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A stability-indicating method for the determination of melphalan and related impurity content by gradient HPLC

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Abstract

A robust gradient high performance liquid chromatographic (HPLC) procedure is described for the simultaneous determination of melphalan content and related impurities in melphalan drug substance. The sample solution is prepared in methanol and injected. A linear gradient from 5 to 60% acetonitrile in water containing 0.05% v/v acetic acid, 0.01% v/v triethylamine, and 0.05% w/v ammonium acetate is applied over 20 min. The chromatographic conditions are capable of separating and quantifying all impurities found in routine production batches of melphalan at above 0.1% area/area. The method has been fully validated and is linear over the column loading range of 0–3 μg of melphalan. All related impurities occurring in routine batches at above 0.1% area/area have been identified, and structures assigned. The method has been applied to melphalan samples stored under stressed conditions, and shown to be stability-indicating.

Keywords: Gradient HPLC; Assay; Impurities; Melphalan; Alkeran®

1. Introduction

Melphalan (or phenylalanine mustard) is an alkylating agent used in the treatment of multiple myeloma and other malignant neoplasms including tumours of the breast and ovary [1]. The drug may be administered as an injection or as a tablet formulation, when 2 mg tablets are typically used.

The British Pharmacopoeia (BP) monograph for melphalan tablets involves an isocratic high performance liquid chromatographic (HPLC) assay procedure, but there is no limit placed on the related impurity content. In addition, the sample diluent is acetonitrile; 0.1 M aqueous hydrochloric acid (4:1, v/v), which leads to rapid hydrolysis

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of the active moeity. The United States Pharmacopeia monograph for melphalan tablets also gives an isocratic HPLC method for determination of melphalan content, but again there is no limit applied to related impurities. Other papers [2–4], have described HPLC procedures for the determination of melphalan in biological samples, but there is no recorded use of such conditions in quantifying manufacturing by-products in melphalan.

Preliminary experimental work to identify a number of related impurities commonly present in routine production batches of melphalan showed these included melphalan dimer and possible polymers, as well as smaller more polar molecules. As a result, attempts to identify a single set of isocratic HPLC conditions which allow resolution and quantitation of these impurities proved unsuccessful.

This work described in this paper relates to the development and validation of a gradient HPLC procedure capable of simultaneous determination of melphalan content and related impurities. The chromatographic conditions have been used in conjunction with mass spectrometry to support the identification and quantitation of related impurities.

In this paper the approach is applied to drug substance, both freshly manufactured batches and stored samples.

2. Experimental

2.1. Apparatus

The column selected was a 5 μm BDS Hypersil C18 (150 × 4.6 mm) column: an equivalent Phenomenex column has also been used successfully. Most of the work described in this paper was carried out using a Hewlett-Packard HP1100 autosampler, gradient HPLC pump and detector, although other autosamplers, pumps and detectors have been used successfully. The UV detector was set at a wavelength of 260 nm with a sensitivity of 0.1 a.u.f.s. An injection volume of 10–20 μl was used. The column temperature was maintained at 20–30°C. The flow rate employed was 1.5 ml min^{-1} throughout the run. The run time employed was typically 30 min, including re-equilibration.

2.2. Gradient high performance liquid chromatographic conditions

Buffer concentrate	Acetic acid–triethylamine–ammonium acetate–water (10:2:10:88, v/v/w/v)
Eluent 1	Buffer concentrate–acetonitrile–water (10:100:1900, v/v/v)
Eluent 2	Buffer concentrate–acetonitrile–water (10:1200:800, v/v/v)

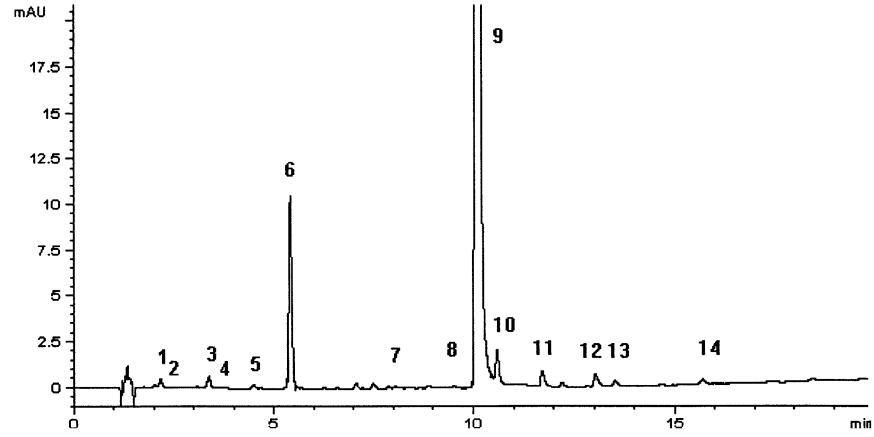


Fig. 1. Typical chromatogram for freshly prepared solution of melphalan.

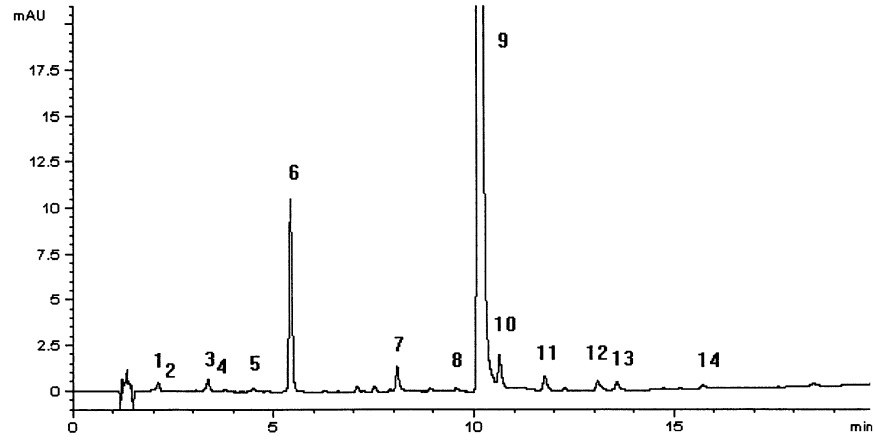


Fig. 2. Typical chromatograms for a solution of melphalan stored for 10 h at UKRT.

Table 1
Selectivity of the HPLC assay procedure[a]

Peak number	Compound name[b]	Relative retention time[c]	Class
1	Dihydroxymelphalan	0.21	s+d
2	Phthalic acid	0.28	s
3	Morpholino derivative	0.34	s
4	Methoxyhydroxymelphalan	0.35	s
5	Chloroethylamino melphalan	0.44	s
6	Monohydroxymelphalan	0.52	s+d
7	Methoxymelphalan	0.79	d
8	Ethoxymelphalan methyl ester	0.95	s
9	Melphalan	1.00	
10	Chloroethoxymelphalan	1.05	s
11	3-Chloro analogue of melphalan	1.16	s
12	Melphalan dimer	1.27	s+d
13	Melphalan methyl ester	1.31	s
14	Melphalan ethyl ester	1.44	s

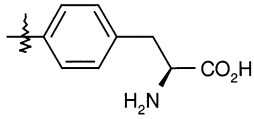
[a] s, Impurity of synthesis; d, degradation product.
[b] Structures of these compounds are given in Table 2.
[c] Relative retention time of the peak relative to the peak due to melphalan.

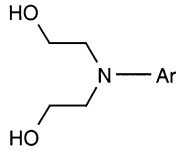
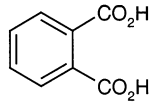
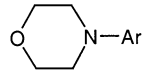
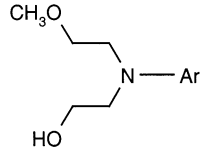
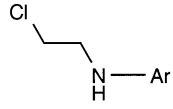
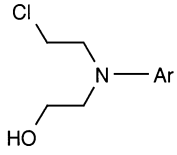
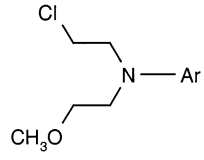
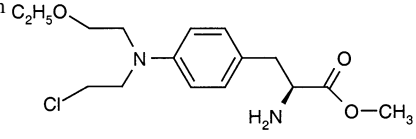
Gradient	0–20 min	Linear gradient from 100% Eluent 1 to 100% Eluent 2
	20–25 min	Hold at 100% Eluent 2
	25–26 min	Linear gradient to 100% Eluent 1
	26–30 min	Hold at 100% Eluent 1

2.3. Standard and drug substance test solutions

A suitable batch of melphalan drug substance was selected as the reference standard material. The melphalan reference standard and samples (in duplicate) were dissolved in methanol and injected onto the chromatographic system using a target

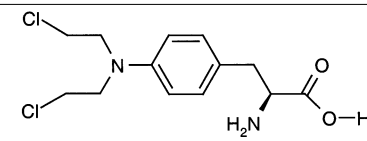
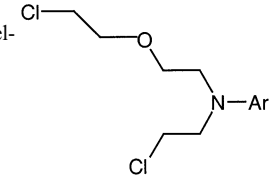
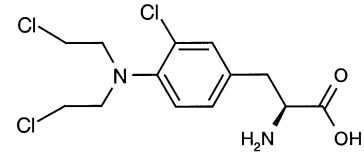
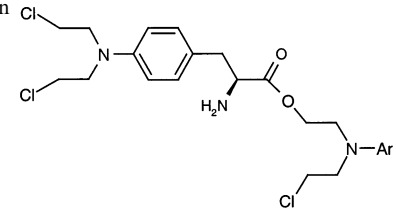
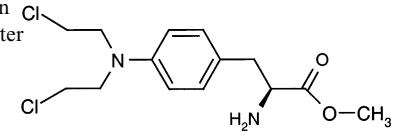
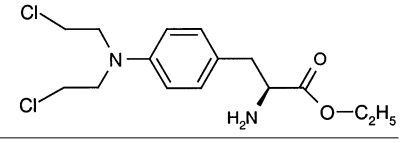
Table 2
Structures of the common related impurities of melphalan

Ar = 

Potential impurity	Structure of impurity
Dihydroxymelphalan	
Phthalic acid	
Morpholino derivative	
Methoxyhydroxymelphalan	
Chloroethylamino melphalan	
Monohydroxymelphalan	
Methoxymelphalan	
Ethoxymelphalan methyl ester	

column loading of 2 μg melphalan. A typical solution contained 40 mg melphalan in 200 ml of methanol.

Table 2 (Continued)

Potential impurity	Structure of impurity
Melphalan	
Chloro-ethoxymel-phalan	
3-Chloro analogue of melphalan	
Melphalan dimer	
Melphalan methyl ester	
Melphalan ethyl ester	

2.4. Procedure

Standard and sample solutions were injected, ensuring that the retention times of the peaks observed compare favourably with those given in the table of typical retention times and that the system suitability criteria are met.

On completion of the sample analysis, it was confirmed that the resolution requirements of the system suitability criteria are met by the final standard solution, and that the retention times of

the peaks observed in the chromatograms compare favourably with those in the table of typical retention times.

Typical chromatograms for a freshly prepared melphalan solution and for a solution stored for 10 h at UKRT are shown in Figures 1 and 2.

3. Results and Discussion

3.1. Validation

3.1.1. Specificity

To demonstrate the specificity of the chromatographic system and to demonstrate that the method is stability indicating, known potential drug-related impurities of synthesis and degradation were chromatographed using the defined HPLC system. The structures were either elucidated or confirmed by combination of the defined HPLC conditions with mass spectrometry, or by use of supercritical fluid chromatography-mass spectrometry (SFC-MS). The relative retention times obtained are given in Table 1. Structures of the common related impurities are given in Table 2. The specificity of the analytical procedure is demonstrated by the absence of interference to the peak due to melphalan from the other potential impurities listed. Figures 1 and 2 demonstrate that all commonly occurring impurities of synthesis and degradation are resolved from melphalan.

In aqueous solution, melphalan hydrolyses to form monohydroxymelphalan and subsequently to dihydroxymelphalan. This hydrolysis has also been shown to occur in vivo [2]. The stability of melphalan is a function of pH, solvent polarity, and temperature. The rate of hydrolysis is highly dependent on the degree of protonation of the basic moiety. Melphalan exhibits greater stability in acidic aqueous solution, i.e. in 0.1 M HCl, than in neutral or basic conditions.

In the solid state, the primary degradation product observed is the melphalan dimer: other polymers (e.g. trimer) are also possible. Several other unidentified decomposition products have also been observed, especially in melphalan tablets undergoing stress testing. Levels of monohydroxymelphalan observed in stressed samples of the drug substance screened remained constant or decreased, indicating that monohydroxymelphalan is not a primary decomposition product of melphalan in the solid state.

3.2. Linearity of the method

The linearity of detector response was determined for both melphalan and the most abundant related impurity, monohydroxymelphalan.

3.3. Linearity with respect to melphalan

The linearity of the method over the column loading range of 0–3 μg of melphalan (equivalent to 0–150% of the nominal column loading of 2 μg) was confirmed by injecting a series of standard solutions at levels of 0, 50, 80, 90, 100, 110, 120 and 150% of the nominal concentration. A linear response of peak area (corrected for blank response) versus concentration was obtained: $y = 51.46x - 19.7$ (correlation coefficient = 0.9998, intercept = −0.4% of nominal column loading).

Table 3
Reproducibility and comparison of assay procedures

Batch	Assay by HPLC (%w/w)			Assay by isocratic HPLC (%w/w)	Assay by titrimetry (w/w)
	Site 1	Site 2	Mean		
1	94.6	94.7, 95.8	95.0	94.1	95.8
2	94.8	94.8	94.8	94.8	95.4
3	94.6			94.3	95.3

Table 4
Reproducibility of the related impurities procedure[a]

Impurity content (% area/area)	Site 1 (using loading of 1 μg)	Site 2 (using loading of 2 μg)
Batch 1		
Dihydroxymelphalan	0.1	0.1
Phthalic acid	ND	ND
Morpholino derivative	0.2	0.1
Chloroethylamino melphalan	<0.1	<0.1
Monohydroxy melphalan	2.4	2.3
Unknown RRT 0.70	<0.1	0.1
Unknown RRT 0.74	<0.1	0.1
Methoxymelphalan	0.2	<0.1
3-Chloro analogue of melphalan	<0.1	<0.1
Chloroethoxymel phalan	0.3	0.3
Methoxymelphalan methyl ester	0.2	0.2
Unknown RRT 1.21	<0.1	<0.1
Melphalan dimer	0.2	0.4
Melphalan methyl ester	0.1	ND
Batch 2		
Dihydroxymelphalan	<0.1	<0.1
Phthalic acid	ND	ND
Morpholino derivative	0.2	0.1
Chloroethylamino melphalan	ND	<0.1
Monohydroxymelphalan	2.5	2.4
Unknown RRT 0.74	<0.1	<0.1
Methoxymelphalan	0.3	<0.1
3-Chloro analogue of melphalan	<0.1	<0.1
Ethoxymelphalan methyl ester	<0.1	ND
Chloroethoxymelphalan	0.3	0.4
Methoxymelphalan methyl ester	0.1	0.1
Melphalan dimer	0.2	0.5
Melphalan methyl ester	0.1	ND
Batch 3		
Dihydroxymelphalan	ND	ND
Phthalic acid	ND	ND
Morpholino derivative	0.2	0.1
Monohydroxymelphalan	2.4	2.4
3-Chloro analogue of melphalan	<0.1	<0.1
Methoxymelphalan	ND	ND
Chloroethoxymelphalan	0.3	0.4
Methoxymelphalan methyl ester	<0.1	<0.1
Melphalan dimer	0.4	0.6
Melphalan methyl ester	ND	<0.1

[a] ND = not detected with LOD = 0.05%; <0.1% = between LOD of 0.05% and LOQ of 0.1%.

3.4. Linearity with respect to monohydroxymelphalan

The linearity of detector response to monohydroxymelphalan was investigated. Solutions of monohydroxymelphalan were injected onto the chromatographic system and the area of the peak due to monohydroxymelphalan measured. A series of solutions containing known levels of monohydroxymelphalan at 0, 1.2, 1.9, 2.2, 2.4, 2.7, 2.9, and 3.6% (% relative to the nominal melphalan column loading of 2 μg) were injected on-column and the area of the peak attributable to monohydroxymelphalan measured. A satisfactory linear response of peak area versus column loading was obtained: $y = 42.12x + 10.02$ (correlation coefficient = 0.9854, intercept = + 7% of nominal column loading).

Table 5
Stability of melphalan in methanolic solution (assay)

Sample details	Melphalan content	
	Peak area ($\times 10^6$)	% of initial area
Initial	2.264	100.0
After storage for 5 h at ambient temperature	2.276	100.5
After storage for 11 h at ambient temperature	2.269	100.2

3.5. Repeatability of injection

The repeatability of injection of the method was determined for both melphalan and the most abundant related impurity. The major impurity chloroethoxymelphalan was selected, on the basis that this impurity peak elutes very close to the main peak, and thus quantitation of this impurity peak was considered to be the most likely to be compromised.

3.6. Repeatability of injection (melphalan)

The repeatability of injection for the assay procedure was assessed by carrying out 10 injections of a standard solution onto the chromatographic system. A relative standard deviation (RSD) = 0.98% was obtained for the peak area of the main peak.

3.7. Repeatability of injection (monohydroxymelphalan)

The repeatability of injection for the related impurities procedure was determined by making ten replicate injections of a single solution onto the chromatographic system. The RSD of peak area was 4.2%: this was regarded as acceptable.

3.8. Reproducibility of analytical procedure

3.8.1. Assay

The reproducibility of the analytical procedure was determined by determining the melphalan content of three batches of drug substance by two different sites. The batches were also tested by a titrimetric procedure and by the BP isocratic HPLC assay method, for comparison purposes. The results (Table 3) show the reproducibility of the analytical procedure to be acceptable, and comparable to those obtained by the isocratic method. The assay results obtained by either HPLC procedure were slightly lower than those obtained by titrimetry; this was to be expected, as many of the related impurities listed in Table 2 will respond to the titrimetric method.

3.8.2. Related impurities

The reproducibility of the analytical procedure was determined by analysing the drug related impurities content of three batches of drug substance by two different sites, using two different loadings of melphalan on column. Assessment of the results showed that the reproducibility of the analytical procedure was acceptable (Table 4).

3.9. Stability of test solutions

3.9.1. Assay

A solution of Melphalan working standard was prepared and analysed using the defined analytical procedure. After storage at ambient temperature (20–30°C) for 6 and 11 h the solution was re-examined. The data (Table 5) showed that the melphalan peak area was essentially unchanged and therefore that solutions of melphalan were sufficiently stable over this period to allow their use in the assay procedure.

3.9.2. Related impurities

A solution of melphalan working standard was prepared and analysed using the defined analytical procedure. After storage at UK ambient temperature ($\approx$ 20°C) protected from light for 1, 4, and 11 days, the solution was re-examined. The data (Table 6) showed that the principal change observed was an increase in methoxymelphalan, due to solvolysis. Levels of the potential degradation products dihydroxymelphalan, monohydroxymelphalan, and dimer did not significantly increase.

3.9.3. Application to stored samples of melphalan

The related impurities test method has been

Table 6
Stability of melphalan in methanolic solution (related impurities)

	Elapsed time (days)			
	Initial	1	4	11
Dihydroxymelphalan	ND	ND	ND	ND
Phthalic acid	ND	ND	ND	ND
Morpholino derivative	0.2	0.2	0.2	0.4
Chloroethylaminomelphalan	0.1	0.1	<0.1	ND
Monohydroxymelphalan	2.5	2.5	2.5	2.4
3-Chloro analogue of melphalan	<0.1	<0.1	0.1	ND
Methoxymelphalan	ND	0.4	1.2	5.3
Ethoxymelphalan methyl ester	0.3	0.2	ND	ND
Chloroethoxymelphalan	0.4	0.4	0.4	0.2
Methoxymelphalan methyl ester	0.1	0.1	ND	ND
Melphalan dimer	0.1	ND	ND	ND
Melphalan methyl ester	0.3	0.2	0.5	0.5
Total	4.2	4.7	6.1	11.0

Table 7
Examination of stored samples of melphalan at ambient conditions[a]

Stability Trial Number	0781 (batch 41418)		0782 (batch 41419)	
Date put on test	June 1992		June 1992	
Time point	Initial	57 months	Initial	57 months
Storage conditions (°C)	5	5	5	5
Related impurities by LC (% area/area)				
Dihydroxymelphalan	NQ	<0.1	NQ	<0.1
Phthalic acid	NQ	ND	NQ	ND
Morpholino derivative	NQ	0.1	NQ	0.2
Monohydroxymelphalan	1.5	1.5	1.7	1.7
Methoxymelphalan	NQ	0.2	NQ	0.2
Ethoxymelphalan methyl ester	NQ	<0.1	NQ	<0.1
Chloroethoxymelphalan	<0.1	0.4	1.1	0.8
Methoxymelphalan methyl ester	NQ	ND	NQ	ND
Melphalan dimer	0.2	0.6	0.1	0.5
Melphalan methyl ester	0.1	0.3	0.1	0.2
Total	1.9	2.9	3.0	3.4

[a] NQ = not quantifiable by existing BP isocratic method; ND = not detected with LOD = 0.05%.

applied to stored samples of melphalan, on formal stability test at 5°C/ambient humidity. At the initial timepoint, the samples were subjected to the isocratic HPLC method defined in the BP monograph for melphalan. At the 57-month time point, samples were taken and the gradient HPLC method for related impurities was applied. The data (Table 7) showed slight increases in levels of melphalan dimer. There was no other significant change in impurity content, including the potential degradation products dihydroxymelphalan and monohydroxymelphalan.

4. Conclusions

A robust and sensitive HPLC procedure has been

developed to allow rapid and automatable determination of the melphalan content of drug substance. Given that the known related impurities of melphalan include polymeric species, it is considered unlikely that an isocratic method will be capable of accurately quantifying the entire range of impurities found. The method has been validated and is demonstrated to be selective and stability indicating. The method can also be applied to bulk assay and impurity testing of solid oral dosage forms such as Melphalan (Alkeran®) Tablets.

Monohydroxymelphalan is the only impurity which occurs at levels in excess of 1%—this impurity has also been shown to be present in vivo due to metabolic conversion from the parent compound melphalan.

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