Biochemical Pharmacology, Vol. 27, pp. 887–890. Pergamon Press, 1978. Printed in Great Britain.

PLASMA PROTEIN BINDING AND INTERACTION STUDIES WITH BENOXAPROFEN

DAVID H. CHATFIELD, JOHN N. GREEN, JACQUELINE C. KAO, MALCOLM E. TARRANT and TREVOR J. WOODAGE

Lilly Research Centre Ltd., Erl Wood Manor, Windlesham, Surrey, U.K.

(*Received* 19 *August*; *accepted* 3 *October* 1977)

Abstract—A high proportion of the acidic anti-inflammatory compound benoxaprofen is bound to the plasma proteins of humans and rats (99.8 and 99.3 per cent respectively). The binding did not vary significantly up to total levels of about 120 μg/ml in human plasma or about 40 μg/ml in rat plasma. Above these levels the proportion bound decreased. At 50 μg/ml, more than 90 per cent of benoxaprofen was associated with the albumin fraction of normal human serum. At higher levels, this binding site became saturated and non-specific binding to all proteins appeared to occur. There was no difference between normal and arthritic subjects. In normal rat serum, there was much less bound to albumin. Total binding was reduced in adjuvant arthritic rats, due to decreased binding to α and β_1 globulins. At plasma levels in the range required for therapeutic activity, benoxaprofen did not displace warfarin, salicylate, prednisolone or dexamethasone from their binding to human plasma proteins. In contrast, therapeutic levels of phenylbutazone displaced warfarin.

The majority of acidic anti-inflammatory drugs are extensively bound to plasma proteins and may displace other bound drugs thus increasing their pharmacological effect [1–3]. The use of a new anti-inflammatory agent such as benoxaprofen, structure I, which is known to bind extensively to plasma proteins in animals and man, requires consideration of possible interactions with other drugs at therapeutic concentrations [4, 5]. This report describes further work on the degree and nature of its binding in normal and arthritic rats and humans, and on the extent to which it might displace other protein bound drugs. These included an anti-coagulant (warfarin), an anti-inflammatory (salicylate) and synthetic steroids (prednisolone and dexamethasone).

MATERIALS AND METHODS

Materials. [^{14}C]Warfarin (23.5 mCi/m-mole), [^{14}C]-salicylic acid (61 mCi/m-mole), [^{3}H]dexamethasone (19.1 Ci/m-mole) and [^{3}H]prednisolone (53 Ci/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. [^{14}C]Benoxaprofen (96.5 μCi/m-mole) was synthesised by Mr. T. A. Hicks in these laboratories from *p*-chlorobenzoic acid–carboxyl ^{14}C (9 mCi/m-mole) obtained from Flurochem Limited, Glossop, Derby.

Serum or heparinised plasma was obtained from healthy female volunteers and from seven rheumatoid arthritic female patients with various degrees of severity of the disease. Rat plasma or serum was obtained from normal and adjuvant-arthritic female Sprague–Dawley rats, 200 g. Samples from arthritic rats were taken between days 11 and 14 after induction of arthritis [6].

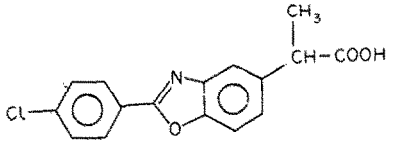

Fig. 1. I, benoxaprofen (2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid).

Measurement of binding. This was performed by equilibrium dialysis at 37°. In most experiments dialysis sacs were made from Visking tubing ($\frac{3}{8}$" × 7"), boiled in 0.5% v/v acetic acid, and thoroughly washed and equilibrated with 0.05 M phosphate buffer in isotonic saline, pH 7.4. (One part of this buffer had been washed with chloroform and then mixed with nine parts of unwashed buffer. This procedure was shown to prevent bacterial contamination, but not to affect the protein-binding of benoxaprofen.) Serum or plasma was diluted to 2% (v/v) with the buffer. The diluted sample (1.5 ml) was placed inside a dialysis sac placed in a tube containing buffer (3.5 ml). Eight replicates were used with the drug(s) under test added alternately inside or outside the dialysis sac unless specified. The sacs were dialysed for 48 hr with periodic mixing by inversion. The levels of radioactivity inside and outside the dialysis sacs were measured by scintillation counting. Results were corrected for the dilution of the serum, quenching by proteins and non-attainment of equilibrium.

In some experiments equilibrium cells (Dianorm, M.S.E., Crawley, Sussex) were used, permitting equilibrium to be reached in 1–3 hr. Results were found to be comparable with the dialysis sac method.

Electrophoresis. Aliquots (10 μl) of solutions of [^{14}C]benoxaprofen at various concentrations were added to 100 μl of serum and allowed to stand for 1 hr at room temperature. Duplicate 5 μl aliquots were applied to cellulose acetate electrophoresis membranes (Oxoid) previously saturated with 0.05 M barbitone buffer, pH 8.6. The serum proteins were separated at a gradient of 0.5 mA per cm for 2 hr (human serum) or 3 hr (rat serum). The membranes were then immersed for 1 hr in denaturant stain (sulphosalicylic

Table 1. Binding of benoxaprofen in the plasma of normal and arthritic humans and rats

Species	Status	No. of samples	Total concn. (μg/ml)	% free ±SEM
Human	Normal	4	32	0.21 ± 0.02
Human	Normal	4	62	0.23 ± 0.02
Human	Normal	4	116	0.26 ± 0.01
Human	Normal	4	297	0.47 ± 0.02
Human	Normal	7	39	0.19 ± 0.01
Human	Arthritic	7	39	0.20 ± 0.01
Rat	Normal	4	20	0.67 ± 0.02
Rat	Normal	4	42	0.67 ± 0.03
Rat	Normal	4	67	0.97 ± 0.03
Rat	Normal	4	196	1.08 ± 0.04
Rat	Normal	4	72	0.71 ± 0.06
Rat	Adjuvant-arthritic	4	46	1.63 ± 0.31

acid, 30 g and Lissamine Green, 2 g per litre). Excess stain was removed by several changes of 2% acetic acid. The relative protein content of each visible band was measured by cutting up one of the membranes, eluting the stain into buffer (10.2 g potassium hydrogen phthalate, 45.8 ml N NaOH per litre, pH 6) and reading the optical density of the solution at 635 nm. The total protein content of the serum was measured by the Biuret Method [7]. The duplicate membrane was cut similarly and the radioactive content of each protein band measured in a scintillation counter.

In preliminary studies the recovery of [^{14}C]benoxaprofen from membranes was found to be unaffected by the staining and washing procedure.

RESULTS

Total binding studies. At low concentrations of benoxaprofen, more than 99.7 per cent was bound to normal human plasma proteins. No important decrease in the extent of this binding was apparent until the total level exceeded 116 μg/ml (Table 1). There were no differences in binding between normal and arthritic human plasma, even in severe cases of arthritis.

In contrast, the proportion bound in normal rat plasma decreased at levels above 42 μg/ml. The plasma of adjuvant arthritic rats contained more than twice as much unbound benoxaprofen as that of normal rats, even though the effective total concentration was lower.

Electrophoretic studies. At a concentration of 50 μg/ml, benoxaprofen was associated almost completely with the albumin fraction of normal human serum (Table 2). When the benoxaprofen concentration was raised to 400 μg/ml, this binding appeared to become saturated so that about half of the compound was associated with the other protein fractions. There was little effect of rheumatoid arthritis on this pattern, even in the most severely-affected subjects, beyond a small fall in the content of albumin and its associated binding.

In rats however, very little benoxaprofen was bound to albumin at 50 μg/ml, and almost none at

Table 2. Binding of benoxaprofen to serum protein fractions of normal and arthritic humans and rats

Species: Protein fraction	Normal subjects % distribution: Protein	Benoxaprofen 50 μg/ml	Benoxaprofen 400 μg/ml	Arthritic subjects % distribution: Protein	Benoxaprofen 400 μg/ml
Human:					
Albumin	64	91	56	56	44
α_1-globulin	3	2	13	4	14
α_2-globulin	8	3	12	10	15
β-globulin	10	3	10	12	13
γ-globulin	14	2	9	18	13
Total	67 mg/ml			65 mg/ml	
Rat:					
Albumin	53	20	2	25	1
α_1-globulin	11	16	14	15	1
α_2-globulin	7	12	23	18	2
β_1-globulin	6	21	21	11	11
β_2-globulin	13	19	15	22	33
γ-globulin	11	12	25	10	51
Total	63 mg/ml			51 mg/ml	

Results at 400 μg/ml are the means of seven values, at 50 μg/ml are the means of two values.

Table 3. Binding of drugs in normal human plasma, in the absence and presence of benoxaprofen

Drug	Drug concn. (μg/ml)	Benoxaprofen concn. (μg/ml)	% Drug free: Benoxaprofen absent	Benoxaprofen present
Warfarin	0.5	20	0.80 ± 0.03	0.71 ± 0.05
	2	20	0.74 ±).02	0.75 ± 0.01
	5	20	0.86 ± 0.01	0.85 ± 0.01
	20	20	0.88 ± 0.01	0.88 ± 0.02
	2	200	0.74 ± 0.01	0.71 ± 0.01
Salicylic acid	500	20	19.1 ± 0.2	19.0 ± 0.9
Prednisolone	0.3	125	5.5 ± 0.1	5.7 ± 0.1
	0.3	500		6.0 ± 0.1
Dexamethasone	0.1	125	12.0 ± 0.1	11.8 ± 0.1
	0.1	250		11.6 ± 0.1

Results are given as the mean ± S.E.M. of three or four (for steroids) replicates.

400 μg/ml. It was associated with all the globulin fractions, with little specificity being apparent. In adjuvant-arthritic rats there was a marked decline in the albumin content of the serum. The reduced binding of benoxaprofen in these rats was however due to impairment of its binding to α and β_1 globulins, in spite of their increased content.

Interaction studies with warfarin, salicylate, prednisolone and dexamethasone. At a total concentration of 20 μg/ml, benoxaprofen did not displace either warfarin or salicylate from normal human plasma proteins (Table 3). The concentrations of these drugs were in the range normally associated with therapeutic activity [5, 8]. Benoxaprofen did not displace warfarin even at a concentration of 200 μg/ml, whereas in the same experiment phenylbutazone at a concentration of 150 μg/ml increased the proportion of free warfarin to 1.29 ± 0.03 per cent. In the converse experiment, displacement of benoxaprofen (at 100 μg/ml) from its binding was not observed when warfarin was added to a final concentration of 5 μg/ml or salicylate to 100 μg/ml or 500 μg/ml.

The interaction of benoxaprofen with two synthetic steroids used in anti-inflammatory therapy, prednisolone and dexamethasone, was also studied. At concentrations of benoxaprofen up to 250 μg/ml and with therapeutic levels of the steroids there was no significant displacement of steroid (Table 3) [9].

DISCUSSION

Many drugs, particularly acidic anti-inflammatories, are bound to plasma proteins and are especially associated with the albumin fraction. The binding of benoxaprofen in human plasma appeared to follow the same pattern. At 50 μg/ml, which is within the range of anticipated therapeutic concentrations, 99.8 per cent of the compound was bound and over 90 per cent of this was associated with albumin. At concentrations of 300–400 μg/ml, the proportion of free benoxaprofen rose and comparatively non-specific binding was observed. This suggests the presence of a single high-affinity site on human albumin, the calculated capacity of which would be about 190 μg of benoxaprofen per ml of plasma.

In contrast, rats did not show this close association with albumin and bound, at the most, about 20 per cent of the total drug to that fraction. Other workers have shown that the binding of acidic drugs to rat albumin is atypical and that binding characteristics in this species often differ from man [10]. In agreement with previous studies marked changes were seen in the serum protein electrophoretic pattern of adjuvant-arthritic rats, and in particular a fall in albumin content [11]. This was not, however, responsible for the reduction in the extent of binding of benoxaprofen, also seen in these animals. This was due rather to the impairment of its binding to α and β_1 globulins, in spite of the increased levels of these protein fractions.

In arthritic humans the changes in electrophoretic pattern were comparatively slight and the binding of benoxaprofen was not affected. Although the changes in blood proteins of the rat in experimental adjuvant disease are similar to those observed in rheumatoid arthritis it must be remembered that the disease states are not identical [11–13]. The impairment in binding to globulins observed in rats did not occur in arthritic humans and even if it had, it would have scarcely influenced the total binding because of the predominance of albumin binding in the probable therapeutic range.

The therapeutic levels of benoxaprofen are not yet closely defined. Early predictions, based on animal studies, suggested that levels of between 5 and 35 μg/ml may be appropriate [14, 15]. Preliminary clinical experience with benoxaprofen in patients with rheumatoid arthritis indicates that serum levels between 100 μg/ml and 200 μg/ml may be necessary for optimum benefit (Ridolfo, unpublished observations). The interaction studies described in this work have used concentrations within the probable therapeutic range. The results suggest that clinical hazards resulting from the displacement of other drugs from their binding to plasma proteins by benoxaprofen are not expected. No important displacement of warfarin, salicylate, prednisolone or dexamethasone was seen. The albumin binding site for benoxaprofen appears to be distinct from that occupied by warfarin. In contrast phenylbutazone, which is known to interact with warfarin because of competition for a common receptor site on albumin, did displace warfarin under identical experimental conditions [3].

Acknowledgements—We wish to thank Mrs. R. A. Goulbourn for obtaining the serum samples from rheumatoid arthritic patients and Mr. C. H. Moore for assistance with the electrophoresis studies.

REFERENCES

1. M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.* **57**, 895 (1968).
2. P. G. Welling, in *Specialist Periodic Report on Foreign Compound Metabolism in Mammals. Chemistry Society* (Ed. D. E. Hathway) **3**, 127 (1975).
3. H. M. Solomon and J. J. Schrogie, *Biochem. Pharmac.* **16**, 1219 (1967).
4. C. H. Cashin, W. Dawson and E. A. Kitchen, *J. Pharm. Pharmac.* **29**, 330 (1977).
5. D. H. Chatfield and J. N. Green, *Xenobiotica* (in press).
6. B. B. Newbould, *Br. J. Pharmac.* **21**, 127 (1963).
7. T. E. Weichselbaum, *Am. J. clin. Path., Tech. Sect.* **10**, 40 (1946).
8. E. Mongan, P. Kelly, K. Nies, W. W. Porter and H. E. Paulus, *J. Am. Med. Assn.* **226**, 142 (1973).
9. R. Leclerg and G. Copinschi, *J. Pharmacokin. Biopharm.* **2**, 175 (1974).
10. D. T. Witiak and M. W. Whitehouse, *Biochem. Pharmac.* **18**, 971 (1969).
11. H. E. Weimer, F. D. Wood and C. M. Pearson, *Can. J. Biochem.* **46**, 743 (1968).
12. R. W. Payne, *Ann. N.Y. Acad. Sci.* **94**, 284 (1961).
13. H. G. M. Clarke, T. Freeman and W. E. M. Pryse-Phillips, *Br. J. Exp. Path.* **51**, 441 (1970).
14. D. H. Chatfield, C. H. Cashin, E. A. Kitchen and J. N. Green, *J. Pharm. Pharmac.* **29**, 371 (1977).
15. G. L. Smith, R. A. Goulbourn, R. A. P. Burt and D. H. Chatfield, *Br. J. clin. Pharmac.* **4**, 585 (1977).