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Stability studies of anticancer agent bis(4-fluorobenzyl)trisulfide and synthesis of related substances

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ABSTRACT

Bis(4-fluorobenzyl)trisulfide, fluorapacin, has been extensively developed as a promising new anticancer drug candidate. Its degradation products were identified and verified by the newly synthesized compounds bis(4-fluorobenzyl)disulfide (A) and bis(4-fluorobenzyl)tetrasulfide (B) which were resulted from the disproportionation of fluorapacin under forced conditions. A stability-indicating HPLC method was used for the stability evaluation of active pharmaceutical ingredient (API) fluorapacin and finished pharmaceutical product (FPP) under various conditions. High recovery (99.57%) of API was found after three freeze–thaw cycle processes of fluorapacin FPP. Susceptibility of fluorapacin to oxidative degradation was studied by treating fluorapacin and FPP in 30% hydrogen peroxide aqueous solution, and the result verified the oxidative stability of fluorapacin. However, treatment of this drug candidate under strong light (4500 Lx ± 500 Lx) for 10 days showed substantial effect on the recovery of fluorapacin, especially from fluorapacin FPP. Strong acid (1.0 M, HCl) did not affect the recovery of fluorapacin while strong basic condition (1.0 M, NaOH) accelerated the disproportionation of fluorapacin to its related substances A and B. The stability of fluorapacin in its aqueous media at a pH range of 2.0–10.0 for up to 6 h was further investigated, and 4.0–8.0 was found to be the most stable pH range. Fluorapacin and FPP were exposed to the elevated temperatures of 40 and 60 °C for 10 days without obvious impact on their stability. The thermal stability of fluorapacin API and FPP under constant humidity with light protection was also thoroughly investigated under accelerated (40 ± 2 °C, RH 75 ± 5%, 6 months) and long-term (25 ± 2 °C, RH 60 ± 10%, 24 months) conditions. There was no significant change except minor color change of fluorapacin FPP. Therefore, fluorapacin has excellent stability as a potential drug candidate for further clinical development investigation.

1. Introduction

Various naturally occurring polysulfide derivatives demonstrated antibacterial, antimicrobial, anticancer, receptor tyrosine kinase, anti-radiation, and other important biological activities [1–8]. Diallyl trisulfide demonstrated interesting anti-tumor activity [9,10]. Dibenzyl trisulfide was isolated from *Petiveria alliacea* L. [11] which has been used as an herb medicine for the treatment of pains, tumor, inflammation, bacteria, and other diseases [12]. Dibenzyl trisulfide exhibited anti-proliferative, cellular differentiation, and anti-tumor activities [13–16]. The drug discovery team in our laboratories generated an advanced lead compound, bis(4-fluorobenzyl)trisulfide (Fig. 1) [16]. This anti-microtubule agent exhibited a broad spectrum of anti-tumor activity against a series of breast, non-small cell lung, skin, ovarian, kidney, stomach, cervical, colon, and other tumor cell lines. Further pre-clinical studies of bis(4-fluorobenzyl)trisulfide, named as fluorapacin, verified the *in vivo* anti-tumor efficacy in human xenograft mice models of colon, ovarian, breast, non-small cell lung, and stomach tumors. The promising biological and pharmacological results of fluorapacin have encouraged further development research toward the clinical trial as a new investigational anticancer drug.

The identity, physiochemical properties, crystal structure, solid state, and pharmacokinetic properties of fluorapacin have been investigated [16–18]. The characteristic structure of fluorapacin has drawn our attention to its intrinsic stability as a potential new drug. Polysulfide derivatives are generally susceptible to oxidative, reductive or other extreme conditions although trisulfide is normally more stable than the corresponding mono-, di-, and tetra-sulfide derivatives under various conditions, and the disproportionation of polysulfide derivatives often occurs [19–21]. It was noticed during our early drug discovery research that the long-term exposure of dibenzyl trisulfide derivatives to strong light affected the surface appearance of the crystalline material. Early pharmacokinetic

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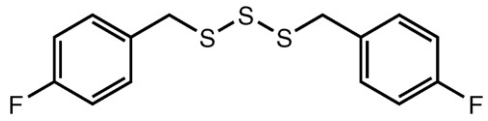


Fig. 1. Chemical structure of fluorapacin, bis(4-fluorobenzyl)trisulfide.

study of fluorapacin also indicated its possible *in vivo* instability [18]. Therefore, it is extremely critical to systematically evaluate the stability of active pharmaceutical ingredient (API) fluorapacin and its finished pharmaceutical product (FPP), fluorapacin injection. The stability assessment for API and FPP of any promising drug candidate plays an important role in the process of new drug development [22]. A variety of environmental conditions, such as light, heat, humidity, and freeze/thaw cycle, could significantly affect the stability of drugs during storage and handling, especially for liquid dose drug products. Identification of stability-affecting factors would facilitate the selection of packaging material and the definition of storage and handling conditions. Therefore, regulatory agencies and the International Conference of Harmonization (ICH) set the mandatory requirements in the associated guidelines for stability testing of drug substances and pharmaceutical products [23,24]. The stability information is essential to define storage and handling conditions of drug substance and finished product to ensure drug quality. The exposure of a drug to the extremely harsh conditions would also reveal degradation products, also called related substances. Meanwhile, identification, synthesis, and verification of related substances become essential for further quality, pharmacokinetic, toxicological, and related studies during the development of new drug fluorapacin.

Herein, we report the identification of related substances bis(4-fluorobenzyl)disulfide (A) and bis(4-fluorobenzyl)tetrasulfide (B) degraded from fluorapacin under extreme conditions. Related substances A and B were synthesized through different synthetic routes, and further utilized for the verification of the degraded related substances. Drug substance fluorapacin and FPP, fluorapacin injection, were thoroughly investigated for their stability assessments in different solvents and dilution schemes, different freeze–thaw cycles, and under various stress conditions such as high temperature, strong acid and base, strong light, and oxidizing agent. The stability of fluorapacin in aqueous solutions at different pH values was also investigated. In addition, the thermal stability of fluorapacin and FPP was also investigated under the accelerated (40 °C, 6 months) and long-term (25 °C, 24 months) conditions, which provided solid stability information. An isocratic, stability-indicating HPLC method [25] was utilized for the quantitative determination of fluorapacin in all of the stability studies. The stability related results facilitated the definition of storage and handling conditions, and prediction for the shelf-life of drug substance fluorapacin and FPP.

2. Experimental

2.1. Material and instrumentation

2.1.1. Chemicals and reagents

Drug substance bis(4-fluorobenzyl)trisulfide, fluorapacin (Fig. 1), was synthesized with a purity of 99.8% according to our reported procedure [16]. The reference sample of fluorapacin was prepared with a purity of 99.90% in our laboratories. The FPP, fluorapacin injection, was manufactured based on our discovered recipe [26]. DL-α-tocopherol (EP grade) was purchased from Sigma–Aldrich–Fluka. The purified Cremophor EL (Cremophor ELP) was purchased from BASF Corporation. 4-Fluorobenzyl mercaptan was manufactured at Shoufu Chemicals, Inc. All solvents used were HPLC grade. Other chemicals and reagents were analytical grade and used as purchased. Water was purified with Millipore system. All solvents used for mobile phase were filtered through membrane (0.22 μm pore size) and degassed before use.

2.1.2. Chromatographic conditions and method

Analyses and stability evaluation were performed using an HP 1100 high performance liquid chromatographic system (Agilent Technologies, Palo Alto, USA) which consisted of an isocratic pump, a vacuum degasser, a thermostated column compartment, an auto-sampler, and a variable wavelength UV–vis detector. The chromatographic data were processed using HP ChemStation 2004 (Agilent Technologies). A reverse phase Spherosorb ODS II (250 mm × 4.6 mm, 5 μm) column was used. All analyses were performed at the column temperature of 40 °C under isocratic conditions with a mobile phase of acetonitrile–water (85:15, v/v), an injection volume of 10 μL, and a flow rate of 1.0 mL/min. The UV absorbance of eluent was measured at 218 nm. The chromatographic conditions and isocratic HPLC method have been developed and validated [25]; therefore, our reported standard solution preparation, calibration curve, and related procedures were utilized for current stability studies.

2.1.3. Other instrumentation

^{1}H- and ^{13}C NMR spectra were recorded on a Varian-500 spectrometer, and trimethylsilane (TMS) was used as an internal reference. FT-IR spectrum was recorded on an AVATAR 360 infrared spectrometer. Ultraviolet-visible absorption spectra were recorded on a TU-1810SPC UV-VIS spectrophotometer. Mass spectrum was recorded on a Traces mass spectrometer. PHS-3C pH meter was used for pH determination. Melting points were measured using an X-6 microscopic melting point apparatus without correction.

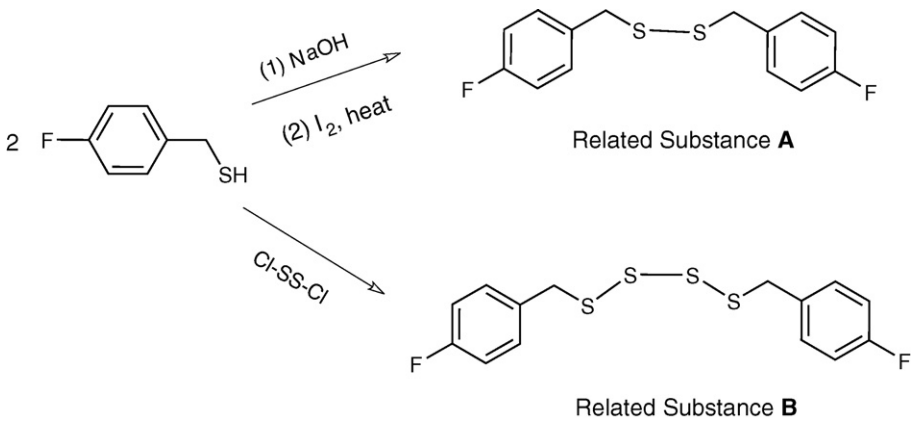
2.2. Synthesis of related substances

2.2.1. Synthesis of bis(4-fluorobenzyl)disulfide (related substance A, Scheme 1)

A mixture of 4-fluorobenzyl mercaptan (5.0 mL, 40.0 mmol) and 15% aqueous sodium hydroxide solution (11.4 mL, 43.0 mmol) was stirred until becoming a clear solution. Iodine (4.36 g, 17.2 mmol) was added in portions. Large amount of precipitate appeared in the reaction mixture, and 10 mL of THF was added to facilitate solubility. The reaction mixture was stirred for 2 h, and extracted with ether. The organic phase was washed with 15% aqueous sodium hydroxide solution, and then washed twice with water. The organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated. The resulting yellow residue was re-crystallized from *n*-hexane to provide 3.16 g (54.9%) white crystal product; m.p. 62.0–63.7 °C. ^{1}H NMR ($CDCl_3$) δ 3.58 (s, 4H, CH_2), 6.97–7.03 (m, 4H, =CH), 7.17–7.24 (m, 4H, =CH); ^{13}C NMR ($CDCl_3$) δ 42.71 ($ArCH_2$), 115.57 (=CH), 115.74 (=CH), 131.13 (=CH), 131.19 (=CH), 133.35 (=CH), 133.38 (=CH), 162.50 (d, J = 246 Hz, =C—F; 161.52, 163.48); FT-IR (KBr, cm^{-1}) 3067, 3038, 1888, 1598, 1507, 1419, 1215, 1156, 839, 649, 532, 473; MS (EI) m/z 282 (M^+, 5%), 109 ($4\text{-}FC_6H_4CH_2^+$, 100%); UV–vis (CH_3CN) λ_{max} 194 nm (ω = 31,988); HPLC purity = 99.94%.

2.2.2. Synthesis of bis(4-fluorobenzyl)tetrasulfide (related substance B, Scheme 1)

A solution of sulfur monochloride (2.6 g, 18.5 mmol) in 5 mL of anhydrous ether was added dropwise to a stirred solution of 4-fluorobenzyl mercaptan (5.0 g, 35.2 mmol) in 15 mL of anhydrous ether at −78 °C under a nitrogen atmosphere. The reaction mixture was stirred at low temperature for 30 min and then stirred for



Scheme 1. Synthesis of related substances A and B.

3 h after removing cooling bath. The reaction mixture was washed twice with brine, and dried over anhydrous sodium sulfate. The drying agent was filtered off, and the solvent was evaporated under reduced pressure. The resulting bis(4-fluorobenzyl)tetrasulfide, related substance B, was obtained as a pale yellow sticky oil which solidified at −78 °C; ^{1}H NMR ($CDCl_3$) δ 4.12 (s, 4H, CH_2), 7.00–7.05 (m, 4H, =CH), 7.26–7.32 (m, 4H, =CH); ^{13}C NMR ($CDCl_3$) δ 42.95 ($ArCH_2$), 115.73 (=CH), 115.91 (=CH), 131.30 (=CH), 131.36 (=CH), 132.23 (=CH), 132.25 (=CH), 162.61 (d, J = 246 Hz, =C—F; 161.63, 163.59).

2.3. Stability studies

2.3.1. Stability in different solvents and dilution schemes

Fluorapacin solutions (200 μg/mL) were prepared by dissolving 20 mg of drug substance fluorapacin in 100 mL of anhydrous ethanol, acetonitrile, ethyl acetate, and mobile phase of acetonitrile–water (85:15, v/v), respectively. For the stability studies of fluorapacin FPP, 200 μg/mL solutions of fluorapacin were prepared by diluting appropriate volume of fluorapacin injection with anhydrous ethanol, acetonitrile, and mobile phase of acetonitrile–water (85:15, v/v), respectively. The resulting solutions were stored in brownish vials at 25 °C to protect from light, and analyzed by HPLC at 0, 1, 2, 3, 4, and 6 h to determine the recovery of fluorapacin for its stability evaluation in different solvents.

The stability of fluorapacin injection solution (FPP) was also studied by diluting fluorapacin FPP 10- and 20-fold with saline. The resulting solutions were stored in brownish vials at 25 °C, and analyzed by HPLC at 0, 0.5, 1, 2, 4, 6, 8, and 24 h to determine the recovery of API for the stability evaluation in different dilution schemes.

2.3.2. Freeze–thaw stability of fluorapacin FPP

Fluorapacin FPP in original vials were kept at 2–8 °C for 2 days, and then heated at 40 °C for 2 days per cycle. Three cycles of freeze–thaw were conducted to examine appearance, special matter, purity, related substances, and pH. The samples were diluted to the testing concentration with acetonitrile, and analyzed by the HPLC method. The freeze–thaw stability was evaluated by comparing the results after each cycle to the results before freeze–thaw studies.

2.3.3. Oxidative and photo-stabilities

Fluorapacin API (20 mg) was treated with 10 mL of 30% aqueous hydrogen peroxide solution at 25 °C for 2–3 h. The sample was transferred to a 50-mL volumetric flask which was then filled to mark with acetonitrile for HPLC analysis. Fluorapacin FPP solution (1 mL) was directly treated with 10 mL of 30% aqueous hydrogen peroxide solution at 25 °C for 2–3 h. The sample was diluted to 100 mL with acetonitrile and analyzed by the HPLC methods.

A uniformly outlaid drug substance fluorapacin (~3 mm thick) in a sample dish was treated under strong light (4500 Lx ± 500 Lx) at room temperature. At the time points of 5 and 10 days, 20 mg of the sample was dissolved in 50 mL of acetonitrile for HPLC analysis. Fluorapacin FPP (1 vial) was treated under strong light (4500 Lx ± 500 Lx) at room temperature. The treated FPP solution (1 mL) was diluted to 100 mL with acetonitrile, and analyzed by the HPLC method at 5 and 10 days. Oxidative and photo-stabilities were evaluated by the recovery of fluorapacin API.

2.3.4. Stability in aqueous solutions of different pH

Fluorapacin API and FPP were first evaluated for their stability under strong base and acid conditions. Fluorapacin solid sample (20 mg) was directly treated with 10 mL of aqueous hydrochloric acid solution (1.0 M) at room temperature for 2–3 h. The sample was then neutralized with the same volume of aqueous sodium hydroxide solution (1.0 M). Thus treated sample was diluted to 50 mL with acetonitrile and analyzed by the HPLC method. The fluorapacin FPP sample (1.0 mL) was treated with 5 mL of aqueous hydrochloric acid solution (1.0 M) at room temperature for 2–3 h. The sample was then neutralized with the same volume of aqueous sodium hydroxide solution (1.0 M). Thus treated sample was diluted to 100 mL with acetonitrile and analyzed by the HPLC method.

The preliminary stability studies indicated that fluorapacin is less stable under basic conditions; therefore, its stability under basic conditions was further evaluated in the different time intervals. Fluorapacin crystalline sample (20 mg) was directly treated with 10 mL of aqueous sodium hydroxide solution (1.0 M) at room temperature. At the time points of 1, 2, 3, 4, and 5 h, a portion of the sample was taken out and neutralized with the same volume of aqueous hydrochloric acid solution (1.0 M). Thus treated samples were diluted to the testing concentration with acetonitrile and analyzed by the HPLC method. The fluorapacin FPP sample (1.0 mL) was treated with 5 mL of aqueous sodium hydroxide solution (1.0 M) at room temperature. At the time points of 1, 2, 3, 4, and 5 h after treatment, a portion of the sample was removed and neutralized with the same volume of aqueous hydrochloric acid solution (1.0 M). Thus treated samples were diluted to the testing concentration with acetonitrile and analyzed by the HPLC method. The stability was evaluated by the recovery of fluorapacin.

The stability of fluorapacin FPP solution under various pH was further investigated in order to evaluate the influence of pH on its stability. Fluorapacin FPP (2 mL each) was diluted to 20 mL with saline. Such samples (11) were adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0,

6.5, 7.0, 7.5, 8.0, 9.0, and 10.0 using aqueous hydrochloric acid or sodium hydroxide solution (1.0 M) and kept for 0, 0.5, 1, 2, 3, 4, and 6 h. Thus treated samples (1 mL each) were diluted to 10 mL with acetonitrile, and analyzed by the HPLC method. The recovery data of fluorapacin at each pH and time point were used to evaluate its stability under various pH.

2.3.5. Thermal (stress, accelerated, and long-term) stabilities

Fluorapacin API and FPP were evaluated for their thermal stability under the stress conditions at different temperatures. Fluorapacin crystalline samples (~3 mm film) and fluorapacin FPP vials were maintained at 4, 25, 40, and 60 °C for 0, 5, and 10 days. The stability samples were dissolved in acetonitrile to their testing concentration, and analyzed by the HPLC method.

Fluorapacin API and FPP were also evaluated for their thermal stability under the accelerated conditions for 6 months. Fluorapacin and FPP samples were stored in the closed desiccators with the constant relative humidity (RH) of 75 ± 5%, maintained by saturated NaCl solution. The desiccators were maintained at 40 ± 2 °C in a thermostat without direct light. At the time points of 0, 1, 2, 3, and 6 months after treatment, appearance, melting point (API), and pH (FPP) were analyzed. The samples were dissolved in acetonitrile to the testing concentration for the quantitative analysis of fluorapacin.

The long-term stability of fluorapacin API and FPP was also studied at 25 ± 2 °C by the similar strategy as described above for accelerated stability. The samples were stored in the closed desiccators with the constant relative humidity (RH) of 60 ± 10%, maintained by saturated $NaNO_2$ solution for 0, 3, 6, 9, 12, 18, and 24 months. The stability samples were examined for appearance, melting point (API), and pH (FPP). The samples were dissolved in acetonitrile to the testing concentration for the quantitative analysis of fluorapacin.

3. Results and discussion

The isocratic and stability-indicating HPLC method was developed and validated for the quantitative determination of fluorapacin API and finished pharmaceutical product (FPP) [25]. Therefore, the validated HPLC method was utilized for the quantitative determination of fluorapacin in current stability investigation.

3.1. Related substances and verification

Drug substance bis(4-fluorobenzyl)trisulfide (Fig. 1), fluorapacin, was synthesized in high purity (99.9%) with good stability under normal conditions. However, the preliminary stability studies under stress conditions indicated that it degraded substantially under strong basic condition. Fig. 2 shows the HPLC chromatogram of fluorapacin treated in strong base aqueous NaOH solution. Part of the drug at the retention time of 5.49 min degraded into two impurities at the retention times of 4.61 and 6.43 min, which were named as related substances A and B, respectively. They were all well separated from the drug peak with the similar separation coefficients. We predicted that related substances A and B were resulted by removing and adding the same moiety from fluorapacin molecule, therefore, they still have structurally similar properties to fluorapacin. The most likely source of instability in the fluorapacin molecule is at the trisulfide linkage. We hypothesized that removal and addition of one sulfur atom in fluorapacin would result in the corresponding di- and tetra-sulfide analogues. Such a disproportionation reaction also occurs in other polysulfide molecules [20]. Therefore, fluorapacin disproportionates to its analogues bis(4-fluorobenzyl)disulfide (related substance A) and bis(4-fluorobenzyl)tetrasulfide (related substance B). Related substance A is the major degradation product, and it is more stable than related substance B. Bis(4-fluorobenzyl)disulfide (related substance A) was also found to be the major metabolite in the *in vivo* pharmacokinetic studies [18].

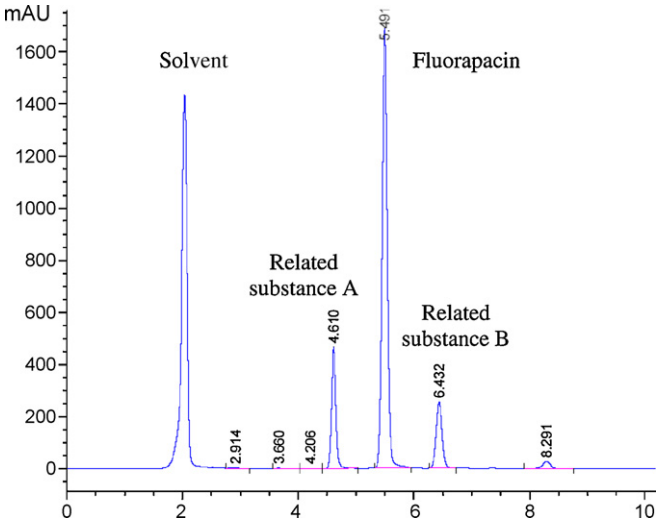


Fig. 2. HPLC chromatogram of fluorapacin treated under strong basic condition.

In order to verify the hypothesis, we designed two new synthetic routes for the synthesis of the proposed related substances A and B (Scheme 1). 4-Fluorobenzyl mercaptan was reacted with iodine under alkaline condition providing a white crystalline product bis(4-fluorobenzyl)disulfide with 99.94% chromatographic purity. It was characterized by NMR, FT-IR, UV–vis, and MS spectroscopic analyses. 4-Fluorobenzyl mercaptan was also coupled with sulfur monochloride at low temperature. The resulting bis(4-fluorobenzyl)tetrasulfide was characterized by NMR spectroscopic analyses. Pure related substance A was utilized for the structure verification and reference standard during quality control and pharmacokinetic studies of fluorapacin. However, bis(4-fluorobenzyl)tetrasulfide disproportionates easily in solution, therefore, it was only used for structure verification.

Newly synthesized bis(4-fluorobenzyl)disulfide and -tetrasulfide were used for the verification of related substances A and B in degraded samples. The impurity A in the degraded fluorapacin sample has the retention time of 4.50 min, which is very close to the retention time of pure related substance A at 4.389 min (see b and a, Fig. 3). When pure related substance A was mixed with the degraded sample in 1:1 ratio, related substance A was overlapped at 4.498 min (c, Fig. 3). The peak area of related substance A in chromatogram c (3039) is very close to the total peak areas of related substance A in chromatograms a and b (3035). The results indicated that the related substance A in the degraded sample from fluorapacin is the same compound as the newly synthesized bis(4-fluorobenzyl)disulfide. Therefore, new compound bis(4-fluorobenzyl)disulfide was utilized as a reference standard for further quantitative and bio-analytical analysis during further development of this anti-tumor agent fluorapacin.

The synthesized bis(4-fluorobenzyl)tetrasulfide was characterized by spectroscopic analysis. It was used for the verification of impurity B in the degraded sample from fluorapacin (Fig. 4). Bis(4-fluorobenzyl)tetrasulfide overlapped very well with related substance B in the degraded fluorapacin sample. Therefore, related substance B is the newly synthesized bis(4-fluorobenzyl)tetrasulfide.

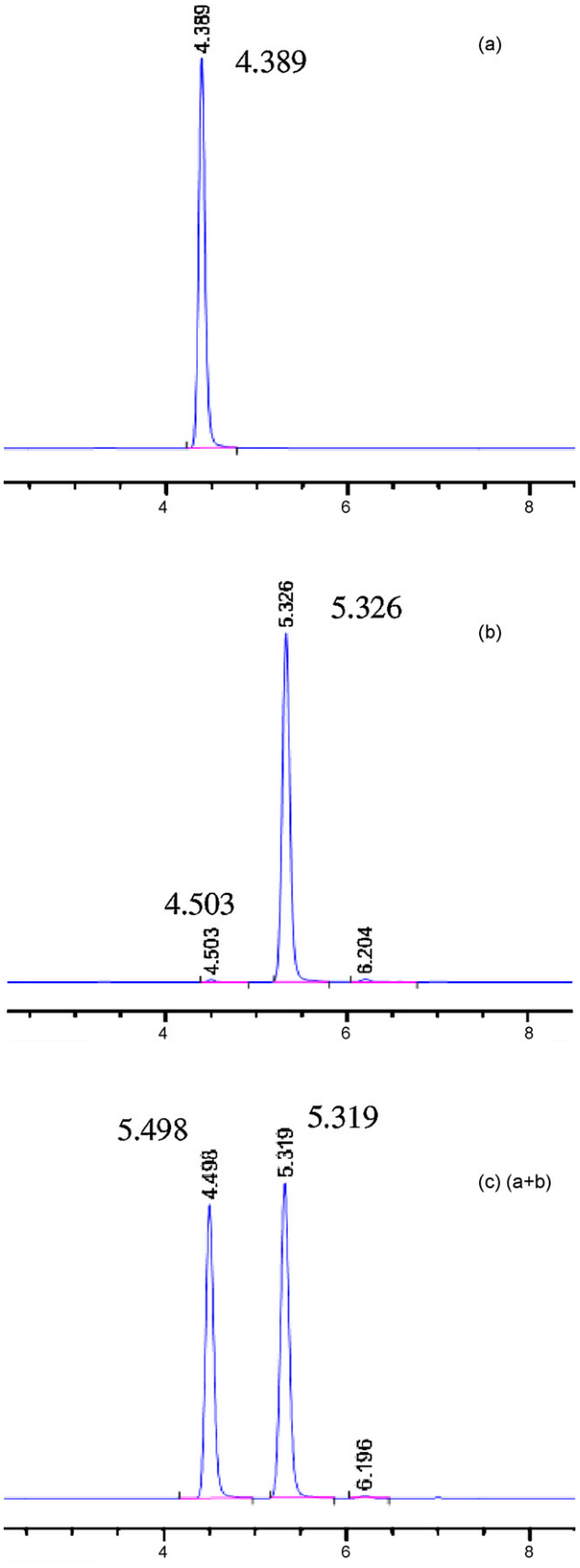


Fig. 3. HPLC comparison of related substance A and the degraded sample ((a) pure related substance A; (b) degraded sample of fluorapacin; (c) 1:1 mixture of related substance A and the degraded sample).

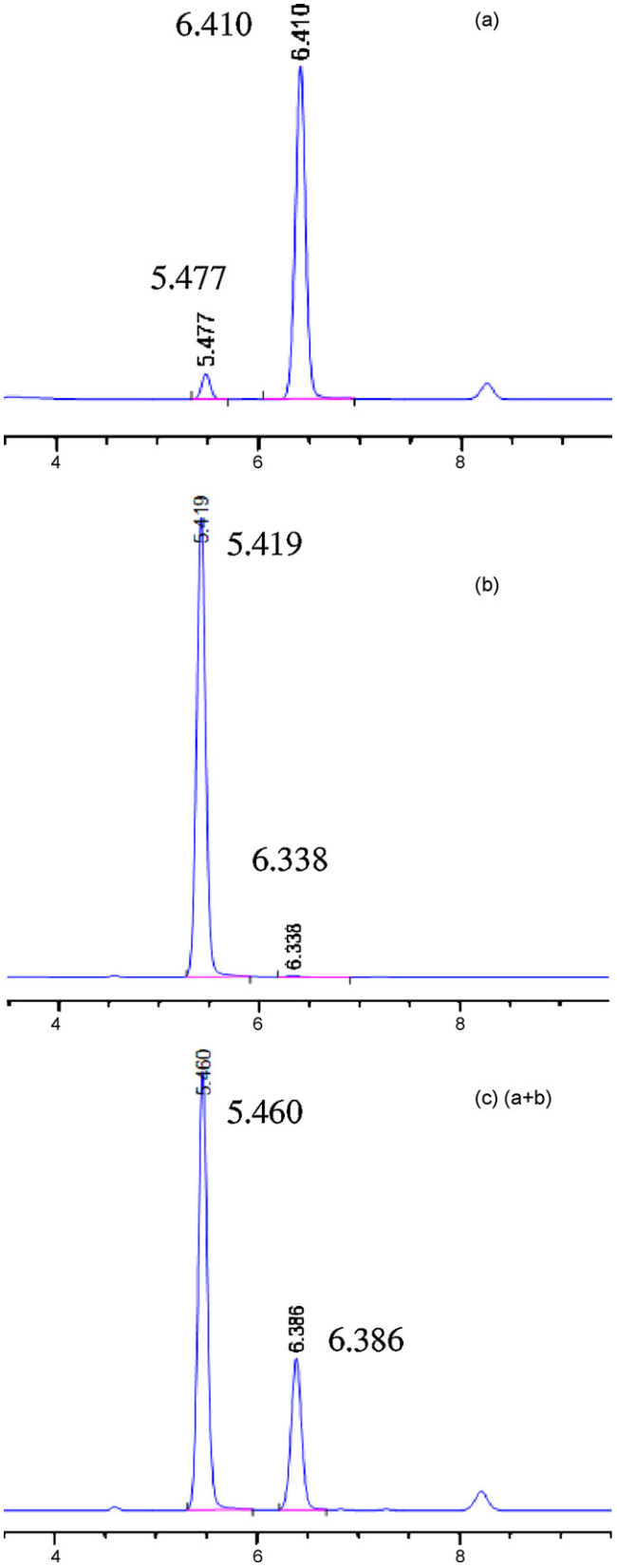


Fig. 4. HPLC comparison of related substance B and the degraded sample ((a) related substance B; (b) degraded sample of fluorapacin; (c) 1:1 mixture of related substance B and the degraded sample).

3.2. Stability of fluorapacin API and FPP

The stability assessment of a promising drug candidate plays an important role in the process of drug development. Various environmental, handling, and storage conditions could seriously affect the stability and quality of a drug substance or the excipients in FPP. Polysulfide derivatives are known to be sensitive to oxidative and other conditions [20,21]. Our preliminary studies also showed that this anti-tumor agent bis(4-fluorobenzyl)trisulfide might be susceptible to light. In order to comply with the requirements of drug regulatory agencies and fully support the development of this drug candidate, we thoroughly investigated the stability of fluorapacin API and FPP under various conditions, such as heat, light, freeze and thaw, strong acid and base, and oxidizing agent. Their accelerated (6 months) and long-term (24 months) formal stability studies under constant temperature and humidity were also investigated to define the storage and handling conditions during the proposed period of validity.

3.2.1. Stability in different solvents and dilution schemes

The storage of fluorapacin solutions in various solvent systems may affect its stability and quantitative determination. Therefore, the stability of fluorapacin in anhydrous ethanol, acetonitrile, ethyl acetate, and mobile phase of acetonitrile–water (85:15, v/v) at 0, 1, 2, 3, 4, and 6 h was first studied. The mean recoveries of fluorapacin from the solutions of drug substance in these solvents were found to be 99.87, 99.91, 99.90, and 99.65% with the relative standard deviations (R.S.D.s) of 0.05, 0.02, 0.02, and 0.21%, respectively (detail data not shown). The stability of fluorapacin FPP in anhydrous ethanol, acetonitrile, and mobile phase was also studied at the same time intervals as for drug substance. The mean recoveries of fluorapacin from the solutions of FPP in these solvents were found to be 99.58, 99.55, and 99.30% with relative standard deviations (R.S.D.s) of 0.12, 0.12, and 0.28%, respectively (detail data not shown). The R.S.D.s of <2.0% for all tests meet the analytical requirement. Therefore, fluorapacin drug substance and FPP were stable in these solvents except a slight decrease in mobile phase.

The mobile phase contains water. Therefore, we further studied the stability of fluorapacin injection in various dilution schemes with water to explore whether the low concentration of fluorapacin in water would accelerate its disproportionation. Fluorapacin FPP was diluted 10- and 20-fold with saline, and the resulting solutions were analyzed at the time points of 0, 0.5, 1, 2, 4, 6, 8, and 24 h. The mean recoveries of fluorapacin from the 10- and 20-fold diluted solutions were 99.67 and 99.63% with the R.S.D.s of 0.02 and 0.06%, respectively. The diluted solutions of fluorapacin FPP are stable for 24 h; therefore, it is stable for the quantitative determination of fluorapacin.

3.2.2. Freeze–thaw stability of fluorapacin FPP

The freeze–thaw occurs during storage, handling, and use of injectable drugs. The freeze–thaw stability of fluorapacin FPP was tested. Appearance, special matter, purity, related substances, and pH were determined after each of the three cycles of freeze–thaw processes (Table 1). The freeze–thaw stability was evaluated by comparing the results after each cycle to the results before freeze–thaw studies. The results indicated that fluorapacin FPP is stable for the freeze–thaw processes.

Table 1
Freeze–thaw stability data of fluorapacin FPP

Freeze–thaw cycle	pH	Related substance (%)			Fluorapacin recovery (%)
		Impurity A	Impurity B	Total impurity	
0	5.60	0.03	0.04	0.30	99.79
1	5.70	0.02	0.03	0.29	99.95
2	5.70	0.02	0.04	0.28	99.69
3	5.70	0.03	0.03	0.27	99.57

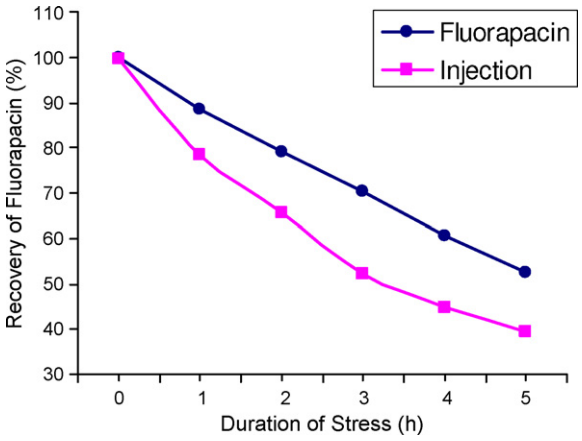


Fig. 5. Recovery profiles of fluorapacin and FPP under strong basic condition (1.0 M, NaOH).

3.2.3. Oxidative and photo-stability

Sulfide derivatives, especially monosulfide, are generally sensitive to oxidative conditions [20]. However, the oxidative stability of trisulfide derivatives has not been systematically investigated. To ensure quality and verify the developability of this promising anti-tumor agent, fluorapacin API and FPP were treated at 25 °C with large excess amount of aqueous hydrogen peroxide (30%) solution for 2–3 h. HPLC assay indicated that fluorapacin is stable against oxidative condition with fluorapacin recovery of 99.78 and 99.36% for fluorapacin API and FPP, respectively.

Drug substance fluorapacin and FPP were treated under strong light (4500 Lx ± 500 Lx) at room temperature for photolysis stability assessment. At the time points of 5 and 10 days after treatment, 97.85 and 95.28% of fluorapacin were recovered from drug substance, while 92.56 and 80.98% of API were recovered from fluorapacin FPP, respectively. The results indicated that fluorapacin and FPP were affected by strong light. Therefore, the fluorapacin API and FPP are recommended to be light protected for long-term storage.

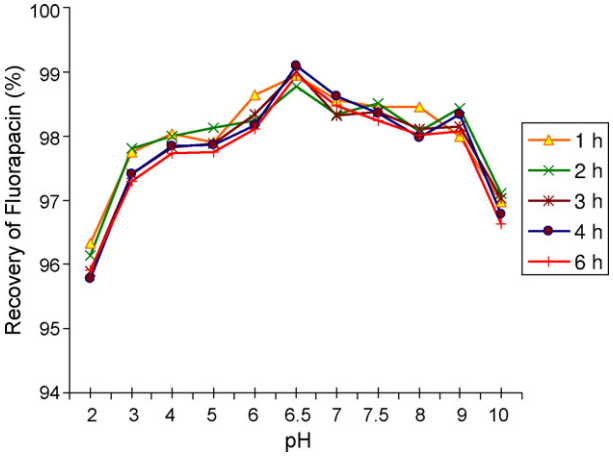


Fig. 6. pH-recovery profile of fluorapacin FPP aqueous solutions.

Table 2
Recovery results of fluorapacin API and FPP under stress conditions

Storage temperature (°C)	Fluorapacin API (assay, %)		
	Day 0	Day 5	Day 10
4	99.82	99.80	99.71
25		99.79	99.73
40		99.77	99.52
60		99.77	99.36
Storage temperature (°C)	Fluorapacin FPP (assay, %)		
	Day 0	Day 5	Day 10
4	99.77	99.75	99.72
25		99.75	99.71
40		99.72	99.50
60		99.67	99.29

3.2.4. Stability in aqueous solutions of different pH

The stability of fluorapacin under strong acidic and basic conditions as well as at different pH was thoroughly investigated. Both drug substance and injection were treated with aqueous hydrochloric acid solution (1.0 M) for 2–3 h. 99.91 and 99.01% of fluorapacin were recovered from fluorapacin API and FPP, which indicated that fluorapacin has high intrinsic stability under strong acidic condition. When fluorapacin API and FPP were treated with aqueous sodium hydroxide solution (1.0 M), the treated samples were maintained at room temperature, and analyzed at the time points of 0, 1, 2, 3, 4, and 5 h. Fig. 5 illustrates the recovery profiles of API from sodium hydroxide treated drug substance and FPP solutions. Therefore, strong base has substantial impact on the stability of fluorapacin, and slightly higher influence on the stability of fluorapacin FPP. The useful information suggested that basic condition shall be avoided during analysis, formulation, dosing preparation, and other studies of this anticancer drug candidate.

In addition, the stability of fluorapacin FPP in its aqueous solution at pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10.0 was further investigated. The samples were maintained at room temperature for 0, 0.5, 1, 2, 3, 4, and 6 h, and then analyzed. Fig. 6 shows the stability of fluorapacin FPP aqueous solution in a pH range of 2.0–10.0 at the different time frames. Fluorapacin is stable in a pH range of 3.0–9.0. The most stable pH range is 4.0–8.0, and the most stable pH is 6.5. Its stability gradually decreases at pH <3.0 and >9.0. Thorough stability investigation indicated that fluorapacin is stable under acid, neutral, and slightly basic conditions up to pH 9, but its stability is affected under stronger basic conditions. Therefore, fluorapacin is expected to be stable in its finished pharmaceutical product (pH 4.5–7.0, established criteria for fluorapacin injection) as well as during the formulation and administration processes.

3.2.5. Thermal (stress, accelerated, and long-term) stabilities

To comply with the requirements of food and drug administration regulatory agencies, drug substance fluorapacin and its finished pharmaceutical product were stored under various conditions for their stress, accelerated, and long-term formal stability studies. Fluorapacin API and FPP were treated at 4, 25, 40, and 60 °C for 5 and 10 days. HPLC assay results (Table 2) indicated that both fluorapacin API and FPP are all stable under the tested conditions.

To recommend the storage conditions and handling requirements for fluorapacin, and ensure drug quality within the recommended period of validity, three primary batches of fluorapacin API and FPP were further investigated for their stability under accelerated (40 °C, 75% RH for 6 months) and long-term (25 °C, 60% RH for 24 months) conditions. As mentioned above, strong light affects the stability of this new drug; therefore, the testing samples were maintained under light protection. Appearance, melting point, drug content, and total impurity were examined for drug substance at the designated time intervals. Appearance, pH, drug content, and total impurity were examined for fluorapacin FPP. The assay results were summarized in Table 3. There was no noticeable change on the appearance and melting point of fluorapacin drug substance for the accelerated and long-term stability investigations (data not shown). HPLC assay results also indicated that the fluorapacin content at 6-month and 24-month testing periods was almost the same as that at the initial point with R.S.D.s of <0.80% though the total impurity in drug substance at the end of tests slightly increased to 0.40%. Accelerated and long-term conditions had minor effect on fluorapacin FPP. The appearance of FPP solution kept constant for 1 month at 40 °C, and for 3 months at 25 °C, while its color gradually changed to pale yellow thereafter. There was also a slight change on pH value of FPP solution. HPLC assay results also verified that the fluorapacin content in FPP still maintained almost the same with slight increase on the total impurity at the final time point. Therefore, both fluorapacin API and FPP are stable with no significant change under accelerated and long-term stability testing conditions. The shelf-life of fluorapacin API and FPP can be predicted as 24 months based on stability results. Further stability testing is still in progress to accumulate additional information and prepare for the possible change of shelf-life for fluorapacin and its finished pharmaceutical product to ensure their quality.

Table 3
Accelerate and long-term stability results of fluorapacin API and FPP

Storage interval (month)	Fluorapacin API		Fluorapacin FPP		
	Assay (% ±S.D., R.S.D.) (n = 3)	Total impurity (%)	pH	Recovery (% ±S.D., R.S.D.) (n = 3)	Total impurity (%)
Accelerated stability (6 months, 40 ± 2 °C, RH 75 ± 5%)					
Initial	99.93 ± 0.6689, 0.67	0.08	5.50	99.66 ± 0.1595, 0.16	0.30
1	99.79 ± 0.5285, 0.53	0.02	5.63	99.80 ± 0.4279, 0.43	0.48
2	99.49 ± 0.3785, 0.38	0.18	5.72	99.83 ± 0.3066, 0.31	0.54
3	99.47 ± 0.2427, 0.24	0.28	5.81	99.37 ± 0.4524, 0.46	0.58
6	99.70 ± 0.3573, 0.36	0.40	5.89	98.11 ± 0.7357, 0.75	0.92
Long-term stability (2 years, 25 ± 2 °C, RH 60 ± 10%)					
Initial	99.93 ± 0.6689, 0.67	0.08	5.50	99.66 ± 0.1595, 0.16	0.30
3	99.59 ± 0.2558, 0.26	0.02	5.54	100.20 ± 0.7143, 0.71	0.36
6	99.69 ± 0.3630, 0.36	0.06	5.63	99.62 ± 0.2873, 0.29	0.40
9	99.45 ± 0.3855, 0.39	0.12	5.69	99.74 ± 0.3926, 0.39	0.57
12	99.63 ± 0.5194, 0.52	0.21	5.73	99.67 ± 0.2818, 0.28	0.49
18	99.60 ± 0.4857, 0.49	0.29	5.94	99.40 ± 0.3262, 0.33	0.61
24	99.13 ± 0.1735, 0.18	0.39	5.96	99.22 ± 0.3024, 0.30	0.89

4. Conclusions

Two major degradation products were identified and verified by the newly synthesized bis(4-fluorobenzyl)disulfide and bis(4-fluorobenzyl)tetrasulfide. These related substances A and B were formed by the disproportionation reaction of fluorapacin. Only strong base and strong light have obvious impact on this degradative reaction; therefore, affect the stability of this drug candidate. Fluorapacin was stable in its aqueous solution of pH 3.0–9.0 though a pH range of 4.0–8.0 was found to be the best range for its storage, handling and administration. Fluorapacin is stable in hydrogen peroxide (30%) solution, and the freeze–thaw processes did not affect its stability. Fluorapacin API and FPP demonstrated excellent oxidative, freeze–thaw (FPP), and thermal stabilities under stress, accelerated, and long-term stability study conditions. The information presented here facilitated the establishment of drug criteria for the quality control and assurance of current drug substance and FPP. The information has also been utilized to define optimal storage and handling conditions as well as the use of pharmaceutical excipients to ensure further development of this promising drug candidate.

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