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Degradation of busulfan in aqueous solution

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Abstract: The influence of pH, phosphate buffer components and temperature on the degradation rate of busulfan was studied. The analysis was performed using gas chromatography with electron capture detection and reversed-phase liquid chromatography with radioactivity monitoring. The degradation rate of busulfan showed no pH dependence in the range pH 1.5–11 and increased at higher pH values. The degradation rate constant was 0.034 ± 0.001 h^{-1} (S.E.M.) for the degradation of busulfan in pure water and 0.45 ± 0.01 $h^{-1}M^{-1}$ (S.E.M.) for the reaction of busulfan with the hydroxide ion at 37°C. The reactivity of HPO_4^{-2} was six times higher than the reactivity of $H_2PO_4^{-1}$ towards busulfan. The hydrolysis products were identified as tetrahydrofuran and methanesulphonic acid by nuclear magnetic resonance spectroscopy.

Keywords: *Busulfan; aqueous degradation; influence of pH and buffer components; reversed-phase HPLC; radioactivity detection.*

Introduction

Busulfan, 1,4-bis(methanesulphonoxy) butane, is a bifunctional alkylating agent, which has been in clinical use for more than 30 years for the treatment of chronic myeloid leukemia. The drug differs from other alkylating agents such as the nitrogen mustards (chlorambucil and melphalan) in the mechanism of alkylation. Busulfan reacts according to an S_N2 mechanism, while chlorambucil and melphalan undergo S_N1 reactions [1, 2].

The degradation rate of busulfan in aqueous solution has been studied by several investigators [3–5] with conflicting results. The results concerning the structure of the degradation products in aqueous solution are also contradictory. Hudson *et al.* [3] have shown that 1,4-butanediol is the only hydrolysis product, while Feit and Rastrup-Andersen [6] have reported that tetrahydrofuran is formed. In both studies the liberation of methanesulphonic acid was reported.

The present study considers the stability of busulfan within the pH range 1.5–13.5 and the effect of phosphate buffer components and temperature on the rate of degradation. The quantitative analysis has been carried out using gas chromatography with electron capture detection and reversed-phase liquid chromatography with radioactivity monitoring. The hydrolysis products were identified by nuclear magnetic resonance spectroscopy.

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Experimental

Chemicals

Busulfan was obtained from EGA-Chemie, Steinheim, F.R.G. The internal standard, 1,5-bis(methanesulphonoxy) pentane, was prepared according to the method described in [7]. [1,4-^{14}C] Succinic acid (sp. act. 118 mCi/mmol) was obtained from Amersham International plc, Buckinghamshire, U.K. Deuterium oxide for NMR spectroscopy, tetrahydrofuran and 1,4-butanediol were obtained from Merck, Darmstadt, F.R.G. All other solvents and reagents were of analytical grade.

Apparatus

A Varian 3700 gas chromatograph equipped with electron capture detector (GC–ECD) and a Varian 1400 gas chromatograph equipped with flame ionization detector (GC–FID) were used. The GC–ECD analysis was carried out on a fused-silica capillary column as described in [8]. The standard curve using aqueous solution was linear within the range 10–400 ng/ml. Least squares analysis gave a slope of $2.12 \times 10^{-2} \pm 0.03 \times 10^{-2}$ (S.E.M.), an intercept of $4.7 \times 10^{-2} \pm 7.6 \times 10^{-2}$ (S.E.M.) and a correlation coefficient of 0.9997 (n=7). The column used in the GC–FID analysis (1.5 m × 2 mm i.d.) was packed with Carbowax 20 M (10%) on Gas Chrom Q (100–120 mesh). The instrument was operated with injector, detector and oven temperatures at 200°, 270° and 60°C, respectively.

The liquid chromatographic (LC) equipment consisted of a LiChrosorb RP-2 (5 μm), column (150 × 4 mm i.d.), a Constametric I pump and a Rheodyne injector (70–10) with a 100 μl loop. The mobile phase was methanol/water (3:7 v/v).

The detection was performed using a Berthold LB 503 radioactivity detector with a solid scintillator cell (50 μl).

The pH was measured with an Orion pH meter model 701, equipped with a glass combination electrode No. 91-03.

The nuclear magnetic resonance (NMR) spectra were obtained on a Jeol FX 200 (200 MHz). The instrument was operated at room temperature and a pulse of 45°.

Synthesis of [1,4-^{14}C] 1,4-*bis(methanesulphonoxy) butane* (^{14}C-*busulfan*)

Succinic acid (1 mmol) was mixed with [1,4-^{14}C] succinic acid (250 μCi) and the esterification of the acid to [1,4-^{14}C] diethyl succinate was carried out as described in ref. [9]. The ester was reduced to [1,4-^{14}C] butanediol using lithium aluminum hydride (10 mmol) in ether. The ether layer was separated and evaporated. The residue was dissolved in pyridine (5 ml). Methanesulphonic anhydride (10 mmol) in dichloromethane (15 ml) was added dropwise. The temperature was kept at 0°C during the reaction (40 min). The organic phase was washed with ice-cold sulphuric acid (0.1 M) and evaporated to dryness. ^{14}C-Busulfan was recrystallized from acetone/n-hexane (yield 70%), m.p. 118°C (lit.[3]118–119°C). ^{14}C-Busulfan was identified after conversion to [1,4-^{14}C] 1,4-diiodobutane as described in ref. [8].

Synthesis of [1,4-^{14}C] *tetrahydrofuran*

[1,4-^{14}C] Butanediol prepared as above was converted to [1,4-^{14}C] tetrahydrofuran as described in ref. [10]. The product eluted with the same retention time as a reference of tetrahydrofuran when analysed by GC–FID.

Degradation studies

Busulfan in acetone (0.1 ml) was mixed with phosphate buffer or sodium hydroxide (10 ml) to give a final busulfan concentration of 2.84×10^{-6} M. Samples were withdrawn at appropriate times and analysed by GC–ECD [8]. ^{14}C-Busulfan in acetone (0.1 ml) was mixed with 10 ml of distilled water, sulphuric acid (0.1 M) or sodium hydroxide (0.1 M) to give a final concentration of 6.10×10^{-6} M. The mixture was incubated at 37.0 ± 0.1°C and at appropriate times samples were withdrawn and analysed by LC with radioactivity monitoring. All studies were performed at 37.0 ± 0.1°C unless otherwise stated.

Identification of the hydrolysis products

A suspension of busulfan (5 mg/ml) in deuterium oxide (10 ml) was kept in a sealed tube under stirring at 60°C for 30 h to give complete degradation. The solution was analysed by NMR.

Results and Discussion

Hydrolysis of busulfan

The hydrolysis of busulfan in aqueous solution was studied by LC using ^{14}C-busulfan (Fig. 1). The time course for ^{14}C-busulfan and its labelled hydrolysis product in pure water is given in Fig. 2. The sum of busulfan and its hydrolysis product was 104% ± 5% (S.D., n=13) during the course of the reaction.

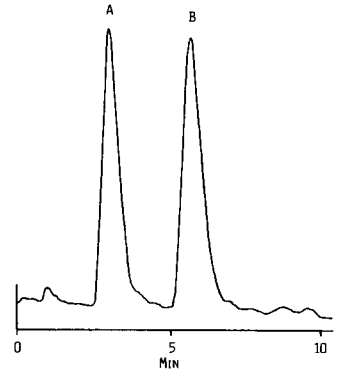

Figure 1
Chromatogram of ^{14}C-busulfan and ^{14}C-tetrahydrofuran. Column: LiChrosorb RP-2 (5 μm). Mobile phase: Methanol–water (3:7 v/v). Detection: A Berthold LB 503 radioactivity detector. Key: (A) ^{14}C-tetrahydrofuran; (B) ^{14}C-busulfan.

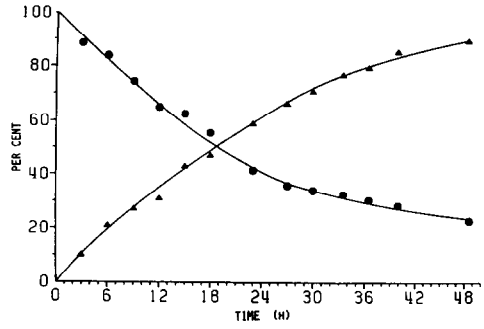

Figure 2
Time course for ^{14}C-busulfan and its labelled hydrolysis product. Temperature: 37.0 ± 0.1°C. The degradation of busulfan was carried out in distilled water. Key: ● Busulfan; ▲ ^{14}C-labelled hydrolysis product.

In sulphuric acid (0.1 M) and in sodium hydroxide (0.1 M), the sum of busulfan and its hydrolysis product was 105% ± 5% (S.D., n=14) and 104% ± 4% (S.D., n=12), respectively. The results show that busulfan has one main ^{14}C-labelled hydrolysis product. This compound has the same retention time as that obtained from ^{14}C-tetrahydrofuran. The identity of the degradation products obtained after complete hydrolysis of busulfan in deuterium oxide was established by NMR (Table 1). The methylene protons in the degradation product of busulfan gave the same shifts as those obtained from a reference of tetrahydrofuran. A signal (singlet) was also observed for the methanesulphonic acid protons. The addition of 1,4-butanediol to a sample containing hydrolysed busulfan gave two new peaks, which shows that tetrahydrofuran and methanesulphonic acid are the only hydrolysis products for busulfan. The same results have been reported by Feit and Rastrup-Andersen [6], who also discussed the hydrolysis mechanism and could isolate the intermediate (4-methanesulphonoxybutanol) which has a half life of about 12 min. However, no indication of the 1,4-butanediol reported by Hudson *et al.* [3], in the hydrolysis of busulfan was found here.

Table 1
NMR shifts [δ ppm]

Busulfan degradation products	(1) multiplet 1.2–1.3 4 H	(2) singlet 2.2 6 H	(3) multiplet 3.08–3.16 4 H
Tetrahydrofuran	(1) multiplet 1.2–1.3 4 H	(2) multiplet 3.08–3.16 4 H	
1,4-Butanediol	(1) multiplet 0.9–1.0 4 H	(2) multiplet 2.95–3.03 4 H	

The spectrum was obtained on a Jeol FX 200 (200 MHz) at room temperature, with 45° pulse and deuterium oxide as solvent.

Influence of pH on the degradation rate of busulfan

The observed rate constant, K_{obs}, for the degradation of busulfan in phosphate buffer solution can be expressed as:

$$K_{obs} = K_o + K_{H^+}[H^+] + K_{OH^-}[OH^-] + \Sigma K_B[B], \quad (1)$$

where K_o is the degradation rate constant in pure water, K_{H^+} is the second-order reaction rate constant for the proton-catalysed degradation and $[H^+]$ is the hydrogen ion concentration. The term K_{OH^-} represents the second-order reaction rate constant for the degradation catalysed by the hydroxide ion and $[OH^-]$ is the concentration of this ion. The last term in equation (1) is the sum of several terms representing the products of the second-order reaction rate constants for the degradation catalysed by each of the buffer components and $[B]$ is the concentration for each of these components. The pseudo-first order reaction rate constant at zero buffer concentration for different pH values was evaluated as the intercept from a plot of K_{obs} versus buffer concentration as illustrated in Fig. 3. The degradation rate of busulfan showed no pH dependence in the range pH 1.4–pH 11 and increased at higher pH values (Fig. 4). Since the rate of degradation is

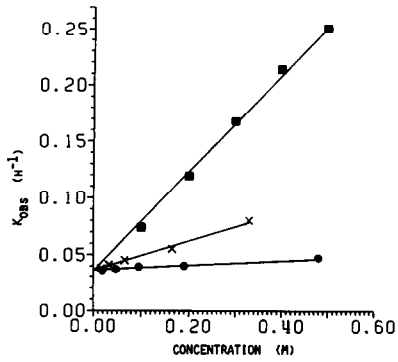

Figure 3
Influence of phosphate buffer concentration and hydroxide ion concentration on K_{obs}. Temperature: 37.0 ± 0.1°C. Key: ● pH 4.8; × pH 9.6; ■ sodium hydroxide.

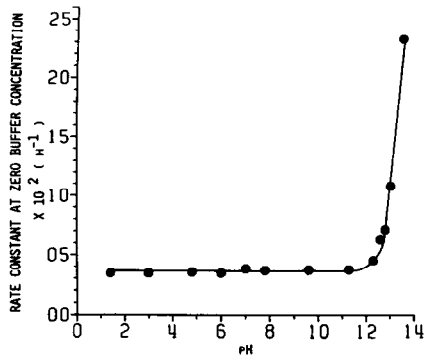

Figure 4
pH profile for the degradation rate of busulfan. Temperature: 37.0 ± 0.1°C.

unaffected by pH down to pH 1.4, the term K_{H^+} $[H^+]$ in equation (1) is $\ll K_o$ and can be neglected in the studied pH interval.

The second-order reaction rate constant for the reaction of busulfan with hydroxide ion (Table 2) was determined from the equation:

$$K_{obs} = K_o + K_{OH^-} [OH^-] \quad (2)$$

by plotting K_{obs} versus OH^- concentration (Fig. 3).

The ratio of the rate constant for the hydroxide ion and the rate constant for water (Table 1, corrected for water molarity) was 7.14×10^2. Hudson *et al.* [3] reported that the ratio was 5.26×10^3 when the reaction was carried out in acetone–water (50:50) at

Table 2
Rate constants for busulfan degradation at 37°C

Rate constant	$h^{-1} M^{-1}$ ± SEM
K_o*	0.034 ± 0.001
K_{OH^-}	0.45 ± 0.01
$K_{H_3PO_4}$	0.006 ± 0.005
$K_{H_2PO_4}$	0.022 ± 0.002
K_{HPO_4}	0.125 ± 0.009

* K_o unit is h^{-1}.

61°C. The difference is probably due to the presence of acetone in the reaction mixture, which should increase the relative reactivity of hydroxide ion [11, 12].

Influence of phosphate buffer components on the degradation rate of busulfan

The degradation rate of busulfan is affected by the phosphate buffer components as illustrated in Fig. 5. Equation (1) can be rewritten as:

$$K_{obs} = K_o + K_{OH^-}[OH^-] + K_{H_3PO_4}[H_3PO_4] + K_{H_2PO_4}[H_2PO_4^{-1}] + K_{HPO_4}[HPO_4^{-2}] + K_{PO_4}[PO_4^{-3}]. \quad (3)$$

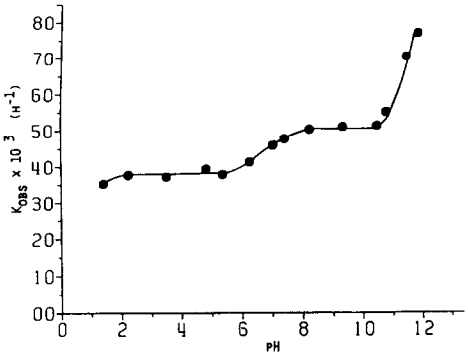

Figure 5
Influence of phosphate buffer components on the degradation rate constant of busulfan. Temperature: 37.0 ± 0.1°C. Phosphate buffer concentration: 0.095 M.

Phosphoric acid has pk_{a1} 2.21, pk_{a2} 7.18 (37°C) and pk_{a3} 12.3 (25°C) [13]. The influence of H_3PO_4 on the degradation rate of busulfan was studied at pH 1.4, where the buffer solution consists mainly of H_3PO_4. If the contribution from the other buffer compounds on the degradation rate can be neglected, equation (3) simplifies to:

$$K_{obs} = K_o + K_{H_3PO_4}[H_3PO_{4d}]. \quad (4)$$

No significant change in K_{obs} was observed when phosphoric acid concentration was varied in the range 1×10^{-2} to 2.5×10^{-1} M, which means that the term $K_{H_3PO_4}[H_3PO_4]$ is $\ll K_o$.

Phosphate buffer consists almost exclusively of $H_2PO_4^{-1}$ in the range $3.2 < pH < 6.2$ and of HPO_4^{-2} in the region $8.2 < pH < 11.3$.

As can be observed from Fig. 5, the rate of degradation is unaffected by pH in these intervals which implies that $H_2PO_4^{-1}$ and HPO_4^{-2}, respectively, are the major phosphate-reacting ions in these pH regions.

The second-order reaction rate constant for $H_2PO_4^{-1}$ was evaluated at pH 4.8, from the equation:

$$K_{obs} = K_o + K_{H_2PO_4}[H_2PO_4^{-1}] \quad (5)$$

by plotting K_{obs} versus the concentration of $H_2PO_4^{-1}$ (Fig. 3).

The second-order reaction rate constant for HPO_4^{-2} was determined at pH 9.6 (Fig. 3) from the equation:

$$K_{obs} = K_o + K_{HPO_4}[HPO_4^{-2}]. \quad (6)$$

Table 3
Influence of temperature on the observed degradation rate constant (K_{obs}) for busulfan

Temperature (°C)	K_{obs} (h^{-1}) ± S.E.M.
30	0.023 ± 0.003
37	0.043 ± 0.002
40	0.058 ± 0.004
51	0.183 ± 0.009
55	0.31 ± 0.03
60	0.39 ± 0.01

Phosphate buffer (0.05 M) pH 7.00.

The values of $K_{H_2PO_4}$ and K_{HPO_4} are given in Table 2, which shows that HPO_4^{-2} ions are about six times more reactive towards busulfan than $H_2PO_4^{-1}$. The evaluation of K_{PO_4} was not possible, since pk_{a3} for phosphoric acid is 12.3 and in this region the reaction with hydroxide will dominate.

Influence of temperature

The influence of temperature on the degradation rate of busulfan in phosphate buffer pH 7.00 is given in Table 3. An Arrhenius plot of the data gave an activation energy of 80 kJ/mol.

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