Tissue and Perfusate Pharmacokinetics of Melphalan in Isolated Perfused Rat Hindlimb

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ABSTRACT
Melphalan is commonly used as a cytotoxic agent in isolated limb perfusion for locally recurrent malignant melanoma. The time course of melphalan concentrations in perfusate and tissues during a 60-min melphalan perfusion and 30-min drug-free washout in the single-pass perfused rat hindlimb was examined using a physiologically based pharmacokinetic model. The rat hindlimbs were perfused with Krebs-Heinseleit buffer containing 4.7% bovine serum albumin (BSA) or 2.8% dextran 40 at a constant rate of 3.8 ml/min. The concentration of melphalan in perfusate and tissues was determined by high-performance liquid chromatography. The tissue concentrations of melphalan were significantly higher with the perfusate containing dextran than BSA during the 60-min perfusion. During the washout period, the melphalan concentration in the perfusate decreased rapidly in first few minutes, followed by a slower monoexponential decline. The estimated half life (t1/2) for melphalan removal from skin and fat was 59 ± 2 min for both BSA and dextran perfusates. However, the estimated t1/2 for melphalan removal from muscle was 79 and 96 min for BSA and dextran washout perfusates, respectively. The predicted concentration-time profiles obtained for melphalan with BSA and dextran perfusates appear to correspond closely to the observed data. This study showed that the uptake of melphalan into perfused tissues is impaired by the use of perfusates in which melphalan is highly bound. Melphalan washout from muscle, but not skin and fat, was facilitated by the use of perfusates in which melphalan is highly protein bound.

ILP is a form of regional chemotherapy for cancer that involves the exposure of a tumour-bearing limb to a high concentration of anticancer agent, although sparing the patient from systemic toxicity (Ghussen et al., 1989; Kremetz et al., 1985; Kremetz et al., 1987; Scott et al., 1992a, Thompson and Gianoutos, 1992). Creech et al. (1958) first used the isolated cytotoxic limb perfusion technique to treat a patient with multiple superficial recurrences of melanoma localised to the leg. The efficacy and safety of the procedure has improved considerably and ILP is now widely accepted as the treatment of choice for locally recurrent melanoma restricted to the limbs, with response rates of 50 to 80% having been reported (Hartley and Fletcher, 1987; Baas et al., 1988; Kremetz et al., 1987; Scott et al., 1992a, Hafstrom et al., 1991; Thompson et al., 1994). ILP has also been successfully used in treatment of sarcomas of the extremities (Englund et al., 1971; Lejeune et al., 1988; Stehlin et al., 1984; Di Filippo et al., 1988; Kettelhack et al., 1990). The drug of choice in ILP for melanoma is melphalan. Although this drug has been in use for many years, an understanding of the pharmacokinetic behaviour of the melphalan in the perfused tissues, on which an optimal dosage and treatment schedule could be based, has not been available (Hafstrom et al., 1984; Briele et al., 1985; Benchhuysen et al., 1986; Scott et al., 1992b).

Benchhuysen et al. (1988) estimated the time course of tissue levels of melphalan during ILP using a biexponential model for the perfusate melphalan concentration versus time curves. Three previous studies (Scotter et al., 1987; Scott et al., 1992a; Klaase et al., 1994) have reported melphalan concentrations in the limb perfusate and tissues during ILP. However, the tissue concentrations of melphalan in these studies were generally lower than those calculated by Benchhuysen et al. (1988) who also suggested that the uptake of melphalan into the normal tissues during ILP greatly exceeded uptake by the tumor tissue. Quantitative data on the amount of drug taken up by limb tissues during perfusion for different perfusate conditions appears undefined but relevant in determining the optimal composition of perfusate to be used in ILP.

Leakage of drug-containing medium into the systemic circulation after ILP is unavoidable, even if limited in extent.

ABBREVIATIONS: PK, pharmacokinetics; IPRH, isolated perfused rat hindlimb; ILP, isolated limb perfusion; t1/2, fraction unbound; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; RBC, red blood cell.
(Hafstrom et al., 1984; Lejune and Ghanem, 1987). Systemic melphalan concentrations are about 1 to 4% of peak concentrations in perfusate (Hafstrom et al., 1984; Minor et al., 1985; Scott et al., 1992b). Rauschecker et al. (1991) suggested that between 1.4 and 18% of the total melphalan dose reaches the systemic circulation after reconnection. They suggested that a careful washing-out procedure was needed to ensure that the unwanted escape of melphalan into the systemic circulation was minimised (Rauschecker et al., 1991; Scott et al., 1992b). This washout procedure is undertaken after disconnection of the limb from the perfusion circuit, with removal of the melphalan-containing perfusate by a single-pass perfusion using drug-free perfusate. The washout solutions that have been used are of variable compositions and include plasma expanders mixed with electrolyte solutions (Rauschecker et al., 1991), Ringer’s lactate (Scott et al., 1992a&b) and dextran (Krementz et al., 1987; Egerton, 1982). Currently, there is no pharmacokinetic data describing the kinetics of melphalan removal during the washout period.

In our study, we have investigated the tissue and perfusate concentrations of melphalan during and after melphalan perfusion in the IPRH model (Wu et al., 1993). Given that melphalan can exhibit variable protein binding in the perfusate (Wu et al., 1995a), we examined whether melphalan perfusate binding was a determinant of melphalan tissue concentrations during ILP and washout. An overall aim of this study was to examine whether melphalan would be best administered with a perfusate containing either albumin or dextran during ILP and similarly for melphalan removal during the washout period. We therefore quantified tissue and perfusate concentrations of melphalan using BSA and dextran perfusates during both perfusion and washout period. This data enabled us to design a physiologically based pharmacokinetic model which may assist in understanding the processes of melphalan distribution and removal during ILP.

**Methods**

**Rat Hindlimb Perfusion Preparation**

The details of the single-pass rat hindlimb perfusion system have been described previously (Wu et al., 1993, 1995b). Briefly, rats (male Wistar, 320 ± 20 g) were anaesthetized, the abdomen opened and the right femoral artery cannulated (PE50) via the dorsal aorta. A second cannula (PE205) was placed in the dorsal vena cava, and the hindlimb perfused in a humidicrib with oxygenated (95% O2/5% CO2) Krebs-Henselit buffer (pH 7.4, 37°C), containing 4.7% BSA (Fraction V, Sigma Chemical Co., Sydney, Australia) or 2.8% dextran 40 (Sigma Chemical Co.). The oncostic pressures of perfusate containing 4.7% BSA or 2.8% dextran 40 (2.7, with 1-octanesulfonic acid added at a concentration of 50 mg/100 ml). The flow rate was 2 ml/min and the injection volume 20 μl.

Tissue samples (100 mg) were minced using scissors and suspended in 200 μl of methanol. The mixture was sonicated, on ice, for 1 min using an ultrasonic microtip and centrifuged at 10,000 × g for 15 min. The supernatant was removed and 20 μl injected into the HPLC system. The limit of quantitation of this assay for melphalan was 7.2 ng for tissue sample on column (Wu et al., 1995a).

**Determination of Melphalan Concentrations**

Melphalan concentrations in perfusate and tissue samples were analyzed by a HPLC assay previously developed in our laboratory (Wu et al., 1995a). Briefly, perfusate samples were analysed following methanol precipitation (100 μl sample with 200 μl methanol containing internal standard, dansyl-arginine), using a phenyl column and fluorescence detection. The detector was programmed to 265 nm excitation and 360 nm emission for melphalan and 265 nm excitation and 575 nm emission for the internal standard. The mobile phase consisted of methanol-water-glacial acetic acid (25:75:2, v/v/v), pH 2.7, with 1-octanesulphonic acid added at a concentration of 50 mg/100 ml. The flow rate was 2 ml/min and the injection volume 20 μl.

**Experimental Procedures**

**Methods**

Before each experiment, melphalan powder (donated by Welcome Australia) was dissolved in HPLC-grade methanol to give a stock solution of 1 mg/ml. Melphalan solution (15.26 μg/ml) was prepared in the perfusate containing 4.7% BSA or 2.8% dextran 40 immediately prior to melphalan perfusion. Rat hindlimbs were perfused with melphalan perfusate for 60 min, followed by a drug-free either BSA or dextran perfusate washout for 30 min. Perfusion inflow and outflow samples (0.5 ml) were taken at 0, 4, 8, 16, 20, 30, 40, 50, 60, 61, 62, 65, 70 and 90 min. Additional groups of rats were also sacrificed at various times (2, 5, 10, 15, 20, 30, 60, 70 and 90 min) during melphalan perfusion and hindlimb dissected. The perfused rat hindlimb was depilated with commercial Nair hair-removal cream and tissue samples (skin, fat and muscle) (500 mg) were taken in duplicate from selected points in the hindlimb for melphalan analysis.

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**Determination of Tissue Vascular Space**

To calculate the vascular space in each tissue of the perfused rat hindlimb, 125I-labeled albumin was mixed with melphalan in the perfusate of the IPRH. After 60 min perfusion, assuming the albumin had reached steady-state, an inflow sample was taken to measure the dpm/ml of albumin in the perfusate (C_{perfusate}). The tissue samples (skin, muscle and fat) were taken to determine the concentration of albumin (dpm) per g of tissue (C_{tissue}). The total weight (g) of each tissue type (Wt) present in the perfused limb was then determined by complete dissection. The skin hair was removed by the commercial Nair hair-removal cream. The quantity of 125I-labeled albumin was determined in a Cobra II gamma-counter (Packard, Meriden, CT). The vascular space of each tissue (V_p) (ml) in the perfused rat hindlimb was then determined from the following formula:

\[
V_p = \frac{C_{tissue}}{C_{perfusate}} \times Wt
\]

**Determination of Tissue Blood Flow**

The blood flow in skin, muscle and fat during perfusion at flow rate of 3.8 ml/min in the IPRH were determined using the radiolabeled microsphere (51Cr, 10 μl) method described in an earlier study (Wu et al., 1995b). The total blood flow to each tissue type in the perfused hindlimb was assumed to equal the blood flow of tissue (ml/min/g of tissue) multiplied by the weight of each tissue type in the perfused hindlimb.
Theoretical Section

Model development. The physiological pharmacokinetic model proposed to describe melphalan distribution into the perfused tissues (skin, fat and muscle) after isolated perfused rat hindlimb is presented in figure 1. Each tissue can be described as a single compartment, connected to a single central plasma compartment (fig. 1). The differential mass balance equations can then be written for four compartments assuming: 1) a pseudo equilibrium exists between a given tissue and the perfusate in the hindlimb; 2) each tissue acts as a well-stirred compartment; 3) lateral spread of melphalan from one tissue to another tissue is negligible; 4) each tissue is a noneliminating organ, except for hydrolysis, where the rate constant in each tissue is the same.

Given that a monoexponential decline was observed in the concentration of melphalan due to hydrolysis in inflow perfusate solution (fig. 2), the concentration of melphalan before reaching the perfused rat hindlimb at any time is defined by

\[ C = C_0 \exp(-K_h t) \] (2)

The rate of amount change of melphalan in the perfusate \( (dA_p/dt) \) at any time during the perfusion period \( (t \leq 60) \) is defined by

\[ \frac{dA_p}{dt} = C_0 Q \exp(-K_h t) - \frac{f_{fu}}{V_p} (PS_s + PS_m + PS_f) A_p + \frac{f_{fu}}{V_{ts}} PS_s A_{ts} \]

\[ + \frac{f_{ts}}{V_{ts}} PS_m A_{ts} + \frac{f_{fu}}{V_{tf}} PS_f A_{tf} - QC_p \] (3)

where \( A_p \) is the amount of melphalan in perfusate, \( A_s, A_m, \) and \( A_f \) are the amounts of melphalan in skin, muscle and fat; \( PS_s, PS_m, \) and \( PS_f \) are the permeability-surface area products for the movement of melphalan from the perfusate into skin, muscle and fat, respectively; \( V_p \) is the vascular space in whole hindlimb; \( V_{ts}, V_{ts} \), and \( V_{tf} \) are the apparent distribution volume of melphalan in each tissue and \( f_{ts}, f_{ts}, \) and \( f_{fu} \) are fractions unbound of melphalan in perfusate and each tissue, respectively. \( C_0 \) is the initial concentration of melphalan in the perfusate reservoir, \( K_h \) is the melphalan hydrolysis rate constant in the perfusate and \( Q \) is the perfusion flow rate (ml/min).

Equation 3 can also be expressed in terms of the rate of change of melphalan concentration in the perfusate with time \( (dC_p/dt) \):

\[ \frac{dC_p}{dt} = C_0 \frac{Q}{V_p} \exp(-K_h t) - \frac{f_{fu}}{V_p} (PS_s + PS_m + PS_f) C_p + \frac{f_{ts}}{V_{ts}} PS_s C_{ts} \]

\[ + \frac{f_{ts}}{V_{ts}} PS_m C_{ts} + \frac{f_{fu}}{V_{tf}} PS_f C_{tf} - QC_p \]

During the 30-min drug-free washout period after melphalan perfusion \( (t > 60) \), the input concentration of melphalan was effectively zero, and equation 4 reduces to:

\[ \frac{dC_p}{dt} = \left( \frac{f_{ts}}{V_{ts}} PS_s + f_{ts} PS_m \right) C_{ts} + \left( \frac{f_{ts}}{V_{tf}} PS_f \right) C_{tf} - QC_p \] (5)

The change in the amount of melphalan in the perfused skin with time can be expressed as

\[ \frac{dA_s}{dt} = f_{fu} PS_s A_p - f_{ts} PS_s A_{ts} - KS_m A_s \] (6)

where \( A_p = C_0 V_p \) and \( KS_m = \) is the rate constant of melphalan hydrolysis, and thus equation 6 can be modified to:

\[ \frac{dA_s}{dt} = f_{fu} PS_s C_p - f_{ts} PS_s A_{ts} - KS_m A_s \] (7)

The corresponding expressions for the change in the amount of melphalan with time in perfused muscle and fat are defined by equations 8 and 9:

\[ \frac{dA_m}{dt} = f_{fu} PS_m C_p - f_{ts} PS_m A_{ts} - KS_m A_m \] (8)

\[ \frac{dA_f}{dt} = f_{fu} PS_f C_p - f_{ts} PS_f A_{tf} - KS_m A_f \] (9)

Data analysis. The concentration of melphalan in the inflow perfusate at time zero is defined as \( C_0 \). The rate constant of melphalan hydrolysis in inflow perfusate was calculated from a semilogarithmic plot of concentration versus time (equation 2). The total amount of melphalan in the perfused perfusate was calculated from the concentration of melphalan in each tissue \((\mu g/g \text{ tissue})\) multiplied by the weight of each tissue. The total vascular space was taken as the sum of the estimated vascular spaces for skin, muscle and fat.

The nonlinear regression program MINIM V3.08 (Shen et al., 1989), was used to numerically integrate four differential equations defined by the sum of equations 4 and 5, together with equations 7, 8 and 9 to fit the melphalan data obtained from the outflow perfusate, skin, muscle and fat using weighted \((1/y_{obs})\) least squares with the Hartley modification of the Gauss-Newton algorithm. The final model fitting was deemed acceptable on the basis of the regression goodness-of-fit criteria that included the Akaike information criteria (AIC) (Landlaw and Distefano, 1984), a lack of systemic deviations in...
the residuals, and the percentage of data accounted for by the regression ($R^2 > 0.99$).

Simulations of the model were performed using the equations defined earlier for the nonlinear regression of eqs 4, 5, 7, 8 and 9, with varying fractions unbound of melphalan in the perfusate (0.01–1), corresponding to possible perfusates of plasma, dextran, red blood cell solution and other solutions. The perfusate and tissue profiles were also simulated for variable amount of melphalan hydrolysed in tissues ($K_m$ from 0–0.1). In addition, the simulations were used to estimate the speed at which melphalan could be removed from each tissue during washout.

Each observation is the mean ± S.D. of three or four determinations. Statistical analyses were carried out using Student’s $t$ test for two groups and analysis of variance and Tukey test for more than two groups. Statistical significance was accepted at $P < .05$.

Results

Tissue vascular volume and blood flow. Individual perfusate flow rates in skin, muscle and fat determined in the rat hindlimb by perfusion with microspheres and perfused tissue vascular volumes determined by $^{125}$I albumin perfusion are given in table 1. The volume and blood flow of each tissue were in the order of muscle > skin > fat, the vascular volume ratio of skin to fat and muscle to fat were 6.7 and 21.4, respectively, in correspondence with perfusate flow ratios of skin/fat and muscle/fat of 7.8 and 23.0, respectively.

Perfusate kinetics of melphalan. Melphalan hydrolysis in the perfusate followed an apparent monoexponential decline (fig. 2) with a hydrolysis rate constant of $0.011 ± 0.001$ (4.7% BSA perfusate) or $0.011 ± 0.0004$ min$^{-1}$ (2.8% dextran 40 perfusate).

Figures 3A and 4A show the outflow profiles of melphalan concentration obtained using both BSA and dextran perfusate and dextran perfusate during melphalan perfusion. Also shown in figures 3A and 4A are the washout profiles obtained from the drug-free perfusate during removal of melphalan from IPRH. It was observed that the melphalan concentration in both perfusates (BSA and dextran) decreased rapidly during the first few minutes of the washout period, followed by a slower monoexponential decline. The estimated half-life for melphalan removal during this latter washout phase was 71 ± 9, and 86 ± 12 min, for BSA and dextran perfusates respectively.

Distribution and pharmacokinetics of melphalan in perfused tissues. The total amount of melphalan in skin, muscle and fat in the IPRH increased with the time during melphalan (15.26 µg/ml) perfusion. The relative amounts of melphalan in each tissue was in the order: muscle > skin > fat for both BSA and dextran perfusions (figs. 3 and 4). A comparison of figures 3 and 4 shows that the amount of melphalan in each tissue was significantly higher using dextran perfusate than using BSA perfusate ($P < .05$). The amount of melphalan washed out from skin and fat using drug-free perfusate was characterized by an estimated apparent $t_{1/2}$ of 59 ± 2 min for both BSA and dextran perfusate (fig. 5A). However, the amount of melphalan removal from muscle was much slower and characterized by an estimated $t_{1/2}$ of 79 and 96 min for both BSA and dextran washout perfusates respectively (fig. 5A). The melphalan concentration remaining in the tissues after 30 min washout with dextran drug-free perfusate was slightly higher but not significantly different from a washout with BSA perfusate after 60 min melphalan perfusion with 4.7% BSA perfusate ($P > .05$) (fig. 5B). A similar result was obtained following perfusion of melphalan for 60 min using 2.8% dextran 40 perfusate ($P > .05$) (fig. 5C).

The nonlinear regression of experimental data with equations 4, 5, 7, 8 and 9 yielded predicted concentrations or amounts of melphalan in perfusate and each tissue which corresponded closely to the observed data (figs. 3 and 4). The percentage of data accounted for by the regression, $R^2$, for melphalan in both BSA and dextran perfusate exceeded 0.99. Table 2 shows the estimated parameters in the pharmacokinetic model for melphalan disposition for both BSA and dextran perfusates respectively (fig. 5A). The melphalan concentration remaining in the tissues after 30 min washout with dextran drug-free perfusate was slightly higher but not significantly different from a washout with BSA perfusate after 60 min melphalan perfusion with 4.7% BSA perfusate ($P > .05$) (fig. 5B). A similar result was obtained following perfusion of melphalan for 60 min using 2.8% dextran 40 perfusate ($P > .05$) (fig. 5C).

![Fig. 3. Concentration or amount-time profiles for melphalan (•) in the outflow perfusate (A), skin (B), muscle (C) and fat (D) during 60 min melphalan perfusion followed by 30-min drug-free washout with 4.7% BSA containing perfusate. The solid line is the prediction from the model shown in figure 1, according to equations 4, 5, 7, 8 and 9. Values are reported as mean ± S.D. (n = 3).](image-url)

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td><strong>The perfused hindlimb tissue vascular space and blood flow during isolated perfused rat hindlimb with perfusion flow rate of 3.8 ml/min (n = 4)</strong></td>
</tr>
<tr>
<td><strong>Skin</strong></td>
</tr>
<tr>
<td><strong>Blood flow (ml/min)</strong></td>
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<tr>
<td><strong>Vascular volume (ml)</strong></td>
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in albumin perfusate is 0.52. Comparable profiles were observed for the simulated, nonlinear regression and the experimental data for both perfusate and tissues (fig. 4).

**Discussion**

This work has shown that melphalan concentrations differ significantly in muscle, skin and fat after perfusion in the IPRH. Scott *et al.* (1992a), in measuring the concentration of melphalan in skin and fat during in ILP, had previously shown a significantly lower melphalan concentration in fat (1.48 mg/g) than in skin (3.83 mg/g). Klaase *et al.* (1994) reported that the uptake of melphalan into muscle (6.5 mg/g) was higher than into skin (3.2 mg/g) after ILP. Our results are consistent with these reports in that the melphalan concentration in each tissue after melphalan perfusion with 4.7% BSA perfusate were of the order: muscle (18.38 mg/g) > skin (12.94 mg/g) > fat (9.76 mg/g). The differences in melphalan tissue concentration in this study compared to earlier work in humans arises in part from the use of the constant infusion input in this study as distinct from the bolus input used in the previous studies (Scott *et al.*, 1992a; Klaase *et al.*, 1994).

In the IPRH, the amount melphalan in each tissue increased during the time of perfusion (60 min) (figs. 3 and 4). The physiological pharmacokinetic model proposed appears to adequately describe the amount of melphalan in each tissue during this perfusion period. The only other data on melphalan tissue concentration-time profiles in a single subject have been reported by Benckhuijsen *et al.* (1988). However, these authors deduced, rather than measured, the tissue concentrations using a biexponential representation of the melphalan perfusate concentration-time data obtained during 60 min ILP after an initial bolus input of melphalan. They suggested a peak tissue concentration of melphalan at 15 min and the model assumed a single homogeneous tissue compartment in the ILP. In present work, melphalan concentrations had not reached steady-state during the 60 min infusion. This result is consistent with a report by Thompson *et al.* (1995), who used a noncompartmental moment analysis to show that the mean residence time of melphalan is 43 to 51 min during ILP.

A limitation in the analysis of data shown in figures 3 and 4 is the use of a compartmental model, in which each plasma or tissue compartment is regarded as being well-stirred (Wagner, 1988). The model is consistent with the physiologically based pharmacokinetic model developed by Bischoff and Dedrick (1968) which depicts the body as a number of well-stirred vascular compartments representing individual
TABLE 2
Pharmacokinetic parameters of melphalan during isolated rat hindlimb perfusion using 4.7% BSA or 2.8% dextran perfusate

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BSA perfusate</th>
<th>Dextran perfusate</th>
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<tbody>
<tr>
<td>Q (ml/min)</td>
<td>3.67</td>
<td>3.67</td>
</tr>
<tr>
<td>( C_0 ) (\mu g/ml)</td>
<td>15.26 ± 0.29</td>
<td>15.33 ± 0.21</td>
</tr>
<tr>
<td>( V_f ) (ml)</td>
<td>0.66</td>
<td>0.66</td>
</tr>
<tr>
<td>( K_u ) (min(^{-1}))</td>
<td>0.010 ± 0.0010</td>
<td>0.010 ± 0.0010</td>
</tr>
<tr>
<td>( PS_i ) (ml/min)</td>
<td>0.62 ± 0.05</td>
<td>0.72 ± 0.01</td>
</tr>
<tr>
<td>( f_{ut}/V_t ) (ml(^{-1}))</td>
<td>0.030 ± 0.0030</td>
<td>0.030 ± 0.0030</td>
</tr>
<tr>
<td>( f_{ut}/V_t ) (ml(^{-1}))</td>
<td>1.74 ± 0.10</td>
<td>1.71 ± 0.09</td>
</tr>
<tr>
<td>( f_{ut}/V_t ) (ml(^{-1}))</td>
<td>0.010 ± 0.0010</td>
<td>0.010 ± 0.0010</td>
</tr>
<tr>
<td>( PS_i ) (ml/min)</td>
<td>0.090 ± 0.020</td>
<td>0.10 ± 0.020</td>
</tr>
<tr>
<td>( f_{ut}/V_t ) (ml(^{-1}))</td>
<td>0.18 ± 0.06</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>( f_{ut}/V_t ) (ml(^{-1}))</td>
<td>0.52 ± 0.04*</td>
<td>0.87 ± 0.07*</td>
</tr>
<tr>
<td>( K_m ) (min(^{-1}))</td>
<td>0</td>
<td>0</td>
</tr>
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\(^*\) Wu et al., 1995a.

As an alternative to \( PS \) and \( f_{ut}/V_t \), the rate constant of influx in each tissue could also be expressed as \( K_{21i} = (f_{ut}/V_t) \cdot PS_i \) and the rate constant of efflux from each tissue by \( K_{12i} = (f_{ut}/V_t) \cdot PS_i \), where \( i \) represents each tissue. Whereas \( PS_i \) and \( f_{ut}/V_t \) are not significantly different between 4.7% BSA and 2.8% dextran 40 perfusate, for the \( K_{21i} \), the dextran 40 perfusate is significantly higher than the BSA perfusate. The differences in \( K_{21i} \) arise from the presence of \( f_u \) in this parameter. The fraction of melphalan unbound in the dextran perfusate (0.87 ± 0.10) is significantly higher than in BSA perfusate (0.52 ± 0.04) perfusate. In contrast, \( f_u \) is not a component of \( K_{21i} \) and similar values for \( K_{21i} \) were obtained for perfusates containing BSA and dextran.

By definition (equations 7, 8 and 9), the amount of melphalan in each tissue is related to the melphalan of \( f_u \) in perfusate, \( PS \) product and \( f_{ut}/V_t \). The change of \( f_u \) in perfusate will therefore affect melphalan uptake into perfused tissues. The outcome is that a perfusate with low binding capacity for melphalan will facilitate melphalan uptake into the perfused tissues.

Rauschecker et al. (1991) reported that the majority of melphalan in the systemic circulation appeared immediately after reconnection of the vasculature when ILP finished, and that it is of major importance in the consideration of melphalan induced systemic side effects. Melphalan from the perfused limb tissues and vasculature will wash into the systemic circulation after reconnection of the ILP. At the end of the 60-min melphalan perfusion in the IPRH, the vascular space contained only 1.1% of the melphalan remaining in the tissues (figs. 3 and 4). In addition, whereas the perfusate is rapidly cleared of melphalan during first few min of the washout (figs. 3A and 4A), the release of melphalan from tissue is much slower. The slow efflux is consistent with previous studies (Scott et al., 1992a; Rauschecker et al., 1991), reporting that a major fraction of the melphalan was retained in the perfused tissue and that redistribution from the tissue compartment into the systemic circuit was slow. Rauschecker et al. (1991) suggested that 1.4 to 18% of the total melphalan dose reaches the systemic circulation after reconnection in ILP. In present study, approximately 14.5% of total melphalan dose would reach the systemic circulation if a washout procedure was not used.

A number of melphalan-free perfusates have been used in the washout procedure during ILP. They include plasma expander mixed with electrolyte solution (Rauschecker et al., 1991), Ringer’s lactate (Scott et al., 1992a) and dextran (Kremetz et al., 1987; Egerton, 1982). To minimize the later distribution of melphalan from the perfused limb into the systemic circulation, techniques for the washing-out phase need to be optimised (Rauschecker et al., 1991). We had hypothesized that a drug free washout solution containing BSA and longer washout periods may better facilitate melphalan removal from the perfused limb than a perfusate without any binding capacity for melphalan. In present study, no difference in melphalan removal from skin and fat were found between BSA and dextran perfusates (fig. 5A). This result suggests removal from tissue is effectively into perfusate under sink conditions, i.e., there is no substantial redistribution back to these tissues from the perfusate. Redistribution may be important for muscles as the melphalan removal from muscle was faster using 4.7% BSA than with using 2.8% dextran perfusate. Given that 72 to 74% of the melphalan is in perfused muscle for both BSA and dextran perfusate (figs. 3 and 4), the use of washout perfusate
containing BSA to reduce the amount of melphalan reaching the systemic circulation will be that primarily from the perfused muscle.

In this work, a comparison of drug-free BSA or dextran perfusates during the washout period was conducted following identical melphalan perfusion conditions. With either 4.7% BSA or 2.8% dextran perfusate with melphalan perfusion, the amount of melphalan in each tissue after 30 min washout was less for the 4.7% BSA drug free perfusate than for the 2.8% dextran perfusate (fig. 5). This result suggests that using washout solutions that bind melphalan highly may facilitate melphalan removal from the perfused limb. However, the removal of 50% of the melphalan from each tissue by 4.7% BSA perfusate would require more than 1 hr washout time, especially for muscle. At present, the washout time in clinical practice is between 3 min (Scotter et al., 1987) and 4 min (Rauschecker et al., 1991).

A variety of media has been used that are likely to influence oxygen supply, arteriolar recruitment, drug binding in blood and perfusion pressure (Wu et al., 1993). What the desirable composition of the perfusate should be was raised by Kroon (1988) in his review of ILP procedures. Our work has suggested that protein binding influences both the input and output of melphalan in muscle during perfusion and washout. Melphalan has been reported (Greig et al., 1987) to bind to red blood cells (36.7%), plasma (84.1%) and human albumin (46.1%). Wu et al. (1995a) reported that melphalan binding to 4.7% BSA was 47.9%. In figure 6, the effect of changing fu in the perfusate to the melphalan concentration-time profiles in outflow perfusate, skin, muscle and fat after the application of a constant input source to the IPRH is examined using the pharmacokinetic model (fig. 1) derived in this work. The conditions used were an initial applied concentration of 15.26 μg/ml and a perfusion flow rate of 3.8 ml/min with perfusate containing BSA, dextran, RBC, plasma or other solutions. The melphalan concentrations in the outflow perfusate with varying fu of melphalan in the perfusate are in the order: fu = 0.01 > fu = 0.16 (plasma) > fu = 0.52 (4.7% BSA) > fu = 0.63 (RBC) > fu = 0.87 (2.8% dextran 40) > fu = 1. Consistent with model predictions, the amount of melphalan uptake into each tissue was highest for the highest fu with an order: fu = 1 > fu = 0.87 (2.8% dextran 40) > fu = 0.63 (RBC) > fu = 0.52 (4.7% BSA) > fu = 0.16 (plasma) > fu = 0.01. It is apparent that a perfusate with a high protein content perfusate impairs melphalan uptake into perfused tissues.

Because the commonly applied process of hyperthermia may enhance melphalan hydrolysis, the effect of potential hydrolysis in tissues was examined in figure 7. The simulated melphalan concentration-time profile in the outflow perfusate was constant for Km ranging from 0 to 0.1. However, the amount of melphalan in each tissue decreased proportionately as Km increased.

**Conclusion**

A physiological pharmacokinetic model has been developed to describe the concentration-time profile of melphalan in both perfusate outflow and tissues in the isolated perfused rat hindlimb during perfusion with melphalan and washout periods. The predicted concentration-time profiles obtained appear to correspond closely to the observed data. Further simulations enabled the prediction of melphalan perfusate and tissue profiles with a dextran perfusate, after correction for difference in melphalan binding to perfusate components. The work also shown that higher melphalan protein binding in the perfusate impairs its uptake into tissue. However, the
effect of protein binding on the washout kinetics was small, a comparable rate of elimination being observed for dextran and albumin perfusates. It is implied throughout that this physiological pharmacokinetic model will have use in the understanding of limb exposure to a chemotherapeutic agent such a melphalan during a local limb perfusion. The description of washout of drug from the perfused limb assists in understanding systemic drug exposure.

References


