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Development and validation of an LC–MS/MS sulfonylurea assay for hypoglycemia cases in the emergency department

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ABSTRACT

Background: Sulfonylureas are antidiabetic agents widely prescribed for the treatment of type-II diabetes. Detection of the presence of sulfonylureas in cases of unexplained hypoglycemia rules out other underlying pathophysiological conditions. The goal of this study was to develop and validate a qualitative liquid chromatography tandem mass spectrometry (LC–MS/MS) assay for the rapid identification of sulfonylureas in serum.
Methods: An LC–MS/MS assay was developed using an Agilent HPLC with an AB Sciex 3200 LC–MS/MS operating in ESI positive mode. Linearity, LOD, precision, matrix effect, recovery, carry-over and stability of the final method were evaluated for method validation. Concordance with another clinically validated LC–MS/MS method was evaluated using remnant samples from patients prescribed a sulfonylurea.
Results: The assay performed well with all validation data meeting pre-determined criteria. The method comparison study showed a correlation coefficient of 0.99 for glipizide, the most common sulfonylurea, despite both methods being validated as qualitative methods. To date the validated assay has been utilized in 19 cases of unexplained hypoglycemia presenting to the emergency department, in which 5 were sulfonylurea positive.
Conclusion: We have developed a rapid and sensitive LC–MS/MS sulfonylurea assay, and utilized this assay to assist in the differential diagnosis of unexplained hypoglycemia.

1. Introduction

Sulfonylurea derivatives are a class of oral antidiabetic drugs that are commonly used in the treatment of type-II diabetes mellitus [1,2]. Sulfonylureas inhibit ATP-sensitive potassium channels in the pancreatic beta cell membrane and increase endogenous insulin release from the beta cells [3]. Unlike other classes of oral antidiabetics such as the biguanides and thiazolidinediones, sulfonylureas are more likely to cause hypoglycemia with overdose or when ingested by nondiabetic patients [4–6]. According to the 2013 Toxic Exposure Surveillance System, 3950 sulfonylurea overdoses were reported to the poison control centers within a year in the US, with greater than a half occurring in the pediatric population due to unintentional ingestion [7]. In most cases, patients with sulfonylurea poisoning present with unexplained hypoglycemia, and symptoms include confusion, difficulty speaking, hemiparesis, anxiety, sweating, palpation, seizures and coma. These symptoms can be life-threatening and even fatal [8]. However, these signs and symptoms can also be caused by other diseases that increase insulin secretion, such as insulinoma [9], or decrease insulin availability due to insulin auto-antibodies or insulin receptor antibodies [10]. In addition, sepsis, hepatic failure, and Addison's disease could also lower blood glucose to dangerous levels. Since the consequences of hypoglycemia can be devastating, a rapid differential diagnosis and treatment is essential for patients. Detection of the presence of a sulfonylurea allows for the correct identification of the underlying cause of hypoglycemia and rules out the other pathological causes.

Cases of sulfonylurea overdose as a result of a drug purchased on the streets that was presumed to be a benzodiazepine have been reported [11,12]. These patients had a history of recreational drug abuse and ingested benzodiazepines to counter the later effects of stimulants such as cocaine or amphetamines. Some glyburide tablets mimic the appearance of street-purchased diazepam, and give the drug users a sedative feeling from drug-induced hypoglycemia [11]. After ingestion, one patient presented to the ED with a serum glucose measurement of less than 40 mg/dL and suffered from prolonged altered mental status [12]. Moreover, sulfonylurea intoxication has been reported when chlorpropamide was accidentally substituted for acetaminophen with codeine in a pharmacy dispensing error [13]. In this case, the patient's serum glucose was less than 20 mg/dL. In our clinical laboratory, we commonly get requests for the rapid detection of sulfonylureas in cases of unexplained hypoglycemia. Sending the samples out to a reference laboratory results in long turn-around times such that the results do not impact clinical care in real time. Therefore, we decided to develop a rapid and reliable assay to screen for sulfonylureas to enhance our clinical service.

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A number of separation and detection methodologies have been used for the identification of anti-diabetic drugs, such as capillary electrophoresis [14,15], high-performance liquid chromatography [16], photodiode array [17], ultraviolet or fluorescent detection [18], and mass spectrometry [19,20]. Here, we describ the development and validation of a liquid–chromatography tandem mass spectrometry (LC–MS/MS) assay to detect eight sulfonylurea compounds in serum. We also discuss the results obtained in real-time from this assay for 19 cases of unexplained hypoglycemia.

2. Materials and methods

2.1. Chemicals and reagents

LC–MS or HPLC grade water, methanol and acetonitrile were obtained from Honeywell Burdick & Jackson Research Chemicals (Muskegon, MI, USA). The following reagents and analytical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): formic acid, ammonium acetate, chlorpropamide, acetohexamide, tolazamide, tolbutamide, glipizide, glyburide, gliclazide, and glimepiride. The internal standard (IS), glipizide-d11, was purchased from the C/D/N isotope Inc. (Pointe-Claire, Quebec, Canada). Lyophilized drug-free human serum was supplied from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Calibrators, controls, and patient samples

A stock solution of each sulfonylurea was prepared in methanol and 0.1% formic acid at a concentration of 1 mg/mL. A working solution of four first-generation sulfonylureas (chlorpropamide, acetohexamide, tolazamide and tolbutamide) at 100 μg/mL and four second-generation sulfonylureas (glipizide, glyburide, gliclazide, and glimepiride) at 10 μg/mL was made. Calibrators were prepared by appropriate dilution of the working solution with drug-free serum at levels of 10/1, 50/5, 100/10, 250/25, 500/50, 1000/100, 2,500/250, 5,000/500, and 10,000/1000 ng/mL of the first- and second-generation sulfonylureas. Quality control specimens were prepared with drug-free serum at three levels: 200/20, 2,000/200, 8,000/800 ng/mL. The internal standard, glipizide-d11, was prepared at a concentration of 1 mg/mL. The stock solutions, IS, quality controls, and calibrators were aliquoted and stored at −20 °C.

Patient prescription information was checked on samples submitted to our laboratory for routine hemoglobin A1c examination. Remnant blood samples were obtained from 51 patients who were prescribed sulfonylureas. These samples were then tested using the newly developed LC–MS/MS sulfonylurea assay. A de-identified aliquot of each sample was sent to an outside laboratory and tested by another clinical validated LC–MS/MS sulfonylurea method. Deming regression analysis and bias plot were used to compare the two different methods. This study was approved by the University of California San Francisco Committee on Human Research who deemed that patient consent was not required.

2.3. Sample preparation

Serum samples, calibrators and controls (250 μL) were spiked with 10 μL of IS (100 μg/mL) and extracted by protein precipitation using 750 μL of acetonitrile. The mixture was agitated for 15 s, and then centrifuged at 10,000 rcf for 10 min. The supernatant was separated from the protein pellet and evaporated under nitrogen in a 37 °C water bath. The drugs were reconstituted in 100 μL of the LC–MS/MS buffer (80% mobile phase A and 20% mobile phase B). The total sample preparation time was approximately 60 min.

2.4. Liquid chromatography and mass spectrometry

Separation of eight sulfonylurea compounds was performed using an Agilent (Santa Clara, CA, USA) high-performance liquid chromatography (HPLC) with a Phenomenex Luna C8(2) 100 Å, 50 × 2.0 mm LC column. The column was used at 50 °C with a gradient elution at a flow rate of 0.4 mL/min. Mobile phase A consisted of 20 mM ammonium acetate in 95:5 water/methanol, and mobile phase B consisted of 100% methanol. The column was equilibrated at 30% B for 1 min. The gradient started at 30% B, was linearly increased to 50% B from 1 to 2 min, then increased from 50–85% B from 2 to 6.5 min, held at 95% B to 8.5 min, and re-equilibrated at initial condition for 2.5 min. The injection volume was 20 μL. A 3200 QTrap (ABSciex, Redwood City, CA) was utilized with an electrospray ionization source (ESI) operated in the positive mode. The source parameters included curtain gas of 40 psi, ion spray voltage of 5000 V, ion source temperature of 700, and medium collision gas. Multiple reaction monitoring (MRM) mode was utilized, with two transitions monitored for each compound and one transition for the internal standard (IS). The raw signal of peak area for each compound was normalized to the IS and the concentrations were calculated from the calibration curve.

2.5. LC–MS/MS assay validation

Validation of the final method included determining the lower limit of detection (LOD), linearity, imprecision, matrix effect, recovery, and carryover for each analyte. A standard calibration curve was prepared using nine calibration points and a blank. Concentration levels were 10/1, 50/5, 100/10, 250/25, 500/50, 1000/100, 2,500/250, 5,000/500, and 10,000/1000 ng/mL of the first- and second-generation sulfonylurea, respectively. The expected concentrations were plotted against responses. The calibration curves were repeated five days in a row. The LOD was defined as the lowest concentration at which the signal-to-noise ratio was greater than 10:1. Intra-assay imprecision was estimated by analyzing five independent aliquots of each of the three QC samples (200/20, 2,000/200, 8,000/800 ng/mL) on the same day. Inter-day imprecision was estimated by analyzing five specimens of each level per day over five days. Coefficient of variation (CV) was calculated by dividing the standard deviation by the mean. Matrix effects were determined by spiking drug standards into water and six healthy and drug-free donor serum matrices at 100 and 500 ng/mL. It was calculated using the following equation: $(B-A)/A * 100\%$, where B is the mean peak area in donor serum matrix and A is the peak area intensity in water. To calculate the percentage of extraction recovery, 500 ng/mL

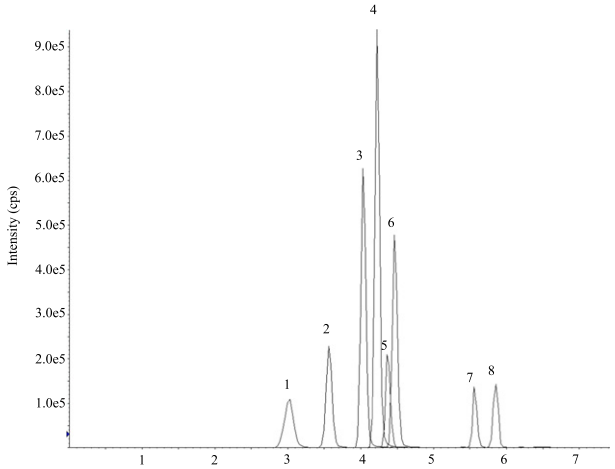

Fig. 1. A representative chromatogram of the 8 sulfonylureas (500 ng/mL): chlorpropamide (1), acetohexamide (2), tolbutamide (3), tolazamide (4), glipizide (5), gliclazide (6), glyburide (7), glimepiride (8).