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# Detection of Gliclazide in Aqueous Samples using Liquid Chromatography/Time-of-Flight/Mass Spectrometry

(Penentuan Gliklazida dalam Sampel Akues Menggunakan Kromatografi Cecair/Masa Penerbangan/Spektrometri Jisim)

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ABSTRACT

*The big challenge for the detection of pharmaceutical residues in water samples is the type of ionization mode in terms of positive or negative ionization which plays an important role to identify and quantify the analytes using liquid chromatography/mass spectrometry. An analytical method was applied to analysis of gliclazide (diabetic drug) in surface water and wastewater from sewage treatment plants and hospitals. The proposed analytical method allows simultaneous isolation and concentration procedure using solid phase extraction (Oasis HLB) prior to separation using high-performance liquid chromatography. The detection and confirmation was achieved by applying time-of-flight analyzer. The limits of quantification were as low as 1.4 ng/L (deionized water), 4 ng/L (surface water), 27 ng/L (hospital influent), 10 ng/L (hospital effluent), 6 ng/L (sewage treatment plant effluent) and 21 ng/L (sewage treatment plant influent), respectively. On average, good recoveries of higher than 87% were obtained for gliclazide in the studied samples. The proposed method successfully determined and quantified gliclazide in surface water and wastewater. The results showed that gliclazide is a persistent compound in sewage treatment effluents as well as in the recipient rivers. Gliclazide was detected in all samples and the highest concentration was 130 ng/L in influent of sewage treatment plant.*

*Keywords: Effluent hospital; gliclazide; positive ionization; solid phase extraction*

ABSTRAK

*Antara cabaran besar untuk mengesan sisa farmasi dalam sampel air menggunakan kromatografi cecair/spektrometri jisim adalah jenis mod pengionan, dengan pengionan positif atau negatif memainkan peranan yang penting untuk mengenal pasti dan menilai kandungan analit. Suatu kaedah analisis telah diaplikasikan untuk menganalisis gliklazida (ubat diabetik) dalam air permukaan dan juga air buangan dari loji rawatan kumbahan dan hospital. Kaedah analisis yang digunakan membolehkan prosedur pengasingan dan pemekatan dijalankan serentak menggunakan pengekstrakan fasa pepejal (Oasis HLB), iaitu teknik pemisahan dengan menggunakan kromatografi cecair berprestasi tinggi. Pengesanan dan pengesahan telah dicapai dengan menggunakan penganalisis masa penerbangan. Had kuantifikasi adalah serendah 1.4 ng/L (air ternyahion) 4 ng/L (air ternyahion), 27 ng/L (influen hospital), 10 ng/L (efluen hospital), 6 ng/L (efluen loji rawatan sisa kumbahan) dan 21 ng/L (influen loji rawatan kumbahan). Secara keseluruhannya, peratusan perolehan semula yang baik diperoleh untuk gliklazida dalam sampel yang dikaji iaitu melebihi nilai 87%. Kaedah yang dicadangkan berjaya menentukan secara kuantitatif kandungan gliklazida dalam air permukaan dan air sisa buangan. Hasil kajian menunjukkan bahawa gliklazida adalah sebatian kekal dalam efluen rawatan kumbahan dan juga di dalam sungai. Gliklazida telah dikesan dalam kesemua sampel dan kepekatan maksima ialah 130 ng/L dalam influen loji rawatan sisa kumbahan.*

*Kata kunci: Efluen hospital; gliklazida; pengekstrakan fasa pepejal; pengionan positif*

## INTRODUCTION

Pharmaceuticals have become recognized as relevant environmental contaminants in the course of the last decade. However, gliclazide is one of the diabetic-pharmaceuticals which are used to treat diabetes type 2 (non-insulin dependent diabetes mellitus) (Drugbank 2012). In Malaysia, gliclazide ($C_{15}H_{21}N_3O_3S$) was used increasingly in last few years hence; the consumption rate was 11373 kg/year in 2008 compared with 2005 which was 3083 kg/year (MOH 2012). Human pharmaceuticals enter the environment mainly through their therapeutic use. Metabolites of pharmaceuticals (parent or conjugated compounds) are excreted via urine or feces will play a main role to pollute the aquatic environment because the end point of all these compounds will be the surface water (Al-Aukidy et al. 2012; Grabic et al. 2012; Koo et al. 2010; Payán et al. 2010). Because of the high to mid polarity of pharmaceuticals, liquid chromatography (LC) is the best

choice to separate the individual compounds. To detect the pharmaceuticals in the environment, powerful analytical methods are needed because the very low concentration of these compounds in water (nano gram per liter – few micro gram per liter). For the detection purposes, modern advanced mass spectrometry is needed to discriminate between multiple pharmaceuticals and interfering matrix compounds and to obtain sufficient sensitivity. At present, a high selectivity, sensitivity and accuracy instrument was used for this purpose which is liquid chromatography/ time-of-flight/ mass spectrometry (LC-TOF/MS) (Ferrer & Thurman 2012; Martin et al. 2011; Petrovic et al. 2006). One challenge accompanied analysis of gliclazide in water is type of ionization mode either positive or negative modes. However, the chemical structure of analyte and functional group on the structure will help to determine the type of ionization. The aim of this study is to develop a sensitive and efficient method for the analysis of gliclazide in influent and effluent of wastewater as well as surface water using LC-TOF/MS.

## Materials and Methods

### Chemicals and Materials

Pure standard (≥98%) of gliclazide (GLZ) (CAS no. 21187-98-4), was purchased from Sigma-Aldrich (USA). Deionized water (DW) used was supplied by EASYPure RODI (USA). HPLC-grade methanol (MeOH), acetonitrile (ACN), acetone and formic acid (FA) were supplied by Merck (Germany). Methyl tertiary butyl ether (MTBE) was supplied by J. T. Baker (USA). The cartridges used for solid phase extraction were Oasis HLB (3cc, Waters, USA). Individual stock standard solutions (1000 mg/L) were prepared in HPLC-grade MeOH and stored at 18°C to minimize the degradation of the standard. A working solution of gliclazide standard was prepared by appropriate dilution of the stock solution. Further dilutions of the working solution were prepared in MeOH-DI (1:9, v/v) before each analytical run. Prior to use, all glassware were boiled with water at 100°C, rinsed with distilled water, dried in an oven at 200°C for 2 h, subsequently rinsed with MeOH, and dried in the oven at 200°C for 2 h.

### Sample Preparation

Samples of influent and effluent of sewage treatment plants (STPs) and hospitals (HSPs), and surface water (SW) were collected from Negeri Sembilan state which is one of the thirteen states in Malaysia as presented in Figure 1. All samples were collected in 1 L amber glass bottles with Teflon-lined and sequentially preserved by adding 1 g/L of sodium azide to prevent microbial degradation. Samples were filtered through a 0.7 µm GF/F filter from Whatman (UK) to remove particulate matter in water samples. The samples were stored at 4°C until SPE extraction, which was performed within 24 h in order to avoid any degradation. The extraction protocol was provided by Al-Qaim et al. (2014). SPE cartridges with different packing materials namely HLB, SAX, ENV and MCX were tested for the best recovery of the analyte. The cartridges were installed on a vacuum manifold and sequentially pre-conditioned with

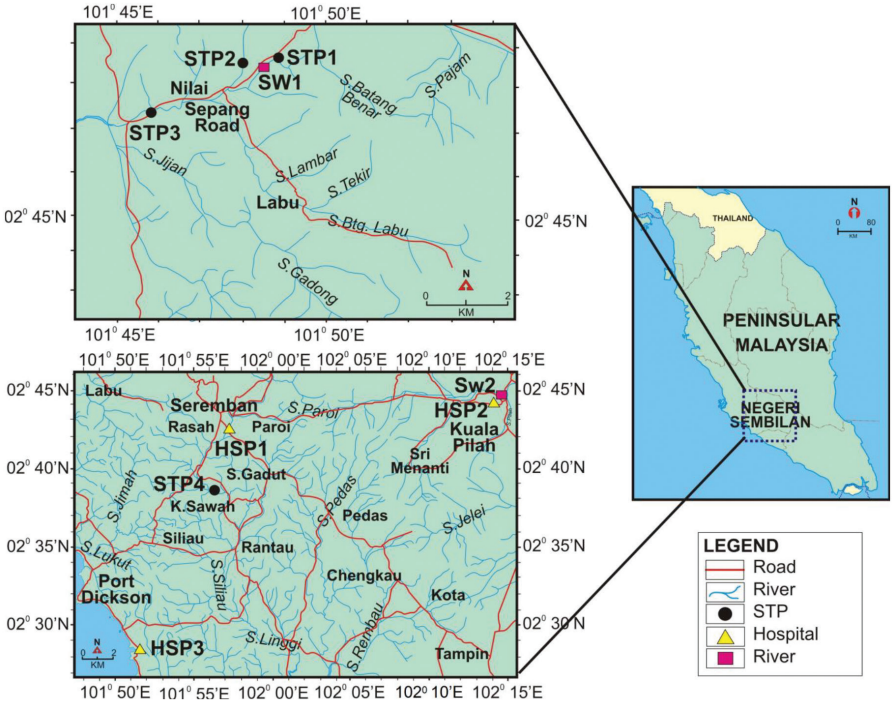


FIGURE 1. Map to describe all sampling points

2 mL MTBE, 2 mL MeOH, and 2 mL DI at flow rate 1.0 mL/min. Then, the samples (100 mL STP, HSP influent, 250 mL STP, HSP effluent and 1000 mL surface water) were passed through the cartridges at a flow rate of 9 mL/min under vacuum conditions. Subsequently, the cartridges were rinsed with 2 mL DI prior the elution. Cartridges were dried under vacuum at 15 mL/min for 25 to 30 min to remove residual water. Finally, cartridges were eluted using three successive aliquots of 5×1 mL MTBE, 2×1 mL acetone-MeOH (21:9, v/v), and 3×1 mL acetone-MeOH (9:21, v/v). The elute solution were collected in a 12 mL collection tube and concentrated to dryness under a gentle stream of $N_2$ gas. Dry extracts were reconstituted to 500 µL with MeOH-DIW (10:90, v/v) and then transferred to 250 µL deactivated glass insert with polymer feet inserted in amber glass vials from Agilent Technologies (USA). The extract (30 µL) was automatically injected into LC-ESI-TOF/MS system for analysis.

### LIQUID CHROMATOGRAPHY-TIME-OF-FLIGHT/MASS SPECTROMETRY

Gliclazide was separated using a Dionex Ultimate 3000/LC 09115047 (USA) system equipped with a vacuum degasser, a quaternary pump, and an autosampler. Sample aliquots of 30 µL were injected to 5 µm, 2.1 mm × 250 mm Thermo Scientific C18 column. ESI source was utilized as ionization source. Gliclazide was analyzed in positive ionization (PI) mode and eluted off the column with a mobile phase consisting of (A) 0.1% FA in DI and (B) ACN-MeOH (3:1, v/v) at 0.3 mL/min. The elution started at 5% B and was then linearly increased to 60% B over 3 min, further increased to 97% B over 3 min and then kept isocratic for 5 min. Next, the elution was returned to its starting conditions over 11.1 min and allowed to equilibrate for 5 min prior to the next run. Mass spectrometry was performed on ESI(+)/TOF instrument (µTOF-Q, Bruker/Germany). The results were obtained with the following settings: MS capillary voltages, 4000/3500 (PI/NI); drying-gas flow rate, 8.0 L/min; drying gas temperature, 190°C; and nebulizer pressure, 4.0 bar. The accurate mass was calculated using software *Daltonics DataAnalysis* incorporated in the instrument.

### METHOD VALIDATION

Gliclazide was identified based on its mass value (m/z) and retention times. Quantification was carried out using the TOF mode by extracting the narrow window extracted ion chromatogram (nwXIC) of the molecular ion for gliclazide (typically extracted using a 20 mDa window) compared with previous studies that used 20 and 50 mDa windows (Ferrer & Thurman 2012; Petrovic et al. 2006). Positive identification of gliclazide was based on (a) accurate mass measurement of the base ion with an error of –0.6 ppm for gliclazide which falls within the widely accepted accuracy compared with a previous study (Petrovic et al. 2006) and (b) LC retention time of the analyte compared with that of a standard with an error of ≤ ± 0.1% for gliclazide. The instrumental quantification limit (IQL) was estimated from the injection of a standard solution successively diluted until reaching a concentration level corresponding to an S/N ratio ≥10. Calibration curve was generated by injecting pooled solutions prepared from the standard solution (0.3 µg/L-3 mg/L). The curve was constructed for gliclazide by plotting the peak area of against the concentration of gliclazide using linear regression analysis and the concentration range that gave good fit (determination coefficients, $R^2 = 0.993$). The limit of quantification (LOQ) for the entire method in the different matrices was calculated using the following (1) (Vieno et al. 2006).

$$\mathrm{LOQ} = \frac{\mathrm{IOL} \times 100}{R\% \times \mathrm{CF}}, \quad (1)$$

where IQL is the instrumental quantification limit (ng/L); R% is is the recovery of the analyte in the corresponding matrix; and CF is the concentration factor (2000, 1000, 500 and 200 for DW, SW, STP and HSP effluent and STP and HSP influent, respectively). In order to determine the recoveries in different matrices, DW, SW, STP effluent and HSP influent were spiked with a standard solution of gliclazide and enriched using SPE. Six replicates were performed on the different days and the spiking level was 0.5, 1, 2 and 5 µg/L for DW, SW, STP effluent and HSP influent, respectively. The different spiking level was applied because different matrix sample was tested. The varying in spiking levels was due to type of the sample in terms of complexity of the sample so low spiking level for less complexity sample and vice versa. The recoveries were evaluated by comparing the solid phase extracted samples to non-extracted standard solutions (i.e. standards dissolved in the solvent). Recoveries (R%) was calculated based on (2) below.

$$R\% = \frac{A_{sp} - A_{un}}{A_{sol}} \times 100, \quad (2)$$

where $A_{sp}$ is the peak area of the analyte in the solid phase extracted sample; $A_{un}$ is peak area in the un-spiked water sample; and $A_{sol}$ is the peak area in the solvent.

## RESULTS AND DISCUSSION

### OPTIMIZATION OF IONIZATION MODE AND COLLISION ENERGY

The optimized modes of positive ionization (PI) and negative ionization (NI) were properly selected to quantify gliclazide in real samples. It was found that at positive ionization mode the signal to noise ratio of gliclazide was very high compared with negative ionization mode as presented in Figure 2(a).

This fact is approved by our previous study which means gliclazide was not detected in surface water and waste water samples using negative ionization analysis (Al-Qaim et al. 2014). However, the positive ionization mode

is the most appropriate choice to analysis of gliclazide in water samples.

Collision energy was studied from 2 to 30 eV to identify the optimum value for gliclazide. Figure 2(b) shows that signal to noise ratio for gliclazide was increased steadily until 10 eV then decreased with increasing the collision energy. Hence, the optimum collision energy is 10 eV. The variation in fragmentation of gliclazide at 10 and 30 eV was presented in Figure 2(c). At 30 eV, some fragmentations were observed in terms of extra peaks with different m/z which mean high collision energy force gliclazide to be more fragmented resulting to reduce limit of detection.

### EVALUATION OF DIFFERENT CARTRIDGES AND OPTIMIZATION OF ELUTING SOLVENT AND FLOW RATE OF SAMPLE LOADING

Different cartridges were used to study the recovery of gliclazide, including mixed-mode cationic exchange (MCX), Supelclean™ ENVI-Chrom P (highly cross-linked, neutral, specially cleaned styrene divinylbenzene co-polymer resin used to retain hydrophobic compounds with some hydrophilic functionality under reversed phase conditions), Oasis HLB cartridges (universal polymeric reversed-phase sorbent) and Supelclean™ LC-SAX (quaternary amine, $Cl^-$ counter-ion, ion exchanger and reverse–phase sorbent cartridge). As can be observed from Figure 3(a), the highest recovery was accompanied by Oasis HLB cartridge may due to the suitability of this sorbent to extract a wide range of acidic, basic and neutral compounds as reported in different previous papers (Gómez et al. 2007; Lajeunesse & Gagnon 2007; Petrovic et al. 2014; Weigel et al. 2004). Otherwise, MCX cartridge exhibited the lowest recovery to retain gliclazide at neutral pH. Gliclazide was extracted efficiently with MCX at acidic conditions may due to protonation of amino group on its structure as reported by previous study (Al-Odaini et al. 2010) to make it suitable for MCX cartridges.

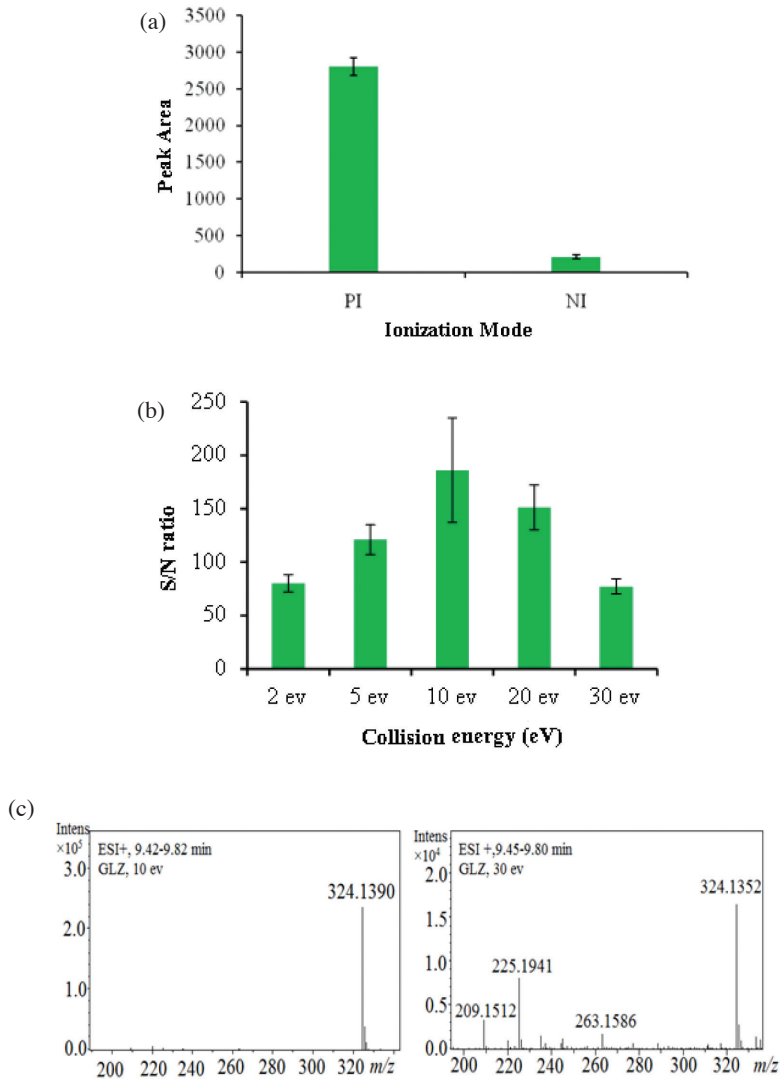


FIGURE 2. Optimization of (a) ionization mode (b) collision energy and (c) mass fragment for detection of gliclazide

The extraction of gliclazide using Oasis HLB cartridge requires an efficient elution solvent to elute it. Hence, four elution solutions were optimized by using combination of different ratio solvents namely elution A (Elu A), B (Elu B), C (Elu C) and D (Elu D). Elu A consist of mixture MTBE (5×1 mL) plus MeOH (5×1 mL); Elu B: DCM (5×1 mL) plus acetone-MeOH (21:9, v/v) (2×1 mL) plus acetone-MeOH (9:21, v/v) (3×1 mL); Elu C: MTBE (5×1 mL) plus acetone-MeOH (21:9, v/v) (2×1 mL) plus acetone-MeOH (9:21, v/v) (3×1 mL); Elu D: acetone-MeOH (21:9, v/v) (5×1 mL) plus acetone-MeOH (9:21, v/v) (5×1 mL). As noticed from Figure 3(b), Elu C was selected as the best eluent solvent in which gliclazide gave the highest recovery, wherein, the lowest recovery was achieved with Elu A and Elu D. Hence, a moderately polar such as acetone is considered more effective with non-polar solvent such as MTBE to get a good recovery for further analysis of real samples. Therefore, Elu C was selected as the best elution solvent.

Flow rate of loading was also considered in this study. Indeed, the principle of optimization of flow rate to elute the analytes from SPE cartridge is based on same principle of using chromatographic column i.e. a relation between flow rate and binding of analytes on sorbent. In order to evaluate this issue, three flow rates were tested: 1-1.5, 3-3.5 and 9-9.5 mL/min, respectively. The increasing flow rate will reduce the force between the analyte and the sorbent, then increasing the elution strength. Figure 3(c) shows the best recovery for gliclazide at 9-9.5 mL/min.

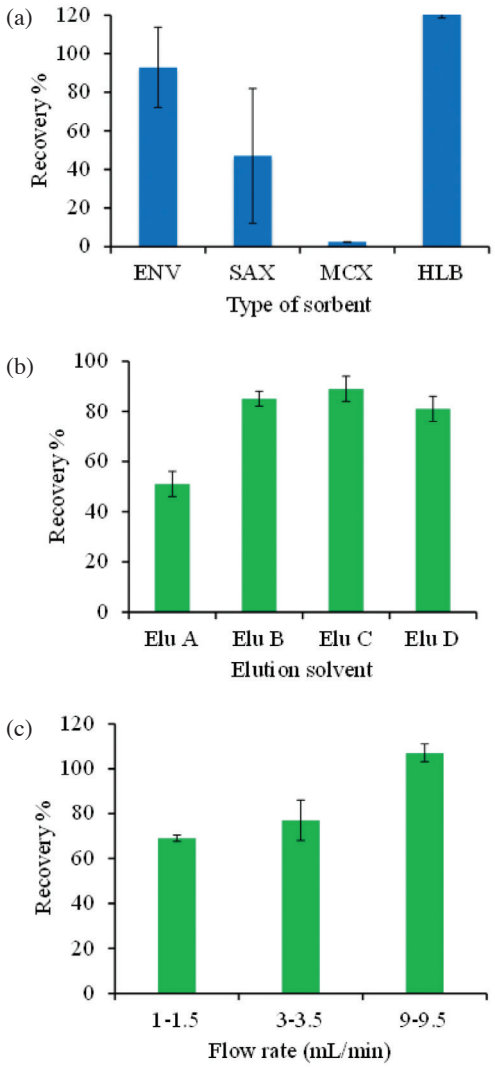


FIGURE 3. Detection of gliclazide based on selection (a) cartridge (b) elution solvent and (c) flow rate

## STUDY ON THE MATRIX EFFECTS AND EXTRACTION EFFICIENCY

The entire method was validated for DW, SW and the HSP and STP effluents and influents. Recovery studies were performed by spiking standard gliclazide to DW, SW, STP effluent and HSP influent at different concentration levels of 0.5, 1, 2 and 5 µg/L, respectively, because the water samples are different in terms of pollution level and matrix effects. Therefore, 0.5 µg/L was selected for very clean water (DW), in contrast 5 µg/L was selected to very polluted sample (influent of HSP and/or STP). The recoveries and other validation parameters are presented in Table 1.

Gliclazide was relatively well-recovered in all sample matrices (% Recovery ≥ 72%) for all samples except for influent in both STP and HSP which was 142 and 45%, respectively. The differences in the recoveries among sample matrices are considered reasonable because the samples are varied in terms of level of pollution. Thus, this method can be considered acceptable for extracting gliclazide using solid phase extraction as compared with other previous studies (Al-Odaini et al. 2010). Evaluation on the matrix effects (ME%) on signal intensity using SPE extracts of water samples spiked with pharmaceuticals (spike level of 200 µg/L). The spiked samples were injected to LC-ESI-TOF/MS. The matrix effect was calculated using (3).

$$ME\% = \frac{A_{sp} - \left(A_{sp} - A_{un}\right)}{A_{sol}} \times 100, \qquad (3)$$

where $A_{sol}$ is the peak area of the analyte in the solvent; $A_{sp}$ is the peak area in the spiked matrix extract (after drying with $N_2$ gas prior to injection); and $A_{un}$ is the peak area in the un-spiked matrix extract. In this procedure, the analyte losses caused by ionization can be evaluated, but any losses caused by SPE and further sample preparation are excluded. From (3), the effect of sorbent is negligible, so that only the effect of matrix is considered. All results were presented in our previous study (Al-Qaim et al. 2010), which indicates a varying matrix effects ranged from 31-66% was observed based on the type of sample.

## METHOD VALIDATION

The linearity of the external calibration curve was evaluated for gliclazide standard by statistical methods measuring the coefficient of determination ($R^2$), which quantify the goodness of fit of the linear regression. The LC-ESI-TOF/MS method exhibits excellent linearity ($R^2$ =

0.993) for gliclazide with range of 0.3 µg/L to 3 mg/L. Gliclazide showed low instrument quantification limits (IQL) in the aqueous samples using positive ionization mode compared to negative ionization mode which exhibits high IQL. The IQL was determined to be the concentration with S/N ratio ≥10 which is 0.3 µg/L. The LOQs over the entire method were calculated using (1), it was ranged from 1.4 to 27 ng/L in all water samples as shown in Table 1. Higher LOQs were observed for gliclazide in STP and HSP influent and effluent because of the signal suppression within these matrices.

## ANALYSIS OF REAL SAMPLES

In order to evaluate the applicability of the developed method, water samples collected from STP influent and effluent, HSP influent and effluent and surface water were analyzed. Figure 4 shows the powerful of LC-TOF/MS in terms of the extracted ionic chromatogram (ESI) of gliclazide against total ionic chromatogram (TIC).

Results of real samples analysis are presented in Figure 5. Gliclazide was found in surface water at relatively high concentrations of 15 ng/L. The lowest detected concentration was 6 ng/L in the surface water. For the

TABLE 1. Method validation parameters

| Method validation | | | | | | |
|---|---|---|---|---|---|---|
| | Type of samples | | | | | |
| | DW | SW | INF STP | EFF STP | INF HSP | EFF HSP |
| LOQ (ng/L) | 1.4 | 4 | 21 | 6 | 27 | 10 |
| Recovery % ± SD $n = 6$ | 93±18 | 72±19 | 142±8 | 75±28 | 45±11 | 97±14 |
| Instrumental validation parameters | | | | | | |
| Linearity ($R^2$) | Equation | | | | IQL (µg/L) | |
| 0.993 | y = 2687× – 9402 | | | | 0.3 | |

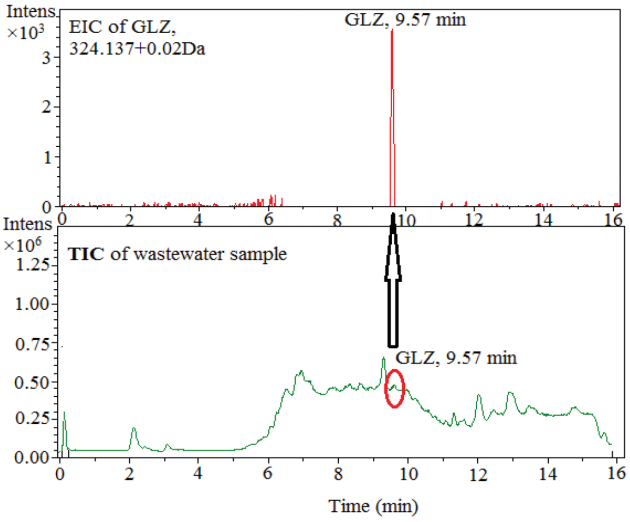


FIGURE 4. Extracted ion chromatogram (EIC) *vs* total ion chromatogram (TIC) for analysis of gliclazide in real sample using LC-ESI (+) TOF/MS

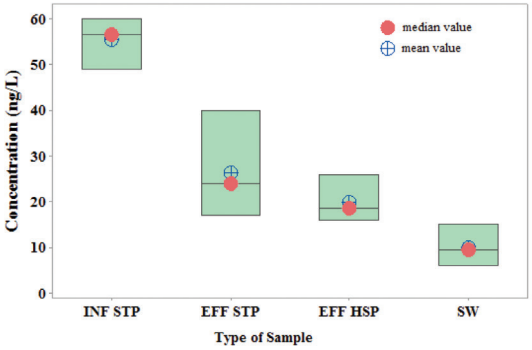


FIGURE 5. Concentration (ng/L) of gliclazide in all water samples using PI mode

hospital effluents, gliclazide was detected at the highest concentrations of 26 ng/L. The lowest concentration detected was 16 ng/L. In case of effluent of sewage treatment plants, the range of concentration was 17-40 ng/L while in the influent of sewage treatment plant, the range of concentration was 49-60 ng/L. These results showed gliclazide was persistent after treatment either in sewage treatment plants (ditch and pond oxidation treatment) or in hospital sewage treatment plants (biological treatment). In our previous study, gliclazide was not detected in all water samples using negative ionization mode, hence the high frequency of detection for gliclazide may be attributed to the best selection of ionization which means positive ionization mode in this present study (Al-Qaim et al. 2014, 2013).

## Conclusion

A methodology using LC-TOF/MS for the analysis of gliclazide was developed and successfully applied to determine gliclazide in different environmental aquatic samples. Ionization mode is considered a power tool to enhance the detection limit. Consequently, gliclazide was detected in positive ionization mode but it was not detected in negative ionization mode. TOF/MS is a very sensitive detector and can detect extremely low concentrations (ng/L) in actual samples with high accuracy and high resolution in terms of m/z value. LOQ varied broadly depending on the water sample which is ranged from 1.4 to 27 ng/L.

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