

Original Research Article

Solid-phase extraction and validated spectrofluorimetric quantification of pamidronate in human plasma

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Abstract

Purpose: To design a simple and sensitive quantification procedure for pamidronate disodium (PAM) after its solid phase extraction from plasma.

Methods: The procedure was based on derivatization of PAM using a suitable fluorogen, 4-chloro-7-nitro-2,1,3-benzoxazole. The product was determined spectrofluorometrically at excitation and emission wavelengths of 390 and 535 nm, respectively. The method was optimized for all factors that affect the reaction between PAM and the fluorogen. These factors include diluting solvent, pH of the reaction medium, volume of fluorogen solution, buffer pH, buffer volume, temperature and heating time. The method was fully validated according to US-FDA guidelines with respect to linearity, accuracy, precision, recovery, robustness and stability.

Results: PAM was successfully extracted from human plasma with solid-phase extraction technique. A linear response was obtained in the concentration range of 10 – 100 ng/mL, with correlation coefficient of 0.998. Mean maximum plasma concentration of PAM was $9.73.2 \pm 3.2 \mu\text{mol/L}$, which was within the linear range of the proposed method, thereby confirming its sensitivity for the determination of plasma PAM.

Conclusion: The proposed procedure is suitable for the quantification of PAM in human plasma after its solid phase extraction. The method is sensitive enough for use in PAM determination in pharmacokinetic studies. Moreover, it is likely a more cost-effective and simpler alternative method than high performance liquid chromatograph (HPLC) methods.

Keywords: Pamidronate disodium, Derivatization, Spectrofluorimetry, Fluorogen, Quantification

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INTRODUCTION

Pamidronate disodium (PAM) is a bisphosphonate drug used for reducing cancer-induced hypercalcemia. The drug is used in combination with chemotherapy for treating bone disease caused by metastasis of multiple myeloma or breast carcinoma. Pamidronate

disodium (PAM) is also useful in the treatment of Paget's disease [1-3]. The determination of bisphosphonates is a daunting process because these compounds do not bear chromophores. Moreover, they are extremely polar, on account of numerous functional groups, and they are not appreciably retained on HPLC columns [4]. Most of the methods applied for the quantification of

PAM belong to the categories of liquid chromatography [5-7] and gas chromatography [8].

Spectrofluorimetric methods are advantageous in many aspects. They are highly sensitive [9]. In addition, spectrofluorimetric methods offer a high degree of selectivity because not all substances that absorb in the UV-region emit fluorescence. This high degree of selectivity may be attributed to the presence of different wavelengths of maximum excitation and/or emission for the fluorescent species [10,11].

It is obvious from literature review that there is need for a sensitive and affordable analytical method for PAM, as an alternative to the current chromatographic methods for pharmacokinetic studies on the drug, most of which depend on fluorescence detector for quantification [12]. Therefore, the main objective of this study was to develop a suitable, sensitive and affordable method for the quantification of PAM in human plasma after its solid phase extraction, and for monitoring of PAM in pharmacokinetic and bioequivalence studies.

EXPERIMENTAL

Raw material and human plasma samples

Pamidronate disodium (PAM, $C_3H_9NO_7P_2Na_2 \cdot 5H_2O$); molecular weight 396.1 g/mol, was kindly provided by Novartis Pharmaceuticals, San Carlos, CA 94070, United States of America. Its purity was 99.90 %. Human plasma was obtained from King Khaled Hospital, Al-Kharj, Kingdom of Saudi Arabia (KSA).

Chemicals and reagents

Methanol, ethanol, HCl and acetonitrile were purchased from Sigma Aldrich. Phosphate buffer solution (pH 8.5 \pm 0.2) was prepared by dissolving specific amounts of potassium dihydrogen orthophosphate and sodium hydroxide (Sigma Aldrich) in distilled water. The reagent 4-chloro-7-nitro-2,1,3-benzoxazole (NBD-ClO) was purchased from E. Merck Darmstadt, Germany. A 0.1 % (w/v) solution of NBD-ClO was made by solubilizing 100 mg of the salt in methanol, and the solution volume was brought up to 100. Distilled water was product of "Aquatron" Water Still (Bibby Sterillin Ltd., UK).

Instrumentation

Spectrofluorometer Model RF-1501 coupled to an Epson LX-300 printer and 1-cm quartz cuvette

was product of Shimadzu, Japan. The apparatus was equipped with xenon discharge lamp, excitation and emission grating monochromators.

Preparation of stock and working standard solutions

A stock solution of PAM (100 μ g/mL) was made by solubilizing 10 mg of bulk PAM in 50 mL of de-ionized H_2O , followed by bringing up the volume to 100-mL mark using de-ionized H_2O . A working standard solution of PAM (1 μ g/mL) was made by diluting 1 mL of stock standard solution to 100 mL in a 100-mL volumetric using de-ionized distilled water. Calibration standards and quality controls were prepared in a biological matrix similar to that of samples for analysis. Calibration curves were constructed from blank sample (matrix sample prepared without any additions) and eight non-zero samples that fall within the anticipated concentration range to be quantified. Plasma calibration standards were prepared by spiking control human plasma with PAM standard solutions. Plasma calibration samples were prepared at concentrations of 10, 20, 30, 40, 50, 60, 80 and 100 ng/mL. Quality control (QC) specimens were prepared at concentrations of 20 ng/mL (low QC, LQC); 50 ng/mL (medium QC, MQC), and 90 ng/mL (high QC, HQC). All calibration standards were vortex-mixed for 5 min to ensure proper mixing.

Extraction of PAM from plasma samples

On the day of assay, plasma samples were removed from a freezer and allowed to thaw at room temperature for 1 h. Some test tubes were set up, and 40 μ L of 1 M HCl was added to each tube and vortexed. Solid phase extraction cartridges (SPEs) were positioned on the extraction manifold, and each cartridge was conditioned with 2 mL methanol. Thereafter, each cartridge was equilibrated with 2 mL of 20 mM HCl and washed with 2 mL of distilled water. Plasma containing PAM was loaded onto the cartridges and then washed with 1 mL of 20 mM HCl, followed by rinsing twice with 3 mL of methanol. The eluate collected was evaporated to dryness with nitrogen at 50 $^{\circ}C$, and reconstituted in 1 mL distilled water.

Scanning of the excitation and emission spectra

An aliquot (0.5 mL) of working standard solution of PAM (1 μ g/mL) was transferred into a 20-mL stoppered test tube, followed by addition of 2 mL phosphate buffer solution (pH 8.5 \pm 0.2). Then, 1.5 mL of 0.1 % (w/v) NBD-Cl reagent was added. The mixture was heated in a thermostatic

water bath at 70 °C for 20 min, and cooled to ambient temperature. Then, the tube contents were transferred to a 10-mL calibrated measuring flask, and the volume was made up to mark with methanol. The excitation and emission spectra were recorded against a reagent blank prepared under the same conditions.

Determination of the effect of various analytical factors

Effect of diluting solvent

The procedure under "Scanning of the excitation and emission spectra of the reaction product" was repeated using methanol, ethanol and acetonitrile as diluting solvents.

Influence of volume of NBD-Cl solution

The procedure described under "Scanning of the excitation and emission spectra of the reaction product" was repeated, but with different volumes of the NBD-Cl reagent ranging from 0.5 - 3 mL in different tubes. Then, fluorescence intensity was read at excitation λ of 390 nm and emission λ of 535 nm.

Effect of buffer pH

The previous procedure was followed but, in this case, phosphate buffer solution with pH values ranging from 7-9.5 was used. Fluorescence intensity was measured at same excitation and emission wavelengths as in the previous subsection.

Influence of volume of buffer

The procedure used under 'Effect of buffer pH' was adopted but, in this case, with varying volumes of phosphate buffer solution (pH 8.5 \pm 0.2) ranging from 0.5 - 3 mL in different tubes. Then, fluorescence intensity was measured as before.

Effect of temperature

The impact of temperature on the reaction between PAM and NBD-Cl was studied by following the procedure described under "Scanning of the excitation and emission spectra of the reaction product", except that variable temperatures were applied, ranging from 40 - 90 °C in a thermostatic water bath.

Effect of heating time

The procedure described under "Scanning of the excitation and emission spectra of the reaction

product" was followed but, in this case, the absorbance of the prepared colored solutions was read within 45 min after various heating times.

Influence of time on stability of reaction product PAM and NBD-Cl

The previous procedure was applied on 0.5 mL aliquot of PAM standard solution (1 μ g/mL) and the fluorescence intensity was read as indicated earlier.

Validation of spectrofluorometric method

Validation was done in line with US-FDA guidelines [13].

Linearity

Nine samples (one blank and eight non-zero samples) of PAM ranging in concentration from 10 to 100 ng/mL were used. The samples included lower limit of quantification (LLOQ), low quality control (LQC), medium QC (MQC), high QC (HQC), and upper limit of quantification (ULOQ). Each sample was put in a 20-mL stoppered test tube. Then, the procedure described under "Scanning of the excitation and emission spectra of the reaction product" was applied. Fluorescence intensity (I) was read as indicated earlier.

A calibration curve was drawn by plotting fluorescence intensity against concentration. The percentages of variations between PAM levels obtained from calculation and the corresponding nominal levels were used for validation of correlation. The model was accepted if the variation was not above 15% for PAM concentrations, with the exception of LLOQ. The calibration curve was validated by determination of correlation coefficient, slope and intercept.

Intra- and inter-day accuracy and precision

These were determined at LQC, MQC, HQC, and LLOQ in six replicates, while the corresponding inter-day values were determined for 3 consecutive days with quality control specimens.

Selectivity

Method selectivity was performed by obtaining blank human plasma samples from 6 persons. All blank specimens were subjected to testing for interference utilizing the proposed extraction method, and compared with standard solutions of the studied drug.

Recovery

Recovery of the studied drug using the extraction method was determined at concentrations corresponding to LQC, MQC and HQC. It was assessed via comparison of the fluorescence intensities of the extracted specimens which were spiked prior to extraction, and the peak areas of the un-extracted specimens.

Robustness

The effect of slight variations on the method parameters was studied with respect to variations in buffer pH, temperature and volume of the fluorogenic agent used.

Stability

Investigations on stability were done by storing plasma specimens at laboratory temperature for 24 h. Freeze-thaw stability was investigated over 3 freezing-thawing cycles, while prolonged stability assays were done following 30-day freezing of samples at -28°C . The concentration of PAM at each period was compared with the corresponding level of PAM at the outset.

RESULTS

Pamidronate disodium (PAM) is an anti-osteoporotic drug which lacks native fluorescence due to the fact that structurally, it lacks nearly all the requisite criteria for fluorescence emission. Therefore, in this study, NBD – Cl served as a fluorogenic agent which reacted with PAM to produce a fluorescent product with excitation and emission wavelengths of 390 and 535 nm, respectively, as shown in Figure 1. This method was applied for the quantification of PAM in human plasma following solid phase extraction.

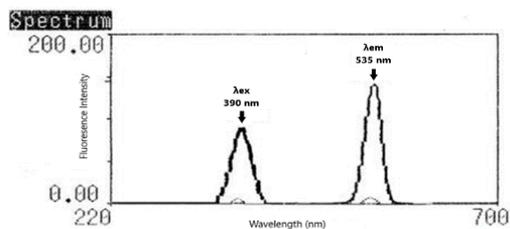


Figure 1: Excitation and emission spectra of PAM-NBD-Cl reaction product () and a corresponding reagent blank (.....)

Method optimization

Different parameters affecting the reaction between PAM and NBD-Cl were studied. This was with a view to optimizing the reaction

conditions and getting maximum fluorescence, and the corresponding maximum sensitivity of the assay. Different solvents were tested, namely methanol, ethanol, and acetonitrile. The highest fluorescence intensity was evoked when methanol was used as a solvent. Thus, methanol was used in subsequent studies (Figure 2). In addition, the other conditions affecting the reaction were optimized. Maximum fluorescence intensity was attained using 1.5 mL of 0.1% NBD-Cl solution (Figure 3). The reaction was carried out at different pH values. The results indicated that the reaction was optimum at a slightly alkaline medium ($\text{pH } 8.5 \pm 0.2$), as shown in Figure 4. The optimum volume of the buffer solution ($\text{pH } 8.5 \pm 0.2$) was 2 mL (Figure 5), while maximum color intensity was attained at a temperature of 70°C (Figure 6). The optimum heating time was 20 min (Figure 7), while the reaction product was stable for up to 90 min (Figure 8).

Validation results

The parameters of the method were validated in line with US-FDA guidelines.

Linearity

The linearity of the method was carefully studied, and a calibration graph of fluorescence intensities (I_f) against PAM concentrations ranging from 10 to 100 ng/mL.

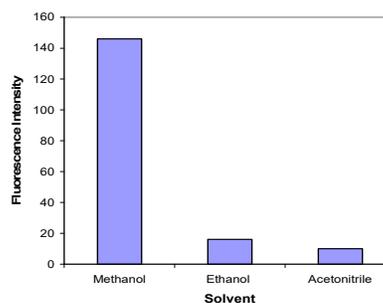


Figure 2: Effect of diluting solvents on fluorescence intensity

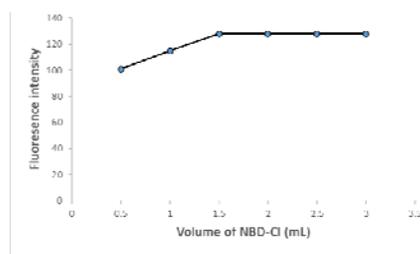


Figure 3: Influence of volume of NBD-Cl (0.1%) on fluorescence intensity

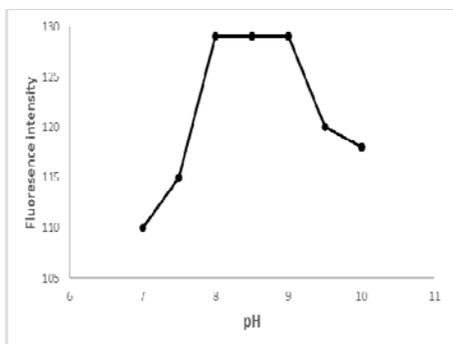


Figure 4: Effect of buffer pH on fluorescence intensity

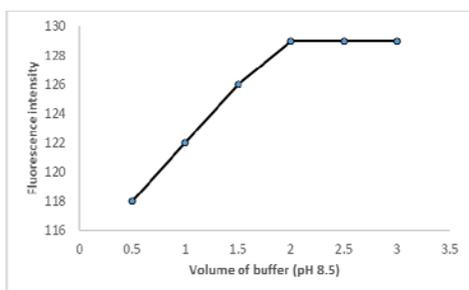


Figure 5: Effect of buffer volume on fluorescence intensity

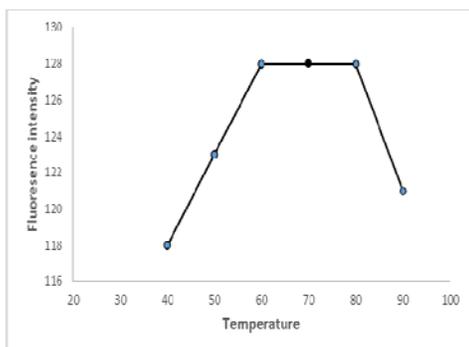


Figure 6: Effect of temperature on fluorescence intensity

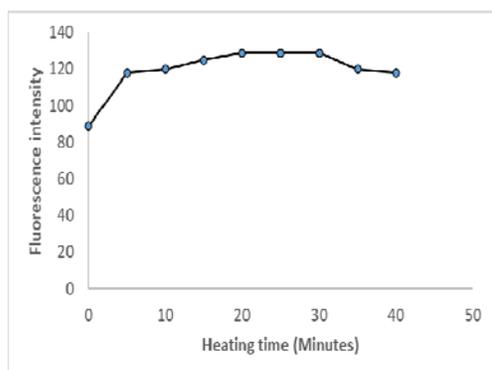


Figure 7: Effect of heating time on fluorescence intensity

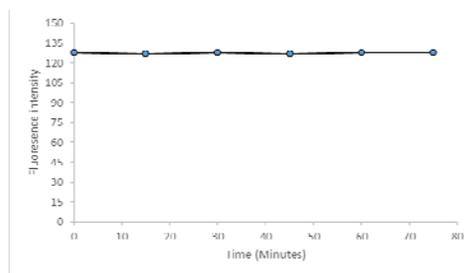


Figure 8: Effect of time on fluorescence intensity of the reaction product

A linear correlation was obtained; the regression equation is as shown in Eq 1.

$$I_f = 2.9182C - 0.80; r = 0.998 \dots\dots\dots (1)$$

where I_f is the fluorescence intensity of the reaction product at excitation λ of 390 nm and emission λ of 535nm, C is concentration (ng/mL), and r is correlation coefficient.

Accuracy and precision

Accuracy and precision were measured by analyzing 6 replicates at concentrations corresponding to HQC, MQC, LLOQ and LQC. The results are presented in Table 1.

Recovery

Recovery of PAM was evaluated by comparing 6 replications of 3 quality control specimens (LQC, MQC and HQC) with those of the corresponding un-extracted specimens of similar concentrations. As indicated in Table 2, the recovery showed acceptable results.

Robustness

Robustness was measured by determination of the influence of deliberate changes in assay conditions on the results. The findings are presented in Table 3. Deliberate changes in assay conditions had no significant effect on the results.

Stability

It was revealed that PAM was stable in human plasma for a minimum of 24 h at laboratory temperature. Moreover, freeze-thaw stability was tested for 3 freeze-thaw cycles. Long term stability was evaluated at -28°C for 30 days for the same quality control concentration levels. All data on the drug stability tests are shown Table 4.

Table 1: Accuracy and precision (inter-day and intra-day; n = 6)

Added concentration (ng/mL)		10 ng/mL (LLOQ)	20 ng/mL (LOQ)	50 ng/mL (MQC)	90 ng/mL (HQC)
Day 1	Mean	10.03	19.91	48.92	91.06
	SD*	0.17	0.21	0.31	0.26
	RSD (%) ^a	1.69	1.05	0.63	0.29
	Mean accuracy (%)	100.30	99.55	97.84	101.18
Day 2	Mean	9.95	20.08	49.21	89.74
	SD	0.21	0.42	0.17	0.42
	RSD (%)	2.11	2.09	0.35	0.47
	Mean accuracy (%)	99.50	100.40	98.42	99.71
Day 3	Mean	10.06	20.89	50.06	90.56
	SD	0.26	0.53	0.62	0.42
	RSD (%)	0.58	2.54	1.24	0.46
	Mean accuracy (%)	100.60	104.45	100.12	100.62

* Standard deviation; ^a Relative standard deviation (RSD, %)

Table 2: Extraction recovery from plasma samples

Nominal concentration (ng/mL)	Recovery (%)	RSD (%)
20 (LQC)	81.46	1.01
50 (MQC)	89.65	0.97
90 (HQC)	83.94	0.86

Table 3: Robustness of the proposed method

Concentration (ng/mL)		20 ng/mL (LOQ)	50 ng/mL (MQC)	90 ng/mL (HQC)
Change in buffer pH	Mean accuracy	100.69	100.89	101.18
	± SD (n=3)	±0.32	± 0.76	± 0.71
Temperature change	Mean accuracy	98.98	101.87	99.47
	± SD (n=3)	± 0.65	± 0.58	± 0.79
Volume of NBD-Cl	Mean accuracy	99.32	99.43	101.84
	± SD (n=3)	±0.84	± 0.64	± 0.59

Table 4: Stability data for PAM in human plasma

Storage condition	20 ng/mL (LOQ)	50 ng/mL (MQC)	90 ng/mL (HQC)
Bench-top stability	94.89 ± 0.89	96.64 ± 0.75	104.75 ± 0.99
Freeze-thaw stability (three cycles)	103 ± 0.85	97.36 ± 1.12	105.43 ± 0.91
Stability at -28°C (prolonged stability)	93.91 ± 1.07	95.55 ± 1.32	103.95 ± 1.24

Data are mean ± SD (%; n = 6)

DISCUSSION

All methods reported for the determination of PAM in biological samples utilize chromatographic techniques. Thus, there are no reported spectroscopic methods. Therefore, the

present study was aimed at developing a simple, sensitive and accurate procedure that can be applied for routine analysis of PAM in human plasma, and for monitoring of PAM in pharmacokinetic studies. The method was based on solid-phase extraction which can be considered a simple and environment-friendly technique for selective extraction of PAM from human plasma samples. The new procedure is robust and it has acceptable recovery without matrix interference. The results for recovery fell within acceptable range, and its linearity range was between 10 to 100 ng/mL, which is very suitable for the sensitive quantification of human plasma PAM (C_{max} of PAM was 9.7 $\mu\text{mol/L}$) [12]. Stability studies indicated very good bench-top, freeze-thaw and prolonged stability profiles. Moreover, the new method has the advantages of sensitivity, reliability and affordability, and it can be effectively applied in studies on pharmacokinetics.

CONCLUSION

The results obtained in this study indicate that the new procedure was effectively optimized to achieve maximum sensitivity. The studied drug was efficiently extracted from human plasma using solid-phase extraction method which is a simple and environmentally friendly technique. The developed procedure was subjected to validation as per US-FDA recommendation guidelines regarding linearity, accuracy, precision, recovery, robustness and stability. Published chromatographic methods for determination of PAM use fluorescence detector which is sufficiently sensitive. In contrast, although the new spectrofluorimetric method can determine plasma pamidronate with the same level of sensitivity, it does so in a simpler and more economical way. Thus, the developed method has the advantage of being suitable for

application in laboratories lacking fluorescence detector-coupled HPLC.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

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