SIMULTANEOUS BIOANALYSIS OF NAPROXEN AND DIPHENHYDRAMINE IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY**

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A simple, sensitive, and rapid bioanalytical method was developed for the first time for simultaneous estimation of naproxen sodium (NPX) and diphenhydramine hydrochloride (DPH) in human plasma using liquid chromatography-tandem mass spectrometry (LC–MS/MS). The isotope-labelled analogs, naproxen $13CD_3$ and diphenhydramine D_5 hydrochloric acid, were used as the internal standards. The analytes were extracted from 50 μL of human plasma employing a simple protein precipitation technique. The separation of analytes was carried out on a Zodiac C_{18} column (50×4.6 mm, 3 μm) using a mixture of HPLC grade acetonitrile and 5 mM ammonium acetate buffer in 0.025% formic acid (60:40, v/v) at a flow rate of 1.0 mL/min. The method showed linearity within the concentration ranges 400 to 120,000 ng/mL for NPX and from 0.80 to 240 ng/mL for DPH with $r^2 > 0.99$. The method was validated as per the US FDA guidelines and the results were found to be within the acceptance limits. The method was successfully applied for the pharmacokinetic study of both drugs simultaneously after an oral dose of two caplets, each containing NPX 220 mg and DPH 25 mg under fed conditions in human volunteers. Incurred sample reanalysis was also performed to authenticate the reproducibility of the method.

Keywords: *naproxen, diphenhydramine, liquid chromatography-tandem mass spectrometry, simultaneous bioanalysis, pharmacokinetic study.*

ОДНОВРЕМЕННЫЙ БИОАНАЛИЗ НАПРОКСЕНА И ДИФЕНГИДРАМИНА В ПЛАЗМЕ КРОВИ ЧЕЛОВЕКА С ИСПОЛЬЗОВАНИЕМ МЕТОДА ЖИДКОСТНОЙ ХРОМАТОГРАФИИ–ТАНДЕМНОЙ МАСС-СПЕКТРОМЕТРИИ

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Разработан простой и чувствительный биоаналитический метод для одновременной оценки напроксена натрия (NPX) и гидрохлорида дифенгидрамина (DPH) в плазме человека с использованием жидкостной хроматографии–тандемной масс-спектрометрии (LC-MS/MS). В качестве внутренних стандартов использованы меченные изотопами аналоги — напроксен $13CD_3$ и дифенгидрамин D_5 соляная кислота. Аналиты экстрагировали из 50 мкл плазмы крови человека методом белковой преципитации. Разделение аналитов проведено на колонке Zodiac C_{18} (50×4.6 мм, 3 мкм) с ис-

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пользованием смеси градуированного ацетонитрила и 5 мМ ацетатно-аммонийного буфера в 0.025 %-ном растворе муравьиной кислоты (60:40, об./об.) при скорости потока 1.0 мл/мин. Метод показал линейность в диапазоне концентраций 400—120000 нг/мл для NPX и 0.80—240 нг/мл для DPH при $r^2 > 0.99$. Результаты проверки метода на валидацию в соответствии с рекомендациями FDA (США) в допустимых пределах. Предлагаемый метод успешно применен для фармакокинетического исследования обоих препаратов одновременно после перорального приема испытуемыми двух капсул, каждая из которых содержит 220 мг NPX и 25 мг DPH. Для подтверждения воспроизводимости метода проведен повторный анализ проб.

Ключевые слова: *напроксен, дифенгидрамин, метод жидкостной хроматографии–тандемной масс-спектрометрии, синхронный биоанализ, фармакокинетическое исследование.*

Introduction. Naproxen (NPX) is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic, anti-inflammatory, and antipyretic properties, and is used to manage acute pain [1, 2]. The drug shows its anti-inflammatory effects by inhibition of cyclooxygenase enzymes and thereby decreasing prostaglandin synthesis [3–5]. Diphenhydramine (DPH) is an H_1-receptor antagonist, used mainly to treat seasonal allergies [6]. Because of its H_1-receptor antagonistic properties in the central nervous system, it is also used to induce sedation [7, 8]. The combination of NPX and DPH is available as an over-the-counter (OTC) medication to treat minor aches and pains associated with sleeplessness (Aleve PM: Label claim naproxen sodium 220 mg and diphenhydramine HCl 25 mg) [9].

As per the literature, few LC-MS/MS methods have been reported for estimation of NPX and DPH individually [10–12] or in combination with other drugs [13–19] in biological samples. To date, there have been no LC-MS/MS methods reported for the simultaneous quantification of NPX and DPH in any biological sample. The simultaneous analysis of both analytes in a single separation mode remains difficult owing to their different physicochemical properties. A sensitive and specific method is necessary for the simultaneous determination of NPX and DPH in human plasma to address their pharmacokinetics in the combined formulation. Hence, we felt that this method would help the researchers as the fixed-dose combination of the two drugs is available on the market.

The present work describes a sensitive, rapid, and simple LC-MS/MS method for the simultaneous quantification of the two analytes, employing isotope-labeled analogs as internal standards and protein precipitation for the sample preparation. The method is successfully applied to a clinical pharmacokinetic study of NPX and DPH following oral administration in healthy male volunteers under fed conditions. The authenticity of the method is established through incurred sample reanalysis.

Experimental. The working standards of naproxen sodium (99.94%), diphenhydramine hydrochloride (99.75%), naproxen $13CD_3$ (99.01%) and diphenhydramine D_5 hydrochloric acid (99.98%) were procured from Vivan Lifesciences Pvt. Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were acquired from J.T. Baker (Phillipsburg, USA). HPLC grade water was purchased from Rankem (Gurugram, India). Analytical grade formic acid and ammonium acetate were procured from Merck Ltd. (Mumbai, India). The control K_2-EDTA human plasma was obtained from Deccan's Pathological Labs (Hyderabad, India).

Analyses of the plasma samples were performed with a Shimadzu LC-20 AD (Kyoto, Japan) liquid chromatography system coupled to a AB Sciex 4500 (Foster City, CA, USA) triple quadrupole mass spectrometer. The chromatographic separation of the analytes was achieved on a Zodiac C_{18} column (50×4.6 mm, 3 μm) using a mixture of HPLC grade acetonitrile and 5 mM ammonium acetate buffer in 0.025% formic acid (60:40, v/v) at a flow rate of 1.0 mL/min.

The mass spectrometer was operated in positive ionization through a multiple reaction monitoring mode for the acquisition of the mass transition pairs. The ion transitions were monitored from *m/z* 231.1 (precursor ion) to 184.9 (product ion) for NPX, *m/z* 256.1 (precursor ion) to 167.0 (product ion) for DPH, *m/z* 235.2 (precursor ion) to 189.1 (product ion) for IS-1 and *m/z* 261.0 (precursor ion) to 172.1 (product ion) for IS-2. The ion spray voltage used was 5000 V. The source-dependent parameters viz., nebulizer gas, auxiliary gas, curtain gas, and collision gas were set at 35, 50, 45, and 6 psi, respectively. The compound-dependent parameters viz., declustering potential, collision energy, collision cell exit potential and entrance potential were set at 40, 20, 7, and 10 V, respectively. The dwell time set was 200 ms. The data acquisition and analysis were performed on Analyst software™ (version 1.7.1).

The primary stock solutions were prepared in HPLC grade water at a concentration of 5 mg/mL for NPX and 1 mg/mL each of DPH and IS-2. The primary stock solutions of IS-1 at a concentration of 1 mg/mL were prepared in HPLC grade methanol. The working standard solution mixture of the analytes

was prepared by appropriate dilution of primary standards using the diluent (mixture of HPLC grade acetonitrile and water; 60:40, v/v). The working standard solution mixture of the internal standards was prepared using the same diluent at a concentration of 40 μg/mL for IS-1 and 4 μg/mL for IS-2.

Calibration curve standards (CC STD) and quality control samples (QC SPL) were prepared by spiking 950 μL of K_2-EDTA human blank plasma with 50 μL of the appropriate working standard solution mixture of the analytes. The CC STD were prepared at concentrations of 399, 798, 1996, 4810, 12,024, 24,049, 48,098, 72,147, 96,195, and 120,119 ng/mL for NPX and 0.80, 1.61, 4.02, 9.69, 24.2, 48.4, 96.9, 145, and 241 ng/mL for DPH. The QC SPL were made at 400 (lower limit of quantitation quality control, LLOQ QC), 1170 (low quality control, LQC), 12,449 (medium quality control, MQC-1) and 54,127 (MQC-2), and 80,188 ng/mL (high quality control, HQC) for NPX and at 0.80 (LLOQ QC), 2.35 (LQC), 25.0 (MQC-1), 109 (MQC-2), and 160 ng/mL (HQC) for DPH.

For the preparation of the sample, a 50-μL aliquot of the human plasma sample was spiked with 10 μL of the IS working standard solution mixture. To this, 600 μL of acetonitrile followed by 400 μL of 5 mM ammonium acetate buffer in 0.025% formic acid was added and vortexed on a multi-tube vortexer at 2000 rpm for 5 min. After centrifuging the samples at 4000 rpm for 15 min at 40°C, the supernatant was collected and 10 μL was injected into the LC-MS/MS instrument.

A complete and thorough validation of the method developed was carried out as per the recent US FDA guidelines [20]. The parameters evaluated were selectivity, sensitivity, linearity, precision, accuracy, matrix effect, extraction recovery, dilution integrity, stability, robustness, carry-over test, and run size evaluation.

The proposed method was applied successfully to a pharmacokinetic study in South Indian healthy male subjects (n = 6). The study protocol was approved by the Prudent Ethics committee, and the volunteers provided written informed consent. After an overnight fast of 10 h and exactly 30 min after serving a high-fat, high-calorie breakfast, the volunteers were administered orally with a dose of NPX 440 mg and DPH 50 mg. The blood samples were collected at pre-dose (0 h) and at 0.33, 0.67, 1.00, 1.50, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 7.00, 8.00, 12.00, 18.00, 24.00, 36.00, 48.00, and 72.00 h after the administration of the tablets into K_2-EDTA vacutainer 5 mL collection tubes (BD, Franklin, NJ, USA). The plasma was collected by centrifugation at 2500 rpm and 4°C for 10 min and stored at −70±10°C until the analysis. The pharmacokinetic (PK) parameters of both analytes were estimated simultaneously using WinNonlin® software version 6.4 (Pharsight Corporation, Mountain View, CA, USA). Incurred sample reanalysis (ISR) was performed to check the authenticity of the method. Re-assay of the subject samples at Cmax and near the elimination phase of plasma concentration–time profiles of the analytes was done, and the % variability was calculated by comparing with the initial assay concentrations.

Results and discussion. The standard solutions were infused into the mass spectrometer, and the mass parameters were optimized by automatic tuning in both positive and negative ionization modes. NPX, being the acidic drug, showed a good response in the negative ion mode and DPH, being the basic drug, showed a good response in the positive ion mode. To develop a method for simultaneous estimation of both analytes, the positive ion mode was selected as it gives a reduced response for high-dose (440 mg), highly bioavailable (~95%) NPX and a good response for low-dose (50 mg), moderately bioavailable (~40–60%) DPH. The most sensitive mass transitions for NPX were monitored from *m*/*z* 231.1 to *m*/*z* 184.9, DPH from *m*/*z* 256.1 to *m*/*z* 167.0, IS-1 from *m*/*z* 235.2 to *m*/*z* 189.1, and IS-2 from *m*/*z* 261.0 to *m*/*z* 172.1 (Fig. 1).

Owing to the different physico-chemical properties of NPX and DPH, obtaining an appropriate peak shape and an adequate peak response for both analytes simultaneously was tried by optimizing the chromatography conditions such as the column type, mobile phase composition, and its flow rate. Different columns such as Kromasil 100-C_{18} (150×4.6 mm, 3.5 μm), Zorbax SB C_{18} (50×4.6 mm, 3.5 μm), Zorbax XDB–Phenyl (75×4.6 mm, 3.5 μm), Zodiac C_{18} (50×4.6 mm, 3 μm), and Ace Phenyl column (150×4.6 mm, 5 μm) were tried and finally, the Zodiac C_{18} column (50×4.6 mm, 3 μm) gave a satisfactory peak shape and response for both the analytes. The mobile phase consisting of methanol and 5 mM ammonium acetate in 0.025% formic acid was chosen for the enhancement of the response at a flow rate of 1.0 mL/min. Ammonium acetate and formic acid were added to improve the resolution of the peaks.

In the bioanalytical method development, the sample preparation step is crucial to achieve the maximum recovery of analytes with the minimal matrix effect. A simple protein precipitation technique with different precipitants such as methanol, acetonitrile, and ethanol was investigated. Acetonitrile was finally optimized as precipitant owing to good and reproducible recovery. The stability of the analytes in the extract was improved by adding 5 mM ammonium acetate buffer to 0.025% formic acid to mimic the mobile phase composition.

Isotope-labelled analogs as internal standards have the same extraction recovery, ionization response in ESI mass spectrometry and the same chromatographic retention times. Hence, naproxen $13CD_3$ and diphenhydramine D_5 hydrochloride were used as internal standards.

Eight lots of blank plasma from different sources, including one lipemic and one hemolyzed plasma, were evaluated for selectivity. Figures 2 and 3 demonstrate the selectivity of the method with no interference peaks in the blank plasma at the retention times of the analytes.

The sensitivity of the method was established at the concentrations of 399 and 0.80 ng/mL for NPX and DPH respectively.

The calibration curves of NPX and DPH were linear over the established concentration range 399 ng/mL to 120,119 and 0.80 to 241 ng/mL, respectively, with a correlation coefficient $r^2 > 0.99$.

The intra- and inter-day accuracy and precision results of the plasma samples for NPX and DPH are presented in Table 1. The assay results on both intra-day and inter-day accuracy were found to be within the accepted limits for both NPX and DPH.

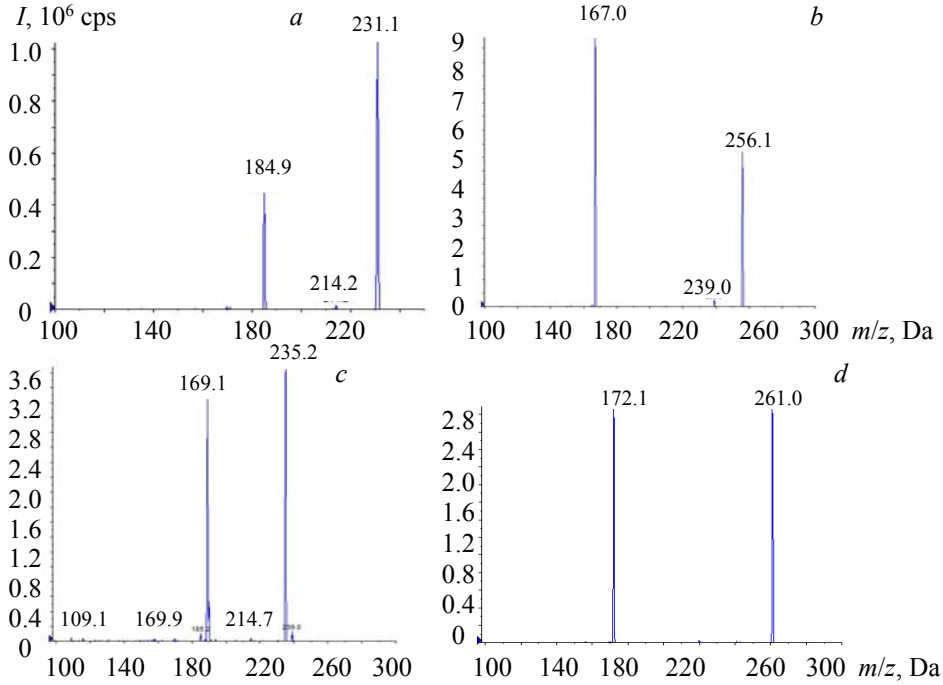

Fig. 1. Product ion mass spectra of NPX (a), DPH (b), IS-1 (c), IS-2(d).

TABLE 1. Intra- and Inter-day Precision and Accuracy Data

Analyte	QC	Concentration spiked, ng/mL	Inter-day accuracy and precision (n=30; 6 from each batch)			Intra-day accuracy and precision (n=12; 6 from each batch)		
			Concentration found (mean; ng/mL)	Accuracy, %	Precision, %CV	Concentration found (mean; ng/mL)	Accuracy, %	Precision, %CV
NPX	LLOQ QC	400.31	402.98	100.67	3.97	395.31	98.75	5.76
	LQC	1170.23	1126.62	96.27	6.53	1111.53	94.98	7.70
	MQC-1	12,449.20	12,254.06	98.43	5.32	12,087.95	97.10	3.60
	MQC-2	54,126.95	55,266.69	102.11	3.94	55,709.21	102.92	4.44
	HQC	80,188.08	77,886.57	97.13	6.32	77,325.51	96.43	7.60
DPH	LLOQ QC	0.80	0.80	99.65	5.10	0.79	98.10	6.80
	LQC	2.35	2.27	96.62	4.56	2.31	98.17	3.51
	MQC-1	25.02	24.52	97.99	4.64	25.01	99.94	2.56
	MQC-2	108.79	109.00	100.20	3.85	110.94	101.97	2.59
	HQC	161.17	159.88	99.20	4.64	165.49	102.68	2.83

The matrix effect was performed with eight different (including one lipemic and one hemolyzed) lots of K_2-EDTA plasma. The average IS-normalized matrix factors calculated for NPX were 0.977 and 1.005 and for DPH were 0.987 and 0.997, respectively, at LQC and HQC levels, proving that the method has no interference from matrix ions.

The extraction efficiency for both analytes and internal standards was found to be good and reproducible. The mean recovery of NPX and DPH was 65.15% and 54.85% with the simple protein precipitation method. The extraction efficiency of ISs was found to be 69.06% and 54.95% for IS-1 and IS-2, respectively.

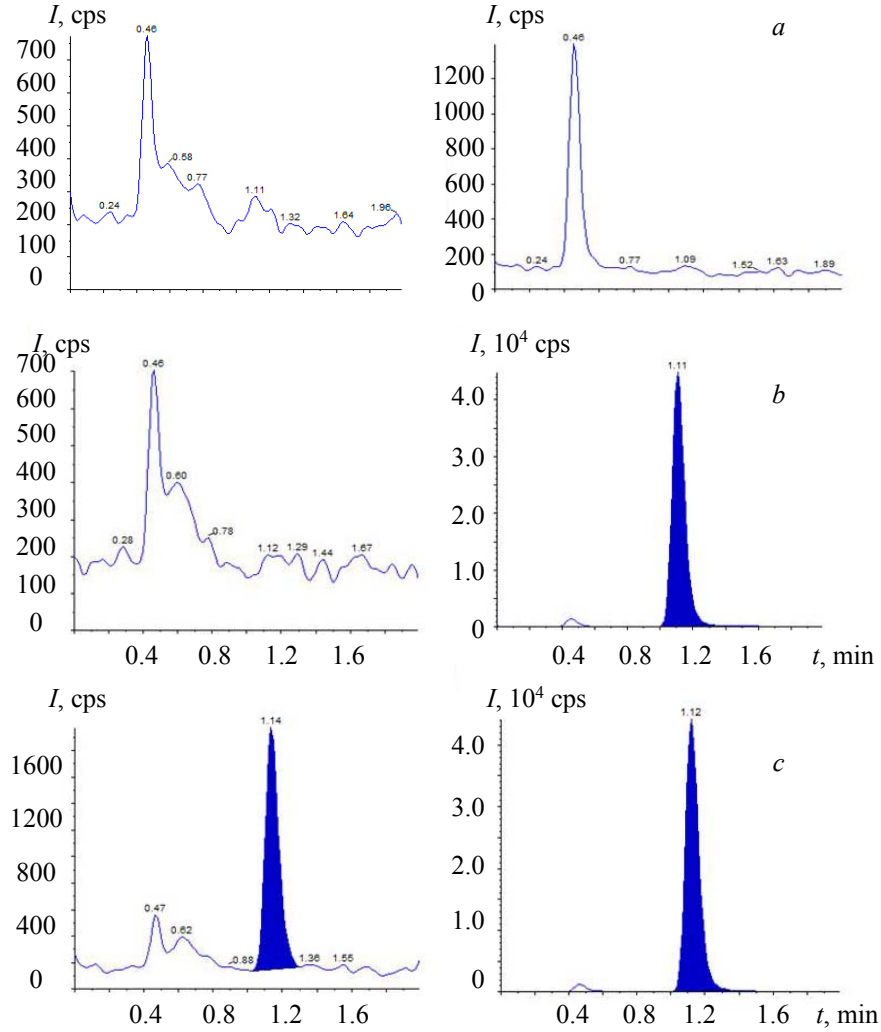

Fig. 2. Typical MRM chromatograms of NPX (left panel) and IS-1 (right panel) in blank plasma (a), blank plasma spiked with ISs (b), LLOQ QC sample (c).

To evaluate the stability of the analytes under different conditions exposed during processing and analysis, stability studies were performed by simulating the same conditions. The analytes were found to be stable in stock solutions for 18 h at room temperature and 6 days at refrigerator temperature. The analytes were found to be stable for 61 h at room temperature in wet extract, 66 h in an auto-sampler at 15°C, 5 freeze–thaw cycles, 11 h on a bench top at room temperature, 3 days at −20±5°C, 38 days at −70±10°C, and 57 h during re-injection. The detailed stability results are shown in Table 2.

The dilution reliability of the samples with analyte concentration above the Upper Limit of Quantitation Quality Control was checked during the dilution integrity test. At four times dilution (192,999 ng/mL for NPX and 388 ng/mL for DPH), the dilution integrity samples showed the accuracy of 97.89 and 101.27% and precision (%CV) of 1.82 and 2.61% for NPX and DPH, respectively.

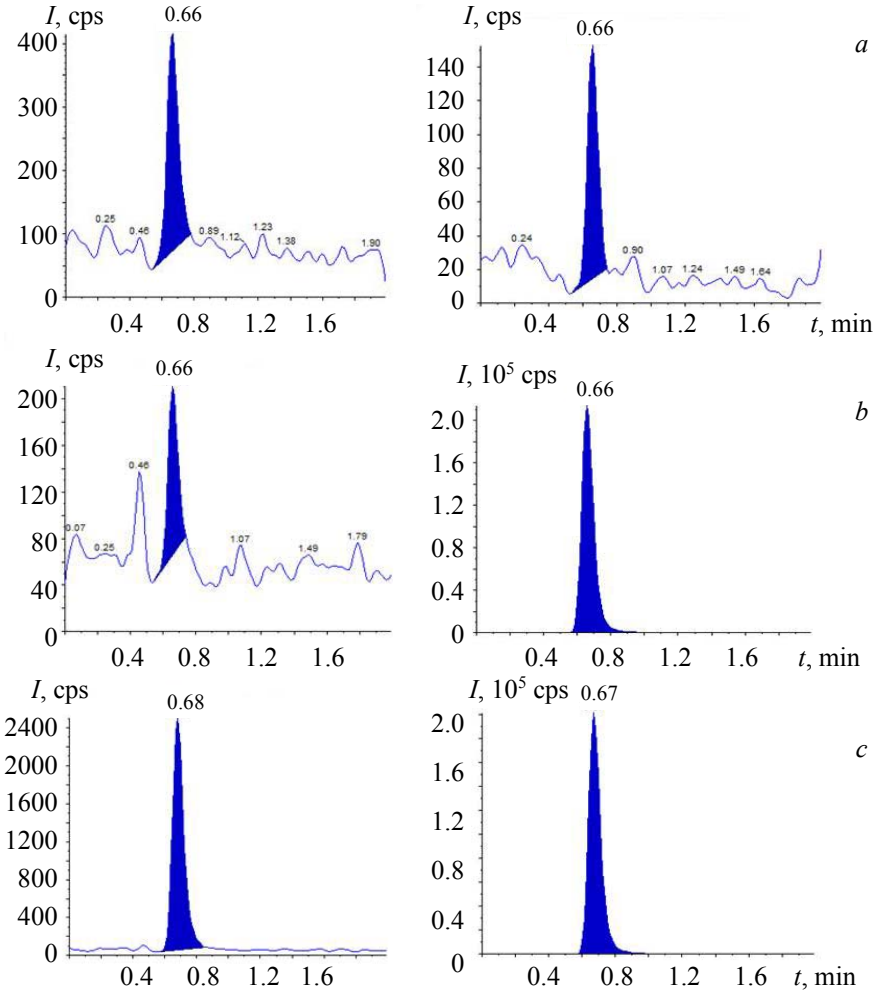

Fig. 3. Typical MRM chromatograms of DPH (left panel) and IS-2 (right panel) in blank plasma (a), blank plasma spiked with ISs (b), LLOQ QC sample (c).

TABLE 2. Stability Studies Under Different Conditions (n = 6)

Stability	Storage condition	NPX				DPH			
		Concentration spiked, ng/mL	Concentration found, ng/mL	% Stability	Precision, %CV	Concentration spiked, ng/mL	Concentration found, ng/mL	% Stability	Precision, %CV
Wet extract stability	Room temperature (61 h)	1170.23 80,188.08	1151.23 79,146.24	101.45 98.43	4.20 1.64	2.35 161.17	2.29 158.46	96.13 94.22	3.92 4.91
Reinjection stability	57 h at 15°C	1170.23 80,188.08	1178.99 80,376.19	108.11 100.62	2.94 2.78	2.35 161.17	2.33 160.88	102.10 95.77	3.70 6.11
Autosampler stability	Autosampler temperature (66 h, 15°C)	1170.23 80,188.08	1119.83 79,840.01	98.68 99.30	1.72 3.46	2.35 161.17	2.34 161.61	98.21 96.09	6.02 1.97
Freeze–thaw stability	After 5th FT cycle	1170.23 80,188.08	1155.21 80,444.69	101.80 100.05	3.26 2.51	2.35 161.17	2.30 157.83	96.80 93.84	4.07 2.54
Short-term stability	3 days at −20±5°C	1170.23 80,188.08	1127.25 82,698.28	99.34 102.85	6.21 3.93	2.35 161.17	2.17 157.09	91.19 93.40	2.79 5.68

Continue Table 2

Stability	Storage condition	NPX				DPH			
		Concentration spiked, ng/mL	Concentration found, ng/mL	% Stability	Precision, %CV	Concentration spiked, ng/mL	Concentration found, ng/mL	% Stability	Precision, %CV
Long-term stability	38 days at −70±10°C	1170.23 80,188.08	1187.75 80,868.85	99.07 98.97	2.86 1.76	2.35 161.17	2.39 165.48	106.61 104.45	3.94 4.07
Bench-top stability	Room temperature (11 h)	1170.23 80,188.08	1157.53 80,631.67	102.00 100.28	2.87 2.46	2.35 161.17	2.33 169.06	97.98 100.52	4.17 4.86

The run size evaluation (RSE) test was performed at LQC, MQC-1, MQC-2, and HQC levels of both the analytes using 40 sets of RSE samples and 6 sets of fresh samples to assess the integrity of the method during the analysis of larger batch sizes. All the 160 RSE samples of DPH and 158 out of 160 RSE samples of NPX and all the 24 fresh samples of the analytes were within 15% of their respective nominal concentrations.

During the assessment of the robustness of the method using different sets of reagents and columns of different batches by different analytes on different instruments of the same make, the accuracy and precision (%CV) were found within the range from 93.97 to 102.35% and from 1.26 to 7.89%, respectively, for NPX and from 94.29 to 100.01% and from 3.16 to 3.65%, respectively, for DPH.

The proposed method was utilized for calculating the PK parameters of both analytes after a single oral dose of NPX and DPH tablets under the fed conditions to six volunteers (Fig. 4). The mean plasma concentration–time profiles of the analytes are shown in Fig. 5. The PK parameters are presented in Table 3.

The % variability of 36 samples analyzed for ISR was found to be <10%, establishing the reproducibility of the proposed method (Table 4).

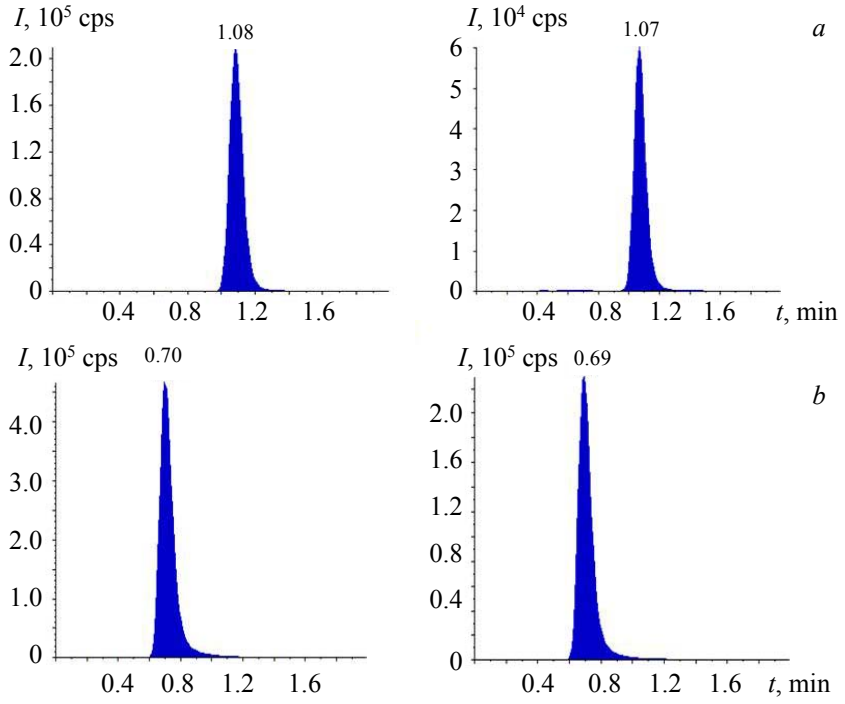

Fig. 4. Typical MRM chromatograms of NPX (left panel) and IS-1 (right panel) (a), DPH (left panel) and IS-2 (right panel) (b) in the subject plasma after the oral administration of a single dose of NPX 440 mg and DPH 50 mg.

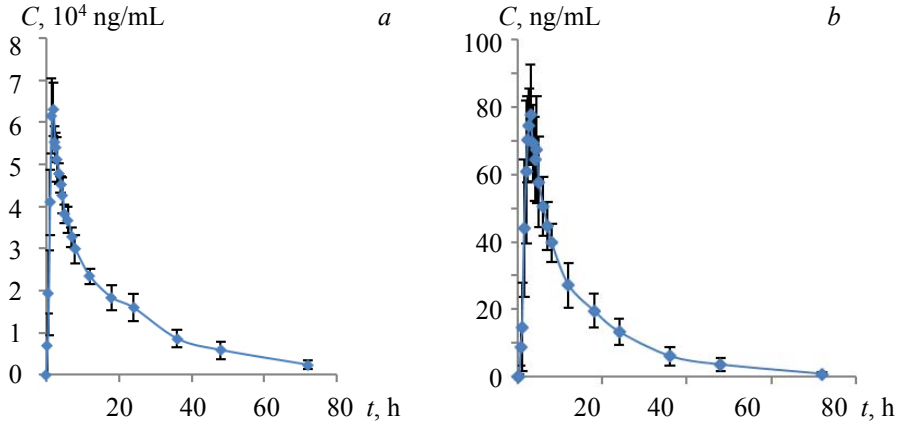

Fig. 5. Mean plasma (±SD) concentration-time profiles of NPX (a), DPH after the oral administration of a single dose of NPX 440 mg and DPH 50 mg (b).

TABLE 3. Pharmacokinetic Parameters (n=6)

Parameter	NPX	DPH
C_{max} (ng/mL)	64,212.06	82.11
t_{max} (h)	1.75	3.28
$AUC_{0\rightarrow t}$ (ng · h/mL)	987,421.25	982.26
$AUC_{0\rightarrow \infty}$ (ng · h/mL)	1,051,442.22	1015.09
$t_{1/2}$ (h)	18.15	12.41
Kel (h^{-1})	0.04	0.06

TABLE 4. Incurred Sample Reanalysis Data

NPX						DPH					
Subject No.	Sampling point, h	Initial concentration, ng/mL	Re-assay concentration, ng/mL	Mean	% Difference	Subject No.	Sampling point, h	Initial concentration, ng/mL	Reassay concentration, ng/mL	Mean	% Difference
1	2.00	65,801.61	64,681.35	65,241.48	1.72	1	3.00	71.111	74.513	72.812	4.67
1	2.67	59,423.27	55,696.19	57,559.73	6.48	1	5.00	72.472	74.285	73.379	2.47
1	72.00	3684.97	3465.24	3575.11	6.15	1	48.00	4.874	5.021	4.948	2.97
2	2.00	62,742.39	65,811.61	64,277.00	4.78	2	2.67	74.422	70.576	72.499	5.30
2	2.33	60,995.43	60,698.06	60,846.74	0.49	2	3.00	65.305	68.267	66.786	4.44
2	72.00	3428.45	3336.85	3382.65	2.71	2	48.00	3.426	3.330	3.378	2.84
3	1.50	55,696.09	54,179.54	54,937.82	2.76	3	2.33	84.055	83.812	83.934	0.29
3	2.33	52,841.54	53,761.19	53,301.36	1.73	3	3.00	85.710	80.141	82.926	6.72
3	72.00	1445.30	1390.10	1417.70	3.89	3	48.00	6.809	6.894	6.852	1.24
4	2.33	50,439.58	51,885.07	51,162.32	2.83	4	2.67	63.729	62.392	63.061	2.12
4	2.67	48,880.80	47,545.39	48,213.10	2.77	4	3.00	58.458	59.689	59.074	2.08
4	72.00	1664.59	1766.70	1715.65	5.95	4	48.00	2.284	2.104	2.194	8.20
5	2.00	67,879.32	69,238.16	68,558.74	1.98	5	2.00	90.685	88.521	89.603	2.42
5	2.33	57,063.66	56,781.39	56,922.53	0.50	5	2.33	76.388	77.320	76.854	1.21
5	72.00	2085.40	1964.64	2025.02	5.96	5	36.00	3.905	3.954	3.930	1.25
6	1.50	63,184.50	60,735.42	61,959.96	3.95	6	2.67	91.341	93.413	92.377	2.24
6	2.33	56,881.39	56,421.48	56,651.44	0.81	6	3.50	80.019	79.154	79.587	1.09
6	72.00	1856.25	1811.64	1833.95	2.43	6	36.00	3.754	3.821	3.788	1.77

Conclusions. The LC-MS/MS assay method reported for the simultaneous determination of NPX and DPH is simple, rapid, and sensitive. The developed method is fully validated as per the commonly acceptable US FDA guidelines. To the best of our knowledge, this is the first time that both analytes have been simultaneously estimated in any of the biological matrices. The simplicity of the assay and the usage of PPT for the sample extraction and a sample turnover rate of 2.0 min per sample make it an attractive procedure in the high-throughput bioanalysis of NPX and DPH. From the validation parameter results, we can conclude that the developed method can be applied for bioavailability/bioequivalence studies and therapeutic drug monitoring of NPX and DPH with the desired precision and accuracy.

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