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Quantitative determination of formononetin and its metabolite in rat plasma after intravenous bolus administration by HPLC coupled with tandem mass spectrometry☆

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ABSTRACT

A new simple, rapid, sensitive and accurate quantitative detection method using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for the measurement of formononetin (FMN) and daidzein (DZN) levels in rat plasma is described. Analytes were separated on a Supelco Discovery C18 (4.6 × 50 mm, 5.0 μm) column with acetonitrile: methanol (50:50, v/v) and 0.1% acetic acid in the ratio of 90:10 (v/v) as a mobile phase. The method was proved to be accurate and precise at linearity range of 5–100 ng/mL with a correlation coefficient (r) of ≥0.996. The intra- and inter-day assay precision ranged from 1.66–6.82% and 1.87–6.75%, respectively; and intra- and inter-day assay accuracy was between 89.98–107.56% and 90.54–105.63%, respectively for both the analytes. The lowest quantitation limit for FMN and DZN was 5.0 ng/mL in 0.1 mL of rat plasma. Practical utility of this new LC–MS/MS method was demonstrated in a pharmacokinetic study in rats following intravenous administration of FMN.

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1. Introduction

Natural isoflavones, widely distributed in Leguminosae family, are plant chemicals with estrogenic activity, and represent the main class of phytoestrogens of current interest in clinical nutrition and disease prevention [1,2]. Isoflavones have been associated with a variety of human health benefits, including prevention of cancer, cardiovascular diseases, and osteoporosis. Soy bean and soy products are known as the richest food sources of isoflavones. However, the available literature suggests that another leguminous plant, red clover (*Trifolium pratense*) also contains significantly higher concentration of isoflavones. Formononetin (FMN), a methoxylated isoflavone, is one of the major isoflavones in red clover and in commercially available extracts of this plant. Red clover extracts are available in the market (e.g. Promensil from Novogen) as dietary supplements for relieving postmenopausal symptoms such as hot flashes and in preventing bone loss and for maintaining men's prostate health. Formononetin causes vascular relaxation via endothelium/NO-dependent mechanism and endothelium-independent mechanism which involves the activation of BK(Ca) and K(ATP) channels [3]. Formononetin also promotes early fracture healing through angiogenesis activation in the early stage of fracture repair, and osteogenesis acceleration in the later stages, and thus may be beneficial for fracture healing [4]. The water soluble sodium formononetin-3′-sulfonate has been found to exhibit good lipid-lowering and liver-protection activities in rat high fat model. [5].

FMN is reported to be metabolized into isoflavone daidzein (DZN) *in vitro* and *in vivo* [6,7]. These isoflavones containing products have raised much public interest; therefore, to assess the potential benefits, or adverse effects and drug interaction potential of their consumption, it is necessary to develop analytical methodologies which are capable of the sensitive and accurate quantification of isoflavones and their metabolites in low volume of biofluids and which are also capable of high throughput analysis of samples. Up to now, several HPLC-UV and LC–MS/MS methods have been reported for the quantification of FMN along with other isoflavones in various plants, dietary supplements, urine and plasma [8–20]. However to the best of our knowledge, there is no validated method available for quantitative determination of FMN and its metabolite DZN in rat plasma. Therefore, we developed a new validated LC–MS/MS method and applied it to the determination of FMN and its metabolite DZN concurrently in rat plasma for the first time. We attained a limit of quantitation of 5.0 ng/mL. The speed of sample preparation and analysis, selectivity and sensitivity

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proved to be satisfactory. For the first time, the plasma concentration versus time profile of FMN in rats has been determined following intravenous administration and it has been shown that FMN metabolizes to DZN *in vivo*.

2. Experimental

2.1. Chemicals and reagents

FMN and DZN were purchased from Indofine Chemical Co. Inc. (Hillsborough, USA). 4-hydroxymephenytoin (IS) was purchased from Sigma Aldrich Ltd. (St Louis, USA). β-glucuronidase (from *Helix pomatia*, type H-1; 577,900 units/g) was purchased from Sigma Aldrich Ltd. (St. Louis, USA). Chemical structure of FMN, DZN and IS are shown in Fig. 1. HPLC grade acetonitrile and methanol were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, India). Diethyl ether was purchased from TKM Pharma (Hyderabad, India). Glacial acetic acid (GAA) AR was purchased from E Merck Limited (Mumbai, India). Milli-Q pure water was obtained from a Millipore Elix water purification system purchased from Millipore India Pvt. Ltd. (New Delhi, India). Heparin sodium injection I.P. (1000 IU/mL) was purchased from Gland Pharma (Hyderabad, India). Blank, drug-free plasma samples were collected from adult, healthy female *Sprague-Dawley* rats at Division of Laboratory Animals (DOLA) of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinized blood (25 IU/mL) at 13000 rpm for 10 min. Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcass of animals.

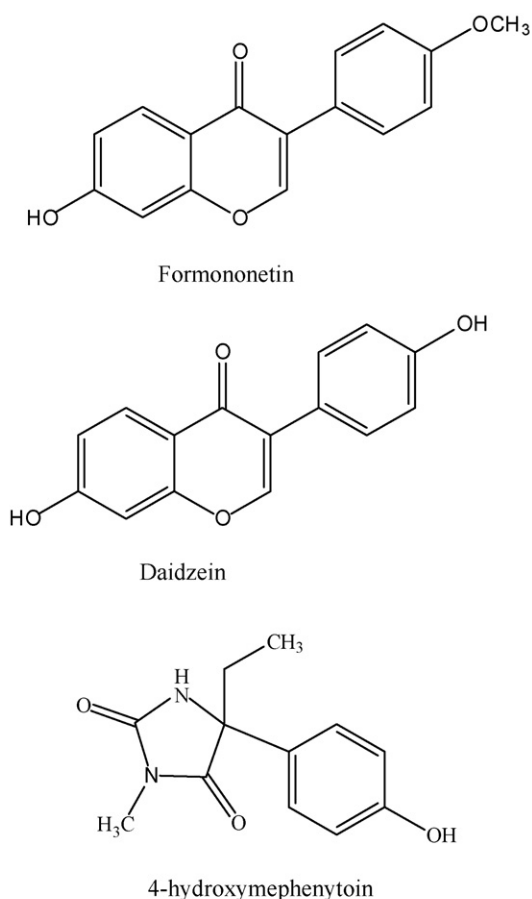

Fig. 1. Structural representation of FMN, DZN and 4-hydroxymephenytoin.

2.2. Instrumentation and chromatographic conditions

HPLC system consists of Series 200 pumps and auto-sampler with temperature controlled Peltier-tray (Perkin- Elmer instruments, Norwalk, USA) was used to inject 10 μL aliquots of the processed samples on a Supelco Discovery C18 column (4.6 × 50 mm, 5.0 μm). The system was run in isocratic mode with mobile phase consisting of acetonitrile: methanol (50:50, v/v) and 0.1% acetic acid in the ratio of 90:10 (v/v) at a flow rate of 0.7 mL/min. Mobile phase was duly filtered through 0.22 μm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min prior to use. Separations were performed at room temperature. Auto-sampler carry-over was determined by injecting the highest calibration standard then a blank sample. No carry-over was observed, as indicated by the lack of FMN, DZN and IS peaks in the blank sample.

Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The ion spray voltage was set at −4000 V. The instrument parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 50, 15, 40 and 10, respectively. Compounds parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were −80, −28, −8, −10 V, −102, −51, −8, −10 V and −65, −40, −8, −10 V for FMN, DZN and IS, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated at ESI negative ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of *m/z* 267 precursor ion $[M–H]^-$ to the *m/z* 252 product ion for FMN, *m/z* 253 precursor ion $[M–H]^-$ to the *m/z* 132 product ion for DZN and *m/z* 233.1 precursor ion $[M–H]^-$ to the *m/z* 161 product ion for IS. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada).

2.3. Preparation of stock and standard solutions

Primary stock solutions of FMN, DZN and IS (1 mg/mL) were prepared in DMSO. Working standard solutions of FMN and DZN were prepared by combining the aliquots of each primary stock solution and diluting with methanol. A working stock solution of IS (0.1 mg/mL) was prepared by diluting an aliquot of primary stock solution with methanol. Calibration standards of FMN and DZN (5, 10, 20, 50, 75, 100 and 200 ng/mL) were prepared by spiking the working standard solutions into pooled drug-free rat plasma. All the stock solutions were stored at 4 °C until analysis. Quality control (QC) samples were prepared by individually spiking control rat plasma at four concentration levels [5 ng/mL (lower limit of quantitation, LLOQ), 15 ng/mL (QC low), 80 ng/mL (QC medium) and 180 ng/mL (QC high)] and stored at −70 ± 10 °C until analysis.

2.4. Recovery

The extraction recovery of analytes, through liquid–liquid extraction procedure, was determined by comparing the peak areas of extracted plasma (pre-spiked) standard QC samples (n = 6) to those of the post-spiked standards at equivalent concentrations [21–23]. Recoveries of FMN and DZN were determined at three concentration levels QC low, QC medium and QC high concentrations viz., 15, 80, and 180 ng/mL, whereas the recovery of the IS was determined at a single concentration of 100 ng/mL.

2.5. Sample preparation

A simple liquid–liquid extraction method was followed for extraction of FMN and DZN from rat plasma. To 100 μL of plasma

in a tube, 10 μL of IS solution (1 μg/mL in methanol), was added and mixed for 15 s on a cyclomixer (Spinix Tarsons, Kolkata, India). Then 2 mL of diethyl ether was added and the mixture was vortexed for 3 min, followed by centrifugation for 5 min at 2000 × *g* on Sigma 3-16K (Frankfurt, Germany). The organic layer (1.6 mL) was separated and evaporated to dryness under vaccum in speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200 μL of the mobile phase and 10 μL was injected onto analytical column. For determination of total FMN and DZN, to account for free circulating FMN, DZN and their glucuronide conjugates, 0.1 mL plasma was incubated with 2000 unit of glucuronidase at 37 °C for 4 h [24] and then prepared as described above.

2.6. Validation procedures

A full validation according to the FDA guidelines was performed for the assay in rat plasma [25].

2.6.1. Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples collected from six different rats to investigate the potential interferences at the LC peak region for analytes and IS using the proposed extraction procedure and chromatographic-MS conditions.

2.6.2. Matrix effect

The effect of rat plasma constituents over the ionization of FMN, DZN and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ($n = 6$) with the response of analytes from neat standard samples at equivalent concentrations [21–23]. The matrix effect for FMN and DZN was determined at QC low, QC medium and QC high concentrations, viz., 15, 80 and 180 ng/mL whereas the matrix effect over the IS was determined at a single concentration of 100 ng/mL. A value of >100% indicates ionization enhancement and a value of <100% indicates ionization suppression. The post-extracted samples were the drug-free control plasma spiked with working standard stock solutions after extraction.

2.6.3. Calibration curve

The calibration curve was acquired by plotting the ratio of sum of peak area of FMN and DZN to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 5, 10, 20, 50, 75, 100, 200 ng/mL. The results were fitted to linear regression analysis using $1/X^2$ as weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.995 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [25].

2.6.4. Precision and accuracy

The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 5, 15, 80 and 180 ng/mL. The inter-day assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% standard deviation (S.D.) from the nominal values and a precision of within ±15% relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed ±20% of accuracy as well as precision [25].

2.6.5. Stability experiments

All stability studies were conducted at two concentration levels, i.e. QC low and QC high, using six replicates at each concentration levels. Replicate injections of processed samples were analyzed up to 20 h to establish auto-sampler (AS) stability of analytes and IS. The peak areas of analytes and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. The stability of FMN and DZN in the biomatrix during 6 h exposure at room temperature in rat plasma (bench top, BT) was determined at ambient temperature (25 ± 2 °C). Freeze/thaw (FT) stability was evaluated up to three cycles. In each cycle samples were frozen for at least 12 h at −70 ± 10 °C. Freezer stability of FMN and DZN in rat plasma was assessed by analyzing the QC samples stored at −70 ± 10 °C for at least 15 days. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% S.D.) and precision (i.e., ±15% R.S.D.).

2.6.6. Dilution integrity

Dilution of biological matrix is required if some study sample concentrations are expected to be higher than the upper limit of quantitation. Dilution integrity experiments were carried out by 20 times dilution of plasma samples containing 3600 ng/mL of FMN and DZN with blank plasma to obtain samples containing 180 ng/mL (QC high) of FMN and DZN.

2.7. Application to a pharmacokinetic study in rats

A pharmacokinetic study was performed to show the applicability of newly developed and validated bioanalytical method. Study was performed in female *Sprague-Dawley* rats ($n = 4$, weight range 200–220 g). FMN was administered intravenously at a dose of 10 mg/kg. Blood samples were collected from the retro-orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at 0.08, 0.5, 1, 3, 5, 7, 9, 10, 11 and 24 h post-dosing. Plasma was harvested by centrifuging the blood at 13000 rpm for 10 min and stored frozen at −70 ± 10 °C until analysis. Plasma (100 μL) samples were spiked with IS, and processed as described above. Along with the plasma samples, QC samples were distributed among calibrators and unknown samples in the analytical run.

3. Results

3.1. Liquid chromatography

Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, acetic acid and formic acid with variable pH range of 4.5–6.5, along with altered flow-rates (in the range of 0.3–0.8 mL/min) were tested for complete chromatographic resolution of FMN, DZN and IS (data not shown). Mobile phase consisting of acetonitrile: methanol (50:50, v/v) and 0.1% acetic acid in the ratio of 90:10 (v/v) at a flow rate of 0.7 mL/min was found to be suitable during LC optimization and enabled the determination of electrospray response for FMN, DZN and IS. Experiments were also performed with different C18 columns and found that chromatographic resolution, speed, selectivity and sensitivity were good with Supelco Discovery C18 column (4.6 × 50 mm, 5.0 μm). The overall analysis time was only 3 min.

3.2. Mass spectrometry

In order to optimize ESI conditions for FMN and IS, quadrupole full scans were carried out in negative ion detection mode. During a direct infusion experiment, the mass spectra for FMN, DZN and IS revealed peaks at *m*/*z* 267, 253 and 233.1 respectively as deprotonated molecular ions $[M–H]^-$. Fig. 2 shows the product ion mass spectrum for FMN, DZN and IS. Following detailed optimization of mass spectrometry conditions (provided in Section 2.2), *m*/*z* 267

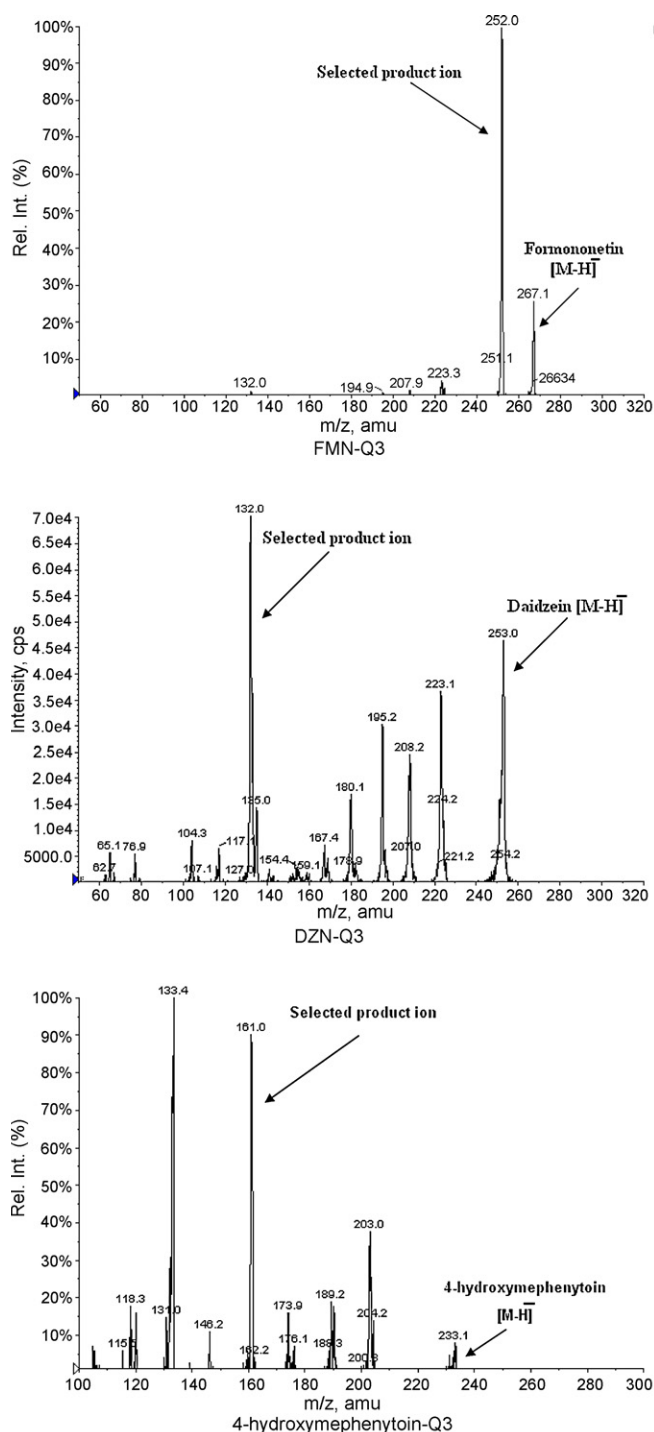

Fig. 2. MS/MS spectra of FMN, DZN and 4-hydroxymephenytoin showing prominent precursor to product ion transitions.

precursor ion $[M-H]^-$ to the m/z 252 product ion for FMN, m/z 253 precursor ion $[M-H]^-$ to the m/z 132 product ion for DZN and m/z 233.1 precursor ion $[M-H]^-$ to the m/z 161 product ion for IS was used for the quantitation purpose.

3.3. Validation procedures

3.3.1. Specificity, recovery and matrix effect

In the present study, the specificity and selectivity has been studied by using independent plasma samples from six different rats.

Fig. 3 shows a typical chromatogram for the drug-free plasma (Fig. 3a), drug-free plasma spiked with FMN and DZN at LLOQ and IS (Fig. 3b), and an *in vivo* rat plasma sample after intravenous administration of FMN (Fig. 3c). As shown in Fig. 3a, there is no significant interference from plasma found at retention time of either the analyte or IS. The retention time of FMN, DZN and IS were 1.69, 1.60 and 1.50 min, respectively.

The extraction recovery was determined by comparing the peak areas of pre-spiked standards at 15, 80 and 180 ng/mL with those of post-extraction blank plasma standards spiked with corresponding concentrations. The extraction recoveries of the FMN and DZN ranged from 95.31–117.83%, and the extraction recovery of the internal standard was 98.39%.

In this study, the matrix effect was evaluated by analyzing QC low (15 ng/mL), QC medium (80 ng/mL) and QC high samples (180 ng/mL). Average matrix effect values were 104.25%, 98.40% and 96.38% for FMN and 94.94%, 90.55% and 101.88% for DZN at QC low, QC medium and QC high, respectively. Matrix effect on IS was found to be 90.98% at tested concentration of 100 ng/mL. It is evident from these values that the ion suppression or enhancement by plasma constituents is less than 10% for analytes and IS, which seems non-significant, as it has not affected validation parameters like precision and accuracy.

3.3.2. Calibration curve

The plasma calibration curve was constructed using seven calibration standards (viz., 5–200 ng/mL). The calibration standard curve had a reliable reproducibility over the by determining the best fit of peak–area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression ($n = 3$) was found to be ≥ 0.996. The lowest concentration with R.S.D. <20% was taken as LLOQ and was found to be 5 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for three calibration curves was within 89.87–108.17; while the % precision values ranged from 0.97 to 5.74 for both the analytes (Table 1).

3.3.3. Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples are presented in Tables 2 and 3. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

3.3.4. Stability

The predicted concentrations for FMN and DZN at 15 and 180 ng/mL samples deviated within the nominal concentrations in a battery of stability tests, viz., AS (20 h), BT (6 h), repeated three freeze/thaw cycles (FT-3) and at $-70 \pm 10\,^{\circ}C$ for at least for 15 days (Table 4). The results were found to be within the assay variability limits during the entire process.

3.3.5. Dilution integrity

Dilution integrity experiments carried out at four replicates by 20 times dilution with blank plasma and assay precision and accuracy were determined in a similar manner as described in Section 2.6.4. The % accuracy of diluted QCs was in the range of 106–108.67; while % precision values ranged from 1.89 to 4.54 for both the analytes. The results suggested that samples whose concentrations were greater than the upper limit of calibration curve could be re-analyzed by appropriate dilution.

3.4. Application of the method

This LC–MS/MS method developed was used to quantitatively determine the FMN and DZN concentrations in plasma samples

Table 1
Precision and accuracy data of back-calculated concentrations of calibration samples for FMN and DZN in rat plasma ($n = 3$).

Nominal Conc. (ng/mL)	FMN				DZN			
	Mean	S.D.	Precision [a] (%)	Accuracy [b] (%)	Mean	S.D.	Precision [a] (%)	Accuracy [b] (%)
5	5.18	0.05	0.99	103.67	5.15	0.07	1.26	103.07
10	9.12	0.15	1.62	91.20	9.80	1.13	11.55	98.03
20	21.63	0.76	3.50	108.17	20.70	0.20	0.97	103.50
50	46.63	1.01	2.16	93.27	44.93	2.58	5.74	89.87
75	73.00	1.25	1.71	97.33	76.37	1.38	1.81	101.82
100	101.50	1.80	1.78	101.50	102.80	2.99	2.91	102.80
200	210.67	3.06	1.45	105.33	207.00	3.00	1.45	103.50

[a] Expressed as % R.S.D. (S.D./Mean) × 100.
[b] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 2
Intra-day assay precision and accuracy for FMN and DZN in rat plasma ($n = 6$).

	FMN (ng/mL)				DZN (ng/mL)			
	5	15	80	180	5	15	80	180
Day-1								
Mean	5.08	15.92	72.23	173.83	5.10	16.13	73.67	177.83
S.D.	0.17	0.27	1.57	4.79	0.34	0.80	2.18	3.31
Precision[a] (%)	3.33	1.71	2.18	2.76	6.61	4.94	2.95	1.86
Accuracy[b] (%)	101.67	106.11	90.29	96.57	102.07	107.56	92.08	98.80
Day-2								
Mean	4.86	15.92	73.08	181.00	5.09	15.78	72.70	178.60
S.D.	0.11	0.37	1.24	6.60	0.27	0.42	1.65	4.83
Precision[a] (%)	2.35	2.33	1.69	3.64	5.34	2.64	2.26	2.70
Accuracy[b] (%)	97.23	106.11	91.35	100.56	101.80	105.22	90.88	99.22
Day-3								
Mean	4.49	14.98	71.98	174.60	4.76	15.62	72.22	173.80
S.D.	0.21	0.50	1.20	6.11	0.32	1.06	2.35	6.72
Precision[a] (%)	4.70	3.36	1.66	3.50	4.33	6.82	3.26	3.87
Accuracy[b] (%)	91.00	99.89	89.98	97.00	97.32	104.11	90.27	96.56

[a] Expressed as % R.S.D. (S.D./Mean) × 100.
[b] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 3
Inter-day assay precision and accuracy for FMN and DZN in rat plasma .

	FMN (ng/mL)				DZN (ng/mL)			
	5	15	80	180	5	15	80	180
Mean[a]	4.81	15.61	72.43	176.31	4.98	15.84	72.86	176.81
S.D.	0.30	0.58	1.36	6.32	0.34	0.79	2.05	5.14
Precision[b] (%)	6.22	3.75	1.87	3.59	6.75	4.97	2.81	2.91
Accuracy[c] (%)	96.21	104.04	90.54	97.95	99.68	105.63	91.08	98.23

[a] $n = 3$ days with six replicates per day.
[b] Expressed as % R.S.D. (S.D./Mean) × 100.
[c] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 4
Stability of FMN and DZN in rat plasma.

	FMN				DZN			
	Mean[a]	S.D.	Precision[b] (%)	Accuracy[c] (%)	Mean[a]	S.D.	Precision[b] (%)	Accuracy[c] (%)
15 ng/mL								
0 h (for all)	15.92	0.27	1.71	106.11	16.13	0.80	4.94	107.56
20 h (AS)	15.72	1.72	10.94	98.74	14.94	0.92	6.18	92.60
6 h (BT)	16.05	1.55	9.66	100.84	14.68	0.50	3.42	90.99
FT-3	15.35	0.95	6.18	96.44	14.62	1.20	8.19	90.60
15 days at −80 °C	15.67	0.76	4.87	98.43	15.05	0.79	5.23	93.29
180 ng/mL								
0 h (for all)	173.83	4.79	2.76	96.57	177.83	3.31	1.86	98.80
20 h (AS)	185.17	4.58	2.47	106.52	183.33	4.41	2.41	103.09
6 h (BT)	171.67	2.07	1.20	98.75	168.17	4.36	2.59	94.56
FT-3	166.67	9.67	5.80	95.88	169.00	8.81	5.21	95.03
15 days at −80 °C	162.17	2.86	1.76	93.29	165.33	3.01	1.82	92.97

[a] Back-calculated plasma concentrations.
[b] Expressed as % R.S.D. (S.D./mean) × 100.
[c] Calculated as (mean determined concentration/nominal concentration) × 100.

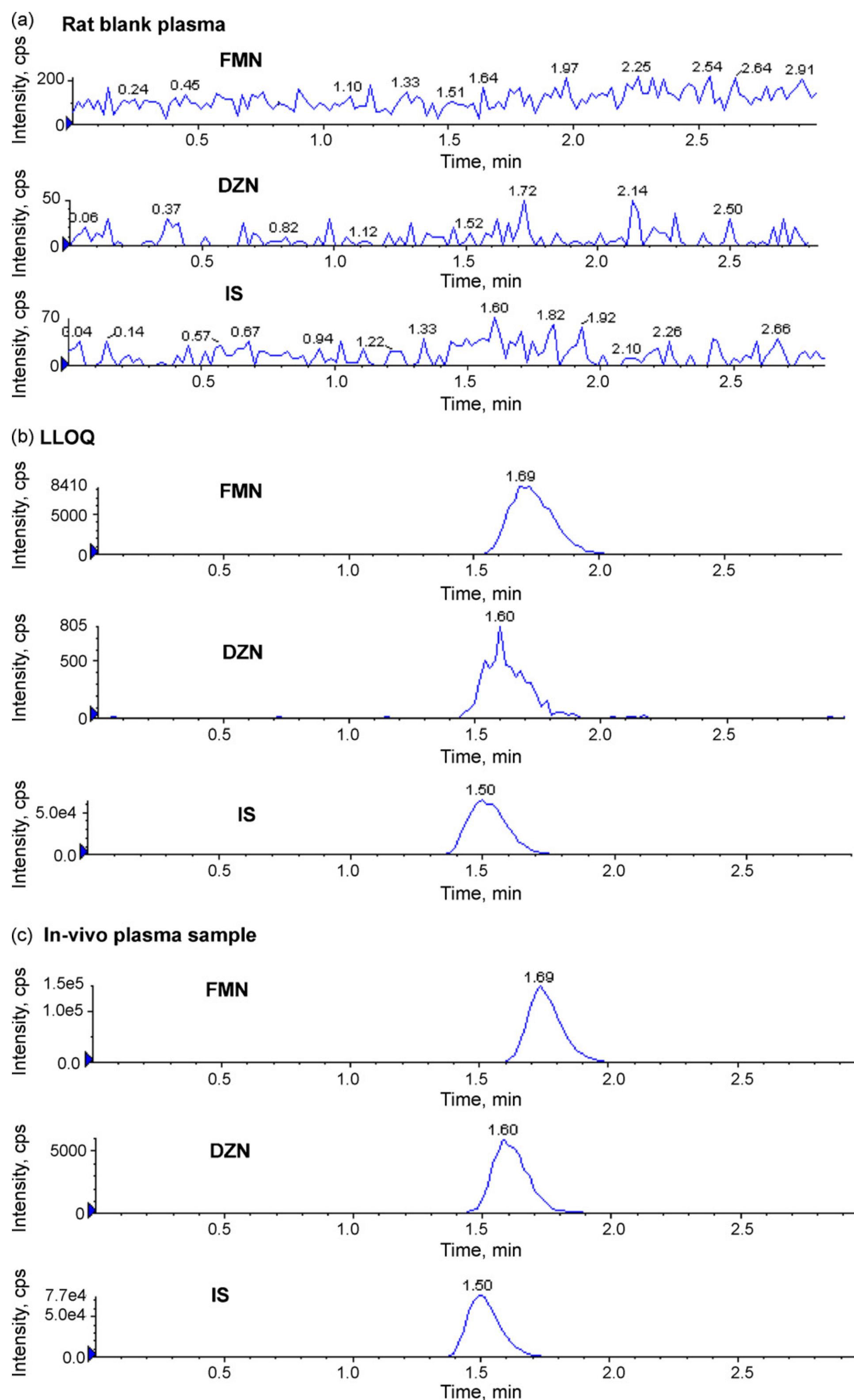

Fig. 3. Typical MRM chromatograms of FMN and DZN in rat plasma (a) a drug-free plasma, (b) drug-free plasma spiked with FMN and DZN at LLOQ (5 ng/mL) and IS and (c) an *in vivo* rat plasma sample showing FMN and DZN peak obtained following intravenous administration of FMN.

from female *Sprague-Dawley* rats after an intravenous administration of FMN. During samples analysis it was found that some sample concentrations were falling above the calibration range, these samples were re-analyzed after dilution along with the diluted QCs. All the QCs met the acceptance criteria (data not shown). Fig. 3c shows the typical representative chromatograms of treated rat plasma. The results indicate that FMN and DZN could be quantitatively detected from rat plasma after intravenous administration of FMN. The mean plasma concentrations versus time profiles are shown in Figs. 4 and 5. FMN was rapidly converted to DZN and conjugates of

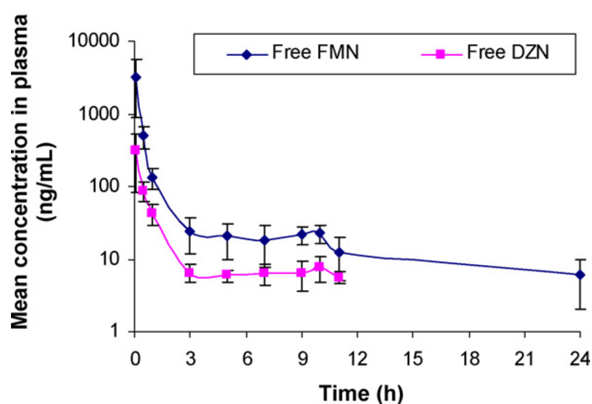

Fig. 4. Mean plasma concentration–time profile of free FMN and DZN in rat plasma following intravenous administration of FMN (Mean ± S.D., $n = 4$).

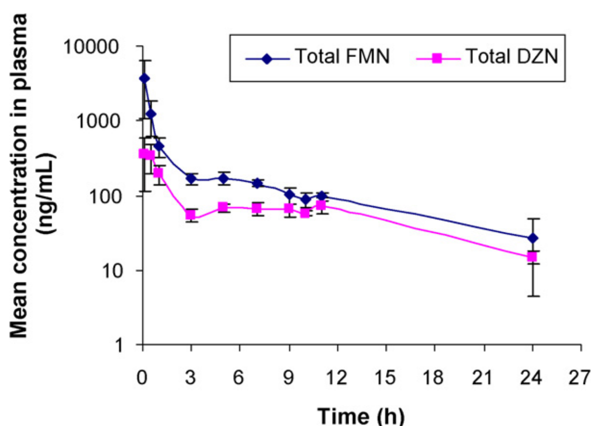

Fig. 5. Mean plasma concentration–time profile of total FMN and DZN in rat plasma following intravenous administration of FMN (Mean ± S.D., $n = 4$).

Table 5
Selected pharmacokinetic parameters of FMN and DZN in female SD rats following intravenous administration of FMN at 10 mg/kg ($n = 4$).

Parameters	Free FMN	Total FMN	Free DZN	Total DZN
AUC_{0-t} (h ng/mL)	1683.86	4317.62	243.68	1625.02
$AUC_{0-\infty}$ (h ng/mL)	1773.40	4595.71	263.86	1773.89
$t_{1/2}$ (h)	10.34	7.18	2.53	6.85
V_z (L/kg)	84.14	22.54	–	–
Cl (L/h kg)	5.63	2.17	–	–
C_{max} (ng/mL)	3207	3676	305.73	347.73

FMN and DZN. The pharmacokinetic parameters of FMN and DZN in rats were determined by noncompartmental analysis and are presented in Table 5.

4. Conclusion

For the first time, an LC–ESI-MS/MS method for simultaneous determination of FMN and its metabolite DZN in rat plasma has been developed. The developed method has acceptable sensitivity, precision, accuracy, selectivity and stability. The method gave consistent and reproducible recoveries for analytes and IS from plasma, with minimum interference and ion suppression. The method was successfully applied to a pharmacokinetic study of FMN after intravenous administration of FMN. Free as well as total FMN and DZN, which were generated after the enzymatic hydrolysis with β-glucuronidase, were also quantified to account for glucuronide conjugates of FMN and DZN.

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