Research article

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Determination of metformin and other biguanides in forensic whole blood samples by hydrophilic interaction liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT: Metformin is an anti-diabetic drug in the biguanide class which also includes phenformin and buformin. Because of the potential adverse effects of the biguanides, a reliable liquid chromatography–tandem mass spectrometry method using pneumatically assisted electrospray ionization was developed for the quantification of the drugs in both live and post-mortem human whole blood. The blood proteins were precipitated by the addition of a mixture of methanol and acetonitrile, and the extract was cleaned up by cation-exchange solid-phase extraction to eliminate ion suppression effects. The separation was performed by hydrophilic interaction liquid chromatography. Matrix-matched calibrants combined with isotope dilution of metformin were used for calibration. The detection limits were 0.01 mg/L for metformin and phenformin and the relative intra-laboratory reproducibility standard deviations were less than 6% at concentrations of 1–10 mg/L. The mean true recoveries were greater than 86%. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: metformin; phenformin; biguanides; whole blood; LC-MS/MS

Introduction

Metformin is an orally administered drug that is widely used in the treatment of type II diabetes mellitus. The drug belongs to the biguanide class of compounds; this class also includes phenformin and buformin, which are earlier generations of orally administered anti-diabetic drugs (Table 1). Phenformin and buformin have both been withdrawn from the market, and metformin has been substituted for these drugs because it has a lower associated risk of drug-induced lactic acidosis. The potential risk of lactate accumulation is the most serious life-threatening complication related to biguanides. Although phenformin and buformin have been generally withdrawn from the market, phenformin may still be legally available in a few countries. The therapeutic blood concentration ranges of metformin and phenformin span 1–4 and 0.03–0.1 (0.3) mg/L, respectively (The International Association of Forensic Toxicologists, 2010).

In forensic toxicology, it is pertinent to monitor body fluids for metformin because of its widespread use and the potential adverse effects related to the use of this drug. Highly specific methods based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) have been published for the determination of metformin in plasma samples (Chen *et al.*, 2004; Ding *et al.*, 2007; Georgita *et al.*, 2007; Heinig and Bucheli, 2004; Koseki *et al.*, 2005; Liu and Coleman, 2009; Mistri *et al.*, 2007; Wang *et al.*, 2004; Zhang *et al.*, 2007). All of these methods apply simple protein precipitation techniques as the only sample treatment, and separation is typically performed by traditional reverse-phase chromatography. Unfortunately, simple protein precipitation is often not sufficient for the treatment of post-mortem blood samples, as the natural blood constituents, such as proteins, carbohydrates and lipids, may have been decomposed to varying degrees by enzyme activities. This decomposition increases the possibility of severe ion suppression effects during detection, leading to poor sensitivities for some samples, which are especially apparent when polar analytes, such as the biguanides, are analysed.

The present LC-MS/MS method based on hydrophilic interaction liquid chromatography (HILIC) analysis of extracts cleaned-up by cation-exchange solid-phase extraction (SPE) was developed and validated as a robust technique suitable for the determination of metformin in both live and post-mortem whole blood samples. Furthermore, the method was validated for the detection of phenformin and buformin.

Experimental

Chemicals and reagents

Metformin HCl, phenformin HCl and 1-phenylbiguanide HCl were obtained from Sigma-Aldrich (Schnelldorf, Germany). Buformin HCl

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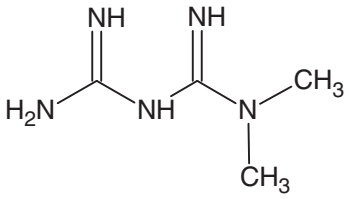
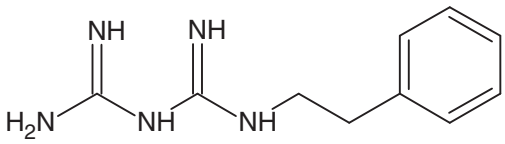
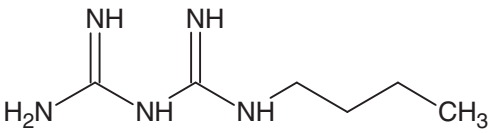
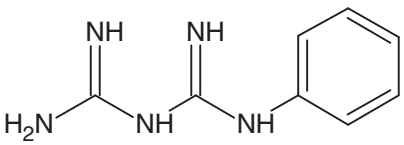
Abbreviations used: HILIC, hydrophilic interaction liquid chromatography; SPE, solid-phase extraction.

Table 1. Biguanides included in the validated method

Substance	Molecular structure
Metformin	
Phenformin	
Buformin	
1-Phenylbiguanide (IS)	

was obtained from Wako Chemicals (Neuss, Germany). Metformin-D6 was purchased from Toronto Research Chemicals Inc. (North York, Canada). Blank whole blood samples for calibration were obtained from the blood bank of Aarhus University Hospital (Skejby, Denmark). The blank samples of live and post-mortem whole blood used for method validation were obtained from the Institute of Forensic Medicine, University of Aarhus. Live blood samples were collected and preserved in Venosafe tubes containing a mixture of sodium fluoride (NaF) and potassium oxalate (Terumo Europe, Leuven, Belgium). Post-mortem blood samples were preserved with 200 mg of NaF per 30 ml of blood. Formic acid and ammonium acetate (NH_4Ac) were purchased from Merck (Darmstadt, Germany). Methanol (MeOH) and acetonitrile (MeCN) were purchased from Sigma-Aldrich. Water was purified using a Direct-Q 3 apparatus (Millipore, Bedford, MA, USA).

Separate stock solutions, each containing 1 mg/mL of the active substance, were prepared in MeOH. The combined standard solutions for the fortification of the samples and for the preparation of the calibrants were prepared by diluting the stock solutions with MeOH. An internal standard solution (IS) containing 4 mg/L of metformin-D6 and 1 mg/L of 1-phenylbiguanide was also prepared in MeOH. The mobile phases A and B consisted of water and MeCN, respectively, which were both acidified with 0.1% formic acid.

Equipment

The liquid chromatographic system was a Waters 2695 Separations Module consisting of a binary pump, a solvent degasser and an autosampler with a sample compartment thermostatted at 10 ± 2°C and a column oven thermostatted at 30 ± 2°C (Waters, Milford, MA, USA). The mass spectrometer was a Micromass Quattro Micro API triple quadrupole instrument with an ESCi ion source (Waters). The separation was performed on a SeQuant ZIC HILIC (5 µm, 200 Å, 2.1 mm i.d. × 150 mm) column (Merck SeQuant, Umeå, Sweden) connected to a ZIC HILIC pre-column cartridge (5 µm, 2.1 mm i.d. × 20 mm; Merck SeQuant). Solid-phase extraction was performed on a 3 mL Oasis weak cation-exchange cartridge containing 60 mg of sorbent (Waters, Milford, MA). Disposable 2 mL polypropylene Safe-Lock tubes (Eppendorf, Hamburg, Germany) were used for the extractions. Other equipment used included pipettes (Biohit, Helsinki, Finland) and a Heraeus Biofuge Pico (Thermo Scientific, Langenselbold, Germany).

Extraction

A 100 µL volume of blood was transferred to a disposable 2 mL centrifuge tube. Subsequently, 300 µL of water, 100 µL of the IS and 100 µL of MeOH were added, and the tube contents were mixed gently. To this solution, 900 µL of MeCN was added, and the sample was mixed immediately for a few seconds. After a total standing time of 15 min, the mixture was centrifuged at 3000 ***g*** for 5 min. A volume of 200 µL of the sample was mixed with 400 µL of 50 mM NH_4Ac and was then eluted through a weak cation-exchange SPE cartridge that had been previously conditioned with 1 mL of MeOH, followed by 1 mL of water, by gravity. The cartridge was washed with 1 mL of the NH_4Ac solution followed by 1 mL of MeOH. Next, the analytes were eluted with 2 mL of an MeOH–MeCN mixture (20:80) acidified with 2% formic acid.

Calibration

Calibrants based on blank donor blood were used for the construction of five-point calibration curves. The samples were treated according to the

above procedure, except that 100 µL of MeOH was replaced by 100 µL of the mixed standards of the drug substances. Sample concentrations were obtained at 0.025, 2, 4, 8 and 12 mg/L of metformin; 0.025, 0.1, 0.2, 0.4 and 0.6 mg/L of phenformin; and 0.025, 0.4, 0.8, 1.6 and 2.4 mg/L of buformin in the original blood sample. The calibration curves were created by weighted (1/*x*) linear regression analysis on the IS-normalized peak areas (analyte area/IS area). Metformin-D6 was used as the IS for metformin, and 1-phenylbiguanide was used as the IS for phenformin and buformin.

LC-MS/MS conditions

All sample extracts were kept at 10 ± 2°C until analysis. A 10 µL volume was injected onto a SeQuant ZIC HILIC column running with 20% mobile phase A and 80% B. A linear gradient was used to change the mobile phase to 40% A and 60% B over 3 min. After a total time of 9 min, the gradient was returned to 20% A and 80% B over 1 min, and the column was equilibrated for 5 min before the next injection. The eluent was diverted to the waste during the intervals 0–3 and 9–15 min after the injection using a post-column switch. The column flow rate was 200 µL/min, and the column temperature was kept at 30 ± 1°C. The source and desolvation temperatures were set at 140 and 350°C, respectively, and the cone and desolvation gas flows were set at 50 and 800 L/h, respectively. The mass spectrometer was operated in positive ion mode with a probe voltage of 4000 V and an extractor potential of 3 V. The dwell time was 200 ms for all ion transitions. Selected reaction monitoring (SRM) was applied under the conditions shown in Table 2. Argon was used for collision-induced dissociation (CID). Data acquisition and processing were performed using MassLynx 4.1 (Waters).

Limit of detection

The limits of detection (LODs) were determined using a random selection of 20 different blank control samples of live and post-mortem whole blood (10 of each type). The samples were fortified prior to extraction with the individual substances to obtain concentrations that were approximately three times the signal/noise ratio. The LODs were calculated as three times the standard deviation (SD) of the measured results.

Precision, trueness and recovery

The repeatability standard deviation (SD_r; i.e. the variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample and in a short interval of time) and the intra-laboratory reproducibility standard deviation ($SD_{R,intra\text{-}lab}$; i.e. the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators under different experimental conditions) were determined on blank control samples of live and post-mortem whole blood fortified to levels of 1 and 10 mg/L of metformin; 0.05 and 0.5 mg/L of phenformin and 0.2 and 2 mg/L of buformin. Furthermore, the precision was determined on a post-mortem blood sample from a person who used metformin. Duplicate analyses were performed on each sample on eight different days. The repeatability and intra-laboratory reproducibility parameters were calculated in accordance with ISO standard 5725-2 (ISO, 1994).

The true recoveries and ion suppression effects were investigated on 20 fortified blank samples of both live and post-mortem blood. The samples were fortified with 2 mg/L of metformin, 0.1 mg/L of phenformin and 0.5 mg/L of buformin. The standards used for the determination of the true recoveries were the same blank samples that had been fortified after extraction and SPE. The stabilities of metformin, phenformin and buformin in samples of live blood and post mortem blood stored at 5 and 20°C were tested at the concentration levels of 2, 0.1 and 0.4 mg/L, respectively.

Results and discussion

Precursor ions and transition products

Electrospray ionization was achieved in positive mode (ESI^+) for all substances, and the dominant Q1 ions obtained were the protonated molecular ions ($[M + H]^+$). The common fragmentation pattern of biguanide precursor ions produced *m/z* 60 $\{[C(NH_2)_3]^+\}$ and *m/z* 85 $\{[CNH_2(NH)_2CNH]^+\}$ product ions in high abundance (Table 2). For all substances at least two product ions of acceptable abundance were obtained. The relative abundances of the transition products were constant in the calibrated range, and the mean difference in the relative abundance between the pure reference standards and the matrix-matched calibrants was less than 5% for each substance. The most sensitive transition products were used for the quantitative measurements.

Extraction and cleanup

The blood samples were diluted four times with water, and the active substances were extracted using a mixture of MeOH and MeCN. The methanol was added to obtain a disperse precipitate of the protein-containing blood components. The denatured proteins were precipitated by centrifugation, and the supernatant was cleaned up by SPE using a weak cation exchange sorbent. Some of the supernatant was also filtered through a 30 kDa regenerated cellulose membrane instead of performing the SPE clean-up. Here, strong ion suppression effects were observed for some of the post-mortem samples, reducing the signal intensities of the metformin to less than 20% of their corresponding signal intensities in live blood. Although these decreases in signal may not affect the trueness of the results when an isotope dilution technique is used, the method sensitivity would vary from sample to sample. Furthermore,

Table 2. Mass spectrometry conditions and relative retention times (RRTs). Bold and underlined ions were used for quantification, and underlined ions were used as primary qualifiers

Drug	Transition		Cone voltage (V)	Collision energy (eV)	Relative abundance	RRT
	Q1 (*m/z*)	Q3 (*m/z*)				
Metformin	130	**60**/71/85/88	20	12/20/12/14	100/72/27/26	0.99
Phenformin	206	**60**/105/85	20	15/24/20	100/38/10	0.56
Buformin	158	**60**/116/85/43	20	14/16/18/25	100/8/7/7	0.68
Metformin-D6 (IS)	136	**60**/77/85/94	20	12/20/12/14	100/77/26/25	1.00
1-Phenylbiguanide (IS)	178	**60**/119/85	20	14/22/15	100/44/3	0.62

samples showing strong ion suppressions produced distorted peak shapes, which could result in poorer precision.

Chromatography

Biguanides are polar substances, which commonly have short retention times on traditional reverse-phase column packings. Using the HILIC separation technique, better retention properties were achieved. In addition, the HILIC technique made it possible to inject the SPE eluate directly onto the analytical column without evaporation of the solvent or aqueous dilution. A typical chromatogram is shown in Fig. 1.

Method performance parameters

The mean ion suppression effects were less than 5% for all investigated biguanides and were not significantly different for the live and post-mortem blood samples (Table 3). The mean true recoveries were greater than 89% for the live blood samples and greater than 86% for post-mortem blood samples (Table 3). The LODs were in the range of 12–54 µg/L for the qualifier ions and 6–11 µg/L for the quantifier ions (Table 4).

In the precision study, the RSD_r and $RSD_{R,intra\text{-}lab}$ values obtained were not significantly different for the two types of blood samples. The $RSD_{R,intra\text{-}lab}$ determined using fortified samples was found to be below 6% for metformin in the therapeutic concentration range (Table 5). Furthermore, the $RSD_{R,intra\text{-}lab}$ determined for the post-mortem sample with a natural content of metformin 3.5 mg/L was 6%. According to a general rule (Horwitz, 1982), the obtained precision figures were considered to be acceptable. The Horwitz equation states that the RSD_R between laboratories should be below 11 and 22% at concentration levels of 10 and 0.1 mg/L, respectively.

The calibration curves created during the precision study using weighted linear regression analyses gave R^2 values better than 0.995 for the transitions used for quantification. The stabilities of

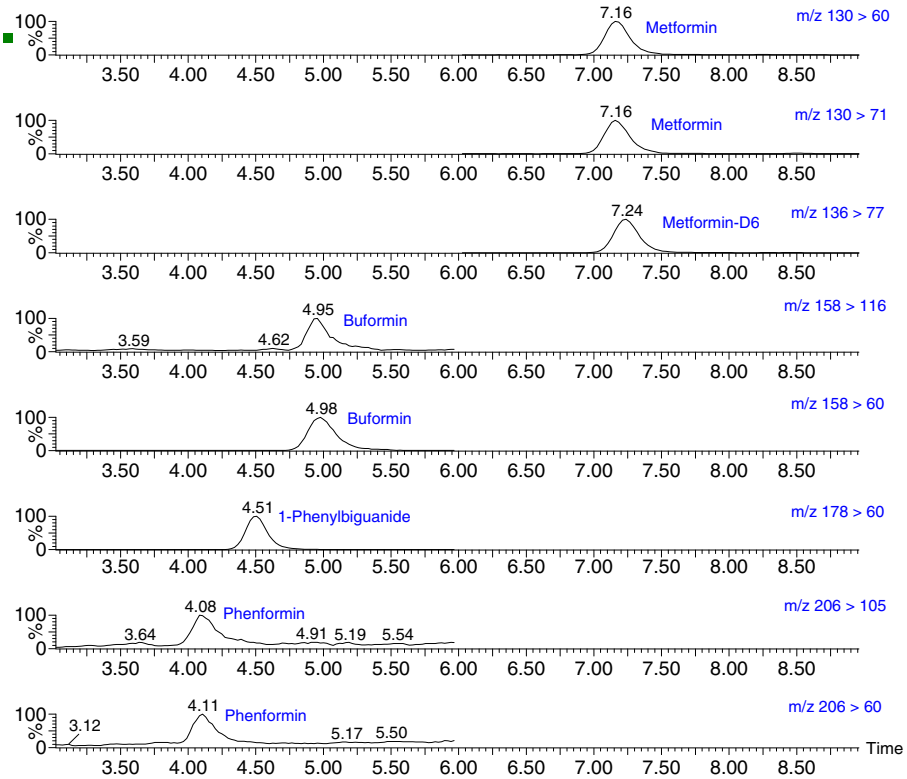


Figure 1. Chromatograms of the ESI(+) product ions in the extract of live whole blood fortified with 1 mg/L of metformin, 0.05 mg/L of phenformin and 0.2 mg/L of buformin. The analytical column used was a SeQuant ZIC HILIC (5 µm, 2.1 mm i.d. × 150 mm).

Table 3. Ion suppression effects and true recoveries obtained from the single analyses of the fortified live and post-mortem blood samples ($n = 20$ for each level and each sample type). Matrix-matched standards used in the calculation of the recoveries were the samples fortified after the extraction and clean-up

Drug	Fortification level (mg/L)	Ion suppression, mean (± SD), %		True recovery, mean (± SD), %	
		Live blood	Post-mortem blood	Live blood	Post-mortem blood
Metformin	2	0 (±5)	2 (±7)	91 (±4)	88 (±6)
Phenformin	0.1	−2 (±6)	3 (±9)	89 (±6)	86 (±6)
Buformin	0.5	−1 (±5)	5 (±6)	93 (±4)	89 (±6)

Table 4. Limits of detection determined in live and post-mortem whole blood samples ($n = 20$ for each group)

Drug	Transition Q1/Q3 (*m/z*)	Fortification level (μg/L)	Result, mean (± SD) (μg/L)		LOD (μg/L)	
			Live	Post-mortem	Live	Post-mortem
Metformin	130/60	18	18 (±3.2)	16 (±2.5)	10	8
	130/71	18	17 (±3.9)	16 (±4.7)	12	14
Phenformin	206/60	18	20 (±3.6)	15 (±2.3)	11	7
	206/105	18	16 (±4.3)	16 (±4.9)	13	15
Buformin	158/60	6	8 (±2.0)	8 (±2.4)	6	7
	158/116	60	45 (±10)	50 (±18)	30	54

Table 5. Average method precisions estimated for different drug concentration levels in live and post-mortem whole blood

Drug	Fortification level (mg/L)	Live blood			Post-mortem blood		
		Mean result (mg/L)	RSD_r (%)	$RSD_{R,intra\text{-}lab}$ (%)	Mean result (mg/L)	RSD_r (%)	$RSD_{R,intra\text{-}lab}$ (%)
Metformin	1	0.95	3.6	6.0	0.94	1.9	3.7
	10	9.9	3.4	3.9	10.1	2.0	4.5
Phenformin	0.05	0.049	6.3	9.0	0.049	5.7	7.7
	0.5	0.50	2.8	3.6	0.50	2.0	3.6
Buformin	0.2	0.19	3.1	4.8	0.18	2.9	3.8
	2	2.0	4.3	4.3	2.0	2.0	4.3

the final sample extracts and calibrants stored at 10 ± 2°C were tested over a period of seven days, and no significant changes in the results were observed. The biguanides were also found to be stable in the preserved live and post-mortem blood samples for at least seven days when stored at 5 ± 2°C. When stored at 20 ± 2°C for 7 days, the reduction was less than 5%.

Conclusion

A simple and rugged LC-MS/MS method was developed and validated for the determination of metformin, phenformin and buformin in live and post-mortem whole blood. Moreover, this method has the potential to be used as a template for the determination of other low-molecular-weight biguanides.

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