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Short communication

Qualitative and quantitative studies on impurities in G004, a potential hypoglycaemic agent, using liquid chromatography, nuclear magnetic resonance and mass spectrometry

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1-(4-(2-(2-Bromobenzenesulphonamino) ethyl) phenylsuphonyl)-3-(trans-4-methylcyclohexyl) urea

ABSTRACT

G004, 1-(4-(2-(4-bromobenzenesulphonamino) ethyl) phenylsuphonyl)-3-(trans-4-methylcyclohexyl) urea, is being developed as a potential hypoglycaemic agent. In this study, the related compounds in the bulk drug substance of G004 were analyzed both qualitatively and quantitatively. An unknown compound in the drug, which has never been reported, was isolated using preparative liquid chromatography and characterized as 1-(4-(2-(2-bromobenzenesulphonamino) ethyl) phenylsuphonyl)-3-(trans-4-methylcyclohexyl) urea using nuclear magnetic resonance and mass spectrometry. Moreover, a reversed-phase liquid chromatography method was developed for quantification of both the major impurities and the main constituent. The proposed method was validated and applied during impurity studies and quality control analysis of the bulk drug and laboratory-prepared samples of G004.

1. Introduction

G004 (Fig. 1a) is described chemically as 1-(4-(2-(4-bromobenzenesulphonamino) ethyl) phenylsuphonyl)-3-(trans-4-methylcyclohexyl) urea. It is designed and synthesized as a novel sulphonylurea and thiourea derivative that is substituted with benzenesulphonamide groups and may be a hypoglycaemic agent [1]. It was reported that G004 could induce a noninsulin-dependent increase in glucose consumption in insulin-resistant HepG2 cells at high, medium and low glucose levels. Additionally, G004 showed an excellent ability to protect collagen–epinephrine-induced mice mortality and plasma glucose-lowering activity *in vivo* in comparison to the model group. The preliminary pharmacological profile of G004 showed that it might be useful in the treatment of diabetes patients that experience cardiovascular and nephropathy complications [2–6].

During the analysis of the laboratory batches of crude G004, an unknown processed compound (Comp G) with area percentage ranging around 0.1% was detected. To commercialize the active pharmaceutical ingredient (G004), it is mandatory to identify and characterize all of the unknown impurities that are present at levels as low as 0.05% [7]. A high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method has been established to analyze G004 in biological fluids [8]. However, no information is available in the literature on either the impurity profile or the quality-indicating analytical method for G004.

Hence, the aim of the present investigation is to develop qualitative and quantitative methods for the isolation, characterization and analysis of the impurities obtained during the process development of the bulk and laboratory preparations of G004.

2. Experimental

2.1. Chemicals and reagents

Samples of G004 (purity 98.5%), compound D (Comp D, 4-(2-acetamide) ethyl benzenesulphonamide, mol. wt. 242.3,

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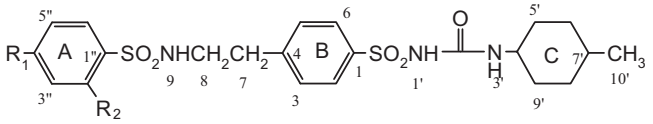
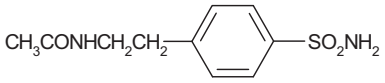
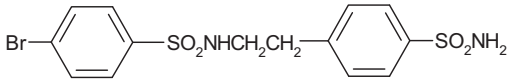


a. G004: R_1=Br R_2=H

b. Comp G: R_1=H R_2=Br



c. Comp D



d. Comp F

Fig. 1. Chemical structures of G004 and its impurities.

purity 98.0%) (Fig. 1c) and compound F (Comp F, 4-(2-(4-bromobenzenesulphonamino) ethyl) benzenesulphonamide, mol. wt. 419.3, purity 98.5%) (Fig. 1d) were kindly provided by the Centre of Drug Discovery, China Pharmaceutical University (Nanjing, China). The unknown impurity (Comp G, purity 95.0%) was isolated and characterized in our laboratory. HPLC-grade methanol was purchased from Merck Limited (China). Other analytical grade reagents were purchased from Nanjing Chemical Co. (Nanjing, China). Water was purified using a Millipore Milli Q-Plus system (Millipore Corp., Billerica, MA, USA).

2.2. Instrumentation and conditions

2.2.1. Elucidation of unknown compound

Structure elucidation of the unknown compound was based on the analysis of its melting point and the NMR and MS spectra data. The NMR spectra were obtained using a Bruker 500 MHz spectrometer (AC, AVANCE, Bruker Daltonics GmbH, Fällanden, Switzerland) using dimethyl sulphoxide (DMSO-d_6) as the solvent and tetramethylsilane (TMS) as an internal standard. Melting points were measured using a RY-1 micro-melting point apparatus (Tianjin Analytical Instrument Corp., Tianjin, China). The electron impact (EI) mass spectra (70 eV) were obtained using a Shimadzu MS-2010 system.

A TSQ Quantum Ultra AM triple-quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an ESI source, was used in negative ionisation mode in full scan and product scan, which allows for further identification. The spray voltage was set to −3.5 kV, and the capillary temperature was maintained at 350 °C. Nitrogen was used as the sheath gas (30 Arb) and the auxiliary gas (10 Arb) for nebulisation. For collision-induced dissociation (CID), argon was used as the collision gas under a pressure of 1.5 mTorr. During full scan MS analysis, the spectra were recorded in the range of m/z 200–1000. The collision energy of the in-source CID mode was set at 10 eV. Analytical data were acquired using the Xcalibur 1.4 software.

2.2.2. Isolation of unknown impurity

Isolation of Comp G was performed using a Shimadzu LC-8A Preparative Liquid Chromatography equipped with a SPD-10AVP UV-vis detector (Shimadzu Corp., Kyoto, Japan). A Shim-Pack Prep ODS column (250 mm × 20 mm i.d., 15 μm) was employed. Water–methanol (30:70, v/v) was the mobile phase, which was set at a flow rate of 12 mL min^{-1}. Analyte detection used a wavelength of 233 nm.

To increase the concentration of the sample during the isolation of the target impurity, methanol containing 2% ammonia (w/w) was used as diluent and subsequently the sample solution was adjusted to pH 4 using formic acid. As a result, a 6 mg mL^{-1} test solution of G004 can be made for impurity preparation. Approximately 2 mL of the test solution was subjected to Prep-LC separation. The separation was performed using a mobile phase of water–methanol (30:70, v/v) at flow rate of 12 mL min^{-1}, and the unknown compound (Comp G) was collected at the time region between 15 and 20 min. Fifty sample injections were made in total. The eluted samples were collected in glass vials, evaporated to dryness and 10 mg of residue of Comp G was obtained, which had a m.p. of 158–160 °C. The purity of Comp G was 95% based on the HPLC analysis using an area normalization method.

2.2.3. Quantitative analysis of G004 bulk drug substance and its impurities

A Shimadzu LC-2010 series system equipped with a quaternary pump, vacuum degasser, autosampler, column heater-cooler and UV detector. The data were acquired and processed using the ClassVP software. The chromatographic separation was achieved on a LiChrospher C_{18} column (250 mm × 4.6 mm i.d., 5 μm) (Hanbon, Jiangsu, China) and a guard column (4 mm × 3.0 mm i.d., 5 μm). Water (containing 0.1% acetic acid and 0.1% triethylamine)–methanol (20:80, v/v) was used as the mobile phase set at a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 30 °C, and the UV detection wavelength was set at 233 nm. The sample injection volume was 10 μL.

2.2.4. Sample preparation

A degassed mixture of water and methanol in the ratio of 20:80 (v/v) was used as a diluent. The test concentrations of G004 were about 500 μg mL^{-1} for related substances, 200 μg mL^{-1} for assay determination and 500 μg mL^{-1} for stock solutions of impurities, which were prepared in the diluent.

3. Method validation for quantitative analysis

3.1. Specificity

The specificity of the related compounds and G004 was assessed using the forced degradation studies under conditions described in the literature [9,10]. Basic, acidic, oxidative, photolytic and thermal degradation were conducted in 1 M sodium hydroxide at 60 °C for 4 h, 1 M hydrochloric acid at 60 °C for 4 h, 30% hydrogen peroxide at 60 °C for 4 h, illumination of 1.2 million lux hours for 5 days and bath of 100 °C for 4 h, respectively. Samples were withdrawn at the appropriate times, the pH was adjusted to neutral and the samples were subjected to LC analysis after suitable dilution (approximately 200 μg mL^{-1}) to evaluate the ability of the proposed method to separate G004 from its potential impurities.

3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for the target compounds were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentrations.

3.3. Linearity

Linearity test solutions for G004 and its related substances were prepared by diluting the stock solutions of the analytes to the

Table 1
NMR assignments for G004 and Comp G.

Position	Comp G			G004		
	δ_C	δ_H (J/Hz)	HMBC (H–C)	δ_C	δ_H (J/Hz)	HMBC (H–C)
1	139.57			138.32		
2, 6	126.9	7.70 (2H, d, 8.0)	C4, C3/C5	127.15	7.78 (2H, d, 8.3)	C4, C3/C5
3, 5	128.73	7.27 (2H, d, 8.0)	C1, C2/C6, C7	129.17	7.38 (2H, d, 8.3)	C1, C2/C6, C7
4	143.12			144.22		
7	35.07	2.79 (2H, t, 7.1)	C8, C3/C5, C4	34.95	2.79 (2H, t, 7.2)	C8, C3/C5, C4
8	43.41	3.15 (2H, q, 7.1)	C7, C4	43.21	3.03 (2H, q, 7.2)	C7, C4
9					7.83 (1H, t, 7.2, NH)	
1′					10.29 (1H, br.s, NH)	
2′	152.59			150.49		
3′		6.32 (1H, br.s, NH)			6.26 (1H, d, 7.6, NH)	
4′	48.43	3.17 (1H, m)		48.50	3.19 (1H, m)	C5′/C9′
5′, 9′	32.46	1.07 (2H, br.q, 12.7) 1.68 (2H, d, 12.7)	C6′/C8′, C4′	32.23	1.08(2H, br.q, 12.8, 3.2) 1.68 (2H, br.d,12.8)	C6′/C8′, C4′ C7′
6′, 8′	33.46	0.89 (2H, br.q, 12.1)1.60 (2H, br.d, 12.1)		33.29	0.89 (2H, br.q, 12.0, 3.2)1.60 (2H, br.d, 12.0)	C5′/C9′ C4′
7′	31.25	1.20 (1H, m)		31.14	1.20 (1H,m)	C5′/C9′, C6′/C8′
10′	22.01	0.82 (3H, d, 6.5)	C5′/C9′, C6′/C8′, C7′	21.93	0.82 (3H, d, 6.5)	C5′/C9′, C6′/C8′, C7′
1″	139.89			139.59		
2″	119.09			132.21	7.79 (1H, d, 8.6)	C3″/C5″, C1″, C4″
3″	130.31	7.94 (1H, dd, 7.4, 1.0)	C2″, C4″	128.45	7.69 (1H, d, 8.6)	C2″/C6″, C1″
4″	128.05	7.50 (1H, td, 7.4, 1.0)	C2″	126.09		
5″	133.75	7.54 (1H, td, 7.4, 1.0)	C1″	128.45	7.69 (1H, d, 8.6)	C2″/C6″, C1″
6″	135.25	7.79 (1H, dd, 7.4, 1.0)	C1″, C5″	132.21	7.79 (1H, d, 8.6)	C3″/C5″, C1″, C4″

Multiplicity is indicated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (brand).

required concentrations. The calibration curves were drawn by plotting the peak area of the target analyte versus their corresponding concentration using a least-squares linear regression analysis.

The linear range of the G004 assay was from 40 to 200% with respect to its concentration of 200 μg mL^{-1}; while Comp D and F were about 1% of the concentration levels in the G004 sample. The calibration solutions of Comp D and F were prepared with respect to their specification level of 1% (i.e., 0.6, 0.8, 1.0, 1.6 and 2.0%) in G004 (500 μg mL^{-1}).

3.4. Precision

The precision of the method was assessed by the determination of six independent assays of G004 against qualified working standards, and the precision for the related substances was checked by adding 1.0% level of Comp D and F into the reference standard and injecting six individual prepared samples. The precision was expressed by the relative standard deviation (RSD). The precision of the method was also verified by different analysts who tested the samples on different days using different instruments in the same laboratory.

3.5. Accuracy

The accuracy of the method was evaluated in triplicate by adding appropriate amounts of the standard solution of the target compound into the test solution of G004 samples at three different concentrations (i.e., 80, 100 and 120%) with respect to their corresponding concentrations, and the mean recoveries were calculated.

3.6. Robustness

To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolutions were evaluated. Flow rates of 0.8 and 1.2 mL min^{-1} instead of the actual flow of 1.0 mL min^{-1}, column temperatures of 25 and 40 °C instead of 30 °C and, variations in the pH of 0.5 units, were employed and the resolution was studied.

4. Results and discussion

4.1. Qualitative analysis of related compounds

4.1.1. Structural elucidation of Comp G

The NMR data of Comp G (white powder, $C_{22}H_{28}O_5N_3S_2Br$) are shown in Table 1. LC–MS analysis showed similar pseudomolecular ions at *m/z* 558/556 $[M-H]^-$ (isotope peaks with abundance in the ratio of 1:1 approximately) (Fig. 2) with that of G004, suggesting that the unknown compound may be an isomer of G004. In order to fully understand the structure of Comp G, the data of ^1H, ^{13}C and 2D NMR were compared to that of G004, which were obtained under the same conditions. It was found that the signals in the high-field region of their ^1H NMR were identical for the two compounds, which suggested that ring C was identical in both compounds. Significant differences in their ^1H NMR spectra

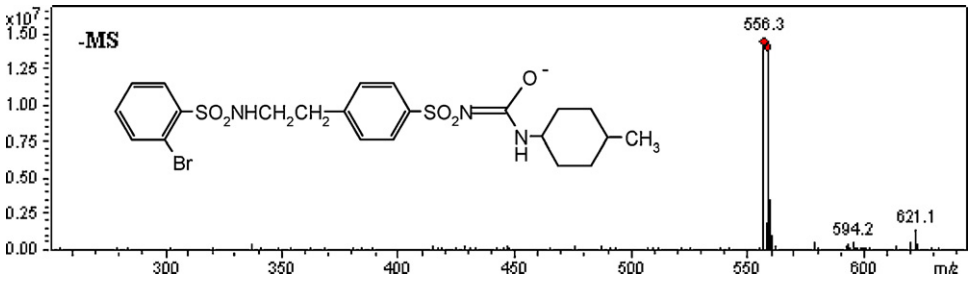


Fig. 2. The molecular ion peak at *m/z* 558/556 $[M-H]^-$ (isotope peaks) in negative ionisation mode using LC–MS/MS analysis.

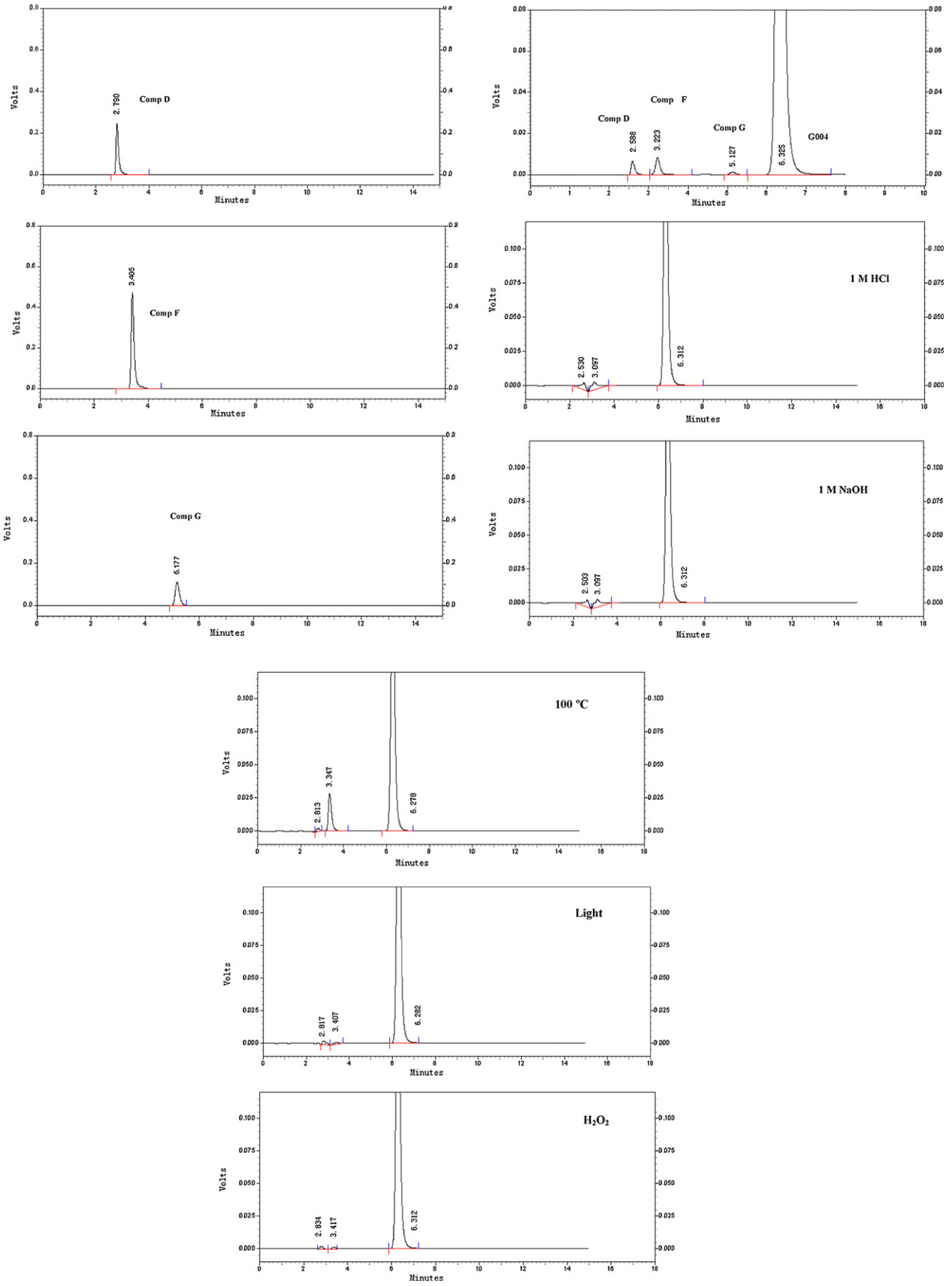


Fig. 3. Typical chromatograms obtained during specificity studies.

Table 2
The results of percent recovery, LOD, LOQ and linearity curve of the method for determination of G004 and its impurities.

Compound	Added (μg)	Found (μg)	Recovery (%)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Slope (b)	Intercept (a)	Correlation coefficient
	4.32	4.36	100.9	0.072	0.30	29,800	−33,513	0.9996
Comp D	5.40	5.46	101.1	0.070	0.30			
	6.48	6.50	100.3	0.075	0.31			
	4.24	4.30	101.4	0.105	0.35	29,758	−7656.6	0.9999
Comp F	5.30	5.32	100.3	0.102	0.36			
	6.36	6.39	100.4	0.108	0.35			
	81.2	82.0	100.9	0.075	0.35	51,497	219,008	0.9999
G004	101.5	101.4	99.95	0.079	0.35			
	121.8	122.5	100.6	0.071	0.37			

were found in the low-field region (unsaturated zone), especially for the protons of benzene ring A. For drug G004, the ^{1}H NMR spectrum showed two doublets at 7.79 ppm (J = 8.6 Hz) and 7.79 ppm (J = 8.6 Hz), both integrating for 2H, which corroborated with the presence of a para-substituted benzene ring (ring A). As for compound G, the four distinct ^{1}H NMR signals, each integrating for 1H, were more typical of an ortho-substituted benzene ring (ring A). Two of the signals appeared as two doublets, and the other two showed three doublets (δ 7.94 (1H, dd, J = 7.4, 1.0 Hz), 7.79 (1H, dd, J = 7.4, 1.0 Hz), 7.54 (1H, td, J = 7.4, 1.0 Hz) and 7.50 (1H, td, J = 7.4, 1.0 Hz)).

In the EI-MS spectra of Comp G, fragment ions at m/z 444/446, 418/420, 248/250, 219/221 and 155/157 could be obtained and attributed to the loss of $C_7H_{15}N$ (−113 Da), $C_8H_{13}NO$ (−140 Da), $C_{15}H_{21}N_2O_3S$ (−309 Da), $C_{16}H_{24}N_3O_3S$ (−338 Da) and $C_{16}H_{24}N_3O_5S_2$ (−402 Da), successively, which agreed with the proposed structure 1-(4-(2-(2-bromobenzenesulphonamino) ethyl) phenylsuphonyl) -3-(trans-4-methylcyclohexyl) urea.

Based on their 2D NMR spectra, the assignments of the ^{1}H and ^{13}C NMR data for G004 and Comp G are shown in Table 1.

4.1.2. Identification of other related compounds

The other two related compounds (Comp D and F) were identified by comparison of the retention time and mass spectra to those of reference standards using LC–MS/MS. The specific molecular ions for two compounds were m/z 241 $[M-H]^-$ and m/z 418 $[M-H]^-$ for Comp D and F, respectively, which were consistent with their reference standards obtained under the same conditions.

The typical chromatograms are shown in Fig. 3. The typical retention times of Comp D, F, G and G004 were approximately 2.8, 3.4, 5.2 and 6.3 min, respectively.

4.2. Optimization of chromatographic conditions for quantitative analysis

G004 is weak acid with an UV absorbance maximum at 233 nm. Therefore, an acidic mobile phase, which can help to improve the symmetry of the chromatographic peak, and a detection wavelength set at 233 nm was selected. Mobile phases consisting of methanol and water (contained 0.1% acetic acid and 0.1% triethylamine) (with pH ranging from 2 to 7) were compared to determine the optimum separation of G004 from the related compounds. It was found that the retention time of G004 did not show any significant changes, while peak symmetry decreased as pH was changed from 2 to 7. However, the retention time of Comp D and F increased as the pH of the mobile phase was changed from 2 to 7.

For the development of the method, several variations of the mobile phase were studied: changes in buffer nature (i.e., phosphate and acetate), percentage of organic solvent and ionic strength of the buffer. Optimum separation was achieved using methanol and water (containing 0.1% acetic acid and 0.1% triethylamine) (80:20, v/v) as the mobile phase.

Under the selected conditions, the retention times of Comp D, F, G and G004 were about 2.8, 3.4, 5.2 and 6.3 min, respectively. Tailing factor for the G004 peak was 1.1, the number of theoretical plates (N) was over 5000 and % RSD for five replicate injections was 0.9%.

4.3. Results of the forced degradation

The chromatograms from the stress studies are shown in Fig. 3. G004 was stable under stress conditions such as photolytic and acid/base hydrolysis; but heating to 100 °C led to an increase in Comp F. Peak purity data obtained from PDA analysis confirmed that the G004 peak was pure in all the stress samples analyzed. The mass balance of the stressed samples was between 99.0 and 99.8% of G004. The assay of G004 was unaffected by the presence of the related compounds, which confirms its stability during the LC method.

4.4. Results of method validation

The LODs of G004, Comp D and F were 0.075, 0.072 and 0.105 μg mL^{-1}; and their LOQs were 0.35, 0.30 and 0.35 μg mL^{-1}, respectively.

A linear series of working solutions of Comp D (2.70, 4.32, 5.40, 8.10 and 10.80 μg mL^{-1}) and Comp F (2.65, 4.24, 5.30, 7.95 and 10.80 μg mL^{-1}) were prepared and detected, respectively. The linearity test for the assay of G004 was completed by injecting a series of G004 solutions with concentrations (C) at 79.2, 118.8, 198.0, 297.0 and 396.0 μg mL^{-1} and the peak area (Y) was recorded. Strong linear relationships were obtained, and the typical equations of calibration curves are shown in Table 2.

The precision study (% RSD) of G004, Comp D and F were 1.5, 1.2 and 1.7%, respectively, confirming the high level of precision of the developed LC method.

The accuracy of the method was evaluated in triplicate at three different concentration levels. The mean recoveries of all the impurities and G004 were found to be in the range of 98.0–102.0% (Table 2).

Deliberate alteration of the chromatographic conditions (mobile phase composition, flow rate, pH and column temperature) revealed that the resolution between Comp D, Comp F and G004 was always greater than 3.0. These results illustrate the robustness of the method.

4.5. Application

The assay and related substance detection were performed on different batches of G004 samples. The results showed that the concentration of Comp D and Comp F was from 0.3 to 0.5% and 0.3 to 0.7% in the G004 samples, respectively.

5. Conclusions

In this paper, the related compounds in the G004 bulk drug substance were identified. An unknown processed compound was isolated using preparative liquid chromatography and was characterized as 1-(4-(2-(2-bromobenzenesulphonamino) ethyl) phenylsuphonyl)-3-(trans-4-methylcyclohexyl) urea using NMR and MS spectrometry. The ^{1}H, ^{13}C NMR and 2D NMR spectra data for G004 and Comp G were recorded, compared and assigned. Moreover, a reversed-phase liquid chromatographic method was developed for quantification of the major impurities as well as the main constituent of the drug. The method was validated and proved to be suitable for quality studies of the drug G004 both at the time of batch release and during stability studies.

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