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Review

# Determination of bisphosphonate active pharmaceutical ingredients in pharmaceuticals and biological material: A review of analytical methods

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ABSTRACT

Bisphosphonates is a class of chemical compounds finding extensive medical applications against bone disorders including osteoporosis, Pagets' disease, etc. Non-N-containing members include etidronate, clodronate and tiludronate, while N-containing bisphosphonates include active pharmaceutical compounds such as pamidronate, neridronate, olpadronate, alendronate, ibandronate, risedronate and zoledronate. The present study covers 20 years of analytical research on this group of compounds, focusing on bioanalytical and pharmaceutical QC applications. A wide range of analytical techniques is presented and critically discussed including among others liquid and gas phase separations, electrophoretic, electroanalytical, automated and enzymatic approaches.

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## 1. Introduction

Bisphosphonates is a class of pharmacological active chemical compounds that inhibit osteoclast action and the resorption of bone. Initially synthesized in the 19th century, these compounds were originally used as water softeners [1]. The first studies of their action against bone disorders started during the 1960s' due to their potential in preventing the dissolution of hydroxylapatite and hence arresting bone loss. From a clinical point of view, bisphosphonates are used for the treatment of osteoporosis, bone metastasis, Paget's disease and other conditions that feature bone fragility. Among bisphosphonates, the most popular first-line drugs are alendronate and risedronate. In certain cases, intravenous pamidronate can be used as an alternative approach [2,3].

From a chemical point of view, the pharmacological function of these active compounds is determined by the P-C-P configuration, where two phosphate groups are covalently linked to a carbon atom as shown in Fig. 1. The groups linked to the carbon atom of the P-C-P chain influence the pharmacokinetics and the mode of action/strength of the drugs, respectively. In general, bisphosphonates can be categorized as the non-N-containing (etidronate, clodronate and tiludronate) and the N-containing (pamidronate, neridronate, olpadronate, alendronate, ibandronate, risedronate and zoledronate). More details on the relative potency of bisphosphonates can be found in Table 1 [4].

The exact mechanism of bisphosphonates action was proved only recently [2,3]. They are all related to pyrophosphate (PPi), which is formed as a by-product of cellular metabolism and during formation of nucleotides according to the following reaction scheme:

$$ATP \rightarrow AMP + PPi$$

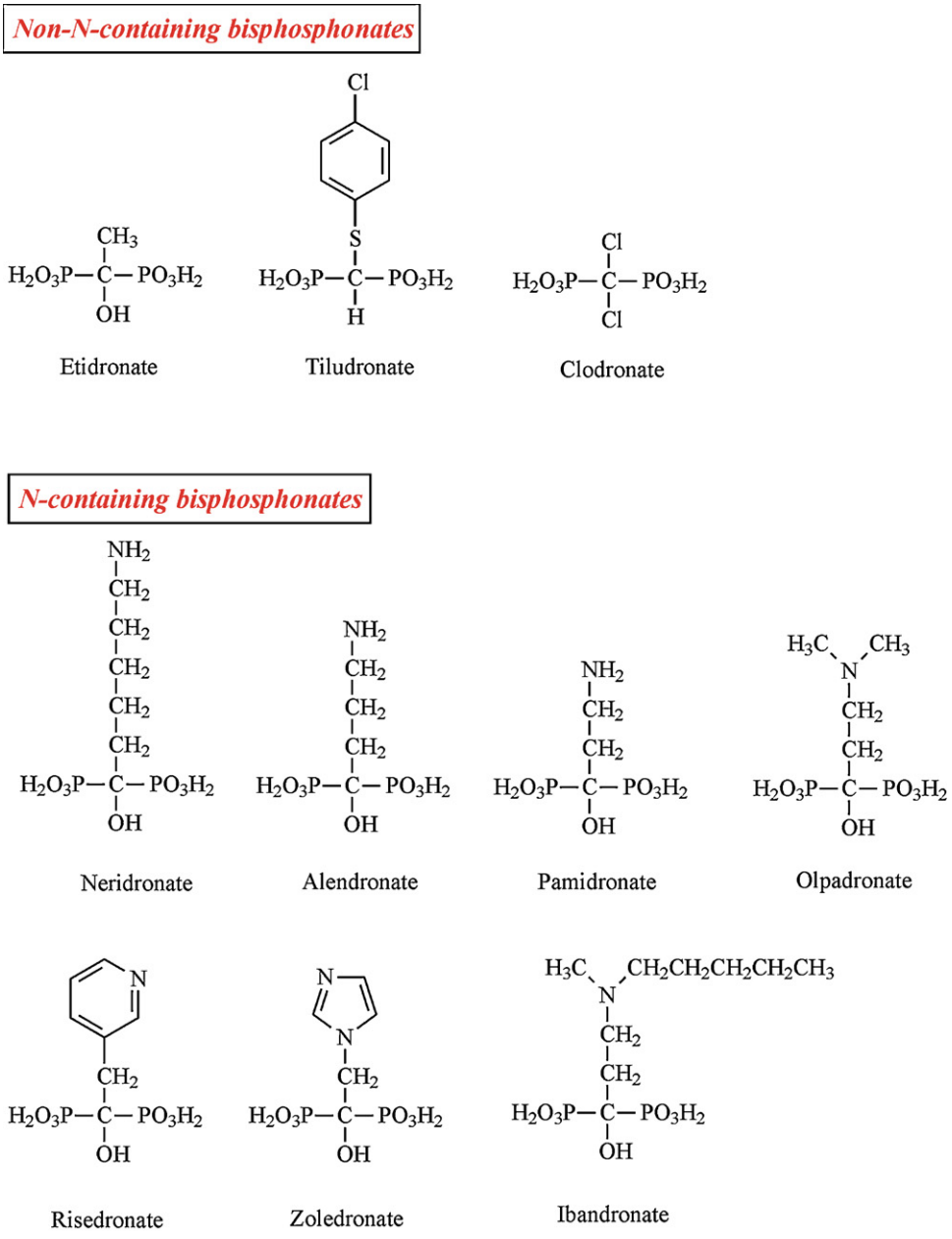


**Fig. 1.** Chemical structures of bisphosphonates.

Pyrophosphate is a natural inhibitor of the mineralization process in the bones which are protected by alkaline phosphatase. People with genetic problems of this enzyme have soft bones due to the action of pyrophosphates as they can get into the bone. By substituting an oxygen atom by a carbon, then a bisphosphonate is formed. Bisphosphonates not only get inside the bone, but they attach strongly to the bone mineral.

The most common side-effect of orally administered N-containing preparations is stomach upset and inflammation of the esophagus. The patients should therefore be seated in an upright position for at least 30–60 min after taking the medication. Bisphosphonates have also been associated with osteonecrosis of the jaw [5] and in some cases of severe bone and joint pain [6]. Less serious side effects include fever or flu symptoms for intravenous administration, while slightly increased risk for electrolyte disturbances has also been reported. However, the possible long-term effects of bisphosphonates are still under investigation.

Separation analytical methods dedicated to the analysis of bisphosphonates have previously been reviewed by Sparidans and den Hartigh, covering the time frame up to 1997 [7]. The scope of this study was to present a more extended and updated review of the analytical methods reported for the determination of this very important group of chemical compounds. Assays intended for both the quality control of bisphosphonate pharmaceutical formulations and analysis of biological material such as urine, plasma and bone tissue, are discussed. The review covers and critically discusses a wide selection of instrumental analytical techniques ranging from liquid and gas chromatography to electrophoretic, enzymatic and automated approaches. Special attention is paid on sample preparation protocols focusing on biological material.

**Table 1**
Relative potency of bisphosphonates APIs.

| Active pharmaceutical ingredient (API) | Potency[a] |
|---|---|
| Non-N-containing bisphosphonates | |
| Etidronate | 1 |
| Clodronate | 10 |
| Tiludronate | 10 |
| N-containing bisphosphonates | |
| Pamidronate | 100 |
| Neridronate | 100 |
| Olpadronate | 500 |
| Alendronate | 500 |
| Ibandronate | 1,000 |
| Risedronate | 2,000 |
| Zoledronate | 10,000 |

[a] Potency relative to that of etidronate [4].

## 2. Liquid chromatography

Liquid chromatography (LC) is a well established group of analytical techniques with extensive applications in bioanalysis and pharmaceuticals quality control [8,9]. LC generally offers reliable methods characterized by sensitivity, ruggedness and accuracy. The separation efficiency of these techniques makes them a useful tool not only for assay purposes, but impurities profiling and metabolites analyses as well. LC modes applied to the determination of bisphosphonates include RP-HPLC, ion-pair HPLC and ion chromatography (IC). The majority of these assays employs pre- or post-column derivatization reactions. Derivatization is often considered a necessary evil and for many scientists is the worst-case scenario. In many circumstances, it is necessary to convert the analyte molecule to a form that is better separated or is detected with much higher detection sensitivity. For HPLC, derivatization often aims at the introduction of a fluorescent (or other) label in the analyte molecule to enhance analytical sensitivity (Fig. 2). It should be stressed that derivatization is not only used prior to chromatographic separations, but also prior to capillary electrophoresis, gas chromatography and mass spectrometry [10].

### 2.1. Reversed-phase liquid chromatography

Reversed-phase high pressure liquid chromatography (RP-HPLC) is based on the use of solid particulate (usually suitably functionalized/chemically modified silica or polymeric materials) or monolithic support as stationary phases. Mixtures of organic solvents with buffers containing acidic or basic additives employed as mobile phases have been widely applied to separate moderately hydrophilic and hydrophobic compounds.

On this basis, a RP-HPLC assay with fluorimetric detection has been proposed by Kline et al. [11] for the quantification of alendronate in urine samples by derivatization with 2,3-naphthalene dicarboxyaldehyde (NDA). The approach includes three steps of sample pretreatment: (a) isolation of drug from urine sample by precipitation with $Ca^{2+}$, (b) sample clean-up using SPE and (c) pre-column derivatization with NDA in the presence of $CN^-$. High fluorescent yield of derivative was achieved at excitation and emission wavelengths of 420 and 490 nm, respectively. A mixture of methanol, citrate and phosphate buffer was used as mobile phase. Satisfactory linearity in the range of 5–100 ng ml$^{-1}$ was achieved. Additional validation parameters included accuracy and precision. Two years later, the same authors improved the previous method by automating the derivatization procedure by using a suitable autosampler [12]. Electrochemical detection was employed for compassion versus the fluorescent detector. Although both setups showed comparable analytical figures of merit, the latter detection setup was preferred due to its more reliable day-to-day performance in long-term applications. The achieved LOQ was improved five-fold when nucleophilic $CN^-$ was replaced by *N*-acetyl-D-penicillamine (NAP). The same reagent (NDA) has been applied to the automated determination of pamidronate in bone samples after pre-column derivatization [13]. The mobile phase consisted of acetonitrile and citrate/phosphate buffer while a RP-$C_{18}$ column was used throughout the study. Bone samples were firstly dissolved in HCl and then alkalized with NaOH while the calcium salt of the analyte is precipitated by the addition of $Ca_3(PO_4)_2$. EDTA was used to remove the excess of $Ca^{2+}$ ions, and the sample was finally derivatized with NDA prior to injection. The validity of the method has been evaluated in terms of precision and accuracy. A potential disadvantage includes the necessity for precise time-controlled reaction due to the instability of the derivatives.

Four different approaches based on derivatization of alendronate with 9-fluorenylmethyl chloroformate (FMOC) have been reported and applied to pharmaceuticals [14], human plasma [15], human urine [16] and bioequivalence studies [17]. In the first method, a simple isocratic elution was utilized with UV detection at 266 nm. Calibration curve was found to be linear in the range of 1–100 μg ml$^{-1}$ while the precision of method was varied between 1% and 3%. The figures of merit of the assay were sufficient for quality control (QC) of pharmaceutical solutions, capsules and tablets, including dissolution tests [14]. Yun and Kwon [15] applied a gradient elution program of acetonitrile, methanol and pyrophosphate buffer on a $C_{18}$ column (Fig. 3A). The LOQ (1 ng ml$^{-1}$) was adequate to determine the pharmacokinetic parameters of alendronate. A similar mobile phase was employed by Ptacek et al. [16] for the determination of alendronate in human urine. The derivative was detected fluorimetrically at $\lambda_{ex}$ = 260 nm, $\lambda_{em}$ = 310 nm. The approach is less sensitive than the previous one while the achieved LOQ was 3.5 ng ml$^{-1}$. The method was validated and applied to the of freeze-thaw stability studies of samples at short- and long-term

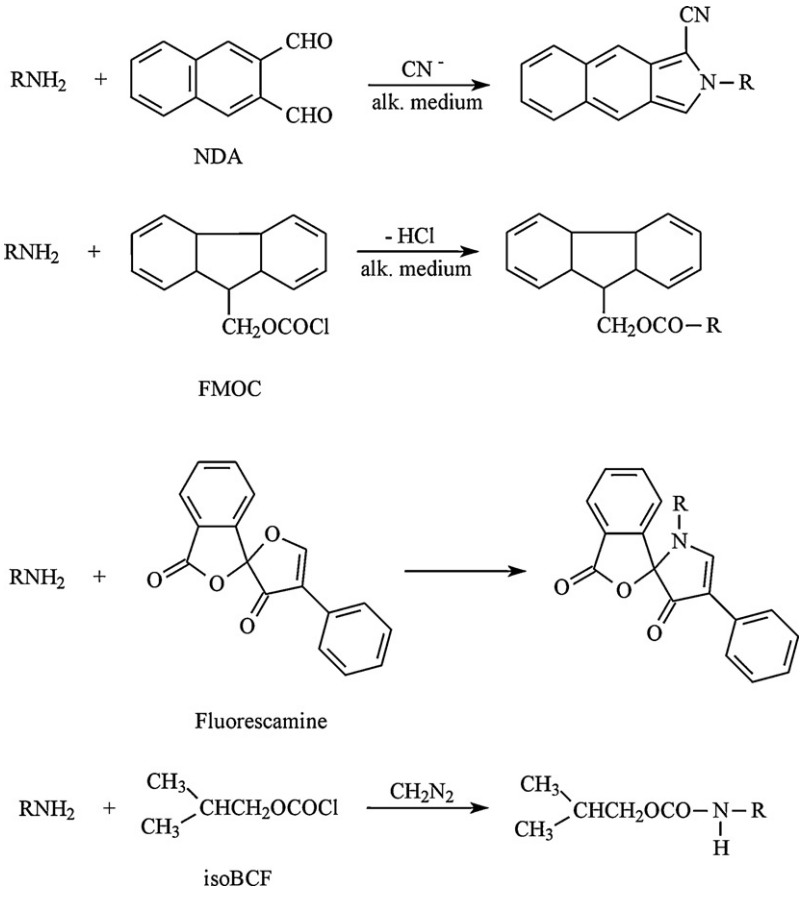


**Fig. 2.** Representative derivatization schemes of primary amino-group.

modes. Finally, a semi-automated workstation has been utilized for assaying human urine during bioequivalence studies [17]. Sample pretreatment involved solid phase extraction (SPE) in 96-well plates format and analysis was carried out by column switching via a 10-port valve. The method was validated according to the US Food and Drug Administration (FDA) protocol.

Fluorescamine was utilized as derivatizing reagent for the determination of pamidronate in human whole blood [18], plasma [19] and urine [18–20] after separation with RP-HPLC. Wong et al. [18] used a mixture of EDTA and methanol as mobile phase on a $C_{18}$ nucleosil column. An aliquot of human whole blood was deproteinized by the addition of trichloroacetic acid following precipitation of the analyte as its calcium salt. After alkalization, the sample was centrifuged and EDTA was added for complexing excess calcium. The calibration curve was linear over a range of 1–10 $\mu g\,ml^{-1}$ and the LODs were 0.1 and 0.5 $\mu g\,ml^{-1}$ in urine and blood samples, respectively. A similar approach was followed by Flesch and Hauffe [20] to determine pamidronate in urine samples. In this case, the fluorescent derivative was detected at $\lambda_{ex}$ = 395 nm, $\lambda_{em}$ = 480 nm selecting neridronate as the internal standard. The LOD and LOQ were 11.6 and 233 $ng\,ml^{-1}$, respectively. The same research group improved their method by applying an additional precipitation step during sample pretreatment [19]. In the latter approach, a five-fold lower LOD (2.3 $ng\,ml^{-1}$) was achieved, while the analysis time was reduced to 5 min.

Recently, LC-MS [21] and LC-$MS^2$ [22] were applied to the determination of N-containing bisphosphonates in serum and urine samples. In the former method [21], alendronate was doubly derivatized firstly with isobutyl chloroformate (IBCF) and then with trimethyl orthoacetate in order to avoid the tedious calcium precipitation step of analyte. A binary gradient elution with acetonitrile and formic acid was employed to achieve successful separation of the analyte and the internal standard (pamidronate). Sensitive detection was enabled in the range of 6.66–4860 $ng\,ml^{-1}$. Extensive validation in terms of accuracy and precision, sample's stability, selectivity, LOD and LOQ was carried out. In the latter approach [22], diazomethane was employed for derivatization of phosphoric groups of analytes converting into a less polar compound. The novelty of the proposed method lies on that the derivatization step was carried out on-cartridge using an anion-exchange sorbent, providing in this way higher recovery values. Multiple reaction monitoring (MRM) in positive mode was selected to quantitate risedronate and alendronate at transitions $m/z$ 340 → 214 or $m/z$ 348 → 163, respectively. The approach was characterized by high sensitivity with an LOD of 0.05 $ng\,ml^{-1}$.

### 2.2. Ion-pair HPLC

In some cases, manipulation of the pH of the mobile phase in RP-HPLC fails to separate mixtures of very polar organic compounds with ionic character. In these circumstances, ion-pair chromatography (IPC) is one of the most popular approaches to achieve efficient separations of such species. IPC can be easily performed by modifying the mobile phases used in the RP-HPLC using the same $C_{18}$ columns. An amphiphilic anion or cation, usually an alkyl sulphonic acid or salt and alkyl quaternary amine, respectively, is added to the mobile phases to enhance the retention of analytes bearing opposite charges. Various retention mecha-

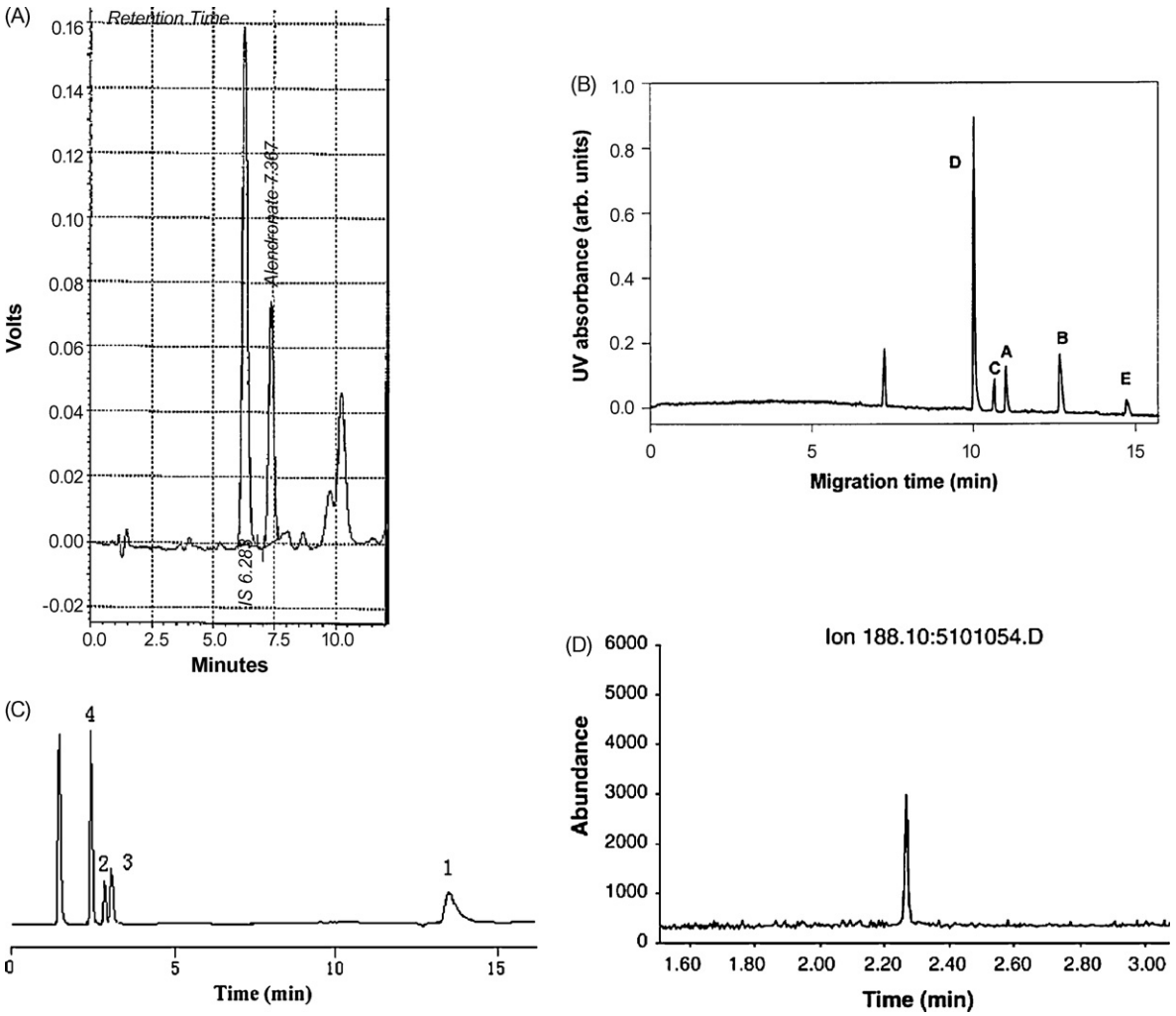


**Fig. 3.** Typical applications of separation techniques to the analysis of bisphosphonates. (A) Analysis of alendronate (44.26 ng ml$^{-1}$) using RP-HPLC (from ref. [15]), (B) capillary electrophoretic separation of clodronate (peak A) at 2.5 μg ml$^{-1}$ and its impurities (peaks B–E) (from ref. [67]), (C) separation of ibandronate (peak 1), phosphate (peak 2), phosphite (peak 3) and 3-methylpentylaminopropionic acid (peak 4) by IPC (from ref. [25]) and (D) GC-MS analysis of a fragmented ion of ibandronate monitored at $m/z$ = 188 (from ref. [80]).

nisms of IPC have been reviewed thoroughly [23]. Regardless of the retention mechanisms, IPC has been extensively used to selectively separate ionizable and ionic organic compounds in various samples.

Several biphosphonates such as alendronate, pamidronate, clodronate, etidronate and ibandronate, lack of chromophore groups. Therefore, quantitation of these compounds by IPC is possible after conversion into a detectable compound through derivatization protocols or directly using alternative detection modes such as evaporative light-scattering detection (ELSD).

### 2.2.1. Direct IPC assays

ELSD is a mass detector and thus it responds to all compounds that are sufficiently non-volatile in the detector chamber. The amount of light scattered is dependent on the size, shape and of course proportional to the analyte concentration. ELSD is based on the entirely volatilization of the mobile phase [24].

An IPC-ELSD method has been proposed by Jiang and Xie for the determination of ibandronate [25]. The method also enabled the simultaneous determination of phosphate, phosphite and 3-methylpentylaminopropionic acid which are considered potential impurities of the analyte. It is capable of determining the analyte in the range of 352–1760 μg ml$^{-1}$, with satisfactory precision and accuracy. The mobile phase consisting of $CH_3COONH_4$ + amylamine/$CH_3CN$/$CH_3OH$, was pumped through a $C_8$ Inertsil column in isocratic mode (Fig. 3C). A possible drawback of this approach is the relatively high LOD of ibandronate (176 μg ml$^{-1}$) but it is still acceptable for routine pharmaceutical quality control. The same authors expanding their work reported a similar method for the simultaneous determination of four bisphosphonates, namely alendronate, pamidronate, zoledronate and etidronate [26]. After investigating the parameters that affected the separation, successful determination of the analytes was achieved in 10 min using isocratic elution on a $C_{18}$ column. Similar eluent composition was selected providing a robust and precise method for routine pharmaceutical analysis.

An IPC-ELSD method has also been reported by Niemi et al. [27] for the determination of clodronate. In this method, while isocratic elution was selected for assaying the active ingredient, gradient elution has been proposed for the simultaneous determination of clodronate and its partial ester derivatives (monomethyl-, monopropyl-, monohexyl-, and monophenyl-) produced by chemical or enzymatic hydrolysis of human or rabbit biological specimens. In both cases, mobile phases were pumped

through a Kromasil $C_8$ column while butylamine was employed as ion-pair reagent. Calibration curves of the analytes were constructed following a quadratic relationship between analyte concentration and peak area. The achieved LOD for clodronate was 37.5 μg ml$^{-1}$.

Tetrabutyl ammonium hydrogen sulphate was proposed as ion-pair reagent for the separation of zoledronate from its impurities and degradation products generated from forced decomposition of the pharmaceutical samples [28]. The analytes were monitored using UV detection at 215 nm. The degradation of zoledronate-containing samples was investigated under the effect of light, heat, acid, alkali and water hydrolysis conditions. A 10% oxidative degradation of the drug was observed by $H_2O_2$ at room temperature. The same by-product was identified at longer time oxidation periods (48 h). A simple IPC method was developed by Jiang et al. [29] for the separation of zoledronate and its related compounds. Efficient separation was achieved on $C_{18}$ column using tetrabutyl-ammonium bromide as ion-pair reagent. Chemical parameters affecting the separation such as pH of mobile phase and concentration of the ion-pair reagent and the buffer were also studied and optimized. The achieved LOD (8 μg ml$^{-1}$) was adequate for the quality control of pharmaceutical samples.

Alternative IPC methods were developed for the quantitation of risedronate in pharmaceutical formulations [30,31], in rat plasma [32] and human urine [33]. Aluoch et al. [30] utilized tetrabutyl-ammonium phosphate as counter-ion to improve the chromatographic peak of risedronate detected at 262 nm. The method was validated in terms of selectivity, linearity (50–150% of 100 μg ml$^{-1}$), precision, accuracy, LOD (1.2 μg ml$^{-1}$ injected volume), LOQ and robustness. The procedure was applied for stability studies of risedronate formulations under acid, alkaline, thermal and light degradation. A more sophisticated work has been published by Kyriakides and Panderi [31] for the determination of the same analyte in pharmaceuticals. One-way analysis of variance (ANOVA) was used to evaluate the repeatability (R.S.D. 2.2–5.9%) and the reproducibility (R.S.D. < 1.3%) of the method. The slope and the intercept of the calibration curve were validated by measuring the S.D. of four curves. Compared to the previous work [32], the latter method provides lower analysis time (less than 6 min) and higher sensitivity (LOD: 0.48 μg ml$^{-1}$). In addition, the applicability of the method was examined on the degradation studies of the samples. The research group of Zhao and co-workers proposed a direct method for the determination of risedronate in rat plasma samples [32]. Sample preparation employed deproteinization of the sample, co-precipitation with $Ca^{2+}$, acidic hydrolysis at 90 °C, co-precipitation with $Ca^{2+}$ at alkaline medium and finally complexation of excess $Ca^{2+}$ with EDTA prior to the analysis. Tetrabutyl-ammonium bromide was used as ion-pair reagent. The linearity of the work was 10–500 ng ml$^{-1}$. The LOD and LOQ were 7 and 10 ng ml$^{-1}$, respectively. The method was applied to the pharmacokinetic study on rats. A "heart-cut" column-switching method was exploited by Vallano et al. [33] for the quantitation of risedronate in human urine using IPC with UV detection. 1-Octyltrimethylammonium phosphate and etidronate were employed as mobile phase additives to improve peak symmetry. Two analytical columns (column I: X-Terra $C_{18}$, column II: Synergi Polar RP Phenomenex) were connected on a 10-port valve improving in this way separation efficiency. The detector (λ = 262 nm) was connected to column II. The sample was injected directly on column I. Prior to the elution of the analyte, the valve switched automatically and the effluent was pumped towards to the column II. A potential drawback of the work is the complicated setup accompanied with high operation cost. On the other hand, it is promising for analysis of risedronate in biological matrixes due to its enhanced sensitivity and relative simple sample handling.

#### 2.2.2. Derivatization-based IPC methods

The research group of den Hartigh and co-workers published two methods for the determination of pamidronate in urine samples [34] and pharmaceutical formulations [35], both after derivatization with 1-naphthylisothiocyanate (NITC) and phenylisothiocyanate (PITC), respectively. In the former, the derivative was detected spectrofluorimetrically ($\lambda_{ex}$ = 285 nm and $\lambda_{em}$ = 390 nm) and in the latter spectrophotometrically at 240 nm. Tetraoctylammonium-bromide was used as ion-pair reagent. In method [34], a laborious pretreatment for urine samples was involved including co-precipitation as calcium salt, SPE, preconcentration, derivatization with NITC and finally double ion-pair liquid–liquid extraction. The method was validated in terms of precision (intra- and inter-day), LOD, LOQ (3 ng ml$^{-1}$) and linearity. The same research group worked further on this topic semi-automating the above-mentioned procedures and applying them in the determination of pamidronate in urine [36], serum and citrate plasma [37] and of olpadronate in urine and serum samples [38]. Using the semi-automatic instrumentation, the labour in solution handling was reduced considerably while the sample throughput was improved. In the case of olpadronate [38], similar protocol has been followed for its determination in biological samples. An expanded study has also been reported by the same authors where pamidronate, alendronate and neridronate were determined simultaneously after derivatization with NITC, DNTC, PITC and fluorescein isothiocyanate (FITC) [39]. The retention of the derivatives was studied using tetramethyl-, tetraethyl-, tetrabutyl-, tetrahexyl-, tetraoctyl-, ammonium as ion-pair reagents. Furthermore, fast atom bombardment mass spectrometry (FAB-MS) was utilized in order to identify the derivatization products of the analytes with above-mentioned isothiocyanates reagents.

Finally, al Deeb et al. [40] reported an IPC method for the quantitation of alendronate in pharmaceuticals tablets and urine samples, by reaction of the primary amino-group of the analyte with *o*-phthalaldehyde (OPA) in basic medium. Spectrophotometric detection at 333 nm was employed for the quality control of pharmaceuticals (LOQ = 14 μg ml$^{-1}$), while the more sensitive fluorimetric detection was selected for urine analysis ($\lambda_{ext}$ = 333 nm, $\lambda_{em}$ = 455 nm) with an LOQ of 0.3 μg ml$^{-1}$. Separations were carried out using a RP Hamilton column with tetrabutyl-ammonium perchlorate as ion-pair additive in the mobile phase.

### 2.3. Ion chromatography

Ion chromatography (IC), like other chromatographic techniques, is a separation technique used to analyze anions and cations in solution. The method is based on the same principles as HPLC where high-pressure pumps and columns are used for sample separation and analysis. During ion chromatography, a sample is injected into an eluent stream and carried through an ion-exchange column under high pressure. Different ions in the solution are separated based on their relative affinity to the column packing material [41]. Ion chromatography has been applied extensively to the determination of pharmaceuticals in various matrices [42–44].

Tsai et al. reported a simple IC procedure for the direct determination of alendronate in pharmaceuticals using a single-column approach with conductivity detection [45]. Diluted nitric acid was employed as the mobile phase, while a Waters IC-Pak HR anion-exchange column was used for analyte separation from the sample matrix. Validation of the assay included accuracy, precision, specificity, linearity and ruggedness.

A similar assay utilizing the same ion-exchange column and detection system was developed for the quality control of

pamidronate pharmaceuticals [46]. Nitric or alternatively succinic acids were proposed as mobile phases. The method offers a detection limit of 25 μg ml$^{-1}$ when the analyte is dissolved in water, but can be improved five-fold by dissolving pamidronate in the mobile phase. The assay was adequately validated for pharmaceuticals quality control applications.

Alternative IC methods for the determination of alendronate [47] and pamidronate [48] include the use of a refractive index detection system. In a similar approach as in ref. [46], a Waters IC-Pak anion-exchange column combined with diluted nitric acid as eluent were applied for the separation of alendronate [47]. Several variables including the pH, the type of acid and its concentration, the addition of organic modifiers and the column temperature were optimized. The retention mechanism of the analyte was also investigated in detail. The method is adequately sensitive for pharmaceutical applications (LOD = 0.4 μg ml$^{-1}$, S/N = 4). Refractive index detection coupled to a hydroxyethyl-methacrylate polymer column with quaternary amine functionalities enabled the separation of pamidronate from potential impurities such as phosphate, phosphite and β-alanine [48]. The effect of the composition and pH of the mobile phase was studied.

Several IC methods are focused on the determination and purity control of clodronate [49–51]. Kosonen adopted a post-column derivatization approach using acidic Fe(III) as complexing reagent and photometric detection at 300 nm, avoiding the conductivity detection. The ion chromatographic system also included a polystyrene-divinylbenzene copolymer column with diluted nitric acid as mobile phase [49]. The specificity of the method was evaluated against potential degradation products of the analyte, while further validation including accuracy, precision and ruggedness was carried out. Post-column derivatization based IC has also been proposed for the separation of clodronate from some of its esters. A more complicated thorium-EDTA-xylenol orange mixed ligand complex was used as a post-column reagent ($\lambda_{max}$ = 550 nm) [50]. The reported LOD of 0.3–1.4 μg ml$^{-1}$ is adequate for the determination of clodronate in urine as the expected levels range between 2 and 100 μg ml$^{-1}$. A probable disadvantage of the method is the time-consuming preparation of the post-column reagent. On the other hand, Taylor [51] reported a non-derivatization method for the determination of impurities in clodronate by IC. Suppressed conductivity detection was employed instead, using diluted sodium hydroxide as the eluent and an anion-exchange IonPac AS5 column. The author also reported the necessity of using a transition metal-ion trap column to avoid contamination and deterioration of the peaks after prolong use of the analytical column.

The complex-formation reaction between bisphosphonates and metal ions has been utilized for the IC determination of olpadronate and pamidronate in pharmaceuticals [52]. In a different approach compared to ref. [49] where Fe(III) was used in a post-column mode, in-line complexation with Cu(II) ions was adopted. UV detection at 245 nm offered limits of quantitation as low as 0.9 and 0.5 μg ml$^{-1}$ for the analytes, respectively. A potential disadvantage of the method is the inability to separate both analytes in a single run.

Post-column derivatization was also employed by Daley-Yates et al. [53] for the determination of bisphosphonates in human urine and plasma. The method based on the oxidation of the analytes with ammonium persulphate giving orthophosphate which then react with molybdenum-ascorbate yielding a coloured product detected at 820 nm. The method was linear up to 500 μg ml$^{-1}$ with an LOD of 10 ng ml$^{-1}$. Diluted nitric acid was used as mobile phase while two additional pumps were used to deriver ammonium persulphate and molybdenum-ascorbate reagents. The complexity of the instrumentation setup makes the proposed method unattractive for routine analysis.

A recent, more sophisticated method for the determination of alendronate and etidronate involved hyphenation of IC with inductively coupled plasma mass spectrometry (ICP-MS) [54]. The method is based on the use of ICP-MS as an element-specific technique for the determination of phosphorus at $m/z$ = 31. The main part of the study was focused on the optimization of the plasma conditions in order to avoid previously reported polyatomic interferences in phosphorus analyses when quadropole MS is used. The achieved LODs were 0.2 and 0.05 μg ml$^{-1}$ for alendronate and etidronate, respectively, while no applications to real samples were reported.

Indirect UV detection has proven to be a popular approach for the determination of bisphosphonates by ion chromatography [55–58]. In the majority of the cases, diluted nitric acid has been used as the eluent and Waters IonPac HR anion exchange as the separation column. Detection wavelength was set at 240 nm for the determination of etidronate [55], at 235 nm for the simultaneous determination of olpadronate and potential impurities [56] and at 220 nm for the determination of alendronate, etidronate and clodronate [57]. A more recent approach reported by Fernades et al. utilized diluted citric acid as the eluent and indirect UV detection at 226 nm for the individual determination of etidronate, clodronate, pamidronate and alendronate [58]. A potential advantage of the latter approach is the usage of silica-based columns instead of the more expensive polymer-based analogues. All the above-mentioned approaches have been optimized and validated for pharmaceutical quality control applications.

Finally, indirect fluorescence detection has been employed for the analysis of bisphosphonates in biological samples [59]. The analytes destroy the Al(III)-morin complex used as a post-column reagent and reduce the fluorescence intensity. Based on the combined selectivity of the fluorescence detection and the IC separation, biological samples can be analyzed without interferences. The LODs ranged between 4 and 10 ng. A disadvantage of the approach is the necessity for elevated temperature (70 °C) for optimum reaction conditions.

## 3. Capillary electrophoresis

The term capillary electrophoresis (CE), describes a family of techniques used to separate a variety of compounds. These analyses, all driven by an electric field, are performed in narrow tubes and can result in the rapid separation of great many different compounds. The versatility and number of ways that CE can be used means that almost all molecules and even whole organisms can be separated using powerful modes such as capillary zone electrophoresis (CZE), isotachophoresis, micellar electrokinetic chromatography (MEKC), isoelectric focussing and capillary electrochromatography (CEC). This makes them useful in situations where other liquid phase separation techniques are limited or impractical. The main advantages of capillary electrophoretic techniques include: high separation efficiencies, minute amounts of sample are required; it is easily automated and consumes limited amounts of reagents, generating low volumes of waste [60,61]. Table 2 contains a summary of the applications of electrophoretic techniques to the analysis of bisphosphonates.

Zeller et al. reported the determination of pamidronate by capillary isotachophoresis using a PTFE separation capillary and a fused silica detection capillary [62]. The proposed method enabled the simultaneous determination of phosphate and phosphite which are considered potential impurities of the analyte. Validation of the procedure included accuracy ($R$ = 102.57%) and precision (R.S.D. = 1.15%).

**Table 2**
Determination of bisphosphonates by CE

| Analyte | Method principle | Detection | LOD | Ref. |
|---|---|---|---|---|
| Pamidronate | Capillary isotachophoresis | Conductivity | – | [62] |
| Alendronate | CZE using in-capillary complexation with $Cu^{2+}$ | UV (254 nm) | – | [63] |
| Clodronate | CZE with indirect UV detection using dyes containing electrolyte | UV (254 nm) | 1 μg ml$^{-1}$ | [64] |
| Clodronate | CZE with indirect UV detection using dyes containing electrolyte | UV (254 nm) | 0.1 μg ml$^{-1}$ | [65] |
| Clodronate | CE-MS using electro-spray ionization | MS | 80–220 μg ml$^{-1}$ | [66] |
| Clodronate impurities | CZE with direct UV detection | UV (200 nm) | 0.25 μg ml$^{-1}$ | [67] |
| Ibandronate impurities | CZE with indirect UV detection using $K_2CrO_4$ containing electrolyte | UV (254 nm) | 2 μg ml$^{-1}$ | [68] |
| Alendronate, pamidronate | Multidimensional CE (ITP-ITP, ITP-CZE) | Conductivity | 0.8/2.8 ng ml$^{-1}$ | [69] |

Capillary zone electrophoresis has been applied to the determination of alendronate in pharmaceutical formulations by Tsai et al. [63]. The authors took advantage of the complexation reaction between the analyte and Cu(II) ions present in the electrolyte solution in order to apply direct UV detection at 240 nm. Validation experiments yielded poorer – but still acceptable – precision compared to routine HPLC assays, but significantly higher separation efficiency and dramatic reduction in mobile phase consumption. The addition of an internal standard is recommended as a potential solution to improve the precision of the method.

An indirect UV-based CZE approach has been developed and applied to the separation and determination of by-products formed during clodronate synthesis, namely phosphate and phosphite [64]. Background electrolyte solutions contained dye reagents, while the "dips" were recorded at 254 nm. Inorganic impurities could be detected effectively at the 0.1% level, whereas clodronate could be determined quantitatively at concentrations up to 2000 μg ml$^{-1}$. Based essentially on the same principles, Perjesi et al. developed a CE approach for the determination of clodronate in liposomal formulations [65]. Nitroso-R salt was employed as background electrolyte, enabling indirect UV detection at 254 nm. The within and day-to-day reproducibility of the assay in terms of both migration time and peak areas was satisfactory being <4% in all cases. Data from application to real samples were also included.

A more detailed study also on the purity control of clodronate has been reported by Huikko and Kostiainen, using a more sophisticated setup such as CE coupled to electrospray ionization mass spectrometry (CE-ESI-MS) [66]. Compared to ref. [64], this study was focussed on bisphosphonate impurities of the analyte. Careful investigation and optimization of chemical and instrumental parameters resulted in high separation efficiency and adequate repeatability. However, a critical disadvantage of the method is its poor sensitivity as the LODs ranged between 80 and 220 μg ml$^{-1}$, thus limiting its applicability only to bulk material. Possible explanations for the low sensitivity include the hydrophilic nature of the analytes and their dilution at the CE-MS interface. The same research group continuing their work on the purity control of clodronate, reported a CE method based on direct UV detection at low wavelengths (198–200 nm) [67]. After optimizing the parameters that affected the separation, an analytical cycle of 15 was achieved using phosphate buffer (pH 7.40) as the background electrolyte (Fig. 3B). The method is more sensitive compared to the previously reported based on CE-ESI-MS [66], as all analytes could be detected at the 0.25 μg ml$^{-1}$ level.

Another recent application of capillary electrophoresis to the purity control of bisphosphonate pharmaceuticals involves ibandronate and its inorganic impurities [68]. Successful separation of the drug from phosphate and phosphite was achieved using tetradecyl-trimethyl-ammonium bromide (TTAB) as the eluent in an uncoated fused silica capillary column. Indirect UV detection at 254 nm was enabled employing potassium chromate as background electrolyte. Efficient separation of the analytes was completed within 3 min, while chloride and acetate ions caused no interferences. The LODs for phosphate and phosphite were 2 μg ml$^{-1}$ in both cases.

An interesting multidimensional electrophoretic approach with significant potentials has been reported for the determination of alendronate and pamidronate [69]. Capillary isotachophoresis (cITP) was applied as a pre-separation technique for sample clean-up and preconcentration, prior to analytical determination using either cITP or CZE with conductivity detection. The developed setup enables the use of increased injection volumes at the 30–300 μl level, increasing this way the sensitivity of the method. The reported LODs for alendronate and pamidronate were 0.747 ng ml$^{-1}$ and 2.8 μg ml$^{-1}$, respectively ($V$ = 300 μl). Additional validation parameters included accuracy, precision and linearity. The method is promising for analysis of bisphosphonates in biological samples due to its enhanced sensitivity and simple sample handling.

## 4. Gas chromatography

Gas chromatography (GC) is undoubtedly the most widely used technique for the separation and analysis of volatile compounds. GC is one of the workhorse's techniques of many laboratories because it provides high separation efficiency, fast analysis, automation capabilities and generally requires a small sample injection volume. Additionally, the wide spectrum of commercially available specific or universal detectors provides the possibility of efficient detection schemes [70]. In typical GC analysis, a defined sample volume is injected into the pre-heated inlet and almost instantaneously volatilized. The volatilized compounds are moved by the carrier gas towards the column for separation. The separation process is based on their relative affinity to the stationary phase of the column (capillary or packed) under a thermal gradient program [71,72].

GC analysis of bisphosphonates is generally problematic. This originates from the ionic nature of these compounds resulting in broad and asymmetric chromatographic peaks. A typical approach to overcome this limitation is application of derivatization protocols using various reagents in organic medium [73–75]. In this way, the analytes' volatility and thermal stability are increased and the mass fragmentation pattern is improved when MS detectors are employed.

Muntoni et al. reported a specific method for the determination of clodronate in human plasma and urine using GC with nitrogen–phosphorus detector (NPD). The analyte was extracted from biological samples using anion-exchange resin and derivatized with bis-trimethyl-silyl-trifluoroacetamide (BSTFA) prior to the GC analysis. The assay was validated in terms of accuracy, precision, specificity and linearity and it was applied to pharmacokinetic studies [76]. An alternative GC approach coupled to electron ionization mass spectrometry has been proposed by Leis et al. [77]. The authors performed a similar sample extraction and derivatization procedure as mentioned above [76], achieving wider linear range in plasma samples (10–1280 ng ml$^{-1}$). The use of iso-

tope $^{18}O_3$–clodronate as internal standard at the latter method was found to be advantageous in terms of accuracy and precision.

A flame photometric (FPD)-based GC method has been proposed and applied to the separation and determination of pamidronate in human biological fluids [78]. Separation and detection of analyte was achieved after its conversion to a *N*-isobutoxycarbonyl (isoBCF) methyl ester derivative. Using an HP-1 capillary column the analysis of pamidronate was accomplished within 30 min, while the achieved LOD was 100 ng ml$^{-1}$. The same research group expanding their work reported a similar procedure for the simultaneous determination of pamidronate and neridronate [79]. Almost the same instrumental parameters were adopted producing similar analytical performance in terms of linear range, LOD, LOQ, etc. The phosphonate derivatives produced were identified performing GC-MS experiments.

GC-MS or GC-MS$^2$ has been proved to be powerful analytical techniques capable for efficient separation and identification of compounds. These hyphenated techniques have been applied by Endele et al. [80] and Auriola et al. [81] to the determination of (dichloromethyl)bisphosphonate and ibandronate, respectively. In Auriola's method the trimethyl silylated derivatives of the analytes are determinated using two different mass spectrometric modes: (i) electron ionization (EI) and (ii) chemical ionization (CI) employing ammonia and methane as reagent gases. In terms of sensitivity, the CI was proved to be advantageous compared to EI because it produces higher $m/z$ values when a single quadropole MS was utilized. Furthermore, the ammonia CI-MS$^2$ method provides adequate selectivity and sensitivity (LOD = 25 ng ml$^{-1}$, S/N = 5) [81]. Endele et al. [80] reported a GC-MS method for the determination of ibandronate in biological specimens. The GC-MS method based on the conversion of analyte to its methyl ester and monitor the $MH^+$ ion ($m/z$ = 188) formed by CI with ammonia (Fig. 3D). The developed assay has been validated in terms of precision, accuracy, linearity, LOD and LOQ.

## 5. Various analytical methods

Less common applications reporting the determination of bisphosphonate drugs include techniques such as inductively coupled plasma (ICP) [82], anodic stripping voltammetry [83], potentiometric titration [84], batch spectrophotometry [85] and sequential injection analysis (SIA) [86] for the determination of alendronate, enzymatic [87] and radioimmunoassay [88] for the determination of zoledronate acid, batch spectrophotometry for risedronate, etidronate and alendronate [89] and ELISA for the quantitation of ibandronate [80].

Reed et al. from Merck Research Laboratories took advantage of the phosphorus content of alendronate acid in order to develop an ICP-based assay for its determination in pharmaceutical tablets. The proposed method is simple, rapid and requires no derivatization steps. It is capable of determining the analyte in the range of 1.7–115 μg ml$^{-1}$, with satisfactory precision and accuracy. The main disadvantage of this method is the sophisticated instrumentation employed, which might be not available to most pharmaceutical analysis laboratories [82].

A voltammetric method reported recently for the determination of alendronate in pharmaceuticals and human plasma is based on the reaction of the analyte with Cu(II) released from a copper phosphate suspension, to form a soluble complex which is measured by anodic stripping voltammetry. The assay is adequately sensitive for bioanalytical purposes (LOD = 8.9 ng ml$^{-1}$), but potential drawbacks for pharmaceutical quality control applications are the rather limited determination range of 96–228 ng ml$^{-1}$ and that it requires 40 min for complex formation including an additional filtration step prior to analysis [83].

Potentiometric setup was utilized by de Haro Moreno et al. [84] for the determination of alendronate in pharmaceuticals. The method based on the titration of alendronate with NaOH. The approach is simple, rapid with low-operational cost, while the LOD achieved was 20 μg ml$^{-1}$ adequate enough for the quality control of pharmaceutical formulations.

UV–vis spectrophotometric assays are frequently employed to pharmaceutical analysis due the wide availability, simplicity and cost-effectiveness of the required instrumentation. On this bases, Kuljanin et al. developed a method for the determination of alendronate based on the formation of a colored complex with Fe(III) in perchloric acid medium. The proposed assay is simple, adequately sensitive (LOD = 2 μg ml$^{-1}$) and selective for pharmaceutical applications and offers a satisfactory determination range of 8.1–162.5 μg ml$^{-1}$. An additional advantage is that color formation proceeds instantaneously while the complex is stable for 3 h [85]. Alternative spectrophotometric approaches have been proposed by Taha and Yussef. These authors reported that risedronate could be determined quantitatively by measuring the absorbance difference in acidic and basic solutions at 262 nm. The approach is simple but it requires two measurements for evaluating the results. Taking advantage of the existence of a primary amino group, they employed the classic ninhydrin reaction for the determination of alendronate. A potential drawback of the method for routine applications is the requirement for heating at 90 °C for 20 min. Finally, they determined risedronate, alendronate and etidronate by oxidizing the analytes using Ce(IV) in sulphuric acid medium at room temperature and measuring the unreacted oxidant at 320 nm. Although a reaction time of 60 min is necessary for completion of the reaction, it is assumed that several samples could be processed simultaneously [89].

Automation is a key demand in modern analytical chemistry, especially in the cases of the quality control of pharmaceutical formulations were several samples have to be analyzed in the minimum of time. Continuous flow techniques offer certain advantages such as high-throughput assays, increased precision and accuracy and simple instrumentation and operation [90]. Tzanavaras et al. reported a normal spectrophotometric and a stopped-flow spectrofluorimetric assays for the determination of alendronate in pharmaceutical formulations using sequential injection analysis. The former was based on the reaction of the analyte with Cu(II) ions in acidic medium ($\lambda_{max}$ = 240 nm) and the latter on the well-known derivatization reaction of primary amino groups with *o*-phthalaldehyde (OPA) in basic medium to form a highly fluorescent derivative. Both approaches offer rapidity (60 and 30 samples h$^{-1}$, respectively), low reagents consumption and waste generation, single-channelled configurations and sufficient selectivity and sensitivity for pharmaceutical quality control. The low detection limit of the fluorimetric assay of 40 ng ml$^{-1}$ is adequate for bioanalytical applications if coupled to HPLC for selectivity enhancement [86].

Two bioanalytical assays have been reported for the determination of zoledronate in biological material [87,88]. In the first, Risser et al. have found that zoledronate can inhibit the multistep synthesis of cholesterol by $^{14}$C-labeled mevalonic acid lactone. Radiometric detection was employed and the achieved LOQ of 25 ng ml$^{-1}$ was sufficient for plasma analysis, providing acceptable recoveries in the range of 95–107% [83]. The latter approach was based on a competitive radioimmunoassay adapted in a microliter plate format. The assay utilized rabbit polyclonal antisera against a zoledronate-BSA conjugate. Compared to the previous method the limit of quantitation was 5-fold lower in urine and more than 50-fold lower in plasma or serum (LOQ = 0.4 ng ml$^{-1}$) [88].

An ELISA method based on the use of biotinylated anti-phosphonate antibody attached to streptavidine-coated tubes for the ibandronate quantitation has been published by Endele et al. [80]. A colour monitored at 422 nm when the peroxidase-conjugated analyte incubated with the antibody. The developed GC-MS and ELISA assays have similar analytical characteristics in terms of precision and sensitivity depending of sample volume injected ($LOD_{GC}$: 1–2 ng ml$^{-1}$ at 1 μl sample, $LOD_{ELISA}$: 0.01–0.05 ng ml$^{-1}$ at 100–500 μl sample volume).

## 6. Sample pretreatment techniques

Generally, biological samples such as urine, blood serum or plasma, saliva, etc., cannot be directly injected into a separation system due to matrix complexity. To avoid this problem, a sequence of sample treatment steps is required prior to analysis. The main goals of sample pretreatment are to suppress matrix effects and enhance selectivity, convert the analytes to a compatible form for analysis depending on the technique and if possible increase sensitivity through preconcentration.

In bisphosphonates pharmaceutical analysis and quality control, the pretreatment of the samples is relatively simple. Typically, it includes grounding, dilution and filtration (see references in Table 3). When it comes to bio-analytical applications, more complicated protocols are needed in order to achieve the above-mentioned goals such as deproteinization, precipitation of the analytes, solid phase and liquid–liquid extraction and combinations of the former.

### 6.1. Precipitation of bisphosphonates as calcium salts

Precipitation as calcium salts is an efficient and widely applied protocol for separation and isolation of bisphosphonates from biological matrices such as urine, plasma, serum and bones [11–13,15,16,18–20,32–34,36,38,40,53,80]. This process is based on complexation of Ca(II) with bisphosphonates in alkaline medium producing insoluble calcium salts. This is a process, analogous to the binding of bisphosphonates to the hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] bone matrix [7]. A typical pretreatment scheme based on precipitation is depicted in Fig. 4. This scheme is followed in the majority of the studies reported so far with slight or moderate modifications.

In order to enhance the efficiency of the procedure, double [11,15,16,18,19,30,32,40,80] or triple [33,34,36,38] precipitation cycles are often applied. Other modifications employ the use acetic acid [12,15,16,40] or phosphoric acid [13] for the dissolution of the formed pellets. While $CaCl_2$ is usually the reagent of choice, $Ca_3(PO_4)_2$ is used occasionally [13,36–38]. Final dilution of the samples can be carried out either in water [33,53] or EDTA solution in order to complex excess of calcium ions [11–13,18–20,32,40].

**Table 3**
Determination of bisphosphonates in pharmaceutical or bulk material samples

| Active pharmaceutical ingredient (API) | Description of method | LOD (μg ml$^{-1}$) | Ref. |
|---|---|---|---|
| Alendronate | RP-HPLC analysis after derivatization with FMOC | – | [14] |
| Ibandronate | IPC coupled to ELS detection | 176 | [25] |
| Alendronate, pamidronate, zoledronate, etidronate | IPC coupled to ELS detection | 16 (alendronate)<br>18 (pamidronate)<br>17 (zoledronate)<br>15 (etidronate) | [26] |
| Zoledronate | IPC with direct UV detection | – | [28] |
| Zoledronate | IPC with direct UV detection | 8 | [29] |
| Risedronate | IPC with direct UV detection | 1.2 | [30] |
| Risedronate | IPC with direct UV detection | 0.48 | [31] |
| Pamidronate | IPC after precolumn derivatization by phenylisothiocyanate and UV detection | 0.1 | [35] |
| Alendronate | Batch spectrophotometric method based on reaction with OPA | – | [40] |
| Alendronate | IC coupled to conductivity detection | – | [45] |
| Pamidronate, Etidronate | IC coupled to conductivity detection | – | [46] |
| Pamidronate | IC coupled to refractive index detection | – | [48] |
| Clodronate | IC with post-column derivatization with $Fe^{3+}$ and UV detection | – | [49] |
| Clodronate | IC coupled to suppressed conductivity detection | – | [51] |
| Olpadronate, pamidronate | IC with in-line complexation with $Cu^{2+}$ and UV detection | 0.4 (Pamidronate)<br>– | [52] |
| Etidronate | IC with indirect UV detection | 1 | [55] |
| Alendronate, etidronate, clodronate | IC with indirect UV detection | 1 | [57] |
| Alendronate | CZE method based on-line complexation with $Cu^{2+}$ and UV detection | – | [63] |
| Clodronate | CZE with MS detection | 80, –220 | [66] |
| Clodronate | CZE with direct UV detection | – | [67] |
| Ibandronate | MEKC with indirect UV detection | 2 | [68] |
| Alendronate, pamidronate | Multidimensional CE (ITP-ITP, ITP-CZE) | 0.8/2.8 (× 10$^{-3}$) | [69] |
| Alendronate | Determination of the phosphorus content by ICP | – | [82] |
| Alendronate | Voltammetric method based on complexation with $Cu^{2+}$ and subsequent reduction at -153 mV. | 0.009 | [83] |
| Alendronate | Batch spectrophotometric determination after complexation with $Fe^{3+}$ and UV detection | 2 | [85] |
| Alendronate | Sequential injection methods based on:<br>(i) Complexation with $Fe^{3+}$ and UV detection<br>(ii) Derivatization with OPA and FL detection | 0.3 (UV)<br>0.04 (FLD) | [86] |
| Alendronate, risedronate, etidronate | Risedronate: UV detection based its native absorbance in acidic medium.<br>Alendronate: derivatization with ninhydrin and vis detection.<br>Etidronate: oxidation reaction by Ce(IV) and UV determination of the consumed oxidant. | 4.8 (risedronate)<br>1.2 (alendronate)<br>0.66 (etidronate) | [89] |

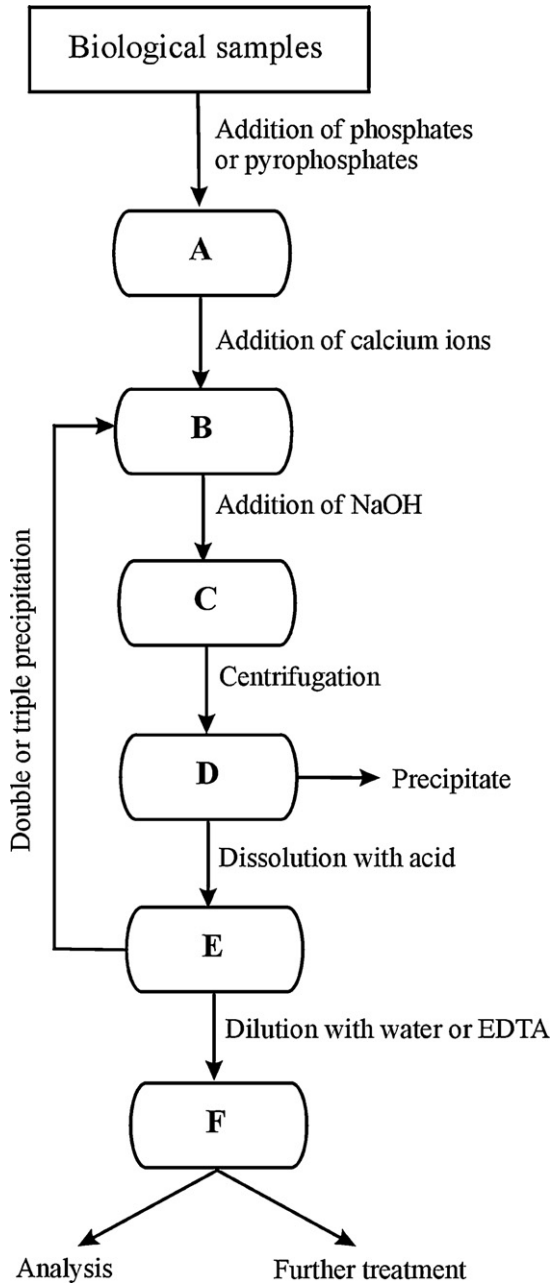


**Fig. 4.** Typical pretreatment protocol for the analysis of bisphosphonates in biological samples using calcium precipitation.

Following precipitation, the resulting solutions can be analyzed directly [32,53], or after suitable derivatization [13,19,40]. However, in most of the cases further sample clean-up is required prior to analysis, involving mainly SPE [11,12,15,16,33,34,36–38,80].

### 6.2. Solid phase extraction of bisphosphonates

Solid phase extraction is widely used modern sample preparation technique as it offers significant advantages over traditional liquid–liquid extraction, automation capabilities, high recoveries, selectivity, preconcentration of the analytes, easy handling and higher throughput.

Due to the ionic nature of bisphosphonates ion-exchange resins are the packings of choice in order to achieve efficient purification of the samples. The most widely applied functional group is the weak anion exchange diethyl amino group (DEA), either in conventional [11,12,15,16] or automated configurations [17]. Elution of the analytes is carried out by aqueous citrate [15–17] or citrate/phosphate buffers [11,12]. Alternative packings include quaternary amine exchangers [34,36,37] using nitric acid as eluent, Dowex $1 \times 2$ anion-exchange resins with HCl as eluent [76,81].

Less common but interesting approaches include the utilization of the silica based ion-exchange sorbent SAX for on-cartridge derivatization prior to LC-MS/MS [22], ion-pair based SPE after modification of the column with 1-octyltriethylammonium phosphate [33] and the use of cation-exchange materials in order to remove excess calcium after the precipitation process [80].

### 6.3. Liquid–liquid extraction

Liquid–liquid extraction (LLE) is maybe the most classic sample preparation technique. Despite of its significant shortcomings including the use of toxic organic solvents, frequent formation of emulsions and lower sampling rate compared to SPE, LLE is still in many cases a technique of choice because of its simplicity and low-operational cost.

LLE is applied to the analysis to the bisphosphonates in order to serve one main purpose. That is removal of the derivatizing reagent excess or its by-products prior to analysis. Typical examples include the cases of fluorescamine [18,20] and isoBCF [78] using dichloromethane and diethylether as extractants, respectively. In the case of derivatization by FMOC [38] or NITC [36,37], LLE was carried out in ion-pair mode using tetrabutylammonium bromide in chloroform.

## 7. Conclusions

Bisphosphonates is a group of active pharmaceutical compounds that generally "gives a headache" to analytical chemists. The two phosphonic groups per molecule lend a strongly ionic character to these compounds and increased polarity. Additionally, the majority of the members of this family lacks of chromophores excluding convenient direct UV detection.

Analysis of bisphosphonates can be classified in two major groups. QC of bisphosphonates-containing pharmaceuticals, including API assay and impurities profiling and the second the analysis of biological material such as urine, blood, plasma and bone samples. Each of these groups has its own demands in terms of sensitivity, selectivity, sample preparation and throughput. These demands more or less specify the techniques of choice.

QC of pharmaceuticals requires usage of robust analytical techniques, preferably capable of both assay and impurities analysis. The LOD is generally not of primary importance, but simplicity, no complicated sample preparation, increased sampling rate and automation possibilities are desirable. A unique feature of bisphosphonates is the fact that their main impurities are ionic (phosphates and phosphites), limiting the applicability of traditional RP-HPLC. Ion chromatography is therefore an obvious solution to this analytical problem. Ion-exchange columns coupled to conductivity detection systems are an interesting approach, offering simplicity, avoidance of derivatization steps, adequate sensitivity and simultaneous separation of ionic impurities. Alternative approaches include indirect UV or fluorescence detection and ICP-MS although the availability of the latter in most QC laboratories is questionable. An interesting alternative to IC for impurities profiling of bisphosphonates is provided by capillary electrophoresis. The high separation efficiency of CE enables separation of both ionic and neutral species with simple modifications. Typical examples include indirect UV detection by adding suitable chromophore compounds in the background electrolyte.

**Table 4**
Determination of bisphosphonates after derivatization

| Derivatizing reagent | Active pharmaceutical ingredient (API) | Analytical technique | LOD | LOQ | Detection | Sample | Ref. |
|---|---|---|---|---|---|---|---|
| | Alendronate | RP-HPLC | – | – | FLD[a] | Urine | [11] |
| NDA | Alendronate | RP-HPLC | 1 ng ml$^{-1}$ (using FLD), 5 ng ml$^{-1}$ (using ED) | – | FLD, ED[b] | Urine and plasma | [12] |
| | Pamidronate | RP-HPLC | – | – | FLD | Bone | [13] |
| | Alendronate | RP-HPLC | – | – | UV | Pharmaceuticals | [14] |
| FMOC | Alendronate | RP-HPLC | – | 1 ng ml$^{-1}$ | FLD | Plasma | [15] |
| | Alendronate | RP-HPLC | – | 3.5 ng ml$^{-1}$ | FLD | Urine | [16] |
| | Alendronate | RP-HPLC | – | 5 ng ml$^{-1}$ | FLD | Urine | [17] |
| Fluorescamine | Pamidronate | RP-HPLC | 500 ng ml$^{-1}$ (in blood), 100 ng ml$^{-1}$ (in urine) | – | FLD | Whole blood and urine | [18] |
| | Pamidronate | RP-HPLC | 2.3 ng ml$^{-1}$ | 186 ng ml$^{-1}$ (in plasma), 163 ng ml$^{-1}$ (in urine) | FLD | Urine, plasma | [19] |
| | Pamidronate | RP-HPLC | 11.6 ng ml$^{-1}$ | 233 ng ml$^{-1}$ | FLD | Urine | [20] |
| IBCF | Alendronate | RP-HPLC | – | 6.66 ng ml$^{-1}$ | MS | Urine | [21] |
| Diazomethane | Risedronate | RP-HPLC | 0.05 ng ml$^{-1}$ (in serum) | – | MS-MS | Serum and urine | [22] |
| | Pamidronate | IPC | – | 3 ng ml$^{-1}$ | FLD | Urine | [34] |
| NITC | Pamidronate | IPC | – | 1–300 ng ml$^{-1}$ | FLD | Urine | [36] |
| | Pamidronate | IPC | – | 20 ng ml$^{-1}$ | FLD | Serum and citrate plasma | [37] |
| PITC | Pamidronate | IPC | – | 0.1 μg ml$^{-1}$ | UV | Pharmaceuticals | [35] |
| NITC, DNTC, PITC, FITC | Pamidronate, alendronate, neridronate | IPC | 1–100 ng ml$^{-1}$ | – | UV, FLD | – | [39] |
| FMOC | Olpadronate | IPC | 5 ng ml$^{-1}$ | – | FLD | Urine and serum | [38] |
| OPA | Alendronate | IPC | | 14 μg ml$^{-1}$ (using UV), 0.3 μg ml$^{-1}$ (using FLD) | UV, FLD | Pharmaceuticals and urine | [40] |
| Molybdenum-ascorbate | Pamidronate | IC | 10 ng ml$^{-1}$ | – | Vis | Urine and plasma | [53] |
| $Fe^{3+}$ | Clodronate | IC | – | – | UV | Pharmaceuticals and bulk material | [49] |
| | Alendronate | Batch UV | 2 μg ml$^{-1}$ | – | UV | Pharmaceuticals | [85] |
| Thorium-EDTA-xylenol orange | Clodronate | IC | 1.26 μg ml$^{-1}$ | – | Vis | Urine | [50] |
| $Cu^{2+}$ | Olpadronate, pamidronate | IC | – | 0.5 μg ml$^{-1}$, 0.9 μg ml$^{-1}$ | UV | Pharmaceuticals | [52] |
| | Alendronate | CE | – | – | UV | Pharmaceuticals | [63] |
| BSTFA | Clodronate | GC | – | 0.3 μg ml$^{-1}$ (in plasma), 0.5 μg ml$^{-1}$ (in urine) | NPD | Plasma and urine | [76] |
| | Clodronate | GC | – | 10 ng ml$^{-1}$ | EI-MS | Plasma | [77] |
| isoBCF | Pamidronate | GC | 40 ng ml$^{-1}$ (in plasma), 80 ng ml$^{-1}$ (in urine) | – | FPD | Urine and plasma | [78] |
| | Pamidronate, neridronate | GC | – | 20 ng ml$^{-1}$ | MS | Plasma | [79] |
| Methanolic HCl | Ibandronate | GC | – | 5 ng ml$^{-1}$ | MS | Serum, plasma and urine | [80] |
| Ninhydrin | Alendronate | Batch UV–vis | 1.2 μg ml$^{-1}$ | – | Vis | Pharmaceuticals and bulk powder | [89] |

[a] FLD: Fluorescence detection.
[b] ED: Electrochemical detection.

**Table 5**
Automated assays for the determination of bisphosphonates

| Analyte | Technique | Method principle | Detection | Sample | Ref |
|---|---|---|---|---|---|
| Alendronate | RP-HPLC | Automated pre-column derivatization with NDA | ED or FLD | Plasma | [12] |
| Pamidronate | RP-HPLC | Automated pre-column derivatization with NDA | FLD | Bone | [13] |
| Alendronate | RP-HPLC | Semi-automated 96-well format SPE coupled to column switching | FLD | Urine | [17] |
| Risedronate | IPC | Automated column switching | UV | Urine | [33] |
| Pamidronate | IPC | Automated SPE and pre-column derivatization with NITC | FLD | Urine | [36] |
| Pamidronate | IPC | Automated SPE and pre-column derivatization with NITC | FLD | Serum and citrate plasma | [37] |
| Olpadronate | IPC | Automated SPE and pre-column derivatization with FMOC using a robotic workstation | FLD | Urine and serum | [38] |
| Clodronate | IC | Post-column complexation reaction with Fe(III) | UV | Pharmaceuticals | [49] |
| Clodronate | IC | Post-column reaction with thorium-EDTA-xylenol orange mixed ligand complex | Vis | Urine | [50] |
| Neridronate, pamidronate, etidronate | IC | Post-column reaction with persulphate and molybdenum-ascorbate | Vis | Urine and plasma | [53] |
| Etidronate alendronate, clodronate | IC | Post-column reaction with Al(III)-Morin system | FLD | Bone | [59] |
| Alendronate | SIA | On-line complexation with Cu(II) and derivatization with OPA | UV/FLD | Pharmaceuticals | [86] |

When it comes to assay analysis of pharmaceuticals the options are broadened. Practically, all the approaches reviewed in this article are more or less suitable for this purpose (Table 3). Ideally, the same analytical technique should be applied to both assay analysis and impurity profiling and if possible determination of both parameters in a single run. Among non-separation techniques, an alternative solution could be sequential injection analysis, offering automation and an increased sampling rate of 60 $h^{-1}$ which is useful when several samples have to be assayed in the minimum of time, e.g. dissolution studies.

On the other hand, determination of bisphosphonates in biological samples such as urine, plasma and bones requires highly sensitive and selective methods. In such protocols, sample preparation is of key importance as it influences both selectivity trough effective matrix removal and sensitivity through preconcentration. The most widely accepted and applied strategy for pretreatment of bisphosphonates-containing biological samples prior to liquid chromatographic analysis is precipitation as calcium salts from alkaline solutions. Such protocols have been proven quite effective, but require several steps and are time consuming. Alternative approaches include SPE or combination of both when increased selectivity is required. When it comes to gas chromatographic analysis, SPE through anion-exchange columns seems to be the technique of choice. An interesting method requiring minimal sample pretreatment is two-dimensional capillary electrophoresis, where a first isotachophoresis step is used for sample preparation coupled to analytical CE for quantitative analysis.

From the sensitivity point of view, pre-column derivatization offers many possibilities for determination of bisphosphonates at the low-ppb range using liquid or gas chromatography (Table 4). Typical reagent examples include NDA coupled to fluorimetric or electrochemical detection and FMOC coupled to fluorimetric detection with LODs and LOQs in the range of 1–5 ng $ml^{-1}$. Strict control of the derivatization conditions especially time is necessary in order to obtain precise and accurate results (Table 5).

Sensitive approaches also include mass spectrometric detectors coupled either to reversed phase or ion pairing chromatography. Although LC-MS and LC-MS/MS nowadays predominate in bioanalyses, applications of these techniques to the determination of bisphosphonates are limited and relevant research should be expected in the future. The most obvious reason is the incompatibility of the mobile phases used in ion-exchange chromatography and ion-pair HPLC to the mass-spectrometric detectors. The limited choice of volatile additives generally results in poor peak shape and retention [91]. An approach to overcome these limitations could be the incorporation of an on-line desalting device acting as a suppressor between the IC column and the MS detector [92]. Gas chromatography also generally requires a derivatization step due to the relatively low volatility of the analytes. In the latter, mass spectrometric detection is the most promising for analysis of biological samples in terms of sensitivity.

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