Talanta 84 (2011) 437–442

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fluorimetric quantification of clodronate and alendronate in aqueous samples and in serum

Wiebke Ulbrich[a], Alf Lamprecht[a,b,c,*]

[a] Laboratory of Pharmaceutical Technology and Biopharmaceutics, University of Bonn, Germany
[b] Laboratory of Pharmaceutical Engineering, University of Franche-Comté, Besançon, France
[c] Institut Universitaire de France, Paris, France

ARTICLE INFO

Article history:
Received 17 October 2010
Received in revised form
22 December 2010
Accepted 16 January 2011
Available online 3 February 2011

Keywords:
Clodronate
Alendronate
Bisphosphonates
Fluorimetric quantification
Plasma
Serum

ABSTRACT

The bisphosphonates clodronate and alendronate are drugs in the therapy of osteoporosis or Paget's disease. They are highly hydrophilic and therefore of low oral bioavailability. Determination methods for bisphosphonates are often laborious and expensive equipment is needed. The presented quantification method based on kinetic measurement of the fluorescence decrease of an Al^{3+}-morin complex can be used to determine the bisphosphonate content in aqueous and plasma samples. The intra- and inter-assay accuracies were found to be within 98.8% and 102.3% of the target samples for clodronate and within 97.2% and 105.0% of the target samples for alendronate. The LOQ was defined as 15.6 ng/ml for clodronate and 62.5 ng/ml for alendronate. In serum samples, intra- and inter-assay accuracy was found to be within 99.0% and 101.6% of the target samples for clodronate and within 97.8% and 102.6% of the target samples for alendronate. In serum samples, the LOQ was defined as 1.55 mg/ml for clodronate and 0.39 mg/ml for alendronate. Though less sensitive in serum, the presented method could support research on the development of drug delivery systems in vitro and in vivo for the investigated and other structurally related bisphosphonates.

1. Introduction

The bisphosphonate drugs clodronate and alendronate are structurally related to the naturally occurring pyrophosphate like other compounds of this family. As highly hydrophilic drugs with extensive ionization bisphosphonates are hardly able to cross biological membranes [3,7]. Therefore, absorption from the gastrointestinal tract is poor leading to low bioavailability [5]. Being an inhibitor of bone resorption and general calcium metabolic disorders they are used for the treatment of Paget's disease, malignant hypercalcemia, osteolytic metastasis as well as osteoporosis.

Although the bisphosphonates clodronate and alendronate are rather old-fashioned, new therapeutic applications are going to be found due to their inhibitory action on cytokine secretion by macrophages on the molecular level [12]. Macrophages are efficiently targeted by colloidal drug carriers and the macrophage suppressive potency of encapsulated clodronate and alendronate has been shown either in vitro or in vivo [4,12,20]. In previous studies, the use of colloidal drug carriers for the delivery of clodronate enhanced the macrophage-suppressive effect up to 20-fold [12] and also the restricted bioavailability of clodronate might increase using its encapsulated forms. In general, this cytokine inhibition could be beneficial in local inflammatory diseases, where the inflammation is sustained by the excessive amounts of inflammatory cytokines produced by activated macrophages. Recently, new insights have been brought into the principally different cellular mechanism of action of both clodronate and alendronate [14] and therefore the development of a fast and easy determination method especially for these two bisphosphonates becomes desirable.

Hence, research into this class of drugs can prominently be supported by the development of a sensitive and reproducible analytical method [15] which could be used in biological samples with regard to bioavailability measurements and additionally for quality control of bisphosphonate containing pharmaceuticals [23].

Although the newer bisphosphonates are more potent, the use of clodronate can be favoured because its restricted ability to cross biological membranes might lead to potentially low adverse effects of the free drug. In detail, alendronate already showed mitigating effects on colitis severity administered in its free form.

For several reasons the analysis of bisphosphonates is difficult. UV-based technologies cannot be applied directly because chromophores are missing. High water solubility and extensive ionization restrict the use of a simple solvent extraction method [11]. Bisphosphonates can be titrated as acids or with a Th^{4+}-diaminocyclohexanetetraacetate complex titrant with

* Corresponding author at: Gerhard-domagk-Str. 3, D-53121 Bonn, Germany. Tel.: +49 (0) 228 73 52 33; fax: +49 (0) 228 73 52 68.
E-mail address: alf.lamprecht@uni-bonn.de (A. Lamprecht).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.01.049

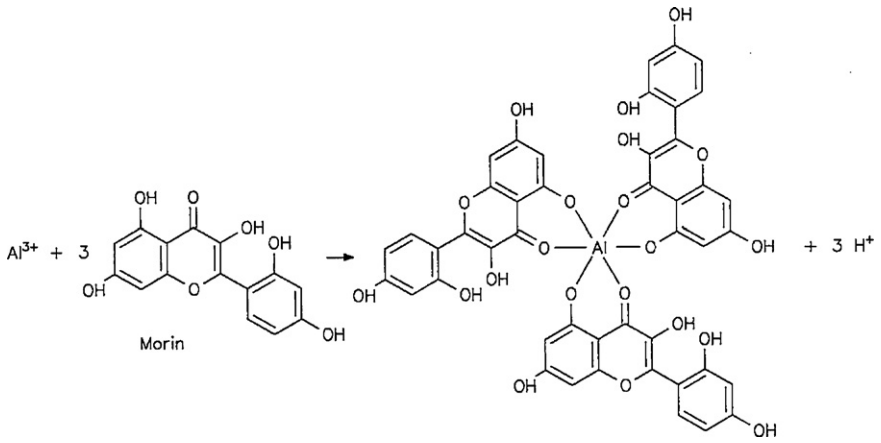

Fig. 1. Structure of the Al^{3+}–morin complex.

spectrophotometric end point detection [10] and spectrophotometrically after conversion of the bisphosphonate to phosphate followed by the formation of phosphomolybdate [1]. These methods are very laborious but sensitive enough to measure etidronate concentrations in the investigated biological media [15]. Nowadays, mostly chromatographic methods are presented for the determination of bisphosphonates because more and more separation methods have been developed since the first clinical experiments with bisphosphonates were started [17–19]. Though high sensitivity could be shown for chromatographic methods, often derivatization and advanced instrumental equipment is afforded [23].

The fluorescent substance morin (2′,3,4′,5,7-pentahydroxyflavone) coordinates Al^{3+} and other metal ions and a more intense fluorescent complex is formed (Fig. 1) which enables the detection of the metal ions [21]. When phosphate ions are added to an Al^{3+}-morin solution, morin is released because of the formation of the stronger Al^{3+}-phosphate complex leading to a fluorescence decrease. Correlation of the fluorescence decrease to the phosphate concentration forms the basis for a quantitative determination of the anion.

Here, we would like to evaluate a simple, fast and non-toxic determination method for bisphosphonates based on kinetic measurement of the fluorescence decrease of an Al^{3+}-morin complex. For the development of the measurement procedure both the non-nitrogen-containing bisphosphonate clodronate and the nitrogen-containing bisphosphonate alendronate have been used and the consequential method has been validated in aqueous solution and in serum using both clodronate and additionally alendronate as a representative of the nitrogen-containing bisphosphonates. The applicability of this method without the need of chromatographic instrumentation and time-consuming sample preparation has been tested in aqueous solution and in serum.

2. Materials and methods

2.1. Materials

Clodronate disodium was a kind gift from Salutas Pharma (Barleben, Germany) and alendronate disodium was purchased from Sigma Aldrich (Deisenhofen, Germany).

Morin hydrate (2′,3,4′,5,7-pentahydroxyflavone) was received from Fluka (Deisenhofen, Germany). Aluminium nitrate was obtained from Honeywell Riedel-de Haën (Seelze, Germany). Absolute ethanol was received from Merck (Darmstadt, Germany) and trichloro acetic acid was received from Sigma Aldrich (Deisenhofen, Germany). All other chemicals were of analytical grade.

2.2. Preparation of the aluminium-morin complex-solution

Morin was solved in 10 ml of a mixture of ethanol: water 4:1 and aluminium nitrate hexahydrate was solved in 100 ml distilled water. The two solutions were mixed and diluted with distilled water to receive a final concentration of 2 μM Al^{3+} and 10 μM morin. Using acetate buffer, the pH of the complex-solution was adjusted to 4.5. The solution was stored overnight to allow the formation of the complex. Excitation and emission maxima were investigated recording the spectra between 300 and 600 nm wavelength. Subsequently, to determine the integrity of the complex fluorescence intensity was read at 410/495 nm (Fig. 2).

2.3. Measurement procedure

To receive clodronate and alendronate standard solutions, either clodronate disodium or alendronate disodium have been dissolved in distilled water. Known aliquots of these stock solutions were diluted and used to prepare solutions of analyte test samples and calibration curves. 150 μl of the fluorescent complex was filled in 96-well microplates. Afterwards, 50 μl of the bisphosphonate standard solution per well was added. Each concentration was measured simultaneously in three wells using a microplate reader (Victor X, Perkin Elmer, USA). At one time, the maximum of three different concentrations was measured. For the determination of the fluorescence decrease, the fluorescence detection was started immediately and performed altogether for 20 times every second. Finally, the slope of the fluorescence decrease was calculated for each concentration and a linear calibration curve for bisphosphonate concentrations between 250 and 1250 μg/l was created.

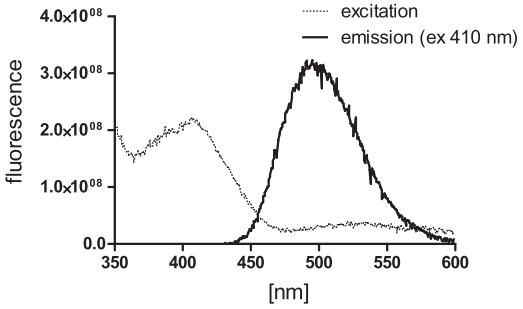

Fig. 2. Excitation/emission scan of the Al^{3+}–morin complex from wavelength 350 to 600 nm.

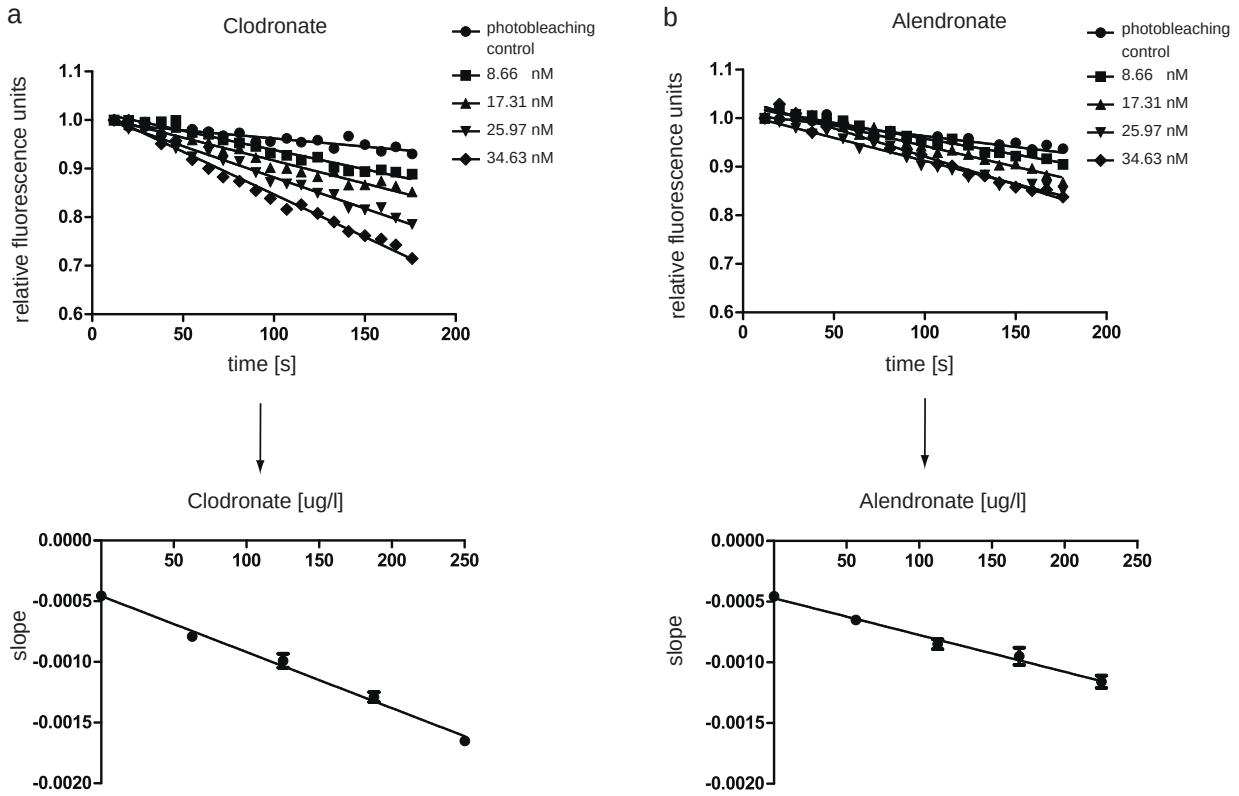

Fig. 3. (a) and (b) Time-dependent fluorescence decrease of an Al^{3+}–morin complex after addition of clodronate solutions (a) respectively alendronate solutions (b) at different concentrations. A linear correlation can be found between the bisphosphonate concentration and the slope.

2.4. Preparation of serum samples

Serum samples were obtained from male Wistar rats by cardiac puncture. Frozen serum samples were thawed in a water bath at 37 °C and the 5-point calibration curve was built by spiking blank (drug-free) plasma samples (100 μl) with aqueous clodronate or alendronate standard solutions to give concentrations ranging from 31 to 76 mg/l. The samples were homogenized by vortex mixing and afterwards, proteins were precipitated by vortex mixing with 100 μl of 6% trichloroacetic acid (TCA) for 4 min [21]. After centrifugation at 19,000 × g for 10 min at room temperature, the supernatant was diluted 1:40 with distilled water and assayed for its bisphosphonate content as described above. The samples for the determination of the intra- and inter-day accuracy and precision were prepared accordingly using three different bisphosphonate standard solutions giving the concentrations 35, 50 and 65 mg/l.

3. Results and discussion

3.1. Development of the measurement procedure using clodronate

Combination of the highly fluorescent Al^{3+}-morin complex with clodronate or alendronate leads to a fluorescence decrease because of the formation of the non-fluorescent Al^{3+}-bisphosphonate complex. It has been suggested that the decrease in fluorescence is proportional to the amount of present bisphosphonates [11]. Therefore, also the reached fluorescence minimum should be proportional to the bisphosphonates concentration. We investigated that the fluorescence always decreased to the same intensity, no matter which concentration of bisphosphonate was added. Nevertheless, the decrease in fluorescence was determined to be time-dependent and the speed was closely related to the bisphosphonate-amount added. For this reason, kinetic measurements for the determination of clodronate and alendronate are performed here.

It has been detected that a rather short time period is necessary for the determination of the fluorescence decrease. It can be assumed that one aluminium-ion is complexed first by one clodronate molecule. With increasing time one molecule of either clodronate or alendronate complexes an increasing amount of aluminium-ions leading to a change in the complexation speed (Reaction (1) and Fig. 3(a).

$$[\text{Bisphosphonate}(Al^{3+})_1] \rightleftharpoons [\text{Bisphosphonate}(Al^{3+})_{1+x}] \quad (1)$$

Accordingly, it was determined to be sufficient enough to read the fluorescence decrease for not longer than 150 s (Fig. 3(a) and (b)). The performance of 20 consecutive measurements every second has been shown to be suitable when a microplate reader is used.

With the intention to use a microplate reader for the measurements, the use of the highly inflammable solvent ethanol which has been recommended in other reports for the preparation of the complex [11] has been avoided. Instead, only morin which is hardly soluble in water has been solved in a mixture of ethanol:water 4:1 to receive a stock solution and the complex has been prepared with distilled water. Consequent investigation of the excitation and emission spectra showed a distinct peak for the excitation wavelength (Fig. 2). To decrease the detection limit to an optimum, the ratio of aluminium: morin was chosen to be 1:5 with an aluminium concentration of 2 μM [11]. With this choice, the background fluorescence decreases allowing for a more favourable detection limit.

The bisphosphonate concentration of the test solution should be less than 60% of the used aluminium-concentration. If higher bisphosphonate concentrations are chosen, the fluorescence decreases much faster because more than one aluminium-ion gets complexed by the bisphosphonate at one time leading to an alternating reaction speed (Reaction (1) and Fig. 3(a) and (b)).

Table 1
Main validation parameters for the determination of clodronate and alendronate by kinetic fluorescence decrease in aqueous and in serum samples fortified with standard solutions.

	Cladronate		Alendronate	
	Aqueous samples	Serum samples	Aqueous samples	Serum samples
Slope ± SD	0.004 ± 0.0002	0.017 ± 0.001	0.001 ± 0.000006	0.018 ± 0.002
Intercept ± SD	0.428 ± 0.029	0.846 ± 0.056	0.086 ± 0.004	0.938 ± 0.062
Determination coefficient r^2	.9883	.9705	.9831	.9671
LOQ	15.6 μg/l	1550.0 μg/l	62.5 μg/l	387.5 μg/l

Table 2
Inter- and intra-day accuracy and precision for clodronate and alendronate at different concentrations in aqueous solution.

	Cladronate			Alendronate		
Concentration	350.0 μg/l	700.0 μg/l	1100 μg/l	350| μg/l	700 μg/l	1100 μg/l
Intra-assay						
Mean	346.0	702.4	1125.2	354.8	733.5	1106.0
SD	22.7	7.6	19.4	17.9	57.6	4.2
% of target	98.9	100.3	102.3	101.2	104.8	100.7
Inter-assay						
Mean	345.8	712.1	1091.8	341.2	680.1	1154.8
SD	11. E	12.4	27.8	8.8	47.4	77.6
% of target	98.8	101.7	99.3	97.3	97.2	105.0

3.2. Linearity, the limit of detection (LOD) and the limit of quantification (LOQ)

In the investigated concentration range the calibration curve was linear.

The limit of quantification (LOQ), defined as the lowest concentration of clodronate or alendronate which can be quantified, was established by serial dilutions with decreasing concentrations of each bisphosphonate. The slopes were compared with the slopes of blank samples using student's *t*-test ($p \leq 0.05$) and the lowest concentration giving a slope significantly different from the slope of the blank sample was defined as the LOD respectively as the LOQ. As soon as a slope statistically different from the slope of the blank sample could be calculated it already correlates with the bisphosphonate concentration. For this reason, only the concentrations defining the LOQ are listed here.

The results are summarized in Table 1. In serum, the LOQ was almost 100 fold higher for clodronate and about 6 fold higher for alendronate than in aqueous samples. The calculation of these parameters showed clearly the inferiority of the investigated determination method for the use in serum compared to other methods.

3.3. Accuracy and precision

In order to evaluate the accuracy and precision of the assay in aqueous solution, three quality control samples with clodronate or alendronate concentrations of 350, 700 and 1100 μg/l were prepared and analysis was performed. Results are shown in Table 1. For the determination of the intra-assay accuracy and precision, each of the three concentrations was analysed four times. Inter-assay accuracy and precision were determined for each of the three concentrations over a period of four days. The three quality control samples were analyzed only once. The mean and standard deviation for clodronate were calculated at each concentration. The accuracy of each concentration (shown as % of target in Table 2) was determined by comparing the mean calculated concentration with the target concentration of the quality control sample. The intra- and inter-assay accuracy was found to be within 98.8% and 102.3% of the target samples for clodronate and within 97.2% and 105.0% of the target samples for alendronate.

The determination of the intra- and inter-assay accuracy and precision in serum samples was performed accordingly. Three quality control samples were prepared by spiking serum samples with clodronate or alendronate standard solutions giving concentrations of 35, 50 and 65 mg/l. After protein precipitation and centrifugation, the supernatant was diluted 1:40 with distilled water and the intra- and inter-assay accuracy was found to be within 99.0% and 101.6% of the target samples for clodronate and within 97.8% and 102.6% of the target samples for alendronate (Table 3). In conclusion, all of the values for the accuracy and precision were satisfying in aqueous solution and serum for both bisphosphonates.

3.4. Listing of possible interferences in aqueous samples

In principle, interferences can be expected with ions that compete with aluminium for complexation by morin. The same is the case for anions which react with aluminium. Therefore, the use of buffers containing phosphate or citrate is not possible and

Table 3
Inter- and intra-day accuracy and precision for clodronate and alendronate at different concentrations in serum samples.

	Clodronate			Alendronate		
Concentration	35.0 mg/l	50.0 mg/l	65 mg/l	35.0 mg/l	50.0 mg/l	65 mg/l
Irfra-assay						
Mean	34.8	50.3	64.4	34.8	51.1	63.6
SD	2.0	0.7	2.9	1.1	4.6	2.8
% of target	99.8	100.6	99.0	99.5	102.2	97.8
Inter-assay						
Mean	35.3	50.8	65.6	34.8	51.3	64.2
SD	1.1	0.9	2.2	1.0	1.8	0.8
% of target	100.6	101.6	100.9	99.3	102.6	98.7

also ethylenediaminetetraacetate (EDTA) and anionic polymers like alginate should not be present in the sample. Bisphosphonates are precipitated by calcium ions [15] and get complexed by iron [9]. Sodium hydroxide solution fluoresces intensive with morin and should accordingly not be used to adjust the pH-value [16]. The ions of beryllium, indium, gallium, thorium, scandium and zirconium react like silicates with morin to fluorescent dyes, whose formation and consistency strongly depends on the pH-value [16]. However, sensitivity can be easily ensured by the investigation of the original fluorescence intensity of the complex. The presence of compounds which react with either morin or aluminium can be realized immediately by a change of the usual fluorescence intensity. Additionally, the time-dependent decrease of the fluorescence intensity by clodronate or alendronate proceeds very characteristically and thus even minor deviations potentially provoked by other negatively charged drugs can be detected quickly. Therefore, selectivity of the presented method is given. Nevertheless, the presence of disturbing compounds has to be avoided because interferences can easily be detected but not prevented like it is the case with chromatographic methods separating the analytes before detection [11].

3.5. Evaluation of the applicability of the presented method in serum

In the concentration range of the quality control samples all validation parameters of the calibration curve were in an acceptable range. Nevertheless, the limit of quantification was rather high and other established methods are shown to be clearly superior despite their higher instrumental effort. The further increase of the bisphosphonate concentration in the quality control samples cannot be suggested as an alternative considering the already high concentrations in the present samples.

A dilution of the supernatant after protein precipitation of 1:40 is necessary because otherwise several pH-adjusting steps must be performed. A suitable possibility to determine the bisphosphonate concentration directly from the supernatant without further dilution might be precipitation of the bisphosphonate as calcium salt [15]. However, this procedure requires further time-consuming centrifugation-, pH-adjusting- and redissolving steps. It remains doubtful if these extensive sample preparation steps might be able to lower the detection limits without further loss of accuracy and precision. Additionally, calcium would definitely compete with aluminium for complexation of the bisphosphonate. Subsequently, the validation of the presented method should be restricted to diluted serum samples in the present work.

The concentration of phosphate ions or other substances which potentially react with aluminium or morin varies in each biological sample. Therefore, further sample pre-treatment like precipitation of the bisphosphonate is required. To avoid pre-treatment steps, the construction of calibration curves for each individual biological sample using spiked blank samples might be a feasible but time-consuming approach.

TCA has been shown to be a suitable reagent for protein precipitation of plasma samples at a concentration of 6% and a plasma to TCA ratio of 1:1 [22]. It has been suggested by Blanchard in 1981 [2] that TCA can precipitate 99% of the proteins with a concentration of 0.02% in plasma. This rather low concentration has not lead to quantitative protein precipitation in the present report. Therefore, the concentration and ratio suggested by Yun et al. [22] was maintained here.

The effect of the TCA-concentration on the linearity and standard deviation of the calibration curve has been investigated in the absence of serum and all parameters were in an acceptable range when the control samples were diluted appropriately. Without dilution, the Al^{3+}–morin complex dissociates due to the decreased pH-value. A dilution of 1:40 was determined to be necessary to adjust the pH-range and to increase the correlation coefficient of the calibration curve closely to $r^2 = 1$.

The preparation of plasma samples requires the addition of anticoagulants like citrate, EDTA or unfractionated heparin. These classical complexation reagents also react with aluminium-ions with partially higher affinity than the bisphosphonates and thus disturb the measurement procedure. The use of low molecular weight heparins seems to be possible to a limited extent (data not shown).

Serum samples have been used here because the preparation does not require any additional compounds. It can be assumed that haemolysis of eventually remaining erythrocytes has lead to the presence of haemoglobin at low concentrations disturbing an optic determination method due to its wide absorption spectrum with a peak around 450 nm [13]. Haemoglobin has been reported to absorb the fluorescence emitted by PicoGreen® and therefore, the fluorescence emission of the aluminium–morin complex probably also interferes with haemoglobin. Additionally, the higher values for the intercept in serum could confirm this suggestion. Also the iron delivered by haemoglobin might be complexed by clodronate [6] decreasing the LOD of this assay, although the serum samples used here were not even slightly red.

The presented method has also been validated for the determination of alendronate as a representative of the nitrogen-containing bisphosphonates. Similar results with regard to accuracy and precision have been received and it can be concluded, that kinetic measurement of the fluorescence decrease can also be considered as a determination method for many other bisphosphonates because equal functional groups responsible for the complexation of Al^{3+} are present. This potentially broad application spectrum to non-nitrogen- and also nitrogen-containing bisphosphonates is not self-evident since for example the Th^{4+}-diaminocyclohexanetetraacetate complex titrant-principle is applicable for clodronate but not for alendronate [18,19].

4. Conclusions

Indirect kinetic fluorescence measurement enables the detection and quantification of clodronate and alendronate in aqueous samples with high accuracy and precision. Without a large instrumental effort and without toxicity risks by organic solvents, the presented method shows a distinct ease of handling research on delivery systems for bisphosphonates. Though the investigated method was shown to be less sensitive in serum samples it is more interesting for an application like in vitro analytics such as formulation design or quality control.

References

[1] S. Bisaz, R. Felix, H. Fleisch, Clin. Chim. Acta 65 (1975) 299–307.
[2] J. Blanchard, J. Chromatogr. 226 (1981) 455–460.
[3] A. Ceponis, E. Waris, J. Monkkonen, L. Laasonen, M. Hyttinen, S.A. Solovieva, R. Hanemaaijer, A. Bitsch, Y.T. Konttinen, Arthritis Rheum. 44 (2001) 1908–1916.
[4] H.D. Danenberg, I. Fishbein, H. Epstein, J. Waltenberger, E. Moerman, J. Monkkonen, J. Gao, I. Gathi, R. Reichi, G. Golomb, J. Cardiovasc. Pharmacol. 42 (2003) 671–679.
[5] A. Ezra, G. Golomb, Adv. Drug. Deliv. Rev. 42 (2000) 175–195.
[6] A.W. Fitchett, A. Woodruff, LC, 1, 1993.
[7] E. Haber, H.D. Danenberg, N. Koroukhov, R. Ron-El, G. Golomb, M. Schachter, Hum. Reprod. 24 (2009) 398–407.
[9] J.P. Kosonen, J. Pharm. Biomed. Anal. 10 (1992) 881–887.
[10] S.J. Liggett, Biochem. Med. 7 (1973) 68–77.
[11] M.J. Lovdahl, D.J. Pietrzyk, J. Chromatogr. A 850 (1999) 143–152.
[12] N. Pennanen, S. Lapinjoki, A. Urtti, J. Monkkonen, Pharm. Res. 12 (1995) 916–922.
[13] N.B. Quashie, H.P. de Koning, L.C. Ranford-Cartwright, Malar J. 5 (2006) 95.
[14] Y. Shikama, Y. Nagai, S. Okada, T. Oizumi, H. Shimauchi, S. Sugawara, Y. Endo, Toxicol. Lett. 199 (2010) 123–128.

[15] R.W. Sparidans, J. den Hartigh, Pharm. World Sci. 21 (1999) 1–10.
[16] J. Strähle, E. Schweda, Lehrbuch der analytischen und präparativen anorganischen Chemie, 14th ed., S. Hirzel Verlag, Stuttgart, Germany, 1995.
[17] E.W. Tsai, S.D. Chamberlin, R.J. Forsyth, C. Bell, D.P. Ip, M.A. Brooks, J. Pharmaceut. Biomed. Anal. 12 (1994) 983–991.
[18] V. Virtanen, L.H. Lajunen, Talanta 40 (1993) 661–667.
[19] V. Virtanen, L.H. Lajunen, J. Chromatogr. 617 (1993) 291–298.
[20] N. Watanabe, K. Ikuta, K. Okazaki, H. Nakase, Y. Tabata, M. Matsuura, H. Tamaki, C. Kawanami, T. Honjo, T. Chiba, Dig. Dis. Sci. 48 (2003) 408–414.
[21] F.J. Welcher, Org. Anal. Reagents 4 (1948) 193.
[22] M.H. Yun, K.I. Kwon, J. Pharm. Biomed. Anal. 40 (2006) 168–172.
[23] C.K. Zacharis, P.D. Tzanavaras, J. Pharm. Biomed. Anal. 48 (2008) 483–496.