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RESEARCH ARTICLE

The Determination of Alendronate Sodium in Microparticular Systems by High Performance Liquid Chromatography

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The Determination of Alendronate Sodium in Microparticular Systems by High Performance Liquid Chromatography

Summary : *Alendronate sodium (AS) is an aminobisphosphonate that inhibits especially the osteoclast-related bone resorption. In orthopaedics, in order to make the total joint prostheses stay in the body for a long time without causing bone tissue loss, microparticular system formulations loaded with AS for implantation were prepared to be applied locally on those regions to prevent osteolysis. The purpose of this study was to apply a sensitive and reliable high performance liquid chromatography (HPLC) method for the determination of drug content in microparticular system formulations and the amount of drug present in the release medium. A validated pre-column HPLC method using 9-fluorenylmethyl chloroformate (FMOC) derivatization was used to determine the amount of AS from the microparticular systems prepared using different polymers. A reverse phase (PRP-1) column (10 µm particle size, 250 x 4.1 mm, i.d. (internal diameter), Hamilton, NV, USA) was used. The mobile phase, a mixture of 0.05 M sodium citrate and sodium phosphate buffer (pH 8)-acetonitrile-methanol (75:20:5, v/v/v) was delivered at a flow rate of 1.0 ml/min at room temperature. Parameters examined for the analytical method validation were linearity, accuracy, precision, sensitivity, selectivity and stability. Calibration curve of AS was found to be linear over a concentration range of 0.5-20 µg/ml (R^2 : 0.999). The precision and accuracy of the method were found to be lower than 2 % by statistical evaluation, and AS was found to be stable during release studies. This HPLC method was applied successfully to the analysis of the drug in microparticular systems. It was found that the amount of drug loaded in microspheres and the amount of released drug into the medium during in vitro release studies were determined precisely by this HPLC method.*

Keywords: Alendronate sodium, HPLC, method validation, microspheres, beads.

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Yüksek Performanslı Sıvı Kromatografisi ile Mikropartiküler Sistemler İçerisindeki Alendronat Sodyumun Tayini

Özet : *Alendronat sodyum osteoklastlara bağlı kemik erimesini inhibe eden bir aminobifosfonattır. Ortopedide, total eklem protezlerinin vücutta uzun süre kemik doku kaybına neden olmadan kalabilmesi için özellikle osteolizisin önlenmesinde, bu bölgelere lokal uygulanmak üzere implantasyon amaçlı olarak alendronat sodyum yüklü mikropartiküler sistem formülasyonları hazırlanmıştır. Bu çalışmanın amacı; mikropartiküler sistem formülasyonları içerisinde ve in vitro salım ortamında bulunan etkin madde miktarının duyarlı bir analitik yöntem ile tespit edilmesidir. Alendronat sodyumun değişik biyoparçalanabilir polimerlerle hazırlanan mikropartiküler sistem formülasyonlarından yapılan miktar tayini yönteminde, 9-fluorenilmetil kloroformat (FMOC) ile türevlendirilmesine dayalı ön-kolon HPLC yöntemi kullanılmış ve analitik yöntemin validasyonu yapılmıştır. Kullanılan kolon; uzunluğu 250 mm, iç çapı 4.1 mm olan 10 µm partikül boyutuna sahip PRP-1 (ters faz) (Hamilton, NV, ABD) özelliklerinde bir kolondur. Mobil faz, 0.05 M sodyum sitrat ve sodyum fosfat tamponu (pH 8)-asetonitril-metanol (75:20:5, h/h/h)'den hazırlanmıştır ve akış hızı 25° C'de 1.0 ml/dk'dır. Analitik metod validasyonunda incelenen parametreler: Doğrusallık, kesinlik, doğruluk, seçicilik, duyarlılık ve stabilitedir. Alendronat sodyumun kalibrasyon doğrusunun 0.5–20 µg/ml konsantrasyon aralığında doğrusal olduğu saptanmıştır (R^2: 0.999). Metodun kesinlik ve doğruluk parametreleri istatistiksel değerlendirmede % 2'den düşük çıkmış ve salım çalışmaları boyunca alendronat sodyumun dayanıklı kaldığı bulunmuştur. Bu çalışmada, mikropartiküllerin içerisinde bulunan alendronat sodyumun analizi için yöntem başarılı olarak uygulanmış ve kullanılan HPLC yöntemi ile mikroküreler içindeki etkin madde miktarının ve in vitro salım çalışmaları sırasında ortama salınan alendronat sodyum miktarının yeterli hassasiyette saptanabildiği belirlenmiştir.*

Anahtar kelimeler: Alendronat sodyum, HPLC, yöntem validasyonu, mikroküreler, boncuklar.

I. INTRODUCTION

Alendronate sodium (AS), which binds to bone surfaces and inhibits bone resorption by osteoclasts[1], is a nitrogen containing bisphosphonate (Figure 1) with potential utility in the treatment of diseases characterized by abnormal turnover, such as metastatic bone disease, hypercalcemia of malignan-

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cy, Paget's disease, periodontal disease and osteoporosis[2,3].

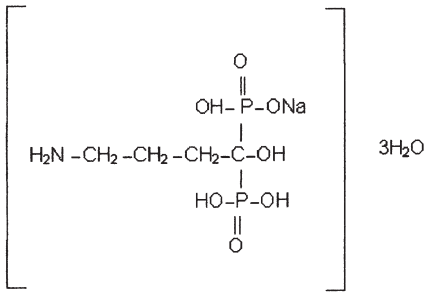

Figure 1. Alendronate sodium trihydrate

The direct chromatographic analysis of AS, as well as of many other bisphosphonates, is complicated due to the lack of a suitable UV chromophore for high performance liquid chromatography (HPLC) analysis with spectrophotometric detection[4-6]. Most of the analytical methods for determination of bisphosphonates require specialized equipment or involve a derivatization step in order to introduce a chromophore into the molecule[5]. AS contains a primary amino group which can be derivatized with a number of reagents to form a compound with chromophoric properties, thus facilitating HPLC analysis[6].

Assays which have been reported previously in the literature for the determination of the amount of AS performed in *in vitro* and *in vivo* studies involved HPLC[4,5,7].

A reversed phase HPLC method utilizing pre-column derivatization of the primary amine group of alendronate with 9-fluorenylmethyl chloroformate (FMOC) for UV detection has been reported in the literature for the determination of AS in solid dosage forms as tablets and capsules in *in vitro* medium[5]. Ion chromatography with indirect UV detection has also been mentioned for the assay of AS[7].

In addition, ion chromatography with post-column derivatization[8] and an ion exchange HPLC method with conductivity detection[9] have been developed and used for the quantitative determination of bisphosphonates in pharmaceutical dosage forms as tablets and solutions.

It has been reported in the literature that a study was carried out to examine the formation of a chromophoric complex between AS and copper (II) ions[6]; another study examined such a formation between AS and Fe (III) ions by UV spectrophotometry[10]. Spectrophotometric determination of AS in pharmaceutical formulations via complex formation with Fe (III) ions was found to be simple and non-sensitive in the literature. Therefore, a non-separative method would not be useful or sufficiently accurate for the determination of AS in microspheres nor for the determination of impurities[10].

In *in vivo* studies, quantitation of AS in human urine was performed by derivatizing the AS with FMOC and by operating a fluorimetric detector[4]. Some studies have been previously reported in the literature regarding the measurement of AS in human urine and in human plasma, including the methods based on co-precipitation of the bisphosphonate with calcium phosphates; automated pre-column derivatization of the primary amino group of the bisphosphonic acid with 2,3-naphthalene dicarboxyaldehyde (NDA)-N-acetyl-D-penicillamine (NAP) or cyanide (CN^-) reagents; and HPLC with electrochemical (ED) or fluorescence detection (FD)[11].

Following this thorough literature survey, the purpose of this study was to apply an HPLC method for the quantitative determination of AS in microparticular system formulations that were designed for the treatment of bone tissue loss around the prosthesis, which occurs in parallel to the aseptic loosening problem of a total joint prosthesis, and for the treatment of periodontal diseases and for use in the field of dental prostheses. Pre-column HPLC method based on FMOC derivatization was used after modification to determine the amount of AS loaded in the prepared microspheres and beads as well as the amount of AS released into the medium during *in vitro* release studies.

II. MATERIALS and METHODS

II.1. Instrumental Parameters and Chromatographic Conditions

Chromatographic analyses were performed using an HPLC system (Agilent Technologies HPLC 1100) with a reverse phase (PRP-1) column (10 µm particle size, 250 x 4.1 mm, i.d., Hamilton, NV, USA).

The mobile phase, a mixture of 0.05 M sodium citrate and sodium phosphate buffer (pH 8) - acetonitrile - methanol (75:20:5, v/v/v) was delivered at a flow rate of 1.0 ml/min at room temperature. Detection of the FMOC derivative was realized using spectrophotometric detector (Diode Array Detector-DAD) at 266 nm. Under these conditions, the retention time of the AS was determined to be approximately 8.1 min (Figure 2).

II.2. Materials

a. Reagents

Alendronate sodium trihydrate was supplied from Dabur India Limited (India) (Batch No.: MAT-2/F04/01). Acetonitrile, methanol, dichloromethane (HPLC grade), tri-sodium citrate dihydrate, citric acid anhydrous, potassium chloride, hydrochloric acid and pepsin (1200 E/g) were obtained from Merck (Germany). FMOC used as derivatization reagent was supplied from Fluka (Switzerland). Orthophosphoric acid was obtained from Riedel-de Haen (Germany), sodium tetraborate decahydrate and sodium phosphate dibasic anhydrous were supplied from Sigma (USA). All other chemicals were of analytical grade.

Chitosan medium and high molecular weight (m.w.) were supplied from Fluka (Switzerland). Chitosan high m.w. (Protasan Up CL 213) was obtained from FMC Biopolymer (Norway). Bovine serum albumin (BSA) was supplied from Sigma (USA), poly (lactide-co-glycolide) acid (PLGA) 50:50 (m.w.: 34000) from Medisorb, Merck Dupont (USA), and sodium alginate from BDH Chemicals Ltd. (England).

b. Standard Solutions

For the *in vitro* studies, a stock standard solution of AS (100 µg/ml) was prepared in 0.1 M sodium citrate solution and diluted to give a series of solutions in concentrations of 0.5, 1, 4, 6, 10, 14, 18, and 20 µg/ml, respectively.

In our study, 0.1 M sodium borate and FMOC solution (0.1 %, w/v) were prepared for the derivatization of AS. FMOC solution (0.1 %, w/v) was also prepared as mentioned before: 250 mg of FMOC dissolved in 250 ml of acetonitrile (this reagent was prepared freshly before HPLC analysis) and 0.05 M sodium citrate-sodium phosphate buffer (adjusted to pH 8 using orthophosphoric acid) was used to prepare mobile phase.

The mobile phase was a mixture of 0.05 M sodium citrate and sodium phosphate buffer (pH 8) - acetonitrile - methanol (75:20:5, v/v/v) and it was filtered through a 0.45 µm membrane filter.

II.3. Assay Procedure

II.3.1. Derivatization Procedure

1 ml of the sample or standard solution (in 0.1 M sodium citrate solution) was added to the 15 ml polypropylene centrifuge tube and to this 1 ml of 0.1 M sodium borate solution was added to adjust the pH of the solution to 9. Accurately measured 0.8 ml of the FMOC solution was then added. The tube was capped and vortexed for 30 seconds and the derivatization reaction was allowed to proceed for 30 minutes at room temperature. At the end of the reaction time, 5 ml of dichloromethane was added and the tube was vortexed for 30 seconds again, and then was centrifuged at 2000 rpm for 10 minutes to remove excessive reagent. Finally, the aqueous layer was transferred to an HPLC vial[5].

II.3.2. Sample Preparation

II.3.2.1. Determination of the Amount of Alendronate Sodium Loaded in Microspheres and Beads

In orthopaedics, in order to make the total joint prostheses stay in the body for a long time without causing bone tissue loss, "chitosan, BSA, PLGA (50:50) microspheres" loaded with AS, "sodium alginate beads" and "sodium alginate beads treated with chitosan" for implantation were prepared to be applied locally on those regions[12].

In order to determine the amount of AS in the microspheres prepared using chitosan biopolymer, 40 mg of microspheres were placed in a 10 ml volumetric flask and 5 ml of 1 % citric acid solution was added. The solution was mixed and at the end of the 24-hour period this solution was centrifuged for 15 minutes at 5000 rpm and the aqueous phase (supernatant) (active substance was extracted to the aqueous phase) was completely removed and placed in a volumetric flask. 10 ml of 0.1 M sodium citrate solution was then added onto this phase (to adjust the pH value of solution). Afterwards, 1 ml of this solution was taken, filtered through a 0.22 µm filter and then this solution was transferred to polypropylene tubes for derivatization. After the derivatization, this solution was injected into the HPLC column and peak areas were obtained. Concentrations were calculated by placing these areas on a calibration curve[12].

For determination of the amount of AS in the microspheres prepared using BSA, 10 mg of microspheres were present in tubes and 25 ml KCl/HCl buffer (pH 2) and 100 mg pepsin were added. Later, the solutions were stirred magnetically for 24 hours. At the end of this time, aqueous phase was taken and 0.1 M sodium citrate solution was added (to adjust the pH value of solution). Then, 1 ml of mixture was taken, transferred to polypropylene tubes after being filtered through a 0.22 µm filter, and then derivatized[12].

In order to determine the amount of AS entrapped in PLGA microspheres (o/w emulsion solvent evaporation technique PLGA-1, PLGA-2, PLGA-3 and PLGA-4 formulations), 50 mg of microspheres were weighed and 5 ml of dichloromethane was added to dissolve the polymer. 0.1 M sodium citrate solution was added on this phase, mixed on the magnetic stirrer for 2 hours and then the AS was transferred to the buffer. In the final phase, to evaporate the dichloromethane at room temperature, it was mixed for 1 more hour. It was then centrifuged for 15 minutes at 5000 rpm. 1 ml of the aqueous phase on the surface was removed, filtered through a 0.22 µm filter and derivatized[12].

The amount of AS entrapped in microspheres in PLGA-5 (w/o/w multiple emulsion technique) formulation was determined by weighing 50 mg of microspheres and adding 1 ml of dichloromethane to dissolve the polymer. Onto this, 0.5 ml of 0.1 M sodium citrate solution was added and AS was transferred to aqueous phase by means of an extraction lasting 2 hours. Then, 1 ml of solution phase was removed and filtered through a 0.22 µm filter and derivatized[12].

In order to determine the amount of AS entrapped, 50 mg of the sodium alginate beads were weighed and placed in a volumetric flask. 6 ml of 0.1 M sodium citrate solution was added and mixed on the magnetic stirrer. At the end of this process, 1 ml of the aqueous phase was removed, filtered through a 0.22 µm filter and transferred to polypropylene tubes and derivatized. This solution was injected into the HPLC column and peak areas were obtained. For the determination of the amount of AS loaded in alginate beads treated with chitosan, the method by which the amount of AS had been determined in alginate beads was used. In all of the above given procedures the same stages were applied to the empty beads and microspheres for control purpose[12].

III. RESULTS and DISCUSSION

III.1. Quantitative Analysis of Alendronate Sodium with High Performance Liquid Chromatography (HPLC)

Pre-column HPLC method based on FMOC derivatization was used in order to determine the amount of AS loaded in the prepared microspheres and beads. The same method was also used to determine the amount of AS released into the medium during in vitro release studies.

In the quantitative analysis of AS by HPLC, pre-column HPLC method which employs FMOC derivatization was used after modification[5]. In the method mentioned in the literature, column oven had been used with temperature adjusted to 35° C. As for the HPLC system used in this study, analyses were carried out at room temperature. Table 1 shows the HPLC conditions of the method.

Table 1. The chromatographic conditions of HPLC method used for quantitative analysis of alendronate sodium.

Column	Hamilton – USA; PRP Length: 250 mm; Internal Diameter: 4.1 mm; Particle size: 10 µm
Mobile Phase	0.05 M Sodium Citrate and Sodium Phosphate Buffer (pH 8): Acetonitrile: Methanol (75:20:5 v/v/v)
Injection Volume	50 µl
Flow Rate	1 ml/min
Detector	Spectrophotometric Detector (Diode Array Detector)
Wavelength	266 nm
Temperature	Room Temperature (25°C)

AS is not volatile and does not have a suitable chromophoric group in its molecule. Therefore, the direct analysis of AS by chromatographic method with spectrophotometric detector is not possible[5]. AS has an amino group which could be easily derivatized with reagents to form a compound having chromophoric properties, thus making the HPLC analysis possible[5]. FMOC can easily react with AS even at room temperature to give a compound containing a chromophoric group, so this can be analyzed using HPLC[5].

The citrate ions in the reaction media have critical importance to produce quantitative results during the derivatization process. Therefore all of the AS solutions used throughout the study were prepared in 0.1 M sodium citrate solution. In literature, citrate ions act as a sequestrant of metal ions in solution, thus allowing the transformation of AS to its non-complex form, which was considered suitable for derivatization[5]. During derivatization, pH of the medium should be nearly 9 to allow the reaction to take place between the derivatization reagent (FMOC) and the active substance. For this reason, 0.1 M sodium borate solution was added in AS solution to adjust the pH of the medium to 9 before adding the derivatization reagent (FMOC). Then, FMOC was added to the solution to complete the derivatization. It was left for 30 minutes, at the end of which time, the excessive amount of derivatization reagent (FMOC) was removed from the medium by the addition of dichloromethane.

Finally, quantitative analysis of AS was realized and HPLC chromatogram of AS was obtained. Retention time belonging to AS was determined to be 8.1 minutes. Peak obtained belonging to AS is shown in Figure 2.

III.2. Validation of the Analytical Method

Validation of the analytical method is the indicator of the reliability of the analytical method used. It is also a procedure to prove that the analytical method is correct, original and could be repeated under determined conditions. Parameters examined for the analytical method validation can be stated as: linearity, accuracy, precision, sensitivity, selectivity, and stability[13].

III.2.1. Linearity

To examine the linearity parameter in the validation of the analytical method, six stock solutions of AS were prepared in 0.1 M sodium citrate solution at a concentration of 100 µg/ml, and dilutions were made with 0.1 M sodium citrate solution to obtain AS in concentrations of 0.5, 1, 4, 6, 10, 14, 18, and 20 µg/ml, respectively. 1 ml of each AS solution in concentrations of 0.5-20 µg/ml was taken and derivatized. Then the aqueous phase was removed from the surface. Finally, it was injected into the HPLC column after having been filtered through the 0.22 µm filter. The calibration curve was obtained by plotting the average value of peak areas calculated in the six repeated studies versus the concentrations of solutions; the calibration equation was also calculated.

R^2 belonging to the calibration curve was found to be 0.999. The values belonging to slope and intercept are shown in Table 2.

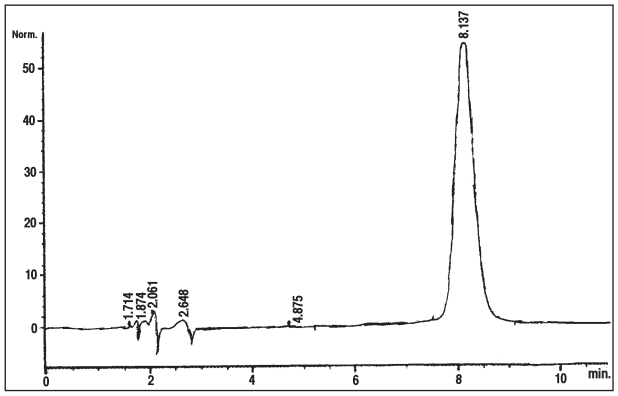

Figure 2. The chromatogram of alendronate sodium at a concentration of 16 µg/ml. (**Mobile phase:** 0.05 M sodium citrate and sodium phosphate buffer (pH 8): Acetonitrile: Methanol, (75:20:5), **Injection volume:** 50µl, **Flow rate:** 1ml/min, **Detector:** Spectrophotometric detector (DAD), **Wavelength:** 266 nm, **Temperature:** Room temperature (25° C), **Column:** PRP; Length: 250 mm, Internal diameter: 4.1 mm, Particle size: 10 µm, Hamilton – USA)

Table 2. The linear regression values of alendronate sodium obtained by HPLC (n=6)

PARAMETER	CONCLUSION
Concentration Range	0.5 µg/ml –20 µg/ml
Slope	73.15 ± 0.39
Intercept	-12.39 ± 0.26
R^2	0.999 ± 0.3 x 10^{-4}

III.2.2. Accuracy and Precision

In the determination of intra-day precision and accuracy, standard solutions in groups of six were prepared in eight different concentration values (0.5, 1, 4, 6, 10, 14, 18, 20 µg/ml) and consecutive measurements were carried out in the same day.

In order to determine the inter-day precision and accuracy, standard solutions were prepared in eight different concentration values (0.5-20 µg/ml) and analyzed daily for six days.

At the end of the experiments, to determine the intra-day and inter-day precision and accuracy, bias values were calculated to obtain accuracy findings and RSD values were calculated to obtain precision findings. The results are shown in Table 3. To determine the precision of the system, six consecutive measurements were performed on the standard solution of 10 µg/ml (standard concentration in the middle of the calibration curve) and the mean, standard deviation and RSD value of concentrations were calculated respectively.

For the determination of the intra-day accuracy, bias values of each concentration were calculated. Those values varied between -0.82 and 0.59 %. RSD values of each concentration for intra-day precision were found to be between 0.99 - 1.76 %.

For inter-day accuracy, bias values of concentrations were obtained. While these values varied between -0.12 and -1.29 %, RSD value was used to express inter-day precision as it had been used for intra-

day precision. The lowest and the highest values were found to be 0.41 % and 1.78 % respectively.

10 µg/ml concentration chosen from the calibration curve was used to evaluate the precision of the system and was injected into the HPLC column six times consecutively. RSD value calculated between the determined concentrations was found to be 0.56%. A RSD value of lower than 2 % indicates the precision of the system is acceptable.

Table 3. Intra-day and inter-day precision and accuracy values of alendronate sodium (n=6)

	Concentration (µg/ml)	Determined Concentration (µg/ml)	Accuracy bias* (%)	Precision RSD** (%)
Intra-day	0.5	0.51	-1.26	1.76
	1	0.99	0.59	1.64
	4	4.02	-0.59	1.72
	6	6.03	-0.45	1.06
	10	10.18	-1.82	1.03
	14	14.13	-0.90	0.99
	18	17.98	0.09	1.23
	20	20.15	-0.75	1.39
	Concentration (µg/ml)	**Determined Concentration (µg/ml)**	**Accuracy bias* (%)**	**Precision RSD** (%)**
Inter-day	0.5	0.50	-0.64	1.78
	1	1.00	-0.12	1.60
	4	4.01	-0.23	0.47
	6	6.05	-0.85	0.61
	10	10.13	-1.29	1.02
	14	14.10	-0.68	0.41
	18	18.16	-0.87	0.44
	20	20.11	-0.57	0.47

* bias = (Determined Concentration – True Concentration)/True Concentration x 100

** RSD = (SD / $\bar{X}$) x 100

III.2.3. Sensitivity

The sensitivity of the method used for the quantitative analysis of AS by HPLC was determined[12].

a) Limit of Quantitation is described as the lowest concentration which can be determined with acceptable precision and accuracy for the substance being analyzed within the limits according to the specified conditions of the method. 0.5 µg/ml, the lowest concentration from which the AS could be determined with acceptable precision and accuracy, was selected by considering the RSD value (1.76 %) determined in the studies.

b) Limit of Detection is the lowest concentration that can be determined qualitatively. This value can be expressed in concentration, the signal/noise ratio of which is 3/1. The signal: noise ratio was calculated, and the determination limit was found to be 0.3 µg/ml.

III.2.4. Selectivity

Selectivity is the capability of an analytical method to determine only the targeted compounds. Solutions of the excipients (polyvinyl alcohol-PVA, chitosan, BSA and glutaraldehyde) in the same concentration values as in the formulations of the prepared microspheres were prepared. After preparing the solutions of chitosan, BSA, glutaraldehyde, PVA, and sodium oleat in the same ratio as in the microsphere formulations, derivatization was carried out and those solutions were injected into the HPLC column. At the end of the analysis, no peaks were observed at the retention time of AS. However, PLGA, Tween 80 and Poloxamer could not be injected as a consequence of the turbidity and viscosity of the solution.

III.2.5. Stability

In order to demonstrate the stability of AS during the analysis, AS solutions in a concentration of 10 µg/ml were derivatized after preparation and subsequent injection. The RSD value of the stability results of the derivatized solutions was found to be 0.56 % and it was determined that derivatized solutions were stable during analysis at auto sampler for 1.5 hours.

III.3. Evaluation of the Stability of the Active Substance During In Vitro Release Studies

Sodium citrate solution having a pH 7.4 (pH value of human body) was chosen as the *in vitro* release medium. However, phosphate buffer at pH 7.4 was not used as the release medium since one of the necessities for performing the required derivatization is to use sodium citrate solution. In the literature, citrate ions act as a sequestrant of metal ions in solution, thus allowing the transformation of AS to its non-complex form, which was considered to be suitable for derivatization[5].

pH value of the AS solution prepared in 0.1 M sodium citrate solution was measured and found to be 8.6. For this reason, 0.1 M sodium citrate solution was selected as the *in vitro* release medium, and was adjusted to pH 7.4 with 1 % citric acid solution. It was tested whether it would be fixed at pH 7.4. Measurements were done after 24 hours and at the end of five days, it was demonstrated that the pH value of the *in vitro* release medium remained unchanged.

In order to demonstrate whether or not the AS was stable during *in vitro* release studies, AS solution at a concentration of 100 µg/ml was prepared in 0.1 M sodium citrate solution at pH 7.4. It was derivatized immediately after preparation and the quantitative analysis by HPLC was performed thereafter. The solution was kept at room temperature and on the 1st, 3rd, 6th, 7th, 9th, 10th, and 12th days it was derivatized, following which the quantitative analysis was performed. Concentrations obtained by quantitative analysis by HPLC were compared with the initial concentration (100 µg/ml) and RSD value was found to be 1.53 %, which again was below 2 %. Stability results of AS in *in vitro* release studies are shown in Table 4.

Table 4. The stability data of alendronate sodium related to in vitro release studies.

Measurement Time (Day)	Concentration (µg/ml)	Determined Concentration (µg/ml)
0	100	98.90
1	100	99.78
3	100	98.66
6	100	97.71
7	100	100.19
9	100	102.11
10	100	101.43
12	100	101.21
	$\bar{X}$	100.01
	SD	1.53
	RSD	1.53 %

III.4. Data Concerning Loaded Alendronate Sodium in Microspheres and Beads

The values concerning drug loading are presented in Table 5[12].

Derivatization was required to realize the quantitative analysis of AS by HPLC. In order to determine the amount of AS present in microspheres prepared using chitosan biopolymer, chitosan microspheres were first degraded with 5 ml 1 % citric acid solution. Since the pH value of the obtained eluent was acidic, 0.1 M sodium citrate solution was added to make it suitable for derivatization. It was observed that both in chitosan-1 and chitosan-2 formulations, the amount of loaded drug had decreased in contrast with the increased amount of glutaraldehyde, and drug loading was found to be lower in both formulations.

For BSA microspheres, acidic medium was changed to basic medium and then derivatization was performed. The amount of the loaded AS in BSA microspheres was found to be higher than in the other formulations. High binding affinity of AS to albumin is a possible explanation.

In the o/w emulsion solvent evaporation technique, it is stated that the loading is lower for active sub-

stances with hydrophilic characteristic. AS is a hydrophilic active substance and in our study, in order to increase the loading, it was aimed to change the solubility of AS by changing the pH value of the external phase. However, the solubility characteristic of AS was completely unchanged although it had five different pKa values. For all PLGA formulations, drug loading was found to be lower.

For sodium alginate beads, adjusting the pH value of the external phase to 2 and increasing the amount of sodium alginate also increased the loaded amount of AS. Since AS is a hydrophilic substance, drug loading was increased by changing the pH value of the aqueous phase. In chitosan-treated sodium alginate beads, it was observed that the loaded amount of AS increased with the increased amount of chitosan used in chitosan-treated alginate beads-3 and chitosan-treated alginate beads-4 formulations.

The above-mentioned procedures were also applied to empty microspheres for control purposes, and no extra peaks were observed at the retention time of AS at the end of the analysis.

IV. CONCLUSION

In quantitative analysis methods, studies performed by HPLC appear to be superior to those performed by UV spectrophotometry with respect to more sensitive and reliable results, as amounts could be determined at the µg level and all of the loaded drug amount and even the released substance (in very small quantities) could be determined.

An HPLC system utilizing pre-column derivatization with FMOC was used to determine the amount of AS. The system was applied successfully to the analysis of the drug in microparticular systems and was used to assay the amount of loaded drug and the released drug in *in vitro* release medium.

Analytical validation of the applied method was performed and the released amount from microspheres and beads was measured precisely. As a result, it can be concluded that pre-column HPLC method based on derivatization is a promising method in the determination of drug amount present in microparticular systems and *in vitro* release medium.

Table 5. Data related to drug loading and loading efficacy in microspheres and beads (n=3)

Formulation	Targeted Drug Loading (%)	Total Loading (%)	Loading Efficacy (%)
Chitosan-1 Microspheres	10	0.10	1.10
Chitosan-2 Microspheres	10	0.30	3.30
BSA Microspheres	10	3.94	43.34
PLGA-1 Microspheres (o/w)	10	0.70	7.70
PLGA-2 Microspheres (o/w)	10	0.20	2.20
PLGA-3 Microspheres (o/w)	10	0.15	1.65
PLGA-4 Microspheres (o/w)	10	0.65	7.15
PLGA-5 Microspheres (w/o/w)	10	0.66	7.26
Sodium Alginate-1 Beads	20	1.30	7.80
Sodium Alginate-2 Beads	20	0.70	4.20
Sodium Alginate-3 Beads	20	2.71	16.20
Chitosan-Treated Alginate Beads - 1	20	3.80	22.80
Chitosan-Treated Alginate Beads - 2	20	4.45	26.70
Chitosan-Treated Alginate Beads - 3	20	4.01	25.00
Chitosan-Treated Alginate Beads - 4	20	6.26	37.50

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References

1. Fleisch H. Bisphosphonates, *Drugs,* 42 (6), 919-944, 1991.
2. Sharpe M, Noble S, Spencer CM. Alendronate: An update of its use in osteoporosis, *Drugs,* 61, 999-1039, 2001.
3. Ezra A, Golomb G. Administration routes and delivery systems of bisphosphonates for the treatment of bone resorption, *Adv. Drug. Deliver. Rev.,* 42, 175-195, 2000.
4. Ptácek P, Klíma J, Macek J. Determination of alendronate in human urine as 9-fluorenylmethyl derivative

by high-performance liquid chromatography, *J. Chromatogr.* B, 767, 111-116, 2002.

5. De Marco JD, Biffar SE, Reed DG, Brooks MA. The determination of 4-amino-1-hydroxybutane-1,1-diphosphonic acid monosodium salt trihydrate in pharmaceutical dosage forms by high-performance liquid chromatography, *J. Pharm. Biomed. Anal.,* 7 (12), 1719-1727, 1989.
6. Ostovic D, Stelmach C, Hulshizer B. Formation of a chromatographic complex between alendronate and copper (II) ions, *Pharm. Res.,* 10 (3), 470-472, 1993.
7. Tsai EW, Chamberlin SD, Forsyth RJ, Bell C, Ip DP, Brooks MA. Determination of bisphosphonate drugs in pharmaceutical dosage formulations by ion chromatography with indirect UV detection, *J. Pharm. Biomed. Anal.,* 12 (8), 983-991, 1994.
8. Kosonen JP. Determination of disodium clodronate in bulk material and pharmaceuticals by ion chromatography with post-column derivatization, *J. Pharm. Biomed. Anal.,* 10, 881-887, 1992.
9. Den Hartigh J, Langebroek R, Vermeij P. Ion-exchange liquid chromatographic analysis of bisphosphonates in pharmaceutical preparations, *J. Pharm. Biomed. Anal.,* 11(10), 977-983, 1993.
10. Kuljanin J, Jankovic I, Nedeljkovic J, Prstojevic D, Marinkovic V. Spectrophotometric determination of alendronate in pharmaceutical formulations via complex formation with Fe (III) ions, *J. Pharm. Biomed. Anal.,* 28, 1215-1220, 2002.
11. Kline WF, Matuszewski BK. Improved determination of the bisphosphonate alendronate in human plasma and urine by automated precolumn derivatization and high-performance liquid chromatography with fluorescence and electrochemical detection, *J. Chromatogr.,* 583, 183-193, 1992.
12. Şamdancıoğlu S. Studies on developing a microparticular system formulation with bisphosphonates, Hacettepe University Health Sciences Institute, M.Sc. Thesis in Pharmaceutical Technology Program, Ankara, 2004.
13. Shabir GA. Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization, *J. Chromatogr. A.,* 987, 57-66, 2003.