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Development of LC-MS/MS method for the determination of dapiprazole on dried blood spots and urine: application to pharmacokinetics

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ABSTRACT: A rapid and highly sensitive liquid chromatography–tandem mass spectrometric (LC-MS/MS) method for determination of dapiprazole on rat dried blood spots and urine was developed and validated. The chromatographic separation was achieved on a reverse-phase C_{18} column (250 × 4.6 mm i.d., 5 μm), using 20 mM ammonium acetate (pH adjusted to 4.0 with acetic acid) and acetonitrile (80:20, v/v) as a mobile phase at 25 °C. LC-MS detection was performed with selective ion monitoring using target ions at *m/z* 326 and *m/z* 306 for dapiprazole and mepiprazole used as internal standard, respectively. The calibration curve showed a good linearity in the concentration range of 1–3000 ng/mL. The effect of hematocrit on extraction of dapiprazole from DBS was evaluated. The mean recoveries of dapiprazole from DBS and urine were 93.88 and 90.29% respectively. The intra- and inter-day precisions were <4.19% in DBS as well as urine. The limits of detection and quantification were 0.30 and 1.10 ng/mL in DBS and 0.45 and 1.50 ng/mL in urine samples, respectively. The method was validated as per US Food and Drug Administration guidelines and successfully applied to a pharmacokinetic study of dapiprazole in rats. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: dapiprazole; dried blood spots; rat urine; LC-MS/MS; pharmacokinetics

Introduction

Dapiprazole (DPZ) is (3-{2-[4-(2-methylphenyl)piperazin-1-yl] ethyl}-5,6,7,8 tetrahydro-[1,2,4]triazolo[4,5-a]pyridine) a potent α-adrenergic blocking drug (Mastropasqua *et al*., 1996a, Nencini *et al*., 1992; Eltze, 1997; Fig. 1A). It is a white, crystalline powder that is freely soluble in methanol and water, but partially soluble in acetonitrile. It melts about at 162 °C and it was reported to be stable at ambient temperature (Ramesh and Nageswara Rao, 2013). DPZ is used in topical eye therapy for the treatment of chronic simple glaucoma, for induction of pre-operative mitosis and for reversion of pharmacologically induced mydriasis (Allinson *et al*., 1990; Hogan *et al*., 1997; Tammy, 1996; Mastropasqua *et al*., 1996b). DPZ causes the pupil of the eye to constrict. It reverses pupil dilation caused by other drugs given during an eye examination (Marx-Gross *et al*., 2005). The drug is also endowed with a unique psychopharmacological profile in mice and rats (Monti *et al*., 1995; Valeri *et al*., 1989; Lisciani *et al*., 1982). It inhibits amphetamine toxicity and alcohol and morphine withdrawal syndromes, it produces sedation, blocks conditioned avoidance reflexes and reduces the response to noxious stimuli. Human tests have proved the efficacy of DPZ in psychotic conditions, such as the withdrawal syndrome from opiods (Valeri *et al*., 1986; Bianchi and Segre, 1990; Canovetti *et al*., 2009). Mepiprazole (MPZ) {1-(3-chlorophenyl)-4-[2-(5-methyl-1H-pyrazol-3-yl)ethyl]piperazine; Fig. 1B} was used as internal standard (IS) for determination of DPZ on rat dried blood spot and urine samples.

A thorough literature search revealed that only a few analytical methods for determination of DPZ in bulk drug preparations (Jaya Prasanthi and Syama Sundar, 2012) and stability-indicating HPLC method (Ramesh and Nageswara Rao, 2013) have been reported. However, the bioanalytical assays require a relatively large blood volume (typically >0.5 mL) to generate a sufficient plasma volume for analysis. This requirement considerably reduces their suitability for pediatric pharmacokinetic studies, particularly in premature newborns, where ethical considerations prohibit large-volume sampling. Dried blood spots (DBS) introduced by Guthrie and Susi (1963) was an innovative blood sampling technique involving the collection of small volumes of human blood from heel/finger pricks, spotting on an appropriate filter paper, drying and transporting to the laboratory for analysis. Initially it was used for neonatal screening of phenylketonurea. Now it has been successfully adopted and is widely practiced by clinical laboratories in newborn screening for inherited metabolic disorders. It requires a micro volume (typically ≤50 μL) blood sample for drug quantification. Recently, it has gained interest in drug discovery and development processes because it offers several advantages over conventional whole blood, plasma or serum sample collection (Nageswara Rao *et al*., 2010, 2011; Uyeda *et al*., 2011; Mather *et al*., 2011). To the best of the authors' knowledge, methods for the determination of DPZ on rat dried blood spots and urine have not been reported in the literature.

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Abbreviations used: DBS, dried blood spots; DPZ, dapiprazole; ESI, electrospray ionization.

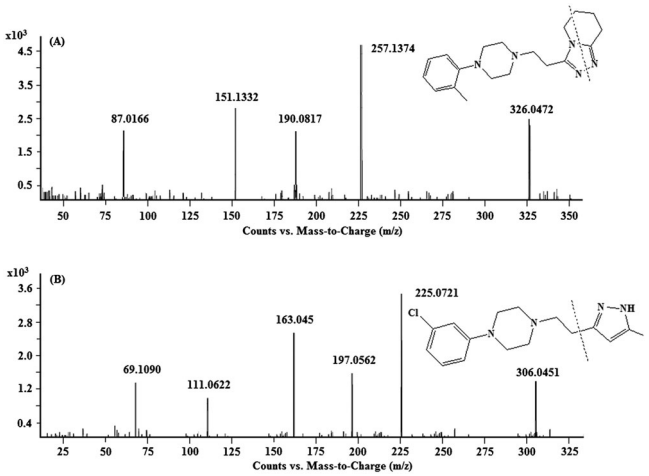

Figure 1. MS^n ($n = 2$) spectra and molecular structure of (A) dapiprazole (DPZ) and (B) internal standard (IS).

The present work describes the development and validation of a LC-MS/MS method for the quantitative determination of DPZ in rat DBS and urine. The method was successfully applied to a pharmacokinetic study of DPZ after oral administration of 30 mg/kg DPZ to rats.

Experimental

Chemicals and reagents

HPLC-grade methanol, acetonitrile (Qualigens, Fine-chem. Mumbai, India), ammonium acetate and acetic acid (SD Fine-Chemicals, Mumbai, India) were used. DPZ and MPZ (Mepiprazole) were gifts from by local industries. Glass-distilled and Millipore water (Nanopure, Barnsted, USA) was used. FTA blood spot cards (Whatmann, Sanford, ME, USA), heparin-coated capillaries (Sangius, Counting, Nümbrecht, Germany), blood collection tubes (Sarstedt Leicester, UK), 0.22 μm nylon syringe filters (Millipore, Mumbai, India), a centrifuge model 2-16P (Sigma, Zurich, Switzerland) and a multipipette for spotting blood (Tarsons, Kolkata, India) were used. Silica gel sachets and sealable plastic bags for the storage of blood spot cards were purchased from the local market.

Animals

Wistar rats (200–220 g) were procured from M/S Mahavir Enterprises, Hyderabad, India. Throughout the experimental period, the animals were housed under standard conditions in cages at room temperature (20 ± 2 °C) and relative humidity 60–70%, and were exposed to a 12/12 h light–dark cycle. They were fed with standard laboratory diet supplied by M/S Rayans Biotechnologies Pvt Ltd, Hyderabad, India. Food and water was allowed *ad libitum* during the experiment. After a single dose by oral administration of 30 mg/kg DPZ to healthy Wistar rats ($n = 6$), at regular time intervals, blood was collected from finger pricks and urine samples were collected into the processed test tube, and the concentrations of DPZ were determined from calibration curve. Statistical analyses were performed using Microsoft Excel 2000 while 'Ramkin' software (Drug and Statistics, Mathematical Pharmacology Professional Committee of China, Shanghai, China) was used for calculation of pharmacokinetic parameters.

Liquid chromatography–mass spectrometry

The analysis was performed on an Agilent 1200 series HPLC instrument (Agilent Technologies, USA) equipped with a quaternary pump (G13311A, USA), a degasser (G1322A, USA), a diode-array detector (G1315D, USA), an autosampler (G1329A, USA) and a column compartment (G1316A, USA). The chromatographic separation was achieved on a Phenomenex (Luna) C_{18} (250 × 4.6 mm i.d.; 5 μm) analytical column at 25 °C using a mobile phase containing 20 mM ammonium acetate (pH adjusted to 4.0 with acetic acid) and acetonitrile (80:20, v/v) at a flow rate of 1.0 mL/min. The run time was 15.0 min. The injection volume was 20 μL and before injection the solution was filtered through a 0.22 μm nylon filter. LC-MS analysis was performed on quadrupole time-of-flight mass spectrometer (Q-TOF LC/MS 6510 series classic G6510A, Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source. The data acquisition was under the control of Mass Hunter workstation software. Typical operating source conditions for MS scan in positive ESI mode were optimized: ionization voltage, 80 V; capillary voltage, 3000–3500 V; skimmer, 60 V; and nitrogen as the drying (300 °C; 9 L/min) and nebulizer (45 psi) gas. For collision-induced dissociation experiments, keeping MS^1 static, the precursor ion of interest was selected using the quadruple analyzer and the product ions were analyzed using a time-of-flight analyzer. Ultra high pure nitrogen was used as collision gas, and the pressure in the collision cell was maintained at 18 Torr. All the spectra were an average of 20–25 scans recorded under identical experimental conditions.

Preparation of working, calibration and quality control standards

Stock standard solutions of DPZ and the IS were prepared in mobile phase at a concentration of 1 mg/mL and stored at 5 °C. The DBS and urine calibration standards were prepared at concentrations of 1,10,100, 500, 1000, 2000 and 3000 ng/mL of DPZ and 20 ng/mL of IS by spiking appropriate aliquots of working solutions to blank pooled drug-free rat blood and urine respectively. Low, medium and high concentration quality control (QC) samples at concentrations of 50, 1000 and 2000 ng/mL, respectively, of DPZ and IS were prepared in a similar fashion.

Blood spotting and sample preparation

The DBS were prepared by spotting 30 μL of the respective spiked CS/QC or whole blood from the DPZ treated rats onto sampling paper using a calibrated pipette. The samples were left to dry in the dark for at least 3 h before storing a controlled room temperature (25 °C) until analysis. Sample were prepared by punching a 10 mm disk from the DBS into a clean tube using the 500 μL of extraction solvents, viz. acetonitrile–water (50:50), methanol–water (50:50), 100% methanol and acetonitrile. Of the solvents tested, 100% methanol offered good recoveries, so all the samples were extracted with methanol followed by vortexing and centrifuging at 4000 ***g*** for 20 min to obtain a clean supernatant which was transferred to a clean tube for analysis by LC-MS.

Urine sample preparation

Rat urine (0.2 mL) was made up to 1 mL with water and loaded onto the solid-phase extraction cartridges (ODS-SPE, 100 mg/1 mL, Agela technologies), which had previously been washed with 2 × 1 mL of methanol and 2 × 1 mL of water. After washing with 2 mL of water, the drug was eluted with 2 × 1 mL of methanol in a clean tube. The eluent was transferred into an autosampler vial and 20 μL injected into the chromatographic system. The same procedure was used for method validation and pharmacokinetic studies.

Results and discussion

Liquid chromatography–mass spectrometry

Ionization of DPZ was carried out with ESI because it provided a more stable and stronger mass spectral signal. In positive ESI mode, DPZ and IS formed protonated molecules $[M + H]^+$ at *m/z*

326 and *m/z* 306, respectively. The $[M+H]^+$ ions were selected as the precursor ions and subsequently fragmented in MS/MS mode (Fig. 1). The precursor and product ions of DPZ and IS are given in Fig. 1. The typical chromatograms of rat blank, spiked DBS and urine samples with 1000 ng/mL, lower limit of quantification, extracted DBS and urine sample collected at 1.5 and 3.0 h after oral administration are shown in Fig. 2. The retention times were 7.15 and 10.26 min for IS and DPZ, respectively. To obtain chromatograms with good resolution and symmetric peak shapes, different mobile phase composition methanol–water and acetonitrile–water binary solvent systems using different buffers, such as 20 mM ammonium formate and acetate, were investigated. As a result, good separation, peak shape and resolution were obtained on a Phenomenex (Luna) C_{18} column (250 × 4.6 mm i.d., 5 μm) using 20 mM ammonium acetate (pH adjusted to 4.0 with acetic acid) and acetonitrile (80:20, v/v) as a mobile phase in an isocratic mode at a flow rate of 1.0 mL/min at 25 °C. The run time was 15 min. These optimized conditions were transported to LC-MS/MS later in order to ensure that all the compounds, including metabolites present in DBS and urine, had been eluted. However, metabolites were not detected in any of the DBS as well as urine samples.

Validation procedures

The validation experiments were performed according to the *Guidance for Industry – Bioanalytical Method Validation*, by the US Food and Drug Administration (2001).

Specificity and selectivity

The specificity of the method was assessed by comparing the chromatograms of six different batches of blank DBS samples and urine. Each blank DBS sample and urine was tested under the chromatographic conditions described above to ensure no interference of the analyte from DBS sample and urine (Fig. 2). The selectivity of the method was established by the analysis of blank DBS samples of control blood from both individual subjects and pooled samples.

Linearity of calibration curves and lower limit of quantification

Quantification was achieved from seven-point calibration curves covering a range between 1 and 3000 ng/mL in DBS and urine samples for DPZ. The calibration curves were obtained by plotting the peak-area ratios of DPZ to the IS versus the concentrations of DPZ, using a weighted least-squares linear regression (the weighting factor used was $1/x^2$). The limits of detection (LOD) and quantification (LOQ) were determined as the concentration with a signal-to-noise ratio of 3 and 10, respectively. The linearity, LOD and LOQ data are given in Table 1.

Precision and accuracy

The intra-day precision expressed as relative standard deviation (RSD) and the accuracy expressed as relative error (RE), were

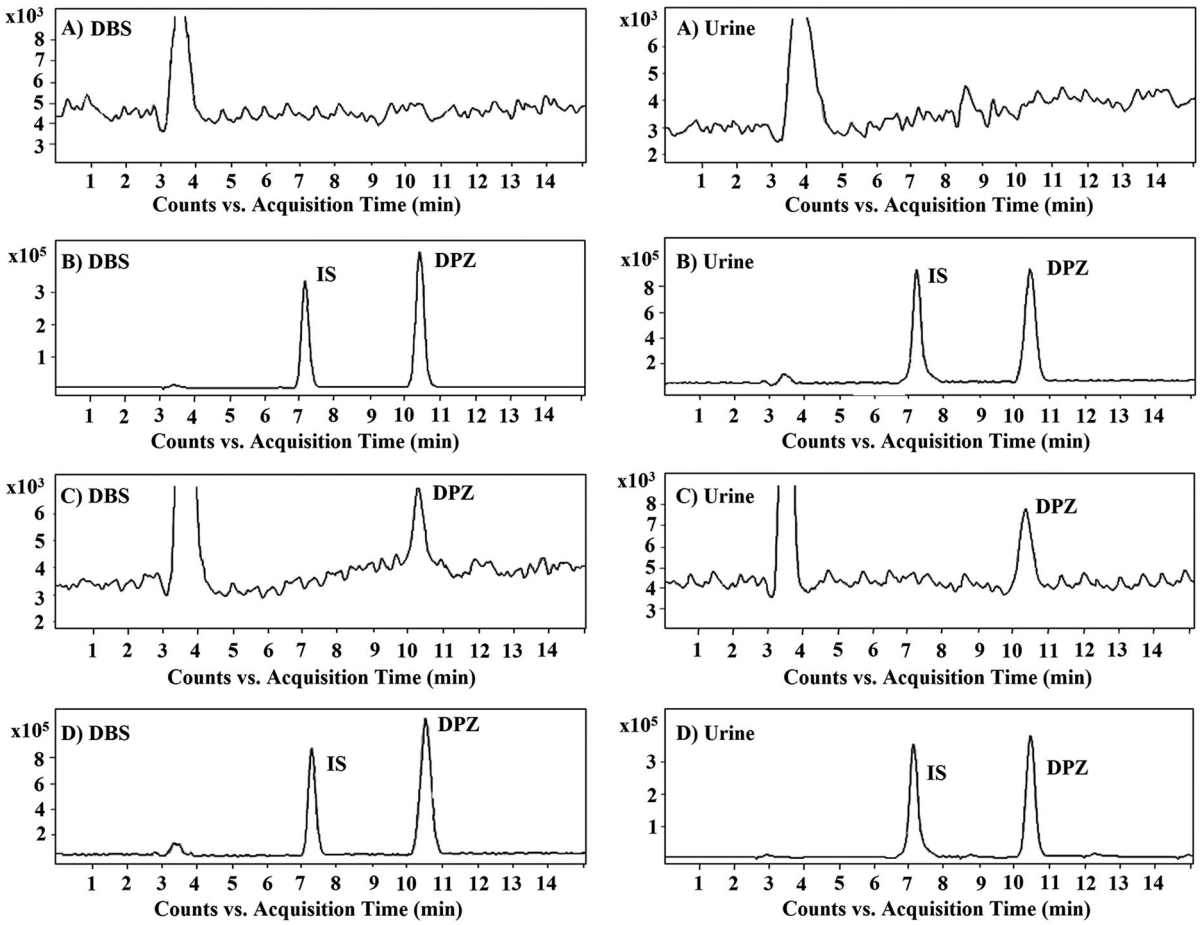

Figure 2. Representative LC-MS chromatograms of rat DBS and urine samples: (A) blank chromatogram, (B) spiked with 1000 ng/mL of DPZ and IS, (C) spiked with DPZ at LLOQ and (D) after 1.5 and 3.0 h of a 30 mg/kg oral dose of DPZ and IS.

Table 1. Linearity, limit of detection (LOD) and limit of quantitation (LOQ) data

Matrix	Regression equation	r^2	LOD (ng/mL)	LOQ (ng/mL)
Rat DBS	$y = 2471.3x + 1948$	0.9997	0.30	1.10
Rat urine	$y = 1057x + 3197$	0.9989	0.45	1.50

r^2, Correlation coefficient.

evaluated by analyzing six different QC samples ($n = 6$) at each of the low, medium and high concentrations of the analyte on the same day. The inter-day precision and accuracy were assessed by analyzing six batches of all QC samples on three different days. Intra- and Inter-day precision and accuracy of quality control data are given in Table 2.

Recovery and matrix effect

The extraction recoveries of DPZ and IS were determined by comparing the peak areas of extracted clear supernatant of DBS and urine samples from the QC samples ($n = 6$) with those obtained from the direct injection of the aqueous QC standards at same concentrations. The recoveries of DPZ and IS were determined at three concentration levels of QC at low, medium and high concentrations and are given in Table 3. The matrix effect was evaluated by comparing the chromatographic peak areas of neat solution of analytes and IS spiked into extracted blank DBS samples ($n = 6$) at low, medium and high concentration levels with those for the clean standard solutions at the same concentrations and was found to be within acceptable limits (95–105%). Thus the matrix effects were found to be insignificant and did not affect the accuracy of the proposed LC-MS/MS method.

Stability

The stability of DPZ was evaluated in DBS and urine samples by exposing to different conditions (time and temperature) at three QC concentration levels (500, 1000 and 2000 ng/mL) in six replicates. These results were compared with those obtained for freshly prepared DBS samples. Freeze–thaw stability was evaluated after three complete free–thaw cycles (−20 to 25 °C) on consecutive days. Short-term temperature stability was assessed by analyzing samples that had been kept at ambient temperature (25 °C) for 6 h. Long-term stability was performed by analyzing samples that had been stored at −20 °C for 30 days. To evaluate the post-preparative stability, QC samples were extracted and kept in the autosampler (10 °C) for 10 h before the injection. The results from all the stability tests are given in Table 4. The results were found to be well within the acceptance limits.

Table 3. Recovery data ($n = 6$)

Analyte	Matrix	Added concentration (μg/mL)	Recovery (%)	RSD (%)
DPZ	Rat DBS	0.05	94.17	3.71
		1.0	92.41	3.85
		2.0	95.06	4.63
	Rat urine	0.05	90.01	3.22
		1.0	91.62	2.85
		2.0	89.25	3.44
IS	Rat DBS	0.05	91.88	4.19
		1.0	93.58	4.63
		2.0	88.63	2.99
	Rat urine	0.05	95.61	3.57
		1.0	89.45	3.85
		2.0	87.67	4.68

DPZ, dapiprazole.

Hematocrit effect

Hematocrit (Hct) has a significant effect on blood viscosity. Variability in viscosity leads to differences in flux and diffusion properties of blood through the DBS card used for sample collection. This can directly affect the accuracy of determinations of analytes in DBS samples (Denniff and Spooner, 2010). Hematocrit is normally about 0.31–0.50 for rats (O'Mara *et al.*, 2011). At a high Hct value, the distribution of blood sample through the paper/card might be poor, resulting in a smaller blood spot when compared with the blood sample with a low Hct. The effect of 20, 35 and 50% hematocrit were tested at 1000 ng/mL of DPZ. The measured DPZ concentrations were compared with the results obtained from DBS samples with Hct of 35% and are given in Table 5. The percentage difference was calculated by subtracting the percentage relative error of Hct 20% and 50% from percentage relative error of 35% Hct, which was taken as standard Hct value. These results revealed that there was an apparent impact of Hct (1.5–1.3%) on the quantification of DPZ.

Table 2. Accuracy and precision data ($n = 6$)

Matrix	Added concentration (μg/mL)	Intra-day			Inter-day		
		EC (μg/mL)	RSD (%)	RE (%)	EC (μg/mL)	RSD (%)	RE (%)
Rat DBS	0.05	0.049	3.73	4.1	0.051	1.98	3.66
	1.0	0.97	4.19	3.3	1.05	1.86	2.7
	2.0	2.11	2.83	0.7	2.07	3.54	3.0
Rat urine	0.05	0.048	2.20	2.7	0.049	1.54	3.8
	1.0	1.02	3.47	2.6	1.02	2.85	4.7
	2.0	1.98	2.94	3.5	2.09	2.40	2.8

EC, Experimental concentration; RSD, relative standard deviation; RE, relative error.

Table 4. Stability data ($n = 6$)

Storage conditions		Rat DBS		Rat urine	
	Added concentration (μg/mL)	Calculated concentration (μg/mL) (mean ± S.D)	RE (%)	Calculated concentration (μg/mL) (mean ± S.D)	RE (%)
RT (25 °C) for 6 h	0.5	0.49 ± 0.07	2.7	0.51 ± 0.06	2.8
	1.0	0.99 ± 0.11	1.6	0.98 ± 0.13	2.5
	2.0	2.10 ± 0.29	2.8	1.97 ± 0.26	1.9
10 °C for 10 h	0.5	0.49 ± 0.09	3.9	0.50 ±0.02	3.7
	1.0	0.97 ± 0.13	3.4	0.96 ± 0.14	4.2
	2.0	1.99 ± 0.17	4.3	2.01 ± 0.22	3.9
−20 °C for 30 days	0.5	0.51 ± 0.07	2.2	0.46 ± 0.06	1.9
	1.0	0.96 ± 0.10	3.6	0.98 ± 0.09	4.5
	2.0	1..98 ± 0.23	4.1	1.92 ± 0.18	3.6
Freeze–thaw −20–25 °C for 5 h	0.5	0.47 ± 0.05	3.7	0.46 ± 0.03	4.0
	1.0	0.99 ± 0.11	2.6	0.94 ± 0.14	2.2
	2.0	1.98 ± 0.27	2.5	1.93 ± 0.29	3.4

Application to a pharmacokinetic study in rats

The developed assay method was applied to a pharmacokinetic study after oral administration of DPZ to rats at a dose of 30 mg/kg and mean concentrations in DBS and urine–time profiles are shown in Fig. 3. At pre-determined time intervals (0, 0.4, 0.8, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0, 20.0 and 24 h post administration), a 50 μL blood sample was taken from the epicanthic veins and transferred to EDTA-coated tubes. After gentle mixing with anti-coagulant, DBS samples were prepared from each time point. Urine samples were collected at 0, 0.6, 1.5, 3, 5, 6, 8, 10, 12, 16, 20 and 24 h and the maximum concentration of drug of 1320 ng/mL was observed at 3 h after oral administration. The pharmacokinetic parameters, peak plasma concentration (C_{max}) and the time to C_{max} (t_{max}), elimination half-life ($t_{1/2}$) and AUC from 0 to infinity ($AUC_{0-\infty}$) were calculated for each subject using 'Ramkin' software. The pharmacokinetic parameters of DPZ are given in Table 6.

Conclusions

A simple and rapid LC-MS/MS method for determination of DPZ on rat DBS and urine has been developed and validated. Its application to pharmacokinetics was demonstrated. The developed DBS method has several advantages, such as being noninvasive, requiring only a microvolume blood sample (typically ≤50 μL) and being simple to perform compared with conventional venipuncture. These advantages would make it efficient for the analysis of large numbers of blood samples for PK/PD studies.

Table 5. Effect of hematocritic on extraction recovery ($n = 6$)

Analyte	DPZ 1000 ng/mL		
Hematocrit	20%	35%	50%
EC	966	981	994
RSD	2.57	3.63	2.85
RE (%)	4.2	2.7	1.4
Percentage difference from 35% Hct	1.5	0	1.3

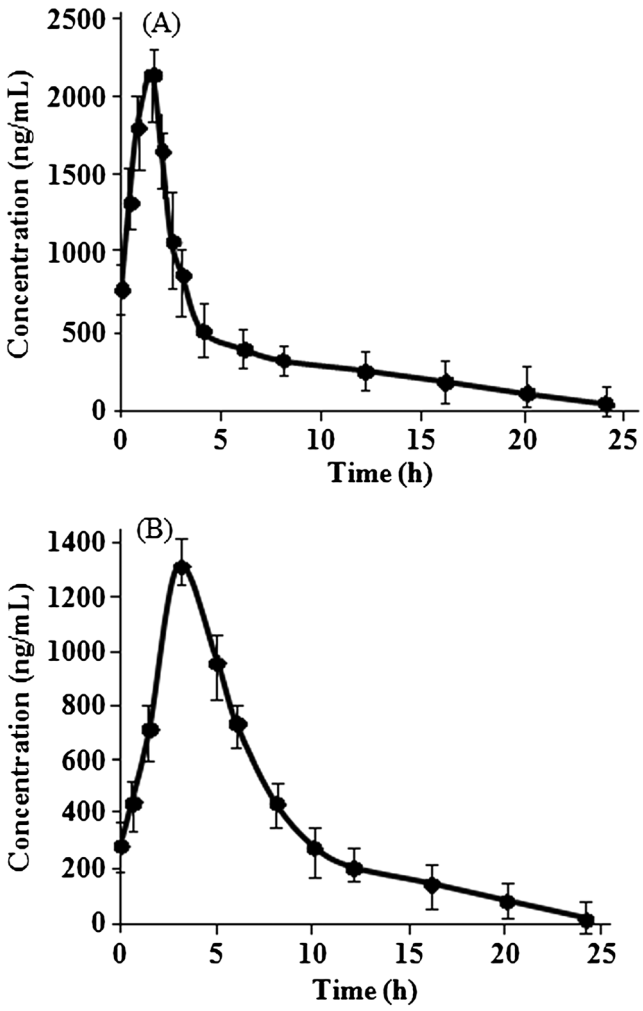

Figure 3. Concentration–time profiles of DPZ of rat DBS (A) and urine (B), after single dose by oral administration of 30 mg/kg DBZ to healthy Wister rats ($n = 6$).

Table 6. Pharmacokinetic data

Parameter	Rat DBS	Rat urine
t_{max} (h)	1.5	3.0
C_{max} (ng/mL)	2147.0	1320.0
AUC_{0-t} (ng/mL/h)	5423.74	2176.25
$AUC_{0-\infty}$ (ng/mL/h)	5569.33	2041.81
$T_{1/2}$ (h)	3.89	5.67

C_{max} (ng/mL), maximum plasma concentration; t_{max}, time to C_{max}; AUC, area under plasma concentration–time curve; $t_{1/2}$, half-life.

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References

Allinson RW, Gerber DS, Bieber S and Hodes BL. Reversal of mydriasis by dapiprazole. *Annals of Ophthalmology* 1990; **22**: 131–133.

Bianchi E and Segre G. Ocular pharmacokinetics of radioactive dapiprazole after topical and parenteral administration. *European Journal of Pharmacology* 1990; **183**: 396.

Canovetti A, Nardi M, Figus M, Fogagnolo P and Benelli U. Aceclidine, brimonidine tartrate, and dapiprazole: Comparison of miotic effect and tolerability under different lighting conditions. *Journal of Cataract and Refractive Surgery* 2009; **35**: 42–46.

Denniff P and Spooner N. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drug. *Bioanalysis* 2010; **8**: 1385–1395.

Eltze M. Affinity of the miotic drug dapiprazole at α1-adrenoceptor subtypes A, B and D. *Journal of Pharmacy and Pharmacology* 1997; **49**: 1091–1095.

Guthrie R and Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *The Journal of Pediatrics* 1963; **32**: 338.

Hogan TS, Mcdaniel DD, Bartlett JD, Hart KK and Paggiarino DA. Dose–response study of dapiprazole HCl in the reversal of mydriasis induced by 2.5% phenylephrine. *Journal of Ocular Pharmacology and Therapeutics* 1997; **13**: 297–302.

Jaya Prasanthi K and Syama Sundar B. Development and validation of an HPLC method for quantifying dapiprazole in bulk preparations. *Analytical and Bioanalytical Techniques* 2012. http://dx.doi.org/10.4172/2155-9872.1000143

Lisciani R, Baldini A and Silvestrini B. General pharmacological properties of dapiprazole, a potential psychotropic agent. *Arzneimittel-Forschung* 1982; **32**: 674–678.

Marx-Gross S, Krummenauer F, Burkhard DH and Pfeiffer N. Brimonidine versus dapiprazole: Influence on pupil size at various illumination levels. *Journal of Cataract and Refractive Surgery* 2005; **31**: 1372–1376.

Mastropasqua L, Carpineto P, Ciancaglini M, Lobefalo L, Costagliola C and Gallenga PE. Effect of dapiprazole an alpha-adrenergic blocking agent on aqueous humor dynamics in pigmentary glaucoma. *Ophthalmic Research* 1996a; **28**: 312–318.

Mastropasqua L, Carpineto P, Ciancaglini M and Gallenga PE. The usefulness of dapiprazole an alpha-adrenergic blocking agent in pigmentary glaucoma. *Ophthalmic Surgery and Lasers* 1996b; **27**: 806–809.

Mather J, Rainville PD, Spooner N, Evans CA, Smith N and Plumb RS. Rapid analysis of dried blood spot samples with sub-2-μm LC-MS/MS. *Bioanalysis* 2011; **3**: 411–420.

Monti D, Saettone MF, Giannacini B and Angeli DG. Enhancement of transdermal penetration of dapiprazole through hairless mouse skin. *Journal of Controlled Release* 1995; **33**: 71–77.

Nencini P, Valeri P and Pimpinella G. The α1-blocker dapiprazole inhibits diuresis but not drinking and feeding induced by U-50,488H. *Brain Research Bulletin* 1992; **29**: 401–405.

Nageswara Rao R, Pawan Kumar M, Ramesh M, Srinivas R and Sachin BA. Development of a validated high-throughput LC-ESI-MS method for determination of sirolimus on dried blood spots. *Biomedical Chromatography* 2010; **24**: 1356–1364.

Nageswara Rao R, Mastan Vali R, Ramachandra B and Pawan Kumar M. Rapid determination of rifaximin on dried blood spots by LC-ESI-MS. *Biomedical Chromatography* 2011; **25**: 1201–1207.

O'Mara M, Hudson-Curtis B, Olson K, Yueh Y, Dunn J and Spooner N. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis* 2011; **20**: 2335–2347.

Ramesh T and Nageswara Rao R. Development and validation of a stability-indicating RP-HPLC assay method and stress degradation studies on dapiprazole. *Journal of Chromatographic Science* 2013; **59**: 856–860.

Tammy PT. Comparison of the cycloplegic and mydriatic effects of 0.5% tropicamide and 2.5% phenylephrine to paremyd and the counteracting effects of dapiprazole. *Clinical Eye and Vision Care* 1996; **8**: 209–213.

US Food and Drug Administration. *FDA Guidance for Industry: Bioanalytical Method Validation*. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research: Rockville, MD, 2001.

Uyeda C, Pham R, Fide S, Henne K, Xu G, Soto M, James C and Wong P. Application of automated dried blood spot sampling and LC-MS/MS for pharmacokinetic studies of AMG 517 in rats. *Bioanalysis* 2011; **3**: 2349–2356.

Valeri P, Palmery M, Severini G, Piccinelli D and Catanese B. Ocular pharmacokinetics of dapiprazole. *Pharmacological Research Communications* 1986; **18**: 1093–1105.

Valeri P, Martinelli B, Pimpinella G and Severini C. Effects of dapiprazole, clonidine and yohimbine on the development of dependence and withdrawal behaviour in mice. *Drug and Alcohol Dependence* 1989; **23**: 173–177.