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Stability-indicating HPLC-PDA assay for simultaneous determination of paracetamol, thiamine and pyridoxal phosphate in tablet formulations

AMIR ALI[1]
MUHAMMAD MAKSHOOF ATHAR[1]
MAHMOOD AHMED[1]
KASHIF NADEEM[2]
GHULAM MURTAZA[2]
UMAR FAROOQ[1*]
MUHAMMAD SALMAN[1]

[1] *Institute of Chemistry University of the Punjab, Lahore 54590 Pakistan*

[2] *Schazoo Pharmaceutical Laboratories Lahore- Jaranwala Road, Sheikhupura Pakistan*

With the increased number of multi-drug formulations, there is a need to develop new methods for simultaneous determinations of drugs. A precise, accurate and reliable liquid chromatographic method was developed for simultaneous determination of paracetamol, thiamine, and pyridoxal phosphate in pharmaceutical formulations. Separation of analytes was carried out with an Agilent Poroshell C18 column. A mixture of ammonium phosphate buffer (pH = 3.0), acetonitrile and methanol in the ratio of 86:7:7 (*V*/*V*/*V*) was used as the mobile phase pumped at a flow rate of 1.8 mL min^{-1}. Detection of all three components, impurities and degradation products was performed at the selected wavelength of 270 nm. The developed method was validated in terms of linearity, specificity, precision, accuracy, *LOD* and *LOQ* as per ICH guidelines. Linearity of the developed method was found in the range 17.5–30 μg mL^{-1} for thiamine, 35–60 μg mL^{-1} for pyridoxal phosphate and 87.5–150 μg mL^{-1} for paracetamol. The coefficient of determination was ≥ 0.9981 for all three analytes. The proposed HPLC method was found to be simple and reliable for the routine simultaneous analysis of paracetamol, thiamine and pyridoxal phosphate in tablet formulations. Complete separation of analytes in the presence of degradation products indicated selectivity of the method.

Keywords: paracetamol, thiamine, pyridoxine phosphate, degradation, HPLC-PDA

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With advancement in research and development in the pharmaceutical industry, the role and production of multicomponent drug formulations is increasing steadily. Recent advances in pharmaceutical sciences have proven that the one drug-one target lock and key model is limited (1). In many complicated and refractory diseases hardly any single drug has shown satisfactory benefits. However, using a combination of drugs has offered more satisfactory results (2). Multidrug pharmaceutical fixed dose combinations contain

* Correspondence; umar.chem@pu.edu.pk

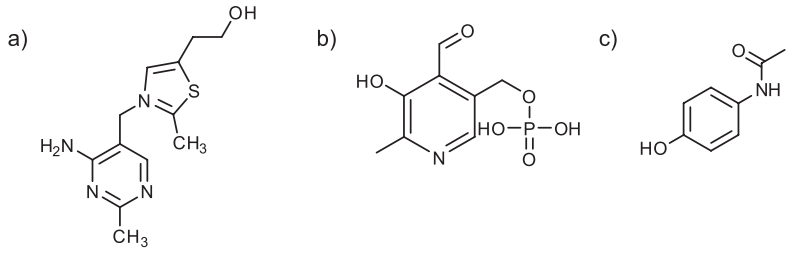

Fig. 1. Chemical structures of: a) thiamine, b) pyridoxal phosphate, and c) paracetamol.

different active pharmaceutical ingredients (API) for the treatment of pain; the components mostly include aspirin and paracetamol with codeine, barbiturates, caffeine, derivatives of pyrazolones, pentazocine and vitamins that can enhance the pharmacological effectiveness of these fixed dose combinations (3). The different API components have different modes of action; they sometimes act as synergists with more efficiency. In multidrug fixed dose combinations each API is usually present in a dose lower than in the single-component formulation, lowering the organ burden (4). Paracetamol (acetaminophen, *N*-acetyl-*p*-aminophenol), thiamine (2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl, B1] ethanol, $C_{12}H_{17}N_4OS^+$) and pyridoxal phosphate ([(4-formyl-5-hydroxy-6-methylpyridin-3-yl)methoxy] phosphonic acid, $C_8H_{10}NO_6P$, B6) are the analytes of interest in the mixture in our research (5–7).

A literature survey revealed that different analytical methods were available for the estimation of paracetamol, individually or in fixed dose combinations other than vitamins B_1 and B_6, by various methods including voltammetry, LC-MS-MS, micellar electrokinetic capillary chromatography and flow injection solid phase spectrometry (8–10). It was also found that vitamins B_1 and B_6 were estimated individually or in pharmaceutical combinations other than paracetamol using spectroscopic and chromatographic techniques coupled to various detectors such as UV-visible, fluorometric and electrochemical ones (11–16).

To the best of our knowledge, several RP-HPLC methods are available for the estimation of paracetamol, thiamine and pyridoxal phosphate (Fig. 1), individually or in combination with other drugs in fixed dose pharmaceutical formulations but no stability indicating assay method has been reported (17–21). The present research is aimed at developing a rapid, sensitive and stability indicating liquid chromatographic (HPLC) method for the simultaneous determination of paracetamol, thiamine, and pyridoxal phosphate. The proposed HPLC method was validated according to ICH guidelines and forced degradation studies were performed to show the stability-indicating nature of the method (22–24).

EXPERIMENTAL

Chemicals and reagents

Paracetamol (PRML), thiamine (B1), and pyridoxal phosphate (B6) working standards were donated by Schazoo Pharmaceutical Laboratories Ltd. (Pakistan). Diammonium hydrogen phosphate and phosphoric acid (85 %) were obtained from Sigma Aldrich, USA. Methanol and acetonitrile were purchased from Merck, Germany. In experimental work,

distilled water (18 μS cm^{-1}) was used, prepared in our own lab using the GenPure water system (Thermo Scientific, USA). The solvents and chemicals used during analysis were of HPLC grade. Multi drug formulations in tablet form, containing paracetamol, thiamine, and pyridoxal phosphate were collected from the local market shelves (Neuro Bedoxine Plus tablets, each film coated tablet containing: vitamin B1 50 mg, vitamin B6 100 mg, vitamin B12 100 mg, paracetamol 250 mg, Schazoo Pharmaceutical Laboratories, Pakistan) in Lahore, the capital city of Pakistan.

Chromatography

An LC-20 liquid chromatograph (Shimadzu, Japan) with diode array detector (SPD-M20A), online degasser (DGU-20A5) equipped with Agilent Poroshell C18 column (5 μm, 4.6 × 250 mm) was employed to separate the components. A mixture of ammonium phosphate buffer (pH = 3.0), acetonitrile and methanol, 86:7:7 volume ratio, was used as the mobile phase. Mobile phase was sonicated for 10 min to degas before use. The finally selected optimized conditions were as follows: injection volume 10 μL, adjusted flow rate 1.8 mL min^{-1}, column temperature 40 °C, API detection at 270 nm. Standard and sample solutions were filtered using a nylon filter (0.45 μm, Sartorius, Germany) before analysis.

Standard and working solutions

Standard stock solutions (1000 μg mL^{-1}) of PRML, B1 and B6 were prepared in methanol and sonicated for 10 min. The stock standard solution was diluted with the mobile phase to prepare individual working standard solutions. Amounts of 250 mg PRML, 50 mg B1 and 100 mg B2 were dissolved in methanol to a 100 mL volume; 5.0 mL of the above solution was diluted with the mobile phase to 100 mL to prepare a mixed standard solution with the final concentration of 125 μg mL^{-1} PRML, 25 μg mL^{-1} B1 and 50 μg mL^{-1} B6.

Sample preparation

Ten tablets (Neuro Bedoxine Plus tablet containing 250 mg PRML, 50 mg B1 and 100 mg B6, Schazoo Pharmaceutical Laboratories, Pakistan) were ground and the mass equivalent to one tablet was dissolved in methanol and the volume was made up to 100 mL with methanol. The working sample solution was prepared by 20-fold dilution with the mobile phase.

Forced degradation studies

Forced degradation studies were performed with standard solutions of PRML, B1 and B6 having final concentrations of 125, 25, 50 μg mL^{-1}, resp. Ten (10.0) mL of each standard solution was added into separate measuring flasks and treated with 10.0 mL of NaOH (0.1 mol L^{-1}), HCl (0.1 mol L^{-1}), and H_2O_2 (15 %, *V*/*V*) for 12 h to study the basic and acidic hydrolysis and oxidative stress, resp. Thermal and photolytic stress studies were performed after keeping the solid tablets at 100 °C for 6 h and exposing them to UV-light (254 nm) for 24 h, resp.

Method validation

Validation of the developed method included precision, linearity, specificity, accuracy, limit of detection (*LOD*), limit of quantification (*LOQ*) and robustness (22). Conformity of

chromatographic parameters, including the selectivity factor, tailing factor, resolution, theoretical plates, were also assessed while robustness was checked by deliberate changes in chromatographic conditions such as the flow rate (± 0.1 mL min^{-1}), column temperature (± 2 °C), wavelength (± 2 nm) and pH (± 0.1).

For accuracy of the proposed method, the tablet formulation was spiked with known amounts of API at 70 % (175 mg PRML, 35 mg B1 and 70 mg B6), 100 % (250 mg PRML, 50 mg B1 and 100 mg B6) and 120 % (300 mg PRML, 60 mg B1 and 120 mg B6) and analysis was performed after 20-fold dilution with the mobile phase. Percentage recoveries of PRML, B1 and B6 were assessed in triplicate and the results are presented as mean ± SEM. Precision of the proposed method was checked by injecting the standard solutions of PRML, B1 and B6 at three concentration levels (70, 100 and 120 % as defined in recovery studies). To measure intra-day and inter-day precision, standard solutions were injected on the same day and on three consecutive days, resp.

Linearity of the proposed method was determined for PRML, B1 and B6 using standard solutions having concentrations 87–150 μg mL^{-1} PRML, 17.5–30 μg mL^{-1} B1 and 35–60 μg mL^{-1} B6 and injected in triplicate to construct the calibration curve. *LOD* and *LOQ* were also determined from the calibration curves as 3.3 σ/S and 10 σ/S, resp., with σ being the standard deviation and *S* the slope of the regression line.

Analysis of a placebo solution including gelatin (40.0 mg), lactose (14.0 mg), Avicel (102.0 mg), primogel (25.0 mg), talc (15.0 mg) and magnesium stearate (4.0 mg) was made to check the specificity of the proposed HPLC method. Specificity was also measured by forced degradation studies.

Solution stability

Stability of the sample and standard solutions was estimated against freshly prepared standard solutions at intervals of 12, 24 and 48 h at room temperature.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The first step in method development was the selection of appropriate wavelengths for different compounds having different wavelength absorption maxima. A spectrum in the range of 200–400 nm showed acceptable absorbance at a wavelength of 270 nm for each analyte (Fig. 2) under investigation, which was chosen for quantification.

Optimization of the mobile phase was performed with a solution containing water and acetonitrile or methanol. It was observed that peaks of these three analytes were not completely separated. Different combinations of methanol, acetonitrile and phosphate buffer of different pH (3.0, 4.0, 5.0, 6.0, and 7.0) were then tested to optimize the chromatographic conditions including the tailing factor, resolution, theoretical plates and retention times. Different C_{18} and C_8 reversed phase columns were also tested including Venusil XBP C8, Hypersil ODS C18, ACE C8 and Agilent Poroshell C18 (Table I). Agilent Poroshell C18 (5 μm, 4.6 × 250 mm) reversed phase column with a mobile phase comprising phosphate buffer (pH = 3.0)/methanol/acetonitrile (86:7:7, *V*/*V*/*V*) pumped at a flow rate of 1.8 mL min^{-1}

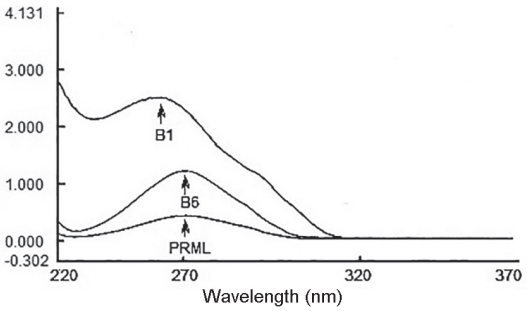

Fig. 2. Overlaid spectra of thiamine (B1), pyridoxal phosphate (B6), and paracetamol (PRML). mAU – milli absorbance units.

afforded the best separation of PRML, B1 and B6. The respective retention times found were 1.89, 2.41 and 6.29 min. Chromatographic parameters obtained under final conditions are summarized in Table II.

Method validation

For linearity assessment, six concentrations ($n = 3$) in the range of 70–120 % of the label claim of B1, B6 and PRML were employed (Table III). Coefficients of determination (R^2) were ≥ 0.998. The results demonstrate linearity of the developed method over the specified ranges. The placebo sample, namely excipients, showed no peak. Therefore, the proposed method is specific for APIs in the tablet, as shown in the chromatogram (Fig. 3).

Accuracy of the proposed method was estimated by recovery experiments. Recovery values (Table IV) for B1, B6 and PRML ranged from 100.4–100.6, 100.2–101.2 and 99.3–99.6 %, resp.

Table I. Stationary phase testing

Column	Analyte	R_s	T_f	α	N
Hypersil ODS C18 (250 × 4.6 mm, 5 μm)	B1	–	1.34	–	3487
	B6	5.34	1.23	–	7634
	PRML	18.79	1.54	8.752	4123
Venusil XBP C8 (250 × 4.6 mm, 5 μm)	B1	–	1.33	–	3452
	B6	8.96	1.02	5.623	8976
	PRML	21.98	1.32	8.534	3576
ACE C8 (250 × 4.6 mm, 5 μm)	B1	–	1.11	–	6954
	B6	7.97	1.42	7.986	4532
	PRML	27.13	1.12	5.987	7684
Agilent Poroshell C18 (250 × 4.6 mm, 5 μm)	B1	–	1.35	–	3452
	B6	8.21	1.27	–	6645
	PRML	34.99	1.16	6.987	7345

α – selectivity factor, B1 and B6 – vitamins B1 and B6, N – number of theoretical plates per meter, PRML – paracetamol, R_s – chromatographic resolution, T_f – tailing factor

Table II. Chromatographic parameters of the optimized HPLC procedure on the Agilent Poroshell C18 column

Parameter	B1	B6	PRML
Retention time (t_R, min)	1.89	2.41	6.29
Tailing factor (T_f)	1.48	1.39	1.52
Chromatographic resolution (R_S)	–	4.18	17.18
Separation factor (α)	–	–	8.75
Theoretical plates per meter (N)	1316	1497	4633
Retention time RSD (%)	0.003	0.002	0.002

B1 and B6 – vitamins B1 and B6, PRML – paracetamol

Table III. Regression and limiting values data for vitamins B1 and B6 and PRML

Parameter	B1	B6	PRML
Linearity range (μg mL^{-1})	17.5–30.0	35.0–0.0	87.0–150.0
Slope (mV mL mg^{-1})	81930	34352	52895
Intercept (mV)	–3645	–3145	23562
Standard error of slope (mV mL mg^{-1})	952.8	386.3	689.8
Standard error of intercept (mV)	22991	18646	83070
Coefficient of determination (R^2)	0.9995	0.9995	0.9993
Limit of detection (*LOD*, μg mL^{-1})	0.40	0.78	2.26
Limit of quantification (*LOQ*, μg mL^{-1})	1.20	2.33	6.77

B1 and B6 – vitamins B1 and B6, PRML – paracetamol

Table IV. Accuracy studies of vitamins B1 and B6 and PRML by the new HPLC method

Analyte	Spiked concentration (μg mL^{-1})	Concentration found (μg mL^{-1}, mean ± SEM; RSD, %)[a]	Recovery (%)
B1	17.5	17.6 ± 0.4; 0.6	100.4
	25.0	25.1 ± 0.5; 0.9	100.6
	30.0	30.2 ± 0.3; 0.4	100.6
B6	35.0	35.3 ± 0.4; 0.6	100.9
	50.0	50.1 ± 0.5; 0.9	100.2
	60.0	60.7 ± 0.3; 0.4	101.2
PRML	87.0	86.4 ± 0.4; 0.6	99.3
	125.0	124.4 ± 0.5; 0.9	99.6
	150.0	149.4 ± 0.25; 0.5	99.6

B1 and B6 – vitamins B1 and B6, PRML – paracetamol
[a] $n = 3$

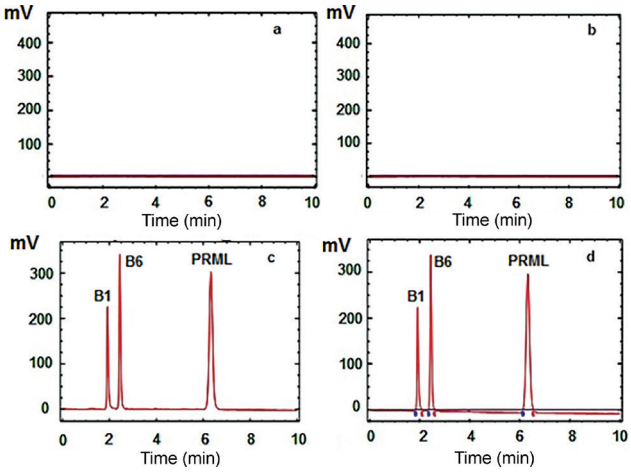

Fig. 3. Typical HPLC chromatograms of: a) mobile phase, b) placebo sample, c) standard mixture (125 μg mL^{-1} PRML, 25 μg mL^{-1} B1 and 50 μg mL^{-1} B6), and d) Neurobedoxine plus tablets (125 μg mL^{-1} PRML, 25 μg mL^{-1} B1 and 50 μg mL^{-1} B6) after dilution with the mobile phase. B1 and B6 – vitamins B1 and B6, PRML – paracetamol.

Intra-day and inter-day precision were assessed on the same day and on three consecutive days, resp. The results were within acceptable limits (RSD < 2 %), as shown in Table V.

Table V. Model precision and accuracy of vitamin B1, B6 and PRML analyses by the new HPLC method

Analyte	Within-a-day[a]		Inter-day[a]		
	Concentration (μg mL^{-1})	Concentration found (μg mL^{-1}, mean ± SEM; RSD, %)[a]	Concentration found (μg mL^{-1}, mean ± SEM; RSD, %)[a]		
			Day 1	Day 2	Day 3
B1	17.5	17.6 ± 0.2; 0.3	17.8 ± 0.6; 1.0	17.3 ± 0.5; 0.8	17.6 ± 0.3; 0.5
	25.0	25.3 ± 0.3; 0.5	25.2 ± 0.5;0.8	24.8 ± 0.5; 0.8	25.3 ± 0.7; 1.3
	30.0	29.9 ± 0.3; 0.5	30.7 ± 0.3; 0.5	30.4 ± 0.6; 1.0	30.1 ± 0.2; 0.4
	35.0	35.4 ± 0.5;0.8	35.6 ± 0.7; 1.2	35.3 ± 0.7; 1.2	35.2 ± 0.5; 0.8
B6	50.0	50.8 ± 0.5; 0.9	49.8 ± 0.6; 1.1	50.8 ± 0.8; 1.4	50.8 ± 0.7; 1.1
	60.0	61.3 ± 0.5; 0.8	61.7 ± 0.5; 0.9	60.6 ± 0.9; 1.4	59.6 ± 0.5; 0.9
	87.0	89.3 ± 0.1; 0.2	88.2 ± 0.9; 1.5	88.2 ± 0.9; 1.4	88.6 ± 0.6; 1.0
PRML	125.0	128.4 ± 0.7; 1.2	127.4 ± 0.5; 0.9	126.8 ± 0.6; 0.9	128.1 ± 0.3; 0.5
	150.0	153.0 ±0.3; 0.6	153.8 ± 0.8; 1.4	150.4 ± 1.0; 1.7	152.9 ± 0.5; 0.8

B1 and B6 – vitamins B1 and B6, PRML – paracetamol
[a] n = 5.

Table VI. Method robustness for vitamins B1, B6, and PRML in tablets

Chromatographic conditions	B1			B6			PRML		
	Assay (%)	t_R (min)	N	Assay (%)	t_R (min)	N	Assay (%)	t_R (min)	N
Flow rate 1.9 (mL min^{-1})	100.3	1.86	3564	100.9	2.38	6645	99.3	6.26	7350
Flow rate 1.7 (mL min^{-1})	100.2	1.93	3576	100.3	2.45	6623	99.7	6.31	7324
Column temp. (45 °C)	101.3	1.92	3579	99.5	2.44	6635	101.3	6.33	7367
Column temp. (35 °C)	101.7	1.93	3591	99.9	2.39	6667	101.6	6.32	7354
Wavelength (272 nm)	100.8	1.89	3518	101.3	2.46	6645	100.7	6.34	7329
Wavelength (268 nm)	100.7	1.90	3542	101.0	2.44	6668	100.4	6.33	7366
pH 3.1	99.3	1.90	3555	100.3	2.38	6623	100.1	6.29	7365
pH 2.9	99.9	1.92	3563	100.6	2.40	6634	100.4	6.27	7325

B1 and B6 – vitamins B1 and B6, PRML – paracetamol, N – number of theoretical plates per meter

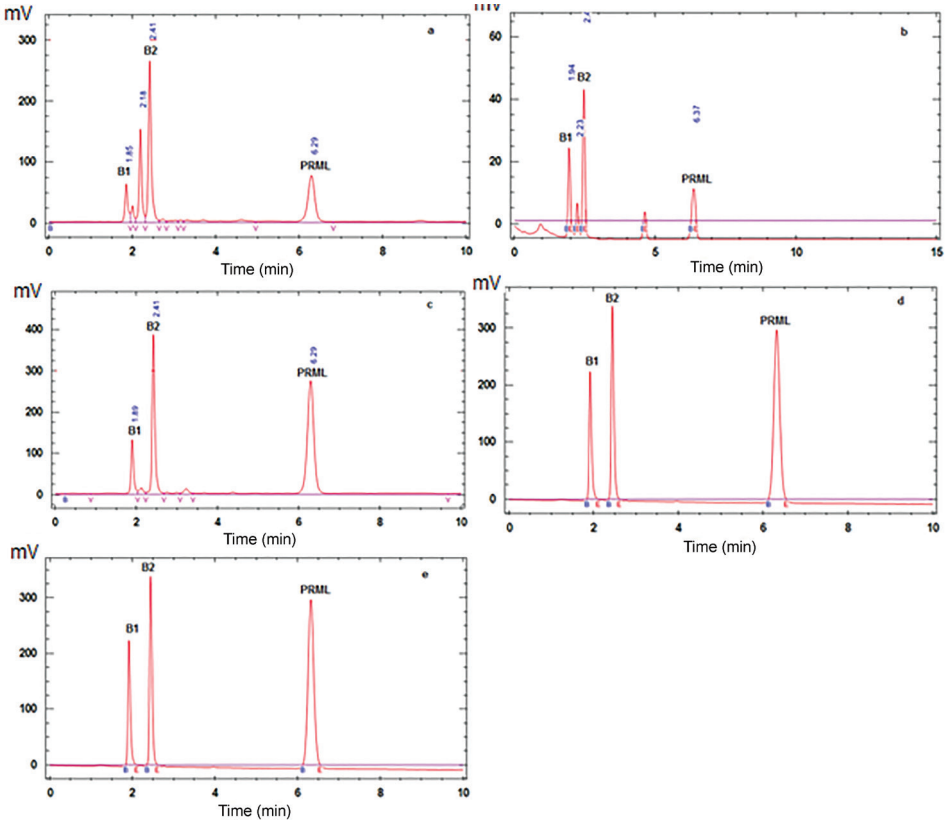

Fig. 4. Typical HPLC chromatograms under stress conditions: a) acidic, b) basic, c) oxidative, d) photolytic, and e) thermal. B1 and B6 – vitamins B1 and B6, PRML – paracetamol.

Table VII. Force degradation studies for vitamins B1 and B6 and PRML in tablets

Stress conditions	Component	Assay (%)	Extent of degradation
HCl (0.1 mol L^{-1}, 12 h)	B1	53.3	Substantial
	B6	88.3	Substantial
	PRML	60.3	Substantial
NaOH (0.1 mol L^{-1}, 12 h)	B1	98.2	None
	B6	87.3	Substantial
	PRML	70.9	Substantial
H_2O_2 (3 %, 12 h)	B1	94.3	Slight
	B6	97.5	None
	PRML	94.3	Slight
UV light (254 nm, 24 h)	B1	99.4	None
	B6	98.8	None
	PRML	100.2	None
Heating (70 °C, 6 h)	B1	99.3	None
	B6	98.9	None
	PRML	99.9	None

B1 and B6 – vitamins B1 and B6, PRML – paracetamol

Table VIII. Commercial tablet assay for B1, B6 and PRML

Product	Analyte	Taken from tablet (μg mL^{-1})	Found in tablet (μg mL^{-1})[a,b]	RSD (%)[b]	Analyte added (μg mL^{-1})	Total conc. found (μg mL^{-1})	Recovery (%)[a,b]	RSD (%)[b]
Neuro Bedoxine Plus tablets[c]	B1	50	50.5 ± 0.1	0.5	40.0	90.9	100.5 ± 0.2	0.6
					50.0	100.9	100.3 ± 0.2	0.7
					60.0	111.1	100.5 ± 0.2	0.5
	B6	100	101.6 ± 0.3	0.9	80.0	181.5	99.9 ± 0.2	0.7
					100.0	201.3	99.8 ± 0.2	0.5
					120.0	222.8	100.5 ± 0.3	0.9
	PRML	250	248.9 ± 0.7	0.9	200.0	450.1	100.3 ± 0.3	0.8
					250.0	500.7	100.4 ± 0.1	0.4
					300.0	551.5	100.5 ± 0.2	0.6

B1 and B6 – vitamins B1 and B6, PRML – paracetamol

[a] Mean ± SEM.

[b] $n = 10$.

[c] Neuro Bedoxine Plus tablet label claim: 250 mg PRML, 50 mg B1 and 100 mg B6.

Concentration of three APIs in the commercial tablet formulation was estimated by the proposed HPLC method. It was evidenced that the proposed HPLC method was precise (RSD 0.4–0.9 %) and accurate enough (recovery 99.8–100.5 %) for successful routine analyses (Table VIII).

CONCLUSIONS

In the literature, no stability indicating HPLC method is found available for simultaneous determination of paracetamol, thiamine, and pyridoxal phosphate. Stress testing under ICH specifications indicated that the proposed HPLC method is stability indicating and selective. The total run time of analysis was found to be less than 7 min. The proposed method showed high recovery and precision in the presence of excipients used in the formulation. It might be assumed that the proposed HPLC method is simple, precise, accurate, linear and sufficiently selective for routine simultaneous determination of paracetamol, thiamine and pyridoxal phosphate.

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