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High-throughput bioanalytical method using automated sample preparation and liquid chromatography–atmospheric pressure ionspray mass spectrometry for quantitative determination of glybenclamide in human serum

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Abstract

A liquid chromatographic–mass spectrometric (LC–MS) method with rapid automated sample preparation was developed and validated for determination of glybenclamide in human serum. Glybenclamide and its deuterated labelled internal standard were extracted from human serum samples by automated solid-phase extraction. The extract was injected into the LC–MS system for analysis. Glybenclamide and its internal standard were measured in multiple ion monitoring mode. The method was validated over a range of 10–1000 ng/ml with good accuracy and precision and was applicable for pharmacokinetic studies.

Keywords: Glybenclamide

1. Introduction

Glybenclamide (also known as glyburide) is a compound from a family of second generation sulfonylurea agents used for treatment of adult onset diabetes. Unlike the earlier class of antidiabetic agents, glybenclamide is administered in lower doses and is cleared from the body quickly, as soon as 5 h. Because of low serum glybenclamide concentration, analytical methods must be specific and sensitive enough to adequately measure low ng/ml levels. An HPLC–MS method using a single quadrupole mass spectrometer reported a lower limit of quantitation of 50 ng/ml [1,2]. More recently, an HPLC–MS–MS method using a triple quadrupole reported a lower limit of quantitation of 1.01 ng/ml [3].

LC–MS, in particular, LC–MS–MS, has become one of the most sensitive and selective analytical technique for quantitative analysis of drugs and metabolites in biological fluids. MS–MS has the obvious advantage over MS in the increased level of selectivity and less chemical noise, hence, higher signal-to-noise ratio. However, MS–MS is less sensitive because a large portion of the ion transmission is lost during fragmentation from parent to products. In addition, tandem MS instruments are more expensive than single quadrupole mass spectrometers. Although single quadrupole mass spectrometers are less selec-

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tive than the triple quadrupoles, the selectivity of single MS mode can be improved by judicious selection of the ion to be monitored to ensure that no background ion contributes to the chosen m/z value.

We have developed and validated a method using HPLC-single quadrupole mass spectrometer to quantitate glybenclamide using low serum sample volumes (200 μl). LC–MS–MS was not necessary because LC–MS was capable of providing an adequate method for the intended purpose. The LC–MS method has a lower limit of quantitation of 10 ng/ml which was adequate to provide pharmacokinetic dosing profile from patients dosed as low as 7.5 mg of Semi-Daonil®, a glybenclamide product available in Europe. We demonstrate here that with some scientific rationale in method development, sensitive and selective bioanalytical methods can be obtained and validated with intra- and inter-assay precision and accuracy under 15% using single quadrupole mass spectrometer.

2. Experimental

2.1. Reagents and chemicals

All solvents were HPLC grade and were purchased from EM Sciences, Merck KGaA (Darmstadt, Germany). Glybenclamide was purchased from Sigma (St. Louis, MO, USA). Isotopically labeled glybenclamide (D_{11}-cyclohexylamine-glybenclamide) was used as internal standard and was purchased from Cambridge Isotopes Laboratories (Andover, MA, USA). Blank human serum free of glybenclamide was purchased from Bioreclamation (Hicksville, NY, USA).

2.2. Equipment

The chromatography consisted of an Inertsil ODS3 (2.1×50 mm I.D., 3 μm; Metachem, Torrance, CA, USA) analytical column with a guard column (2.1× 10 mm I.D., 3 μm). The HPLC system was a Shimadzu LC-10ADvp pump with an SCL-10ADvp controller (Shimadzu, Columbia, MD, USA). The autosampler was a Shimadzu SCL-10AD (Shimadzu, Columbia, MD, USA). The mass spectrometer was a Sciex API 150 EX (PE Sciex, Concord, ON, Canada) single quadrupole equipped with Turbo Ionspray source interface. Total ion chromatograms from HPLC separations were collected in multiple ion monitoring mode using PE-Sciex Mass Chrom 1.1 software (PE Sciex, Concord, ON, Canada). The peaks were integrated using data processing software package MacQuan 1.6 (PE Sciex, Concord, ON, Canada). Statistical regression and macro analyses were performed with an in-house validated software.

2.3. Standard and quality control (QC) sample preparation

All standard and QC samples were prepared manually by spiking glybenclamide solution into human serum. Stock solutions of glybenclamide were prepared at a nominal concentration of 400 μg/ml in acetonitrile. From the stock solutions, dilutions were made in acetonitrile–water (10:90, v/v) to obtain working standard solutions of 20 μg/ml. An internal standard stock solution containing isotopically labeled glybenclamide was prepared at a nominal concentration of 50 μg/ml in acetonitrile.

The calibration curve consisted of seven concentrations ranging from 10 to 1000 ng/ml. The calibration standard solutions were prepared by manually pipetting specified volumes of glybenclamide working standard solution (20 μg/ml) into standard volumetric flasks and diluting to mark with human serum to obtain nominal concentrations of 10, 20, 44, 100, 200, 440 and 1000 ng/ml. QC samples were prepared by pipetting specified volumes of glybenclamide working standard solution from a separate weighing into standard volumetric flasks and diluting to mark with human serum. QC samples at four levels with nominal concentrations of 10, 30, 100 and 1000 ng/ml (VC1, VC2, VC3 and VC4, respectively) were prepared for validating the method. For batch analyses of clinical study samples, QC samples at three levels with nominal concentrations of 30, 100 and 800 ng/ml were prepared. In a pharmacokinetic study of 600 samples, a batch of standard and QC samples consisting of 25 ml at each

concentration was prepared, stored frozen at −70 °C and was used throughout the study.

2.4. Sample processing

For LC–MS analyses, all study samples, standards and QC samples were processed using automated solid-phase extraction (SPE) in a 96-well 100 mg Isolute C_{18}(EC) SPE block (International Sorbent Technology, Ystrad Mynach, Mid Glamorgan, UK). The automation system consisted of a central Zymark robotic arm (Zymark Corporation, Hopkinton, MA, USA) and several components: a Tecan RSP 8051 sample processor and liquid dispensing system (Tecan US, Research Triangle Park, NC, USA), a Zymark Rapid Plate (Zymark Corporation, Hopkinton, MA, USA) and a custom built SPE station consisting of a reagent addition station (RAS) and a vacuum manifold fitted for a 96-well plate. The procedure consisted of six steps. First, the 96-well block was conditioned with 400 μl acetonitrile per well. Second, the 96-well block was conditioned by 400 μl water per well. Third, the wells were conditioned each with 300 μl acetic acid (0.1%). The RAS dispensed the reagents into the wells and the vacuum manifold eluted the liquid from the wells. Following plate conditioning by the RAS, the 96-well plate was transferred by the Zymark robotic arm to the Tecan system, which was equipped with four tips. In the fourth step, four aliquots of internal standard (I.S.) solution (100 μl of 200 ng/ml in 0.1% acetic acid) were aspirated in parallel along with four aliquots of serum samples (200 μl of either study samples, standards or QC samples) and dispensed simultaneously into individual wells by Tecan tips. After dispensing was complete, the 96-well plate was transferred by the arm to the vacuum manifold. In the fifth step, the serum samples were eluted by vacuum and each well was washed with 300 μl acetic acid (0.1%). In the sixth step, the final extracts were obtained by elution with 300 μl acetonitrile–water (80:20, v/v) into a 1-ml deepwell 96-well plate. The extracts were evaporated under a heated gentle stream of nitrogen. The dried extracts were then reconstituted with 300 μl acetonitrile–water (10:90, v/v), sealed with a mat and vortexed prior to analysis by LC–MS.

2.5. Chromatographic conditions

An analytical column (2.1×50 mm) with a guard column (2.1×10 mm) packed with 3 μm Inertsil ODS-3 was used for chromatographic separation. The mobile phase consisted of acetonitrile–ammonium acetate buffer, 5 m*M* (45:55, v/v) without any pH adjustment and was operated isocratically at a flow-rate of 0.4 ml/min. The column eluent was connected into a 4-port switching valve (Valco, Houston, TX, USA) before entering into the mass spectrometer source. Immediately after injection of a sample (typically, 30 μl), the valve toggled to divert the column eluent to waste for the first 2 min, thus minimizing the amount of unretained matrix interferences from building up inside the mass spectrometer source. While column eluent was diverted to waste, a solvent consisting of acetonitrile–water (50:50, v/v) flowing at 0.2 ml/min was diverted to the mass spectrometer source. After 2 min, the valve toggled so that the column eluent now entered into the mass spectrometer source with glybenclamide and internal standard eluting simultaneously at about 2.5 min.

2.6. Mass spectrometric conditions

The eluent was directed to a Turbo Ionspray heated at 450 °C using air as the nebuliser gas and nitrogen as the auxiliary gas. The capillary electrode was set at negative 4500 V to generate glybenclamide and internal standard molecular ions $[M–H]^-$ at m/z 492 and 503, respectively. Orifice voltage and initial rod offset were optimized for ion optic potentials that yielded the most abundant $[M–H]^-$ ions. The quadrupole was calibrated with solutions of polypropylene glycol and the ion optics were adjusted to operate at unit mass resolution. Optimization and calibration were achieved with PE Sciex LCZ Tune (PE Sciex, Concord, ON, Canada) software to obtain a state file for mass spectrometric conditions.

2.7. Data analysis

Glybenclamide and internal standard were monitored at m/z 492 and 503 with a dwell time of 500

ms each. The peaks were collected using PE-Sciex Sample Control (PE Sciex, Concord, ON, Canada) software. At the end of data collection, the chromatograms were processed with PE Sciex processing software MacQuan 1.6. Calibration curves were constructed by plotting peak area ratios of glybenclamide to its internal standard against concentration, using a weighted $(1/x)$ linear regression model. Concentrations of study samples and QC samples were interpolated using two calibration curves that bracket the batch analysis. A one-way statistical analysis of variance (ANOVA) for a random model was performed using an Excel macro. Accuracy was expressed as % bias. Precision was calculated by variance component analysis, a standard statistics technique. Precision was expressed as two components: intra- and inter-day. Intra-day precision is the pooled variability (standard deviation) of replicates within each occasion. Inter-day precision is the variability among the four occasions.

3. Results and discussion

3.1. Mass transition selection

Full scan spectra of glybenclamide were obtained in positive and negative ion modes using Turbo Ionspray mass spectrometry. Fig. 1 shows the full scan spectrum of glybenclamide in positive ion mode. Protonated molecular ion $[M+H]^+$ of glybenclamide appeared at m/z 494 as well as a fragment ion at m/z 369. The fragment ion m/z 369 was produced in-source as its abundance increased at higher orifice voltages. Because these two different mass transitions (494 or 369) can be used to monitor for glybenclamide, selection of the appropriate mass depends on presence of other matrix interferences occurring at the same mass transition and chromatographic retention as glybenclamide and its internal standard. Therefore, serum from various sources were processed by solid-phase extraction and injected into the LC–MS system to evaluate for any matrix interferences at these transitions. There were matrix interferences coeluting with glybenclamide at m/z 494. However, no interferences were observed from serum extracts at m/z 369. The fragment ion with m/z 369 results from the loss of the cyclohexylamine moiety containing the isotope labels. Therefore, the internal standard molecule produces the same fragment ion with m/z 369. Because of this, glybenclamide and its internal standard could not be distinguished if monitored at m/z 369. Structures of glybenclamide and its internal standard are shown in Fig. 2. A full scan spectrum of glybenclamide was obtained in negative ion mode as shown in Fig. 3. Again, the serum extracts were injected and no matrix interferences were observed to coelute with glybenclamide at m/z 492, corresponding to deprotonated molecular ion $[M-H]^-$. The mass transition corresponding to the internal standard at m/z 502 was also monitored and no interferences from serum extracts were observed to coelute. Total ion chromatograms of an extract from a glybenclamide-free human serum at m/z 494 and 502 are shown in Fig. 4.

3.2. Chromatographic separations

In tandem MS, analytes exhibit unique mass transitions from parent to product. Because of the specificity of tandem MS, there is a tendency to reduce chromatographic capacity factor to elute the analyte as quickly as possible. However, it has been described that to obtain good accuracy and precision in LC–MS–MS electrospray methods, higher capacity factor (k') may be needed to separate analyte away from unobserved matrix interferences that otherwise would compete for ionization [4]. In LC–MS, where a single mass transition is monitored, interferences were observed near the void volume at m/z transitions 492 and 502. Therefore, the solvent strength was adjusted for glybenclamide and its internal standard to elute shortly after interferences have eluted near solvent front. In this method, shortly after 2 min, all the interferences with m/z transitions 492 and 502 have eluted. Therefore, a solvent strength of about 45% acetonitrile was adjusted to elute glybenclamide and its internal standard at about 2.5 min. A representative total ion chromatogram of a serum extract spiked at 100 ng/ml is shown in Fig. 5.

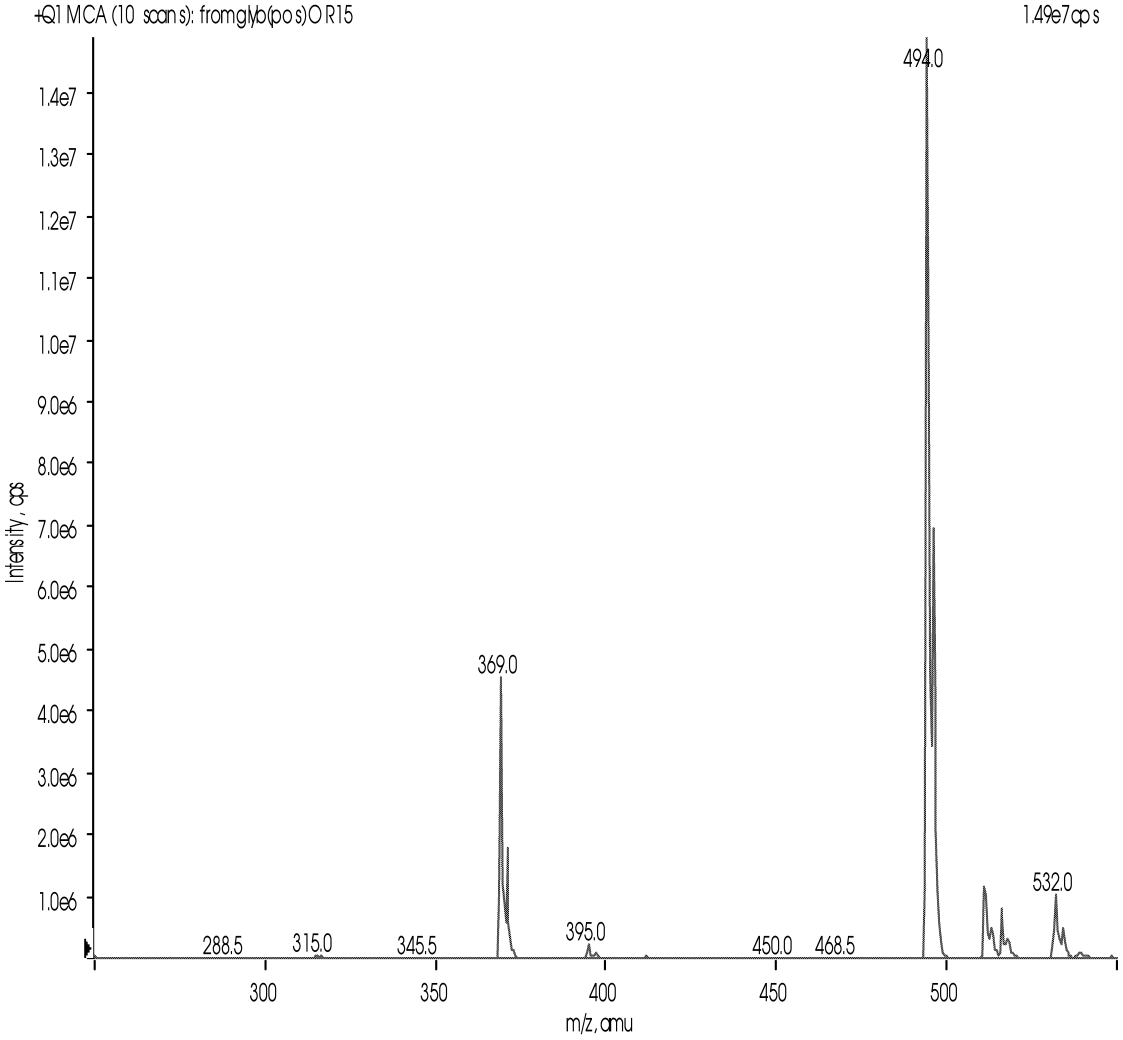


Fig. 1. Full scan spectrum of glybenclamide in positive ion mode.

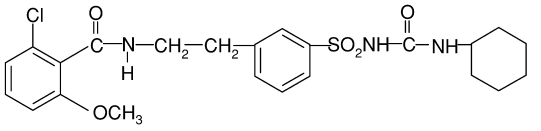
3.3. Mass ionization enhancement

Glybenclamide is a weakly acidic compound with a pK_a of 5.3. At a pH above its pK_a, the amine on the sulfonyl group deprotonates and the molecules convert from neutral to anionic species in solution. By maintaining the solution pH above its pK_a, glybenclamide molecules in solution are anionic in nature and thus aid in creating more anionic species in gas phase. Therefore, the mobile phase was maintained at a pH above its pK_a to enhance ionization and sensitivity. A signal enhancement of about 50% was observed for glybenclamide molecular ions when a mobile phase with pH above its pK_a was used.

3.4. Sample preparation and processing

Good sample preparation of biological samples is essential to perform LC–MS–MS. It has been dem-

Glybenclamide



Glybenclamide Internal Standard

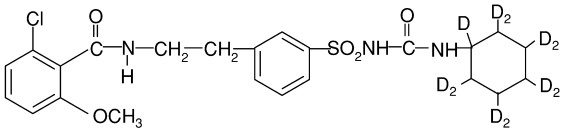


Fig. 2. Structures of glybenclamide and its isotopically labeled internal standard.

onstrated that lack of appropriate sample preparation can lead to signal suppression and poor accuracy and precision [4,5]. SPE is an effective technique to clean up samples. It is also available in 96-well format for high throughput parallel sample processing by commercially available automated workstations (e.g. Zymark, Tecan). Because the SPE (Isolute C_{18}) used has a hydrophobic octadecyl phase bonded on silica particles, appropriate buffer condition for sample loading was chosen to achieve higher retention on the bed. At a pH below the pK_a of glybenclamide, the analyte is neutral, which enhances its retention on the SPE bed while other matrix components are washed away. Therefore, a volume of acetic acid buffer containing internal standard is mixed with an equal volume of serum sample prior to loading on the conditioned SPE bed. After loading the sample, an aliquot of acetic acid

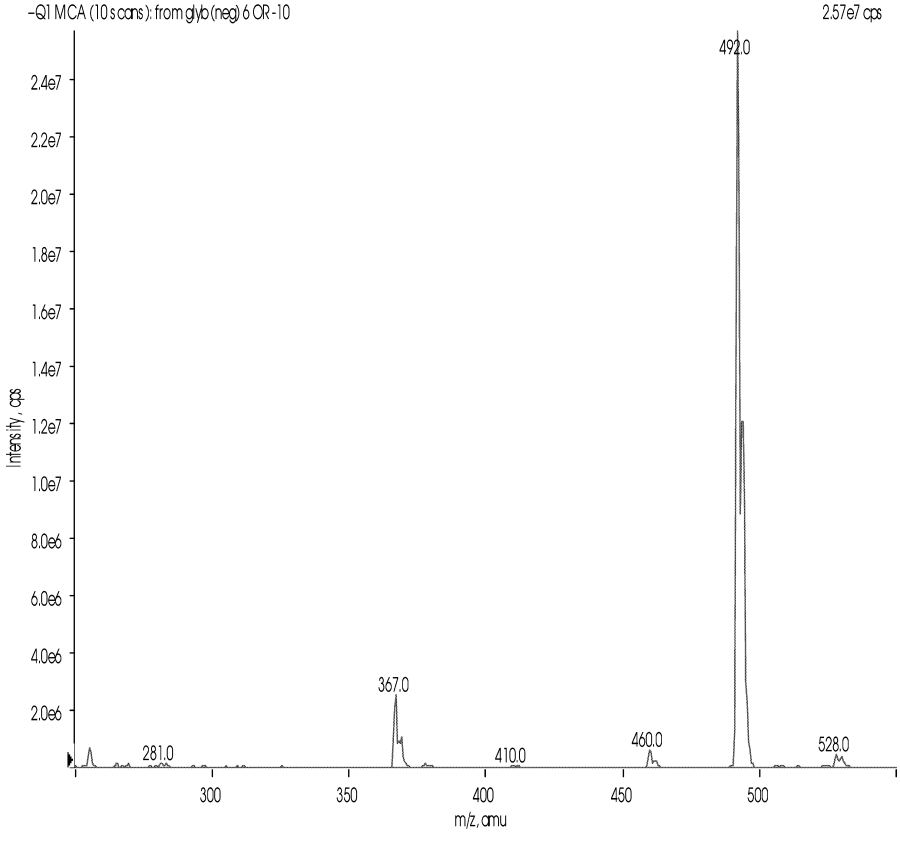


Fig. 3. Full scan spectrum of glybenclamide in negative ion mode.

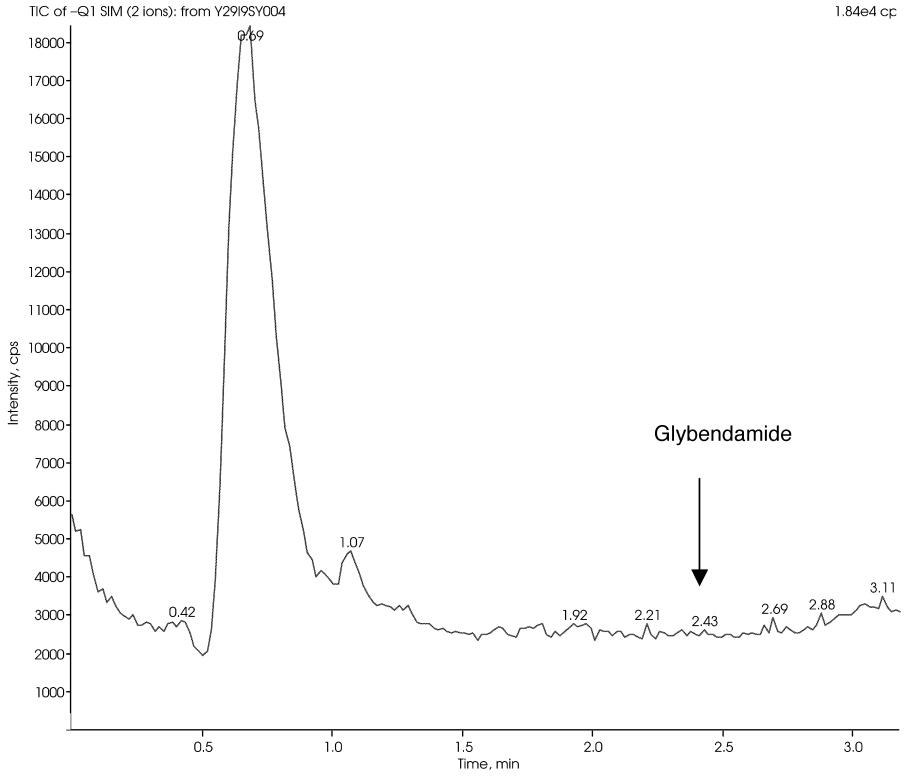


Fig. 4. Total ion chromatogram of a glybenclamide-free human serum extract monitored in negative ion mode at m/z 494 and 502.

buffer was used to wash other matrix components away followed by an aliquot of solvent (80% acetonitrile–20% water) to elute the analyte.

3.5. Method validation

A method is validated when aspects such as specificity, accuracy, precision and stability at intended storage and processing conditions meet minimum acceptable criteria (e.g. accuracy and precision $\leq$15%) [6]. Four separate runs were executed to assess specificity, intra and inter-day precision and accuracy, as well as limit of quantitation and linearity of the range. Each run consisted of a 96-well plate with two standard curves, one at the beginning and one at the end, interspersed with quality control (QC) samples.

3.5.1. Specificity

Human serum samples were obtained from six volunteers free of glybenclamide. The samples were processed and analyzed by LC–MS. Acceptable specificity was noted for all six samples from lack of any ion current trace at the same retention time as glybenclamide. A representative total ion chromatogram trace from a human serum extract is shown in Fig. 4.

3.5.2. Accuracy and precision

Six replicates of glybenclamide samples at four different concentrations, VC1 (10.4 ng/ml), VC2 (29.9 ng/ml), VC3 (104 ng/ml) and VC4 (996 ng/ml) were analyzed in each run. The values obtained for accuracy and precision is listed in Table 1. In four runs, the average inaccuracy for each

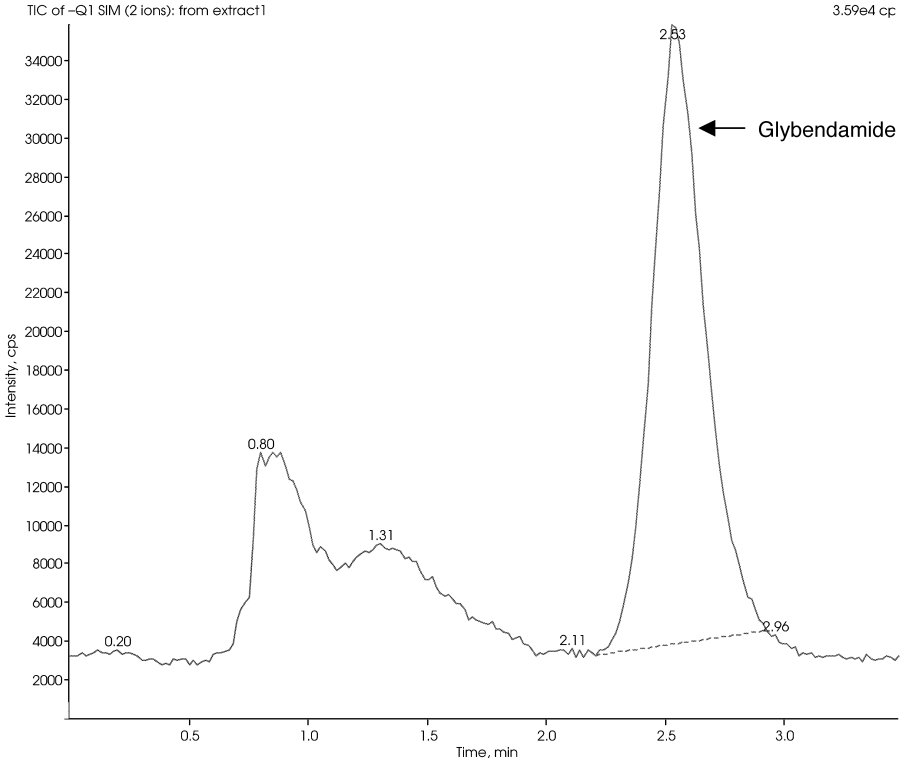


Fig. 5. Total ion chromatogram of a processed human serum extract at 100 ng/ml monitored in negative ion mode at m/z 494 and 502.

concentration level was <15%. The intra and inter-day precision over four runs were <15%. Table 1 shows the values obtained for inter and intra-day precision and accuracy for the four VC levels.

3.5.3. Linearity

The calibration curve consisted of seven standards ranging from 10 to 1000 ng/ml. Linear curves were generated by linear least-squares regression analysis according to the equation $y = a + bx$, where y was the peak area ratio of glybenclamide to its internal standard, x was the concentration of glybenclamide, a was the intercept and b was the slope of the line. Using a $1/x$ linear fit, the equation of line from eight runs (16 standard curves) can be expressed as $y = 0.0002740 + 0.0060597x$ with an intercept standard

Table 1
Accuracy, intra- and inter-day precision of VC1, VC2, VC3 and VC4

	VC1 (10.4 ng/ml)	VC2 (29.9 ng/ml)	VC3 (104 ng/ml)	VC4 (996 ng/ml)
Accuracy (% bias)	−0.1	−3.9	−8.1	−6.9
Intra-day precision (%)	10.0	9.2	6.7	6.8
Inter-day precision (%)	Negligible[a]	Negligible	3.3	2.9

[a] Negligible means variance estimate is negative.

error 0.00726454 and a slope standard error 0.00007330.

3.5.4. Limit of quantitation

The lowest concentration in this method was 10 ng/ml. After sample processing and analysis by LC–MS, the lowest standard typically gave a peak of signal-to-noise (peak-to-peak noise) of about 20. This suggests that even lower concentration can be detected and quantified if needed. A single ion monitoring trace at m/z 492 of a serum extract spiked at 10 ng/ml is shown in Fig. 6.

3.5.5. Stability

Glybenclamide is shown to be stable under conditions of storage and processing. It is stable in serum extract at room temperature for at least 24 h. The analyte is also stable in human serum when stored at −70 °C for at least 10.5 months and at room temperature for at least 24 h. It is stable under the influence of three freeze–thaw cycles. Table 2 shows the stability data of glybenclamide subsequent to various storage conditions and freeze thaw cycles.

Table 2
Stability data for glybenclamide in human serum

Conditions	% Recovery[a]
Storage in serum at RT for 24 h	96
Storage in serum at −70 °C for 10.5 months	99
Storage in processed serum extract at RT for 24 h	105
Freeze–thawed three times	98

[a] % Recovery is expressed as the response of glybenclamide stored in serum following stability conditions compared to the response of glybenclamide freshly spiked in serum. RT, room temperature.

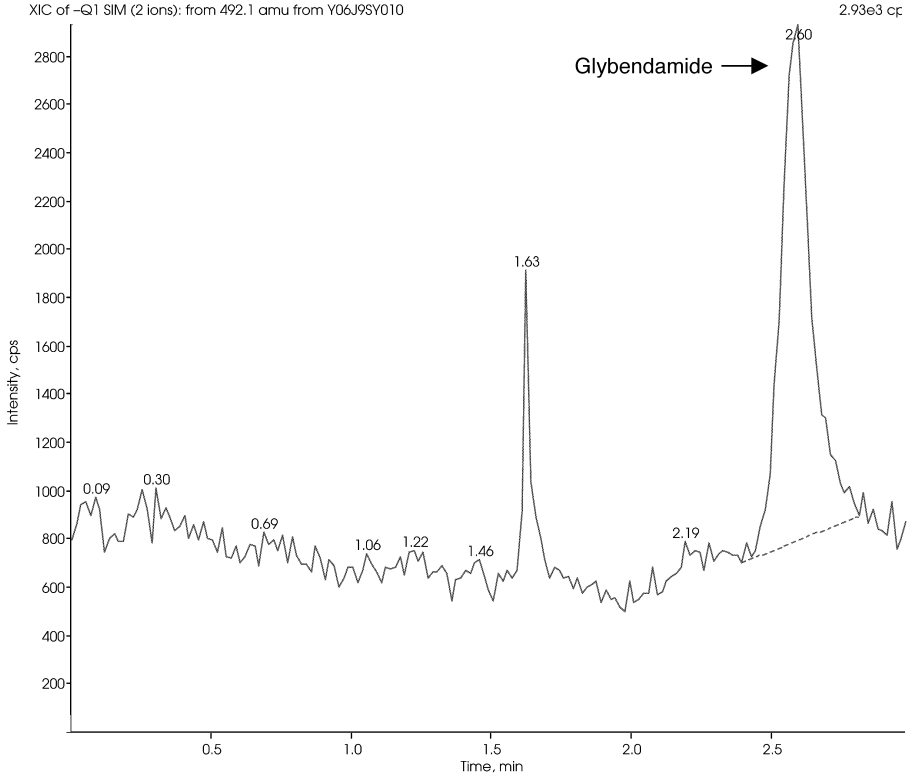


Fig. 6. Single ion monitoring LC–MS at m/z 492 of a processed human serum extract at the limit of quantitation (VC1, 10.4 ng/ml).

3.6. Application in clinical pharmacokinetic investigations

This method has been successfully validated and applied for analysis of about 600 human clinical samples. An example of a concentration vs. time profile of randomly chosen patients with adult onset diabetes after an oral dose of 7.5 mg Semi-Daonil® is shown in Fig. 7. The sensitivity of this method was adequate for profiling during the 12-h sampling period.

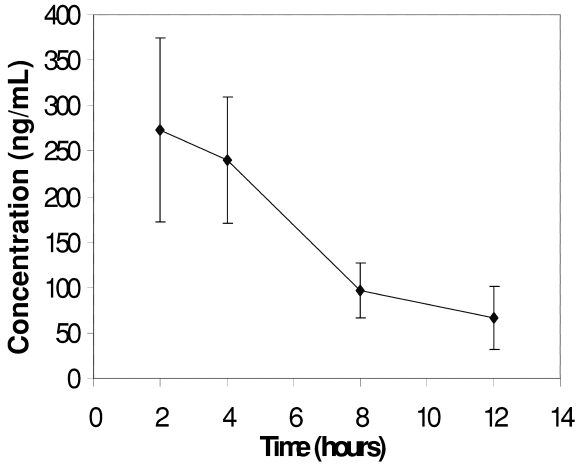


Fig. 7. Glybenclamide serum concentration–time profile for 12 randomly chosen patients with adult onset diabetes after a single dose administration of 7.5 mg of Semi-Daonil®.

4. Conclusion

A rapid technique for analysis of glybenclamide in human serum was successfully validated using LC–MS. The method was specific, fast (under 3 min per analysis), accurate and precise. We have shown here that by using information from the physicochemical properties of the compound, a sensitive and selective method can be developed using LC coupled to a single quadrupole mass spectrometer.

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