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Automated stability determination of anticancer drugs incubated in cell culture medium

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Summary

An automated high-performance liquid chromatographic method for the determination of the stability of various anticancer drugs incubated in RPMI 1640 tissue culture medium with fetal calf serum is presented. We have used the anionic surfactant sodium dodecyl sulphate (SDS, 0.1 M) as a mobile phase additive to allow direct injection of samples without the usual problem of on-column serum protein precipitation. We have also developed a novel but simple plumbing arrangement for our autosampler that enables consecutive injections of sample (kept at 37 °C) and external standard (kept at 0 °C). Half-lives have been determined for the following drugs incubated in medium: melphalan (mean ± SD = 86.6 ± 1.1 min), tauromustine (32.6 ± 1.1 min), fludarabine (202.8 ± 7.5 min). For chlorambucil, degradation followed a biexponential curve with an initial half-life of 42.4 ± 4.8 min. Anthrapyrazolone was stable for > 10 h under these conditions.

Introduction

Much is known on the stability of anticancer drugs in simple aqueous solution (Bosanquet, 1985, 1986, 1989; Trissel, 1988, 1990). However many in vitro test systems used to study such drugs may involve incubation with cultured cells in an appropriate growth medium. The differential staining cytotoxicity (DiSC) assay used by us requires incubation of the drug with human tumour cells in culture medium consisting of RPMI-1640 and fetal calf serum (FCS) (Bird et al., 1985). Interpretation of such in vitro tests requires a knowledge of the chemical stability of the drugs under similar conditions. Little has been published in the scientific press on the stability of drugs under such conditions. What has been published is in some cases quite contradictory (Hildebrand-Zanki and Kern, 1984; Ludwig and Alberts, 1984; Pavlik et al., 1984).

We have developed a fully automated high-performance liquid chromatographic system to determine the stability of solutions of several common anticancer drugs incubated in medium at 37°C. To enable direct injection of the medium samples without on-column precipitation of serum proteins, we have used micellar mobile phases

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based on the anionic surfactant sodium dodecyl sulphate (Koenigbaur and Curtis, 1988). We have also developed a novel but simple plumbing arrangement which allows fully automated injection of samples maintained at two temperatures using a single autosampler and switching valve.

Materials and Methods

Chemicals

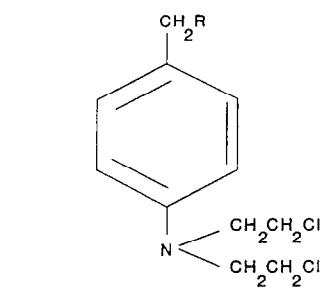
Methanol was HPLC grade from Rathburn Chemicals Ltd (Walkerburn, U.K.). Sodium dodecyl sulphate was specially pure grade and potassium dihydrogen orthophospate analar (both BDH, Poole, U.K.). Anhydrous sodium acetate and disodium hydrogen orthophosphate dihydrate were both analar grade (Fisons, Loughborough, U.K.) as was dimethyl sulphoxide (DMSO, Sigma, Poole, U.K.). RPMI 1640 medium (×1) and fetal bovine serum (FCS) were purchased from Gibco Europe Ltd (Uxbridge, U.K.). Phosphate-buffered saline (PBS) was obtained from Oxoid Ltd (Basingstoke, U.K.). Water was deionised and distilled.

Drugs

Scheme 1 details the chemical structures of the drugs tested. The drugs were initially made up at 100 × required concentration in either DMSO (tauromustine), methanol (chlorambucil, melphalan, anthrapyrazolone) or 1 : 1 methanol : water (fludarabine). External standards were prepared by dilution of the concentrated solutions with the appropriate mobile phase and were stored at −20°C until required. Samples were prepared by dilution with RPMI-1640 cell culture medium containing 11% FCS.

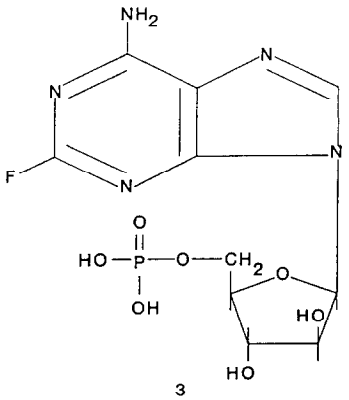
HPLC system

Our system consisted of a Waters 600E HPLC pump, Gilson 232-401 autosampler and a Hewlett Packard 1040A diode array detector. Cooling of the autosampler tray to 0°C was achieved with a



1a: R=CH_2CH_2COOH

1b: R=$CH(NH_2)COOH$



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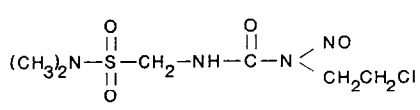

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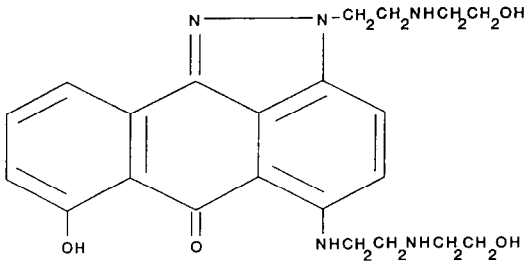

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Scheme 1.

Techne C-400/FC-200 refrigerated circulator. All HPLC columns were Spherisorb (Phase Separations, Clwyd, U.K.) maintained at 40°C by a Waters column heater.

Fig. 1 shows the layout of autosampler injector and remote sampling valve. The autosampler injector waste outlet port is connected via 0.010 inch i.d. stainless-steel tubing to a two-way Universal Valve Switching Module (Anachem, Luton, U.K.) which directs the flow to either waste or to the remote sampling vessel. With the switching valve directed to waste the autosampler works in the normal way transferring external standard from the cooled sample tray, dispensing it into the loop and then injecting. Alternatively, with the switching valve directed to the remote sampling vessel a heated sample can now be aspirated via the loop filler port. The valve is then switched back to the waste position and the requisite volume dispensed into the loop and injected.

Due to the inherent dead volume within the system the autosampler must be capable of aspirating quite large volumes (~ 300 μl) via its loop filler port. A suitable contact closure output should also be available to enable direct control over the switching valve for fully automated operation. The Gilson autosampler fulfills both of these requirements. The remainder of our HPLC system consisted of a Waters 600E pump and a Hewlett Packard 1090 diode array detector. A Hewlett Packard Chemstation controlling the diode array detector was programmed to record elapsed time from zero for each sample enabling degradation half-lives to be calculated easily.

The remote sampling vessel consisted of a glass test tube sitting in a 37°C hot block (Grant Instruments Ltd, Cambridge, U.K.). Through the tubes septum top we inserted a length of 0.01 inch i.d. flexible PEEK tubing and connected the free end to the switching valve. To allow easy withdrawl of sample a venting needle was also inserted through the septum. The valve used was a six port unit with the port adjacent to the remote sampling vessel fitted with a 1 ml syringe. With the switching valve directed to waste the syringe allows initial priming of the system. A four port valve would have been just as suitable.

Table 2 summarizes the process involved in making consecutive injections of sample then standard using this system.

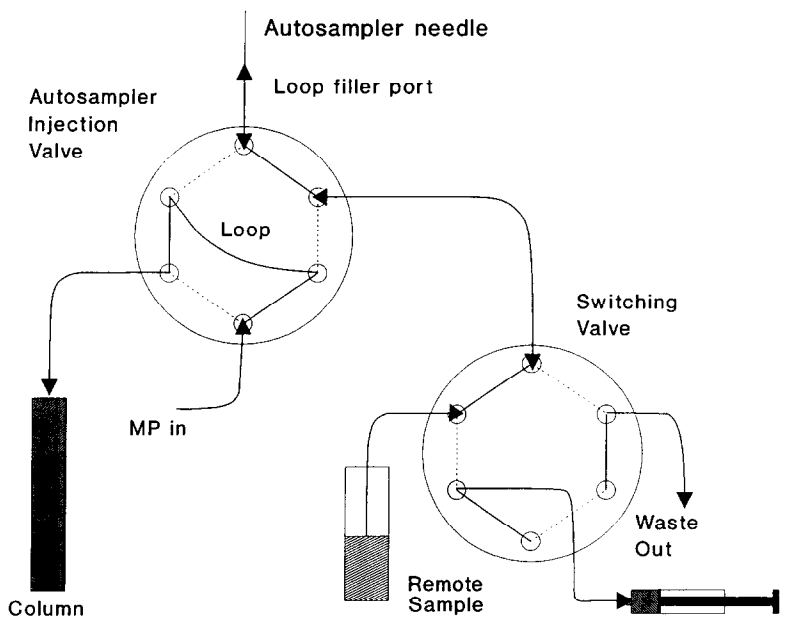

Fig. 1. Diagram showing plumbing of the autosampler injector to the remote sampling valve.

System precision

To check the precision of the automated system a test solution was sampled consecutively from autosampler tray and remote vessel both kept at room temperature. Initial experiments showed that simply aspirating the sample directly into the loop gave poor precision. With the autosampler in inject mode, aspirating sample, switching to load and then dispensing gave much better precision. The volume aspirated should take into account the volume of the connecting tubing plus the volume of the sample to be injected. Using the full loop method (5 × overfilling), repeat injections of the test sample resulted in little deviation from the autosamplers reported precision with relative standard deviations of between 0.33 and 0.70% ($n = 6$ for each experiment).

Drug analysis

Table 1 lists the test conditions used for the analysis of each drug. All mobile phases were based on the anionic surfactant sodium dodecyl sulphate. It was found that the methanol concentration of the mobile phase could be a maximum of 80% and the pH a minimum of 4 before on-column serum protein precipitation became a problem.

Calibration

Calibration curves were constructed for each drug in mobile phase at concentrations of 2 ×, 1 ×, 0.5 × and 0.25 × the concentration of each solution tested. The relationship between concentration and peak area was linear in all cases ($r^2 >= 0.99$, Table 1). Coefficients of variation (% CV) for six replicate injections of a standard solution of each drug are listed in Table 1. Blank medium samples were also analysed to ensure that medium peaks were not eluting within the same time window as the requisite drug peak.

TABLE 2

Summary of autosampler commands for consecutive injections of sample then standard

Step	Autosampler action
1	Move needle to loop filler port.
2	Turn switching valve to remote vessel position.
3	Aspirate sample from remote vessel.
4	Turn switching valve to waste position.
5	Turn autosampler injection valve to load position.
6	Dispense sample into loop.
7	Turn autosampler injection valve to inject position.
8	Start data acquisition.
9	Stop data acquisition.
10	Aspirate standard from autosampler tray.
11	Move needle to loop filler port.
12	Turn autosampler injection valve to load position.
13	Dispense standard into loop.
14	Turn autosampler injection valve to inject position.
15	Start data acquisition.
16	Stop data acquisition.

TABLE 1

Analysis conditions and calibration data for drugs tested

Drug	Structure (Scheme 1)	Drug concentration (μg/ml)	HPLC column type [a]	Mobile phase (methanol: aqueous)	Analysis wavelength (nm)	Drug retention (min)	Standard calibration (r^2)	Standard precision (% CV)
Chlorambucil	**1a**	100	ODS1	3:1 [b]	254	4.6	0.998	0.18
Melphalan	**1b**	50	ODS1	3:2 [c]	254	6.9	0.998	0.10
Tauromustine	**2**	250	ODS2	2:3 [b]	230	4.6	0.988	0.09
Fludarabine	**3**	60	NH_2	1:1 [d]	254	7.3	0.999	0.37
Anthrapyrazolone	**4**	12	NH_2	4:1 [d]	500	5.2	0.999	3.09

[a] All columns: Spherisorb 5 μm, 25 cm × 4.6 mm, held at 40 °C, with a pump flow rate of 1 ml/min.
[b] Aqueous phase 0.1 M SDS in 0.01 M acetate buffer (pH 4).
[c] Aqueous phase 0.1 M SDS in 3:1 0.01 M acetate buffer (pH 4):0.1 M acetic acid.
[d] Aqueous phase 0.1 M SDS in 0.01 M phosphate buffer (pH 7).

Stability-indicating tests

Experiments were performed to determine that the HPLC methods were stability-indicating for each drug. Drugs were stressed by heating solutions overnight at 60°C. Chromatography of these solutions revealed no changes in the upslope, apex or downslope UV spectra for remaining drug peaks and decomposition peaks were clearly resolved from their respective parent. In the case of anthrapyrazolone however the decomposition was too slow to pass even one half-life at this temperature over a period of 10 h. Even at 80°C in 0.1 M sulphuric acid or 0.1 M sodium hydroxide for 18 h, decomposition was only 34 and 14%, respectively. To confirm further that no coeluting peak was forming, stressed anthrapyrazolone solution was chromatographed using a cyano column with a mobile phase of 4 : 1 methanol : SDS (pH 4 with orthophosphoric acid). Calculation of absorbance ratios for anthrapyrazolone at 254, 360, 385, 410, 465 and 515 nm relative to its λ_{max} at 492 nm were identical to standard samples and very similar to reported values (Graham et al., 1989) (data not shown).

Data analysis

The concentration vs time data for the degradation studies were fitted to the first-order exponential equation of the type:

$$y = Ae^{-\alpha t}$$

For chlorambucil, however, we found a better fit was obtained using a biexponential equation of the type:

$$y = Ae^{-\alpha t} + Be^{-\beta t}$$

and have calculated an initial alpha half-life based on this.

Results and Discussion

Fig. 2 shows normalized decay profiles for each of the drugs tested. Half lives were determined and are listed in Table 3. Initially all drugs were fitted to an exponential equation assuming first order kinetics.

Chlorambucil

For Chlorambucil, the fitted data produced shorter half-lives than previously observed (Bosanquet and Clarke, 1986) and the half-life lengthened with the length of experiment. Our first suspicion was that the pH of the solution was changing over the period of the experiment. To control the pH we buffered the solution by incubating samples under an atmosphere of 5% carbon dioxide in air. Results, however, did not differ from our initial findings. This led us to believe that the degradation did not follow simple first-order kinetics. The data better fitted the biexponential model. From this we were able to determine an initial half-life $t_{1/2\alpha}$ of 42.4 ± 4.8 min, although due to the slight nature of the curve different methods of calculation gave slightly different results. Chlorambucil aqueous stability is reported to increase approx. 100-fold by the presence of serum proteins (Ehrsson et al., 1981). This is due to high levels of protein binding protecting the drug from normal aqueous solvation processes. We may therefore be observing an initial rapid decay of unbound drug followed by a slower decay as the drug dissociates from the complex.

Melphalan

The aqueous stability of melphalan is also increased by the presence of serum proteins. In this case only a 3-fold increase in half-life was found

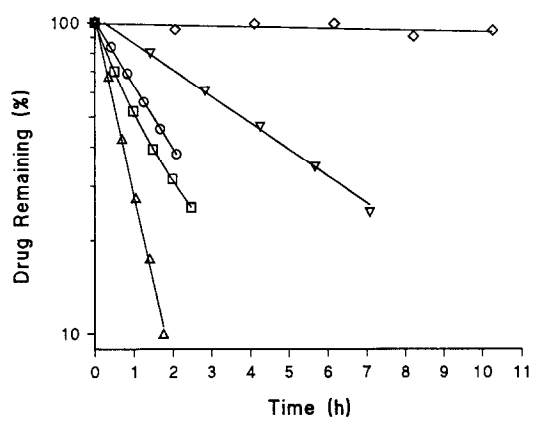

Fig. 2. Normalized decay profiles for solutions of chlorambucil (□), melphalan (○), tauromustine (△), fludarabine (▽), and anthrapyrazolone (◇) incubated in RPMI-1640 medium containing 11% fetal calf serum.

TABLE 3

Degradation half-lives of drugs incubated in RPMI-1640 medium containing 11% fetal calf serum

Drug	$t_{1/2}$ [a] (min)	r^2 [a]
Chlorambucil	42.4 [b] ± 4.8	0.9998 ± 0.0001
Melphalan	86.6 ± 1.1	0.9995 ± 0.0002
Tauromustine	32.6 ± 1.1	0.9996 ± 0.0003
Fludarabine	202.8 ± 7.5	0.9956 ± 0.0002
Anthrapyrazolone	(93.8%) [c]	

[a] Values are expressed as mean ± SD.
[b] $t_{1/2\alpha}$.
[c] % remaining after 10.25 h, $t_{1/2}$ not determined.

when melphalan was incubated in phosphate buffer containing up to 60 mg/ml human serum albumin (Ehrsson and Lönroth, 1982). A half-life of 1.13 ± 0.10 h for melphalan incubated in medium with 10% FCS has been reported (Bosanquet, 1984). In these experiments, however, we found that melphalan degrades at a slightly slower rate with a $t_{1/2}$ of 1.44 ± 0.02 h when incubated in medium with 11% FCS.

Tauromustine

For tauromustine degradation in medium at 37°C a mean half-life of 32.6 ± 1.1 min was determined; this value agrees closely with earlier experiments of ours (Betteridge et al., 1989). In phosphate buffered saline alone the stability of tauromustine is much greater with a $t_{1/2}$ of over 16 h. This phenomenon of aqueous degradation being accelerated by cell culture medium has also been observed during a study on the stability of anthracycline antineoplastic agents (Beijnen et al., 1986).

Fludarabine

Fludarabine has been reported to be quite stable in aqueous buffer over a pH range of 4.5–8 with only 4% degradation observed after heating at 65°C for 24 h (Trissel et al., 1988). However, when incubated in medium we found a mean degradation half-life of only 3.4 ± 0.1 h; this corresponds to 5% loss of the drug in only 15 min. We have also incubated fludarabine in PBS with and without FCS. In PBS alone, in agreement with Trissel et al. (1988), no degradation was observed over the period of the experiment whilst with FCS; in a single experiment fludarabine degraded with a half-life of 7.9 h. This is another example of drug degradation being accelerated by medium, but more specifically by the presence of FCS in the medium.

Anthrapyrazolone

The anthrapyrazoles are a new class of DNA-complexing agents based on the anthracenediones with the addition of a fused pyrazole ring in place of one of the carbonyl groups (Leopold et al., 1985; Scheme 1). Unusually for an anticancer drug, anthrapyrazolone showed very little degradation during incubation in medium, with only 6.2% degradation over 10.25 h. The degradation of anthrapyrazolone was too slow to pass even one half-life within the period of the experiment. Hence, in Table 3 only the percentage of the initial concentration of anthrapyrazolone remaining after incubation is shown.

Conclusion

Our new fully automated method for the analysis of samples at two different temperatures allows the stability of anticancer drugs in cell culture medium to be determined rapidly and, if required, outside normal working hours. Suprisingly one of the drugs (anthrapyrazolone) was quite stable, however two drugs (melphalan, tauromustine) had half-lives of < 90 min meaning that their activity in our in vitro experiments would have virtually finished after 6 h. We have also found that the stability of a drug in aqueous buffer may not be related to its stability in medium, as we have seen degradation may either increase or decrease significantly in medium.

References

Beijnen, J.H., Van der Nat, J.M., Labadie, R.P. and Underberg, W.J.M., Decomposition of mitomycin and anthracycline cytostatics in cell culture media. *Anticancer Res.*, 6 (1986) 39–44.

Betteridge, R.F., Culverwell, A.L. and Bosanquet, A.G., Stability of tauromustine (TCNU) in aqueous solutions during preparation and storage. *Int. J. Pharm.*, 56 (1989) 37–41.

Bird, M.C., Bosanquet, A.G. and Gilby, E.D., In vitro determination of tumour chemosensitivity in haematological malignancies. *Hematol. Oncol.*, 3 (1985) 1–10.

Bosanquet, A.G., Stability of melphalan solutions during preparation and storage. *J. Pharm. Sci.*, 74 (1984) 348–351.

Bosanquet, A.G. and Clarke, H.E., Chlorambucil: stability of solutions during preparation and storage. *Cancer Chemother. Pharmacol.*, 18 (1986) 176–179.

Bosanquet, A.G., Stability of solutions of antineoplastic agents during preparation and storage for in vitro assays. General considerations, the nitrosoureas and alkylating agents. *Cancer Chemother. Pharmacol.*, 14 (1985) 83–95.

Bosanquet, A.G., Stability of solutions of antineoplastic agents during preparation and storage for in vitro assays. II. Assay methods, adriamycin and other antitumour antibiotics. *Cancer Chemother. Pharmacol.*, 17 (1986) 1–10.

Bosanquet, A.G., Stability of solutions of antineoplastic agents during preparation and storage for in vitro assays. III. Antimetabolites, tubulin-binding agents, platinum drugs, amsacrine, L-asparaginase, interferons, steroids and other miscellaneous antitumour agents. *Cancer Chemother. Pharmacol.*, 23 (1989) 197–207.

Ehrsson, H. and Lönroth, U., Degradation of melphalan in aqueous solution - influence of human albumin binding. *J. Pharm. Sci.*, 71 (1982) 826–827.

Ehrsson, H., Lönroth, U., Wallin, I., Ehrnebo, M. and Nilsson, S.O., Degradation of chlorambucil in aqueous solution – influence of human albumin binding. *J. Pharm. Pharmacol.*, 33 (1981) 313–315.

Graham, M.A. Newell, D.R. and Calvert, A.H., Determination of the anthrapyrazole anticancer drug CI-941 in plasma and urine by solid-phase extraction and high-performance liquid chromatography. *J. Chromatogr.*, 491 (1989) 253–261.

Hildebrand-Zanki, S.U. and Kern, D.H., A new bioassay for in vitro drug stability. In Salmon, S.E. and Trent, J.M. (Eds), *Human Tumor Cloning*. Grune & Stratton, Orlando, 1984, pp. 451–458.

Koenigbauer, M.J. and Curtis, M.A, Use of micellar mobile phases and microbore column switching for assay of drugs in physiological fluids. *J. Chromatogr.*, 427 (1988) 277–285.

Leopold, W.R., Nelson, J.M., Plowman, J. and Jackson, R.C., Anthrapyrazoles, a new class of intercalating agents with high level, broad band spectrum activity against murine tumours. *Cancer Res.*, 45 (1985) 5532–5539.

Ludwig, R. and Alberts, D.S., Chemical and biological stability of anticancer drugs used in a human tumor clonogenic assay. *Cancer Chemother. Pharmacol.*, 12 (1984) 142–145.

Pavlik, E.J., Kenady, D.E., Van Nagell, J.R., Hanson, M.B., Donaldson, E.S., Casper, S., Garrett, D., Smith, D., Keaton, K. and Flanigan, R.C., Stability of doxorubicin in relation to chemosensitivity determinations: loss of lethality and retention of antiproliferative activity. *Cancer Invest.*, 2 (1984) 449–458.

Trissel, L.A., Flora, K.P., Vishnuvajjala, R., Davignon, J.P. and Quinn, F.R., *NCI Investigational Drugs – Pharmaceutical Data*. National Institutes of Health, Bethesda, 1988.

Trissel, L.A., *Handbook on Injectable Drugs*. American Society of Hospital Pharmacists, 1990.