J. Pharm. Pharmacol. 1983, 35: 383–384
Communicated November 5, 1982

COMMUNICATIONS

Protein binding and hepatic extraction of diazepam across the rat liver

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Many drugs are highly bound to plasma proteins, the extent of which can vary in disease and in the presence of other drugs that compete for the binding sites. The likely effect of such changes in binding on drug elimination is perhaps best thought of in terms of the extraction ratio of the drug across an elimination organ (Rowland & Tozer 1980). For drugs of low extraction, clearance is expected to vary in direct proportion to the fraction of drug unbound in blood, and there are many data supporting this expectation (Levy & Yacobi 1974; Gugler et al 1975; Trenk & Jahnchen 1980). At the other extreme are drugs of high extraction. For these elimination is perfusion-rate limited and clearance is considered to be relatively insensitive to changes in binding within blood. Some evidence supporting this last statement is also forthcoming (Guentert & Øie 1980). In most studies to date, however, the absolute change in binding has been relatively small. In the present study we report changes in the extraction ratio (and clearance) of diazepam across the rat liver, when protein binding is varied over a very wide range.

Methods

Experiments were performed in a single-pass isolated perfused in-situ rat liver preparation (Pang & Rowland 1977a). The perfusion medium consisted of Krebs bicarbonate buffer, containing approximately 500 ng diazepam ml^{-1}. Perfusate flow was fixed at 15 ml min^{-1}. The degree of binding was varied by changing the concentration of human serum albumin in the perfusate (0–40 g $litre^{-1}$). Binding was determined by equilibrium dialysis against Krebs bicarbonate (pH 7·4) at 37 °C using radiolabelled diazepam; 1 ml each of perfusate and buffer were used and the dialysis time was 3 h, by which time an equilibrium had been achieved.

The concentration of diazepam in the perfusate immediately on both sides of the liver was determined by electron-capture gas liquid chromatography using a modification of the procedure developed by Rutherford (1977), in which toluene instead of butyl acetate is used as the organic solvent. In each preparation a condition of no albumin in the perfusate was followed by periods of differing albumin concentration. Each treatment period was 20 min, a time shown to ensure that a steady-state effluent diazepam concentration was achieved. The extraction ratio and clearance at steady state were calculated in the standard manner (Rowland & Tozer 1980).

Diazepam is highly bound to albumin. By reference to a predetermined binding isotherm, the fraction of diazepam unbound can be adjusted to different values (0·05–1) by varying the albumin concentration in the perfusate. Based on the output concentration of diazepam, preliminary experiments have shown that the rat liver is viable for considerable periods in the absence of protein (when extraction of drug is at its highest), and that an input diazepam concentration below 800 ng ml^{-1} ensured that linear conditions always prevailed. Mass balance experiments using radiolabelled diazepam in the perfusate also showed that at steady state, loss of drug across the liver is due entirely to elimination.

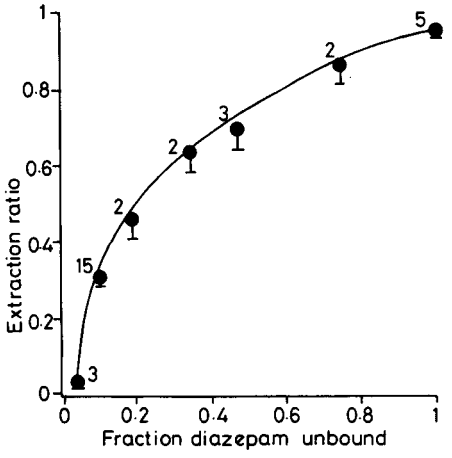

FIG. 1. The extraction ratio of diazepam (●, ± s.e.m.) varies with the fraction of unbound drug in the perfusate. The numeral above each circle refers to the number of treatment periods performed.

* Correspondence.

Results and discussion

Fig. 1 summarizes the data and illustrates the dramatic change in extraction ratio (and clearance) of diazepam with a change in protein binding. In the absence of protein, with no binding, the extraction ratio of diazepam is very high, being close to one. Whereas, in the presence of 35 g litre^{-1} albumin (when only 5% of drug is unbound) the extraction ratio is virtually zero. That is, the drug has changed from one of high extraction to one of low extraction simply by changing the degree of protein binding. Such profound changes have not been reported previously to our knowledge.

Two models of hepatic clearance have been proposed Pang & Rowland (1977b). Both models predict that if the degree of binding in the perfusate can be made high enough, then eventually the extraction ratio, of even those drugs which have a high intrinsic clearance, will become low and limited by binding. Our data support this view and stress the need to quote not only the value of the extraction ratio or clearance but also the associated degree of binding of drug within blood.

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J. Pharm. Pharmacol. 1983, 35: 384–386
Communicated December 15, 1982

Monobromobimane: a substrate for the fluorimetric assay of glutathione transferase

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The glutathione *S*-transferases (EC 2.5.1.18) are a family of isoenzymes that play a vital role in the protection of cells from toxic metabolites of drugs and environmental chemicals (Jakoby 1978; Grover 1982; Ketterer et al 1982). The enzymes conjugate these electrophilic xenobiotic materials to glutathione.

We wish to report that the compound monobromobimane (mBrB) (Kosower & Pazhenchevsky 1980) is a useful new substrate for glutathione transferase(s). It is a non-fluorescent compound that reacts slowly (non-enzymically) with thiols (Kosower et al 1979; Fahey et al 1980), such as glutathione (GSH), to give fluorescent products (Fig. 1). We have discovered that glutathione transferase catalyses the reaction of mBrB with GSH, with the result that glutathione transferase activity can be conveniently assayed with this new substrate. This is achieved by direct measurement of the rate of appearance of the fluorescent conjugate formed within the reaction mixture, i.e. in the presence of an excess of unreacted non-fluorescent mBrB.

Materials and methods

Monobromobimane (mBrB, 3-bromomethyl-2,5,6-trimethyl-lH, 7H-pyrazole[1,2-a]pyrazole-1,7-dione) was synthesized, and purified by chromatography (to remove fluorescent material), as described by Kosower & Pazhenchevsky (1980). Stock solutions were: (a) mBrB (1.0 mM in dry acetonitrile), (b) GSH (3.0 mM in Na_2EDTA, 10 mM, pH 4·5, prepared in de-aerated water and stored at 4 °C), and (c) phosphate buffer (0·05 M, pH 6·5).

Rat liver supernatants, obtained after $CaCl_2$ precipitation of microsomes, served as a source of cytosolic glutathione transferases (Benson et al 1979). When desired, GSH could be removed from this enzyme

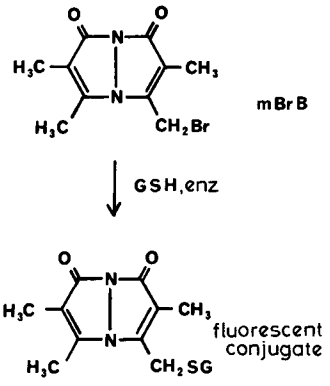

FIG. 1. Structures of mBrB and its fluorescent glutathione conjugate.

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