



# Simultaneous quantitation of metformin and sitagliptin from mouse and human dried blood spots using laser diode thermal desorption tandem mass spectrometry

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## ABSTRACT

A simple, rapid and robust high-throughput assay for the simultaneous analysis of metformin and sitagliptin from mouse and human dried blood spot samples using laser diode thermal desorption interfaced with atmospheric pressure chemical ionization tandem mass spectrometry (LDTD–APCI–MS/MS) was developed for use in a pharmaceutical discovery environment as an alternative to traditional plasma analysis. Analytes were extracted from dried blood spots using a simple punch disc and solvent extract procedure. Details of the method development and optimization of the instrumental parameters are presented. The method was successfully applied to spiked mouse and human dried blood spot samples. Analyte stability was determined in dried blood spots on FTA cards and as extracts of dried blood spots. The method was subsequently used to determine the oral pharmacokinetics of metformin and sitagliptin after dosing to male mice. Metformin and Sitagliptin results are compared to data generated by more traditional liquid chromatography–mass spectrometry methods. Intra-assay and inter-assay accuracy and precision across the analytes and species deviated by less than 30% at all calibration levels and less than 20% at all quality control levels.

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## 1. Introduction

Metformin (*N,N*-dimethylbiguanide), originally marketed as Glucophage™ by Bristol-Myers Squibb, is now available in many generic formulations. Metformin is a biguanide type insulin sensitizing drug for the treatment of diabetes. The drug's mode of action is by activation of adenosine monophosphate activated protein kinase (AMPK) a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. Activation of AMPK exerts an inhibitory effect on the production of glucose by liver cells [1]. Metformin is the most prescribed anti-diabetic drug in the world and forms the primary first line therapy for treatment of type II diabetes.

Sitagliptin ((2*R*)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4] tri azolo[4,3-*a*]pyrazin-7(8*H*)-yl]butan-2-amine), marketed as Januvia™ by Merck and Co., is a relatively new oral antihyperglycemic drug used to treat type II diabetes. Sitagliptin competitively inhibits dipeptidyl peptidase IV (DPPIV), an enzyme involved in the breakdown of incretins such as glucagon-like particle-1 (GLP-1) which potentiate insulin secretion *in vivo*. Inhibition of DPPIV reduces the breakdown of

GLP-1 and increases insulin secretion; this suppresses the release of glucagon from the pancreas and drives down blood sugar levels [2]. Merck and Co. also market Sitagliptin in combination with metformin in a single dosage form as Junumet™.

Metformin and Sitagliptin are used in drug discovery *in vivo* models to assess the anti-diabetic potential of new chemical entities, commonly as a comparison compound in oral glucose tolerance tests (OGTT) or other pharmacokinetic/pharmacodynamic studies [3]. The drugs are often prescribed in combination in the clinic when monotherapy of either drug does not improve glycaemic control. Measurement of systemic concentrations of metformin and sitagliptin from a single sample is thus of interest both pre-clinically and clinically for therapeutic drug monitoring of diabetic patients to prevent toxicity and ensure adherence to prescribed medications [4].

Plasma or whole blood samples are often taken to measure drug exposure within the pharmaceutical industry and the clinic. However, the nature of our global industry means that storage and shipping of such samples is often problematic and expensive. The collection of blood samples as dried blood spots (DBS) has been a routine technique in the screening of inherited metabolic disorders in neonates for many years [5]. Beaudette and Bateman have reported the application of DBS to discovery stage pharmacokinetic analysis [6] and Spooner et al. has reported application of DBS in clinical pharmacokinetic studies [7], both these studies concentrate

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on single compound or monotherapy analysis. AbuRuz et al. has published details of a DBS assay for the therapeutic drug monitoring of metformin as a monotherapy in the clinic using HPLC with ultra violet detection [8].

Metformin is a highly polar molecule that is difficult to measure by methods based on reversed phase HPLC in combination with mass spectrometric detection. Liu and Coleman have published a ESI-MS/MS based method using hydrophilic interaction liquid chromatography (HILIC) that adequately assayed metformin with a lower limit of quantification of 0.5 ng/ml from 50  $\mu$ l of human plasma. The use of the HILIC chromatography system leads to cycle times of 2 min per injection [9]. Swales et al. published a robust analytical method utilizing laser diode thermal desorption (LDTD) atmospheric pressure chemical ionization tandem mass spectrometry to analyze metformin concentrations in mouse, rat, dog and human plasma [10]. This technique reduces cycle times to  $\sim$ 20 s per sample.

Sitagliptin is a relatively polar molecule ( $\log D_{7.5}$  octanol/water 0.31). Analysis of plasma samples by traditional LC-MS/MS is well documented both preclinically [11] and clinically [12], the reported methodology uses complex extraction schemes and the common use of HPLC leads to analytical cycle times of 2–2.5 min per sample. At the time of writing this paper no published material could be found documenting the analytical techniques used to measure sitagliptin in combination with metformin or utilizing DBS and LDTD technology.

Laser diode thermal desorption (LDTD) is a direct sample introduction source that does not require HPLC separation prior to detection via tandem mass spectrometry. Wu et al. highlighted the ability of LDTD to introduce multiple analytes to a tandem mass spectrometer [13], although to date no one has reported quantitative analysis of multiple analytes from dried blood spots or pharmacokinetic samples. LDTD uses an infrared laser to thermally desorb analytes coated onto the metallic surface of specially designed plates (LazWell™) by evaporation of an organic solvent, usually the methanolic final product of an analytical extraction scheme. Each desorption releases neutral gas phase molecules that can be efficiently ionized in combination with a atmospheric pressure chemical ionization interface. The ions produced are then selectively detected by tandem mass spectrometry [14]. The direct nature of LDTD gives rise to analysis times that are typically <30 s per desorption. The laser is applied to the LazWell plates in a power gradient, giving rise to a MS response reminiscent of a chromatographic peak (Fig. 1), any subsequent quantitation can be done through standard peak integration software.

This paper explores the development of the extraction procedure and LDTD-APCI-MS/MS method for the optimized measurement of metformin and sitagliptin concentrations from mouse and human dried blood spots and goes on to assess the stability of the analytes after storage at either ambient temperature as dried blood spots or at +4 °C as extracted dried blood spot samples. A sensitive and robust high throughput LDTD-APCI-MS/MS method for the simultaneous determination of metformin and sitagliptin from mouse and human dried blood spot samples is described, data is supplied for the subsequent application of the method to mouse pharmacokinetic study samples in support of drug discovery. The results obtained by LDTD-APCI-MS/MS are compared to those generated by more traditional HPLC-MS/MS methods.

## 2. Materials and methods

### 2.1. Chemicals, reagents and materials

Metformin hydrochloride and sitagliptin were synthesized at AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Phen-

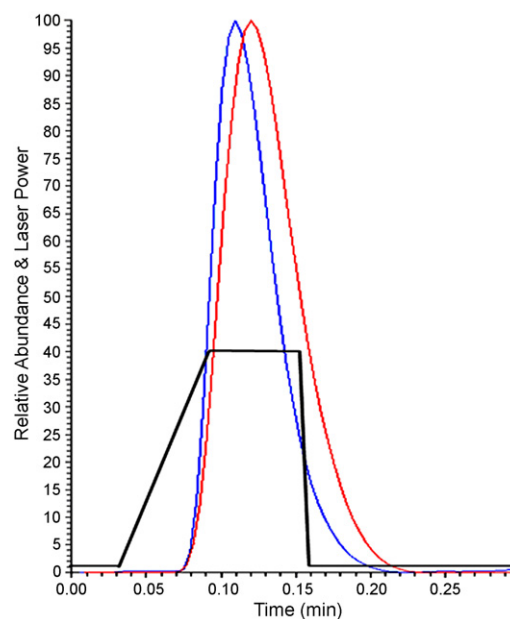


Fig. 1. A typical MS response from an LDTD desorption of metformin (blue) and sitagliptin (red) and the laser power gradient pattern applied to promote desorption (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

formin hydrochloride, the internal standard and ammonium formate was obtained from Sigma-Aldrich (Poole, Dorset, UK). HPLC grade methanol, acetonitrile and formic acid were purchased from Thermo Fisher Scientific (Loughborough, Leicestershire, UK). Control mouse blood was obtained from the AstraZeneca breeding colonies. Control human blood was obtained from Quintiles Drug Research Unit (Guys Hospital, London, UK).

### 2.2. Equipment

FTA DMPK-C blood spot cards were supplied by GE Healthcare UK Limited (Little Chalfont, Bucks, UK). Sample tubes were supplied by Thermo Fisher Scientific (Loughborough, Leicestershire, UK). The centrifuge was a Eppendorf 5415C (Hamburg, Germany). A Harris punch and cutting matt was supplied by GE Healthcare UK Limited (Little Chalfont, Bucks, UK). Gilson pipette man and Gilson Microman pipettes were supplied by Anachem Limited (Luton, Bedfordshire, UK).

### 2.3. Instrumental

The LDTD source was manufactured by Phytronix Technologies (Quebec, QC, Canada). Dried samples are loaded into the LDTD sample manager on specially designed LazWell™ 96-well plates 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 (Arbitrary units), auxiliary gas off, sheath gas off. The carrier gas was compressed air at a flow rate of 3.5 L/min. Laser power was ramped from 0 to 40% over 3 s and held at 40% power for 4 s before shutting off, the data system was allowed to acquire for a further 9 s giving a total desorption time of 18 s per sample.

The LDTD source was mounted on a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer was operated in positive ion selected reaction monitoring (SRM) mode. Metformin was monitored at a parent mass of 130.1 Da and a daughter mass of 71.1 Da with a tube lens voltage of 54 V and a collision energy of 22 V. Sitagliptin was moni-

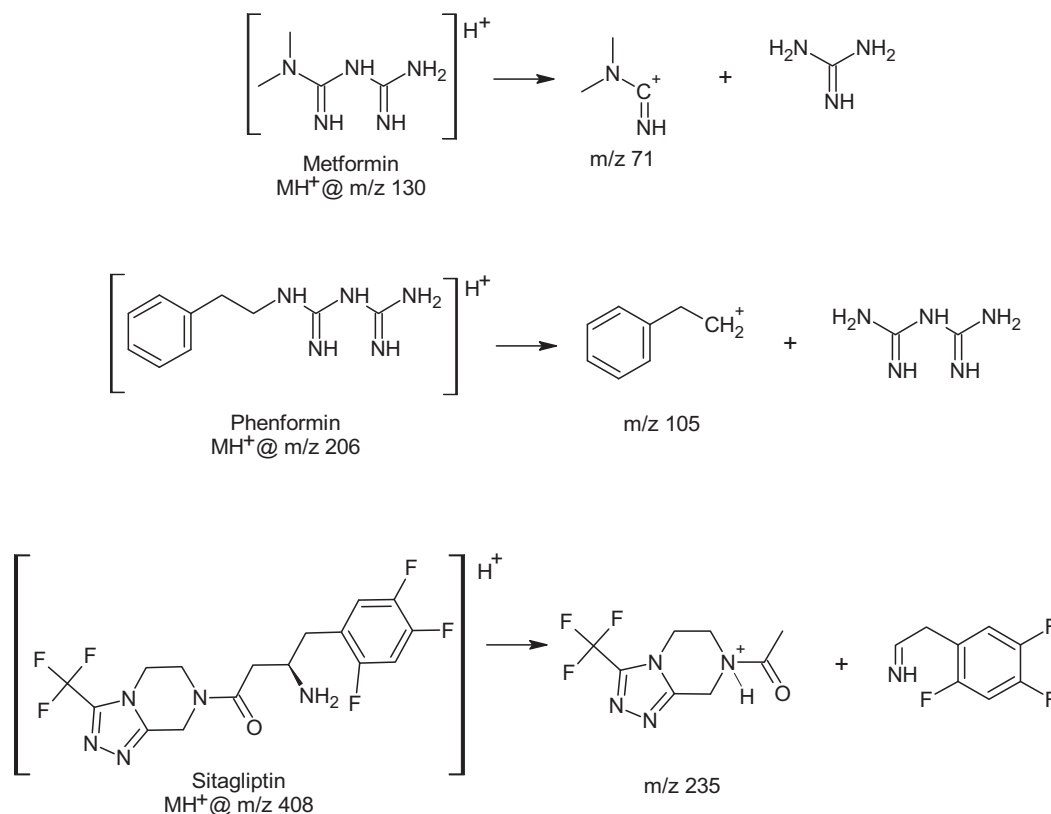


Fig. 2. Structures of metformin, sitagliptin and phenformin and the corresponding fragment ions monitored by MS/MS.

tored at a parent mass of 408.1 Da and a daughter mass of 235.0 Da with a tube lens voltage of 88 V and a collision energy of 17 V. Phenformin, the internal standard, was monitored at a parent mass of 206.2 Da and a daughter mass of 105.1 Da, the tube lens and collision energy were 58 V and 105 V, respectively. Parent molecules and fragments are displayed in Fig. 2. The capillary temperature was set at 270 °C, the collision pressure was set at 1.5 mTorr.

An Accela UHPLC pump (Thermo Fisher Scientific, Hemel Hempstead, UK) and a CTC Analytics HTC PAL autosampler (Presearch Ltd., Basingstoke, UK) were used to introduce samples to the mass spectrometer for the comparison of metformin concentrations by UHPLC–MS. The chromatographic system was comprised of a Kinetix HILIC (100 mm × 2.1 mm ID, 2.6 μm) UHPLC column (Phenomenex, Macclesfield, UK) kept at a constant temperature of 50 °C. The mobile phase consisted of eluent (A) 5 mM ammonium formate in acetonitrile containing 0.1% formic acid and (B) 0.5 mM ammonium formate in water containing 0.1% formic acid. The linear gradient used was (*T* = minutes): at *T* = 0.0, 90%A:10%B at 500 μL/min, *T* = 2.5, 55%A:45%B at 500 μL/min, *T* = 3.5, 2%A:98%B at 500 μL/min, *T* = 4.9, 90%A:10%B at 700 μL/min. An LTQ XL Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA) mass spectrometer operating with an electrospray ionization interface and monitoring positive ions in a mass range of 110–500 Da at 30k resolution (50% valley definition) was used for the UHPLC–MS metformin in mouse blood samples analysis. The measured mass accuracy of the protonated Metformin molecular ion (MH<sup>+</sup>) was within ±1.0 mDa of the theoretical accurate mass of 130.1086 Da throughout all the analyses.

A Surveyor MS Pump Plus HPLC pump (Thermo Fisher Scientific, Hemel Hempstead, UK) and a CTC Analytics HTS PAL autosampler (Presearch Ltd., Basingstoke, UK) were used to introduce the samples to the mass spectrometer for the HPLC comparison of sitagliptin. Chromatography was performed on a Max-RP

(50 mm × 2.1 mm ID, 5 μm) HPLC column (Phenomenex, Macclesfield, UK) with mobile phase consisting of eluent (A) water containing 0.1% formic acid and (B) methanol containing 0.1% formic acid. The linear gradient used was (*T* = minutes): at *T* = 0.0, 95%A:5%B, *T* = 3.0, 5%A:95%B, *T* = 4.0, 5%A:95%B, *T* = 4.1, 95%A:5%B, *T* = 5.0, 95%A:5%B. The flow rate used was 750 μL/min. A TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA, USA) mass spectrometer operating with a heated electrospray ionization interface was used for the HPLC–MS/MS determination of sitagliptin from mouse blood samples. The mass transitions monitored were identical to those used for LDTD–APCI–MS/MS, as were the MS conditions with the exception of sheath gas and auxiliary gas which were set at 80 and 40, respectively (Arbitrary units).

The mass spectrometer software used for data capture was Xcalibur 2.1 (with QuickQuan 2.4) and Xcalibur 2.0 (Thermo Fisher Scientific, San Jose, CA, USA) on the TSQ Quantum Ultra and the LTQ XL Orbitrap, respectively.

#### 2.4. Method development

Compound optimization was performed using the newly developed LDTD auto-optimize function in the QuickQuan 2.4 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 in an incubation chamber thermostatically controlled to a constant 37 °C. Each sample was then systematically desorbed by LDTD into the mass spectrometer, the auto-tune algorithm captured the relevant instrument parameters (tube lens voltage, adjusted parent mass, collision energy and daughter ion) throughout the optimization process.

In order to ensure maximum sensitivity and extraction of metformin and sitagliptin from the DBSs several different spot

sizes, extraction solution mixtures and volumes were compared.

### 2.5. Solutions and standards

Stock solutions of metformin, sitagliptin and phenformin were prepared in methanol to give a final concentration of 1 mg/mL. Subsequent working solutions containing both metformin and sitagliptin for use in calibration curve and quality control construction were prepared by serial dilution of the stock solutions in methanol. Solutions used to prepare quality controls were prepared from a separate weighing of the analytical reference materials. An extraction solvent was prepared using the phenformin stock solution diluted to a concentration of 0.1 µg/mL in methanol/water (80/20 v/v).

Samples for the standard curves and quality controls were prepared by spiking control blood with the appropriate metformin/sitagliptin working solution. The calibration standards were prepared at 5, 10, 50, 100, 500, 1000, 2000 and 5000 ng/mL. Quality controls were prepared at 25, 250 and 2500 ng/mL.

### 2.6. Sample preparation and extraction

FTA DMPK-A and DMPK-B cards were not tested as part of this paper. They contain chemical additives to aide cell lysis and stabilize DNA, the manufacturers claim these additives may interfere with mass spectrometric detection and due to the inherent direct ionization of LDTD–MS/MS the authors deemed the cards unsuitable for this technique.

30 µL of blood was spotted directly onto FTA DMPK-C cards (untreated with additives), this filled the entire sample spot area and ensured that samples did not contaminate each other on the cards; each sample was labeled with any relevant sample information. The cards were then left at ambient temperature for 24 h or heated at 37 °C in an incubation chamber for 2 h prior to extraction. A 6 mm disk was punched from each DBS and was placed in a fresh sample tube, each tube was then extracted using 200 µL of the phenformin extraction solvent, the samples were then mixed for 90 s. The extraction solvent from each sample was directly applied (2 µL per sample) to the LazWell™ plates and allowed to evaporate to dryness in an incubation chamber at 37 °C prior to LDTD–APCI–MS/MS analysis.

### 2.7. Data analysis and method validation

All data was processed using QuickCalc™ (Gubbs Inc., Alpharetta, GA, 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 5 and 5000 ng/mL ( $n = 6$  at each level) were prepared in mouse and human blood. Quality controls were spiked at 25, 250 and 2500 ng/ml ( $n = 6$  at each level) in mouse and human blood 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 ( $n = 6$ ) at each calibration and quality control level with their nominal concentrations. Intra-assay precision was calculated

based on the coefficient of variation of each set of calibration standards and quality controls ( $n = 6$  at each concentration level). The assay was deemed acceptable for the analysis of samples in each species of blood (as a DBS) if the intra-assay accuracy and precision deviated by less than  $\pm 30\%$  (acceptable for discovery bioanalysis).

Inter-assay accuracy was evaluated by comparing the mean measured concentration of each quality control level assayed on six different occasions with their nominal concentrations throughout the validation process. Inter-assay precision was calculated based on the coefficient of variation at each quality control level assayed on six separate occasions. The assay was deemed acceptable for the analysis of samples in each species of blood if the inter-assay accuracy and precision deviated by  $\pm 30\%$ .

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

### 2.9. Stability

The stability of samples as dried blood spots after storage in a desiccated plastic bag at ambient temperature and as extracted DBS samples at +4 °C was assessed over 40 days.

#### 2.9.1. Method application

Metformin and sitagliptin were co-administered orally to two C57Bl/6 strain mice at a dose level of 50 and 5 mg/kg, respectively. The dose formulation was HPMC/Tween (1% w/v polysorbate). Blood samples (30 µL) were taken with a capillary tube via tail prick at 0.25, 0.5, 1, 2, 3, 6, 12 and 24 h post administration, all animals were sampled at each time point. Blood was spotted directly from the capillary onto fresh FTA DMPK-C cards which were subsequently dried in an incubation chamber for 2 h and stored in desiccated plastic bags at ambient temperature.

For the analysis of the mouse pharmacokinetic DBS samples, quality control 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  $\pm 30\%$  of nominal concentration, with the curve constructed from no less than 5 points. The accuracy of at least 75% of the quality controls was within  $\pm 30\%$  of nominal concentration. The linearity of the calibration curve was no less than 0.9801. These acceptance criteria values are deemed acceptable within a drug discovery bioanalytical environment.

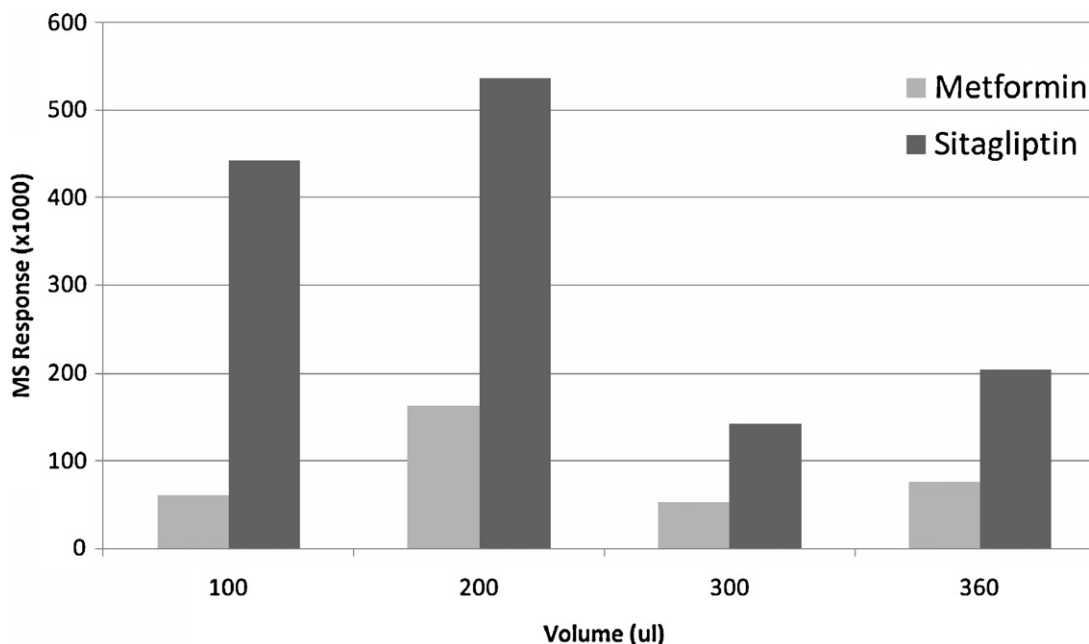
The blood concentration–time results generated for metformin and sitagliptin by LDTD–APCI–MS/MS were compared to results generated by liquid chromatography/mass spectrometry methods (UHPLC–MS for metformin and HPLC–MS/MS for sitagliptin). Analytes were extracted for comparison from whole blood (50 µL) by protein precipitation with 200 µL of acetonitrile containing internal standard and subsequent centrifugation. 50 µL of supernatant was diluted with 300 µL of water prior to analysis by HPLC–MS/MS (50 µL injection). UHPLC–MS analysis comprised of direct injection of 50 µL of the supernatant onto the chromatographic system.

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

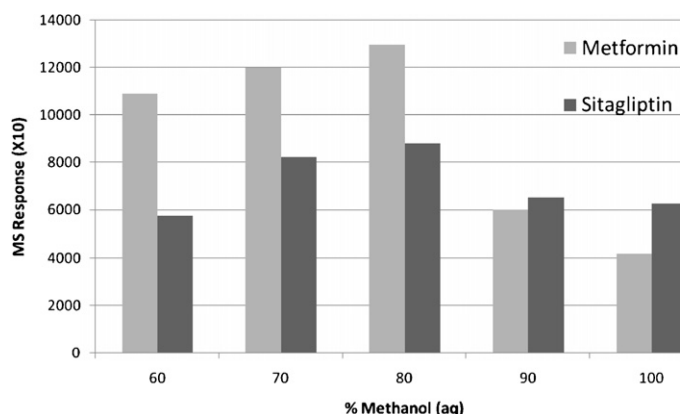
## 3. Results and discussion

### 3.1. Optimization of extraction from DBS samples

Consideration of the dilution factor when extracting samples for analysis by LDTD is important due to the direct nature of the technique. If too much analyte or endogenous material is present ion suppression can occur, this can usually be seen by a drop in the internal standard response at the top of the calibration range. The



**Fig. 3.** The LDTD–APCI–MS/MS responses of a 2000 ng/ml DBS calibration standard containing metformin and sitagliptin after extraction with different volumes of methanol. An extraction volume of 200  $\mu$ l gives the highest area response.



**Fig. 4.** The LDTD–APCI–MS/MS responses of 100 ng/ml DBS calibration standards containing metformin and sitagliptin after extraction with different aqueous methanol mixtures. A 80% methanol/water mixture give the highest area response.

authors have had success using a 12 fold sample to solvent dilution when using plasma samples. DBS samples are different in several ways (i) not all of the sample is removed from the DBS card when a disc is cut out (ii) DBS samples appear visibly cleaner when compared to samples prepared from plasma extraction. DBS samples spiked at 2000 ng/mL in mouse blood were extracted with 100, 200, 300 and 360  $\mu$ L of methanol containing phenformin (0.1  $\mu$ g/mL), this is equivalent to a 3, 6.7, 10 and 12 fold dilution of the blood sample used to form the DBS. The results demonstrated that extraction with a 200  $\mu$ L volume of solvent (or 6.7 fold dilution) gave the greatest metformin and sitagliptin response, see Fig. 3. A calibration curve was run using the 200  $\mu$ L volume; this did not show any ion suppression effects in terms of reduced internal standard response at the higher calibration levels.

Extraction efficiency was further explored using different mixtures of methanol. Mouse DBS calibration curves were extracted using 60, 70, 80, 90 and 100% methanol. Fig. 4 shows the area response given by the 100 ng/mL calibration standard from each curve. The recoveries obtained were relatively close between the different percentages of methanol, although 80% methanol gave the

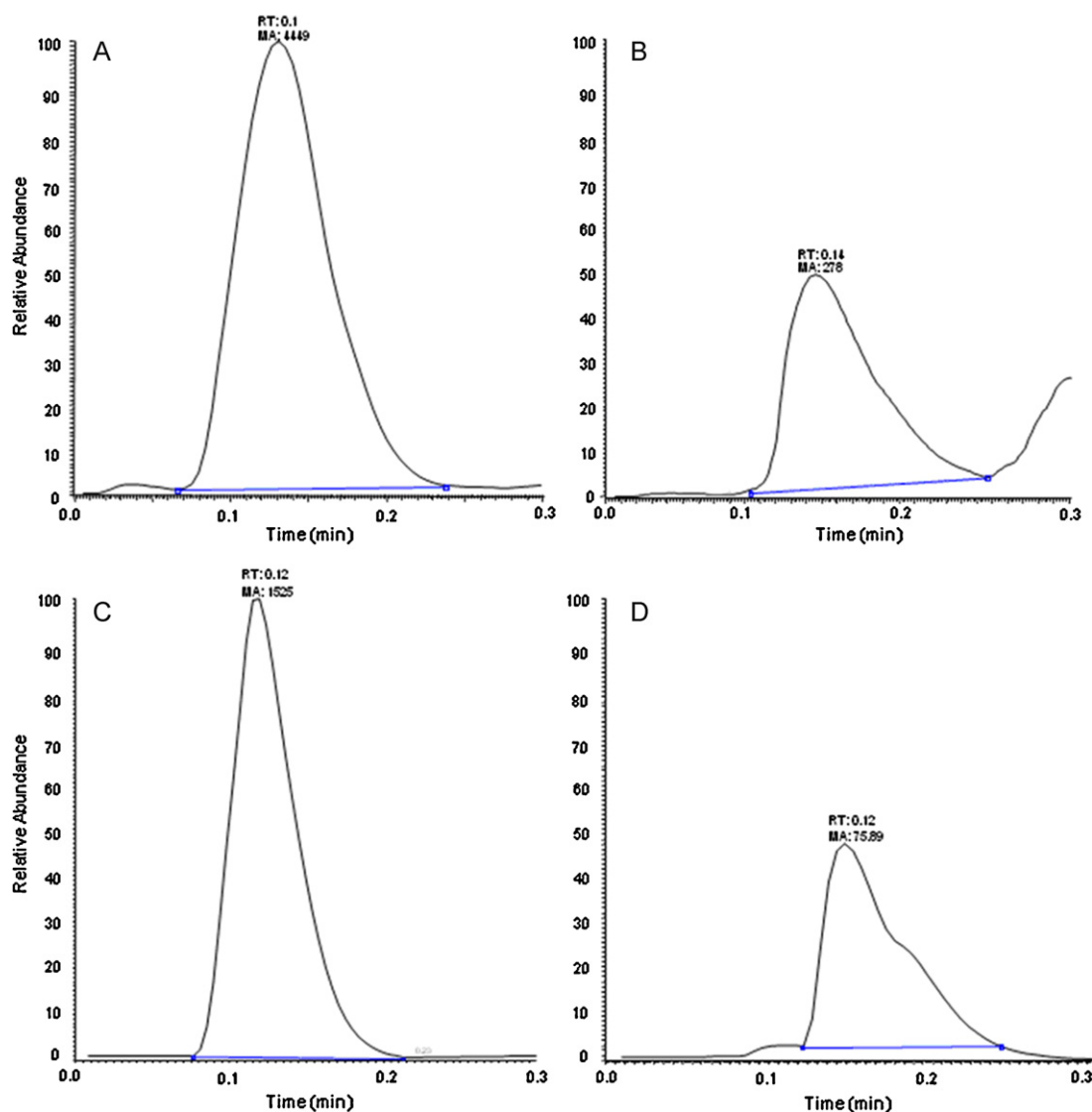
greatest response for both metformin and sitagliptin. This result was not unexpected and could be the result of improved extraction of the compounds or the formation of a more coherent droplet in the centre of the LazWell™ plate when a small amount of aqueous is present in the organic extraction solution. Highly organic solvents spread out across the base of the LazWell™ plates more evenly due to lower surface tension, this effectively dilutes the amount of analyte available for desorption by the laser and leads to lower MS responses.

### 3.2. Specificity

The assay was specific for both metformin and sitagliptin in both mouse and human DBS samples. Fig. 5 shows representative raw data obtained upon desorption of an extracted mouse DBS blank sample compared to desorption of a calibration standard in the same matrix at the LLOQ. A response was observed for both metformin and sitagliptin in the blank DBS sample, although, comparison with the response at the LLOQ shows that the 'blank' response was around 70-fold less for metformin and 20-fold less for sitagliptin. The peaks in the blank DBS sample responses was not caused by the FTA cards and could be due to several factors including the inherent nature of the direct sample introduction process employed by LDTD, cross contamination during the extraction procedure or in the case of metformin it may be attributed to the low mass region that metformin falls into and endogenous background noise from blood.

### 3.3. Accuracy and precision

Both accuracy and precision were acceptable for use in a discovery drug metabolism and pharmacokinetics environment. Intra-assay accuracy and precision (Table 1), expressed as percentage of nominal concentration and coefficient of variation, respectively, was  $\pm 22.4\%$  and 12.7% in mouse DBS samples and  $\pm 11.5\%$  and 13.2% in human DBS samples across the metformin calibration range. Intra-assay accuracy and precision for sitagliptin was  $\pm 21.5\%$  and 27.6% in mouse DBS samples and  $\pm 19.4\%$  and 17.2% in human DBS samples across the calibration range. The lower limit



**Fig. 5.** Typical LDTD-APCI-MS/MS responses for: (A) metformin  $m/z$  130  $\rightarrow$   $m/z$  71 transition in a extracted mouse DBS sample at the LLOQ, (B) metformin  $m/z$  130  $\rightarrow$   $m/z$  71 transition in extracted control mouse DBS sample, (C) sitagliptin  $m/z$  408  $\rightarrow$   $m/z$  235 transition in a mouse DBS sample at the LLOQ and (D) sitagliptin  $m/z$  408  $\rightarrow$   $m/z$  235 transition in a control mouse DBS sample.

**Table 1**

Summary of intra-assay accuracy and precision (%) for metformin and sitagliptin calibration standards in mouse and human dried blood spot (DBS) samples.

	Metformin		Sitagliptin	
	Mouse	Human	Mouse	Human
Cal range (ng/mL)	10–5000	5–5000	5–5000	5–5000
Accuracy (%)	86.1–122.4	88.5–105.9	78.5–117.7	87.7–119.4
Precision (%)	2.6–12.7	1.9–13.2	6.6–27.6	3.9–17.2

of quantitation (LLOQ) for the assays was 10 ng/mL in mouse and 5 ng/mL in human DBS samples for metformin. The corresponding sitagliptin LLOQ was 5 ng/mL for mouse and human DBS samples.

The intra-assay accuracy (and precision) of quality control samples (Table 2) inserted randomly throughout the analytical run ( $n=6$ ) was less than 15% (21.1%) and 5% (12.8%) for metformin in mouse and human DBS samples, respectively. The corresponding accuracy (and precision) of sitagliptin quality controls within the same samples was less than 20% (17.0%) and 25% (10.3%) in mouse and human DBS samples, respectively.

Table 3 summarizes the inter-assay accuracy and precision (in parenthesis) of quality control samples at three concentrations

spanning the calibration range and taken from different analytical batches assayed on different days ( $n=6$ ). Metformin was less than 20% (19.3%) and 16% (18.2%) in mouse and human DBS samples, respectively and sitagliptin was less than 20% (14.6%) and 11% (14.5%) in mouse and human DBS samples, respectively.

### 3.4. Stability

The stability of metformin and sitagliptin in DBS samples is summarized in Table 4. Mouse and human DBS samples containing metformin and sitagliptin at 25, 250 and 2500 ng/ml ( $n=3$ ) were stored long term at ambient temperature in a sealed plastic bag

**Table 2**  
Intra-assay accuracy and precision (%) for metformin and sitagliptin quality controls in mouse and human dried blood spot (DBS) samples.

Concentration	Metformin				Sitagliptin			
	Mouse DBS		Human DBS		Mouse DBS		Human DBS	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
25 ng/mL	93.9	21.1	99.6	12.8	92.8	17	75.9	10.3
250 ng/mL	86.1	8.6	95.6	5.5	82.9	7	99	4
2500 ng/mL	97	3.9	100.4	7.1	108.4	4.6	103.4	4.8

**Table 3**  
Inter-assay accuracy and precision (%) for metformin and sitagliptin quality controls in mouse and human dried blood spot (DBS) samples.

Concentration	Metformin				Sitagliptin			
	Mouse DBS		Human DBS		Mouse DBS		Human DBS	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
25 ng/mL	90.9	19.3	109.1	14.5	107.9	9.3	90.6	12.4
250 ng/mL	83.1	5.3	84.1	13.2	83.4	12.5	89.5	14.5
2500 ng/mL	99.3	5.3	99.5	18.2	96.3	14.6	98.9	14.3

**Table 4**  
Stability of metformin and sitagliptin under various storage conditions as Dried Blood Spots (DBS) on FTA cards or as extracted dried blood spot samples from Mouse (top) and Human samples (bottom).

Samples	Concentration	Metformin			Sitagliptin		
		Mean $\pm$ SD	% CV	% Bias	Mean $\pm$ SD	% CV	% Bias
<b>Mouse DBS samples</b>							
Long term (DBS @ RT 40 days)	25 ng/mL ( $n=3$ )	21.8 $\pm$ 3.7	17.1	-12.8	20.9 $\pm$ 3.5	16.6	-16.5
	250 ng/mL ( $n=3$ )	280.3 $\pm$ 15.3	5.5	12.1	232.8 $\pm$ 20.3	8.7	-6.9
	2500 ng/mL ( $n=3$ )	2436.4 $\pm$ 88.5	3.6	-2.5	2448.6 $\pm$ 102.2	4.2	-2.1
Short term (extracts @ +4 °C 7 days)	25 ng/mL ( $n=3$ )	24.9 $\pm$ 3.8	15.3	-0.5	19.6 $\pm$ 1.1	5.5	-21.5
	250 ng/mL ( $n=3$ )	233.5 $\pm$ 41.6	17.8	-6.6	223.1 $\pm$ 26.4	11.8	-10.8
	2500 ng/mL ( $n=3$ )	2682.6 $\pm$ 54.2	2.0	7.3	2786.3 $\pm$ 162.5	5.8	11.5
Long term (extracts @ +4 °C 40 days)	25 ng/mL ( $n=3$ )	86.6 $\pm$ 14.4	16.6	246.5	27.2 $\pm$ 1.2	4.2	8.8
	250 ng/mL ( $n=3$ )	263.7 $\pm$ 24.5	9.3	5.5	168.9 $\pm$ 10.4	6.2	-32.4
	2500 ng/mL ( $n=3$ )	2139.8 $\pm$ 310.2	14.5	-14.4	1904.2 $\pm$ 90.8	4.8	-23.8
<b>Human DBS samples</b>							
Long term (DBS @ RT 40 days)	25 ng/mL ( $n=3$ )	24.2 $\pm$ 2.7	11.2	-3.3	26.9 $\pm$ 2.4	8.8	7.7
	250 ng/mL ( $n=3$ )	306.0 $\pm$ 36.8	12.0	22.4	296.6 $\pm$ 10.5	3.5	18.6
	2500 ng/mL ( $n=3$ )	2633.4 $\pm$ 63.8	2.4	5.3	2107.0 $\pm$ 129.4	6.1	-15.7
Short term (extracts @ +4 °C 7 days)	25 ng/mL ( $n=3$ )	29.6 $\pm$ 2.4	8.1	18.5	18.7 $\pm$ 1.7	9.3	-25.1
	250 ng/mL ( $n=3$ )	241.3 $\pm$ 7.5	3.1	-3.5	249.0 $\pm$ 18.4	7.4	-0.4
	2500 ng/mL ( $n=3$ )	2888.3 $\pm$ 154.3	5.3	15.5	2835.1 $\pm$ 310.9	11	13.4
Long term (extracts @ +4 °C 40 days)	25 ng/mL ( $n=3$ )	23.3 $\pm$ 5.0	21.3	-6.7	34.7 $\pm$ 7.1	20.4	38.9
	250 ng/mL ( $n=3$ )	323.1 $\pm$ 23.0	7.1	29.3	377.2 $\pm$ 51.4	13.6	50.9
	2500 ng/mL ( $n=3$ )	3311.3 $\pm$ 21.0	0.6	32.5	3957.4 $\pm$ 158.0	4.0	58.3

containing silica gel desiccant prior to extraction and analysis by LDTD-APCI-MS/MS. The % bias of metformin and sitagliptin was less than  $\pm 30\%$  of nominal concentration at all levels, indicating the samples were suitable for storage under these conditions for 40 days.

Stability of metformin and sitagliptin was also assessed after storage of extracted quality control DBS samples ( $n=3$  at each level) at +4 °C in sealed sample tubes both short term and long term. The % bias of metformin and sitagliptin was less than  $\pm 30\%$  of nominal concentration at all levels after 7 days of storage. This indicates short term stability, however, after 40 days of storage the % bias was greater than  $\pm 30\%$  for metformin at 25 and 2500 ng/mL in mouse and human DBS samples, respectively and for sitagliptin at 250 ng/mL in mouse DBS samples and at 25, 250 and 2500 ng/mL in human DBS samples, indicating storage of extracts for this length of time was not possible. The increase in % bias between 7 and 40 days was probably due to the highly organic nature of the extraction solvent evaporating over time.

### 3.5. Mouse pharmacokinetic study analysis

Samples from a oral pharmacokinetic study of metformin, dosed at 50 mg/kg with sitagliptin, dosed at 5 mg/kg Simultaneously to

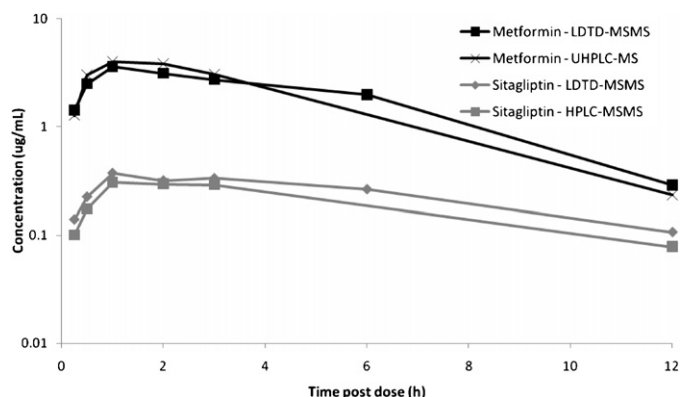
male C57Bl/6 mice were evaluated by LDTD-APCI-MS/MS. The mean blood concentration-time profiles for the analytes are shown in Fig. 6. The pharmacokinetic parameters derived from the analysis are listed in Table 5. The maximum sample concentration ( $C_{max}$ ) was calculated to be  $3.60 \pm 0.34 \mu\text{g/mL}$  and  $0.40 \pm 0.04 \mu\text{g/mL}$  for metformin and sitagliptin, respectively. The time at which the maximum concentration occurs ( $T_{max}$ ) was reached at 1 h post dose for both compounds. The oral half-life of metformin ( $T_{1/2}$ ) was  $4.4 \pm 0.346$  h with an area under the blood concentration-time curve ( $AUC_{0-\infty}$ ) of  $25.6 \mu\text{g h/mL}$ , sitagliptin had an oral half-life of  $3.4 \pm 0.147$  h and an area under the blood concentration-time curve of  $3.6 \pm 0.250 \mu\text{g h/mL}$ .

The DBS concentration-time profiles for metformin and sitagliptin were compared to data from fresh whole blood taken from the same animals as the DBS samples but analysed by conventional liquid chromatography/mass spectrometry (UHPLC-MS for metformin and HPLC-MS/MS for sitagliptin). The % bias between the LDTD-APCI-MS/MS results and those generated by liquid chromatography/mass spectrometry was less than  $\pm 30\%$  at all time points, further validating the LDTD-APCI-MS/MS results. The comparative pharmacokinetic parameters are listed in Table 5 and show good agreement between LDTD-APCI-MS/MS and the more conventional liquid chromatography/mass spectrometry approach.

**Table 5**

Pharmacokinetic parameters of metformin and sitagliptin after a combined single oral dose of 50 and 5 mg/kg, respectively. Metformin and sitagliptin parameters generated by LDTD-MS/MS are compared with those generated in the same sample by UHPLC-MS (metformin) and HPLC-MS/MS (sitagliptin).

	Metformin (50 mg/kg dose)		Sitagliptin (5 mg/kg dose)	
	LDTD-MS/MS	UHPLC-MS	LDTD-MS/MS	HPLC-MS/MS
$C_{max}$ ( $\mu\text{g/ml}$ )	3.6	3.9	0.4	0.3
$T_{max}$ (h)	1	1	1	1
$T_{1/2}$ (h)	4.4	5.1	3.4	3.8
$AUC_{0-\infty}$ ( $\mu\text{g h/mL}$ )	25.6	26.3	3.6	3.0



**Fig. 6.** Blood concentration–time profiles for the simultaneous analysis of metformin dosed at 50 mg/kg and sitagliptin dosed at 5 mg/kg orally to male C57Bl/6 mice and comparative profiles generated by the liquid chromatography/mass spectrometry methods.

The data generated for metformin and sitagliptin from this pharmacokinetic study was subsequently used to predict efficacious doses for a mouse acute efficacy test.

#### 4. Conclusion

A high throughput, sensitive and robust assay is reported for the simultaneous quantitation of metformin and sitagliptin from mouse and human dried blood spot samples by LDTD-APCI-MS/MS. The assay was validated in terms of intra and inter-assay accuracy and precision for use in a pharmaceutical drug discovery environment. Stability of the analytes was established when stored over 40 days as DBS samples on FTA cards or over 7 days as extracted DBS samples. Dried blood spots were used to simplify the ‘sampling’ phase of discovery pharmacokinetic/pharmacodynamic studies by removing the need to harvest plasma and reducing the volume of sample needed for analysis; furthermore a simple and effective generic extraction scheme was demonstrated which reduces analytical sample preparation time.

The use of LDTD reduces analytical run times due to its inherently rapid nature. Combination of the technique with the selectivity of a triple quadrupole mass spectrometer to quantify multiple analytes in a single desorption increases efficiency and utilizes mass spectrometry hardware more effectively compared to conventional liquid chromatography/mass spectrometry methods. LDTD could be combined with modern accurate mass instruments operating in full scan mode, removing the need to optimize compounds for parent and product ion transitions, further increasing efficiency. The technique has great potential within the pharmaceutical industry not only for *in vivo* analysis but also for use as a rapid sample introduction technique for *in vitro* experiments such as absorption and solubility assays which often screen many hundreds of compounds per week.

The potential of the method to be applied to therapeutic drug monitoring of metformin and sitagliptin concentrations in the clinic was explored. The ease of sample handling and rapid turnaround of samples from patient to final result could be a significant step forward in ensuring clinical compliance to prescribed dosing regimens.

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