Simultaneous Determination of Dofetilide and Amlodipine in Plasma by HPLC

Nisrin Kaddar1,2, Sylvie Pilote1, Simon Wong1,2, Bertrand Caillier1,2, Dany Patoine1, Benoit Drolet1,2 and Chantale Simard1,2*

1University Institute of Cardiology and Pneumology Quebec, Research Centre, 2725, Chemin Sainte-Foy, Quebec QC, G1V 4G5, Canada
2Faculty de pharmacie, Pavillon Ferdinand-Vandry, 1050, Avenue of Medicine, Laval University, Quebec QC, G1V 0A6, Canada

Abstract

Dofetilide is a Class III antiarrhythmic drug useful in the management of atrial fibrillation but also known to prolong the QT interval on the ECG and to induce malignant ventricular tachyarrhythmias such as torsades de pointes (TDP). Drugs with calcium channel-blocking properties contribute to decrease the incidence of TDP. Amlodipine is a dihydropyridine calcium channel antagonist. Considering the potentially safe and useful combined use of dofetilide and amlodipine, we undertook drug biotransformation studies. To perform studies aiming at evaluating the possible pharmacokinetic interaction between these drugs, a unique sensitive HPLC assay able to quantify both drugs simultaneously was required. A sensitive and specific HPLC method using a tandem of UV/fluorescence detection was described for the analysis of amlodipine and dofetilide in plasma. The within-day and between-day precision studies showed good reproducibility with coefficients of variation less than 10% for all the analytes. The limits of detection were 0.5 ng/mL and 0.25 ng/mL and the limit of quantification were 1.7 ng/mL and 0.8 ng/mL for dofetilide and amlodipine respectively. This new method could be of great value in many applications such as in vitro and in vivo pharmacokinetic and drug-drug interactions studies, as well as therapeutic drug monitoring.

Keywords: High performance liquid chromatography; Tandem UV/fluorescence detection; Amlodipine; Dofetilide

Abbreviations: APD: Action Potential Duration; CV: Coefficient of Variation; EAD: Early after Depolarization; EC50: 50% Effective Concentration; ICa,L: Rapid Component of the Delayed Rectifier Cardiac Potassium Current; IS: Internal Standard; LOD: Limit of Detection; LOQ: Limit of Quantification; Qc: Concentrations of Qualifiers; RSD: Relative Standard Deviation; TDP: Torsades De Pointes

Introduction

Dofetilide (N-[4-[2-[methyl[2-[4-(methylsulfonyl)amino]phenoxy]ethyl][amino][ethyl][phenyl]-methanesulfonamide) is a Class III antiarrhythmic drug (TIKOSYN®) that selectively inhibits the rapid component of the delayed rectifier cardiac potassium current (Ikr) with high potency (50% effective concentration (EC50) in the nanomolar range) [1-3]. Dofetilide is considered to be a good adjunct to catheter-based ablation and alternative pharmacological approach for the treatment of atrial arrhythmias [4]. Dofetilide’s chemical structure is shown in Figure 1.

Dofetilide prolongs the refractory period and action potential duration (APD) without having sodium channel-blocking effect, beta-blocking action or calcium channel-blocking activities [5,6]. Excessive prolongation of APD, an extension of dofetilide’s pharmacological effect, presumably results from an abnormal increase of cytosolic Ca2+ concentration ([Ca2+]i) [7] and initiation of torsades de pointes (TDP), a malignant polymorphic ventricular tachycardia predisposing to ventricular fibrillation and sudden death [8,9]. Dofetilide is known to prolong the QT interval on the ECG and to induce malignant ventricular tachyarrhythmias including TDP [6]. QT interval prolongation is directly related to the dose and plasma concentration of the drug [7]. Huang et al. [10], showed that CPU228, a derivative of dofetilide with calcium (Ca2+) antagonist properties, is able to block (ICa,L), contributing to decreased TDP while leaving Ikr blockade unaffected. Blockade of both Ikr and ICa,L contributes to the major characteristics of complex Class III agents, such as amiodarone, dronedarone and azimilide [11-13]. All these agents cause a lower incidence of TDP and are more potent at suppressing ventricular arrhythmias. Along those lines, our laboratory has recently shown that amlodipine, a calcium channel blocker, reduces the monophasic APD-prolonging effect of dofetilide [14], suggesting a potential reduction of its proarrhythmic properties and the possible rationale for using both drugs simultaneously.

Amlodipine besylate (NORVASC®) is chemically described as 3-ethyl-5-methyl (4-[2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylatemonobenzenesulphonate. Its structure is shown in Figure 2. Amlodipines a dihydropyridine calcium antagonist that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle [15,16].

Keeping in mind the potentially useful and safe combined use of dofetilide and amlodipine, we undertook biotransformation studies to test if there is any significant drug interaction between these two drugs.

*Corresponding author: Chantale Simard, University Institute of Cardiology and Pneumology Quebec, Research Centre, 2725, Chemin Sainte-Foy, Quebec QC, G1V 4G5, Canada, Tel: 418-656-8711 ext. 3072; Fax: 418-656-4508; E-mail: chantale.simard@pha.ulaval.ca

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It has been shown that the majority of adverse drug-drug interactions, which have a pharmacokinetic origin, can be understood in terms of alteration of cytochrome P450-catalyzed reactions [17]. The human CYP3A subfamily plays a dominant role in the metabolic elimination of more than 50% of commercially available drugs [17-19]. The CYP3A4 enzyme is expressed in the liver and small intestine and contributes to first-pass effect [18,20]. The major metabolic route of dofetilide (N-demethylation) is mediated by CYP3A4 in human liver microsomal preparations [21]. Moreover, it has been shown in vivo and in vitro that amloidine can be an inhibitor of CYP3A4 [22,23]. In order to study the possible pharmacokinetic interaction between dofetilide and amloidine, a unique sensitive HPLC assay that is able to quantify both drugs was then required.

Several analytical methods for quantifying dofetilide (LCMS [24], HPLC [21,25]) and RIA [26] or amloidine (HPLC [9,27-31], HPLC with radioactivity detection [32], HPLC with electrochemical detection [33], LCMS [34-36], GCMS [37] and GC with electron capture detection [38] in biological fluids have been reported. Unfortunately, all of these described methods do not allow the quantification of both drugs in the same run. In contrast, our work described a rapid and sensitive HPLC method, which enables to determine plasma concentrations of dofetilide and amloidine simultaneously, using fluorescence detection.

**Experimental**

Experiments were performed in accordance with our institutional guidelines on animal use in research. Animals were housed and maintained in compliance with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

**Chemicals and reagents**

Dofetilide and amloidine besylate were kindly provided by Pfizer (Groton, CT, USA). Nortryptiline, used as internal standard (IS), phosphoric acid, dimethylsulfoxide (DMSO), triethylamine, and ammonium phosphate 20 mM, 1-heptane-sulfonate 5 mM filtered with methanol for activation and 1 mL of H2O for equilibration. Then, the cartridges were washed with 1 mL (H 2O:NH4OH, 95:5). After low vacuum application (<300 mmHg), samples were eluted with 1 mL (methanol: formic acid, 98:2) and evaporated to dryness under light stream of nitrogen at 37°C. The residue was reconstituted in 150 µL of buffer A of the mobile phase and centrifuged at 4000 rpm for 10 min at 4°C. An aliquot (50 µL) of the supernatant was injected into the HPLC system.

**Instrumentation**

Apparatus and chromatographic conditions: The HPLC was performed at room temperature with a complete Prominance Shimadzu system (Columbia, MD, USA) consisting of an automatic sample injector model SIL-20ACHT, a pump model LC-20AT, a fluorescence detector models RF-10Ax1 and an ultraviolet (UV) detector model SPD-20A. The software EZ-Start (Columbia, MD, USA) was used for acquisition, storage and analysis of data. UtraspHERE ODS 5 µ column, 250 mm×4.6 mm (Phenomenex, Torrance, CA, USA) and Bondapak C18 guard column (Waters, Mississauga, ON, Canada) were used to perform the separation.

Chromatographic separation was achieved at room temperature and was based on a method previously described [31]. The modified mobile phase consisted of buffer A: methanol/ammonium phosphate 20 mM, 1-heptane-sulfonate 5 mM filtered through 0.45 µm filter/acetoniitrile (19/70/11) containing 0.08% (wt/vol) triethylamine and adjusted to pH 7.1 with phosphoric acid and buffer B: methanol/ammonium phosphate 20 mM, 1-heptane-sulfonate 5 mM filtered through 0.45 µm filter/acetoniitrile (38/42/20) containing 0.08% (wt/vol) triethylamine and adjusted to pH 7.1 with phosphoric acid. The buffers were degassed by ultrasonication before use. A flow-rate of 1.1 mL/min was imposed. The elution gradient used was as follows: 0-15 min, 0-100% buffer B; 15-35 min, 100% buffer B; 35-38 min, 100-0% buffer B; 38-43 min 100% buffer A. The peaks of dofetilide and amloidine were monitored in fluorescence at wavelengths determined by scanning spectrophluorimeter: 228 nm excitation/325 nm emission and 366 nm excitation/457 nm emission respectively. To optimize the detection, the following program of wavelengths monitoring was used: 0-8 min, 228 nm excitation/325 nm emission, sensitivity magnification X1; 8-16 min, 228 nm excitation/325 nm emission, sensitivity magnification X2; 16-43 min, 366 nm excitation/457 nm emission, sensitivity magnification X5. The peak of internal standard was monitored in UV at 238 nm.
Recovery and matrix effect

Extraction recoveries were determined for three Qc points of dofetilide and amlodipine from the ratio of the analyte peak height in samples spiked before extraction compared to the corresponding peak height in untreated samples prepared in neat solution. Briefly, 10 µL of standard solutions of dofetilide (10, 100, 500 ng) and amlodipine (25, 500, 1000 ng) were added to two sets of three tubes containing the IS (50 µL). To the first set, 400 µL of guinea pig plasma were added, vortexed and extracted according to the sample procedures described above. The second set was adjusted to the same volume with mobile phase. After injection of 40 µL into the HPLC system, the heights of the peaks of the unextracted and extracted samples were compared. The extraction efficiency was calculated as the percent difference between the mean observed concentration and the nominal concentration. The recovery was also evaluated in sample containing 1000 µL of plasma.

Stability

Stability of the analytes in plasma was determined using triplicates samples at three concentrations (10, 50, 100 ng/mL for dofetilide and 25, 100, 500 ng/mL for amlodipine) which were analyzed immediately after preparation and after the applied storage conditions. Three storage conditions were assessed: short-term storage at room temperature (4 and 24 hours), long-term storage at 4°C and -20°C (over 5 weeks). The autosampler stability of the extract was determined by analyzing the samples after 24 h of storage in the autosampler (set at +15°C). The concentrations of the samples were calculated against a calibration curve obtained from freshly prepared calibration standards.

Recovery

Recoveries of dofetilide, amlodipine and nortryptiline were determined by analyzing 5 blank plasma samples from three different species to evaluate possible endogenous interference at the retention time of the analytes and of the internal standard. The sample preparation and chromatographic conditions for detection (box) and buffer gradient (dotted line) were optimized to guarantee that no interference incurred at the retention times of the tested compounds. The specificity was also demonstrated in another matrix by analyzing the supernatant of plasma materials were detected. The run time of 43 min allowed a good separation of the three peaks with complete return to baseline.

Chromatographic analysis

A representative chromatogram of a sample of guinea pig plasma containing dofetilide, amlodipine and the IS is shown in Figure 3. The average retention times were 13.5, 29 and 32 min for dofetilide, amlodipine and the IS (nortryptiline), respectively. Under the chromatographic conditions described, no endogenous interfering plasma materials were detected. The run time of 43 min allowed a good separation of the three peaks with complete return to baseline.

Recovery

Recoveries of dofetilide, amlodipine and nortryptiline were determined by comparing the peak heights of the solid-phase extracted standards from plasma and those from the unextracted samples. The percent recovery of the method was found to be 95.8 ± 3.0% for

![Figure 3: Typical chromatograms of a blank plasma spiked with 5 ng dofetilide, 10 ng amlodipine and internal standard (plain line) and a blank plasma spiked with 50 ng dofetilide, 75 ng amlodipine and internal standard (dash line) (upper chromatogram) and a control blank plasma (lower chromatogram). The chromatographic conditions for detection (box) and buffer gradient (dotted line) were pointed out.](image-url)
Additionally, concentrations of analytes and internal standard were stable in processed (extracted) plasma samples at +15°C for 24 hours.

No statistically significant degradation of dofetilide and amlodipine was observed in stock and working solutions stored at −20°C for 8 weeks.

**Linearity and sensitivity:** Five series of eight-point calibration curves (prepared on five different days) were constructed for both drugs over the range of 5 to 500 ng for dofetilide and 10 to 1000 ng for amlodipine. Equations were determined by least-squares linear regression analyses. The mean equation were $y = 0.0060x ± 0.0003 (r²=0.999 ± 0.001)$ for the determination of dofetilide and $y = 0.0017x ± 0.0002 (r²=0.998 ± 0.001)$ for the determination of amlodipine. The relative standard deviation values of the slope were 5.2% for dofetilide and 12.7% for amlodipine. For each calibration curves, the intercept was not significantly different from zero ($\chi^2<3.84$). The method was linear across the whole range of concentrations allowing the possibility to extend the method to different purposes.

**Accuracy and precision:** The limits of detection (LOD) defined for dofetilide and amlodipine were 0.5 ng/mL and 0.25 ng/mL and the limits of quantification (LOQ) were 1.7 ng/mL and 0.8 ng/mL for dofetilide and amlodipine respectively.

Intraday and interday expressed as a coefficient of variation were less than 5.4% and 10.1% for dofetilide and amlodipine respectively. For both intraday and interday, the mean accuracy value was 99.0 ± 4.9% for dofetilide and 95.4 ± 9.7 for amlodipine (Tables 2 and 3).

**In vivo pharmacokinetic application**

Typical chromatogram of guinea pig plasma after a single oral gavage of dofetilide 0.1 mg/kg and amlodipine 0.5 mg/kg is shown in Figure 4. It shows the measured concentrations of both dofetilide and amlodipine at three different sampling times in three different guinea pigs after a single oral gavage dose of dofetilide 0.1 mg/kg and amlodipine 0.5 mg/kg. These in vivo data are validating the method described in the present paper and confirming its usefulness for pre-clinical and eventually, clinical purposes.

**Discussion**

We developed a new HPLC method with tandem fluorescence/UV detection, allowing the simultaneous quantification of two structurally different drugs (dofetilide and amlodipine) in plasma. The validity and usefulness of this new method was further confirmed by in vivo animal experiments.

Other more sensitive methods use GC and LC-MS techniques.
The thermal instability of the drugs under GC conditions represents a serious concern. The present HPLC assay has the advantage of eliminating the risk of thermal degradation of drugs. Methods using MS are very sensitive and have low LOQ. However, these methods are not available for most laboratories due to cost and equipment/expertise requirements.

As previously mentioned, our laboratory showed that amlodipine, a calcium channel blocker, reduces the cardiac action potential-prolonging effect of dofetilide [14], suggesting a potential reduction of its proarrhythmic properties, thus suggesting a rationale for using these two drugs simultaneously. This HPLC method was therefore developed in order to further study ex vivo and in vivo drug-drug interaction between dofetilide and amlodipine in guinea pigs. Due to the limited plasma volume in the guinea pig, we developed this method requiring only 400 µL of plasma. The method was not affected by increasing plasma volumes up to 1 mL. The previously reported methods for quantifying amlodipine and dofetilide often require elaborated extraction procedures and/or provide low compound recovery [25,28,31,33]. In contrast, our extraction procedure requires minimal sample preparation and allows a recovery >75% of amlodipine, dofetilide and nortryptiline from the same sample.

The present method was based on previous results described by Walker et al. and Yeung et al. [25,31] to quantify dofetilide and amlodipine respectively. Walker et al. [25] had a LOD of 2.5 ng/mL for dofetilide and Yeung et al. [31] had a LOQ of 2.5 ng/mL for amlodipine. Our method offers better results with a LOD of 0.5 ng/mL and 0.25 ng/mL and LOQ of 1.7 ng/mL and 0.8 ng/mL for dofetilide and amlodipine respectively.

Bahrami and Mirzaeei [30] evaluated a HPLC method for the determination of amlodipine in human and its use in a pharmacokinetics study. This method was applied to the determination of the drug in plasma following a single amlodipine oral dose of 10 mg in 12 healthy volunteers. The LOD is 0.1 ng/mL and the LOQ is 0.25 ng/mL. With similar detection capabilities for amlodipine, we suggest our method could also be used for pharmacokinetics studies. Moreover, following repeated oral doses of amlodipine (10 mg once daily), Stangier and Su [40], showed that the C_{min} is 17.7 µg/mL and the C_{max} is 10 µg/mL. On the other hand, following an oral twice daily dosing of dofetilide (500 µg BID), Allen et al. [41] observed a C_{max} of 3.8 ng/mL and a C_{min} of 1.24 ng/mL. Our method has a LOD of 0.25 ng/mL and a LOQ of 0.8ng/mL. We are therefore confident that our method could be used for pharmacokinetic interaction studies following coadministration of repeated doses of amlodipine and dofetilide in humans.

This newly developed assay is selective, presents a simple drug extraction procedure, requires a reduced plasma volume and allows the simultaneous detection and quantification of both dofetilide and amlodipine with better LOD and LOQ for both drugs than the previously described methods. Moreover, it is readily accessible without the use of more sophisticated analytical techniques requiring costly GC or MS installations.

As we demonstrated ex vivo in guinea pig hearts, the coadministration of both drugs could be beneficial in reducing the cardiac proarrhythmic potential of dofetilide [14]. Therefore, it could be very interesting to conduct a study in humans to confirm these exciting results. This newly developed high recovery HPLC method will allow the routine analysis of biological samples in pharmacokinetic studies involving the coadministration of dofetilide and amlodipine in humans.

Conclusion

In summary, the herein described assay is the first HPLC method that permits the determination of dofetilide and amlodipine in the same sample. This HPLC method provides a quick, simple, reproducible, and sensitive assay for the detection of both drugs in plasma. This method could be valuable in many applications; in vitro and in vivo pharmacokinetic and drug-drug interactions studies, as well as therapeutic drug monitoring.

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Page 6 of 6


