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Simultaneous determination of clodronate and its partial ester derivatives by ion-pair reversed-phase high-performance liquid chromatography coupled with evaporative light-scattering detection

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Abstract

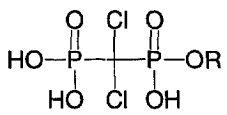
A new ion-pair HPLC method coupled with evaporative light-scattering detection (ELSD) for the simultaneous determination of clodronate and its partial esters has been developed. The simultaneous chromatographic separation was achieved on a reversed-phase C_8 column with a gradient system and butylamine as an ion-pair reagent. This method provides good enough reproducibility and sensitivity for in vitro determinations of clodronate and its ester derivatives. The method is applied for hydrolysis studies of clodronate monoesters which have been described as possible prodrugs of clodronate.

Keywords: Clodronate

1. Introduction

Clodronate, Cl_2MBP (**I**, Fig. 1), belongs to the group of methylenebisphosphonates (MBP). MBPs, containing a stable P–C–P bridge, are widely used in the treatment of various bone and tooth diseases and as regulators in calcium metabolism [1]. Bisphosphonates, including clodronate, inhibit bone resorption, both in vitro and in vivo, and are under clinical investigation for the treatment of osteoporosis ([2] and references cited therein).

The major drawback in the clinical use of clodronate is its poor oral bioavailability; only 2% of clodronate is absorbed after oral dosing [3]. The poor oral bioavailability of clodronate is mostly attributed to its very low lipophilicity because clodronate is completely ionized and negatively charged at pH of



Compound	Name	R
I	Clodronate	H
II	Clodronate monomethyl ester	$-CH_3$
III	Clodronate monopropyl ester	$-(CH_2)_2-CH_3$
IV	Clodronate monohexyl ester	$-(CH_2)_5-CH_3$
V	Clodronate monophenyl ester	$-C_6H_5$

Fig. 1. Chemical structures of clodronate and its monoesters.

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the intestine. Clodronate esters have been studied in order to modify the physicochemical properties of clodronate [4]. However, authors are not concluding whether these novel clodronate derivatives are prodrugs or analogs of clodronate. The prodrug approach would be a technique to improve the physicochemical properties and absorption characteristics of clodronate by masking an ionized group (or many groups) with suitable pro-moieties. A prodrug of clodronate should release the active drug in the body after absorption by chemical or enzymatic hydrolysis [5].

Clodronate, like other bisphosphonates, is nonchromophoric which makes the use of direct UV detection without pre- or post-column derivatization impossible. The pre- or post-column derivatization methods require extensive sample preparation or complicated and specialized equipment. In the literature, liquid chromatographic methods using UV [6–8] or fluorescence [9] detection based on derivatization of clodronate have been reported. An indirect UV detection which monitors the decrease in UV absorption of nitric acid eluent (negative signal) has been reported recently [10]. Also ion-pair and ion-exchange chromatography with refractive index [11] and flame photometric [11,12] detection have been reported for the determination of clodronate.

An evaporative light-scattering detector (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 number of particles, and therefore proportional to the concentration of analyte. ELSD requires the complete volatilization of mobile phase. Nonvolatile components would increase the background due to solid particles and would decrease sensitivity of ELSD [13–15]. ELSD has been used for determination of, e.g. phospholipids [16], fats and fatty acids [17] and carbohydrates [18].

The aim of the present study was to develop a selective and sensitive HPLC method using evaporative light-scattering detection for the simultaneous determination of clodronate and its prodrug candidates. The method was applied to determine susceptibility of clodronate esters for chemical and enzymatic hydrolysis in vitro.

2. Experimental

2.1. Chemicals

HPLC-grade methanol was obtained from Lab-Scan (Dublin, Ireland). Butylamine was purchased from Fluka (Buchs, Switzerland). Acetic acid and ammonium acetate were from Riedel-de-Haën (Seelze, Switzerland). Clodronate, disodium (dichloromethylene)bisphosphonate tetrahydrate (**I**), was obtained from Leiras OY (Turku, Finland). Monoesters of clodronate (monomethyl (**II**), monopropyl (**III**), monohexyl (**IV**) and monophenyl (**V**) clodronate trisodium salts) were prepared by the method described previously [19,20].

2.2. Instrumentation

Isocratic and gradient elutions were performed with a Merck LaChrom HPLC system consisting of Model L-7250 programmable autosampler, Model L-7100 HPLC pump, Model D-7000 interface module and Model D-7000 HPLC system manager (all from Hitachi, Tokyo, Japan). The loop used was a 100-μl stainless steel loop and injection volumes of 20 μl were used. A Sedex 55 evaporative light-scattering detector (Sedere, Vitry-Sur-Seine, France) was used. The detector temperature was 70°C, gain 8 and pressure of nebulizing gas (dried and filtered air) 2.2 bar. The analytical column was a Kromasil 100 RP-C8 (250×4.6 I.D., 5 μm) (Higgins Analytical Inc., Mountain View, CA, USA). An Orion SA 520 pH meter (Boston, USA) equipped with a combination pH electrode was used for pH determination.

2.3. Chromatography

The mobile phase for isocratic elution was a mixture of methanol (3%) and 0.10 *M* ammonium acetate buffer (97%) containing 0.23 *M* butylamine, adjusted to pH 4.60 with acetic acid and delivered at a flow-rate of 1.2 ml/min. Isocratic method was used for determination of clodronate.

In the gradient elution for simultaneous detection of clodronate and clodronate monoesters the amount of methanol was raised linearly from 3 to 40–60% (depending on analytes) for between 1.0 and 6.0 min,

and the flow-rate was kept as a constant (1.2 ml/min).

2.4. Hydrolysis in aqueous solution

The hydrolysis of monophenyl (**V**) and monomethyl (**II**) clodronate was studied in aqueous buffer solution (pH 7.4) at 37°C. Phosphate buffer (50 m*M*) at an ionic strength (μ) of 0.15 *M* was used. An appropriate amount of the compound was dissolved in 10.0 ml of pre-heated phosphate buffer. The solutions were placed in a thermostated water-bath and, at suitable intervals, 0.5-ml samples were withdrawn. The samples were analyzed by the described HPLC method.

2.5. Enzyme hydrolysis in human serum

Clodronate monoesters (40–70 μmol) were dissolved in 0.6 ml of phosphate buffer (50 m*M*, μ= 0.15, pH 7.4) at 37°C. Pre-heated human serum (2.4 ml) was added and the solutions were kept in a water-bath at 37°C. At suitable intervals, 250-μl samples were withdrawn and added to 250 μl of methanol in order to deproteinize the serum. After mixing and centrifugation, 300 μl of the supernatant was evaporated to dryness under a stream of nitrogen at 20°C and redissolved with 400 μl of water. The solutions were analyzed by the described HPLC method.

2.6. Enzyme hydrolysis in rabbit liver homogenate

The hydrolysis of clodronate phenylester (**V**) was studied at 37°C in 10% (m/v) rabbit liver homogenate. The rabbit liver was homogenized with 4 volumes of isotonic phosphate buffer, pH 7.4, using a X-1020 homogenizer (Ystral, Germany). The homogenate was centrifuged for 90 min at 9000 *g* at 4°C with a Biofuge 28 RS-centrifuge (Heraeus Instruments, Germany). The supernatant was stored at −80°C. Before use, 20% liver homogenate was diluted to 10% with isotonic phosphate buffer, pH 7.4. An appropriate amount of compound was dissolved in 4.0 ml of pre-heated 10% liver homogenate. The solution was incubated at 37°C. At appropriate intervals, samples of 400 μl were withdrawn and added to 400 μl of methanol. After mixing and centrifugation, 450 μl of the supernatant was evaporated to dryness under a stream of nitrogen at 20°C and redissolved with 600 μl of water. The solutions were analyzed by the described HPLC method.

3. Results and discussion

A chromatogram of the simultaneous determination of clodronate and its phenylester by gradient elution is shown in Fig. 2a. The first two peaks in the chromatogram belong to sodium and phosphate ions.

3.1. Calibration

In the isocratic elution the linearity of the method was determined by analyses of 10 different concentrations of clodronate (0.0375–1.40 mg/ml) in phosphate buffer (50 m*M*, pH 7.4). Each concentration was analysed three times. The peak area vs. analyte concentration graph is shown in Fig. 3a.

In the gradient elution the linearity was determined for clodronate and clodronate phenylester in phosphate buffer. Ten different concentrations (for clodronate 0.0375–1.40 mg/ml and for clodronate phenylester 0.054–2.50 mg/ml) were used. Each concentration was analyzed three times. The peak area vs. analyte concentration graphs are shown in Fig. 3b,c.

All the calibration curves were nonlinear and followed the equation of the type $y=ax^2+bx+c$ with high correlation (r^2=0.999–1.000). The response of ELS detector is usually reported to increase sigmoidally with increasing sample size [13,14,21], but has also been described as linear or exponential [22]. In addition to the chromatographic conditions and detector parameters, the chemical structure of the sample also affects the detector response [23].

3.2. Reproducibility

The reproducibility was determined for two concentrations of clodronate in isocratic elution, and for two concentrations of both clodronate and clodronate phenylester in gradient elution with six subsequent injections. In isocratic elutions, the R.S.D. values for

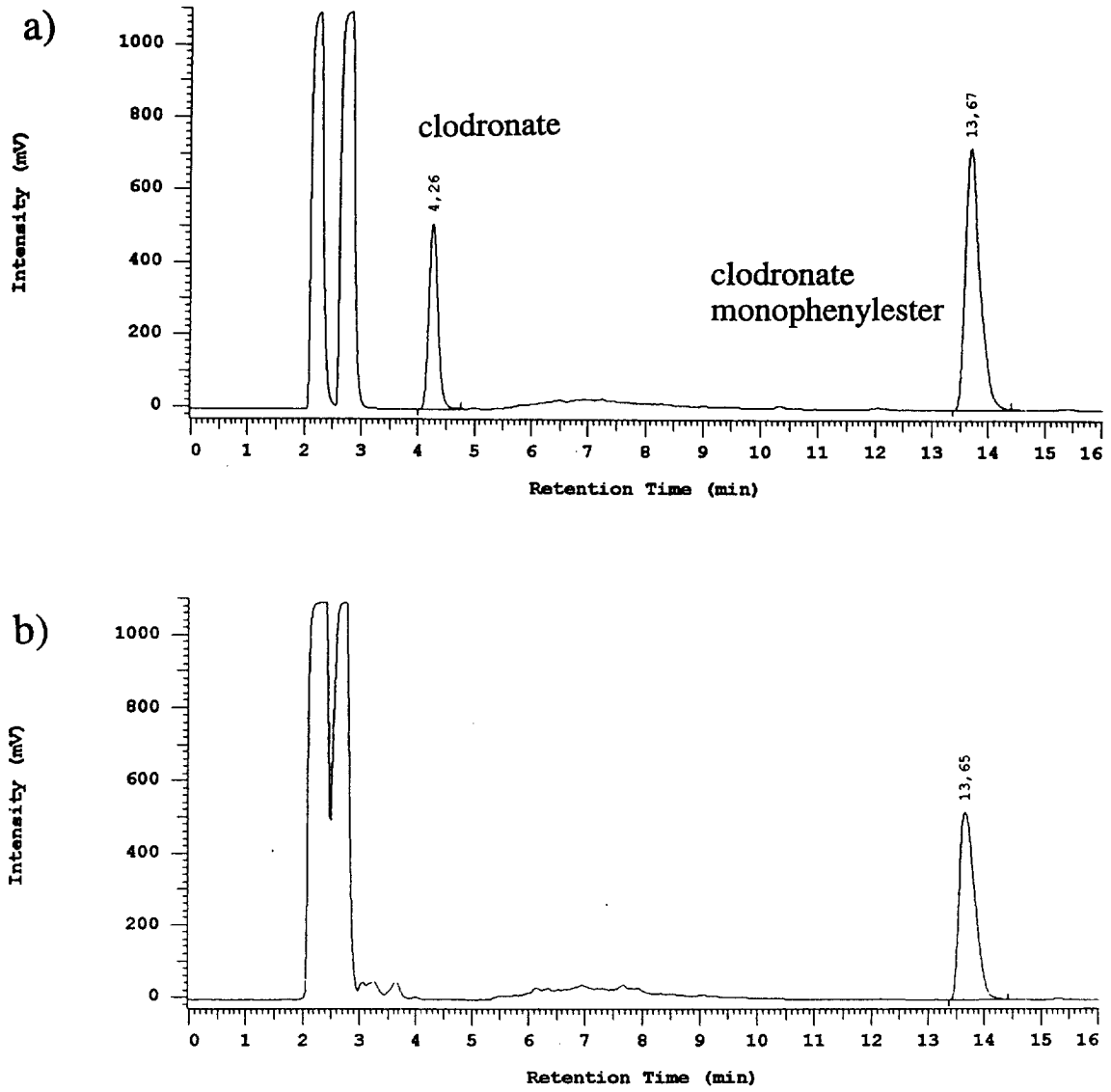

Fig. 2. (a) Chromatogram of simultaneous determination of clodronate (0.96 mg/ml) and clodronate phenylester (1.72 mg/ml) with gradient elution. (b) Chromatogram of a sample (2 h) from enzymatic hydrolysis studies of clodronate monophenylester.

0.10 and 1.20 mg/ml clodronate in phosphate buffer were 4.6 and 1.0%, respectively. In gradient elutions the R.S.D. values for the same clodronate concentrations were 5.5 and 1.0%, and for 0.18 and 2.15 mg/ml solutions of clodronate phenylester 1.4 and 1.0%, respectively.

3.3. Limit of detection and limit of quantitation

In simultaneous determination of clodronate and its ester, the limit of detection for clodronate was 37.5 μg/ml (0.75 μg/injection) and for clodronate phenylester 54 μg/ml (1.1 μg/injection) with a

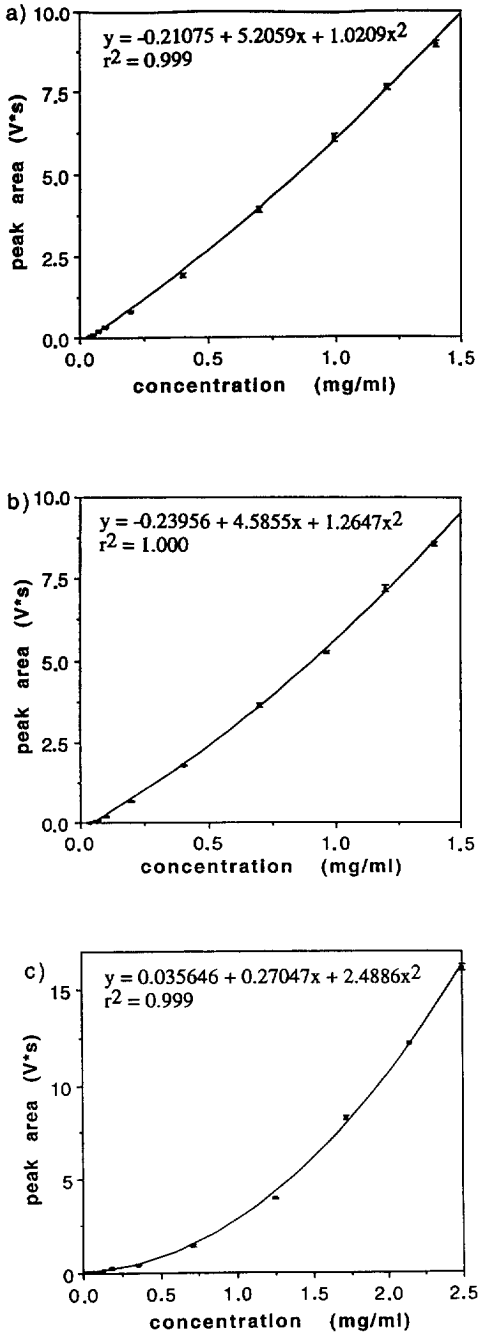

Fig. 3. Standard curves of clodronate in isocratic elution (a), and clodronate (b) and clodronate monophenylester (c) in gradient elution.

signal-to-noise ratio of 3:1. The limit of quantitation for clodronate was 50 μg/ml (1.0 μg/injection) and for clodronate phenylester 89 μg/ml (1.8 μg/injection) with a signal-to-noise ratio of 20:1. The injection volume was 20 μl.

3.4. Application of the method to hydrolysis assays of clodronate monoesters

Clodronate methylester (**II**) and phenylester (**V**) did not release clodronate via chemical hydrolysis during 28 days in phosphate buffer at pH 7.4 and at 37°C. The susceptibility of clodronate methylester (**II**), propylester (**III**), hexylester (**IV**) and phenylester (**V**) to undergo enzymatic hydrolysis was studied in 80% human serum. The clodronate monoesters (**II**, **III**, **IV**, **V**) did not release clodronate via enzymatic hydrolysis in 80% human serum during 4 h at pH 7.4 and at 37°C. A typical chromatogram of a sample withdrawn from the enzymatic hydrolysis assay of clodronate phenylester (**V**) in human plasma is shown in Fig. 2b. The susceptibility of clodronate phenylester (**V**) to liver enzymes was studied in 10% rabbit liver homogenate. In this assay clodronate phenylester did not release clodronate within 6 h at pH 7.4 and at 37°C.

Prodrugs are pharmacologically inactive derivatives of a drug molecule that require a chemical or enzymatic transformation to release the parent drug (in this case clodronate) in vivo. Orally administered prodrugs might be activated in the gut wall, plasma or liver. The present study shows that simple esters of clodronate are not prodrugs due to their resistance to chemical and enzymatic hydrolysis.

4. Conclusions

The present ion-pair HPLC method coupled with evaporative light-scattering detection (ELSD) enables simultaneous determination of clodronate and its ester derivatives. The method can be fully automated and it provides good enough sensitivity for in vitro determinations. Clodronate esters do not release active clodronate via chemical or enzymatic hydrolysis, and thus they do not fulfil the criteria of prodrug approach.

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