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Semi-automatic liquid chromatographic analysis of olpadronate in urine and serum using derivatization with (9-fluorenylmethyl)chloroformate

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Abstract

The semi-automatic bioanalytical assays for olpadronate [(3-dimethylamino-1-hydroxypropylidene)bisphosphonate] involves a protein precipitation with trichloroacetic acid and a double co-precipitation with calcium phosphate for serum samples and a triple calcium co-precipitation for urine samples. These manual procedures are followed by an automated solid-phase extraction on a cation-exchange phase. The procedure is continued either directly, at high olpadronate levels in urine, or after off-line evaporation under nitrogen and reconstitution in water on the same robotic workstation. The continued automatic procedure comprehends derivatization with (9-fluorenylmethyl)chloroformate, ion-pair liquid–liquid extraction and ion-pair HPLC with fluorescence detection at 274/307 nm. The intra- and inter-day precisions for urine and serum samples are typically in the 5–8% range for different olpadronate concentrations [levels near the lower limit of quantification (LLQ) excluded]. The LLQ is 5 ng/ml olpadronate for a 2.5-ml urine sample and 10 ng/ml for a 1-ml serum sample, respectively.

Keywords: Derivatization, LC; Olpadronate

1. Introduction

Olpadronate [(3-dimethylamino-1-hydroxypropylidene)bisphosphonate] is a recently introduced representative of the bisphosphonates, a group of drugs applied in the treatment of calcium metabolic disorders like Paget's disease, hypercalcaemia or osteoporosis [1,2]. Olpadronate is a potent amino-substituted bisphosphonate, undergoing phase III clinical evaluation since several years. The actual mode of action of bisphosphonates is still not known; however, the first intracellular target of nitrogen containing bisphosphonates has recently been discovered [3]. A bioanalytical assay, which is not yet available for olpadronate, can support the clinical pharmacological investigations in obtaining data on pharmacokinetics or bioavailability. Because of their high potency, olpadronate and other new bisphosphonates are administered in lower doses than previously introduced bisphosphonates. Therefore, the

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demands on the sensitivity of bioanalytical bisphosphonate assays are strongly increased. The number of developed assays with an appropriate sensitivity is limited; the methods with a lower limit of quantification (LLQ)<100 ng/ml will be discussed briefly.

Only recently, a very sensitive enzyme-linked immunosorbent assay has been reported briefly for risedronate [4]. Previously, sensitive bioanalytical assays of bisphosphonates were all based on chromatography and, to achieve low detection limits, four different approaches can be distinguished between these assays [4–6]: (1) derivatization prior to gas chromatography (GC) [7], (2) ion-exchange chromatography (IEC) with post-column decomposition of the bisphosphonate into orthophosphate and derivatization of the phosphate into a colored complex, followed by spectrophotometric detection [8], (3) reversed-phase liquid chromatography (RPLC) with selective, UV [9], fluorescence [10] or electrochemical [11] detection; this approach can only be followed if the bisphosphonate has any selective detectable property, (4) RPLC after derivatization of a primary amino group of the bisphosphonate into a fluorescent moiety; reported derivatization agents are 2,3-naphthalene dicarboxyaldehyde [12–14] and 1-naphthylisothiocyanate [15–17]. The advantage of the first two approaches is the applicability to all types of bisphosphonates, although the more complex chemical structure of the latest generation of bisphosphonates may complicate the derivatization reactions in such methods.

The successes of the last approach for the development of sensitive bioanalytical assays for pamidronate [(3-amino-1-hydroxypropylidene)bisphosphonate] and alendonate [(4-amino-1-hydroxybutylidene)bisphosphonate] challenged us to develop a derivatization reaction for olpadronate, a bisphosphonate containing a tertiary amine, based on the synthesis of a fluorescent derivative suitable for high-performance liquid chromatography (HPLC). Only one type of reaction is known to synthesize a tertiary amine derivative: the reaction with a chloroformate; 2-naphthylchloroformate (NCF) [18,19] and (9-fluorenylmethyl)chloroformate (FMOC) [20] have been employed in the past in HPLC methods as a reagent for this type of components. Since NCF is not commercially available, FMOC was chosen as a reagent for olpadronate. In this paper, the development and validation of a bioanalytical assay for olpadronate, the first one ever described for this type of bisphosphonate, in both urine and serum is reported. The method is based on derivatization with FMOC, including optimal automation with a Cartesian coordinate robot. This robotic workstation is especially designed for solid-phase extraction (SPE) with disposable extraction columns but can also perform other laborious pre-treatment procedures of a chromatographic method [16,17,21].

2. Experimental

2.1. Chemicals

Disodium olpadronate was obtained from Henkel (Düsseldorf, Germany) and etidronate (monosodium salt) originated from Nogepha (Alkmaar, The Netherlands). Monosodium olpadronate as a concentrate for infusion (20 mg/5 ml) was kindly provided by Gador (Buenes Aires, Argentina). (4-Dimethylamino - 1 - hydroxybutylidene)bisphosphonic acid (DABD) was generously provided by Dr. C.W.G.M. Löwik (Department of Endocrinology, Leiden University Medical Center, The Netherlands). FMOC, tetrabutylammonium-bromide (TBA-Br) and TBA-hydroxide (20%, w/w, in water) were of synthetic quality and were purchased from Merck–Schuchardt (Hohenbrunn, Germany). Tetraoctylammonium-bromide (TOA-Br) and tetra(decyl)ammonium-bromide (TDA-Br) originated from Sigma (St. Louis, MO, USA). Water was distilled at the production facility of our department, acetonitrile (HPLC-grade) was supplied by Rathburn (Walkerburn, UK) and trichloroacetic acid (20%, w/w) was obtained from UCB (Leuven, Belgium). All other chemicals were of analytical grade originating from Merck (Darmstadt, Germany).

2.2. Equipment

Chromatographic analyses were performed on the following configuration: a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, USA) with an ASPEC XL sample processor (Gilson, Villiers-le-Bel, France). The processor was equipped with a 402 syringe pump (Gilson) with one 5-ml

syringe, a 7010-80 Rheodyne injection valve (Rheodyne, Cotati, CA, USA) with a 100-μl sample loop and a vial rack (Gilson) for 32×12 mm vials, thermostated by a Thermomix BU thermostatic bath (B. Braun Biotech, Melsungen, Germany). The detector was a Jasco 821-FP spectrofluorometric detector (Jasco, Hachioji City, Japan) with a 16-μl flow cell. Data were recorded on an IPC Dynasty HE 486DX personal computer (IPC, Singapore), equipped with a Gynkosoft chromatographic data system (Softron, Gräfelfing, Germany). To obtain UV spectra, a UVD 340 S diode array detector (Gynkotek, Germering, Germany) was coupled to the LC system.

Isolute propyl sulfonic acid (PRS) modified silica SPE columns (1 ml/100 mg, IST, Mid-Glamorgan, UK) and Bakerbond aromatic sulfonic acid (ASA) modified silica SPE columns (1 ml/100 mg, J.T. Baker, Phillipsburg, NJ, USA) were used for SPE. Further, an Eppendorf 5416 centrifuge (Eppendorf, Hamburg, Germany) and a Zymark TurboVap LV evaporator (Zymark, Hopkinton, MA, USA) were employed.

2.3. Chromatographic conditions

Flushed loop injections (100 μl) were made on a Microspher C_{18} column (100×4.6 mm, d_p=3 μm, average pore diameter=13 nm, Chrompack, Middelburg, The Netherlands) with a reversed-phase (R2) guard column (10×2 mm, Chrompack). The column temperature was ambient. For urine analysis, the eluent comprised 72.5% (v/v) of a 30 m*M* phosphate buffer (pH 7.0), containing 5 m*M* TBA-hydroxide and 2 m*M* etidronate as an adsorption suppressor, and 27.5% (v/v) acetonitrile. The eluent flow-rate was 0.8 ml/min. For serum analysis, the acetonitrile content of the eluent was 25% and the flow-rate 1.0 ml/min. The fluorescence detection wavelengths were 274 nm for excitation and 307 nm for emission, respectively.

2.4. Sample preparation

Stock solutions of 494 μg/ml disodium olpadronate and 130 μg/ml DABD (I.S.) in water were stored at 4–6°C. The olpadronate stock solution was stored for maximum 6 months. Aqueous dilutions, if required, were prepared daily. Urine and serum samples were stored at −20°C. Flow tables of all three analytical procedures are shown in Fig. 1.

2.4.1. Method A

A 2.5-ml sample of urine was transferred into a 10-ml conical glass tube and, in case of a calibration sample, spiked with the appropriate olpadronate solution to give final drug concentrations in the range of 2 to 20 ng/ml; 100 μl of 1 mg/ml etidronate in water and 50 μl of 1.30 μg/ml DABD were added.

The urine procedure was started with a triple co-precipitation with calcium phosphate; between all additions the sample was mixed by vortex-mixing. A 100-μl volume of 0.1 *M* calcium chloride and 100 μl of 0.1 *M* sodium dihydrogenphosphate were added, followed by addition of subsequent portions of 25 μl of 1 *M* sodium hydroxide; when after adding and vortex-mixing of one of these additions a precipitate was formed, one final portion of sodium hydroxide was added to ensure the formation of sufficient precipitate. A pellet was formed by centrifugation for 1 min at $3.9 \cdot 10^3$ *g* and, after removal of the liquid phase, redissolved in 25 μl of 1 *M* hydrochloric acid. Next, a sequence of sample handlings, comprising dilution with 2.5 ml water, precipitation with 25 μl of 1 *M* sodium hydroxide, centrifugation and redissolution in 25 μl of 1 *M* hydrochloric acid, was carried out twice. The precipitate was completely redissolved after the third precipitation. The resulting samples were processed further on the ASPEC system with an automatic SPE using the disposable PRS columns; water was employed in the syringe for automatic liquid displacement by the syringe pump. The SPE column was preconditioned, at 0.5 ml/min, with 0.5 ml water and 0.25 ml aqueous 1 mg/ml etidronate, respectively. After dilution with 0.5 ml water and mixing by aspiration and dispensing, the column was loaded with the sample and washed with 0.25 ml water, both at a 1 ml/min flow-rate. Sample and washing liquid were collected together in one polypropylene (PP) tube. This eluate was then evaporated off-line until dryness at 80°C under 1 bar nitrogen for 35 min. The residue was redissolved in 250 μl water by vortex-mixing before the automatic procedure was continued. In this part, acetone was used in the syringe for liquid displacement. For the derivatization, 250 μl of 0.25 *M* phosphate buffer

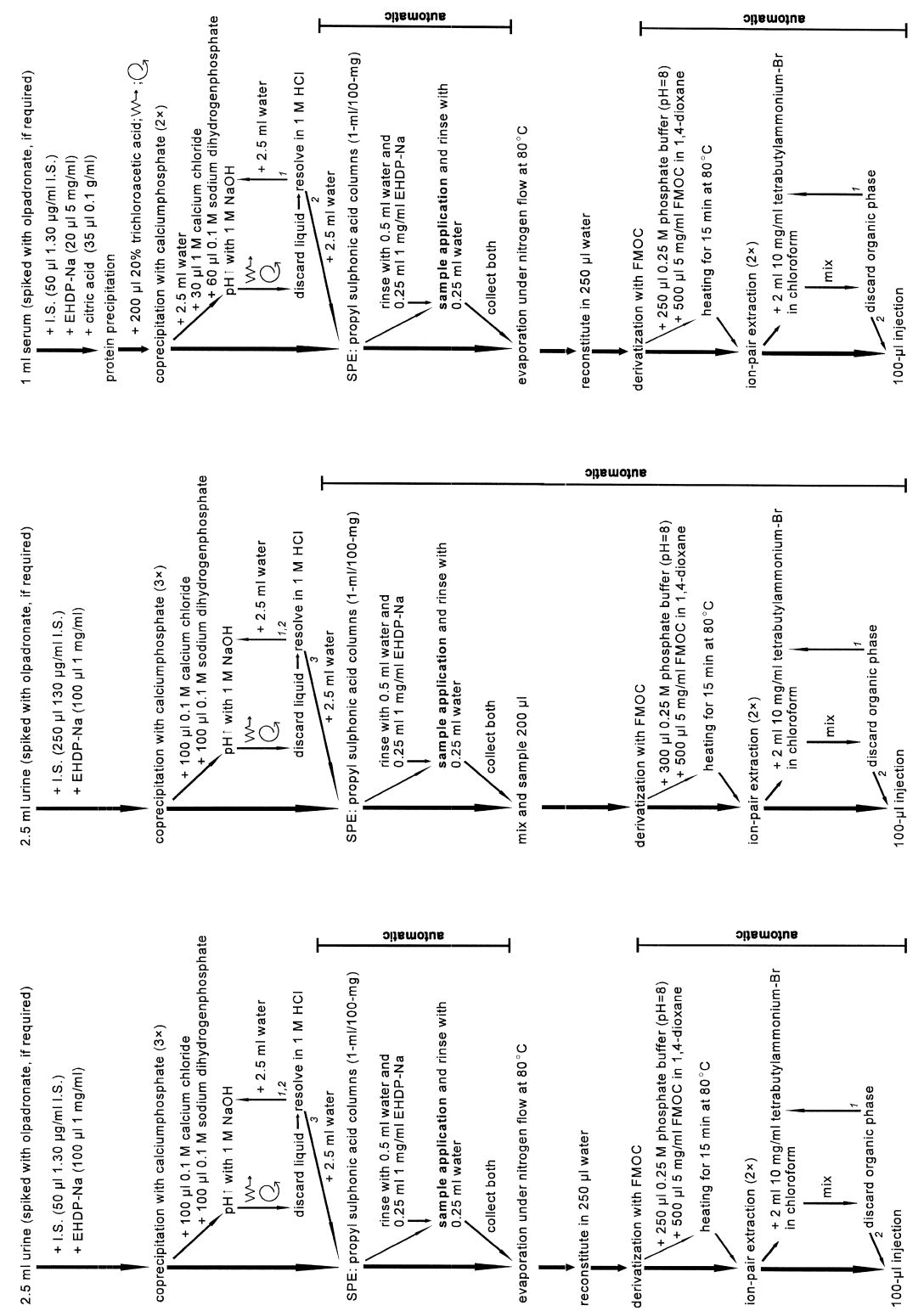

Fig. 1. Flow tables for the three analytical procedures. Vortex mixing; Centrifugation.

(pH 8.0) and 500 μl of 5 mg/ml FMOC in 1,4-dioxane were added to the sample and mixed by air bubbling to obtain a clear solution. Next, this mixture was transferred to an open borate glass sample vial (32×12 mm) and heated at 60°C for 15 min. The reaction mixture was then transferred to a 10 mm I.D. glass tube and cleaned-up by a two-fold ion-pair liquid–liquid extraction with 2 ml of 10 mg/ml TBA-Br in chloroform. The two phases were mixed by bubbling 5 ml air at a 5 ml/min flow-rate through the liquid and after waiting for 1 min, in which the two phases were allowed to separate, the lower organic layer was removed and rejected. Finally, 100 μl of the aqueous phase was transferred directly onto the HPLC column by flushed-loop injection, 40 min after the previous injection.

2.4.2. Method B

This method for urine analysis is a more automated version of method A, particularly developed and validated for higher olpadronate concentrations. Essentially, method B is a combination of the two automated parts of method A, where the off-line evaporation and redissolution is omitted. Consequently, a few modifications had to be made. Urine samples were spiked, if required, with the appropriate olpadronate solution to give final drug concentrations in the range of 20 to 200 ng/ml; next, 250 μl of 130 μg/ml DABD is added. After elution of the analyte from the SPE column, the eluate is mixed by bubbling 1 ml air at a 5 ml/min flow-rate through the liquid; an aliquot of 200 μl is taken and transferred into the sample vial, together with 300 μl of the phosphate buffer and 500 μl of the FMOC solution. Method B is carried out with acetone as the dispensing liquid in the syringe. Starting with the derivatization, the procedure is continued similar to method A.

2.4.3. Method C

A 1-ml sample of serum was transferred into a 1.5-ml PP micro test tube and, in case of a calibration sample, spiked with the appropriate olpadronate solution to give final drug concentrations in the range of 5 to 50 ng/ml; 20 μl of 5 mg/ml etidronate, 35 μl of 0.1 g/ml citric acid and 50 μl of 1.30 μg/ml DABD (I.S.) were added. The procedure was started with a protein precipitation step by adding 200 μl of 20% trichloroacetic acid; the tube was closed, strongly shaken by vortex-mixing and centrifuged for 10 min at $14 \cdot 10^3$ *g*. From the supernatant, pipetted into a 10-ml conical glass tube, the bisphosphonates were co-precipitated with calcium phosphate by adding 30 μl of 1 *M* calcium chloride, 60 μl of 0.1 *M* sodium dihydrogenphosphate, 2.5 ml water and 200 μl of 1 *M* sodium hydroxide; the sample was vortex-mixed before and after the last addition. A pellet was formed by centrifugation for 1 min at $3.9 \cdot 10^3$ *g* and, after removal of the liquid phase, redissolved in 30 μl of 1 *M* hydrochloric acid. Next, the sample was diluted with 2.5 ml water and a precipitate was formed again by adding 25 μl of 1 *M* sodium hydroxide. After vortex-mixing, centrifugation and redissolution in 25 μl of 1 *M* hydrochloric acid, the procedure was continued similar to the SPE, concentration through evaporation, derivatization and liquid–liquid extraction of method A.

2.5. Validation

The bioanalytical methods were validated analyzing several series of samples. Serum samples from six individual patients and six urine samples, donated by different volunteers, were tested as independent blanks. Serum of one volunteer and pooled urine of seven volunteers was applied for preparing calibration samples. The intra- and inter-day precision and accuracy were determined using pooled serum portions of about seven patients each and three individual urine samples of different volunteers. The lower limit of detection (LLD) is the concentration level where the presence of olpadronate is certain for more than 95% and was calculated from the independent blanks and the calibration line. At the LLQ, the RSD of the repeatability, as well as the deviation of the accuracy, are not more than 20% of the concentration. The LLQ was calculated from the intra-assay data at the lowest level and the calibration line. The recoveries of the consecutive pretreatment steps were determined by adding 100 μl of 494 ng/ml olpadronate to blank urine or serum samples in between the different steps before derivatization, of procedures A and C, both in duplicate.

Olpadronate was determined in serum and 24-h urine sampled from a 56-year-old male patient

suffering Paget's disease of bone who had been treated with olpadronate 2 years before. Urine samples were collected before and during treatment with 8 mg monosodium olpadronate in a 4-h infusion of 500 ml 0.9% (w/v) sodium chloride on 5 consecutive days, and were analyzed according to methods A and B, respectively. The renal excretion was calculated from the olpadronate concentration in urine and the excreted volume. Blood samples were taken at 0, 1, 2, 4, 4.5, 6, 7, 10, 14, 20 and 24 h after the start of the infusion on the first day of treatment. Serum was separated from the blood cells by centrifugation at $2.8 \cdot 10^3$ *g* for 10 min.

3. Results and discussion

3.1. Method development and optimization

In order to investigate the potential use of FMOC as a fluorescent reagent for olpadronate, the optimal reaction conditions were determined. The derivatization could be performed in a mixture of 1,4-dioxane–phosphate buffer, pH 7–8 (1:1, v/v), one of the reaction media reported by Doyle et al. [19] for the derivatization of amines with NCF. A reaction for olpadronate with FMOC is postulated, based on previous literature on this type of derivatizations [18–20], and shown in Fig. 2. The reaction product of this reaction would be FM-olpadronate {[3-{(9-fluorenyl-methylcarbonyl)methylamino}-1-hydroxy-propylidene]bisphosphonate} then. Degradation of the olpadronate derivative, in the reaction mixture in a closed glass tube at ambient temperature and not protected from light, was not observed after overnight storage. The UV and fluorescence spectra of the olpadronate derivative, determined using LC coupled with the diode array detector and performing stop-flow LC–fluorescence measurements, respectively, were comparable to spectra of FMOC and to the FMOC derivative of alendronate, a bisphosphonate containing a primary amino function [22]. Unfortunately, attempts to identify and confirm the postulated structure by off-line mass spectrometric analysis using both fast atom bombardment-MS, matrix-assisted laser desorption ionisation–mass spectrometry (MS) and electrospray MS, and on-line LC–electrospray-MS using reversed-phase chromatography (combined with various sample clean-up procedures) were not yet successful. On the other hand, all other results of the analytical method validation reported in this paper indicate that the chromatographed analyte is any kind of FMOC derivative of olpadronate.

Acetone was investigated as a less toxic alternative for 1,4-dioxane as a solvent for FMOC. Although acetone did not affect the formation rate of the olpadronate derivative compared to the 1,4-dioxane medium, acetone did reduce the efficiency of the ion-pair liquid–liquid extraction of the by-products of the derivatization. Reduction of the amount of reagent lead to a lower yield of FM-olpadronate, while higher amounts of FMOC did not dissolve in the reaction mixture. The reaction rate was optimal

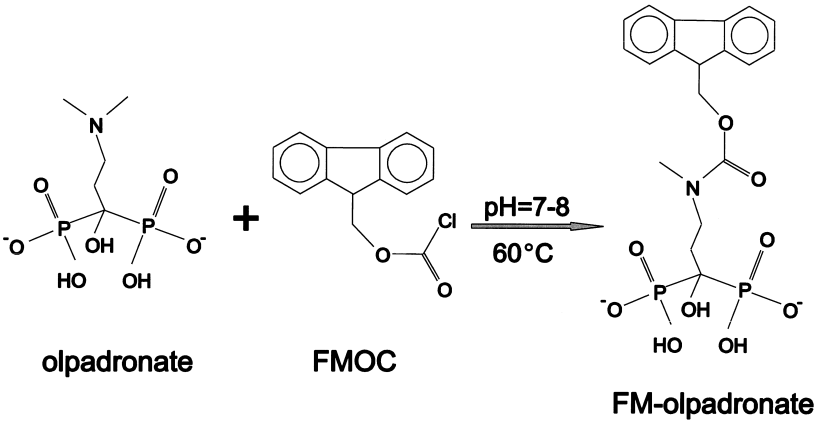

Fig. 2. Postulated derivatization reaction of olpadronate with FMOC.

with a phosphate buffer, pH 8; the pH slightly decreased during the reaction. The relative reaction yield for 250 μl of an aqueous standard solution, containing 0.72 μg/ml olpadronate and 0.52 μg/ml DABD, was measured at 40 and 60°C, applying a 0.1 *M* phosphate buffer. The results are plotted in Fig. 3. This figure clearly indicates that a reaction temperature of 60°C is required for the maximal conversion of both analyte and I.S. within the time range investigated. Since the reaction rate of olpadronate is faster compared to DABD, 15 min was chosen as the reaction time for the final assay, as a compromise between the optimum of both bisphosphonates. For the derivatization of aqueous samples in the optimization experiments an 0.1 *M* buffer concentration was sufficient; however, for biological samples a stronger phosphate buffer (0.25 m*M*) was required to keep the pH, which decreased during derivatization, at the required level during the total reaction time, in order to obtain maximal conversion of the bisphosphonates. In addition, the pH of the buffer was chosen as high as possible (pH 8) in the second buffering pH range of phosphate ($pK_{a,2}=7.2$) to correct for the observed pH decrease during derivatization.

Co-precipitation with calcium phosphate as a selective pre-treatment step in the bioanalysis of bisphosphonates was first reported by Bisaz et al. [23]. In the present study, the calcium precipitation for urine samples was derived from the procedure for pamidronate developed in our laboratory [15]; only a small modification was made: the amount of calcium ions added was reduced and a small amount of phosphate was added to the urine. This resulted in less variation in the amounts of precipitate, formed in the individual urine samples. For serum samples, the protein precipitation and the calcium precipitation was also similar to the procedure for pamidronate [16]. However, a lower background signal at the olpadronate retention time in chromatograms of blank samples could be obtained by performing a double co-precipitation with calcium phosphate, as performed in the presented assay, instead of a single co-precipitation as employed for the serum samples in the pamidronate method [16].

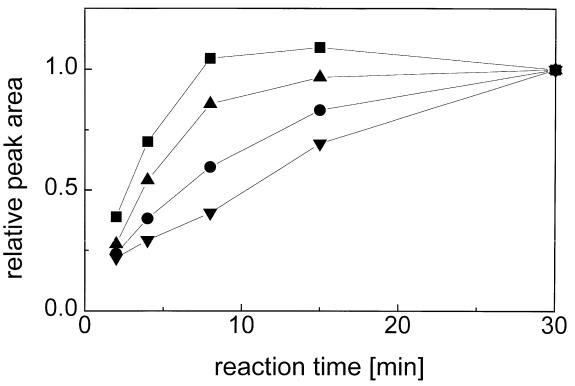

Fig. 3. Formation of the FMOC-derivatives of olpadronate and DABD, relative to the amount at 30 min, as a function of reaction time at two temperatures. (▼) DABD, 40°C; (●) olpadronate, 40°C; (▲) DABD, 60°C; (■) olpadronate, 60°C.

The anion-exchange-based SPE procedure [retaining bisphosphonates and primarily employed for the removal of calcium (II) ions] as reported for pamidronate [15–17] was not applicable in combination with the FMOC derivatization of olpadronate. After evaporation of the anion-exchange SPE eluate almost no derivative appeared to be formed in the residue after derivatization under the reaction conditions described. Therefore, cation-exchange-based SPE (not retaining bisphosphonates) was investigated to remove calcium (II) ions from the sample after calcium precipitation. The ASA and PRS columns were compared and for the PRS columns a higher olpadronate yield and a lower flow resistance was observed; therefore, this column type was chosen for the final assay. Etidronate was employed in the SPE procedure for the suppression of irreversible adsorption of olpadronate and DABD on the column.

The ion-pairing agent in the liquid–liquid extraction after derivatization improved the selectivity of the extraction; nevertheless, several late eluting large peaks still remained in the chromatogram as shown in Fig. 4d. The extraction efficiency could be slightly improved by applying a stronger ion-pairing agent than TBA; however, the strong cohesion of the chloroform containing 10 g/l TOA-Br or TDA-Br hindered the automatic removal of the last droplet of chloroform from the tube after liquid–liquid extraction; therefore, TBA-Br was preferred as the ion-pairing agent. Acetone was chosen as the dispensing liquid in the syringe because of its mixability with all the solvents applied in the procedure. Alcohols, methanol for example, could not be em-

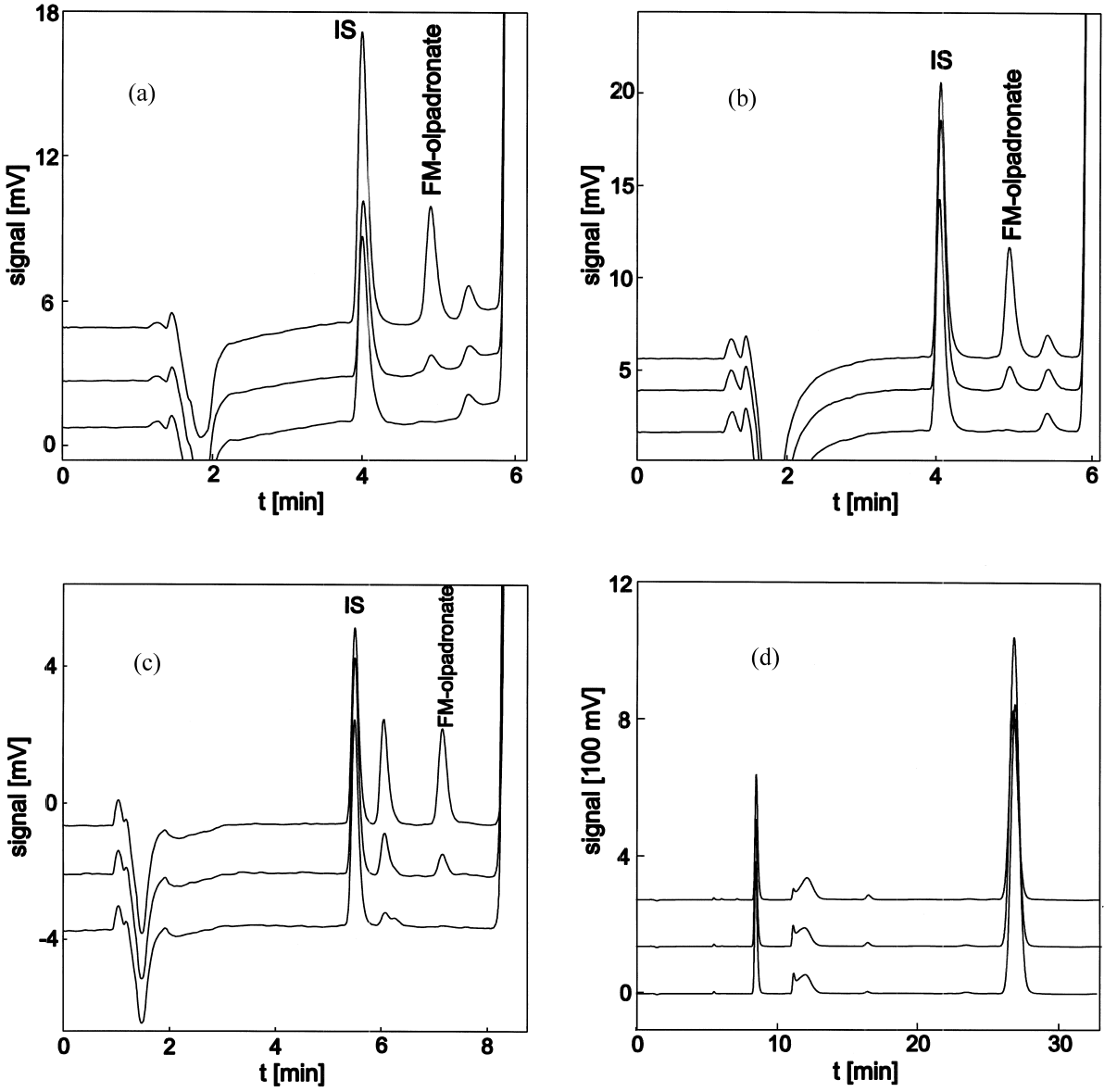

Fig. 4. Chromatograms of olpadronate in urine and serum. (a) Method A: 0, 4 and 20 ng/ml disodium olpadronate in urine, respectively; (b) method B: 0, 20 and 100 ng/ml disodium olpadronate in urine, respectively; (c) method C: 0, 10 and 50 ng/ml disodium olpadronate in serum, respectively; (d) full scale plot of the chromatograms in (c) to show the peaks of the by-products, eluting after the bisphosphonate derivatives.

ployed because they can react with chloroformate reagents.

The chromatographic separation of bisphosphonates could be improved by adding an ion-pairing agent to the eluent, in order to increase the retention of the bisphosphonate and the selectivity of the separation. Of the two ion-pairing agents investigated (TBA and TOA), TBA was selected because only TBA facilitated both the separation between the different bisphosphonate derivatives and the late

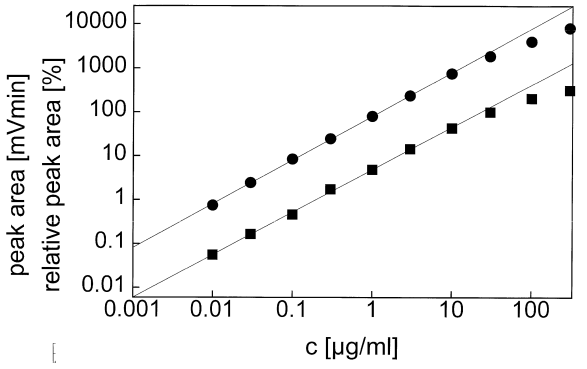

Fig. 5. Formation of FM-olpadronate at different concentrations in aqueous solutions. The lines are fitted through the seven points in the 0.01–10 μg/ml range. (●) Peak area (mV min); (■) peak area, relative to 0.52 μg/ml DABD (%).

eluting by-products and the separation between the olpadronate derivative and a contaminant in the I.S. (Fig. 4).

Table 1
Intra-day precision (repeatability) at different concentrations of disodium olpadronate (*c*) in urine and serum

Sample	Method	*c* (ng/ml)	Relative area	RSD (%)	*n*
Urine	A	0	0.023	36	6
	B	0	0.009	56	6
	A	5	0.158	16	6
	A	20	0.479	5	6
	B	20	0.107	7	7
	B	99	0.472	1.6	7
Serum	C	0	0.025	48	6
	C	10	0.109	8	6
	C	49	0.489	4.5	5

Table 3
Lower detection levels (ng/ml) of the presented assays for disodium olpadronate in urine and serum samples

Sample	Method	Concentration at $S/N=3$	LLD	LLQ
Urine	A	0.2	2	5
	B	1	6	8
Serum	C	1	6	10

3.2. Validation

The derivatization yield was linear with the concentration until approximately 10 μg/ml olpadronate in a 250-μl sample of a diluted aqueous standard solution (Fig. 5). Examples of chromatograms are shown in Fig. 4 for the three methods. The intra- and inter-day validation parameters are tabulated in Tables 1 and 2. The components of interest were well separated from other peaks for both urine and serum; there was only a small disturbing peak at the FM-olpadronate retention time. Therefore, the LLD was higher than expected from the $S/N=3$ level (Table 3). The LLD was calculated from the blank analyses, the LLQ from the inter-day data and the calibration line. Both are reported in Table 4. Typical calibration lines, calculated by (non-weighted) least-squares linear regression analysis were:
method A: $y=0.015(\pm0.007)+0.0260(\pm0.0006)x$ (range=2–20 ng/ml; $n=6$; $r^2=0.998$);
method B: $y=0.002(\pm0.014)+0.00473(\pm0.00011)x$ (range=20–200 ng/ml; $n=6$; $r^2=0.998$);
method C: $y=0.010(\pm0.013)+0.0111(\pm0.0004)x$ (range=5–50 ng/ml; $n=6$; $r^2=0.994$).

The concentration of olpadronate in urine (20 and

Table 2
Inter-day precision (reproducibility) and accuracy at different concentrations of disodium olpadronate (*c*) in urine and serum

Sample	Method	*c* (ng/ml)	Relative area	RSD		Accuracy	
				(%)	*n*	(%)	*n*
Urine	A	5	0.153	15	6	100	2
	A	20	0.481	8	6	93	3
	B	20	0.099	6	6	102	2
	B	99	0.447	7	7	92	3
Serum	C	10	0.123	17	8	112	3
	C	49	0.530	8	6	103	2

Table 4
Average recoveries (%) of individual pre-treatment steps and the total recovery of procedures A and C prior to derivatization ($n=2$)

Pre-treatment	Method A (urine)	Method C (serum)
Protein precipitation		80
Calcium precipitation	90	100
SPE	80	80
Evaporation	90	90
Overall	70	60

100 ng/ml) and serum (10 and 50 ng/ml), respectively, was not significantly affected by five extra freeze–thaw cycles. The results of the experiments for the determination of the recoveries of the individual pre-treatment steps are reported in Table 4, they are all sufficiently high.

Finally, olpadronate was measured in 24-h urine samples originating from two patients suffering from Paget's disease of bone, demonstrating the applicability of the assay for clinical samples. In Fig. 6, the results (method B) are shown during a 5-day intravenous treatment with 8 mg/day olpadronate of these patients. The day before this treatment, no olpadronate was detectable in their urine (method A).

Urinary olpadronate excretion during intravenous treatment, measured using method B, is shown in Fig. 6a; olpadronate was not detectable in the pre-treatment urine sample using method A. In Fig. 6b, olpadronate concentrations in serum during the first day of treatment are shown (method C). The olpadronate concentrations in the samples taken at 20 and 24 h after the start of the infusion were below the LLQ.

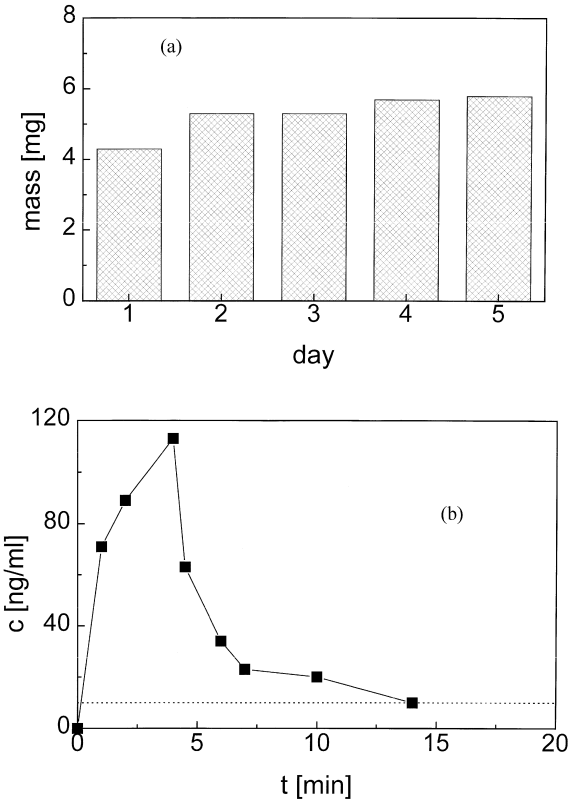

Fig. 6. (a) Daily 24-h urinary excretion of monosodium olpadronate (mg), during a 5-day treatment with daily 4-h infusion of 8 mg monosodium olpadronate in 500 ml 0.9% sodium chloride, measured for a 56-year-old male patient suffering Paget's disease of bone. (b) Monosodium olpadronate concentrations in serum during and after the first infusion for this patient.

4. Conclusions

Selective semi-automatic methods for the analysis of olpadronate in urine and serum have been presented. The extensive pre-treatment procedure, protein precipitation (for serum samples only), co-precipitation with calcium phosphate, SPE, evaporation, derivatization and liquid–liquid extraction, has sufficiently been automated for routine use of these assays. The validation data indicate that the method is appropriate for the determination of olpadronate in these matrices; currently, the methods are extensively employed in clinical pharmacokinetic investigations. For urine method B is preferred, requiring less manual handling compared to method A. Only in the very low ng/ml range method A has to be employed. The presented assays are specifically suited for olpadronate, however, the methodology can possibly be adjusted for analogous compounds, for example ibandronate {[3-(methylpentyl)amino-1-hydroxypropylidene]bisphosphonate} or even bisphosphonates containing another type of amine function.

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