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A digitized impurity database analysis method for determining the impurity profiles of gatifloxacin in bulk materials and injections

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HPLC has become the most important analytical technique for impurity profiling in order to assure the quality of pharmaceutical products. Although HPLC is considered as a well-established technology, it requires CRS (chemical reference substances) of impurities for qualification and quantification of impurity peaks. Many impurity CRS have been widely used for the impurity profile control, which causes a high cost of production in practice. In this study, we developed a new method for impurity profiling control, so called digitized impurity database analysis, which does not directly use impurity CRS. Using a quinolone antibiotic, gatifloxacin as an example, we first analyzed its impurities by DAD (diode array detector) to compile a digitized impurity database and then used the database to analyze the impurities in the samples of domestic gatifloxacin bulk materials and injections in China. We identified the impurities in the chromatogram by combining two-dimensional chromatographic spectral correlation analyses of ultraviolet spectra data and relative retention times. The content of the impurities was determined using relative response factors of impurity to gatifloxacin as normalization factors. The digital impurity database analysis technology we developed is a "green", economic and convenient method that may eliminate the use of impurity CRS in the impurity profile control.

1. Introduction

An impurity is defined as any organic material other than the drug substance or ingredients, arising from the synthesis or unwanted chemicals that remain in APIs (active pharmaceutical ingredients). The drug substance is compromised due to impurity in terms of its pharmaceutical effect, even when the impurities have superior pharmacological or toxicological properties (Nagpel et al. 2011; Pilaniya et al. 2010). Impurity profiling is a general term including structure elucidation/identification and determination of the impurities of a chemical substance (Görög 2006; Kovaleski et al. 2007). Recently, much critical attention from regulatory authorities has been paid to this term. Different pharmacopoeias, such as European pharmacopoeia, United States pharmacopoeia, Chinese pharmacopoeia, are gradually incorporating the limits of the allowable levels of impurities present in APIs or formulations. Typically, the impurities are structurally similar to the drug substance, so HPLC has become the primary technique used for their determination in drug substances and products. Although HPLC is a well-established technology, it requires CRS (the chemical reference substances) of the impurities for identification and quantification of impurity peaks. Thousands of impurity CRS are used worldwide for impurity control, which requires a lot of efforts and materials in preparation. An impurity peak may be qualitatively analyzed by chromatographic parameters such as RRT (relative retention time) when the reference impurity substance is unavailable. However, due to various factors such as the chromatographic column involved, it is very difficult to meet accuracy and quality requirements (Snyder et al. 2007). As a result, the impurity CRS has become a limiting factor in drug impurity profile control.

DAD (diode array detector) is one regular equipment in drug analysis. Recently, three-dimensional HPLC-DAD and spectrum technology related to two-dimensional chromatography have been developed for mutual identification of impurity peaks between independent chromatographic conditions (Li and Hu 2008; Wang et al. 2011). If a three-dimensional standard HPLC-DAD chromatogram of impurity CRS can be stored as a digital format in a standardized pattern and RRFs (relative response factors) between each impurity and the main component of drug can be detected, the digital impurity-control database would be established. With such a database, in the analysis of a specific sample, the impurity peak in the sample can be qualified by the three-dimensional standard HPLC-DAD chromatogram in the digital impurity database; while the RRFs between impurity and main drug component can be used for quantification. Therefore, without directly using impurity CRS, the impurities in the drug can be qualified and quantified by such a "green", economic and convenient method.

GTFX (gatifloxacin) is a fluoroquinolone antibiotic introduced by Bristol-Myers Squibb for the treatment of respiratory tract infections. It was found to have some adverse reactions that can potentially induce hypo- or hyperglycemia in diabetic patients (Li et al. 2009). Therefore, its production has been stopped globally, but since the case numbers of glycometabolism disorders induced by GTFX in China were much lower than in other countries (Guan et al. 2007), various GTFX preparations are still

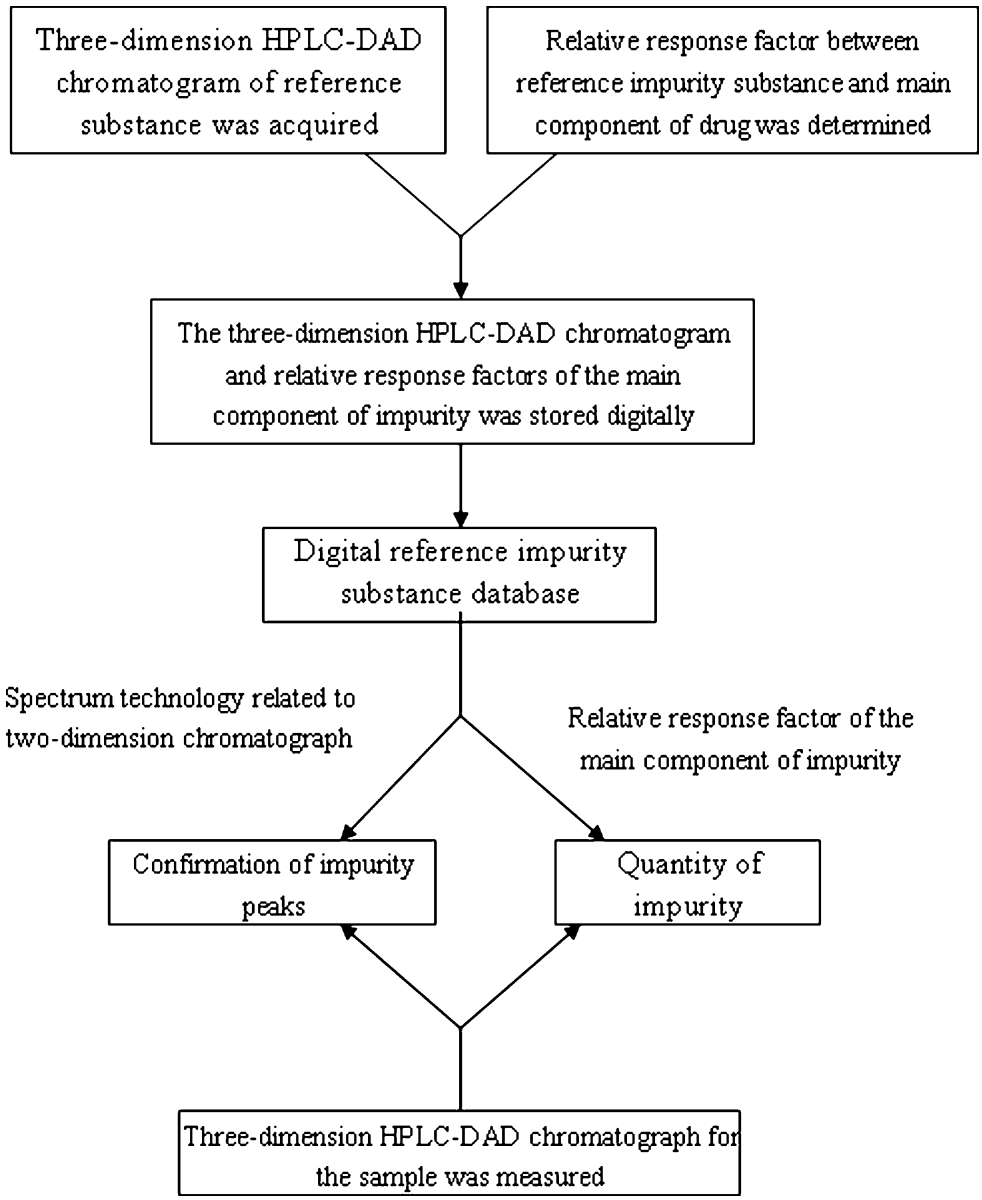

Fig. 1: Digital characterization and its application schematic for reference impurity substance

being prescribed. The raw materials used in GTFX production in China include GTFX hydrate, GTFX hydrochlorate and GTFX sulfate. Although the active ingredients of various raw materials are all GTFX, their impurity profiles may be different due to various factors in the production process. So far, no report has compared different GTFX raw materials and impurity profiles. The impurity profile analysis of drugs prepared by different processes can effectively control various potential impurities and ensure the drug safety.

Here we first established a digital GTFX impurity database (Fig. 1) and then used it to analyze the impurity profiles of domestic GTFX raw materials and injection preparations in China.

2. Investigations, results and discussion

2.1. Digital standard database

We have obtained the three-dimensional chromatogram, chromatogram of the control GTFX and impurities mixture solutions, and the corresponding UV spectra overlay (Fig. 2),

We have summarized the RRTs and RRFs of the impurities relative to GTFX (Table 1). These results formed the basic contents of the digital GTFX impurity standard database.

The UV results (Fig. 2) showed that impurity peak 2 (EDA-GTFX) and impurity peak 4 (MEDA-GTFX) were almost identical, which could be used in combined with the RRT as indicators to quantify the impurities in a chromatogram. The impurities were first divided into four groups, depending on their RRTs as previously described (Li and Hu 2008). Group 1 (Table 1) included the impurity peaks with RRT less than 0.5 based on the similarity between the standard spectra of DC-GTFX and EDA-GTFX in the standard database. Group 2 included impurity peaks with RRTs ranging from 0.6 to 0.8 based on the similarity among the standard spectra of F-GTFX, MEDA-GTFX, and HD-GTFX. Group 3 included impurity peaks with RRTs ranging from 1.1 to 1.3 based on the similarity among the standard spectra of FE-GTFX, NM-GTFX, and MP-GTFX. Group 4 included impurity peaks with RRTs ranging from 1.4 to 1.7 based on the similarity between the standard spectra of EO-GTFX.

The U-values of the RRF of GTFX impurities ($k = 2$ at 95% confidence interval) were ~0.6 (Table 1). The U-values of five CRS

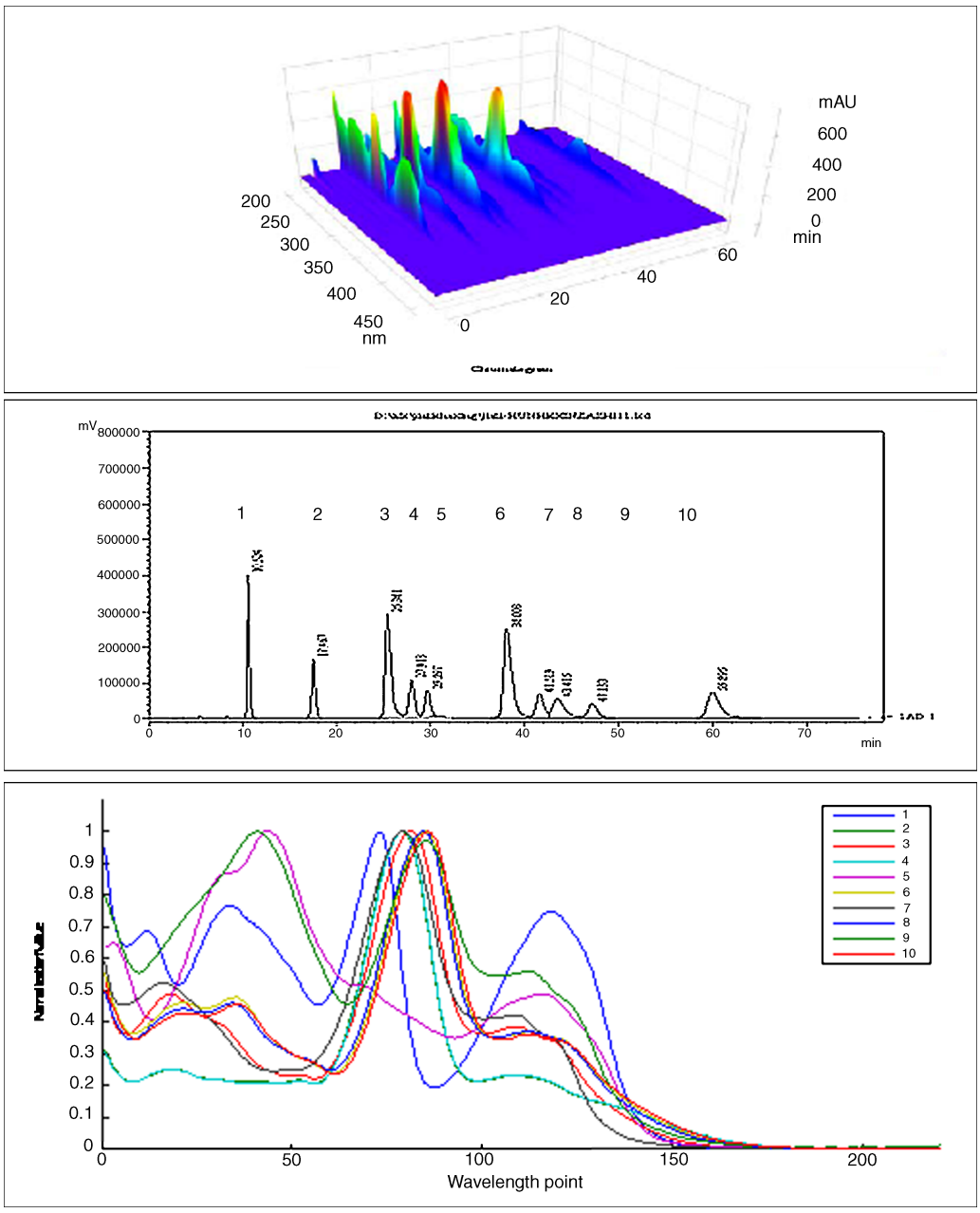

Fig. 2: The 3D and plane chromatograms and the UV spectra of GTFX and its impurity RS 1. DC-GTFX; 2. EDA-GTFX; 3. F-GTFX; 4. MEDA-GTFX; 5. HD-GTFX; 6. GTFX; 7. FEC-GTFX; 8. NM-GTFX; 9. NP-GTFX; 10. EO-GTFX

Table 1: Relative retention times and relative response factors of gatifloxacin impurities

Peak No.	Compound	Relative retention time (RRT)	Concentration-peak area regression equation (y = bx, r, Sy,x*)	Realative response factor (RRF)	Expanded uncertainty (U, k = 2)
1	DC-GTFX	0.277	y = 48.805x, 0.9999, 96.1308	1.473	0.58
2	EDA-GTFX	0.451	y = 30.607x, 0.9926, 62.4042	0.924	0.55
3	F-GTFX	0.667	y = 35.631x, 0.9954, 74.3967	1.076	0.58
4	MEDA-GTFX	0.734	y = 25.629x, 0.9926, 62.4042	0.774	0.52
5	HD-GTFX	0.779	y = 33.894x, 0.9887, 72.5802	1.023	0.72
6	GTFX	1.000	y = 33.125x, 0.9954, 74.3967	1.000	0.55
7	FE-GTFX	1.092	y = 20.988x, 0.8785, 83.8784	0.634	1.60
8	NM-GTFX	1.142	y = 27.414x, 0.9745, 74.5958	0.828	0.93
9	NP-GTFX	1.240	y = 24.354x, 0.9849, 53.3219	0.735	0.58
10	EO-GTFX	1.576	y = 33.73x, 0.9928, 81.4424	1.018	0.67

* Stanadard deviation of y for fixed x; t-test value > 2.015($t_{0.05(5)}$)

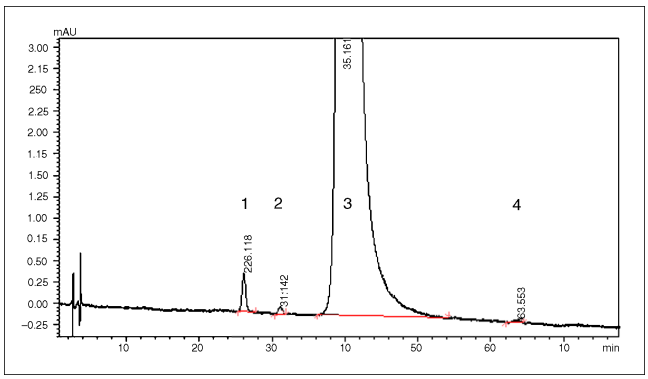

Fig. 3: A typical HPLC chromatogram of GTFX mesylate raw material

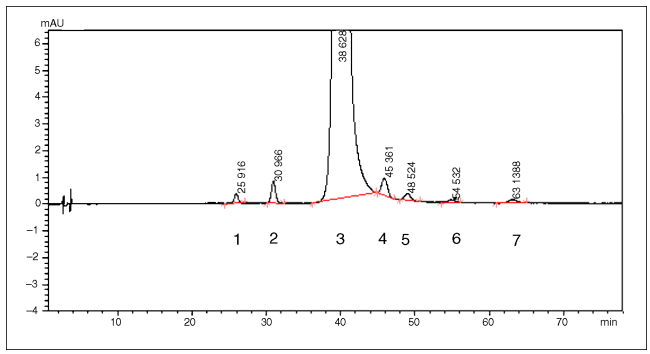

Fig. 4: A typical HPLC chromatogram of GTFX injection

of macrolide antibiotics by NMR quantitative calibration and mass balance calibration were about 2.0% (Liu and Hu 2007), while the U-values of 17 CRS of β-lactam antibiotics by collaborative calibration and mass balance calibration were 1.18% and 1.34%, respectively (Yao et al. 2010). These results suggested that the U-value of the RRF around 0.6 met the need of quantitative analysis for pharmaceutical impurities. U-value of the RRF is an important indicator of the quality of RRF in the Digital Impurity Standard Database. The greater U-values of the RRF from FE-GTFX and NM-GTFX were associated with greater errors of the concentration-peak area regression equation, leading to greater u_b values of both slopes. Based upon the data form Table 1, U-values of the RRF were ranged form 0.47 to 0.73 ($\overline{U} \pm 2SD$).

2.2. *HPLC method validation*

The analysis method on impurity profiling of GTFX was validated according to the basic requirements of ICH (ICH 1997). The impurities were well separated by this method (Fig. 2). Moreover, all impurities generated from GTFX in stress degradation test (acid, alkaline, oxidative and photo-degradations) did not interfere with the determination. The intra-day and inter-day reproducibility of the method were tested by analyzing 6.5 μg*mL^{-1} of GTFX solution; the relative standard deviation for the peak area was 0.9999 (n = 6) and 0.9998 (n = 3), respectively. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.25 μg*mL^{-1} and 1.0 μg*mL^{-1}, respectively. When the minimum amount of injection used for two-dimensional correction was 0.1 μg (about 10 times of the limit of quantification), the resulting correlation coefficient was greater than the threshold value (0.995).

2.3. *Analysis of impurity chromatogram profiles*

Fig. 3 shows a typical HPLC chromatogram of GTFX raw material, while Fig. 4 shows a typical chromatogram of GTFX injection.

The impurities were identified by our method using the RRT in combination with the degree of spectral matching. Fig. 5 shows the results of the two-dimensional chromatographic correlation spectrum for the impurity peaks in the GTFX injection chromatogram (Fig. 4) and those in GTFX Impurity Standard Database.

The similarity between impurity peaks 1$^{\#}$, 2$^{\#}$, 4$^{\#}$, 5$^{\#}$ and 7$^{\#}$ in the UV spectra of GTFX injection and those from the corresponding impurities in GTFX Impurity Standard Database was greater than the threshold value of 0.995, whereby the impurities could be uniquely identified. However, the impurity peak 6$^{\#}$ (RRT = 1.40) had a greater difference in RRT and different UV spectrum from the impurities in the database, suggesting that it was an unknown impurity.

We identified the impurities in gatifloxacin mesylate raw material (Fig. 3) using the same method, and Fig. 6 shows the analysis result of their two-dimensional chromatographic correlation spectra.

Based on GTFX Impurity Standard Database, impurity peak 1$^{\#}$ in Fig. 4 was identified as F-GTFX. The correlation calculation could not be performed for impurity peaks 2$^{\#}$ and 4$^{\#}$ due to their very low response; therefore, an increased volume of injection was required for their identification.

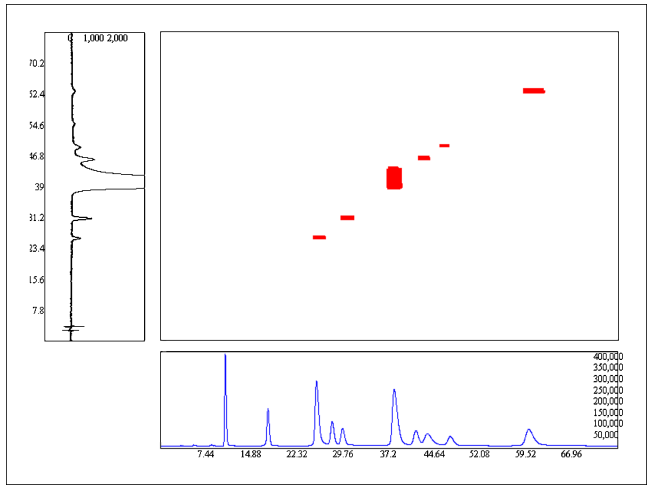

Fig. 5: The two-dimensional chromatographic correlation spectra of GTFX injection with GTFX impurities standard baseline

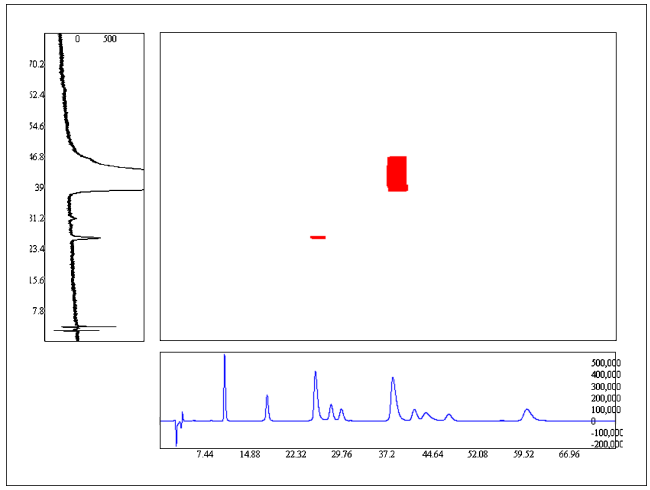

Fig. 6: The two-dimensional chromatographic correlation spectra of GTFX mesylate raw material with GTFX impurity standard baseline

Table 2: Results of analysis on impurity spectra of gatifloxacin raw material

Classification	Manufacturer	RRT	Impurity content %	Impurity peak No. in database	Name of impurity
	A	0.66	0.22%	3#	F-GTFX
Gatifloxacin mesylate		1.361	0.02%	\	Unknown
		1.59	0.03%	10#	EO-GTFX
	B	0.654	0.03%	3#	F-GTFX
		1.296	0.10%	9#	MP-GTFX
Gatifloxacin and a half hydrate	C	0.782	0.02%	5#	HD-GTFX
		1.23	0.06%	9#	MP-GTFX
	D	0.657	0.07%	3#	F-GTFX
		0.783	0.02%	5#	HD-GTFX
	E	0.575	0.08%	\	Unknown
		0.655	0.43%	3#	F-GTFX
		0.78	0.06%	5#	HD-GTFX
		1.158	0.16%	8#	NM-GTFX
		1.233	0.04%	9#	MP-GTFX
		1.691	0.09%	\	Unknown
	F	0.655	0.21%	3#	F-GTFX
Gatifloxacin hydrochloride		0.783	0.13%	5#	HD-GTFX
		1.16	0.09%	8#	NM-GTFX
		1.239	0.04%	9#	MP-GTFX
	G	0.646	0.39%	3#	F-GTFX
		1.144	0.10%	8#	NM-GTFX
	H	0.655	0.07%	3#	F-GTFX
		0.781	0.19%	5#	HD-GTFX
		1.157	0.19%	8#	NM-GTFX
		1.235	0.09%	9#	MP-GTFX
		1.386	0.04%	\	Unknown
		1.592	0.05%	10#	EO-GTFX

We performed the analysis of impurity profiles from different GTFX raw materials (Table 2).

Gatifloxacin and a half hydrate mainly contained HD-GTFX and NP-GTFX, where the content of any individual impurity was < 0.07% and total impurities content was < 0.1%. Gatifloxacin mesylate from two different manufacturers mainly contained F-GTFX and NP-GTFX, but EO-GTFX was detected in some samples. The content of any individual impurity was < 0.1% and the total impurities content was < 0.3%. Gatifloxacin hydrochloride samples from five different manufacturers contained a heterogeneous group of impurities at different concentrations, which mainly consisted of F-GTFX, HD-GTFX, and FE-GTFX. The content of any individual impurity was between 0.1% and 0.5%. NM-GTFX and EO-GTFX could be detected from some samples at < 0.1%. The total impurities content in these samples were < 0.8%. In addition, we detected other unknown impurities in some samples, but their contents were < 0.1%. All the impurities found in raw materials were process impurities and mainly associated with the purification processes used by different manufacturers, and the process for gatifloxacin sesquihydrate was optimal.

Through analyzing the impurities form 70 batches of GTFX injection in the market, we found that the major impurities were process impurities (F-GTFX and NP-GTFX) with a mean content of 0.08%. The changes in content values were greater in samples from different batches, so their levels in the preparations may reflect differences in raw material quality. In addition, the mean contents for EDA-GTFX, MEDA-GTFX, and HD-GTFX were about 0.03%, where EDA-GTFX and MEDA-GTFX were the major photodegradation impurities. Therefore, their contents in the preparation may reflect the manufacture and the storage processes.

In sum, we developed a method based on HPLC-DAD technology for analyzing pharmaceutical impurity profiles without direct using CRS of impurities by establishing a digital standard database of impurity reference substances. The calculation for the two-dimensional chromatographic correlation spectroscopy is rapid and convenient by the commercially available software; the qualification by RRT and UV spectra double indicators in the HPLC analysis ensures the accuracy of impurity identification; the impurity contents are accurately determined by using the RRFs of impurity to the API as normalization factors. Our method may eliminate the need of large CRS quantities of impurities for impurity analysis, which would greatly facilitate the control of impurities in pharmaceutical drugs.

3. Experimental

3.1. Instruments

HPLC: (1) Shimadzu LC-10AT pump, SIL-10AVP automatic sample injector, SPDM10AVP DAD; (2) Waters 2690 chromatography separation unit, PDA996 DAD. Chromatographic column: Phenomenex Prodigy C18 (250 mm × 4.6 mm, 5 μm). Beckman Φ45pH meter.

3.2. Materials

The following GTFX reference substances were used: gatifloxacin decarboxylate (DC-GTFX), 7-ethylenediamine-gatifloxacin (EDA-GTFX),7-(2-methyl-ethylenediamine)-gatifloxacina (MEDA-GTFX), 8-hydroxy gatifloxacin (HD-GTFX), gatifloxacin N-methylate (NM-GTFX), and 7-(2-methyl piperazine)-gatifloxacin (MP-GTFX) were provided by Sino-American Shanghai Squibb Pharmaceuticals Ltd.; 8-fluoro-gatifloxacin (F-GTFX) and 8-fluoro-3-ethyl -gatifloxacin (FE-GTFX) were provided by Zhejiang Medicine Co. Ltd. and Xinchang Pharmaceutical Factory; 8-ethoxy-gatifloxacin (EO-GTFX) was provided by Henan Kangtai Pharmaceutical Group. Fig. 7 shows the chemical structures of these reference substances.

GTFX semihydrate, GTFX methylsulfonate and GTFX hydrochlorate were obtained from nine different companies in China. GTFX injections provided by different companies were the sampling products in a national market evaluation in 2009. Acetonitrile was of HPLC grade and obtained from Fisher.

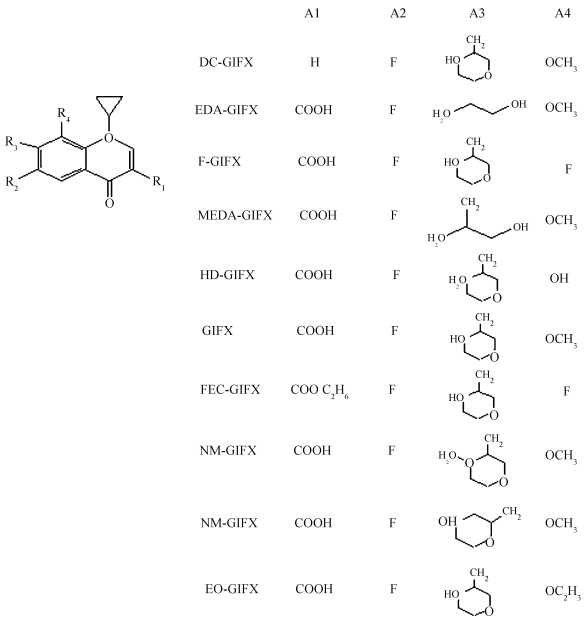

Fig. 7: Chemical structures of GTFX and impurities

Triethylamine and phosphoric acid of analytical grade were purchased from Beijing Chemical Company (China).

3.3. Methods

3.3.1. Analytical method

The HPLC conditions were the same as previously described (Li and Hu 2008). The mobile phase consisted of 1% triethylamine solution (pH was adjusted to 4.3 with phosphoric acid) and acetonitrile (87:13, v/v). The flow rate was 1.0 mL*min^{-1}, the column temperature was 30 °C, and the injection volume was 10 μL. The wavelength range for the DAD detector was set at 200–500 nm and the UV detection wavelength was 325 nm. The samples were weighted precisely, which was equivalent to about GTFX 25 mg, and they were put into a 50-mL measuring flask. Subsequently, an appropriate amount of 6 mL of diluted phosphate acid solution (2 mL→1000 mL) was added, which was dissolved by sonication; 0.0025 mol*L^{-1} of sodium dihydrogen phosphate solution, which was prepared by adding water into 390 mg of sodium dihydrogen phosphate dihydrate to 1000 mL, was added and then mixed as test solution; an appropriate amount of the above solution was measured precisely, and 0.0025 mol*L^{-1} of sodium dihydrogen phosphate was added to into the solution containing GTFX 1 μg per mL, which was used as control solution.

3.3.2. Digital impurity database

The GTFX and various reference impurity samples were dissolved and diluted as previously described (Li and Hu 2008), and then they were further diluted to 0.1 mg*L^{-1} of impurity and 0.25 mg*L^{-1} of GTFX. The mixture solution was injected and the three-dimensional HPLC-DAD chromatogram was collected. The data was exported in ASC II format and the database was used for peak identification. Each of GTFX and various reference impurities was diluted into six concentrations ranging from 0.25 mg*L^{-1} to 6.5 μg*mL^{-1}. To obtain the concentration-response curves, the sample concentration was plotted on the X axis, the peak area was plotted on the Y axis, and linear regression was performed. The ratio of the slope of the equation obtained from impurity to the slope of the equation obtained from GTFX was used as the RRF ($b_{impurity}/b_{GTFX}$), which was stored in the database for quantifying the impurity peak in a sample.

3.3.3. Application of the digital standard database

The three-dimensional HPLC-DAD chromatogram data for GTFX test was exported by the format of ASC II code. Based on the RRT of impurities, the impurity peaks in the sample were classified preliminarily, and then based on the measures previously described (Li and Hu 2008) two-dimensional correlation calculation with the chromatograph and spectra data in the digital impurity database was performed. The RRT of two chromatograms was shown in the X axis and Y axis, respectively, and the correlation coefficient was shown the Z axis based on which three-dimensional correlation diagram was plotted, and the threshold of correlation coefficient was set as 0.995. The similarity calculation was conducted using the Drug Chromatography Correlation software designed by Beijing Sijiaxinda Technology Co. Ltd (China). For the confirmed impurity peaks, the content was calculated when the RRF between this impurity and GTFX was used as the normalization factor for impurity.

3.3.4. Uncertainty evaluation for relative response factors

Based on the definition of RRF, the mathematical model for uncertainty evaluation (National Institute of Metrology 1999) is as follows:

$$RRF = \frac{b_{impurity}}{b_{\mathrm{GTFX}}} \tag{1}$$

where $b_{impurity}$ is the slope of the linear regression from the impurity concentration-response curve, and b_{GTFX} is the slope of the linear regression from the GTFX concentration-response curve. Since the concentration-response curve for impurity and GTFX are the straight lines passing across the origin, the standard deviation for b can be used as the standard uncertainty of linear regression equation (u_b) which is calculated with Eq. (2). Therefore, the uncertainty components for each RRF are all 2, and there is no correlation among all uncertainty components.

$$u_b = \sqrt{\frac{\sum_{j=1}^{m}\left[y-(a+bx)\right]^2}{n-2}} \tag{2}$$

where n is the number of data points for linear regression,
The relative standard uncertainty ($u_{rel(b)}$) for b was calculated with Eq. (3), while the relative standard uncertainty (u_{rel}) for RRF was calculated with Eq. (4). The combined standard uncertainty (u_c) for RRF was calculated with Eq. (5). The coverage factor k was set as 2 and the expended uncertainty (U) was calculated with Eq. (6).

$$u_{rel(b)} = u_b/b \tag{3}$$

$$u_{rel} = \sqrt{u^2_{rel(b_{impurity})} + u_{rel^2(b_{GTFX})}} \tag{4}$$

$$u_c = u_{rel} \times \frac{\mathrm{b_{impurity}}}{\mathrm{b}_{GTFX}} \tag{5}$$

$$U = \mathrm{k} \times u_c = 2 \times u_c \tag{6}$$

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