Br. J. clin. Pharmac. (1980), **9**, 61–65

THE EFFECT OF SEMISYNTHETIC PENICILLINS ON THE BINDING OF BILIRUBIN BY NEONATAL SERUM

LOUISE A. FRIEDMAN* & P.J. LEWIS*

Clinical Pharmacology Unit, Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London W6 0XG

1 The effect of ampicillin, cloxacillin, flucloxacillin and sulphafurazole on bilirubin binding by pooled human umbilical cord serum and bovine serum albumin was studied *in vitro* using Sephadex gel filtration.

2 Sulphafurazole displaced bilirubin from binding to both bovine serum albumin and human pooled cord serum.

3 Ampicillin had no effect on bilirubin binding; both cloxacillins displaced bilirubin from pooled cord serum but not bovine serum albumin.

4 No displacement of bilirubin by the cloxacillins from pooled cord serum could be detected at therapeutic plasma concentrations of these drugs.

5 Scatchard analysis of the interactions showed that displacement of bilirubin by these drugs occurred principally at the primary, high affinity, low capacity binding site.

Introduction

Human neonates conjugate bilirubin poorly during the first week of life due to the immaturity of hepatic glucuronyl transferase. Neonatal jaundice is potentially hazardous in that it may precipitate kernicterus or bilirubin encephalopathy due to uptake of bilirubin by the central nervous system (CNS). Only bilirubin not bound to circulating albumin can be taken up by the CNS. Serum albumin possesses one high affinity low capacity primary binding site for bilirubin and one or more low affinity sites (Krasner, Giacoia & Yaffe, 1973).

In 1956 the sulphonamide sulphafurazole was observed to precipitate kernicterus in preterm neonates (Silverman, Andersen, Blanc & Crozier, 1956) and this was later shown to be due to displacement of bilirubin from serum albumin (Odell, 1959). The possibility that other highly protein bound drugs might also displace bilirubin from albumin binding has been suggested (Bratlid, 1972). Semisynthetic penicillins are highly bound to circulating albumin and are widely used in neonatal medicine. Treatment of preterm infants with cloxacillin has been reported to result in elevated concentrations of free bilirubin even at low total bilirubin levels (Satge, Colin, Voyer, Crumiere & Charlas, 1974). Flucloxacillan administration to newborn Gunn rats has been reported to cause neurological damage (Hanefeld & Ballowitz, 1976).

The objective of the present study was to investigate the activity of cloxacillin, flucloxacillin and ampicillin in this regard using an *in vitro* method of measuring bilirubin binding by neonatal serum. The effect of sulphafurazole was also studied, as this drug has a kernicterogenic effect in jaundiced neonates. Similar binding studies were undertaken with bovine serum albumin to see whether this could be used as a model for human neonatal serum.

Methods

Blood was collected from the placental end of the umbilical cord at unselected deliveries. The serum was pooled, divided up and stored at −20°C until required. Bovine serum albumin was dissolved in 0.066 M phosphate buffer and a solution of 30 g/l used. Sodium flucloxacillin, sodium cloxacillin and ampicillin trihydrate were supplied by Beecham Research Laboratories. Sulphafurazole was obtained from Sigma Chemical Co.

Bilirubin binding was studied using a method based on that of Kaufmann, Kapitulnik & Blondheim (1969). Plastipak syringes (5 ml) plugged with cotton wool containing 2 cm columns of Sephadex G-25M (Pharmacia) were used as separation columns. The Sephadex was soaked for 24 h in 0.066 M phosphate buffer, pH 7.4.

* Present address: Department of Clinical Pharmacology, Royal Postgraduate Medical School, Hammersmith Hospital, Ducane Road, London W12 0HS.

0306–5251/80/010061–05 $01.00

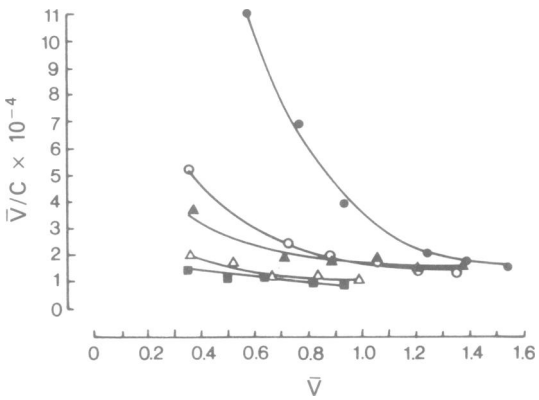

Figure 1 Scatchard plots showing the effect of increasing sulphafurazole concentrations on bilirubin binding by bovine serum albumin. ● control, ○ 1 mM, ▲ 2 mM, △ mM and ■ 8 mM.

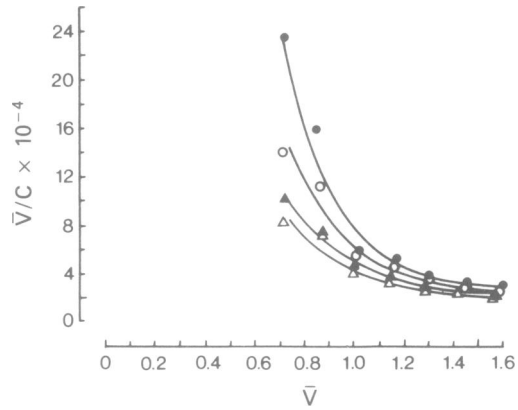

Figure 2 Scatchard plots showing the effect of increasing sulphafurazole concentrations on bilirubin binding by human pooled cord serum. ● control, ○ 1 mM, ▲ 2 mM and △ 4 mM.

Anhydrous bilirubin (10 mg) was dissolved in 1 ml 0.1 N NaOH. Buffer (1 ml, 0.45% Na_2CO_3, 0.45% NaCl, pH 7.8) was then added. This preparation was kept in the dark and used within 30 min. The cloxacillins were dissolved directly in the bovine serum albumin solution and pooled cord serum before the addition of bilirubin. Sulphafurazole was dissolved in a minimal amount of 0.05 N NaOH and neutralized with phosphate buffer before use. Various concentrations of bilirubin were added to 100 µl aliquots of bovine serum albumin or pooled cord serum over the range 85 µmol/l to 850 µmol/l. Bound and free bilirubin in the albumin solutions were then separated. The buffer in the columns was drained off and the treated sera were layered onto the Sephadex columns and allowed to absorb into the gel matrix. The columns were washed with 5 ml phosphate buffer and free bilirubin, if present, was recovered by subsequent elution with 10 ml 0.1 N NaOH. The bilirubin was collected in 15 ml glass-stoppered centrifuge tubes containing 200 µl 1% ascorbic acid (freshly prepared) acidified with 1 ml 1 N HCl and extracted into 2 ml chloroform. Separation was effected by centrifuging for 5 min at 2000 rev/min. The absorbance of free bilirubin was determined at 450 nm in a Pye Unicam SP 1800 spectrophotometer using chloroform as blank.

Results were calculated by Scatchard analysis (1949) using a curve fitting programme based on the method of least squares.

Results

Bovine serum albumin

Ampicillin, cloxacillin and flucloxacillin had no effect on bilirubin binding by bovine serum albumin. Sulphafurazole, however, was found to displace bilirubin.

Figure 1 shows the effect of 1–4 mM sulphafurazole on bilirubin binding by bovine serum albumin plotted as Scatchard plots, where $\bar{V}$ is the number of moles bilirubin bound per mol of albumin and (C) is the molar concentration of free bilirubin. Table 1 gives computed values from the Scatchard plots for N_1, K_1 and K_2 for the control data and in the presence of 1, 2 and 4 mM sulphafurazole. N_1 is the capacity of the primary binding site and K_1 and K_2 are the affinities of the primary and secondary sites. N_1, K_1 and K_2 all decreased with increasing concentrations of sulphafurazole. However, K_2 was found to be very low compared to K_1.

Pooled cord serum

Ampicillin had no effect on bilirubin binding by pooled cord serum. Figures 2, 3 and 4 show the effect of sulphafurazole, cloxacillin and flucloxacillin on bilirubin binding by pooled cord serum plotted on Scatchard plots. Table 2 gives values for N_1, K_1 and K_2 for pooled cord serum control data and in the presence of sulphafurazole, cloxacillin and flucloxacillin. N_1 remained unchanged at around 0.8 mol

Table 1 Relationship between sulphafurazole concentration and bilirubin binding by bovine serum albumin (0.444 mM) as calculated by Scatchard analysis

c (mM)	n_1	K_1^*	K_2^*
0	0.84	31.59	0.28
1	0.51	17.64	0.17
2	0.35	17.35	0.13
4	0.33	10.25	0.05

* (10^4 M^{-1})

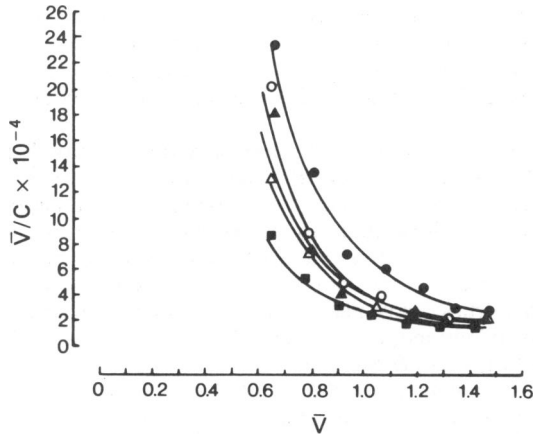

Figure 3 Scatchard plots showing the effect of increasing cloxacillin concentrations on bilirubin binding by human pooled cord serum. ● control, ○ 1 mM, ▲ 2 mM, △ 4 mM and ■ 8 mM.

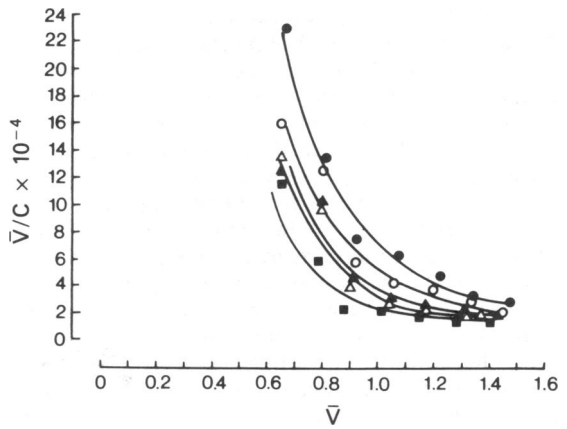

Figure 4 Scatchard plots showing the effect of increasing flucloxacillin concentrations on bilirubin binding by human pooled cord serum. ● control, ○ 1 mM, ▲ 2 mM, △ 4 mM and ■ 8 mM.

bilirubin bound/mol albumin in the presence of all three drugs, even at high concentrations. This value for cord serum is very similar to the value of 0.84 obtained for binding of bilirubin by bovine serum albumin. K_1 decreased from around $9.6 \times 10^5\ M^{-1}$ with increasing drug concentration. However, sulphafurazole exerted the greatest effect. K_2 was found to be very low compared to K_1 and decreased further with increasing concentrations of cloxacillin and flucloxacillin. However, the changes in K_2 in the presence of sulphafurazole were not related to increasing drug concentrations. Since sulphafurazole can be seen to displace bilirubin from the secondary site, this anomaly probably reflects the unreliability of applying Scatchard analysis to data points obtained at low affinity sites, especially when changes in affinity are small.

Discussion

Bilirubin was displaced from either bovine serum albumin or human pooled cord serum *in vitro* by sulphafurazole, cloxacillin and flucloxacillin but not by ampicillin. These results are consistent with Bratlid's (1972) observation that drugs which effectively displace bilirubin from protein binding are themselves strongly protein bound. Ampicillin is less than 25% protein bound (Kunin, 1967) whereas cloxacillin, flucloxacillin (Tan, Trott, Phair & Watanakunakorn, 1972) and sulphafurazole (Anton, 1973) are over 90% bound at therapeutic plasma concentrations.

The apparent primary association constants (K_1) of bovine serum albumin and pooled cord serum for bilirubin were $3.15 \times 10^5\ M^{-1}$ and $9.56 \times 10^5\ M^{-1}$

Table 2 Relationship between drug concentration and bilirubin binding by human pooled cord serum as calculated by Scatchard analysis

	c (mM)	n_1	K_1^*	K_2^*
Sulphafurazole	0	0.90	95.6	0.03
	1	0.91	55.0	0.22
	2	0.79	49.9	0.49
	4	0.86	35.0	0.14
Cloxacillin	0	0.81	97.0	1.18
	1	0.82	85.7	0.03
	2	0.80	70.0	0.02
	4	0.85	50.0	0.005
	8	0.76	41.5	0.005
Flucloxacillin	0	0.81	97.0	1.18
	1	0.78	85.0	1.08
	2	0.80	76.9	0.071
	4	0.78	77.0	0.013
	8	0.72	77.8	0.005

*($10^4\ M^{-1}$)

respectively. These values are somewhat lower than the value of 5.2×10^7 M^{-1} obtained by Krasner *et al.* (1973) by equilibrium dialysis of purified cord albumin. The discrepancy may be due partly to the fact that Sephadex binds bilirubin in competition with albumin (Meuwissen & Heirwegh, 1970).

Bovine serum albumin and pooled cord serum exhibited very similar primary binding capacities (n_1). Approximately 0.8 mol bilirubin were bound/mol albumin. These results are in agreement with Krasner *et al.* (1973) who found that both purified cord albumin and bovine albumin show maximum fluorescence at a bilirubin/albumin molar ratio of 1.

In the presence of drugs, however, bilirubin binding by bovine albumin and human neonatal serum was both quantitatively and qualitatively different. Whereas only sulphafurazole displaced bilirubin from bovine albumin, sulphafurazole, cloxacillin and flucloxacillin all effectively displaced bilirubin from pooled cord serum. Furthermore, the capacity of the primary site on bovine albumin was reduced by sulphafurazole but remained unchanged on pooled cord serum in the presence of all three drugs. Bovine serum albumin is not therefore a suitable model for human neonatal albumin when investigating the kernicterogenic potential of highly protein bound drugs *in vitro*.

It appears that drugs which interfere with bilirubin binding by human cord albumin do so by lowering the affinity of the albumin for bilirubin and that they do not necessarily diminish the capacity of the primary site. Sudlow (1978) carried out studies on specific binding sites on human albumin and found that the affinity of albumin for bilirubin at its primary site is so great as to make competition for binding by other ligands virtually impossible. However, changes in albumin conformation as a result of ligand binding have been frequently observed. One result of this conformational mobility is that binding sites on albumin, although spatially distinct, are unlikely to be independent. Thus the affinity, specificity and number of binding sites for a given ligand may change in the presence of a second ligand bound to a separate site.

Since bilirubin was displaced from its primary high affinity site as measured by Sephadex gel filtration, our results can only be explained by assuming Sudlow's hypothesis to be correct, i.e. it must be assumed that alteration in albumin conformation has occurred. Furthermore, other studies have shown that drugs can displace bilirubin from its high affinity site (Coutinho, Lucek, Cheripko & Kuntzman, 1973; Schiff, Chan & Stern, 1972).

Although cloxacillin and flucloxacillin caused displacement of bilirubin from pooled cord serum, the usual therapeutic serum concentration of these drugs must be taken into account when assessing the clinical significance of these *in vitro* findings. Administration of a single oral dose of cloxacillin (50 mg/kg) to seven neonates resulted in a mean 2 h serum concentration of 0.2 mM (Grossman & Ticknor, 1965). Administration of 25 mg/kg oral flucloxacillin 6 hourly to neonates results in a mean 3 h serum concentration on the second day of treatment of 0.044 mM (Cohen, Raeburn, Devine, Kirkwood, Elliot, Cockburn & Forfar, 1975). However, significant displacement of bilirubin from pooled cord serum was only seen at threshold concentrations of 1 mM or five times higher than therapeutic concentrations. By contrast, the concentration of sulphafurazole which will effectively displace bilirubin from pooled cord serum is very similar to the therapeutic plasma concentration.

These considerations make it unlikely that the cloxacillins, unlike the sulphonamides, are kernicterogenic when used in jaundiced human neonates and we are not aware of any reports of kernicterus resulting from administration of these drugs.

References

ANTON, A.H. (1973). Increasing activity of sulfonamides with displacing agents: a review. *Ann. N.Y. Acad. Sci.*, **226**, 273–292.

BRATLID, D. (1972). The effect of antimicrobial agents on bilirubin binding by human erythrocytes. *Scand. J. clin. Lab. Invest.*, **30**, 331–337.

COHEN, M.D., RAEBURN, J.A., DEVINE, J., KIRKWOOD, J., ELLIOTT, B., COCKBURN, F. & FORFAR, J.O. (1975). Pharmacology of some oral penicillins in the newborn infant. *Arch. Dis. Child.*, **50**, 230–234.

COUTINHO, C.B., LUCEK, R.W., CHERIPKO, J.A. & KUNTZMAN, R. (1973). A new approach to the determination of protein-bound bilirubin displacement and its applications. *Ann. N.Y. Acad. Sci.*, **226**, 238–246.

GROSSMAN, M. & TICKNOR, W. (1975). Serum levels of ampicillin, cephalothin, cloxacillin and nafcillin in the newborn infant. In *Antimicrobial agents and chemotherapy*, ed. Hobby, G.L., pp. 214–219. Ann Arbor, Michigan: Am. Soc. Microbiology.

HANEFELD, F. & BALLOWITZ, L. (1976). Flucloxacillin and bilirubin binding. *Lancet*, **i**, 433–434.

KAUFMANN, N.A., KAPITULNIK, J. & BLONDHEIM, S.H. (1969). The adsorption of bilirubin by Sephadex and its relationship to the criteria for exchange transfusion. *Pediatrics*, **44**, 543–548.

KRASNER, J., GIACOIA, G.P. & YAFFE, S.J. (1973). Drug-protein binding in the newborn infant. *Ann. N.Y. Acad. Sci.*, **226**, 101–114.

KUNIN, C.M. (1967). Clinical significance of protein binding of the penicillins. *Ann. N.Y. Acad. Sci.*, **145**, 282–290.

MEUWISSEN, J.A.T.P. & HEIRWEGH, K.P.M. (1970). Transfer of adsorbed bilirubin to specific binding proteins. *Biochem. J.*, **200**, 19P.

ODELL, G.B. (1959). The dissociation of bilirubin from albumin and its clinical implications. *J. Pediat.*, **55**, 268–279.

SATGE, P., COLIN, J., VOYER, M., CRUMIERE, C. & CHARLAS, J. (1974). Place des dosages de la bilirubine non liee et de l'albumine serique. *Arch. Franc. Ped.*, **31**, 201–213.

SCATCHARD, G. (1949). The attraction of protein from small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672.

SCHIFF, D., CHAN, G. & STERN, L. (1972). Sephadex G-25 quantitative estimation of free bilirubin potential in jaundiced newborn infants sera: A guide to the prevention of kernicterus. *J. Lab. clin. Med.*, **80**, 455–462.

SILVERMAN, W.A., ANDERSEN, D.H., BLANC, W.A. & CROZIER, D.N. (1956). A difference in mortality rate and incidence of kernicterus among premature infants alotted to two prophylactic antibacterial regimens. *Pediatrics*, **18**, 614–624.

SUDLOW, G. (1978). Specific binding sites on albumin. *7th Int. Conf. Pharmac.* Abstracts, **2**, 892.

TAN, J.S., TROTT, A., PHAIR, J.P. & WATANAKUNAKORN, C. (1972). A method for measurements of antibiotics in human interstitial fluid. *J. infect. Dis.*, **126**, 492–497.

(Received March 15, 1979)