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Development and validation of a capillary zone electrophoretic method for the determination of bisphosphonate and phosphonate impurities in clodronate

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Abstract

Capillary zone electrophoresis with direct UV detection at low wavelength and reversed polarity was applied for the separation and quantitation of bisphosphonate and phosphonate impurities in clodronate bulk material. Polyacrylamide-coated capillaries were used to reduce the interactions between the analytes and the electric double layer of the capillary, and to minimize electroosmotic flow. Study was made of the major factors affecting the separation, i.e., pH and ionic strength of the electrolyte solution and various instrumental parameters. The developed method provided reproducible separations of clodronate and related impurities (between-day precision of migration times: RSD<2.3%, 275 runs). Acceptable validation results in the impurity quantitation range of 0.5–7.5 μg ml^{-1} (corresponding to 0.1–1.5% of clodronate working concentration) were obtained in specificity, within-day and between-day precision, accuracy and linearity.

Keywords: Pharmaceutical analysis; Validation; Bisphosphonates; Phosphonates; Clodronate

1. Introduction

Clodronate (dichloromethylene bisphosphonate, as disodium salt, see Fig. 1) belongs to the bisphosphonate class of drugs, which carry a P–C–P bridge in the structure. It has been widely investigated as an inhibitor of bone resorption and dissolution and calcium metabolic disorders and as a potential drug for the treatment of Paget's disease and hypercalcaemia [1,2].

Several high-performance liquid chromatographic

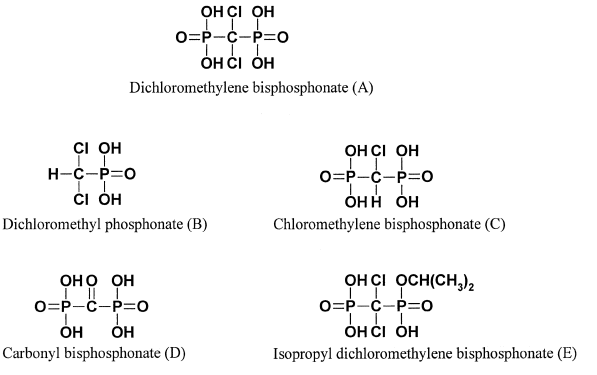

Fig. 1. Structures of clodronate and related impurities.

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(HPLC) methods have been applied for clodronate analysis. For bisphosphonates, however, the technique is problematic owing to their strong metal chelating ability [2,3], which causes adsorption problems, i.e., asymmetric peaks and instrument blocking. Moreover, bisphosphonates do not have strong UV chromophores. Therefore, the HPLC methods reported for clodronate are based upon ion-exchange chromatography (IEC) with indirect UV detection [4,5], UV or fluorescence detection with post-column derivatization [2,6–9] or on-line complexation [10], conductivity detection [11] or flame photometric phosphorus-selective detection [12]. Ion-pair liquid chromatographic methods with flame photometric [13], refractive index [13] or evaporative light-scattering detection [14] have also been described.

Common potential impurities in clodronate drug substance include dichloromethyl phosphonate (B), chloromethylene bisphosphonate (C), carbonyl bisphosphonate (D) and isopropyl dichloromethylene bisphosphonate (E) (see Fig. 1). The methods reported for determining bisphosphonate impurities in clodronate bulk materials and pharmaceuticals are based on IEC with UV detection with post-column derivatization (impurities B and C) [2] or with suppressed conductivity detection (impurities C, D and E) [11]. The use of IEC decreases the adsorption problems of bisphosphonates. On the negative side, specialized equipment is required, and derivatization is an extra complication for the method based on UV detection. Furthermore, a new purity method for the determination of all above-mentioned impurities in clodronate drug substance is needed.

In previous work we applied capillary electrophoresis with electrospray ionization mass spectrometry (CE–ESI-MS) to bisphosphonate analysis [15]. CE offered efficient separation and ESI-MS specific identification for clodronate and related bisphosphonates and phosphonate. However, ESI-MS is too expensive a technique for purity analysis in quality control, and the dynamic range of the CE–ESI-MS method is insufficient for the detection of clodronate with even small amounts of impurities [15].

CE with indirect UV detection has been applied for the separation of clodronate from its phosphate and phosphite impurities [16]. However, the determination of possible bisphosphonate impurities is not described. The indirect UV detection system also tends to be more unstable than direct UV detection, in part because of large baseline drifts and disturbances [17].

A CE method based on direct UV detection with on-line complex formation has been applied to alendronate [18]. In addition, CE with direct UV detection at low wavelength (185 nm) has been reported for the analysis of 2-thioethane-1,1-bisphosphonic acid [19]. CE allows direct UV detection in the low-UV-wavelength region because CE electrolytes are generally aqueous-based, and water itself possesses weak UV absorptivity at wavelengths above 180 nm [20].

This study describes the application of capillary zone electrophoresis (CZE), with direct UV detection at low wavelengths, polyacrylamide-modified capillaries and reversed polarity, to the quantitative analysis of bisphosphonate and phosphonate impurities (B–E, Fig. 1) in clodronate bulk material. The optimization of the most important factors affecting the separation, i.e., pH and ionic strength of the electrolyte solution, and various instrumental parameters, is discussed. Moreover, the validation results including specificity, within- and between-day precision, limits of detection (LODs) and quantitation (LOQs), accuracy, linearity and range, are reported for the method.

2. Experimental

2.1. Chemicals

The compounds studied (Fig. 1) were dichloromethylene bisphosphonate (clodronate, as disodium salt), dichloromethyl phosphonate (as sodium salt), chloromethylene bisphosphonate (as tetrasodium salt), carbonyl bisphosphonate (as tetrasodium salt) and isopropyl dichloromethylene bisphosphonate (as disodium salt). All were obtained from Leiras, Analytical Development (Turku, Finland). Monobasic sodium phosphate and dibasic sodium phosphate were obtained from Merck (Darmstadt, Germany), phenylphosphonic acid from Fluka (Buchs, Switzerland) and sodium hydroxide from Eka Nobel (Bohus, Sweden). Water was treated with

a Milli-RO water purification apparatus (Millipore, Bedford, MA, USA).

2.2. Preparation of buffer and sample solutions

Phosphate buffers were prepared by mixing mono- and dibasic sodium phosphate buffers of the desired ionic strength to give the desired pH. The buffer used in method validation and quantification of real samples was 40 mmol l^{-1} phosphate, pH 7.40, prepared by mixing 40 mmol l^{-1} mono- and dibasic sodium phosphate to give pH 7.40. The pH instrument (PHM 83 Autocal pH meter with the combined glass-reference electrode GK 2401C, Radiometer, Copenhagen, Denmark) was calibrated prior to every buffer preparation with the pH 7.00 (potassium dihydrogenphosphate/disodium hydrogenphosphate, Riedel-de Haën, Seelze, Germany) and pH 10.00 (boric acid/potassium chloride–sodium hydroxide, Merck) buffer solutions. The stock standard solutions were prepared by dissolving clodronate reference or sample substance in water to a concentration of 2.0 mg ml^{-1}, and bisphosphonate or phosphonate impurity reference substances in water to a concentration of 1.0 mg ml^{-1}. A series of working solutions containing compounds at appropriate concentrations were prepared by diluting stock solutions further with water. All solutions were degassed in an ultrasonic bath for 10 min and passed through 0.45-μm membrane filters (Millipore) before analysis.

2.3. Instrumentation

The CE system was a P/ACE System MDQ equipped with a diode array detector (Beckman Instruments, Palo Alto, CA, USA). UV spectra for the compounds were recorded in the wavelength region 190–300 nm. Detection wavelengths of 200 nm and 198 nm were used in the method validation. In the method development, studies were first performed with uncoated fused-silica capillaries (50 μm I.D.×360 μm O.D., Polymicro Technologies, White Associates, Pittsburgh, PA, USA). Coated capillaries used in the further method development and validation were the linear polyacrylamide modified BioCAP LPA capillary [50 cm (effective length 40 cm)×50 μm I.D.×375 μm O.D., Bio-Rad Labs., Hercules, CA, USA] and the e-Cap neutral capillary [60 cm (effective length 50 cm)×50 μm I.D.×360 μm O.D., Beckman Instruments]. Samples were introduced by electrokinetic injection (−5 kV, 8 s). A running voltage of −12 kV was set to the injection end and the capillary was thermostated at 20°C. For quantification of impurities in clodronate samples, one injection was made for sample solutions and two injections for standard solutions at each calibration level.

Capillaries (BioCAP LPA and e-Cap neutral capillary) were conditioned before use and at the beginning of each day by flushing with purified water for 10 min and electrolyte solution (40 mmol l^{-1} phosphate buffer, pH 7.40) for 15 min. Between runs capillaries were flushed with electrolyte solution (2 min) and at the end of the day with purified water (10–15 min). Capillaries were stored in purified water.

2.4. Resolution calculations

Resolution calculations were performed with the equation $R_s=2(t_2-t_1)/(w_{b1}+w_{b2})$, where t_1 and t_2 are the migration times of two adjacent compounds and w_{b1} and w_{b2} their peak widths.

3. Results and discussion

3.1. Detection of clodronate and related impurities

Since clodronate and its related bisphosphonate and phosphonate impurities have absorptivity only in the low-UV-wavelength region, we first studied the suitability of indirect UV detection for the analysis of clodronate purity. Unfortunately, large baseline drifts and co-migration of compounds were observed with several different compositions of UV absorbing background electrolytes. These problems are related to the low electrolyte concentrations needed for indirect detection, as well as to the interactions between bisphosphonates and electrolyte ions. The best resolution and most satisfactory baseline were obtained with use of the polyacrylamide-modified capillary (see Sections 2.3 and 3.2), reversed polarity (cathode as injection end), 10 mmol l^{-1} phenylphosphonic acid (pH 7.5 adjusted with NaOH) as background electrolyte, and detection wavelength of 214

nm. However, in the studies performed with clodronate samples spiked with impurities B–E the resolution between clodronate and the impurity C was not sufficient for quantitative analysis. Moreover, the dynamic range available with indirect UV detection was too limited for purity analysis.

In view of the problems associated with indirect detection and the need for a simple and reproducible method, indirect UV detection was abandoned and direct UV detection at low wavelength was chosen as the detection technique. For wavelength optimization, UV spectra of the compounds of interest were recorded using diode array detection in the wavelength region 190–300 nm. Wavelengths below 190 nm could not be monitored with the detector chosen. The recorded UV spectra of clodronate and the impurities B, C and E were very similar, with maximum absorption at wavelength 190 nm (within 190–300 nm). Impurity D absorbs at slightly higher wavelengths, with a maximum at wavelengths 190–195 nm. No second maximum was observed in the spectra of the analytes in the region 190–300 nm.

Although water exhibits very weak UV absorptivity at wavelengths above 180 nm [20], the mono- and dibasic phosphates used as buffer and the possible impurities in the reagents slightly increase the absorptivity of the CE electrolyte. The wavelengths with maximum absorption for clodronate and the impurities were not optimal for the detection, therefore. To find a single wavelength suitable for all analytes, wavelengths in the range 190–220 nm were examined. Greatest signal-to-noise ratios for the most weakly UV absorbing compounds C and E and a stable baseline were obtained with wavelengths 198 and 200 nm, which were tested in the method validation. Results of the validation indicated that both wavelengths were suitable for the analysis of clodronate purity. Since slightly more reproducible results were achieved at wavelength 200 nm, this was selected for the method.

3.2. Selection of capillary

Uncoated fused-silica capillaries were employed in preliminary method development, with both indirect and direct UV detection. However, electrostatic interaction between analytes and the electric double layer of the capillary caused adsorption of analytes, which was observed as band broadening and poor resolution. Two neutral coated capillaries (BioCAP LPA and e-Cap neutral capillaries) were then tested instead. In both, silanol groups were shielded with polyacrylamide to reduce electroosmotic flow (EOF) and adsorption of analytes. Unfortunately, detailed information about coating procedures was not available from the manufacturers. Both capillaries performed well in separation of clodronate and its related impurities with direct UV detection. Peak shapes and area responses were quite similar and resolution between the analytes sufficient with both capillaries. Although analysis was 5 min faster with the shorter capillary (BioCAP LPA), the e-Cap capillary was selected for the method (Fig. 2a) since it provided slightly better resolution and thus better reliability for the long-term routine analysis.

3.3. Electrolyte composition

The pH of the electrolyte solution is an important selectivity factor in capillary electrophoretic analysis, affecting both the EOF in the capillary and the degree of dissociation, and thus the electrophoretic mobilities of analytes. In this study, neutralization of the capillary surface minimized the EOF, so that the total velocities of analytes were affected almost solely by their electrophoretic mobilities. Since clodronate and its related impurities are polyprotic acids, their dissociation states and electrophoretic mobilities depend strongly on pH.

Neutral capillaries enabled the use of reversed polarity (cathode as injection end) over a wide pH range without affecting the velocity of EOF. However, the usable pH range is 4 to 8 since the coating (e-Cap) is stable only within this range. Fig. 3 shows the effect of pH (7.05–7.70) of the phosphate buffer on the resolution between clodronate and related impurities. In the pH range between 7.30 and 7.60 the resolution was sufficient for all compounds, but at pH lower than 7.30 it was unsatisfactory for clodronate and the impurity C. Moreover, with increasing pH in the range 7.05–7.70, resolution between the fastest migrating impurities C and D decreased, being insufficient at pH 7.70. When the pH was increased from 7.05 to 7.70, the electrophoretic mobility of compound C increased more than that of compounds A and D. The same was

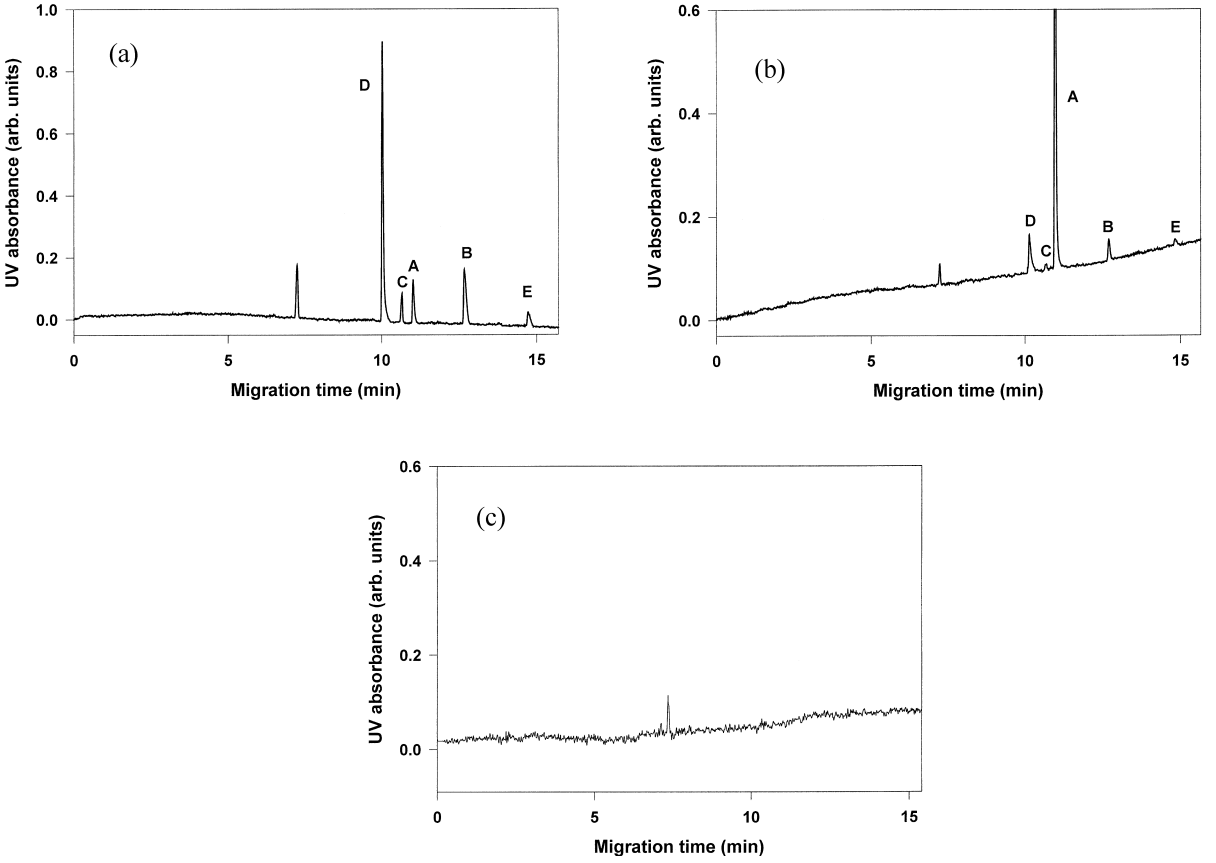

Fig. 2. (a) Electropherogram obtained from reference standard solution containing 2.5 μg ml^{-1} of clodronate and related impurities; A=clodronate, B=dichloromethyl phosphonate, C=chloromethylene bisphosphonate, D=carbonyl bisphosphonate and E=isopropyl dichloromethylene bisphosphonate. (b) Electropherogram obtained from the clodronate sample solution of 100% (500 μg ml^{-1}) spiked with the impurities B–E at the 0.1% level of the clodronate drug substance. (c) Electropherogram obtained from the blank sample. Method of analysis: detection wavelength 200 nm; e-Cap neutral capillary [60 cm (effective length 50 cm)×50 μm I.D.×360 μm O.D., Beckman Instruments]; injection −5 kV, 8 s; running voltage −12 kV set to the injection end; capillary temperature 20°C; electrolyte 40 mmol l^{-1} phosphate, pH 7.40.

observed in our earlier study done with uncoated capillary when pH was increased from 6.9 to 8.0 [15]. The results can be explained in terms of a dissociation state shift of impurity C in the pH range 7.05–7.70. It follows that the pK_{a3} value of C is close to pH 7.05–7.70. As concluded in our earlier study, the pK_{a3} values of compounds A (pK_{a3}=6 [7]) and D (pK_{a3} unknown) are assumed to be lower than the value for compound C since the two chlorine atoms attached to carbon atom in compound A and the carbonyl group attached to carbon atom in compound D stabilize the negative charge more than the chlorine and hydrogen atoms of compound C do (Fig. 1) [15].

Resolution in pH range 7.30–7.60 was sufficient for all the compounds. pH 7.40 provided good resolution for both the most closely migrating compounds, clodronate and impurity C, and for the impurities C and D (Fig. 3), and it was thus used in the method validation.

The ionic strength of the phosphate buffer was varied in the range 20–50 mmol l^{-1} in order to achieve optimal resolution (Fig. 4) and sensitivity for the compounds. With increasing ionic strength in the

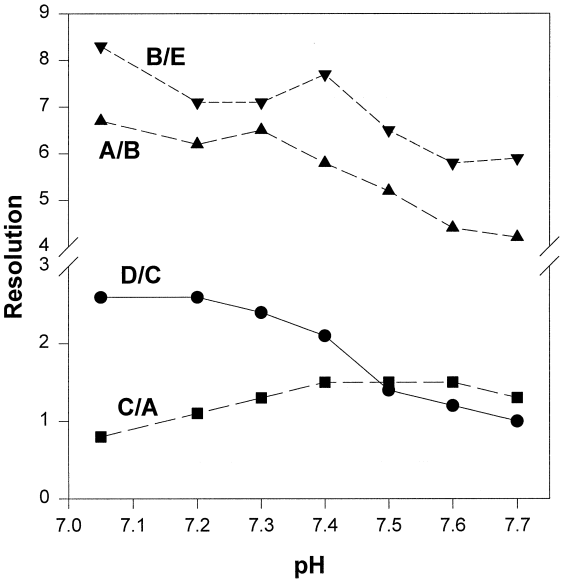

Fig. 3. Effect of pH on resolution. Sample: reference standard solution of 15 μg ml^{-1} of clodronate and impurities B–E. Electrolyte: 40 mmol l^{-1} phosphate buffer in pH range 7.05–7.70. Other separation conditions as in Fig. 2.

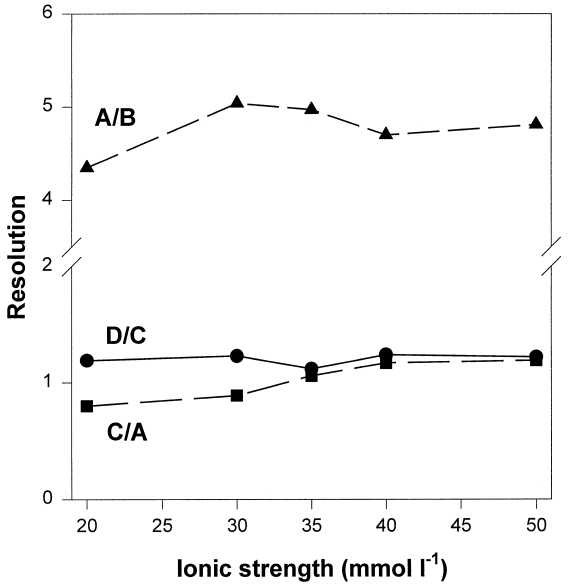

Fig. 4. Effect of ionic strength on resolution. Sample: clodronate (100%) spiked with impurities B–D (3%). Electrolyte: 20–50 mmol l^{-1} phosphate buffer, pH 7.40. Other separation conditions as in Fig. 2.

range 20–40 mmol l^{-1}, resolution improved slightly, between the most closely migrating compounds, clodronate and impurity C, since peaks were narrower. In addition, sensitivity increased considerably for all compounds when the ionic strength was increased from 20 mmol l^{-1} to 40 mmol l^{-1}. Since the samples were dissolved in water, sample stacking probably improved when the ionic strength of the buffer was higher. An increase from 40 mmol l^{-1} to 50 mmol l^{-1} had no notable effect on the resolution and even resulted in loss of sensitivity for the impurities. Thus, the 40 mmol l^{-1} phosphate buffer was considered optimal.

With the optimized electrolyte composition (40 mmol l^{-1} phosphate buffer, pH 7.40), proper separation of clodronate and related impurities (B–E) was achieved within 15 min (Fig. 2a). The compounds migrated in decreasing charge/molecular mass ratio. Impurity B, the smallest in molecular mass, but as a phosphonate with lower degree of dissociation than bisphosphonates, migrated after bisphosphonates D, C and A. However, the impurity E possessed the slowest electrophoretic mobility in consequence of the clearly larger molecular mass than that of the other bisphosphonates.

3.4. Instrumental parameters

Pressure and electrokinetic injection modes were compared for sample introduction. Injection times of 4–40 s with 3.45 kPa pressure were tested in pressure injection mode. Quantitation of impurity traces was found to require high clodronate concentrations (>2.5 mg ml^{-1}), which resulted in band broadening of clodronate and unsatisfactory resolution between clodronate and impurity C. Thus, a dynamic range adequate for purity analysis was not achieved with the pressure injection mode.

In electrokinetic injection, the amounts of analytes that can be introduced to the capillary are generally affected by EOF as well as electrophoretic mobility [21,22]. The effect of reversed EOF with the use of reversed polarity was minimized with the coated capillary and negatively charged analytes were introduced and focused more effectively into a zone in the capillary inlet. The amount of introduced ions with electrokinetic injection was also increased with injecting the samples in water instead of high-con-

ductivity electrolyte solution, which provided higher electric field strength for the injection end. Thus, electrokinetic injection provided about five-fold better detection sensitivity than obtained with pressure injection and sufficient dynamic range for the method.

With electrokinetic injection, ions with higher electrophoretic mobility are introduced in greater amount to the capillary [21,22]. In our case, only slight differences in the response ratios of the compounds were observed between electrokinetic and pressure injection.

Injections for 4–20 s with voltages of −5 and −10 kV were tested in electrokinetic mode. Injections longer than 8 s with −5 kV voltage and 4 s with −10 kV resulted in band broadening probably due to increased heat formation in a sample zone. Increase of 30% in peak widths was observed with 20-s injections. However, greater sample loading was not required for sufficient detection sensitivity and dynamic range. Injections for 8 s with −5 kV voltage and 4 s with −10 kV resulted in optimal resolution and peak-area response. Nevertheless, longer injection time with lower electric field produced better repeatability. Thus, injection for 8 s with −5 kV voltage was selected.

3.5. Method validation

The suitability of the optimized method for the analysis of clodronate purity was evaluated with validation studies, including specificity, within-day precision (repeatability), between-day precision (reproducibility), LODs and LOQs, accuracy, linearity and range. Validation results were evaluated with use of the specifications for HPLC purity methods, which are recommended for CE validation [23].

3.5.1. Specificity

The specificity of the method for clodronate purity analysis was studied to ensure a confident separation of potential impurities from clodronate. The set of samples in specificity studies was blank samples, clodronate spiked with known potential impurities, non-spiked clodronate and individual impurity solutions. The peak purity technique with diode array detection was used to confirm consistent spectra of the obtained peaks. With the optimized method using 40 mmol l^{-1} phosphate buffer, pH 7.40 and conditions described in Experimental, clear separation of clodronate and related impurities (B–E) was achieved within 15 min (Fig. 2a and b). No interfering peaks were observed in an analysis of the blank sample (Fig. 2c). Unknown peak was observed before 8 min in electropherograms of both blank and reference samples but this does not interfere with the peaks of the analytes. For clodronate sample solutions spiked with impurity reference standards in the impurity quantitation range (0.1–1.5%, see Section 3.5.6), resolution values better than 1.4 were obtained for all compounds (Table 1).

3.5.2. Within-day precision

The within-day precision of the system was studied with 6–8 repeated injections of one standard solution containing all reference compounds. The within-day precision of the system was studied at the concentration levels of 5, 7.5, 15 and 20 μg ml^{-1} [1, 1.5, 3 and 4% of clodronate working concentration (500 μg ml^{-1}), respectively]. The results (Table 2)

Table 1
Resolution between adjacent peaks (R_s) obtained under optimal CE conditions for clodronate (A) and impurities (B–E) ($n=6$)[a]

Analyte	Migration time (min)	Adjacent peaks	R_s (LOQ)	R_s (0.5%)	R_s (1.5%)
A	11.0	C/A	1.4	1.4	1.5
B	12.7	A/B	5.6	5.4	6.6
C	10.7	D/C	2.2	2.3	2.1
D	10.0	System peak/D	11.5	10.1	9.9
E	14.7	B/E	10.9	7.7	9.0

[a] Samples: clodronate sample solutions spiked with impurity reference standards to yield 0.1% (LOQ), 0.5% and 1.5% of clodronate working concentration. Method of analysis: detection wavelength 200 nm; e-Cap neutral capillary [60 cm (effective length 50 cm)×50 μm I.D.×360 μm O.D., Beckman Instruments]; injection −5 kV, 8 s; running voltage −12 kV set to the injection end; capillary temperature 20°C; electrolyte 40 mmol l^{-1} phosphate, pH 7.40.

Table 2
Within-day precision of the system and the method, and between-day precision of the method (relative standard deviations, RSDs, %, of migration time and peak-area)[a]

	Level	*n*	A		B		C		D		E	
	(%)		Migration time RSD (%)	Area RSD (%)	Migration time RSD (%)	Area RSD (%)	Migration time RSD (%)	Area RSD (%)	Migration time RSD (%)	Area RSD (%)	Migration time RSD (%)	Area RSD (%)
Within-day precision (system)	1	6	0.05	3.3	0.03	2.2	0.06	8.3	0	3.9	0.04	4.1
	1.5	6	0.06	3.8	0	3.8	0	4.0	0.06	3.7	0.09	6.8
	3	6	0.61	1.9	0.63	2.4	0.61	3.1	0.56	2.3	0.63	2.2
	4	8	0.11	2.3	0.11	1.8	0.13	5.8	0.12	1.5	0.12	6.2
Within-day precision (method)	A: 100 B–E: 0.10	6	1.2	1.1	1.5	3.3	1.1	15.3	1.3	3.0	1.7	9.9
Between-day precision (method)	A: 100 B–E: 0.10	18	1.1	2.8	1.4	5.8	1.2	14.1	1.2	5.5	2.5	7.8

[a] Samples (within-day precision of the system): 6–8 repeated injections of mixtures of reference standard solutions at concentration levels of 1–4% of clodronate working concentration. Samples (within-day and between-day precision of the method): clodronate sample solutions (100%) spiked with impurity reference standards to yield 0.1% (LOQ) of clodronate working concentration; one sample/injection. Method of analysis as in Table 1.

show that migration times were repeatable (RSDs= 0–0.63%, n=6–8) for all compounds and concentration levels studied. Repeatability of peak areas was also sufficient for purity analysis (RSD=1.5–8.3%, n=6–8).

The effect of injection vial size on within-day precision of the system was studied by transferring final sample solutions to 200-μl and 1.6-ml vials. Greater sample volume was found to provide better precision.

The within-day precision of the method was tested by analyzing replicate sample solutions (six different clodronate samples of 500 μg ml^{-1} spiked with impurity solutions to yield 0.5 μg ml^{-1}=0.1% of clodronate working concentration). As can be seen in Table 2, the RSD of 1.1% was obtained for the peak-area of clodronate. For the impurities, the lowest RSD (3.0%) was obtained for the peak-area of impurity D (S/N=35) and the highest RSD (15.3%) for impurity C (S/N=10). The within-day precision meets the specification for purity analysis with all the impurities (RSD<20%).

3.5.3. Between-day precision

The between-day precision of the method was studied by analyzing six replicate sample solutions (clodronate samples of 500 μg ml^{-1} spiked with impurity working solutions to yield 0.1% of clodronate working concentration) on different days (Table 2). The RSDs for migration times were 1.1–2.5% and for peak-areas 2.8–14.1%. The results indicate acceptable between-day precision of the method for all the compounds studied.

The between-day precision of migration times was also studied for a validation period consisting of 275 runs. RSD values <2.3% were obtained for all the compounds studied. Thus, reproducible separation for clodronate and related impurities was achieved with the coated capillary under optimized conditions.

3.5.4. Limits of detection and quantitation

The LOD of the method (0.25 μg ml^{-1} corresponding to 0.05% of clodronate working concentration) was determined as the concentration producing a peak-area at S/N ratio of 3 for impurity E with the highest LOD. The LOQ of the method (0.5 μg ml^{-1}; 0.1%) was defined as a concentration level, in which impurity E with the highest LOQ has an acceptable precision and accuracy. The LOQ was confirmed for all impurities by precision and accuracy

Table 3
Accuracy of the method [*E* (%)=mean recovery of the related impurity; n.d.=not determined][a]

Level	*n*	B		C		D		E	
		E (%)	RSD (%)	*E* (%)	RSD (%)	*E* (%)	RSD (%)	*E* (%)	RSD (%)
LOQ	6	87.1	3.0	71.7	10.9	74.4	3.5	122.1	5.6
0.2%	6	97.0	12.8	89.5	23.3	93.8	18.6	n.d.	
0.5%	8	102.2	6.9	95.7	5.5	81.8	8.1	110.8	8.3
1%	6	74.4	6.6	79.8	19.9	78.5	1.5	n.d.	
2%	6	95.4	9.1	95.5	20.5	86.0	7.9	97.6	17.0

[a] Samples: clodronate sample solutions spiked with impurity reference standards to yield 0.1% (LOQ)–2.0% of clodronate working concentration; one sample/injection. Method of analysis as in Table 1.

studies, in which an acceptable RSD of <20% was achieved for all impurities studied.

3.5.5. Accuracy

The accuracy of the method was studied with clodronate samples spiked with the impurities at concentration levels of 0.5, 1, 2.5, 5 and 10 μg ml^{-1} (0.1, 0.2, 0.5, 1 and 2% of clodronate working concentration, respectively). The mean recoveries of impurities at the LOQ level (0.1%) were 71.7–121.1%, with RSDs of 3.0–10.9%. At levels of 0.2–2% the mean recovery of the impurities was slightly better (74.4–110.8%) with somewhat greater RSDs (1.5–23.3%) than at the LOQ level. The accuracy results are shown in Table 3.

Table 4
Regression data for clodronate (A) and related impurities (B–E) obtained for 0.1–1.5% clodronate working concentration[a]

Analyte		*r*	Slope	Intercept
A	Mean	0.9988	1.21	0.53
	SD	0.0005	0.27	0.32
B	Mean	0.9969	2.44	0.82
	SD	0.0019	0.70	0.74
C	Mean	0.9924	0.79	0.54
	SD	0.0074	0.17	0.29
D	Mean	0.9960	6.85	5.63
	SD	0.0029	1.79	2.65
E	Mean	0.9961	0.59	0.10
	SD	0.0022	0.18	0.22

[a] The calibration curves were constructed for 4 days with use of five calibration points. Method of analysis as in Table 1.

3.5.6. Linearity and range

The linearity of the method was studied for all reference compounds over the range 0.5–10 μg ml^{-1}, corresponding to a range of 0.1–2% clodronate working concentration. The lowest limit of the range was defined as the LOQ level of impurity E with the lowest LOQ (0.5 μg ml^{-1}). On the basis of systematic error (%) measured as the differences between nominal and mean concentrations determined at calibration points, the highest calibration point (2%) in between-day analysis was not always within the linear range. Since a level of 2% is not necessary in purity analysis of clodronate, however, the range 0.1–1.5% was chosen for the range of method. Within that range, correlation coefficients better than 0.9924 were obtained for all compounds studied, which meets the acceptance criteria for linearity in purity studies. Regression data for clodronate and related impurities are presented in Table 4.

4. Conclusions

CZE with direct UV detection at low wavelength (200 nm) provided effective separation and quantitation of bisphosphonate and phosphonate impurities in clodronate bulk material. The developed method enables determination of clodronate purity with simple sample preparation and standard, low-cost instruments and reagents.

Optimal separation of clodronate and related impurities was achieved within 15 min by using reversed polarity and 40 mmol l^{-1} phosphate buffer, pH 7.40. The use of polyacrylamide-modified capil-

laries reduced electrostatic interactions between analytes and the electric double layer of the capillary, and minimized EOF, which resulted in reproducible separations (between-day precision of migration times: RSD values <2.3%, 275 runs). Electrokinetic injection enhanced the detection sensitivity and all compounds could be detected at a concentration of 0.25 μg ml^{-1}. Satisfactory validation results in the range 0.5–7.5 μg ml^{-1} [corresponding to 0.1–1.5% of clodronate working concentration (500 μg ml^{-1})] were obtained in specificity, within-day and between-day precision, accuracy and linearity. On the basis of these results, the method is concluded to be suitable for quality control of bisphosphonate and phosphonate impurities in clodronate drug substance.

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