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Development and validation of a UPLC-MS/MS analytical method for dofetilide in mouse plasma and urine, and its application to pharmacokinetic study

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Highlights

- We first developed a LC/MS-MS analytical method for the quantification of dofetilide in plasma and urine.
- This analytical method is more accurate and precise which requires very small sample volume (10 µL for plasma, and 2 µL for urine)
- We used isotope labeled dofetilide-D4 as an internal standard drug which increases method's stability and accuracy.
- We first reported the pharmacokinetic profile of dofetilide in FVB strain mice administered by oral and intravenous route.

Abstract

A novel method using UPLC with tandem mass-spectrometric detection (UPLC-MS/MS) with positive electrospray ionization was developed for the detection of the antiarrhythmic drug, dofetilide, in mouse plasma and urine. Protein precipitation was performed on 10 µL of plasma and 2 µL of urine samples using dofetilide-D4 as an internal standard, and separation of the analyte was accomplished on a C18 analytical column with the flow of 0.40 mL/min. Subsequently, the method was successfully applied to determine the pharmacokinetic parameters of dofetilide following oral and intravenous administration. The calibration curve was linear over the selected concentration range ($R^2 \geq 0.99$), with a lower limit of quantitation of 5 ng/mL. The intra-day and inter-day precisions, and accuracies obtained from a 5-day validation ranged from 3.00-7.10%, 3.80-7.20%, and 93.0-106% for plasma, and 3.50-9.00%, 3.70-10.0%, 87.0-106% for urine, while the recovery of dofetilide was 93.7% and 97.4% in plasma and urine, respectively. The observed pharmacokinetic profiles revealed that absorption is the rate-limiting

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step in dofetilide distribution and elimination. Pharmacokinetic studies illustrate that the absolute bioavailability of dofetilide in the FVB strain mice is 34.5%. The current developed method allows for accurate and precise quantification of dofetilide in micro-volumes of plasma and urine, and was found to be suitable for supporting *in vivo* pharmacokinetic studies.

Keywords: Dofetilide, UPLC-MS/MS, pharmacokinetics, mouse plasma, mouse urine

1. Introduction

Dofetilide is a class III antiarrhythmic drug that inhibits the delayed rectifier potassium channel and is used to treat the recurrence of atrial fibrillation [1]. This agent does not cause any negative inotropic, dromotropic, and chronotropic effects observed with other commonly prescribed antiarrhythmic drugs, such as amiodarone, dronedarone, or sotalol [2]. However, the clinical use of dofetilide is associated with extensive inter-individual pharmacokinetic variability, in which mechanisms of variability remains to be elucidated. Furthermore, dofetilide has a narrow therapeutic window, and elevated plasma concentrations have been associated with QTc prolongation changes and increasing the risk of *torsade de pointes*, a potentially lethal cardiac arrhythmia [3]. Dofetilide undergoes extensive renal tubular secretion [4], and the initial dofetilide dose is individualized based on an individual's creatinine clearance. Taken together, the initiation of dofetilide therapy requires inpatient hospice monitoring due to the variable response, and the potential risk of proarrhythmia. Despite the presence of its narrow therapeutic index, mechanistic

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details of dofetilide elimination and the underlying causes of recurrent or drug-induced arrhythmias remain poorly understood [5]. Prior studies have suggested that certain drug transporters play an important role in mediating drug-drug interactions through inhibition of renal tubular secretion of dofetilide and thus, elevating drug levels in systemic circulation that may exacerbate adverse events [6, 7].

In parallel of a project to define the renal tubular secretion mechanisms involved in the elimination of dofetilide, we developed a new analytical method for dofetilide that is adaptable to micro-volumes of plasma and urine obtained through serial sampling in individual mice. In order to avoid the use of antisera required in previously used radioimmunoassays [8, 9, 10] and improve on the specificity of methods based on HPLC with fluorescence detection [11], our method utilizes UPLC technology with tandem mass-spectrometric detection (UPLC-MS/MS). The developed method was found to provide enhanced speed, resolution, and accuracy, and was successfully applied to a pharmacokinetic application of dofetilide in mice.

2. Materials and methods

2.1. Chemical and reagents

Dofetilide was purchased from Selleck Chemicals (100% purity, Houston, TX, USA). An internal standard of dofetilide (dofetilide-D4) was purchased from Toronto Research Chemicals Inc. (>99% purity, North York, ON, Canada). HPLC-grade acetonitrile, methanol, and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Blank plasma and urine samples were obtained from untreated inbred wild-type mice on an FVB strain (Taconic Biosciences, Cambridge City, IN, USA).

2.2. UPLC-MS/MS conditions

The analysis was performed on a Vanquish UHPLC system, a TSQ Quantiva triple quadrupole mass spectrometer from Thermo Fisher Scientific, and Thermo Trace Finder General

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Quan system software (version 3.3). Chromatographic separation was accomplished on an Accucore Vanquish C18 column (2.1 mm x 100 mm, dp = 1.5 µm, Thermo Fisher Scientific) shielded by an XBridge®BEH C18 guard column (dp = 5 µm) by injecting sample volumes of 5 µL. The temperature of the auto-sampler rack and the column was maintained at 4°C and 40°C, respectively. The mobile phase A contained water with 0.1% (v/v) formic acid; whereas mobile phase B contained a mixture of acetonitrile and methanol (1:3) with 0.1% (v/v) formic acid. Gradient elution (5 min) was as follows: 0 - 0.5 min, 10% of mobile phase B; 0.5 - 3.0 min, 95% mobile phase B; 3.0 - 4.0 min, 95% mobile phase B; 4.0 - 4.1 min, 10% mobile phase B; and 4.1 - 5.0 min, 10% mobile phase B. The flow rate was maintained at 0.4 mL/ min. The positive voltage applied to the ESI capillary in mass spectrometer assay was 3500 V, and the capillary temperature was 342°C with a vaporizer temperature of 358°C. The collision gas argon was used at a pressure of 1.5 mTorr. The precursor molecular ions, as well as product ions for confirmation and detection, are shown in **Table 1**.

2.3. Working solution, calibration and quality control samples

Stock solutions of dofetilide were prepared by dissolving 10 mg of dofetilide with 10 mL methanol, and working solutions were prepared by diluting the stock with methanol. The calibration standard concentrations ranged from 5 to 1000 ng/mL. The lower limit of quantitation (LLOQ), low (QC 1), medium (QC 2), and high-quality controls (QC 3) were prepared at dofetilide concentrations of 5, 25, 75, and 750 ng/mL, respectively. A stock solution of the internal standard dofetilide-D4 was prepared at a concentration of 100 ng/mL in a mixture of acetonitrile: water (3:1, v/v). All the solutions were stored at -20°C and brought to room temperature immediately prior to use.

2.4. Method validation

2.4.1. Calibration curve, accuracy, and precision

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The method validation was carried out according to recommendations outlined in the United States Food and Drug Administration (FDA), and the European Medicines Agency (EMA) guidelines [12, 13]. To evaluate the linearity of the dofetilide concentration-response relationships, a regression model was used by plotting data from the calibration curves as measured by the peak area ratios of analytes to IS. In the calibration curves, seven concentrations (5, 10, 50, 100, 250, 500, and 1000 ng/mL) were measured, and weighting of $1/area^2$ was used in every case. The coefficient of variance (CV) for each concentration was <15%, and <20% for the LLOQ. The intra-day precision, inter-day precision, and accuracy of LLOQ and QC samples were measured in quintuplicate at each concentration on 5 different days [14, 15].

2.4.2. Selectivity, dilution, and recovery

Plasma samples from 6 different untreated mice were used to confirm selectivity, to evaluate the accuracy of sample dilution, plasma containing dofetilide at a concentration of 500 ng/mL was diluted 1:10 (v/v) in blank mouse plasma, and results within ±15% of nominal values were considered sufficiently accurate. The extraction recoveries of dofetilide were calculated by comparing the peak area of QC samples with those of reference QC samples in blank plasma. The matrix effects were measured by continuous post-column infusion of the analytes in plasma from untreated mice [15, 16].

2.4.3. Freeze-thaw, bench, short-term stability

The influence of freeze-thawing cycles from -80°C to room temperature on the stability of analytes was measured by analyzing plasma QC samples at low, medium, and high concentrations during three consecutive cycles. The bench stability was evaluated by leaving the QC samples in the autosampler at 4°C for a period of 24 hours, followed by re-analysis. Short-term stability was assessed for the QC samples at room temperature for 8 h.

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2.5. *In vivo* pharmacokinetics studies

2.5.1. Animal studies

In vivo experiments were performed in male mice from an inbred FVB strain (25-30 g) aged 8-10 weeks. All studies were approved by the University Laboratory Animal Resources (ULAR) Animal Care and Use Committee at The Ohio State University (2015A00000101-R1). Animals had free access to a standard diet and water, and were housed in a temperature- and light-controlled environment. Prior to drug administration, dofetilide was dissolved in a mixture of a sterile saline and HCl solution (399:1) with the pH adjusted to 7.4. Mice were randomly divided into two experimental groups of equal size (n=5), and received dofetilide by oral gavage at the dose of 5 mg/kg or intravenously at a dose of 2.5 mg/kg.

For pharmacokinetic studies, whole blood samples of 30 µL were collected in heparinized capillary tubes from each mouse at 5 min, 15 min, 30 min, 1 h, 3 h, and 6 h post administration of dofetilide. The samples were centrifuged at 11,000 rpm for 5 min, and the plasma (supernatant) was placed immediately on dry ice and then stored at -80°C. Samples collected between 5 and 30 min were obtained from the submandibular vein using a 5 mm Goldenrod™ sterile animal lancet. For samples at 1 h and 3 h, mice were anesthetized under 2% isoflurane and whole blood was taken from the retro-orbital venous plexus using heparinized capillary tubes. A final sample was obtained at 6 h by cardiac puncture using a syringe and needle. This protocol was followed and adapted according to Leblanc, *et al* (2018) [17].

In case of metabolic studies, a group FBV male wild-type mice (n=5) were placed in Nalgene single mouse metabolic cages 3-days prior the intravenous administration of dofetilide at a dose of 2.5 mg/kg. Animals had free access to a standard diet and water, and were housed in a temperature- and light-controlled environment. Urine samples were collected in sterile 1.5 ml Eppendorf tubes at 8 h, 24 h, 48 h, and 72 h post administration of dofetilide and stored at -80°C.

2.5.2. Sample preparation

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Prior to analysis, frozen plasma and urine samples were thawed at room temperature. Dofetilide was extracted by protein precipitation; in brief, an aliquot of 10 µL of plasma was transferred into a 0.5 mL Eppendorf tube, followed by the addition of 20 µL of methanol, and mixed with 50 µL of the internal standard, dofetilide-D4, at a concentration of 100 ng/mL in acetonitrile for 5 min. The tubes were then centrifuged at 13,000 rpm for 5 min at 4°C. Supernatants of the mixture (60 µL) and Milli-Q water (25 µL) were added to autosampler vials and vortex-mixed. Subsequently, 5 µL of the above mixture was injected into the UPLC-MS/MS system for drug quantification.

Since dofetilide is extensively eliminated through the kidneys, the accumulation in the urine samples was highly concentrated. Thus, in case of preparing urine samples, a similar procedure was followed where 2 µL of urine (versus 10 µL of plasma) were used and samples were further diluted (1:5) in 8 µL of Milli-Q water. Despite the micro-volume of urine, dofetilide was still detectable within the linear concentration range.

2.5.3. Pharmacokinetic data analysis

A non-compartmental analysis using Phoenix WinNonlin® version 8.0 (Certara, USA) was used to determine the pharmacokinetic parameters of dofetilide in mice receiving an oral and intravenous dose. From the slope of the log plasma concentration vs time curve, the elimination rate constant (K_{el}) was calculated following the least squares method. The terminal half-life ($T_{1/2}$) was calculated by $0.693/K_{el}$. The linear trapezoidal rule was followed to estimate the area under the plasma concentration-time curve (AUC). Peak plasma concentration (C_{max}), and time to reach peak plasma concentration (T_{max}) were determined by visual inspection of the data from the log concentration vs time curve. The absolute bioavailability (F_{abs}) was calculated by $F_{abs} = 100 \times (AUC_{po} \times D_{iv}) / (AUC_{iv} \times D_{po})$, where D_{iv} and D_{po} are the absolute doses after intravenous and oral administration of dofetilide, respectively.

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Urinary excretion of dofetilide was determined based on the concentration in diluted urine measured by UPLC-MS/MS at each individual time points, collected urine volume, normalized to body weight, and expressed as a percentage to the total amount of dofetilide administered per animal.

3. Results

3.1. UPLC-MS/MS spectrometry and specificity

Mass-spectra results of dofetilide and dofetilide-D4 generated as protonated molecular ions $[M + H]^+$ are shown in **Table 1** and **Fig 1.** To obtain the highest intensity of fragmentation, mass-spectra parameters were optimized, and the collision-induced dissociation energy was enhanced. Both dofetilide and dofetilide-D4 showed a retention time of 2.16 min under optimal separation conditions. Representative chromatograms for untreated, treated, and spiked plasma and urine samples are shown in **Fig 2 (A-D)**. The lack of interference in the blank plasma and urine samples supports the specificity of the method.

3.2. Linearity, accuracy, precision, and selectivity

Dofetilide calibration curves were demonstrated to be linear in the concentration range of 5 to 1000 ng/mL ($R^2 \geq 0.99$). The signal to noise ratios for dofetilide at 5 ng/mL was >1000-fold in blank plasma and urine samples. Calibration curves of dofetilide in plasma and urine, assessed by performing back-calculated concentrations, showed <15% deviation from nominal values at all concentrations, and the deviation was <20% for the LLOQ. The results from a 5-day validation study are presented in **Table 2.** Intra- and inter-day accuracy values of dofetilide in plasma and urine ranged 93.0-106%, and 87.0-106%, respectively. The intra- and inter-assay precision values for the LLOQ, QCs, and diluted QC samples were 4.30-5.00%, 3.00-7.20%, and 3.80-5.80% in plasma, and 7.60-10.5%, 3.50-10.0%, 3.50-3.60% in urine, respectively. Selectivity was

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evaluated by analyzing plasma samples from 6 different untreated mice. The interfering peak areas were less than 10% for the LLOQ analyte in plasma.

3.3. Recovery, matrix effect, and stability

The recovery and matrix effect of dofetilide in plasma and urine were measured at 25, 75, and 750 ng/mL. Both dofetilide in plasma and urine QCs samples showed >90% recovery. By comparing the response signal with those of standard solutions, matrix effects of dofetilide in plasma and urine were determined (**Table 3**). However, in the post-column infusion process, no matrix effects were observed. The stability data obtained during method validation are shown in **Table 4** and demonstrate that the exposure of plasma or urine samples to room temperature for a period of up to 8 hours is not associated with significant degradation of dofetilide (<15% deviation from nominal concentrations). Plasma and urine samples in the autosampler were also found to be stable at 4°C for up to 24 h.

3.4. Pharmacokinetic studies

The developed analytical method was subsequently applied to establish the concentration-time profile of dofetilide. The observed mean plasma concentration-time curves after oral (5 mg/kg) and intravenous (2.5 mg/kg) administration of dofetilide are shown in **Fig 3**, and pharmacokinetic parameters derived from a non-compartmental analysis are shown in **Table 5**. The concentration-time profiles associated with oral administration of dofetilide exhibit typical flip-flop kinetics, indicating that dofetilide distribution and elimination is rate-limited by absorption. The analysis of dofetilide excretion in urine by administering a single-dose (2.5 mg/kg; i.v.) is shown in **Fig 4**.

4. Discussion

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The elimination of dofetilide is predominantly mediated by glomerular filtration and tubular secretion, yet the mechanism by which this occurs remains poorly understood. Since clinical drug interactions with dofetilide pose increased risk for patients developing proarrthymia, drug-drug interaction studies are further warranted to fully establish patient covariates such as renal transport function, genetic polymorphisms, and concomitant use of other medications which negatively impact the narrow therapeutic window of dofetilide. Such studies require analyzing plasma and urine samples in rodent animals by administering single and multiple doses of dofetilide along with other contraindicated drugs. Therefore, to elucidate the mechanism(s) contributing to pharmacokinetic variability, a rapid and accurate analytical method was developed to quantify dofetilide concentrations in both mice plasma and urine.

Several analytical methods were previously developed to measure plasma concentrations of dofetilide in different animal species using radioimmunoassays [8, 9], and HPLC with fluorescence detection technique [11]. However, in spite of having lower LOQ of 1.7 ng/mL and 2.5 ng/mL for dofetilide quantification by Kaddar et. al. 2013 [11] and Walker et. al. 1991 [8], respectively, the drawbacks of these techniques were limited to using high plasma (400 µL) in guinea pig [11], and urine volume (100 µL) [8, 9]. Both of these analytical methods [11, 9] require high sample retention time of 43 and 44 min, respectively. In addition, some analytical method also requires the usage of radioactive biohazard conducting radioimmunoassays that involve in using the selective antibody for each analyte to be measured [8, 9]. As a result, analyzing plasma and urine samples could be expensive, and likely to cause cross-reactivity among antibodies which could lower the specificity of an assay.

In contrast, our method uses UPLC technology with tandem mass-spectrometric detection (UPLC-MS/MS) for the first time which requires less run time (5 min), provides accurate and cost-effective analysis of dofetilide using very low sample volume (2-10 µL). This method also reduces

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the usage of radioactive chemicals and immunological reagents in analyzing plasma and urine samples.

In this analytical method, chromatographic separation of dofetilide was performed in a gradient elution mode having water with 0.1% (v/v) formic acid as a mobile phase A, and a mixture of acetonitrile and methanol (1:3) with 0.1% (v/v) formic acid as a mobile phase B. A mixture of acetonitrile-methanol (3:1, v/v) was used for an effective protein precipitation in plasma which illustrates a high linearity, intra-day, and inter-day within the concentration range of 5-1000 ng/mL. This method also exhibits a good recovery (>90%), intra-day and inter-day accuracy, and precision in both plasma and urine.

Pharmacokinetic analysis of dofetilide (5 mg/kg, oral; 2.5 mg/kg, intravenous) demonstrated that absorption is the rate-limiting step in dofetilide distribution and elimination. This phenomenon is also known as flip-flop kinetics, an exception to the usual case ($k_{el} > k_a$), in which rate of elimination (k_{el}) is greater than the rate of absorption (k_a) [18, 19]; thus, an increase in oral half-life (1.46 ± 0.50 h) compared to intravenous half-life (0.75 ± 0.40 h) was observed. Our pharmacokinetic studies illustrate that the absolute bioavailability of dofetilide is 34.5% in the FVB strain mice. Furthermore, the 72-hour urinary excretion profile shows that 25.3% of dofetilide (2.5 mg/kg) dose is eliminated through urine post intravenous administration.

5. Conclusion

A sensitive UPLC-MS/MS method was developed and validated for the quantification of dofetilide in plasma and urine from mice. The analysis requires micro-volume aliquots of 2-10 µL, yet retains adequate sensitivity (LLOQ, 5 ng/mL), resolution, and speed (run time, 5 min). The developed method was also found to be sufficiently accurate and precise, independent of the matrix, to support pharmacokinetic studies in mice undergoing serial sampling. The results from preclinical pharmacokinetics study illustrate that absolute bioavailability of dofetilide is 34.5% FVB

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strain mice. The method is currently being implemented in studies that focus on the role of renal tubular solute carriers in the urinary excretion of dofetilide as a critical determinant of drug clearance. This analytical method is also being applied to investigate the transporter-mediated drug-drug interactions and evaluate the changes in pharmacokinetic parameters using contraindicated drugs of dofetilide such as cimetidine, verapamil, and ketoconazole.

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Figure Legends

Fig. 1. MS/MS chromatograms and proposed fragmentation pathways of dofetilide.

Fig. 2. Representative chromatograms spiked with (A) a concentration of 5 ng/mL dofetilide in plasma, (B) blank plasma, (C) a concentration of 5 ng/mL dofetilide in urine, (D) blank urine, (E) a concentration of 100 ng/mL dofetilide-D4 in plasma, (F) a concentration of 100 ng/mL dofetilide-D4 in urine, (G) a dofetilide plasma sample in 5 min after oral administration, (H) a dofetilide urine samples in 24 h after oral administration.

Fig. 3. Mean plasma concentration-time profiles of dofetilide after an oral (5 mg/kg) and intravenous (2.5 mg/kg) administration in FVB male mice (mean ± SEM, n=5).

Fig. 4. Urinary excretion-time profile of dofetilide after an intravenous (2.5 mg/kg) administration in FVB male mice (mean ± SEM, n=5).

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Figure 1

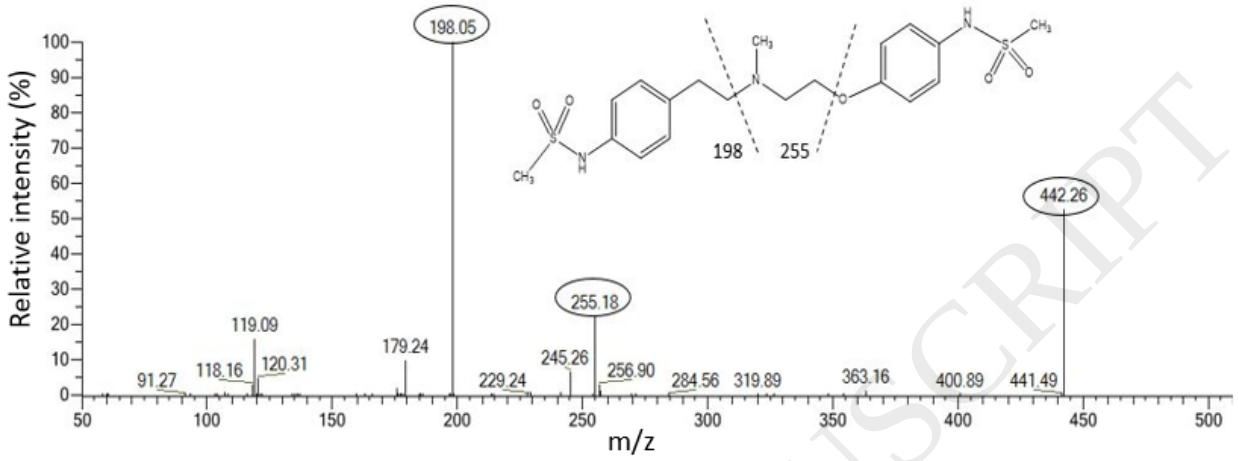

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Figure 2

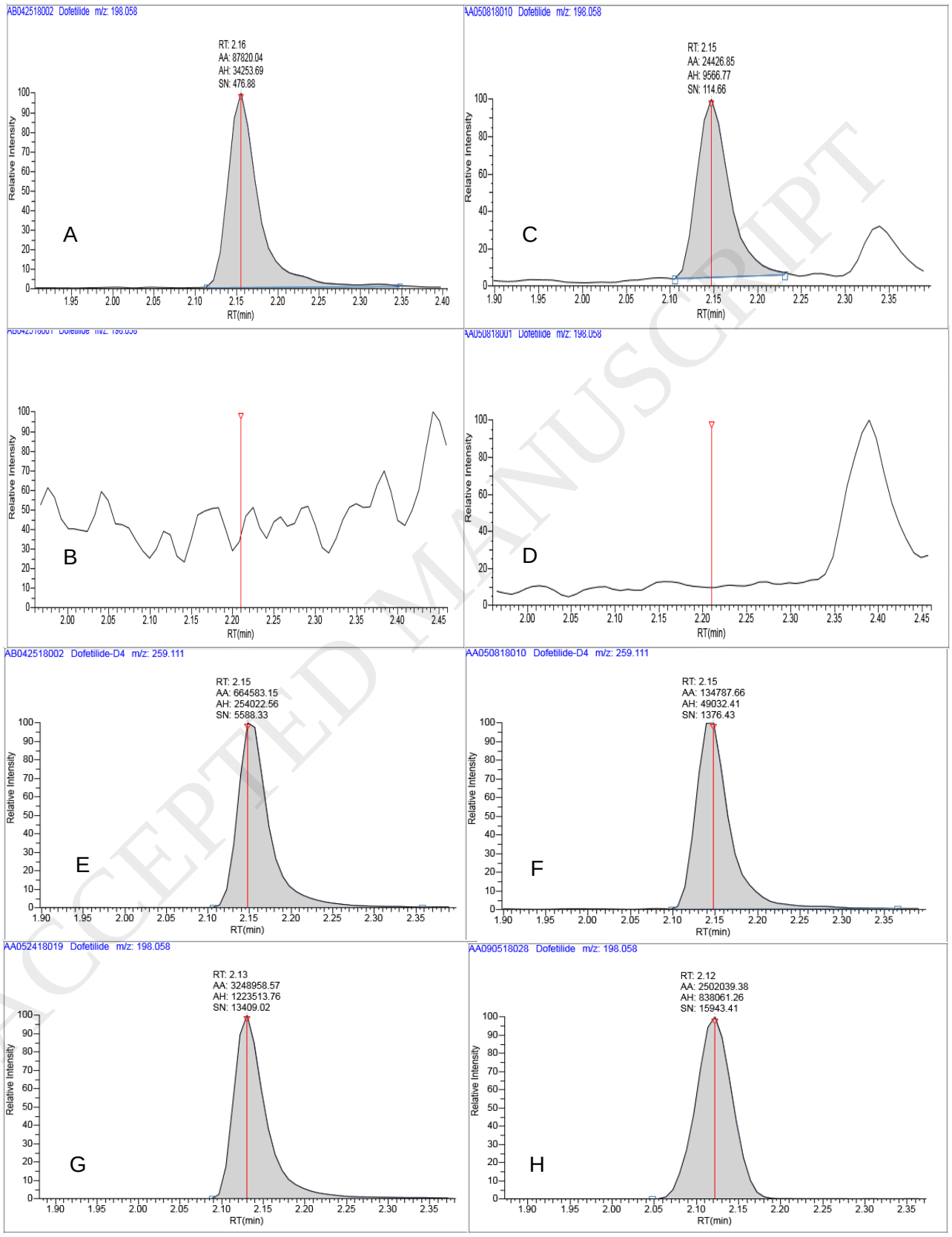

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Figure 3

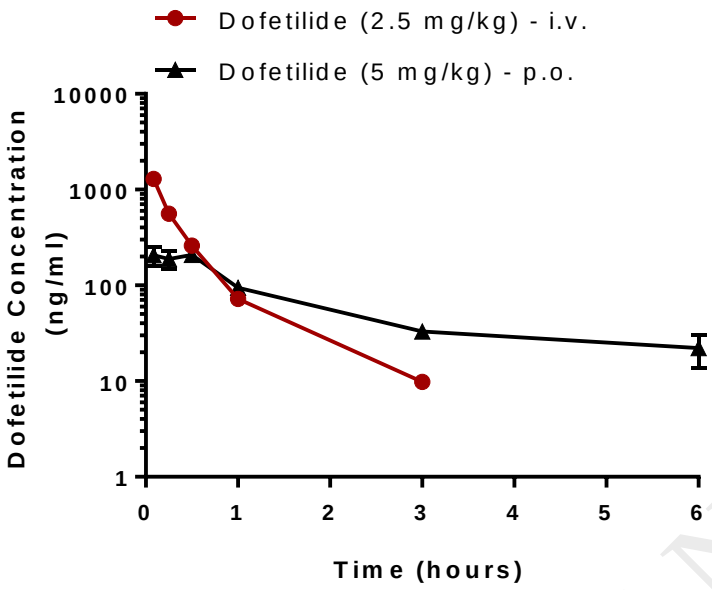

Figure 4

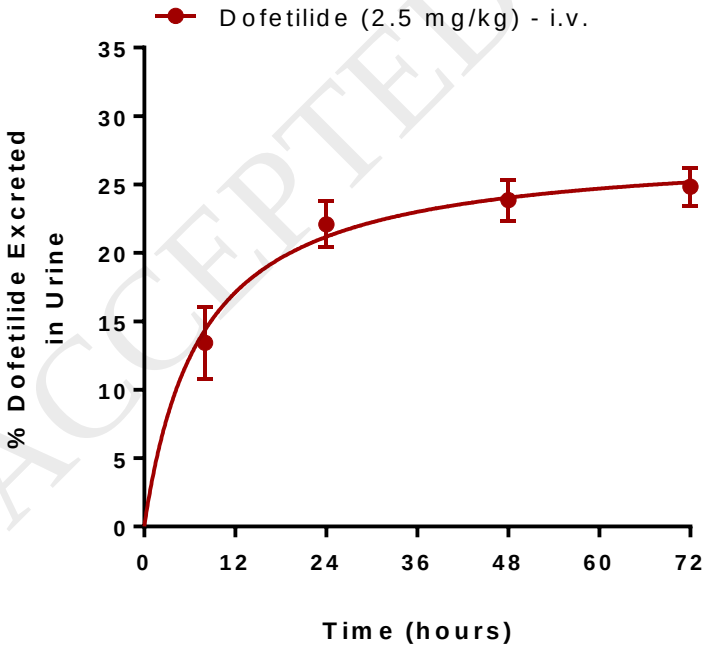

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Table

Table 1: Precursor molecular/product ion for quantification, confirmation and detection parameters for each analyte.

Analyte	Polarity	Precursor (m/z)	Product (m/z)	Confirmation (m/z)	Collision Energy (V)	RF Lens (V)
Dofetilide	Positive	442	198	255	26.4	86.0
Dofetilide- D4	Positive	446	259	N/A	21.4	88.0

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Table 2: Assay performance data for the quantitation of dofetilide in mouse plasma and urine (n=5).

	Concentration (ng/ml)	Intra-assay (%)	Inter-assay (%)	Accuracy (%)
In plasma				
LLOQ	5.00	4.30	5.00	94.0
QC 1	25.0	3.00	3.80	93.0
QC 2	75.0	7.10	7.20	103
QC 3	750	5.00	4.90	106
Dilution (1:10)	500	3.80	5.80	108
In urine				
LLOQ	5.00	7.60	10.5	107
QC 1	25.0	3.60	3.70	87.0
QC 2	75.0	3.50	4.50	105
QC 3	750	9.00	10.0	106
Dilution (1:10)	500	3.60	3.50	108

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Table 3: Recovery and matrix effect of dofetilide and its metabolites in mouse plasma (n=5).

Concentration (ng/mL)	Dofetilide in plasma			Dofetilide in urine	
	Absolute Recovery (%)	Matrix Effect (%)		Absolute Recovery (%)	Matrix Effect (%)
25	93.0 ± 3.00	-0.30 ± 9.00		93.0 ± 17.0	3.00 ± 10.0
75	91.0 ± 5.00	-1.44 ± 5.00		98.0 ± 12.0	1.40 ± 4.00
750	97.0 ± 9.00	-0.06 ± 15.0		110 ± 5.00	-3.20 ± 2.00

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Table 4: Short-term stability of dofetilide and its metabolites in mouse plasma and urine (n=5).

Concentration (ng/ml)	Dofetilide in plasma			Dofetilide in urine	
	CV (%)	Accuracy (%)		CV (%)	Accuracy (%)
25	3.40	106		6.00	107
75	1.50	113		3.60	112
750	1.40	111		0.90	114

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Table 5: Pharmacokinetics parameters of dofetilide oral and IV administration in mice (n=5).

Parameter	Dofetilide (p.o., 5 mg/kg)		Dofetilide (i.v., 2.5 mg/kg)
C_{max} (ng/mL)	245 ± 74.0		1300 ± 400
$T_{1/2}$ (h)	1.46 ± 0.50		0.75 ± 0.40
AUC_{last} (ng*h/mL)	329 ± 41.0		475 ± 120
T_{max} (h)	0.29 ± 0.20		
F (%)	34.5		

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