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Long-term Stability of 5-Fluorouracil and Folinic Acid Admixtures

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5-Fluorouracil (5-FU) and d,l-folinic acid (FA) are used in association to treat a wide variety of malignancies. The stability and the compatibility of 5-FU and FA in combination in intravenous admixtures were studied under various storage conditions and with drug concentrations matching their clinical use (0.9% sodium chloride, 5% dextrose, protected from light or not). 5-FU and FA concentrations (mg/ml) were 6.5 or 50 and 4.0 or 30.8, respectively. Successive aliquots of the drugs mixtures were withdrawn during 60 h from 500 ml glass bottles and 500 ml polyvinyl chloride (PVC) bags (at room temperature) and during 120 h from cassettes (at 32°C). Drug concentrations were measured by high performance liquid chromatography. For all conditions tested, the changes in 5-FU and FA relative to the initial concentrations remained within the assay reproducibility (10%). In complement, infrared Fourier transformation spectrophotometry has not shown a significant fixation of FA or 5-FU on the PVC bags, in all tested conditions. Under the conditions examined above 5-FU and FA can be mixed in the same container for their use in cancer chemotherapy. This can have practical consequences by simplifying the widely used treatment protocols associating 5-FU and FA.
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INTRODUCTION

EXPERIMENTALLY, the addition of folinic acid (FA) to 5-fluorouracil (5-FU) has been shown to increase the cytotoxicity of this anti-cancer agent [1, 2]. This has been confirmed clinically, as this drug combination has proven superior to the use of 5-FU alone in the treatment of advanced colorectal carcinoma [3]. This association is also very active in the treatment of head and neck cancer [4], breast cancer [5] and lung cancer [6]. 5-FU/FA combinations are currently used on a wide scale to treat various types of cancer and are usually administered by continuous venous infusion. To determine whether these two drugs remain stable when mixed together, serial 5-FU and FA concentrations were measured over a prolonged period under various conditions (containers, diluents, etc.). This information is of practical importance for both the patient and the medical staff because it determines the suitability of administration of these admixtures in the same infusion device.

MATERIALS AND METHODS

Drug solutions

Drugs were assayed under different physical conditions corresponding to those encountered clinically: 500 ml glass bottles or polyvinyl chloride (PVC) plastic bags (Aguettant), and 100 ml cassettes (Pharmacia) to simulate ambulatory treatment. To determine drug concentrations, the average body surface area was considered to be 1.7 m^2, and daily doses of 5-FU and FA were respectively 800 and 500 mg/m^2. These high doses were used to detect any possible incompatibilities between the drugs. These doses also simulate actual clinical situations (5-day continuous infusion) [4]. The study period of 60 h corresponded to the half-cycle period of a 5-day continous infusion. The corresponding cumulative doses of 5-FU and FA were thus 3.4 g and 2.125 g, respectively. 5 ml vials of 5-FU (50 mg/ml, Fluorouracile Roche) and FA vials (200 mg of lyophylised powder, Lederfoline, d,l-calcium folinate, Lederle) were used. In order to reach the above-mentioned 5-FU and FA concentrations, and to use an entire number of 5-FU and FA vials, 13 vials of 5-FU and 10 vials of FA were mixed in the 500 ml containers. This gave theoretical initial drug concentrations of 6.5 mg/ml for 5-FU and 4 mg/ml for FA. Drug solutions were prepared with 0.9% sodium chloride or 5% dextrose in the 500 ml containers. FA powder was diluted by the 5-FU solution from the ampoules in 100 ml cassettes; the total volume was thus 65 ml (13 × 5) in each cassette; the corresponding theoretical initial concentrations for 5-FU and FA were thus 50 mg/ml and 30.8 mg/ml, respectively.

Sampling

Glass bottles and PVC bags were maintained for 60 h at room temperature (21°C), in the presence or absence of light. Cassettes were placed in the dark at 32°C to simulate ambulatory conditions with patients. 15 ml were taken in containers at the time of the drug mixtures (T_0) as well as 8, 24, 48 and 60 h later. The timing was identical for cassettes, except for the last sampling time (120 h instead of 60 h).

Measurements of study parameters

At the time of each sampling, 5 ml of each solution was used for an immediate determination of the pH value; the remaining volume was immediately stored at −20°C until drug analysis. Specific conditions with the addition of ascorbic acid (2 mg/ml) were used in order to prevent any degradation of FA. All experiments were performed in duplicate, and each sample was analysed twice within the same assay. d,l FA was measured by a

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Table 1. Evolution of 5-FU and FA concentrations in glass containers

Container/diluent Parameter		Sampling times										
		T_o		8h		24h		48h		60h		Mean
		L	D	L	D	L	D	L	D	L	D	(CV%)
Glass/NaCl												
pH	I	7.96	8.00	8.11	8.11	8.04	8.04	8.06	8.05	8.07	8.06	8.05(0.6)
	II	7.99	8.02	8.10	8.11	8.02	8.01	8.05	8.07	8.07	8.06	8.05(0.5)
5-FU conc.	I	6.90	6.82	6.84	6.89	6.80	7.03	6.69	7.00	6.84	7.13	6.89(1.9)
(mg/ml)	II	6.69	7.11	6.82	6.58	6.79	6.95	7.03	7.05	6.90	6.92	6.88(2.4)
FA conc.	I	3.84	4.22	4.08	4.31	4.04	4.29	4.00	4.28	4.03	4.41	4.15(4.3)
(mg/ml)	II	3.93	4.10	3.93	4.40	3.99	4.29	3.97	4.40	3.84	4.29	4.11(5.2)
Glass/dextrose												
pH	I	8.04	7.97	8.09	8.10	8.00	8.02	8.07	8.07	8.11	8.11	8.06(0.6)
	II	8.00	7.99	8.09	8.11	8.01	8.03	8.08	8.07	8.11	8.11	8.06(0.6)
5-FU conc.	I	6.76	6.53	6.79	6.45	6.74	6.37	6.58	6.84	6.66	6.92	6.66(2.7)
(mg/ml)	II	6.71	7.13	6.63	7.13	6.61	7.00	6.66	7.16	6.73	7.03	6.88(3.3)
FA conc.	I	3.62	4.14	3.74	4.06	3.71	3.83	3.94	4.31	3.79	4.18	3.93(5.9)
(mg/ml)	II	3.81	4.16	3.59	4.13	3.70	4.02	3.70	4.17	3.72	3.95	3.90(5.6)

Theoretical 5-FU and FA concentrations in glass containers were 6.5 and 4 mg/ml, respectively. I, II represent separate experiments; L = light, D = dark.

high performance liquid chromatography (HPLC), technique with ultraviolet detection at 313 nm, as we described previously [7]. 5-FU was measured by a HPLC technique with ultraviolet detection at 254 nm, according to Christophidis *et al.* [8]. The coefficients of variation (CV) for interassay reproducibility were 8% for 5-FU and 10% for FA.

In order to determine if FA or 5-FU have reacted and/or fixed on PVC plastic bags, infrared Fourier transformation spectrophotometry (IRFT) has been performed on the bags. The spectrophotometer (resolution 4 cm^{-1}) is a Nicolet 5SXC equiped with a KRS5 cristal at 45°. The IRFT spectra are recorded following this attenuated total reflexion (ATR) method.

An original HPLC method has been developed to assay di(ethyl-2 hexyl)phthalate in infusions having contained FA, 5-FU or the drugs mixture. The HPLC method is performed on a Spherisorb ODS-2 (C-18,5μm) 250 mm × 4.6 mm (Interchim) thermostated at 40°C. The mobile phase A (0.01 mol/l) consists of 1.42 g disodium hydrogen phosphate anhydrous (Merck) dissolved in 1000 ml of water (MilliQ) adjusted to pH 3.5 with 20% phosphoric acid. The mobile phase B is methanol (Carlo Erba). The detection is set at 275 nm and the flow rate is 1 ml/min. The HPLC conditions were 80 : 20, A : B to 100% B over 5 min, then 100% methanol until t = 10 min. A 10 min re-equilibration step was performed with the initial mobile phase. In these conditions, di(ethyl-2 hexyl)phthalate is eluted at around 12 min 30 s. Assay solutions (20 μl) are directly injected into the chromatograph. The standard solutions were prepared by dissolution of di(ethyl-2 hexyl)phthalate in methanol. Linearity was checked from 1 μg/ml to 100 μg/ml. The coefficient of correlation is r = 0.999. The precision is 16.2% (1μg/ml), 5.0% (10μg/ml) and 3.4% (100μg/ml). The limit of quantification is 20 ng. No interfering peak has been detected.

Statistics

The effect of time on the drug concentration was analysed by the Spearman rank correlation test. Drug levels measured in light and dark conditions were compared by the Wilcoxon matched-pairs signed-ranks text.

RESULTS

As detailed in Tables 1, 2 and 3, 5-FU and FA concentrations in all drug mixtures remained significantly stable throughout the 60 h survey. All the coefficients of variation remained below the intrinsic variability of the analytical methods for both 5-FU and FA. Interestingly, even with the highest concentrations tested (those in the cassettes), the drug levels were still unchanged after 120 h at a physiological temperature. No evidence of precipitate formation was observed in any of the test conditions. As corollary, the IRFT spectrophotometry has not shown a significant fixation of FA or 5-FU on the PVC bags, in all tested conditions (dark or light exposure after 48 h of contact).

Light had a significant effect only on FA concentrations: respective mean FA losses were 7.7% ($P = 5 \times 10^{-3}$) in glass/NaCl, 8.8% ($P = 5 \times 10^{-3}$) in glass/dextrose, and 5.3% ($P = 5 \times 10^{-3}$) in plastic/NaCl. Light had no effect on concentrations in plastic/dextrose.

We also noted a change in the colour of the drug solutions, which were initially lemon-yellow; they darkened progressively with time, regardless of the nature of the container, the presence or the absence of light, or the diluent used. In addition, the found quantity of di(ethyl-2 hexyl)phthalate was, in all cases, less than 1 ppm. PH values remained fairly stable in all tested conditions.

DISCUSSION

The potentiation of 5-FU cytotoxicity by FA is based on a scientific demonstration [1, 2] and has demonstrated its clinical utility [3 – 6]. It was justified to investigate the compatibility of 5-FU and FA in mixture in order to know whether their prolonged administration is feasible in the same container. This has a practical importance for both patient and clinical staff. Moreover there is a tendency to administer 5-FU with multiple potentially synergistic compounds such as not only FA but also interferon and cisplatin [12]. We thus measured the evolution of 5-FU and FA concentrations under different physical conditions corresponding with those encountered clinically (doses, duration of administration, containers, diluants, presence/absence of

Table 2. Evolution of 5-FU and FA concentrations in plastic containers

Container/diluent Parameter		Sampling times										
		T_0		8h		24h		48h		60h		Mean
		L	D	L	D	L	D	L	D	L	D	(CV%)
Plastic/NaCl												
pH	I	8.35	8.35	8.39	8.39	8.40	8.40	8.38	8.40	8.43	8.43	8.39(0.3)
	II	8.36	8.36	8.38	8.39	8.40	8.39	8.39	8.40	8.44	8.44	8.40(0.3)
5-FU conc.	I	6.50	6.28	6.68	6.47	6.83	6.64	6.85	6.43	6.90	6.64	6.62(3.1)
mg/ml	II	6.90	6.69	6.73	6.78	6.83	6.74	6.75	6.81	6.90	6.77	6.79(1.0)
FA conc.	I	3.80	3.97	4.03	4.06	3.99	4.22	4.05	4.08	4.05	4.31	4.06(3.4)
(mg/ml)	II	4.12	4.33	3.92	4.44	4.09	4.30	4.03	4.41	4.12	4.35	4.21(4.2)
Plastic/dextrose												
pH	I	8.39	8.33	8.35	8.35	8.40	8.38	8.40	8.39	8.44	8.44	8.39(0.4)
	II	8.36	8.35	8.35	8.37	8.39	8.38	8.40	8.39	8.43	8.43	8.39(0.3)
5-FU conc.	I	6.38	6.41	6.55	6.43	6.75	6.67	6.90	6.59	6.65	6.69	6.60(2.5)
(mg/ml)	II	6.73	6.69	6.55	6.47	6.90	6.50	6.90	6.57	6.53	6.72	6.66(2.4)
FA conc.	I	4.16	4.11	4.24	4.09	4.40	4.28	4.21	4.24	4.16	4.28	4.22(2.2)
(mg/ml)	II	4.18	4.29	4.21	4.14	4.27	4.24	4.30	4.25	4.17	4.29	4.23(1.3)

Theoretical 5-FU and FA concentrations in plastic containers were 6.5 and 4 mg/ml, respectively. I, II represent separate experiments; L = light, D = dark.

Table 3. Evolution of 5-FU and FA concentrations in cassettes

Container/diluent		Sampling times					
		T_0	8h	24h	48	120h	Mean (CV%)
Cassettes							
pH	I	8.48	8.36	8.44	8.48	8.39	8.43(0.6)
	II	8.49	8.37	8.43	8.48	8.39	8.43(0.6)
5-FU conc.	I	50.00	51.56	50.24	53.91	51.43	51.43(3.0)
(mg/ml)	II	50.71	50.00	52.38	50.47	51.19	50.95(1.2)
FA conc.	I	31.80	31.62	31.80	33.10	33.26	32.32(2.5)
(mg/ml)	II	33.26	33.08	36.03	32.72	33.64	33.75(3.9)

Theoretical concentrations in cassettes were 50 mg/ml for 5-FU and 30.8 mg/ml for FA. I, II represent separate experiments.

light). The analytical methods we used (HPLC) are those allowing both sensitivity and specificity in drug analysis. 5-FU and FA concentrations in all drug mixtures remained significantly stable throughout the 60 h survey. More interestingly, even with the highest doses tested in the cassettes, the drug levels were still unmodified after a period of 120 h at a physiological temperature. In addition, IRFT spectrophotometry confirmed the absence of significant fixation of FA or 5-FU on the PVC bags. No physical evidence of precipitate formation was noted in any of the test conditions.

Using a similar experimental protocol other investigators have shown that 5-FU and FA tested alone remain stable in solution over prolonged periods of time [9, 10]. However, a slight loss of FA (approx. 10%) has also been reported when this drug was used alone over 4 days at low concentrations (0.1 – 0.5 mg/ml) in plastic bags with 0.9% NaCl [10]. Smith *et al.* [11] investigated the stability of floxuridine and FA in admixtures of 0.9% sodium chloride at various concentrations and temperature conditions. In their study, the concentrations of both drugs remained stable (within USP limits) at all conditions tested for a minimum of 48 h. However, the chemical stability of FA tested at the lowest concentrations (30 μg/ml) exhibited the highest degree of degradation (less than 10% of loss). Along with a clinical trial, Anderson *et al.* [13] checked the stability of a single 5-FU–FA admixture (0.2 mg/ml FA and 10 mg/ml 5-FU, 0.9% sodium chloride, PVC bags protected from light). After storage for 14 days at 40°C, they noted no significant changes in the 5-FU and FA concentrations. These authors did not evaluate the effect of exposure to light.

In the present study light had a moderate and significant effect only on FA concentrations excluding conditions were plastic/dextrose was used. As observed by Smith *et al.*[11]. pH values remained fairly stable in all tested conditions. In agreement with the study by Smith *et al.* [11] we noted a progressive darkening in the drug mixture colour, whatever the condition tested. We have presently no clear explanation about this observation and more scrutinity on this point appears advisable.

We have recently been informed of some cases of catheter obturation observed during treatment when high concentrations of both 5-FU and FA were used in cassettes at a very low flow rate. This was occurring in the distal part of the catheter. This could be due to a direct contact of drugs mixture with blood. For high drug concentrations in cassettes we suggest the use of two-way catheters. In view of the results obtained in clinically compatible conditions the stability of both FA and 5-FU in admixtures appears very satisfactory. When solutions are exposed to light, use of plastic containers with dextrose as a diluent appears advisable.

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Tumour Cell Proliferation is Abolished by Inhibitors of Na^+/H^+ and HCO_3^-/Cl^- Exchange

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Cell membrane-associated ion transporters, Na^+/H^+ exchanger and Na^+-dependent HCO_3^-/Cl^- antiport, were shown to be important in the regulation of acidic intracellular pH in different cell types. This study investigated the role of the ion exchangers and their inhibitors in the serum-induced proliferation of two murine tumour cell lines, P815 and L929. The presence of Na^+/H^+ exchanger [inhibited by amiloride and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA)] and Na^+-dependent HCO_3^-/Cl^- antiport [inhibited by 4,4′diisothiocyanostilben-2,2-disulphonic acid (DIDS)] was shown on the tumour cell line tested. EIPA suppressed tumour cell proliferation more strongly than amiloride, and its effect was further increased after intracellular acidification by nigericin. DIDS slightly inhibited proliferation of L929 cell line and did not influence proliferation of P815 cells. However, in nigericin acidified cells DIDS had a dose dependent antiproliferative effect. Furthermore, DIDS significantly increased antiproliferative effects of amiloride and EIPA, suggesting the activity of Na^+-dependent HCO_3^-/Cl^- antiport in tumour cell proliferation. These results demonstrate the importance of Na^+-dependent HCO_3^-/Cl^- exchange in addition to Na^+/H^+ antiport, in tumour cell proliferation and indicate the possibility that ion exchange inhibitors could act as antitumour reagents.
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INTRODUCTION

THE MICROENVIRONMENT in tumours is generally more acidic than in normal tissues, approximately 0.5 pH units below the pH of normal tissues. The reduction of tumour pH is caused by the increased production of lactic acid and hydrolysis of ATP under hypoxic conditions [1, 2]. Maintaining the viability of tumour cells under such conditions requires regulation of intracellular pH (pHi) [2, 3]. Major transport mechanisms in regulation of decreased pHi involve Na^+/H^+ antiport and sodium-dependent HCO_3^-/Cl^- exchanger [4, 5].

Na^+/H^+ exchange seems to be important in initiation of growth and proliferation of different cell types, in addition to its role in regulation of cytoplasmic concentrations of Na^+, H^+ and cell volume [6–8]. The activation of Na^+/H^+ exchange by various mitogens and growth factors leads to the intracellular alkalinisation followed by cell growth and proliferation [6, 7]. Recent investigations have shown that mutant cells which lack Na^+/H^+ exchange have either absent or reduced ability to generate tumours [9, 10]. Although Na^+/H^+ antiport seems to be the major cellular H^+-extruding mechanism, sodium-dependent HCO_3^-/Cl^- antiport has been detected in a number of cell lines [11]. This exchanger was shown to be important in the regulation of small reduction of pHi and recovery of steady-state pHi [12]. However, mechanism of activation of Na^+-dependent HCO_3^-/Cl^- antiport is still poorly understood and its role in cell proliferation has not been previously shown.

We have demonstrated the presence of Na^+/H^+ antiport and Na^+-dependent HCO_3^-/Cl^- exchanger in a tumour cell line and

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