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Preparation and determination of desethylamiodarone in dog lung by HPLC–MS

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A novel method was developed for the preparation and determination of desethylamiodarone in dog lung by high-performance liquid chromatography (HPLC) with amiodarone as a standard. The selected dog was orally given amiodarone, then executed and the active metabolite desethylamiodirone in lung tissue was isolated, concentrated and purified by Waters C_{18} column (25 mm × 250 mm,10 μm) with mobile phase of acetonitrile-100mmol/L acetic acid containing 15 mmol/L diethylamine (55:45 v/v) at a flow rate of 10.0 mL/min. Hypersil ODS_2 column (4.6 mm × 250 mm 5 μm) was used to analyze amiodarone and desethylamiodarone, with the same mobile phase at a flow rate of 1.0 mL/min, the detection wavelength was 237.5 nm. Atmosperic pressure electronic spray ionization (AP-ESI) and ion mass spectral (m/z) of 618.1(M + H) were selected to validate desethylamiodarone. The f_i of desethylamiodarone and amiodarone were 1.04 ± 0.02 and 1.020 ± 0.01, respectively. It indicated that desethylamiodarone can be separated and purified by preparational HPLC after Mass Spectrometry (MS) validation and quantified according to f_i of amiodarone indirectly. The proposed method enables the preparation and determination of desethylamiodarone in dog lung successfully.

1. Introduction

Amiodarone (AM) is an antiarrhythic drug (Zahno et al. 2011), which is metabolized by a polymorphic enzyme to its active metabolite desethylamiodarone (DEA) in the human body, with the same biological activities and electrophysiology effects as the original. Although there is a linear relationship between oral doses of AM and its tissue concentrations, the concentrations in patients receiving the same dose vary markedly. AM's therapeutic use is limited because of its numerous side effects (Jessurun et al. 1998; Lewis et al. 1990; Morse et al. 1988), so it is necessary to modify and adjust an AM dosage regimen based on the blood concentration of AM and DEA (Hutchings et al. 1986; Kuhn et al. 2010) to increase the curative effect and decrease the side effects.

The metabolite of AM cannot be commercially obtained which confines related investigations. Several methods, such as HPLC assays (Juenke et al. 2004; Pérez-Ruiz et al. 2008), capillary electrophoresis (Zhang et al. 1996) and LC-MS method (Kollroser et al. 2002; Maes et al. 2006; Shayeganpour et al. 2007), have been described for the measurement of AM and DEA in human plasma. Because of the uniform structures of AM and DEA, a method using HPLC-MS was established for quantification of AM and DEA with AM as a standard.

In this study, when fed-dog was administrated with large dosage of AM, AM was metabolized to DEA by liver enzymes which are generally distributed in most tissue and concentrated in lung of the AM fed dog (Brien et al. 1990). This experiment introduced the method for the preparation and determination of DEA in dog lung by HPLC-MS, which established the foundation of the research, exploitation and application of the active metabolite of AM.

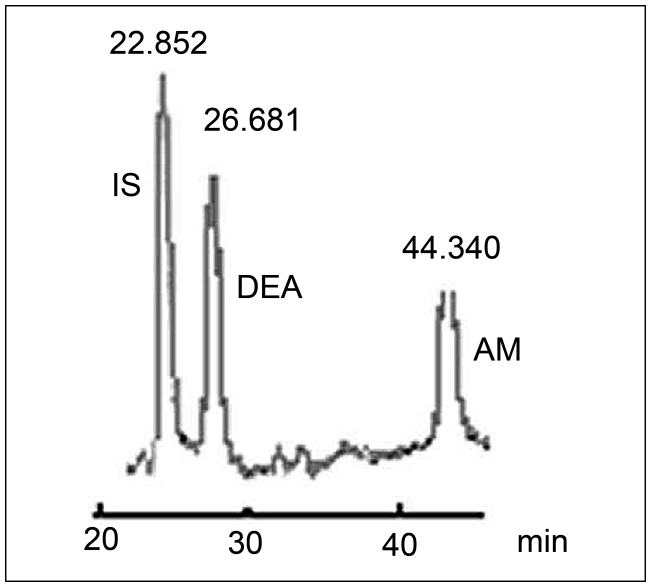

Fig. 1: Chromatogram of standard AM, DEA and IS

2. Investigations and results

2.1. Determination of fi of control article of DEA and AM

The chromatograms of standard AM, DEA and internal standard (IS) are shown in Fig. 1. The retention times are 44.340, 26.681 and 22.852 min for AM, DEA and tamoxifen, respectively. So the fi of AM and DEA were 1.014 ± 0.02 and 1.020 ± 0.01, respectively ($a = 0.05$, $p > 0.05$). The retention time ratio of DEA

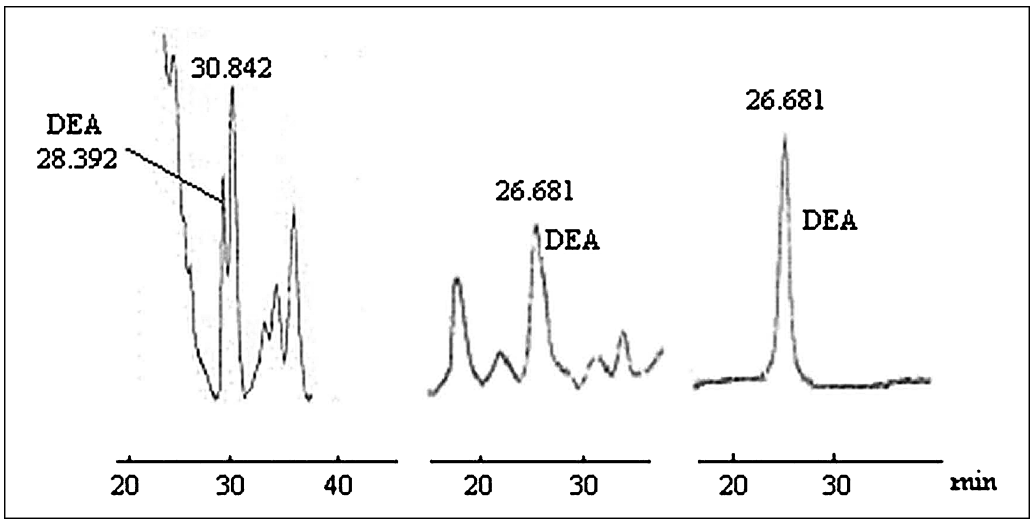

Fig. 2: Chromatograms of DEA (DEA extracted before and after purified DEA)

Table 1: Recovery and precision validation of AM and DEA

Expected concentration μg /mL	Recovery (%)		Intraday precision (CV %)		Interday precision (CV %)	
	AM	DEA	AM	DEA	AM	DEA
0.1	90.3 ± 4.126	94.4 ± 3.958	3.303	4.875	10.134	5.972
0.8	95.8 ± 3.586	97.5 ± 2.176	3.428	2.789	4.972	8.136
3.2	99.4 ± 3.466	101.9 ± 3.032	5.436	3.369	5.614	4.746

is 0.602 corresponding to AM. There was no statistical significance between AM and DEA, so it can be presumed that the fi of DEA is equal to that of AM, so the $C_{DEA} = C_{AM}{}^{*} A_{DEA}/A_{AM}$. According to the standard curves, the regression equation of AM and DEA was: $A_{AM}/A_{IS} = 0.02982 + 1.85633C_{AM}$, $A_{DEM}/A_{IS} = 0.02612 + 1.86961C_{DEM}$. It demonstrated that DEA could be purified with AM as a standard, and a new, simple and applicable method was provided for the determination of the blood concentration of DEA.

The average extraction recoveries in plasma were 90.3, 95.8 and 77.7% for AM, respectively. The average extraction recoveries in plasma were 94.4, 97.5 and 101.9% for DEA, respectively. For AM and DEA, the assay CV for both the intraday and interday assessments were equal to or less than 11% (Table 1).

2.2. Preparation and purification of DEA

Figure 2 shows the chromatogram of DEA in the extract of dog lung tissue, before and after purified DEA. The retention times were 28.392, 26.681 and 26.681 min for DEA in DEA extracted, before and after purified DEA, respectively. In the MS mode the most prominent product ion for all compounds is the protonated molecular ion $[M+H]^+$: at *m/z* 618.3 for DEA. The chromatograms of DEA after purification in Fig. 2, mass spectrum as in Fig. 3, showed that DEA was highly purified.

DEA was isolated and prepared from an AM fed-dog. DEA and foreign substance was isolated completely, with well peak shape and 70% recovery rate. The liposolubility of DEA was maximal at pH 3.5∼5.5, therefore the sample should be acidified before extraction. Because the surface active material of biological sample would be destroyed by isopropyl alcohol, isooctane:isopropyl alcohol (80:20) was selected for extraction to avoid emulsification, so the recovery rate was high and endogenous foreign matter from tissue could be removed. These additives can be easily dissolved in acidic medium and affect abstraction and determination, thus hard PVC and glass tubes were applied to avoid the interference and expenses of composition from plastisites (Peters et al. 1990).

3. Discussion

Here, we report validation of the first HPLC–MS method to determine AM and its main metabolite DEA in dog lung.

The coupling of HPLC to MS with atmospheric pressure ionization leads to a much more specific and sensitive analytical technique. The presented assay is a HPLC–MS method that allows the simultaneous determination and quantitation of AM and DEA in dog lung. The combination of HPLC and MS leads to specificity and sensitivity of drug identification, which is crucial in both clinical and forensic applications. The HPLC–MS

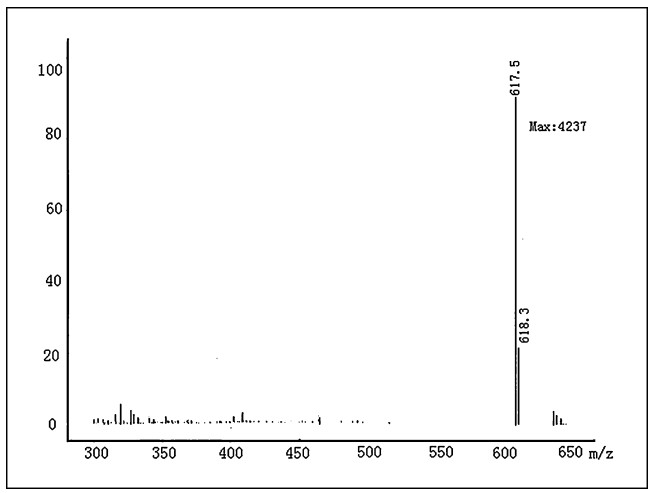

Fig. 3: DEA mass spectrum after purified

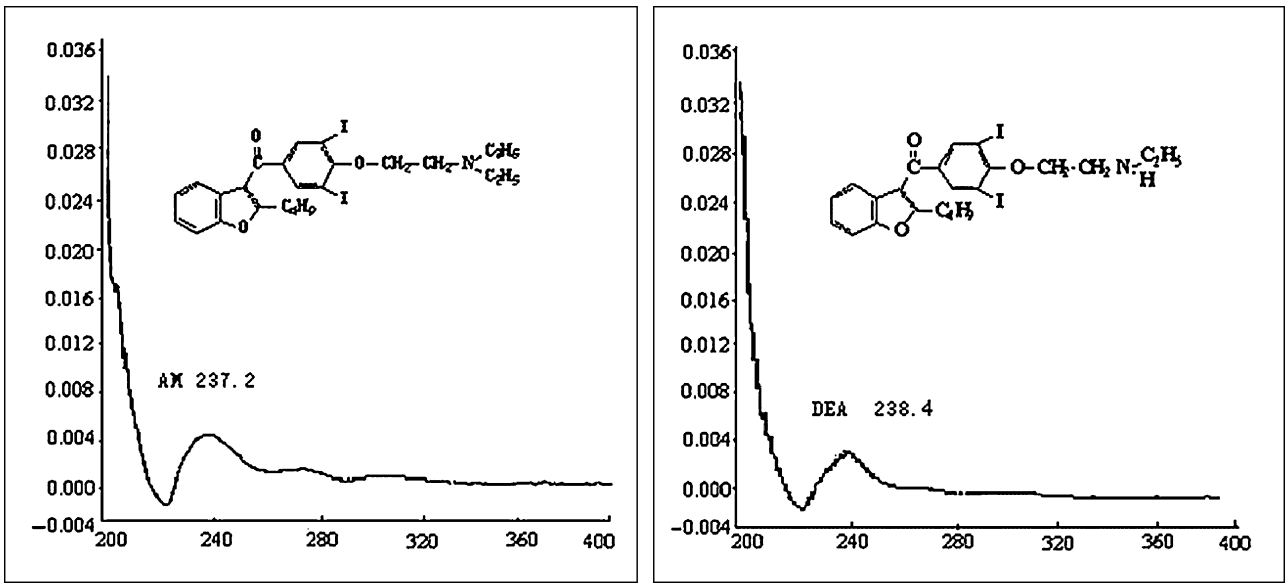

Fig. 4: Chemical structure and UV spectrograms of AM and DEA

method described in this paper for the quantitation of AM and DEA is a fast procedure. The minimal sample preparation, namely a simple deproteinization step, allows the extraction of many samples in a day. Combined with solid phase extraction, the HPLC-MS method is ten times more sensitive although it is more time consuming and more expensive than the HPLC–UV method. Hence, further efforts should be made to develop more inexpensive, simple and convenient methods.

The pharmacokinetics and pharmacodynamics nature of AM and DEA are special. If the blood concentrations of the both are monitored simultaneously, the curative effects could be utmost educed and toxicity could be decreased (Pollak 2001). To obtain the active metabolism product of AM conduces to further research of pharmacologic action, relative activity, clinical effect and drug monitoring.

4. Experimental

4.1. Equipment and agents

Waters 600E HPLC, Millennium 32 Data Processing System (the USA Waters Analytical Apparatus Corporation). HP1100LC-MSD LS-MS, 1100 Series Chromatographic Work Station (the USA Agilent Analytical Apparatus Corporation). J-type High-Speed Centrifuge (Shanghai Analytical Apparatus Station). CX-205 Ultrasonic Cleaning Apparatus (Beijin Medical Treatment Facility Station), AX205Equi-Armbalance (Meitele toydo Shanghai Limited Company).

AM hydrochloride capsules were obtained from Jiangsu Chenpai pharmaceutical limited company(200 mg/capsule, Batch No. 011113). AM standard (purity over 99.5%) was provided by Pinyuan pharmaceutical Factory (Shandong, China). DEA standard (purity over 99.7%) was provided by Sailofi pharmaceuticals. Tamoxifen (purity over 99.5%), as internal standard was purchased from Dongfeng pharmaceutical company, (Shandong, China). Chromatographic grade methyl alcohol and methyl cyanide were purchased from TEDIA of USA. Acetic acid, ethylendiamine, isooctane, isopropyl alcohol, potassium dihydrogen phosphate and dibasic potassium phosphate analytical grade. A Milli-Q Plus water purification system (Millipore-Iberica, Madrid, Spain) was used to obtain purified water.

Solutions: Certain amounts of AM, DEA and tamoxifen standard were dissolved by methyl alcohol in a 50 mL measuring flask and 1 mg/mL standard solutions were obtained and stored at 4 °C for analysis.

4.2. Chromatographic conditions

Mobile phase was acetonitrile-100 mmol/L acetic acid (contain 15 mmol/L ethylendiamine) (55:45). Waters 996 diode array detector, column temperature was the room temperature (Hanioka et al. 2002). Analysis condition OSD2 column (4.6 mm × 250 mm, 5 μm), flow rate 1.0 mL/min, volume of sample injection 50 μl. preparation condition Waters C_{18} column (25 mm × 250 mm, 10 μm), flow rate 10.0 mL/min, volume of sample injection 15 mL, nitrogen gas 50 mL/min on-line degassing. LC-MS condition, flow rate 1.0 ml/min, split ratio 2:1, volume of sample injection20 μL, mode of positive ion, ion source AP-ESI, temperature of ion source 100 °C, pressure of atomization 30 psi, protective gas of nitrogen gas 10 L/min, APCI pressure of corona discharge styporoct is 4000 V, pressure of chip is 130 V, collection mode SIM, ion collection of DEA (m/z)-618.1(M + H), scanning extent 300∼700 aum.

4.3. Wavelength choice

The structures of AM and DEA are shown in Fig. 4. The scan range of AM and DEA was 195 nm to 400 nm. It is indicated that 237.5 nm was the maximum absorption wave for both AM and DEA.

4.4. Determination of fi of control article of DEA and AM

AM and DEA standard solutions were diluted to 1.5 nmol/mL, and 20 μL tamoxifen was added as internal standard. Parallel operation was executed 30 vices according to the chromatographic condition mentioned before, and the data of chromatographic figure and peak area were collected. Standard curves and regression equation were prepared by spiking appropriate amounts of AM (0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μg/mL) and DEA (0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μg/mL) in 1.0 mL of rat plasma. The fi of control article of DEA and AM was calculated by the formula: $fi = As^* mi/Ai^* ms$ (i: AM or DEA, s: internal standard of tamoxifen, A: peak area, m: amount of component or standard substance).

4.5. Recovery and precision

The plasma recoveries were determined at AM and DEA concentrations of 0.1, 0.8 and 3.2 μg/mL, using five replicates for each concentration containing 1 μg tamoxifen. The recovery rate was calculated by: $(C_{AM1}/C_{AM2}) \times 100\%$ and $(C_{DEM1}/C_{DEM2}) \times 100\%$, the C_{AM2} and C_{DEM2} was the actual concentration, the C_{AM1} and C_{DEM1} was calculated by the regression equation mentioned above.

Intraday and Interday precision of the assay were determined using three different concentrations of DEA and AM in rat plasma. For both AM and DEA, the concentrations were chosen at 0.1, 0.8 and 3.2 μg /mL. Each concentration had a replicate of five samples. To permit the assessment of interday precision, the assay was repeated on five separate days. Precision was assessed by percentage coefficient of variation (CV%).

4.6. Preparation and purification of DEA

4.6.1. Animal experiments

A male ripe hybrid dog, which was raised in an ordinary animal laboratory room, was fed AM at a dose of 40 mg/kg/day for 10 days, and 30 mg/kg/day for 4 days. Seven hours after administration on the 14th day, 3% pentobarbital were injected (40 mg/kg) for intravenous anesthesia, then the lung tissue was obtained. After washed-out with sodium chloride, the lung tissue was immediately frozen in liquid nitrogen and stored at −20 °C.

4.6.2. Tissue abstraction

A piece of lung tissue of the dog was obtained and 8 times the volume of methanol was added. After homogenated for 5 min, concentrate was harvested by centrifugating at 4500 r/min for 20 min and dried. The constituent was dissolved in a 60 °C water-bath and 0.1 mol/L buffer phosphate and an equal volume of methanol was added. After vortexed for 5 min, a proper amount of acid liquid was got and 5 times isooctane: isopropyl alcohol (80:20, v/v) was added. Then the mixture was vortexed for 2 min and centrifuged at 4500 r/min for 15 min, the supernatant was removed and concentrate was dried.

4.6.3. Preparation and purification of sample

The above-mentioned crude product was taken in a 60 °C water-bath and a moderate amount of methanol was added. After centrifuged at 4500 r/min for 15 min, supernatant was obtained and purified with preparative HPLC under the chromatographic conditions mentioned before. The component was collected which contain the aim point of DEA, and purified DEA was obtained by vacuum concentration and drying.

4.6.4. Sample validation by HPLC-MS

The chromatographic data of DEA standard substance, extract of dog lung tissue and purified DEA were recorded. The mass-spectrogram of purified DEA was also recorded to validate DEA.

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