Metabolism of $^{14}$C-busulfan in isolated perfused rat liver

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SUMMARY

$^{14}$C-Busulfan gave one main metabolite in the isolated perfused rat liver during 4 hr cyclic perfusion. The cumulative bile excretion contained about 38% of the total radioactivity. About 1% of unchanged $^{14}$C-busulfan was excreted in the bile. The metabolite was identified as $\gamma$-glutamyl-$\beta$-(S-tetrahydrothiophenium) alanyl-glycine (sulfonium ion of glutathione) by $^{252}$Cf-plasma desorption time-of-flight mass spectrometry. The formation of the metabolite was drastically decreased when the glutathione-S-transferase was inhibited, which indicates that the major reaction of busulfan with glutathione is enzymatic in nature. The sulfonium ion was more stable in the perfusate ($t_{1/2}=22.4$ hr, $37^\circ$C) than in the bile ($t_{1/2}=3.2$ hr, $37^\circ$C) at pH 7.4.

INTRODUCTION

Busulfan, 1,4-bis(methanesulfonyl)butane, is an alkylating agent which has been in clinical use for the last 35 years. The drug is mainly used in the treatment of chronic myelogenous leukemia and polycythemia vera.

Studies in animals have suggested that busulfan reacts in vivo with thiol groups (1), and the reaction mechanism with different thiol groups has been studied in vitro (2-4). In addition, after administration of radiolabelled drug to rats, high concentrations of the radioactivity have been found in the well-perfused tissues, particularly in the liver, kidneys and lungs (5). Only scant information about the metabolic pathway of busulfan in man and animals is available due to the lack of suitable analytical methods for the determination of the drug and its metabolites in biological fluids.

In the present study, the metabolic fate of busulfan has been investigated in the isolated perfused rat liver during a cyclic perfusion. The metabolite has been identified by $^{252}$Cf-plasma desorption time-of-flight mass spectrometry and the stability of the metabolite has been investigated.

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MATERIALS AND METHODS

Chemicals

$^{14}$C-Busulfan was synthesized by the method described previously (6) with a specific activity of 1 mCi/m mole. The purity was 97-98% as established by gas chromatography with electron-capture detection (7). The internal standard, 1,5 bis-(methanesulfonyl)pentane, was prepared as described in a previous work (8). Bovine albumin fraction V was purchased from United States Corp. (Cleveland, Ohio). Ethacrynic acid was obtained from Merck Sharp & Dohme (Haarlem, The Netherlands). All other chemicals were of analytical grade and purchased from E. Merck (Darmstadt, F.R.G.).

Animals

Male Sprague-Dawley rats (300-400 g) were obtained from Anticimex laboratories (Sollentuna, Sweden).

The abdomen in the anaesthetized animal (I.P. sodium pentobarbitial, 50 mg/kg) was opened and the portal vein as well as the common bile duct were cannulated and tied. The liver was transferred to a perfusion chamber which was maintained at 37.0°C ± 0.1. The perfusate consisted of Krebs-Ringer bicarbonate containing 0.1% glucose and 4.5% bovine albumin. The pH was kept at 7.35-7.43 with NaOH (1M).
Perfusion with $^{14}$C-busulfan

The liver was perfused for 4 hr in a cyclic mode with 60 ml of perfusion solution. The perfusate was aerated with 95% O$_2$ and 5% CO$_2$. The flow rate through the liver was maintained at 25-30 ml/min. $^{14}$C-Busulfan (3-6 mg) in acetone/perfusate, 1:4 (v/v) was added after the liver had been equilibrated for 20-30 min. The principal indices of liver viability were steady oxygen consumption, sustained bile production and constant perfusion pressure as described in (9).

Radioactivity measurements

At timed intervals, samples (50 ul) were withdrawn from perfusate. Insta-Gel (10 ml, Packard, Switzerland) was added and the total radioactivity was determined by liquid scintillation (LS) LKB Model 1217 (Turku, Finland). The bile excretion was collected in an ice bath and the total radioactivity was measured by LS.

Inhibition of glutathione S-transferase (GSH-S-transferase)

Ethacrynic acid was added to the perfusate after the equilibration of the liver to give a final concentration of 0.2 mM. The perfusion was continued for 30 min and $^{14}$C-busulfan was subsequently added by the method described above.

Analysis of bile and perfusate

Samples (50 µl) from the perfusate or from the cumulative bile excretion were analyzed for busulfan by gas chromatography with electron capture detection as described previously (7).

Samples (50 µl) from bile excretion were filtered using a 22 µm Millipore filter (Molsheim, France) and analyzed by liquid chromatography (HPLC). After addition of trichloracetic acid (TCA) (10%, 250 µl), samples from the perfusate (250 µl) were centrifuged and 100 µl of the supernatant used for the analysis. The HPLC consisted of a constametric I pump (Milton Roy Co., Florida), a Rheodyne 7125 (Cotati, California) with a 100 µl loop, a refractometer ERC 7510 (Erma Inc., Japan), a radioactivity monitor Berthold LB 503 (Wildbad, F.R.G.), a gradient equipment LKB 11300 Ultragrad (Bromma, Sweden) and a Hibar RP-8 column (10 µm) 150 × 4.6 mm i.d. obtained from E. Merck (Darmstadt, F.R.G.). To follow the metabolites in bile or perfusate, the HPLC was run in the gradient mode. Solvents I and II were methanol and acetic acid $5 \times 10^{-2}$M containing $5 \times 10^{-3}$ M n-octyl sodium sulfate. The gradient was 0-100% solvent I using a linear gradient profile. The time course of the metabolite in perfusate was followed by the same HPLC system as described above operated in the isotatic mode. The mobile phase was 25% methanol in $5 \times 10^{-3}$M acetic acid containing $5 \times 10^{-3}$M n-octyl sodium sulfate with a flow rate of 0.7 ml/min.

Isolation and identification of the metabolite

The bile was filtered and analyzed by HPLC on a Partisil 10 (10 µm) column 200 × 4.6 mm i.d. The mobile phase was 0.1 M acetic acid and the flow rate was 1 ml/min. The radioactive peak was collected and the solvent was removed under reduced pressure at room temperature. The metabolite was identified by $^{252}$Cf-plasma desorption time-of-flight mass spectrometry as described in (10).

Synthesis of reference compound

$^{14}$C-Busulfan (2 mM) was added to phosphate buffer (pH = 8.25) containing glutathione (GSH) (1 mM). The mixture was kept at 50°C for 20 min and TCA (10%) was added to stop the reaction. HPLC, fraction collection and mass spectrometry was performed as described above.

The stability of the metabolite

Bile was collected and the pH was adjusted by either HCl (1 M) or NaOH (1 M) to give a pH in the range 2-10. The bile was incubated at 37.0°C ± 0.1. At timed intervals samples were removed and analyzed by HPLC. The metabolite peak was collected and the radioactivity was measured by LS. The stability of the metabolite was studied in the perfusate at pH 7.4 and 37.0°C ± 0.1.

RESULTS

A gradient HPLC analysis of the bile (Fig. 1) showed that busulfan is excreted into the bile as one major radioactive metabolite. Recovery of the radioactivity injected on to the column was better than 95%. The metabolite was non-retarded on a LiChrosorb RP-8 column using an aqueous mobile phase (0.1 M acetic acid), but had a capacity factor of 6.6 on a Partisil column with the same mobile phase which indicates the polar character of the metabolite. However, the retention time could be regulated on the LiChrosorb column when n-octyl sodium sulfate was added to the mobile phase as a counter ion (Fig. 2). The perfusate also contained only one radioactive peak which eluted with the same retention times as that found in the bile on the same HPLC systems as above. The radioactive