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Short communication

Quantitative determination of anti-tumor agent bis(4-fluorobenzyl) trisulfide, fluorapacin and its pharmaceutical preparation by high-performance liquid chromatography

Yimei Bao [b], Yuyu He [b], Xiaoying Xu [b], Xiaopeng Mo [b], Xiao Xu [a], Xiaobo Wang [a], Haoyun An [a,*]

[a] *ACEA Biosciences, Inc., 6779 Mesa Ridge Road, Suite 100, San Diego, CA 92121, USA*
[b] *ACEA Bio (Hangzhou) Co., Ltd., Building 3, West Lake Technical & Economic Park, 2# Xiyuan 5 Road, Sandun, Hangzhou 310030, China*

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Abstract

A simple isocratic and stability-indicating HPLC method was developed and validated for the quantitative determination of anti-tumor agent fluorapacin and its pharmaceutical preparation. A Spherisorb ODS II C_{18} (250 mm × 4.6 mm, 5 μm) column was eluted with a mobile phase consisting of acetonitrile/water (85:15, v/v). The analyses were performed at 40 ± 1 °C with a flow rate of 1.0 mL/min and UV detection at 218 nm. The calibration curve was linear over a concentration range of 160–240 μg/mL with the correlation coefficient of 0.9997. The LOD and LOQ were determined to be 1.4 and 7.0 ng/mL, respectively. Average recoveries were 98.27% and 100.40% for fluorapacin API and its drug product with corresponding relative standard deviations (R.S.D.) of 0.41% and 0.30%, respectively. Good repeatability (precision and intermediate precision), accuracy and tolerability were obtained with R.S.D. of <1.0%. This specific and reliable method has been successfully applied for quality control of fluorapacin API and drug product.

Keywords: Fluorapacin; Pharmaceutical preparation; Analytical method; Isocratic; HPLC; Quantitative determination

1. Introduction

Petiveria alliacea L. has been used as a herb medicine (named Anamu or apacin) for the treatments of pains, tumor, inflammation, bacteria and other diseases in Caribbean, Latin America, West Africa and other regions [1]. Dibenzyltrisulfide isolated from this subtropical shrub [2] exhibited biological activities [3–7]. Systematic screens against a wide range of tumor cell lines and structural modification of this compound in our laboratories generated an advanced lead compound bis(4-fluorobenzyl)trisulfide (Fig. 1) [8,9]. The spectroscopic and thermal properties as well as crystal structure of this natural product derivative were extensively investigated [9]. This advanced lead was named as fluorapacin during preclinical studies. Fluorapacin demonstrated a broad spectrum of cellular anti-tumor activities by cell cycle specifically working on microtubule dynamics as an antimicrotubule agent [9]. It also exhibits potent *in vivo* anti-tumor activity in human xenograft mice models with good safety and pharmacokinetic profiles. The therapeutic potential of fluorapacin encouraged us to develop and validate an analytical method to meet the regulatory requirements for an investigational new drug [10–13].

Fluorapacin is a small molecule natural product derivative (Fig. 1). The special trisulfide character of this structure represents a new class of anti-tumor agent. No method was reported in the literature to analyze and quantitate this type of compounds, and no method was compiled in any pharmacopoeia for the analysis of this new anti-tumor agent. From the quality control perspective in pharmaceutical industry, it is important to develop and validate an analytical method for quantitative analysis of an active pharmaceutical ingredient, quality control and stability testing during pharmaceutical research and development processes [10–13]. The International Conference

* Corresponding author. Tel.: +1 858 724 0928; fax: +1 858 724 0927.
E-mail address: han@aceabio.com (H. An).

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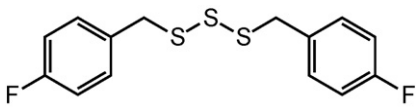

Fig. 1. Chemical structure of fluorapacin, bis(4-fluorobenzyl)trisulfide.

on Harmonization (ICH) and its associated guidelines also set a mandatory requirement to develop stability-indicating assay.

Herein, we describe the development and validation of an HPLC method for the quantitative determination of fluorapacin in drug substance and the corresponding pharmaceutical product, fluorapacin injection. This simple isocratic method is stability-indicating and has desirable sensitivity, specificity, accuracy and precision for efficient quality control and stability studies during the development of new anti-tumor agent fluorapacin as well as its pharmaceutical preparation.

2. Experimental

2.1. Chemicals and reagents

All solvents used were HPLC grade, and reagents were analytical grade. Water was purified using a Mili-Q Academic A10 water purification system (Millipore, France). Solvents used for mobile phase were filtered through membrane (0.22 μm pore size) and degassed before use. DL-α-Tocopherol (EP grade) was purchased from Sigma–Aldrich. The purified Cremophor EL (Cremophor ELP) was purchased from BASF Corporation. Anhydrous ethanol was obtained from Ante Biochemical Co., Ltd. (Anhui, China).

2.2. Samples

Drug substance bis(4-fluorobenzyl)trisulfide, fluorapacin, was synthesized according to our reported procedure [9]. Its identity and structure were verified by elemental analysis, FT-IR, ^{1}H, ^{13}C and ^{19}F NMR, EI mass spectral and X-ray single crystal analyses. The standard sample of fluorapacin was prepared with a purity of 99.9% in our laboratories. Pharmaceutical preparation, 20 mg/mL fluorapacin injection, was manufactured at ACEA Bio (Hangzhou) Co., Ltd. according to its formulation in 40% Cremophor ELP (w/v) and 60% anhydrous ethanol (v/v) containing 0.1% DL-α-tocopherol (w/v).

2.3. HPLC and chromatographic conditions

The analyses were performed using high-performance liquid chromatographic system HP 1100 (Agilent Technologies, USA) which consisted of an isocratic pump, a vacuum degasser, a thermostated column compartment, an autosampler and a variable wavelength UV–vis detector. HPLC systems HP1100 and HP1050 were used as equipments I and II for intermediate precision studies. The chromatographic data were processed using an HP ChemStation 2004 (Agilent Technologies). A reverse phase Spherosorb ODS II (250 mm × 4.6 mm, 5 μm) column (Waters, Ireland) was used. All analyses were performed at the column temperature of 40 ± 1 °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.

2.4. Standard solution preparation

Accurately weighed approximately 50.4 mg of standard sample bis(4-fluorobenzyl)trisulfide was transferred to a 50 mL volumetric flask which was then filled to mark with acetonitrile. Final concentration of the resulted stock solution was 1.008 mg/mL. Accurate aliquots 1.60, 1.80, 1.90, 2.00, 2.20 and 2.40 mL of this stock solution were diluted to 10 mL with ace-

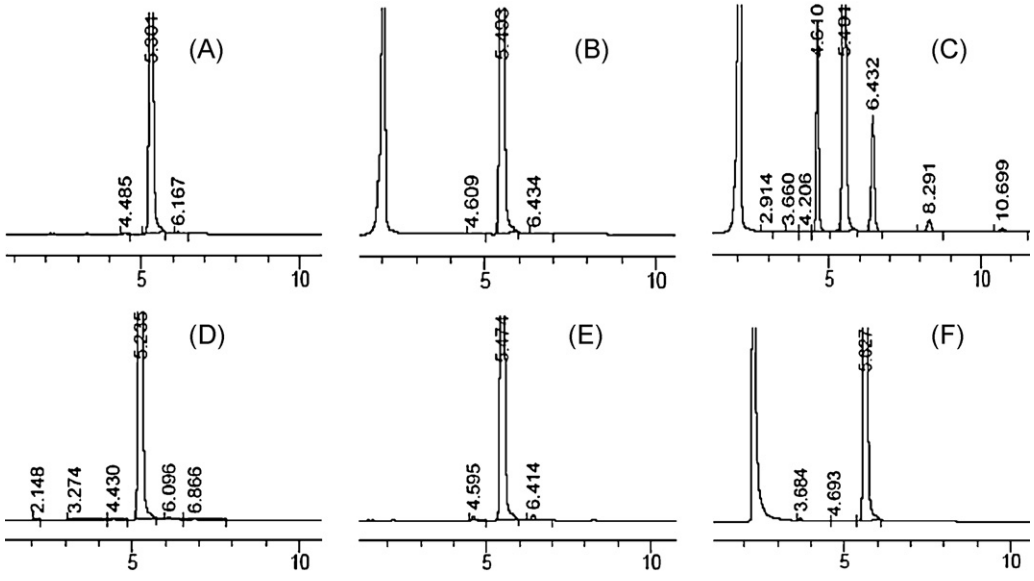

Fig. 2. HPLC chromatograms of fluorapacin (A) and stressed samples (B–F). ((A) fluorapacin; (B) strong acid; (C) strong base; (D) high temperature; (E) strong light; (F) H_2O_2).

Table 1
Accuracy of the method

Amount added (mg ± S.D., R.S.D.) ($n = 3$)	Amount found (mg ± S.D., R.S.D.) ($n = 3$)	Recovery (%)
Fluorapacin		
3.94 ± 0.0208, 0.53	3.889 ± 0.0035, 0.09	98.71
4.95 ± 0.0208, 0.42	4.847 ± 0.0133, 0.27	97.92
5.90 ± 0.0306, 0.52	5.793 ± 0.0294, 0.51	98.19
Average		98.27 ± 0.3999, 0.41
Fluorapacin injection		
16.73 ± 0.1528, 0.91	16.855 ± 0.0178, 0.11	100.75
20.40 ± 0.1000, 0.49	20.440 ± 0.0288, 0.14	100.20
24.43 ± 0.1528, 0.63	24.494 ± 0.0358, 0.15	100.26
Average		100.40 ± 0.3010, 0.30

tonitrile in volumetric flasks to generate corresponding standard solutions.

3. Results and discussion

In order to develop and validate an efficient method for the analyses of drug substance fluorapacin and API in its pharmaceutical product, fluorapacin injection, we explored the detection wavelength, different solvent systems and the compositions of mobile phase. According to preliminary results, we finalized the detection wavelength of 218 nm and the mobile phase of acetonitrile/water (85:15, v/v). Before fully implemented in the quantitative determination of drug substance and pharmaceutical preparation, this method was thoroughly validated for its linearity, specificity, accuracy, precision and intermediate precision, robustnesses under various modified conditions, limits of detection and quantification.

3.1. Calibration curve and linearity

The calibration curve was generated from six concentration levels of 161.28, 181.44, 191.52, 201.60, 221.76 and 241.92 μg/mL and the corresponding peak areas. It demonstrated an excellent linearity in a range of 160–240 μg/mL for both fluorapacin and fluorapacin injection. The linear equation for calibration curve was $y = 43.35x + 99.624$ with a correlation coefficient of 0.9997.

3.2. Selectivity and specificity

In order to study specificity of the current method, we conducted the decomposition of fluorapacin and its injection under stress conditions including strong acid, strong base, oxidation, high temperature and strong light [10–15]. Thus, obtained samples provided enough degradants to investigate the relative separation coefficients (Rs) of the drug fluorapacin.

Acetonitrile was injected and used as a blank. A drug substance peak at 5.30 min was used as a reference (see Fig. 2A). Fluorapacin samples (20 mg) were treated in parallel with hydrochloric acid (1.0 M, 10 mL), sodium hydroxide (1.0 M, 10 mL) and 30% hydrogen peroxide (10 mL) aqueous solutions for 2–3 h. The acid and base treated samples were neutralized with the same volume of base and acid, respectively. Fluorapacin was treated at 60 °C for 15 days or under strong light (5200 Lx) for 24 h. The forced samples were dissolved or diluted to the testing concentration with acetonitrile for HPLC analysis. Fig. 2 shows the HPLC chromatograms of fluorapacin (A) and the stressed solutions (B–F). The fluorapacin injection samples were treated under the similar forced conditions to generate the stressed solutions for HPLC analysis.

The HPLC chromatograms in Fig. 2 demonstrated that the peaks of the resulted degradants were all well resolved from the drug peak (retention time = 5.30 min) with relative separation coefficients $Rs > 1.5$. The stressed solutions from fluorapacin injection showed the similar HPLC chromatograms (data not shown), and the degradants were well resolved with $Rs > 1.5$. Therefore, the current method was proven to be selective and specific for the analyses of fluorapacin in drug substance and injection.

3.3. Accuracy

We thoroughly studied the recovery of fluorapacin and fluorapacin injection in order to evaluate the accuracy of the current method. The fluorapacin solution samples with known concentration and containing 80%, 100% or 120% of fluorapacin standard ($n = 3$) were analyzed. The recovery and statistic data of fluorapacin drug substance and injection are presented in Table 1. The average recovery of fluorapacin was 98.27%, and the average recovery of fluorapacin injection was 100.40%. The relative standard deviations (R.S.D.) were 0.41% and 0.30%, within the acceptance criterion (<2%) [10,11].

3.4. Repeatability and intermediate precision

Precision of the proposed method for the analysis of drug substance fluorapacin and injection was evaluated through the intra-day repeatability of the response of sample solutions. Intermediate (inter-day) precision of the method was evaluated through two different analysts (A and B) by working on two different HPLC instruments (I and II). All solutions were prepared fresh. The repeatability and intermediate precision were determined and expressed as relative standard deviation (R.S.D.) (Table 2). The intra-day precisions (repeatability) for fluorapacin and injection were examined at three different concentrations, and the R.S.D.s were found to be in the ranges of 0.21–0.34%

Table 2
Precision of the method

Concentration added (μg/mL)	Retention time (min)	Recovery (%)
Intra-day ($n=6$) (±S.D., R.S.D.)		
Fluorapacin		
153	5.39 ± 0.0396, 0.74	99.60 ± 0.3359, 0.34
193	5.40 ± 0.0040, 0.07	100.21 ± 0.3187, 0.32
234	5.40 ± 0.0036, 0.07	100.24 ± 0.2126, 0.21
Fluorapacin injection		
161	5.40 ± 0.0151, 0.28	101.00 ± 0.0806, 0.08
202	5.39 ± 0.0033, 0.06	100.11 ± 0.0722, 0.07
242	5.39 ± 0.0052, 0.10	100.08 ± 0.1195, 0.12

	Retention time (min)	Recovery (%)
Inter-day ($n=6$) (%) (± S.D., R.S.D.)		
Fluorapacin		
Equipment I	100.30 ± 0.4942, 0.49	
Equipment II	99.99 ± 0.1409, 0.14	
Average	100.15 ± 0.3832, 0.38	
Fluorapacin injection		
Equipment I	99.78 ± 0.1624, 0.16	
Equipment II	99.96 ± 0.1643, 0.16	
Average	99.87 ± 0.1808, 0.18	

and 0.07–0.12% for fluorapacin and injection, respectively. The inter-day intermediate precision for fluorapacin and injection were found to be 0.38% and 0.18% R.S.D.s, respectively. Therefore, the current assay method has good repeatability and intermediate precision for the quantitative analysis of fluorapacin drug substance and its pharmaceutical preparation.

3.5. Robustness

Robustnesses of the current method for the analyses of fluorapacin and fluorapacin injection were evaluated under intentionally altered conditions including the proportion of mobile phase (±5%), column temperature (±5%), flow rate (±20%) and wavelength (±1 nm). R.S.D.s were found to be in the range of 0.16–0.42%. Therefore, the current method has high tolerability against separation conditions, and is reliable for the analyses of fluorapacin and its injection.

3.6. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was tested by successive dilution of a standard solution with acetonitrile and determined as the concentration that resulted in a signal to noise ratio of 3:1 ($S/N=3$). The LOQ was determined as the concentration that resulted in a signal to noise ratio of 10:1 ($S/N=10$). The LOD and LOQ of this method were determined to be 1.4 and 7.0 ng/mL, respectively.

3.7. Interference of supplementary materials

The possible interference of supplementary materials in fluorapacin injection was also investigated. Blank solutions of DL-α-tocopherol, Cremophor ELP and anhydrous ethanol were prepared according to their corresponding compositions in fluorapacin injection, diluted to 100 times with acetonitrile, and then filtered through 0.22 μm membrane. The diluted solutions were analyzed by HPLC under the proposed conditions. There was no detectable chromatographic peak for DL-α-tocopherol and anhydrous ethanol blank samples. Cremophor ELP sample showed peaks at 10.467, 11.356, 11.773, 12.279 and 13.252 min. Fluorapacin was well resolved from these peaks with $Rs>1.5$. Therefore, the assay procedure is specific for fluorapacin without interference of supplementary materials.

4. Conclusion

We have developed and validated a stability-indicating assay for the quantitative determination of drug substance fluorapacin and its injection by high-performance liquid chromatography. The current simple isocratic method has high specificity, accuracy and precision. The method is also robust against the possible alteration of mobile phase, column temperature and flow rate during analysis. All relative standard deviations (R.S.D.) obtained during method validation were less than 1.0%. Therefore, the current method can be used efficiently for the quantitative determination of fluorapacin drug substance and its finished product fluorapacin injection without interference from excipients. This reliable and accurate method is being routinely used for quality control and stability testing.

References

[1] http://www.rain-tree.com/anamu.htm.
[2] J.R. de Sousa, A.J. Demuner, J.A. Pinheiro, E. Breitmaier, B.K. Cassels, Phycochemistry 29 (1990) 3653–3655.

[3] L. Johnson, L.A.D. Williams, E.V. Roberts, Pest. Sci. 50 (1997) 228–232.
[4] E. Mata-Greenwood, A. Ito, H. Westenburg, B. Cui, R.G. Mehta, A.D. Kinghorn, J.M. Pezzuto, Anticancer Res. 21 (2001) 1763–1770.
[5] H. Rosner, L.A.D. Williams, A. Jung, W. Kraus, Biochim. Biophys. Acta 1540 (2001) 166–177.
[6] L.A.D. Williams, H. Rosner, W. Moller, W. Kraus, Jamaican J. Sci. Technol. 15 (2004) 54–60.
[7] S. Kim, R. Kubec, R.A. Musah, J. Ethnopharmacol. 104 (2006) 188–192.
[8] X. Xu, H. An, X.B. Wang, PCT /WO 2005/112933, December 1, 2005; United States Patent Application 2005/0261321, November 24, 2005; Chinese Patent Application 200580012460.5, November 17, 2006.
[9] H. An, J. Zhu, X.B. Wang, X. Xu, Bioorg. Med. Chem. Lett. 16 (2006) 4826–4829.
[10] Guidance for Industry: Analytical Procedures and Methods Validation: CMC Documentation, United States Department of Health and Human Services, Food and Drug Administration, Rockville, Maryland, August 2000.
[11] Guidance for Analytical Method Validation of Chemical Drugs, State Food and Drug Administration, Beijing, March 2005.
[12] J.M. Green, Anal. Chem. (1996) 305A–309A.
[13] M.M. Dantus, M.L. Wells, J. Liq. Chromatogr. Rel. Technol. 27 (2004) 1413–1442.
[14] M. Bakshi, S. Singh, J. Pharm. Biomed. Anal. 28 (2002) 1011–1040.
[15] S. Klick, P.G. Muijselaar, J. Waterval, T. Eichinger, C. Korn, T.K. Gerding, A.J. Debets, C.S. van de Griend, C. van den Beld, G.W. Somsen, G.J. de Jong, Pharm. Technol. (2005) 48–66.