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Binding of busulfan to plasma proteins and blood cells

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The reversible and non-reversible binding of busulfan to plasma proteins and to blood cells has been evaluated in man. The reversible binding to plasma proteins was insignificant in both healthy subjects and in patients with chronic myelocytic leukaemia. The percentage of busulfan irreveribly bound to plasma ligands was 32·4. Busulfan was distributed evenly between blood cells and plasma. The fraction of busulfan irreversibly bound to the blood cells was 46·9%. The major portion of busulfan that is degraded in whole blood reacts with the blood cells and the main part of that binds to macromoelcular components in the cells.

Busulfan has been in clinical use since the 1950s for the treatment of chronic myelocytic leukaemia and has been recently proposed as the treatment of choice for polycythaemia vera (Brodsky 1982). Despite its widespread and long use, scant information is available concerning the disposition of the drug in man. Recently, the kinetics of the parent drug was studied for the first time in man (Ehrsson et al 1983). In the present study the reversible and non-reversible binding of busulfan to plasma proteins and to blood cells has been evaluated in man.

Materials and methods

Protein binding. The reversible binding was studied by equilibrium dialysis using the equipment and procedure described previously (Eksborg et al 1982). Blood samples from healthy drug-free subjects and patients with neoplastic diseases were collected in glass test tubes containing 150 IU of freeze-dried heparin. The addition of heparin did not affect the protein binding, as was established by comparative binding studies in serum samples. The plasma fractions were separated by centrifugation and were immediately used for protein binding studies. The plasma (5 ml) was mixed with busulfan (EGA-Chemie, Steinheim, GFR) in acetone (0·01 ml) to give a final busulfan concentration in the range 20–200 ng ml^{-1}. The equilibrium dialysis was carried out for 5 h at 25·0 ± 0·1 °C. The concentration of busulfan was determined by gc with ecd (Hassan & Ehrsson 1983).

The irreversible binding was studied using [^{14}C]busulfan (spec. act. 0·9 μCi mg^{-1}) synthesized from [^{14}C]1,4-butanediol and methanesulfonic anhydride according to the principles described for 1,5-pentanediol dimethanesulfonate (Ehrsson & Hassan 1983). Busulfan in acetone, 0·01 ml, was added to plasma (5 ml) to give a final busulfan concentration of 25 μg ml^{-1}. The plasma was left at 37·0 ± 0·1 °C for 50 h and the protein binding was determined by equilibrium dialysis at 37·0 ± 0·1 °C. Radioactivity was assayed by liquid scintillation counting (LKB 1217) as described previously (Ehrnebo et al 1974).

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Distribution between plasma and blood cells. Blood was collected as described above and the haematocrit values were determined (Adams Autocrite Centrifuge, Clay-Adams, N.J., USA). The reversible distribution was studied by mixing blood (5 ml) with busulfan in acetone (0·01 ml) to give a final busulfan concentration of 200 ng ml^{-1}. The blood was incubated at 37·0 ± 0·1 °C for 0·5 h unless otherwise stated. The concentration of busulfan was determined in whole blood and in the separated plasma fraction after dilution 5 times with water by gc with ecd (Hassan & Ehrsson 1983).

The irreversible binding to blood cells was studied using [^{14}C]busulfan as described above for plasma. The blood was incubated for 20 h at 37·0 ± 0·1 °C and the blood cells were washed six times with isotonic phosphate buffer, pH 7·35. The cells were finally suspended in the buffer to restore the initial volume of the whole blood. The radioactivity was corrected for the amount of busulfan that did not decompose during the incubation procedure (about 20%).

Degradation studies. Busulfan was mixed with whole blood, plasma and plasma water (prepared by ultrafiltration of plasma) to give an initial busulfan concentration of 100 ng ml^{-1}. The mixture was incubated at 37·0 ± 0·1 °C. At appropriate times, aliquots were separated and analysed by gc with ecd (Hassan & Ehrsson 1983).

Results and discussion

Busulfan is an alkylating agent which is supposed to react by an S_N2 mechanism (Ross 1953). Due to its alkylating activity the compound might be bound both reversibly and irreversibly (covalently) to blood components. The reversible binding of busulfan to plasma proteins was studied by equilibrium dialysis at a concentration range which is clinically relevant (Ehrsson et al 1983). The chemical degradation of busulfan in the dialysis system was minimized by performing the study at 25 °C. The equilibrium between plasma and buffer compartments was obtained after about 4 h. Less than 10% of busulfan decomposed within 5 h in the dialysis system. The degree of protein binding was insignificant in both healthy subjects (3·3 ± 3·1%, s.d., n = 6) and in patients with chronic myelocytic leukaemia (2·7 ± 2·5%, s.d., n = 7) and the protein binding was unaffected by the concentration of busulfan in the

range studied (20–200 ng ml^{-1}). Busulfan differs in this respect from alkylating agents as chlorambucil (Ehrsson et al 1980) and melphalan (Ehrsson & Lönroth 1982) where a considerable fraction in plasma is bound to proteins (99 and 69% respectively).

The irreversible binding of busulfan was investigated by adding [^{14}C]busulfan to plasma followed by incubation at 37 °C for 50 h (busulfan remaining unchanged <10%). The binding of busulfan was then evaluated by equilibrium dialysis. Due to the low specific activity of the radiolabelled busulfan it was necessary to use a high concentration (25 μg ml^{-1}). The percentage of busulfan bound to plasma ligands was 32·4 ± 2·2 (s.d., n = 5, healthy volunteers). This figure must represent busulfan covalently bound since control experiments comprising equilibrium dialysis of [^{14}C]busulfan completely degraded in plasma water against plasma revealed that the degradation products did not bind to plasma.

Furthermore, the major portion of busulfan must be bound to albumin since studies using human albumin dissolved in plasma water (45 mg ml^{-1}) gave an irreversible binding of 28·4 ± 0·4% (s.d., n = 6). The degree of binding in plasma is in general agreement with the value obtained (22%) from calculations based on the pseudo first-order degradation rate constants in plasma (0·055 ± 0·002 h^{-1}, s.e., n = 10, 37 °C) and plasma water (0·043 ± 0·003 h^{-1}, s.e., n = 10, 37 °C) according to the equation:

$$F_r = \frac{k_p - k_{pw}}{k_p} \quad (1)$$

where F_r is the fraction of busulfan that reacts with plasma proteins and k_p and k_{pw} are the pseudo first-order rate constants for the degradation of busulfan in plasma and plasma water respectively. However, it should be pointed out that the value obtained by equation 1 is uncertain since the numerator represents a difference between two large numbers and no correction has been made in k_{pw} for the volume occupied by the plasma proteins.

The reversible distribution of buslfan in whole blood was studied by incubating the blood with busulfan (37 °C) followed by determination of the drug concentration in whole blood (C_b) and the separated plasma fraction (C_p). The distribution ratio (D) between the concentration in the blood cells and in the plasma can be expressed by the equation:

$$D = \frac{C_b - C_p(1 - H)}{H \cdot C_p} \quad (2)$$

where H is the haematocrit value.

The equilibrium between plasma and blood cells was rapidly obtained (<2 min). Busulfan was distributed evenly between blood cells and plasma (D = 1·05 ± 0·20, s.d., n = 12). There was no statistically significant difference between the distribution ratios obtained at 37 and 25 °C. The fraction of busulfan in plasma (λ_p) was calculated by the equation (Ehrnebo et al 1974):

$$\lambda_p = \frac{C_p(1 - H)}{C_b} \quad (3)$$

The relationship between λ_p and haematocrit values was studied in healthy subjects and in cancer patients (Fig. 1) and shows a variation of λ_p in the range 0·71–0·51 (haematocrit: 0·28–0·48). The precision in the determination of λ_p was about 7% (cv). The large proportion of busulfan in blood cells may have consequences for the pharmacokinetics of the drug in situations where the blood cells counts are pathologically high or low.

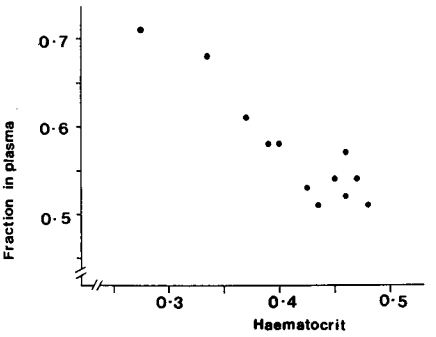

FIG. 1. Relationship between haematocrit values and busulfan fraction in plasma.

The irreversible binding of busulfan to blood cells was studied after degradation of [^{14}C]busulfan in whole blood (haematocrit: 0·43–0·48) at 37 °C followed by extensive washing of the cells and measurements of the radioactivity retained. The fraction of busulfan irreversibly bound to the blood cells was 46·9 ± 3·5% (s.d., n = 5). The fraction of busulfan that reacts with the blood cells (F_r) can be expressed by equation:

$$F_r = \frac{k_b - k_p(1 - H)}{k_b} \quad (4)$$

where k_b and k_p are the pseudo first-order rate constants for the degradation in blood and plasma, respectively. Substituting k_b = 0·080 ± 0·002 h^{-1} (s.e., n = 10, 37 °C), k_p = 0·055 ± 0·002 h^{-1} (s.e., n = 10, 37 °C) and H = 0·45 gives F_r = 0·62. Thus, it can be concluded that the major portion of busulfan that is degraded in whole blood reacts with the blood cells and that the main part of that binds to macromolecular components in the cells. The extensive irreversible binding of busulfan to plasma proteins and blood cells might explain the prolonged retention of radioactivity in plasma and blood observed after administration of radiolabelled busulfan (Nadkarni et al 1959; Vodopick et al 1969).

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Inhibition by diethylcarbamazine of acetylcholine-induced endothelium-dependent relaxation of rabbit aorta: are leukotrienes involved?

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Acetylcholine caused relaxations of preconstricted rabbit aortic strips if the endothelium was intact. These relaxations were reversed in a concentration-dependent manner by diethylcarbamazine, an inhibitor of slow-reacting substance and leukotriene formation. However, when exogenous leukotrienes (LTA_4, LTB_4, LTC_4 and LTD_4) were added to the precontracted arterial strips none of them caused relaxation, showing that they are unlikely to be involved as mediators in the acetylcholine-induced relaxation of rabbit aorta.

Acetylcholine (ACh) causes relaxations of isolated blood vessels by a mechanism dependent upon intact endothelial cells (Furchgott & Zawadzki 1980; Chand & Altura 1981). This effect of ACh is resistant to cyclooxygenase inhibition but sensitive to inhibitors of phospholipase A_2 and lipoxygenases (Furchgott & Zawadzki 1980; Chand & Altura 1981; Singer & Peach 1983; Förstermann & Neufang 1984). It has therefore been postulated that lipoxygenase products (hydroperoxy/hydroxy fatty acids or leukotrienes) might be mediators of the endothelium-dependent relaxation (Furchgott & Zawadzki 1980; Chand & Altura 1981).

Diethylcarbamazine has been shown to inhibit the synthesis of slow-reacting-substance or cysteinyl-leukotriene-like material in several systems (Orange et al 1971; Engineer et al 1978; Piper & Temple 1981; Mathews & Murphy 1982). We therefore tested the effect of this drug on the ACh-induced endothelium-dependent relaxation of rabbit aortic strips.

Methods

Helically cut strips of rabbit thoracic aorta (15 × 2 mm, about 30 mg wet wt) were set up in organ baths (3·5 ml) as previously described (Förstermann et al 1984), care being taken to avoid damage of the intimal surface. In some experiments the endothelium was removed by careful abrasion with a razor blade. The bath medium (Krebs-bicarbonate solution of the following composition (mM): NaCl 120·0, KCl 4·75, $NaHCO_3$ 25·0, KH_2PO_4 1·2, $MgSO_4$ 1·2, CaCl 1·7 and glucose 6·4) was changed every 12 min throughout. Changes in tissue length were recorded with an isotonic lever system. Drugs were added to the organ bath in a volume of 50 µl. Contractions of the strips were elicited at 1 h intervals with 10^{-7} M noradrenaline (NA). Seven min after NA, when a stable contraction plateau had been reached, different concentrations of ACh were added to the organ bath to produce relaxations. Different concentrations of diethylcarbamazine (Sigma, Munich, FRG) were always added to the bath medium 36 min before the next contraction–relaxation period. When the effect of leukotrienes (LTs) was tested, they were added to the organ bath instead of ACh (7 min after NA). LTA_4 was obtained as the methylester (Paesel, Frankfurt, FRG) and hydrolysed to the sodium salt with 1 mM NaOH pH 11·0. Since LTA_4 is unstable at neutral pH, the pH of the solution was readjusted to 7·4 5 s before adding it to the organ bath. In some experiments the stable LTA_4-methylester was given directly to the aortic tissue. LTB_4 (Paesel, Frankfurt, FRG) and LTC_4 and LTD_4 (both generous gifts of Dr J. Rokach, Merck-Frosst, Pointe-Claire - Dorval, Canada) were obtained and tested as free acids. All concentrations of drugs given refer to the free bases or acids respectively.

Results and discussion

ACh induced concentration-dependent relaxations of rabbit aortic strips that had been contracted by NA. The maximal effect was reached with 10^{-6} M (relaxation of

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