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Analysis of bisphosphonates by capillary electrophoresis–electrospray ionization mass spectrometry

Katri Huikko, Risto Kostiainen*

Department of Pharmacy, Division of Pharmaceutical Chemistry, P.O. Box 56, FIN-00014, University of Helsinki, Helsinki, Finland

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Abstract

Capillary electrophoresis–electrospray ionization mass spectrometry (CE–ESI-MS) was applied to the direct identification and quantitation of clodronate and its four common impurities. The coaxial interface technique and negative ion mode were used in the detection. Ion source parameters and sheath liquid composition were optimized to produce maximum abundance of singly charged deprotonated molecules used in monitoring. In addition, the effects of electrolyte composition and instrumental parameters of CE on separation were studied. The developed method provides high separation power and specificity to bisphosphonate analysis. In quantitative analysis, the method showed good linearity (r=0.9946–0.9989), satisfactory repeatability (migration time variation: RSD=0.43–1.0%, peak area variation: RSD=2.2–9.4%) and sufficient sensitivity (detection limits: 0.08–0.22 mg ml^{-1}) for identification of bisphosphonates from bulk material.

Keywords: Bisphosphonates; Clodronate

1. Introduction

Bisphosphonates are a broad class of drugs possessing a P–C–P bridge structure. They have been widely investigated for their role in inhibiting bone resorption and dissolution, and in calcium metabolic disorders. Clodronate (dichloromethylene bisphosphonate as a disodium salt, see Fig. 1) belongs to the bisphosphonate family and has been used in the treatment of Paget's disease and hypercalcaemia. It has also been presented as a potential drug for the treatment of osteoporosis [1].

Several chromatographic methods have been reported for the determination of clodronate. A method based on gas chromatography–mass spectrometry (GC–MS) has been described for the analysis of clodronate in urine samples. However, this method requires a time-consuming derivatization procedure [2]. High-performance liquid chromatography (HPLC) analysis of bisphosphonates, in turn, is complex since compounds easily form chelates with metal ions, which causes adsorption problems, such as tailing peaks and precipitation of compounds in instruments [1,3,4]. In addition, direct UV detection is complicated due to the lack of strong UV chromophore. Therefore, the HPLC methods developed for clodronate analysis are based upon anion-exchange chromatography with indirect UV detection [5,6], UV or fluorescence detection with post-column derivatization [1,7,8], conductivity detection [9] or

*Corresponding author. Tel.: +358-9-1915-9134; fax: +358-9-1915-9556.

E-mail address: risto.kostiainen@helsinki.fi (R. Kostiainen)

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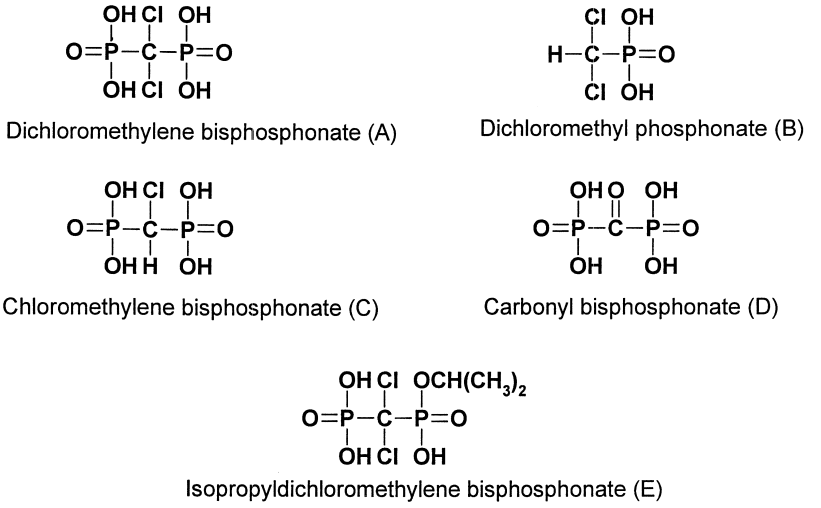


Fig. 1. Structures of the compounds studied.

flame photometric phosphorus-selective detection [10]. Ion-pair chromatographic methods with either refractive index detection [11] or evaporative light-scattering detection [12] have also been presented.

Most HPLC methods described for other bisphosphonates involve anion-exchange or ion-pair chromatography and on-line, pre- or post-column derivatization with UV or fluorescence detection [13–18]. Furthermore, conductivity detection [3,19], refractive index detection [20], mass spectrometric detection [21] and electrochemical detection [22,23] have been applied to HPLC analysis of bisphosphonates. Other methods reported in literature involve the application of inductively coupled plasma (ICP) [24] or capillary isotachophoresis [25] in bisphosphonate determination.

Capillary electrophoresis (CE) is becoming an increasingly popular, complementary technique to HPLC because of its high separation power. A capillary zone electrophoretic method using indirect UV detection for the analysis of clodronate has already been developed [26]. This report does not, however, describe the separation of the bisphosphonate impurities of clodronate drug substance. Moreover, the indirect UV detection system is generally more unstable than direct UV detection partly due to large baseline drifts and disturbances [27]. Recently, CE methods based on direct UV detection using complex formation or detection of low wavelength have also been reported for analyzing alendronate and 2-thioethane-1,1-bisphosphonic acid [28,29].

The above-mentioned methods are complex and time-consuming, or their specificity is limited. Thus, there is still a need for a direct and specific bisphosphonate screening method. In this study, we applied capillary electrophoresis–electrospray ionization mass spectrometry (CE–ESI-MS) to the identification and quantitation of clodronate (A) and its common bisphosphonate and phosphonate impurities (B–E) (Fig. 1). The use of CE in separation reduces the adsorption problems described earlier and MS detection offers direct and highly specific identification. CE–MS has already been applied to many kinds of analytical problems. So far, the method has not been used on clodronate or the other compounds determined in our study, although a few applications have already been reported for other phosphonates and bisphosphonates [4,30]. Here, we use coaxial interface technique and negative ion mode for the detection. The effects of ion source parameters and pH of the sheath liquid to the mass spectral behavior are described. In addition, the effects of electrolyte composition and various instrumental parameters of CE on the separation are discussed.

2. Experimental

2.1. Chemicals and sample solutions

The compounds studied (Fig. 1) were dichloromethylene bisphosphonate (clodronate; as a disodium salt), dichloromethyl phosphonate (as a sodium salt), chloromethylene bisphosphonate (as a tetrasodium salt), carbonyl bisphosphonate (as a tetrasodium salt) and isopropyl dichloromethylene bisphosphonate (as a disodium salt), were obtained from Leiras, Analytical Development (Turku, Finland). Phenylphosphonic acid was tested as an internal standard and it was obtained from Fluka (Buchs, Switzerland). Ammonium acetate, formic acid and acetic acid were purchased from Merck (Darmstadt, Germany), sodium hydroxide from Eka Nobel (Bohus, Sweden), methanol (HPLC grade) from Rathburn (Walkerburn, UK) and ammonium hydroxide from J.T. Baker (Phillipsburg, NJ, USA). Water was treated with a Milli-RO water purification apparatus (Millipore, Bedford, MA, USA).

Stock solutions of the compounds studied were prepared by dissolving compounds in water to a concentration of 10 mg ml^{-1}. A series of working solutions containing all analytes at concentrations varying between 0.08 and 1.2 mg ml^{-1} were prepared by further diluting stock solutions with water. All solutions were passed through the 0.45-μm membrane filters (Millipore) before analysis.

2.2. Instrumentation

A Beckman P/ACE System 2200 capillary electrophoresis system (Beckman Instruments, Palo Alto, CA, USA) was employed for the MS coupling. Fused-silica capillaries of 50 μm I.D.×186 μm O.D. were supplied by Polymicro Technologies (White Associates, Pittsburgh, PA, USA). The length of the capillary was 80 cm. Optimal sensitivity and stability were achieved when the fused-silica capillary outlet was set 2 mm outside the ESI needle. Samples were introduced using pressure injection (3.45 kPa, 6 s). A voltage of +20 kV was set to the anode. A pressure of 3.45 kPa was applied to cut down analysis time.

Capillaries were conditioned once before use by flushing with 1 *M* NaOH for 15 min, followed by 0.1 *M* NaOH and purified water (15 min of each), and finally with electrolyte solution (20 m*M* ammonium acetate, pH 8.0 adjusted with NH_4OH) for 30 min. At the beginning of each analysis day the capillary was rinsed with 0.1 *M* NaOH for 10 min, followed with water (5 min) and electrolyte solution (15 min). Between different runs, the capillary was flushed with electrolyte solution (3–4 min). When not in use, the capillary was stored in water. Direct coupling of capillary electrophoresis with mass spectrometry was accomplished using a sheath liquid coaxial coupling. The pH of the sheath liquid (20 m*M* ammonium acetate–methanol, 1:3, v/v) was 5.0, adjusted with formic acid. Both the electrolyte solution and the sheath liquid were degassed for 10 min in a ultrasonic bath to avoid bubble formation. Sheath liquid was delivered by a micro syringe pump (Harvard Apparatus, USA) with a flow-rate of 5 μl min^{-1}.

The mass spectrometer used was a PE Sciex API 300 triple quadrupole LC–MS–MS system fitted with an electrospray ionization source (PE Sciex, Toronto, Canada). Data were collected with a Macintosh computer (Model 8500/180) using PE Sciex API 100/300 software, version 1.2. Synthesized air (99.998%, AGA, Finland) was used as a nebulizing gas and nitrogen generated with a Whatman 75-720 generator (Whatman, Haverhill, MA, USA) was used as a curtain gas. The instrument was operated in the negative ion mode. The spray needle voltage was set to −4.5 kV and the orifice lens voltage to 30 V. The quadrupole Q1 was scanned in the range of m/z 50–500 for optimization of ESI-MS conditions. Dwell time was 0.85 ms. In the CE–ESI-MS experiments, detection was based on selected ion monitoring (SIM). The ions monitored were deprotonated molecules, i.e., m/z 189 for carbonyl bisphosphonate and deprotonated molecules with their M+2 isotopes, i.e., m/z 163 and 165 for dichloromethylene phosphonate, m/z 209 and 211 for chloromethyl bisphosphonate, m/z 243 and m/z 245 for dichloromethylene bisphosphonate and m/z 285 and 287 for isopropyl dichloromethylene bisphosphonate. In the CE–MS method optimal sensitivity for the detection was achieved with the dwell time of 7.5 ms.

3. Results and discussion

The direct coupling of capillary electrophoresis with mass spectrometry was done using a coaxial sheath-flow interface described in detail in an earlier study by Smith et al. [31]. This kind of interface provides stable performance and easy operation, which is necessary in high throughput routine analysis. In the development of the CE–MS method, the most important parameters, i.e., composition of sheath liquid and electrolyte solution, operation parameters of the mass spectrometer and CE system, were studied.

3.1. Optimization of sheath liquid

The proper performance of the ESI process is dependent on the sheath liquid composition. A high methanol content in the sheath liquid was chosen since the low surface tension and good conductivity of methanol promote the formation of gas-phase ions, improving sensitivity and stability. In addition, background noise is decreased when the amount of methanol in water is increased. To achieve sufficient conductivity and the compatibility with CE, 20 m*M* ammonium acetate in water was added to methanol (1:3, v/v).

The compounds studied are polyprotic acids and therefore capable of forming multiply charged ions in ESI process. Sodium adduct ions can also be observed in the MS spectra of analytes (Table 1). The degree of dissociation in solution phase is naturally dependent on pH. This is reflected in the relative abundances of singly and multiply charged ions in the MS spectra, thus optimization of sheath liquid pH in CE–ESI-MS is essential. The optimization of sheath liquid composition was established in order to produce maximum abundance of singly charged deprotonated molecules used in SIM. The choice of $[M-H]^-$ ions for monitoring was preferred since they are observed at higher m/z values than multiply charged ions and chemical noise is reduced in the higher m/z region. Sodium adduct ions were not chosen because the total amount of sodium in the charged droplets formed in ESI process is difficult to control. There are several sources of sodium ions, for example, the samples in form of sodium salts as well as vials and transfer lines of the instrument.

The optimization of pH was done with eluent composition of methanol–water (3:1, v/v) in the pH range of 2.5–11.0 for all analytes studied (Fig. 2). Formic acid was used in adjusting the desired pH to acidic solutions and ammonium hydroxide to basic solutions. The maximum abundance of $[M-H]^-$ ions in all compounds studied was achieved at pH 5.0. The pK_a values of clodronate are about 1, 2, 6 and 9.5 [32]. It follows that the compound is approximately 90% doubly charged at pH 5.0 in solution phase. However, $[M-H]^-$ ion of clodronate appeared in the spectra at pH range of 2.5–10. This is partly due to the neutralization of multiply charged

Table 1
Observed masses of the compounds studied in negative ESI-MS obtained under optimal MS conditions [spray needle voltage −4.5 kV; orifice voltage 30 V; scan: m/z 50–500, dwell time 0.85 ms; eluent composition: methanol–water (3:1, v/v), pH 5.0, flow-rate 5 μl min^{-1}][a]

	m/z (rel. int. %)				
	A	B	C	D	E
$[M-H]^-$	243 (100), 245 (68), 247 (9)	163 (100), 165 (72), 167 (10)	209 (100), 211 (34)	189 (100)	285 (100), 287 (64), 289 (13)
$[M-2H+Na]^-$	265 (16), 267 (12), 269 (3)	185 (1)	231 (31), 233 (8)	211 (55)	307 (19), 309 (14), 311 (3)
$[M-3H+2Na]^-$				233 (4)	
$[M-2H]^{2-}$	121 (47), 122 (32), 123 (7)		104 (18), 105 (7)	94 (24)	142 (82), 143 (69), 144 (12)
$[M-3H+Na]^{2-}$	143 (3)				
$[PO_4H_2]^-$	97 (13)		97 (5)	97 (41)	97 (12)
$[PO_3]^-$			79 (3)	79 (37)	79 (13)
$[PO_2]^-$		63 (48)			
Other ions		209 (3)	173 (37)	159 (32)	
				143 (14)	
				125 (8)	

[a] The samples (20 μg ml^{-1}) were introduced by direct injection.

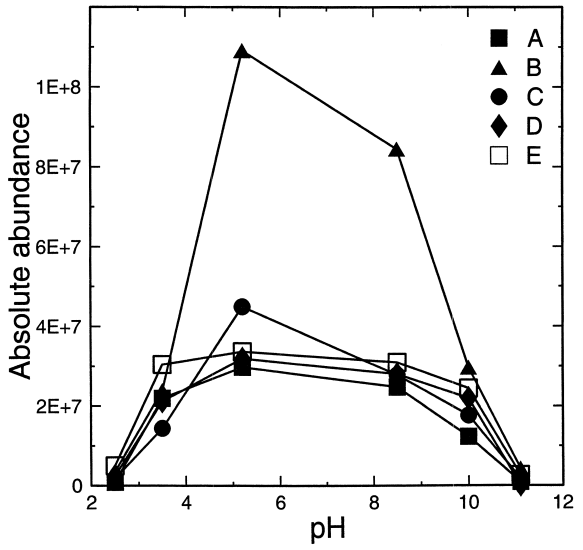


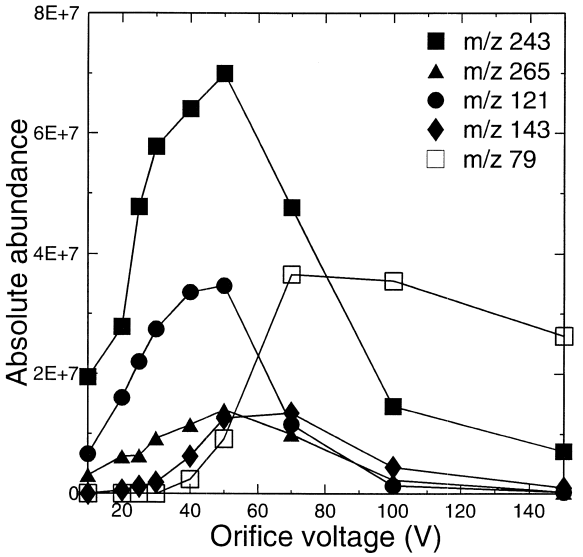
Fig. 2. Effect of pH on the abundance of singly charged deprotonated molecules of the compounds studied. The samples (20 μg ml^{-1}) were introduced by direct injection. ESI-MS conditions: spray needle voltage −4.5 kV; orifice voltage 30 V; scan: *m/z* 50–500, dwell time 0.85 ms; eluent composition: methanol–water (3:1, v/v), pH 2.5–11.0, flow-rate 5 μl min^{-1}.

ions in gas phase. It is also possible that the pH of the droplets in the ESI process differs from the pH of solution phase due to the solvent evaporation during the ion evaporation process.

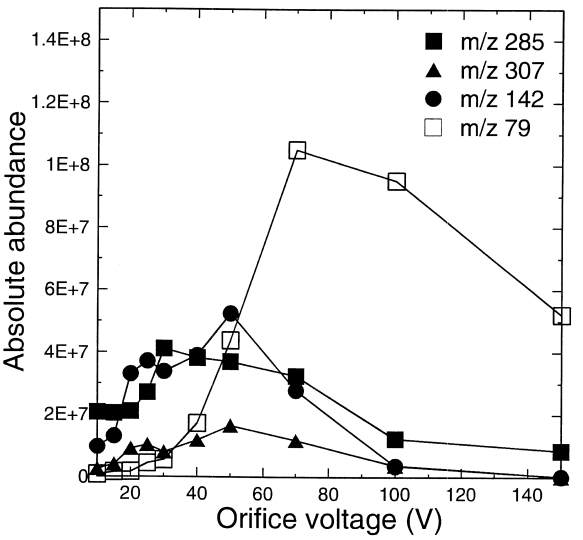
Since the maximum abundance of all $[M-H]^-$ ions was achieved at pH 5.0, this pH was chosen for further experiments. The optimum composition of the sheath liquid was 20 m*M* ammonium acetate–methanol (1:3, v/v), pH 5.0, which provided good sensitivity and stability in the CE–MS operation.

3.2. Effect of orifice voltage

The most important mass spectrometric parameter, orifice voltage, was optimized using compounds A and E in order to produce the maximum abundance of $[M-H]^-$ ions (Fig. 3a and b). The recorded breakdown curves of compounds resembled each other. In the MS spectra of both compounds, the singly and doubly charged deprotonated molecules produced the most abundant peaks at low orifice voltages (10–50 V). At high orifice voltages (60–150 V), the deprotonated molecules fragmented, producing ion $[PO_3]^-$ (*m/z* 79). Weak sodium adduct



(a)



(b)

Fig. 3. Effect of orifice voltage on absolute abundance of compounds A (a) and E (b). The samples (20 μg ml^{-1}) were introduced by direct injection. ESI-MS conditions: spray needle voltage −4.5 kV; orifice voltage 10–150 V; scan: *m/z* 50–500, dwell time 0.85 ms; eluent composition: methanol–water (3:1, v/v), pH 5.0, flow-rate 5 μl min^{-1}. (a) *m/z* 243=$[M-H]^-$, *m/z* 265=$[M-2H+Na]^-$, *m/z* 121=$[M-2H]^{2-}$, *m/z* 143=$[M-3H+Na]^{2-}$, *m/z* 79=$[PO_3]^-$. (b) *m/z* 285=$[M-H]^-$, *m/z* 307=$[M-2H+Na]^-$, *m/z* 142=$[M-2H]^{2-}$, *m/z* 79=$[PO_3]^-$.

ions (see Table 1) were recorded at whole orifice voltage range. An orifice voltage of 30 V was chosen for the CE–ESI-MS method since it produced maximum and near maximum abundances of $[M-H]^-$ ion for compounds E and A, respectively.

3.3. ESI-MS spectra

The ESI-MS spectra of compounds studied recorded in optimal MS conditions are presented in Table 1. Since the singly charged deprotonated molecule was the most abundant ion in all the spectra, it was selected for monitoring in the final CE–MS method. The doubly charged deprotonated molecules $[M-2H]^{2-}$ were abundant in the MS spectra of all the bisphosphonates, but not recorded for compound B which, as a phosphonate, possesses lower degree of dissociation than tetraprotic bisphosphonic acids. The spectra of bisphosphonates also showed abundant $[M-2H+Na]^-$ ions and weak $[M-3H+2Na]^-$ and $[M-3H+Na]^{2-}$ ions. In contrast to bisphosphonates, only the weak $[M-2H+Na]^-$ adduct ion was recorded for the phosphonate. Different MS behavior between bisphosphonates and the phosphonate was also seen in fragmentation patterns. Under optimal MS conditions, fragment $[PO_4H_2]^-$ (m/z 97) was observed in the MS spectra of all bisphosphonates, whereas fragment $[PO_2]^-$ (m/z 63) appeared in the MS spectrum of phosphonate.

3.4. Optimization of separation for the CE–MS method

The effects of most important factors, i.e., type, ionic strength, and pH of the electrolyte, on the CE separation of compounds studied were examined. The choice of electrolyte type is quite restricted because only volatile electrolytes are suitable for long-term routine analysis with ESI-MS. In this work, acetic acid, ammonium hydroxide and ammonium acetate were used in the optimization of separation.

The influence of pH on the separation was studied at pH 5.0, 6.9, 8.0 and 10.0. When acetic acid in water at pH 5 was used as an electrolyte, no peaks were detected in the ion electropherogram. Because the anode was at the injection site, the electroosmotic flow (EOF) needed to be higher than the electrophoretic mobility of the anions studied in order for the anions to migrate towards the ESI source (cathode). However, at pH 5 or lower, the EOF decreased and the analytes migrated towards the anode. At pH 6.9 or higher, using 20 m*M* ammonium acetate as an electrolyte, the EOF was high enough to carry the analytes to the ESI source and peaks were detected in the ion electropherogram. However, initial analysis times were over 30 min. In order to achieve faster analysis, the flow-rate was increased with low pressure (3.45 kPa), which was used for the remainder of the studies.

Fig. 4 shows the effect of pH (6.9–10) on the resolution of compounds studied. Since the satisfactory resolution between all analytes was achieved at pH 8.0, this pH was chosen for further studies. Compound E migrated first due to its largest radius and, therefore, lowest electrophoretic mobility. Compound B, the smallest in radius, but as a phospho-

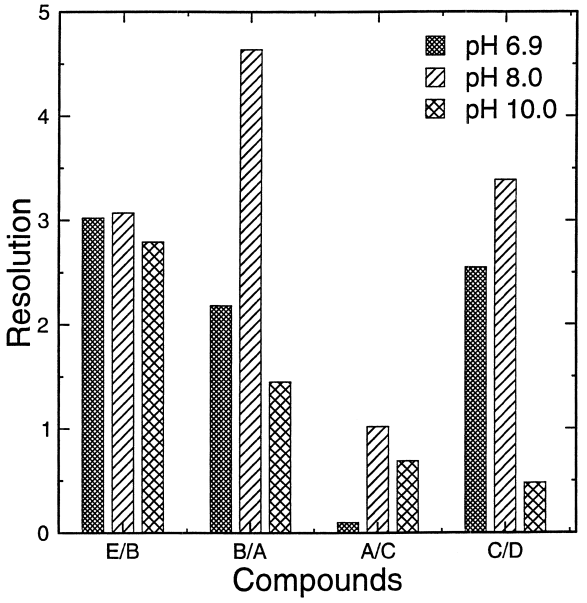


Fig. 4. Effect of pH on the resolution between adjacent compounds in CE. Conditions: 20 m*M* ammonium acetate as an electrolyte, pH 6.9, 8.0 and 10.0; voltage +20 kV (anode) and pressure 3.45 kPa; pressure injection 3.45 kPa, 6 s. Resolution was calculated with the equation $R_s=2(t_2-t_1)/(w_{b1}+w_{b2})$, where t_1 and t_2 are the migration times of two adjacent compounds, and w_{b1} and w_{b2} their peak widths measured at baseline of selected ion electropherograms.

nate, with a lower degree of dissociation than bisphosphonates, migrated second. At pH 6.9 compounds A and C, and at pH 10.0 the last migrating compounds C and D were not separated from each other. It is therefore likely that at pH higher than 6.9, the electrophoretic mobility of compound C increases more than that of compounds A and D. The dissociation state of compound C has probably shifted towards pK_{a3}, in the pH range from 6.9 to 10.0. The pK_{a3} values of compounds A ($pK_{a3}=6$) and D (pK_{a3} unknown) are assumed to be lower than that of compound C, since the two chlorine atoms or the carbonyl group attached to the carbon atom of compounds A and D, respectively, stabilize negative charge more than the chlorine and hydrogen atoms of compound C (Fig. 1). Therefore, pH variations in the range of 6.9–10.0 do not have as great an effect on the electrophoretic mobility of compounds A and D as on compound C.

The effect of ionic strength (20–30 m*M*) on the resolution of compounds studied is shown in Fig. 5. Analysis times were slightly shorter with higher ionic strength. The reason for this may be that both the EOF and the electrophoretic mobility of ions are expected to decrease as ionic strength and thus the viscosity of solution increases; though, the electrophoretic mobility decreased more than EOF. However, an increase in ionic strength produced higher current and, thus, loss of resolution. Adequate resolution between all adjacent compounds was achieved with 20 m*M* ammonium acetate.

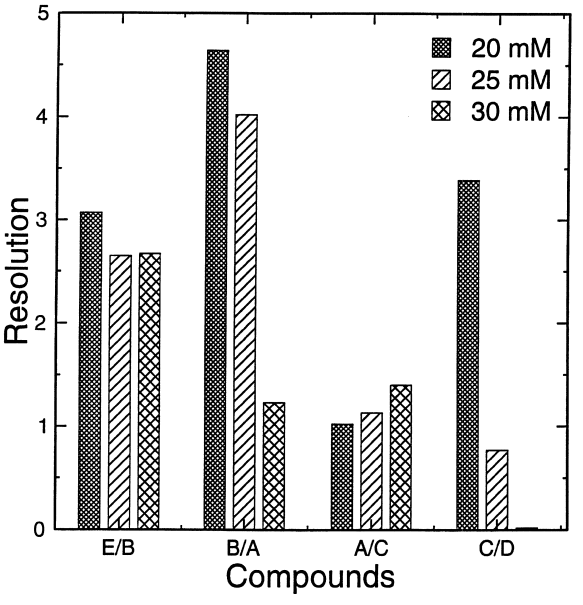


Fig. 5. Effect of ionic strength on the resolution between adjacent compounds in CE. Conditions: 20–30 m*M* ammonium acetate as an electrolyte, pH of the electrolyte solution adjusted to 8.0 with ammonium hydroxide; voltage +20 kV (anode) and pressure 3.45 kPa; pressure injection 3.45 kPa, 6 s. Resolution calculated as in Fig. 4.

Optimization of instrumental parameters of CE, i.e., injection time and the voltage in the capillary inlet, was established in order to achieve the optimal resolution and sensitivity for the compounds studied using the optimal electrolyte composition. Samples were introduced to the capillary inlet using pressure injection mode. Injection times of 4–12 s with 3.45 kPa pressure were tested, and a time of 6 s was chosen as a compromise between peak sharpness and sensitivity. Raising the voltage in the capillary inlet from +20 kV to +25 kV resulted in weaker resolution, which could be explained by increased Joule heating due to the increased current.

Fig. 6 shows total and selected ion electropherograms obtained under optimized CE–MS conditions. Proper separation and clear detection for all analytes studied was achieved within 21 min.

3.5. Quantitative analysis

The suitability of the method for quantitative analysis was studied by testing the linearity and repeatability of the method. In addition, the limits of detection were determined. The results are presented in Tables 2 and 3.

Calibration curves were obtained by plotting the peak areas of singly charged deprotonated molecules against the concentrations of the calibration solutions. All calibration curves showed good linearity with correlation coefficients $r=0.9946–0.9989$ in the ranges studied (Table 2).

The limits of detection were determined with a signal-to-noise ratio of 3. Detection sensitivity was quite low (detection limits varied between 0.08 and 0.22 mg ml^{-1}, see Table 2), which is a result of the highly hydrophilic nature of the compounds studied, leading to poor emission of gas phase ions. Moreover, a dilution of samples occurs when using this

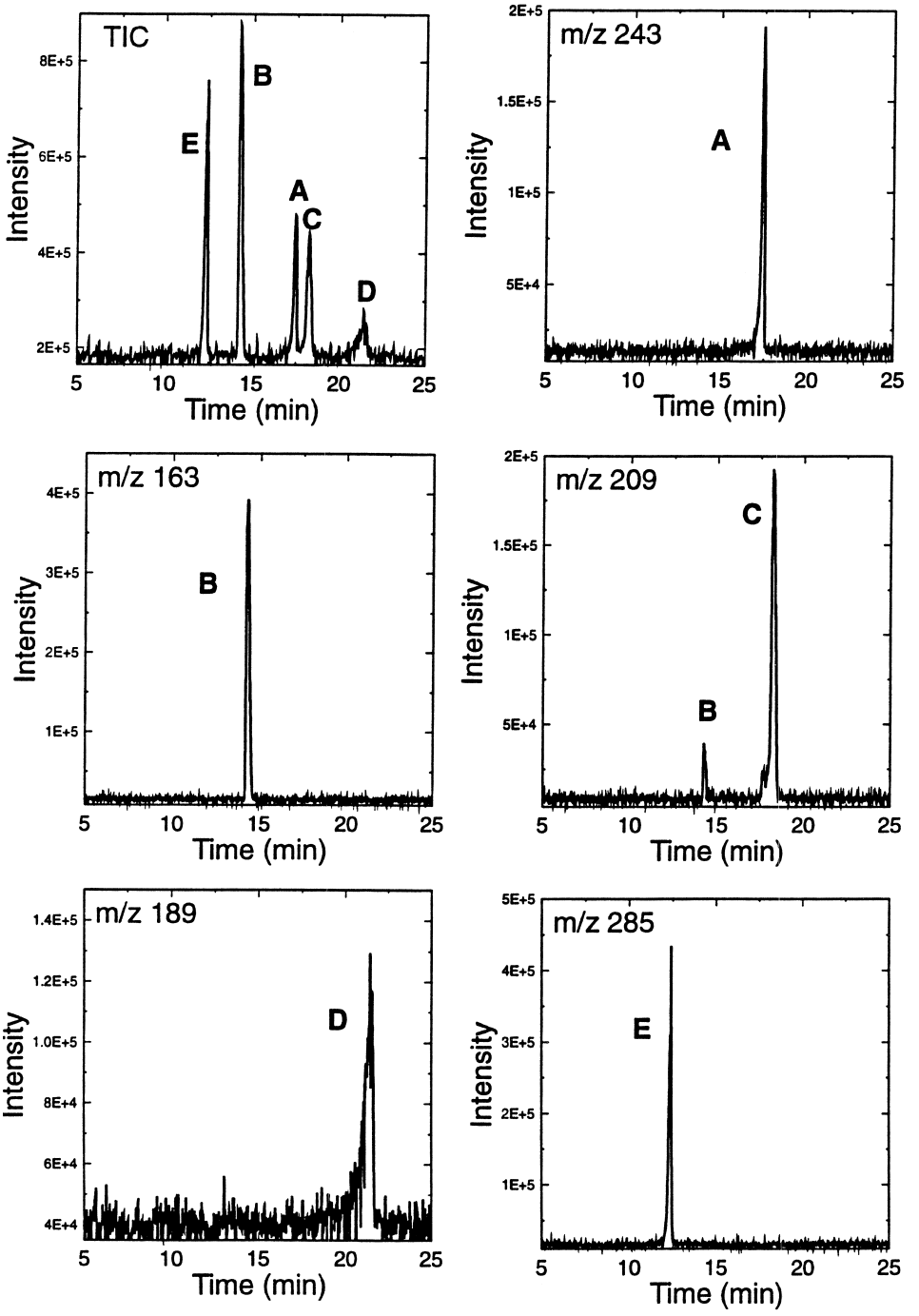


Fig. 6. Total and selected ion electropherograms obtained under optimal CE–MS conditions from a mixture of compounds studied (A: 0.4 mg ml^{-1}, B: 0.4 mg ml^{-1}, C: 0.3 mg ml^{-1}, D: 0.35 mg ml^{-1}, E: 0.4 mg ml^{-1}). CE–MS conditions: spray needle voltage −4.5 kV; orifice voltage 30 V; selected ion monitoring (see text), dwell time 7.5 ms; sheath liquid composition: 20 m*M* ammonium acetate–methanol (1:3, v/v), pH 5.0, flow-rate 5 μl min^{-1}; 20 m*M* ammonium acetate, pH 8.0, as an CE electrolyte; separation: voltage +20 kV (anode) and pressure 3.45 kPa; pressure injection 3.45 kPa, 6 s.

Table 2
Calibration data and detection limits for the compounds studied[a]

Analyte	Range (mg ml^{-1})	Regression line	Correlation coefficient (r)	Detection limit (mg ml^{-1})
A	0.33–1.20 (n=6)	$y=-1.5+8.8x$	0.9989	0.22
B	0.33–0.98 (n=5)	$y=0.55+11.5x$	0.9989	0.08
C	0.27–0.81 (n=5)	$y=-1.4+13.5x$	0.9968	0.18
D	0.24–0.57 (n=4)	$y=-6.7+31.6x$	0.9946	0.17
E	0.24–0.98 (n=7)	$y=-1.6+14.0x$	0.9970	0.08

[a] CE–MS conditions as in Fig. 6.

kind of coaxial CE–MS interface due to the addition of sheath liquid.

Repeatability studies were performed with compound mixtures at concentration levels approximately in the middle of linear range for each compound (0.5–0.6 mg ml^{-1}). Migration time variation was very good (RSD=0.43–1.0%, n=7) and peak area variation satisfactory (RSD=2.2–9.4%, n=7). Repeatability was also tested by using phenylphosphonic acid (0.4 mg ml^{-1}) as an internal standard (I.S.). In the optimized CE–MS conditions, the I.S. migrated slightly before the compounds studied. However, the repeatability of peak areas was slightly better without I.S. addition (Table 3).

4. Conclusions

CE–ESI-MS proved to be an applicable technique for simple direct determination of bisphosphonates. Efficient separation of clodronate and its common phosphonate and bisphosphonate impurities was achieved with 20 m*M* ammonium acetate as the CE electrolyte at pH 8. The negative ion ESI-MS with optimized ion source parameters provided highly specific identification of the compounds studied. Moreover, use of coaxial interface with optimized sheath liquid composition (20 m*M* ammonium acetate–methanol, 1:3, v/v, pH 5.0) provided stable performance of CE–MS system for routine analysis.

The developed method showed good linearity (r= 0.9946–0.9989) and adequate repeatability (migration time variation: RSD=0.43–1.0%, peak area variation: RSD=2.2–9.4%). However, due to the highly hydrophilic nature of compounds studied, and dilution of samples with sheath-flow interfacing, the sensitivity of the method is too low (detection limits: 0.08–0.22 mg ml^{-1}) for its practical application to bioanalytical studies. Nevertheless, the detection sensitivity is sufficient for the confident identification of bisphosphonates from bulk material. Thus, the method could probably also be more widely applied to direct screening of different bisphosphonates.

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Table 3
Migration time and peak area repeatability (n=7)[a]

Analyte	Migration time (min)	Migration time RSD (%)	Peak area RSD (%)	Peak area ratio[b] RSD (%)
A	17.5	0.43	2.2	5.9
B	14.3	0.48	6.9	9.5
C	18.2	0.54	5.3	9.9
D	21.4	0.83	9.4	14.1
E	12.4	1.0	4.0	4.3

[a] Studies were performed with mixtures of compounds (A: 0.6 mg ml^{-1}, B: 0.5 mg ml^{-1}, C: 0.5 mg ml^{-1}, D: 0.5 mg ml^{-1}, E: 0.5 mg ml^{-1}, I.S.: 0.4 mg ml^{-1}). CE–MS conditions as in Fig. 6.

[b] Peak area ratio is defined as the analyte peak area divided by the internal standard (phenylphosphonic acid) area.

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