J. Pharm. Pharmac., 1969, **21**, 293–296 *Received January* 16, 1969

The binding of salicylate to plasma protein from several animal species

J. L. KUCERA AND F. J. BULLOCK

Arthur D. Little, Inc., Cambridge, Massachusetts 02140, *U.S.A.*

A comparison has been made of the binding of salicylate to plasma from man, green monkey, rabbit, rat, dog, and guinea-pig. Since the total protein content of normal, citrated, pooled plasmas of these species was not identical, comparisons were made with samples adjusted to the same total protein concentration. The % binding to plasmas of 5·3% total protein at 50 μg of drug/ml was found to be: man, 72%; green monkey, 70%; guinea pig, 64%; rabbit, 64%; rat, 47%; dog, 45%. Results for 150 μg and 500 μg of salicylate/ml are also reported. The albumin : globulin ratio as determined by electrophoresis varied widely among the species. Fractionation of the plasmas and comparison of binding, using 3% solutions of the various albumin fractions in buffer, indicated that the low binding of dog and rat plasmas was due primarily to the low binding affinity of the albumins. Adequate characterization of plasma samples is needed when comparative binding studies are made.

The numerous and difficult problems associated with achieving an understanding of species variations in drug response have been the object of several recent summaries (Brodie, Cosmides & Rall, 1965; Proceedings, 1967; Williams, 1967). One aspect of this problem is related to variations in drug binding to plasma protein among species, which may play a role in determining differences in tissue levels of drug (Brodie & Hogben, 1957; Gillette, 1965), in drug toxicity (Loomis, 1968), and in overall drug kinetics, particularly for highly bound drugs (Martin, 1965; Krüger-Thiemer, 1967). A most striking species variation in drug binding was recently reported for salicylate (Sturman & Smith, 1967). The work reported here was undertaken both to confirm these results and, if possible, find the basis for the species variations. Differences in total protein of plasmas among these species are a significant variable and might therefore account in part for many apparent species differences in binding of salicylate. This variable was significant for a number of other drugs, as demonstrated by work in these laboratories as well as by a report of Dayton, Perel & others (1967). Consequently, comparisons reported here were made with samples of identical total protein content.

EXPERIMENTAL

Plasma pools. Fresh pooled citrated plasmas (100–150 ml) were prepared and analysed for protein by the biuret method. The pools were then quantitatively diluted with pH 7·4, 0·075 M phosphate buffer, ionic strength 0·15, to give the same total protein, 5·3%. The pools were divided into 25 ml batches which were frozen for storage. No sample was carried through more than one cycle of freezing and thawing and the results from similar experiments were comparable before and after one cycle. Electrophoresis was done on cellulose acetate strips in pH 8·8, 0·025 M, barbitone buffer. After the protein bands had been stained (Ponceau S) and the strips dried, the

albumin and globulin fractions were cut out and eluted with 3·0 ml of 0·1N NaOH. Eluates were clarified as necessary by gentle centrifugation and the solutions read at 525 nm with a Beckman DU spectrophotometer. Using a standard curve, the ratio of albumin to globulins was calculated. The accuracy of these ratios is estimated as $\pm 0{\cdot}2$.

Plasma fractionations. The globulins were precipitated by repeated dialysis of the plasma sample against 6 litre volumes of 0·001M citrate buffer, pH 5·0 at 5°. The precipitated globulins were removed by centrifugation. The supernatant which was largely albumin was adjusted to pH 7·4 with 0·075 M phosphate buffer and checked for purity by electrophoresis. The major component corresponded to the albumin of the whole plasma and was contaminated with only traces (<5%) of globulins. Each albumin solution after analysis by the biuret method was diluted to 3% with pH 7·4, 0·075 M phosphate buffer.

Methods and materials. Binding studies were made by equilibrium dialysis using 0·25 inch diameter Visking cellulose casing prepared for use as described by Hughes & Klotz (1959). Casings were stored refrigerated in buffer. After addition of the appropriate amount of drug to plasma, 1·0 ml of the drug-plasma mixture was dialysed against 4·0 ml of pH 7·4, 0·075 M phosphate buffer in a screw cap vial. The vial contents were rotated gently at 37 $\pm$ 0·1° until equilibrium (5–6 h). An aliquot of the buffer was then pipetted into Bray's solution (Bray, 1960) for scintillation counting. Sufficient counts were obtained to achieve the 98% confidence limit. The salicylic acid was a [^{14}C]carboxyl-labelled sample obtained from New England Nuclear Corporation, Medford, Mass. It had a specific activity of 2·31 mCi/mg and was chromatographically pure.

RESULTS

Binding studies were made at salicylate levels of 50, 150 and 500 μg/ml. The results, expressed in terms of percent drug bound, are presented in Table 1 along with the ratio of albumin to globulins for our particular plasma pools.

Table 1. *Salicylate bound (%) to various plasmas of constant total protein**

Drug level (μg/ml)	Man	Green monkey	Rabbit	Guinea-pig	Rat	Dog
50	72	70	64	64	47	45
150	60	58	55	53	36	41
500	40	42	37	41	33	37
Albumin/globulin ratio	1·8	1·6	2·0	1·1	0·8	1·2

* Total protein was adjusted to 5·3% with 0·075 M phosphate buffer, pH 7·4, ionic strength 0·15.

The results of a similar binding study using 3% solutions of the various albumin fractions in pH 7·4 buffer are given in Table 2. Percentages in both Tables are rounded to the nearest whole integer and are $\pm$3%. Although our albumin fractions contained traces of globulins, these samples were judged suitable for comparative studies. We have confirmed the report of Reynolds & Cluff (1960) that globulins bind salicylate much less than albumin in human plasma. We find that salicylate at 50 μg/ml is 25% bound to a 3% solution of human globulins in pH 7·4, 0·075 M phosphate buffer, ionic strength 0·15. It is 21% bound at 150 μg/ml and 16% bound at 500 μg/ml.

DISCUSSION

Our results for salicylate binding indicate genuine species differences which cannot be accounted for in terms of different protein concentration in the samples. Broadly speaking, our results support some of the conclusions of Sturman & Smith (1967). We agree that the species man, monkey, rabbit and guinea-pig can be considered together as having a higher proportion of salicylate bound than rat and dog which fall into a clearly differentiated group having a lower proportion of salicylate bound at the same total drug concentration. The data of Kurtz & Friemel (1967) for salicylate binding to various plasmas also show rat and dog plasmas to be clearly differentiated from those of man, rabbit and guinea-pig. Some of our absolute values for binding are at large variance with those of Sturman & Smith (1967). Agreement of values for binding to man and monkey plasmas is good and consistent with other reports for human plasma (Gutman, Yü & Sirota, 1955; Moran & Walker, 1968). We do not find rabbit

Table 2. *Salicylate bound (%) to albumin fractions of various plasmas**

Drug level (μg/ml)	Man	Green monkey	Rabbit	Guinea-pig	Rat	Dog
50	73	58	53	57	42	31
150	60	45	46	40	30	30
500	44	32	35	34	31	29

* Albumins were 3% solutions in pH 7·4, 0·075 M phosphate buffer, ionic strength 0·15.

plasma to have an intrinsically higher binding affinity than human at 50 μg/ml as previous work suggests. Our values for dog and rat plasma at this drug level are considerably different from the reported respective values of 14% and 2%. The value for salicylate binding to dog plasma reported by Potter & Guy (1964) also seems low, but the 60% binding value at 2–35 μg/ml (Weiner, Washington & Mudge, 1959) is somewhat more consistent with our data. We cannot explain these differences but note that all workers who have reported very low binding used gel filtration methods. We have observed many acidic drugs, including salicylate, to be reversibly but highly bound to Sephadex. This complicating factor introduces the possibility of competitive phenomena between column support and protein in quantitative studies. McArthur & Smith (1968) have demonstrated that the column length of gel influences the apparent extent of binding of salicylate to bovine serum albumin when it is determined by the gel filtration method. With short columns (90 mm), higher apparent binding was observed than with long columns (240 mm). This is consistent with the possibility of competitive binding of salicylate by the column support.

Kurtz & Friemel (1967) have made the qualitative observation that variations in the binding of salicylate to various animal plasmas are better correlated with the percentage of albumin in the sample than with total protein. Our binding results (Table 2) for the various albumin fractions differentiate rat and dog albumins as having lower binding affinity for salicylate than the other albumins studied. The low binding affinity of this fraction seems the likely cause of the low binding observed with these species.

Acknowledgement

This work was carried out under contract No. PH43–66–1139 from the National Institute of General Medical Sciences, Bethesda, Maryland.

REFERENCES

BRAY, G. A. (1960). *Analyt Biochem.*, **1**, 279.

BRODIE, B. B. & HOGBEN, C. A. M. (1957). *J. Pharm. Pharmac.*, **9**, 345–380.

BRODIE, B. B., COSMIDES, G. J. ,& RALL, D. P. (1965). *Science, N.Y.*, **148**, 1547–1554.

DAYTON, P. G., PEREL, J. M., LANDAU, M. A., BRAND, M. & MARK, L. E. (1967). *Biochem Pharmac.*, **16**, 2321.

GILLETTE, J. R. (1965). Drugs and Enzymes, Editors: Brodie, B. B. & Gillette, J. R., pp. 9–22, New York: Macmillan and Co.

GUTMAN, A. B. YU, T. F. & SIROTA, J. H. (1955). *J. clin. Invest.*, **34**, 711–721.

HUGHES, T. R. & KLOTZ, L. M. (1956). *Methods of Biochemical Analysis*, Editor: Glick, D., p. 265, New York: Interscience.

KRÜGER-THIEMER, D. (1967). *Pharmacologic Techniques in Drug Evaluation*, Editors: Siegler, P. E. & Moyer, J. H., III, pp. 224–230, Chicago: Yearbook Medical Publishers.

KURTZ, H. & FRIEMEL, G. (1967). *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **257**, 35.

LOOMIS, T. R. (1968). *Essentials of Toxicology*, p. 34, Philadelphia: Lea and Febiger.

MARTIN, B. K. (1956). *Nature, Lond.*, **207**, 959–960.

MCARTHUR, J. N. & SMITH, M. J. H. (1968). *FEBS Letters*, **1**, 332.

MORAN, C. J. & WALKER, W. H. C. (1968). *Biochem. Pharmac.*, **17**, 153.

POTTER, G. D. & GUY, J. L. (1964). *Proc. Soc. exp. Biol. Med.*, **116**, 658–660.

Proceedings of an International Symposium on Comparative Pharmacology (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **26**, 964–1262.

REYNOLDS, R. C. & CLUFF, L. E. (1960). *Bull. Johns Hopkins Hosp.*, **107**, 278–290.

STURMAN, J. A. & SMITH, M. J. H. (1967). *J. Pharm. Pharmac.*, **19**, 622.

WAGNER, J. G. (1968). *Ann. Rev. Pharmac.*, 81–82.

WEINER, I. M., WASHINGTON, J. A., II, & MUDGE, G. H. (1959). *Bull. Johns Hopkins Hosp.*, **105**, 284–297.

WILLIAMS, R. T. (1967). *Drug Responses in Man*, Ciba Symposium. Editors: Wolstenholme, G. & Porter, R., pp. 71–82, Boston: Little, Brown and Co.