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Determination of clodronate content in liposomal formulation by capillary zone electrophoresis

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Abstract

Liposome-mediated intracellular delivery of clodronate is reported to selectively deplete mononuclear phagocytic cells such as macrophages that are important effector cells involved in the pathogenesis of neuropathies associated with demyelination and destruction of neuronal cells. Application of liposome-encapsulated clodronate (dichloromethylenediphosphonic acid disodium salt) is a method of choice to deplete macrophages to prevent such a neurodegeneration. In the present work, a comparison of an ion-exchange chromatography (IEC) and a capillary zone electrophoresis (CZE) method with indirect UV detection was performed and, based on the results of this comparison, a CZE assay was developed for quantitation of clodronate in mannosylated liposomes. This CZE method employed Nitroso-R salt (1-nitroso-2-naphthol-3,6-disulphonic acid disodium salt) as background electrolyte with UV detection of the analyte at 254 nm. The assay for the determination of clodronate in mannosylated liposomes after their solubilization in 10 mM Triton X-100 showed acceptable within-day precision (repeatability), day-to-day precision (reproducibility) and linearity in the target quantitation range of 0.5–10.0 mg ml^{-1}. The method reported here can be used as part of the quality control during the preparation of liposome-encapsulated clodronate as a drug formulation for macrophage-mediated diseases.

Keywords: Clodronate; Mannosylated liposomes; Ion-exchange chromatography; Capillary zone electrophoresis; Indirect UV detection

1. Introduction

Clodronate disodium (dichloromethylenediphosphonic acid disodium salt) belongs to the bisphosphonate class of drugs that is currently the most important class of antiresorptive drugs used for treatment of diseases with exessive bone resorption

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[1,2]. Clodronate encapsulated in liposomes is also toxic to macrophages in vivo and may therefore be of use in the treatment of autoimmune/inflammatory diseases [3,4]. Its selective depletion of macrophages in the periphery by intravenous (iv) or subcutaneous (sc) injection of clodronate liposomes has been previously reported [5–7]. Liposomal uptake by macrophages is further enhanced by mannosylation [8,9]. Mannosylated clodronate liposomes were reported to act as 'macrophage suicide vehicles' [7] by facilitating intracellular delivery of clodronate into peripheral macrophages by phagocytosis of the liposomes. In amyotrophic lateral sclerosis (ALS) that is a fatal neurodegenerative disease, activated macrophages are believed to play a significant role in altered immune regulation, leading to loss of motor neurons in the central nervous system (CNS). In experimental demyelinating diseases (e.g. experimental allergic encephalomyelitis) serving as models for human autoimmune/inflammatory diseases such as multiple sclerosis, inhibition of macrophage function with mannosylated liposomes of clodronate can prevent or ameliorate the development of demyelination [10,11]. Macrophage/microglia activation in the CNS leading to neuronal loss is also implicated in Alzheimer's and several other neurodegenerative diseases (e.g. schrizophrenia, HIV-associated dementia) [12–14]. Depletion of activated macrophages in the CNS by intraventricular injection of liposome-encapsulated clodronate may be a means to arrest neuronal death in these cases [15]. To further develop clodronate liposomes as a potential drug for macrophage-mediated neurodegenerative diseases, it was necessary to have a reliable analytical method to quantify clodronate content in liposomes for preclinical (animal) studies. This also ensured quality control of the liposome-mediated intracellular delivery of clodronate in the experiments performed. The development of a chromatographic assay appeared to be an appropriate approach.

Development of a high performance liquid chromatographic (HPLC) method is challenging due to the strong metal chelating ability of clodronate [16], which causes adsorption problems; i.e. asymmetric peaks and increasing back-pressure. Moreover, clodronate does not have strong enough UV chromophore for conventional UV or fluorescent detection. Therefore, the HPLC methods reported for clodronate are based upon ion-exchange chromatography (IEC) with indirect UV detection [17,18], UV or fluorescent detection with post-column derivatization [16,17,19–22] or on-line complexation [23], conductivity detection [24] or flame photometric phosphorous-selective detection [25]. Ion-pair liquid chromatographic methods with flame photometric [26] and evaporative light-scattering detection [27] have also been reported. As an alternative to HPLC, capillary zone electrophoresis (CZE) methods with indirect UV detection [28], direct UV detection [29] and electrospray ionization mass spectrometry [30] have been reported.

In order to develop a simple, easy-to-perform assay to quantitate clodronate in liposome preparations, we only considered methods without derivatization reactions. Among them, the IEC [17] and the CZE [28] methods with indirect UV detection seemed to fulfill these requirements. In this paper, we present a comparison of the two methods for measurement of clodronate, and report the development of the CZE–indirect UV detection method for the quantitative analysis of clodronate in mannosylated liposomes. Our present study focused on sample preparation, within-day precision, day-to-day precision and linearity for formulation stability and preclinical studies.

2. Experimental

2.1. Instrumentation

HPLC was performed with a ThermoSeparations/SpectraPhysics chromatography system (Fremont, CA) consisting of an SP8810 precision isocratic pump, SP8450 UV/Vis detector and SP4290 integrator. Samples were injected by Rheodyne model 7125 injector (Cotati, CA). The injection volume was 20 μl. Measurements were performed on a 5 cm × 4.0 mm i.d. 50GLC4-SAX-8/5 ion exchange column (SGE, Australia) at a flow rate of 0.9 ml min^{-1} with 10 mM HNO_3 as

an eluent. The detection wavelength was 240 nm and the polarity of the detector output was reserved for signal processing by the computing integrator. Analyses were performed at ambient temperature.

CZE was performed on a Waters Quanta 4000E capillary electrophoresis system (Waters Chromatography Division, Milford, MA) equipped with a fixed-wavelength (254 nm) UV detector connected to the data system with the output polarity reversed. The dimensions of the non-coated fused-silica capillaries (Agilent Technologies, Wilmington, DE) were 50 μm i.d. × 360 μm o.d. × 73 cm analytical length ($L_{det} = 65.0$ cm). The working electrolyte solution contained 0.5 mM of Nitroso-R salt (1-nitroso-2-naphtol-3,6-disulfonic acid disodium salt hydrate) and its pH was adjusted to 8.0. The injection time was 3 s (hydrostatic, at 10 cm height), and the voltage was 20 kV during the separations. The temperature during the measurements was maintained at 30 °C. A Millenium (Waters Chromatography Division, Milford, MA) chromatographic data system was used to record and evaluate the electropherograms.

A Corning pH meter and a Corning combined electrode (Corning, Acton, MA) were used to adjust the pH of the electrolyte solutions. Centrifugations were performed by Sorvall RC2-B automatic refrigerated centrifuge (Ivan Sorvall Inc., Newtown, CT) and a Fisher Compac Micro 7 microcentrifuge (Fisher Scientific, Inc., Pittsburgh, PA).

2.2. Chemicals and reagents

Clodronate (dichloromethylenediphosphonic acid disodium salt), cholesterol, *p*-aminophenyl-α-D-mannopyranoside, Triton X-100 and phosphate buffered saline (PBS), pH 7.4, were purchased from Sigma (St. Louis, MO). L-α-Phosphatidylcholine was purchased from Avanti (Alabaster, AL). Nitroso-R salt was purchased from Aldrich (Milwaukee, WI). Nitric acid (69%, certified) and sodium hydroxide solution (1 N, certified) were obtained from Fischer Scientific, Inc. (Pittsburgh, PA).

All solvents and reagents were used without further purification. Deionized water, with at least 18 MΩ, filtered through a 0.45 μm membrane filter (Osmonics, Minnetonka, MN) was used for mobile phase, sample and standard preparations.

2.3. Preparation of liposomes

Multilamellar mannosylated liposomes were produced from phosphatidylcholine, cholesterol and *p*-aminophenyl-α-D-mannopyranoside as previously described [5]. Briefly, 70.9 mg of phosphatidylcholine and 10.8 mg of cholesterol were dissolved in 10 ml of chloroform and added to 2 ml of methanol containing 3.6 mg *p*-aminophenyl-α-D-mannopyranoside in a round-bottom flask. The thin film formed on the interior of the flask after low-vacuum rotary evaporation was dispersed in 10 ml of PBS containing 1.89 g of clodronate by gentle rotation for 10 min. For preparation of control (empty) liposomes, the lipid film was dispersed in 10 ml of PBS. Free clodronate was removed by rinsing the liposomes with 10 ml of PBS and centrifuging (Sorvall RC2-B centrifuge) the suspension for 30 min at 25,000 × *g* three times. Finally, the liposomes were resuspended in 4.0 ml of PBS.

2.4. Electrolyte solutions

The 10-mM HNO_3 solution was prepared by appropriate dilution of concentrated HNO_3 with filtered deionized water. The 10-mM Triton X-100 (average molecular weight 628 Da) solution was prepared by dissolving 628 mg of the surfactant in 100.0 ml filtered deionized water. A standard stock electrolyte solution (20 mM) of Nitroso-R salt was prepared by dissolving 754.5 mg salt in 100.0 ml filtered deionized water. Before water was added to the final volume, the pH was adjusted to 8.0 with 1 N NaOH. The working electrolyte solution (0.5 mM) for indirect UV detection was made from the stock solution by dilution it with deionized water.

2.5. Standard solutions

A 10.0 mg ml^{-1} clodronate stock solutions for the IEC and the CZE measurements were prepared by dissolving 100.0 mg clodronate in 10.0 ml 10

mM aqueous HNO_3 and in 10 mM aqueous Triton X-100, respectively. Appropriate volumes of the stock solutions were diluted to obtain 5.0, 2.0, 1.0 and 0.5 mg ml^{-1} clodronate concentrations. All the solutions were freshly prepared on the day of analysis.

2.6. Analysis of liposomes

Samples were prepared by centrifuging (Micro 7 microcentrifuge) of 50 μl PBS suspension of liposomes diluted with 500 μl of PBS at 10,000 rpm for 5 min. The supernatant was discarded. Then, 100 μl of 10 mM Triton X-100 was added to the pelleted liposomes and the mixture was sonicated for 10 min. Calibration samples were prepared by solubilization of the pellet obtained from 50 μl of control (empty) liposomes in 100 μl of 0.5, 1.0, 2.0, 5.0 and 10.0 mg ml^{-1} clodronate in 10 mM Triton X-100 solution. All solutions were freshly prepared on the day of analysis. For day-to-day measurements, the solutions were stored at 4 °C.

2.7. Assay validation

Injection precision was measured by integrating peak areas of five replicate injections. For determination of within-day precision of migration time and injection precision, one set of calibration samples with 0.5, 1.0, 2.0, 5.0 and 10.0 mg ml^{-1} clodronate content was analyzed in the morning, 2, 4, 6 and 8 h later, respectively. Day-to-day precision was evaluated by the analysis of the same set of calibration samples prepared and measured on three different working days (on the 3rd and 6th days after starting the study) over a 6-day period. Critical, detection and determination levels of the assay were evaluated as described in the literature [31].

3. Results and discussion

3.1. IEC of clodronate with indirect UV detection

Our IEC study was based on the previous publication in which clodronate was quantitated on a Waters IC-Pack HR anion exchange column using 12 mM HNO_3 as eluent [17]. In our experiments, clodronate was eluted from the column giving a rather broad and asymmetric peak (Fig. 1A) under the optimized IEC conditions (10 mM HNO_3, 240 nm detection wavelength, 0.9 ml min^{-1} flow rate). An increase of the eluent's HNO_3 concentration resulted in an even shorter retention time causing interference with the unretained sample constituents, while decreasing the acid concentration resulted in an increase of the retention time accompanied by further broadening of the clodronate peak. We could only use peak heights to construct a calibration curve using clodronate solutions in the 0.5–10.0 mg ml^{-1} concentration range. For the injection precision, performed by making five replicate (20 μl) injections of 0.5 and 2.0 mg ml^{-1} clodronate standard solutions, relative standard deviations (RSD) of 2.67 and 1.74%, respectively, were found. Based on peak heights of the corresponding peaks of 0.5, 1.0, 2.0, 5.0 and 10.0 mg ml^{-1} clodronate standard

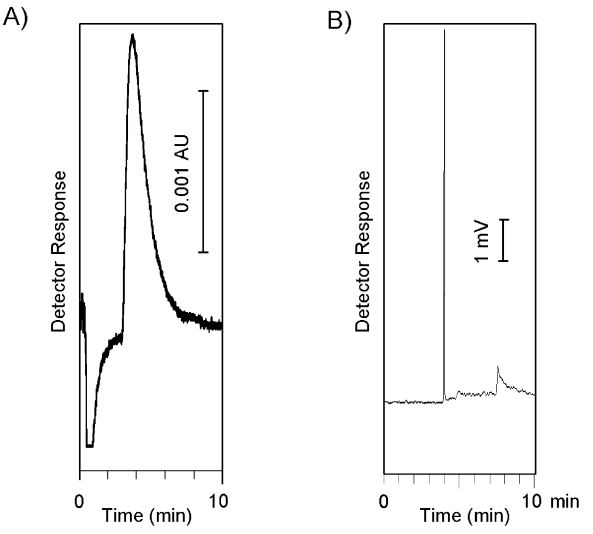

Fig. 1. (a) HPLC chromatogram of 2.0 mg·ml^{-1} clodronate sodium solution in 10 mM HNO_3. Conditions: column, 50GLC4-SAX-8/5 I.E. column; eluent, 10 mM HNO_3; flow rate, 0.9 ml·min^{-1}; λ, 240 nm. Instrument: ThermoSeparations/SpectraPhysics system. (b) Electropherogram of 2.0 mg·ml^{-1} clodronate sodium in 10 mM Triton-X solution. Conditions: buffer, 0.5 mM Nitroso-R salt, pH 8.0; injection, 3 s (hydrostatic); voltage, 20 kV; T, 30 °C; λ, 254 nm. Instrument: Waters Quanta 4000E.

solutions, an acceptable calibration curve ($y = 148.96x$) could be obtained ($R^2 = 0.9892$).

3.2. CZE of clodronate with indirect UV detection

As a second choice of our assay development, we took the previously reported CZE with indirect UV detection [28] into consideration. This method was carried out in 0.5 mM Nitroso-R salt solution of pH 8.0 with UV detection at 254 nm. Under the optimized CZE conditions (20 kV current, $T = 30$ °C, injection time: 3 s) clodronate dissolved in 10 mM Triton X-100 solution gave a sharp peak at about 3.8 min migration time (Fig. 1B) all over the target 0.5–10.0 mg ml^{-1} concentration range. The migration times were found quite reproducible ($n = 15$; RSD = 1.37%), especially when the daily column conditioning (see below) were implemented and the working temperature were kept constant (30 °C). For injection precision measured by integrated peak areas of five replicate injections, the RSD values of the 0.5 and 2.0 mg ml^{-1} clodronate solutions were 1.71 and 1.98%, respectively. The detector response was found to be linear ($y = 12{,}893x + 7440$) and showed R^2 of 0.9992 for the investigated 0.5–10.0 mg ml^{-1} concentration range using 0.5, 1.0, 2.0, 5.0 and 10.0 mg ml^{-1} clodronate standard solutions.

3.3. Quantitative determination of clodronate content of mannosylated liposomes by CZE

Since the CZE method provided better chromatographic performance, we chose this latter method to adapt for the quantitation of the clodronate content in the mannosylated liposomes. To construct a calibration curve for determination of clodronate in mannosylated liposomes, control liposomes were solubilized in duplicate with appropriate dilutions of clodronate stock solutions. The amount of drug in the calibration samples was equivalent to 25 (0.5 mg ml^{-1}), 50 (1.0 mg ml^{-1}), 100 (2.0 mg ml^{-1}), 250 (5.0 mg ml^{-1}) and 500% (10.0 mg ml^{-1}), respectively, of the anticipated clodronate content of our mannosylated liposome preparations (2.0 mg ml^{-1}, after solubilization). The clodronate content of the standard solutions with solubilized liposomes gave sharp peaks similar to those without liposomes. Baseline stability, reproducibility of migration time and the integrated areas were found to be the highest when the daily work followed a standard conditioning of the column starting with consecutive washing with 0.1 M NaOH, distilled water and the Nitroso-R salt buffer (for 10 min each). During the day, the column was washed with replenished electrolyte for 10 min after four to five injections.

The percent RSD values ($n = 5$) for within-day migration times and injection precisions measured by integrated peak areas of the above standard solutions are shown in Table 1. The migration times were found reproducible and were found to fall into the region of 1.23–1.40%. Reproducibility of integrated peak areas was acceptable with RSD values falling into the range of 1.89–2.99%. In order to obtain such reproducible results, the samples were vortexed (for 30 s) before each chromatographic run. The detector response was found to be linear and showed R^2 of 0.9997 for the investigated 0.5–10.0 mg ml^{-1} concentration range. For the calibration curve obtained ($y = 11{,}978x + 9329$), relative standard errors of the slope and intercept were 3.5 and 6.6%, respectively. The critical level, detection level and determination limit were 0.1, 0.4 and 0.2 mg ml^{-1}, respectively.

The percentage RSD values for day-to-day migration time and injection precision (based on integration areas) obtained from the measurements are summarized in Table 1. The day-to-day precision of migration times was found acceptable (RSD = 2.97–3.34%); however, it was higher than the within-day values. Similar observation could be done on comparison of the within-day (RSD = 1.89–2.99%) and day-to-day precision (RSD = 4.26–5.03%) of the integrated peak areas (Table 1). This observation might be due to the different solubilization efficacy of the samples, which varied on storage (Sonication and vortexing of the samples on the 2nd and 3rd days of measurements resulted in slightly opaque solutions even after longer sonication times.). Consequently, clodronate content of each batch of liposome preparation was analyzed by using freshly prepared calibration series.

Table 1
Within-day and day-to-day precisions of migration times and peak areas of clodronate standard solutions

Concentration of standard solution (mg ml^{-1})	Within-day precision of migration times (RSD, %) ($n = 5$)[a]	Within-day precison of peak areas (RSD, %) ($n = 5$)[a]	Day-to-day precision of migration times (RSD, %)[b]	Day-to-day precision of peak areas (RSD, %)[b]
0.5	1.40	2.99	3.00	4.93
1.0	1.34	2.81	2.97	5.00
2.0	1.23	2.58	3.22	4.26
5.0	1.24	2.87	3.34	5.03
10.0	1.32	1.89	3.25	4.34

[a] Number of determinations.
[b] Results are based on means of five measurements over a period of 6 days.

The results obtained for analysis of three mannosylated clodronate liposome preparations are summarized in Table 2. Clodronate contents of the prepared liposomes were in the 3.5–4.2 mg ml^{-1} range, which was in good agreement with previously published results [7,32]. It should be emphasized, however, that measurement of freshly prepared analytical and standard samples completely solubilized (upon visual inspection) according to the procedure described in Section 2 was found to be an absolute necessity to obtain such reproducible measurements.

4. Conclusions

Based on a CZE method with indirect UV detection [28], a simple assay was developed to measure clodronate content in mannosylated liposomes, which showed acceptable results in the target quantitation range of 0.5–10.0 mg ml^{-1} for within-day precision (repeatability), day-to-day precision (reproducibility) and linearity. The method has proved to be simple and easy-to-perform for quantitation of clodronate in liposomal formulation. This straightforward and reproducible method may be routinely applied as quality control during the preparation of liposomal formulation of clodronate, a potential drug for macrophage-mediated neurodegenerative diseases.

Table 2
Results of clodronate-content of mannosylated liposome preparations

Liposome preparation	Mean clodronate content (mg ml^{-1})[a]	RSD (%)
No. 1	4.09	3.93
No. 2	3.48	3.44
No. 3	3.85	3.39

[a] Results expressed as means of three determinations.

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References

[1] H. Fleisch, Bisphosphonates: mechanisms of action, Endocr. Rev. 19 (1998) 80–100.
[2] G.A. Rodan, H.A. Fleisch, Bisphosphonates: mechanisms of action, J. Clin. Invest. 97 (1996) 2692–2696.
[3] A.M.J. Buiting, N. van Rooijen, Liposome-mediated depletion of macrophages—an approach for fundamental studies, J. Drug Target. 2 (1994) 357–362.
[4] J. Monkkonen, M. Taskinen, S.O. Auriola, A. Urtti, Growth inhibition of macrophage-like and other cell types by liposome-encapsulated, calcium-bound, and free bisphosphonates in vitro, J. Drug Target. 2 (1994) 299–308.
[5] N. Van Rooijen, A. Sanders, Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications, J. Immunol. Methods 174 (1994) 83–93.
[6] J. Biewenga, M.B. Van der Ende, L.F. Krist, A. Borst, M. Ghufron, N. Van Rooijen, Macrophage depletion in the rat after intraperitoneal administration of liposome-encapsulated clodronate: depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by

administration of Freund's adjuvant, Cell Tissue Res. 280 (1995) 189–196.
[7] T. Liu, N. Van Rooijen, D.J. Tracey, Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury, Pain 86 (2000) 25–32.
[8] S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita, M. Hashida, Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes, Gene Ther. 7 (2000) 292–299.
[9] P. Opanasopit, Y. Higuchi, S. Kawakami, F. Yamaschita, M. Nishikawa, M. Hashida, Involvment of serum mannan binding proteins and mannose receptors in uptake of mannosylated liposomes by macrophages, Biochim. Biophys. Acta 1511 (2001) 134–145.
[10] I. Huitinga, N. van Rooijen, C.J.A. DeGroot, B.M.J. Uitdehaag, C.D. Dijkstra, Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages, J. Exp. Med. 172 (1990) 1025–1033.
[11] J. Bauer, I. Huitinga, W. Zhao, H. Lassmann, W.F. Hickley, C.D. Dijkstra, The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis, Glia 15 (1995) 437–446.
[12] H.A. Smits, L.A. Boven, C.F. Pereira, J. Verhoef, H.S. Nottet, Role of macrophage activation in the pathogenesis of Alzheimer's disease and human immunodeficiency virus type 1-associated dementia, Eur. J. Clin. Invest. 30 (2000) 526–535.
[13] T.A. Bayer, R. Buslei, L. Havas, P. Falkai, Evidence for activation of microglia in patients with psychiatric illnesses, Neurosci. Lett. 271 (1999) 126–128.
[14] P.J. Gebicke-Haerter, K. Lieb, P. Illes, M. Berger, Mikroglia: Mechanismen der Aktivierung und Bedeutung bei der Pathogenese neuropsychiatrischer Erkrankungen, Nervenarzt 69 (1998) 752–762.
[15] M.M.J. Polfliet, P.H. Goede, E.M.L. van Kesteren-Hendrikx, N. van Rooijen, C.D. Dijkstra, T.K. van den Berg, A method for the selective depletion of perivascular and meningeal macrophages in the central nervous system, J. Neuroimmunol. 116 (2001) 188–195.
[16] J.P. Kosonen, Determination of disodium clodronate in bulk material and pharmaceuticals by ion chromatography with postcolumn derivatization, J. Pharm. Biomed. Anal. 10 (1992) 881–887.
[17] E.W. Tsai, S.D. Chamberlin, R.J. Forsyth, C. Bell, D.P. Ip, M.A. Brooks, Determination of bisphosphonate drugs in pharmaceutical dosage formulations by ion chromatography with indirect UV detection, J. Pharm. Biomed. Anal. 12 (1994) 983–991.
[18] R. Thompson, N. Grinberg, H. Perpall, G. Bicker, P. Tway, Separation of organophosphonates by ion chromatography with indirect photometric detection, J. Liq. Chromatogr. 17 (1994) 2511–2531.
[19] V. Virtanen, L.H.J. Lajunen, High-performance liquid-chromatographic method for simultaneous determination of clodronate and some clodronate esters, J. Chromatogr. Biomed. Appl. 617 (1993) 291–298.
[20] V. Virtanen, L.H.J. Lajunen, Determination of clodronate in aqueous-solutions by HPLC using postcolumn derivatization, Talanta 40 (1993) 661–667.
[21] S.E. Meek, D.J. Pietrzyk, Liquid-chromatographic separation of phosphorous oxo acids and other anions with postcolumn indirect fluorescence detection by aluminium morin, Anal. Chem. 60 (1998) 1397–1400.
[22] M.J. Lovdahl, D.J. Pietrzyk, Anion-exchange separation and determination of bisphosphonates and related analytes by post-column indirect fluorescence detection, J. Chromatogr. A 850 (1999) 143–152.
[23] R.W. Sparidans, J. den Hartigh, P. Vermeij, High-performance ion-exchange chromatography with in-line complexation of bisphosphonates and their quality control in pharmaceutical preparations, J. Pharm. Biomed. Anal. 13 (1995) 1545–1550.
[24] G.E. Taylor, Determination of impurities in clodronic acid by ion-exchange chromatography, J. Chromatogr. A 770 (1997) 261–271.
[25] T.L. Chester, E.C. Lewis, J.J. Benedict, R.J. Sunberg, W.C. Tettenhorst, Determination of (dichloromethylene)diphosphonate in physiological fluids by ion-exchange chromatography with phosphorous-selective detection, J. Chromatogr. 225 (1981) 17–25.
[26] T.L. Chester, Dual flame photometric phosphorous-selective detector for high-performance liquid-chromatography, Anal. Chem. 52 (1980) 1621–1624.
[27] R. Niemi, H. Taipale, M. Ahlmark, J. Vepsäläinen, T. Järvinen, Simultaneous determination of clodronate and its partial ester derivatives by ion-pair reversed-phase high-performance liquid chromatography coupled with evaporative light-scattering detection, J. Chromatogr. B 701 (1997) 97–102.
[28] H. Sirén, A. Määttänen, M.-L. Riekkola, Determination of small anions by capillary electrophoresis using indirect UV detection with sulphonated nitrosonaphtol dyes, J. Chromatogr. A 767 (1997) 293–301.
[29] K. Huikko, R. Kostiainen, Development and validation of a capillary zone electrophoretic method for the determination of bisphosphonate and phosphonate impurities in clodronate, J. Chromatogr. A 893 (2000) 411–420.
[30] K. Huikko, R. Kostiainen, Analysis of bisphosphonates by capillary electrophoresis-electrospray ionization mass spectrometry, J. Chromatogr. A 872 (2000) 289–298.
[31] L. Oppenheimer, T.P. Capizzi, R. Weppelman, H. Mehta, Determining the lowest limit of reliable assay measurement, Anal. Chem. 55 (1983) 638–643.
[32] A.M.J. Buiting, F. Zhou, J.A.J. Bakker, N. van Rooijen, L. Huang, Biodistribution of clodronate and liposomes used in the liposome mediated macrophage 'suicide' approach, J. Immunol. Methods 192 (1996) 55–62.