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Review

Stability of solutions of antineoplastic agents during preparation and storage for in vitro assays

General considerations, the nitrosoureas and alkylating agents

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Summary. *In vitro drug sensitivity of tumour biopsies is currently being determined using a variety of methods. For these chemosensitivity assays many drugs are required at short notice, and this in turn means that the drugs must generally be stored in solution. There are, however, a number of potential problems associated with dissolving and storing drugs for in vitro use, which include (a) drug adsorption; (b) effects of freezing; (c) drug stability under the normal conditions of dilution and setting up of an in vitro assay; and (d) insolubility of drugs in normal saline (NS) or phosphate-buffered saline (PBS). These problems are considered in general, and some recommendations for use of solutions of drugs in in vitro assays are suggested. The nitrosoureas and alkylating agents are also investigated in greater detail in this respect.*

The nitrosoureas are found to be very labile in PBS at pH 7, with 5% degradation ($t_{0.95}$) occurring in 10–50 min at room temperature. These values are increased about 10-fold on refrigeration and about 5- to 10-fold on reduction of the pH of the medium to pH 4–5.

At pH 7 and room temperature, $t_{0.95}$ is observed in under 1 h with the alkylating agents nitrogen mustard, chlorambucil, melphalan, 2,5-diaziridinyl-3,6-bis(2-hydroxyethylamino)-1,4-benzoquinone (BZQ), dibromodulcitol, dibromomannitol, treosulphan, and procarbazine. Of the other alkylating agents, 4-hydroperoxycylophosphamide (sometimes used in vitro in place of cyclophosphamide), busulphan, dianhydrogalactitol, aziridinylbenzoquinone (AZQ), and dacarbazine have a $t_{0.95}$ of between 2 and 24 h, while ifosfamide and pentamethylmelamine are both stable in aqueous solution for > 7 days.

About half the drugs studied in detail have been stored frozen in solution for in vitro use, although very little is known about their stability under these conditions.

Introduction

In vitro determinations of chemosensitivity on biopsy material from tumours, unlike most other in vitro drug experiments, require a number of different drug solutions to be rapidly accessible. To provide these by making up fresh solutions each time, although ideal, is usually not feasible, since the assay is best set up as soon as possible after the specimen is received. Most researchers in this field overcome the problem by freezing aliquots of the drug solutions and thawing them just prior to their addition to the assay [10, 17, 36, 71, 73, 117–119, 145–147, 155–157]. However, even under these conditions,

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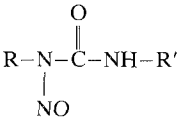
Table 1. Structures and NSC numbers of the nitrosoureas



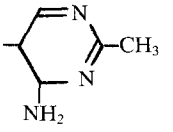
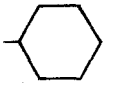
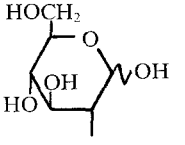
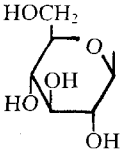
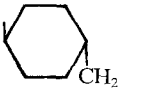
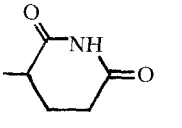
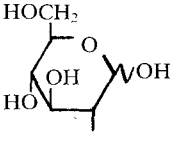
Drug	R	R′	NSC No.
ACNU	$ClCH_2CH_2-$		245382
BCNU	$ClCH_2CH_2-$	$-CH_2CH_2Cl$	409935
CCNU	$ClCH_2CH_2-$		79037
Chlorozotocin	$ClCH_2CH_2-$		178248
GANU	$ClCH_2CH_2-$		
Methyl-CCNU	$ClCH_2CH_2-$		95441
PCNU	$ClCH_2CH_2-$		95466
Streptozotocin	CH_3-		85998

Table 2. Structures and NSC numbers of alkylating agents

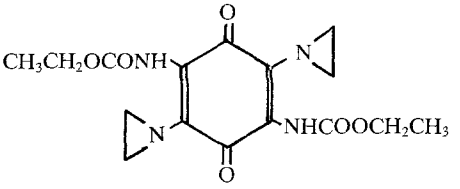
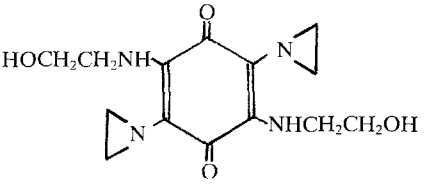
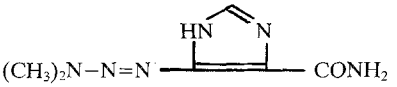
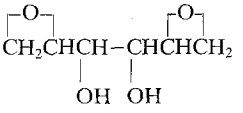
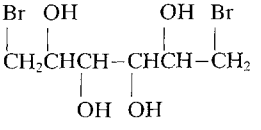
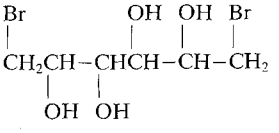
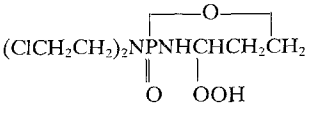
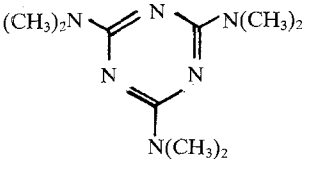
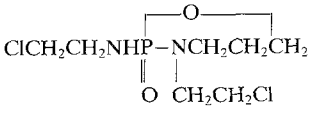
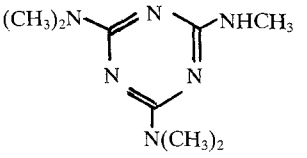
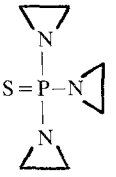
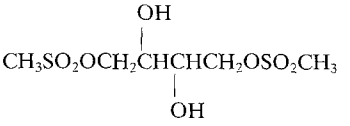
Drug	Structure	NSC No.
AZQ (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone)		182986
BZQ (2,5-diaziridinyl-3,6-bis(2-hydroxyethylamino)-1,4-benzoquinone)		224070
Busulphan	$CH_3SO_2O(CH_2)_4OSO_2CH_3$	750
Chlorambucil	$(ClCH_2CH_2)_2NpC_6H_4CH_2CH_2CH_2COOH$	3088
Dacarbazine		45388
Dianhydrogalactitol		132313
Dibromodulcitol		104800
Dibromomannitol		94100
4-HC (4-hydroperoxycyclophosphamide)		181815
Hexamethylmelamine		13875
Ifosfamide		109724
Melphalan	$(ClCH_2CH_2)_2NpC_6H_4CH_2CH(NH_2)COOH$	8806
Nitrogen mustard	$(ClCH_2CH_2)_2NCH_3 \cdot HCl$	762

Table 2 (continued)

Drug	Structure	NSC No.
Pentamethylmelamine		118742
Procarbazine	$CH_3NHNHCH_2pC_6H_4CONHCH(CH_3)_2$	77213
Thio-TEPA		6396
Treosulphan		39069

some drugs have been found to be unstable [16, 52, 157, 163].

The crucial question is: will the drug still be at least 95% potent by the time it is added to the cells in the assay? The answer depends on the drug and the conditions under which it is stored and handled. It is, therefore, the object of this review (a) to pinpoint some of the areas where drug instability could inadvertently be caused; (b) to bring together pertinent information about the stability of solutions of the nitrosoureas and alkylating agents; and (c) to make specific suggestions about the storage and handling of some of these antineoplastic agents. It is hoped that some of the work that so far has had to be undertaken empirically in each laboratory working with chemosensitivity assays might thereby be avoided, although a great deal more work is required in this area. No attempt has been made to identify or investigate drug metabolities, or the stability of the drugs once in the assay. This latter problem has been investigated for melphalan [14] and a number of other drugs [3, 93].

Structures and NSC numbers are presented for the nitrosoureas and alkylating agents in Tables 1 and 2, respectively.

Calculations

In a few instances, drug stability data have been calculated at lower temperatures than those reported by using the Arrhenius equation [80, 120]:

$$\log k = \log Z_0 - \frac{a}{2.303\,k}\left(\frac{1}{T}\right), \tag{1}$$

where k is the apparent first-order decay constant measured in time^{-1}, T is the temperature in degrees absolute (° C + 273), a is the activation energy in kJ/mol, Z_0 is the collision number and $\boldsymbol{k}$ is a constant ($= 8.31 \times 10^{-3}$).

The value of k_x at temperature T_x can either be calculated from two known values of k (k_1 and k_2) at temperatures T_1 and T_2 using Eq. (2):

$$\log k_x = \log k_1 - \left(\frac{1}{T_x} - \frac{1}{T_1}\right)\frac{(\log k_1 - \log k_2)}{\left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \tag{2}$$

or from one known value of k and a:

$$\log k_x = \log k_1 - \frac{a}{0.01914}\left(\frac{1}{T_x} - \frac{1}{T_1}\right). \tag{3}$$

The resultant values of k must obviously be treated with a certain amount of caution, and a better method of calculating them, where original data are available, would be that described by King et al. [75]. The time for 5% of the drug to degrade ($t_{0.95}$) has been calculated using Eq. (4):

$$t_{0.95} = \frac{-\log_e 0.95}{k} = \frac{0.05129}{k} = \frac{\text{half-life}}{13.5134}. \tag{4}$$

General considerations and potential problems with drug preparation and storage

Potential problems may be encountered with drug preparation and storage in a number of different areas.

Drug adsorption

For a few drugs, adsorption is a significant problem, and for all drugs it must be considered [89]. Drugs can adsorb to filters [25, 106, 107, 116, 122] (one filter set is stated to be able to adsorb up to 5 mg drug substance [129]) or containers [42, 135], and can dissolve into plasticisers used in the manufacture of flexible polyvinylchloride (PVC) (sorption) [81, 103]. This

Table 3. Binding of drugs to cellulose ester membranes[a]

Negligible binding < 5%	Slight binding 5–15%	Considerable binding > 15%
Bleomycin	Chlorozotocin	Actinomycin D[b]
cis-Platinum	Daunorubicin[b]	Adriamycin[b]
Cytosine arabinoside	Vincristine[b]	Dihydroxy-anthracenedione[b]
Dexamethasone		ICRF 187
5-Fluorouracil		Maytansine[b]
Melphalan[c]		Razoxane
Pentamethylmelamine		
Streptozotocin		
Thio-TEPA		
Vinblastine		

[a] In most cases, a 1-ml sample of drug solution was filtered through a cellulose acetate/nitrate sterile disposable filter unit. Post-filtration concentration was then compared with control [14, 106, 107]

[b] These drugs also bound significantly to PTFE filters [106, 107]

[c] Did not bind significantly to PTFE or polysulfone membranes [14]

latter problem may be part of the cause of reduced stability of some drugs when stored in PVC containers [8, 9, 76].

Twelve antineoplastic drugs tested by Pavlik et al. [106, 107] showed > 5% adsorption with sterile filtration units made from cellulose nitrate/cellulose acetate esters (Millex OR), and the results of these and other experiments [14, 25, 116, 129] are shown in Table 3. Seven of the 12 drugs tested by Pavlik et al. also showed binding of > 10% to polytetrafluoroethylene (PTFE) filters (Millex FG) [106, 107]. Greater than 95% of the two drugs most affected, actinomycin D and adriamycin, was adsorbed by the cellulose filters, and they were also adsorbed to a considerable extent by the PTFE units. It is of interest to note that whilst vincristine showed significant adsorption, vinblastine, with a very similar structure, showed negligible binding [25, 106, 107]. Similarly, adriamycin adsorbs far more strongly than daunorubicin [106, 107].

Actinomycin D [21] and adriamycin [61, 135] have also been shown to adsorb to a number of other materials, and this may be the cause of part of the latter drug's 'degradation' in solution [135]. Neither siliconised glass nor polypropylene binds adriamycin [135]. However, this is not necessarily a general phenomenon, as a number of antibiotics and other drugs bind more strongly to siliconised glass than to glass [100, 135]. Bleomycin [24] and mithramycin [39] may adsorb to metals as, like adriamycin [5, 59], they form complexes with them.

Because of the potential problem of drug adsorption, we do not sterile-filter drug solutions, but simply make them up aseptically.

Effects of freezing

Although the obvious temperature for storage of solutions of the more labile drugs would seem to be as low as possible (i.e., −70° C or in liquid nitrogen), it has been suggested that this is unwise unless stability studies have been undertaken [142]. A number of drugs have been reported to be unstable when frozen including BCNU [157], BZQ [16], 5-fluorouracil and melphalan [163]. Adriamycin has also been found to be unstable when frozen in plasma [47]. Other workers, however, have disagreed with these findings on BCNU, 5-fluorouracil [52] and melphalan [14], and further research is therefore required in this important area.

A number of drugs are packaged in solution and, according to the manufacturer's instructions, should not be refrigerated and/or frozen [113, 136]. This is usually due to the possibility of precipitation or crystallization at lower temperatures, although the low concentrations generally required for in vitro assays will obviously reduce this problem.

In general, repeated freezing and thawing should be avoided, and most workers in the field never refreeze drug solutions.

All drugs should be frozen in tubes that are not much larger than the solution volume and are tightly capped to reduce evaporation of liquid both within and out of the tube. We routinely use 1.2-ml polypropylene liquid nitrogen tubes (Nunc, from Gibco Europe, Uxbridge, England) and store the drugs in 0.5-ml or 0.7-ml aliquots.

Drug stability

A number of drugs are sensitive to light, and therefore should not be exposed to light for extended periods. These include dacarbazine (DTIC) [6, 70, 78, 97, 98, 126, 136], 5-fluorouracil [99, 136], methotrexate [30], neocarzinostatin (NSC 69856) [23], *cis*-retinoic acid [96, 132], bleomycin [40, 41, 134], adriamycin, and daunorubicin [37, 131], BZQ [16], and possibly BCNU [136].

The stability of a number of drugs is pH-dependent, and caution is required when making up, storing or investigating the stability of these drugs in unbuffered solutions such as water or 150 m*M* sodium chloride solution (NS). We have found that glass-distilled water, and NS from PVC containers (Travenol Laboratories Ltd., Norfolk, England) have a pH of 4–5, whereas water for injection and NS, both from glass containers (Phoenix Pharmaceuticals, Gloucester, England) have a pH close to 7. This would make a dramatic difference to the stability of drugs such as the nitrosoureas (5–10× more stable at pH 5 [31, 82], azacytidine [72] or BZQ (about 20× less stable at pH 5 [16]. This problem may be the cause of discrepancies in published BCNU stability [52, 163], and another potential source of the differences seen in the stability of drugs stored in plastic or glass containers [8, 9].

In general, the two most labile classes of drugs are the alkylating agents and nitrosoureas. For many of these drugs, more than 5% of the drug will have degraded when a solution is left at room temperature in Dulbecco's phosphate-buffered saline (PBS) or NS for 1 h. On the whole, the steroids, anthracyclines, antibiotics, antimetabolites, and vinca alkaloids are more stable.

Low solubility or stability of drugs in PBS or NS

6-Mercaptopurine and 6-thioguanine will only dissolve at alkaline pH (in weak solutions of alkali hydroxides), and even when diluted with PBS, will still be alkaline when added to an in vitro assay. A number of other drugs need to be dissolved in nonaqueous solvents such as dimethyl sulphoxide (DMSO) or ethanol due to their low solubility in PBS or NS. Most of these solutions can subsequently be diluted with PBS.

Additions of solvent or solutions of nonneutral pH can obviously have significant effects on in vitro assays. For example, the cytotoxicity of chlorambucil [20] and BCNU [62, 162] and the incorporation of ^{3}H-uridine after 5-fluorouracil treatment [104] have all been found to be strongly pH-de-

Table 4. Stability of nitrosoureas in PBS or similar solution at room temperature

Drug	Approx $t_{0.95}$ at room temperature (min)	Membrane filtration[a]	Drug stability reduced by	References	References to drug stored in solution frozen[b]
ACNU[c]	10/30[d]	...	...	[68, 121]	...
BCNU[c, e]	40	...	Freezing?[f]; Serum proteins	[9, 31, 35, 52, 78, 82, 90, 101, 157]	[10, 36, 108, 118, 145, 147][f]
CCNU[c]	50	...	Serum proteins	[31, 101, 121, 154, 158, 161]	...
Chlorozotocin	20	+	...	[31, 121, 137, 158, 161]	...
GANU	10	...	...	[121]	...
MeCCNU[g]	50	...	Serum proteins	[31, 111, 154]	...
PCNU	25	...	...	[137, 154, 158]	...
Streptozotocin	50	0	...	[31, 139, 158]	[108, 147]

[a] Binding to cellulose acetate/nitrate filters: 0, none; +, slight; ..., no data available (see Table 3)
[b] References are tabulated here when the authors have reported that 'all drugs were stored frozen in solution', and the drug has then been used in their work. It is conceivable, however, that this might have been an over-simplification for the purposes of writing the paper due to the large numbers of drugs involved, and occasionally all the drugs mentioned in a publication may not have been stored frozen
[c] Additional information can be found in the text
[d] Results at variance [68, 121] (see text)
[e] Other precautions for BCNU: cytotoxicity (a) affected by serum concentration [61,], (b) greatly affected by temperature [61–63], reduced by rat hepatocytes [4]
[f] One author [157] reported instability, others have frozen the drug successfully
[g] Cytotoxicity reduced by rat hepatocytes [4]

pendent. However, we have found surprisingly little effect on adding 10% of solutions of nonneutral pH (0.01 *M* citrate buffer pH 4.0, 1.26% $NaHCO_3$, or 0.04 *M* NaOH) to dye-exclusion assays [10] both in terms of the pH of the medium and the control viability (Bosanquet AG, Bird MC, unpublished observations).

In other experiments using the same assay [10], we have found that 0.1% DMSO or 0.05% ethanol are the maximum solvent concentrations that have no effect on control cells (Bosanquet AG, Bird MC, unpublished observations). A concentration of 0.1% (in upper agar layer, or 0.05% allowing for diffusion; continuous solvent exposure) of DMSO or ethanol has also been found to be acceptable in the clonogenic assay (Alberts DS, Einspahr J, personal communication) although the limit may be as high as 0.5% for ethanol [107].

These preliminary experiments suggest that drugs which cannot be dissolved in neutral aqueous solutions can usually be readily incorporated into in vitro assays, and that drugs that are more stable at nonneutral pH, such as BZQ (pH 9 [16]) and BCNU (pH 5 [82]) can often be made up at the more stable pH and added to the assay without detrimental effects.

Other potential problems

Drugs need to be checked carefully if powder is to be weighed, or even when a vial is made up according to the manufacturer's instructions. Many drugs are formulated with other materials (such as mannitol) and Pavlik et al. [107] have suggested that all drugs should be made up from the pure powders. It has also been pointed out that 1 g cyclophosphamide is either 1.069 g of the monohydrate (= 1 g cyclophosphamide) or 1 g of the monohydrate (= 0.935 g cyclophosphamide), depending on the source of the material [66, 148].

The presence or absence of a preservative in the water has also been found to have a considerable effect on the stability of cyclophosphamide [18] and actinomycin D [39]. This is a reminder that small details which can seem insignificant may be quite important for drug stability.

Wheeler et al. [160] reported a shorter half-life for BCNU when incubated at a lower concentration. Such concentration-dependent stability (or adsorption) should also be borne in mind when very dilute solutions of drugs are stored.

For work on drug stability, stability-indicating assays should be used [138]. Where this is not done major errors can occur [8] and may be the cause of discrepancies between results.

Drug incubation cautions

Two groups have found that drug exposure at increased temperatures often affects cytotoxicity greatly [61–65]. Although methotrexate [62] and vindesine [64] showed little temperature effect, adriamycin [63, 65], bleomycin [62, 63, 65], mitoxantrone (NSC 301739) [64], *cis*-platinum [63], and BCNU [61–63] all showed far greater cytotoxicity at higher temperatures. At pH 6.6 Chinese hamster cells showed a 28,000-fold decrease in surviving fraction on incubation with BCNU (3.3 μg/ml) at 43° C compared with 37° C [62]. This approximates a 5-fold increase in cytotoxicity per centigrade degree. Amsacrine (NSC 249992), on the other hand, showed a large decrease in cytotoxicity with increased temperature [64]. This indicates that drug incubation temperature and timing should be very carefully controlled, and this problem is one reason why we now use continuous drug incubation [10].

Recently it has been found that cell density has an effect on cytotoxicity [26, 79] with the LD_{50} for 4-HC rising 5-fold when the cell concentration was raised from 10^5 ml^{-1} to 2.2×10^7 ml^{-1} [79]. Drug exposure should therefore preferably be undertaken at a constant cell concentration.

Stability of the nitrosoureas

Much work has been undertaken on the nitrosoureas (Table 1) in attempts to determine the alkylating species of these drugs [31, 82, 83, 90, 101, 111, 151, 158, 161]. This and other work [7, 9, 27, 34, 35, 78, 84, 121, 150, 152–154, 159, 160] has shown

Table 5. Summary of the stability of alkylating agents

Drug	Approx $t_{0.95}$ at room temperature in PBS	Most stable pH	Membrane filtration[a]	Effect of light[b]	Drug stability reduced by
AZQ	21 h	6.3	...	0	↑ buffer strength ↑ ionic strength
BZQ	1 h	9	0[d]	+	↑ buffer strength ↑ ionic strength
Busulphan	~ 3 h[c]	...	...	...	Plasma
Chlorambucil	~ 15 min	< 3	? +	...	...
Dacarbazine	24 h[f]	?4	...	+	...
Dianhydrogalactitol	> 8 h	...	...	...	...
Dibromodulcitol	~ 30 min[e]	...	...	...	...
Dibromomannitol	~ 30 min[e]	< 7	...	+	↑ pH
4-HC	~ 2.5 h	< 5	...	...	↑ ionic strength Phosphate Bicarbonate
Hexamethylmelamine	? > 7 days	...	...	...	...
Ifosfamide	> 7 days	...	...	...	...
Melphalan	50 min	< 3	0	0	...
Nitrogen mustard	Short (? < 10 min)	...	...	...	...
Pentamethylmelamine	> 7 days	...	0	...	...
Procarbazine	1.5 min	...	...	+	Oxygen UV light
Thio-TEPA	> 12 h	...	0	...	...
Treosulphan	5 min (pH 8.5)	...	...	...	...

[a] Drug binding to cellulose acetate/nitrate membranes (see Table 3): 0, none; +, slight; ..., no data available
[b] 0, not photolabile; +, photolabile; ..., no data available, but presumably not photolabile as the photolabile drugs are usually well documented
[c] See Table 4 footnote[b]
[d] Bosanquet AG, unpublished observations
[e] Calculated from values in *Comments*
[f] pH ~ 4.0, not protected from light [78]. Although the photodegratation products of dacarbazine may cause unpleasant side effects in vivo [6], light may be the best method of activating this drug [98, 147]

that nitrosoureas are very labile in aqueous media. In general, the stability of these compounds is better in acid medium (pH 3–5) [31, 82, 137, 139], or in ethanol solution [7, 38, 83, 90, 101]. Table 4 summarises the stability data of eight nitrosoureas and further details on BCNU, CCNU, and ACNU are given below.

BCNU

BCNU is most stable at about pH 5, with a slight decrease in stability at lower pH, and an approximately 3-fold decrease in half-life for each pH unit from pH 5 to 8 [82]. It is marginally more stable in Tris or citrate buffers at pH 7 than in phosphate [82, 90, 101], but buffer strength does not seem to affect its half-life [31, 82]. The degradation of BCNU in PBS will, therefore, not be much different from that in other buffers at pH 7.3, with a half-life at 22° C of about 9 h, and $t_{0.95}$ of 40 min (interpolated from Fig. 2 of Laskar and Ayres [82]). At 5° C the $t_{0.95}$ becomes 9 h. These figures approximately agree with those of Kleinman et al. [78]. At pH 5 the values of $t_{0.95}$ at 22° C and 5° C are 5 h and 60 h, respectively. Montgomery et al. [101] found greatest stability of BCNU in water, but this may well have been the coincidence that distilled water has a pH similar to the most stable pH for the drug. Similarly, bicarbonate reduces the stability of BCNU [35], presumably because of the increase in pH.

The storage of solutions of BCNU in glass (as opposed to PVC) containers may be beneficial to the drug's stability [9], although both pH or sorption [103] could also be the cause of this difference (see above).

The two studies reporting the stability of frozen solutions of BCNU have reached opposite conclusions [52, 157], and further work is require to clarify these conflicting data.

The drug is very much more soluble in ethanol [38] and stable in nonaqueous solutions [7, 83, 90, 101]. In 95% ethanol the $t_{0.95}$ at 22° C has been reported at 5.5 [101] and 10 days [7,

Drug	Comments	References to stability	References to storage frozen[c]
AZQ	$t_{0.95}$ at pH 6.3 in 0.01 *M* phosphate buffer = 48 h at room temperature [109]	[109, 110, 137]	Unstable frozen[d]
BZQ	Dissolve in H_2O pHed to 9.0 or 1.26% $NaHCO_3$ for longer $t_{0.95}$ [16]	[16]	Polymerises (?) on freezing [16]
Busulphan	$t_{1/2}$ = 16 h at 37° C [43]	[43]	...
Chlorambucil	See text	[32, 45, 88, 105, 122, 149, 164]	[10, 17, 145, 147]
Dacarbazine	Probably requires activation [97, 98, 147]; Cytotoxicity reduced by rat hepatocytes [4]	[70, 78, 126]	[36]
Dianhydrogalactitol	...	[141]	...
Dibromodulcitol	$t_{1/2}$ = 2.3 h at 35° C [143]	[143]	...
Dibromomannitol	$t_{1/2}$ = 15 min at 60° C [48]	[48, 112]	...
4-HC	See text	[12, 13, 91, 92, 166]	[10, 17, 36]
Hexamethylmelamine	...	...	[36, 147]
Ifosfamide	$t_{0.95}$ > 6 weeks at 4° C [140]; 4-Hydroxyfosfamide stability similar to that of 4-hydroxycyclophosphamide [92]	[140]	...
Melphalan	See text	[14, 28, 29, 33, 44, 49, 51, 55, 69, 114]	[10, 36, 118, 147, 155, 157, 167, 168]
Nitrogen mustard	Cytotoxicity reduced by albumin [130]; $t_{1/2}$ also recorded as 1–2 months [133]	[53, 54, 133]	[155, 157]
Pentamethylmelamine	...	[106, 137]	...
Procarbazine	Activated by cytochrome P_{450} [127], and rat hepatocytes [4]	[22, 60, 115]	[36]
Thio-TEPA	Protect solid from temperatures over 10° C and from light [112]	[53, 77, 106]	[147, 156]
Treosulphan	At pH 7.5 drug degrades to dianhydrogalactitol within 3 h [95]	[50, 95]	...

83]. Calculations using Eq. (2) and data from Table 1 of Laskar and Ayres [83] suggest that at −20° C and −40° C the $t_{0.95}$ would be approximately 1.5 and 22 years, respectively.

The degradation of the nitrosoureas BCNU, CCNU, and MeCCNU has been shown to be catalysed by protein [154] and to be protected by the addition of lipoproteins to protein-containing medium [153]. For these reasons it is possible that different batches of serum used in in vitro assays could produce different half-lives for these drugs.

CCNU

Although CCNU is slightly more stable in aqueous solutions than BCNU [31, 101, 121, 158, 161], it degrades six times as quickly as BCNU in 95% ethanol [101], which suggests a $t_{0.95}$ of approximately 3 months at −20° C and 3 years at −40° C (assuming the same activation energy for the degradation of BCNU and CCNU).

We found that when CCNU was dissolved at 5 mg/ml in ethanol, diluted to 100 μg/ml with PBS (possible without precipitation), frozen and then thawed for an in vitro assay, it was in the form of a crystalline suspension. Gentle warming brought it back into solution [Bosanquet AG, Bird MC, unpublished observations]. Davignon et al. [38] also found that CCNU was more difficult to keep in solution than BCNU when attempting to formulate the drug.

ACNU

ACNU is one of the water-soluble nitrosoureas developed in recent years. The drug information booklet (Sankyo Co., Tokyo) says that, when dissolved in distilled water at 5 mg/ml pH 3–4 (the normal formulation), the drug degrades with a half-life of about 3 days at 25° C ($t_{0.95}$ = 5 h), and at 5° C it has a $t_{0.95}$ of 60 h – these values are almost identical with those for BCNU at acid pH. Hisazumi et al. [68] reported that 'ACNU

dissolved in neutral fluid of pH 7.4 has a half-life of approximately 25 min over a time course of 3 h at 37° C', whereas Schein et al. [121] quote a figure of 75 min. It is suggested that ACNU and other investigational nitrosoureas be treated with a caution greater than that afforded to BCNU.

Stability of the alkylating agents

The alkylating agents are a diverse group of drugs (Table 2) with a very large range of stability in solution. Table 5 summarises the data on 17 alkylating agents, and further information on 4-HC, chlorambucil, and melphalan is given below.

4-Hydroperoxycyclophosphamide (4-HC)

Aqueous solutions of cyclophosphamide are reasonably stable at room temperature [9, 18, 19, 58, 67, 74, 76, 165]. For instance, in sterile water for injection the half-life of the drug is 25 days at room temperature, giving a value for $t_{0.95}$ of 43 h [18]. It can also be stored frozen for 4 weeks [76]. However, the drug is not active in vitro, as it requires hydroxylation by liver microsomes [128]. Because of this, a number of other alkylating agents have been used in in vitro chemosensitivity assays to predict for response to cyclophosphamide in vivo, including melphalan [102, 118], chlorambucil [145] and nitrogen mustard [157]. Microsomal activating systems have also been incorporated into in vitro assays to overcome the problems of the inactivity of cylcophosphamide in vitro [85, 97].

An activated form of cyclophosphamide, 4-HC, which seems to degrade to the same products as those formed by microsomal activation of cyclophosphamide, is now often being used to predict clinical response to cyclophosphamide therapy [3, 10, 17, 123, 124, 144], and has been used to eliminate tumour cells from marrow suspensions in vitro [125].

Recently much work has been published on the degradation of 4-HC and 4-hydroxycylophosphamide [12, 13, 91, 92, 166]. Low et al. showed that the degradation of 4-hydroxycyclophosphamide was mediated by bifunctional catalysts such as phosphate, bicarbonate, and glucose-6-phosphate [91] as well as hydroxyl ion [92]. In 0.5 *M* Tris buffer at pH 7.4 and 37° C, 4-HC was converted to 4-hydroxycylophosphamide with a half-life of 43 min; at 0° C the half-life was > 8 h [91]. Without a bifunctional catalyst present, there was negligible degradation to phosphoramide mustard. Interpolation of the figures of 4-HC stability from charts 3 and 4 of Low et al. [91] to pH 7.3 and 0.007 *M* phosphate (the approximate values for PBS) at 37° C suggested a half-life of about 16 h, giving a $t_{0.95}$ of about 70 min. Thus, at room temperature, the value of $t_{0.95}$ could be expected to be around 2.5 h (using the rule of thumb described by Kostenbauder [80]), and in NS these values should be larger.

Chlorambucil

Chlorambucil is one of the most labile antineoplastic agents when in aqueous solution [32, 45, 105, 164], degrading via a cyclic aziridinium ion [32, 45, 88, 105, 114, 149]. The ring structure cannot form at low pH when the nitrogen becomes protonated [105] and thus chlorambucil is more stable at pH ≤ 3. The hydrolysis reaction is reversible and liberates chloride ion, and so chlorambucil is also more stable in chloride solutions. Chatterji et al. [32] found that the half-life of chlorambucil at pH 7 and 37° C was increased from 17 to 42 min on addition of 0.2 *M* chloride (approximately the chloride concentration in PBS), giving a $t_{0.95}$ of about 3 min. Degradation of chlorambucil in PBS at 25° C should therefore have a half-life of about 3.4 h and $t_{0.95}$ of about 15 min (calculated using Eq. (3) and values of k from Chatterji et al. [32] and a from Ehrsson et al. [45]. Chlorambucil was also found to bind to membranes designed to allow molecules of $< 30{,}000$ daltons through [122].

A number of reports have shown a marked increase in stability of chlorambucil in the presence of albumin [45, 46, 69, 86, 87], with the bound form being approximately 100 times more stable than free chlorambucil [46]. Thus Ehrsson et al. [46] found the half-life was nearly 10 times as long with 4 mg/ml human albumin (an albumin concentration approximating that in an in vitro assay in medium containing 10% serum); and with 40 mg/ml albumin, chlorambucil stability increased by a factor of approximately 35 compared with the phosphate-buffered control. Albumin binding did not, however, seem to affect the antitumour activity of the drug [69]. Micellar aggregation of chlorambucil with consequent increase in stability has also been noted [11].

The cytotoxicity of the drug against P388 murine tumour cells was markedly pH-dependent. A 3-fold increase in drug concentration was required to kill the same proportion of cells when the pH was increased from 7.2 to 7.8 [20]. Linford [88] also found a similar influence of pH on the alkylation of haemoglobin by chlorambucil. This illustrates the importance of pH control in such experiments.

The hydrolysis of phenyl acetic mustard, the one major metabolite of chlorambucil which is also active [114], was found to be very similar to that of the parent drug [45].

Melphalan

Melphalan is also a labile drug in aqueous solution [14, 28, 29, 33, 49, 51, 55, 114], degrading via an aziridinium ion, but it is not as unstable as chlorambucil. As with the latter drug, little difference in stability of melphalan was noted in various buffers over the pH range 3–9 at 25° C, with half-lives in the order of 4–5 h [51]. The presence of the chloride ion in NS or PBS (with chloride concentrations of 150 and 133 m*M*, respectively) increases the stability of melphalan, giving half-lives (at room temperature and pH 7.0) of 16 and 11 h, respectively, and $t_{0.95}$ of 70 and 50 min, respectively [14]. The presence of other buffer ions makes little difference to the stability of melphalan. For many experiments the drug has been dissolved and stored in HCl [1, 2, 55–57, 94, 167, 168].

When dissolved for IV injection (acid-alcohol solvent and polyethylene glycol diluent), melphalan is relatively stable with $t_{0.95}$ at room temperature of 9 h [51]. Using our HPLC assay for melphalan [15], this formulated product, diluted 1–20 with NS, showed no degradation over 1 month at −35° C (Bosanquet AG, unpublished observation). I also found no degradation when the drug, dissolved in NS or PBS, was stored at −20° C or −35° C for > 6 months, and suggested that it could be frozen for 12 months at these temperatures [14]. Yang and Drewinko [163], on the other hand, found considerable degradation of melphalan over 3 weeks when it was stored at 4° C, −20° C, or −70° C. Many investigators have stored the drug frozen for in vitro use [10, 36, 118, 147, 155, 157, 167, 168].

Melphalan was found to be three times more stable when bound to albumin [28, 44, 69], giving a 2-fold increase in half-life in 40 mg/ml albumin [44] – a far smaller difference than that seen with chlorambucil. It is nevertheless far less stable in serum containing medium than in NS [14].

Conclusions and recommendations

General

1. Much work is still required. Some conflicting data have been published, perhaps due to slightly different experimental conditions. Therefore, procedures should be followed carefully where stable storage of a drug has been shown.
2. The effect of light on drug stability is well documented, and therefore other drugs can be assumed to be unaffected.
3. Where aseptic drug solution make-up is possible, I recommend not filtering the solution unless the drug has been shown not to adsorb to the particular filter material.
4. Be cautious of solution pH (especially unbuffered solutions) with drugs which exhibit pH-dependent stability.
5. Keep final solvent concentrations in the in vitro assay down to about 0.1%.

Nitrosoureas

6. No nitrosourea has been consistently found to be unstable when frozen in solution.
7. Dilute nitrosoureas and add to cells with the minimum of delay.

Alkylating agents

8. Except for AZQ and BZQ, no alkylating agent has been consistently found to be unstable when frozen in solution.
9. Solutions of procarbazine, treosulphan, and nitrogen mustard should be rapidly handled and kept on ice.
10. Solutions of chlorambucil, melphalan, BZQ, dibromodulcitol, and dibromomannitol should be handled with the minimum of delay (at room temperature).
11. Solutions of 4-HC, busulphan, dianhydrogalactitol, AZQ, and dacarbazine have a $t_{0.95}$ at room temperature of between 2 and 24 h.
12. Solutions of ifosfamide and pentamethylmelamine are stable for > 7 days at room temperature.

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