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QUANTITATIVE ANALYSIS OF 5-CHLORO-2-METHOXY-N-[2-(4-SULFAMOYLPHENYL)ETHYL]BENZAMIDE (GLYBURIDE ANALOGUE, GA) IN MOUSE PLASMA AND WHOLE BLOOD USING A MICRO-EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science at Virginia Commonwealth University

By

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Acknowledgement

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Abstract

QUANTITATIVE ANALYSIS OF 5-CHLORO-2-METHOXY-N-[2-(4-SULFAMOYLPHENYL)ETHYL]BENZAMIDE (GLYBURIDE ANALOGUE, GA) IN MOUSE PLASMA AND WHOLE BLOOD USING A MICRO-EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Pharmacokinetic evaluation of 5-chloro-2-methoxy-N-[2-(4-sulfamoylphenyl)ethyl]benzamide in mouse plasma demanded for a suitable bioanalytical method. No reported bioanalytical method exists to-date that can quantify concentration of this compound in any biological matrix. The purpose of this study was 1) to develop and validate a new bioanalytical method using a micro-extraction and LC-MS/MS to quantify the target analyte in mouse plasma and 2) to partially validate the method in whole blood. A bioanalytical method was developed and validated in both matrices for a linear concentration range of 2-1000 ng/ml. For

both matrices, the reverse predicted concentration of calibration standards (-8.95% to 12.16% and -9.54% to 12.90% respectively) and precision and accuracy (QCs) were within ±15% (%RSD and %BIAS). Four-hour bench top stability and post preparative stability results for plasma and whole blood matrices were within ±15% and ±20% respectively.   Blood –plasma concentration correlation co-efficient was 0.9956 with a slope value of 1.018.

CHAPTER 1: INTRODUCTION

1.1 5-Chloro-N-[4-(cyclohexylureidosulfonyl)phenethyl]-2-methoxybenzamide (glyburide) and 5-chloro-2-methoxy-N-[2-(4-sulfamoylphenyl)ethyl]benzamide (glyburide analogue)

Type 2 Diabetes Mellitus (T2DM) is a chronic condition that has affected 29 million people in the US alone by 2012 (Diabetes Latest, 2014). It is an increasingly prevalent disease affecting more people worldwide with no cure to-date. Pathologically, T2DM causes elevated plasma blood glucose levels by beta cell dysfunction in pancreas and insulin resistance to dietary sugars (Lim et al., 2015). Elevated glucose levels further results in heart and blood vessel disease, neuropathy, kidney failure, eye damage and skin conditions (Type 2 Diabetes, 2013). While not curable, current treatments to manage diabetes include diet regulation and physical activity and exercise, in combination with medication and insulin therapy (Type 2 Diabetes, 2013). The first line agent used in the US to manage T2DM is metformin, a drug that belongs to biguanide class which works by reducing the amount of glucose produced by the liver and helping the body respond better to insulin (White, 2010). Next in line are sulfonylureas that stimulate the beta cells of the pancreas to secrete more insulin, which in turn helps lowering free glucose circulating in blood. These include the only currently used first generation drug, chlorpropamide, and three second generation drugs namely: glipizide, glyburide and glimepiride (Lim et al., 2015). Aggressive therapies to treat

T2DM that is not managed by metformin alone include a sulfonylurea in combination with metformin (Lim et al.)

Among available sulfonylureas, glyburide or 5-chloro-N-[4-(cyclohexylureidosulfonyl)phenethyl]-2-methoxybenzamide (**Figure 1.1**) is the most widely used sulfonylurea for the treatment of T2DM in the US (McIntosh et al., 2011). It was first approved by the USFDA in 1984 as a prescription medication to treat T2DM (Electronic Orange Book, 2016). Mechanistically, the cyclohexylurea moiety within glyburide (highlighted in green, **Figure 1.1**) binds to the ATP-sensitive K^+ channels on the surface of sulfonylurea receptor (SUR) of pancreatic β-cells and inhibits them, which eventually regulates insulin secretion and release that further lower plasma glucose concentrations (Lamkanfi et al., 2009).

In 2009, an *in-vitro* study using lipopolysaccharides (LPS) primed BMDM (Bone Marrow Derived Macrophage) cells by Lamkanfi et al. demonstrated that in addition to its glucose lowering effect, glyburide also prevented activation of the cryopyrin inflammasome modulated by cytokines interleukin-1β secretion, thus delaying lipopolysaccharide (LPS) induced lethality in mice. **Figure 1.2** shows expression levels of IL-1β in presence of varying concentration of different compounds (**Figure 1.3**) tested by Lamkanfi et al. Furthermore, the structure-activity relationship experiments performed by Lamkanfi et al. with compounds including glyburide demonstrated that only sulfonyl and benzamido groups (highlighted in red, **Figure 1.1**) within glyburide are required for optimal inhibition of the cryopyrin inflammasome.
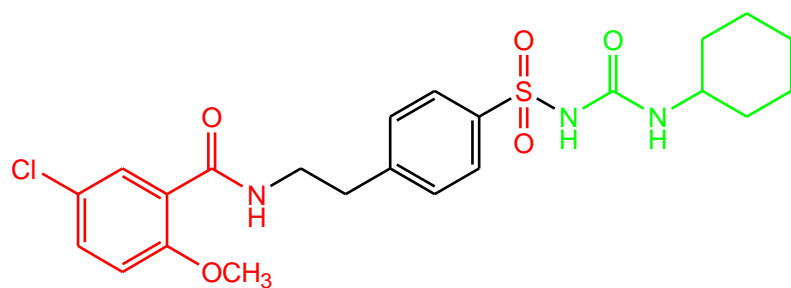
Figure 1.1 Chemical structure of glyburide highlighting important moieties for optimal inhibition of the cryopyrin inflammasome (red) and ATP-sensitive K^+ channels on the surface of pancreatic β-cells (in green). Reprinted from Product Specification, In *Sigma-Aldrich*, Retrieved January, 10, 2016, from http://www.sigmaaldrich.com/catalog/product/sial/g2539?lang=en&region=US
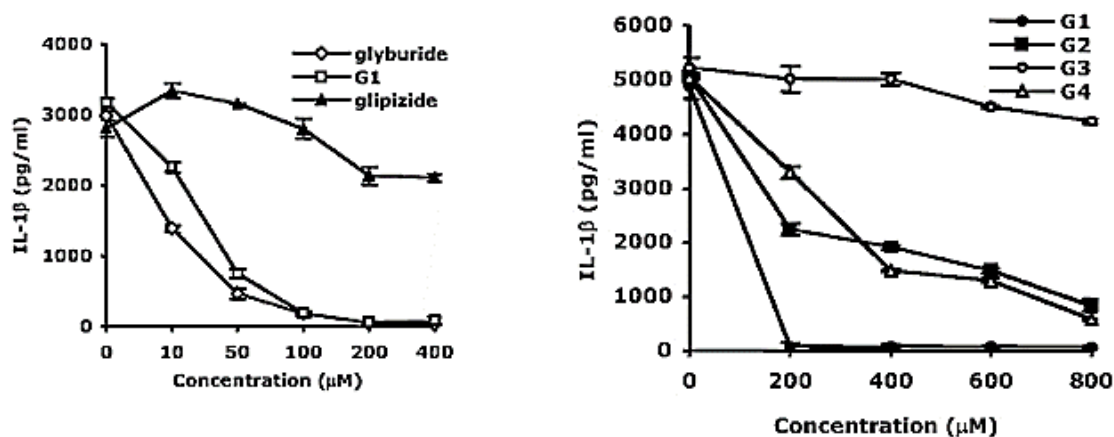
Figure 1.2 Expression levels of IL-1β in LPS-primed BMDM cells in the presence of different sulfonylureas (Lamkanfi et al., 2009).
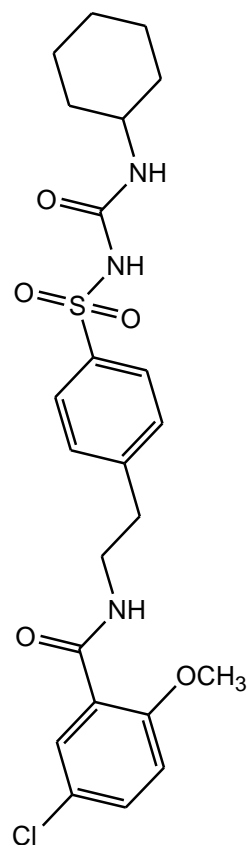
Glyburide
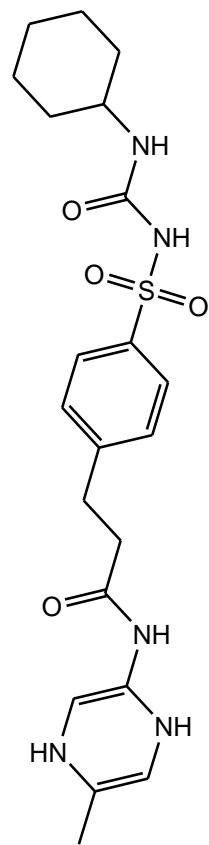
Glipizide
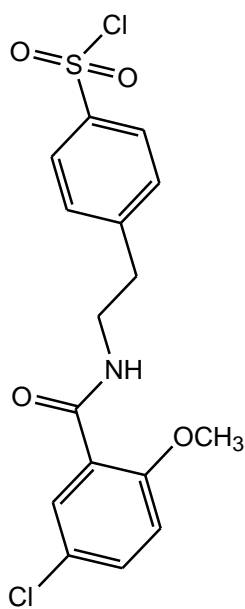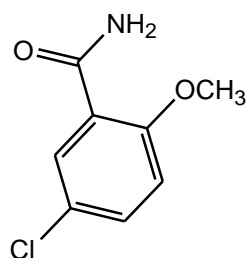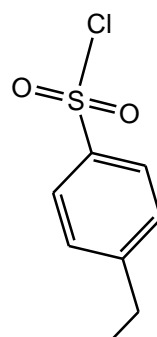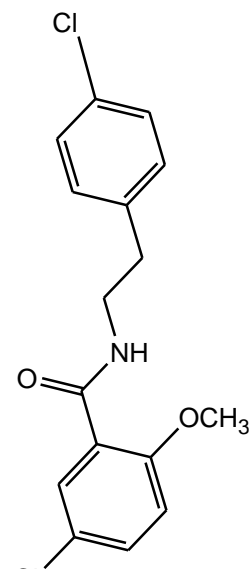
G1   G2   G3   G4

Figure 1.3 Chemical structures of compounds tested by Lamkanfi et al for their effect on IL-1β.

Recently, it was established through an *in-vivo* study using a mouse model that the cryopyrin inflammasome is one of the intracellular protein sensors that amplify the inflammatory response after an experimentally induced acute myocardial infarction (Marchetti et al., 2014). The genetic deletion of protein encoding nucleotides of the cryopyrin inflammasome limited the infarct size in experimental acute myocardial infarctions. This suggested that the protein itself would be a viable target for pharmacologic inhibition via glyburide. Glyburide, however in an, in-vivo (mouse model) to inhibit cryopyrin inflammasome would require 100-fold higher doses than amounts used in the treatment of diabetes, inevitably leading to lethal hypoglycemia. Hence, Marchetti et al., based on conclusions drawn from research efforts by Lamkanfi et al., synthesized 5-chloro-2-methoxy-N-[2-(4-sulfamoylphenyl)ethyl]benzamide (compound 16673-34-0), a glyburide analogue that retained only the sulfonyl and benzamido moieties of glyburide needed for the selective inhibition of the cryopyrin inflammasome (Marchetti et al., 2014). The chemical structure of the glyburide analogue (GA) that contains only the required moieties for the cryopyrin inhibition is shown in **Figure 1.4** (highlighted in red).
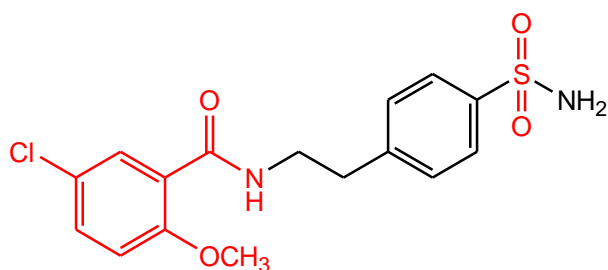
Figure 1.4 Chemical structure of 5-chloro-2-methoxy-N-[2-(4 sulfamoylphenyl)ethyl] benzamide (glyburide analogue) with important moieties (red) synthesized by Marchetti et al.

The chemical synthesis of compound 16673-34-0 or GA was initiated by first reacting 5-chloro-2-methoxybenzoic acid with 2-phenylethylamine in the presence of EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) to form the amide intermediate, 5-chloro-2-methoxy-N-(2-

phenylethyl)-benzamide. This intermediate compound was then treated with chlorosulfuric acid and aqueous ammonium hydroxide to form the target compound, 5-chloro-2-methoxy-N-[2-(4-sulfamoylphenyl)ethyl]benzamide. **Figure 1.5** shows the synthetic pathway of the compound 16673-74-0 (Marchetti et al., 2014).
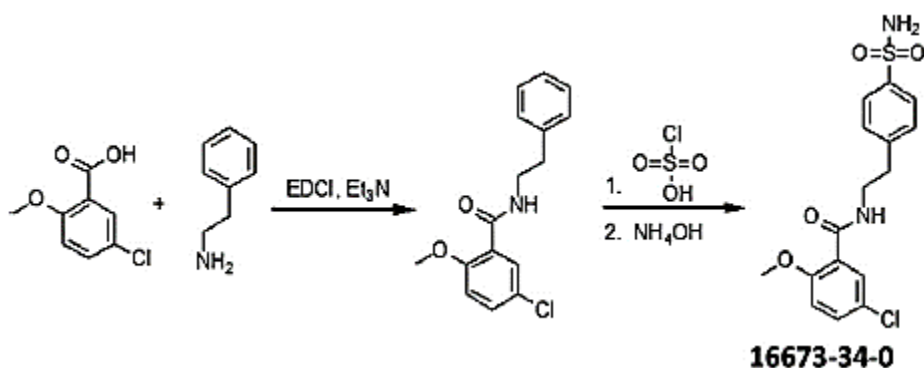
Figure 1.5 Chemical synthesis of compound 16673-34-0 (Marchetti et al., 2014).

Following the synthesis and subsequent use of the glyburide analogue in their in-vivo experiments with mice, Marchetti et al., demonstrated that compound 16673-34-0 significantly reduced both serum cardiac troponin I levels and infarct size in the heart 24 hours after induced ischemia and subsequent reperfusion compared to the saline control. Moreover, it was concluded that compound 16673-34-0 free of cyclohexylurea moiety inhibited the formation of the cryopyrin inflammasome without any adverse anti-diabetic effects in the experimental mouse model, thus disclosing it to be a novel pharmacologic inhibitor of the cryopyrin inflammasome. **Figure**s **1.6A** and **1.6B** show experimental data collected by Marchetti et al.
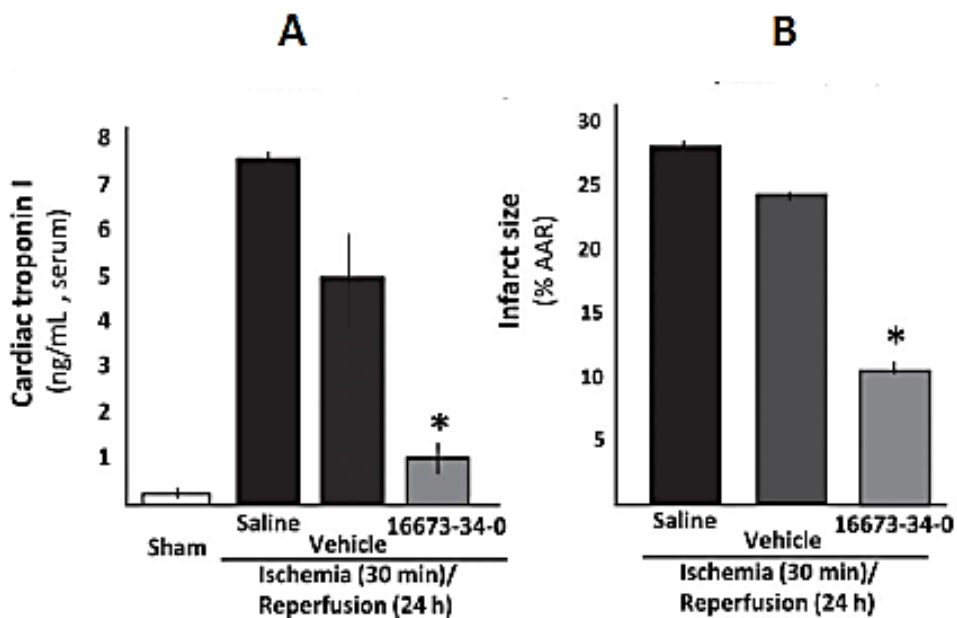
Figure 1.6A (left) and 1.6B (right) Experimental data by Marchetti et al showing significant reductions in cardiac troponin I levels and heart infarct size in their in-vivo experiment using mouse model treated with compound 16673-34-0 (Marchetti et al., 2014).

1.2 Rationale for bioanalysis of GA and specific aims

Marchetti et al. further pursued the *in-vivo* mice study on the inhibitory effects of 5-chloro-2-methoxy-N-[2-(4-sulfamoylphenyl)ethyl]benzamide on the cryopyrin inflammasome with a subsequent pharmacokinetic evaluation of the novel compound in plasma. Prior to this work, initial efforts were made quantify the glyburide analogue in mouse (CD-1 strain adult male) plasma using LC-MS/MS platform. Plasma based methodologies offer ease of handling and storage along with relatively cleaner matrix over whole blood, thus enhancing selectivity and assay sensitivity (Chance, 2002). However, initial results showed intra subject variabilities in plasma drug concentrations for a particular time point and inter subject variabilities in overall PK profiles. Moreover, limited plasma availability from the mouse model resulted in inadequate time points in

the study necessary to determine the terminal half-life of the drug compound. At least three time points during the terminal phase are required for a reliable estimation of the terminal half-life and those three time points should span at least two half-lives (Fan et al., 2014). Furthermore, the variabilities observed in the plasma results could also be explained if glyburide analogue exhibits preferential binding to red blood cells over free distribution in plasma. If true, plasma analysis alone would misrepresent drug exposure in the circulatory system of the mouse model and would yield an overall false PK evaluation of the target compound. No scientific information exits that show blood distribution characteristics of glyburide analogue. Thus, the purpose of this study was 1) to develop and validate a new bioanalytical method that can quantify the levels of the target analyte in mouse plasma with minimal sample volume to obtain adequate time points for an appropriate PK evaluation and 2) to partially validate the method for analysis of the test article directly in whole blood in an event of preferential distribution of the target analyte into erythrocytes over plasma.

To our knowledge, no reported bioanalytical method exists to date that can quantify drug concentrations of this compound in any biological matrix. This led to the challenge and opportunity of developing a new bioanalytical method using LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) as the main platform that can successfully quantify biological concentrations of the test article, both reliably and reproducibly as proposed.

1.3 Bioanalysis of GA and specific challenges

During the developmental phase of developing a suitable bioanalytical method for glyburide analogue, specific challenges were identified and addressed with respect to the overall process of sample quantification via LC-MS/MS platform. These included limited availability of scientific literature on the test article, limited sample volume availability from the animal model,

need for an extraction procedure that used minimal sample, selection of a suitable internal standard and possible blood-plasma partitioning of GA relevant to the proposed direct whole blood analysis. All these issues are discussed further in detail.

1.3.1 Availability of scientific literature

Literature searches on 5-chloro-2-methoxy-N-[2-(4-sulfamoylphenyl)ethyl]benzamide or glyburide analogue (target analyte) yielded very limited information due to the novelty of the compound. Although not a sulfonylurea, it is structurally similar to glyburide and other compounds within the sulfonylurea class. Compounds within the sulfonylurea class are broadly classified as weak organic acids (pKa 5-6) due to the presence of sulfonamide group that is often used as non-classical carboxylic acid bioisostere (Smith, 2010). The molecular mass of glyburide analogue is 368.84 g/mol with the empirical formula of $C_{16}H_{17}ClN_2O_4S$ (Sigma-Aldrich, 2014). It is a small, polar molecular entity soluble in dimethyl sulfoxide (DMSO) (HIMEDIA LAB, 2012). It is commercially available as a reference standard. Like other sulfonylureas, the glyburide analogue possesses both lipophilic and hydrophilic moieties within its structure. The aromatic rings provide lipophilic character while the $-SO_2$, -NH-, -CO- moieties are hydrophilic (DeRuiter, 2003). Similar compounds that belong to same class as glyburide analogue include, but not limited to glyburide, gliclazide, glipizide, gliquidone, glibonuride and glimepiride (DeRuiter, 2003). Henceforth, available literature on these compounds with respect to chromatographic separation and mass spectrometric analysis was used in the development of a suitable bioanalytical method to quantify the glyburide analogue.

1.3.2 Availability of sample volume

The *in-vivo* study by Marchetti et al used adult male mouse (CD-1 strain) as the animal model. Obtaining an adequate amount of blood sample for a typical PK study is a major challenge

in rodent species due to their small size and other physiological considerations. Moreover, established guidelines set limits on maximum blood sample that can be collected from a particular animal based on size (or weight) and to minimize stress on the animal (Dainty et al., 2012). In a study involving repeated draws, separated by weeks, only 1% of animal's total circulating blood can be removed within 24 hours in mice (NIH 2015). Given the blood composition of mice with 39-49% hematocrit, plasma recovery is even less. **Table 1.1** shows maximum available blood volumes that could be collected in 24 hours from mouse (Burnett, 2011).

Table 1.1 Maximum Available Blood Volumes (Mice)

	Avg. weight (g) Day 1	Avg. weight (g) Week 4	Volume (ml) Day 1	Volume (ml) Week 4
Male (Plasma)	30	38	0.30 (0.15-0.18)*	0.38 (0.19-0.23)*
Female (Plasma)	25	30	0.25 (0.13-0.15)*	0.30 (0.15-0.18)*

*Values represent plasma recovery volumes based on 39-49% HCT.

Such limitations often require using multiple animals per time point in a PK study. A seven time point study using four animals per time point with both male and female species would end up using 166-168 animals (Burnett, 2011). This leads to large numbers of animals being sacrificed with both economic and ethical implications. Additionally, studies suffer from high inter-animal variability when several animals are used for a single time point. Overall, these aspects often lead to a compromise in number of time points being evaluated or an incomplete PK profiling altogether. **Table 1.2** shows typical animal numbers required when generating toxicokinetic data from mouse plasma (Burnett, 2011).

Table 1.2 Typical animal numbers required when generating toxicokinetic data from mice plasma (Burnett, 2011)

Mice group/sex (description)	Sample time (h post-dose)						
	Pre-dose	1	2	4	6	8	24
1M (Control)	1–3	4–6	7–9	10–12	13–15	16–18	19–21
2M (Low)	22–24	25–27	28–30	31–33	34–36	37–39	40–42
3M (Intermediate)	43–45	46–48	49–51	52–54	55–57	58–60	61–63
4M (High)	64–66	67–69	70–72	73–75	76–78	79–81	82–84
1F (Control)	85–87	88–90	91–93	94–96	97–99	100–101	102–104
2F (Low)	105–107	108–110	111–113	114–116	117–119	120–122	123–125
3F (Intermediate)	126–128	129–131	132–134	135–137	138–140	141–143	144–146
4F (High)	147–149	150–152	153–155	156–158	160–162	163–165	166–168

Table 2. Typical animal numbers required when generating toxicokinetic data from plasma samples of mice.

F: Female; M: Male; n = 3.

1.3.3 Extraction procedure with minimal sample

Once obtained, most samples are not ready for direct analysis via LC-MS. The target analyte(s) have to be extracted into a solution, which can then be submitted to LC-MS instruments (Mitra et al., 2003). The biological samples should be processed in a way that the final sample extracts have minimal amount of other endogenous components present in the matrix. Thus, it should be ensured that extraction procedure is selective for the target analyte. Selective extraction procedures in turn increases the overall assay sensitivity and help to accurately and reliably quantify the analyte of interest. Finally, the sample extract composition should be compatible with the analytical platform of choice (Bylda et al., 2014). Once a sample is extracted and rendered mostly free from interfering substances, only then chromatographic techniques such as HPLC conjunction with mass spectrometry platform become employable to quantify compounds of interest. In order to overcome the inherent challenge of sampling while ensuring selective extraction of the test article, a microextraction procedure via HybridSPE technology that allowed for sample processing in low microliter volumes was employed in the development of a suitable

bioanalytical method to quantify the glyburide analogue in plasma and whole blood. The benefit of such a method is that with low sample volumes, it is possible to obtain more time points during the PK profiling of the glyburide analogue. In addition, a low sample volume requirement would consume minimal numbers of animals, which in turn reduces the inter-animal variability in the study.

1.3.4 Selection of a suitable internal standard

As mentioned earlier, during LC-MS/MS analysis of drug compounds and/or their metabolites, samples have to be pre-treated prior to being injected onto the instrument. These pre-treatments are necessary for selective extraction of the test article from the biological matrix. Thus, processes such as dilution, extraction, quantitative transfer or evaporation and reconstitution of the representative biological sample may be necessary. During these processes, loss of targeted analytes may occur in variable forms affecting its recovery in the prepared sample to be analyzed (Tan et al., 2012) Moreover, there may be variability within the instrumental analysis such as injection volume and ionization process in form of suppression or enhancement of the signal caused by matrix effects (Tan et al., 2012). Therefore, an internal standard is added to the sample of interest at the very beginning of the sample preparation procedure to account for such losses and analytical variations. It is added in equal amounts to all samples, calibration standards as well as quality control samples, within an assay. In this way, the target analyte losses and instrumental variations can be accounted by taking a ratio of the analyte to the internal standard peak area (or height) versus concentration to form a calibration curve, and ultimately improve accuracy and precision of the assay.

Mainly, there are two types of internal standards that are routinely used in bioanalytical methods that use LC-MS technique for quantification: stable isotope labeled (SIL) internal

standards and structural analogues of the target compound (FDA, 2001). SILs are compounds similar to the actual analyte with atoms being replaced by their respective isotopes i.e. deuterium (^{2}H), ^{13}C, ^{15}N, etc. Normally, SILs are chosen in a LC-MS/MS for two main reasons. First, due to their similar physicochemical properties, ionization inefficiencies are well tracked with similar retention time as the target analyte (Arrivault et al., 2015). Second, SILs possess same stability as the target analyte and thus would be equally affected at various steps of the analysis. On the other hand, the structural analogues used as internal standards are compounds with a different mass than the actual analyte, with key moieties being preserved. Thus, it is ensured that the ionization characteristics are still similar to that of the actual target analyte (Valbuena et al., 2016). It is always preferable to use a SIL compound as the internal standard as they are the most effective. However, they are not always available or maybe extremely expensive. In these cases, structural analogues should be given preference. Possible candidate compounds that could be used may be found from the same therapeutic or chemical class as the analyte of interest. This way the chosen compound would most likely have similar chemical and physical properties as the analyte. In the bioanalysis of the glyburide analogue, glipizide (MW 494 g/mol) (Sigma-Aldrich, 2011), which is a compound that belongs to the same class called sulfonylurea, was chosen as the internal standard. The structural similarity between the glyburide analogue and glipizide can be seen in **Figure 1.7**.

Glyburide Analogue (369 g/mol)   Glipizide (494 g/mol)
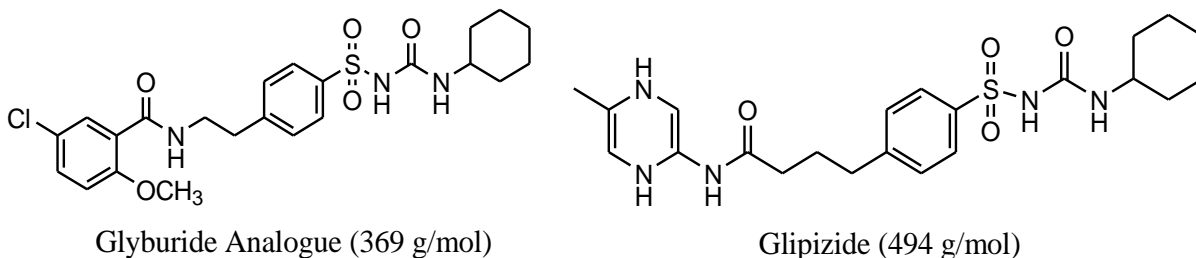
Figure 1.7 Structural similarities between the glyburide analogue and glipizide.

Structurally similar to glyburide analogue, glipizide was predicted to have the same physical and chemical properties as the analyte i.e., solubility, hydrophobicity and ionization

characteristics as well as stability in various solvents and chemicals used during sample preparation. It was predicted that glipizide would not completely resolve from the target analyte during chromatographic separation and thus, have similar retention time as the target analyte. This would ensure similar degree of ionization for both the target analyte and the internal standard during subsequent MS analysis. Finally, the glipizide was predicted to fragment differently than the target analyte in a SRM experiment during the mass spectrometric detection phase of the analysis.

1.3.5 Blood-plasma partitioning of GA

Pharmacokinetic parameters calculated from the plasma data alone may be misleading if there is a difference between concentrations of the drug in the plasma and the red blood cells (Altmayer et al., 1983). Thus, determining blood to plasma ratio for a compound known to partition in red blood cells is an important parameter to determine to make the case for using direct whole blood analysis over plasma. Compounds that are sulfonamide derivative have been known to extensively bind to red blood cells, which typically result in blood to plasma ratio greater than one (Smith, 2010). Moreover, these compounds have shown a concentration dependent partitioning into blood with mechanisms involving not only passive diffusion but also protein binding and active transport (Yu et al., 2004). In the bioanalysis of GA, a blood to plasma distribution study was conducted to determine whether direct analysis of whole blood would be more appropriate than plasma analysis since the target compound possessed the sulfonamide moiety.

This thesis will further elaborate on the choice and rationale for using the HybridSPE technology, a microextraction sample preparation technique in conjunction with LC-MS/MS analysis of the glyburide analogue. In addition, this thesis will also focus on the selection process of a suitable internal standard (structural analogue) for LC-MS/MS analysis and chromatographic

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optimization experiments during method development phase along with several modifications in the extraction procedure of glyburide analogue.

1.4. Microextraction via Hybrid SPE-Phospholipid and Precipitation Technology for GA sample processing and clean up

As discussed earlier, sample preparation is an important step in the process of analysis, as most biological samples cannot be directly introduced into instruments. This step involves sample extraction wherein the drug of interest is isolated from the matrix with the help of extraction solvents, typically organic in nature (Mitra et al., 2003). As emphasized before, an important consideration during development of the analytical assay of glyburide analogue was that the extraction procedure use minimal amount of sample volume. This is due to the fact that the animal model being studied was mouse in which blood volumes become a limiting factor in pharmacokinetic studies. This issue was addressed by employing a microextraction technique using HybridSPE precipitation technology during the extraction phase of the experimental procedure.

Typical sample extraction techniques include liquid-liquid extraction and solid phase extraction (Juhascik et al., 2009). In a liquid-liquid extraction (LLE), compounds are separated from each other based on their relative solubility in two or more immiscible liquids. In a solid phase extraction (SPE), compounds are separated from a liquid using a solid stationary phase through which they are eluted. These methods are conceptually simple, however, both of these techniques are time consuming and often lead to extracted volumes, which exceed the chromatographic needs (Pawliszyn et al., 2006). In contrast, supported liquid extraction (SLE) allows for a traditional liquid-liquid extraction using low solvent volumes followed by elution of organic (extraction) solvent through a supported cartridge (Pan et al., 2010). Thus, SLE helps

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address the issues of bigger sample volume requirement and are much more time efficient than LLE or SPE avoiding column conditioning, equilibration and washing steps, which can add to the complexity of the procedure (Chang, 2013). Thus, a microextraction procedure using this HybridSPE technology in form of solid supported liquid extraction (SLE) offered an alternative method, which was relatively less time consuming and labor intensive.

In addition to sample extraction, removal of other endogenous components from the matrix cleanup is also an important step in processing of samples in a biological matrix such as plasma and whole blood. In particular, matrix components such as proteins, anti-coagulants, fibrinogen and especially phospholipids interfere with drug of interest that may lead to ion suppression; poor peak shape and resolution in MS/MS based quantification approaches. Due to the amphiphilic nature of phospholipids, they can be co-extracted with analytes of interest during sample preparation and lead to variability and overall accuracy of the assay when analyzing small molecules. (Pucci et al., 2009). Several techniques have been used to remove phospholipids and other components within the matrix during sample preparation. Traditionally, these techniques comprise of solid-phase extraction procedures that use strong cation exchange sorbents (Shen et al, 2005). Non-traditional approaches for phospholipid removal have used automated on-line SPE or liquid/liquid extraction with methyl *tert*-butyl ether (Marchese et al., 1998) followed by solid-phase extraction using chemical sorbents with an active lanthanide (Want et al., 2006). Although effective, these procedures have the disadvantage of increased costs related to materials and instrumentation set-up along with introduction of further time consuming steps in the cleanup process.

In the bioanalysis of the glyburide analogue, a relatively new and commercially available HybridSPE technology by Sigma-Aldrich was adopted. HybridSPE precipitation technology in a

96 well plate format is used for fast and efficient sample preparation of plasma samples. It merges both protein precipitation and solid phase extraction techniques for phospholipid removal within plasma matrix (Pucci et al., 2009). It uses a zirconia sorbent that exhibits a high affinity for phospholipids while remaining non-selective toward a wide range of basic, neutral and acidic compounds (Supelco, 2009). This technology allows for a simultaneous execution of SPE and SLE extraction techniques in a convenient way that helps to eliminate matrix effects (i.e., phospholipids) with the ability to extract model compounds in a smaller sample volume LLE. In addition, the miniaturization of sample volume is achieved via this technology, as it only requires loading volumes of less than 100 µl. The 96 well plate format simplifies the assay procedure and contributes to overall efficiency and ease in carrying extraction of 96 samples per plate. **Figure 1.8** shows the working principle of HybridSPE technique.
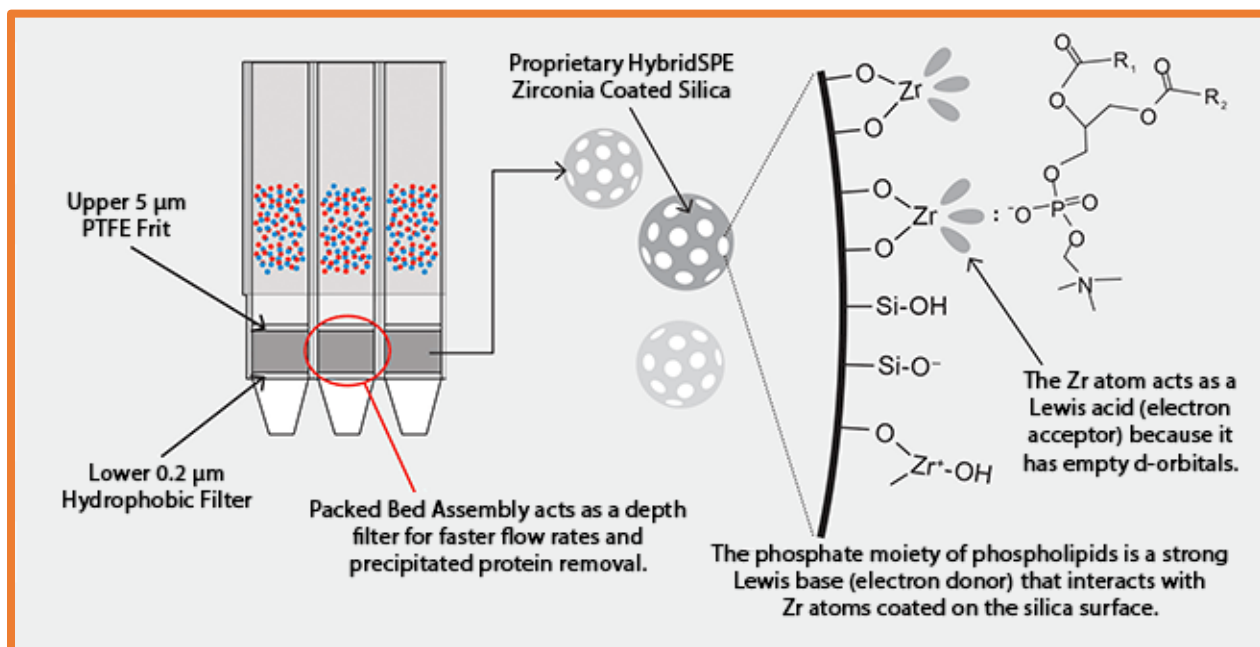
Figure 1.8 Retention mechanism of HybridSPE Phospholipid Technology (Supelco, 2009).

In a typical 96 well plate HybridSPE sample preparation experiment, plasma or serum is first subjected to protein precipitation (in-well precipitation) via the addition and mixing of

acidified acetonitrile or methanol. After a brief mixing step, vacuum is applied. During this step, the solid phase column in the packed bed containing low porosity filters within acts as a depth filter that aids in the removal of both phospholipids and precipitated proteins. The retention of phospholipids is based on a highly selective Lewis acid-base interaction between the zirconia ions functionally bonded to the stationary phase and the phosphate moiety within the phospholipids. The resultant eluent is then free from the interfering phospholipids and ready for LC-MS/MS analysis (Supelco, 2009).

To process smaller volumes of plasma, the plasma sample is first diluted with deionized water so the final volume is around 100 µL. To this, 300 µl of precipitating agent is added (in a ratio of 1:3, plasma: precipitant). Acetonitrile with 1% formic acid is used as the primary precipitating agent. This is because formate acts as a much stronger Lewis base than most carboxylate groups found on acidic compounds, thus inhibiting analyte retention on the HybridSPE phase but not as strong a Lewis base as the phosphate moiety found in phospholipids. For compounds containing nitrogen atoms, ammonium formate in methanol is used as the precipitating agent. Here, NH_4^+ ions act as stronger counter ions then H^+ in inhibiting basic compounds from interacting with HybridSPE silanol groups (Si-O). Also, methanol is a more polar solvent than acetonitrile, further inhibiting any potential hydrophilic interactions between the analyte and silica surface. In the bioanalysis of glyburide analogue, ammonium formate in methanol was used as the precipitating agent after comparing selectivity and recovery of the test article in both variants of the extraction solvent. Such a comparison was necessary due to the fact that the target compound contained amine moieties (basic) while sulfonamide moieties exhibited acid like characteristics (Supelco, 2009).

1.5 LC-MS/MS as the quantitative platform

The combined power of liquid chromatography and tandem mass spectrometry makes LC-MS/MS one of the most powerful quantitative approaches in small molecule drug analysis. With enhanced selectivity and selectivity, it is far more superior to conventional high performance liquid chromatography with UV-Vis detection alone especially for small drug analytes. In the bioanalysis of the glyburide analogue, LC-MS/MS was chosen as the primary platform for quantitative analysis. The target analyte from the sample extract was first isolated on a column using reversed phase liquid chromatography followed by a subsequent MS/MS analysis.

1.5.1 LC-MS method conditions in the bioanalysis of the glyburide analogue

Based on previous literature on sulfonylureas, GA (a sulfonylurea derivative) was predicted to be a suitable candidate for chromatographic separation via reversed-phased liquid chromatography (RP-LC) (Mistri et al., 2007). Chromatographic separation of GA was characterized by sample molecule's strong interaction between the polar mobile phase (dipole interaction and hydrogen bonding) and relatively weaker interactions with the non-polar stationary phase. The non-polar, hydrophobic stationary phase of the column (2.1 x 50 mm, 3 µm, 100 Å) used in the bioanalysis of glyburide analogue consisted of a silica based packing bonded to octadecylsilyl (C-18) functional group moieties. The mobile phase consisted of two miscible solvents: solvent A (95:5 water: acetonitrile + 0.5% formic acid) and solvent B (acetonitrile + 0.5 % formic acid). A suitable gradient scheme with respect to both solvents was achieved through a series of experiments with neat solutions of GA/GP in which the eluent composition was changed from solvent A (weaker solvent) to solvent B (stronger solvent) over 4.5 minutes at a flow rate of 0.300 ml/min. These details are explained in the method development section of this thesis.

Separation of GA was achieved upon its relative amount of time spent interacting with the stationary phase and the mobile phase based on its chemical affinity. Being relatively hydrophobic (Log P ~ 2.5), GA is thought to partition into the non-polar stationary phase from the mobile phase eluent. Initial column loading of GA was achieved by the aqueous phase of the gradient. The hydrophobic moieties present in GA partitioned into the stationary phase by hydrophobic interaction with the non-polar bonded phase. The analyte was retained until a gradient shift to a stronger solvent (mobile phase B). Glyburide analogue, due to its favorable chemical affinity for a more polar solvent, subsequently eluted. **Figure 1.9** shows the relative interaction of GA molecules with mobile and stationary phase inside the column (Patel, 2001).
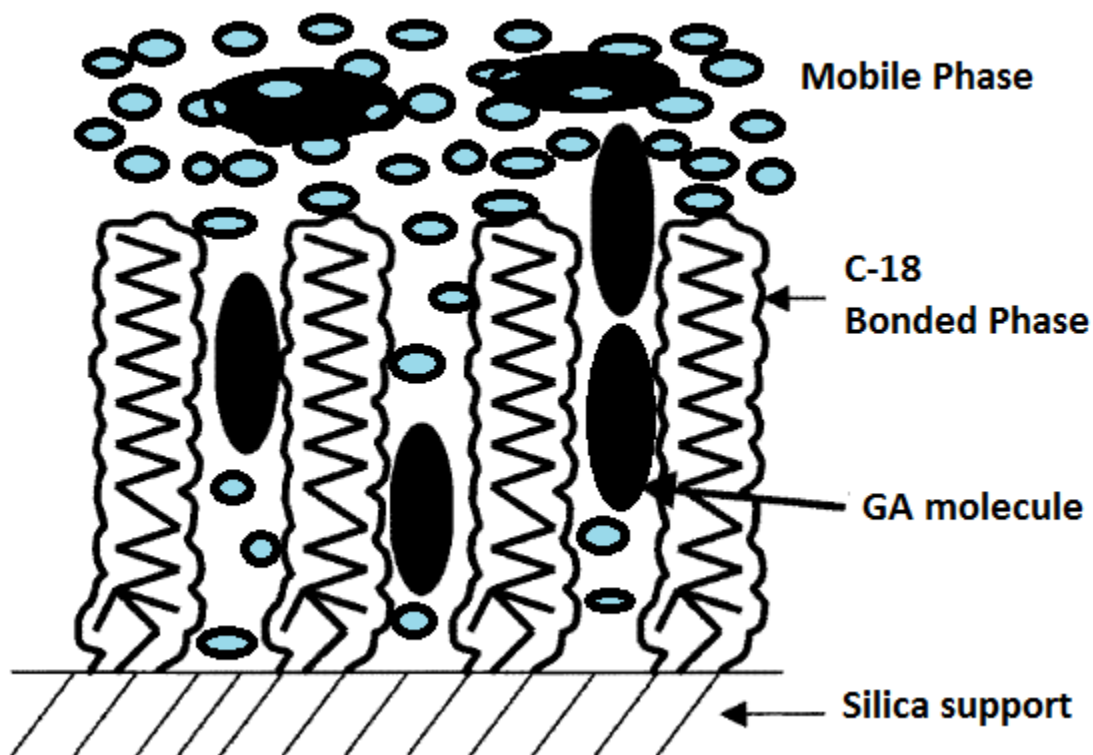
Figure 1.9 Schematic showing GA molecule in mobile and stationary phase (Patel, 2001).

The dimensions of the column were carefully chosen so as to maximize the separation efficiency of the analyte molecule. A shorter column (50 mm) was chosen in the bioanalysis of GA as it is ideal for gradient analyses for a short run time, which was desirable in the LC-MS/MS analysis of GA. The Van Deemter equation, $H = A + \frac{B}{\mu} + C\mu$ describes the relationship between the plate height, H (measure of column efficiency) and the linear velocity, μ (flow rate of the mobile phase). Smaller plate height values or more number of plates correspond to increased column efficiency and reduced band broadening. The constants A, B and C describe the peak broadening governed by eddy diffusion, longitudinal diffusion and mass transfer of the analyte inside a column, respectively. **Figure 1.10** shows the Van Deemter plot relating the effect of each constant to band broadening (column efficiency) (Lake, 2016).
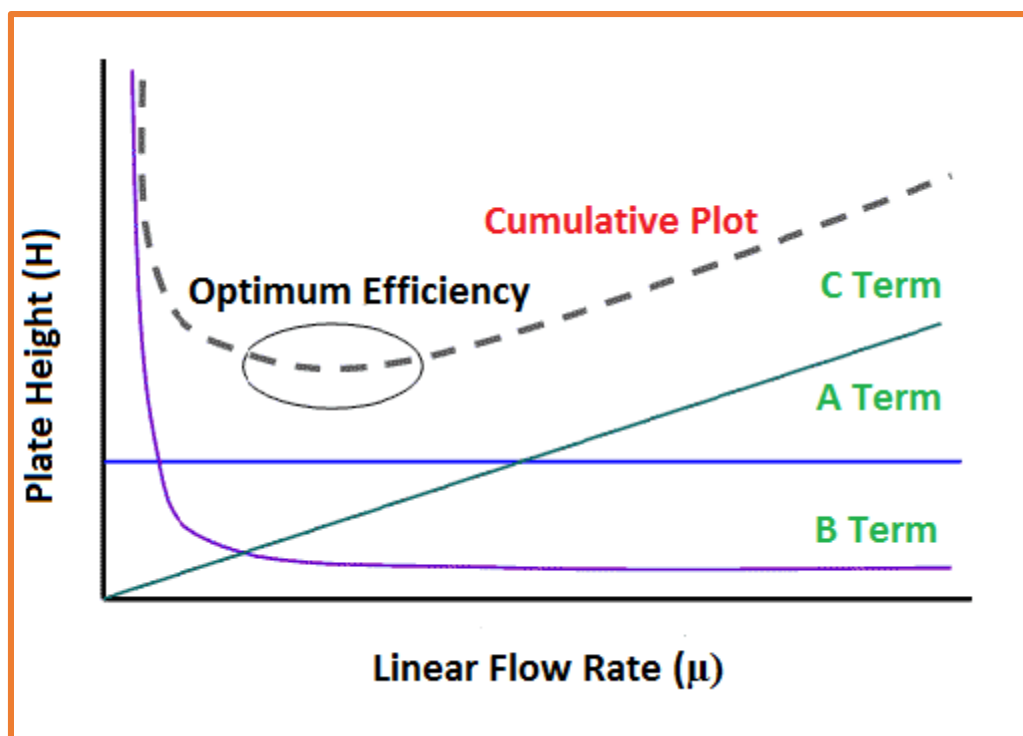
Figure 1.10 Van Deemter plot with graphical representation of each term contributing to band broadening (Lake, 2016).

Eddy dispersion (term A) relates to multiple paths available for analyte molecules to flow through the column. A smaller particle size and homogenous packing of the material reduce the path between particles allowing a solute molecule to travel in and out of the particle faster. As the analyte spends less time inside the particle, peak diffusion is limited, thus increasing the overall column efficiency by reducing contributing effects of the A term. Considering this, a column with 3 µm particle size and nearly homogenous particle size distribution (90%/10% diameter ratio of 1.46) was used. **Figure 1.11** shows the effect of particle size on overall efficiency via a Van Deemter plot.

Longitudinal diffusion relates to the outward movement of analyte molecules from the center of the band due to concentration gradient along the axis of the flow. Practically, increasing mobile phase flow rates minimizes this. However, column efficiency tends to be independent of flow rates with a smaller particle size as seen in **Figure 1.11** allowing for a wider optimum flow rate. As is the case with GA analysis, a column with 3 µm particle size allowed for a wide range of flow rate without significant loss of column efficiency and hence effects of band broadening due to longitudinal were thought to be minimized.
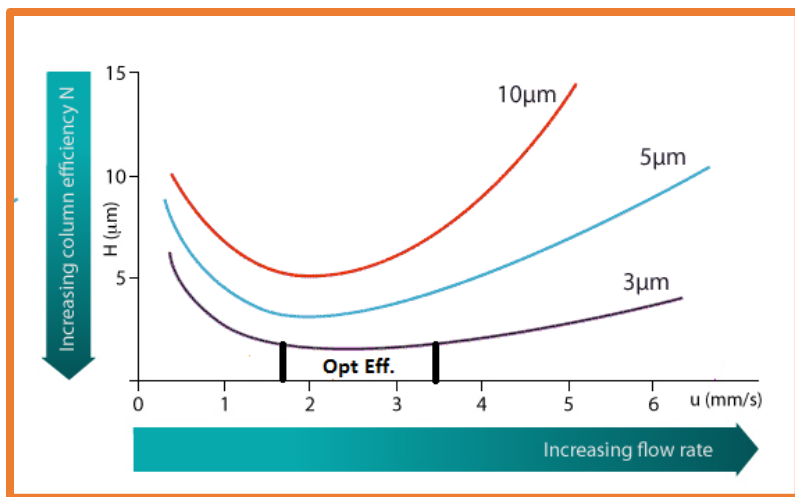
Figure 1.11 Particle size vs column efficiency in a Van Deemter Plot. Reprinted from CHROMacademy.

The mass transfer (C term) relates to the movement of analyte between the mobile phase and porous packing material of the stationary phase. The mobile phase within the pores remains relatively stagnant compared to the free flowing mobile phase. Analyte molecules that penetrate the pores are held for a longer time to the extent causing broadening of the band. In this study, the effects of mass transfer were minimized by selecting a column with smaller pore size (100 Å) and heating the column (40 ℃) to speed up the diffusion process that would eventually reduce the elution time differences of individual analyte molecules from the particle pores.

1.5.2 Mass spectrometric detection and m/z analysis of glyburide analogue and glipizide

Once the target analyte (GA) and the internal standard (GP) were isolated via liquid chromatography, the mass spectrometric analysis was carried out using a triple quadrupole mass analyzer and electrospray ionization in positive mode. The detection and subsequent quantification of GA and GP was achieved through selected reaction monitoring (SRM) specific to GA (369→169) and GP (446→320).

After the chromatographic separation, the sample eluent containing GA/GP was introduced into the ion source. The eluent was passed through a stainless steel capillary held at 3.50 kV. The strong electric field caused the dispersion of the sample solution into an aerosol of highly positively charged electrospray droplets. A flow of dry N_2 gas around the capillary aided in better nebulization of the liquid droplets with simultaneous evaporation of solvent. At high temperature (400 ℃) the pre-formed ions were further desolvated and gas phase production of positively charged ions from liquid droplets was achieved. **Figure 1.12** shows the schematic representation of the sample flow within the ion source.
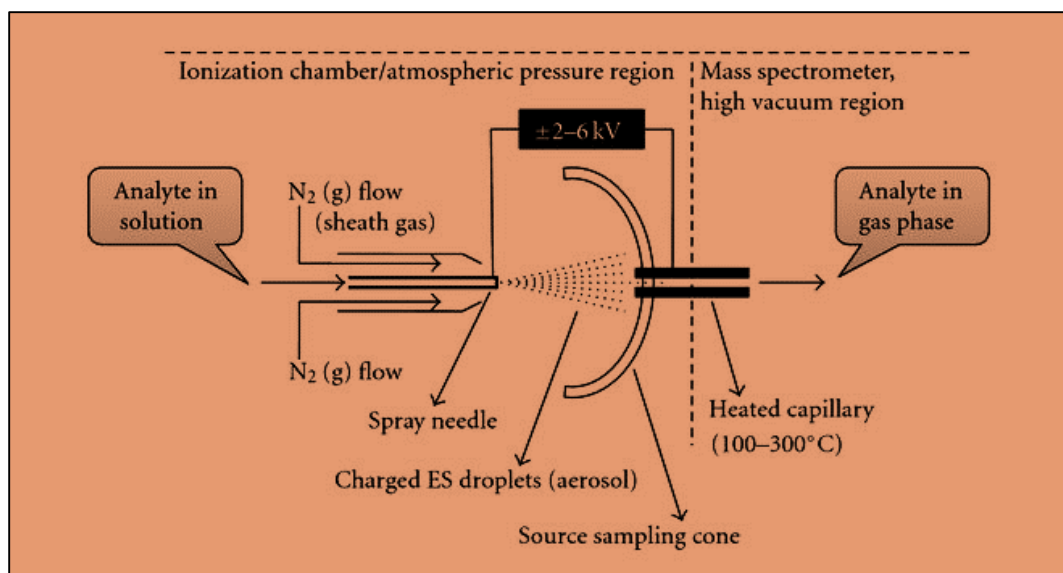
Figure 1.12 Sample flow within the ion source during MS/MS analysis of GA/GP (Banerjee et al., 2012).

ESI was the preferred ionization method for GA/GP molecules as it is best suited for polar ionizable analytes in solutions (Pereira et al., 2008). Moreover, being a soft ionization technique, ESI allows compound with low internal energies to remain intact without causing in-source fragmentation of the analyte molecule generating unwanted ions that could later on interfere with MS/MS analysis (Banerjee et al., 2012). Furthermore, ESI technique could be operated either in positive or in negative mode depending on the nature of the molecule to be analyzed (Chin et al., 2004). With available –N- atoms in the analyte molecule, it was predicted that (GA/GP) would be positively ionized by simply providing a source of generating positive ions in the eluent. Formic acid, a pH-lowering agent was used as the source of positive ion generation within the sample solution. **Figure 1.13** depicts ESI process in the positive mode.
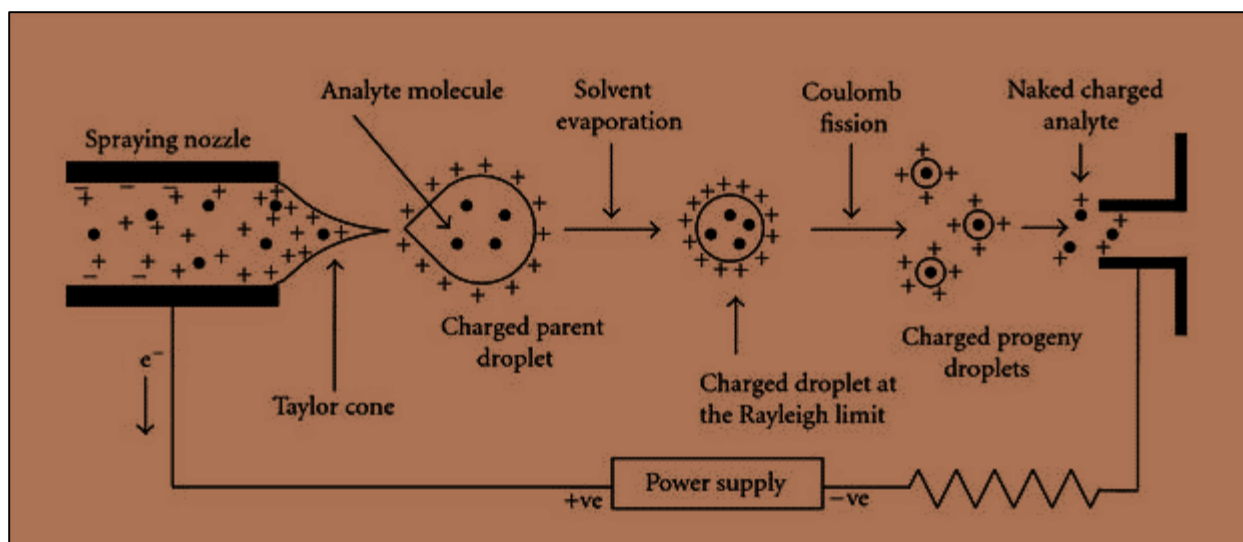
Figure 1.13 Electrospray ionization in positive mode in MS/MS quantification of GA/GP (Banerjee et al., 2012).

The generation of singly charged ions of the target analyte (GA) and the internal standard (GP) occurred via the ion evaporation model of the ESI process. During this process, the positive potential (capillary) causes repulsion of positive ions within the LC-eluent. As the eluent exits the capillary, it is stretched towards the downfield electrical gradient. Upon exiting the stretched solution forms into a Taylor cone at the tip of the metal capillary. Eventually, small liquid droplets break off with net positive charge from the solution. The solvent on the droplets then undergoes rapid evaporation. The process of evaporation is aided by high temperature and continuous flow of N_2 gas. As the solvent evaporates, the surface tension on droplets decreases while charge density increases (Chin et al., 2004). The droplets subsequently disintegrate in a Coulomb fission generating smaller progeny ions with positive charge. This process continues until droplets are broken into singly charged ions, which are then directed towards a metal plate that is being held negative with respect to the ground into the mass analyzer (Grebe et al., 2011). Separation based on *m/z* of ions takes place within the mass analyzer depending on the type of experiment is being carried out. The glyburide analogue and glipizide (ISTD) were analyzed using Full Scan (Q1) and

Selected Reaction Monitoring (Q3) experiments in a triple quadrupole mass analyzer. **Figure 1.14** shows the internal parts of a triple quadrupole mass analyzer.
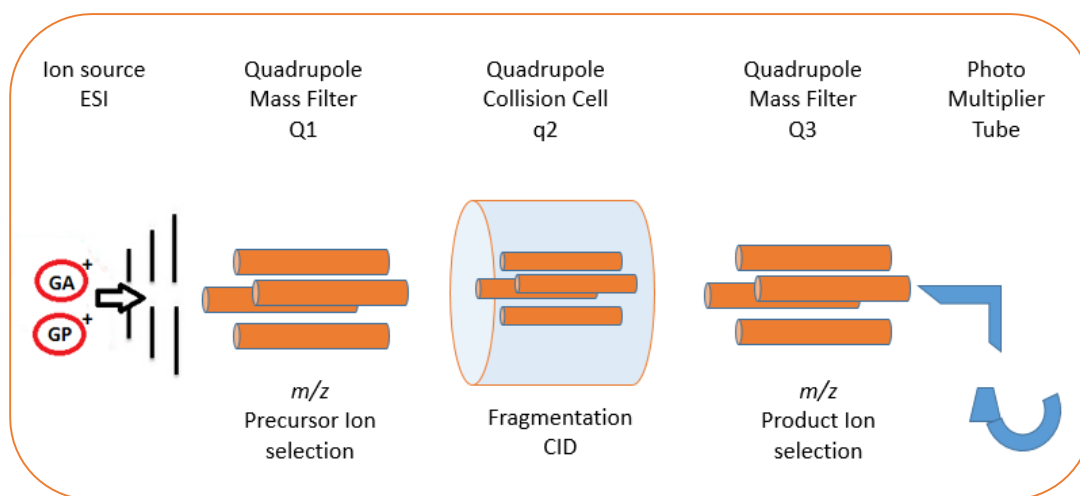
Figure 1.14 Internal parts of a triple quadrupole mass analyzer used in the bioanalysis of GA/GP.

In the full scan experiment, the sample (GA/GP) ions were scanned across the entire mass range within Q1. During this mode, the collision cell was void of collision gas (and energy), which did not generate product fragments of the precursor ions. The $(M+H)^+$ ion representing precursor *m/z* of GA/GP molecules were then selected for the SRM. The selected ions (GA and GP) in Q1 were then allowed to fragment in the collision cell (Q2). The collision cell was innervated by Ar gas with various energies (V) that aided in the fragmentation of the sample ions into product ions through a process called collision-induced dissociation. Generated product ions were then transferred into Q3 using constant DC and RF voltages. Under SRM, $(M+H)^+$ product ions for GA/GP with specific *m/z* fragments generated in Q2 were allowed to pass and subsequently detected. The most intense $(M+H)^+$ peak for each compound were then selected as the specific SRM for GA/GP. This allowed for a very selective detection of the test article and the ISTD. Each ion hit a conversion dynode that generated an electrical signal in form of counts per second, which was further amplified via a photo multiplier tube. A mass spectrum was generated based on the

intensity of the signal with respect to relative abundance of the ions based on *m/z*. **Figure 1.15** shows the schematic representation of SRM as used in the bioanalysis of GA.
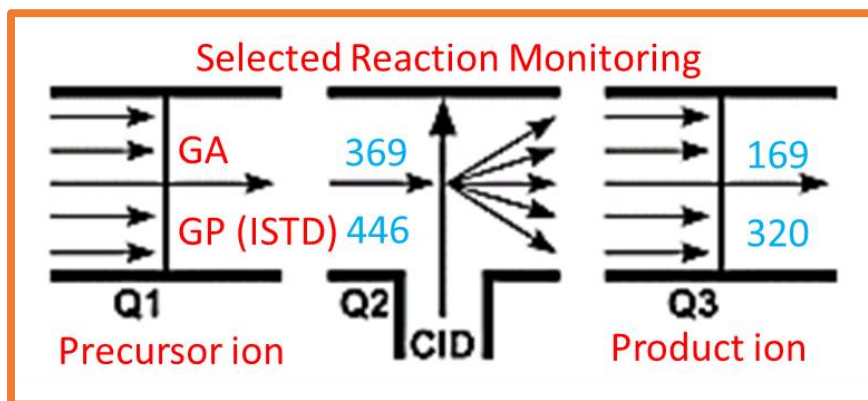
Figure 1.15 SRM transitions used in the bioanalysis of GA and GP (Grebe et al., 2011).

1.5.3 Evaluation of Matrix Effects

The advantages of quantitative LC-MS/MS over traditional HPLC include reduced run times, superior selectivity due to its ability to monitor specific mass ions, and increased sensitivity because of the enhanced signal to noise ratio. However, this technique also comes with a major pitfall called matrix effects especially when ESI is chosen as ionization mechanism (Weng et al., 2002). In its most simple definition, matrix effects are any changes in the ionization process of an analyte due to a co-eluting compound (Lambert, 2004). In a typical LC-MS/MS experiment, large amounts of endogenous matrix components can co-elute with the target analyte. In a SRM experiment these co-eluting components may not be detected. However, they can significantly affect the ionization process of the target analyte. The co-eluting compound may compete with the target analyte during ionization process and contribute to ion suppression (or enhancement). Ion suppression leads to decreased signal intensity of the target analyte causing overall loss in sensitivity of the assay (Lambert, 2004).

Typically, ion suppression is prominent in matrix effects due to phospholipid interference. Glycerophosphocholines and lysophosphatdylchonies constitute about 70% and 10% of total

plasma phospholipids, respectively (Pucci et al., 2009). In a SRM transition experiment carried out in ESI+ mode, these two classes of phospholipids have shown to fragment to form trimethylammonium-ethyl phosphate ions (m/z) 184 in MS/MS (Pucci et al., 2009). **Figure 1.16** shows chemical structures of major class of phospholipids and their corresponding ion fragment of *m/z* of 184 (19).
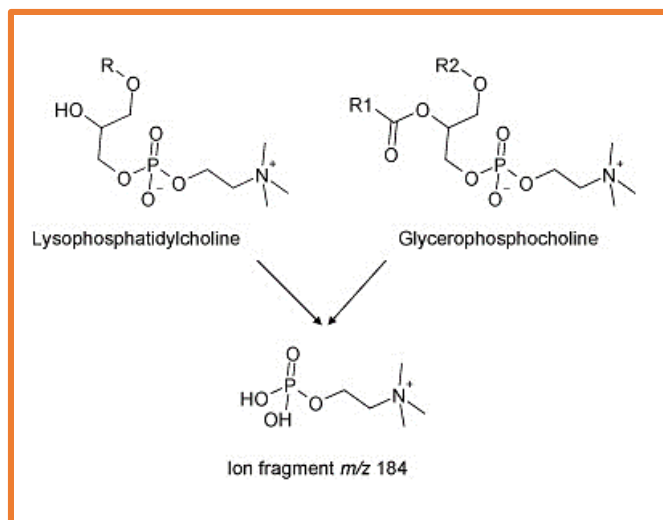
Figure 1.16 Chemical structures of lysophosphatidylcholines and glycerophosphocholines and their trimethylammonium-ethyl phosphate ion fragment (m/z 184) (Pucci et al., 2009).

The molecular structure of both of these phospholipids reveal two major functional groups: a polar head region which consists of an ionizable phosphate moiety and long fatty acid chains (one or two) shown as R, R1 and R2. The highly ionic structure of phospholipids makes them susceptible to co-ionization with the target compound during the ionization process within mass spectrometer.

In the bioanalysis of GA/GP, it was thus important to identify if there was such a loss in sensitivity due to an existing matrix effect. First, a qualitative approach was used to evaluate matrix effect by conducting a post column infusion experiment. **Figure 1.17** shows the post column infusion experiment set up. In this experiment, the analyte was infused at a steady flow while

simultaneously injecting a blank extract. The signal of the analyte was then monitored for variation

caused by co-eluting components from the blank extract in form of suppression in signal intensity

of the analyte. Thus, in this way critical areas (retention time) related to matrix effects were

visualized by either seeing signal suppression of signal enhancement (Weng et al., 2002).
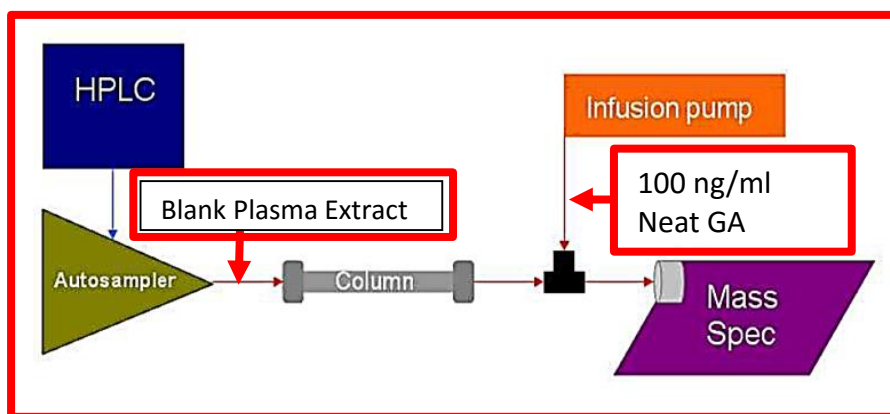
Figure 1.17 Post column infusion experiment Set-Up

Figure 1.18 shows an example of matrix effect in an experiment done elsewhere (Ye et al.,

2015). In this experiment, chromatographic run was carried out using an extracted plasma blank

and 50 % acetonitrile in water while monitoring the phospholipid fragment (m/z of 184) in SRM

mode. The relative intensity of the plasma compared with water is significantly reduced by the co-

eluting phospholipids that cause a drop in the signal (regions of dips in gray, **Figure 1.18**). This

behavior of signal intensity suppression becomes an issue if the analyte peak appears within that

region leading to reduced signal intensity
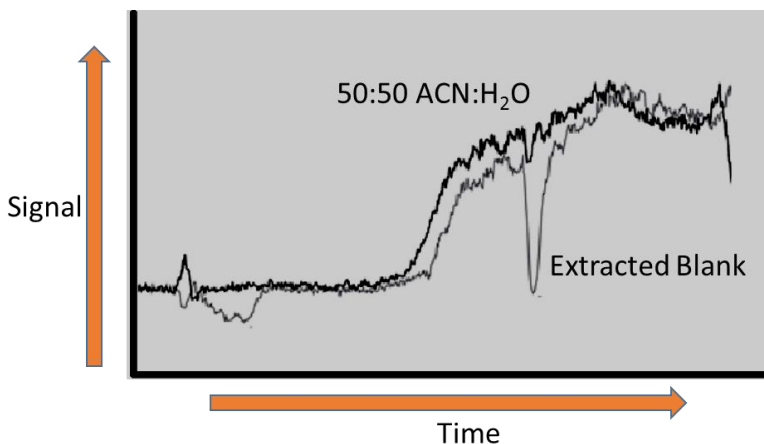
Figure 1.18 Example chromatogram showing matrix effect (Ye et al., 2015).

While a post column infusion experiment is a qualitative way of evaluating matrix effects, a quantitative approach was also carried out by comparing the post extracted spiked sample to the unextracted sample, using the following equation $\%ME = [(A-B)/B]*100$, where A is the area of the post extracted spiked sample and B is the area of the neat standard. A negative value would suggest ion suppression and a positive value would suggest ion enhancement (Pucci et al., 2009). In the bioanalysis of GA, miniaturizations of the sample volume extract and phospholipid removal via HybridSPE (as discussed earlier) during sample process were carried out to minimize matrix effects. Furthermore, chromatographic conditions were optimized to selectively elute peak of interest away from the regions of matrix effect.

CHAPTER 2: METHOD DEVELOPMENT

During the development phase of a suitable bio-analytical method to quantify the glyburide analogue in a biological matrix i.e., plasma, the literature search was expanded to find out what has been already done with this compound from a bioanalytical standpoint. To our knowledge, no reported bioanalytical method exists to date that can quantify concentrations of this compound in any biological matrix. The literature search was therefore further expanded to similarly classified compounds with respect to bioanalysis to form the basis of the method development. An article titled, "Rapid extraction, identification and quantification of oral hypoglycaemic drugs in serum and hair using LC-MS/MS" published in Forensic Science International journal (Binz et al, 2012) was referred to obtain chromatographic and mass spectrometric parameters that could be used in development of the proposed method for the glyburide analogue.

2.1 MS tuning and detection of glyburide analogue via direct infusion

2.1.1   Stock solution preparation of glyburide analogue

Glyburide analogue (GA) reference standard was obtained from Sigma-Aldrich (St. Louis, MO, USA). The GA stock solution was made by dissolving 0.1 mg of the reference standard in 10 mL 50:50 dimethylsulfoxide: acetonitrile + 1% formic acid using a 10 mL volumetric flask. The contents were mixed on a single tube vortexer at the max setting for 30 seconds followed by sonication for one minute in a water bath.

2.1.2   MS tuning with glyburide analogue

The initial detection and subsequent tuning of the glyburide analogue for optimum sensitivity was carried on Waters Micromass Quattro Micro mass spectrometer (Waters Corporation, Milford, MS, USA). The operation of the MS system was carried out by MassLynx 4.1 software in positive electrospray ionization mode (ESI+).

A tuning solution at a concentration of 1 µg/ml (made from 0.1 mg/mL stock solution) was directly infused using a 0.500 mL Hamilton syringe at a flow rate of 0.012 mL/min into the MS source. Tuning parameters for the ESI source and the mass analyzer were adjusted to detect and obtain optimum intensity of the parent ion of glyburide analogue. These parameters under the ESI+ mode were as follows: capillary voltage 3.50 kV, cone energy 28.00 V, collision energy 24.00 V, extractor voltage 2.00 V, RF lens voltage 0.2 V, source temperature 150°C, desolvation temperature 500°C, desolvation gas flow 400 L/hr, cone gas flow 150 L/hr, collision gas (argon) flow 0.15 mL/min), gas cell pirani pressure <1x 10^{-4}mbar, LM1 resolution 12.5 V, HM1 resolution 11.5, LM2 resolution 12.5, HM2 resolution 11.5, ion energy 1 0.5 V, ion energy 2 2.0 V. A total ion chromatogram was obtained for the parent ion (MS 1 or MS full scan) showing the precursor *m/z* of 369.24 representing the (M+H)$^+$ mass. Following precursor ion optimization, collision gas was employed alongside collision energy potential to obtain optimal product ions of the precursor compound. A total ion chromatogram was obtained as MS 2 or product ion scan to see possible fragments of the precursor ion within a range of 100 to 500. The product fragment with *m/z* of 169.02 was found to be the most intense. This observation was confirmatory with respect to theoretical fragmentation pattern within the structure of glyburide analogue as shown in

Figure 2.1. Figures 2.2 and 2.3 show representative mass spectra of precursor and product ions as detected.

5-Chloro-2-methoxy-*N*-[2-(4-sulfamoyl-phenyl)-ethyl]-benzamide
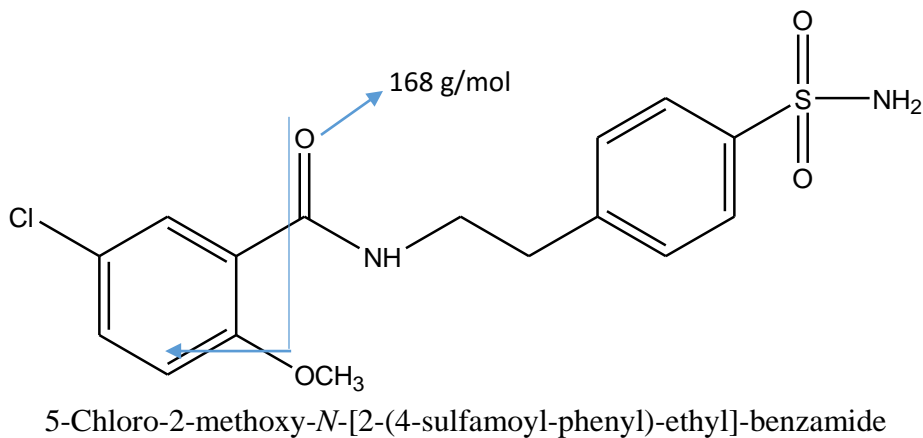
Figure 2.1 Amide bond cleavage leading to the characteristic ion with *m/z* of 169.02 as (M+H)$^+$ product ion.
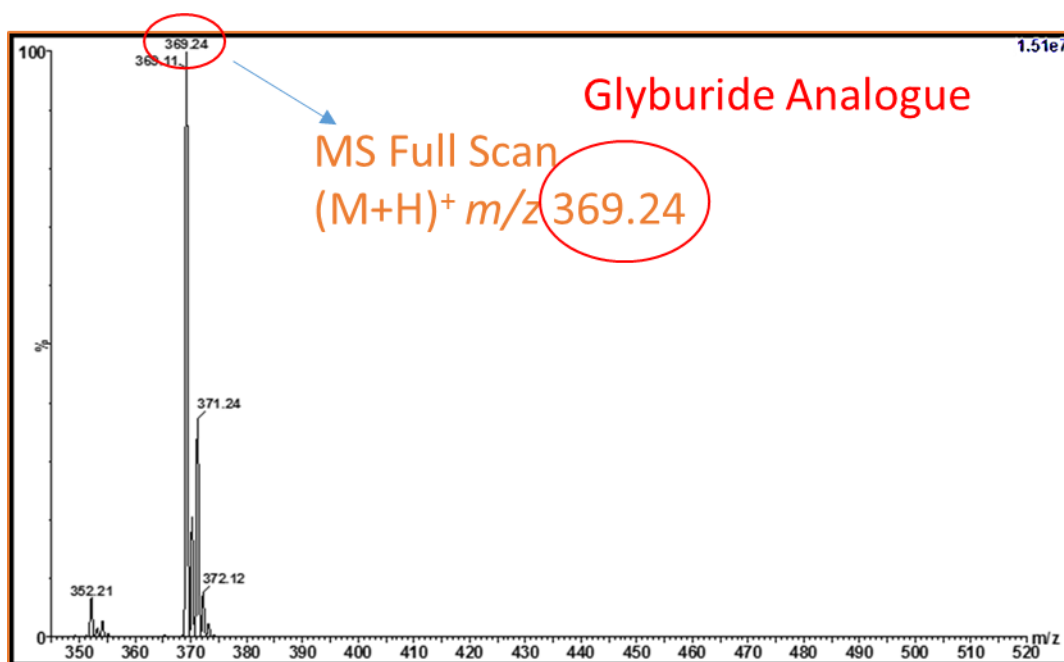
Figure 2.2 (M+H)$^+$ precursor ion peak for glyburide analogue with m/z of 369.24.
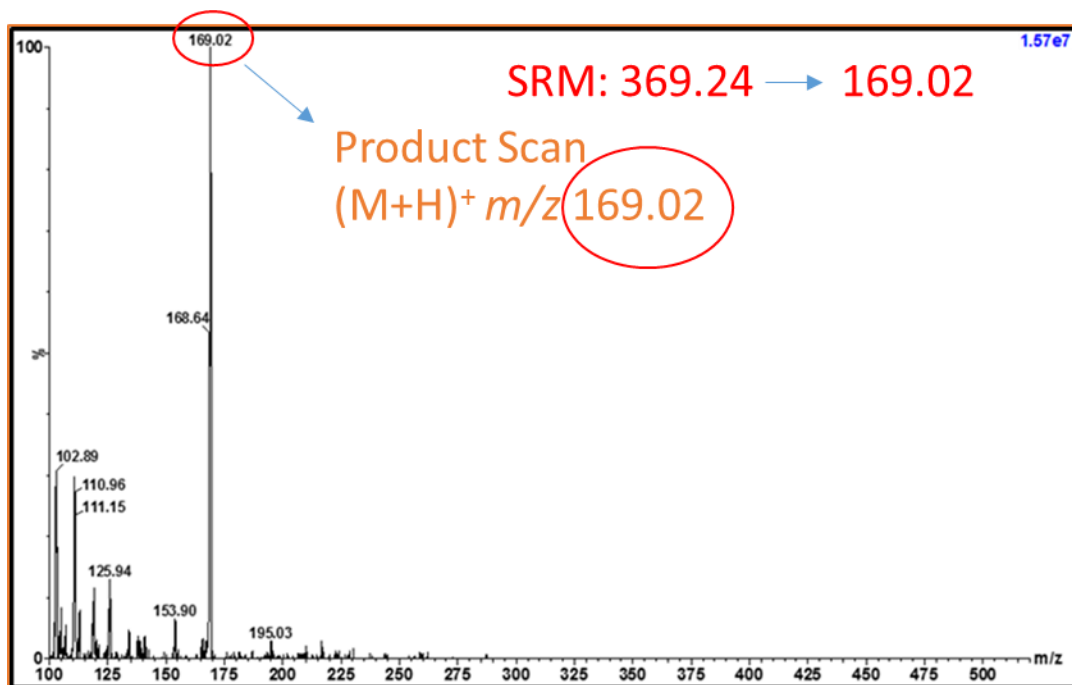
Figure 2.3 (M+H)⁺ product ion peak for glyburide analogue with m/z of 169.02.

2.1.3 *Initial chromatographic detection of glyburide analogue using SRM*

The chromatographic separation of GA (1 µg/ml in 50:50 MeOH: water) was achieved via reversed phase liquid chromatography using a Atlantis dC18 column (2.1 x 50 mm, 3 µm, 100 Å) through a gradient **(Table 2.1)** of mobile phase A (95:5 water:acetonitrile) and mobile phase B (acetonitrile + 0.5% formic acid) at a flow rate of 0.300 mL/min. **Figure 2.4** shows mass chromatograph of GA at 2.77 minutes

Table 2.1. Gradient used during initial chromatographic detection of glyburide

Time	A%	B%
0.00	75.0	25.0
0.20	90.0	10.0
0.50	85.0	15.0
4.50	50.0	50.0
5.50	75.0	25.0
6.50	75.0	25.0
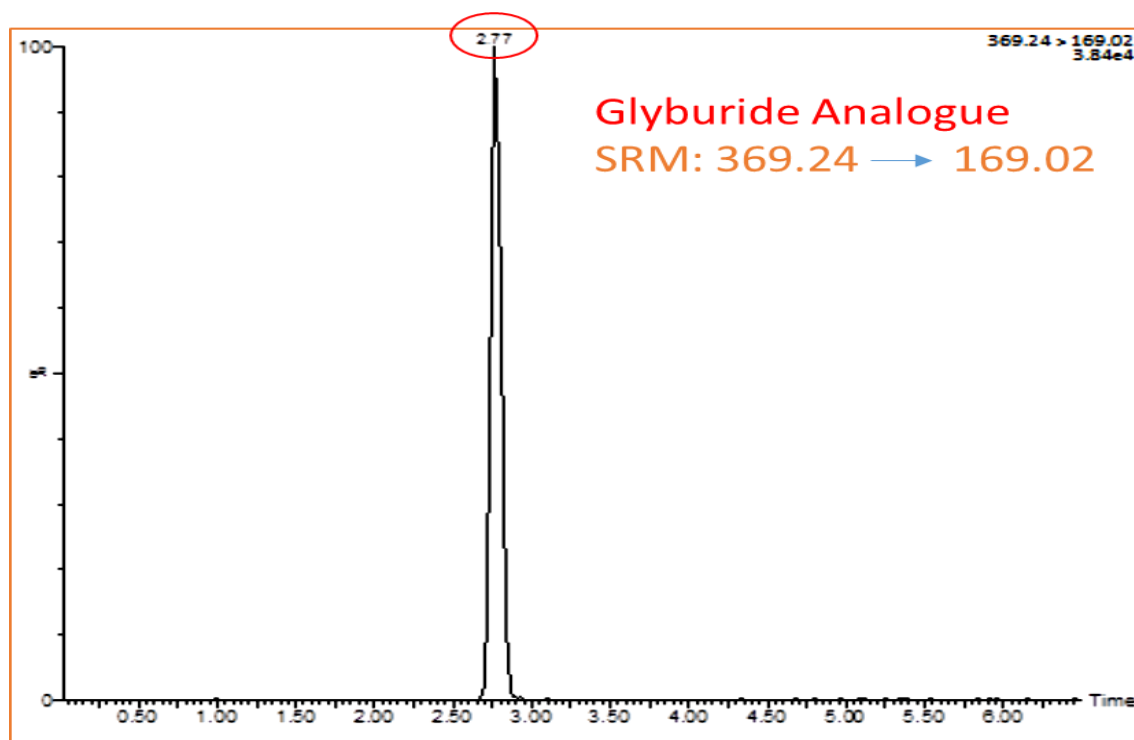
Figure 2.4 Chromatographic detection of GA using SRM (369.24→169.02)

2.2 Internal standard selection and optimization

Currently, no stable isotope labeled internal standard is available for the glyburide analogue; therefore, structurally similar analytes were evaluated as potential internal standards (ISTD) for quantitative analysis of glyburide analogue. **Table 2.2** shows a summary of all compounds tested as potential ISTD.

Table 2.2. List of all compounds tested as potential ISTD in bioanalysis of GA

Compound	Result
Glyburide (MW 494 g/mol)	Failed; in-source fragmentation; cross-talk with GA
Gliclazide (MW 323.41 g/mol)	Failed; Chromatographically resolved, possible Na^+ adduct formation
Glipizide (MW 445.54 g/mol)	Passed; Co-elution with GA; no cross-talk

2.2.1 Evaluation of glyburide

Glyburide, a sulfonylurea, was initially evaluated due its structural similarity. The molecular mass of glyburide is 494 g/mol with a molecular formula of $C_{23}H_{28}ClN_3O_5S$ (5). **Figure 2.5** shows the structure of glyburide.
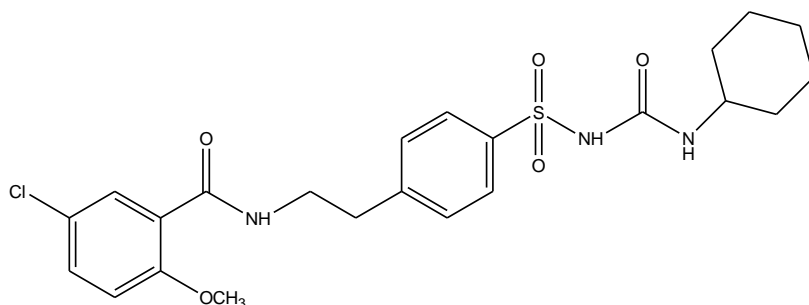
Figure 2.5 Structure of glyburide

The stock solution of glyburide was made using the analytical reference standard obtained from Sigma Aldrich (St. Louis, MO, USA) at a concentration of 0.1 mg/mL in methanol (3). A tuning solution of glyburide was prepared at a concentration of 1 µg/mL in methanol from its stock solution (0.1 mg/mL in methanol). The initial detection and tuning was carried out similarly to the test compound via direct infusion at 12 µl/min. MS 1 or full scan spectrum of glyburide was obtained using the MS tune parameters optimized for the test compound. The cone voltage was changed from 28 V to 25 V and the capillary voltage was changed from 3.50 kV to 3.00 kV while the remaining tune parameters were left unchanged. **Figure 2.6** shows MS 1 scan of glyburide obtained between the masses of 345 to 520. The *m/z* values of the $(M+H)^+$ ion of glyburide was 494.13. However, $(M+H)^+$ ion of the glyburide analogue (*m/z* 369.01) was also detected. This suggested cross-talk between glyburide and the glyburide analogue in their mass spectrometric detection. With varying cone voltage values, the phenomenon of in-source fragmentation of glyburide to glyburide analogue was confirmed. Furthermore, MS 1 scan using precursor mass of

369 was obtained to confirm the presence of the glyburide analogue. **Figure 2.7** shows the confirmatory $(M+H)^+$ ion of glyburide analogue (*m/z* 369.12) during direct infusion of the glipizide tuning solution. Moreover, the MS2 or the daughter scan (**Figure 2.8**) of precursor ion with a *m/z* value of 494 (glipizide) revealed 168.91 $(M+H)^+$ mass ions of the highest relative abundance. This observation suggested that glyburide undergoes two amide bond cleavages first breaking down to yield the parent test compound (glyburide analogue) with $(M+H)^+$ molecular ion of 369.12 *m/z* and subsequently fragmenting to it daughter ion yielding the $(M+H)^+$ molecular ion of 168.91 *m/z*. **Figure 2.9** reveals amide bond cleavages within the chemical structure of glyburide. This observation led glyburide to be a useless candidate as the internal standard because of the prevailing cross talk between the internal standard and the target compound due to a common SRM transition.
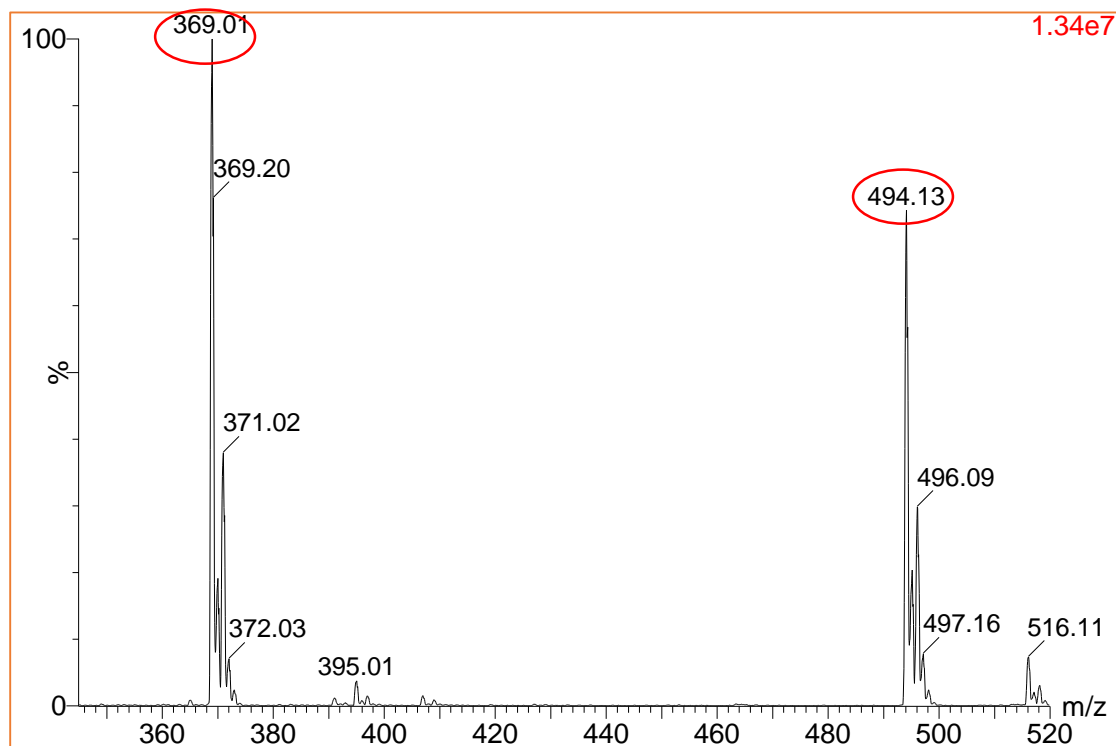
Figure 2.6 $(M+H)^+$ molecular ions for glyburide analogue and glyburide (precursors) with m/z of 369.01 and 494.13 respectively.
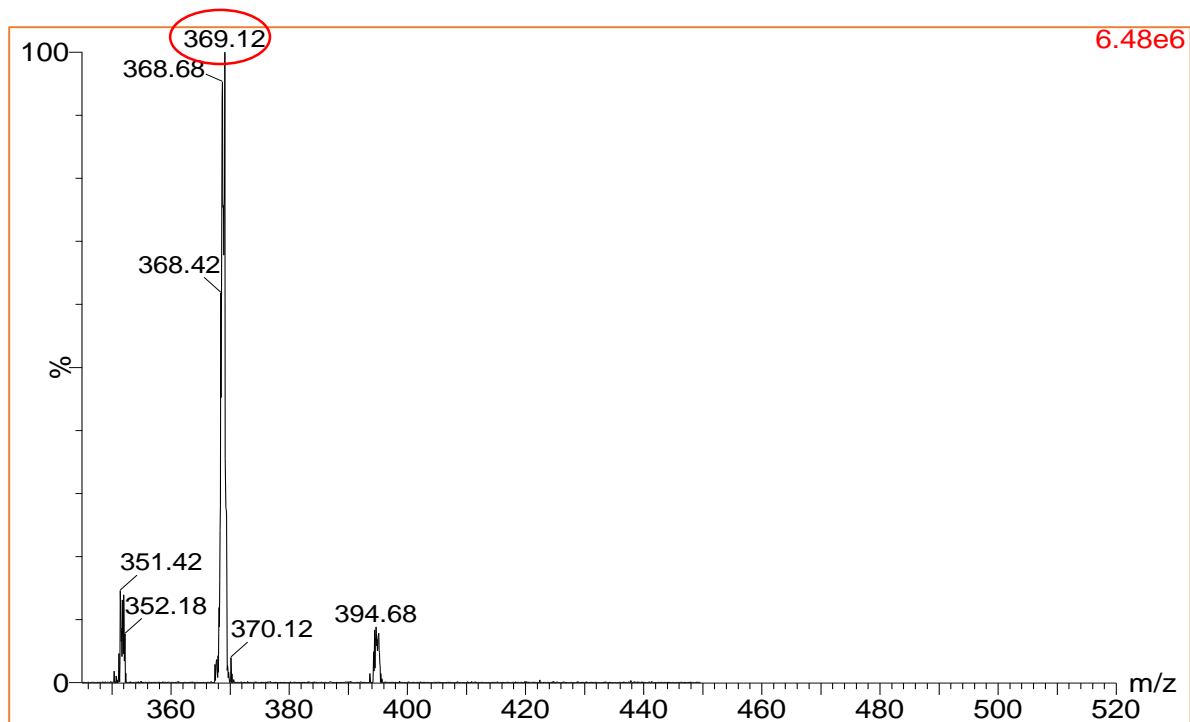
Figure 2.7 (M+H)⁺ molecular ion peak with m/z of 369.12 representing glyburide analogue ion generated from glyburide.
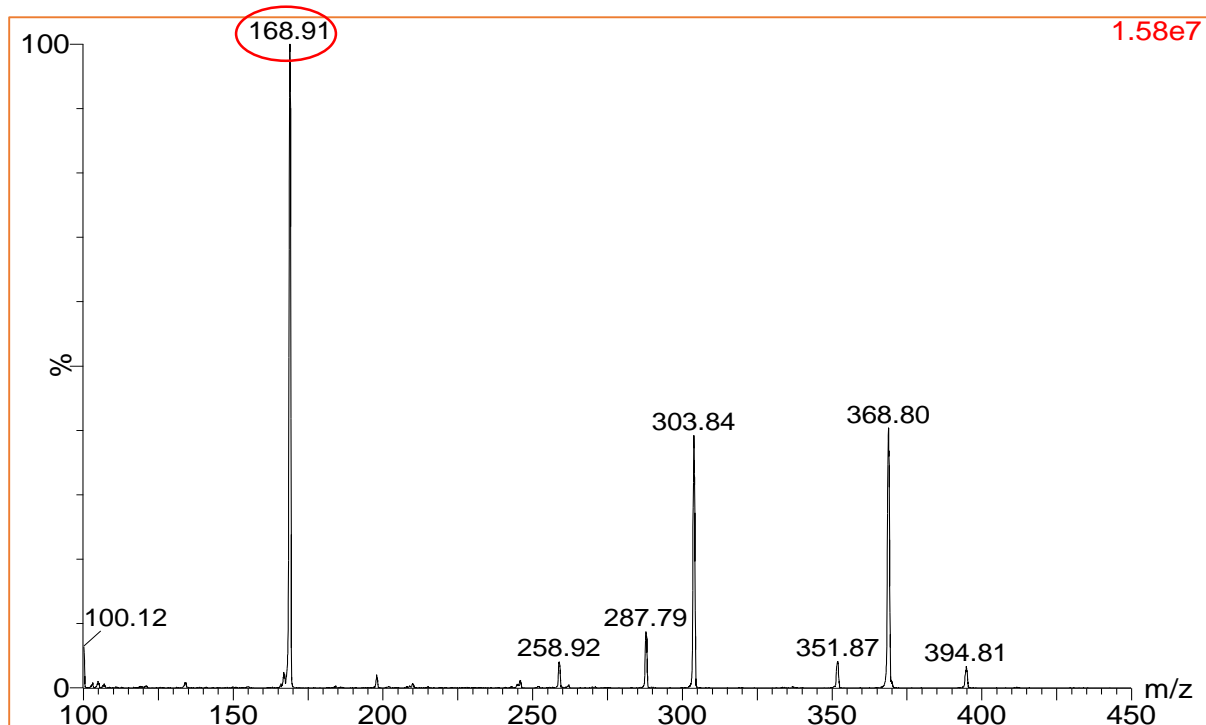
Figure 2.8 (M+H)⁺ molecular ion peak (product) with m/z of 168.91 representing glyburide analogue daughter ion generated from glyburide after multiple amide bond cleavage.
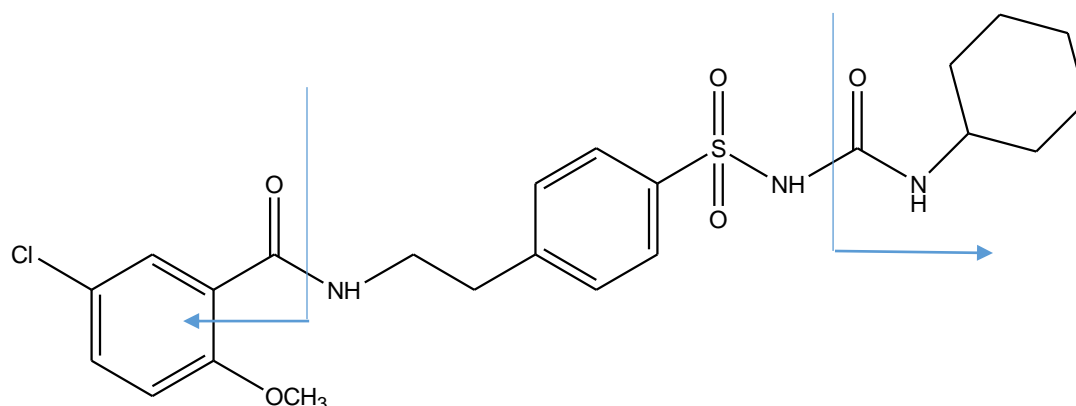
Figure 2.9 Amide bond cleavages within glyburide leading to generation of glyburide analogue and its characteristic product ion fragment

2.2.2 Evaluation of gliclazide

Gliclazide (MW 323.41, linear formula $C_{15}H_{21}N_3O_3S$), another sulfonylurea, was then tested as an alternative internal standard. The MS tuning solution was prepared using the reference standard from Sigma-Aldrich (St. Louis, MO, USA) at a concentration of 1 µg/mL in methanol from the stock solution of 0.1 mg/mL in methanol. **Figure 2.10** below shows the chemical structure of gliclazide (Sigma-Aldrich, 2014).
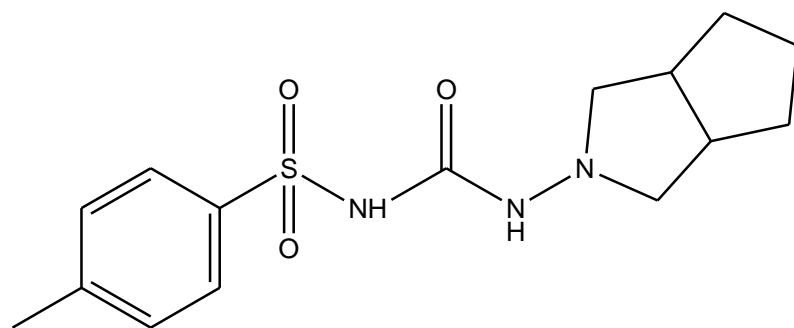
Figure 2.10 Chemical structure of gliclazide. Reprinted from Sigma-Aldrich (2014).

Using previously set tuning parameters for the test compound, initial detection and tuning of gliclazide was carried out in similar fashion. The highest intensity was achieved with the cone voltage and capillary voltage set at 30 V and 3.00 kV, respectively. The MS1 spectra for the $(M+H)^+$ parent ion of mass 323.26 *m/z* was obtained as shown in **Figure 2.11**. Applying a collision

energy of 24 V, the MS2 scan revealed several product ions of $(M+H)^+$ including 90.81, 110.36, and 127.12 m/z as shown in **Figure 2.12**. However, upon further monitoring the 90.81 and 110.36 product fragments were found to be irreproducible even with different cone, capillary and collision energies. The fragment with $(M+H)^+$ at 127.12 was eventually selected as the product ion to be monitored. This was also based on previously known literature that used a similar SRM transition (324.4→127.2) to quantify gliclazide (6). In addition, of 127 was monitored as a specific product for the precursor ion. **Figure**s **2.13** shows a mass chromatogram of $(M+H)^+$ at 127.27 as the product fragment. **Figure 2.14** shows the theoretical fragment pattern which is responsible for generating such a fragment off the parent ion of gliclazide via amide bond cleavage as seen within the test compound as well as glyburide.
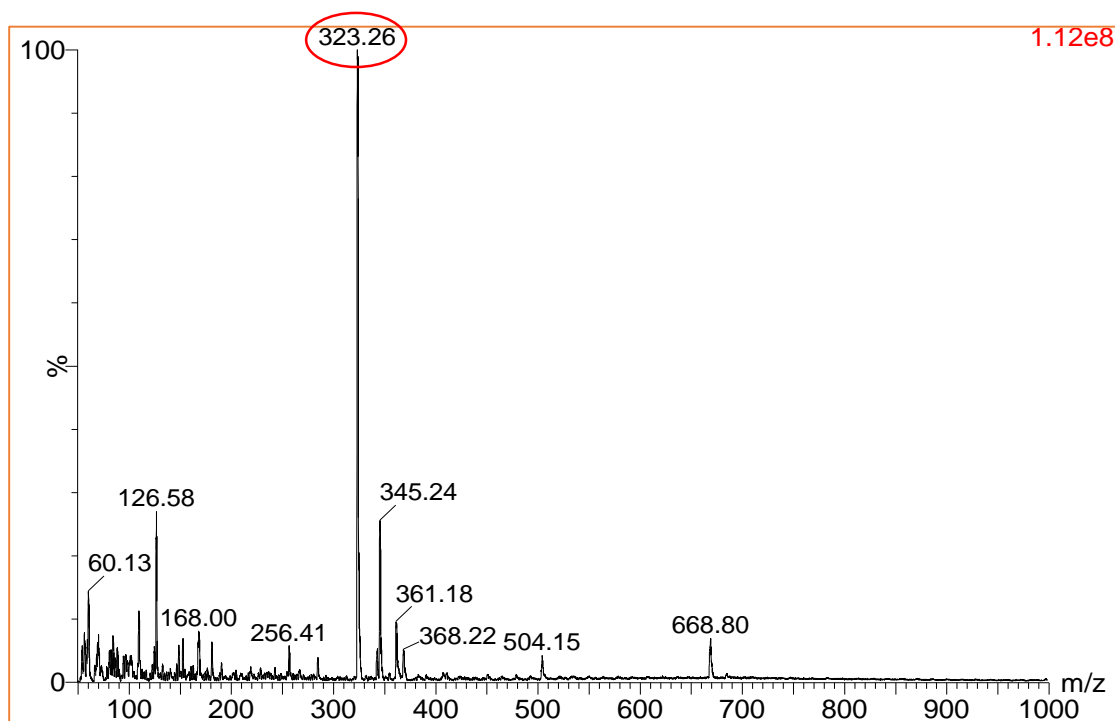
Figure 2.11 $(M+H)^+$ molecular ion peak (parent) with m/z of 323.26 showing gliclazide
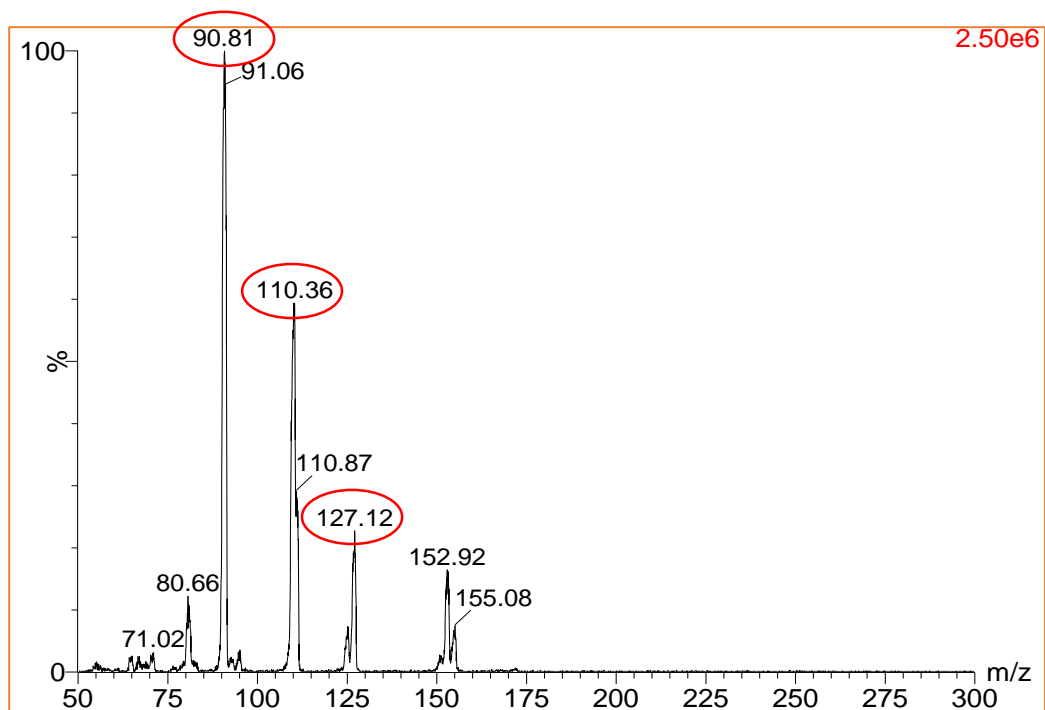
Figure 2.12 Product ion spectrum of ion peaks of gliclazide with m/z of 90.81, 110.36, and 127.12 using 323 as the precursor ion
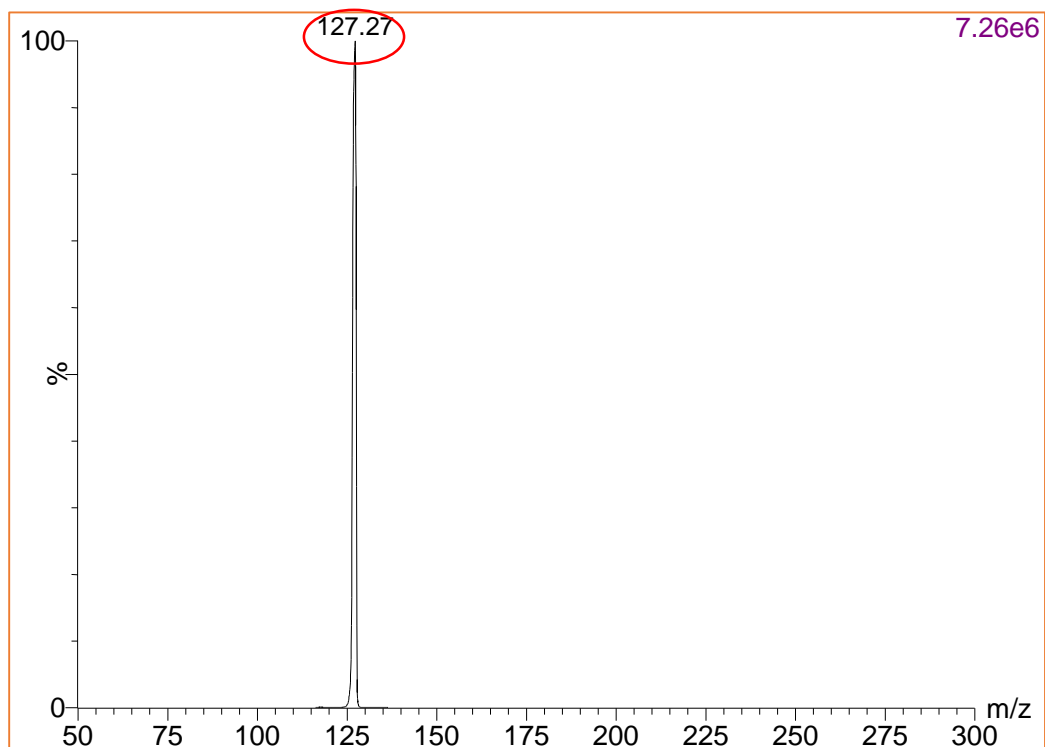
Figure 2.13 (M+H)⁺ product ion peak of gliclazide with m/z of 127.27 upon a specific transition (323→127) being monitored.
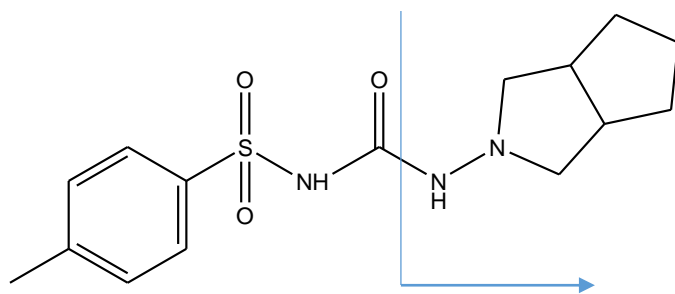
Figure 2.14 Fragment pattern within gliclazide which leads to the generation of daughter ion with (M+H)$^+$ of 127 *m/z* from the parent ion via amide bond cleavage

Upon selecting specific transition, it was necessary to perform a chromatographic run to check the feasibility of simultaneous detection of both, glyburide analogue and gliclazide with their respective SRMs. It was anticipated that both, glyburide analogue and gliclazide co-elute and have similar retention times so matrix effects during plasma and whole blood sample analysis are correctly accounted. To do this, gliclazide solution at a concentration of 1 µg/ml in 50:50 MeOH: water was made from its stock solutions and subsequently injected (30 µl) using similar chromatographic conditions as the glyburide analogue. **Table 2.3** shows specific SRM for gliclazide that was monitored during MS/MS analysis.

Table 2.3. Specific SRM transition being monitored for gliclazide during MS/MS analysis

Q1 (Precursor Ion)	Q3 (Product Ion) (Da)	Cone (V) Energy	Collision (V) Energy	Ionization mode
323.69	127.12	24	28	ESI+

A representative chromatogram of gliclazide is shown in **Figure 2.15**. It was observed that the gliclazide peak (represented by characteristic SRM transition 323.69→127.12) had a different retention time (3.88 minutes) compared to the glyburide analogue (RT 2.77 minutes; **Figure 2.4**). In other words, the compounds were found to be significantly chromatographically resolved. Furthermore, cross-talk between gliclazide and the glyburide analogue was observed when the SRM transition (369.01→169.15) specific to the glyburide analogue was monitored in form of two

peaks detected at retention times of 3.71 and 3.93 minutes. This phenomenon can be explained by possible Na+ adduct formation of the gliclazide molecule. During the ESI+ process, it could have been possible that 2 sodium ions formed an adduct with gliclazide, increasing its mass by 46 Da for a total of 369 Da which then generated a fragment of 169 *m/z* similar to glyburide analogue. It was concluded that even if systematic approaches to address possible adduct formation happen to be successful, it would be difficult to achieve co-elution of both compound given the vast difference in their retention times. Hence, gliclazide was deemed inappropriate as the ISTD in bioanalysis of the glyburide analogue.
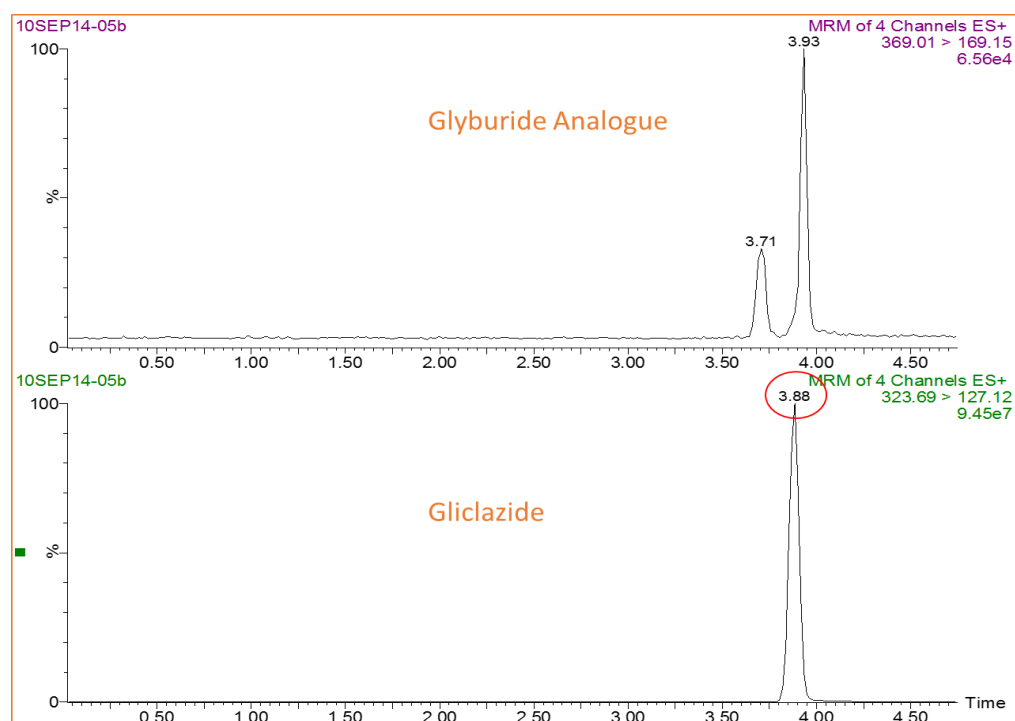
Figure 2.15 Cross-talk between glyburide analogue and gliclazide detected during a chromatographic run of gliclazide only (1 µg/ml)

2.2.3 Evaluation of glipizide

Due to the glyburide cross talk and possible sodium adduct formation along with issue of chromatographic resolution with gliclazide, glipizide, another sulphonylurea, was tested for its use as the IS. The chemical structure of glipizide is shown in **Figure 2.16.**
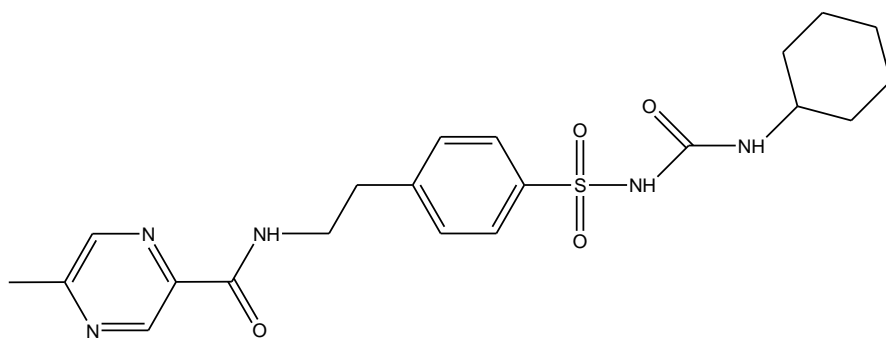
Figure 2.16 Chemical structure of glipizide (Sigma-Aldrich, 2014).

Glipizide reference standard in form of white powder (MW of 445.54 g/mol with a molecular formula of $C_{21}H_{27}N_5O_4S$) was obtained from Sigma Aldrich (St. Louis, MO, USA). The MS tuning solution was prepared using the reference standard at a concentration of 1 µg/ml in methanol from the stock solution of 0.1 mg/ml in dichloromethane. **Figure 2.17** shows the MS 1 spectra obtained via MS tune file settings for the glyburide analogue. The cone voltage and the capillary voltage were set at 17 V and 3.50 kV respectively and rest of the parameters were unchanged. The MS1 scan reveals glipizide $(M+H)^+$ with *m/z* of 445.80. The MS2 scan of glipizide obtained with collision energy of 15 V is shown in **Figure 2.18**. The product ion with *m/z* of 320.62 was most abundant.
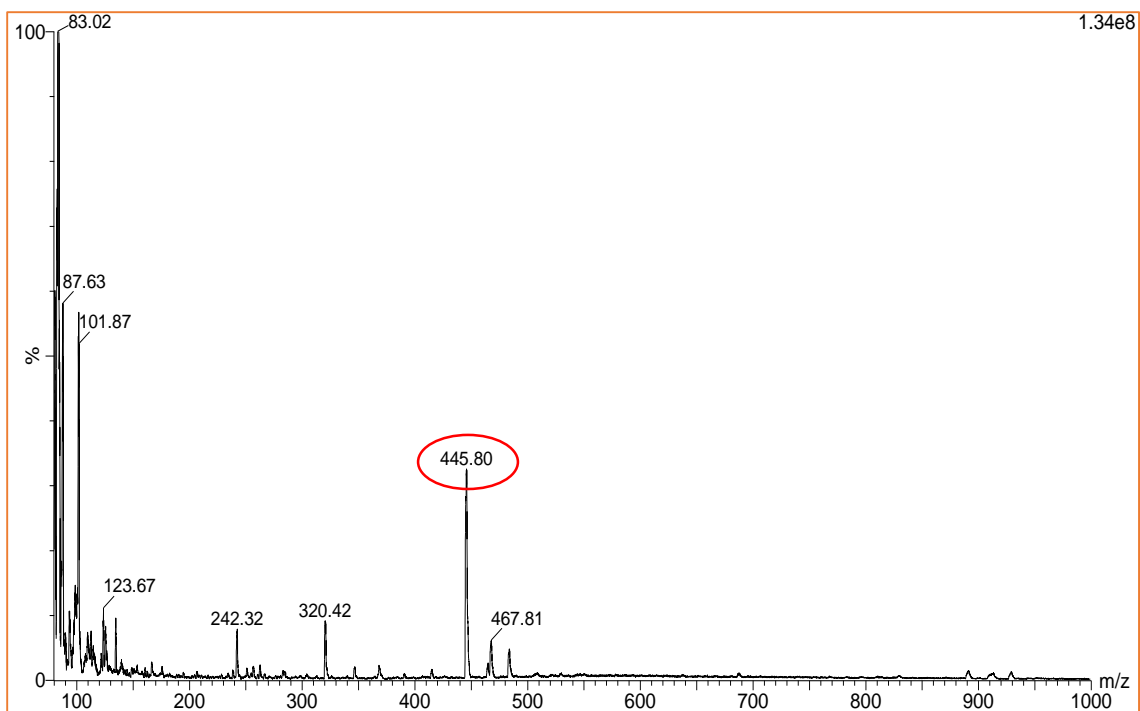
Figure 2.17 (M+H)+ molecular ion peak (precursor) of glipizide with m/z of 445.80
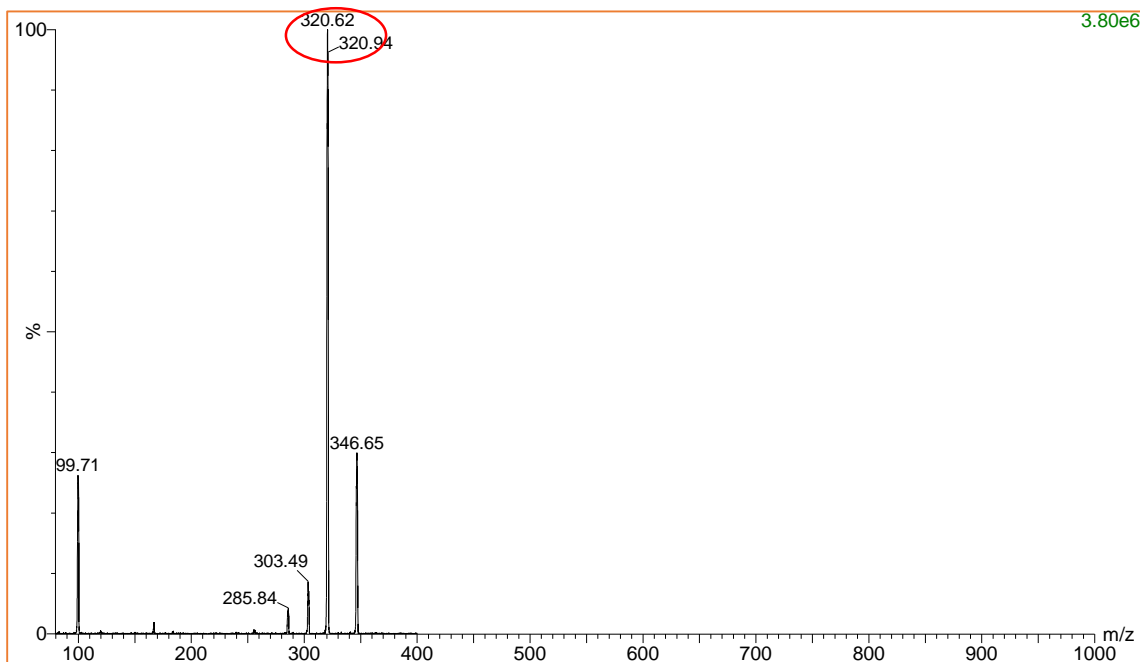
Figure 2.18 Product ion peak of glipizide with *m/z* of 320.62

The chromatographic feasibility and co-elution of glyburide analogue and glipizide were evaluated similarly to the gliclazide study. The initial chromatographic detection of glipizide was carried out using previously used chromatographic parameters for glyburide analogue including mobile phase, column and gradient conditions. Separate solutions of the test compound and the ISTD were made at 1 µg/ml in 50:50 methanol: water using respective stock and intermediate solutions (in methanol). The injection volume for each compound was 30 µL. **Figures 2.19** and **Figure 2.20** show chromatograms of glyburide analogue and glipizide in 50:50 methanol: water. Knowing that these analytes are structural analogues to glyburide, both glyburide analogue and glipizide show similar retention times while free of cross-talk. The IS elutes at 2.81 min while the test compound has a retention time of 2.77 min, which is very favorable for compensation of matrix effects. Based on this observation, glipizide was chosen as the IS in developing a suitable LC-MS/MS based bioanalytical method to quantify glyburide analogue and the solvent of choice was 50:50 methanol: water for future analysis.
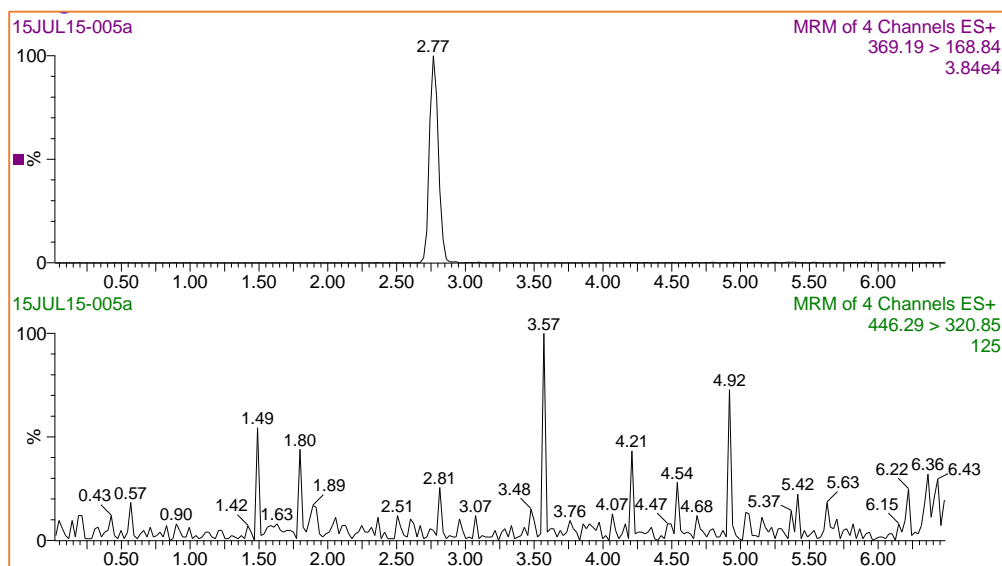
Figure 2.19 Chromatographic detection of the glyburide analogue at 1 µg/ml (top) with SRM (369.19→168.84). No cross talk observed with glipizide upon its SRM (446.29→320.85) in the glyburide analogue solution (bottom)
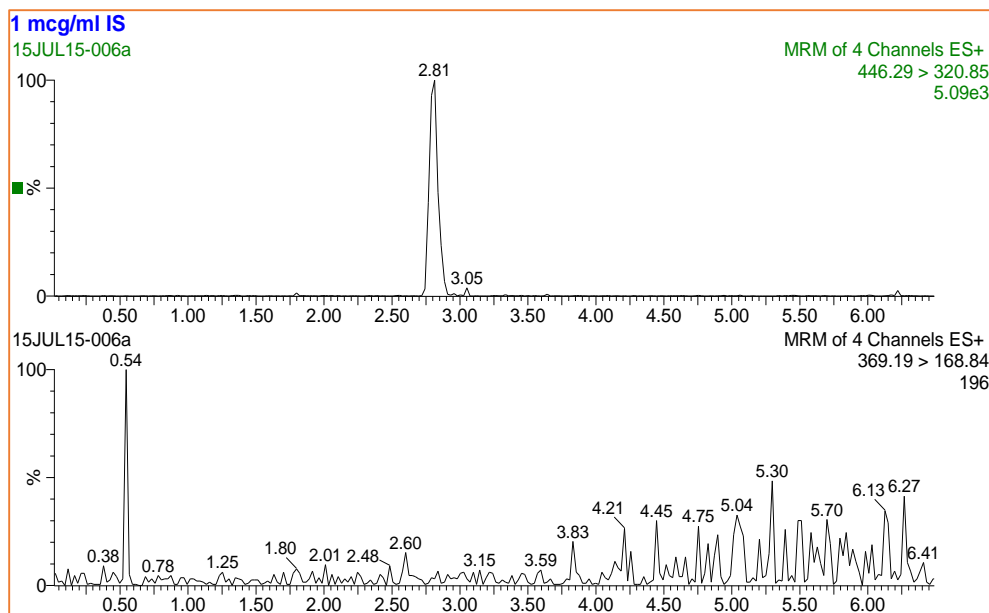
Figure 2.20 Chromatographic detection of glipizide at 1 µg/ml (top) with SRM (446.29→320.85). No cross talk observed with glyburide analogue upon its SRM (369.19→168.84) in the glipizide solution (bottom)

2.3 Post column infusion experiment to evaluate for matrix effects in extracted blank mouse plasma

After the initial detection of the glyburide analogue (test compound) and its structural analogue internal standard, glipizide, a post column infusion experiment was carried out to evaluate the presence of matrix effects, specifically at their retention times. Blank extract (Mouse Plasma, CD-1, sodium heparin, 3 lots pooled gender) was prepared using HybridSPE 96 well precipitation plate (Sigma, St. Louis, MO, USA) as follows: first, a 75 µL aliquot of water was added to the Hybrid SPE plate to pre-wet the bed. To this, 25 µl of blank plasma was added after being vortexed and centrifuged (3000 rpm, 5 minutes). Then, 300 µL of 1% formic acid in acetonitrile was added as the precipitating reagent. The blank plasma samples were then vortexed using a Talboys 96 well plate shaker (Troemner Laboratory Equipments, Thorofare, NJ, USA) for 2 minutes. The samples were then collected onto a Waters 1 mL collection plate (Waters Corporation, Milford, MA, USA) using the Tomtec Quadra (Hamden, CT, USA) vacuum manifold

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and evaporated to dryness using N$_2$ (60 psi) via SPE Dry (Biotage, Charlotte, NC, USA) 96 well plate evaporator at 50 °C. The samples were then reconstituted using 100 µl of 50:50 methanol: water followed by mixing for 1 min on the Talboys 96 well plate shaker. Thirty microliters of sample was injected onto the LC-MS and data were collected for three replicates while performing a post column infusion of glyburide analogue at 100 ng/mL concentration. Phospholipids were monitored at cone energy of 90 V and collision energy of 5. The specific SRM transition monitored to detect phospholipids was 184→184. The inlet file, MS tunes file and the SRM transition being monitored for glyburide were same as used during the initial chromatographic run. **Figure 2.21** shows the post column infusion chromatogram obtained in this experiment.
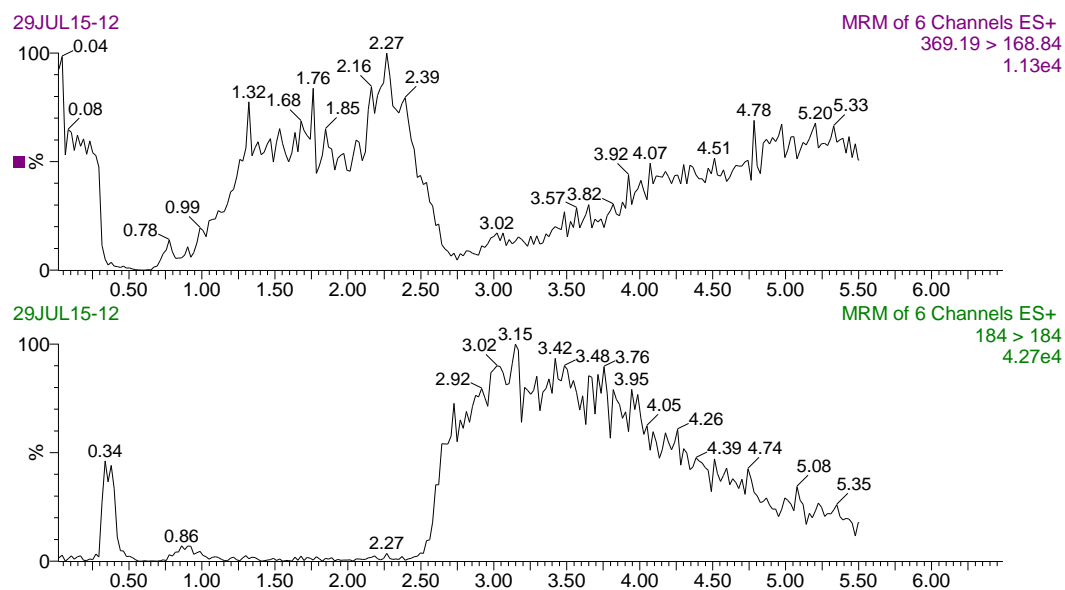
Figure 2.21 Post column infusion chromatogram of 100 ng/ml glyburide analogue showing the glyburide analogue (top), while monitoring characteristic SRM (184→184) for phospholipids (bottom).

The post column infusion experiment revealed areas of signal suppression around the retention times (2.77 – 2.81 minutes) at which both glyburide analogue and the ISTD were detected. Also, it appeared that the HybridSPE plate did not completely remove phospholipids. In

order to minimize matrix effects, the chromatographic optimization experiments were carried out

to cause "peak shift" such that the peaks of interest elute away from the regions of ion suppression

as identified during the post column infusion experiment.

2.4 Chromatographic optimization based on results of post column infusion experiment

2.4.1 Sample Solvents vs Peak Shape

In order to see the effect of solvent composition (reconstitution solution) on peak shape

and signal intensity, glyburide analogue at 1 µg/ml in the baseline composition (50:50 methanol:

water) was subjected to increased organic (60:40 MeOH: H_2O) and aqueous (10:90 MeOH: H_2O)

conditions. The initial gradient at which glyburide analogue was detected remained unchanged.

Figure 2.22 show GA detection at increased organic condition where peak splitting was observed.

In contrast, no such splitting was observed with increased aqueous condition of the sample solvent.

It was thus concluded that increased aqueous component was necessary to retain the peak shape

during chromatographic detection of the test article. Similar results were assumed for the ISTD

due to its similarity in structure and physicochemical properties.
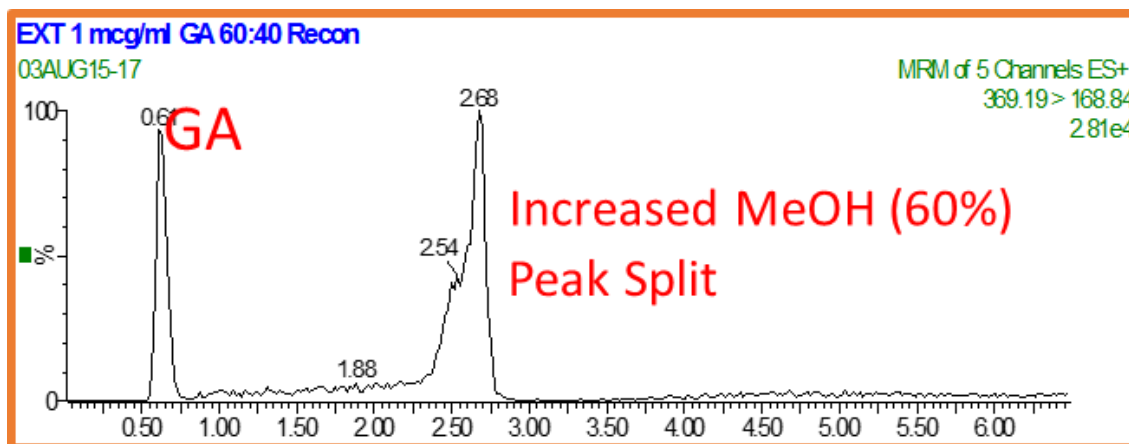
Figure 2.22 Chromatographic detection of GA (1 µg/ml) upon increased organic component in the solvent phase (60: 40 methanol: water).

2.4.2. Gradient Change vs. Peak Shift

A) Modified Gradient 1

A modified gradient with respect to %B composition was employed where aqueous component of mobile phase was changed to organic phase relatively quickly as compared to the baseline composition used during the initial detection of GA. Upon changing the gradient from 15% to 50% between 0.50-4.50 minutes to 15% to 95% between 0.50-4.50 minutes, the retention time changed from 2.77 minutes to 2.70 minutes (**Figure 2.24**). A steeper gradient (**Figure 2.23**) change favored the elution of peak of interest slightly away from the region of suppression. Based on this result, gradient was further modified to improve elution (reduced RT).
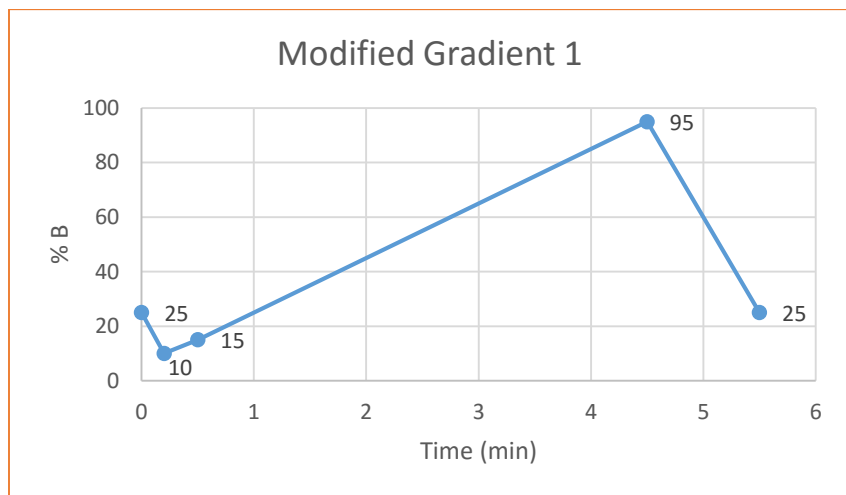
Figure 2.23 Modified gradient 1
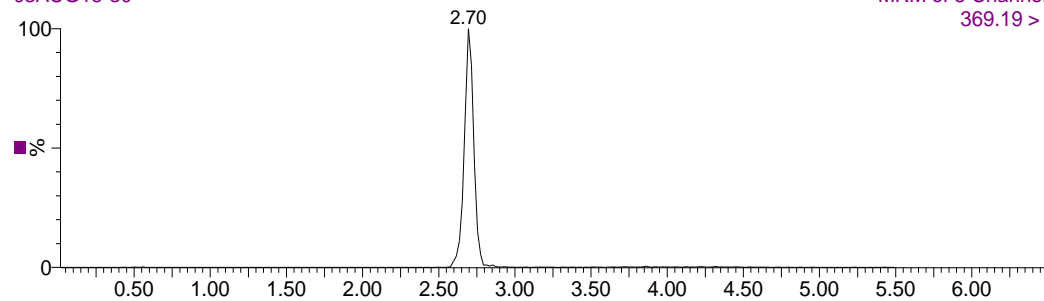
Figure 2.24 Chromatographic shift of GA peak to 2.70 minutes (RT)

B) Modified Gradient 2

A modified gradient 2 was employed where aqueous composition of mobile phase was changed to organic phase even more steeply compared to the modified gradient 1. Upon changing the gradient from 15% to 95% between 0.50-4.50 minutes to 10% to 98% between 0.20-2.50 minutes, the retention time changed from 2.70 minutes to 2.16 minutes (**Figure 2.26**). A steeper gradient change (**Figure 2.25**) favored the elution of peak of interest completely away from the region of suppression.
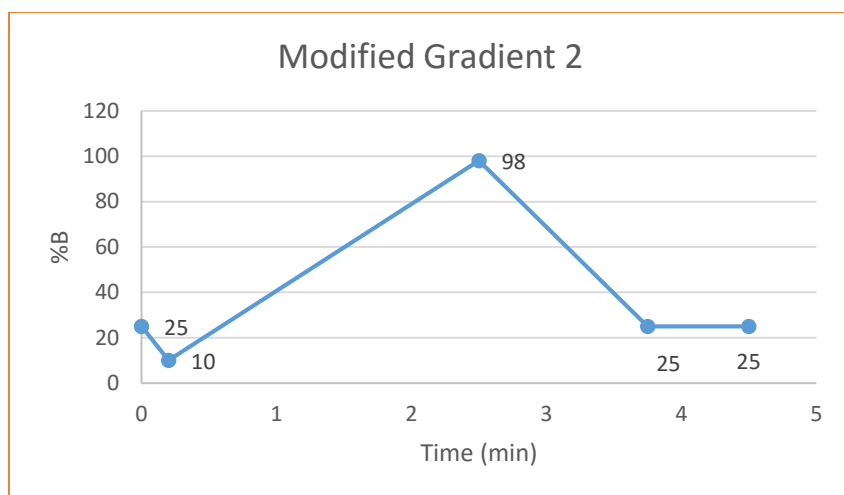
Figure 2.25 Modified gradient 2
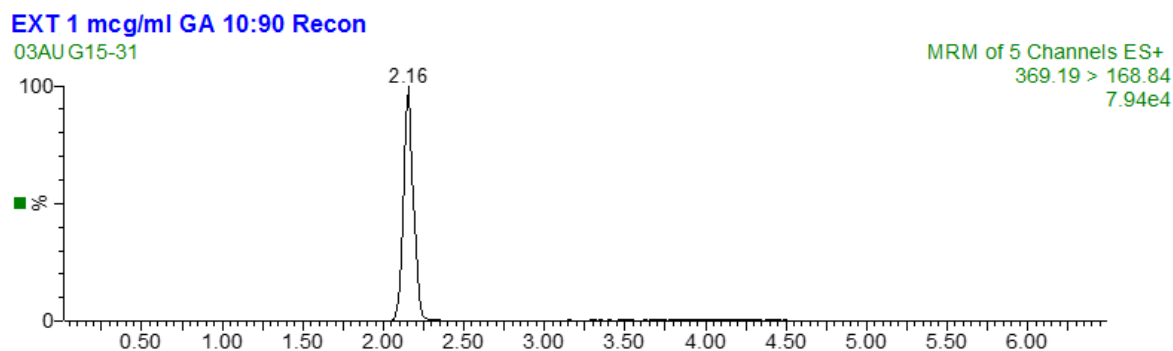
Figure 2.26 Chromatographic shift of GA peak to 2.16 minutes (RT)

C) Modified Gradient 3

Upon changing the gradient from 10% to 98% between 0.20-2.50 minutes to 0% to 98% between 0.20-2.50 minutes (**Figure 2.27**), no change in the retention time was observed. However, a two-fold increase in the signal intensity was observed (**Figure 2.28**). Modified gradient 3 was finalized for subsequent chromatographic separation for future analysis.
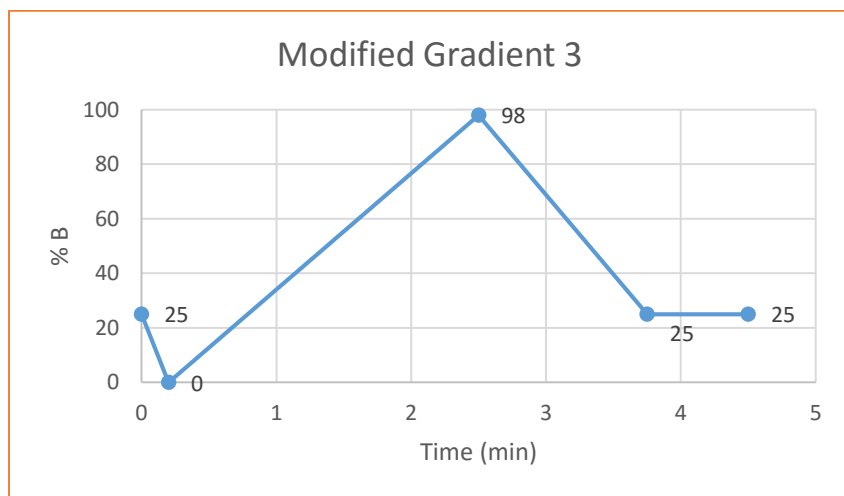
Figure 2.27 Modified gradient 3
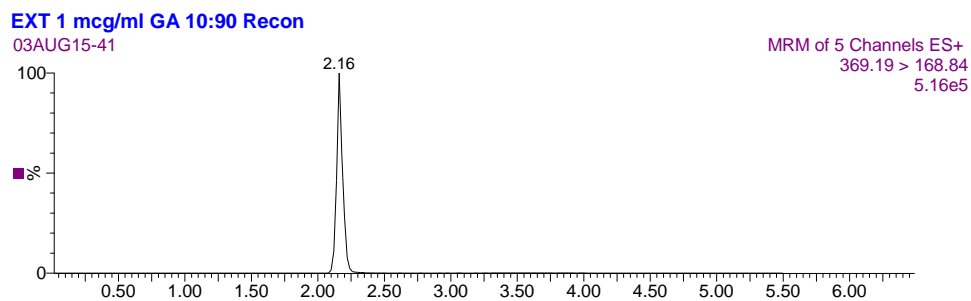
Figure 2.28 Chromatographic detection of GA peak with no change in the retention time and increased signal intensity.

2.4.3 Post chromatographic optimization detection of glyburide analogue and glipizide (ISTD) in a neat solution

After conducting chromatographic optimization experiments with the modified gradient conditions, it was imperative to verify whether the ISTD peak has shown similar behavior (elute

away from the suppression region) as the test compound. Chromatographic detection of GA and

ISTD (both 1 µg/ml) was carried out with modified gradient 3 conditions in a combined solution

in 10:90 methanol: water. **Figure 2.29** shows co-elution of GA and ISTD as expected with

modified gradient conditions and an overall increase in the signal intensity for both compounds

with respect to the baseline gradient conditions.

EXT Combined 1 mcg/ml GA/GP
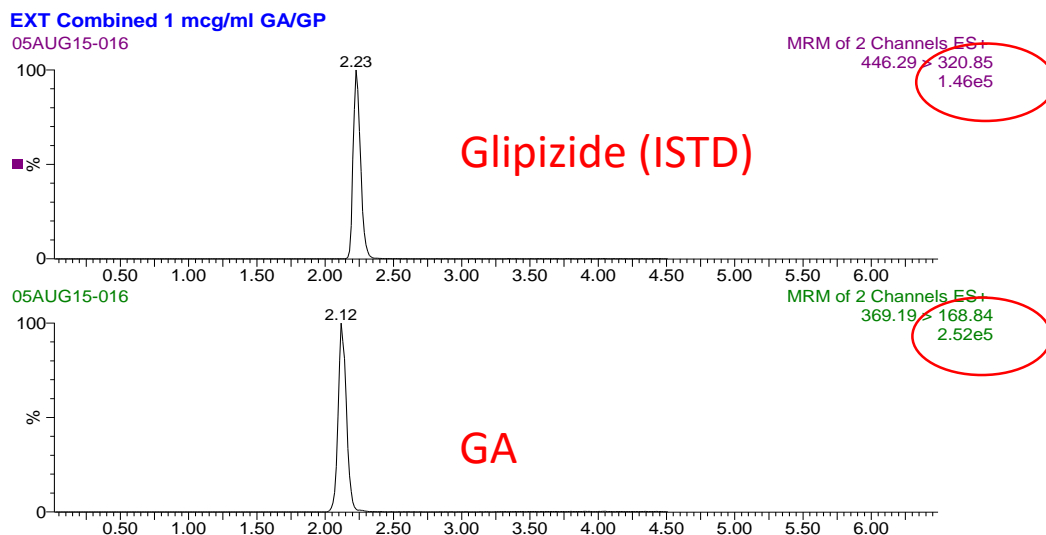
Figure 2.29 Chromatographic detection of GA/GP (1 µg/ml) with finalized gradient conditions

2.4.4 Finalized LC-MS/MS parameters for glyburide analysis

After chromatographic optimization experiments, parameters of the MS Tune File, Inlet

File and MS/MS Method File were finalized as following:

o   *MS Tune File Parameters*

Tuning parameters for the ESI source in the positive mode and the mass analyzer optimized

for the parent compound, the glyburide analogue and the internal standard were as follows:

capillary voltage 3.50 kV, cone energy 28.00 V, extractor voltage 2.00 V, RF lens voltage 0.2 V,

source temperature 150 °C, desolvation temperature 450°C, desolvation gas flow 400 L/hr, cone

gas flow 150 L/hr, collision gas (argon) flow 0.15 mL/min), gas cell pirani pressure $<1 \times 10^{-4}$ mbar,

LM1 resolution 12.5 V, HM1 resolution 11.5, LM2 resolution 12.5, HM2 resolution 11.5, ion energy 1 0.5 V, ion energy 2 2.0 V.

o *Inlet File Parameters/Chromatographic Conditions:*

Chromatographic separation of glyburide analogue and the ISTD were achieved using a Shimadzu LC-10AD VP binary pump and HTC PAL O2-AS Auto-sampler with following inlet parameters and LC conditions: Atlantis C18 column (2.1 x 50 mm, 3 µm, 100 A°), mobile phase A (95:5 water: acetonitrile + 0.5% formic acid), mobile phase B (acetonitrile + 0.5% formic acid), column temperature 40 ºC, sample temperature 5 ºC, sample injection volume 30 µL. The following step gradient conditions were used as shown in **Table 2.4** with a total run time of 4.50 minutes.

Table 2.4. Gradient conditions used in the bioanalysis of glyburide analogue

Time	A%	B%	Flow (ml/min)
0.00	75.0	25.0	0.300
0.20	100.0	0.0	0.300
2.50	2.0	98.0	0.300
3.75	75.0	25.0	0.300
4.50	75.0	25.0	0.300

o *MS/MS Method Parameters:*

MS/MS analysis of glyburide analogue and the ISTD was achieved using following SRM parameters as shown in **Table 2.5** for a total detection time of 4.50 minutes.

Table 2.5. MS/MS parameters used in the bioanalysis of glyburide analogue

Q1 (Precursor Ion)	Q3 (Product Ion) (Da)	Cone (V) Energy	Collision(V) Energy	Dwell Time (s)
369.19	168.84	28.00	24.00	0.35
446.29	320.85	17.00	15.00	0.35

2.5 HybridSPE extraction optimization

2.5.1   Mouse Plasma Evaluation

2.5.1.1 Selectivity based on extraction solvent variants

The HybridSPE technology allowed for two variants of extraction solvent that could be used for selective extraction of compound of interest from the biological matrix (plasma): 1% formic acid in acetonitrile and 1% ammonium formate in methanol. In-well precipitation method using HybridSPE 96-well format was performed to extract blank plasma (3 lots, pooled gender) from mouse (CD-1 strain with sodium heparin) in a similar fashion as the post column infusion experiment. Both extraction solvents namely 1% formic acid in acetonitrile and 1% ammonium formate in methanol were tested separately during the extraction procedure while other parameters remained unchanged. **Figure 2.30** and **Figure 2.31** show chromatograms of blank plasma extract (injection vol. 30 µl) with 1% formic acid in acetonitrile and 1% ammonium formate in methanol with relative signal responses. Selectivity was evaluated based on the relative signal response during the SRM of glyburide analogue and glipizide in each solvent. In blank extracts, a relatively higher signal (from other co-eluents) was observed with glyburide analogue (1320 counts) where extraction was carried out with 1% formic acid in acetonitrile compared to the signal response (756 counts) of the blank extracted with 1% ammonium formate in methanol. Similar differences were observed with glipizide as well. Based on these chromatographic results, it was concluded that a more selective extraction was possible with 1% ammonium formate in methanol as the extraction solvent.
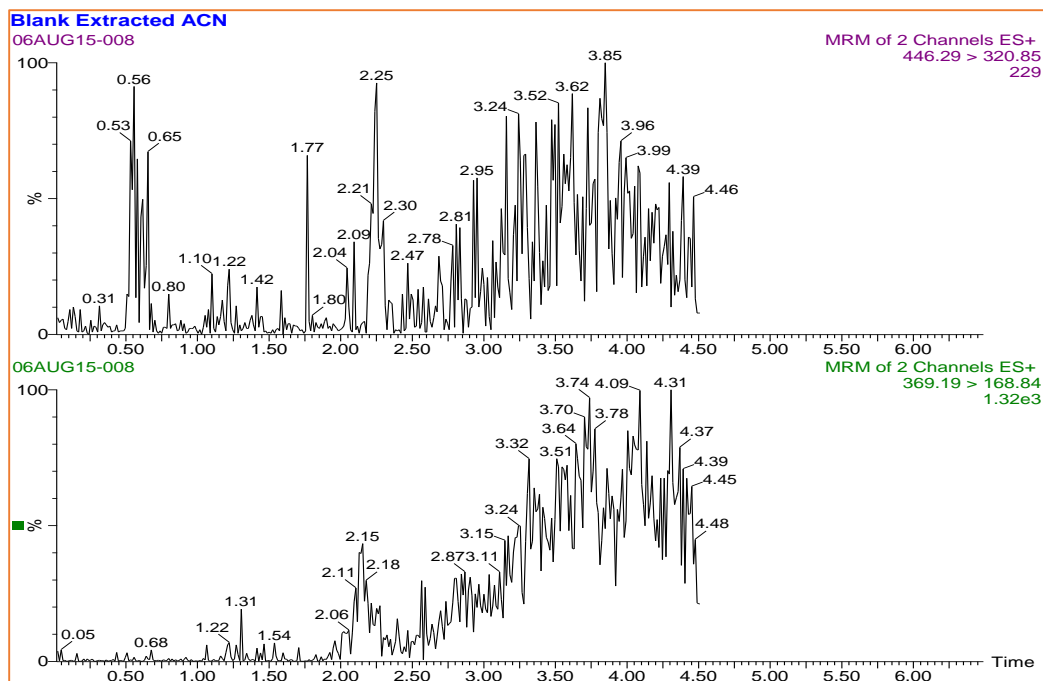
Figure 2.30 Blank plasma extracted with 1% formic acid in acetonitrile. Glipizide (top) and glyburide analogue (bottom) were monitored using specific SRM transitions 446.29→320.85 and 369.19→168.84 respectively.
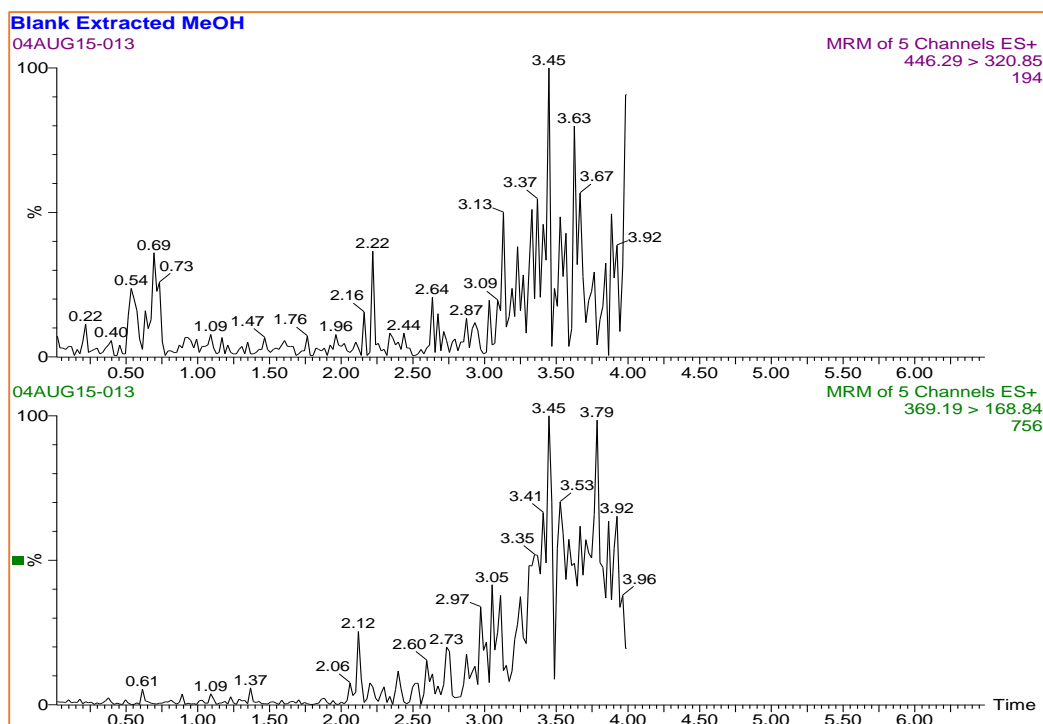
Figure 2.31 Blank plasma extracted with 1% ammonium formate in methanol. Glipizide (top) and glyburide analogue (bottom) were monitored using specific SRM transitions 446.29→320.85 and 369.19→168.84 respectively.

2.5.1.2 Recovery and Matrix Effect Evaluation Post Chromatographic Optimization

I)   Qualitative Assessment: Post Column Infusion Experiment

While post chromatographic experiments enabled favorable results with peak shifting away from regions of signal suppression due to matrix effects, it is however possible that the whole suppression region might have shifted along with the peaks of interest. It was thus imperative to do a qualitative assessment of matrix effects via post column infusion experiment with optimized gradient conditions. To do this, a post column infusion experiment was carried out in a similar fashion as before with glyburide analogue and glipizide directly infused (100 ng/ml, 12 µL/min) during a chromatographic run of blank extract using 1% ammonium formate in methanol as the extraction solvent. **Figure 2.32** shows the chromatographic run of a blank plasma extract where SRM transitions of glyburide analogue, glipizide and phospholipids were monitored. The vertical blue line shows the peak retention time for glyburide and glipizide free from phospholipid presence and minimized matrix effects.
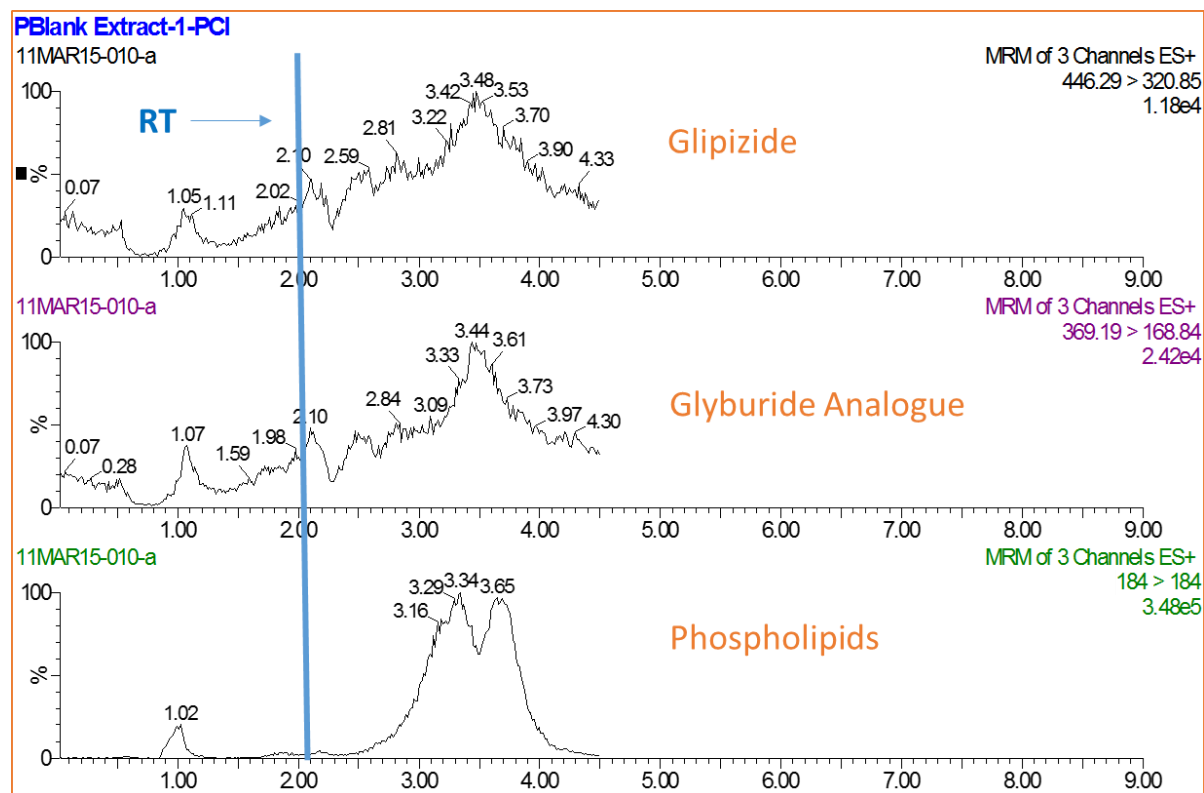
Figure 2.32 Post column infusion chromatogram showing, glipizide (446.29→320.85), glyburide analogue (369.19→168.84) and phospholipids (184→184) from a blank plasma extract. The vertical blue line represents retention time region of GA and GP approximately at 2 minutes.

II) Quantitative Assessment: Matuszewski et al. method (i.e., Post-Extraction Addition)

In this procedure, three sets of samples were prepared and subsequently injected. Set A) consisted of neat standard solutions of glyburide analogue and the internal standard at 50 ng/mL and 200 ng/mL respectively. Set B) consisted of 3 different blank matrices (3 lots, pooled together) fortified at same concentration of standard solutions as in set A post extraction. Finally set C) consisted of 3 different blank matrices (3 lots, pooled together), supplemented before extraction with the same amount of standards as in set A (Matuszewski et al., 1998). The standard solutions were made at desired concentrations from 1 µg/mL stock solutions.

The matrix effect, extraction efficiency and absolute recovery were then calculated using the following equations:

- Matrix Effect
  - %ME = (B-A)/A x 100
- Extraction Efficiency
  - %EE = C/B x 100
- Absolute Recovery
  - %AR = C/A x 100

A: Area of the neat standard solution

B: Area of post extraction spiked blank

C: Area of pre-spiked extracted blank

Table 2.6 and **Table 2.7** show quantitatively assessed parameters (matrix effects, extraction efficiency and absolute recovery) for glyburide analogue and ISTD respectively in comparison with both extraction solvents (1 % formic acid in acetonitrile vs 1 % ammonium formate in methanol).

Table 2.6. Quantitative analysis of % matrix effect, % extraction efficiency and % absolute recovery for glyburide analogue comparing both variants of extraction solvent in mouse plasma

Actual Response (Area GA/Area ISTD)

	Pre-Spiked 50 ng/ml (C)		Post-Spiked 50 ng/ml (B)		Neat (A)
Replicate	**Methanol**	**Acetonitrile**	**Methanol**	**Acetonitrile**	**N/A**
1	0.080	0.071	0.063	0.174	0.080
2	0.066	0.073	0.069	0.122	0.076
3	0.072	0.070	0.068	0.130	0.078
Average	0.073	0.071	0.067	0.142	0.078

Parameter	**Methanol**	**Acetonitrile**
%ME	-15%	82%
%EE	109%	50%
%AR	93%	91%

Table 2.7. Quantitative analysis of % matrix effect, % extraction efficiency and % absolute recovery for glipizide comparing both variants of extraction solvent in mouse plasma

ISTD	Response in Area Counts				
	Pre-Spiked 50 ng/ml (C)		**Post-Spiked 50 ng/ml (B)**		**Neat (A)**
Replicate	**Methanol**	**Acetonitrile**	**Methanol**	**Acetonitrile**	**Neat**
1	7608	5185	13900	13515	10571
2	7828	7509	13993	14660	10246
3	7068	6624	15518	14790	10415
Average	7501	6439	14470	14322	10411

Parameter	**Methanol**	**Acetonitrile**
%ME	39%	38%
%EE	52%	45%
%AR	72%	62%

Based on these results, it was concluded that 1% ammonium formate in methanol performed better than 1% formic acid in acetonitrile as far as matrix effect and recovery of the test analyte and the IS were concerned.

2.5.1.3 Chromatographic detection of glyburide Analogue and glipizide

Following recovery and matrix effects evaluation, glyburide analogue and the ISTD were extracted via HybridSPE using 1% ammonium formate in methanol as the extraction solvent using similar experimental procedure as before. A 30 µL aliquot of each sample extract was submitted for LC-MS/MS analysis. **Figure 2.33** shows chromatographic detection of both the compounds at 1 µg/mL in extracted plasma sample.
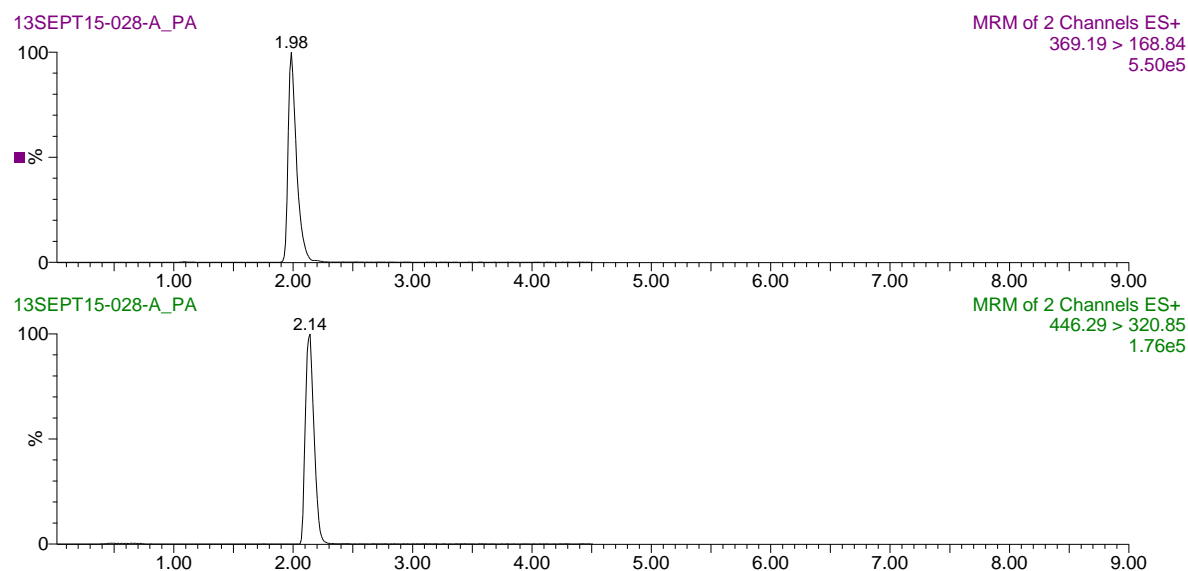
Figure 2.33 Chromatographic detection of GA (top: 369.19→168.84) and ISTD (middle: 446.29→320.85) from a pre-spiked plasma sample followed by HybridSPE extraction with 1% ammonium formate in methanol. TIC (bottom) shows co-elution of the test article and ISTD as desired

2.5.2   *Whole Blood evaluation*

2.5.2.1 Recovery and Matrix Effect Evaluation Post Chromatographic Optimization

I)   Qualitative Assessment: Post Column Infusion Experiment

A post column infusion experiment was conducted to evaluate regions of suppression or enhancement using whole blood blank extract in a similar fashion as mouse plasma. However, based on results from solvent comparison study with mouse plasma, only 1% ammonium formate in methanol was tested in this evaluation. **Figure 2.34** shows the chromatographic run of a blank whole blood extract where SRM transitions of glyburide analogue, glipizide and phospholipids were monitored. The vertical blue line shows the peak retention time for glyburide and glipizide free from phospholipid presence and minimized matrix effects.
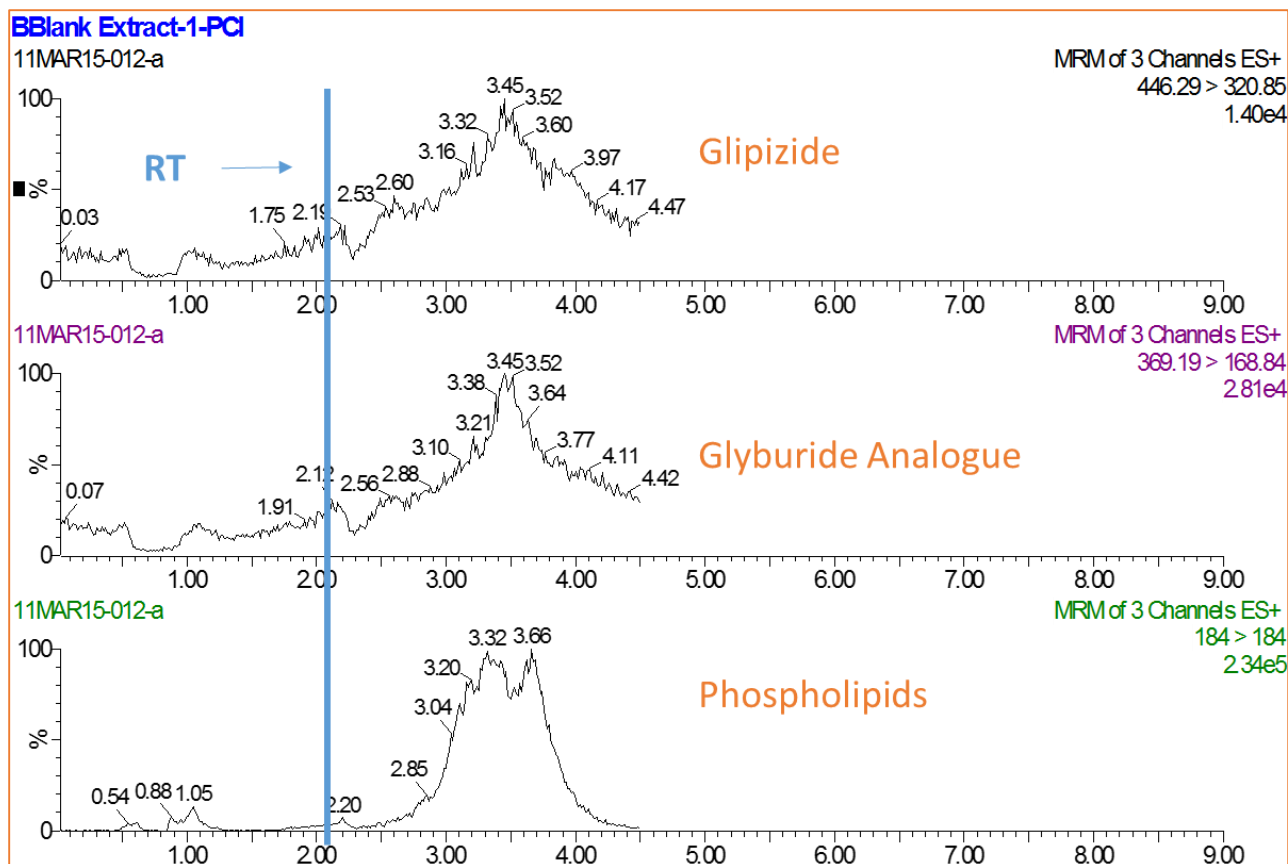
Figure 2.34 Chromatographic detection of GA (top: 369.19→168.84) and ISTD (middle: 446.29→320.85) from a pre-spiked plasma sample followed by HybridSPE extraction with 1% ammonium formate in methanol. TIC (bottom) shows co-elution of the test article and ISTD as desired

II) Quantitative Assessment: Matuszewski et al method (i.e., post extraction addition)

A quantitative assessment of matrix effect, extraction efficiency and absolute recovery was done using mouse whole blood in a similar fashion as plasma for glyburide analogue and glipizide. Based on recovery results obtained for GA and ISTD in plasma, only 1% ammonium formate in methanol was used as the extraction solvent. However, three different concentration levels were tested i.e. 10, 100 and 500 ng/ml of GA. **Table 2.8** and **Table 2.9** show quantitatively assessed parameters (matrix effects, extraction efficiency and absolute recovery) for glyburide analogue and ISTD respectively.

Table 2.8. Quantitative analysis of % matrix effect, % extraction efficiency and % absolute recovery for glyburide analogue comparing both variants of extraction solvent in whole blood

GA	Actual Response (Area GA/Area ISTD)								
	Pre-Spiked (ng/ml) (C)			**Post-Spiked (ng/ml) (B)**			**Neat (A)**		
Replicate	**10**	**100**	**500**	**10**	**100**	**500**	**10**	**100**	**500**
1	0.029	0.27	1.5	0.036	0.28	1.6	0.040	0.32	1.6
2	0.032	0.28	1.6	0.034	0.29	1.6	0.040	0.32	1.6
3	0.035	0.28	1.6	0.035	0.28	1.5	0.038	0.31	1.6
Average	0.032	0.28	1.6	0.035	0.28	1.6	0.039	0.32	1.6

Parameter	**10**	**100**	**500**
%ME	-11%	-11%	-3%
%EE	91%	98%	100%
%AR	81%	87%	97%

Table 2.9. Quantitative analysis of % matrix effect, % extraction efficiency and % absolute recovery for glyburide analogue comparing both variants of extraction solvent in whole blood

IS	Response in Area Count								
	Pre-Spiked (ng/ml) (C)			**Post-Spiked (ng/ml) (B)**			**Neat (A)**		
Replicate	**10**	**100**	**500**	**10**	**100**	**500**	**10**	**100**	**500**
1	9489	8213	10164	15096	20016	21577	18108	18099	17339
2	12241	4684	11632	16758	10406	22106	17588	17615	17574
3	9922	5133	18108	19517	19957	20895	17776	17968	17486
Average	10550	6010	13301	17123	16793	21526	17824	17894	17466

Parameter	**10**	**100**	**500**
%ME	-4%	-6%	23%
%EE	62%	36%	62%
%AR	60%	34%	76%

2.5.2.2 Chromatographic detection of glyburide Analogue and glipizide

Following recovery and matrix effects evaluation, glyburide analogue and the ISTD were

extracted via HybridSPE using 1% ammonium formate in methanol as the extraction solvent using similar experimental procedure as before. A 30 µL aliquot of each sample extract was submitted for LC-MS/MS analysis. **Figure 2.35** shows chromatographic detection of both the compounds at 1 µg/ml in extracted whole blood sample.
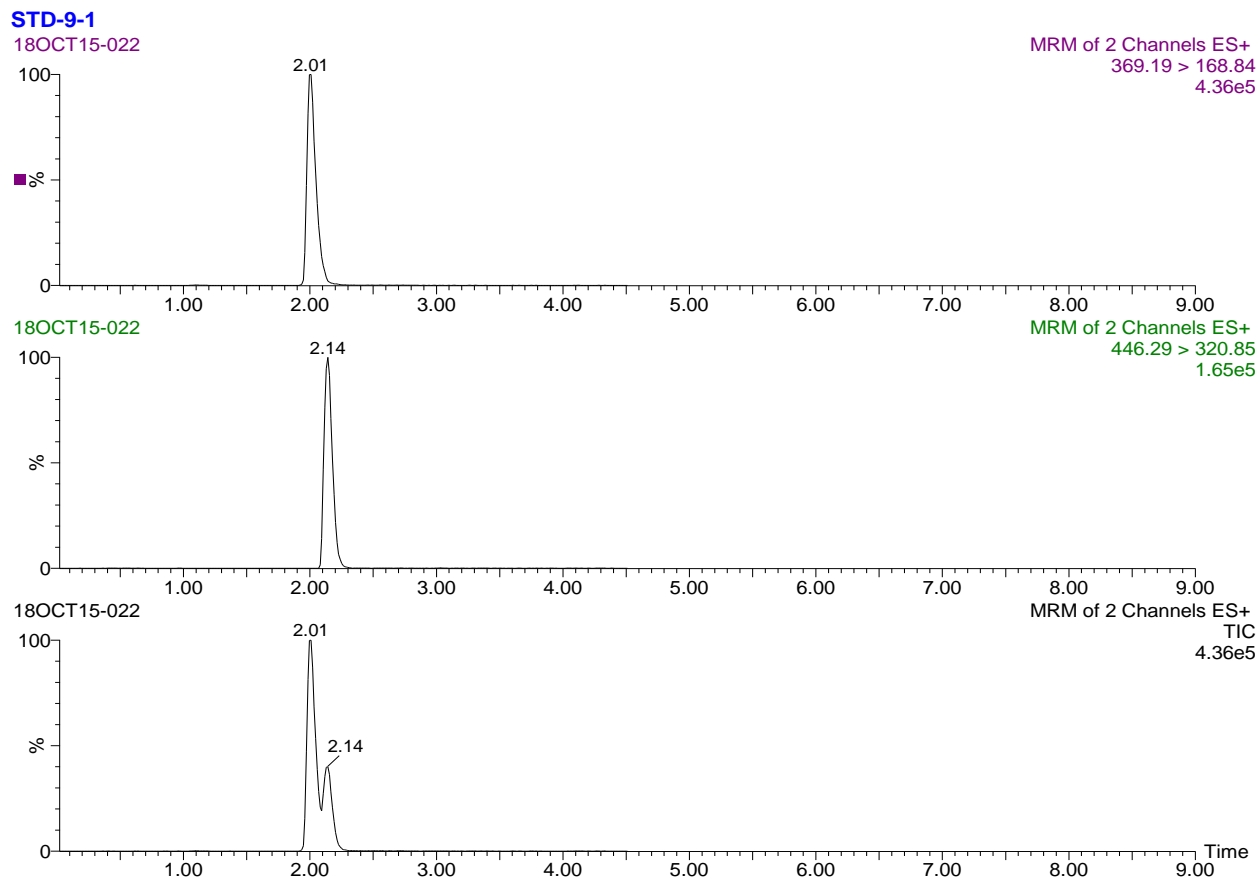
Figure 2.35 Chromatographic detection of GA (top: 369.19→168.84) and ISTD (middle: 446.29→320.85) from a pre-spiked whole blood sample followed by HybridSPE extraction with 1% ammonium formate in methanol. Total Ion Chromatogram (bottom) shows co-elution of the test article and ISTD as desired.

2.5.2.3 Blood to plasma ratio of glyburide analogue

In order to assess whether, direct whole blood analysis of glyburide analogue is more appropriate versus plasma analysis, a blood to plasma partitioning study was conducted using a method from Yu et al. without reference red blood cells. To do this experiment, test compound

was spiked into fresh CD-1 mouse whole blood with sodium heparin at two different concentrations: 2.50 ng/ml and 800 ng/ml. The whole blood samples were then vortexed and centrifuged at 3000 rpm for 5 minutes at 5 °C. A 25 µl aliquot from plasma layer on top and red blood cell layer at bottom were extracted using HybridSPE and analyzed by LC-MS/MS. The blood to plasma ratio was calculated from the following equation:

$$K\frac{b}{p} = \left(K\frac{e}{p} * H\right) + (1 - H);$$

In the above equation, $K\frac{b}{p}$ is the relative whole blood to plasma ratio, $K\frac{e}{p}$ is the erythrocyte (red blood cell) to plasma partition coefficient defined by the response for whole blood to plasma and H is the hematocrit level in mice. **Table 2.10** shows the blood to plasma ratio at two different concentration levels using spiked test compound.

Table 2.10. Blood to plasma ratio at two concentration levels in spiked samples of glyburide analogue.

Blood to Plasma Ratio	Low QC (2.5 ng/ml)	High QC (800 ng/ml)
$K\frac{b}{p}$	1.11	1.17

The glyburide analogue showed fairly even whole blood-to-plasma distribution for two concentrations tested. Hence, it can be inferred that drug concentration levels calculated using direct whole blood analysis should show a good correlation with plasma concentration values and could be used as an alternative to plasma values which would be simpler and faster.

CHAPTER 3: EXPERIMENTAL

3.1 Chemicals and reagents

Glyburide analogue and glipizide were purchased from Sigma Aldrich (St. Louis, MO, USA). Pooled gender mouse plasma and whole blood (CD-1 strain with sodium heparin) were purchased from Bioreclamation IVT (Westbury, NY, USA). HPLC grade water was purchased from Acros Organics (Fairlawn, NJ, USA). HPLC grade methanol and formic acid were purchased from EMD Chemicals (Gibbstown, NJ, USA). Ammonium formate was purchased from Sigma Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Spectrum Chemicals (New Brunswick, NJ, USA). Dichloromethane and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA).

3.2 Materials and equipment

Plasma and whole blood samples were aliquoted into 1.5 mL microcentrifuge tubes purchased from VWR International (Westchester, PA, USA). Prior to sample preparation, plasma was centrifuged using an Allegra X-15R centrifuge by Beckman Coulter, Inc. (Brea, CA, USA). All aliquoting was carried out using Biohit mechanical pipettes with adjustable volumes from Sartorius AG (Goettingen, Germany) using Neptune pipette tips from Biotix Inc. (San Diego, CA, USA). All repeat aliquots were carried out using Eppendorf Repeater M4 mechanical pipettes and Combitips advanced pipette tips from Eppendorf (Hamburg, Germany). Plasma and whole blood samples were mixed using a multi-tube vortexer from VWR International (Westchester, PA, USA).

Plasma and whole blood samples were extracted using HybridSPE Phospholipid Precipitation 96 well plates from Sigma Aldrich (St. Louis, MO, USA). Extracted samples were mixed using a Talboys 96 well plate shaker from Troemner Laboratory Equipments (Thorofare, NJ, USA) and collected into a Waters 1 ml 96 well collection plate purchased from Waters Corporation (Milford, MA, USA) under vacuum using Tomtec Quadra vacuum manifold from Tomtec (Hamden, CT, USA). All samples were evaporated to dryness using SPEDry 96 well plate evaporators from Biotage (Charlotte, NC, USA).

3.3 Instruments and HPLC conditions

HPLC separations during method development and validation experiments were carried out using following equipment: Shimadzu system controller SCL-10A VP, pumps LC-10 AD VP, solvent degasser DGU14A (Shimadzu, Kyoto, Japan). An HTS PAL Autosampler from CTC Analytics (Zwingen, Switzerland and a CH-30 column heater from Eppendorf (Westbury, NY, USA) were used. Reversed phase liquid chromatography was carried out using an Atlantis C-18 column (2.1 x 50 mm, 3 µm) as the loading and elution column purchased from Waters Corporation (Milford, MA, USA). A binary gradient using two Shimadzu pumps operated with one controller was carried out using mobile phase A consisting of 95:5 water: acetonitrile with 0.5% formic acid and mobile phase B consisting of acetonitrile with 0.5% formic acid. Modified gradient 3 was used at flow rate of 0.300 mL/min. The column temperature and the sample temperature were maintained at 40 C and 5 C respectively. Intermittent washes between sample injections were made using two cycles of methanol.

3.4 Mass Spectrometer Parameters

The mass spectrometric detection was carried out by using a Micromass Quattro micro mass spectrometric system by Waters (Milford, MA, USA) equipped with a triple quadrupole mass

analyzer operated in the positive electrospray ionization mode with selected reaction monitoring (SRM) of the glyburide analogue and glipizide. Initial tuning of the target analyte and internal standard using stock solutions (1 µg/ml) was performed via direct infusion method through a 500 µl Hamilton syringe. LC-MS/MS evaluations of glyburide analogue and glipizide (ISTD) during method development and validation experiments were carried out using Q1 full scan and Q3 product ion scan. The mass spectrometer parameters to achieve maximum sensitivity of both compounds were finalized as follows: capillary voltage 3.50 kV, cone energy 28.00 V, extractor voltage 2.00 V, RF lens voltage 0.2 V, source temperature 150°C, desolvation temperature 450°C, desolvation gas flow 400 L/hr, cone gas flow 150 l/hr, collision gas (argon) flow 0.15 ml/min), gas cell pirani pressure $<1 \times 10^{-4}$ mbar, LM1 resolution 12.5 V, HM1 resolution 11.5, LM2 resolution 12.5, HM2 resolution 11.5, ion energy 1 0.5 V, ion energy 2 2.0 V. **Table 3.1** shows the SRM transition and MS/MS analysis parameters.

Table 3.1. MS/MS parameters used for glyburide analogue and glipizide

Q1 (Precursor Ion)	Q3 (Product or "Daughter" Ion) (Da)	Cone (V)	Collision (V)	Dwell Time (s)
369.19	168.84	28.00	24.00	0.35
446.29	320.85	17.00	15.00	0.35

3.5 Stock solution and working solution preparation

The glyburide analogue stock solution was made in 50:50 dimethylsulfoxide: 1% formic acid in acetonitrile at a concentration of 100 µg/mL. The glipizide internal standard stock solution was prepared in dichloromethane at a concentration of 100 µg/mL. Intermediate working stock solution for both compounds were made at 10 µg/mL in methanol followed by a final dilution to 1 µg/mL in 10:90 methanol: water. All stock solutions and subsequent working solutions were prepared fresh throughout the validation experiments.

3.6 Preparation of calibration standards and quality control samples in mouse plasma and whole blood

Pooled mouse plasma and whole blood from at least two donors (CD-1 strain with sodium heparin) were used to prepare the calibration standards and quality control samples. A 50 µL aliquot of a 10 µg/mL intermediate solution of Glyburide was spiked into 450 µL of blank plasma or whole blood to obtain the highest calibration standard at 1000 ng/mL. The high standard was then used to spike into 1.5 mL micro centrifuge tube to prepare remaining eight calibration standards (2.0, 4.0, 10.0, 25.0, 50.0, 100.0, 250.0 and 500.0 ng/mL). A 20 µL aliquot of 10 µg/mL intermediate solution was spiked into 230 µL of plasma or whole blood to obtain the highest quality control sample at 800 ng/mL. Similarly, a 12.5 µL aliquot of 1 µg/mL intermediate solution was spiked into 237.50 µL blank plasma or whole blood to obtain the medium QC. Finally, 25.00 ng/ml standard calibrator was used to obtain the low QC at a concentration of 2.5 ng/mL by spiking 25.0 µL aliquot into 225.0 µL blank of whole blood. Calibration standards and quality control samples were freshly prepared for all analytical runs. The calibration standards and QC samples were prepared in a similar fashion to contain less than 10 % (v/v) of the spiking solution to comply with the SOP to stimulate real matrix samples as much as possible.

3.7 Extraction via HybridSPE

In order to overcome the challenge of limited sample volume from mice and render samples free of phospholipids that become cause of concern due to matrix effects in a LC-MS/MS study, selective extraction of standard calibrators and quality control samples was carried out via HybridSPE precipitation technology. The goal of this method was to maximize target analyte recovery while minimizing recovery of background phospholipids and endogenous proteins found within plasma or whole blood. In the pre-extraction phase of the sample preparation, a 25 µL

aliquot of standard calibrators and QC samples was mixed with 75 µL of deionized water in a 1.5 mL microcentrifuge tube. To this, a 50 µL aliquot of glipizide ISTD was added at a concentration of 200 ng/mL. The sample mixture was then vortexed on a multi tube vortexer for 30 seconds. The entire content of the microcentrifuge tube was then transferred to a single well on the 96 well plate HybridSPE precipitation plate. Following this, a 300 µL aliquot of 1 % ammonium formate in methanol was added as the extraction solvent. All standard calibrators and QC samples were extracted in a similar fashion. The 96 well extraction plate was then covered with a polypropylene mat (hard plastic) with edges taped to ensure a thorough fit. The extraction plate was then put on Talboys shaker for 1 minute at 800 rpm to ensure thorough extraction. The mixture was allowed to sit for 1 minute. Using Tomtec Quadra's vacuum manifold, the samples were pulled through the depth filter of the HybridSPE plate over a time of 3 minutes with a pressure of -20 mm/Hg. The samples were collected onto a Waters 1 mL collection plate and submitted to SPEDry for evaporation of the extraction solvent. The samples were dried at 50 degrees C for roughly 10 minutes at 60 psi until the all wells were dried. After that, the plate was allowed to cool for about 2 minutes at room temperature. Samples were then reconstituted with 100 µl aliquot of 90:10 water: methanol solution. The samples were mixed for 1 minute on Talboys shaker. A 30 ul sample was injected onto LC-MS for further evaluation.

3.8 Plasma Validation parameters

3.8.1 *Linearity*

Nine calibration standards were prepared by serial dilution at concentrations of 2, 4, 10, 25, 50,100, 250, 500, and 1000 ng/mL in CD-1 mouse plasma with sodium heparin. Stock solutions of glyburide analogue and glipizide (1 µg/mL) were used to make necessary intermediate solutions to carry out serial dilution. All standards were analyzed in duplicates. Standard curves were

constructed using linear regression with a $1/x^2$ weighting factor to determine concentration of glyburide analogue.

3.8.2 *Precision and Accuracy*

Precision and accuracy were assessed using quality control samples at three different concentrations and were analyzed in four separate runs for plasma. Intra- and inter-assay precision and accuracy were determined by extracting LLOQ, low, medium and high QC in six replicates to determine the intra assay performance of the analysis. Concentration of quality control samples were calculated from the calibration curve analyzed in the same run.

3.8.3 *Selectivity*

Mouse plasma and whole blood samples from three different lots (pooled together) were analyzed in triplicate to evaluate selectivity with regard to interferences. The mixed lot was extracted using similar extraction procedure as described in section 3.7 with the addition of the internal standard. The selectivity criterion was that the peak area response co-eluting with glyburide analogue must be less than 20% of the average peak area of LLOQ samples of glyburide for all replicates.

3.8.4 *Stability*

Stock solution stability study was dispensable and adverse effects were minimized due to fresh preparation for each inter assay run. Four-hour (bench side extraction time) bench top stability was evaluated by preparing six additional replicates of each QC during the first validation run with freshly prepared calibration standards and quality control samples for precision and accuracy assessment. Post preparative stability was determined to evaluate extracted samples stored in the autosampler beyond 24 hours at 5 ℃ in the event of an instrument malfunction that would require re-injection of samples. Bench top stability was assessed by extraction of low and

high QC samples whereas post preparative stability was assessed via re-injection reproducibility with respect to precision and accuracy.

3.9 Whole Blood Partial Validation parameters

A partial validation with whole blood samples containing spiked glyburide analogue and ISTD at same concentration as plasma was conducted through evaluation of linearity, precision and accuracy and stability in similar fashion as plasma samples from a single run. Standard calibrators and QC samples were prepared at similar concentration to assess linearity, precision and accuracy and stability. In addition, whole-blood to plasma correlation was carried out to check the applicability of the developed method for direct analysis of GA in whole blood.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Plasma Validation Results

4.1.1 Limit of Detection, Limit of Quantification and Linearity

The peak area response of glyburide analogue in blank extract sample spiked with internal standard was used to determine the limit of detection. A total of 12 blank replicates from pooled plasma (three lots, mixed gender) were extracted and peak area response were obtained. Standard deviation of these samples was calculated and LOD in concentration units (ng/mL) was obtained by taking the ratio of $3SD_{blank}$ to the slope of the calibration curve. The LOD was found to be 423 pg/mL

The LLOQ for glyburide was established at 2 ng/mL. The peak area ratios of glyburide analogue to glipizide internal standard in mouse plasma linearly correlated to concentration over the range of 2 to 1000 ng/mL. The calibration curve ($1/X^2$ weighted; linear) r^2 values of three assays were 0.994, 0.992, and 0.991 with a mean of 0.992, SD 0.002 and %RSD 0.154. The slope values of the same were 0.00163, 0.00224, and 0.00202 with a mean of 0.00196, SD 0.00031 and %RSD 14.6. The reverse predicted concentrations for glyburide analogue calibration standard were acceptable ranging from -12.16 % to 8.41 % in term of percent bias. The method precision assessed in term of relative standard deviation ranged from 6.08 % to 14.08 %. **Table 4.1** shows the reverse predicted residuals for glyburide analogue standards from a total of three runs.

Table 4.1. Reverse predicted concentrations for glyburide analogue calibration standards (ng/mL)

| RUN No. | Glyburide Analogue Concentration (ng/ml) | | | | | | | | | r^2 |
	2.00	4.00	10.00	25.00	50.00	100.00	250.00	500.00	1000.00	
1	2.03	3.94	11.14	26.24	55.11	107.46	251.06	505.38	1036.51	0.994
	2.62	3.94	8.95	21.41	51.77	94.72	248.10	454.09	987.64	
2	1.85	4.22	9.57	22.66	48.86	94.23	226.92	480.89	924.93	0.992
	2.09	4.21	9.28	16.64	55.81	111.94	277.65	524.04	1020.79	
3	2.25	4.04	7.96	22.42	63.73	122.52	274.05	639.17	1118.61	0.991
	1.88	3.56	7.73	22.39	49.96	95.17	249.85	534.62	1006.33	
Mean	2.12	3.99	9.11	21.96	54.21	104.34	254.61	523.03	1015.80	0.992
SD	0.285	0.242	1.234	3.092	5.416	11.635	18.725	63.975	63.504	0.002
%RSD	13.45	6.08	13.55	14.08	9.99	11.15	7.35	12.23	6.25	0.154
%BIAS	6.00	-0.38	-8.95	-12.16	8.41	4.34	1.84	4.61	1.58	

4.1.2 Selectivity

The selectivity of the assay was evaluated in three different lots of blank mouse plasma for a total of 12 replicates in four runs. Average apparent blank area count at the retention time of the glyburide analogue was found to be less than 20 % of the average LLOQ area count from all those four runs. **Figure 4.1** shows a representative chromatogram of a blank plasma extract sample with SRM transitions for glyburide analogue (369.19→168.84) and the internal standard, glipizide (446.29→320.85) monitored in a chromatographic run of blank plasma extract.
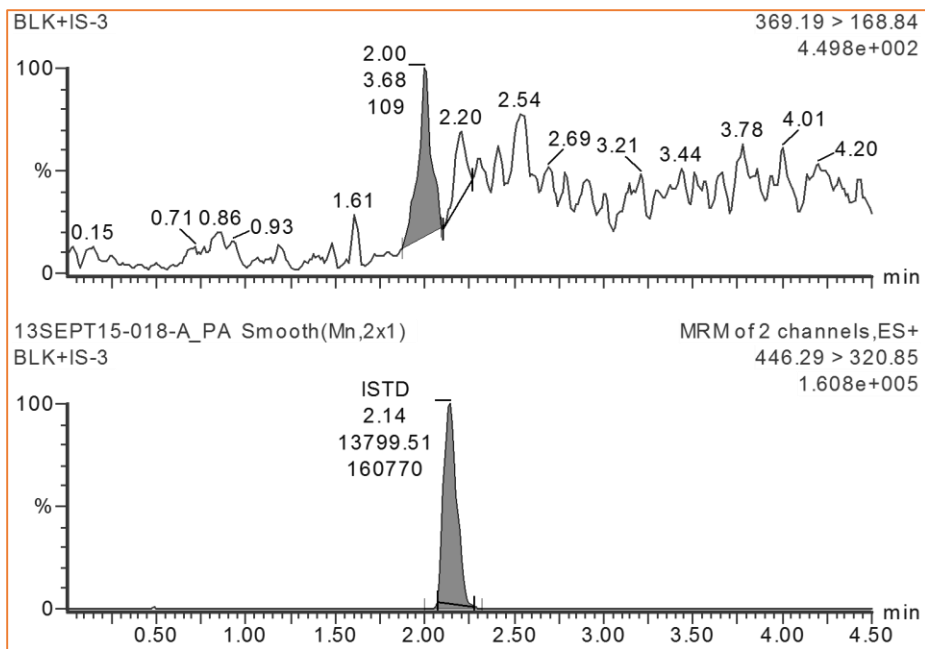
Figure 4.1 Representative chromatogram of a blank sample extract showing peak areas for the glyburide analogue (top) and internal standard (bottom).

4.1.3 Precision and Accuracy

Intra- and inter assay precision and accuracy for the LLOQ, low, medium and high QC samples were determined in four runs. The intra assay accuracy or % bias ranged from -3.74 to 1.83 percent for all concentrations including the LLOQ QC sample. The intra assay precision ranged from 3.16% to 14.6%. Similarly, inter assay accuracy and precision ranged from -11.66 % to 4.60 % and 7.79 % to 14.5 % respectively. These results indicate that assay method to quantify glyburide analogue was both accurate and precise according to the established acceptance criteria. **Tables 4.2 and 4.3** show intra assay and inter assay precision and accuracy calculations respectively.

Table 4.2. Intra Assay Precision and Accuracy (1 Assays, 3 Replicates)

QC (ng/mL)	LLOQ (2 ng/mL)	High QC (800 ng/mL)	Medium QC (50 ng/mL)	Low QC (2.50 ng/mL)
Mean	2.04	781.46	48.13	2.47
SD	0.176	24.7	7.04	0.356
%RSD	8.62	3.16	14.6	14.4
%Bias	1.83	-2.32	-3.74	-1.40

Table 4.3. Inter Assay Precision and Accuracy (3 Assays, 3 Replicates)

QC (ng/mL)	LLOQ (2 ng/mL)	High QC (800 ng/mL)	Medium QC (50 ng/mL)	Low QC (2.50 ng/mL)
Mean	2.26	836.8	44.2	2.64
SD	0.316	73.4	3.44	0.383
%RSD	14.0	8.77	7.79	14.5
%BIAS	13.0	4.60	-11.7	5.67

4.1.4 Stability

Post preparative stability was assessed using re-injection reproducibility of stored QC (LLOQ, high, medium and low) samples at 5 ºC in the auto-sampler. The processed samples were stable for 14 days with a % bias of -16.4% and -15.8 % with %RSD of 6.47% and 10.9 % respectively for high and low QC samples the same (≤20% required for precision and accuracy). Four hour benchtop stability revealed a %bias of -5.70% and -6.00 % with %RSD of 3.65% and 12.9% for high and low QC samples respectively (≤15% required for precision and accuracy).

4.2 Whole Blood Partial Validation Results

4.2.1 Linearity and Precision & Accuracy

Whole blood partial validation was carried out in similar fashion as plasma validation with respect to standard calibrators, quality control samples, exaction procedure and the like. One intra assay run was conducted to assess linearity, precision and accuracy and stability (four hour

benchtop stability). **Table 4.4 and 4.5** show reverse predicted residuals of glyburide analogue standard calibrators and intra assay precision and accuracy.

Table 4.4. Reverse predicted concentrations for glyburide analogue calibration standards (ng/mL). An * suggest excluded value when reporting the range. Linear, $1/X^2$ weighted.

					Glyburide Analogue Concentration (ng/mL)					
RUN No.	**2.00**	**4.00**	**10.00**	**25.00**	**50.00**	**100.00**	**250.00**	**500.00**	**1000.00**	**r^2**
1	2.06	4.26	11.58	27.46	50.98	97.65	261.01	584.09	1089.51	0.993
2	2.38	3.70	12.18	24.67	49.04	90.72	282.31	597.1	1168.87	
Mean	2.22	3.98	11.88	26.07	50.01	94.19	271.66	590.60	1129.19	N/A
SD	0.226	0.400	0.424	1.97	1.37	4.90	15.1	9.20	56.1	
%RSD	10.2	9.95	3.57	7.57	2.74	5.20	5.54	1.56	4.97	
%Bias	11.0	-9.54	18.8	4.26	0.0200	-5.82	8.66	18.1*	12.9	

Table 4.5. Intra Assay Precision and Accuracy (1 Assay, 3 Replicates)

QC (ng/mL)	LLOQ (2 ng/mL)	High QC (800 ng/mL)	Medium QC (50 ng/mL)	Low QC (2.50 ng/mL)
Mean	2.11	880.58	55.35	2.54
SD	0.123	35.8	1.81	0.122
%RSD	5.84	4.07	3.26	4.80
%Bias	5.42	10.1	10.7	1.93

4.2.2 Stability

Post preparative stability was assessed using re-injection reproducibility of stored QC (LLOQ, high, medium and low) samples at 5 ºC in auto-sampler similarly to plasma samples. The processed samples were stable for 7 days with the % bias of -18.0 and -15.8% for high and low QC samples respectively. The %RSD of the same QC samples were 10.2% and 12.6 % respectively. Similarly to plasma, the four hour benchtop stability revealed a %bias of 3.37% and -7.95 % with %RSD of 5.38% and 6.47% for high and low QC samples respectively.

4.3 Whole blood-Plasma Correlation

To evaluate whether plasma concentrations of glyburide analogue correlate with concentration in whole blood, reverse predicted standard calibrator concentration in whole blood were plotted against calibrators in plasma with respect to nominal concentration. It was concluded that plasma concentration of glyburide reflect the whole blood concentration with a correlation coefficient of 0.9956. This evaluation suggests that the validated method can be directly used to determine the analyte concentration in whole blood rendering unnecessary sample loss and procedural burden. **Figure 4.2** shows the correlation of glyburide concentration in plasma against whole blood concentration. A linear relationship was observed with a slope of 1.0819.
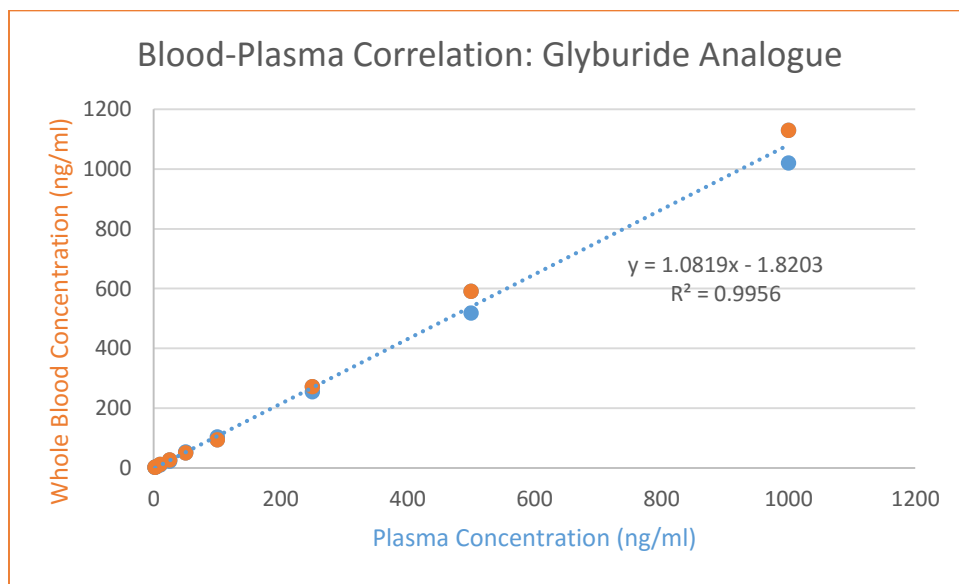
Figure 4.2 Blood-plasma correlation of glyburide analogue in mouse model.

CHAPTER 5: CONCLUSIONS

A bioanalytical method to quantify the glyburide analogue in mouse plasma (CD-1 strain, sodium heparin) using a microextraction procedure and LC-MS/MS was developed and validated for a concentration range of 2-1000 ng/mL. The microextraction procedure was accomplished via HybridSPE technology using 25 µL of plasma which addressed low sample volume issue in mice as well as selective extraction using an optimized extraction solvent. With a limited availability of scientific literature due to the novelty of the test compound, chromatographic separation was carried out by referring to literature of similar compounds within the same class as the glyburide analogue. Prior to MS/MS analysis, reverse phase chromatography was employed to achieve initial separation of the test compound along with the internal standard. LC-MS/MS analysis of the test article was accomplished using a structural analogue internal standard (glipizide). A systematic approach to find a suitable internal standard for further analysis via MS/MS conditions was undertaken where possible candidate compounds from sulfonylurea class were evaluated. Matrix effects were evaluated using post column infusion experiments and minimized using HybridSPE platform via selective extraction of the test article from plasma. Further evaluations of matrix effects led to chromatographic optimization studies using modified gradient LC conditions where the peaks of interests (glyburide analogue and glipizide) were eluted away from possible regions of ion suppression resulting from incomplete removal of phospholipids by the HybridSPE plate.

Selectivity was evaluated using two variants of extraction solvent namely 1% formic acid in acetonitrile and 1% ammonium formate in methanol. Subsequently, quantitative assessment of matrix effects, extraction efficiency and absolute recovery were made to select a suitable extraction solvent for further analysis. Plasma validation experiments determining precision and accuracy, linearity and stability were carried out and obtained results were within the bioanalytical guidance by FDA.

A partial validation of the method was done using whole blood with acceptable results for precision and accuracy, linearity and stability within a concentration range of 2-1000 ng/mL. Blood to plasma ratio study determining relative concentration of the test compound within red blood cells was carried out to better reflect the suitability for a direct whole blood analysis. It was concluded that glyburide analogue evenly distributes between whole blood and plasma and thus, a direct whole blood analysis would be more appropriate to achieve a quick and simpler pharmacokinetic profile of the test article from a bioanalytical standpoint. Finally, blood-plasma correlation was done to show that the validated method can either be used for plasma or whole blood analysis with a correlation coefficient of 0.9993. The utility of the validated method needs to be further studied in pre-clinical study samples of in-vivo experiment using mouse model of CD-1 strain.

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