Research Paper

Simultaneous quantification of multiple anti-diabetic drugs in whole blood by ultra-performance liquid chromatography-tandem mass spectrometry

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Abstract An analytical method was developed and validated for the simultaneous quantification of multiple anti-diabetic drugs in whole blood using ultra-performance liquid chromatography-tandem mass spectrometry. Chromatographic separation was achieved on a CAPCELL PAK INERT ADME-HR column. The mobile phases consisted of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid. Quantification of 10 antidiabetic drugs was performed by selected reaction monitoring with each product ion referenced against 4 isotopically labelled internal standards. Calibration curves exhibited good linear relationships ranging from 0.0002−20 μg/mL, with correlation coefficients exceeding 0.996. The limits of detection were estimated to be 0.0001−0.005 μg/mL. The intra- and inter-day accuracies and precisions were 98.6−105.5% and 0.1−8.0%, respectively. The recovery efficiencies were in the range of 74.4−102.3%. Matrix effects were observed ranging from −29.1 to 534.6%. Stability tests of the analytes revealed that gliclazide was relatively unstable when stored long-term (28 days) at −30°C. We expect that the method described in the present study will be useful for various studies such as clinical and forensic toxicological investigations.

Key words: anti-diabetic drugs, whole blood, ultra-performance liquid chromatography-tandem mass spectrometry, quantification, forensic toxicology

Introduction

Increasing numbers of patients are being diagnosed with diabetes mellitus, constituting a serious health problem[1]. Diabetes mellitus type 2, which is non-insulin dependent diabetes, accounts for the majority of diabetic patients (around 90−95%). It is considered a chronic metabolic syndrome[2]. It includes individuals with insulin resistance and relative insulin deficiency. The primary drug therapies for diabetes mellitus type 2 consist of insulin secretagogues, biguanides, insulin sensitizers, alpha glucosidase inhibitors, incretin mimetics, amylin antagonists and sodium-glucose co-transporter-2 inhibitors[3]. Among them sulfonylureas and biguanides can cause life-threatening hypoglycemia and/or lactic acidosis[4,5].

The serum glucose level is regulated within a narrow range by multiple factors under physiological conditions, but is greatly modified in the death process and after death[6]. Therefore, it is not adequate to determine the blood glucose level to diagnose the life-threating hypoglycemia

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after death. The risk of developing hypoglycemia is also increased in case of renal impairment, reduced food consumption or with simultaneous intake of drugs which potentiate the action of sulfonylureas' (i.e., salicylates)[7]. Furthermore, the measurement of lactate after death is difficult since it has been shown that lactate concentrations in blood and other matrices still increase for some time after death as a result from anaerobic glycolysis[8]. Therefore, it is important to analyze such drugs in biological fluids as a part of forensic toxicological examination.

In forensic toxicology, almost all postmortem analyses are performed on whole blood because of hemolysis[9,10]. To our knowledge, there are no reports of the simultaneous quantification of sulfonylureas and biguanides in whole blood by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). In this study, we aimed to develop a method for the simultaneous quantification of five sulfonylureas (acetohexamide, chlorpropamide, gliclazide, glibenclamide and glimepiride) and two biguanides (buformin and metformin) together with three other drugs (pioglitazone, alogliptine and vildagliptin) used in combination. The analysis focused on whole blood samples using UPLC-MS/MS.

Materials and Methods

Chemicals and materials

Acetohexamide was purchased from MP Biochemicals (Santa Ana, CA, USA). Glibenclamide and metformin hydrochloride were obtained from Sigma-Aldrich (St. Louis, Mo, USA). Buformin hydrochloride, chlorpropa-

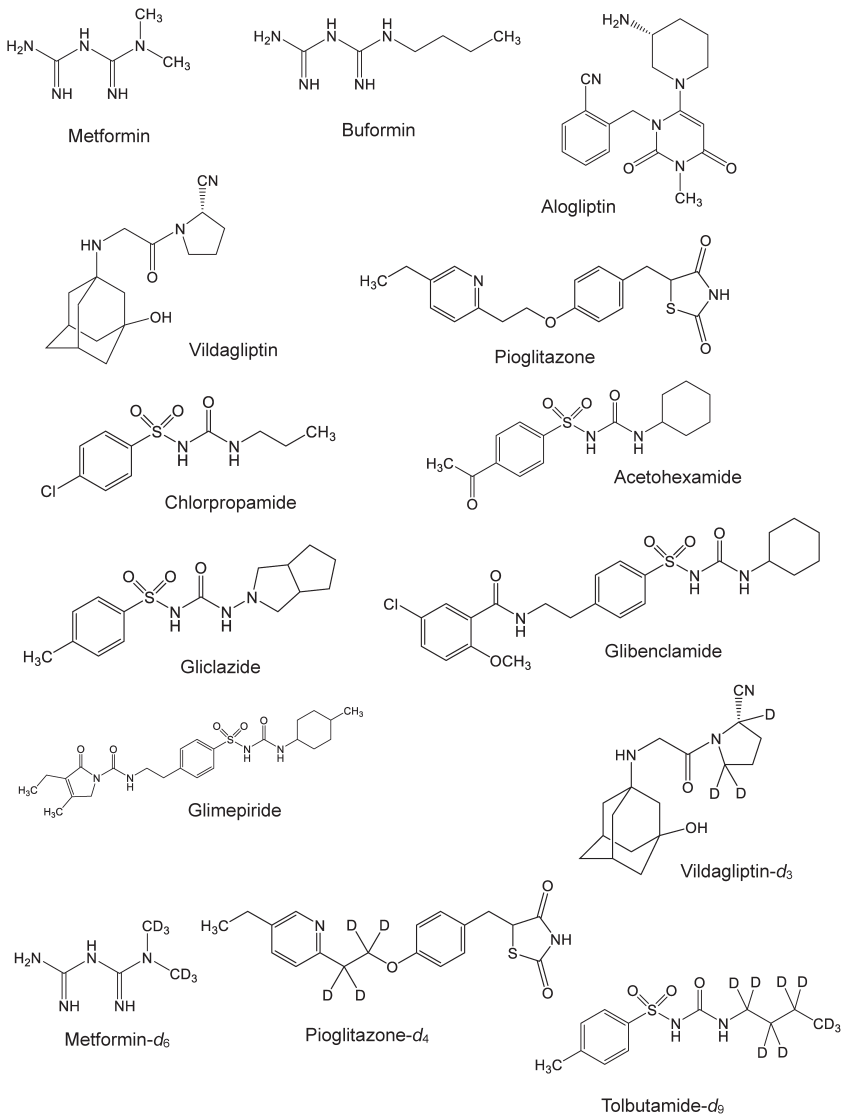

Fig. 1. Chemical structures of the analytes and internal standards.

mide, gliclazide, glimepiride and pioglitazone were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Alogliptin was obtained from Bio Vision Inc. (Milpitas, CA, USA). Vildagliptin and metformin-d_6 hydrochloride came from Cayman Chemical (Ann Arbor, MI, USA). Vildagliptin-d_3 and pioglirazone-d_4 were purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Tolubutamide-d_9 was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The chemical structures of the analytes are shown in Fig. 1. Stock solutions of all substances were prepared at concentrations of 0.4−1 mg/mL in methanol and were stored at −80°C until analysis. The IS stock solutions were prepared in methanol to give a concentration of 100 μg/mL and were stored at −80°C. LC-MS grade acetonitrile, methanol and formic acid were purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). Other common chemicals used in this study were of the highest purity commercially available. Laboratory distilled water was purified using a Direct-Q UV 3 (Millipore, Molsheim, France). Human whole blood was obtained from Tennessee Blood Services (Tennessee, USA).

Sample preparation procedure

A 100 μL whole blood sample was mixed with 10 μL IS solution (1 μg/mL of metformin-d_6 and tolubutamide-d_9 and 0.1 μg/mL of vildagliptin-d_3 and pioglirazone-d_4), 100 μL methanol, and 200 μL acetonitrile. The mixture was vortexed for 60 s and centrifuged at 15,000 *g* for 10 min. The supernatant was transferred to another tube, and evaporated with a centrifugal evaporator (CVE-2000; Tokyo Rikakikai, Tokyo, Japan). The residue was reconstituted in 100 μL of 0.1% formic acid, and used for the analysis by UPLC-MS/MS.

Table 1. SRM transitions and parameters for detection of the analytes and IS

Analyte	Molecular weight	Retention time (min)	SRM transition (*m/z*)	Cone voltage (V)	Collision energy (eV)
Metformin	129.16	1.36	130 → 71*	28	22
			130 → 60	28	14
Buformin	157.22	2.23	158 → 85*	26	20
			158 → 60	26	14
Alogliptin	339.39	2.23	340 → 116*	38	30
			340 → 89	38	58
Vildagliptin	303.40	2.24	304 → 154*	32	18
			304 → 97	32	28
Pioglitazone	356.44	2.43	357 → 134*	44	30
			357 → 119	44	46
Chlorpropamide	276.74	3.36	277 → 175*	22	20
			277 → 111	22	32
Acetohexamide	324.40	3.53	325 → 119*	26	28
			325 → 91	26	44
Gliclazide	323.41	4.01	324 → 110*	28	22
			324 → 91	28	34
Glibenclamide	494.00	4.49	494 → 369*	24	14
			494 → 169	24	42
Glimepiride	490.62	4.56	491 → 352*	26	14
			491 → 126	26	28
Metformin-d_6 (IS)	135.20	1.35	136 → 77*	28	20
			136 → 94	28	14
Vildagliptin-d_3 (IS)	306.42	2.24	307 → 151*	32	26
			307 → 157	32	18
Pioglitazone-d_4 (IS)	360.46	2.42	361 → 138*	48	28
			361 → 123	48	46
Tolbutamide-d_9 (IS)	279.40	3.50	280 → 91*	26	32
			280 → 83	26	14

Notes: *Transitions used for quantification of the compounds.

UPLC-MS/MS conditions

UPLC-MS/MS analysis was run on a Waters ACQUITY UPLC system, which included an ACQUITY UPLC binary pump and a sample manager (Waters, Milford, MA, USA). The LC column used for chromatographic separation was a CAPCELL PAK INERT ADME-HR (2.0 mm I.D. × 100 mm, particle size 3 μm). The column temperature was maintained at 40 °C. Gradient elution was employed using a mobile phase consisting of mobile phase A (water containing 0.1% formic acid) and mobile phase B (acetonitrile containing 0.1% formic acid) at a flow rate of 0.2 mL/min (0−1 min) and 0.3 mL/min (1−10 min). The mobile phase system started at 99% A for 1 min. A linear gradient was used to ramp from 60% A to 1% A within 5 min and maintained at 1% A for 1 min. The mobile phase was then returned to 99% within 0.01 min and maintained at 99% A for 3 min to equilibrate the column for the next sample.

The MS/MS was operated with an electrospray ionization source in the positive ionization mode on a tandem quadrupole mass spectrometer (ACQUITY TQD; Waters). Quantification was performed by selected reaction monitoring (SRM) using peak areas. The parameter settings were as follows: capillary voltage, 3.0 kV; source temperature, 150°C; desolvation temperature, 450°C; desolvation and cone gas, nitrogen with flow rates of 800 and 50 L/h, respectively; and collision gas, argon with flow rate of 0.15 mL/min. Table 1 shows the optimized SRM parameters and retention times for each compound and IS. All data were acquired in the centroid mode and processed using MassLynx NT 4.1 software with the QuanLynx program (Waters).

Method validation

The method was validated according to the US FDA guidelines on bioanalytical method validation and related documents[11]. The intra- and inter-day accuracies and precisions were determined by conducting 5 experiments in the course of a single day and by conducting one experiment on 5 different days, respectively. The precisions were expressed as the coefficient of variation. The accuracies and precisions should be within 20% for the LOQ and 15% for other concentrations.

The matrix effects and recovery efficiencies were determined by the following method. For matrix effects, two sets of samples were prepared by directly spiking the analytes into reconstitution solution with or without the presence of residue extracted from whole blood. The matrix effects were calculated by [(Aeb/Ans −1) × 100], where Aeb and Ans represent the analyte peak area of the extracted blank whole blood that was directly spiked with the analytes and the neat analyte solution, respectively. The recovery rates were calculated by [(Aex/Aeb) × 100], where Aex and Aeb represent the analyte peak area of the extracted analytes in whole blood and extracted blank whole blood directly spiked with the analytes, respectively.

Stability tests, such as freeze−thaw stability, short-term stability and long-term stability were carried out at three different concentrations. We evaluated several storage conditions, including room temperature for 4 h, −30°C and −80°C for 28 days and three freeze−thaw cycles. Each freeze−thaw cycle consisted of thawing the spiked samples at room temperature and refreezing for a minimum of 12 h between each cycle.

Results and Discussion

Optimization of analytical conditions

The first step in method development was the optimization of mass spectrometry parameters by infusing individual standard solutions at concentrations of 0.1−1 μg/mL. The product ion mass spectra of the analytes and IS were obtained from the protonated molecular ions. Using protonated molecular ions as precursor ions, other MS/MS parameters and transitions were determined as shown in Table 1.

The chromatographic elution of the analytes was optimized by examining several analytical columns and mobile phases. We found that a CAPCELL PAK INERT ADME-HR column with a mobile phase of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid provided the best chromatographic separation. SRM chromatograms of an extract from a whole blood sample spiked with 0.02 μg/mL buformin, alogliptin, vildagliptin, pioglitazone, glibenclamide and glimepiride, 0.07 μg/mL gliclazide, 0.2 μg/mL metformin, chlorpropamide and acetohexamide, 0.1 μg/mL pioglitazone-d_4 and 1 μg/mL metformin-d_6, vildagliptin-d_3 and tolbutaminde-d_9 are shown in Fig. 2. The peaks of the analytes were clearly visible with retention times within 5 min, and there were no peaks that interfered with the measurements.

Reliability of the method

The method was validated by characterizing a series of parameters such as the limits of detection, linearity, accu-

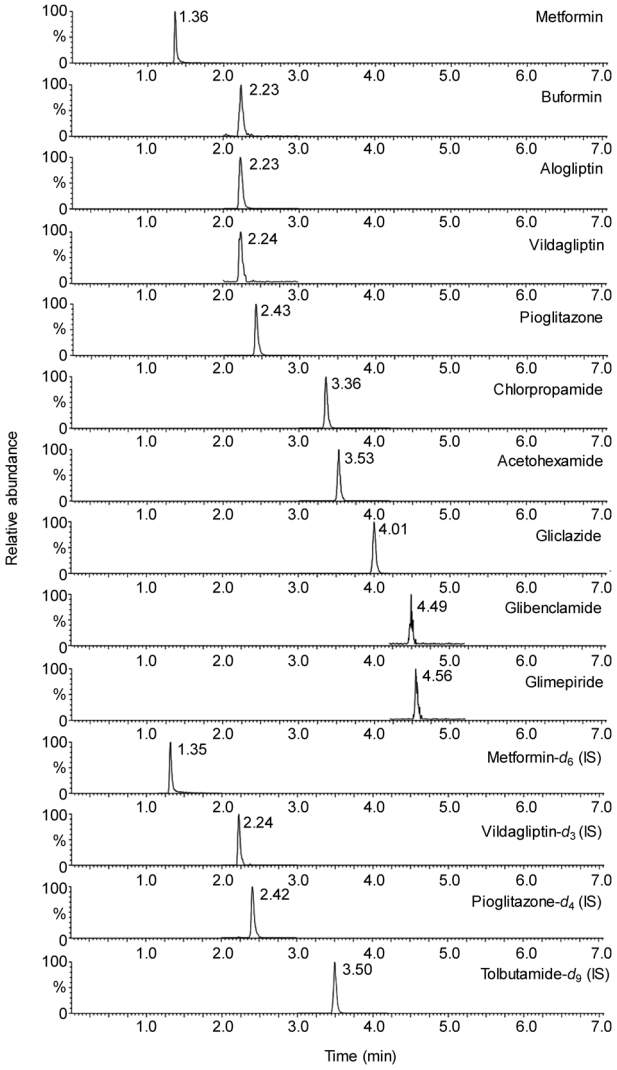

Fig. 2. SRM chromatograms of an extract from whole blood sample spiked with analytes and internal standards.

racy, precision (intra- and inter-day), recovery efficiencies and matrix effects. Calibration curves were prepared by adding appropriate calibrators to blank whole blood. As shown in Table 2, calibration curves were linear in the range from 0.005−20 μg/mL for metformin, 0.005−5 μg/mL for buformin, 0.001−5 μg/mL for alogliptin, 0.0002−2 μg/mL for vildagliptin, 0.0002−5 μg/mL for pioglitazone, 0.01−20 μg/mL for chlopropamide, 0.01−20 μg/mL for acetohexamide, 0.005−10 μg/mL for gliclazide, 0.001−3 μg/mL for glibenclamide and 0.001−3 μg/mL for glimepiride, with correlation coefficients exceeding 0.996. These data established the high degrees of linearity within these ranges. The limits of detection (LOD), defined as the concentrations giving a signal-to noise ratio of 3 : 1, were estimated to be 0.0001−0.005 μg/mL under the UPLC-MS/MS conditions used in this experiment. The sensitivities of the present results were equal to or greater than those of previous reports[12−19].

The intra- and inter-day accuracies and precisions were evaluated at four levels, the results of which are summarized in Table 3. The accuracies and precisions were 98.6−105.5% and 0.1−8.0%, respectively. Their values were acceptable at all concentrations. The recovery efficiencies and the matrix effects of the samples are shown in Table 4. The recovery efficiencies were in the range of 74.4−102.3%. Matrix effects were observed ranging from −29.1 to 534.6%. The wide range of matrix effect observed in the present study might come from the lack of ability of protein precipitation using organic solvent. Further experiments aimed at reducing the amount of matrix compounds using solid phase extraction, QuEChERS and other devices are required.

Table 2. Regression equations, correlation coefficients (r), limits of detection (LOD) and therapeutic concentration for the analytes

Analyte	Range (μg/mL)	Regression equation[a]	Correlation coefficient (r)	LOD (μg/mL)	Therapeutic concentration (μg/mL)
Metformin	0.005−20	y=3.081x+0.3322	0.999	0.002	0.25−1.3
Buformin	0.005−5	y=1.902x+0.0270	0.999	0.002	0.15−0.58
Alogliptin	0.001−5	y=189.2x+1.503	0.999	0.0005	0.025−0.2
Vildagliptin	0.0002−2	y=482.8x+4.636	0.999	0.0001	0.3−0.5
Pioglitazone	0.0002−5	y=108.3x+4.777	0.999	0.0001	0.1−1.5
Chlorpropamide	0.01−20	y=3.551x+0.5240	0.999	0.005	30−363
Acetohexamide	0.01−20	y=2.948x+0.2873	0.999	0.005	21−56
Gliclazide	0.005−10	y=20.17x+4.843	0.997	0.002	0.5−2.0
Glibenclamide	0.001−3	y=7.236x+0.4765	0.996	0.0005	0.05−0.2
Glimepiride	0.001−3	y=9.736x+0.5579	0.997	0.0005	0.09−0.5

[a] Values are the means of five replicate determinations.

Table 3. Intra- and inter-day accuracy and precision data of the analytes in whole blood

Analyte	Concentration added (μg/mL)	Intra-day[a]		Inter-day[a]	
		Accuracy (%)	Precision (CV, %)	Accuracy (%)	Precision (CV, %)
Metformin	0.005	100.1	3.2	99.9	3.2
	0.2	98.6	2.1	100.3	1.2
	2	100.8	2.3	100.7	1.2
	15	98.8	1.4	99.5	0.8
Buformin	0.005	102.3	5.9	103.2	2.6
	0.02	100.4	3.5	99.6	2.4
	0.2	105.5	4.7	102.3	3.5
	3	104.2	3.1	102.8	1.3
Alogliptin	0.001	101.2	3.0	101.0	0.7
	0.02	101.7	1.4	101.5	0.1
	0.2	100.2	1.6	101.0	1.0
	3	99.5	1.6	100.3	0.6
Vildagliptin	0.0002	100.1	2.0	99.9	1.2
	0.02	102.1	0.8	102.5	1.5
	0.2	100.9	0.8	100.7	0.6
	1.5	99.7	0.3	100.2	0.4
Pioglitazone	0.0002	100.6	8.0	99.4	1.5
	0.02	99.9	1.5	103.3	2.5
	0.2	100.7	1.5	101.5	0.7
	3	101.3	0.5	102.2	2.2
Chlorpropamide	0.01	100.9	1.9	101.0	1.8
	0.2	102.3	1.9	102.7	3.2
	2	100.9	4.7	104.5	2.7
	15	99.8	1.4	99.5	1.6
Acetohexamide	0.01	99.5	2.5	99.6	0.3
	0.2	100.6	1.1	101.1	0.9
	2	101.9	0.9	103.5	1.5
	15	99.4	0.7	99.6	2.9
Gliclazide	0.005	100.4	0.7	100.1	0.4
	0.07	100.5	0.9	100.6	0.8
	0.7	100.6	3.3	100.8	3.0
	7	99.2	2.1	100.3	3.8
Glibenclamide	0.001	102.0	5.4	100.4	2.0
	0.02	101.9	1.7	102.2	0.3
	0.2	104.4	2.7	105.1	4.1
	1.5	100.4	2.0	99.4	1.1
Glimepiride	0.001	99.5	2.2	100.0	0.4
	0.02	99.9	0.6	101.3	1.3
	0.2	99.6	1.6	101.7	1.8
	1.5	99.8	0.5	99.9	1.3

[a] Values are the means of five replicate determinations.

Carry-over effect was not observed when washing solvent (methanol) was injected after the analysis of high concentration samples. The phenomenon that a peak area gradually increased was not also observed even though the same sample was analyzed repeatedly. Collectively, there was no evidence of carry-over effect in the analysis of the target compounds.

Table 4. The recovery efficiencies and matrix effects of the analytes in whole blood

Analyte	Concentration added (μg/mL)	Recovery efficiency[a] (%)	Matrix effect[a,b] (%)
Metformin	0.2	75.5±1.1	129.9±11.2
	2	74.4±1.3	39.7±9.1
	15	81.0±1.3	2.6±1.0
Buformin	0.02	76.8±5.2	42.6±3.8
	0.2	78.6±1.5	6.0±11.2
	3	77.8±3.7	−22.2±4.9
Alogliptin	0.02	80.0±0.9	0.7±4.0
	0.2	84.2±2.5	3.7±1.3
	3	83.0±3.5	−16.8±3.6
Vildagliptin	0.02	80.7±2.2	24.6±2.2
	0.2	96.4±2.3	12.9±9.9
	1.5	93.4±2.3	−1.0±1.3
Pioglitazone	0.02	94.5±12.4	534.6±67.5
	0.2	91.5±4.5	237.6±26.5
	3	94.3±3.4	2.3±4.2
Chlorpropamide	0.2	97.1±12.2	70.8±6.2
	2	90.6±18.3	6.3±6.8
	15	92.0±11.0	−6.6±4.1
Acetohexamide	0.2	94.9±14.6	44.6±6.8
	2	90.6±21.7	−5.0±6.7
	15	89.3±7.9	−3.3±3.8
Gliclazide	0.07	99.6±16.1	33.9±6.4
	0.7	92.8±14.7	−9.6±3.0
	7	95.9±4.4	−7.0±5.2
Glibenclamide	0.02	94.3±10.1	7.7±2.2
	0.2	92.7±11.1	−6.6±3.7
	1.5	98.3±18.3	8.0±4.7
Glimepiride	0.02	90.8±15.5	53.7±16.8
	0.2	92.9±12.2	−5.0±6.0
	1.5	93.7±6.2	−5.0±7.2
Metformin-d_6 (IS)	1	99.3±2.0	−29.1±1.4
Vildagliptin-d_3 (IS)	1	102.3±12.1	55.4±17.7
Pioglitazone-d_4 (IS)	0.1	99.0±5.4	−6.8±5.3
Tolbutamide-d_9 (IS)	1	98.7±1.5	2.8±2.7

[a] Values are expressed as the means±SD (n=5).
[b] Positive values account for signal enhancement and negative values for signal suppression.

The percentages of initial concentrations after storage under different conditions are shown in Table 5. The stabilities at the tested concentrations are acceptable except for gliclazide. Gliclazide was relatively unstable when stored long-term (28 days, −30°C) at concentrations of 0.07 and 0.7 μg/mL. Thus, it is necessary to pay attention to stability.

Conclusions

In this study, we developed and validated a method for simultaneous quantification of sulfonylureas and biguanides in whole blood by UPLC-MS/MS. We expect that the

Table 5. Stability of the analytes in whole blood under different storage conditions

Analyte	Concentration added (μg/mL)	Freeze-thaw, 3 cycles[a] (%)	Short-term, 4 h, room temperature[a] (%)	Long-term, 28 days[a] (%)	
				−30°C	−80°C
Metformin	0.2	98.3	102.5	106.0	104.6
	2	98.2	101.0	102.0	99.4
	15	94.7	104.3	100.7	100.4
Buformin	0.02	98.6	96.5	92.4	105.2
	0.2	92.1	98.1	89.8	103.9
	3	93.6	98.1	91.8	100.3
Alogliptin	0.02	99.2	97.5	99.5	102.1
	0.2	99.2	99.1	97.3	97.2
	3	96.0	103.2	97.6	98.7
Vildagliptin	0.02	95.6	100.2	98.8	100.6
	0.2	95.8	99.6	98.6	98.6
	1.5	98.3	101.4	98.8	101.0
Pioglitazone	0.02	95.4	103.1	96.3	97.9
	0.2	92.9	101.4	94.3	97.5
	3	92.5	101.0	92.9	97.6
Chlorpropamide	0.2	103.9	107.2	101.3	103.0
	2	98.6	102.4	100.5	101.2
	15	101.6	104.0	109.6	109.7
Acetohexamide	0.2	96.1	105.9	96.6	96.5
	2	94.0	100.8	96.0	96.4
	15	98.4	102.4	103.2	102.4
Gliclazide	0.07	96.2	100.2	68.8	98.3
	0.7	94.5	99.7	53.4	95.1
	7	98.2	98.9	81.8	98.7
Glibenclamide	0.02	97.7	102.3	93.3	104.0
	0.2	92.6	100.5	97.0	100.1
	1.5	93.8	105.8	97.2	109.6
Glimepiride	0.02	97.3	105.2	95.6	99.6
	0.2	93.3	106.7	95.1	108.2
	1.5	93.3	102.1	97.4	102.6

[a] Values are the means of five replicate determinations.

method described in the current study will be useful for various studies such as clinical and forensic toxicological investigations.

Conflict of Interest

The authors declare no conflict of interest.

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