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Chlorambucil: stability of solutions during preparation and storage*

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Summary. A stability-indicating high-performance liquid chromatographic method has been used to investigate the stability of chlorambucil in solution. Dissolved in ethanol and diluted with 150 m*M* NaCl (NS), the drug was found to be very much more stable frozen, with a value for 5% degraded ($t_{0,95}$) of approximately 8 months at −70 °C. The presence of agar or cells, and freezing and thawing the solution did not reduce the concentration of chlorambucil significantly. The drug seemed to adsorb to polyvinyl chloride (PVC) infusion bags (but to no other container material) and also to all three filtration units tested. Intense light increased the degradation of the drug, whilst the addition of 10% serum to chlorambucil in medium increased the drug's stability four fold. Dilution with NS of stock solutions of chlorambucil in ethanol resulted in supersaturated solutions of the drug.

For preparation of the drug for in vitro drug sensitivity assays, the results suggest that care should be taken (a) in the initial dilution of the drug; (b) not to use filtration units to sterilize solutions; (c) to anticipate different degradation rates (and therefore cytotoxicity) if the serum concentration in medium is altered; and (d) to avoid the use of PVC.

Introduction

Chlorambucil was one of the original anticancer drugs to be developed and has now been in use for over three decades. It is used in the treatment of chronic lymphocytic leukaemia and malignant lymphomas, and also in ovarian cancer. Chlorambucil has been used in in vitro drug sensitivity assays to predict both for chlorambucil sensitivity in vivo [1, 2] and for cyclophosphamide sensitivity in vivo [14], because of the inactivity of the latter drug in vitro. Chlorambucil is a labile drug [8–10, 12], but when used in vitro it has sometimes been stored frozen in solution with little understanding of how stable it is under these conditions [4, 5]. Thus, a stability-indicating high-performance liquid chromatographic (HPLC) method has been used for the measurement of chlorambucil, not only to determine its stability, but also to investigate other pertinent factors that might compromise the efficacy of the drug before it is added to an in vitro assay.

Materials and methods

Many details of materials and experimental methods have already been published [3], and only significant differences from these are presented below.

Chlorambucil was a gift from the Wellcome Foundation Ltd., Beckenham, UK. Polyvinyl chloride (PVC) infusion bags were obtained from Travenol (Thetford, UK), and polypropylene 'Micronic' tubes from Flow Laboratories (Irvine, UK). Methanol (HPLC grade), glacial acetic acid and cetyltrimethylammoniumbromide (CTMAB) were obtained from Fisons Scientific Apparatus (Loughborough, UK). Light intensities were measured using a radiometer (R101; Macam Photometrics, Livingston, UK).

Chlorambucil was dissolved at 5 mg/ml in absolute ethanol and diluted to 20 μg/ml (unless otherwise stated) with NS at room temperature. All dilutions were carried out in the shortest possible time and used immediately. Fresh solutions were made up at the start of each experiment.

Samples were analysed with a previously described HPLC system [6] and a mobile phase consisting of methanol: PBS (1:1 v/v) plus 0.01% glacial acetic acid and containing 2 m*M* CTMAB, which was filtered prior to use and pumped at a rate of 1.0 ml/min.

In general, triplicate experiments were performed following the degradation of chlorambucil in aqueous solution over several half-lives. Solutions were stored in polypropylene tubes at 20 μg/ml in NS under ambient laboratory lighting (for experiments at room temperature and above) or in the dark for experiments at 8 °C, −20 °C and −70 °C.

Three experiments were undertaken at both −20 °C and −70 °C, and every week for 4 months duplicate aliquots were analysed from each of two tubes. Over the first week, samples were analysed daily and the results averaged over the week to give an initial 100% concentration as previously described [3].

The stability of chlorambucil in 1-ml tubes arranged in the microtitre format (the Micronic system) was also investigated, as we now routinely store drugs this way [1, 2]. Chlorambucil in NS (20 μl) at 10, 5, 2, 1, and 0.5 μg/ml was frozen in the Micronic tubes at −20 °C. Immediately

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before HPLC, samples were thawed and 180 µl NS at room temperature was added, giving final concentrations of 1000, 500, 200, 100 and 50 ng/ml (the concentrations used in our differential staining cytotoxicity [DiSC] assay).

For experiments in medium, chlorambucil, at 5 mg/ml in ethanol, was added to RPMI 1640 medium [7] containing various combinations of 10% FCS, 3% agar (Sea Plaque agarose, Miles Laboratories, Slough, UK) and K562 cells (5×10^5/ml), to give a final drug concentration of 20 µg/ml. The solutions were incubated at 37 °C. At intervals, aliquots were removed, the protein precipitated by addition of two volumes of ice-cold methanol and microcentrifugation, and the resulting supernatant analysed by HPLC. When cells were present, decay was followed in both the supernatant (cells removed by centrifugation) and in the solution containing the cells.

The results of all the degradation studies were fitted to an exponential equation asuming first-order kinetics. For calculations involving the Arrhenius equation, the method of King et al. [11] was used, with a slight rearrangement of their Eq [17] to

$$C = C_0 \exp\left\{-t\left(\frac{0.05129}{t_{x,0.95}}\right)\exp\left[\left(\frac{a}{8.31\times10^3}\right)\left(\frac{1}{T_x}-\frac{1}{T}\right)\right]\right\}, \quad \text{Eq.(1)}$$

where C is the concentration at time t and temperature T (K), C_0 is the initial concentration, a is the activation energy in kJ/mol, T and t are the two independent variables, and $t_{x,0.95}$ is the time for 5% of the drug to degrade at temperature T_x. In practice, for calculation of the activation energy, values of C (calculated as percentages of the initial concentration), t and T were fed into the nonlinear regression computer program NONLIN [3], and a value for a calculated. For calculation of the stability of the standard solution of chlorambucil, T_x was given the value 238 (= −35 °C), as this was the temperature at which the standard was kept, and values for a and $t_{x,0.95}$ were calculated.

Results and discussion

A representative sample of the chromatograms produced with the HPLC conditions used in this work is given in Fig. 1. The main degradation products (monohydroxy- and dihydroxychlorambucil) and the major metabolite (phenylacetic mustard) were well separated from the parent drug. Also, chlorambucil decayed exponentially in NS at different temperatures for a minimum of three half-lives, suggesting the absence of any co-eluting degradation product. For these two reasons, the method was considered to be stability-indicating for chlorambucil.

One potential problem with long-term stability work is that the standard may also significantly deteriorate during the period of the study. This would result in the observed stability being greater than the actual stability of the drug. We therefore investigated the stability of chlorambucil in the mobile phase at 25–60 °C and calculated the stability at −35 °C by extrapolation using Eq. (1). The results suggested that chlorambucil in the mobile phase degraded by 1% in 7.5±0.1 months at −35 °C, considerably longer than the duration of the experiment.

Chlorambucil was found to have 25%–30% greater stability in NS, compared with PBS, whether measured at 8.5 °C ($t_{1/2}$ 31.7 h and 23.0 h, respectively) or 31.5 °C (106±9 min and 77±4 min) presumably because of the higher chloride ion concentration of NS. In agreement

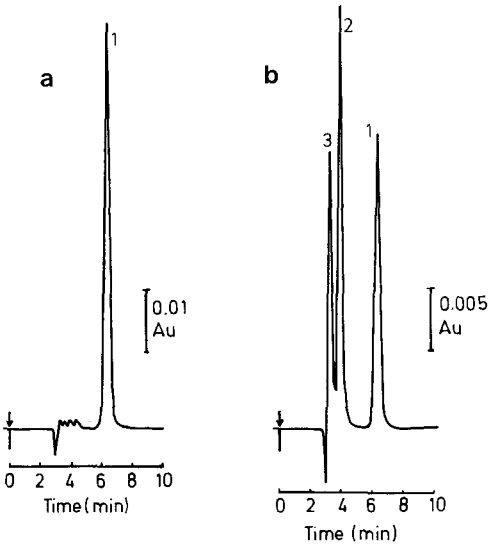

Fig. 1a, b. Chromatography of chlorambucil *(1)*, monohydroxy-chlorambucil *(2)* and dihydroxy-chlorambucil *(3)*. Chlorambucil in NS at 20 µg/ml, **a** at time zero, and **b** after 25 min at 50 °C

with Ehrsson et al. [9], drug concentration did not significantly influence the degradation rate of chlorambucil in NS when tested at 0.05, 1 and 10 µg/ml.

No difference was seen in the half-life of chlorambucil in NS when stored in containers made from glass, siliconized glass, polystyrene, polypropylene, and polyethylene. However, chlorambucil in NS degraded twice as fast when stored at 22 °C in PVC ($t_{1/2}$ = 133 min) than when stored in glass, polystyrene or plypropylene (mean $t_{1/2}$ = 306 ±2 min). In this experiment the peak heights of the degradation products (by HPLC) were also lower than expected, suggesting that chlorambucil probably adsorbed to the PVC, with a resultant increase in apparent degradation rate.

Filtration of aqueous solutions of chlorambucil through cellulose acetate, polysulphone and polytetrafluoroethylene units significantly altered drug concentrations, the rapidly filtered samples (0.5–1.0 s to collect 1 ml) being 85.9%±2.5%, 82.1%±1.8% and 66.4%±3.2%, respectively, of the unfiltered control. These results are in agreement with the observation of Schiffman et al. [13] that chlorambucil binds to membranes. Thus, filtration as a means of sterilization of solutions of chlorambucil is not advised.

Aqueous solutions of chlorambucil were found to degrade faster in the light than in the dark. In ambient lighting this difference was small (11%), but in direct sunlight, chlorambucil degraded considerably faster than in the dark, the difference in half-lives being 41.5%. In these experiments a photodegradation product was observed in the HPLC traces which was distinct from chlorambucil, chlorambucil's degradation products and phenylacetic mustard (Fig. 2), but it was not investigated further.

The stability of chlorambucil in NS (20 µg/ml) at different temperatures is presented in Table 1. All the data fitted apparent first-order rate equations well, with the correlation coefficient, r^2, always >0.99. Using the equation of King et al. [11], the activation energy for chlorambucil hydrolysis in NS was calculated (from data gathered between 8.5 °C and 60 °C) to be 85.4±0.2 kJ/mol. This is smaller

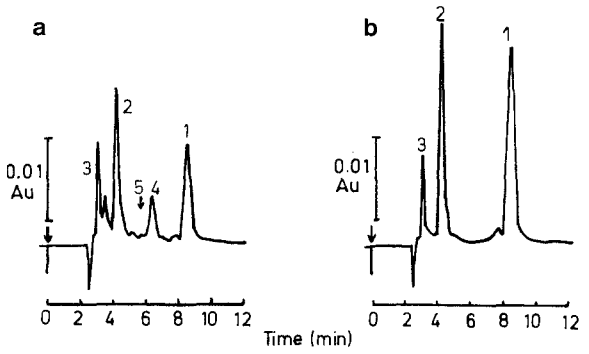

Fig. 2a, b. Chromatography of chlorambucil in NS, **a** after 2.9 h in sunlight (150 W/m^2), and **b** after 2.5 h in the dark at the same temperature. Peaks *1, 2,* and *3* as in Fig. 1; peak *4* is a degradation product only found in the light. The *arrow* labelled *5* is where phenylacetic mustard elutes

Table 1. Stability of chlorambucil at 20 μg/ml in NS at different temperatures

Temperature (°C)	*n*	$t_{0.95}$ (mean ± SD)[a]
60	3	0.47 ± 0.03 min
50	3	1.12 ± 0.03 min
37	3	3.72 ± 0.13 min
31.5	10	7.92 ± 0.28 min
25	1	21.8 min
23	1	23.3 min
22	1	25.8 min
8.5	3	166.0 ± 7.0 min
−20	3	83.4 days
−70	3	250.0 days

[a] Values for $t_{0.95}$ were calculated for the *n* experiments and then averaged, except for the values at −20° and −70°C, where the $t_{0.95}$ was calculated from the pooled data

than the value calculated by Ehrsson et al. [9], possibly because of the presence of NS.

The experiments at −20 °C and −70 °C were run for 4 months, and values for $t_{0.95}$ of 3 and 8 months (respectively) were calculated from the pooled results of the three experiments. Thus the stability of chlorambucil, like that of melphalan [3], is greatly increased by freezing.

One experiment was also undertaken with storage of the drug in "Micronic" tubes for 4 months at −20 °C at 0.5–10 μg/ml. The overall $t_{0.95}$ was calculated to be approximately 30 days, with drug concentration once again not affecting the stability.

In another experiment, up to three cycles of freezing and thawing had no significant effect on chlorambucil concentration (< 3% degraded).

Having previously determined the stability of chlorambucil before it is put into an in vitro assay, an investigation was carried out to determine the drug's stability under typical in vitro assay conditions (RPMI medium containing 10% FCS, 37 °C), and the half-life calculated from three experiments was 123.4 ± 1.3 min. Agar (0.3%) made no difference to the drug's stability with half-lives of 105.6 min (with agar) and 103.9 min (without). The presence of 5×10^5 cells/ml also had no effect whether the half-life was measured in the extracellular fluid or in the fluid plus cells. However, in medium lacking FCS, chlorambucil decayed with a very much shorter half-life (34.2 min), in agreement with the results of Ehrsson et al. [9, 10]. No phenylacetic mustard was observed in any of these experiments even when cells at 5×10^6/ml were present.

Figure 3 shows the results of experiments carried out to investigate the limits of solubility of chlorambucil solutions containing different proportions of ethanol and NS. The results show that the drug is only slightly soluble in NS (approximately 30 μg/ml). It can also be seen that diluting a 5.0 or 0.5 mg/ml stock solution of chlorambucil in ethanol resulted in passing through a supersaturated phase, when precipitation could easily occur. In one routine dilution in a polystyrene container, precipitation *was*

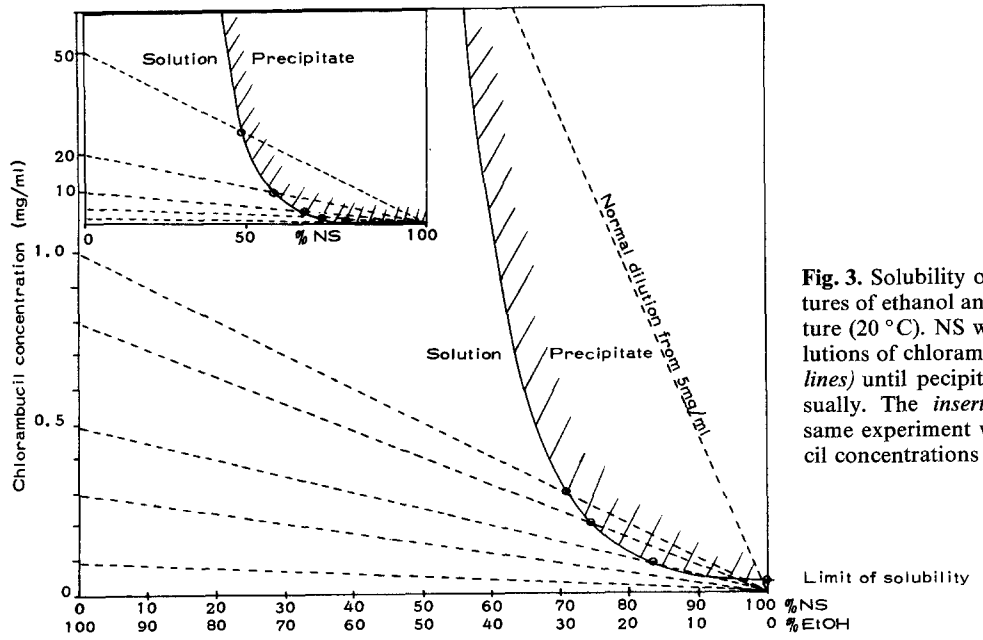

Fig. 3. Solubility of chlorambucil in mixtures of ethanol and NS at room temperature (20 °C). NS was added slowly to solutions of chlorambucil in ethanol *(dotted lines)* until pecipitation was observed visually. The *insert* shows results of the same experiment with higher chlorambucil concentrations

observed to occur (although this is unusual), but cleared within 1–2 min. Analysis of the solution resulted in only 75% of the expected drug concentration, whilst the remainder was found adsorbed to the walls of the container.

Conclusions and recommendations

In this work we have confirmed that chlorambucil is a labile drug, which is more stable in chloride solutions and serum-containing media. Container material (excluding PVC), freezing and thawing, and the presence of agar and cells all have negligable effect on the drug's stability. It can be stored frozen for extended perios ($\geqslant 3$ months), greater stability being seen at $-70\,^{\circ}C$ than at $-20\,^{\circ}C$.

The expected degradation peaks of monohydroxy- and dihydroxychlorambucil have been routinely observed along with a few smaller peaks, including one that only occurred in solutions exposed to light. However, no degradation or metabolism to phenylacetic mustard was observed in any of the experiments, even in the presence of both medium and cells.

Precipitation and adsorption are two possible causes of significant errors in making up solutions of chlorambucil. The drug adsorbs to all filtration units tested and therefore it is recommended that solutions of chlorambucil are not sterilized by filtration, but made aseptically. Precipitation may occur if care is not exercised in the dilution of concentrated stock solutions of chlorambucil with saline, because of the low solubility of the drug in aqueous solutions, and it is therefore recommended that the drug is diluted very rapidly, and that the solution is discarded or the drug concentration checked if any precipitation is observed.

Chlorambucil solutions have also been found to be degraded by intense light, indicating that they should be protected from sunlight. However, as rapid degradation occurs anyway at room temperature, it seems unnecessary to protect the drug from normal laboratory lighting.

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