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Semi-automatic liquid chromatographic analysis of pamidronate in urine after derivatization with 1-naphthylisothiocyanate

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Abstract

An existing sensitive chromatographic assay for pamidronate in urine has considerably been automated. Using the same sample processor, the solid-phase extraction (SPE) was automated separately from the derivatization with 1-naphthylisothiocyanate, the two-fold ion-pair liquid–liquid-extraction and the treatment with hydrogen peroxide for the 2–20 ng/ml concentration range. The automatic procedure was preceded by a triple calcium precipitation and interrupted by evaporation of the SPE eluate under nitrogen. For the 0.5–5 μg/ml concentration range one automatic sequence was used by avoiding evaporation during the sample treatment. In addition to the labour-saving of the semi-automatic procedure, the daily sample-throughput was improved compared to the existing manual assay. Further, the validation showed marginal improvements in the precision, accuracy and lower limit of quantification.

Keywords: Derivatization, LC; Pamidronate; 1-Naphthylisothiocyanate

1. Introduction

Bisphosphonates are a class of drugs typically used to inhibit bone resorption. A sensitive and reliable bioanalytical assay will enormously support clinical investigations with these drugs [1]. HPLC with fluorescence detection after an extensive sample pre-treatment, typically containing the very selective co-precipitation with calcium phosphate, has shown to be the most successful approach for the development of sensitive bioanalytical assays for bisphosphonates [2]. In previous work at our laboratory bioanalytical assays for pamidronate [(3-amino-1-hydroxypropylidene)bisphosphonate, APD], the first representative of the aminobisphosphonates, were presented for both urine [3] and serum [4], suitable for clinical pharmacological research. The method for pamidronate in urine [3] is very labour-intensive and not yet automated like the serum assay. Because it is preferable to have a time saving automated (and validated) method for urine samples as well, the analysis of pamidronate in urine was automated analogous to the semi-automatic method for serum

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samples [4]. Validation results are reported in this paper. In addition, some improvements of the analytical procedure have been made too and the suitability of the assay for both short-term and long-term pharmacokinetics is demonstrated.

2. Experimental

2.1. Chemicals, equipment and chromatographic conditions

All suppliers, apparatus and HPLC conditions have been reported previously [4], only acetonitrile (HPLC-grade) was provided now by Rathburn Chemicals (Walkerburn, UK).

2.2. Sample preparation

Stock solutions of 495 μg ml^{-1} APD and 51.3 μg ml^{-1} neridronic acid, the internal standard (I.S.), in water were stored at 4–6°C. Dilutions, if required, were prepared daily. Urine samples were stored at −20°C.

2.2.1. Method A

A 2.5-ml sample of urine was transferred into a 10-ml conical glass tube and spiked, if required, with the appropriate APD solution to give final drug concentrations in the range of 2 to 20 ng ml^{-1}; 100 μl of 1 mg ml^{-1} etidronate and 30 μl of 2.05 μg ml^{-1} I.S. were added. The procedure was started with a triple co-precipitation with calcium phosphate; between all additions the sample was mixed by vortexing. 100 μl 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 3900 *g* and, after removal of the liquid phase, redissolved in 25 μl of 1 *M* hydrochloric acid, using vortex-mixing. 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 procedure was continued by automatic solid-phase extraction (SPE) using the ASPEC sample processor (Gilson, Villiers-le-Bel, France); water was applied in the syringe for the automatic liquid displacement. The SPE column (Bakerbond 3 ml/500 mg quaternary amine (J.T. Baker, Phillipsburg, NJ, USA)) was pre-conditioned (5 ml min^{-1}) with 2.5 ml water, 1 ml of 1 mg ml^{-1} etidronate, 2.5 ml of 0.1 *M* nitric acid and 5 ml water, respectively. After dilution with 2.5 ml water and mixing by aspiration and dispensing, the sample was loaded onto the column and the column was then washed with 1 ml water (both at 2 ml min^{-1}). The column was washed further (3 ml min^{-1}) with 4 ml water and 2.5 ml of 10 m*M* nitric acid. After elution (2.5 ml min^{-1}) of the bisphosphonates with 2.5 ml of 0.1 *M* nitric acid and collection in a polypropylene tube, the eluate was evaporated off-line until dryness at 80°C under 0.6 bar nitrogen for 50–75 min. The residue was reconstituted in 500 μl water by vortex-mixing.

Next, the automatic procedure was continued with methanol in the syringe for the liquid displacement. For the derivatization 50 μl of 1 mg ml^{-1} etidronate, 75 μl triethylamine and 500 μl of 20 mg ml^{-1} NITC in pyridine were added and mixed with the sample by air-bubbling to form a clear yellow solution. Subsequently, the sample was transferred into an open 2.5-ml polypropylene sample vial (15×45 mm) and heated at ca. 80°C for 15 min. The reaction mixture was transferred into a 10-mm I.D. glass tube and treated with a two-fold ion-pair liquid–liquid-extraction with 2 ml of 10 mg ml^{-1} tetrabutylammonium-bromide in chloroform to clean-up the sample. The two phases were mixed by bubbling 3 ml air (5 ml min^{-1}) through the liquid and were separated after waiting for 1 min; the lower organic layer was subsequently removed and rejected. Of the resulting sample 300 μl was transferred together with 90 μl of 3% hydrogen peroxide into an open 750-μl polypropylene sample vial (12×30 mm) and heated at ca. 80°C for a complete oxidative desulphuration into the naphthylcarbamyl derivatives of

the aminobisphosphonates. After 5 min, 100 μl was transferred directly onto the HPLC column using flushed-loop injection.

2.2.2. Method B

This method is a further automated version of method A, developed and validated for higher pamidronate concentrations. A few modifications were made. Drug-free urine samples were spiked, if required, with the appropriate pamidronate solution to give final drug concentrations in the range of 0.5 to 5 μg ml^{-1}; 100 μl of 51.3 μg ml^{-1} I.S. was added. After elution of the analyte from the SPE column, the eluate was automatically mixed by aspiration and dispensing; an aliquot of 200 μl was then taken and transferred into the polypropylene sample vial (15×45 mm), together with 300 μl water, 50 μl of 1 mg ml^{-1} etidronate, 75 μl triethylamine and 500 μl of 20 mg ml^{-1} NITC in pyridine, for derivatization. The procedure is then continued identically to method A. This combination of the two automated parts from method A was carried out with methanol as the dispensing liquid in the syringe.

2.3. Validation

Both bioanalytical methods were validated by analysing, intra- and inter-day, series of six samples, spiked at 5; 20; 1000 and 4000 ng ml^{-1}, respectively and donated by different volunteers. For the independent blanks, samples from six volunteers were tested. Pooled urine of seven volunteers was used for the preparation of calibration samples and spiked with 2, 4, 8, 12, 16 and 20 ng ml^{-1} APD for method A and 0.5, 1, 2, 3, 4 and 5 μg ml^{-1} APD for method B, respectively. The lower limit of detection (LLD) is the concentration level where the presence of APD is >95% certain and was calculated from the independent blanks and the calibration line in the lower range. At the lower limit of quantification (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 slope of calibration line.

APD was determined in 24-h urine samples of a 47-year-old male and a 42-year-old female suffering from sternocostoclavicular hyperostosis, they were treated 3-monthly with APD. Samples were collected 3 months after an intravenous course with daily 15 mg APD in a 4-h infusion of 0.9% (w/v) sodium chloride on 5 consecutive days, and were analysed according to method A. Samples were also collected daily during the next treatment with 15 mg APD on 5 consecutive days, and were analysed using method B. APD renal excretion (mg per 24 h) was calculated from the APD concentration in urine and the excreted volume.

3. Results and discussion

The manual bioanalytical assay of pamidronate in urine as described previously [3] was automated as far as possible in order to decrease the manual labour, to increase the maximum daily sample-throughput and to achieve a possible improvement of precision and accuracy. Method B was even further automated than method A whereby the evaporation step was avoided to facilitate a single automated procedure, starting with SPE and ending with the chromatographic separation. Consequently, the bisphosphonates had to be derivatized directly in the SPE eluate; sequential evaporation of the eluate using the ASPEC is not a realistic option. Because the nitric acid in the eluate may affect the derivatization reaction, the influence of the amount of SPE eluate, sampled for derivatization, on the derivatization reaction, was investigated. Two hundred microlitres of SPE eluate appeared to contain the maximum allowable amount of nitrate that did not decrease the reaction yield significantly and this amount of SPE eluate was therefore employed in method B.

Examples of chromatograms for both methods are shown in Fig. 1. Two series of six individual drug-free samples were tested as independent blanks for both procedures (Table 1). The intra- and inter-day validation parameters are tabulated in Tables 1 and 2. The LLD is 1 ng ml^{-1} for procedure A and 70 ng ml^{-1} for procedure B; the LLQ is 2 ng ml^{-1} for procedure A and 300 ng ml^{-1} for procedure B.

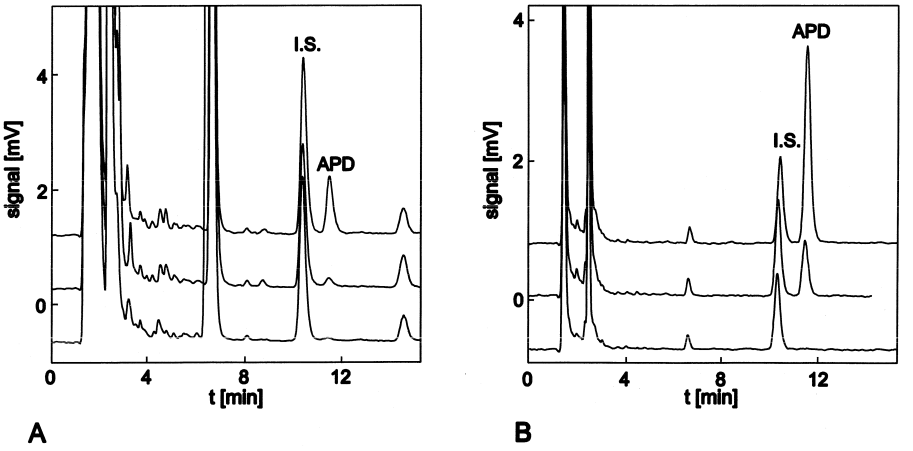

Fig. 1. Chromatograms of pamidronate (APD) in urine. (A) Method A, the APD concentration is respectively 0, 5 and 20 ng ml^{-1}. (B) Method B, the APD concentration is respectively 0, 1 and 4 μg ml^{-1}. Chromatographic conditions: injection volume: 100 μl; column: Microspher C_{18} (100×4.6 mm, d_p=3 μm, average pore diameter=13 nm); guard column: reversed-phase (10×2 mm); column temperature: ambient; eluent: 38% (v/v) 10 m*M* phosphate buffer, containing 10 m*M* tetraoctylammonium-bromide and 2 m*M* EHDP, and 62% (v/v) acetonitrile, pH*=8.0; eluent flow: 0.8 ml min^{-1}. Detection: fluorescence at λ_{ex}=285 nm and λ_{em}=390 nm.

Table 1
Intra-day precision (repeatability) at different concentrations of disodium pamidronate (c) in urine

Method	*c* [ng ml^{-1}]	Relative area	RSD [%]	*n*
A	0	0.013	63	6
B	0	0.0057	81	6
A	5	0.117	6	6
A	20	0.419	5	6
B	1000	0.603	6	6
B	4000	2.35	6	6

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

Method	*c* [ng ml^{-1}]	Relative area	RSD		Accuracy	
			[%]	*n*	[%]	*n*
A	5	0.117	15	6	109	2
A	20	0.391	8	8	111	3
B	1000	0.590	8	6	104	2
B	4000	2.38	6	6	105	2

Typical calibration lines, calculated by (non-weighted) least-squares regression analysis were:

method A: $y = 0.022(\pm 0.003) + 0.160(\pm 0.004)x$

(range = 2–20 ng ml^{-1}; $n = 6$; $r^2 = 0.997$),

method B: $y = 0.000(\pm 0.013) + 0.622(\pm 0.008)x$

(range = 0.5–5 μg ml^{-1}; $n = 6$; $r^2 = 0.9993$).

The concentration of APD (1 μg ml^{-1}) in two spiked urine samples, stored at −20°C was not influenced by storage for 14 months when compared to the present inter-day results.

The semi-automatic method is undoubtedly less labour-intensive and has a higher maximum daily sample-throughput (n=18–20) compared to the manual method (n=12–16). Method B is even less labour-intensive (n=24–26), however, it is only suitable for higher pamidronate concentrations (>0.3 μg ml^{-1}) because only a fraction (ca. 1/15) of the SPE eluate can be processed further. Improvements in the precision, accuracy and LLQ of the APD determination in urine were only marginal.

The results of the measurements of the clinical samples are shown in Fig. 2. Typically, the pamidronate levels are >LLQ, even 3 months after the previous treatment. The APD excretion during the treatment, 49% and 66% of the administered dose for the male and female respectively, was normal for patients without renal impairment and suffering a metabolic disease not characterized by an elevated rate of bone turnover [5–7].

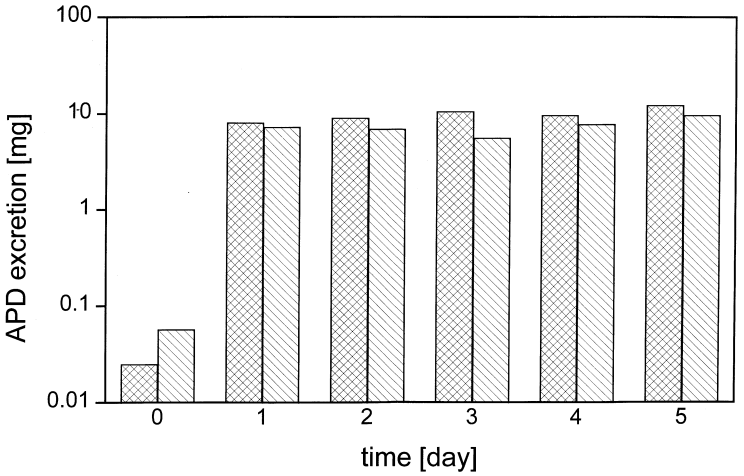

Fig. 2. Pamidronate excretion of a 42-year-old female (××××) and a 47-year-old male (\ \ \ \) before and during a 5-day course of 15 mg d^{-1} pamidronate i.v., as a part of a 3-monthly regimen.

4. Conclusions

The semi-automatic assay for APD in urine is preferred above the manual method [3] because of a reduced labour-intensiveness and an improved sample capacity of both automated methods. Both assays seem appropriate for the determination of pamidronate in urine during and up to 3 months after intravenous treatment; therefore, method A and B have been, and will be, employed in further pharmacokinetic investigations of APD in other metabolic bone disorders like osteoporosis.

References

[1] R.W. Sparidans, I.M. Twiss, S. Talbot, Pharm. World Sci. 20 (1998) 206.
[2] R.W. Sparidans, J. den Hartigh, Pharm. World Sci. 21 (1999) 1.
[3] R.W. Sparidans, J. den Hartigh, J.H. Beijnen, P. Vermeij, J. Chromatogr. B 696 (1997) 137.
[4] R.W. Sparidans, J. den Hartigh, J.H. Beijnen, P. Vermeij, J. Chromatogr. B 705 (1998) 331.
[5] S. Leyvraz, U. Hess, G. Flesch et al., J. Natl. Cancer Inst. 84 (1992) 788.
[6] E. Redalieu, J.M. Coleman, K. Chan et al., J. Pharm. Sci. 82 (1992) 665.
[7] L. Hyldstrup, G. Flesch, S.A. Hauffe, Calcified Tissue Int. 53 (1993) 297.