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Optimization and validation of a high-performance liquid chromatography method for the determination of amlodipine in patients plasma after gastrectomy – pilot study

Optymalizacja i walidacja metody HPLC w celu oznaczenia amlodypiny w osoczu pacjentów po gastrektomii – badanie pilotażowe

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Abstract

Background. A sensitive, rapid and simple high-performance liquid chromatography (HPLC) method has been developed for the assay of amlodipine in human plasma, by off-line solid-phase extraction followed by HPLC coupled with ultraviolet Dual Absorbance Detector (UV). ***Material and methods.*** Separation of compounds was achieved on a Symmetry, C18 (250 mm × 4.6 mm, 5 μm) analytical column using a mobile phase consisting of 0.1 M ammonium octane in water/acetonitrile (40:60, v/v), at a flow rate of 1.0 ml/min, with diltiazem added as internal standard. Linear calibration curves in human plasma were generated over the range of 2-16 ng/ml with values for the coefficient of determination of 0.997. Amlodipine was detected by UV at 239 nm. Plasma (1000 μl) was diluted 1:1 with 2% phosphoric acid subjected to a solid-phase extraction on a C18 cartridge. After matrix components elimination, amlodipine was subsequently eluted with 1000 μl of methanol. The resulting eluate was evaporated under nitrogen at room temperature and the residue was reconstituted in mobile phase and a volume of 50 μl was injected onto the HPLC column. ***Results.*** The calibration curves are linear between 2.0 and 16.0 ng/ml. The method is precise with mean inter-day coefficients of variation (CV) within 5.4-10.6%, and accurate (range of inter-day deviations -9.5 to +8.5%). ***Conclusion.*** This method can be used in routine clinical practice to monitor plasma amlodipine concentrations in patients. *(Farm Współ 2018; 11: 3-7)*

Keywords: amlodipine, HPLC with ultraviolet detection (UV), validation

Streszczenie

Wstęp. Opracowano szybką i prostą metoda analityczna HPLC w nadfiolecie (UV) do oznaczania amlodypiny w osoczu ludzkim z wykorzystaniem ekstrakcji w fazie stałej. ***Materiał i Metody. R***ozdział przeprowadzono na kolumnie analitycznej Symmetry, C18 (250 mm x 4.6 mm, 5μm) wykorzystując fazę ruchomą w składzie 0.1 M wodny roztwór octanu amonu / acetonitryl w stosunku stechiometrycznym 40:60 v/v, przepływ 1ml/min, standard wewnętrzny diltiazem. Zakres krzywej wzorcowej obejmował 2-16 ng/ml, współczynnik korelacji r = 0.997. Amlodypinę oznaczano przy 239 nm. Osocze (1000 μl) rozcieńczono w stosunku stechiometrycznym 1 :1 za pomocą 2% kwasu orto-fosforowego i ekstrahowano w fazie stałej na kolumnach C18. Po rozdzieleniu składników matrycy, amlodypinę wymywano za pomocą metanolu 1000 μl. Po odparowaniu w strumieniu azotu suchą pozostałość rozpuszczono w fazie ruchomej. Objętość nastrzyku wynosiła 50 μl. ***Wyniki.*** Krzywa kalibracyjna liniowa w zakresie stężeń 2-16 ng/ml. Metoda analityczna precyzyjna (współczynnik zmienności CV dla inter-day w zakresie 5.4-10.6%) i dokładna (inter-day -9.5 – +8.5%). ***Wnioski.*** Opracowana metoda może być wykorzystana w laboratoriach klinicznych do monitorowania stężenia amlodypiny u pacjentów. *(Farm Współ 2018; 11: 3-7)*

Słowa kluczowe: amlodypina, HPLC z detekcją w nadfiolecie (UV), walidacja

Introduction

Amlodipine (AML; 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyle-6-methyl-1,4-dihydropyridine) is a calcium-channel blockers that is prescribed for the treatment of high blood pressure [1]. Based on its mechanism of action, amlodipine inhibits the movement of calcium ions into cardiac and vascular smooth muscles. This is a lipophilic molecule, whose absorption occurs in stages and in junctional area of the digestive tract between the stomach and small intestine [2]. After oral administration the bioavailability of amlodipine is 60 to 65% and plasma concentrations rise gradually to peak 6 to 8h after administration. AML is a substrate of cytochrome P450 (CYP), metabolized by CYP3A4 [3]. The elimination half-live in humans ranges from 35 to 45 hr due to a large volume of distribution (21 l/kg), > 95% being bound to plasma proteins [4].

Several methods have been published for the determination of AML, such as HPLC with UV [5-8] or fluorescence detection FL [9], liquid chromatography – tandem mass spectrometry LC-MS/MS [10]. This study intended to develop and validate a new HPLC-UV method for the determination of AML in plasma by liquid chromatography coupled with UV spectroscopy as per European Medicines Agency (EMEA's) bioanalytical guidelines. Subsequently, the validated method was applied to clinical pharmacokinetic study.

The study was conducted at the Department of Clinical Pharmacy and Biopharmacy of the Poznan University of Medical Sciences in Poznan, Poland and at the Oncological and General Surgery Department I of the Wielkopolska Cancer Center in Poznan.

Material and methods

Reagents

Amlodipine, diltiazem (Internal Standard, IS), ammonium octane, acetonitrile were purchased from Sigma-Aldrich, methanol from Merck and orthophosphoric acid from Fluka. All reagents were HPLC grade.

Water used in the mobile phase was deionized, distilled and filtered through a Millipore system before use. Aldan® 5 was purchased (batch number: P177H) from Polfarmex S.A., Kutno, Poland.

Chromatographic assay method

The measurement of amlodipine concentrations in the blood plasma was made by means of the HPLC method with UV detection, which was a modification of the method developed by Zarghi A *et al.* [11].The HPLC system (Alliance, model 2695; Waters Corporation, Milford, MA, USA) was used with a Dual Absorbance Detector (model 2487; Waters Corporation) and a data acquisition and processing module (Empower Pro Software No 1154; Waters Corporation). Separation was achieved by isocratic elution of the mobile phase, 0.1 M ammonium octane in water – acetonitrile (40 : 60, v/v), at a flow rate of 1.0 ml/min through a Symmetry C18 column (250 mm × 4.6 mm, 5 μm particle size) (Waters). The column temperature was maintained at 25°C, and the UV detection wavelength was set at 239 nm. The injection volume was 50 μl. The total analysis time for each run was 7 min.

Standard preparation

A stock solution of amlodipine was prepared from accurately weighed (1 mg) pure powder dissolved in acetonitrile. The solution was kept at 4°C in the dark. Working standard solutions were prepared by appropriate dilutions of the stock solution in acetonitrile to obtain concentrations across the range of 2-16 ng/ml. Quality control (QC) samples were prepared freshly on each day of the experiment. The IS stock solution at 10 ng/ml was diluted with acetonitrile prior use.

Calibration curve

The calibration curve was constructed from plots of peak area *versus* concentration. The linearity of the method was evaluated at six amlodipine concentrations varying from 2 ng/ml to 16 ng/ml (three-fold injections).

Intra- and inter-day precision and accuracy

To evaluate the intra- and inter-day accuracy and precision the QCs at four concentrations were prepared and determined by quantitating five replicates on the same day and on 3 consecutive days. Table I shows intra- and inter-day precision (CV%) and accuracy of this assay method. The precision of the method at each concentration (CV) was calculated as the relative standard deviation (SD) of the mean by means of the following equation:

$$CV = (SD/mean) \times 100.$$

Accuracy was measured as the percentage difference from the theoretical calculation, according to the equation:

$$Bias\ (\%) = (concentration_{measured} - concentration_{theoretical})/(concentration_{theoretical}) \times 100\%.$$

Sample preparation

16 µl of IS and 0.1 ml of standard solution amlodipine were added to 0.384 ml of plasma (QC), (16 µl of IS were added to 0.484 ml of plasma sample). After 10 s of mixing 0.5 ml 4% orthophosphoric acid was added, mixed and submitted to the solid phase extraction (SPE, Oasis HLB, Waters) on the reversed-phase (C18) cartridges (1 ml, 30 mg) under the following steps:

1) *Conditionation*: 1 ml of methanol followed with 1ml of water was drown slowly through the column under taking care for not to let the column dry
2) *Sample application*: mixture of plasma and IS were applied on the column
3) *Washing*: 1 ml of 5% methanol solution (about 10 mmHg vacuum)
4) *Elution*: The analytes were eluted by application of 1ml of methanol solution (99.8%), the eluate was collected into the glass tube and evaporated at 45°C under the stream of nitrogen. The residue was reconstituted in 0.5 ml of acetonitrile. The resulting solutions were carefully vortexed and centrifuged at 2886 g for 10 min. Aliquots of the supernatants were introduced into 150 µl HPLC microvials and volume of 50 µl was injected onto the HPLC column.

Patients samples

Blood plasma concentration of amlodipine was determined in 2 patients (1 men and 1 woman) after partial (n = 1) or complete (n = 1) gastric resection due to gastric cancer. The patients had received amlodipine (*Aldan*®) at a dose of either 5. To determine the steady state concentration of amlodipine, venous blood (2 ml) was collected in the 9th 24-hour period of treatment, in the 6, 8, 10, or 24th hr after receiving that day's dose. The blood samples were transferred into heparinized tubes and they were centrifuged at 2880 g for 10 min at 4°C. Next the plasma was transferred to propylene tubes and stored at -20°C until analysis. All patients were informed and approved the protocol and the sampling in compliance with the ethical principles originating from the revised Declaration of Helsinki and according to French regulations.

Results and discussion

The specificity was assessed in plasma samples from patients, however the selectivity of amlodipine in the presence of other drugs was not analyzed.

The calibration curve of the peak area *versus* concentration was found to be linear over the evaluated range of 2-16 ng/ml in acetonitrile. The mean calibration equations was $y = 5.09 \cdot 10^{-2}x - 8.17 \cdot 10^{-2}$. The linear regression coefficient in plasma was $r^2 = 0.997 \pm 0.025$. Intra- and inter-day precision and accuracy of the low quality control (LQC) 2.0 ng/ml, medium quality control (MQC) 4 ng/ml, and high quality control (HQC) 10 ng/ml were well within the acceptable limit of 15% CV%. The results of intra-day precision and accuracy ($n = 5$) are summarised in Table I. Intra-day precision and inter-day precision were acceptable with all CVs less than 10.6%. The intra-day accuracy and inter-day accuracy were also acceptable with the range of 90.5-108.5%. The CVs, demonstrated in Table I were acceptable for the measurement of amlodipine. Therefore this method can be used in the clinical laboratory for conventional analytical application due to the simple solid – phase extraction procedure and accurate method for determination of AML in patients'

Table I. Intra- and inter-day accuracy and precision of amlodipine ($n = 5$)
Tabela I. Precyzja i dokładność amlodypiny w jednej serii i między seriami (n = 5)

Concentration (ng/ml)	Mean ± SD (ng/ml)	Accuracy (bias %)	Precision (CV%)
Intra-day			
2.0	1.99 ± 0.11	-0.5	5.5
4.0	3.74 ± 0.26	-6.5	6.9
10.0	9.62 ± 1.01	-3.8	10.5
Intra-day			
2.0	2.17 ± 0.23	8.5	10.6
4.0	3.97 ± 0.34	-0.8	8.6
10.0	9.05 ± 0.49	-9.5	5.4

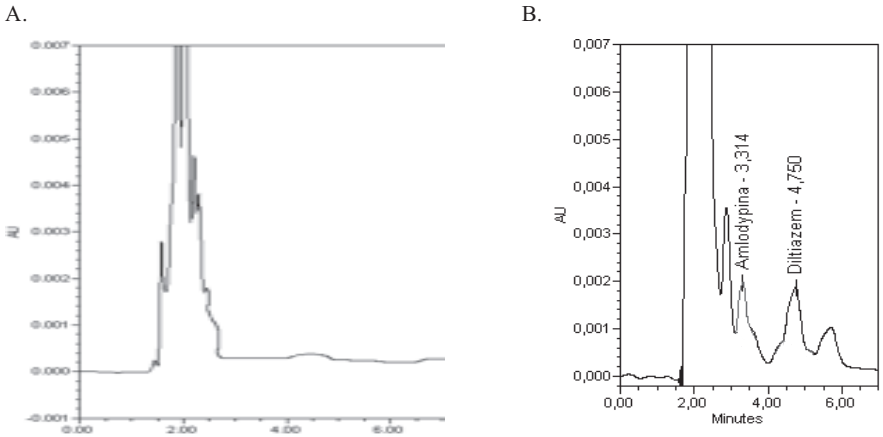

Figure 1. The chromatogram of amlodipine: a drug-free plasma (A), patient's plasma (B)
Rycina 1. Chromatogramy amlodypiny: próbka ślepa (A), próbka osocza krwi pacjenta (B).

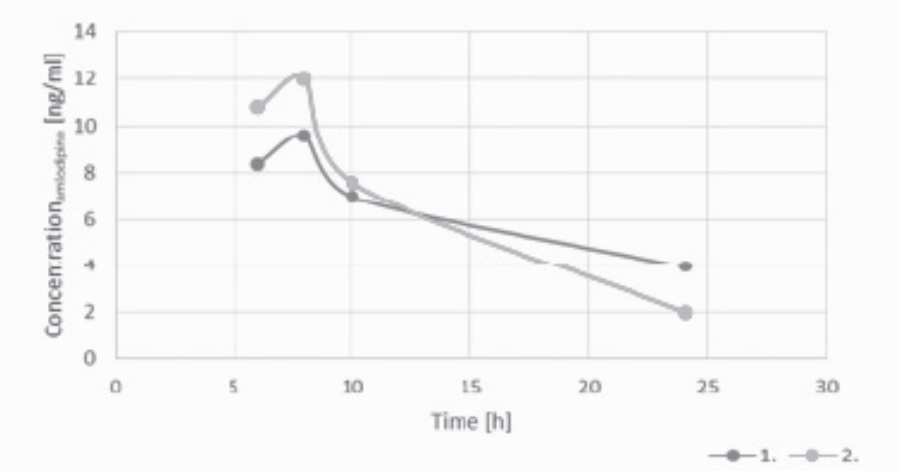

Figure 2. Plasma concentration (at steady state) *vs*. time plot of amlodipine from patients treated with 5 mg AML once daily (1. – first patient, 2. – second patient)
Rycina 2. Wykres zależności stężenia amlodypiny w funkcji czasu (w stanie stacjonarnym), pacjentów leczonych amlodypiną *p.o.* w dawce 5 mg/24 h (1. – pierwszy pacjent, 2. – drugi pacjent)

plasma. This analytical method can be used in clinical laboratory settings for the rapid analysis of biological samples due to simple extraction procedure and good sensitivity. Figure 1 A, B shows typical chromatograms obtained from a drug-free plasma (A) and a plasma sample from patient after oral administration 5 mg of amlodipine (B). The chromatograms show that the separation from matrix constituents is sufficient for reliable quantitation and no endogenous components interfered with the analyte peak. Amlodipine peak was detected with retention time 3.309 ± 0.029 min. Figure 2 shows plasma concentration-time profiles for amlodipine from 2 patients.

Conclusion

A simple, highly sensitive and specific new HPLC-UV method was developed for the determination of AML in human plasma. This technique utilized a solid-phase extraction step. The precision and accuracy of our HPLC assay is adequate for phar-

macokinetic studies. This method may contribute to the AML monitoring in hospital laboratories not having LC-MS/MS.

Conflict of interest
None

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References

1. Choi Y, Lee S, Cho SM, et al. Comparisons of the pharmacokinetics and tolerability of fixed-dose combinations of amlodipine besylate/losartan and amlodipine camsylate/losartan in healthy subjects: a randomized, open-label, single-dose, two-period, two-sequence crossover study. Drug Des Devel Ther. 2016;10:3021-8.
2. Meredith PA, Elliott HL. Clinical Pharmacokinetics of Amlodipine. Clin Pharmacokinet. 1992;22:22-31.
3. Wang R, Zhang H, Sun S, et al. Effect of Ginkgo Leaf Tablets on the Pharmacokinetics of Amlodipine in Rats. Eur J Drug Metab Pharmacokinet. 2016;41(6):825-33.
4. Khodadoustan S, Nasri Ashrafi I, et al. Evaluation of the effect of time dependent dosing on pharmacokinetic and pharmacodynamics of amlodipine in normotensive and hypertensive human subjects. Clin Exp Hypertens. 2017;39(6):520-6.
5. Talele GS, Porwal PK. Development of Validated Bioanalytical HPLC-UV Method for Simultaneous Estimation of Amlodipine and Atorvastatin in Rat Plasma. Indian J Pharm Sci. 2015;77(6):742-50.
6. Sultan F, Shoaib MH, Yousuf RI, et al. Simultaneous quantitation of aspirin, amlodipine and simvastatin in a fixed dose combination of encapsulated tablet formulation by HPLC-UV method. Pak J Pharm Sci. 2014 Sep;27(5 Spec no):1553-8.
7. Chaudhari BG, Patel NM, Shah PB. Stability indicating RP-HPLC method for simultaneous determination of atorvastatin and amlodipine from their combination drug products. Chem Pharm Bull (Tokyo). 2007;55(2):241-6.
8. Naidu KR, Kale UN, Shingare MS. Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benazepril hydrochloride from their combination drug product. J Pharm Biomed Anal. 2005;39(1-2):147-55.
9. Moussa BA, El-Zaher AA, Mahrouse MA, et al. Simultaneous determination of amlodipine besylate and atorvastatin calcium in binary mixture by spectrofluorimetry and HPLC coupled with fluorescence detection. Anal Chem Insights. 2013;8:107-15.
10. Wang B, Sheng L, Li Y. Simultaneous determination of telmisartan and amlodipine in dog plasma by LC-MS-MS. J Chromatogr Sci. 2015;53(10):1708-13.
11. Zarghi A, Foroutan SM, Shafaati A, et al. Validated HPLC method for determination of amlodipine in human plasma and its application to pharmacokinetic studies. Farmaco 2005; 60:789-92.