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Short communication

Determination of metformin in mouse, rat, dog and human plasma samples by laser diode thermal desorption/atmospheric pressure chemical ionization tandem mass spectrometry

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ABSTRACT

A simple, rapid and robust high-throughput assay for the quantitative analysis of metformin in plasma from different species using laser diode thermal desorption interfaced with atmospheric chemical pressure ionization tandem mass spectrometry (LDTD-APCI-MSMS) was developed for use in a pharmaceutical discovery environment. In order to minimize sample preparation a generic protein precipitation method was used to extract metformin from the plasma. Laser diode thermal desorption is a relatively new sample introduction method, the optimization of the instrumental parameters are presented. The method was successfully applied to spiked mouse, rat, dog and human plasma samples and was subsequently used to determine the oral pharmacokinetics of metformin after dosing to male rats in order to support drug discovery projects. The deviations for intra-assay accuracy and precision across the four species were less than 30% at all calibration and quality control levels.

1. Introduction

Metformin is a biguanide type insulin sensitizing drug used to treat type-2 diabetes (Diabetes mellitus) [1]. It is used in drug discovery *in vivo* models to assess the anti-diabetic potential of other drugs, commonly as a comparison compound in oral glucose tolerance tests (OGTT) or other pharmacokinetic/pharmacodynamic studies [2]. Measurement of systemic concentrations of metformin is thus of interest both pre-clinically across various species, and clinically in therapeutic drug monitoring of diabetic patients to prevent toxicity and ensure adherence to prescribed medications [3].

Metformin is a highly polar molecule that is traditionally difficult to measure. Several chromatographic methods have been reported for metformin analysis including normal phase chromatography on silica and cyano columns [4], ion exchange chromatography [5], ion pair chromatography [6] and reversed-phase chromatography [7]. The eluent of these separation techniques can be directed to a tandem mass spectrometer by use of ion sources such as electrospray ionization (ESI) [8,9] and atmospheric pressure chemical ionization (APCI) [10–12]. ESI is not considered the ideal ion source for metformin quantification due to the risk of ion suppression from endogenous material in the sample matrix, this effect is not as prevalent in APCI due to the fundamentally different ionization mechanism. The analytical methods often require large sample volumes (100 μL+) and the fundamental use of chromatography means they are time consuming (each injection taking multiple minutes) and often lack sensitivity (>10 ng/mL) depending on the detection method employed. Liu and Coleman recently published a ESI-MS based method using hydrophilic interaction liquid chromatography (HILIC) [13] that adequately assayed metformin with a lower limit of quantification of 0.5 ng/mL from 50 μL of human plasma, the use of the HILIC chromatography system leads to cycle times of 2 min per injection.

Laser diode thermal desorption (LDTD) is a relatively new sample introduction source that does not require an HPLC step prior to detection via tandem mass spectrometry. LDTD has potentially many applications, however, at the time of writing this paper only a limited number have been reported [14,15]. Thermal desorption of the analyte is initiated by use of an infrared laser. This generates neutral molecules in the gas phase from samples that have been adsorbed onto a metallic surface, in the case of LDTD this is a specially designed stainless steel 96 or 384 well plate. When combined with atmospheric pressure chemical ionization (APCI) these neutral gas phase molecules can be ionized for subsequent detection by MS/MS [16]. The direct nature of LDTD means analysis times can be greatly reduced (typically <30 s per desorption), data can be captured by conventional LC–MS/MS software because laser power can be applied in a gradient that gives rise to a MS response reminiscent of a chromatographic peak (Fig. 1). This paper demonstrates a sensitive and robust high-throughput LDTD-APCI-MSMS method for the determination of metformin in preclinical mouse, rat and

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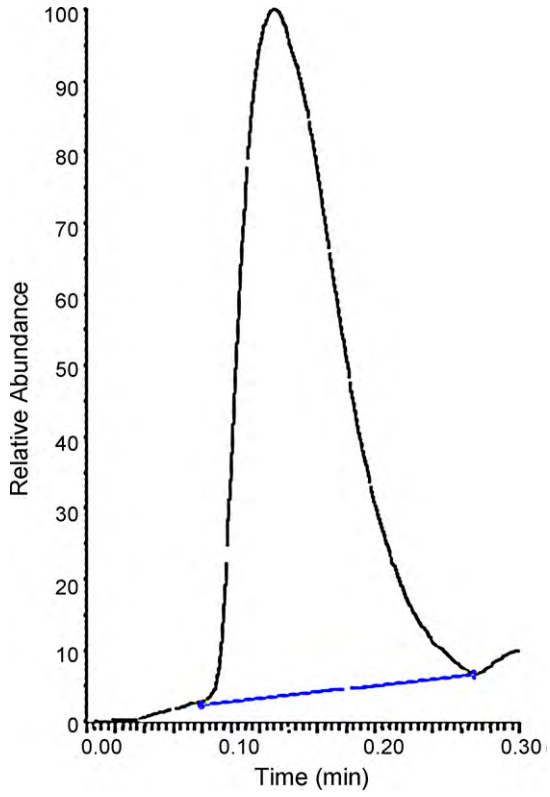

Fig. 1. Raw data showing the typical 'chromatogram' like response for the metformin *m/z* 130 > 71 transition produced by LDTD-APCI-MSMS.

dog plasma samples and its subsequent application to rat pharmacokinetic samples in support of drug discovery. The paper also explores the potential of the technique for use in the analysis of human plasma samples.

2. Experimental

2.1. Chemicals, reagents and materials

Metformin hydrochloride was synthesized at AstraZeneca. Phenformin hydrochloride, the internal standard, was obtained from Sigma–Aldrich (Poole, Dorset, UK). HPLC grade methanol was purchased from Thermo Fisher Scientific (Loughborough, Leicestershire, UK).

2.2. Control plasma

Control mouse, rat and dog plasma was obtained from the AstraZeneca breeding colonies. Control human plasma was obtained from the AstraZeneca clinical trial unit based at Alderley Park, Macclesfield, Cheshire, UK.

2.3. Instrumental

The LDTD source was manufactured by Phytronix Technologies (Quebec, QC, Canada). Dried samples are loaded into the LDTD system on a specially designed LazWell™ 96-well plate manufactured by Phytronix Technologies (Quebec, QC, Canada). The LDTD source had the following settings: corona discharge needle voltage 3000 V, vaporizer temperature ambient, ion sweep gas pressure 0.3, auxiliary gas off, sheath gas off. The carrier gas was nitrogen at a flow rate of 3 L/min. Laser power was ramped from 0% to 35% over 3 s and held at 35% power for 3 s before shutting off.

The LDTD source was mounted on a Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA). The mass spectrometer was operated in positive ion selected reaction monitoring (SRM) mode. Metformin was monitored at a parent mass of 130.097 and a daughter mass of 71.14 with a tube lens voltage of 54.56 V and a collision energy of 22 V. Phenformin, the internal standard, was monitored at a parent mass of 206.167 and a daughter mass of 105.08, the tube lens and collision energy were 58.07 and 105.08 V, respectively. Parent molecules and fragments are displayed in Fig. 2. The capillary temperature was set at 270 °C, collision pressure at 1.5 mTorr.

The mass spectrometer software used for data capture was Xcalibur 2.0.7 and QuickQuan 2.3 (Thermo Fisher Scientific, San Jose, California, USA).

2.4. Method development

Compound optimization was performed using the auto-tune function in the Xcalibur software. Four separate aliquots of a methanolic standard of each compound (10 μg/mL) were spotted (2 μL) onto a LazWell™ plate and evaporated to dryness under a gentle stream of nitrogen. Each sample was then systematically adsorbed by LDTD into the mass spectrometer, the auto-tune algorhythm captured the relevant instrument parameters (tube lens voltage, adjusted parent mass, collision energy and daughter ion) throughout the optimization process.

2.5. Solutions and standards

Stock solutions of metformin and phenformin were prepared in methanol to give a final concentration of 1 mg/mL. Subsequent

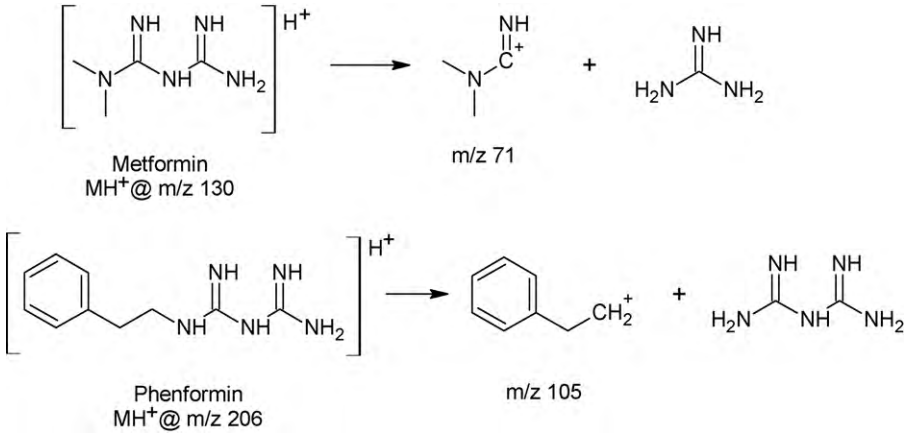

Fig. 2. Structures of metformin and phenformin and the corresponding fragment ions monitored by MS/MS.

working solutions of metformin for use in calibration curve construction were prepared by serial dilution of the stock solution. A protein precipitation solvent was prepared using the phenormin stock solution diluted to a concentration of 0.1 μg/mL in methanol.

Samples for the standard curves and quality controls were prepared by spiking control plasma with the appropriate metformin working solution. The calibration standards were prepared at 5, 10, 50, 100, 500, 1000 and 2000 ng/mL. Quality controls were prepared at 10, 100 and 1000 ng/mL.

2.6. Sample preparation and extraction

To precipitate 50 μL of mouse, dog and human plasma, 500 μL of protein precipitation solvent containing internal standard were added. Ion suppression in terms of a significant drop in internal standard response was observed at the top end of the calibration curve using this ten-fold dilution with rat plasma, the ratio of the precipitation solvent was thus increased to twelve-fold to compensate for this effect. Precipitated samples were then mixed for 30 s prior to centrifugation at 3700 g for 20 min. The supernatant was then directly applied to the LazWell plates (2 μL per sample) and evaporated to dryness under a gentle stream of nitrogen.

2.7. Data analysis and method validation

All data was processed using QuickQuan™ (Gubbs Inc., Alpharetta, Georgia, USA) software. Linear least-squares regression with a $1/x$ weighting of the peak area ratios (analyte/IS) versus the nominal concentration of the calibration standards was used to construct the calibration curves. Eight calibration standards between 1 and 2000 ng/mL ($n = 6$ at each level) were prepared in each species of plasma. Quality controls were spiked at 10, 100 and 1000 ng/ml ($n = 6$ at each level) in each species of plasma and were interspersed between the calibration standards. One set of calibration standards from each species was used to construct calibration curves and quantify the subsequent calibration samples and quality controls. All Calibration standards and quality controls were used to calculate intra-assay accuracy and precision at each level in each matrix to establish the calibration range for each matrix.

2.8. Accuracy, precision and specificity

Intra-assay accuracy was evaluated by comparing the mean measured concentrations of the calibration standards and quality controls with their nominal concentrations. Intra-assay precision was calculated based on the coefficient of variation of each set calibration standards and quality controls ($n = 6$). The assay was deemed acceptable for the analysis of samples in each matrix if the intra-assay accuracy and precision deviated by ±30%.

The specificity of the method was established by assaying 12 lots of blank control plasma and comparing the response of each blank relative to the lowest calibration standard.

2.9. Method application

Two Hans Wistar rats were dosed orally with metformin at a dose level of 50 mg/kg in order to support dose setting of a planned pharmacodynamic study. Plasma samples (50 μl) were taken at 0.25, 0.5, 1, 2, 3, 6, 12 and 24 h post administration.

For the analysis of rat pharmacokinetic samples, quality controls samples (in duplicate) were interspersed throughout the unknowns. The analytical batch was considered acceptable if the accuracy of each calibration standard used to construct the calibration curve was ±30% of nominal concentration, with the curve constructed from no less than 5 points. The accuracy of at least 3/4 of the quality controls should was within ±30% of nominal concentration. The acceptance criteria values are deemed acceptable within a discovery bioanalysis environment.

Pharmacokinetic parameters were calculated using non-compartmental analysis performed in WinNonlin 5.2.1 (Pharsight Corp., Mountain View, California, USA).

3. Results and discussion

Metformin and the internal standard both showed a good MS response when introduced into the mass spectrometer by the LDTD source. Various laser power settings were evaluated and ranged from 25% to 45% in order to achieve the best MS response possible for metformin. 35% laser power gave the most intense response with no improvement being observed using higher laser power, suggesting that at 35% laser power all of the sample was being desorbed from the LazWell™ plate.

As LDTD is a direct introduction technique all of the analytes are desorbed into the mass spectrometer at the same time. It was thus necessary to be vigilant for any ion suppression effects within each assay and this was achieved by monitoring the internal standard variation (typical range 11–21% in these experiments) and calibration curve linearity within each analytical run. Ion suppression was observed in the analysis of rat plasma samples when the protein precipitation method used a ten-fold ratio (sample:precipitation solvent) resulting in variation of the internal standard by 31% throughout the analytical run ($n = 84$). This was largely due to suppression of the IS signal at the higher metformin calibration levels. A repeat experiment was performed using a 1:12 dilution with the precipitation solvent and this improved internal standard variability (18%). Linearity was comparable across the different dilution methods, 0.9989 and 0.9985 for the ten-fold and twelve-fold dilutions respectively.

A response was observed for metformin in extracted plasma blank samples across the species. Fig. 3 shows representative raw data obtained upon desorption of an extracted rat plasma blank sample compared to desorption of a calibration standard in the same matrix at the LLOQ. The response in the calibration standard at the LLOQ was over 3 times that of the blank sample in terms of peak area and over 3 times greater in terms of signal to noise ratio. The peak in the blank sample could be due to several factors including the inherent nature of the LDTD sample introduction process to the mass spectrometer, cross contamination during the extraction procedure or it may be attributed to the low mass region that metformin falls into and endogenous background noise.

The intra-assay accuracy and precision throughout the analytical runs were within acceptable limits for discovery bioanalysis. Accuracy and precision for the calibration standards ($n = 6$) within the validation experiments was ±20%, ±30%, ±30% and ±30% for mouse, rat, dog and human plasma, respectively (Table 1). The lower limits of quantification (LLOQ) were equal to 5, 5, 5, and 1 ng/mL in mouse, rat, dog and human plasma, respectively. The intra-assay accuracy and precision of quality controls was within ±25% across the species (Table 2) with the exception of the 10 ng/mL mouse plasma quality control which had an accuracy of 72.1% and met our acceptance criteria set at ±30%. The bottom standard in mouse, rat and dog plasma showed poor accuracy and precision and the metformin response was not considered adequate in terms of signal to noise to accept the 1 ng/mL standards as the LLOQ.

Raw data was reprocessed in the absence of internal standard (data not shown). Under these conditions the accuracy of the assay

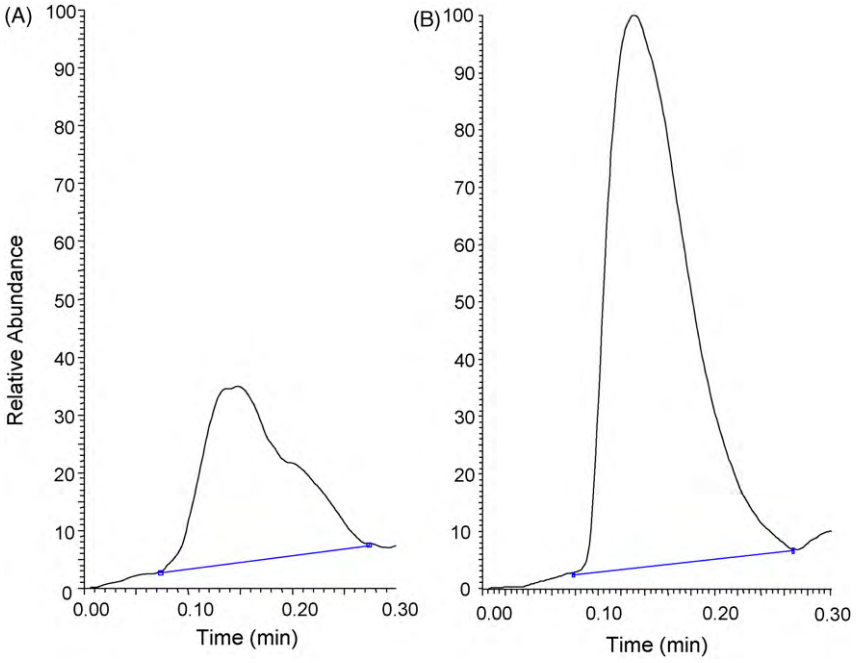

Fig. 3. LDTD-APCI-MSMS raw data of the product ions of metformin at m/z 130 → 71 in (A) a protein precipitated rat plasma blank and (B) a calibration standard at the LLOQ.

Table 1
Summary of intra-assay accuracy and precision (%) of LDTD-APCI-MS calibration standards.

	Mouse plasma	Rat plasma	Dog plasma	Human plasma
Cal range (ng/mL)	5–2000	5–2000	5–2000	1–1000
Accuracy range (%)	82.8–100.9	109.9–127.8	71.6–106.4	96.8–121.0
Precision range (%)	81–19.8	9.6–28.1	6.8–24.8	14.8–27.7

Table 2
Intra-assay accuracy and precision (%) of LDTD-APCI-MS for quality control samples.

	Mouse plasma		Rat plasma		Dog plasma		Human plasma	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
10 ng/mL	72.1	19.8	118.4	19.9	104.8	10.8	80.7	14.4
100 ng/Ml	81.0	12.2	112.8	5.8	96.8	29.8	87.2	16.6
1000 ng/mL	80.1	11.7	102.4	20.5	106.3	8.9	78.2	10.7

remained within the acceptance criteria across the calibration and quality control range for mouse and rat plasma but failed in dog and human plasma. Precision was greatly affected by the omission of the internal standard, each matrix failed the acceptance criteria indicating high variability when an internal standard is not used.

A typical validation run of 84 desorptions was completed in 50 min by elimination of the chromatography step, an equivalent LC–MS/MS analysis would require around 3.5 h of mass spectrometer time based on our in-house chromatography system which has a 2.5 min cycle time.

Having demonstrated that the method was accurate and precise within the defined acceptance criteria the assay was applied to a rat plasma pharmacokinetic study. The mean venous plasma concentration-time profile of metformin after oral administration at 50 mg/kg to male Hans Wistar rats are shown in Fig. 4. The pharmacokinetic parameters derived from the analysis are listed in Table 3. The C_{max} was 3.9 ± 0.651 μg/mL and occurred at 1.3 h. The oral half-life of metformin was 3.2 ± 0.1 h and the area under the plasma concentration-time curve was 16.7 μg h/mL. The results shown in Table 3 compared favorably to values reported in the literature [17] utilizing a reversed-phase HPLC method and ultraviolet detection. The literature method had a LLOQ of 50 ng/mL compared to 5 ng/mL using the LDTD method, intra-assay precision is stated as being below 8.94% compared to 16.0%, 23.3% and 22.9% at the 10, 100 and 1000 ng/mL quality control levels (n = 4) for the LDTD method. The data generated by LDTD have been used to assess the

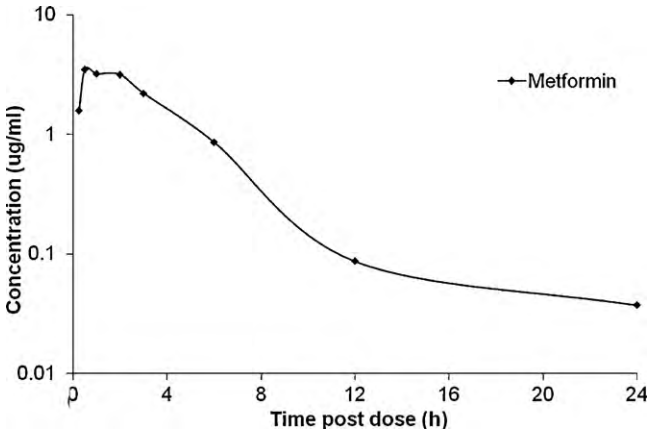

Fig. 4. Mean venous plasma concentration-time profiles of metformin after oral administration of the drug at 50 mg/kg to male Hans Wistar rats.

Table 3
Pharmacokinetic parameters of metformin after oral administration of the drug at 50 mg/kg to male Hans Wistar rats.

Parameter	50 mg/kg ($n = 2$)	50 mg/kg lit. values ($n = 7$)
C_{max} (μg/ml)	3.9 ± 0.651	3.4 ± 0.484
T_{max} (h)	1.3 (0.5–2)	0.5 (0.25–2)
$T_{1/2}$ (h)	3.2 ± 0.1	3.2 ± 1.3
AUC0-α (μg h/mL)	16.7 ± 0.141	10.7 ± 1.4

pharmacokinetic/pharmacodynamic relationship of potential drug candidates in relation to metformin.

4. Conclusion

A high-throughput, sensitive and robust assay is reported for the determination of metformin in mouse, rat, dog and human plasma using LDTD-APCI-MSMS. This method has significant advantages in terms of simplicity and sample analysis time. The assay was validated in terms of accuracy and precision for use in a pharmaceutical discovery environment and the analysis of real pharmacokinetic study samples was demonstrated. Sample preparation time was minimized by the use of a generic protein precipitation extraction technique that enabled metformin to be quantified across the species with a lower limit of quantification of 1–5 ng/mL using 50 μL of plasma.

The rapid nature of the LDTD sample introduction technique is a major step forward for bioanalysis, the speed of the technique allowing more efficient use of mass spectrometer time.

The use of LDTD-APCI-MSMS in the analysis of human plasma samples was demonstrated. Human plasma is not routinely analysed in a discovery environment but the results indicate that the technique could potentially be applied to therapeutic drug monitoring in the clinic.

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