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Instability of the anticancer agent etoposide under in vitro culture conditions*

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Summary. Degradation of etoposide is rapid under in vitro culture conditions. At pH 7.4 and 37° C, the isomerisation of trans-etoposide to the inactive compound cis-etoposide has a half-life of 2 days in Dulbecco's modified Eagle's medium and results in the loss of 90% of the active drug within 1 week. As a consequence, prolonged incubations with etoposide in in vitro assays may lead to erroneous interpretations ignoring the real in vitro situation. The degradation is not influenced by organic compounds such as bovine serum albumin or amino acids but depends strongly on the pH value and, to a lesser degree, on the ionic strength of the medium. Therefore, we propose a mathematical correction based on the pH value so as to obtain the real exposure of cells to trans-etoposide during in vitro assays.

Introduction

The pharmacokinetic behaviour of the anticancer drug etoposide is well characterised for dose regimens of up to 3.5 g/m^2 in humans. The decline of etoposide in plasma after intravenous injection is often described as a biexponential decay, with elimination half-lives varying from 2 to 25 h [3, 7, 10, 14, 16]. In aqueous solution, the degradation of etoposide depends on the pH value beginning with the isomerisation of trans-etoposide to cis-etoposide in alkaline media. The abiotic decomposition is different in acidic solution, including opening of the lactone ring in both media as the final step. The degradation is delayed in the pH range from 4 to 5 [2] and leads to physiologically inactive compounds. Ludwig and Alberts [13] have reported the instability of trans-etoposide under in vitro culture conditions, observing a loss of 60% of the biological activity within 10 days. The absence of trans-etoposide after this period was confirmed by HPLC analysis, indicating fast degradation.

The aim of the present study was the description of the decomposition of trans-etoposide, considering standard cell-culture media such as Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) 1640 with and without bovine serum albumin (BSA), the pH value and various buffer systems and the evaluation of their influence on the real exposure of cells to etoposide during in vitro assays.

Materials and methods

Chemicals

trans-Etoposide was diluted from a commercially available stock solution (Bristol-Myers, FRG) containing 100 mg etoposide, 150 mg benzylic alcohol, 3.25 g polyethylene glycol, 10 mg citric acid, 400 mg polyoxyethylene sorbitan and filled up to 5 ml with ethanol for degradation experiments. Before the start of each experiment the absence of degradation products was proven by HPLC. The stock solution is stable at 4° C in a dark environment for at least several weeks. cis-Etoposide and the cis-hydroxy acid derivative of trans-etoposide were prepared by degradation of the parent drug in commercially available formulations (Vepesid 100 mg, Bristol-Myers). Initially at 45° C in 67 mmol/l phosphate buffer (pH 8) trans-etoposide was degradated until the complete disappearance of trans-etoposide, then the pH value was adjusted to 4 with 1 *N* HCl (combined electrode, type 405/N5; Ingold, FRG). After centrifugation at 0° C (2,000 *g* for 10 min), the precipitated carboxylic acid was collected and washed three times with ice-cold water.

cis-Etoposide was purified by HPLC from the supernatant. The respective fractions were collected, the acetonitrile of the mobile phase was removed under a gentle stream of nitrogen, and the aqueous solutions were further purified by solid-phase extraction on a cyclohexyl sorbent (Bond Elut, Analytichem Int., USA). The fractions were applied to conditioned cyclohexyl sorbents, washed with water and then dried with 1 ml n-hexane. cis-Etoposide was eluted with 2 ml $CHCl_3$: MeOH (2 : 1, v/v) and the organic solvent was evaporated completely under a stream of nitrogen.

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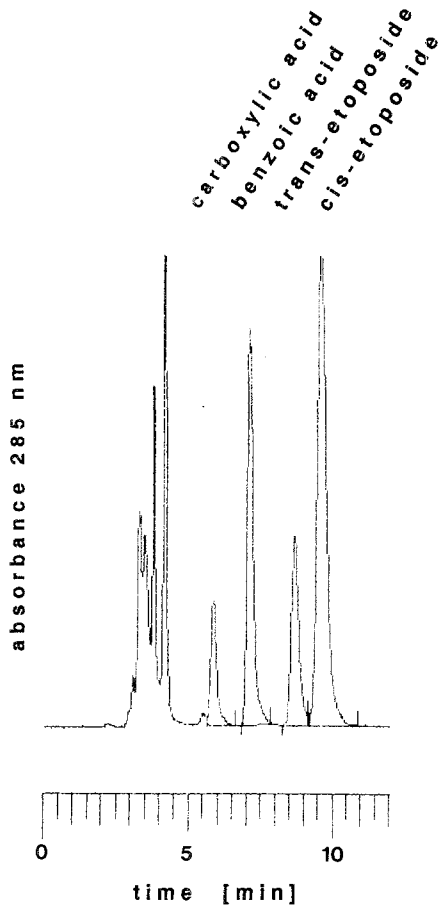

Fig. 1. Representative chromatogram of trans-etoposide and its degradation products in DMEM +1% BSA

The isolated compounds were dried and stored in a light-protected desiccator at –20° C. The purity of the compounds (>99%) was confirmed by HPLC analysis and by a constant ratio of signals using a diode-array detector simultaneously measuring the absorption at 260 and 285 nm. The molecular structure of the substances was confirmed by mass spectrometry.

RPMI 1640 (product number 12-602; Dulbecco Inc., USA) and DMEM (product number 041-01885; Gibco, USA) were used for degradation experiments after the addition of 10 mmol/l HEPES (Sigma Chemie, West Germany). All other buffers were prepared using reagents of p. a. purity from Merck (FRG). For HPLC purposes, water and acetonitrile of chromatographic purity were used (Rathburn Chemicals Ltd., Scotland, and Promochem, Mallinckrodt Inc., USA).

Incubation assays

Cell-culture medium was buffered with 10 mmol/l HEPES (resulting in pH 7.3–7.4 in fitted glass tubes at 37° C) and adjusted either to +0.2 pH values with 1 *N* NaOH or to – 0.2 pH values with 1 *N* HCl (resulting in pH 7.2 and 7.7, respectively, at 37° C in fitted glass tubes for DMEM). Benzoic acid as the internal standard for HPLC analysis and etoposide were added to a final concentration of 250 µg/ml. Of this solution, 10 ml was filtered through a 0.45-µm filter (Acrodisc, Gelman Sciences Inc.) and transferred into sterile Vacutainer glass tubes with tight-fitting rubber caps.

These caps are important for maintenance of a constant partial pressure of CO_2 above the solution and for avoiding a change in the pH value. Because of frequent sampling, the generally used incubation in an atmosphere containing 5% CO_2 is too susceptible to contamination. Glass tubes were incubated in a dark environment in a water bath. Samples for chromatographic analysis were drawn from the tubes with a disposable syringe and injected without further purification onto HPLC columns.

Experiments with BSA were carried out by adding 1% (w/v) purified albumin (Behring, FRG) directly to the cell-culture media. After solubilization of the albumin, the media were filtered two times (Acrodisc, 1.2- and 0.45-µm pore size). Each experiment was performed in five parallel samples.

Analytical procedures

High-performance liquid chromatography. Quantification of the compounds is normally done by HPLC analysis using VM 26 [8, 19, 20], ethyl-*p*-hydroxybenzoate [9], or methyl-*p*-aminobenzoate [6] as an internal standard. For long-term incubations we prefer a non-hydrolysable substance such as benzoic acid as an internal standard. The chromatographic system consisted of a 1090 M liquid chromatograph (Hewlett Packard, USA) equipped with a diode-array detector. Separation of the compounds was obtained using 35 mmol/l ammonium formate buffer (pH 3) + 1 mmol/l ethylenediaminetetraacetic acid (EDTA) : acetonitrile (65 : 35, v/v) as the mobile phase and a µ-Bondapak phenyl stainless-steel column (300 × 3.9 mm, 10-µm particles; Waters Chromatography Division, USA) as the stationary phase. The UV absorption of the compounds was monitored simultaneously at 260 and 285 nm, with a detection limit of 20 ng etoposide.

HPLC quality control. No interferences attributable to synthetic cell-culture media could be detected. Figure 1 shows a representative chromatogram of the parent drug and its degradation products in DMEM. The detector response was linear up to 5 µg trans-etoposide, cis-etoposide and carboxylic acid. In analyses performed on 4 consecutive days, the intraassay variation for a standard mixture of 250 µg/ml in DMEM was in the region of 1% and the interassay variation was <2%.

Mass spectrometry. Mass spectra of the parent drug and its isolated degradation products were recorded on a HSQ 30 Finnigan MAT mass spectrometer (Bremen, FRG) under the following conditions:

1. Electron impact mode (EI): electron energy, 70 eV; electron current, 1 mA; ion source temperature, 230° C
2. Fast atom bombardment (FAB) mass spectrometry: matrix, glycerol; primary atom beam, xenon; gun voltage, 8 kV; scan rate 10 s/decade

trans-Etoposide shows an intense M^+ peak at m/z = 588 (temperature of crucible, 250° C; Fig. 2 a), which amounts to only 10% of the base peak for the isomer cis-etoposide (temperature of crucible, 230° C; Fig. 2 b). No M^+ peak could be observed in the spectrum of the hydroxy carboxylic acid (Fig. 2 c). The dominating fragmentation process leads to the aglycone fragment (m/z = 382) of trans- and cis-etoposide and, prior to ionisation, to the aglycone fragment of the hydroxy carboxylic acid (m/z = 400; temperature of crucible, 220° C). At a temperature of 255° C, the mass spectrum of hydroxy carboxylic acid changes into that observed for cis-etoposide, indicating the recycling of cis-hydroxy carboxylic acid under these conditions. In FAB mass spectrometry, a $(MH)^+$ peak at m/z = 607 definitely confirmed cis-hydroxy carboxylic acid as a degradation product (Fig. 2 d) under in vitro assay conditions.

Analysis of data

Apparent first-order degradation rate constants (k_{deg}) and areas under the concentration-time curve (AUCs) were calculated by PKCALC, a program for pharmacokinetic analysis of data that was written by Shumaker [18]. The coefficient of variation in independent degradation experiments rarely exceeded the 5% limit. We therefore did not indicate the standard deviation in the respective figures.

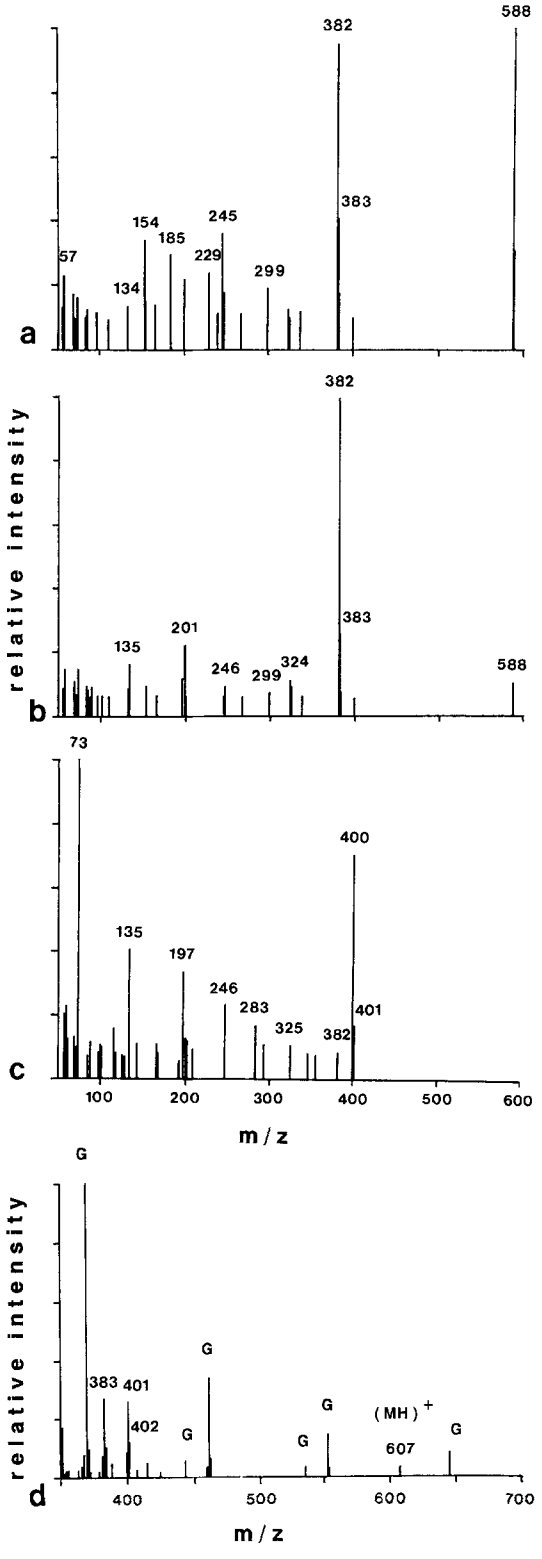

Fig. 2 a–d. Mass spectra of **a** trans-etoposide, **b** cis-etoposide and **c** cis-hydroxy carboxylic acid and **d** FAB mass spectrum of cis-hydroxy carboxylic acid

Results

Degradation at 37 °C in DMEM

The degradation of etoposide is dependent on the pH value, as pointed out by Beijnen and co-workers [2]. The surprising strength of this dependence is represented in Fig. 3. Degradation of trans-etoposide occurs rapidly in untreated medium at pH 7.4. No trans-etoposide was detectable after 17 days. A change from pH 7.4 to pH 7.2 decreases the degradation rate constant from 0.339 to 0.241 day^{-1}. However, even at the slowest rate of decomposition, its disappearance from the medium was completed after 23 days as compared with 10 days at the fastest rate.

The maximal concentration of cis-etoposide in DMEM was reached after 3, 6 and 10 days (at pH 7.7, 7.4 and 7.2, respectively), reaching very similar concentrations in all experiments. The decay of cis-etoposide is caused by the cleavage of the cis-lactone ring to its hydroxy carboxylic acid. Ester hydrolysis is a slow step in the reaction chain and does not yield the final degradation product, as we show later.

Applying concepts of human pharmacokinetics to our data, we calculated the AUC for the monoexponential decay of trans-etoposide in untreated DMEM as being 25% of the theoretical AUC for a stable compound. In other words, the real exposure of cells to trans-etoposide under common in vitro culture conditions is only one-fourth of its nominal value as calculated for several pH values in Table 1. The degradation rate constants of characteristic experiments are summarised in Table 2.

Degradation in DMEM at different temperatures

The Arrhenius equation relates the activation energy of a reaction to its rate constant; in our case, the isomerisation of etoposide from the trans- to the cis-isomer. For the representation of this relationship, we examined the degradation kinetics at 37°, 45°, 60°, 70° and 80°C (Fig. 4). Knowledge of the rate constants at different temperatures enables (a) calculation of the low activation energy of 88 kJ/mol in untreated DMEM at pH 7.3–7.4, which is very similar to that reported by Beijnen and co-workers [2] and explains the reactivity of the drug; and (b) the duration of experiments to be shortened by performing them at higher temperatures if the linearity between 1/temperature and the degradation constant is proven [12]. We extensively used this proven relationship to check the degradation behaviour of trans-etoposide in media in which the decay is not characterised against that in a solution in which these parameters are well defined. The Arrhenius plot, 1/T versus the rate constant, where T is the absolute temperature in degrees Kelvin, is shown in Fig. 5.

At higher temperatures, the decrease in carboxylic acid and the monitoring of unidentified peaks in the chromatograms led us to the conclusion that the carboxylic acid was also degraded. At 70°C we noted a steady state after the disappearance of trans-etoposide. Diminution occurred simultaneously for both remaining substances, cis-etoposide and carboxylic acid. We found no explanation for this reproducible phenomenon, which obviously has no implication for the in vitro assays.

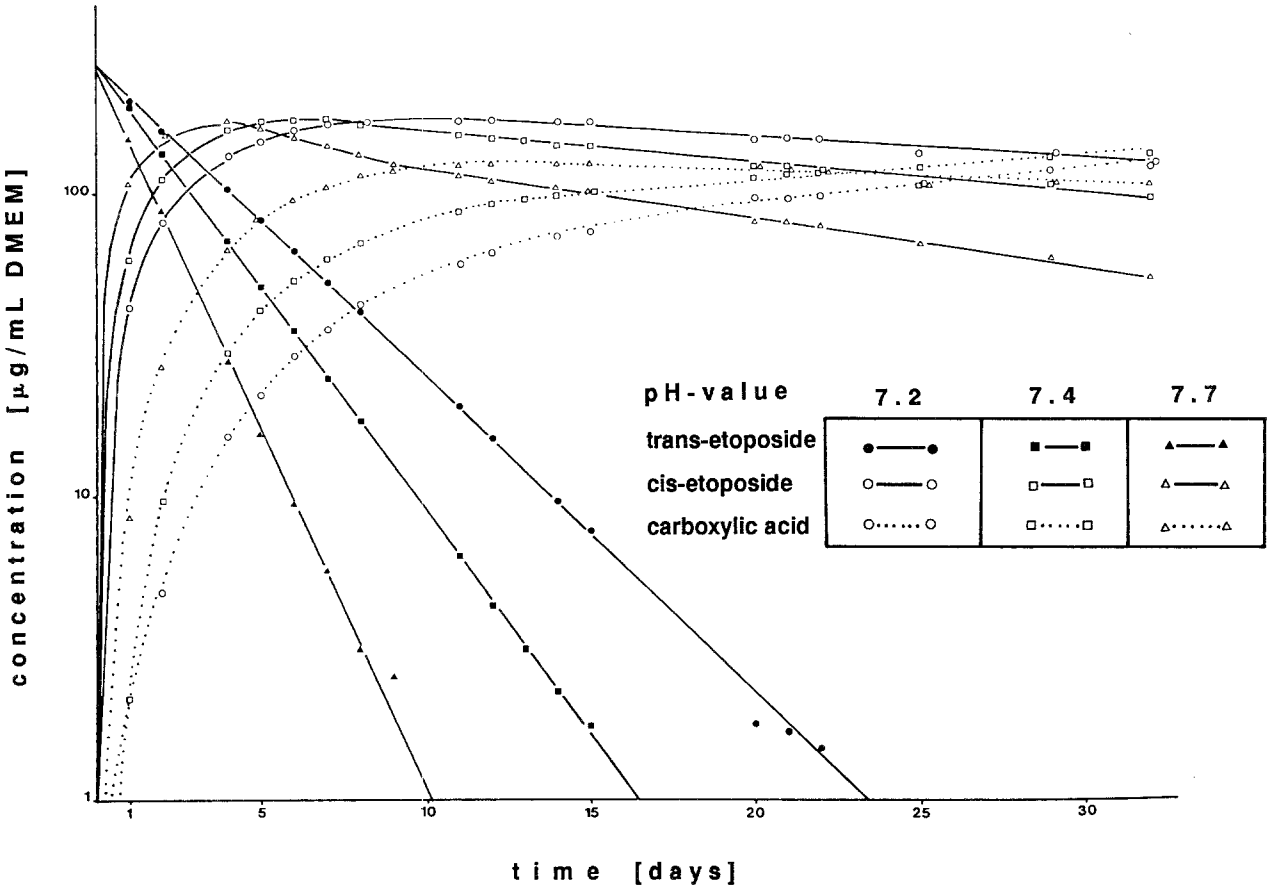

Fig. 3. Degradation kinetics of trans-etoposide in DMEM at 37° C and pH = 7.2, 7.4 and 7.7

Table 1. Dependence of the degradation velocity of trans-etoposide on the pH value

pH value	k_{deg} ($days^{-1}$)	trans-Etoposide remaining after 14 days	Real exposure
7.1	0.126	17%	47%
7.2	0.192	6.8%	35%
7.3	0.257	ND	27%
7.4	0.322	ND	22%
7.5	0.387	ND	18%
7.6	0.453	ND	16%
7.7	0.518	ND	14%

k_{deg}, Degradation constant; trans-etoposide remaining, remaining concentration of trans-etoposide expressed as a percentage of the initial concentration after an incubation period of 2 weeks; real exposure the exposure to trans-etoposide as compared with a theoretically stable compound after an incubation period of 2 weeks; ND, not detectable

Table 2. Degradation rate constants under characteristic experimental conditions

Medium	Temperature (° C)	pH value	k_{deg} ($days^{-1}$)
DMEM	37	7.24	0.241
	37	7.37	0.299
	37	7.45	0.339
	37	7.69	0.527
DMEM	45	7.44	0.697
	45	7.46	0.65
DMEM + BSA	45	7.46	0.673
	45	7.52	0.754
RPMI	45	7.26	0.439
RPMI + BSA	45	7.39	0.593
0.067 M HEPES buffer	45	8	1.64
0.067 M phosphate buffer	45	8	2.08
DMEM	70	7.36	10.2
DMEM salt solution	70	7.45	10.8

DMEM, Dulbecco's modified Eagle's medium; DMEM salt solution, a mixture containing only the inorganic compounds of DMEM; RPMI, Roswell Park Memorial Institute medium 1640; + BSA, addition of 1% (w/v) bovine serum albumin to the medium

Comparison of degradation kinetics in DMEM vs RPMI

Under these test conditions, RPMI 1640 reaches a pH value of approximately 7.15–7.2 within 24 h of incubation at 37° C. The pH value of DMEM remains somewhat higher (7.3–7.4), and one would therefore expect a slower disappearance of trans-etoposide from RPMI. In fact, the isomerisation in RPMI (k_{deg} = 0.442 day^{-1}) is considerably slower than that in DMEM (k_{deg} = 0.658 day^{-1}; temperature in both experiments, 45° C).

Although extremely high serum protein binding is reported for trans-etoposide [1], the addition of bovine serum albumin (BSA) to synthetic cell-culture media had no effect on the decomposition velocity. Unchanged degradation kinetics were observed for both media. Regardless of

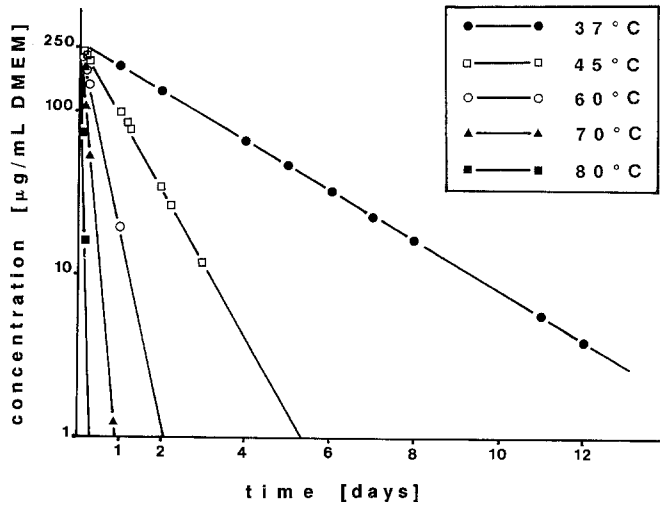

Fig. 4. Degradation kinetics of trans-etoposide in DMEM at various temperatures

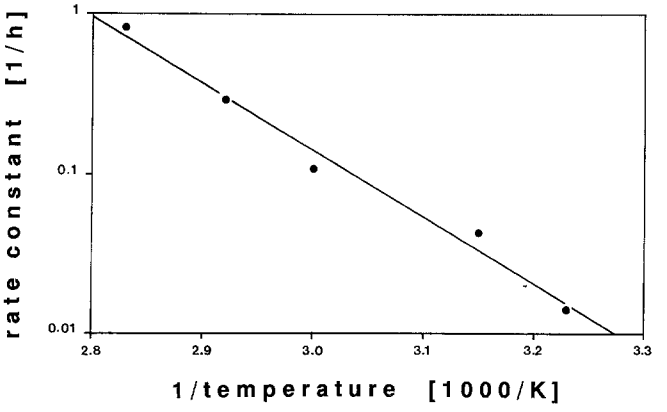

Fig. 5. Arrhenius plot for the degradation of trans-etoposide in DMEM.

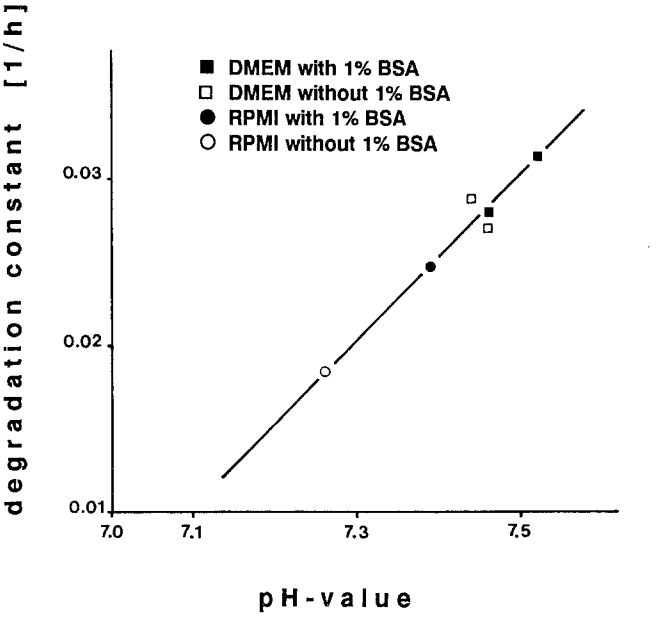

Fig. 6. Relationship between the degradation constant and the pH value in synthetic cell-culture media with and without BSA

the presence or absence of BSA, the rate constants for the isomerisation are in very good accordance, showing a linear dependence on the experimental pH value (Fig. 6).

Degradation in buffer solutions

Degradation is also dependent on the ionic strength of buffer solutions. Acetate, carbonate and phosphate ions are known to influence decomposition. Phosphate buffer accelerates this process as compared with HEPES buffer of the same molarity, as shown in Fig. 7 (buffer strength, 67 mmol/l; pH 8 at 45° C). This phenomenon is caused by the increased ionisation of phosphate buffer as compared with HEPES buffer at a pH value of 8. Under equal pH conditions, a close relationship was found between the degradation rates of trans-etoposide and the ionic strength of the solutions.

Organic compounds such as the amino acids of synthetic cell-culture media did not alter the degradation process. We conclude this from identical decomposition kinetics obtained in DMEM and in a buffer containing only the inorganic compounds of DMEM.

Although 10 mmol/l HEPES buffer per se has a moderate effect on drug instability (data not shown), the addition of DMEM to HEPES buffer increases the decomposition velocity. Excluding the influence of organic compounds, we therefore consider the inorganic ions of the system to be the cause of this phenomenon seen at stable pH value. Concerning the degradation velocity, however, the difference between 10 mmol/l HEPES buffer and DMEM +10 mmol/l HEPES decreases at higher pH values, indicating that the ionic strength is only a secondary factor influencing degradation.

Exposition to trans-etoposide in in vitro assays

We propose the following procedure for the interpretation of data from in vitro assays using etoposide in long-term incubations: (1) measure the pH value at 24 h and at 14 days after the beginning of the incubation to control a possible pH shift; (2) calculate the degradation constant using the formula

$$k_{deg} = 0.65 \times pH_f - 4.5,$$

expressed in days^{-1}, where pH_f is the final pH value (valid in the pH range from 7.1 to 7.7 in common synthetic cell-culture media at 37° C); and (3) interpolate the real exposure of cultured cells after 2 weeks as shown in the last row of Table 1 and correct the nominal drug concentration at time 0 to obtain the estimated mean concentration of exposure. As this correction applies only to the conditions stated, this formula requires verification for a different set of data.

Discussion

The results obtained clearly indicate a direct dependence of the drug stability of etoposide in vitro on cell-culture conditions. Factors such as pH value, CO_2 concentration, tem-

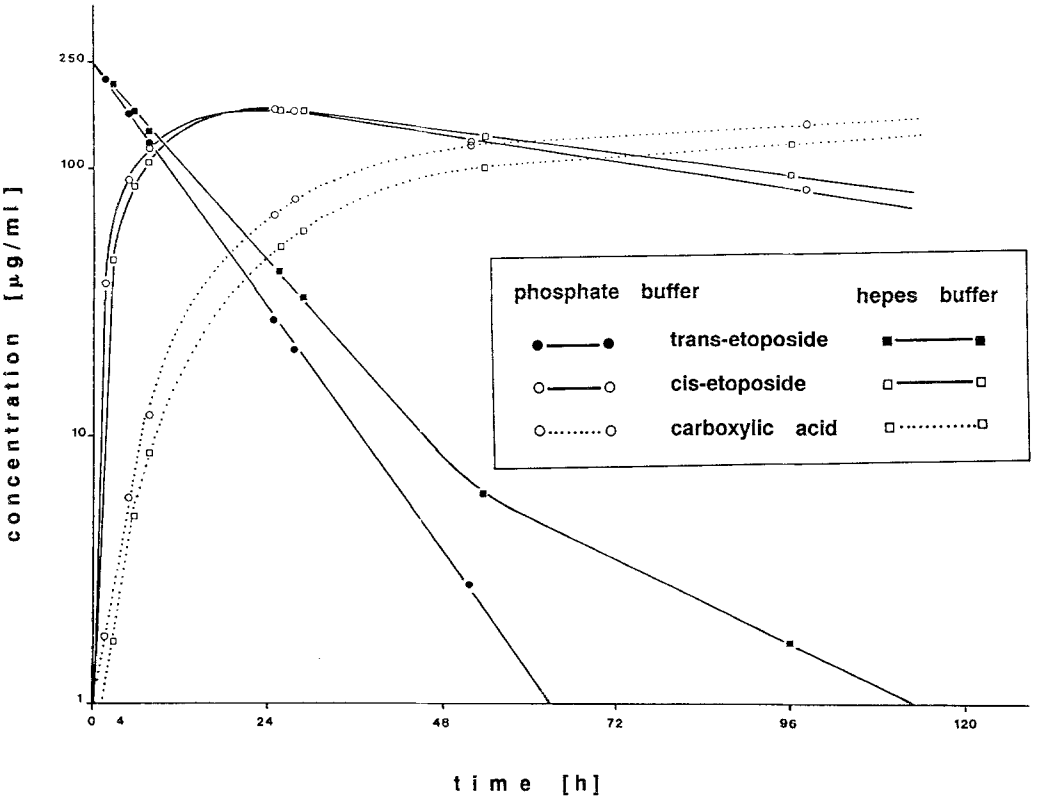

Fig. 7. Degradation kinetics of trans-etoposide in 67 mmol/l phosphate buffer versus 67 mmol/l HEPES buffer at 45° C and pH = 8

perature and ionic strength continuously influence one another. In addition, interactions between cells in culture, cell-culture media and drugs contribute to the complex phenomena of in vitro drug testing, e. g. differential uptake, efflux and binding of etoposide in sensitive and resistant tumour cell lines [17]. A prerequisite for obtaining reproducible in vitro drug-testing data is therefore the maintenance of known culture conditions within a narrow range of fluctuation so as to minimise unexpected effects.

The extremely lipophilic drug trans-etoposide is hardly soluble in aqueous solutions [15]. The formulation for clinical use contains polyethylene glycol and benzyl alcohol and is known to be light-sensitive and degradable in basic and acidic media [6]. Its instability in isotonic fluids, dependent on the concentration of etoposide, has been reported [11]. The first systematic approach of Beijnen and co-workers [2] described the decomposition of etoposide in aqueous solutions, pointing out the instability and (pseudo) first-order degradation kinetics of the parent drug.

Stock solutions of trans-etoposide remain biologically active over several weeks [21], but this is not true for trans-etoposide in synthetic cell-culture media. Ludwig and Alberts [13] reported a loss of 60% of the biological activity of etoposide in Connaught Medical Research Laboratory (CMRL) medium 1066 at 37° C as well as the complete disappearance of the drug and hypothesised that degradation is a mechanism for the loss of activity. As we now know, this disappearance is due to the isomerisation of trans-etoposide to cis-etoposide, which reacts rather slowly to its carboxylic acid.

The degradation rate of trans-etoposide was constant in both cell-culture media tested, as was proven by the monoexponential decay in the range from 250 to 1 µg/ml, a range of more than 2 orders of magnitude. In experiments using concentrations below the detection limit of 1 µg/ml, which are useful for clonogenic assays, investigators can base their considerations only on analogous degradation behaviour.

Evans and co-workers [4, 5] evaluated the potency of the degradation products and found minimal cytotoxicity in their clonogenic assay. Carboxylic acid is known to be completely non-toxic and cis-etoposide is 100-fold less toxic to human leukemia cells (CCRF-CEM) than is the parent drug [5], thus suggesting only a minor contribution of the metabolites to the cytotoxic effect of the parent drug. The data of Ludwig and Alberts [13], on the other hand, indicate remaining biological activity in the endometrial carcinoma cell line HEC-1A. As the cytotoxic effects of the degradation products seems to differ for several cell lines, we desisted from comparisons between trans-etoposide and its degradation products and focused our attention on the parent drug.

For the correct interpretation of in vitro assay data and for optimising in vitro culture conditions, we suggest the following considerations:

1. An exact calculation of the cell exposure is possible without continuous monitoring of the drug, as we can predict the decay of trans-etoposide in synthetic standard cell-culture media by measuring the pH value of the system. However, even this certainly useful mathematical correction fails to reflect the entire in vitro situation equating

a short incubation at high drug concentrations with a long-term incubation at low levels, both resulting in identical AUCs.

2. For incubation assays with trans-etoposide, investigators should minimise degradation by considering the order of the factors influencing the decomposition: pH value and, to a lesser degree, the ionic strength, not the molarity, of the inorganic compounds in the system. RPMI is therefore a better medium to use than DMEM in assessing the cytotoxic effect of etoposide, but the necessity for mathematical correction cannot be ruled out. Even under conditions such as 67 mmol/l HEPES buffer at pH 7.2, a loss of 16% of the trans-etoposide was noted within 72 h, which is, of course, not a negligible quantity.

As exemplified for etoposide, careful examination of a drug's stability and degradation behaviour is a prerequisite for the adequate planning of in vitro culture conditions and enables the correct interpretation of data derived from such experiments.

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