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Original Article

IMPURITY PROFILING OF THIAMINE HYDROCHLORIDE INJECTION BY RP-HPLC AND CHARACTERIZATION OF DEGRADATION PRODUCT BY LC-MS/MS/QTOF

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ABSTRACT

Objective: To propose a comprehensive, simple, and affordable RP-HPLC method for impurity profiling and characterization of unknown degradation products of thiamine hydrochloride injectable formulation.

Methods: The chromatographic separation employs gradient mode using the octadecyl silane column using a mobile phase consisting of phosphate buffer with ion pair reagent, acetonitrile, and methanol delivered flow rate at 1.2 ml/min. The detection was carried out at 248 nm using empower software. LC-MS/MS/QTOF hyphenated technique was used for isolation and characterization of unknown degradation impurity. The performance of the method was systematically validated as per ICH Q2 (R1) guidelines.

Results: Degradation product observed in accelerated stability was characterized by LC-MS/MS/QTOF hyphenated technique and found m/z value 351.1604 and postulated as an oxidative degradation product of thiamine due to excipient interaction. The validated method was sensitive, selective, and specific data proves the method is precise and accurate from LOQ to 150% level and results are within 95-108% and less than 4.5% RSD. The developed method is linear from 0.03-58.83 µg/ml with a correlation coefficient of more than 0.990 and LOD and LOQ value ranged from 0.03 to1.51 µg/ml.

Conclusion: An efficient RP-HPLC method for impurity profiling of thiamine injectable formulation was successfully developed and unknown degradation product observed instability condition samples characterized by LC-MS/MS/QTOF technique. The validated method can be successfully employed for the impurity profiling of thiamine injectable in the quality control department.

Keywords: Thiamine hydrochloride injection, RP-HPLC, Identification, Characterization, Degradation products, Validation, LC-MS-MS

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INTRODUCTION

Thiamine hydrochloride (THI) or Vitamin B1, a water-soluble vitamin, and the B1 family consist of thiamine, a pyrimidyl substitutedthiazole, and its phosphate esters, thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiamine triphosphate. Vitamin B1 is an important cofactor for enzymes involved in amino acid and carbohydrate metabolism in humans. Severe deficiency of vitamin B1 is characterized by cardiovascular (wet) beriberi, neurological (dry) beriberi, and Wernicke–Korsakoff syndrome [1], and less commonly, lactic acidosis (Shoshin beriberi) [2]. Recent studies have suggested that vitamin B1 deficiency may also play a role in patients with diabetes, obesity, and heart failure [3-8].

A survey of literature revealed that some analytical methods, including UV and visible spectrophotometric and multivariate analysis [9-11], fluorimetric [12-24], capillary zone electrophoresis [25], by simultaneous HPLC methods [26-36] and UHPLC/MSMS [37], turbidimetric and nephelometric [38], amperometric [39], voltammetric [40-46] methods have been reported for thiamine and its esters in API, pharmaceutical formulations, food material and in biological fluids. Some reports were also available on the kinetic and thermal behaviour of thiamine [47, 48]. Official methods for the assay of thiamine salts were based on non-aqueous titration [49] and liquid chromatography [50] has been reported. An extensive literature survey revealed that no reports were available for impurity profiling and characterization of degradation products of THI injectable formulation in any journal.

The impurity, which may appear in the final product as a process related or by-products or catalysts or by hydrolyzed or oxidative residues or by adducts or as degradants due to potential drug-drug interactions and drug-excipients interactions. Thus, regulatory guidelines and pharmacopeias extensively emphasized on impurity profiling of pharmaceutical formulations to control the quality of products and guidelines made mandatory to identify and characterize the unknown degradation products [51] if present above the acceptable limit [52]. Regulatory agencies also recommend adopt a scientific approach for method development and perform extensive stress studies followed by validation of ICH Q2 (R1) guidelines [53] to develop stability-indicating methods (SIMs) for the analysis of stability samples [54].

As there is no method published so far, the authors felt it is essential to develop the comprehensive, simple, and affordable chromatographic technique for impurity profiling of THI in an injectable formulation. Therefore, herein report new simple stability-indicating HPLC method, which will serve as a reliable, precise, accurate, and sensitive for impurity profiling of THI impurities in the injectable formulation and successfully validated according to ICH Q2 (R1) guidelines. During routine stability evaluation of the injectable formulation, it was observed one unknown degradant product observed more than the acceptable threshold; hence this is identified by using LC-MS/MS hyphenated technique and structure was correlated. The chemical structures of THI and their known related impurities were shown in fig. 1

MATERIALS AND METHODS

Instruments

Chromatographic separations were performed on Waters HPLC system (Waters Corporation, Milford, USA) equipped with a quaternary pump, auto-sampler, online degasser, Photo-diode array (PDA) detector (e2998, Waters corporation, Milford, USA) and a rheodyne injector with a 100 µl injector loop (Waters corporation, Milford, USA). The liquid chromatography (LC) hyphenated with quadrupole time-of-flight mass spectrometry (Q-TOF-MS) (Waters micro mass, Manchester, UK) operated with MassLynks software was employed for characterization study. Photo-stability studies were carried out using Newtronic photo-stability chamber (Newtronic lifecare equipment Pvt. Ltd., India) and thermal degradation studies were performed in Newtronic hot air laboratory

oven (Newtronic lifecare equipment Pvt. Ltd., India). Ultrasonic bath (PCi analytics, India) was used to dissolve the samples. The pH values of different buffer solutions were checked and adjusted using a Thermo Scientific pH-meter (Orion star series, India) equipped with a combined glass-calomel electrode. Sartorius analytical balance (Sartorius analytical lab, Germany) was used for weighing materials. Evoqua water technologies system (Ultraclear GP, Germany) was used for HPLC grade water for all preparations.

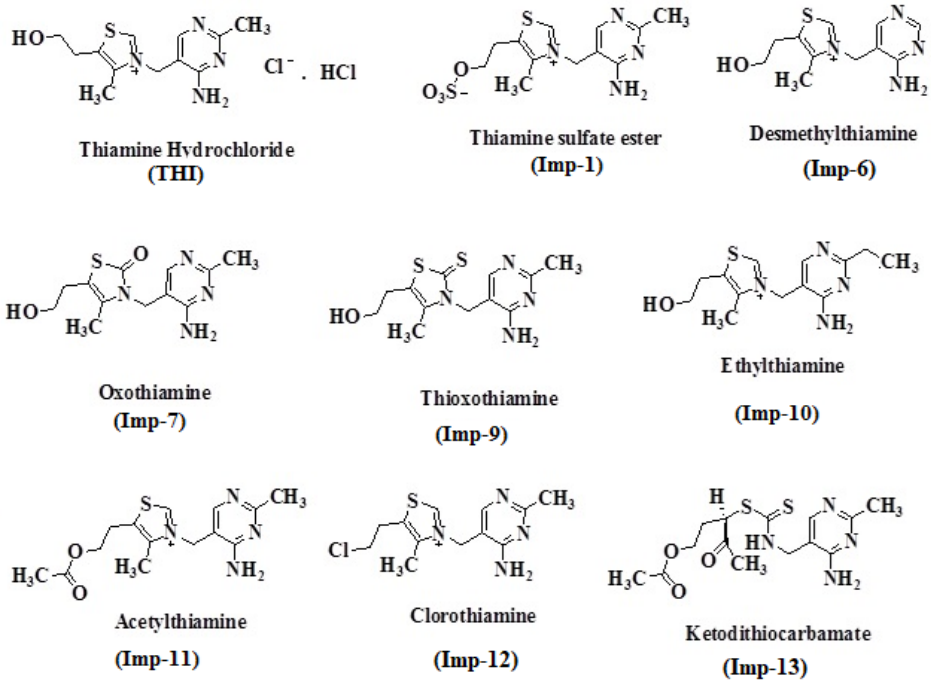

Fig. 1: Chemical structures of THI and known related impurities. Impurities numbering was given according to their elution pattern

Materials and reagents

The working standard of THI provided by Aurobindo Pharma Limited Research Centre, Hyderabad, with a purity of 95.5±1.0 % by applying the official method [53]. Related impurities of THI, namely Thiamine sulfate ester (purity 99.8%), Desmethyl thiamine (purity 99.8%), Oxothiamine (purity 97.8%), Thioxothiamine (purity 98.9%), Ethylthiamine (purity 98.5%), Acetylthiamine (purity 98.0%), Chlorothiamine (purity 98.5%) and Ketodithiocarbamate (purity 94.7%) and injection formulation samples were supplied by APL Research Centre (Aurobindo Pharma Limited, Hyderabad, India). Specified known imp-1 (DP-3, purity 85.7%), Specified known imp-2 (DP-4, purity 76.1%), and Specified known imp-3 (DP-5, purity 88.5%) have been synthesized. The sodium salt of hexane-1-sulfonic acid, Glacial acetic acid, Acetonitrile (HPLC grade), Methanol (HPLC grade), were procured from Merck Inc., Mumbai, India. Orthophosphoric acid (~88%), Hydrochloric acid (~35%), Hydrogen peroxide (~30%) were procured from Rankem, India. HPLC grade water was used for all preparations. A series of buffer solutions of different pH were prepared by following the standard methods [55].

Preparation of buffer solution

Dissolved 3.8 g of the sodium salt of hexane-1-sulfonic acid in 1000 ml of water. Adjusted the pH to 3.1±0.05 with diluted orthophosphoric acid and filtered the solution through a 0.45 µm membrane filter.

Preparation of diluent

Dissolved 10 ml of glacial acetic acid in 1000 ml water and mixed well.

Methods

HPLC system and chromatographic conditions

All separations were performed on Waters HPLC system and operated with Empower-3 software for data acquisition and processing. The analysis was carried out by using octadecylsaline column of make Inertsil (ODS-3V) having dimensions 250 mm x 4.6 mm id 5 µm particle size column ((GL Sciences, UK). The mobile phase consists of pH 3.1 buffer, acetonitrile and methanol in the ratio of 90:8:2 (% v/v/v) (A) and pH 3.1 buffer, acetonitrile and methanol in the ratio of 50:35:15 (% v/v/v) (B) and run with 1.2 ml/min flow rate in gradient mode of program: Time $_{(min)}$/A (v/v): B (v/v); T $_{0.01}$/98:2, T_3/98:2, T_{25}/95:5, T_{35}/89:11, T_{50}/72:28, T_{60}/64:36, T_{80}/5:95. The column oven temperature maintained at 40 °C. Samples were monitored and detected at wavelength maxima 248 nm by injecting sample volume 10 µl and data was acquired for 80 min.

LC-MS/MS instrumentation and chromatographic conditions

The LC-TOF-MS operated with MassLynks software was employed for characterization study. The fragmentation profile was established on positive electrospray ionization (+ve ESI) mode with the following parameters; hexapole RF, capillary voltage 3000 v, cone voltage 25 v, source temperature at 120 °C, desolvation temperature 375 °C, ion energy 1.0 v and the collision energy was set to 8 v. For the MS/MS operation, argon was used as a collision gas with energy 25 v and mass spectra was acquired over an m/z range of 100–1000 range. The MS settings refer to a previous study [56]. The separation was performed on mass compatible HPLC method using Zorbax SB-Aq, 150 mm × 4.6 mm id, 5 µm particle size column (Agilent Corporation, USA) with mobile phase consists of 0.02 mol formate buffer with 0.1% formic acid and acetonitrile/0.02 mol formate buffer with 0.1% formic acid as solution A and B respectively. The chromatography was developed with flow rate 0.8 ml/min by maintaining column oven temperature at 25 °C and monitoring analytes at 248 nm by injecting 10 µl sample volume in gradient mode program as follows: Time $_{(min)}$/A(v/v):B (v/v); T $_{0.01}$/90:10, T $_{8.0}$/45:55, $T_{8.1}$/90:10, T $_{13.0}$/90:10.

Preparation of standard solution

Accurately weighed and transferred about 20 mg of THI standard into 100 ml volumetric flask. To this added 80 ml of diluent, we sonicated to dissolve and cool to room temperature. Diluted to

volume with diluent and mixed well. Further, diluted 2.0 ml of this solution to 100 ml with diluent to get a concentration of 4 µg/mland mixed well.

Preparation of sample solution

The sample solution was prepared by pooling sufficient THI injection (100 mg/ml) vials and transferring 1 ml of a test sample into a 100 ml volumetric flask. Diluted to volume with diluent to get a concentration of 1.0 mg/ml and similarly placebo solution was prepared.

Forced degradation studies

Stress degradation studies were conducted to determine the stability of products to know the degradation pathways and to observe and identify the likelihood of degradation products under controlled exposure conditions such as hydrolysis, oxidation, dry heat, and photolytic [57]. Stress degradation studies were carried out on THI injectable formulation as per prescribed stress conditions ICH Q1A (R2) [58]. Multiple stressed samples were prepared in this study. Forced degradation of THI samples in the acidic and basic medium was carried out by sample solution containing 1.0 mg/ml of THI was treated with 5M HCl and 5M NaOH at 60 °C for 60 min on water bath respectively. The solutions were neutralized as needed (5M NaOH or 5M HCl) before makeup to volume. Oxidative degradation was carried by subjecting a sample solution containing 1.0 mg/ml of THI was treated with 30% v/v hydrogen peroxide at 85 °C for 120 min on the water bath. During thermal degradation, THI sample was subjected to thermal condition at 85 °C for 72 h in hot air oven and after THI, a thermal degradation sample concentration of 1.0 mg/ml has been prepared. Photolytic degradation was carried by exposing the THI sample to artificial light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200Wh/m^2. Similarly, placebo is also treated in the same way in each condition separately. All stressed samples analyzed by HPLC and samples were evaluated for the appearance of additional impurities and degradants. The impurities were identified on the basis of respective placebos and results were shown in table 3.

RESULTS AND DISCUSSION

The main objective of the study is to propose a simple, selective, sensitive and affordable chromatographic method for the impurity profiling of THI in injectable dosage formulation. The impurity profiling indicates the qualitative and quantitative determination of impurities and degradation products and identification and characterization of unknown degradation products if appear more than the identification threshold. Optimization of chromatographic conditions for THI impurity profiling has been carried out systematically as follows as.

Selection of mobile phase and the effect of pH

The knowledge of physicochemical properties of THI drug such as solubility, partition coefficient (log P), acid dissociation constant (pKa) values are critical parameters which will determine and significantly influence the selecting of a suitable buffer. Drug (THI) is freely soluble in water and has Partition coefficient (logP) value-3.1 indicates hydrophilic in nature and dissociation constant (pKa) value of 5.54 indicates 100% dissociation at this pH and all impurities has more or less similar hydrophilicity and dissociation constant as that of Drug, as these are highly polar, best chromatographic separation can be achieved on non-polar stationary phase column with ion pair reagent as a modifier in mobile phase rather than on polar columns, where polar analytes elute as clusters and achieving separation is very difficult. The advantage with ion pair reagent on a non-polar column is that anionic part [sulfate] interacts with analytes and the non-polar part binds to the non-polar chain of the column hence retains the polar analytes, thereby increases the retention of the polar analytes. Based on this cause someone of the report was available [59]. Hence, ion pair reagents sodium salt of pentane, heptane, and octane sulfonic acids were selected for chromatographic separation. Optimum chromatographic separation was observed with hexane, heptane and octane ion-pair reagents as compared to pentane ion-pair reagent. Therefore, hexane ion pair reagent has been introduced in the buffer system as optimum ion pair reagent. As the dissociation constant value of THI is 5.52, the pH related changes in retention time will not be effective above or below 2 pH units. However at above 2 pH units of pKa, THI is unstable and undergoes degradation and below 2 pH units of pKa i.e.: pH 3.5, THI will be a completely ionized form which is suitable for effective separation of peaks. To obtain effective separation among the analytes, optimization is required with different stationary phases makes, selection of suitable organic modifiers and optimization pH of mobile phase to be considering using hexane sulphonic acid ion-pair reagent.

a. The feature of the stationary phase (octylsilane and octadecylsilane columns with different dimension and makes).

b. Mobile phases containing ion pair reagent with different gradient elution with different pH's like 3.5, 3.2, 3.0, 2.8, and pH 2.5 with organic modifier's acetonitrile and methanol individually and in combination.

Influence of stationary phase

Among the non-polar columns, octylsilane and octadecylsilane stationary phase columns are the choice of columns to retain and control the retention time of polar analytes with strong ion-pair reagents, which are in accordance with available literature data [60]. During optimization with octylsilane stationary phase column, it was noticed that near void region the degradation products are co-eluted and no resolution between Imp-6 and Imp-7 and Imp-11 and Imp-12 peaks. In-case of octadecylsilane column, early eluting peaks have improved resolution among peaks. Therefore, octadecylsilane stationary phase has been finalized. Moreover, the affinity of all impurities toward different commercially available octadecylsilane column has been evaluated to get the optimum resolution between critical pairs of impurities. Among all commercially available octadecylsilane columns, Inertsil ODS–3V column has shown better separation for all impurities and reason might attribute to its high surface area, more hydrophobicity, and carbon load as compare to other commercially available columns. Moreover, separation and resolution among all components also have been improved slightly showing resolution more than 1.5 and tailing factor ≤ 2.0 by using long dimension column i.e.: 250 mm over 150 mm column length. However, particle size and internal diameter (id) have no impact on resolution. Hence, Inertsil ODS-3V, 250 x 4.6 mm id, 5µ particle size selected as a suitable column for better separation and resolution. In addition to the stationary phase, organic modifiers and other method parameters also play an important key role which leads to better separation.

Influence of organic modifier

From the chromatographic perspective, organic modifiers substantially influence (selective of the analytes, most common modifiers are methanol and acetonitrile because of their most compatible properties. Hence, the separation was optimized with a selected buffer with 100% methanol/acetonitrile and different ratios of acetonitrile and methanol as in the mobile phase. When methanol content was more in the mobile phase composition resulted in high retention of the peaks with a poor resolution between critical pair of impurities i.e.: Imp-6 and Imp-7 and Imp-8 and THI. To improve the resolution between critical pairs and reduce the retention time of peaks, the acetonitrile ratio has been increased systematically as it has strong elution strength with low viscosity as compared to methanol. The chromatographic separation was optimized between critical pairs and among other peaks with about 50% ratio of acetonitrile and about 15 to 20% of methanol as an organic modifier in the mobile phase.

Influence of gradient elution and effect of pH

Even though the impurities and the THI have similar polarity, the minute difference in polarity of individual impurities is crucial for separation; hence input of organic modifier ratio is controlled in gradient mode program to get the optimized separation. When the ratio of methanol was more in gradient composition, the separation among early eluting degradation products was critical and not achieved, and peak shapes of Imp-10 and Imp-11 were broad and resulting longer retention time in a specified program. Hence, acetonitrile input in gradient program has increased gradually to

elute the highly retained impurities and improve the resolution of critical pairs. Mobile phase buffer pH is critical for the good separation of early eluting degradant unknown impurities and peaks adjacent to main analytes, when mobile phase pH 3.5, early eluting peaks eluted with a poor resolution less than 1.5 and when pH of the mobile phase is decreased resolution is increased. However, when the pH of the mobile phase is decreased less than 3 the adjacent peaks were co-eluting with the main analyte and at pH 3, there were well separated but Imp-4 and Imp-5 and unknown and THI peaks were not well resolved and a resolution was observed more than 1.5 when pH is adjusted to 3.1. After systematic evaluation, the method was finalized on Inertsil ODS 3V, 250 mm×4.6 mm, 5 µm using a variable composition of solvent A: Buffer (0.02 M Hexane sulfonic, pH 3.1), acetonitrile, methanol in the ratio of 90:8:2 (%v/v/v) and solvent B: buffer, acetonitrile, methanol in the ratio of 50:35:15. The solvent A and solvent B pumped through the column at a flow rate of 1.2 ml/min with gradient elution follows as Time $_{(min)}$/A (v/v): B (v/v); $T_{0.01}$/98:2, T_3/98:2, T_{25}/95:5, T_{35}/89:11, T_{50}/72:28, T_{60}/64:36, T_{80}/5:95 with the column oven temperature was maintained at 45 °C to get the sharpness of the peaks. In the above optimized chromatographic conditions, all peaks are well separated with resolution (Rs)>1.5 among all peaks having symmetrical peak shapes. Lowest and maximum detection peak response with good reproducibility is achieved with injection volume 10 µl without compromising resolution between closely eluted peaks.

Selection of wavelength and optimization of diluent

Spectral absorption maxima scan of THI and its impurities on the PDA detector shown absorption maxima at 248 nm. Hence the same wavelength was selected for identification and quantification. Drug (THI) is in hydrochloride salt form and freely soluble in water and organic solvents. Hence, water or a binary mixture of water and organic solvents are the most probable choice of diluents. However, asymmetrical peak shape for early eluting peaks and main analyte were observed in these combinations. As the compound is basic, peak shapes of analytes have been improved by acidifying diluent with acetic acid. Hence reduced the interaction of the basic molecule with the stationary phase and increased the peak symmetry for all analytes. The same diluent has been chosen for standard and sample preparations in the method development and further validation activity. The typical chromatograms of placebo solution and spiked sample with all known related impurities were given in fig. 2.

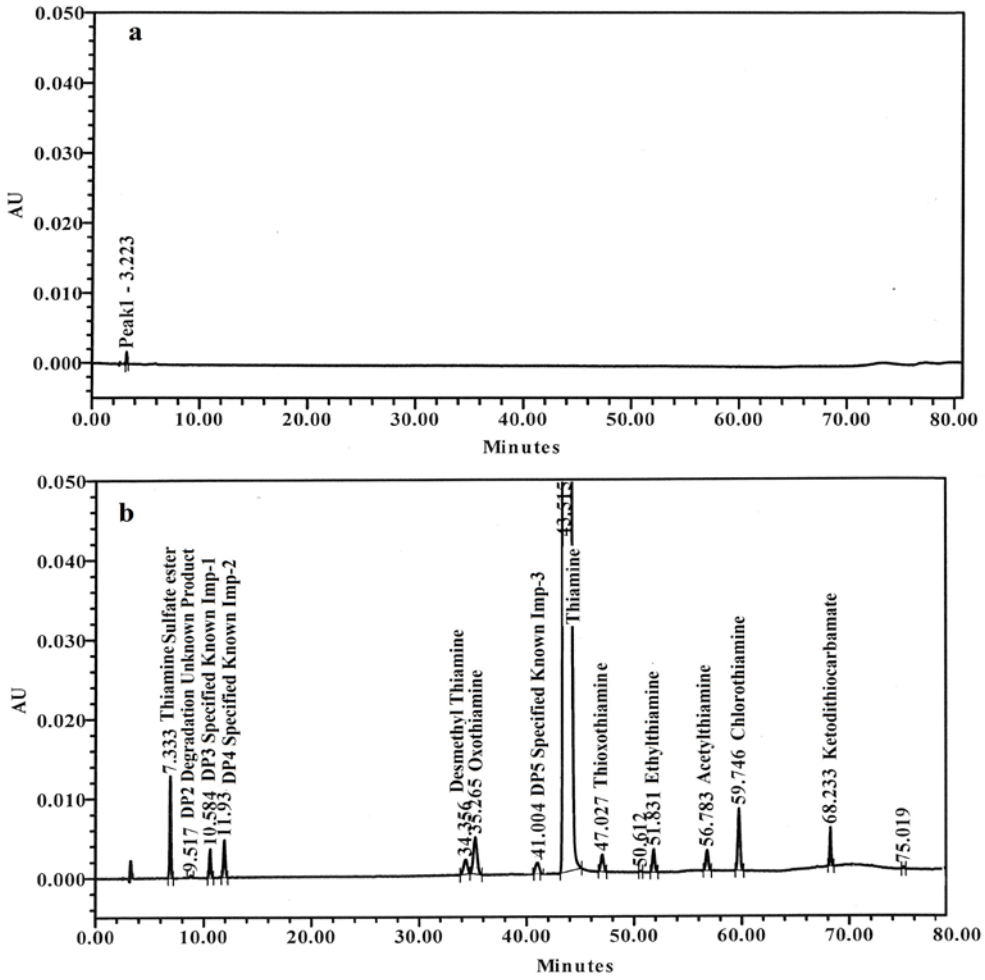

Fig. 2: Typical HPLC chromatograms of (a) placebo solution and (b) spiked sample with all known impurities

Detection of impurities by HPLC

As per stability guidelines ICH Q1A (R2), routine stability evaluation of THI injection formulation was analyzed by using developed HPLC method and resulted few unknown degradation impurities that were observed above the identification threshold. In these, impurity at RT 8.513 min, 10.531 min, 11.766 min and 36.227 min are corresponding to specified known Imp-1, 2, 3 and 4 respectively. The impurity at 9.526 min is an unknown degradation impurity and named as unknown depredation product (DP-2). It is necessary and mandatory to identify unknown degradation impurity in finished dosage forms as per regulatory guidelines. A typical HPLC chromatogram has been displayed in fig. 3. Here, system suitability parameters of impurities from the spiked sample and stability sample were captured in table 1.

Table 1: Results of system suitability testing parameters for spiked sample with known impurities

S. No.	Name	Analytes	RRT*	(Rs)**	(T)	(N)
1	Imp-1	Thiamine Sulfate ester	0.17	--	1.01	11715
2	Imp-2	DP-1-Specified Unidentified impurity#	0.20	--	1.04	11605
3	Imp-3	DP-2-Degradation Unknown product #	0.22	1.64	1.02	11646
4	Imp-4	DP-3-Specified Known Impurity-1	0.27	9.51	0.97	11563
5	Imp-5	DP-4-Specified Known Impurity-2	0.31	3.94	1.0	17379
6	Imp-6	Desmethyl thiamine	0.77	24.23	0.92	16810
7	Imp-7	Oxothiamine	0.81	1.52	0.94	20957
8	Imp-8	DP-5-Specified Known Impurity-3	0.91	5.18	0.99	31256
9	THI	Thiamine	1.00	3.63	1.61	68841
10	Imp-9	Thioxothiamine	1.09	5.67	1.0	54603
11	Imp-10	Ethylthiamine	1.18	8.10	1.01	143874
12	Imp-11	Acetylthiamine	1.29	10.90	0.98	202231
13	Imp-12	Chlorothiamineor	1.35	6.62	0.99	214833
12	Imp-13	Ketodithiocarbamate	1.61	23.65	1.01	117806

#Thiamine degradation impurities have been included from stability sample data, *RRTs of impurities established with respect to THI main peak, ** Resolution determined between each analyte peak and previous one.

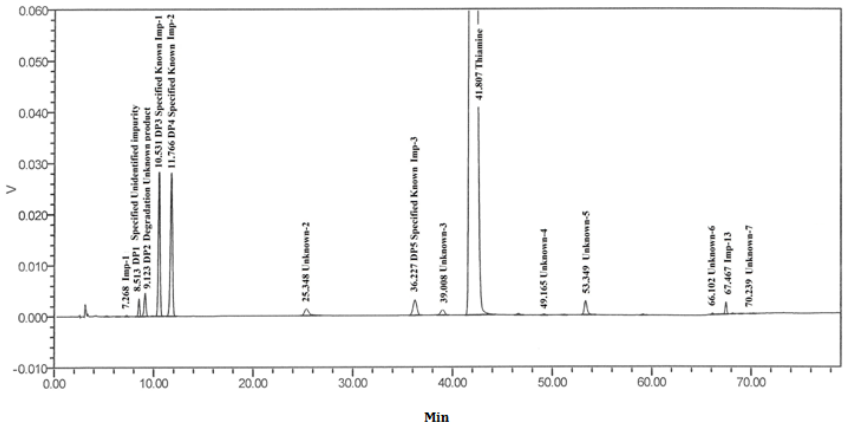

Fig. 3: Typical HPLC chromatogram of THI injection accelerated stability sample with DP-2

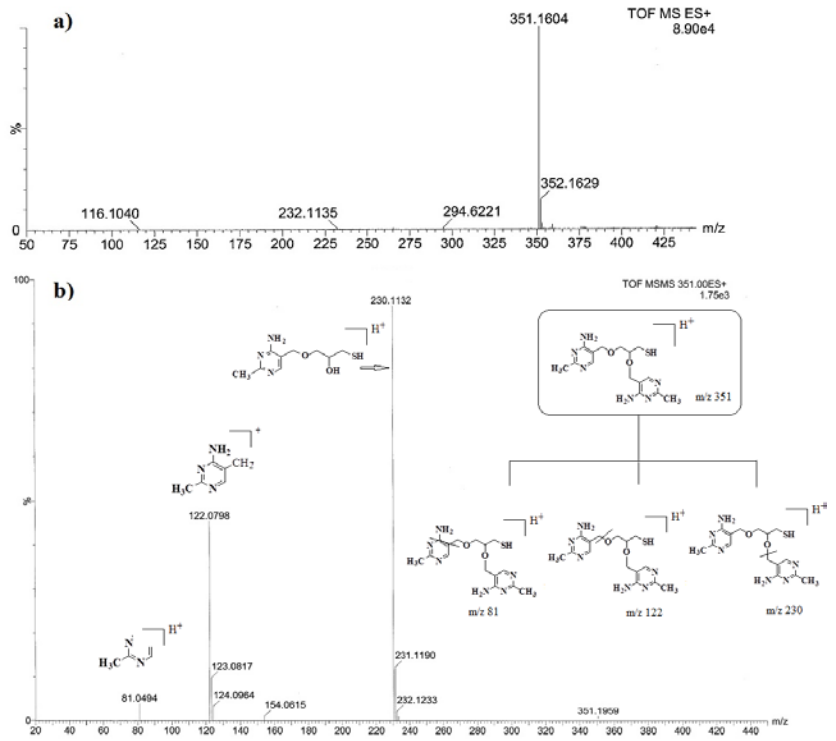

Fig. 4: (a) Mass spectrum of DP-2 and (b) MS/MS spectrum of DP-2

LC-MS and MS/MS characterization and interpretation

Identification of degradation unknown impurity (DP-2) formed in accelerated stability samples has been carried out by LC-MS/MS/QTOF hyphenated techniques to find out for possible structural elucidation. Identification of degradation products has been a general approach for structure elucidation using LC-MS [61, 62]. Since the mobile phase used in the HPLC method for related impurities analysis of THI product was not compatible with the mass instrument, a new LC-MS compatible method was developed as given in LC-MS/MS analysis experiment. The LC-MS scan was recorded in both positive and negative ion modes. However, positive ion mode produced the most intense and stable MS spectrum for DP-2. The positive ion MS-ESI$^+$spectrum of DP-2 shown $[M+H]^+$ion peak at m/z 351.1604 predominantly with fragments at m/z 230.1132, 122.0798, and 81.0494 (fig. 5). The fragments m/z 122.0798 and 81.0494 are corresponding to characteristic fragments pattern of Imp-5 and mass spectra were in given fig. 4 and 5 for structural correlation inspection. To confirm the molecular formulae of the DP-2, these measured masses were plugged into the elemental composition calculator with reasonable limits of elements (i.e. carbon, hydrogen, nitrogen, oxygen, and sulfur) was set. The search revealed several theoretically possible molecular formulae. The closest possible molecular formula for protonated molecular ion was selected based on the lowest difference in mass between theoretical and observed values. Therefore, based on the mass spectral data support and information of the injection formulation process and excipients were taken into account to proceed for impurity structure elucidation. Hence, DP-2 tentatively concluded as an oxidative degradation product of thiamine. The plausible reaction mechanism for the formation of DP-2 was proposed based on the reactivity of the THI present in the injection formulation (fig. 6).

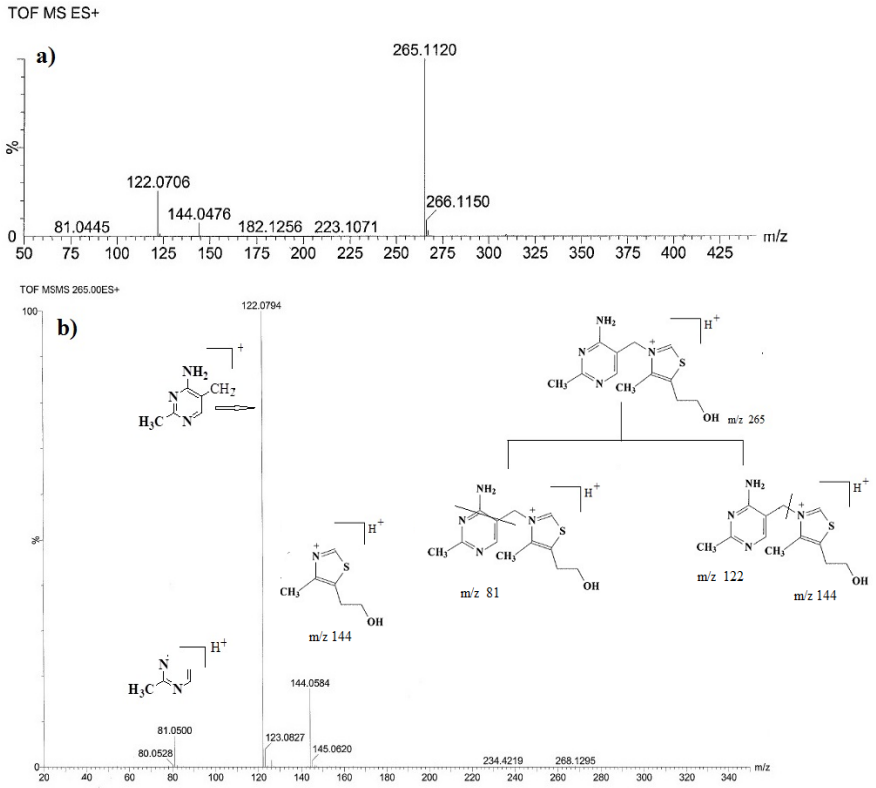

Fig. 5: (a) Mass spectrum of THI and (b) MS/MS spectrum of THI

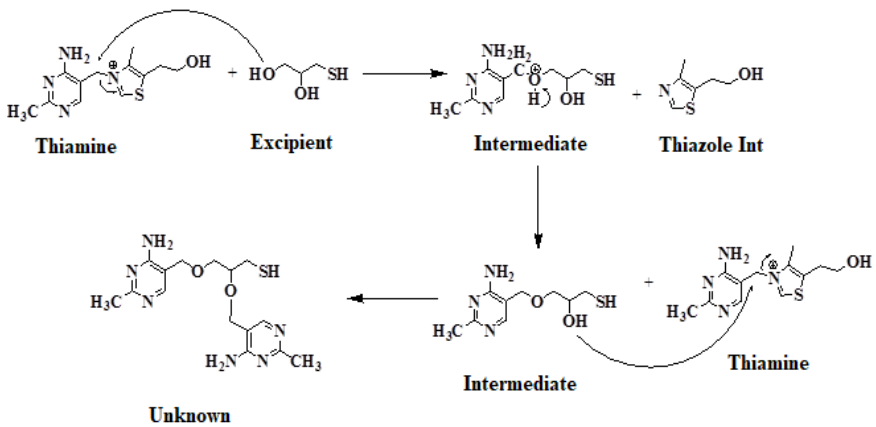

Fig. 6: Scheme for formation of DP-2 in THI injection sample

Method validation

The optimized method was systematically validated according to ICH Q2 (R1) guidelines, which include parameters like system suitability, linearity, accuracy, precision, robustness, and solution stability.

System suitability

The system suitability test is an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed method. System suitability parameters with respect to tailing factor, plate count, and % relative standard deviation (RSD) were assessed by injecting a blank followed by standard solution (n=6) (4 µg/ml). The % RSD of peak areas of standard solution (acceptance limit % RSD<5) has been determined. The results obtained were all well within acceptable limits and given in table 2.

Specificity

Specificity is the ability of the analytical method to measure the analytes accurately in the presence of all other potential impurities, degradation products, and excipients if any. All known impurities, spiked sample, worst case placebo at high concentration level and samples exposed to various specified stress conditions like hydrolysis, oxidation, photolytic, humidity/heat and thermal degradation samples are evaluated in optimized chromatographic conditions on water HPLC system equipped with PDA detector to establish the selectivity, specificity, and stability-indicating power of the developed method. Peak purity test was carried out by using a PDA detector in all stress samples and it is found that all are meeting the acceptance criteria (purity angle<purity threshold), indicating that no peaks are co-eluting. Peak purity test data show that analyte chromatographic peaks are not attributed to more than one component [63, 64]. Hence, the proposed related impurities method is specific and stability-indicating. As stress data show, about more than 5% degradation is observed in specified stress conditions except for humidity condition and photolytic stress indicating that THI was stable under this stress condition [65]. All the degradation products are well separated from each other and the method is found to be stability-indicating specific and selective technique and its results were summarized in table 3.

Table 2: System suitability and statistical parameters of standard solution

Parameter	THI
Retention time (min)	42.9
USP plate count (N)	87126
USP tailing factor (T)	1.0
Mean area*	73859
(%) RSD	0.5
95% Confidence interval (±)	350

*Mean from six replicate injections of standard solution

Table 3: Optimized stress condition of THI Injection

Name of condition	Stress exposure	Time	Temperature (°C)	Related impurities						% Total degradation
				Imp-1	Imp-4	Imp-5	Imp-8	Imp-11	Unknow-1	
Control	--	--	--	0.01	0.23	0.34	0.05	-	-	0.66
Acidic	5.0 M HCl	60 min	60	0.01	0.30	0.4	0.06	0.16	0.05	2.44
Basic	5.0 M NaOH	60 min	60	0.01	0.53	0.8	0.04	-	0.73	7.69
Oxidation	30% H_2O_2	60 min	85	0.15	0.63	0.28	1.7	-	1.95	14.6
Thermal	--	72 h	85	0.03	4.43	3.12	2.42	-	0.42	16.2
Photolytic	250Wh/m2	168 h	--	0.01	0.24	0.347	-	-	0.03	0.68
Humidity	90% relative humidity	72 h	25	0.01	0.23	0.338	-	-	0.03	0.74

Linearity

Linearity test was performed at eight concentration levels in triplicates from 1% to 150% concentration level to demonstrate the detection capability of the developed method. The detailed concentration ranges were shown in table 4 and the linearity graph was plotted with peak areas of the analytes from the chromatogram against the respective concentration of analytes to obtain the analytical curve. The results were subjected to the regression coefficient by the linear least-square regression method and found regression coefficient (r^2)>0.99 for all analytes thus demonstrates good linear response for all impurities across the concentration range of interest. From the respective linearity graph, response factor (RF) of each component has been established and corresponding RF values were tabulated in table 4.

Sensitivity parameters

Sensitivity parameters of the limit of detection (LOD) and limit of quantification (LOQ) concentration was established by using residual standard deviation (σ) and slope (S) of calibration method by using Linearity experiment data and values predicted using formula 3.3 σ/slope for LOD and 10 σ/slope for LOQ and established precision (n=6) at these predicted concentration levels. The results were summarized in table 4.

Precision and accuracy

Method precision was performed on six sample solutions (n=6) prepared individually using spiked sample at specification level and intermediate precision also called ruggedness also evaluated by a different analyst (n=6), by using different column and different HPLC system on different days as per the methodology and found % RSD within the acceptance criteria and results tabulated in table 5. Method precision and ruggedness experiment shows that the related impurities method has repeatability and reproducibility.

The accuracy of the method was assessed by preparing triplicate (n=3) sample solutions spiked with all known related impurities from LOQ level to 150% concentration level at four different levels at LOQ, 50%, 100%, and 150% and analyzed as per methodology. The experimental result revealed that recoveries were well within the acceptance criteria for all the impurities and demonstrates the quantitative assessment power of the method for the intended purpose of routine analysis and results were summarized in table 6.

Table 4: Results of linearity and sensitivity parameters

Analytes*	Regression equation**	(R^2)	(R)	Linearity range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	RF
Imp-1	Y=20176X-197	0.9998	0.9999	0.06–9.12	0.09	0.28	1.05
Imp-4	Y=7706X+233	0.9998	0.9999	0.39–58.83	0.37	1.11	2.66
Imp-5	Y=19850X-21	0.9998	0.9999	0.17–25.55	0.17	0.52	1.03
Imp-6	Y=21517X-1318	0.9996	0.9998	0.06–9.28	0.13	0.40	0.98
Imp-7	Y=27149X-4972	0.9990	0.9995	0.19–9.49	0.39	1.17	0.77
Imp-8	Y=20907X-1028	0.9998	0.9999	0.03–4.52	0.04	0.12	0.98
THI	Y=21091X-3350	0.9998	0.9999	0.30–45.06	0.50	1.51	1.00
Imp-9	Y=15730X-3894	0.9984	0.9992	0.20–9.93	0.53	1.26	1.01
Imp-10	Y=23852X-514	0.9998	0.9999	0.13–6.46	0.36	0.83	0.87
Imp-11	Y=18971X-631	0.9998	0.9999	0.16–7.77	0.10	0.32	1.09
Imp-12	Y=22669X-1146	0.9998	0.9999	0.06–9.09	0.10	0.31	0.93
Imp-13	Y=22072X-1001	0.9994	0.9997	0.22–10.94	0.34	1.02	0.89

*Thiamine Sulfate ester (Imp-1); DP-3 Specified Known Impurity-2 (Imp-4); DP-4 Specified Known Impurity-3 (Imp-5); Desmethyl thiamine (Imp-6); Oxothiamine (Imp-7); DP-5 Specified Impurity-4 (Imp-8); Thioxothiamine (Imp-9); Ethylthiamine (Imp-10); Acetylthiamine (Imp-11); Chlorothiamineor (Imp-12); Ketodithiocarbamate (Imp-13). ** Y is the peak area and X is the concentration injected

Table 5: Results of precision data

Analytes	Method precision (M) (n=6)#		Ruggedness (R) (n=6)#		(M+R) overall
	mean±SD (%w/w)	% RSD	mean±SD (%w/w)	% RSD	% RSD
Imp-1*	0.62±0.003	0.5	0.63±0.003	0.5	2.96
Imp-4	0.21±0.001	0.5	0.24±0.001	0.4	2.92
Imp-5	0.31±0.002	0.6	0.35±0.002	0.6	2.23
Imp-6*	0.62±0.003	0.5	0.60±0.006	1.0	2.68
Imp-7	0.21±0.003	1.4	0.21±0.002	0.8	1.94
Imp-8	0.16±0.003	1.9	0.15±0.001	0.6	1.77
Imp-9	0.21±0.003	1.5	0.22±0.002	1.1	2.56
Imp-10	0.21±0.002	0.8	0.22±0.001	0.6	1.45
Imp-11	0.22±0.003	1.2	0.23±0.002	0.9	1.84
Imp-12*	0.62±0.003	0.5	0.62±0.002	0.3	2.69
Imp-13	0.21±0.003	2.4	0.20±0.003	1.4	2.75

#Number of experiments (n):6, *Impurities were spiked at 0.6% level to test concentration of sample (1000 µg/ml) and remaining at 0.2% level spiking proposed based on the nature of impurity.

Table 6: Results of accuracy from LOQ to 150 % level of sample concentration

Analytes	Mean % recovery (n=3)					Overall statistical parameters for 50%-150% levels	
	LOQ		50%	100%	150%		
	% Recovery	% RSD	% Recovery	% Recovery	% Recovery	% RSD*	95% Confidence interval (±)*
Imp-1	100.7	0.3	102.0	101.2	102.2	0.6	0.5
Imp-4	98.8	4.4	104.1	107.2	105.3	1.3	1.1
Imp-5	95.7	4.0	92.8	95.3	93.8	1.2	0.8
Imp-6	95.1	3.2	100.0	101.1	102.3	1.0	0.8
Imp-7	97.2	4.1	94.2	97.4	96.6	1.7	1.2
Imp-8	102.8	4.5	93.6	99.0	96.8	2.5	1.8
Imp-9	97.6	3.7	101.5	96.8	104.1	3.7	0.9
Imp-10	101.3	2.8	103.6	100.1	102.9	1.8	1.6
Imp-11	96.9	3.9	94.2	98.7	96.9	2.3	1.8
Imp-12	100.3	3.7	101.2	101.3	101.8	0.4	0.3
Imp-13	96.5	4.2	101.9	98.2	98.6	2.1	1.4

Number of experiments (n): 3. *Overall % RSD and 95% Confidence interval (±) of 50%, 100% and 150% levels.

Table 7: Results of robustness on critical pairs from spiked sample of specified known impurities

Name	Resolution (Rs) between critical pair from spiked sample												
	As such	Flow rate variation		Column temperature variation		Methanol variation		Acetonitrile variation		pH variation		Wavelength variation	
	-	-10%	+10%	-5 °C	+5 °C	-1%	+1%	-1%	+1%	-0.2	+0.2	-5 nm	+5 nm
Critical pair (Imp-2 and Imp-3)	2.14	1.71	2.24	2.17	2.05	2.11	1.90	2.28	1.83	1.67	2.19	2.14	2.10
Critical pair (Imp-3 and Imp-4)	4.26	4.05	4.40	4.31	4.28	4.35	4.14	4.41	4.16	3.96	4.27	4.27	4.29
Critical pair (Imp-4 and Imp-5)	3.96	3.91	3.98	3.98	3.91	3.81	3.94	4.01	3.74	3.80	3.98	3.92	3.94
Critical pair (Imp-6 and Imp-7)	1.58	1.52	1.59	1.57	1.60	1.46	1.59	1.64	1.36	1.64	1.59	1.57	1.54
Critical pair (Unknown and THI)	3.58	3.66	3.79	3.62	3.45	3.62	3.54	3.63	3.51	3.54	3.56	3.59	3.57

Robustness

The robustness experiment measures capability of the method parameters to remain unaffected by small and deliberate variation in chromatographic conditions. Hence, the developed operating parameters of the method evaluated for deliberate variations in column oven temperature±5 °C, inflow rate±10%, in pH of the buffer±0.2 units, in organic composition±1% in mobile phase (±1% absolute in gradient composition) and±5 nm wavelength and system suitability parameters like resolution, tailing factor and USP plate count have been accessed. It is found that there is no significant change in the system suitability parameter`s acceptance criteria. Therefore, it is demonstrated that the developed method is robust across different variations studied for the experiment. Robustness results for resolution between critical pairs were given in table 7.

Solution stability in analytical solution

The stability of solutions is a crucial parameter that assesses the diluent effect and also gives flexible allowed time for analysis. Solution stability was evaluated by injecting samples at regular time intervals in the proposed method at room temperature. On verification % difference with initial time interval value, it was found that solutions are stable up to 48 h at room temperature (25 °C). There is no considerable change observed in the responses of standard and impurities in the sample solution and no additional peaks are formed or found indicating that the solution is stable and results were tabulated in table 8.

Table 8: Results of solution stability of standard and known impurities from spike sample

Analytes	6 h	12 h	25 h	48 h
% Difference from initial area to the respective time interval				
THI (standard)	0.9	1.9	2.6	3.5
Imp-1	0.2	0.4	0.9	0.3
Imp-4	0.1	0.2	0.1	0.8
Imp-5	0.1	0.2	0.3	0.3
Imp-6	0.8	0.9	0.6	0.6
Imp-7	0.7	1.6	0.9	3.2
Imp-8	0.6	1.3	3.2	5.4
Imp-9	0.9	1.8	1.2	1.6
Imp-10	1.1	1.3	0.9	1.8
Imp-11	0.6	1.7	2.3	1.2
Imp-12	0.3	0.0	0.6	0.4
Imp-13	1.1	0.7	0.9	2.1

CONCLUSION

A systematic qualitative and quantitative method for impurity profiling of THI injectable formulation was successfully developed and validated. The method has found to be selective, specific, precise, accurate, linear, stable, and having very good sensitivity for the lowest possible impurities if there any. Moreover, the developed HPLC method has a robust and rugged stability-indicating method being fit for its intended purpose. During the life cycle of a product, observed degradation unknown product above identification threshold is identified and characterized by LC-MS/MS hyphenated technique and its degradation pathway and possible structure have been derived. Therefore, the developed method is a simple, efficient, and affordable technique that could be useful for monitoring the impurity profile of THI injectable formulation in quality control departments of manufacturing units.

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Mr. Srinivasu Kondra has generated the research activity and prepared the manuscript. Dr. Bapuji and Dr. Gowri Sankar have given guidance and supervision to carry out this research work. Mr. Potturi Muralikrishnam Raju helped in the compilation of data.

CONFLICTS OF INTERESTS

The authors confirm that this article content has no conflict of interest.

REFERENCES

1. Singleton CK, Martin PR. Molecular mechanisms of thiamine utilization. Curr Mol Med 2001;1:197–207.
2. Abdou E, Hazell AS. Thiamine deficiency: an update of pathophysiologic mechanisms and future therapeutic considerations. Neurochem Res 2015;40:353–61.
3. Teigen LM, Twernbold DD, Miller WL. Prevalence of thiamine deficiency in a stable heart failure outpatient cohort on standard loop diuretic therapy. Clin Nutr 2016;35:1323–7.
4. Kerns JC, Arundel C, Chawla LS. Thiamin deficiency in people with obesity. Adv Nutr 2015;6:147–53.
5. Jain A, Mehta R, Al-Ani M, Hill JA, Winchester DE. Determining the role of thiamine deficiency in systolic heart failure: a meta-analysis and systematic review. J Card Fail 2015;21:1000–7.
6. Shah S, Wald E. Type B lactic acidosis secondary to thiamine deficiency in a child with malignancy. Pediatrics 2015;135:221–4.
7. Al-Daghri NM, Alharbi M, Wani K, Abd-Alrahman SH, Sheshah E, Alokail MS. Biochemical changes correlated with blood thiamine and its phosphate esters levels in patients with diabetes type 1 (DMT1). Int J Clin Exp Pathol 2015;8:13483–8.
8. Page GL, Laight D, Cummings MH. Thiamine deficiency in diabetes mellitus and the impact of thiamine replacement on glucose metabolism and vascular disease. Int J Clin Pract 2011;65:684–90.
9. Abdel Rehman ST, Elbashir AA, El-Mukhtar M, Ibrahim MM. Application of spectrophotometric methods for the determination of thiamine (vb1) in pharmaceutical formulations using 7-chloro-4-nitrobenzoxadiazole (NBD–Cl). J Anal Pharm Res 2016;2:1-6.
10. Lopez-de-Alba PL, Lopez Martinez L, Cerda V, Amador Hernandez. Simultaneous determination and classification of riboflavin, thiamine, nicotinamide and pyridoxine in pharmaceutical formulations by UV-visible spectrophotometry and multivariate analysis. J Braz Chem Soc 2006;17:715–22.
11. Abdel Maaboud M, Horria M, Niveen MA, Marwa EZ. Chemometric methods for the simultaneous determination of some water-soluble vitamins. J AOAC Int 2011;94:467–81.
12. Chen QY, Li DH, Yang HH, Zhu QZ, Zheng H, Xu JG. Novel spectrofluorimetric method for the determination of thiamine with iron (iii) tetrasulfonatophthalocyanine as a catalyst. Analyst 1999;124:771–5.
13. Zhu H, Chen H, Zhou Y. Determination of thiamine in pharmaceutical preparations by sequential injection renewable surface solid-phase spectrofluorometry. Anal Sci 2003;19:289–94.

14. Alonso A, Almendral MJ, Porras MJ, Curto Y. Flow injection solvent extraction without phase separation. fluorimetric determination of thiamine by the thiochrome method. J Pharm Biomed Anal 2006;42:171–7.
15. Tabrizi AB. A cloud point extraction-spectrofluorimetric method for determination of thiamine in urine. B Korean Chem Soc 2006;27:1604–8.
16. Amjadi M, Manzoori JL, M Orooji. Use of crude extract of kohlrabi (brassica oleracea gongylodes) as a source of peroxidase in the spectrofluorimetric determination of thiamine. B Korean Chem Soc 2007;28:246–50.
17. Sun J, Liu L, Ren C, Chen X, Hu Z. A feasible method for the sensitive and selective determination of vitamin B1 with CdSe quantum dots. Microchim Acta 2008;163:271–6.
18. Khan MA, Jin SO, Lee SH, Chung HY. Spectrofluorimetric determination of vitamin B1 using horseradish peroxidase as catalyst in the presence of hydrogen peroxide. Luminescence 2009;24:73–8.
19. Li Y, Wang P, Cao M. An immediate luminescence enhancement method for determination of vitamin B1 using long-wavelength emitting water-soluble CdTe nanorods. Microchim Acta 2010;169:65–71.
20. Zeeb M, Ganjali MR, Norouzi P. Dispersive liquid-liquid microextraction followed by spectrofluorimetry as a simple and accurate technique for determination of thiamine (vitamin B1). Microchim Acta 2010;168:317–24.
21. Mohamed AM, Mohamed HA, Abdel-Latif NM, Mohamed MR. Spectrofluorimetric determination of some water-soluble vitamins. J AOAC INT 2011;94:1758–69.
22. Tarigh GD, Shemirani F. Simultaneous in situ derivatization and ultrasound-assisted dispersive magnetic solid phase extraction for thiamine determination by spectrofluorimetry. Talanta 2014;123:71–7.
23. Zhu J, Liu S, Liu Z, Liu Y, Qiao M, Hu X. Enhanced spectrofluorimetric determination of hypochlorite based on the catalytic oxidation of thiamine to thiochrome in the presence of trace ferrocyanide. RSC Adv 2014;4:5990–4.
24. Tan H, Li Q, Zhou Z. A sensitive fluorescent assay for thiamine based on metal-organic frameworks with intrinsic peroxidase-like activity. Anal Chim Acta 2015;856:90–5.
25. Restani M, Neubert RH. Thiamine analysis in biological media by capillary zone electrophoresis with a high-sensitivity cell. J Chromatogr A 2000;871:351-6.
26. Herve C, Beyne P, Delacoux E. Determination of thiamine and its phosphate esters in human erythrocytes by high-performance liquid chromatography with isocratic elution. J Chromatogr B Biomed Appl 1994;653:217–20.
27. Baines M. Improved high-performance liquid chromatographic determination of thiamin diphosphate in erythrocytes. Clin Chim Acta 1985;153:43–8.
28. Lynch PL, Trimble ER, Young IS. High-performance liquid chromatographic determination of thiamine diphosphate in erythrocytes using internal standard methodology. J Chromatogr B Biomed Sci Appl 1997;701:120–3.
29. Losa R, Sierra MI, Fernandez A, Blanco D, Buesa JM. Determination of thiamine and its phosphorylated forms in human plasma, erythrocytes and urine by HPLC and fluorescence detection: a preliminary study on cancer patients. J Pharm Biomed Anal 2005;37:1025–9.
30. Tang X, Cronin DA, Brunton NP. A simplified approach to the determination of thiamine and riboflavin in meats using reverse-phase HPLC. J Food Composition Anal 2006;19:831–7.
31. Ake M, Soko YN, Malan KA. Liquid chromatographic determination of free thiamine and its esters in whole blood. Dakar Med 2006;51:172–7.
32. Zafra Gomez A, Garballo A, Morales JC, Garcia Ayuso LE. Simultaneous determination of eight water-soluble vitamins in supplemented foods by liquid chromatography. J Agric Food Chem 2006;54:4531–6.
33. Lebiedzinska A, Marszall ML, Kuta J, Szefer P. Reversed-phase high-performance liquid chromatography method with coulometric electrochemical and ultraviolet detection for the quantification of vitamins B (thiamine), B (pyridoxamine, pyridoxal and pyridoxine) and B in animal and plant foods. J Chromatogr A 2007;1173:71–80.
34. Wang XX, Hu Sun YZ, Bie MJ, Sun CJ. Simultaneous determination of five water-soluble vitamins in human serum by high-performance liquid chromatography. Journal of Sichuan University Medical Science Edition 2010;41:158–61.
35. Tan J, Li R, Jiang ZT. Determination of thiamine (vitamin B1) in pharmaceutical tablets and human urine by titania-based ligand-exchange hydrophilic interaction chromatography. Anal Methods 2011;3:1568–73.
36. Dinc E, Kokdil G, Onur F. A comparison of matrix resolution method, ratio spectra derivative spectrophotometry and HPLC method for the determination of thiamine HCl and pyridoxine HCl in pharmaceutical preparation. J Pharm Biomed 2000;22:915–23.
37. Hampel D, York ER, Allen LH. Ultra-performance liquid chromatography-tandem mass-spectrometry (UPLC–MS/MS) for the rapid, simultaneous analysis of thiamin, riboflavin, flavin adenine dinucleotide, nicotinamide and pyridoxal in human milk. J Chromatogr B 2012;903:7–13.
38. Santos VB, Guerreiro TB, Suarez WT, Faria RC, Fatibello Filho O. Evaluation of turbidimetric and nephelometric techniques for analytical determination of n-acetylcysteine and thiamine in pharmaceutical formulations employing a lab-made portable microcontrolled turbidimeter and nephelometer. J Braz Chem Soc 2011;22:1968–78.
39. Akyilmaz E, Yasa I, Dinckaya E. Whole-cell immobilized amperometric biosensor based on saccharomyces cerevisiae for selective determination of vitamin B1 (thiamine). Anal Biochem 2006;354:78–84.
40. Jiang X, Sun T. Indication ion square wave voltammetric determination of thiamine and ascorbic acid. Electrochemistry 2007;40:2589–96.
41. Norouzi P, Garakani TM, Rashedi H, Zamani HA, Ganjali MR. Ultrasensitive flow-injection electrochemical method using fast fourier transform square-wave voltammetry for detection of vitamin B1. Int J Electrochem Sci 2010;5:639–52.
42. Bas B, Jakubowska M, Gorski L. Application of renewable silver amalgam annular band electrode to voltammetric determination of vitamins C, B1 and B2. Talanta 2011;84:1032–7.
43. Tyszczuk Rotko K. New voltammetric procedure for determination of thiamine in commercially available juices and pharmaceutical formulation using a lead film electrode. Food Chem 2012;134:1239–43.
44. Brahman PK, Dar RA, Pitre KS. DNA functionalized electrochemical biosensor for detection of vitamin B1 using electrochemically treated multi-walled carbon nanotube paste electrode by voltammetric methods. Sensor Actuat B-Chem 2013;177:807–12.
45. Antal IP, Bazel YR, Kormosh ZA. Electrochemical methods for determining group B vitamins. J Anal Chem 2013;68:565–76.
46. David IG, Florea MA, Cracea OG. Voltammetric determination of B1 and B6 vitamins using a pencil graphite electrode. Chem Pap 2015;69:901–10.
47. Dwivedi BK, Arnold RG, Libbey LM. Chemistry of Thiamine degradation. Mechanisms of thiamine degradation in a model system. J Food Sci 1972;37:689-92.
48. Mulley EA, Stumbo CR, Hunting WM. Kinetics of thiamine degradation by heat. Effect of pH and form of the vitamin on its rate of destruction. J Food Sci 1975;40:989-92.
49. British Pharmacopoeia, Monograph on 9iamine, Her Majesty's Stationary Office, London, UK; 2016.
50. United States Pharmacopeia 29, Monograph on Thiamine, United States Pharmacopeial Convention, Rockville, MD, USA; 2016.
51. Guidance for Industry, ICH. Q3B (R2): Impurities in New drug product, Conference on Harmonization, Geneva; 2006.
52. Srikanth G, Uttamkumar R, Murali N, Badrinadh Gupta P, Jagadeesh Kumar V, Satheesh D, *et al.* Identification, isolation and characterization of process-related impurities in ezetimibe. J Pharm Biomed Anal 2014;88:385-90.
53. ICH. ICH Harmonized Tripartite Guidelines Q2 (R1): Validation of analytical procedures. ICH, Geneva; 2005.
54. FDA, Guidance for Industry, Analytical Procedures and Method Validation (Chemistry, Manufacturing and Controls Documentation), Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), Rockville, USA; 2000.

55. Britton HTS. Hydrogen Ions. 4th Ed. Chapman and Hall; 1952.
56. Srinivasu K, Bapuji AT, Gowri Shankar D, Vijay Bharathi D, Satyanarayana KVV. Novel analytical method using acquity QDa mass detector coupled with LC-PDA for impurity profiling of amlodipine besylate and olmesartan medoxomil in fixed-dose tablets formulation. Saudi J Med Pharm Sci 2019;5:871-84.
57. Blessy M, Patel RD, Prajapati PN, Agrawal YK. Development of forced degradation and stability indicating studies of drugs-a review. J Pharm Anal 2014;4:159-65.
58. ICH. ICH Harmonized guideline Q1A (R2): Stability testing of new drug substances and products: text and methodology. ICH, Geneva; 2005.
59. Azad IF, Sarmad BD, Retrwar OH. Separation and determination of some water-soluble vitamins in pharmaceutical preparation by IP RP-HPLC. Tikrit J Pharm Sci 2005;1:15-29.
60. Novi Y, Diah W, Wartini, Tiwi A. Validation of HPLC method for determination of thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride in syrup preparation. Canadian J Sci Ind Res 2011;2:269-78.
61. Gadhave RV, Tamnar AB, Bansode AS, Choudhari VP. Stability Indicating RP-HPLC-PDA method for determination of abiraterone acetate and characterization of its base catalyzed degradation product by LC-MS. Int J Pharm Pharm Sci 2016;8:76-81.
62. Mayuri P, Krishna D, Krishnapriya M. Isocratic LC-UV, LC-MS and MS/MS studies on fenoxazoline and its degradation products. Int J Pharm Pharm Sci 2015;7:50-7.
63. Young PM, Gorenstein MV. Tryptic mapping by reversed-phase HPLC with photodiode-array detection incorporating the spectral-contrast technique. LC GC 1994;12:832–8.
64. Manohar CS, Rajput AP. Development and validation of a new stability-indicating analytical method for the determination of related components of brimonidine tartrate in drug substances and drug products using UPLC. Int J Pharm Pharm Sci 2011;3:145-50.
65. Adrienne LV, Jenna M, Cordelia AR, Lynne ST, Lisa JM. Chemical stability and reaction kinetics of two thiamine salts (thiamine mononitrate and thiamine chloride hydrochloride) in solution. Food Res Int 2018;112:443-56.