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High-performance liquid chromatographic assay for the measurement of melphalan and its hydrolysis products in perfusate and plasma and melphalan in tissues from human and rat isolated limb perfusions

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Abstract

A sensitive, specific and rapid reversed-phase high-performance liquid chromatographic (HPLC) assay was developed for the quantitation of melphalan and its hydrolysis products in samples from the isolated perfusion of human and rat limbs. Samples of perfusate, plasma and tissue were analysed, following methanol precipitation, using a phenyl column and fluorescence detection. Dansyl-arginine (38 μg ml^{-1}) was employed as the internal standard. Good resolution was observed allowing quantitation of melphalan, monohydroxymelphalan (MOH) and dihydroxymelphalan (DOH) in perfusate and plasma and melphalan in tissue. The mean recoveries of melphalan, MOH and DOH from perfusate and plasma were all 100 ± 10%. The recovery of melphalan in tissue was 93.5%. A linear response was demonstrated for melphalan in the concentration range 1.8–56.8 μg ml^{-1}, for DOH in the concentration range 0.5–30.0 μg ml^{-1} and for MOH in the range 1.4–25.1 μg ml^{-1}, in perfusate and plasma. The lower limits of quantitation of melphalan, MOH and DOH in perfusate and plasma were 1.4, 2.4 and 1.2 ng on column, respectively, and 7.2 ng of melphalan on column in tissue. Intra-assay coefficients of variation (C.V.) for melphalan, MOH and DOH, at low and high concentrations were all less than 5% and the inter-assay C.V.s were less than 9%. An ultra-filtration study to determine the protein binding of melphalan and the hydrolysis products showed that the unbound fractions (f_u) of melphalan in buffer containing dextran and bovine serum albumin were 0.873 and 0.521, respectively. The assay was used to quantitate melphalan and its hydrolysis products in samples from isolated perfusions in the human limb and rat hindlimb.

1. Introduction

Melphalan (4-[bis(2-chloroethyl)amino]-L-phenylalanine (Fig. 1A(I)) is an antineoplastic agent which has been used to treat multiple melanoma [1] and recurrent melanoma by regional limb perfusion [2] with response rates between 50 and 80% for locally recurrent melanoma [3, 4]. Benckhuijsen et al. [5] described the disposition of melphalan following a

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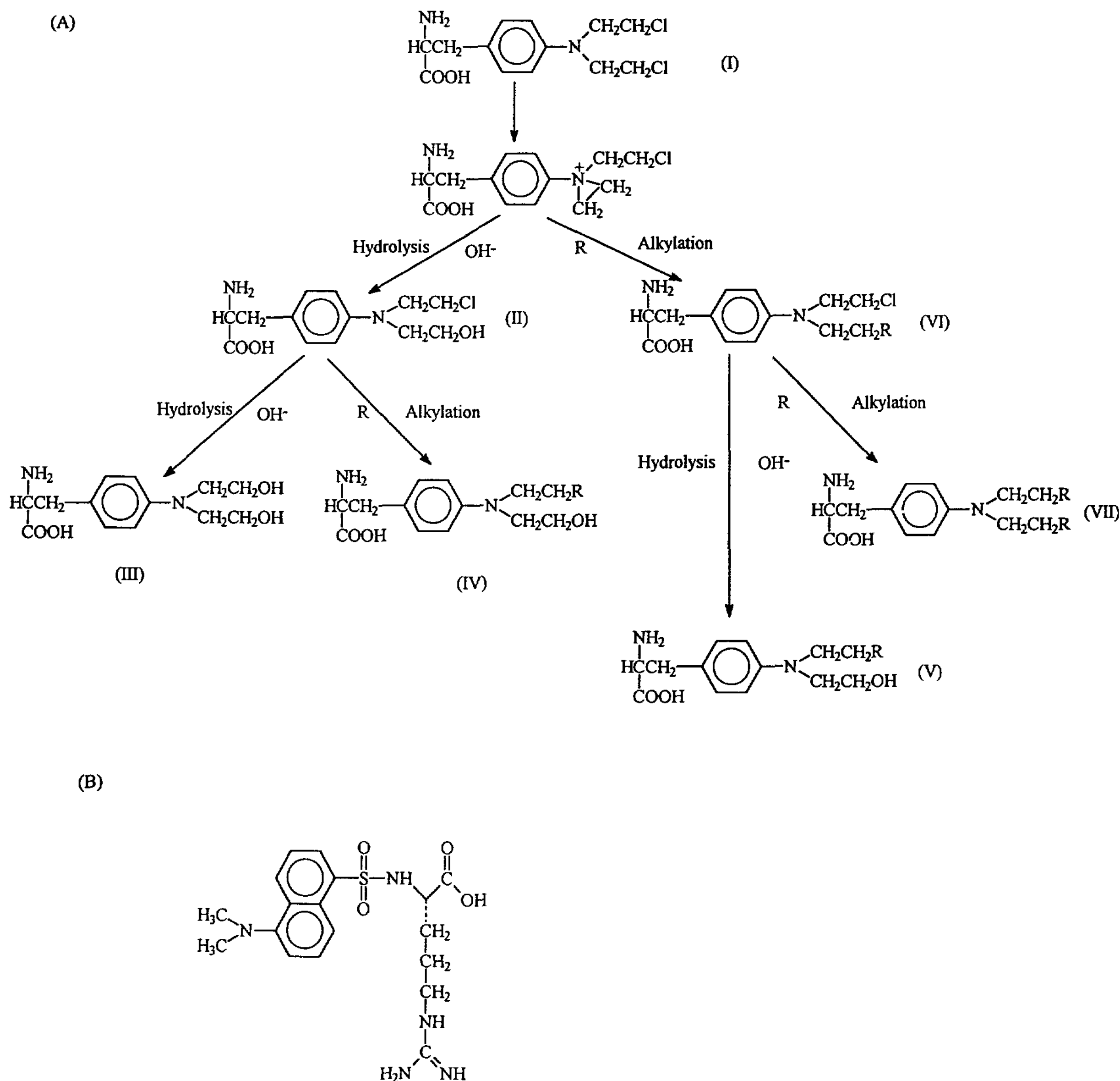

Fig. 1. Molecular structures of analytes and the possible reaction pathways of melphalan. (A) I = melphalan; II = monohydroxymelphalan (MOH); III = dihydroxymelphalan (DOH); IV, V, VI = melphalan-protein adducts (R represents protein). (B) Dansyl-arginine (internal standard).

single bolus dose as a biexponential concentration–time profile and other studies [6,7] have reported the concentration profiles following melphalan administration in three divided doses to a patient undergoing isolated limb perfusion. Another study by Scott et al. [8] recently compared the pharmacokinetics of a bolus dose of melphalan with divided dose administration. The use of melphalan for regional limb perfusion as adjuvant therapy for high risk, stage I melanomas is the basis of an international prospective trial supervised by the European Health Organisation for the Research and Treatment of Cancer and the World Health Organisation

Melanoma Group [9]. If survival benefits are confirmed, then the use of melphalan in regional limb perfusion may increase markedly.

Melphalan undergoes rapid hydrolysis in aqueous solution [10] at room temperature. Firstly the unstable, mono-hydrolysis product (Fig. 1A(II)) is formed, then the stable, di-hydrolysis product (Fig. 1A(III)). Previous methods for the quantitation of melphalan have been published using gas–liquid chromatography [10], HPLC [5–7,11–12] and radiochemical techniques [13]. HPLC with either ultraviolet [5–6, 14–15], fluorescence [7,12,16] or electrochemical [17] detection are the most widely used methods. However, the majority of these methods are not specific for melphalan, are unable to detect it and its hydrolysis products at clinical levels or employ complex and time-consuming techniques [7,14,18–26]. Thus, a new analytical method was developed and validated for use in a kinetic study of melphalan in isolated, perfused limbs.

2. Experimental

2.1. Materials

Melphalan was kindly donated by Wellcome (Sydney, Australia). Dansyl-arginine, bovine serum albumin (BSA) fraction V and dextran 40 were obtained from Sigma (St. Louis, MO, USA). 1-Octanesulphonic acid sodium salt was purchased from Rhône-Poulenc (Clayton South, Australia) and methanol (HPLC grade) from Millipore Waters (Brisbane, Australia). Water was purified and de-ionized using a Waters Milli-Q unit (Millipore Waters).

2.2. HPLC apparatus and conditions

The HPLC system consisted of a Model LC-6AD pump with a SCL-6B system controller and a SIL-6B auto-injector (Shimadzu, Kyoto, Japan). A Model RF-551 programmable spectrofluorimeter was used for detection (Shimadzu). Analysis was performed using an Alltima phenyl column, 5 μm, 250 mm $\times$ 4.6 mm I.D., (Alltech Associates, Deerfield, IL, USA). Detector output was processed and manipulated using a Delta chromatography data system (Digital Solutions, Brisbane, Australia) operating on a 486SX personal computer. The mobile phase consisted of methanol–water–glacial acetic acid (25:75:2, v/v), pH = 2.7, with 1-octanesulphonic acid added at a concentration of 50 mg per 100 ml. The flow-rate was 2 ml min^{-1} and the injection volume 20 μl. The detector was programmed to 265 nm excitation and 360 nm emission for melphalan and its hydrolysis products and 265 nm excitation and 575 nm emission for the internal standard (dansyl-arginine) (Fig. 1B). These maximal wavelengths were pre-determined using standard solutions at pH 2.7 and a series II luminescence spectrometer (Aminco Bowman, IL, USA). The molecular mass of peaks eluted from the HPLC column were confirmed by Mr. P.J. Taylor using an API III Biomolecular LC–MS–MS Mass Analyzer (PE-Sciex, Thornhill, Toronto, Canada). Samples were introduced by flow-injection with the LC–MS–MS operated in positive ionisation mode ($[M+H]^+$). The mobile phase was methanol–water–glacial acetic acid (80:20:0.1, v/v). The flow-rate was 20 μl min^{-1} with an injection volume of 5 μl.

2.3. Extraction procedures

Plasma and perfusate samples

Perfusate and plasma samples from isolated perfused human and rat limbs were stored at −70°C until required for analysis. Samples from human and rat hindlimb studies were thawed and aliquots (100 μl) mixed with methanol (200 μl) containing dansyl arginine, 38 μg ml^{-1}, at 4°C. Samples were vortex-mixed for 30 s and then clarified by centrifugation at 10 000 *g* for 15 min. An aliquot (100 μl) of the supernatant was removed for analysis and 20 μl injected onto the HPLC system. To minimise the hydrolysis reaction all reagents, standards, auto-injector vials and samples were kept on ice or at −20°C during the extraction procedure and prior to injection. Each sample was prepared immediately before use.

Tissue samples

Tissue samples (fat, skin and muscle) were excised from the perfused rat hindlimb on the completion of each experiment and stored at −70°C until analysed. Tissue samples (approx. 100 mg) were minced using scissors and suspended in 400 μl of methanol containing dansyl-arginine (38 μg ml^{-1}). The mixture was sonicated, on ice, for 1 min using an ultra-sonic microtip (3 mm, Sonics and Materials, Danbury, CT, USA) and centrifuged at 10 000 *g* for 15 min. The supernatant was then removed and 20 μl injected onto the HPLC system. Tissue samples from a non-perfused rat hindlimb were also extracted and analysed as "blanks" to check for interfering co-eluting compounds.

2.4. Recovery

Perfusate and plasma

As MOH is very unstable, pure preparations are not available commercially and so standard solutions cannot be prepared in the usual manner for validation. Quantitation of this compound was performed using mass balance equations as described below. Three solutions containing melphalan (1.4, 6.8 and 13.6 μg ml^{-1}), MOH (4.0, 11.4 and 15.2 μg ml^{-1}) and DOH (1.5, 7.3 and 14.6 μg ml^{-1}) were prepared by spiking Krebs Henseleit bicarbonate buffer, perfusate (Krebs Henseleit buffer containing BSA (4.7% w/v) or dextran 40 (2.5% w/v)) and plasma with freshly prepared stock solution. The concentrations of melphalan, MOH and DOH in each of these solutions were calculated from their standard curves as described below. In order to determine their recoveries, samples were extracted ($n = 3$) using the methanol precipitation method and analysed. The recovery was calculated by comparing peak-height ratios of MOH, DOH or melphalan (analyte:IS) for samples extracted from spiked perfusate or plasma with samples prepared in plain buffer and processed in the same manner, according to the following formula:

$$\%\ recovery = \frac{peak\ height\ ratio\ in\ plasma\ or\ perfusate}{peak\ height\ ratio\ in\ plain\ buffer} \cdot 100$$

Tissue

Recovery of the tissue extraction procedure was assessed by multiple extraction of samples of muscle, skin and fat from a rat hindlimb following an isolated perfusion with melphalan as substrate. The extraction was repeated on each tissue sample three times, with the amount of drug removed at each extraction step being expressed as a percentage of the total melphalan recovered.

2.5. Preparation of standard curves of melphalan, MOH and DOH

Stock solutions (1 mg ml^{-1}) of melphalan and dansyl-arginine (38 μg ml^{-1}) were prepared in methanol. Dilutions in the concentration range 1.8 to 56.7 μg ml^{-1} were prepared of the melphalan stock solution with an appropriate volume of plasma or perfusate solution. Aliquots (100 μl) were extracted and analysed as above. For the tissue analyses approximately 100% recovery was demonstrated (see Section 3) and therefore the calibration curve for plasma utilised.

Melphalan is unstable in aqueous solution and is readily hydrolysed to MOH which is also unstable and cannot be isolated in pure form. The DOH is stable and can be prepared by incubating a solution of melphalan in water at 50°C until 100% hydrolysis occurs [18]; this was verified by HPLC analysis. Dilutions were then prepared as for melphalan in the concentration range 0.5–30 μg ml^{-1} with appropriate volumes of plasma or perfusate.

The concentrations of MOH can be determined using mass balance calculations [7,15,18]. A solution of melphalan, of known concentration, was hydrolysed at 50°C in a water bath. Triplicate samples (100 μl) were taken at 5, 10, 20, 30, 40 60 and 80 min, and cooled on ice immediately. These were used to spike plasma and perfusate samples which were extracted and analysed as above. The concentrations of melphalan and DOH were calculated for each time-point from their standard curves. According to the mass balance theory, the sum of the

molarities of all three substances is constant and equal to the initial molarity of melphalan (M^{o}_{Mel}), therefore the molarity of MOH at each time point (M^{t}_{MOH}) can be calculated using the corresponding concentrations of DOH (M^{t}_{DOH}) and melphalan (M^{t}_{Mel}), as shown in Eq. 1.

$$M^{t}_{MOH} = M^{o}_{Mel} - M^{t}_{Mel} - M^{t}_{DOH} \quad (1)$$

Therefore, linearity of MOH was demonstrated in the range 1.4-25.1 μg ml^{-1}

2.6. Precision studies

Two solutions containing melphalan (2.1 and 23.2 μg ml^{-1}), MOH (4.2 and 11.4 μg ml^{-1}) and DOH (0.3 and 23.1 μg ml^{-1}) in perfusate and plasma were used to determine intra-assay and inter-assay coefficients of variation. Six samples were extracted and duplicate injections were made of each concentration. The coefficient of variation (C.V., %) was calculated to assess intra-assay precision. The experiment was repeated every day for five days to determine inter-assay precision. Separate aliquots of the same spiked solutions were stored at −70°C between experiments and defrosted for use on a daily basis.

2.7. Sensitivity

The sensitivity of the assay was defined as the concentration of analyte giving a signal equal to ten times that of the background noise. Although each analyte could be detected at levels below this, this definition allowed precise and accurate quantitation.

2.8. Investigation of the rate of hydrolysis of melphalan and protein binding in different dissolution media

Solutions of Krebs Henseleit buffer alone or with the addition of 2.5% w/v dextran 40 or 4.7% w/v BSA were incubated at 41.5°C for 30 min to attain the required temperature. A known concentration of the methanolic melphalan stock solution was added to each ($t = 0$). Samples were taken (1.2 ml) at 0, 10, 20, 30, 40, 50, 60 min and assayed for DOH, MOH and melphalan. Further samples were collected every 30 min for another 2 h to study the degradation profile, but were only analysed for melphalan. A 100-μl aliquot of the sample was extracted using the protein precipitation method described above and analysed by HPLC to quantify the total melphalan, MOH and DOH present. The unbound fractions (f_u) of melphalan, MOH and DOH were investigated using the following ultra-filtration method. A further 1.0-ml sample was placed in a micropartition system (MPS-1, Amicon, Danvers, MA, USA) and centrifuged at 30 000 g for 10 min. The ultra-filtrate was then extracted using methanol containing dansyl-arginine (in order to keep the dilution factor of all samples the same) and analysed. The f_u was determined as the ratio of the free concentration to total concentration of solutes. Concentration versus time profiles of total and unbound drug were constructed for melphalan and the hydrolysis products in each of the solvents for 60 min (Fig. 3). Plots of log concentration against time were also prepared for total melphalan in the different solutions over 3 h to calculate the rate constant (k) and half life ($t_{1/2}$) for the hydrolysis reaction under each set of conditions.

2.9. The isolated perfused rat hindlimb

The single-pass isolated perfused rat hindlimb [21,27] was used to study the pharmacokinetics of melphalan and its hydrolysis products. Briefly, a rat is anaesthetised, the femoral artery and vena cava are cannulated and the hindlimb perfused with Krebs Henseleit buffer containing 4.7% w/v BSA and 25 μg ml^{-1} of melphalan for 60 min at a temperature of 41.5°C. Samples were taken from the inflow and outflow of the perfused hindlimb at 0, 8, 16, 20, 30, 40, 50 and 60 min. Samples of tissue (skin, fat and muscle) were also taken from the hindlimb on completion of the perfusion. All samples were immediately stored at −70°C until analysed.

2.10. Clinical application in isolated human limb perfusion

Human isolated limb perfusion (ILP) was performed at Royal Brisbane Hospital as treatment for patients with malignant melanoma, with the concomitant study of melphalan kinetics in perfused human limbs. The procedure used for ILP in Brisbane by Dr. W.S. Egerton is briefly as follows [28]. The limb vessels (femoral artery and vein) are cannulated and perfused with bicarbonate buffer containing dextran 40 at 41.5°C using a heart–lung machine and a heat exchanger. The limb is isolated with a tourniquet and arterial injections of melphalan are given in four divided doses at 15 min intervals (total dose 1.5 mg kg^{-1} body weight). After approximately 1.5 h, residual cytotoxic drug within the circulation is drained from the limb. The cannulae are then removed, the artery and vein repaired and flow to the limb from the systemic circulation resumed. Samples of perfusate were taken from the venous outflow at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min and frozen at −70°C until analysed.

3. Results

3.1. Chromatographic behaviour and specificity

Melphalan and its hydrolysis products (MOH and DOH) were eluted from the phenyl column in order of their decreasing polarities: DOH, MOH and melphalan. A representative chromatogram of a partially hydrolysed melphalan sample with internal standard (dansyl-arginine) is presented in Fig. 2A. The total run time of one sample is 14 min, and the DOH, MOH, melphalan and dansyl-arginine eluted from column at 2.4, 3.5, 8.4 and 12.6 min, respectively. The peaks were all well separated under the conditions of the assay, and the identities of these compounds were confirmed by LC–MS–MS. Fig. 2B shows a chromatogram of blank perfusate which displays an endogenous peak at 2.9 min. This is in between the peaks of DOH and MOH, however, and does not affect quantitation. A typical chromatogram obtained from an extracted plasma sample is shown in Fig. 2C. The chromatogram obtained for a blank plasma sample (Fig. 2D) shows that the small endogenous peaks attributable to the plasma do not interfere with quantitation. However, the chromatogram for a tissue sample (Fig. 2E) and the corresponding blank injection (Fig. 2F) show polar, endogenous peaks that co-elute with those of MOH and DOH. Despite numerous attempts at varying experimental conditions, these peaks could not be separated and, therefore, it is not possible to use this method to quantitate the hydrolysis products in tissue.

3.2. Analytical recovery

Table 1 shows the calculated recoveries of three concentrations of melphalan, MOH and DOH in perfusate (containing dextran or BSA) and plasma. The mean recoveries from perfusate of melphalan, MOH and DOH were 92.7%, 103.2% and 105.8% and from plasma were 104.9%, 102.6% and 82.4%, respectively. The mean recoveries of dansyl-arginine (38 $\mu g\ ml^{-1}$) in plasma, perfusate and tissue were 98.1 ± 4.1%, 100.5 ± 4.8% and 90.9 ± 1.4%, respectively. If samples were incubated at 41.5°C for 30 min before extraction, the apparent recovery of MOH and DOH from plasma or BSA solutions was reduced dramatically. This prompted investigation into the protein binding of melphalan and its hydrolysis products.

Multiple extraction of melphalan from tissues (skin, fat and muscle) from isolated rat hindlimb perfusions gave mean values of 93.5% of total recoverable drug after one extraction and 99.9% recovery after a second extraction. No chromatographic peaks for melphalan, MOH and DOH were observed after the third extraction procedure. A single extraction procedure therefore yielded adequate recovery and was employed for all tissue samples.

3.3. Linearity

The perfusate and plasma spiked with melphalan gave excellent linearity with respect to

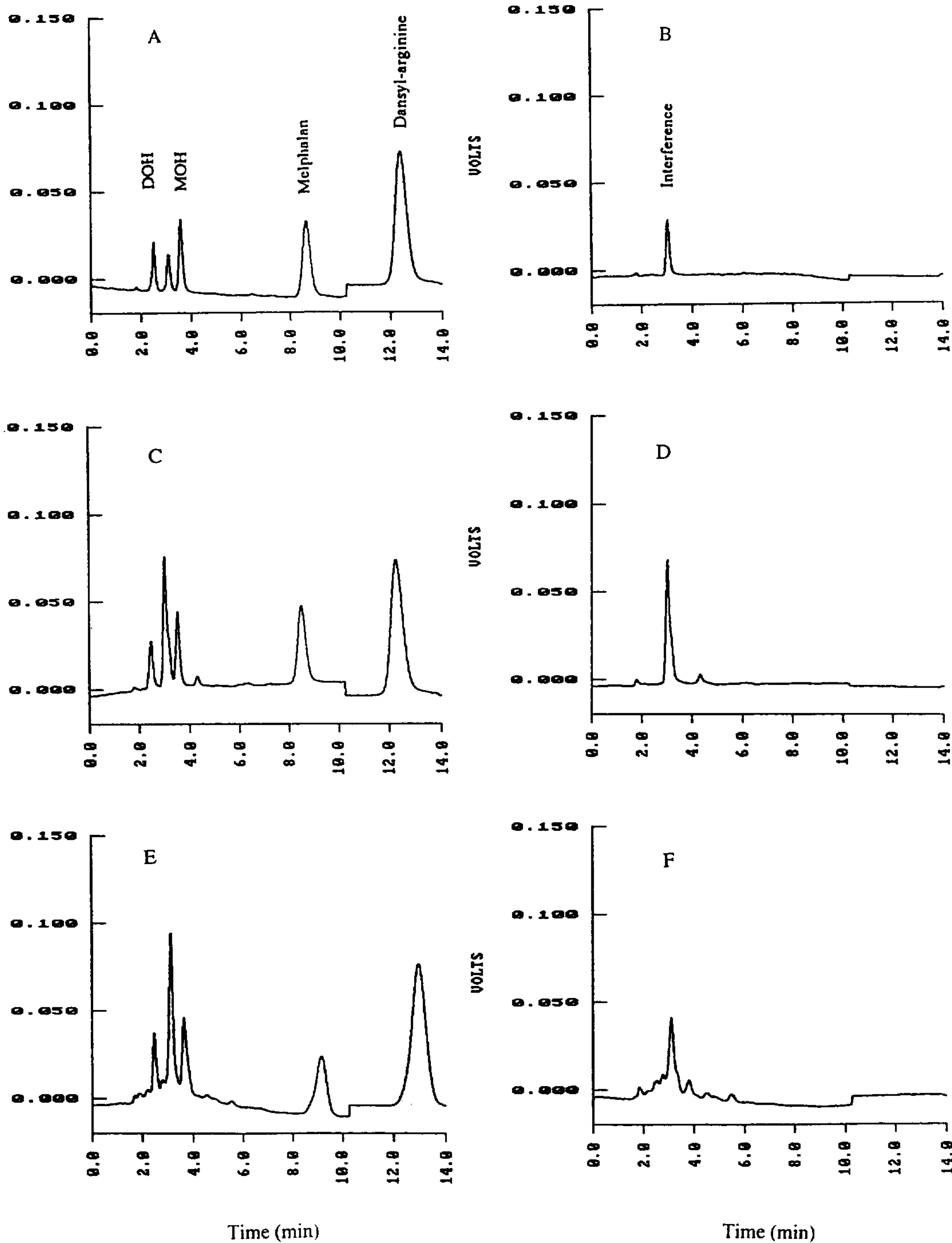

Fig. 2. Typical chromatograms of melphalan and hydrolysis products after extraction and of corresponding blanks. Chromatographic conditions as in text. Change in baseline at 10.5 min corresponds to change in emission wavelength from 360 to 575 nm. (A) Extraction from perfusate. (B) Blank perfusate. (C) Extraction from plasma. (D) Blank plasma. (E) Extraction from rat skin. (F) Blank rat skin.

Table 1
Recoveries of melphalan and its hydrolysis products in plasma and perfusate (with BSA or dextran) samples ($n = 3$)

Compounds	Concentration (μg ml^{-1})	Recovery (mean ± S.D.) (%)		
		Plasma	Perfusate with dextran	Perfusate with BSA
Melphalan	1.4	106.8 ± 12.5	101.9 ± 1.6	104.4 ± 9.3
	6.8	104.6 ± 3.9	105.6 ± 8.4	94.3 ± 1.5
	13.6	105.4 ± 1.3	104.7 ± 3.8	91.1 ± 5.7
MOH	4.2	107.1 ± 0.7	100.2 ± 10.6	103.2 ± 5.4
	11.4	103.4 ± 3.4	104.9 ± 6.7	101.3 ± 5.4
	15.2	97.9 ± 1.8	100.9 ± 2.6	99.4 ± 0.5
DOH	1.5	80.5 ± 11.9	99.3 ± 0.8	107.2 ± 4.6
	7.3	85.9 ± 8.4	102.4 ± 9.0	105.9 ± 2.5
	14.6	78.8 ± 3.1	95.8 ± 2.2	105.8 ± 4.8

peak-height ratios in the range of 1.8–56.8 μg ml^{-1} [$y = 0.0646(\pm 0.0005)x - 0.0152(\pm 0.025)$, $r^2 = 0.999$). A linear response was demonstrated for DOH in the range of 0.5–30.0 μg ml^{-1} ($y = 0.0661(\pm 0.0021)x - 0.0140(\pm 0.0269)$, $r^2 = 0.995$). Based on the mass balance, the MOH also showed good linearity in the range of 1.4–25.1 μg ml^{-1} ($y = 0.1336(\pm 0.0055)x - 0.0159(\pm 0.0412)$, $r^2 = 0.992$).

3.4. Precision and sensitivity

Extraction and measurement of melphalan, MOH and DOH at each of two concentrations in plasma and perfusate over each calibration range was repeated on five occasions. Table 2 shows that the mean intra-assay coefficients of variation (C.V., %) for the three compounds were all less than 5% and that the mean inter-assay coeffi-

Table 2
Inter-assay ($n = 6$) and intra-assay precision ($n = 5$) with low and high concentrations of melphalan and its hydrolysis products in plasma and perfusate samples

Compounds	Concentration (μg/ml)	Inter-assay precision (C.V., %)			Intra-assay precision (C.V., %)		
		Plasma	Perfusate with dextran	Perfusate with BSA	Plasma	Perfusate with dextran	Perfusate with BSA
Melphalan	2.1	2.77	4.97	3.36	3.68	9.21	1.06
	23.2	6.06	2.64	6.99	3.81	2.29	5.72
MOH	4.2	3.79	5.42	4.04	8.48	8.66	7.92
	11.4	2.76	0.58	3.61	4.95	3.12	6.97
DOH	0.3	2.79	5.89	4.06	8.79	5.65	8.01
	23.1	4.80	3.59	5.88	2.72	3.78	2.54
Melphalan	2.1	2.77	4.97	3.36	3.68	9.21	1.06
	23.2	6.06	2.64	6.99	3.81	2.29	5.72
MOH	4.2	3.79	5.42	4.04	8.48	8.66	7.92
	11.4	2.76	0.58	3.61	4.95	3.12	6.97
DOH	0.3	2.79	5.89	4.06	8.79	5.65	8.01
	23.1	4.80	3.59	5.88	2.72	3.78	2.54

cients of variation for three compounds was less than 9%. The lower limit of quantitation for melphalan, MOH and DOH in perfusate and plasma were 1.4, 2.4 and 1.2 ng, on column, and 7.2 ng melphalan on column in tissues. The mean accuracy of the analysis of melphalan was ± 5.5% over the validated range of concentrations measured in three standard curves. This was determined by back-calculating the results obtained for each of duplicate standards at various concentrations in the standard curves to their respective values from the fitted equation.

3.5. The kinetic parameters of melphalan, MOH and DOH in vitro and in vivo

The melphalan degradation time courses in various solutions are shown in Fig. 3. No significant differences were found between the plots for free and total concentration of DOH, MOH and melphalan in buffer solution (Fig. 3A), which suggests negligible binding to the filter of the micropartition system. The unbound fractions (f_u) of DOH, MOH and melphalan in dextran solution (Fig. 3B) were 0.913 (±0.072), 0.832 (±0.055) and 0.873 (±0.068), respectively. The f_u of DOH, MOH and melphalan in BSA buffer were 0.860 (±0.129), 0.938 (±0.064) and 0.521 (±0.044), respectively, confirming that when melphalan is incubated with proteins, there is a significant difference between total and unbound levels (Fig. 3C). When concentration–time profiles are compared for the degradation of melphalan in buffer and BSA buffer (Figs. 3A and 3C), only 24.4% of DOH and 9.7% MOH are observed in the presence of protein with respect to the concentrations observed in plain buffer. However, no significant difference was found for the rate constant for the hydrolysis of melphalan in the different dissolution media (Table 3). The reason for this was thought to be that the melphalan binds to the proteins present to form adducts as shown in Fig. 1A (IV, V, VI and VII); therefore, the hydrolysis products are formed at a much lower rate in BSA than the other two solutions. To investigate whether DOH also interacts with protein, a solution was incubated with 4.7% w/v BSA at 41.5°C. No

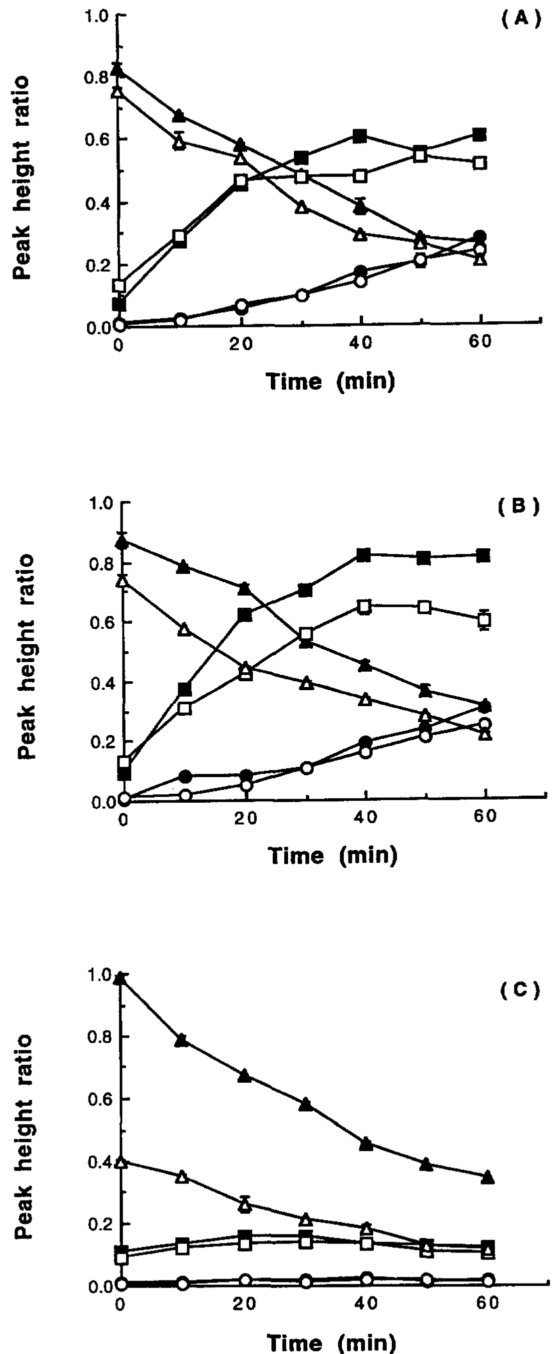

Fig. 3. Concentration versus time profiles for total and unbound levels of melphalan and its hydrolysis products in Krebs Henseleit buffer. (A) Buffer alone. (B) Buffer containing 2.5% dextran 40. (C) Buffer containing 4.7% BSA. ▲ = total melphalan; △ = free melphalan; ■ = total MOH; □ = free MOH; ● = total DOH; ○ = free DOH.

Table 3
In vitro and in vivo kinetic parameters for melphalan

Conditions	k (min^{-1})	$t_{1/2}$ (min)
In vitro		
Buffer only	0.00864 ± 0.00122	73.6 ± 10.4
Dextran buffer	0.00815 ± 0.00131	78.6 ± 11.8
BSA buffer	0.00713 ± 0.00074	88.9 ± 8.9
Free conc.[a]	0.00897 ± 0.00056	70.4 ± 4.2
Human limb perfusion		
1st dose	0.02182 ± 0.00032	28.96 ± 0.426
2nd dose	0.02891 ± 0.00642	22.41 ± 4.98
3rd dose	0.01531 ± 0.00035	41.30 ± 0.93
4th dose	0.00120 ± 0.00800	68.13 ± 45.62
Rat hindlimb perfusion		
Inflow	0.00505 ± 0.00026	125.26 ± 6.37
Outflow	0.00424 ± 0.00063	150.82 ± 22.52

[a] Calculated from the mean free concentration of melphalan versus time plots in buffer, BSA or dextran shown in Fig. 3.

change in recovery was observed after 60 min incubation suggesting that covalent binding between DOH and protein did not occur in this case. It is possible that MOH could also bind to the protein (Fig. 1A(IV)); however, it was not possible to demonstrate this using recovery experiments due to its inherent instability. In all cases except melphalan in BSA buffer, the free concentration is equivalent to the total drug concentration as determined using the protein precipitation method. This validates the use of the chosen extraction procedure.

A melphalan concentration–time profile from an isolated human limb perfusion following four equal doses of melphalan (35 mg per bolus) is shown in Fig. 4A. This shows sharp increases in melphalan concentration corresponding to each bolus of drug and a decrease resulting from the uptake of melphalan into tissue and its degradation to the hydrolysis products. The calculated elimination rates (k) of melphalan are shown in Table 3. It was observed that the calculated value of k is reduced for the third bolus dose when compared to those for the first two doses. The experimental data presented suggest that the concentration of melphalan approaches steady state after approx. 30 min and the administration of three doses. The concentration of free melphalan in the human limb during perfusion was between 4.3 and 12.1 μg ml^{-1}.

The melphalan time profile in an isolated perfused rat hindlimb is shown in Fig. 4B. The calculated value for k was lower than that observed in the human study giving rise to an increased half-life. Drug concentrations in skin, fat and two muscle layers following a 60 min perfusion of melphalan at a constant concentration of 25 μg ml^{-1} are shown in Fig. 4C. Similar levels of melphalan are observed in both the skin and muscle layers.

4. Discussion

As the hydrolysis of melphalan occurs rapidly in biological samples a simple and quick method for sample preparation is required. Previous methods in the literature have employed ion-exchange [15], solid-phase extraction [7], fractional crystallisation [6,14] and derivatisation [11]. Two other extraction methods were attempted: one employing perchloric acid (35%) to precipitate protein and another using solid-phase extraction cartridges [7]. Results from both methods were inferior to those obtained using methanol protein precipitation. The method described in this paper is reproducible, gives good values of recovery and allows rapid sample processing. When compared to other extraction methods, the one-step protein precipitation was found to be superior in terms of recovery and reproducibility.

To understand fully the pharmacokinetic and biochemical pharmacology of melphalan, it is necessary to be able to quantify its metabolites and degradation products. MOH and DOH are the prominent products of spontaneous hydrolysis, and are regarded as the main reaction products in man [10,18,19]. Goodman et al. [29] suggested that melphalan has twenty times the potency of MOH and that DOH is inactive. This would be expected as melphalan is an alkylating agent and thought to produce its cytotoxic effect by forming cross-links between DNA strands [1]. This action is prevented when the molecule loses

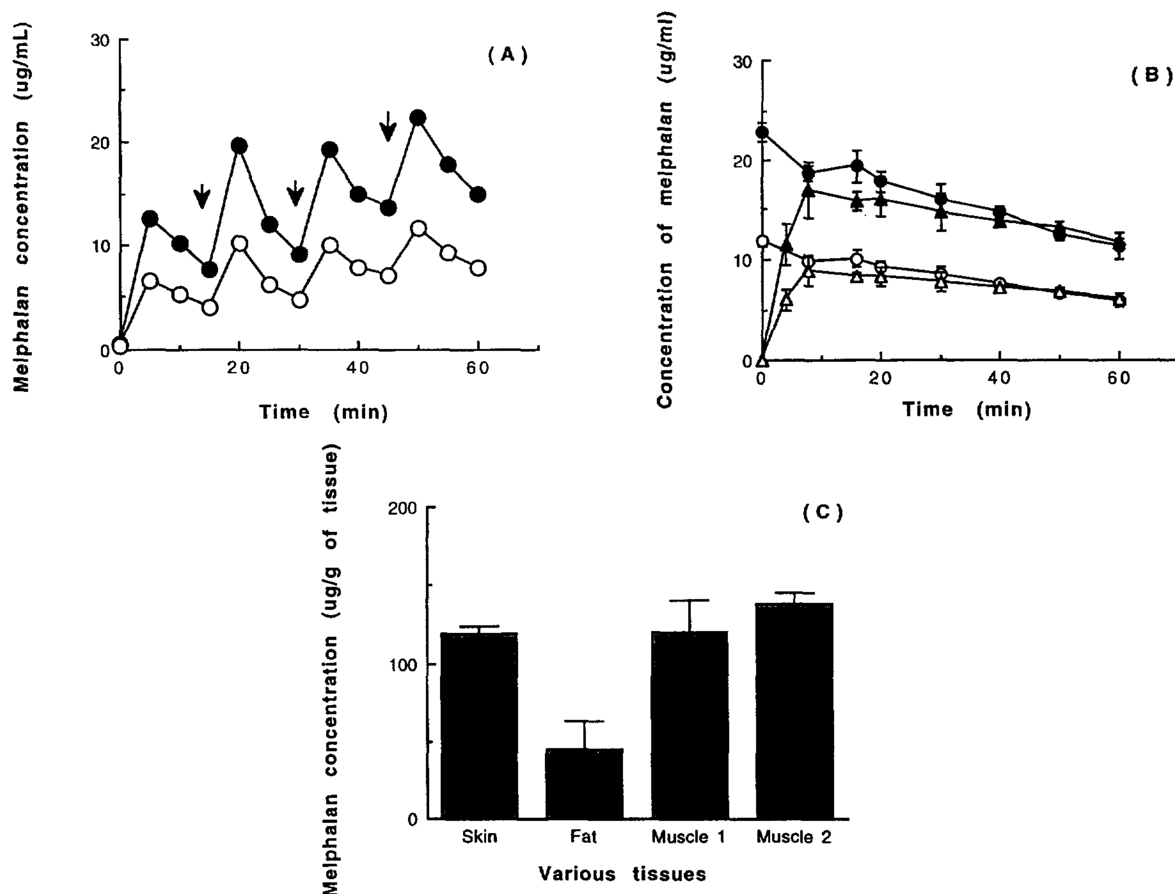

Fig. 4. Concentration versus time curves for melphalan during isolated limb perfusion. (A) In the human limb after four bolus doses (35 mg) of melphalan injected for melanoma treatment at 41.5°C (arrows indicate adminstration of melphalan doses); ● = total melphalan concentration, ○ = free melphalan concentration. (B) The inflow (○, ●) and outflow (△, ▲) profiles in the isolated rat hindlimb during perfusion with 25 μg ml^{-1} melphalan in Krebs Henseleit buffer containing 4.7% BSA at 41.5°C ($n = 3$); open and closed symbols represent the free and total melphalan concentration, respectively. (C) Concentration of melphalan in skin, fat and muscle layers from the rat hindlimb on completion of the perfusion (mean ± S.D., $n = 3$).

its chloroethyl groups. In previous literature there has been little mention of protein binding of melphalan despite the fact that it is the free drug which is active. Figs. 3B and 3C show that the unbound fraction of melphalan is higher in dextran buffer than in the presence of BSA. The perfusion media employed depend on the preference of the surgeon but various types have been reported including Hartmann's solution with red cells [9], whole blood [2], plasma [16] and dextran buffer [28]. The results in this study suggested that the use of perfusate containing dextran rather than albumin would increase the concentration of free drug available to the tissue and possibly increase therapeutic effect.

The comparison of Figs. 3A to 3C shows that the concentrations of the hydrolysis products MOH and DOH are much lower in the albumin solution than in protein-free buffer or that containing dextran. However, there is no difference between the total concentration and the free concentration for the hydrolysis products in either case. Because of this, the MOH and DOH could be quantitated using this extraction method in BSA solutions or plasma. However, the hydrolysis products are present at very low

concentrations as, in the presence of albumin, the melphalan binds covalently to the protein rather than following the hydrolysis degradation pathway (Fig. 1A). Investigations into the protein adducts formed would be required to characterise the mechanism of interaction further.

Figs. 4A and 4B show that the HPLC technique allows the determination of the concentration of melphalan, MOH and DOH in perfusate during isolated human and rat limb perfusion (data for melphalan only presented). Samples must be frozen and the assay should be run within 24 h to minimise any melphalan degradation. Systemic levels of melphalan can be measured using this simple and rapid technique and could alert the surgeon to possible subsequent toxicity due to a systemic leakage during perfusion. Valuable kinetic data can be derived, especially to compare different dosage regimens. This could lead to the optimisation of dosing and improve the understanding of treatment failures. At present the optimal dosage regimen of melphalan to be used in the isolated perfused limb has not been clarified and there are conflicting views world-wide in relation to the most desirable perfusion conditions. This assay will assist in the resolution of many of these questions by examining the pharmacokinetics and pharmacodynamics affecting melphalan, MOH and DOH disposition and tumour cytotoxicity in the limb.

5. Conclusions

The developed assay has been shown to be specific for melphalan, MOH and DOH, and to be accurate and precise through validation experiments. The assay has been applied to the analysis of plasma and perfusate from rat and human limb perfusions to quantify melphalan, MOH and DOH and also to quantify melphalan in perfused rat tissues. Measurement of the hydrolysis products from tissue extracts was not possible due to interference of endogenous tissue compounds; however, the melphalan concentration could be accurately determined in the tissue. This allows the quantitation of unbound melphalan reaching the skin which is an important factor in tumour remission. As the assay is rapid and simple to perform, it minimises any loss of melphalan due to degradation. It is sensitive and suitable for kinetic and metabolic studies of melphalan disposition in human and animal perfusion studies.

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