

Original Research Article

Solid phase extraction and LC-MS/MS quantification of ibandronate in human plasma

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Sent for review: 23 October 2019

Revised accepted: 24 May 2020

Abstract

Purpose: To develop and validate a simple, highly sensitive and accurate method for the quantification of ibandronate (IBN) in human plasma.

Methods: Electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) in positive ion mode was applied to obtain optimum signals. The parent ion was acquired under collision-activated dissociation conditions, and the abundant fragments used to design multiple reaction monitoring experiments for monitoring two ibandronate transitions (m/z 376 to m/z 114, and m/z 376 to m/z 250). The IBN was isolated from plasma with weak anion exchange solid phase extraction columns with 'on-cartridge' derivatization using tri-methylsilyl-diazomethane (TMSDZ) reagent to convert IBN to tetra-methyl derivative.

Results: The studied drug was successfully extracted from plasma samples without any interference at a retention time of 3.2 min. The matrix effect averaged 110 %, indicating that endogenous materials had little effect on ionization. The relationship between plasma analyte concentration and IBN signal area was satisfactorily linear, with correlation coefficient (r^2) ranging from 0.9817 to 0.9942 in the concentration range of 0.5 – 200 ng/ml. The lower and upper limits of quantification (LLOQ and ULOQ) for IBN were 0.5 and 200 ng/ml, respectively. Relative recovery of IBN from plasma after extraction and derivatization at 3 distinct concentrations was 83.93 to 85.06 %, relative to standard solutions. The ranges of intra- and inter-day accuracies of quantification of quality controls were 89.39 - 106.40 %, and 90.50 - 107.96 %, respectively. Processed plasma IBN extracts were stable in autosampler at 4 °C (91.12 to 103.49%). Long-term stability in plasma after 30 days at -24 °C ranged from 89.52 to 113.18 %.

Conclusion: This validated LC-MS/MS method can be successfully applied for determination of IBN in pharmacokinetic studies. It is a sensitive and specific assay for plasma IBN in bioequivalence studies.

Keywords: Ibandronate, LC-MS/MS, Validation, Derivatization, Solid-phase extraction

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INTRODUCTION

Ibandronate sodium (IBN) (Figure 1) is 3-(n-methyl-N-pentyl) amino-1-hydroxypropane-1,1-diphosphonic acid, monosodium salt, monohydrate which suppresses the ability of osteoclasts to resorb bone [1]. The drug is prescribed for the prevention and treatment of osteoporosis, since it can be used to elevate the mineral density of the bone [2].

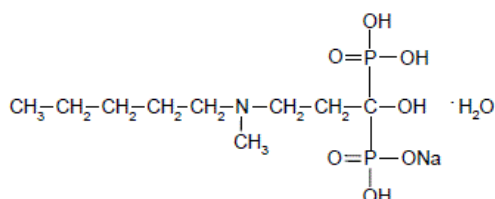


Figure 1: Chemical structure of ibandronate sodium

Analysis of bisphosphonates in pharmaceutical form is very challenging due to a variety of reasons. Most of these compounds lack a chromophore. They are extremely polar, with several functional groups. Moreover, they are not well-retained on HPLC columns. Their ionization efficiency in mass spectrometry, in either negative ion or positive ion modes, is also poor [1]. In a study, HPLC method coupled with analyte derivatization was applied to quantify aminobisphosphonate, and alendronate (ALN) was determined after its extraction with diethylamine (DEA) solid-phase extraction (SPE) cartridge, followed by derivatization with 9-fluorenylmethyl [3]. The technique of GS-MS has been used to measure different aminobisphosphonate members, including ALN after their conversion to the more volatile N-isobutoxycarbonyl methyl ester derivatives [4]. Moreover, capillary electrophoresis has been applied to determine bisphosphonates and their impurities [5]. In one study, ELISA technique was applied in quantification of IBN [6], and IBN has also been determined spectrophotometrically via ligand exchange [7].

The technique of LC-MS/MS was recently utilized for determination of aminobisphosphonates in samples after their liquid-liquid extraction and derivatization [8,9]. Liquid-liquid extraction has many drawbacks: it is a tedious extraction method, being that it results in emulsion formation. In addition, it can be considered an environment-unfriendly protocol because it consumes large volumes of organic solvents [10]. Solid phase extraction (SPE) is the future prospect for green analytical chemistry due to its simplicity, environment-friendly sample-preparation technique, and low cost in terms of time and solvents [11]. Recently, SPE was

applied in the isolation of risedronate and alendronate from serum and urine, followed by derivatization of the four active hydroxyl groups to the corresponding methyl esters using either diazomethane or trimethylsilyl-diazomethane (TMS-DZ) [12].

This study was designed to develop and authenticate an easy, highly sensitive and accurate procedure for the quantification of ibandronate (IBN) in human plasma so as to overcome the drawbacks of liquid – liquid extraction.

EXPERIMENTAL

Chemicals and reagents

Ibandronate (IBN) reference material ($C_9H_{24}NNaO_8P_2$, molecular weight = 359.23 g/mol) was kindly provided by Faculty of Pharmacy, Cairo University, Cairo, Egypt. Formic acid, LC/MS LiChrosolv® Acetonitrile, ammonium formate and tri-methylsilyldiazomethane (2M in hexane) were purchased from TMS-DZ, Sigma-Aldrich; CAS Number 18107-18-1. The LC/MS-grade water used was supplied by LiChrosolv®. Solid phase extraction columns (SPEs) i.e. high-capacity Waters Oasis 6cc SPE columns purchased from Waters Co., USA, were used for combined extraction and derivatization of IBN.

Instrumentation and its optimization

Mass spectrometry optimization

Applied Biosystems API3200Q 3-quadrupole mass spectrometer with electrospray probe and interface was used for MS. Electrospray ionization (ESI) was done in the positive ion MRM mode. The MS/MS parameters were: declustering potential (DP): 48 V; collision energy (CE): 28 eV for m/z 376 to m/z 114 transition; entrance potential (EP): 4 V; collision cell exit potential (CXP): 2 V; CEP = 18 V, and ion spray voltage (ISV) was set at 5500 V. Source temperature was 650°C, curtain gas = 30 psi, GS1 = 55, GS2 = 55, and collision activated dissociation (CAD) gas was set at 5 psi. Dwell time was maintained at 200 ms. Integration of signal areas was done using data system analyst (version 1.5) running under Windows XP environment.

In order to optimize the electrospray ionization (ESI) for IBN tetra-methyl derivative, the derivative was prepared using IBN working solution (50 µg/ml). After appropriate dilution, the product of the reaction was infused into the mass

spectrometer ion source in positive ion mode ESI, using a speed of 20 μ l/60 sec.

LC-MS/MS

Chromatographic analysis of tetra-methyl IBN was performed utilizing Waters Sunfire column preceded by an in-line filter. Chromatography was in the isocratic mode of analysis using CH_3CN and 150 mM NH_4HCOO at a volume ratio of 28:72 (adjusted to pH 3.5) at a speed of 0.75 mL/min. The column temperature was adjusted to room temperature. The autosampler temperature was maintained at 4 $^\circ\text{C}$. The injection volume was 5 μL , and the total run and data acquisition took 5 min. The mass spectrometer ion source temperature and the flow rate of desolvation gas were optimized to obtain the best signal-to-noise ratio for IBN derivative. These parameters were checked and deemed consistent for areas or signal-to-noise ratio, or otherwise adjusted before each run. Furthermore, the mass assignment was periodically checked to ensure calibration of the instrument. The change from mass: charge ratio 376 to mass: charge ratio 114 was utilized for quantification. On the other hand, change from mass: charge ratio 376 to mass: charge ratio 250 was used as a confidence transition to confirm the identity of the analyte peak.

Quality control and calibration standard samples

A stock solution was made by weighing suitable amount of IBN and dissolving it in 5 mL of de-ionized water acidified with HCl to pH 4.0 in a 10-mL volumetric flask. Following vortexing, the volume was brought up to mark with acidified water to yield a final concentration of 5000 $\mu\text{g/mL}$. Working standard solutions of concentrations 50, 5 and 0.5 $\mu\text{g/mL}$ were prepared from the IBN stock solution via serial dilution.

Calibration standards and quality controls were prepared under the same conditions as the samples for analysis. Calibration curves consisted of a blank sample without any additions, and eight samples within the anticipated range of concentrations to be analyzed. To prepare plasma calibration standards, control plasma was spiked with IBN standard solutions. Samples for plasma calibration had concentrations of 0.5, 1, 2.5, 5, 15, 50, 100 and 200 ng/mL. Low, medium and high concentrations of each quality control sample corresponding to 3, 80 and 160 ng/mL, were prepared. All calibration standards were vortex-mixed for 5 min to ensure proper mixing.

The prepared standards were dispensed in 0.5-mL portions in marked tubes and kept at -24 $^\circ\text{C}$ together with QC samples until required for assay. For each assay, one tube of each concentration was thawed for one hour before sample extraction. They were used during the pre-study method validation phase, and also for quality control during the analytical phase.

Extraction procedure of plasma samples

On the day of assay, samples to be extracted were thawed for one hour. To all tubes, 40 μL of 1 M HCl was added and vortexed. Solid phase extraction (SPE) cartridges were positioned on the extraction manifold. Then, each cartridge was equilibrated with 2 mL of 20 mM HCl and washed with 2 mL of water. Plasma containing IBN was loaded onto the cartridges, followed by washing with 2 mL of 20 mM HCl, and rinsing with 2 mL of methanol. An aliquot of 1 mL of TMS-DZ was added to each cartridge, and the eluate was collected into labeled tubes. Another 1 mL portion of TMS-DZ was added and combined with previously collected eluates. The collected eluate was evaporated to dryness with nitrogen at 50 $^\circ\text{C}$, reconstituted in 250 μL of mobile phase and transferred to autosampler vial. A 5- μL portion was injected into the mass spectrometer.

Validation of bioanalytical procedure

Validation was carried out with respect to parameters such as precision and accuracy, linearity selectivity, sensitivity, recovery, matrix effect, and stability according to US-FDA guidelines.

RESULTS

Method development

The mass spectrum of IBN tetra-methyl showed base peak for the protonated molecular ion at m/z 376 (Figure 2). Following the optimization of single quad signal, the collision-activated dissociation spectra (CAD) for the protonated molecular ion was acquired at different collision energies to optimize the output signal. Tetra-methyl IBN yielded two major fragments at m/z 114 and m/z 250. Figure 3 shows the product ion spectrum of tetra-methyl IBN. These two fragments formed the basis for the design of the MRM experiments used for analysis.

Figure 4 and Figure 5 show MRM chromatograms for the two transitions of IBN derivative obtained from plasma calibrators under the experimental conditions mentioned above. The shift from mass: charge ratio 376 to

mass: charge ratio 114 was applied for quantification, while the shift from mass: charge ratio 376 to mass: charge ratio 250 was used as a confidence transition to identify the LC-MS/MS peak for the analyte.

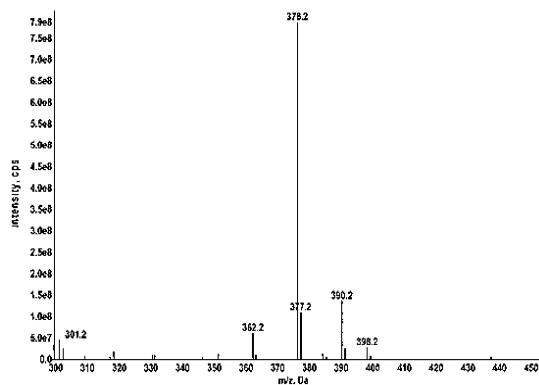


Figure 2: Positive ion ESI spectrum for tetra-methyl IBN prepared from working solution

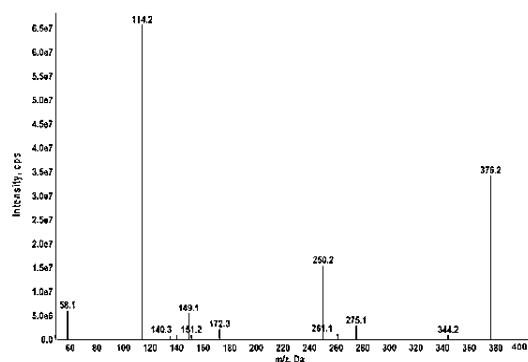


Figure 3: Product ion spectrum of IBN at collision energy of 28 eV

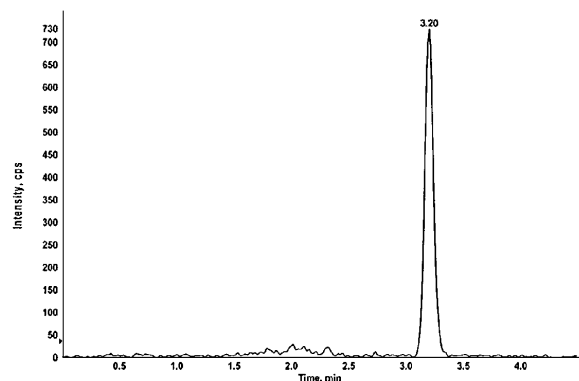


Figure 4: MRM shift of mass: charge ratio 376 to mass: charge ratio 114 for IBN derivative from plasma calibrator

Selectivity

Method selectivity was determined by testing interference from blank human plasma samples from 6 persons utilizing the proposed extraction and derivatization procedure and chromatographic protocols, and comparing with a standard solution of derivatized IBN. No interference was observed in the two transitions for IBN at their respective retention times.

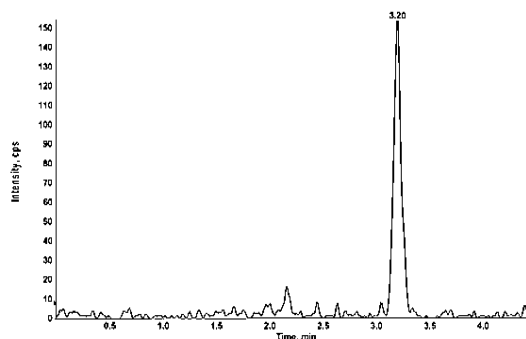


Figure 5: MRM shift of mass: charge ratio 376 to mass: charge ratio 250 for IBN derivative from plasma calibrator

Matrix effect

Six plasma samples from different donors were processed as indicated under sample preparation without the addition of analyte. The final extract of each sample was spiked with IBN standard at two distinct concentrations of 3.0 and 160 ng/mL. Standard solutions of IBN (at the same concentrations) in mobile phase were prepared by the same approach. Each of the plasma sample extracts was then injected into the mass spectrometer, and peak areas for analyte were measured. Each of the standard solutions was injected three times and peak areas were averaged. Table 1 shows that there were no significant differences in peak areas amongst different plasma samples and standard solutions, indicating that if the analyte was co-eluting with any endogenous materials, this did not significantly affect the ESI ionization. The average matrix effect for the IBN concentrations was 110.6 %.

Linearity

This was measured using six calibration curves containing eight non-zero concentrations. The analyte concentrations were calculated using weighted least squares regression analysis.

Table 2 shows summary of the calibration curves obtained during pre-study validation phase. Figure 6 shows six overlaid calibration curves

obtained during pre-study validation phase. The excellent precision and accuracy obtained for such a large number of QC samples clearly show the ruggedness of the assay, despite the lack of an internal standard and the number of days that it took to analyze volunteer samples, involvement of two different analysts in sample extraction and derivatization, and the large number of solid phase columns used. The data also reflect the stability of the mass spectrometer.

Accuracy and precision

Accuracy was calculated in terms of mean, while precision was estimated from the coefficient of variation (% CV). The mean value for accuracy and CV for precision did not surpass 15 % of CV. Table 3 and Table 4 show the inter-day and intra-day precision and accuracy for IBN during pre-study validation phase.

Table 1: Matrix effect analysis at three IBN concentrations

Plasma sample no.	IBN peak area (3 ng/ml)	IBN peak area (160 ng/ml)	
1	3857	78395	
2	3975	74698	
3	3757	72930	
4	3953	82989	
5	4133	77051	
6	4191	78744	
Average peak area	3977	77468	
Average standard solution peak area	3655	68902	
Matrix Effect	108.8	112.4	110.6
CV (%)	4.10	4.52	

Table 2: Summary of the calibration curves obtained in the pre-study validation

Curve no.	Slope	Intercept	Correlation coefficient (r ²)
1	512	29.1	0.9926
2	475	44.8	0.9857
3	556	5.14	0.9938
4	533	119	0.9974
5	550	82.8	0.9817
6	561	211	0.9942
Mean	531.17	81.97	0.9909
SD	32.82	74.97	0.0060
CV (%)	6.18	--	0.60

Lower limit of quantification (LLOQ)

The limits of detection and quantification in plasma were obtained. As the assay progressed, it was found that IBN was detected in all

calibrators at a concentration of 0.5 ng/mL, and consistently acceptable precision values were got when back-calculated, with signal-to-noise ratio at LLOQ consistently >3. Therefore, 0.5 ng/mL was considered the limit of quantification (LLOQ). Levels below LLOQ were considered “below quantification levels”. The upper limit of quantification (ULOQ) was 200 ng/ml.

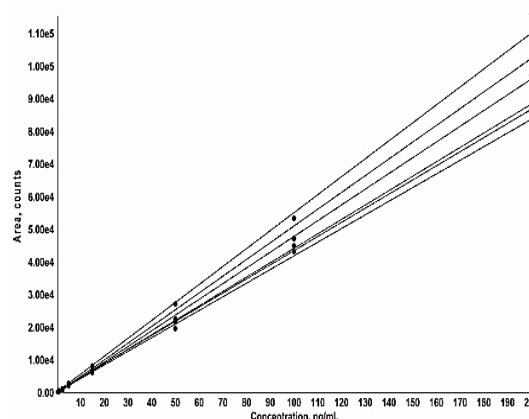


Figure 6: Calibration curves for IBN area (n = 6) at each concentration in the pre-study phase. This data was extracted directly from mass spectrometer analyst software by overlaying the 6 curves

Table 3: Inter-day precision and accuracy for IBN during pre-study validation phase

IBN	Nominal conc.	Calculated conc.		Accuracy (%)	CV (%)
		Mean ^a	SD		
QC-L	3.0	2.72	0.15	90.50	5.41
QC-M	80	86.37	6.25	107.96	7.23
QC-H	160	169.17	9.41	105.73	5.56

^an = six replicates at each concentration (ng/mL)

Recovery

Recovery refers to the effectiveness of IBN extraction using the designed analytical method. It was assessed from 3 plasma concentrations, with the usual extraction procedure. Three plasma samples at each concentration were processed.

Working solutions of IBN were diluted with mobile phase to produce the same concentrations as the plasma samples, and they were derivatized and analyzed. Recovery was calculated as shown earlier. The average recovery across the three concentrations was 84 %, and CV was 6.8 %, as shown in Table 5. Tarcomnicu *et al* [9] reported a recovery of 65.3 % and CV of 5.7 %, with d3-IBN as an internal standard.

Table 4: Precision and accuracy (intra-day) for IBN during pre-study validation phase

IBN	Nominal plasma concentration (ng/mL)	Calculated concentration		Accuracy (%)	Precision (CV, %)
		Mean ^a	SD		
QC-L	3.0	2.68	0.22	89.39	8.31
QC-M	80	85.12	5.35	106.40	6.29
QC-H	160	162.33	9.83	101.46	6.06

^an = six replicates at each concentration**Table 5:** Relative recovery results for IBN

IBN	Area response (SD)		Recovery (%)	CV (%)
	Derivatized extracted plasma ^a	Derivatized standard		
QC-L	1970	2347	83.93	2.95
QC-M	112444	132186	85.064	1.85
QC-H	160929	194610	82.69	0.90

^an = 3 for each concentration, and n = 3 for IBN standard solution

Stability

Stability was assessed in the plasma samples under different timing and temperature conditions. To test stability, a series of standard samples were analyzed. The respective low, medium and high-quality controls were used. Autosampler stability (Table 6) was assessed using the processed quality control plasma samples at 4 °C, and anticipated batch run time up to 12 h. The results indicate that the analyte was stable in the autosampler for up to 12 h without significant loss. The average stability of the concentrations tested was 95.6 %.

Table 6: Auto-sampler stability data

IBN (ng/mL)	Calculated concentration (ng/ml) ^a		
	Fresh	12 h	Stability %
QC-L (3.0)	3.15	3.26	103.49
QC-M (80)	85.72	78.98	92.14
QC-H (160)	187.00	170.40	91.12

^an = 5 at each concentration for each experiment

As shown in Table 7, the analyte in human plasma was stable at RT for up to 24 h.

Table 7: Short-term stability data

IBN (ng/mL)	Calculated concentration (ng/mL) ^a						
	Fresh	6 h (RT)	Stability (%)	24 h (4 °C)	Stability (%)	24 h (RT)	Stability (%)
QC-L (3.0)	2.81	2.58	91.81	2.70	96.38	3.23	115.15
QC-M (80)	81.20	78.27	96.39	73.67	90.72	84.40	103.94
QC-H (160)	167.33	153.67	91.83	157.67	94.22	177.33	105.98

^a n = 3 at each concentration and for each experiment; RT = room temperature

Long term stability (Table 8) was tested at -24 °C for 30 days. The results indicated that the drug in plasma samples was sufficiently stable at all three concentrations tested when kept at -24 °C for a relatively long time and then thawed to room temperature. The average long-term stability at three concentrations was 98.7 %, with CV of 12.8 %.

To evaluate short term and long term IBN stabilities of working solutions, one working solution (IBN-1) was freshly prepared (50 µg/mL) and analyzed immediately. Then, portions were allowed to stand at room temperature for 6 h and 24 h, and then analyzed. A third portion was stored at -24 °C for 30 days prior to analysis. On day 30, two working solutions were freshly prepared and analyzed against the aged solutions. All solutions were analyzed five times, and the results are presented in Table 9. The results were based on the determination of IBN areas. The average working solution stability was 101.9 %.

The results of freeze-thaw stability are shown in Table 10 which indicates the freeze and thaw stability limitations. The 3 freeze-thaw cycles resulted in mean stability difference < 15 %.

DISCUSSION

Ibandronate (IBN) was obtained from plasma with weak anion exchange SPE columns with 'on-cartridge' derivatization using tri-methylsilyl-diazomethane (TMS-DZ) reagent to convert IBN to tetra-methyl derivative. The eluate of SPE was evaporated to dryness, reconstituted in mobile phase, and an aliquot was analyzed using electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) in positive ion mode. Plasma calibrators and quality

Table 8: Long-term stability results

IBN (ng/mL)	Calculated concentration (ng/mL) ^a		
	Fresh	30 days (-24 °C) ^a	Stability (%)
QC-L (3.0)	2.81	3.18	113.17
QC-M (80)	82.37	73.73	89.51
QC-H (160)	167.3 3	156.33	93.43

^a n = 3 at each concentration and for each experiment

Table 9: Stability of IBN working solutions

IBN (50 µg/µL)	IBN peak area ^a	
	Mean (SD)	Stability
Day zero	410263 (6783)	--
6 h (RT)	385578 (3516)	93.98
24 h (RT)	410858 (2744)	100.14
30 Days zero	297020 (11570)	--
30 Days (-24 °C)	331052 (9377)	111.46

^a 2 µl of each solution (100 ng IBN) was injected five times. RT = room temperature

Table 10: Freeze and thaw stability for IBN

IBN	Nominal concentration in plasma (ng/mL)	IBN concentration ^a			
		Fresh	After 3 cycles ^a	Stability (%)	CV (%)
QCM	80	82.0	79.93	97.48	5.0
QCH	160	155.33	147.00	94.64	16

^an = 3. Tests performed indicated that the mean freeze-thaw stability at two concentrations was 96.1%

control (QC) plasma samples were prepared in control human plasma in the expected concentration range, and were treated in the same way. Stringent sample handling and processing enhanced the full validation of this assay and maintained accuracy and precision throughout the work. Bioanalytical method validation was performed with respect to selectivity, matrix effect, linearity, accuracy, precision and stability. Sample preparation technique plays an important role in successful application of the method. The solid phase extraction technique provides substantial advantages over conventional liquid-liquid extraction. These advantages include high selectivity, high recoveries and sensitivity.

CONCLUSION

This work offers a method for extracting IBN from human plasma using SPE, thereby overcoming the drawbacks associated with previously published methods for determination of IBN. Conditions used for LC-MS/MS were optimized to get maximum sensitivity and precision. Validation parameters such as autosampler, freeze and thaw, and short- and long-term stability showed that there was no appreciable degradation at the temperatures and time periods specified and described in the method. This validated LC-MS/MS method is suitable for analysis of IBN in pharmacokinetic studies. It is a sensitive and specific assay for plasma IBN in bioequivalence studies.

DECLARATIONS

Acknowledgement

This project was supported by the Deanship of Scientific Research at Prince Sattam bin Abdulaziz University under Research Group no. 2016/03/6600.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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