ORIGINAL ARTICLE

Reversed-Phase High-Performance Liquid Chromatographic for Quantifying –IBA-HAP Conjugate in Innovative Transdermal Emulgel Formulations

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Abstract

Objectives: This research presents a reversed-phase high-performance liquid chromatographic (RP-HPLC) approach that is easy, precise, accurate, and economical for the quantification off or the estimation of IBA-HAP conjugate (IBA-HAP). The lack of a suitable ultraviolet chromophore makes the conjugate of ibandronate Hydroxyapatite challenging. An important challenge with the absence of a chromophore impacts the process of separation. **Materials and Methods:** It is challenging to preserve the Ibandronate hydroxyapatite conjugates on traditional reversed-phase columns due to their structural makeup. Separation was achieved by column C–18 (pore size – 5 µm and column dimensions (4.6 × 250 mm). The mobile phase comprises of 0.01 M sodium dihydrogen phosphate (NaH_2PO_4) and Acetonitrile at a ratio of 75:25 with orthophosphoric acid being used to adjust the pH to 3.5 with a flow rate of 1 mL/min at 35°C column temperature. The analysis was monitored using a photodiode array detector. **Results:** The results indicated a linear response (R2 = 0.9998) in the range of 0.125 $\mu g/mL^{-1}$–6 $\mu g/mL^{-1}$. The estimated limits of quantification were determined to be 0.167 $\mu g/mL^{-1}$. These techniques were then successfully used to estimate the IBA-HAP content during drug content estimation and *in vitro* through dialysis membrane 60 and *ex vivo* studies through Rat skin. **Conclusion:** the developed approach was precise, robust, sensitive, reliable, and incredibly easy. Retention times are fast and highly sensitive, rendering them suitable for both routine and bioanalysis analyses.

Key words: Calibration curve, IBA-HAP, limit of detection and limits of quantification, reversed-phase high-performance liquid chromatographic

INTRODUCTION

The analytical method development and validation are essential in the drug discovery, research and development, and manufacturing of pharmaceuticals.

Quantitative procedures used to measure quantitative measurements in the dosage forms are used by QC labs to ensure the test methods are in-line with the official monographs and also to ensure the analytical methods developed are accurate and potent and shows its performance on the quality of the dosage form.[1]

Ibandronic acid also known as ibandronate sodium (IBA) [(1-hydroxy-3-(methyl pentyl amino) propylidene bisphosphonic acid monosodium monohydrate)] is a Nitrogen containing synthetic bisphosphonate derivative. Post-menopausal osteoporosis, malignant hypercalcemia, and Paget's disease are among the conditions treated with this novel third-generation bisphosphonate.[2,3] Ibandronate hydroxyapatite (HAP) conjugate was complicated to analyze due to the absence of chromophores in both Ibandronate -Hydroxyapatite conjugate IBA and HAP.[4]

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Currently, there are few analytical techniques that are suitable for testing the purity and impurities in the Ibandronate sodium effectively. For instance, a high-performance ion exchange chromatographic method employed by indirect fluorescence detection developed a non-fluorescent Al^{3+} ibandronate compound with a subsequent post-column addition of fluorescent Al^{3+} -morin reagent.[5] Another study used high-performance ion exchange chromatography to identify ibandronate.

Another study used high-performance ion exchange chromatography to identify ibandronate. After forming a combination with the Cu2+ ion, the compound was detected at 240 nm by ultraviolet light. Ibandronate with related impurities (phosphate, phosphite) was also detected directly at 254 nm using the capillary zone electrophoretic technique. The reported values with a limit of detection (LOD) for phosphate as 5 µg/mL^{-1}, ibandronate as 176 µg/mL^{-1}, and for phosphite as 3 µg/mL^{-1}, and the respective linearity ranges were found to be around 90–500 mcg/mL^{-1}, 350–1800 mcg/mL^{-1} and 20–400 mcg/mL^{-1}. It is also proven that the ion chromatography approach is stable when used to simultaneously determine the drug material ibandronate sodium and its impurities.[6]

There are other techniques as well, such high-performance liquid chromatographic (HPLC) with an anion column and quantification using an HPLC-photodiode array (PDA) detector.[7,8] To identify a specific drug, such as ibandronate; however, all these techniques are expensive and need sample resources.[9] Furthermore, there is no such technique that uses HPLC and the IBA-HAP (IBA-HAP) conjugate complex, which would have been easier to use, less expensive, and resource-efficient. Thus, this research intended to develop an IBA-HAP conjugate in Emulgel dosage form using a straightforward, sensitive, and accurate HPLC technique for the 1st time. In addition, the newly developed method might be used in clinical trials to get precise human plasma pharmacokinetic characteristics.

MATERIALS AND METHODS

Reagents and chemicals

Ibandronate sodium (IBN) was obtained as a gift sample from Fleming Laboratories Ltd. in Hyderabad. Hydroxyapatite was synthesized. IBA-HAP conjugate was developed by adsorption technique [Figure 1].[10,11] Whereas sodium dihydrogen phosphate (NaH_2PO_4): ACN as a mobile phase. Analytical grade solvents are used in the HPLC system were procured from Merck, Mumbai, India., Acetonitrile and HPLC grade water were purchased from Sigma Aldrich, USA.

Instrumentation

The equipment/instrument specified in the current study, HPLC (Waters e2695 separations module, USA) equipped with a binary solvents delivery system, autosampler, and PDA 2998 detector. The separation has been done by using Sun fire column C-18 (pore size-5 µm and column dimensions (4.6 × 250 mm) was used for the chromatographic separation maintained at 25°C, with 1 mL/min flow rate. The sample temperature was maintained at 35°C. All the samples were filtered through a 0.22 µm membrane filter using a vacuum pump before analysis. Waters Corporation's Empower 3 software was used for data acquisition [Table 1].[12]

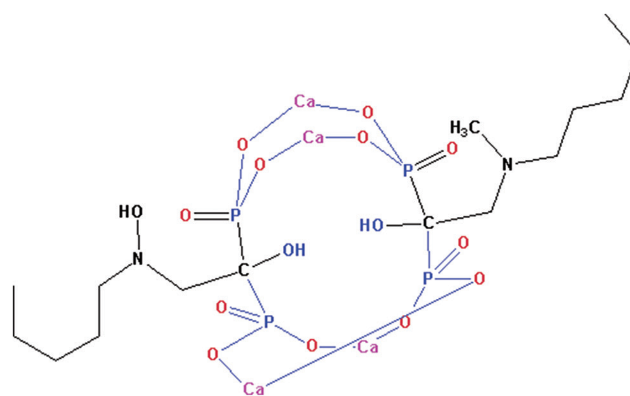

Figure 1: Chemical structure of ibandronate sodium-hydroxyapatite conjugate

IBA-HAP standard solution preparation

10 mg of the IBA-HAP conjugate was accurately weighed and added into a 10 mL volumetric flask. 2 mL of dimethylsulfoxide (DMSO) was added to dissolve the components and the volume was made up to the mark with the addition of HPLC grade water. It was dissolved in 2 mL of DMSO, followed by the addition of HPLC-grade water up to the mark, the primary stock solution was preserved under 2–8 °C until further use.

Mobile phase preparation

1.5601 g of NaH_2PO_4 was accurately weighed and added into a 1000 mL volumetric flask by dissolving the components with HPLC grade water to get 0.01 M NaH_2PO_4. 75:25 ratio of 0.01 M NaH_2PO_4 buffer and acetonitrile mixture was prepared and pH of the resulting solution was maintained at 3.5 using orthophosphoric acid. The pH was adjusted to 3.5 using orthophosphoric acid. It was then degassed in an ultrasonic water bath for 10 min and then filtered through a 0.45 µ filter using vacuum filtration. The solution was kept at 25 ± 5°C and used within 5 days.

Preparation of calibration curve standards

1 mL of the primary stock solution was taken into a 10 mL volumetric flask and 9 mL of HPLC grade water was added (1 mg/mL^{-1}) to get a secondary stock solution. Series of further dilutions were made in concentration ranges from

Table 1: Analytical conditions

Chromatographic conditions and spectroscopic parameters	
Column details	Sun fire column C-18
Mobile phase	Buffer pH 3.5: acetonitrile (75:25)
Flow rate	1 mL/min
Column temperature	35°C
Sample temperature	25°C
Injection volume	2 mL
Relative retention time	2.45
Run time	15 min

0.125 µg/mL^{-1}, 0.25 µg/mL^{-1}, 0.5 µg/mL^{-1}, 1 µg/mL^{-1}, 2 µg/mL^{-1}, 4 µg/mL^{-1}, and 6 µg/mL^{-1}. The calibration curve was constructed by measuring the absorbance at the wavelength of 211 nm and the correlation coefficient (r^2) was determined as 0.998.

Determination of assay content

IBA-HAP conjugate was loaded in Emulgel formulation[13,14] by accurately weighing 1 g of the emulgel formulation equivalent to 0.33 mg of ibandronate and extracted with a 75:25 ratio of 0.1N $NaH_2PO_4$and acetonitrile. The resultant solution was further diluted to 200 mL and filtered. 15 mL of the filtrate was centrifuged and the supernatant was injected into the HPLC system and the absorbance was measured at 211 nm using RP-HPLC.

Sample extraction from rat abdominal skin (*ex vivo* studies)

Ex vivo drug permeation studies were conducted on Rat abdominal skin.[15] First, the rat was sacrificed (IACE approval no: XXI/MSRFPH/P-17/September 12, 2018) by using chloroform in a desiccator for about 10 min. After the euthanasia of the rat, it was made to cervical dislocation and in the abdominal area, the fur was trimmed with a trimmer. The collected skin was then preserved in ringers' solution for 8 h.

A modified Franz diffusion cell or an inverted measuring cylinder was used to perform *ex vivo* skin permeation studies, with an effective diffusional area of 8.04325 cm^2. The excised skin was preserved in simulated skin pH 5.5 or normal saline solution. Later it is washed with plenty of water and positioned between the donor and receptor compartments of the diffusion cell. The skin with stratum corneum was faced toward the donor compartment and the receptor compartment was faced with the dermal side of the skin. The complete sandwiched system is then clamped. 50 mL of the simulated skin pH phosphate buffer was filled into the receptor compartment and pH 5.5 was maintained. The accurately weighed amount of 1 g of Nano emulgel formulation (0.33 mg of IBA-HAP conjugate) was uniformly spread on the skin exposed toward the donor compartment. A tiny magnetic bead was placed in the receptor compartment to maintain constant agitation at 25 rpm, an ambient temperature of 37 ± 2°C during the experiment. Retain sink conditions by, withdrawing 2 mL samples at pre-defined intervals for up to 10 h, and the same quantity of fresh buffer was replaced. The drug samples were analyzed by HPLC at 211 nm and the cumulative percentage of drug release was calculated.

Analytical method validation (AMV)

AMV is a process of creating documentary evidence creating a high level of assurance to the developed analytical procedure will produce a desired outcome and will consistently maintain pre-determined standards and quality attributes.

The developed method was validated using ICH guidelines (ICH, Methodology, May 19th, 1997 and ICH Q2 (R1) September 4th, 2005) including HPLC criteria, such as Linearity, Precision, Accuracy, Ruggedness, Robustness, limit of quantification (LOQ) and LOD.

Linearity

IBA-HAP conjugate exhibits linearity at concentration ranges from 0.125, 0.25, 0.5, 1, 2, 4, and 6 mcg/mL^{-1}, respectively. The IBA-HAP conjugate calibration curve was plotted using concentration versus peak area at 211 nm to determine the correlation coefficient (r^2). It was constructed by replica analysis (n = 6), performed across all the concentration ranges, to determine the linear relationship.

Accuracy

The method's accuracy was determined by performing repeat analyses on 3 sets of spiked samples with 3 different concentration ranges (80%, 100%, and 120%) of IBA-HAP conjugate, and comparing the difference between theoretical value and actual value by employing the developed HPLC method. All the samples were made in triplicate and the % recovery was determined.

Precision

The method's precision is calculated based on analysis of repeatability intraday (within a day), replicate analyses were carried out on 3 sets of samples spiked at 3 different doses of IBA-HAP conjugate 0.5 mcg/mL^{-1}, 2 mcg/mL^{-1}, and 6 mcg/mL^{-1}. The method's reproducibility (day-to-day variance) was validated using the same range of concentrations as indicated above for accuracy. For both approaches, Mean, standard deviation, and relative standard deviation (RSD) were calculated and represented by percentages (%).

Ruggedness/intermediate precision

The method's intermediate precision was determined on different days often referred as robustness. In the intermediate precision analysis, samples were collected exactly the same as in the precision study, and they were injected in HPLC at 3-time intervals on the next day, with the peak areas of all three injections determined. The percentage RSD for the area of six repeated injections was determined and found to be within the limits specified.

Table 2: linear regression equations generated during the validation of the IBA-HAP conjugate. The value of the slope, intercepts, and correlation coefficient of the determination by RP-HPLC

Analyte		
IBA-HAP conjugate		
Concentration (μg/mL^{-1})	Peakarea (m Vs.)	
0.125	145570	Slope: 871139
0.25	265801	Intercept: 34536
0.5	479381	r^2: 0.9998
1	874905	
2	1823119	
4	3536475	
6	5238101	

RP-HPLC: Reversed-phase high-performance liquid chromatographic

Specificity

A specificity analysis was conducted by analyzing the standard solution and blank to determine whether any peak in the blank interfered with the retention time of analytical peaks.

LOD and LOQ

LOD is a process of detecting the least amount of analyte in the sample that can be detected but can't be quantified to an accurate value. LOQ is the process of quantifying the lowest amount in the sample with sufficient accuracy and precision. The quantitation limit parameter helps in quantitative testing of the substances with minimum quantities in samples. The

Table 3: Accuracy determination

Compound	Intake (%)	Drug intake (μg/mL^{-1})	Total amount found (μg/mL^{-1})	Area	Recovery (%)[e]	Average recovery (%)	RSD (%)
IBA-HAP	80	8	8.103	7093698	101.29	100.013	1.1673
(μgmL^{-1})	80	8	7.98	7185362	99.75		
	80	8	7.92	7069356	99.00		
	100	10	10.070	8807291	100.704	100.165	0.4669
	100	10	9.987	8706742	99.87		
	100	10	9.992	8790874	99.92		
	120	12	11.772	1028975	98.101	99.319	1.3402
	120	12	12.089	1156721	100.74		
	120	12	11.894	1067546	99.116		

RSD %: Relative standard deviation

Table 4: Repeatability inter day by RP-HPLC

Compound				
IBA-HAP conjugate				
Concentration (μg/mL^{-1})	Concentration found (μg/mL^{-1})	Average (μg/mL^{-1})	S.D.	RSD (%)[a]
0.5	0.507	100.860	1.085	1.075
0.5	0.498			
0.5	0.507			
2	2.025	102.009	0.653	0.653
2	2.045			
2	2.050			
6	5.950	99.141	0.116	0.117
6	5.940			
6	5.954			

RP-HPLC: Reversed-phase high-performance liquid chromatographic, RSD %: Relative standard deviation. [a]% RSD=SD/Mean*100

calibration curve parameters were used to calculate LOD and LOQ using the below formula:

$$LOD = 3.3 \times S.D/Slope$$

$$LOQ = 10 \times S.D/Slope$$

Where,
S.D = Standard deviation of the response
SC = Slope of the calibration curve.

RESULTS AND DISCUSSION

Calculation of λ_{max} and relative retention time

IBA-HAP conjugate exhibited λ_{max} as 211 nm [Figure 2]. The acceptable retention time range of IBA-HAP conjugate was found to be 2.450 nm.

Calibration curve

The absorbance maxima set at 211 nm, correlation coefficient (r^2) was found to be 0.9998, slope as 871139, and intercept estimated as 34536. A linear response curve was calibrated over the concentration range from the developed method. The developed method therefore precisely used for the estimation of IBA in pharmaceutical dosage forms.

AMV

Linearity

The correlation coefficient (r^2) for IBA-HAP was found to be 0.9998, by the HPLC method, respectively [Table 2].

Accuracy

The solutions were prepared in triplicate, and the accuracy was shown by the percentage recovery, as shown in Table 3.

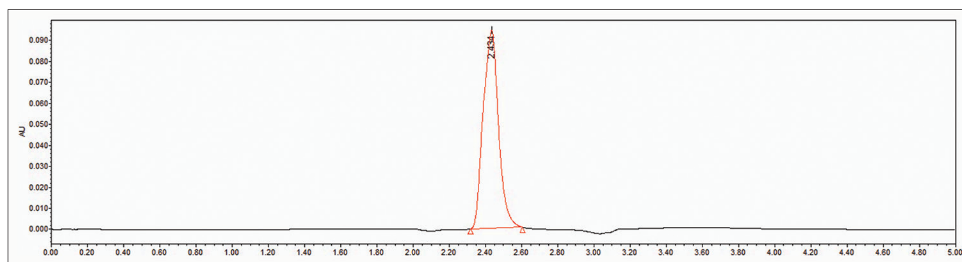

Figure 2: Chromatogram of ibandronate sodium-hydroxyapatite conjugate

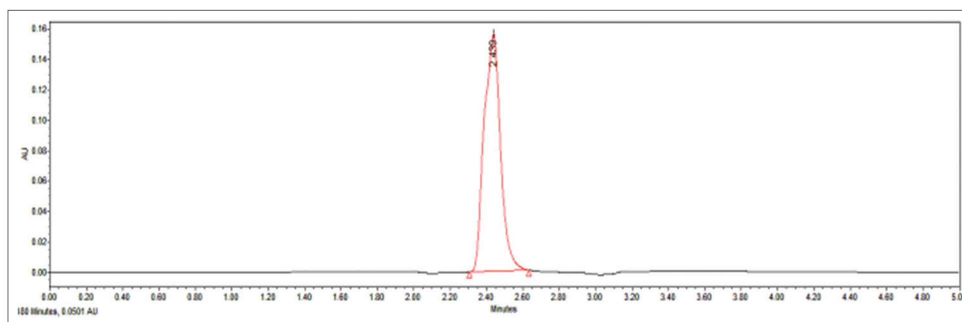

Figure 3: Chromatogram of ibandronate sodium-hydroxyapatite conjugate assay in Nano emulgel formulation

Table 5: Intra-day precision (n=3) by RP-HPLC

		IBA-HAP conjugate			
Day	**Concentration. (µg/mL^{-1})**	**Concentration found (µg/mL^{-1})**	**Average (µg/mL^{-1})**	**S.D.**	**RSD (%)**
Day 0	0.5	0.496	99.359	0.539	0.543
Day 1	0.5	0. 494			
Day 2	0.5	0.499			
Day 0	2	2.041	100.681	1.642	1.631
Day 1	2	1.977			
Day 2	2	2.022			
Day 0	6	5.914	99.123	0.696	0.702
Day 1	6	5.933			
Day 2	6	5.994			

RP-HPLC: Reversed-phase high-performance liquid chromatographic, RSD %: Relative standard deviation. %RSD=SD/Mean*100

The mean percent recoveries of IBA-HAP conjugate ranged from 99.319% to 100.013%. The findings show that the acceptance criteria for each level were within 98.0 and 102.0%, indicating that the determination accuracy was well within acceptable limits.

Table 6: Statistical and spectral data determination for IBA-HAP by developed RP-HPLC method

Experimental parameters	Observed values for IBA-HAP conjugate
Absorbance Maxima λmax (nm)	211 nm
Linearity range (μg/mL^{-1})	0.125–6 μg/mL
Correlation coefficient (r^2)	0.999
Slope (b)	871139
Intercept (a)	34536
Standard error of intercept	14595.95
Intercept standard deviation	1479332
Limit of detection	0.050
Limit of quantitation	0.167

RP-HPLC: Reversed-phase high-performance liquid chromatographic

Table 7: Drug content determination for Nano Emulgels formulations

Formulation code	Peak area	RT	% Drug content
NEGT	308598	2.439	98.53

Precision

The % RSD was found to be <2.0% [Table 4]. The inter day precisions have been expressed as %RSD.

Intermediate precision/ruggedness

The intra-day precision has been expressed as %RSD and found to be <2.0 % [Table 5].

Specificity

To ensure specificity, both blank and standard solutions were injected into the HPLC system. The pressure was found to be constant with no interference at the RRT of the analyte in the blank.

LOD and LOQ

The LOD for IBA-HAP was 0.050 μg, whereas the LOQ was determined and found to be 0.167 μg [Table 6].

Determination of assay content

IBA-HAP conjugate was loaded in Emulgel formulation, which was evaluated and the absorbance of this solution was measured at 211 nm using RP-HPLC and the results were reported in Tables 7 and 8, and the chromatogram is presented in Figure 3.

Table 8: Drug content determination (%) for IBA-HAP conjugate by RP-HPLC

Formulation	Compound	Label claim mg/1 g of gel	Conc. present (mg)	Conc. calculated (mg)	Mean Conc. calculated (mg)	Assay (%) (w/w)±[a]	RSD (%)[b]
IBA-HAP	IBA-HAP	1 mg/g	0.33	0.340	0.325	98.53	3.448
Conjugate	Conjugate		0.33	0.321			
Emulgel			0.33	0.320			
			0.33	0.312			
			0.33	0.320			
			0.33	0.338			

RP-HPLC: Reversed-phase high-performance liquid chromatographic, RSD %: Relative standard deviation. [a]Recovery of IBA-HAP conjugate (%)=Recovery of IBA-HAP conjugate/IBA-HAP present (mg) × 100±[b]S.D% RSD=SD/Mean×100

Table 9: *Ex vivo* permeation study of NEGT emulgel formulation through Rat skin

S. No	Time	RT	Peak area	Conc (mcg/mL)	Amount present in 2 mL (mcg)	Amount present in 50 mL (mcg)	Drug release (mg)	Cumm.% drug release
1	15	2.296	828423	0.9113	1.8226	91.1320	0.0911	27.61
2	30	2.296	1007520	1.1169	2.2338	111.6910	0.1116	33.84
3	60	2.295	1246393	1.3911	2.78223	139.1117	0.1391	42.15
4	120	2.289	1381532	1.5462	3.0924	154.6246	0.1546	46.85
5	240	2.286	1541943	1.7303	3.4607	173.0386	0.1730	52.43
6	360	2.277	2203479	2.4897	4.9795	248.9778	0.2489	75.44
7	480	2.281	2305048	2.6063	5.2127	260.6371	0.2606	78.98
8	600	2.282	2486963	2.8151	5.6303	281.5195	0.2815	85.30

Table 10: System suitability variables for IBA-HAP conjugate

S. No	Variables	IBA-HAP conjugate
Retention time RRT (min)		2.45
HETP (mm)		0.09
Theoretical plates (USP)	1694.6	
Capacity factor (k)		1.612

Ex vivo drug permeation studies

The IBA-HAP conjugate passed through rat abdominal skin samples withdrawn at pre-determined time intervals up to a period of 10 h was analyzed by HPLC at 211 nm and the cumulative percentage drug release found to be 85.30% at the end of 10 h are provided in Table 9 and system suitability parameters were tabulated in Table 10.

CONCLUSION

The linearity of IBA-HAP was validated at varying concentrations, ranging from 0.125 to 0.125 µg mL^{-1} to 6 µg mL^{-1}. The % recovery was found to be in the range of 99.319% and 100.013%, indicating the accuracy of the technique. % RSD values for precision were found to be <2%. The developed method is accurate for all the analytical validation parameters. The results of the Nano emulgel formulation The Nano emulgel formulation % assay yielded findings of 98.53%. The percentage of drug released by the *ex vivo* permeation study at the end of 10 h was determined to be 85.30%. The retention time is less time-consuming.

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