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## Note

# Quantitative determination of melphalan in plasma by liquid chromatography after derivatization with N-acetylcysteine

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Melphalan is an alkylating agent of the nitrogen mustard type used for the treatment of malignant melanoma, multiple myeloma and ovarian carcinoma. During the last few years, several liquid chromatographic procedures have been presented for the determination of melphalan in biological materials [1—7]. However, only a few of these methods are applicable to pharmacokinetic studies after low peroral doses, since these studies require determination of melphalan at the low ng/ml level [8].

Melphalan is rapidly hydrolysed in aqueous solution [9—11]. Hence, conditions during the work-up procedures have to be thoroughly investigated. Furthermore, the liquid chromatographic separations have in many cases been performed at elevated temperatures (40—60°C), which might adversely affect the chromatographic yield of melphalan [3—6]. In the present study, melphalan is converted to a stable N-acetylcysteine derivative by reaction with N-acetylcysteine prior to the liquid chromatographic separation. The use of fluorimetric detection allows quantitative determination of melphalan concentrations exceeding 5 ng/ml plasma.

## EXPERIMENTAL

### *Chemicals*

Melphalan was purchased from The Wellcome Foundation (Dartford, U.K.). Octanesulphonic acid was obtained from Fisons (Loughborough, U.K.) and trichloroacetic acid and N-acetylcysteine from Merck (Darmstadt, F.R.G.).

### *Instrumentation*

The liquid chromatographic system consisted of a LDC 711 pump, a

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Rheodyne (Model 7125) injection valve with a sample loop of 100 μl and a Shimadzu RF-530 fluorescence detector (260/360 nm) or an LDC Spectro Monitor III photometric detector measuring at 260 nm. A radial compression separation column system (Waters) with a Nova-Pak $C_{18}$ Radial-Pak cartridge (particle diameter 5 μm) or a stainless-steel column (150 mm × 4 mm I.D.) packed with μBondapak $C_{18}$ (particle diameter 10 μm) were used. A presaturator column (μBondapak $C_{18}$, 10 mm × 4 mm I.D.) was placed between the pump and the injector.

The mass spectrum of the melphalan derivative was obtained using a $^{252}$Californium plasma desorption time-of-flight mass spectrometer (Bio Ion Nordic, Uppsala, Sweden) [12].

*Synthesis of the bis-N-acetylcysteine derivative of melphalan*

Melphalan (25 mg), N-acetylcysteine (55 mg) and sodium hydroxide (28 mg) in water (10 ml) were mixed (ultrasonic bath) and heated for 15 min at 70°C. The reaction mixture was evaporated to ca. 1 ml and transferred to a cation-exchange column (AG-50W-X8, 50—100 mesh, 150 mm × 20 mm I.D.). The column was washed with 0.1 *M* hydrochloric acid (50 ml) and water (100 ml). Finally, the derivative was eluted with 1 *M* ammonia. Fractions (5 ml) were collected and the fractions containing the derivative (evaluated by liquid chromatography with photometric detection) were pooled and evaporated to dryness. The structure of the derivative was established by $^{252}$Californium plasma desorption time-of-flight mass spectrometry.

*Procedure for the determination of melphalan in plasma*

Plasma (4°C, 1.00 ml) was mixed with 0.25 *M* trichloroacetic acid (4°C, 1.00 ml) and left at 4°C for 30 min and centrifuged for 10 min (1500 *g*). The supernatant (1.00 ml) was mixed with 1 *M* N-acetylcysteine (0.200 ml), 2.65 *M* sodium hydroxide (0.200 ml) and 0.5 *M* phosphate buffer (pH 11.0, 0.200 ml). The solution was heated for 15 min at 70°C, mixed with 2 *M* citric acid (0.200 ml) and filtered (Millex-Fg, 0.2 μm, Millipore). An aliquot of 0.100 ml was injected into the liquid chromatographic system run at room temperature (Support: Nova-Pak $C_{18}$; mobile phase: 0.036 *M* citrate buffer, pH 4.25, containing 4% (v/v) ethanol and $5 \cdot 10^{-5}$ *M* octanesulphonic acid). Quantitation was based on peak-area measurements using fluorimetric detection (260/360 nm). The results were evaluated using a calibration curve obtained by adding melphalan (in methanol) to plasma samples that were subsequently processed as described above.

## RESULTS AND DISCUSSION

*Derivatization conditions*

The derivatization of melphalan in aqueous solution requires the use of a high concentration of a powerful nucleophile to efficiently compete with hydrolysis reactions of the nitrogen mustard group [13]. In this study, melphalan has been derivatized with N-acetylcysteine. Initially, reactions with sodium sulphide [14] and sodium thiosulphate [13] were investigated. However, sodium sulphide was easily oxidized, giving problems owing to clogging of the

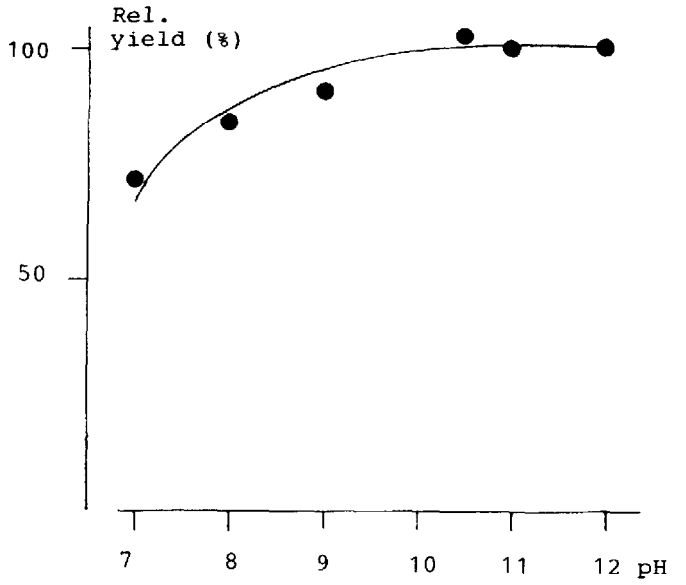


Fig. 1. Influence of pH on the yield of the N-acetylcysteine derivative of melphalan. Concentration of N-acetylcysteine, 0.1 *M*; temperature, 50° C; reaction time, 90 min.

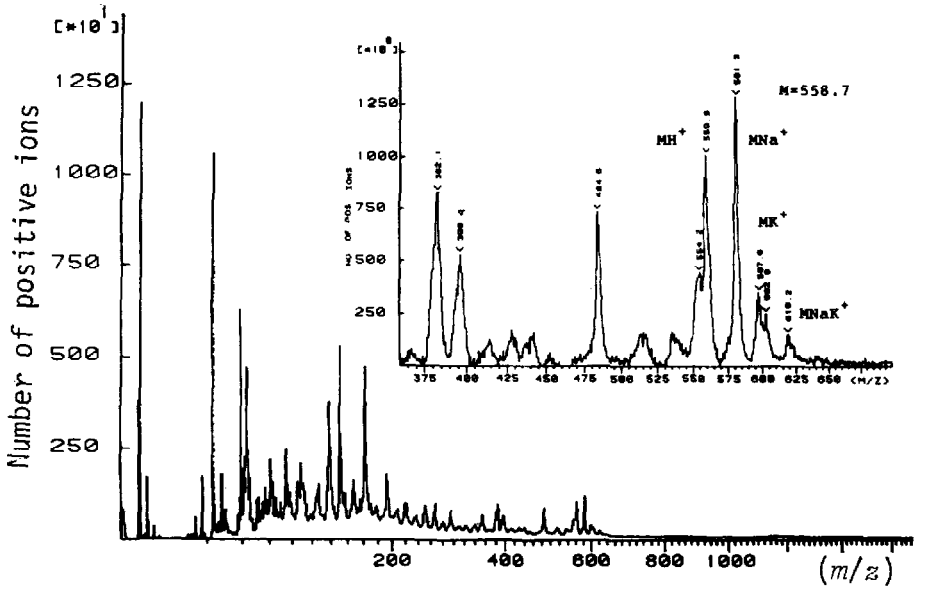


Fig. 2. $^{252}$Californium plasma desorption time-of-flight mass spectrum of the N-acetylcysteine derivative of melphalan.

liquid chromatographic system, and the reaction with thiosulphate gave a derivative that could not be determined with sufficient sensitivity or selectivity using fluorimetric or photometric detection. The influence of pH on the yield of the N-acetylcysteine derivative is given in Fig. 1. A maximum yield is obtained at pH > 10. Obviously, the sulphydryl group ($pK_a$ 9.6 [15]) of N-acetylcysteine must be present as an anion to supress hydrolysis reactions of

the nitrogen mustard group. A complete reaction was obtained within 15 min at 70°C using 0.1 *M* acetylcysteine at pH 11. The absolute yield was 96% as determined by comparison with the purified reference compound. A decrease of the concentration of N-acetylcysteine to 0.01 *M* gave a reduction of the yield of ca. 10%. The Californium plasma desorption time-of-flight mass spectrum (Fig. 2) clearly establishes that the chlorine atoms of melphalan have been substituted with N-acetylcysteine groups.

No degradation of the melphalan derivative was observed, as studied at 25°C in the reaction mixture (0.1 *M* acetylcysteine, pH 11) and in phosphate buffer (pH 7) for 72 h.

*Liquid chromatography*

The retention of the derivative on a μBondapak $C_{18}$ and a Nova-Pak $C_{18}$ column was regulated by pH and the addition of a lipophilic anion to the mobile phase. The capacity factor rapidly increased when lowering the pH below 5 (Fig. 3), which must be due to the fact that an increased fraction of the carboxylic groups of the derivative will be present in an uncharged form at lower pH, rendering it more lipophilic. The influence of the concentration of octanesulphonic acid on retention is given in Fig. 4. The decreased retention at higher octanesulphonic acid concentrations probably reflects the competition between the derivative and the anion with regard to binding sites on the solid support. The Nova-Pak $C_{18}$ column had similar retention characteristics to the μBondapak $C_{18}$ column, but gave considerably higher column efficiency.

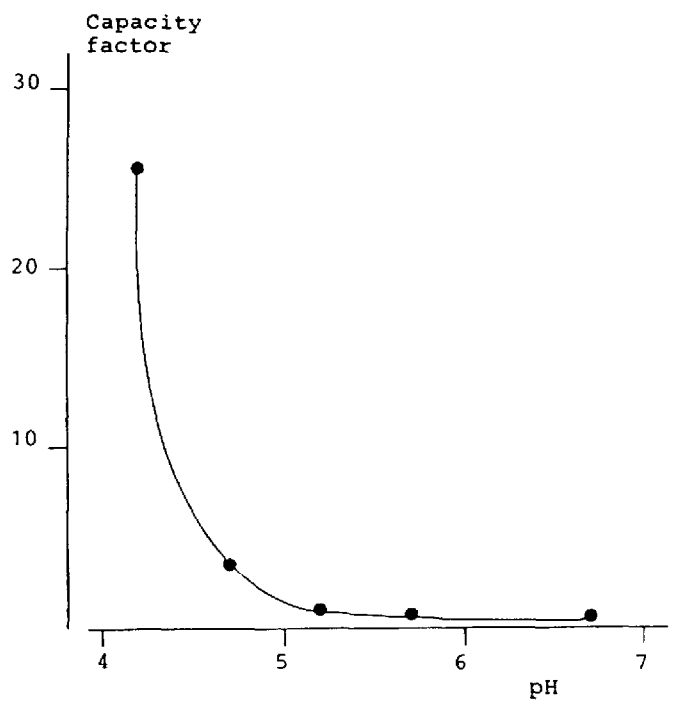


Fig. 3. Influence of pH on the capacity factor. Column, μBondapak $C_{18}$; mobile phase, buffer containing octanesulphonic acid (0.01 *M*).

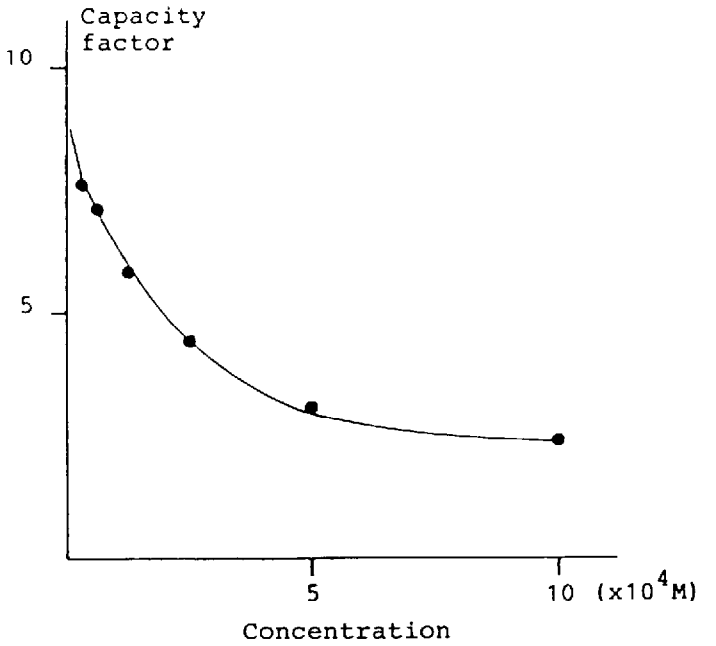


Fig. 4. Influence of the concentration of octanesulphonic acid on the capacity factor. Column, μBondapak $C_{18}$; mobile phase, buffer (pH 4.25) containing 4% (v/v) ethanol.

*Sensitivity and selectivity*

No endogenous compounds interfered in the analysis of melphalan (Fig. 5). It was possible to make quantitative determinations of melphalan exceeding 5 ng/ml in plasma with a precision better than 10% (coefficient of variation) (signal-to-noise ratio at 5 ng/ml = 5). Melphalan is extensively eliminated in vivo by hydrolysis to monohydroxy- and dihydroxymelphalan [16]. Monohydroxymelphalan was isolated by liquid chromatography from an aqueous solution of partially hydrolysed melphalan [9] and was subsequently derivatized with N-acetylcysteine. Neither dihydroxymelphalan ($k'$ = 2.5)

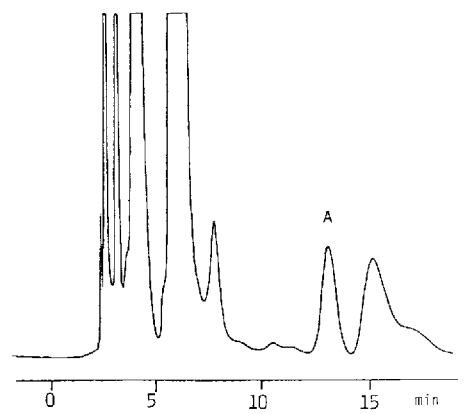


Fig. 5. Chromatogram obtained from plasma containing 90 ng/ml melphalan. A = N-acetylcysteine derivative of melphalan.

(prepared according to Furner et al. [1]) nor the derivatized monohydroxymelphalan ($k'$ = 7.2) interfered with the analysis of the melphalan derivative ($k'$ = 4.0). No N-acetylcysteine derivative was formed in vivo from endogenous sulphydryl compounds as established by analysing plasma from patients on melphalan therapy excluding the N-acetylcysteine derivatization step.

*Linearity and precision*

The standard curve was linear in the concentration range studied (10- 800 ng/ml in plasma). A least-squares analysis give a correlation coefficient of 0.9995, a slope of 8.50 ± 0.11, and an intercept of − 2.4 ± 43.2 ($n$ = 7). The relative standard deviation was 1.4% at the 25 ng/ml level and 3.0% at the 400 ng/ml level.

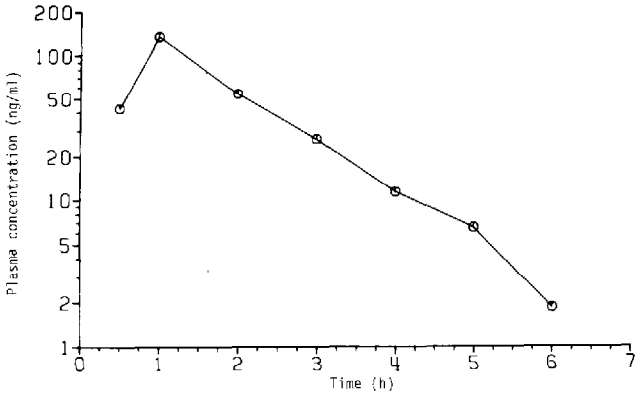


Fig. 6. Plasma concentrations after oral administration of 5 mg of melphalan.

The technique has successfully been applied in studies on the plasma pharmacokinetics of melphalan and the disposition of melphalan in neoplastic cells and tumour tissue, which will be reported in future publications. A plasma concentration—time curve obtained from a patient receiving 5 mg of melphalan by the oral route is given in Fig. 6.

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