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First liquid chromatographic method for the simultaneous determination of amiodarone and desethylamiodarone in human plasma using microextraction by packed sorbent (MEPS) as sample preparation procedure

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ABSTRACT

For the first time a simple and fast high-performance liquid chromatography (HPLC) method using a novel sample preparation procedure based on microextraction by packed sorbent (MEPS) was developed and validated for the determination of amiodarone (AM) and its main metabolite desethylamiodarone (DEA) in human plasma. Chromatographic separation of the analytes (AM and DEA) and tamoxifen, used as internal standard (IS), was achieved within less than 5 min on a LiChroCART Purospher® Star C_{18} column (55 mm × 4 mm, 3 μm). The mobile phase consisting of 50 mM phosphate buffer with 0.1% formic acid (pH 3.1)/methanol/acetonitrile (45:5:50, v/v/v) was pumped isocratically at a flow rate of 1.2 mL/min. The detection was carried out at 254 nm. Calibration curves were linear ($r^2 \geq 0.9976$) in the ranges of 0.1–10 μg/mL for AM and DEA. The limits of quantification were established at 0.1 μg/mL for AM and DEA. The overall imprecision did not exceed 6.67% and inaccuracy was within ±9.84%. The overall mean recovery of AM and DEA ranged from 58.6% to 68.2%. Neither endogenous nor tested exogenous compounds were found to interfere at retention times of the analytes (AM and DEA) and IS. This new MEPS/HPLC method was also applied to real samples obtained from polymedicated patients receiving AM therapy. Thus, this bioanalytical method seems to be a useful tool for therapeutic drug monitoring of patients under AM treatment and also to support other clinical pharmacokinetic-based studies involving this drug, such as bioavailability/bioequivalence studies.

1. Introduction

Amiodarone [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxybenzoyl)-benzofuran; AM] (Fig. 1) is one of the most frequently prescribed antiarrhythmic drugs despite the availability of novel antiarrhythmic agents [1,2]. Nevertheless, the unusual pharmacokinetic properties of AM and its primary metabolite desethylamiodarone (DEA; Fig. 1) complicate the clinical use of the drug. Actually, the high lipid solubility of AM and DEA leads to an extensive and variable accumulation of both compounds in different tissues, resulting in a marked delay in onset of pharmacological action and in a number of safety concerns particularly after several weeks to months of AM therapy [1,3–5]. Therefore, the long-term use of AM is limited by serious or even life-threatening adverse events, such as thyroid dysfunction, pulmonary toxicity and hepatic toxicity [1,6,7].

Indeed, AM has long been recognised as a drug having a narrow therapeutic window (0.5–2.0 μg/mL) [8–12]. Several studies have reported that serious toxicity is more likely at AM serum/plasma concentrations above 2.5 μg/mL [13,14]; Rotmensch et al. [15] also concluded that AM serum concentrations below 2.5 μg/mL significantly improve AM's benefit-to-risk relationship. Furthermore, the therapeutic drug monitoring (TDM) of AM plasma concentrations may differentiate treatment failure from suboptimal dosing and may reduce the incidence of concentration-related adverse effects [13]. On the other hand, the monitoring of AM and DEA plasma concentrations may be of particular interest to compare different routes of administration [16] and to assess the impact of the switching in drug formulation (innovator *versus* generic medicine)

Abbreviations: AM, amiodarone; DEA, desethylamiodarone; HPLC, high-performance liquid chromatography; IS, internal standard; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantification; MEPS, microextraction by packed sorbent; PP, protein precipitation; SPE, solid-phase extraction; TAM, tamoxifen; TDM, therapeutic drug monitoring.

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[17]. Therefore, AM can be used more safely when the clinician is aware of the concentrations the patient is exposed to and adjusts the dose as necessary. For that, the availability of a fast, sufficiently sensitive, and selective bioanalytical method to enable the reliable determination of AM and its pharmacologically active metabolite (DEA) is required.

Until now, a lot of bioanalytical methods have been reported in literature for the determination of AM and DEA in human plasma and other biological fluids. However, in those methods the sample preparation has been carried out by means of liquid–liquid extraction (LLE) [10,18–26], solid-phase extraction (SPE) [27–30], protein precipitation (PP) [31–33] and combining different procedures such as PP and LLE [34,35].

Nevertheless, the recent developments in the field of sample preparation have been directed toward miniaturization and automation, and one of the latest developments was the emergence of microextraction by packed sorbent (MEPS), which was already used for the quantitative analysis of several drugs such as antidepressants [36], methadone [37], acebutolol and metoprolol [38], atorvastatin [39], pravastatin [40], oxcarbazepine [41], risperidone [42,43] and remifentanil [44], among others. This novel approach for sample preparation is essentially a miniaturized version of SPE using 1–4 mg of sorbent packed either inside a syringe (100–250 μL) as a plug or between the barrel and the needle as a cartridge [45,46]. In fact, there is a constant need for the development of faster and more selective sample clean-up procedures and MEPS represents a new approach suitable for the rapid analysis of drugs and/or metabolites from biological fluids. Nevertheless, to the best of our knowledge none bioanalytical assay was previously developed for the determination of AM using MEPS as sample preparation procedure.

Thus, the aim of this work was to develop and fully validate, for the first time, a high-performance liquid chromatography (HPLC) method for the simultaneous quantification of AM and DEA in human plasma using the innovative MEPS technology for sample preparation.

2. Materials and methods

2.1. Chemicals and reagents

AM (lot no. 078K1246) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and DEA (lot no. LB33020) was kindly supplied by Sanofi-Aventis (Paris, France). Tamoxifen as citrate salt (TAM; lot no. 035K1270) was obtained from Sigma–Aldrich (St. Louis, MO, USA) and it was used as internal standard (IS). The chemical structures of these compounds are shown in Fig. 1. Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultra-pure water (HPLC grade, >18 MΩ) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). All other reagents were of analytical grade: sodium phosphate monobasic purris p.a. (Sigma–Aldrich GmbH; Seelze, Germany), sodium phosphate dibasic anhydrous (Sigma–Aldrich; St. Louis, MO, USA), sodium dihydrogen phosphate dehydrate purum p.a. (Fluka Chemie; Buchs, Switzerland) and formic acid (98–100%) (Merck KGaA; Darmstadt, Germany). MEPS 100 μL syringe and MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica – C_{18} material (SGE Analytical Science, Australia) were purchased from ILC (Porto, Portugal). Blank human plasma from healthy blood donors was kindly provided by the Portuguese Blood Institute after the written consent of each subject and in accordance with the principles of Helsinki Declaration.

2.2. Stock solutions, calibration standards and quality control samples

Stock solutions of AM, DEA, and TAM (IS) at the concentration of 1 mg/mL were individually prepared by dissolving appropriate amounts of each compound in methanol. Appropriate volumes of each of the stock solutions of AM and DEA were combined and diluted in methanol to obtain an intermediate solution at 100 μg/mL. Thereafter, stock and intermediate solutions were appropriately used to afford six combined spiking solutions at final concentrations of 0.5, 1, 2.5, 7.5, 20 and 50 μg/mL for AM and DEA, which were used to spike blank human plasma in order to prepare plasma calibration standards at six different concentration levels: 0.1, 0.2, 0.5, 1.5, 4 and 10 μg/mL for AM and DEA. The stock solution of IS was daily diluted with water–methanol (60:40, v/v) in order to obtain a working solution of 25 μg/mL. All solutions were stored protected from light at approximately 4 °C for one month, except the IS working solution which was prepared daily.

Quality control (QC) samples were prepared independently in the same matrix (blank human plasma), at three different concentration levels, representing the low (QC_1), middle (QC_2) and high (QC_3) ranges of the calibration curves. Aliquots of blank human plasma were appropriately spiked to achieve the concentrations for both analytes (AM and DEA) of 0.3 μg/mL in QC_1; 5 μg/mL in QC_2 and 9 μg/mL in QC_3.

2.3. Apparatus and chromatographic conditions

Chromatographic analysis was carried out using an HPLC system (Agilent 1260 Infinity Quaternary LC system) coupled with a

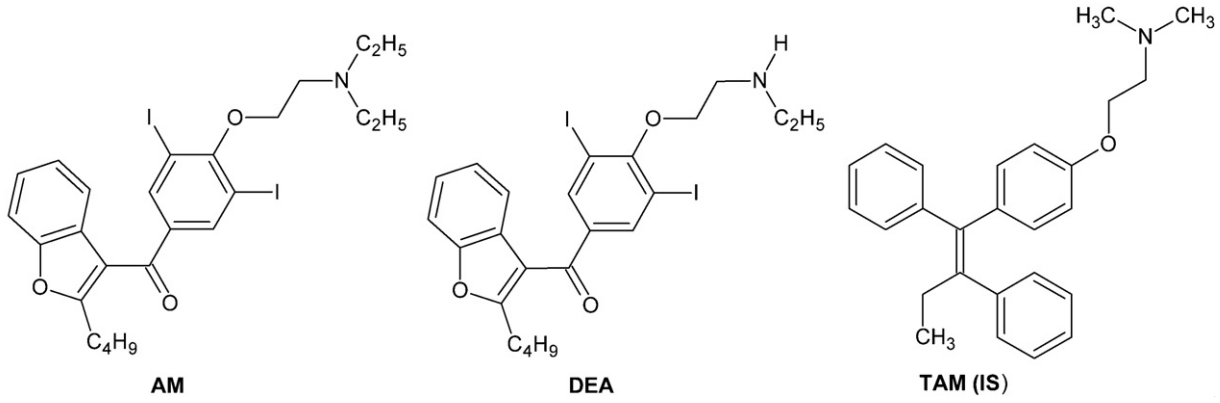

Fig. 1. Chemical structures of amiodarone (AM), desethylamiodarone (DEA) and tamoxifen (TAM) used as internal standard (IS).

diode-array detector (Agilent 1260 Infinity; G1315D DAD VL). All instrumental parts were automatically controlled by Agilent ChemStation software (Agilent Technologies).

The chromatographic separation of AM, DEA and IS was achieved in less than 5 min and it was carried out at room temperature, by isocratic elution with a mobile phase of 50 mM phosphate buffer with 0.1% formic acid (pH 3.1)/methanol/acetonitrile (45:5:50, v/v/v), at a flow-rate of 1.2 mL/min, on a reversed-phase LiChroCART® Purospher Star-C_{18} column (55 mm × 4 mm; 3 μm particle size) purchased from Merck KGaA (Darmstadt, Germany). The mobile phase was filtered through a 0.45 μm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 μL and the wavelength of 254 nm was selected for the detection of all compounds (AM, DEA and IS).

2.4. Sample preparation and extraction

The sample preparation was previously optimized and the final conditions were as follows. Each aliquot (100 μL) of human plasma samples, spiked with 20 μL (0.5 μg) of the IS working solution, was added of 300 μL of ice-cold acetonitrile. The mixture was vortex-mixed for 30 s and centrifuged at 17,000 rpm for 2 min at 4 °C. Afterwards, the supernatant was transferred to a clean vial, diluted with 400 μL of ultra-pure water and this mixture volume was then submitted to MEPS procedure. Briefly, the MEPS sorbent (C_{18}) was manually conditioned with 3 × 100 μL of methanol/formic acid (95:5, v/v) followed by 2 × 100 μL of ultra-pure water. After that, the whole volume of the diluted supernatant was drawn through the sorbent and ejected at a flow rate of approximately 10 μL/s (this procedure was performed twice). The sorbent was washed with 100 μL of ultra-pure water in order to remove interferences, and then the analytes were eluted with 100 μL of methanol/formic acid (95:5, v/v). An aliquot (20 μL) of the eluted sample was injected into the chromatographic system. To avoid the carryover the MEPS sorbent was sequentially washed/reconditioned with 30 × 100 μL of methanol/formic acid (95:5, v/v) and 2 × 100 μL of ultra-pure water before the application of following sample. The carryover was carefully investigate on MEPS sorbent and no effect was evident using the previously referred conditions after the extraction and analysis of successive aliquots at the highest standard concentrations followed by extraction and analysis of aliquots of blank plasma. Each MEPS device was re-used in about 100 extraction cycles before being discarded.

2.5. Method validation

The described method was validated according to internationally accepted recommendations for bioanalytical method validation [47–49].

Selectivity was evaluated by analyzing blank plasma samples from six different sources to ensure the absence of chromatographic interferences from endogenous compounds (matrix effects) at the retention times of DEA, AM and IS. In addition, interferences from other drugs usually co-administered with AM in clinical practice were also evaluated injecting, under the optimized chromatographic conditions, standard solutions of these compounds at a concentration of 10 μg/mL.

To evaluate the linearity of the analytical method, calibration curves were prepared using six calibration standards in the range of 0.1–10 μg/mL and assayed on five different days ($n = 5$). The calibration curves were constructed by plotting analytes (DEA or AM)/IS peak area ratios as function of the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis [50].

The limit of quantification (LOQ) was defined as the lowest concentration of the calibration curve that can be measured with acceptable inter and intraday precision and accuracy, which were assessed respectively by the coefficient of variation (CV) not exceeding 20% and the deviation from nominal concentration value (*bias*) within ±20%. The LOQs for DEA and AM were evaluated by analysing plasma samples which were prepared in five replicates ($n = 5$). The limit of detection (LOD) of the analytes, defined as the lowest concentration that can be distinguished from the noise level, was established as the concentration that yields a signal-to-noise ratio of 3:1.

Intra and interday precision and accuracy were assessed by using plasma QC samples analysed in replicate ($n = 5$) at three concentration levels (low, medium and high QC samples) representative of the calibration range. The concentrations to be tested were 0.3, 5 and 9 μg/mL for DEA and AM. The acceptance criterion for intra and interday precision (expressed as percentage of CV) was a CV value equal to or lower than 15% (or 20% in the LOQ) and for accuracy (expressed as percentage of *bias*) was a *bias* value within ±15% (or ±20% in the LOQ).

The recovery of the analytes from human plasma samples was calculated using three QC samples and the procedure described in the Section 2.4. The recoveries of the analytes (DEA and AM) were calculated by comparing the analytes peak area from extracted samples against the corresponding areas obtained by direct injection of solutions at the same theoretical concentrations. The recovery of the IS was determined at the concentration used in sample analysis by calculating the peak area ratio of the IS in extracted samples and non-extracted solutions.

Human plasma stability of AM and DEA was assessed at low (QC_1) and high (QC_3) concentration levels, in replicate ($n = 5$), at room temperature for 4 h, at 4 °C for 24 h, at −20 °C for 30 days, and at −80 °C for 30 days to simulate sample handling and storage time in the freezer before analysis. The stability of AM and DEA was also studied at 4 °C during 24 h in the processed samples (post-preparative stability) to simulate the time that samples can be in the auto-sampler before analysis. The effect of three freeze-thaw cycles on the stability of the analytes (AM and DEA) was also investigated in plasma at −20 °C and −80 °C. Aliquots of spiked plasma samples (QC_1 and QC_3) were stored at −20 °C and at −80 °C for 24 h, thawed unassisted at room temperature, and when completely thawed samples were refrozen for 24 h under the same conditions until completing the three cycles. Stability was assessed comparing the data of QC samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples); a stability/reference samples ratio of 85–115% was accepted as stability criterion ($n = 5$).

2.6. Method application to the analysis of real plasma samples

To evaluate the validity of the proposed bioanalytical method and its high selectivity for clinical application a set of real plasma samples obtained from ten polymedicated adult patients (5 males and 5 females) admitted at the Coimbra University Hospital and having in common the treatment with AM (200 mg tablets administered *per os* at different dosing regimens: once a day, twice a day or five days a week) were analysed. The blood samples were taken at the morning (7 am) and the informed consent was obtained from each subject. The analysis of these samples also represents an important approach to re-assess the selectivity of the developed method. Indeed, the number of different drugs co-prescribed with AM ranged from 6 (ID_2, ID_3 and ID_5) to 19 (ID_{10}) considering individually the patients, and were sixty-two different active pharmaceutical ingredients taking simultaneously all patients into account (ID_1–ID_{10}) (Table 1).

Table 1
Range of drugs co-prescribed with amiodarone in ten hospitalized patients from which real plasma samples were collected and analysed using the HPLC method described.

Patients	Drugs
ID_1	Acetylcysteine, Allopurinol, Aminophylline, Amiodarone, Darbepoetin Alfa, Digoxin, Enoxaparin Sodium, Folic Acid, Furosemide, Metolazone, Oxazepam, Pantoprazole, Paracetamol, Piperacillin/Tazobactam, Spironolactone
ID_2	Amiodarone, Ceftazidime, Dosulepine, Fluconazole, Lactulose, Lysine Acetylsalicylate, Omeprazole
ID_3	Amiodarone, Ciprofloxacin, Diosmin, Finasteride, Furosemide, Ramipril, Warfarin
ID_4	Amiodarone, Azithromycin, Digoxin, Enoxaparin Sodium, Furosemide, Nitroglycerin, Omeprazole, Oxazepam, Piperacillin/Tazobactam, Spironolactone, Warfarin
ID_5	Amiodarone, Ampicillin, Fentanyl, Lactulose, Metoclopramide, Nystatin, Ranitidine
ID_6	Acetylcysteine, Amiodarone, Bromide Ipatropium, Captopril, Ceftazidime, Folic Acid, Furosemide, Insulin, Levodopa/Carbidopa, Lorazepam, Metronidazole, Omeprazole, Paracetamol, Tramadol
ID_7	Acetylcysteine, Acetylsalicylic Acid, Amiodarone, Atorvastatin, Bromide Ipatropium/Salbutamol, Ciprofloxacin, Digoxin, Enoxaparin Sodium, Flavoxate, Furosemide, Oxazepam, Pantoprazole, Paracetamol, Spironolactone
ID_8	Acetylcysteine, Amiodarone, Bromazepam, Digoxin, Enalapril Maleate, Enoxaparin Sodium, Furosemide, Pantoprazole, Sertraline, Simvastatin, Spironolactone
ID_9	Acetylcysteine, Alprazolam, Amiodarone, Atorvastatin, Carvedilol, Digoxin, Enalapril Maleate, Enoxaparin Sodium, Furosemide, Pantoprazole, Propylthiouracil, Spironolactone, Triflusal
ID_{10}	Acetylcysteine, Allopurinol, Aminophylline, Amiodarone, Bromide Ipatropium/Salbutamol, Bromocriptine, Dopamine, Furosemide, Levofloxacin, Melperone, Meropenem, Metoclopramide, Metolazone, Morphine, Oxazepam, Pantoprazole, Paracetamol, Sertraline, Sucralfate, Zolpidem

ID, individual.

3. Results

3.1. Method validation

3.1.1. Selectivity

The analysis of blank human plasma samples showed no endogenous interferences at the retention times of the compounds of interest (IS, DEA and AM). Typical chromatograms of the extracts obtained from blank and spiked human plasma samples are shown in Fig. 2. Likewise, none of the tested drugs commonly co-prescribed with AM were found to interfere at the retention times of the chromatographic peaks of IS, DEA and AM (Table 2).

3.1.2. Calibration curves, LOQs and LODs

The calibration curves obtained in human plasma for DEA and AM were linear ($r^2 \geq 0.9976$) over the concentration range of 0.1–10 μg/mL. Due to the wide calibration range established, and in order to counteract the heteroscedasticity detected, the use of weighted linear regression analysis was required. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor. Indeed, taking into account the plots and the sums of absolute percentage relative error as statistical criteria, the best-fit weighting factor for both compounds (AM and DEA) was shown to be $1/x^2$ between the weighting factors usually tested under heteroscedasticity conditions ($1/\sqrt{x}$, $1/x$, $1/x^2$, $1/\sqrt{y}$, $1/y$ and $1/y^2$). The weighted regression equations ($n = 5$) of the calibration curves were $y = 0.000238x - 0.000325$ ($r^2 = 0.9987$) for DEA and $y = 0.000222x + 0.000356$ ($r^2 = 0.9976$) for AM, where y represents the analyte/IS peak area ratio and x represents the plasma concentration. The LOQs of the method were set at 0.1 μg/mL for DEA and AM, with good precision (CV ≤6.67%) and accuracy (*bias* ±5.29%) (Table 3). The LODs were established at 0.02 μg/mL for DEA and AM.

3.1.3. Precision and accuracy

The data for intra and interday precision and accuracy obtained from QC plasma samples at three different concentration levels (QC_1, QC_2 and QC_3) are shown in Table 3. The intra and interday CV values did not exceed 5.16%, and the intra and interday *bias* values varied between −9.84 and 0.78%.

Table 2
Retention times of some drugs potentially co-prescribed with amiodarone (AM) examined as possible exogenous interferences.

Drugs	RT (min)	Drugs	RT (min)
Analgesics/Antipyretics		*Antiepileptics (continuation)*	
Acetylsalicylic acid	0.53	Carbamazepine	0.68
Paracetamol	0.40	Phenytoin	0.66
Nimesulide	1.43	Topiramate	ND
Antiarrhythmics		Phenobarbital	0.57
Flecainide	0.52	*Antihypertensives*	
Bepridil	1.59	Propranolol	0.45
Verapamil	0.57	Amiloride	0.34
Diltiazem	0.49	Nifedipine	1.15
Adenosine	0.32	Candesartan	8.28
Anticoagulants		*Antipsychotics*	
Warfarin	1.40	Chlorpromazine	0.72
Antidepressants		Haloperidol	0.57
Fluoxetine	0.65	Droperidol	0.44
Sertraline	0.72	*Sedatives/Hypnotics*	
Imipramine	0.77	Alprazolam	0.76
Trazadone	0.42	Clobazam	1.06
Maprotiline	0.61	Diazepam	1.46
Antiepileptics		Zolpidem	0.42
Lamotrigine	0.39	Promethazine	0.54

RT, retention time; ND, not detected within 20 min after the chromatographic injection.

Table 3
Precision (% CV) and accuracy (% *bias*) for the determination of desethylamiodarone (DEA) and amiodarone (AM) in human plasma samples at the concentrations of the limit of quantification (*) and at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges ($n = 5$).

Analyte	$C_{nominal}$ (μg/mL)	Precision (% CV)	Accuracy (% *bias*)
Interday			
DEA	0.1*	2.34	4.73
	0.3	5.16	−3.96
	5	3.46	0.78
	9	2.51	−0.09
AM	0.1*	4.20	5.21
	0.3	2.09	−9.84
	5	2.55	−7.51
	9	2.74	−6.01
Intraday			
DEA	0.1*	3.39	5.21
	0.3	4.29	0.20
	5	0.94	−0.57
	9	1.06	−1.47
AM	0.1*	6.67	5.29
	0.3	2.53	−4.26
	5	2.63	−7.53
	9	1.44	−6.73

$C_{nominal}$, nominal concentration; CV, coefficient of variation.

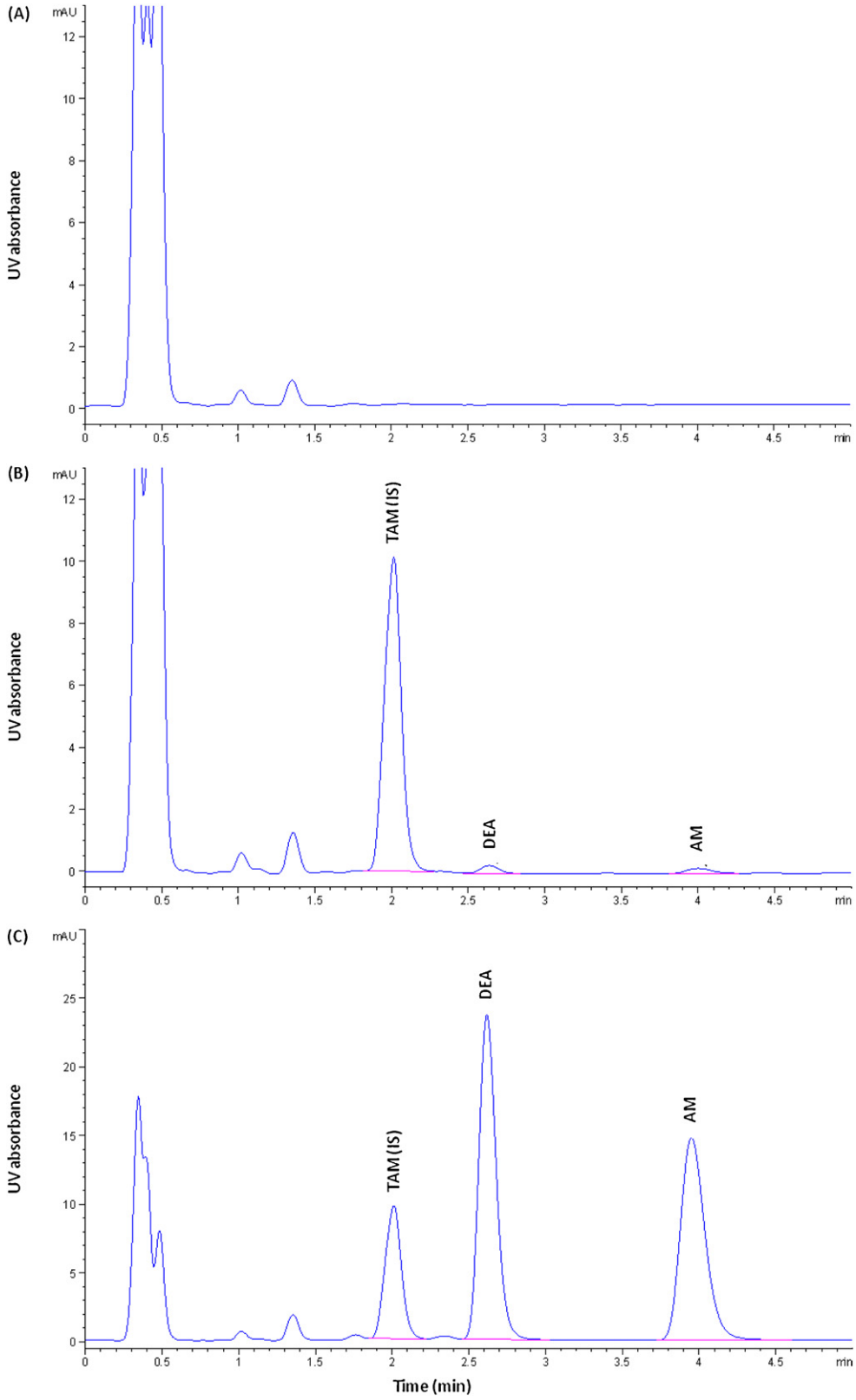

Fig. 2. Typical chromatograms of extracted human plasma samples obtained by the MEPS/HPLC method developed: blank plasma (A); plasma spiked with internal standard [IS; tamoxifen (TAM)] and the analytes [desethylamiodarone (DEA) and amiodarone (AM)] at concentrations of the limit of quantification (0.1 μg/mL) (B) and at concentrations of the upper limit of calibration range (10 μg/mL) (C).

Table 4
Recovery (%) of desethylamiodarone (DEA) and amiodarone (AM) from human plasma samples at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges ($n = 5$).

Analyte	$C_{nominal}$ (μg/mL)	Recovery (%)	
		Mean ± SD	CV (%)
DEA	0.3	65.9 ± 5.38	8.17
	5	64.7 ± 0.83	1.28
	9	68.2 ± 1.50	2.19
AM	0.3	59.7 ± 4.71	7.89
	5	58.6 ± 2.12	3.62
	9	62.3 ± 1.73	2.78

$C_{nominal}$, nominal concentration; SD, standard deviation; CV, coefficient of variation.

3.1.4. Recovery

The recovery of DEA and AM from human plasma samples spiked at three different concentration levels (QC_1, QC_2 and QC_3) was evaluated and the results are presented in Table 4. The mean recoveries of DEA and AM ranged from 64.7 to 68.2% and 58.6 to 62.3% respectively, and showed low CV values. The mean recovery of the IS was 61.0%, with a CV of 9.51%.

3.1.5. Stability

The stability of DEA and AM in human plasma was evaluated under different circumstances, simulating the handling and sample storage conditions likely to be encountered during the analytical process, by analysing low and high QC samples in replicate ($n = 5$). The results of the stability assays showed that no significant loss was observed for AM and DEA in human plasma at room temperature for 4 h, at 4 °C for 24 h, and at −20 °C and −80 °C for 30 days. The analytes also demonstrated to be stable in processed plasma samples at 4 °C during 24 h. The stability data for DEA and AM evaluated after three freeze-thaw cycles at −20 °C and −80 °C indicated that the stability criteria previously established were not fulfilled only at −20 °C for QC_3. The stability data are shown in Table 5.

3.2. Analysis of real plasma samples

The MEPS/HPLC method was applied to the analysis of DEA and AM in plasma samples taken from ten polymedicated patients orally treated with AM and the concentrations obtained are summarized in Table 6, as well as the prescribed AM regimens. In addition, a representative chromatogram (ID_{10}) of the analyses of such real plasma samples is also depicted in Fig. 3. As one can see, no interference from human plasma endogenous compounds or the co-administered drugs is apparent, and it is also clearly

Table 5
Stability (values in percentage) of desethylamiodarone (DEA) and amiodarone (AM) in unprocessed plasma samples at room temperature for 4 h, at 4 °C for 24 h, after three freeze-thaw cycles (−20 °C and −80 °C), and at −20 °C and −80 °C for 30 days; and in processed plasma samples left at 4 °C for 24 h ($n = 5$).

Analyte	DEA		AM	
$C_{nominal}$ (μg/mL)	0.3	9	0.3	9
Unprocessed plasma				
Room temperature (4 h)	94.4	94.9	93.6	93.7
4 °C (24 h)	91.6	91.9	96.1	90.3
Freeze-thaw (3 cycles; −20 °C)	86.0	81.3	91.5	84.6
Freeze-thaw (3 cycles; −80 °C)	97.0	89.1	103.3	93.1
−20 °C (30 days)	97.7	96.4	103.0	101.5
−80 °C (30 days)	97.2	90.8	96.6	97.5
Processed plasma				
4 °C (24 h)	96.6	104.2	98.4	108.5

$C_{nominal}$, nominal concentration.

Table 6
Plasma concentrations of amiodarone (AM) and desethylamiodarone (DEA) in real plasma samples obtained from polymedicated patients taking AM orally (200 mg tablets) at different prescribed regimens. All samples were collected at the morning (7 am).

Patients	Prescribed regimen	$C_{measured}$ (μg/mL)	
		AM	DEA
ID_1	200 mg tablet (bid)/(9 am; 9 pm)	0.333	0.165
ID_2	200 mg tablet (5 days a week)/(7 pm)	0.622	0.234
ID_3	200 mg tablet (id)/(9 am)	BLQ	BLQ
ID_4	200 mg tablet (id)/(9 am)	0.129	BLQ
ID_5	200 mg tablet (5 days a week)/(7 pm)	0.512	0.236
ID_6	200 mg tablet (id)/(9 am)	0.122	0.118
ID_7	200 mg tablet (id)/(9 am)	0.124	BLQ
ID_8	200 mg tablet (id)/(9 am)	0.858	0.624
ID_9	200 mg tablet (id)/(9 am)	0.664	0.408
ID_{10}	200 mg tablet (id)/(9 am)	0.560	0.644

ID, individual; $C_{measured}$, measured concentration; bid, twice a day; id, once a day; BLQ, below the limit of quantification (<0.1 μg/mL).

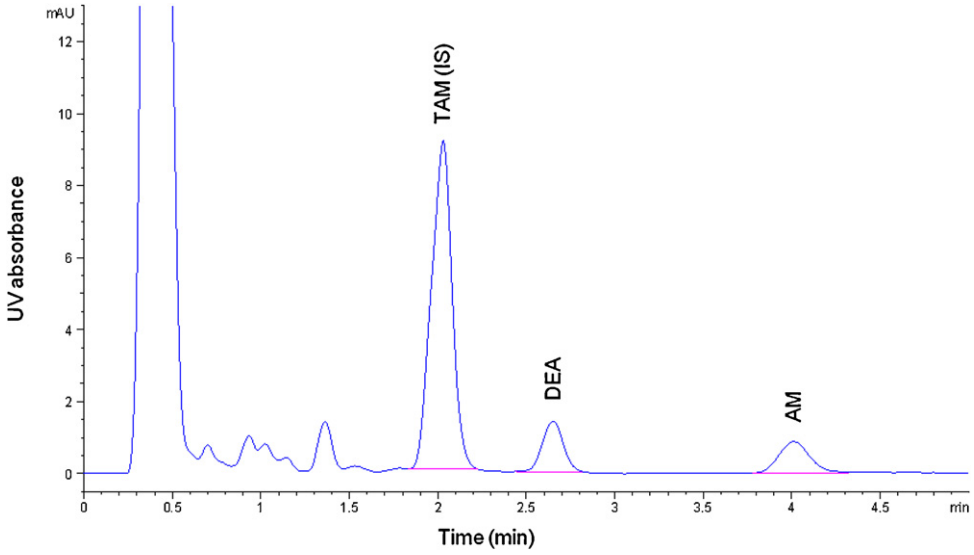

Fig. 3. Representative chromatogram of the analysis of real plasma samples obtained from polymedicated patients under treatment with amiodarone (AM). Specifically, this chromatogram was generated by the analysis of the sample collected from patient ID_{10}, which was also the most polymedicated patient; the plasma concentrations of desethylamiodarone (DEA) and AM measured in this sample were respectively 0.644 μg/mL and 0.560 μg/mL.

seen that the peak shape of the analytes (IS, DEA and AM) and chromatographic resolution are similar to those obtained after the analysis of spiked human plasma samples.

4. Discussion

For the first time a simple, fast and reliable MEPS/HPLC method was developed and fully validated for the simultaneous determination of AM and its main metabolite (DEA) in human plasma. The optimized MEPS procedure enabled the validation of the method for the quantitative analysis of AM and DEA within a wide concentration range (0.1–10 μg/mL), which includes the therapeutic window usually proposed for AM (0.5–2.0 μg/mL), and provided an acceptable extraction recovery (~60%) for all compounds of interest [TAM (IS), DEA and AM].

The MEPS procedure developed presents several advantages in comparison with the sample preparation techniques usually used in bioanalysis. Firstly, the MEPS procedure does not require the evaporation and reconstitution steps of the sample usually needed in most of the LLE or SPE techniques. In addition, after the initial protein precipitation step, the MEPS procedure herein developed could be automatable by using the automated analytical syringe eVol® or connecting MEPS on-line with HPLC. MEPS also presents a cost per analysis minimal compared to conventional SPE because each MEPS sorbent can be re-used several times before being discarded while SPE cartridges are indicated for single use. MEPS also enables the reduction of the solvent volume consumption and the sample preparation time. On the other hand, a small volume of plasma (100 μL) is required in this case in comparison with many other methods published that use larger sample volumes (0.5–2 mL) [10,18–25,27–29,31,33,34]. In fact, our method presents a LOQ for AM and DEA similar or even lower than other methods that employed a larger volume of plasma [10,19,21,23,26,31,32,34]. However, there are also methods reported in literature presenting lower LOQs for AM and DEA but making use of more expensive and sensitive detection systems, such as tandem mass spectrometry [29,30], or using higher volumes of sample [18,20,24,25,28,33,35].

In addition, this bioanalytical method enables the rapid analysis of AM and DEA in human plasma samples (less than 5 min) using the usual detection system and the simplest chromatography conditions found in clinical units. In this method the IS selected was TAM which is commercially available; in contrast, other analytical methods for determination of AM and DEA used as IS a compound that is no longer available L8040 (a brominated analogue of AM) [10,18,20,21,23–28,31,32].

This method was also successfully applied to real plasma samples of highly polymedicated patients receiving treatment with AM. From the analysis of the Table 6 it is evident that in some patients the concentrations of AM are below the proposed therapeutic range (0.5–2.0 μg/mL) and in patient ID_3 the AM plasma concentration was found to be below the limit of quantification (BLQ; < 0.1 μg/mL). These findings may be explained by the short time of treatment with AM, since the patients have initiated AM therapy in the hospital few days (2–10 days) before sample collection. Indeed, as AM and DEA have a huge apparent volume of distribution, it is likely to find BLQ levels at the end of the dosing interval during the first days of AM therapy. On the other hand, these results expose the urgent need for routine TDM of AM plasma/serum concentrations as a guide to individualize dosing regimens, even during the first days/weeks of treatment.

5. Conclusions

A simple, fast and reliable MEPS/HPLC method was developed and validated for the simultaneous determination of AM and DEA in human plasma. This bioanalytical method constitutes an attractive and promising alternative to the existing methodologies for the quantitative analysis of AM and DEA from human plasma, enabling its application for the routine TDM of AM but also in other clinical pharmacokinetic-based studies involving treatment with this drug, such as bioavailability/bioequivalence studies.

Conflict of interest

The authors have declared no conflict of interest.

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