a standard aqueous solution of barbiturate to give a final concentration of about 9 μg/ml. 5 ml portions of the ultrafiltrate were acidified with 0·5 ml of 2 N hydrochloric acid and then analysed as described for blood samples.

* J. W. Brackett and B. S. Finkle, unpublished work.

The difference in the values for ultrafiltered barbiturate obtained when a pH 7·4 phosphate buffer was used in place of plasma or saliva and when plasma or saliva was used was taken as a measure of the protein bound barbiturate.

RESULTS

Analytical methods. Using AnalaR chloroform, which had not been further purified, the normal value for the apparent barbiturate in 32 random fresh blood samples was determined and a value of $-0{\cdot}05 \pm 1{\cdot}53\,\mu$g/ml of barbiturate found (as pentobarbitone). The value of this blank did not usually vary sufficiently during an experiment to affect the determination of clearance rates. The spectra of normal blood extracts at pH 13 and 10·4 were such that the presence of barbiturate (10 μg/ml) was virtually masked and no use of the method for qualitatively identifying barbiturates, as described by Broughton[9] could be made. The recoveries of added pentobarbitone and phenobarbitone from blood were 95 per cent (S.D. 6%, n = 9) and 77 per cent (S.D. 5%, n = 7) respectively at concentrations of between 7·5 and 10 μg/ml.

Barbiturate saliva blanks were constant and depended solely on the ether used for their actual value. Typical values were—0·10 μg/ml (S.D. 0·10 μg/ml, n = 17). Qualitatively the method suffered from the same disadvantages as have been described for blood analysis, particularly as the saliva-barbiturate concentrations worked with were much lower than the blood-barbiturate concentrations. The recoveries of added amounts of the two barbiturates were 92 per cent (S.D. 2·6%, n = 8) and 80 per cent (S.D. 3·9%, n = 7) for phenobarbitone (concentration 3 μg/ml) and pentobarbitone (concentration 1 μg/ml) respectively.

Blood clearances. From six experiments on four ponies a mean clearance rate of 46 per cent/hr (S.D. 8%/hr) was obtained for the blood clearance of pentobarbitone. The mean clearance curve is depicted in Fig. 1.

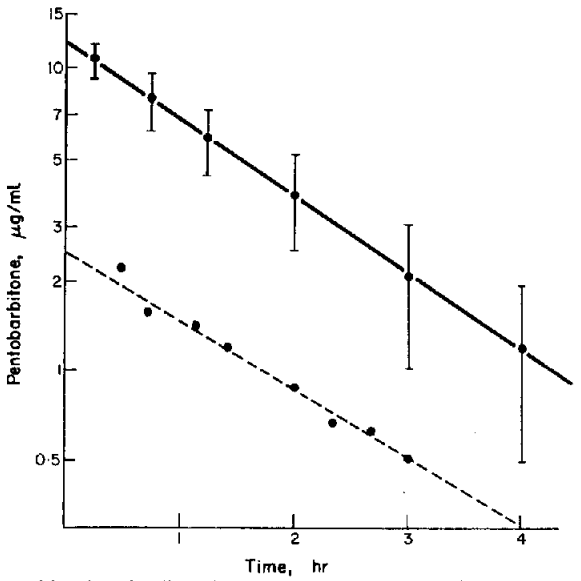

FIG. 1. Pentobarbitone blood and saliva clearances. (———) Plot of mean values (±S.D.) for blood concentrations obtained from six experiments on four ponies; dose 10 mg/kg. (— — — —) Saliva clearance following a single dose of 4 mg/kg. Saliva flow rates:— samples 1–4, <100 ml/7 min; samples 5–8, >100 ml/2·5 min.

With phenobarbitone the blood clearance rates did not appear to be logarithmic. Collection of the results from eleven experiments on five ponies (Fig. 2) and calculation of the lines of best fit, followed by an analysis of variance confirmed that the mean clearance curve was not logarithmic and was more accurately represented by a quadratic function. The calculated best fitting quadratic matched closely the mean curve in Fig. 2.

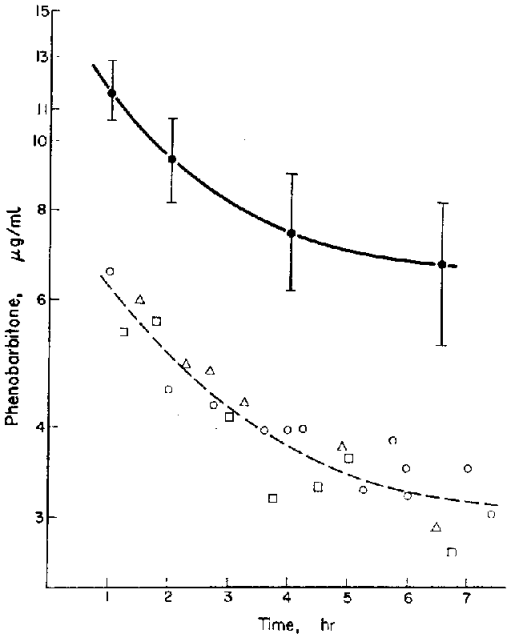

FIG. 2. Phenobarbitone blood and saliva clearances. (———) Plot of mean values (±S.D.) for blood concentrations obtained from eleven experiments on five ponies; dose 8 mg/kg. (— — — —) Saliva clearance results from three experiments on Pony II; dose 6·6 mg/kg. Saliva flow rates:— (○) <100 (□) 100 to 200 and (△) >200 sec/50 ml.

Saliva clearances. In four experiments, two on each fistulated pony, it was found that the saliva-pentobarbitone concentration declined logarithmically with time having a mean rate of 49 per cent/hr (S.D. 4%/hr). A typical clearance is shown in Fig. 1. The saliva-pentobarbitone concentration was independent of saliva flow rate as can be seen from Fig. 1, in which the first four samples were collected at a slow flow rate and the last four were collected at fast flow rate, the same logarithmic clearance curve fitting all eight points. Eight experiments following oral dosage indicated that the concentration in the saliva rose rapidly to a maximum at about 10–20 min after dosage, and then fell quickly until about 40–50 min after dosage when it continued to fall at a rate similar to that found after i.v. injection.

With phenobarbitone three separate experiments were carried out at each dosage rate except the lowest for which four experiments were done. When the results of each experiment were plotted either singly or in groups a wide scatter was observed which was well outside the limit of analytical error assuming a smooth clearance curve. A statistical interpretation of each group of results, identical to that applied to phenobarbitone blood clearances, showed that each clearance was non-logarithmic and was

better fitted by a quadratic equation. A typical clearance is shown in Fig. 2, in which the saliva flow rate of each sample is indicated. As can be seen there appears to be no obvious correlation between flow rate and concentration. Results obtained following oral administration of phenobarbitone were basically the same as those obtained under the same circumstances with pentobarbitone.

Protein binding. Pentobarbitone and phenobarbitone were bound to plasma proteins to the extent of 44 per cent and 15 per cent respectively. Although pentobarbitone was not bound to saliva proteins phenobarbitone was to the extent of 7 per cent with considerable fluctuations between values.

DISCUSSION

The low concentrations of the two barbiturates found in horse blood and saliva, resulting from the relatively small doses which could be given with safety, placed a limit on the applicability of differential ultra-violet spectrophotometry to their quantitative and qualitative analysis. Such analytical methods have been designed to operate with concentrations two or more times greater than the maximum value encountered with horse blood. However, by establishing the blank value of blood from a sample taken just before drug administration, the basic accuracy of the method was increased but clearly this facility would not be available in the detection of 'horse doping'. The possible error for a random sample of blood could be ± 15 per cent at a concentration of 10 μg/ml which is close to the highest concentration measured. The use of u.v. absorption as a means of qualitatively determining barbiturates has been described by Broughton[9] but was not applicable to horse blood samples, especially at low concentrations, due to interference by naturally occurring chromogens.

The high quantitative accuracy with which saliva samples could be analysed was principally due to the availability of large samples and the invariant nature of the saliva blanks, provided that the extracting ether used was the same batch for each sample. As a qualitative method the analysis suffered from the same disadvantages as it did with blood samples. For the practical analysis of saliva samples from normal horses the method described would be of little use since sample size would be only a few ml.

In both blood and saliva analysis it was assumed that the barbiturate determined was in fact the barbiturate administered. The grounds for this assumption were based on the reports of other investigators[10,11] who have not detected metabolites of these barbiturates in the blood of humans and dogs. Furthermore the relative insolubility of the metabolites of both barbiturates in chloroform would be expected to prevent their extraction, to any great extent, from blood. Although Maynert and Dawson[12] have claimed that the hydroxy metabolites of pentobarbitone are insoluble in chloroform this is not strictly true, since in a continuation of this work it was found that these metabolites have a partition coefficient of 0·04 between chloroform and water. This suggested that little interference to the blood-pentobarbitone determination would be encountered from low concentrations of these metabolites. The carboxylic acid metabolite[13] would not be extracted under these conditions. Curry[14] has demonstrated that *p*-hydroxyphenobarbitone, the only identified metabolite of phenobarbitone in man and dog, is much less soluble in chloroform than the parent drug and therefore not so readily extracted by that solvent. Butler[15] has shown that

p-hydroxyphenobarbitone, after i.v. injection, was rapidly cleared from the blood of dogs and concluded that in all probability it was removed from the blood as rapidly as it was formed from phenobarbitone which has a very slow clearance. From these reports it was concluded that if *p*-hydroxyphenobarbitone was present in the blood, which is doubtful in view of Butler's findings, it would not cause much interference in the determination of phenobarbitone. Since it was concluded that metabolic material was not present in blood, it followed that it was not likely to be present in saliva since blood is the source of most of the compounds present in saliva.

A comparison of blood clearance rates obtained for pentobarbitone with those published for other species showed that only the sheep had a similar clearance rate.[20] However, the sheep was much less susceptible to pentobarbitone and could tolerate doses of up to four times as great as those used for the horse with consequently much higher and more easily measured blood concentrations.[20] If the estimated concentration of pentobarbitone in blood at zero time is used to calculate the total quantity of barbiturate in the blood a value of about 10 per cent of the initial dose is found, taking the blood volume as about 60 ml/kg body wt.* This would indicate no localisation of pentobarbitone in blood and, since the clearance curve was logarithmic from 15 min after administration onwards, it follows that the distribution of the barbiturate in the horse was complete within 15 min of i.v. injection. These conclusions agree with the results of other workers who have shown that there is no localisation of pentobarbitone in any single organ or tissue in rats[16] rabbits[7,17] and sheep.[20]

Direct comparison of phenobarbitone blood clearance rates in the horse with clearance rates in other species was not possible because of the non-logarithmic nature of the horse blood clearance. The low blood concentration and possible fluctuations in the blood blank made the accurate measurement of blood concentrations for any length of time impossible, so that if a logarithmic clearance was established after 6·5 hr it could not be measured. It was assumed that the unknown rate of absorption of phenobarbitone through the gut was at least partially responsible for the non-logarithmic clearances.

That the saliva clearances of both barbiturates paralleled the corresponding blood clearance was in agreement with the findings of Alexander *et al.*[21] for chloral hydrate and its metabolites, although the absorption of both barbiturates, judged by the time taken to reach their maximum concentration in the saliva following oral administration was much more rapid. The lack of a noticeable relationship between saliva flow rate and barbiturate administration, and between saliva flow rate and saliva barbiturate concentration conflicted with reports by Guimarais *et al.*[5] and Burgen[22] which dealt with barbiturate salivary secretion rate inhibition and a general study of non-electrolyte salivary secretion respectively. Rasmussen[2] in his work on barbiturates with cows and goats, did not record any note on salivary flow inhibition although he did mention a drop in the saliva-pentobarbitone concentration with an increase in saliva flow rate. Moreover, ruminants have the added peculiarity of constantly secreting saliva, whereas the horse only secretes during mastication (Alexander[6]). It was considered that the relatively high horse saliva flow rate (up to 60 ml/min) and the accuracy of the analysis would allow ready detection of either saliva flow inhibition or a change in the saliva-barbiturate concentration with saliva flow rate.

* F. Alexander, unpublished results.

Comparing the estimated phenobarbitone/pentobarbitone concentration ratio in the saliva at zero time, following equal doses, with the same ratio for the blood concentrations of non-protein bound drugs a close match resulted. Assuming that the molecular and ionic forms exist in equilibrium between blood and saliva this would indicate that the saliva-barbiturate concentration was dependant on the concentration of the non-protein bound barbiturate in the blood and not on the concentration of the unionised drug. If the latter condition was to be obeyed then calculation showed that pentobarbitone (pK_a 8·0) concentration should exceed phenobarbitone (pK_a 7·2) concentration in the saliva if both drugs were present in equal concentration in the blood. This was not the case. That this conclusion conflicts with the theories of other investigators,[2,23,24] who have found that it is the unionised drug concentration in the plasma which governs the saliva concentration, is readily explained by the equality in pH between horse plasma and saliva.[6]

A comparison of the plasma protein binding results with those obtained by other investigators was not considered justified due to the conflicting information available on this subject in the literature,[2,25,26] The lack of protein binding in saliva of pentobarbitone agreed with the findings of Rasmussen[2] but the small degree of binding of phenobarbitone did not.

The wide scatter of results obtained in phenobarbitone saliva clearance experiments was not readily explained but it was considered that there might be some relationship with the wide scatter, relative to pentobarbitone, of plasma protein binding results since it is this latter factor which determines the barbiturate-saliva concentration.

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REFERENCES

1. R. K. Richards and J. D. Taylor, *Anesthesiology* **17**, 414 (1956).
2. F. Rasmussen, *Acta pharmac. tox.* **21**, 11 (1964).
3. G. W. Stavraky, *J. Pharmac. exp. Ther.* **43**, 499 (1931).
4. A. Tournade and E. Joltrain, *C. r. Séanc. Soc. Biol.* **121**, 908 (1936).
5. J. A. Guimarais, A. Malafaya-Baptista, J. Garrette and W. Osswald, *Archs int. Pharmacodyn. Thér.* **102**, 235 (1955).
6. F. Alexander, *J. Physiol., Lond.* **184**, 646 (1966).
7. L. R. Goldbaum, *J. Pharmac. exp. Ther.* **94**, 68 (1948).
8. A. Goldstein and L. Aronow, *J. Pharmac. exp. Ther.* **128**, 1 (1960).
9. P. M. G. Broughton, *Biochem. J.* **63**, 207 (1956).
10. E. J. Algeri and A. J. McBay, *New Engl. J. Med.* **248**, 423 (1953).
11. A. S. Curry, *J. Pharm. Pharmac.* 7, 604 (1955).
12. E. W. Maynert and J. M. Dawson, *J. biol. Chem.* **195**, 389 (1952).
13. E. Titus and H. Weiss, *J. biol. Chem.* **214**, 807 (1955).
14. A. S. Curry, *J. Pharm. Pharmac.* 7, 1072 (1955).
15. T. C. Butler, *J. Pharmac. exp. Ther.* **116**, 326 (1956).
16. J. B. Kahn, *Anesth. Analg. curr. Res.* **29**, 273 (1950).
17. T. Koppanyi, C. R. Linegar and J. M. Dille, *J. Pharmac. exp. Ther.* **55**, 62 (1935).
18. B. B. Brodie, J. J. Burns, L. C. Mark, P. A. Lief, E. Bernstein and E. M. Papper, *J. Pharmac. exp. Ther.* **109**, 26 (1953).
19. J. M. Dille, C. R. Linegar, T. Koppanyi, *J. Pharmac. exp. Ther.* **55**, 46 (1935).
20. J. H. Rae, *Res. vet. Sci.* **3**, 399 (1962).
21. F. Alexander, M. W. Horner and M. S. Moss, *Biochem Pharmac.* **16**, 1305 (1967).
22. A. S. V. Burgen, *J. cell. comp. Physiol.* **2**, 201 (1932).

23. S. A. KILLMANN and J. H. THAYSEN, *Scand. J. clin. Lab. Invest.* 7, 86 (1965).
24. J. H. THAYSON and I. L. SCHWARTZ, *J. exp. Med.* **98**, 261 (1953).
25. D. S. CAMPION and J. D. K. NORTH, *J. Lab. clin. Med.* **66**, 549 (1965).
26. W. WADDELL and T. C. BUTLER. *J. clin. Invest.* **36**, 1217 (1957).