Egyptian Journal of Forensic Sciences

ORIGINAL ARTICLE Open Access

Development and validation of the method for the detection of glimepiride via derivatization employing *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide using gas chromatography-mass spectrometry

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Abstract

Background: Recent advances in the diversified anti-diabetic drugs have appeared in the startling increase in the count of poisoning cases. The epidemics of diabetes mellitus are increasing; hence, the no. of anti-diabetic drug users raised by 42.9%. The use of glimepiride raised to 24%. As the toxicity and drug cases are also escalating with increasing epidemics of diabetes mellitus, a novel gas chromatography-mass spectrometry (GC-MS) method for detecting glimepiride in biological matrices is developed.

Results: Liquid-liquid extraction method was employed by using 1-butanol: hexane (50:50, v/v) under an alkaline medium, and then back extraction was done via acetic acid. Distinct derivatization techniques were employed for the sample preparation for GC-MS analysis, i.e., silylation and acylation. Derivatization approaches were optimized under different parameters, i.e., reaction temperature and reaction time. *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide [MSTFA] was found to be the best sound derivatization reagent for the GC-MS analysis of glimepiride. Total ion current (TIC) mode was selected for the monitoring of ions of trimethylsilyl (TMS) derivative of glimepiride with an m/z ratio of 256. Distinct parameters like specificity, carryover, stability, precision, and accuracy were evaluated for validating the identification method. The GC-MS method is found to be linear and illustrated within the range 500 to 2500 ng/ml with the value of R^2 (coefficient of determination) at 0.9924. The stability of the extracted and derivatized glimepiride was accessed with regard to processed/extracted sample conditions and autosampler conditions, respectively. Accuracy at each concentration level was within the $\pm$ 15% of the nominal concentration. Precision (%) for the interday and intraday analysis was found to be in the respectable spectrum.

Conclusion: Henceforth, the proposed GC-MS method can be employed for the determination of glimepiride in biological matrices.

Keywords: Diabetes mellitus, Glimepiride, MSTFA, Derivatization, Gas chromatography-mass spectrometry, Biological matrices

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Background

1-((p-(2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl)-phenyl)-sulfonyl)-3-(trans-4-methylcyclohexyl)urea, ordinarily known as glimepiride, a derivative of sulphonylurea, is a hypoglycemic drug, oral administration drug, extensively used in the treatment of non-insulin-dependent diabetes mellitus (type 2 diabetes) (Lestari and Indrayanto 2011; Massi-Benedetti 2003). In patients with insulin-dependent diabetes mellitus (type 1 diabetes), the peripheral application of insulin results in 'insulin-like growth factor-I' (IGF-I) insufficiency, sequentially to an insulin-resistant state; wherein, glimepiride efficaciously increases IGF-I and does not expose patients towards hypoglycemia (Wudy et al. 2003).

Glimepiride works as an anti-diabetic drug by exhilarating insulin production in the pancreas, hence enhancing the efficiency of insulin to control the blood glucose level (Müller and Geisen 1996; Seedher and Kanojia 2009).

As the epidemics of diabetes mellitus is increasing, the number of anti-diabetic drug users is also increasing. With this elevated use of glimepiride, drug abuse cases are also increasing. Many patients have been reported due to the sulphonylurea and glimepiride induced toxicity, which includes fatal conditions like euglycemic ketoacidosis, hepatotoxicity, drug-induced cholestatic liver injury, severe hypoglycemia, acute tubulointerstitial nephritis, and tachycardia, which possess forensic toxicological and clinical toxicological relevance (Chounta et al. 2005; Juurlink et al. 2003; Soderstrom et al. 2006; Tarek et al. 2016; Theodore et al. 2018; Zolpidem/glimepiride 2015). Irregular lifestyle leading to drug overdosage, suicidal attempts, and inadvertent drug administration result in escalating the cases corresponding drug poisoning (Ibragimova and Ikramov 2015). As the toxicity and drug cases are escalating with increasing epidemics of diabetes mellitus, there is an urgent need to develop the protocol for the detection of glimepiride in biological matrices. Also, the quantification of glimepiride is required in therapeutic drug supervision, pharmacokinetic examinations, bioequivalence studies, and drug dosing optimization.

Several distinct analytical methods are available for the detection of glimepiride in pharmaceutical preparations and human plasma, which employs thin layer chromatography (TLC) (Rivai et al. 2016), high-performance thin-layer chromatography (HPTLC) (Dhole et al. 2013; Dubey et al. 2013; Kale and Kakde 2011; Parthiban et al. 2013), ion-pair reversed-phase liquid chromatography (Rao and Nikalje 2010), high-performance liquid chromatography (HPLC) (Sane et al. 2004), and spectrophotometry (Salma et al. 2011). Liquid chromatographic techniques coupled with ultra-violet spectroscopy (Ramadan and Zeino 2018; Rezk et al. 2012) and tandem mass spectrometry (Kim et al. 2004). Glimepiride is a highly polar compound having active hydrogens (-NH, -OH). So, it was necessary to derivatize it into a thermally stable form to elute at acceptable temperatures eliminating thermal decomposition and rearrangement of molecules. Active hydrogen (–NH, –OH) present in the glimepiride tends to structure intermolecular hydrogen bonds, which affects the genetic volatility of the analyte compound. The active hydrogens can also interact with the GC column packing material and subsequently decrease the separating efficiency of various compounds. Three distinct derivatization techniques were used to derivatize glimepiride to decrease the analyte adsorption and ameliorate detector response and peak response (Box et al. 2020; Orata 2012; Sigma-Aldrich 1997).

In literature, HPLC, UV, HPTLC, and electrochemical methods are available, which are mainly used for the detection of glimepiride. However, no GC-MS method is available for the detection of glimepiride in forensic toxicological samples, particularly viscera samples; the proposed method helps us to identify glimepiride in viscera samples. GC-MS, as a combined technique, provides exceptional specificity, sensitivity, and mass spectral data for the identification of drugs. Many forensic laboratories widely use GC-MS for routine analysis, as it includes non-targeted data acquisition.

This study intends to explore the potential of GC-MS (which is widely used in forensic laboratories) for the detection of glimepiride to establish a new competent method apart from the other available methods. Therefore, we explore and develop the identification method of glimepiride in pharmaceutical preparations and biological matrices via the GC-MS technique. Different derivatization techniques viz. Acylation, methylation, and silylation are adopted and compared to find an advisable derivatization approach for the GC analysis. The reaction temperature and reaction time subjected to the derivatization process were optimized. Different extraction procedures are investigated and developed for the isolation of glimepiride from biological matrices to find a suitable extraction method. During the literature review, it was found that there is no method available for the extraction of glimepiride from the biological matrices like viscera samples, including the kidney, liver, and spleen. Henceforth, an extraction method, the best suitable derivatization technique, is developed for the GC-derivatization of glimepiride, and a novel GC-MS method is developed for the identification of glimepiride in pharmaceutical preparation and biological matrices.

Methods

Chemicals and reagents

Glimepiride [1-((p-(2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl)-phenyl)-sulfonyl)-3-(trans-4-

methylcyclohexyl)urea], bistrimethylsilyltrifluoroacetamide [BSTFA], *N*-methyl-bis(trifluoroacetamide) [MBTFA], and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide [MSTFA] were of GC-grade and supplied by TCI chemicals (pvt. ltd India). Other chemicals and reagents including butanol, hexane, acetic acid, methanol, toluene, and dimethyldichlorosilane were of analytical grade.

Preparation of standard and working solutions

Glimepiride was weighed accurately to make 1000 μg/ml and 500 μg/ml stock solution. Methanol was used as the solvent system for the standard stock solution. Working solutions of 2500 ng/ml, 2000 ng/ml, 1500 ng/ml, 1000 ng/ml, and 500 ng/ml were prepared by serial dilutions of the standard stock solution using methanol. Working and stock solutions were conserved at 4 °C.

Deactivation of glassware

Usually, the laboratory glassware surface is slightly acidic and tends to adsorb some analytes, notably amines. To forbid this sample loss, laboratory glassware is silanized to mask the polar Si–OH groups present on the glass surface and thus conducted with 5% dimethyldichlorosilane (in toluene) for 25 min. The resultant deactivated glassware is then rinsed with toluene and methanol.

Instrumentation and GC-MS parameters

The research analysis is executed on Perkin Elmer Clarus 500 GC. Elite 5MS of 30 m × 0.32 mm, 0.25 μm, and 1,4-bis(dimethylsiloxy)phenylene dimethylpolysiloxane column is used. Helium gas was employed as the carrier gas with a flow rate of 1.2 ml/min. The injection port temperature was set at 250 °C. The initial column temperature was set at 90° with the hold time of 2 min and then ramped at the rate of 15 °C per minute, and further, the temperature was raised to the final temperature of 300 °C. The final column temperature was held for 5 min. The total run time was 21 min. The retention time of the compound is 3 min 58 s. However, we chose to keep the longer run time to confirm the interference from any endogenous substance. The transfer line temperature was set at 310 °C. The injecting volume of the sample was 2 μL. TIC mode was used for the determination of glimepiride. Mass fragments of derivatized glimepiride were observed at m/z 256. The ion m/z 256 was picked for the quantification of glimepiride.

Optimization of the derivatization reaction

Derivatization was carried out by three reagents: MBTFA, BSTFA, and MSTFA. Derivatization reaction was optimized at different reaction temperatures and reaction times. The reaction was carried out at 80 °C and 100 °C for 45 and 60 min. Five replicate analyses were done for each set. Each respective derivatization reagent was employed at the mentioned reaction temperatures and reaction time.

The final optimized derivatization reaction was performed by taking the extracted sample and get it derivatized by adding 40 μl of MSTFA at 80 °C for 1 h in a dry heat chamber. The resultant is then cooled down at room temperature, and 2 μl of the derivatized sample was injected for the GC-MS analysis.

Extraction procedure

As no extraction method is available for the glimepiride from the biological matrices, we have developed these two extraction procedures. These were followed and compared to the approach for the best suitable methods.

1. A 300 μl of the urine sample/300 μg spiked tissues sample (a portion of the kidney, liver, spleen, and intestine) were taken in a centrifugation tube, 4.5 ml of acetonitrile was added, and the resulting mixture was vortexed for 3 min and then centrifuged for 7 min at 3500 pm. The supernatant was taken to evaporate to the dryness.
2. A 300 μl of the urine sample/300 μg spiked tissues sample was taken in a centrifugation tube, 5 ml of 1-butanol: hexane (50:50, v/v) was added under an alkaline medium. The mixture was vortexed for 5 min and then centrifuged for 7 min, followed by back extraction into 5 ml acetic acid. The resulting supernatant was taken to evaporate to the dryness.

Extraction method 2 was found to be a capable and suitable method for the extraction of glimepiride in the biological matrices.

Method validation for the GC-MS

The proposed method was validated following Bioanalytical Method Validation by Food and Drug Administration (FDA) and Center for Drug Evaluation and Research (CDER) (Fda and Cder 2018).

Linearity

The calibration curve is made to determine the linearity of the proposed method in the range of concentration 500 ng/ml–2500 ng/ml. Calibration curves between the concentration of the glimepiride versus peak area were plotted.

Specificity

The specificity of the proposed GC-MS method was evaluated by testing 5 negative control. The procedure was done to inspect if there is any kind of intervention from the endogenous substance. Interference was

assessed by adding other commonly used prescribed medicines (biguanide: metformin, other sulphonylureas: glyburide and glipizide) to the negative control via monitoring the analytes at m/z 256 for glimepiride through GC-MS analysis.

Carryover

Carryover peaks might be ascertained after injecting glimepiride's higher concentrations. Blank samples were run after every injection of a higher concentration of glimepiride.

Stability

During method development, the determination of the chemical stability of the glimepiride in the biological matrices was important. The stability of derivatized glimepiride in autosampler was assessed under different conditions. Glimepiride's stability after the derivatization procedure was evaluated under distinct conditions: short term (in the autosampler at room temperature) and long term (up to 1 week at 4°). Inferences procured from these conditions were compared with freshly processed samples. Glimepiride was observed to be stable under all the mentioned conditions. Results attained after putting samples under distinct conditions were compared with the freshly processed samples.

Limit of detection and quantification

A signal to noise (S/N) ratio of 10 has taken to arbitrate the limit of quantification. Limit of detection was ascertained concerning the concentration of the analyte, for which signal to noise (S/N) ratio of 3 has taken.

Precision and accuracy

Precision and accuracy were tested at two distinct strata, i.e., interday and intraday evaluation. The variant concentration of glimepiride was spiked in the tissues (2500 ng/ml, 2000 ng/ml, 1500 ng/ml, 1000 ng/ml, and 500 ng/ml) and assessed on the very same day and after 1 week. The process was replicated five times.

Recovery

Recovery was investigated at five concentrations, i.e., 500 ng/ml, 1000 ng/ml, 1500 ng/ml, 2000 ng/ml, and 2500 ng/ml. The working solution of the respective concentration of glimepiride was spiked into the 300 μl of the blank viscera samples. Then the samples were extracted with the abovementioned procedure and derivatized for the sample preparation for GC-MS.

Robustness and ruggedness

During method development and validation, it is necessary to assess the ability of an analytical method to remain unaffected by minute variations in method parameters. So, Placket-Burman experimental design was used to test the robustness and ruggedness of the proposed GC-MS method for the viscera samples. We have used an 8-run design that is efficient and reliable. Four variables were selected to assess the robustness and ruggedness of the method (Table 1). Eight experimental runs and four significant variables were selected for the Placket-Burman experimental Design (Table 2). The peak area of glimepiride was considered as the response, and the statistical significance was determined using MS Excel software.

Result and discussion

Glimepiride should be converted to its thermally stable and volatile derivative to be analyzed by GC-MS. Therefore, two different approaches were used to derivatize glimepiride. Firstly, derivatization was approached via acylation employing MBTFA. We have used MBTFA to derivatize the glimepiride present in the sample at different temperatures and reaction time to introduce the acyl group to glimepiride and form a trifluoroacetic (TFA) derivative of glimepiride. But we have not observed any TFA derivative of glimepiride because of the production of acid-by products, which acts as a big source of interference, making acylation an incompetent approach to derivatize glimepiride before GC-MS analysis.

Secondly, derivatization was approached via silylation employing BSTFA to eliminate the problem of acid-by products produced during acylation. Introduction of silyl group to the glimepiride, specifically in substitution for active hydrogens present in the glimepiride to reduce the polarity of the compound and abates the hydrogen bonding. The derivatization process was performed under the presence of a distinct catalyst (pyridine, dimethylformamide, and dilute hydrochloric acid.) to obtain the results as BSTFA generally requires catalysts for the sterically hindered compounds. Even in the presence of catalysts, BSTFA did not produce any trimethylsilyl (TMS) derivative of glimepiride. With MSTFA, results were found to be suitable to approach the sound derivatization of

Table 1 List of variables for investigating robustness and ruggedness for GC-MS

S. No.	Variables	Low level (-)	High level (+)
1.	Helium flow rate	1 ml/min	1.2 ml/min
2.	Injection port temperature	240 °C	250 °C
3.	Derivatization reaction time	50 min	60 min
4.	Derivatization reaction temperature	90 °C	100 °C

Table 2 Plackett-Burman experimental design for GC-MS

Experiment No.	Variables			
	Helium flow rate	Injector port temperature	Derivatization reaction time	Derivatization reaction temperature
1	+	−	−	+
2	+	+	−	−
3	+	+	+	−
4	−	+	+	+
5	+	−	+	+
6	−	+	−	+
7	−	−	+	−
8	−	−	−	−

glimepiride. In this reaction, the silyl group was introduced to the glimepiride to form the TMS derivative. The probable reaction mechanism is summarized in Fig. 1. MSTFA is the consummate volatile of the TMS acetamides, and a powerful TMS donor even when the process of measuring is long time series. BSTFA and MSTFA are both silylation reagents, but MSTFA forms the TMS derivative of glimepiride, but BSTFA did not because of the volatility of MSTFA by-products. MSTFA is the most versatile and volatile TMS-amide available.

During optimization and method development, the signal strength, when derivatization of glimepiride processed at 80 °C, was found to be more than the signal strength observed at 100 °C. For the reaction time optimization for the derivatization process, the TMS derivative of glimepiride formed at 80 °C was found to give better intensity in the mass spectra when heated for 1 h. Ions were monitored at m/z 256 (Figs. 2 and 3) under these derivation conditions, viz, reaction temperature of 80 °C and reaction time of 1 h. The retention time of glimepiride is 3 min 58 s.

During the literature review, we found that no extraction method of glimepiride from biological matrices is available. We have developed two extraction methods for the extraction of glimepiride from the viscera samples. Generally, the viscera samples received at the forensic science laboratory contain a lot of fats, mucous, and other viscous substances, which makes the extraction process very exhausting and challenging. During the development of the extraction method, we have found that the 1st method using acetonitrile was not capable enough to vanish the emulsification that occurred during liquid-liquid extraction. However, in the 2nd method, wherein we have used 1-butanol: hexane (50:50, v/v) under alkaline medium including back extraction with acetic acid, we did not face such a problem, and thus, it is found to be the most suitable approach for the extraction of glimepiride from biological matrices.

Method validation

Linearity

The proposed method for the detection of glimepiride is found to be linear in the range of concentration 500 ng/ml to 2500 ng/ml with the value of R^2; Coefficient of determination at 0.9924 (refer to Fig. 4).

Specificity

Gas chromatograms and mass spectra obtained were assessed for the interference test at the retention time and m/z ratio. Interference from the endogenous substances and the similar type of drugs and other medicines (commonly used in the treatment of diabetes mellitus; metformin, glyburide, and glipizide) was not observed via

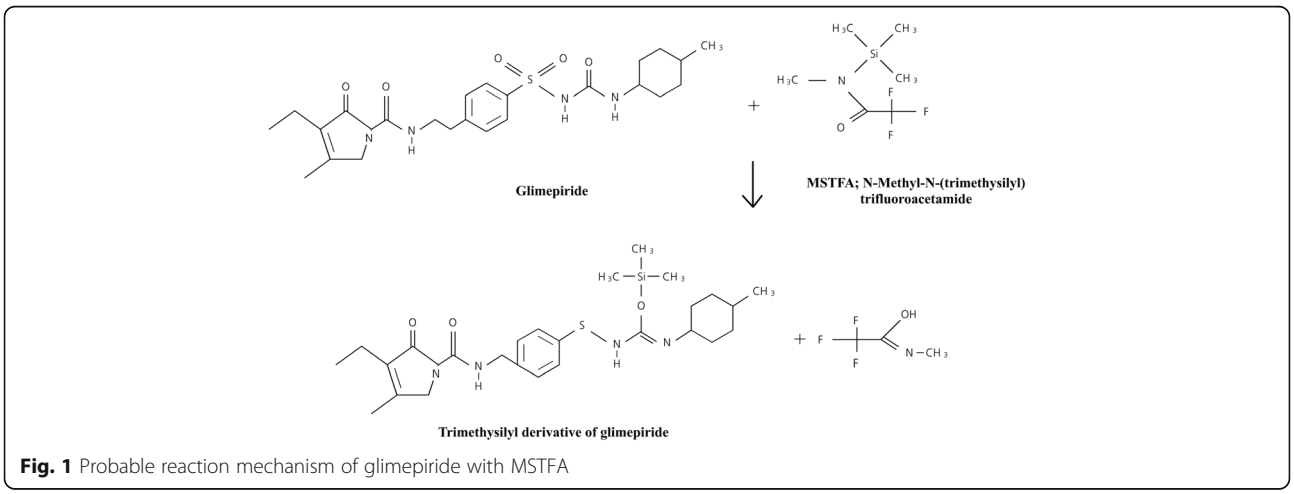

Fig. 1 Probable reaction mechanism of glimepiride with MSTFA

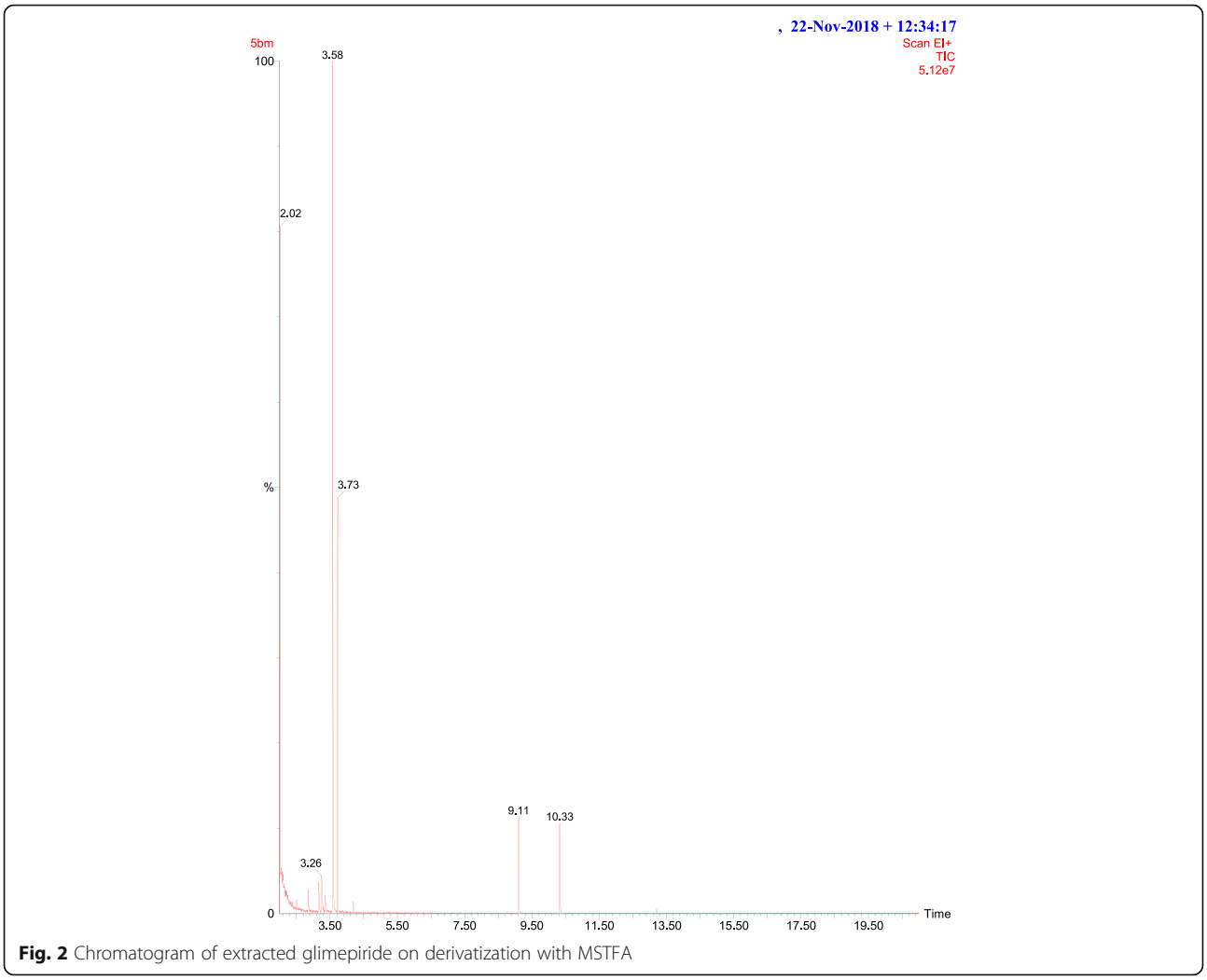

Fig. 2 Chromatogram of extracted glimepiride on derivatization with MSTFA

monitoring the analytes at m/z 256 for glimepiride through GC-MS analysis. Hence, the proposed method is specific.

Carryover

No carryover peaks were observed after glimepiride's high concentration injections.

Precision and RSD (related standard deviation) %

Inferences for the precision/RSD%, mean, the standard deviation for the GC-MS method is compiled in Table 3. The values for the interday and intraday precision (%) lie in the spectrum 0.70 to 1.003 and 0.74 to 0.99, respectively.

Stability and recovery

The stability of glimepiride in the biological matrices is examined via calculating the recovery by changing the sample's environment. Results demonstrate the glimepiride's stability after extraction and derivatization in the autosampler at room temperature and for the long term at 4 °C (and then analyzed by GC-MS). Recovery for the different concentrations lies in the range of 93.91 to 95.63% (refer to Table 4).

Robustness and ruggedness

Statistical significance was tested with the T test (one-tailed, type 2 error) and Plackett-Burman experimental design. It is used to recognize the effects of different variables on the peak area of the glimepiride. The results showed that the effect of the chosen variables on the response was not significant at $p > 0.05$. Henceforth, the selected variables for the Plackett-Burman experimental design showed no significant effects on the peak area of the glimepiride. Hence, the proposed method is robust and rugged.

Application of the derivatization and GC-MS method in forensic toxicology

The developed and validated GC-MS method can be employed in the pharmaceutical laboratories and

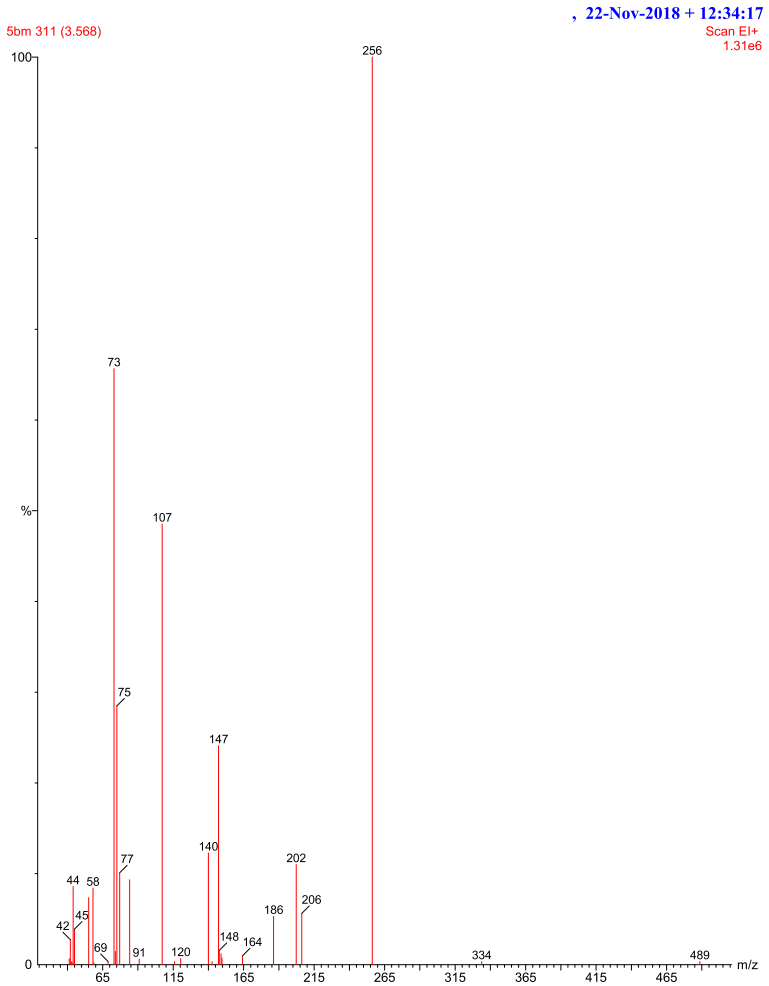

Fig. 3 Mass spectra of glimepiride on derivatization with MSTFA

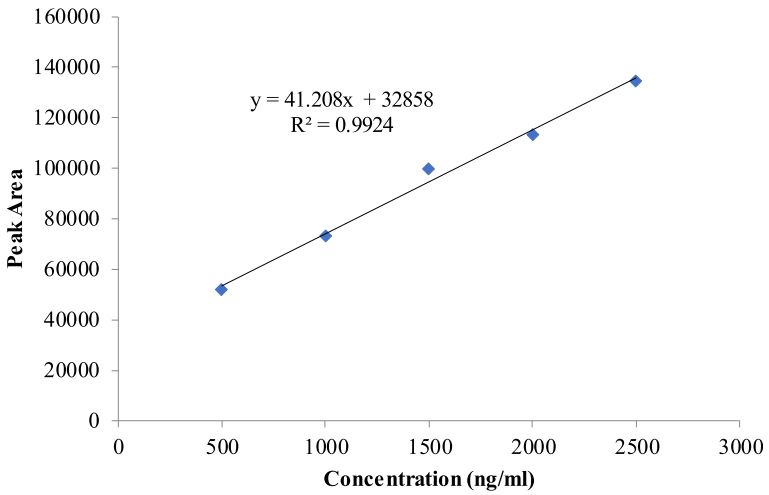

Fig. 4 Calibration curve between peak area and concentration of glimepiride

Table 3 Interday and Intraday precision results for GC-MS

Concentration (ng/ml)	Intraday			Interday		
	Mean (*x*)	*X* ± SD	Precision (RSD) (%)	Mean (*x*)	*X* ± SD	Precision (RSD) (%)
500	317.2	317.2 ± 3.162	0.74	315	315 ± 3.16	0.70
1000	996.4	996.4 ± 7.82	0.75	985.2	985.2 ± 7.85	0.77
1500	1327.6	1327.6 ± 11.01	0.82	1320.4	1320.4 ± 11.34	0.85
2000	1987.6	1987.6 ± 15.07	0.78	1980.2	1980.2 ± 15.4	0.79
2500	2314.4	2314.4 ± 17.32	0.99	2307.4	2307.4 ± 16.31	1.003

forensic science laboratories for the detection of glimepiride in the biological matrices and pharmaceutical preparations. During the post mortem, tissues and other biological matrices are collected specifically for the toxicological analysis, which is a complex and time-consuming process that requires sophisticated technique. These shreds of evidence majorly help investigate the cases and potential cause of death for the administration of law and justice in the court of law. From extraction procedure to derivatization approach to the confirmatory method, this research will help in toxicological and forensic analysis of glimepiride in viscera samples in the cases of drug-induced poisoning and drug abuse cases.

Conclusion

A derivatization method and the novel GC-MS method for the identification of glimepiride in biological matrices have developed. Distinct methods for the extraction of drugs from the matrices, i.e., viscera samples, were applied to find the appropriate approach. Before GC-MS testing, samples were converted to a volatile and thermally stable form via applying the silylation derivation technique. MSTFA was found to be the successful derivatization technique for the GC-MS analysis of glimepiride. Thus, the identification of glimepiride can be done by monitoring the ion at m/z 256. Henceforth, the novel GC-MS method was manifested and validated for the identification of glimepiride in biological matrices.

Table 4 Stability and recovery results for GC-MS

Concentration (ng/ml)	Recovery	
	Short term (3–5 h) at room temperature	Long term (1 week) at 4 °C
500	94.26	93.97
1000	94.66	94.03
1500	95.63	94.93
2000	94.22	94.07
2500	94.12	93.91

Abbreviations

GC-MS: Gas chromatography-mass spectrometry; MSTFA: *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide; TIC: Total ion current; IGF-I: Insulin-like growth factor-I; TLC: Thin layer chromatography; HPTLC: High-performance thin-layer chromatography; HPLC: High-performance liquid chromatography; BSTFA: Bistrimethylsilyltrifluoroacetamide; MBTFA: *N*-Methyl-bis(trifluoroacetamide); TFA: Trifloroacetyl; TMS: Trimethylsilyl; RSD: Relative standard deviation

Acknowledgements

Not applicable.

Authors' contributions

PV contributed to the conceptualization, investigation, data curation, formal analysis, and original manuscript writing. PV and AB contributed to the methodology. AB and RMT contributed to the project administration and resources. AB, RMT, SKS, and SN contributed to the supervision, validation, and visualization. The authors read and approved the final manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Ethical approval reference number CFSL/38(119)/Estt/4547, Central Forensic Science Laboratory, Directorate of Forensic Science Services, Ministry of Home Affairs, Govt. of India.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 21 February 2020 Accepted: 28 January 2021
Published online: 15 February 2021

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