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LC determination of glimepiride and its related impurities

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Abstract

Five impurities in glimepiride drug substance were detected and quantified using a simple isocratic reverse phase HPLC method. For the identification and characterization purpose these impurities were isolated from a crude reaction mixture of glimepiride using a normal phase HPLC system. Based on the spectroscopic data like NMR, FTIR, UV and MS these impurities were characterized and used as impurity standards for determining the relative response factor during the validation of the proposed isocratic reverse phase HPLC method. The chromatographic separation was achieved on a Phenomenex Luna C8 (2) 100 Å, 5 μm, 250 mm × 4.6 mm using a mobile phase consisting of phosphate buffer (pH 7.0)–acetonitrile–tetrahydrofuran (73:18:09, v/v/v) with UV detection at 228 nm and a flow rate of 1 ml/min. The column temperature was maintained at 35 °C through out the analysis. The method has been validated as per international guidelines on method validation and can be used for the routine quality control analysis of glimepiride as active pharmaceutical ingredient (API).

Keywords: Glimepiride; API; Related impurities; Characterization; HPLC

1. Introduction

Glimepiride,1-[[4-[2[(3-ethyl-4-methyl-2-oxo-3pyrroline-1-carboxamido) ethyl] phenyl]-sulphony]-3-trans-(4-methyl cyclohexyl) urea], a third generation sulphonylurea, has a three-fold faster rate of association and nine-fold faster rate of dissociation than glabenclamide [1,2]. The molecule showed a rapid onset of action and prolonged duration of action permitting once daily administration; though its initial action is stimulation of insulin [3,4]. Glimepiride exerted a marked blood glucose lowering effect in controlling the non-insulin dependent diabetes mellitus (NIDDM). Several methods have been described in the literature for the determination of assay of glimepiride and its metabolite in biological fluids [5–10]. Since the active glimepiride is a *trans* molecule, methods for the determination of *cis*- and *trans*-isomers of glimepiride have also been found in the literature [11,12]. However, a very little information is available for the determination of its related impurities and the degradation products in drug substance [13,14].

Recently appeared methods for the determination of related substances of this molecule proposed by the PHARMAEUROPA and USP 28 official monograph of glimepiride [15,16] are based on the two different methods. A method where the *cis*-isomeric impurity is determined based on a normal phase chromatographic system and the determination of other related compounds and the assay is carried out using a reverse phase HPLC method. This method also recommends that the analysis should be carried out at a temperature not exceeding 12 °C and the solutions under the described experimental conditions should not be stored more than 15 h at this temperature.

The presence of impurities in an API can have a significant impact on the quality and safety of the drug products. Therefore, it is necessary to study the impurity profile of the API to be used in the manufacturing of a drug product. ICH guidelines recommend identifying and characterizing all impurities, which are present at a level of ≥0.10% [17,18]. Therefore, it becomes a primary responsibility of a development chemist to develop a simple, accurate and precise analytical method for the quantitative determination of these impurities for the routine quality control monitoring of API.

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The present manuscript describes the development and validation of an isocratic reverse phase HPLC method. The proposed method can determine the *cis*-isomer and other *isomeric impurities and degradation products* using a single method at room temperature. Based on the detection of these isomers and the other related impurities in a commercial lot of glimepiride, these related compounds were isolated and characterized using various spectroscopic techniques. A relative response factor for all these impurities with respect to glimepiride was determined for the quantitative determination of these known impurities and other unknown impurities were determined using diluted standard method. The determination of relative substances and degradation product in an API at a lower level of 0.10% or below often leads to misleading interpretation of results in the absence of a relative response factor. It has been observed in many cases that the relative substance or the degradation product generated during the manufacturing of an API may not have the same UV response at the wavelength of the determination of these tests. Therefore, there is a need either to use the impurity standards or the relative response factor (RRF) for the quantitative determination of these low levels of impurities and degradation product.

2. Experimental

2.1. Chemicals and standard

Glimepiride samples from laboratory batches (Batch #GRC1500/0210/03) and crude reaction mixtures (Batch #GRC1500/RM0210/03) were received from Process Research and Development Division of the Glenmark Research Centre. In-house *Reference Standard* of glimepiride Batch #GPL1500A3001/AC-I was used for the assay determination of glimepiride drug substance. Commercial lot #A15003023 was obtained from Glenmark Pharmaceuticals Ltd., Ankleshwar, Gujrat. HPLC grade acetonitrile, tetrahydrofuran, tertiary-butyl methyl ether were obtained from J.T. Baker. HPLC grade *n*-hexane and isopropyl ether was used from Rankem (India). Triethyl amine and trifluoroacetic acid were obtained from Fluka. *Ortho*-phosphoric acid and potassium dihydrogen phosphate (GR grade) were used from Merck. Water was obtained from Milli-Q Gradient water purification system.

2.2. Analytical mode high-performance liquid chromatography

An Agilent 1100 series HPLC system equipped 1100 series quaternary gradient pump, auto sampler with cooler and DAD system and a Shimadzu LC 2010CHT HPLC module equipped quaternary gradient pump, column oven, auto sampler and DAD system were used for the analysis and validation of the proposed method of analysis. The data was recorded using Chemstation and LC solution software for Agilent 1100 series and Shimadzu, respectively.

2.2.1. Method-I chromatographic conditions for determination of related substances

The analysis was carried out on Phenomenex Luna C8 (2) 100 Å, 5 μm, 250 mm × 4.6 mm using a mobile phase consisting of phosphate buffer (pH 7.0)–acetonitrile–tetrahydrofuran (73:18:09, v/v/v) with UV detection at 228 nm at a flow rate of 1 ml/min. The column was maintained at 35 °C through out the analysis. A 25 μl sample of concentration as described in the sample preparation was injected and the chromatogram was recorded for 90 min. Buffer solution was prepared by dissolving 6.80 g potassium dihydrogen phosphate in 1000 ml water and 10 ml of triethyl amine was added to this. The final pH was adjusted to 7.0 ± 0.05 with dilute *ortho*-phosphoric acid and filtered through 0.45 μm before use.

2.2.1.1. Preparation of sample, standard and the system suitability solutions. Diluent A was prepared by mixing acetonitrile and THF in a ratio of 65:35 (v/v). Buffer solution of pH 7.0 as described above was used as diluent B and the mobile phase corresponding to the composition described for assay and related substances was used as diluent C.

A stock solution of all five impurities was prepared in 100 ml volumetric flask by accurately weighing 8 mg of glimepiride–sulphonamide, 4 mg of *glimepiride-cis-isomer*, 3 mg of *glimepiride-meta-isomer*, 3 mg of *glimepiride-ortho-isomer* and 4 mg of *glimepiride-urethane* and dissolving in a 30 ml of diluent A with occasional sonication and volume was made up to mark with diluent B. This solution was marked as *Reference Solution A*.

About 60 mg of glimepiride reference standard was dissolved in 30 ml of diluent A in a 100 ml volumetric flask and the volume was made up to the mark with diluent B. This solution was marked as *Reference Solution B*. A 10 ml aliquot of this solution was further diluted to 100 ml with diluent C and 2 ml of this solution was further diluted to 100 ml with diluent C to get a final concentration of 1.2 μg/ml equivalent to 0.2% of test solution concentration. This final solution was marked as *Reference Solution C*.

For the preparation of test solution, about 60 mg of glimepiride sample was dissolved in 30 ml of diluent A in 100 ml volumetric flask and made up to the mark with diluent B. This solution was marked as *Reference Solution D*. An aliquot of this was filtered through 0.45 μm Acrodisc (LC 13 PVDF Gelman) and loaded on to HPLC vial. A blank solution was prepared by mixing 30 ml of diluent A and 70 ml of diluent B.

For the preparation of system suitability solution, about 60 mg of glimepiride reference standard was dissolved in 30 ml of diluent A in a 100 ml volumetric flask and 3 ml of *Reference Solution A* was added to this, the volume was made up to the mark with diluent B to give a final concentration of

Table 1
RRT and RRF of the impurities

Impurities	RRT	RRF
Impurity A (*glimepiride–sulphonamide*)	0.35	0.71
Impurity B (*glimepiride-cis*-isomer)	0.95	1.17
Impurity C (*glimepiride-meta-isomer*)	1.1	1.29
Impurity D (*glimepiride-ortho-isomer*)	1.3	1.37
Impurity E (*glimepiride–urethane*)	0.12	0.82

sulphonamide impurity (2.4 μg/ml), *cis* impurity (1.2 μg/ml) and *ortho*, *meta* and urethane impurity (0.9 μg/ml), respectively, equivalent to 0.4% for sulphonamide, 0.2% *cis* and 0.15% of each of *meta*, *ortho* and urethane with respect to test concentration.

Equal volumes of blank solution, system suitability solution and six replicate injections of *Reference Solution B* were injected separately on to the chromatograph and test solution in duplicate and all the peaks were integrated using valley to valley integration.

All the known and unknown impurities were calculated against the area obtained from replicate injections of *Reference Solution B* and a RRF given in the Table 1 were applied for the calculation of known impurities.

2.2.1.2. System suitability parameter. Resolution between *glimepiride-cis-isomer* and glimepiride should not be less than 1.2, resolution between glimepiride and *glimepiride-meta-isomer* should not be less than 2.4 and resolution between *glimepiride-meta-isomer* and *glimepiride-ortho-isomer* should not be less than 4.5 in the chromatogram obtained with system suitability solution. The relative standard deviation determined from the *Reference Solution B* for six replicate injections should not be more than 5.0%. In *Reference Solution B*, the number of theoretical plates of glimepiride peak should not be less than 3000 and peak symmetry should be in between 0.7 and 1.2.

2.2.2. Method-II chromatographic conditions the determination of assay of glimepiride

The analysis was carried out on Phenomenex Luna C8 (2) 100 Å, 5 μm, 250 mm × 4.6 mm using a mobile phase consisting of phosphate buffer (pH 7.0)–acetonitrile–tetrahydrofuran (65:25:10, v/v/v) with UV detection at 228 nm and a flow rate of 1 ml/min. The column was maintained at 35 °C through out the analysis. A 25 μl sample of concentration as described in the sample preparation was injected and the chromatogram was recorded for 17 min. Buffer was prepared by as described in Method-I and filtered through 0.45 μm before use.

2.2.2.1. Preparation of sample, standard and the system suitability solutions. All the diluents were prepared as described in Method-I

For the preparation of standard and test solutions, a 5 ml aliquots of *Reference Solutions B* and *D* as described in Method-I were diluted to 100 ml with diluent C separately. Aliquots of these solutions were filtered through 0.45 μm Acrodisc (LC 13 PVDF Gelman) and loaded on to HPLC vial labeling as standard and test. A blank solution was prepared by mixing 30 ml of diluent A and 70 ml of diluent B. A 5 ml of this solution was further diluted to 100 ml with diluent C.

Equal volumes of blank preparation and five replicate injections of standard preparation and duplicate injections of test preparation were injected on to the chromatograph and chromatograms were recorded.

2.2.2.2. System suitability parameters. The relative standard deviation determined from the five replicate injections of standard solution should not be more than 2.0%.

2.3. Preparative mode high-performance liquid chromatography

A Shimadzu LC-8A equipped with UV–vis detector SPD10A VP series and high-pressure binary pump and a Rheodyne Injector Model 7725i with 1 ml loop was used for the isolation of the impurities. Following chromatographic conditions were used for the isolation of impurities. The elution was carried out on Kromasil silica, 250 mm × 10 mm, 7 μm column using a mobile phase consisted of [*n*-hexane–{(tBME–IPA) (95:5, v/v)}], (60:40, v/v) +0.1% TFA at a flow rate 20 ml/min with a detection 228 nm.

2.4. NMR spectroscopy

The ^{1}H and ^{13}C spectra were recorded on Mercury 300 MHz Varian NMR spectrometer using Vnmr 6.1 software.

2.5. Mass spectrometry

ESI mass spectra of all isolated impurities were recorded on a PE-Sciex API 3000 mass spectrometer. The samples were introduced to the, mass spectrometer through the direction injection mode in methanol.

2.6. FTIR spectroscopy

FTIR spectra were recorded on Perkin-Elmer model Spectrum One using KBr disc method.

3. Synthetic routes of glimepiride

The reaction scheme used for the synthesis of glimepiride has been shown in Fig. 1.

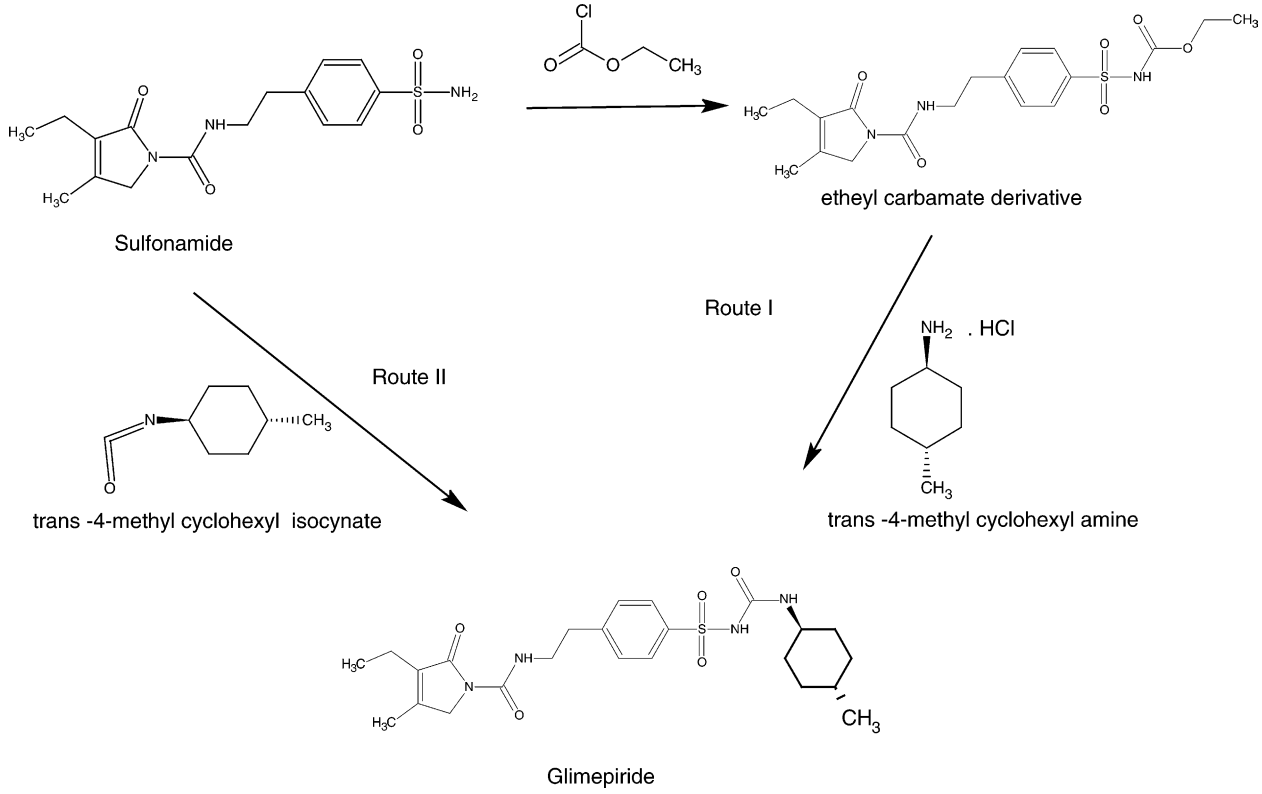

Fig. 1. Synthetic routes of glimepiride.

4. Results and discussion

4.1. Detection and isolation of impurities

Analysis of related impurities of glimepiride drug substance using the Method-I described in Section 2.2 has been shown in Fig. 2. The chromatogram obtained revealed the presence of four impurities above 0.10% level. These impurities were isolated and characterized for their identification. The relative response factors and relative retention times (RRT) given in Table 1 were determined for the quantitative estimation of these impurities in glimepiride drug substance. Structure and chemical name of glimepiride and these impurities have been given Fig. 3a–f.

Since the molecule shows poor solubility in water, it was found to be difficult to isolate these impurities using a reverse phase HPLC method. Therefore, an isocratic HPLC method using normal phase chromatographic was developed to see the elution pattern of these impurities. A crude reaction mixture was used to identify all the peaks of impurities. This reaction mixture showed five major peaks. For the confirmation of these peaks, glimepiride reference standard and the glimepiride sulphonamide precursor were injected under the same experimental condition and it was found that the peaks observed at RT about 8.8 and 16.6 min were from the glimepiride and the sulphonamide precursor, respectively. The remaining three peaks were labeled as unknown peaks. To isolate all these peaks the method was slightly modified to be suited to the preparative mode of HPLC. These experimental conditions are described in the Section 2.3. All four major peaks other than the glimepiride were collected individually in multiple fractions and concentrated at $25 \pm 2\,^{\circ}C$ on a rota evaporator. These dried fractions were checked for their chromatographic purities and the confirmation of relative retention time using the proposed reverse phase method for the determination of related substances described in *Method-I* of Section 2.2. These isolated impurities were subjected to spectroscopic analysis, characterized and named as described in Fig. 3a–f. The structure elucidation details of these impurities are presented in the following sections.

4.2. Structure elucidation of impurities

4.2.1. Structure elucidation of impurity A

ESI mass spectrum of impurity A showed a protonated molecular ion $(M+H)^+$ peak at m/z 352.1. The MS/MS of this molecular ion peak shows a fragmentation pattern at m/z 335.4, 167.4 and 126.2, which is similar to the fragmentation pattern observed for sulphonamide intermediate. FTIR and NMR spectrum assignments of the isolated impurity are shown in Table 2. These values were found to be in accordance with the those of recorded for sulphonamide intermediate. Since the process of synthesis of glimepiride followed by Glenmark is based on the sulphonamide Route I

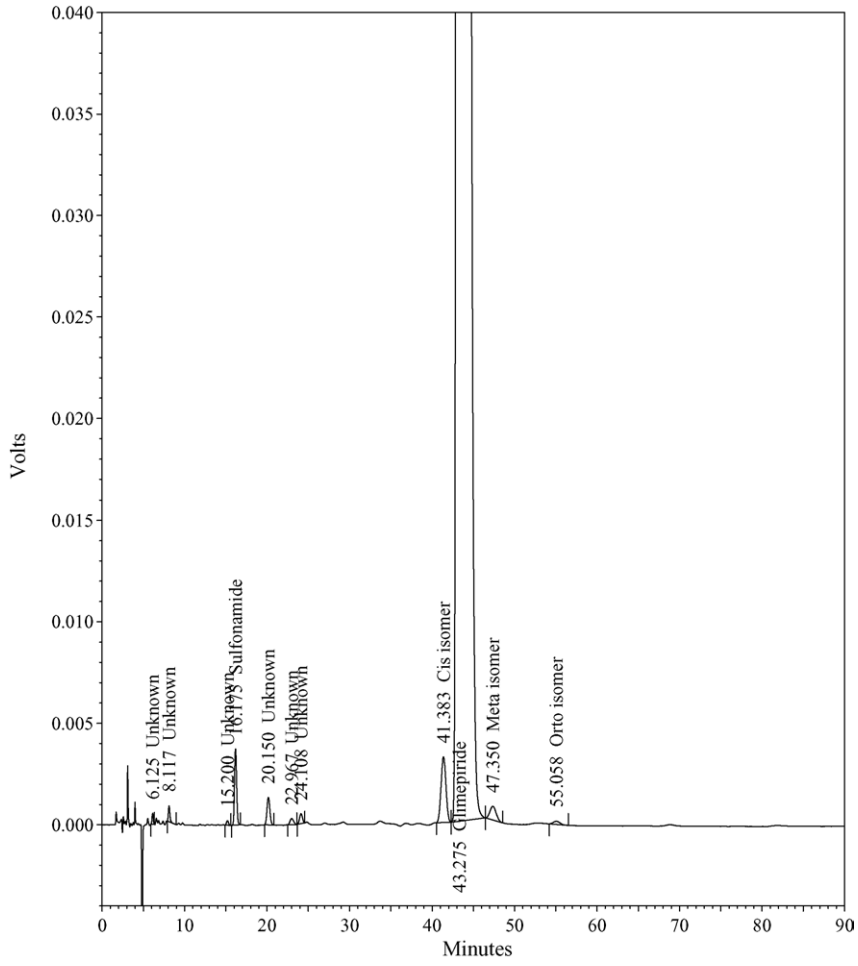

Fig. 2. Related substances analysis using Method-I.

as shown in the Fig. 1, there is a possibility that some amount of this intermediate may remain unreacted during the course of synthesis and appears as an impurity in the final material. During the development of method, we also observed that at mobile phase pH between 2.1 and 2.5 at a temperature $25 \pm 2\,^{\circ}C$, glimepiride degrades to the sulphonamide over a period of time. This degradation phenomenon can also be supported by the degradation of glimepiride into its precursor sulphonamide when it was refluxed in 2N HCl during the forced degradation study. Thus, this impurity A is not only the process impurity but also a degradation product and is named as *glimepiride–sulphonamide*.

4.2.2. Structure elucidation of impurities B–E

Isolated dried fractions of impurities B–D were characterized for structure elucidation using spectroscopic analysis. ESI mass spectra of these isolated fractions of impurities B–D shows a similar pattern with a protonated molecular ion peak at about *m*/*z* 491.3 and the product ion scan shows the peaks at about *m*/*z* 352.5, 335 and 126 which are exactly similar to that of obtained for glimepiride thus showing that these are the isomeric forms of glimepiride. FTIR, ^{1}H and ^{13}C NMR spectra were recorded for these impurities. A comparison of shifts in chemical shifts (δ, ppm) recorded for ^{1}H and ^{13}C NMR spectrum of these impurities has been shown in Tables 3 and 4. This data clearly suggest that these impurities are the possible isomers of glimepiride.

Since the synthesis of glimepiride uses the condensation of sulphonamide with *trans*-4-methyl cyclohexyl isocynate (Route I) or the condensation of ethyl carbamate derivative of sulphonamide with *trans*-4-methyl cyclohexyl amine (Route II) as shown in Fig. 1, there is a possibility that this *trans*-isocynate or amine may contain some amount of *cis*-isomer as a impurity and thus leading to the formation of *glimepiride-cis-isomer* which can also be corroborated with a change in the chemical shift from δ ppm value 3.17 at 19 carbon position for NH–CH– shifts to 3.5 and at position carbon 22 from 1.24 to 1.024 observed for glimepiride standards and impurity B, respectively.

^{1}H NMR spectrum of impurity C shows *meta* substitution in the aromatic ring with the two multiplet peaks for two protons each at δ 7.744 (m, 2H) and 7.541 (m, 2H) and the

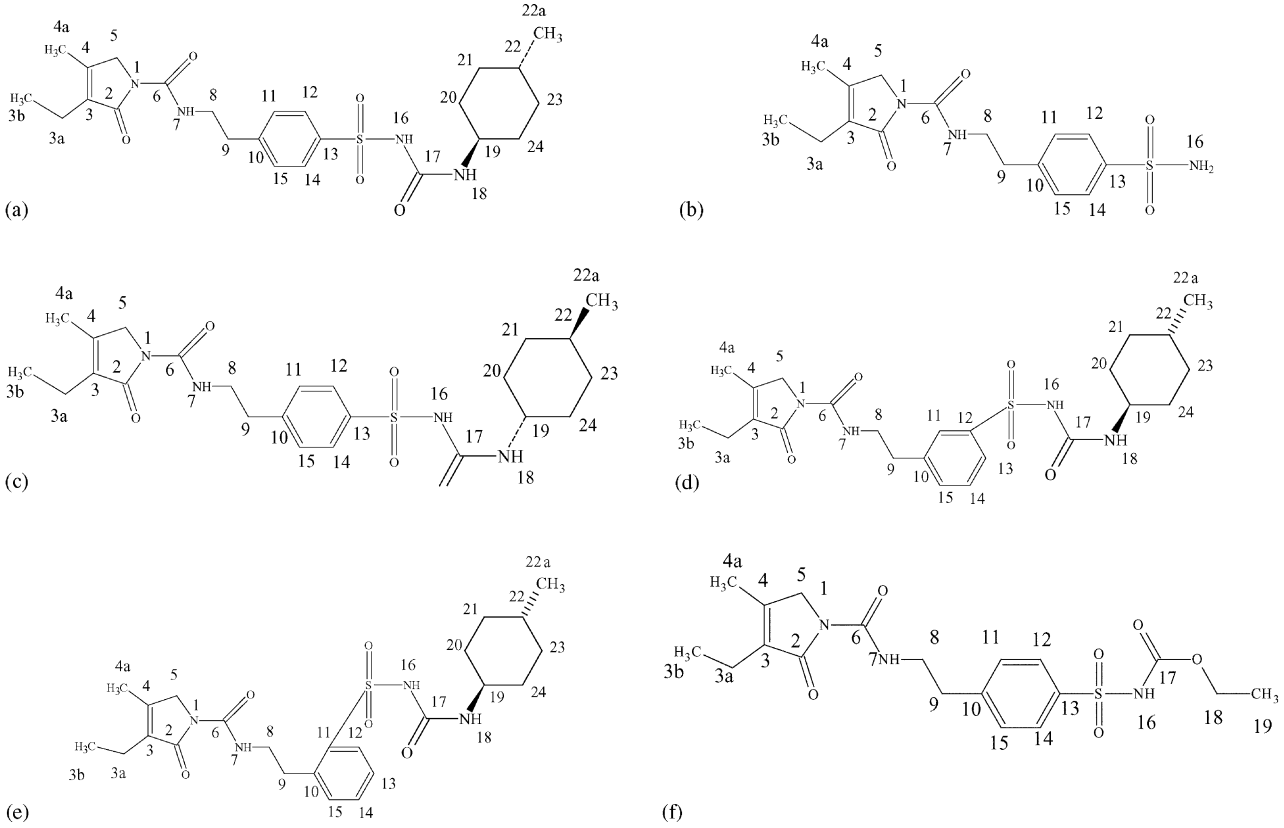

Fig. 3. Structures and chemical names of glimepiride and its impurities: (a) glimepiride: 1-[[4-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl]phenyl]-sulphonyl]-3-trans-(4-methylcyclohexyl)urea; (b) impurity-A: [*N*-(4-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl] benzene sulphonamide; (c) impurity-B: 1-[[4-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl]phenyl]-sulphonyl]-3-cis-(4-methylcyclohexyl)urea; (d) impurity-C: 1-[[3-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl]phenyl]-sulphonyl]-3-(4-methylcyclohexyl)urea; (e) impurity-D: 1-[[2-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl] phenyl]-sulphonyl]-3-(4-methylcyclohexyl)urea; (f) impurity-E: *N*-[4-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl]-benzenesulphonyl]ethyl carbamate.

multiplicity of these protons shows that they are not symmetrical where as the position of these protons were found to be symmetrical as expected for *para* substitution in glimepiride observed at δ 7.793 (d, 2H) 12, 14 and δ 7.441 (d, 2H) 11, 15. Thus, this impurity C was named as *glimepiride-meta-isomer* The ^{1}H NMR spectrum of impurity D also showed a different splitting pattern at δ 7925 (d, 1H), δ 7.584 (t, 1H) and δ 7.432 (t, 2H) with that of glimepiride standard, indicating that D impurity is a *glimepiride-ortho-isomer*.

Apart from these impurities, one more impurity E, which is an ethyl carbamate derivative of sulphonamide (*glimepiride–urethane*), was also considered as a process impurity because the synthesis of glimepiride is also possible by the second route as given in Fig. 1. It was assumed that a fraction of this intermediate may remain unreacted and appear as one of the impurity in the final material. ^{1}H, ^{13}C NMR and FTIR spectra recorded for *glimepiride–urethane* have been shown in Table 5. Structure and chemical name of all these impurities are given in Fig. 3. These impurities were used as in-house standards for the determination of a relative response factor was calculated to report the level of these impurities with a higher accuracy in the commercial lots of glimepiride.

5. Validation of HPLC method for the determination of related impurities

Based on the information on the impurity profiling, an HPLC method for the determination of these impurities was developed to elute all possible known and unknown impurities in a single run with the best possible resolution between these impurities and the main peak. Under the experimental conditions described in *Method-I* of Section 2.2, a condition for system suitability parameter for the proposed method was established where the resolution between *glimepiride-cis-isomer* and the glimepiride was found to be more than 1.3. The resolution of more than 2.4 between glimepiride and *glimepiride-meta-isomer* and the resolution between *glimepiride-ortho-isomer* and *glimepiride-meta-isomer* more than 4.5 was achieved under these experimental conditions. To our knowledge, no method is available in the literature where the determination of *cis-isomer, glimepiride* and other impurities can be determined in a single run (please note that the active glimepiride is a *trans* molecule). However, methods recently appeared in glimepiride USP 28 monograph and PHARMAEUPROPA [15,16] use a normal phase chromatographic system for

Table 2
(a) ^{1}H and ^{13}C assignments for impurity A (*glimepiride–sulphonamide*). (b) FTIR assignments for impurity A (*glimepiride–sulphonamide*)

^{1}H	δ, ppm	J (Hz)	DEPT	^{13}C (δ, ppm)
Part (a)				
1H	8.343	t	qC	172.108
2H	7.725	d	qC	152.273
2H	7.419	d	qC	151.922
2H	7.294	s	qC	143.653
2H	4.159	s	qC	142.409
2H	3.337	q	qC	132.22
2H	2.874	t	CH	129.35
2H	2.189	q	CH	126.023
3H	2.008	s	CH_2	52.14
3H	0.972	t	CH_2	40.448
			CH_2	35.346
			CH_2	16.259
				13.05
				12.951

Wave number (cm^{-1})	Functional group
Part (b)	
3366	─N─H stretching
3315	─N─H stretching due to H─ bonding
3189	NH─C═O
3095, 2965, 2938, 2878	C─H stretching aromatic and aliphatic
1690	N─C═O stretch
1661	Amide─NH─C═O stretch
1537	NH deformation
1439	$-CH_3$ antisymmetric deformation
1340	Asymmetric SO_2
1160	Out-of-plane CH deformation
688	N─C═O bend
593	SO_2 scissoring

the determination of *cis*-isomer and a reverse phase HPLC method for the other related impurities. Also, these methods recommend the analysis to be performed at a temperature not exceeding 12 °C and the solutions prepared are not stable for more than 15 h under the conditions described. The proposed method can resolve all the possible impurities mentioned in one single run and thus save the time and cost of analysis using two different modes of chromatography. Moreover, the analysis can be performed at a higher temperature between 30 and 40 °C and all the solutions prepared under the proposed experimental conditions were found to be stable up to 72 h at room temperature (25 ± 2 °C) against a freshly prepared solution.

Typical chromatogram showing the separation of all the impurities A–E under the system suitability parameter is shown in Fig. 4. The system suitability parameters were designed on the basis of the approximate concentration of the glimepiride to be used in the test sample and the expected level of each individual impurity to get a good correlation between the chromatogram obtained from system suitability and the test sample. The chromatograms from one commercial lots of glimepiride have been shown in Fig. 5.

To determine the specificity of the method all impurities were initially injected under the proposed chromatographic conditions to determine the individual retention time of these impurities with respect to the glimepiride peak. The peak purity of each peak was determined using the DAD detection and it was found that there is no interference from any of the impurity at the retention time of glimepiride. To check the stability indicating characteristics of the method, a laboratory validation batch (lot #GRC1500/0210/03) of glimepiride was subjected to forced degradation under various stress condition like temperature, humidity, acid, base, oxidation and photo-degradation. The data obtained was compared with

Table 3
Comparative ^{1}H NMR assignments for glimepiride and its impurities

Position	^{1}H	Glimepiride		^{1}H	Impurity B		^{1}H	Impurity C		^{1}H	Impurity D	
		δ, ppm	J (Hz)		δ, ppm	J (Hz)		δ, ppm	J (Hz)		δ, ppm	J (Hz)
16	1H	10.315	s	1H	10.218	s	1H	10.319	s	1H	10.621	s
7	1H	8.357	t	1H	8.349	t	1H	8.401	t	1H	8.448	t
Ar	2H	7.793	d	2H	7.793	d	2H	7.744	m	1H	7.925	d
Ar	2H	7.441	d	2H	7.443	d	2H	7.541	m	1H	7.584	t
Ar	–	–	–	–	–	–	–	–	–	1H	7.423	t
18	1H	6.273	d	1H	6.441	d	1H	6.312	d	2H	6.619	d
5	2H	4.16	s	2H	4.155	s	2H	4.158	s	2H	4.203	s
8	2H	3.49	q	3H	3.55, 3.48	m, q	2H	3.475	q	2H	3.464	q
19	1H	3.17	m		3.55, 3.48	m	1H	3.187	m	3H	3.191	t
9	2H	2.894	t	2H	2.829	t	2H	2.903	t	–	3.191	–
4a	2H	2.179	q	2H	2.177	q	2H	2.187	q	2H	2.206	q
–	–	–	–	–	–	–	2H	2.894	–	–	–	–
3a	3H	2.008	s	3H	2.005	s	2H	2.016	s	3H	2.301	s
–	4H	1.639	m	–	–	–	9H	1.696–0.856	m	8H	1.677–0.822	m
–	–	–	–	8H	1.419	m	–	–	–	–	–	–
22	1H	1.024	m	2H	1.024, 0.971	–	–	–	–	–	–	–
–	4H	1.087	m	–	–	–	–	–	–	–	–	–
3b	3H	0.973	m	3H	1.024, 0.971	m, t	3H	0.979	t	3H	0.994	t
22a	3H	0.8195	d	3H	0.857	d	3H	0.819	d	3H	0.811	d

Table 4
Comparative ^{13}C NMR assignments for glimepiride and its impurities

Glimepiride		Impurity B		Impurity C		Impurity D	
DEPT	^{13}C (δ, ppm)	DEPT	^{13}C (δ, ppm)	DEPT	^{13}C (δ, ppm)	DEPT	^{13}C (δ, ppm)
CH_3	12.931	CH_3	12.920	CH_3	13.421	CH_3	12.790
CH_3	13.023	CH_3	13.023	CH_3	13.521	CH_3	13.206
CH_2	16.225	CH_2	16.221	CH_2	16.696	CH_2	16.591
CH_3	22.223	CH_3	21.620	CH_3	22.713	CH_3	22.040
CH	31.439	CH	30.431	CH	31.898	CH	31.736
CH_2	32.511	CH_2	29.195	CH_2	32.944	CH_2	32.892
CH_2	32.511	CH_2	29.195	CH_2	32.944	CH_2	33.026
CH_2	33.579	CH_2	29.389	CH_2	34.031	CH_2	33.026
CH_2	33.579	CH_2	29.389	CH_2	34.031	CH_2	33.640
CH_2	35.407	CH_2	35.407	CH_2	35.775	CH_2	33.640
CH_2	40.253	CH_2	40.253	CH_2	40.957	CH_2	41.417
CH	48.770	CH	45.092	CH	49.226	CH	49.098
CH_2	52.102	CH_2	52.094	CH_2	52.565	CH_2	52.247
CH	127.576	CH	127.511	CH	125.868	CH	126.866
CH	127.576	CH	127.511	CH	127.769	CH	130.507
CH	129.381	CH	152.212	CH	129.753	CH	132.167
CH	129.381	CH	152.212	CH	132.634	CH	133.350
qC	132.174	qC	132.159	qC	134.115	qC	133.967
qC	138.421	qC	138.291	qC	140.911	qC	136.967
qC	145.164	qC	145.259	qC	141.056	qC	138.474
qC	150.716	qC	150.559	qC	151.187	qC	150.800
qC	151.876	qC	151.865	qC	152.377	qC	151.143
qC	152.208	qC	152.212	qC	152.683	qC	153.933
qC	172.035	qC	172.028	qC	172.495	qC	172.688

that of obtained without stress condition. Chromatograms were checked for the appearance of any extra peak due to the degradation of the analyte under these conditions and their respective retention times were recorded. The data were checked for the interference of the any degraded peak at the retention time of the glimepiride and the known impurities by checking the peak purity using the DAD detection. This data has been shown in Table 6. It was observed that under acidic condition, there is an increase in the level of sulphonamide impurity when the analyte was refluxed in 2N HCl at 80 °C for 8 h. However, a higher degradation (total degradation about 22%) was found when the sample was refluxed in 2N NaOH at 80 °C for 8 h. No degradation was observed up to 12 h in 2N HCl and 1N NaOH. This higher degradation in alkali can be attributed to the base hydrolysis of the molecule resulting in the breaking of the amide linkage of the molecule and the

Table 5
^{1}H, ^{13}C and FTIR assignments for impurity E

Position of protons	^{1}H	δ, ppm	J (Hz)	DEPT	13C (δ, ppm)	FTIR assignments	
						Wave number	Functional
16	1H	11.965	s	qC	172.051	2373	─N─H stretching
7	1H	8.373	t	qC	152.300	3243	─N─H stretching due to H─ bonding
Ar	2H	7.812	d	qC	151.872	3056, 2981, 2936, 2878	C─H stretching aromatic and aliphatic
Ar	2H	7.419	d	qC	151.304	1750	C═O stretch ester
5	2H	4.168	s	qC	154.767	1713	N─C═O stretch
17	2H	3.992	q	qC	137.376	1659	Amide─NH─C═O stretch
4a	2H	3.517	q	qC	132.140	1551	NH deformation
9	2H	2.914	t	2CH	129.63	1460	─CH_3 antisymmetric deformation
8	2H	2.195	q	2CH	127.763	1352	Asymmetric SO_2
3a	2H	2.014	s	CH_2	62.126	1162	Symmetric SO_2
18	3H	1.095	t	CH_2	52.098	686	Out-of-plane CH deformation
3b	3H	0.973	t	CH_2	40.185	615	N─C═O bend
				CH_2	35.399	580	SO_2 scissoring
				CH_2	16.217		
				CH_3	14.164		
				CH_3	13.065		
				CH_3	12.951		

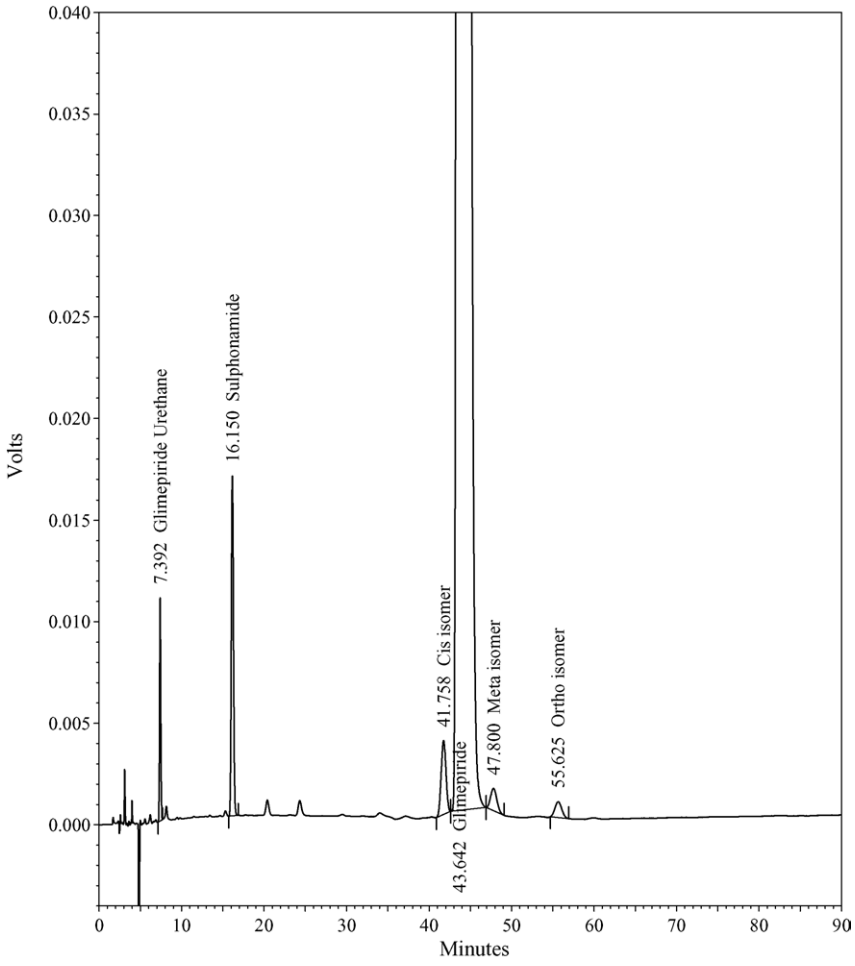

Fig. 4. System suitability solution showing the resolution between critical isomers.

possible generated product will be the sulphonamide, which under basic condition may further degrade. No degradation was observed under humidity and temperature condition but a slight degradation was observed under oxidation condition. No degradation was observed when the material was subjected to photo-degradation. All the degradation products were found to be well separated from the main and the known impurity peaks suggesting that the method is stability

Table 6
Specificity data for related substances and assay

Related substances			Assay
Stress conditions	Total degradation found	Observation	Assay dried basis (%)
0.1N HCl (12 h)	No degradation	–	101.45
2N HCl (12 h)	No degradation	–	99.53
2N HCl (reflux for 8 h at 80 ± 2 °C)	About 3.5% degradation	Increase in the sulphonamide impurity and at an extra peak 6.048 min.	101.67
0.1N NaOH (12 h)	About 1.5% degradation	–	101.61
2N NaOH (12 h)	About 3.0% degradation	Extra peak at 7.5 min.	96.05
2N NaOH (reflux for 8 h at 80 ± 2 °C)	About 22% degradation	Increase in the sulphonamide impurity. Extra peaks at 5.53 min, 6.18 min, 4.13 min, 13.09 min and 19.584 min.	65.51
Oxidation (20% hydrogen peroxide for 12 h)	About 2% degradation	–	96.78
Humidity (>75% relative humidity for 24 h)	No degradation	–	100.26
Temperature (105 ± 2 °C for 24 h)	No degradation	–	101.49
Photo-degradation (1.2 million lx h)	No degradation	–	100.55

Note: None of degradation peak interfere at the retention time of impurities or the main peak and the peak purity of the main peak checked by PDA was found to be >0.9999.

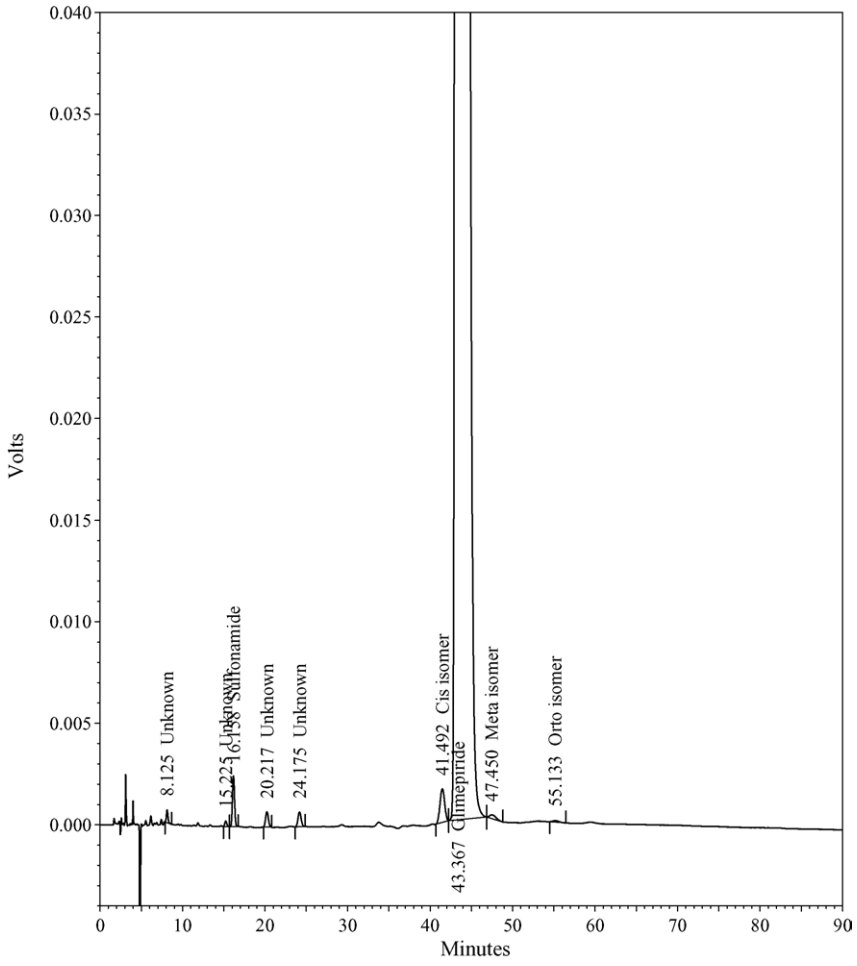

Fig. 5. Typical chromatogram showing the detection of impurities in the commercial lot.

indicating method and can very well be used to study the stability of the API as per ICH guidelines on stability [19].

Selectivity of the method was checked by using five different columns of different manufacturers, which are equivalent in dimensions and the stationary phase. Since the resolution separation between the *glimepiride-cis-isomer* and the glimepiride and glimepiride and *glimepiride-meta-isomer* is critical, a mixture described in the system suitability parameter was injected containing all the impurities and the resolution between the desired isomers and the glimepiride was compared. A comparison of the resolution, capacity factor, number of theoretical plate and the asymmetry has been presented in Table 7. This data suggest that most suitable column is Phenomenex Luna and Waters Symmetry C8 can be used as an alternative.

The linearity of glimepiride and all other impurities were evaluated over the range of 0.24–6.00 μg/ml equivalent to 0.04–1.00% with respect to test sample concentration. Five replicate sets of each concentration level were prepared and checked for linearity. A calibration curve was established between the average response and the concentration of the analyte. A correlation of more than 0.9999 was achieved and the data is presented Table 8.

The RRF and the RRT for all impurities with respect to glimepiride were determined and have been given in Table 1. These RRFs will be used while calculating the levels of these known impurities in the commercial lots and all other impurities will be calculated using the diluted standard (*Reference Solution B* in Section 2.2, Method-I).

Limit of detection (LOD) and limit of quantification (LOQ) was determined for all the impurities and the diluted standard of glimepiride from the linearity experiments. The LOD was found to be 0.02% (with respect to test sample) for glimepiride and all five related impurities with signal to noise (S/N) ratio of more than 3 and LOQ was found to be 0.04% (with respect to test sample) with S/N ratio of more than 10.

Accuracy of the method was determined by spiking all the impurities at three different concentration levels of 50, 100 and 150% each in triplicate of the specified limit. The recovery of all these impurities were found to be with in the pre-defined criterion of 90–110% and the data is presented in Table 9.

Further, the precision of the method was studied for repeatability and intermediate precision. Method precision was studied by estimating the related substances in one lot of glimepiride using six different weighing. All the samples

Table 7
Selectivity of the column for related substances

Column	Related compound	Retention time (min)	Resolution	Capacity factor	Asymmetry	Theoretical plates
Phenomenex Luna C8 (2), 100 Å, 5 μm, 250 mm × 4.6 mm	Urethane	7.17	–	1.87	1.15	10268
	Sulphonamide	15.41	22.28	5.16	1.07	18285
	Cis-isomer	39.54	31.62	14.82	1.06	21832
	Glimepiride	41.34	1.42	15.54	2.32	10241
	Meta-isomer	45.36	2.64	17.14	1.08	16604
	Ortho-isomer	52.84	5.08	20.14	1.08	18821
Waters Symmetry C8, 5 μm, 250 mm × 4.6 mm	Urethane	7.13	–	1.85	1.12	8372
	Sulphonamide	15.98	22.04	5.39	1.02	16939
	Cis-isomer	39.70	29.65	14.88	1.00	20519
	Glimepiride	41.58	1.33	15.63	1.89	11600
	Meta-isomer	45.23	2.46	17.09	1.06	16204
	Ortho-isomer	52.41	4.79	19.96	1.04	17681
MN Nucleodor C8, 100 Å, 5 μm, 250 mm × 4.6 mm	Urethane	6.59	–	1.64	1.10	7701
	Sulphonamide	16.44	23.53	5.58	1.12	15020
	Cis-isomer	32.90	22.77	12.16	0.91	20950
	Glimepiride	34.11	1.09	12.64	1.74	10937
	Meta-isomer	36.55	1.96	13.62	1.23	15322
	Ortho-isomer	41.52	3.92	15.61	1.12	15038
Waters Spherisorb C8 (R), 5 μm, 250 mm × 4.6 mm	Urethane	5.34	–	1.14	1.11	7025
	Sulphonamide	15.32	28.97	5.13	0.99	19947
	Cis-isomer	26.85	19.82	9.74	0.99	21640
	Glimepiride	28.01	1.34	10.20	1.87	12686
	Meta-isomer	30.14	2.24	11.06	1.11	17438
	Ortho-isomer	34.26	4.21	12.70	1.11	17330
BDS Hypersil C8, 5 μm, 250 mm × 4.6 mm	Urethane	6.24	–	1.50	1.08	9609
	Sulphonamide	11.14	15.71	3.46	0.99	14582
	Cis-isomer	28.81	30.83	10.52	0.86	22000
	Glimepiride	29.87	1.04	10.95	1.89	8907
	Meta-isomer	32.50	2.19	12.00	1.03	13011
	Ortho-isomer	37.60	4.31	14.40	0.99	15011

were analyzed in a single session. The method was found to be precise with a R.S.D. of NMT 10%. To determine the intermediate precision of the method, six sample of the same lot were prepared by a different analyst and were analyzed using a different reagent preparations and instruments on a different day. The comparative data of the analysis by two different analysts is described in Table 10.

The robustness of the method was demonstrated by the ability of the method to remain unaffected by small changes in parameters like percentage of organic content, flow rate

Table 8
Average linearity of impurities and the diluted reference solution of glimepiride

Concentration (μg/ml)	Average area of glimepiride urethane	Average area of glimepiride sulphonamide	Average area of glimepiride *cis*-isomer	Average area of glimepiride *meta*-isomer	Average area of glimepiride ortho-isomer	Average area of glimepiride diluted reference solution
0.24	23796.75	19002.50	27057.75	16145.50	13011.00	13313.50
0.48	46307.00	36305.00	53511.00	33639.00	28306.25	27349.25
0.60	59270.00	48323.75	68473.50	38179.75	36598.00	35814.25
1.20	116294.00	97343.25	134595.25	87895.00	75437.50	70484.75
2.40	233239.00	191389.75	269509.50	177054.50	150546.25	141026.75
3.60	362122.75	298607.50	417751.75	282297.50	234967.00	221842.00
6.00	576318.50	474410.75	665945.00	457155.50	374893.50	355336.75
Intercept	1620.278	843.925	−1772.428	−4567.284	836.029	−515.400
Standard	6146.069	5437.882	6843.960	4719.420	4081.991	3588.514
Error of intercept						
Slope	96832.373	79853.652	111875.809	77433.729	63334.139	59832.993
Standard	1189.675	1052.593	1324.763	913.523	790.138	694.617
Error of slope						
r-Value	0.999	0.999	0.999	0.999	0.999	0.999

Table 9
Accuracy of the related substances method

Related substances	Amount recovered (%)	Amount spiked (%)	Recovery (%) ($n = 3$)	Mean (%)	% R.S.D.
At 50% level					
Sulphonamide	0.217	0.208	104.51		
	0.225	0.208	108.13	106.77	1.85
	0.224	0.208	107.68		
	0.431	0.435	99.16		
Cis-isomer	0.425	0.435	97.80	98.38	0.71
	0.428	0.436	98.19		
	0.109	0.100	108.53		
Meta-isomer	0.103	0.100	102.97	104.01	3.94
	0.101	0.101	100.53		
	0.102	0.102	100.40		
Ortho-isomer	0.105	0.102	103.09	101.45	1.42
	0.103	0.102	100.86		
	0.050	0.050	100.24		
Glimepiride–urethane	0.051	0.050	101.98	100.69	1.13
	0.050	0.050	99.84		
At 100% level					
Sulphonamide	0.428	0.416	102.73		
	0.431	0.415	103.80	103.97	1.29
	0.439	0.417	105.39		
	0.921	0.871	105.71		
Cis-isomer	0.890	0.869	102.40	103.78	1.66
	0.900	0.872	103.23		
	0.207	0.201	102.97		
Meta-isomer	0.203	0.201	101.17	99.65	4.30
	0.191	0.202	94.82		
	0.211	0.205	103.01		
Ortho-isomer	0.216	0.204	105.46	104.00	1.24
	0.212	0.205	103.52		
	0.102	0.100	102.15		
Glimepiride-urethane	0.102	0.100	102.26	102.58	0.63
	0.104	0.100	103.32		
At 150% level					
Sulphonamide	0.670	0.623	107.56		
	0.669	0624	107.16	107.53	0.33
	0.673	0.624	107.86		
	1.412	1.305	108.21		
Cis-isomer	1.414	1.306	108.28	108.22	0.05
	1.413	1.306	108.17		
	0.295	0.302	97.92		
Meta-isomer	0.300	0.302	99.34	99.09	1.07
	0.302	0.302	100.00		
	0.306	0.307	99.60		
Ortho-isomer	0.313	0.307	101.81	102.74	3.60
	0.328	0.307	106.81		
	0.157	0.150	104.82		
Glimepiride–urethane	0.156	0.150	103.70	104.50	0.67
	0.158	0.150	104.99		

and pH of the mobile phase, change in temperature, change in the wavelength of detection and different lot of the columns from the same make.

To determine the robustness of the method, experimental conditions were subjected to challenge and the effects of these positive and negative changes in the experimental conditions were evaluated for chromatographic characteristics. The change in the organic content form the proposed condition of buffer–acetonitrile–THF, 73:18:09 (v/v/v) to a ratio of 75:16:09 resulted in slight loss of resolution

Table 10
(a) Intermediate precision related substances determination. (b) Intermediate precision assay method

Chemist/instrument/day	Sample	Sulphonamide (%)	*Cis*-isomer (%)	*Meta*-isomer (%)	*Ortho*-isomer (%)	Unknown max impurities (%)	Total impurity (%)
Part (a)							
I	1	0.085	0.220	0.039	0.015	0.048	0.536
	2	0.084	0.213	0.042	0.015	0.048	0.532
	3	0.089	0.204	0.041	0.016	0.047	0.526
	4	0.083	0.205	0.042	0.013	0.046	0.521
	5	0.086	0.203	0.043	0.014	0.048	0.540
	6	0.086	0.221	0.043	0.015	0.048	0.550
Mean (%) ($n = 6$)		0.085	0.211	0.042	0.015	0.047	0.534
% R.S.D.		2.42	3.87	3.61	7.04	1.76	1.93
II	1	0.086	0.202	0.044	0.015	0.048	0.579
	2	0.085	0.202	0.046	0.017	0.048	0.552
	3	0.090	0.201	0.041	0.017	0.049	0.529
	4	0.084	0.199	0.039	0.017	0.048	0.525
	5	0.089	0.206	0.041	0.016	0.047	0.546
	6	0.086	0.209	0.043	0.014	0.049	0.494
Mean (%) ($n = 6$)		0.087	0.203	0.042	0.016	0.048	0.537
% R.S.D.		2.70	1.80	5.91	7.90	1.76	5.34
Overall mean ($n = 12$)		0.086	0.207	0.042	0.015	0.048	0.536
% R.S.D.		2.55	3.52	4.76	8.49	1.86	3.85

Chemist/instrument/day	Sample	Assay w/w (%)	Mean (%) ($n = 6$)	% R.S.D.
Part (b)				
I	1	100.13		
	2	100.06		
	3	99.94	99.96	0.11
	4	99.88		
	5	99.91		
	6	99.86		
II	1	99.81		
	2	99.92		
	3	99.95	99.95	0.09
	4	99.93		
	5	100.01		
	6	100.06		
Overall mean ($n = 12$)	99.96			
% R.S.D.	0.09			

between *glimepiride-cis-isomer* and g*limepiride* from a value of 1.50–1.4 where as an improved resolution was observed between *glimepiride* and *glimepiride-meta-isomer* from a value of 2.47 to 4.68. With a mobile phase ratio of buffer–acetonitrile–THF of 71:18:11 (v/v/v), there was no significant change in the resolution of the two isomers with glimepiride. With a mobile phase of 75:18:07 (v/v/v), the *glimepiride* peak shifted to a retention time of 71 min. But a significant loss of resolution was observed between these isomers when the THF content was decreased by 2%. However, in the ratio of 71:18:11 (v/v/v) a significant change in the elution behavior was observed for one of the unknown impurity present at a level of 0.06% in test sample. This impurity eluted out just after sulphonamide peak, which was appeared before sulphonamide peak under the proposed experimental conditions. By changing the flow rate of the mobile phase to 0.8–1.2 ml form the proposed 1 ml/min, there is no change in the estimated results of the total related substances.

No change in the results was observed by changing the mobile phase to pH 6.8 and 7.2 from the proposed of pH 7.0. No significant change in the estimated results of total related substances was observed by altering the wavelength of determination by 2 nm to positive and negative side both. The change in temperature of the column by $\pm 5\,^{\circ}C$ does not affect the estimation of related substances. The results were fond to be reproducible using two different lots of the columns. Precisely, the method was found to be robust under the experimental condition studied.

To study the stability of the glimepiride in the solution, a sample was studied for the individual and total impurities at every 10 h interval for 72 h against a freshly prepared

standard. It was found that there is no change in the impurity level of this sample against a freshly prepared sample. The solution is stable up to 72 h under the proposed experimental conditions.

6. Validation of HPLC method for the determination of assay of glimepiride

For the determination of assay of glimepiride drug substance, chromatographic conditions were slightly modified to reduce the analysis time by changing the organic content of the mobile phase. Experimental conditions and the sample preparations are described in the Section 2.2, Method-II. The proposed method was also validated studied for specificity, linearity, precision, accuracy and robustness.

Specificity of the method was demonstrated by subjecting it to various stress conditions to study the stability indicating characteristics of the method. The study at various condition of stress like thermal, photo acid, base and oxidation reveals that glimepiride shows degradation under acid, base hydrolysis and oxidative degradation and the assay values are more affected under the base and oxidative hydrolysis. The peak purity of the glimepiride peak does not show any interference from these degradation products. The assay values calculated under each condition of stress are shown in Table 6.

Selectivity of the method was demonstrated by spiking all the impurities into the assay preparation and it was found that none of the known impurities interfere at the retention time of the main peak. Fig. 6 shows the resolution between various impurities and the *glimepiride* under the experimental conditions described in Method-II for the determination of assay of glimepiride.

The response for the detection of *glimepiride* was found to be linear ranging from 15 to 45 μg/ml. A calibration curve was drawn between the response and the concentration and correlation coefficient, slope and intercept were calculated using the regression analysis and was found to be 0.9998, 70080.64 and 20712.80, respectively.

Precision of the method was determined by estimating the method precision and the intermediate precision. Method precision was determined by estimating the assay of one lot

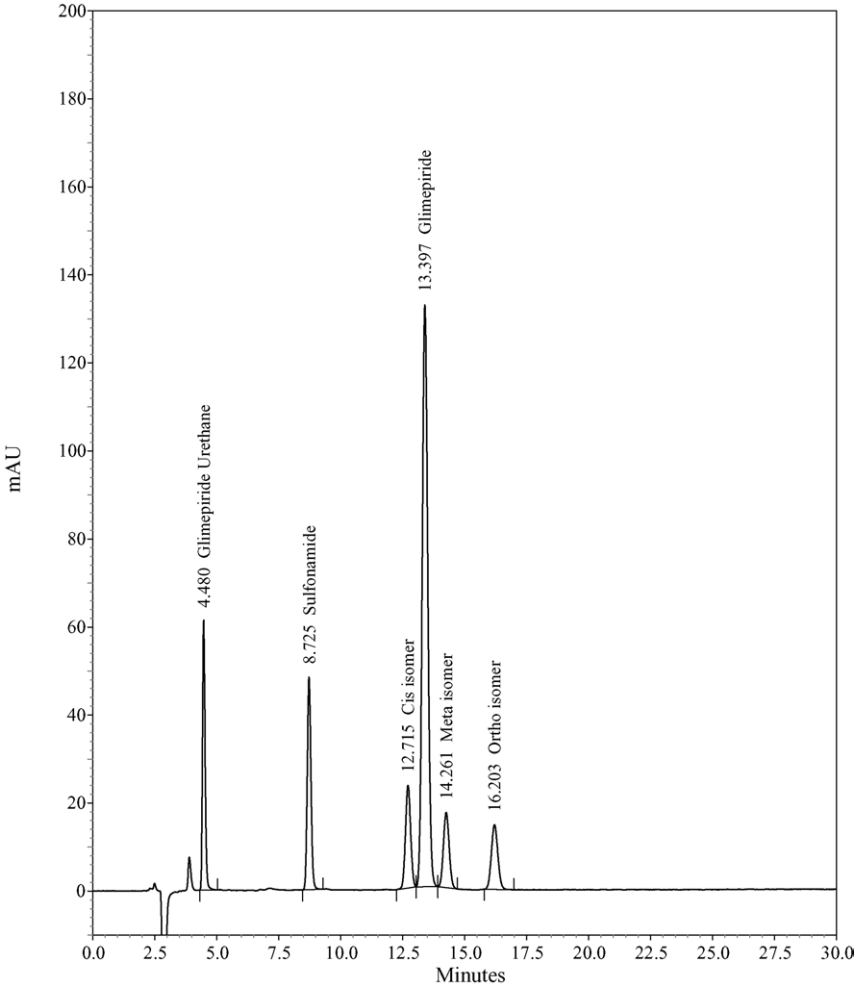

Fig. 6. Selectivity for assay method.

Table 11
Accuracy of assay method

Level (%)	Amount added (μg/ml)	Amount recovered	% Recovery (μg/ml)	Mean (%)	% R.S.D.	Mean
80	24.16	24.04	99.54			
	24.43	24.46	100.16	99.86	0.31	
	24.13	24.10	99.89			
100	30.05	29.79	99.13			
	30.38	30.33	99.84	99.73	0.56	0.09
	30.48	30.54	100.23			
120	36.18	36.07	99.69			
	36.48	36.65	100.47	99.90	0.50	
	36.13	35.96	99.53			

of glimepiride using six different weighing of the sample on the same day. The mean assay value was found to be 99.97% with R.S.D. of 0.11%. Intermediate precision was determined by estimating the assay of the same lot using six different weighing by a different analyst using the different instrument, different column on a different day. The mean assay value for two different analysts and twelve determinations was found to be 99.96% with a R.S.D. of 0.01%. The data is presented in Table 10b.

Accuracy of the method was determined at three different concentration levels, i.e. at 80, 100 and 120% (w/w) of the assay concentration as described in Method-II Section 2.2 for three different determinations each in triplicate. For each determination fresh samples were prepared and the assay values calculated. The data presented in Table 11 demonstrate the accuracy of the method.

Robustness of the method was determined by changing the experimental conditions like percentage of organic content, flow rate and pH of the mobile phase, change in temperature, change in the wavelength of detection and different lot of the columns from the same make. Change in the organic content of mobile phase was studied by varying the percentage of the acetonitrile to a ratio of buffer–acetonitrile–THF (63:27:10, v/v/v) and 67:23:10 (v/v/v) from an original ratio of 65:25:10 (v/v/v). It was observed that by increasing 2% of the acetonitrile, the glimepiride retention time comes to 10 min and by decreasing it goes to 17 min without affecting the assay value. The mean assay value between the two sets was found to be 100.22% with a R.S.D. of 0.35%. With the increase and decrease in the percentage of THF by 2%, the retention time of the glimepiride shifts to 10 and 19 min, respectively, as compared to the original retention time of 13 min. However, the assay values remain unaffected by this change with a mean assay value of 100.13% with a R.S.D. of 0.09%. The change in flow rate to 0.8 and 1.2 ml/min from 1.0 ml/min does not show any change in the assay value. The mean assay for the two different sets was found to be 99.94% (w/w) with a R.S.D. of 0.04%. However, the retention time was shifted to 16 and 11 min, respectively. It was observed that the change in the column temperature results in the change in the retention time of the glimepiride. The retention time of *glimepiride* was found to be about 13 and 12 min at 30 and 40 °C, respectively, without affecting the results of assay of glimepiride. The mean assay value for said change was found to be 99.935 with an R.S.D. of 0.04%. Different lot column of the similar make does not affect the results of assay of the glimepiride. The mean assay between the two sets using two different lot of the column was found to be 100.03% (w/w) with an R.S.D. of 0.05%.

The sample preparation of glimepiride was found to be stable for 72 h and no significant change was observed under proposed experimental conditions.

7. Conclusions

An HPLC method for the determination of related impurities and assay of glimepiride was developed and validated as per the ICH guidelines. The method was found to be accurate, simple, sensitive and robust for the estimation of related impurities and assay of glimepiride for routine quality control monitoring of the glimepiride API. All the possible isomers, degradation products and the related unknown and known impurities can be determined using a single isocratic method. The method can be successfully used as stability indicating method for studying the stability of the molecule.

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