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Determination of amiodarone and desethylamiodarone in human plasma by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry with an ion trap detector

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Abstract

A sensitive and specific high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS–MS) method has been developed for the simultaneous determination of amiodarone and desethylamiodarone in human plasma. After the addition of the internal standard tamoxifen, plasma samples were extracted using Oasis® MCX solid-phase extraction cartridges. The compounds were separated on a 5 μm Symmetry C_{18} (Waters) column (150×3.0 mm, internal diameter) with a mobile phase of acetonitrile–0.1% formic acid (46:54, v/v) at a flow-rate of 0.5 ml/min. The overall extraction efficiency was more than 89% for both compounds. The assay was sensitive down to 1 μg/l for amiodarone and down to 0.5 μg/l for desethylamiodarone. Within-run accuracies for quality-control samples were between 95 and 108% of the target concentration, with coefficients of variation <8%. The proposed method enables the unambiguous identification and quantitation of amiodarone and desethylamiodarone in both clinical and forensic specimens.

Keywords: Amiodarone; Desethylamiodarone

1. Introduction

Amiodarone, a Class III antiarrhythmic agent, is used in the treatment of various supraventricular and ventricular arrhythmias [1,2]. It has a long duration of action, permitting once-daily dosing, and a very long serum elimination half life ranging from 30 to 120 days [3–5] with typical therapeutic plasma concentrations of 0.5 to 2.5 mg/l [6]. There is a linear relationship between oral doses of amiodarone and its concentrations in plasma and myocardial tissue, however, serum concentrations of patients administered with the same dose vary considerably due to a wide inter-individual variability in half life. The main metabolite of amiodarone is desethylamiodarone, which can approach the concentration of the parent drug during long term therapy [4]. The known adverse effects of amiodarone include hypo- and hyperthyroidism [7], pulmonary fibrosis [8], as

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well as cutaneous, neurological, and gastrointestinal toxicity [9–11]. Some of these side effects are dose-dependent, emphasizing the need to apply the lowest effective dose and to perform conscientious drug monitoring. Hence, the unambiguous identification and quantitation of amiodarone and its major metabolite desethylamiodarone require a specific method that is suitable for routine analysis.

Several HPLC assays [12–23] as well as a capillary electrophoresis (CE) method [24], all of which use UV detection, have been described for the determination of amiodarone and desethylamiodarone in human plasma. The shortcomings of all of these methods are their unreliability in regard to specificity. The coupling of HPLC to MS with atmospheric pressure ionization (API) leads to a much more specific and sensitive analytical technique. A second stage of mass analysis (MS–MS) further enhances specificity and provides an improved signal-to-noise ratio compared to single stage MS. One HPLC–MS–MS method has been described for the assignment of the structures of metabolites and impurities of amiodarone [25].

The purpose of this work was to exploit the high selectivity and sensitivity of an ion trap detector operated in tandem MS mode with an ESI interface for the detection and quantitation of amiodarone and desethylamiodarone in human plasma. The limitations of alternative HPLC methods were overcome by combining a new simple solid-phase extraction procedure with the power of a benchtop tandem MS system.

2. Experimental

2.1. Drugs and reagents

Amiodarone and desethylamiodarone (both over 99.8% pure) were obtained from Sanofi (Basel, Switzerland). Tamoxifen (purity over 99%) was supplied by Promochem (Herts, UK). HPLC grade acetonitrile and analytical-grade concentrated formic acid (HCOOH) were obtained from Promochem (Wesel, Germany). Isopropanol, ammonia (33%), methylene chloride, and orthophosphoric acid (85%) were of analytical grade and were purchased from Merck (Darmstadt, Germany). A Milli-Q® Plus water-purification system (Millipore Corp., Vienna, Austria) was used to obtain purified water for the HPLC solvent. Oasis® MCX solid-phase extraction cartridges were supplied by Waters (Vienna, Austria).

2.2. LC–ESI-MS–MS

The LC–MS–MS analyses were performed using a TSP LC system consisting of a vacuum degasser, a P4000 quaternary pump, an AS3000 autosampler, a UV6000LP diode-array UV detector, and a Finnigan LCQ^{DUO} ion trap mass spectrometer equipped with an ESI source (Finnigan MAT, USA) run by XCALIBUR software.

HPLC separations were performed on a Symmetry C_{18} (Waters, USA), 5 μm, 150×3.0 mm internal diameter (I.D.) HPLC column, operated at ambient temperature and protected by a Sentry guard column Symmetry C_{18} (Waters), 5 μm, 20×3.9 mm I.D. Each 5 min chromatographic run was carried out at a flow-rate of 0.5 ml/min with a mobile phase of acetonitrile–0.1% HCOOH (46:54, v/v).

Operating conditions for the ESI source used in the positive ionization mode were optimized by constantly adding a mixture of amiodarone, desethylamiodarone, and tamoxifen each at a concentration of 1 mg/l in methanol to the HPLC flow by a syringe pump via a T connector in the infusion mode. The signal was optimized on the total ion current in MS mode, resulting in a transfer capillary temperature of 270°C, a spray voltage of 4.5 kV, and a sheath gas flow of 80 units (units refer to arbitrary values set by the LCQ software). At the same time, the selection of ions and the collision voltages were optimized using LCQ software.

In the MS–MS experiments, the protonated precursor pseudomolecular ions $[M+H]^+$ of amiodarone (m/z 646), desethylamiodarone (m/z 618), and tamoxifen (m/z 372) were selected (isolation width was set to m/z 2) and fragmented by helium gas collision in the ion trap at a relative collision energy of 40%. The mass spectra resulting from these fragmentations were acquired in the full scan mode from m/z 100 to 700. Several product ions were observed for each compound. The most abundant product ions, m/z 573 for amiodarone, m/z 547 for desethylamiodarone, and m/z 327 for tamox-

ifen, respectively, were extracted and chosen for quantitation.

2.3. Calibration standards and quality control samples

Stock solutions of amiodarone, desethylamiodarone, and tamoxifen (internal standard, I.S.), were prepared by dissolving 1 mg of each analyte in 1 ml of methanol. The stock solutions of amiodarone, and desethylamiodarone were combined and diluted with methanol to obtain working solutions containing 1, 10, and 100 mg/l, respectively. The stock solution of the I.S. was also diluted in methanol to a final concentration of 10 mg/l. To prepare calibration samples, drug free plasma was spiked with the appropriate volume of the working solutions to contain amiodarone and desethylamiodarone, respectively, at concentrations of 50, 100, 200, 400, 800, 1600, 3200, and 5000 μg/l. The calibration curves were constructed by linear regression using the peak area ratios of amiodarone and desethylamiodarone, respectively, to I.S., plotted against the corresponding concentrations.

A similar dilution procedure was used to make additional working solutions containing amiodarone and desethylamiodarone each at concentrations of 10 and 100 mg/l. These solutions were used to prepare quality-control samples in drug free plasma at concentrations of 100, 800 and 3500 μg/l of each analyte.

2.4. Sample preparation

One milliliter of each calibration and quality-control plasma sample was pipetted into labeled glass tubes. A 20 μl volume of orthophosphoric acid and 20 μl of the diluted internal standard solution (10 μg/l) were added to each sample. The tubes were vortex mixed and samples were allowed to equilibrate for at least 30 min.

Solid phase extraction was performed using Oasis® MCX cartridges (1 cm^3/30 mg), a vacuum manifold device, and a vacuum source. SPE cartridges were conditioned and equilibrated with 1 ml of methanol and 1 ml of water. Each spiked and acidified specimen was applied to the cartridge and passed through the bed at a constant flow-rate (1 ml/min). Cartridges were sequentially washed with 1 ml of 0.1 N hydrochloric acid and 1 ml of methanol. Analytes were eluted with 1 ml of methylene chloride:isopropanol:ammonia (78:20:2), collected into appropriately labeled tubes and evaporated to dryness under a stream of nitrogen at 35°C. The residues were reconstituted with 100 μl of mobile phase and were transferred to autosampler vials. A 20 μl volume of the reconstituted extract were injected.

2.5. Extraction efficiency

The extraction efficiency for amiodarone and desethylamiodarone was experimentally determined at concentrations of 100, 1000, and 3000 μg/l. The extraction recoveries were evaluated by comparing the analyte areas to external standard calibration curves that were obtained from unextracted reference standards at concentrations ranging from 50 to 3500 μg/l.

3. Results and discussion

3.1. Chromatography and mass spectra

ESI is a ''gentle'' ionization technique which produced high mass-to-charge $[M+H]^+$ precursor ions with minimal fragmentation of the analytes. Fig. 1 shows the full scan mass spectra (m/z 100–700) of the compounds investigated. The major ions observed were m/z 646 for amiodarone, m/z 618 for desethylamiodarone, and m/z 372 for tamoxifen. The product ion chromatograms and the corresponding full scan product ion spectra of amiodarone, desethylamiodarone, and tamoxifen extracted from spiked plasma are depicted in Fig. 2. The retention times of amiodarone, desethylamiodarone, and tamoxifen were 3.22, 2.44, and 2.24 min, respectively. The total HPLC–MS–MS analysis time was 5 min per sample. The most intense product ions in MS–MS spectra, m/z 573 for amiodarone, m/z 547 for desethylamiodarone can be explained by the loss of the diethylamino group. For tamoxifen, with its most intense product ion at m/z 327, the loss of the dimethylamino group is proposed.

No matrix interference with the analytes was observed due to the acquisition in the precursor ion

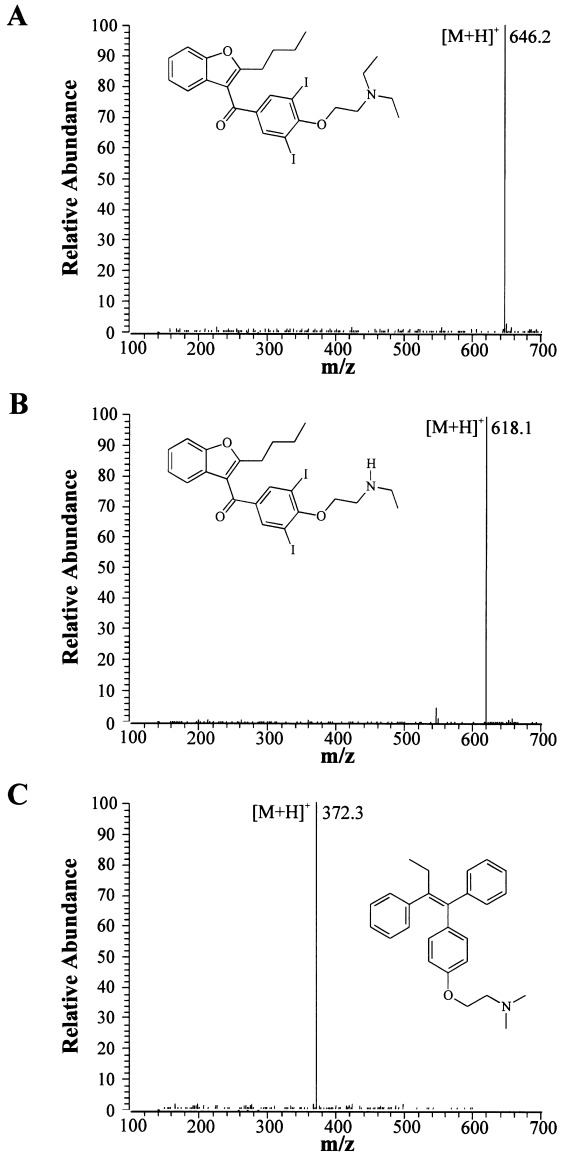

Fig. 1. Electrospray ionization mass spectra of (A) amiodarone, (B) desethylamiodarone, and (C) tamoxifen.

selection mode followed by fragmentation. The processes that occur in the ion trap detector can be broken down into the following steps: ionization of the molecules, storage of the ions formed in the ion source, selection of ions of a single mass-to-charge ratio (precursor ions) and ejection of all other ions, collision induced dissociation of the precursor ions, and detection of the product ions formed. In comparison to LC–MS, this technique produces a higher signal-to-noise ratio, which is hardly affected by the matrix. Hence, analytical background noise has significantly less influence on product ion chromatograms and mass spectra obtained by the LC–MS–MS technique than on those generated by LC–MS. The main advantage of the LC–MS–MS technique compared to the CID-LC–MS interface, where analyte fragments can also be detected, is that there are no uncertainties as to the origin of the fragments observed in the product ion spectra. In comparison to a triple-quadrupole mass spectrometer, the ion trap has the advantage that full scan product ion spectra can be acquired without any loss of sensitivity.

3.2. Extraction efficiency

The extraction recoveries were calculated at analyte concentrations of 100, 1000, and 3000 μg/l, by comparing the analyte areas to external standard calibration curves that were obtained from unextracted reference standards at concentrations ranging from 50 to 3500 μg/l.

The recovery for all compounds was greater than 89% at all three concentrations tested (Table 1).

3.3. Linearity, limit of detection (LOD), and limit of quantitation (LOQ)

Calibration lines for amiodarone and desethylamiodarone were linear in the concentration range investigated with coefficients of determination (r^2 values) ≥0.996. Slopes and intercepts, the standard deviations of the slope and the intercept, and the coefficients of determination were as follows: for amiodarone, $y = (0.0033 \pm 0.0001)x + (0.9114 \pm 0.2302)$, $r^2 = 0.996$; desethylamiodarone, $y = (0.1425 \pm 0.0036)x + (34.50 \pm 8.825)$, $r^2 = 0.997$.

The limit of detection (LOD), defined as the lowest concentration of the analyte which can be detected with a signal-to-noise ratio greater than 7:1, was established by serial extraction of plasma samples spiked with decreasing concentrations of amiodarone and desethylamiodarone. The LOD was

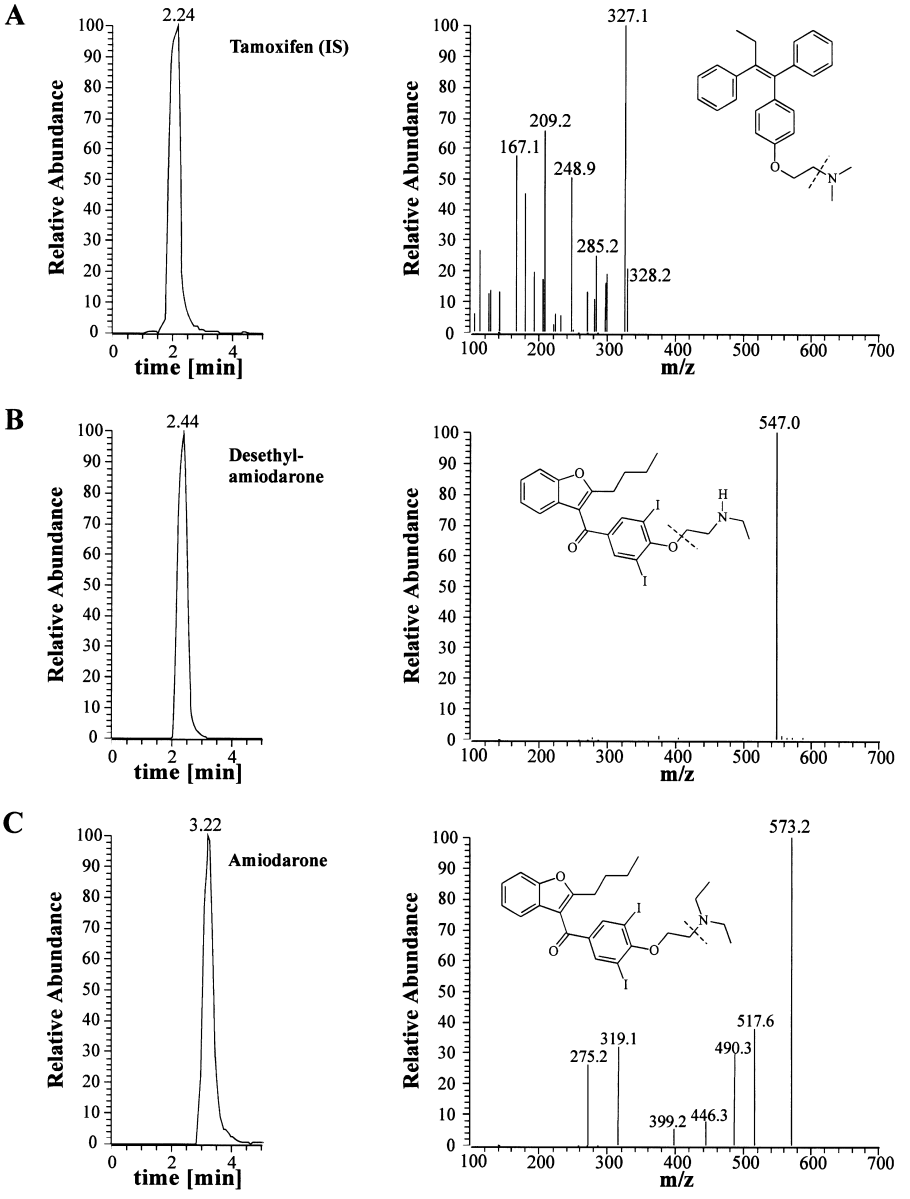

Fig. 2. Product ion chromatograms obtained from an extract of human plasma spiked with 500 μg/l of (A) tamoxifen, (B) desethylamiodarone, and (C) amiodarone. The product ion traces are *m*/*z* 327 for tamoxifen, *m*/*z* 547 for desethylamiodarone, and *m*/*z* 573 for amiodarone. On the right of each chromatogram the corresponding full scan product ion spectra and the proposed pattern of fragmentation are shown.

1 μg/l for amiodarone and 0.5 μg/l for desethylamiodarone.

The limit of quantitation (LOQ) was defined as the lowest concentration of the analyte at which the coefficient of variation (C.V.) was ≤20% and the accuracy was within ±20% of the true value. The limit of quantitation was determined as 50 μg/l for both drugs.

Table 1
Absolute extraction efficiencies ($n=5$) of amiodarone and desethylamiodarone

Amiodarone (mg/l)	Recovery (%)	Desethylamiodarone (mg/l)	Recovery (%)
0.1	91.4	0.1	92.4
1.0	89.9	1.0	94.6
3.0	93.2	3.0	96.8

3.4. Accuracy and precision

In order to evaluate the accuracy and precision of the assay, analyses of the prepared quality control samples containing amiodarone and desethylamiodarone at concentrations of 100, 800, and 3500 μg/l were performed. Results are summarized in Table 2. To determine intra-assay accuracy and precision, five replicate analyses were performed at each of the three concentrations. Inter-assay accuracy and precision were determined at each of the three concentrations over a period of 10 days, by establishing calibration curves for the analytes on eight different days. The three quality control samples were analyzed once.

The mean, standard deviation, and percent coefficient of variation (%C.V.) for amiodarone and desethylamiodarone were calculated at each concentration. The intra-assay %C.V. for amiodarone and desethylamiodarone were ≤7.9%. All inter-assay %C.V. were below 14.6%. The accuracies, referred to as % of Target in Table 2, were determined by comparing the mean calculated concentration with the spiked target concentration of the quality control samples. The intra- and inter-assay accuracies for all analytes were found to be within 93.94 and 107.9%, respectively, of the target values. The relative error (%RE) calculated as % of target − 100% showed that the mean calculated concentrations where within ±7.9% of the nominal values (Table 2).

3.5. Clinical applicability of the assay

Plasma samples of 15 patients taking 400 mg of amiodarone three times daily were analyzed with this procedure. Plasma concentrations ranged from 1478 to 1983 μg/l of amiodarone and from 522 to 1008 μg/l of desethylamiodarone. The product ion chromatograms and corresponding spectra obtained from an extracted plasma sample of one of the patients are given as an example in Fig. 3. Measured plasma concentrations were 1703 μg/l of amiodarone and 705 μg/l of desethylamiodarone.

4. Conclusion

The presented assay is the first HPLC–ESI-MS–MS method that allows for the simultaneous de-

Table 2
Inter- and intra-day accuracy and precision data for amiodarone and desethylamiodarone

	100 μg/l Amiodarone	800 μg/l Amiodarone	3500 μg/l Amiodarone	100 μg/l Desethylamiodarone	800 μg/l Desethylamiodarone	3500 μg/l Desethylamiodarone
Intra-assay ($n=5$)						
Mean	95.78	770.8	3600	97.74	860.6	3703
SD	4.90	31.81	194.2	7.14	58.02	294.6
%C.V.	5.12	4.13	5.39	7.31	6.74	7.96
% of Target	95.78	96.35	102.9	97.74	107.6	105.8
%RE	−4.2	−3.6	2.9	−2.3	7.6	5.8
Inter-assay ($n=8$)						
Mean	107.9	791.3	3750	106.5	751.5	3303
SD	12.71	73.26	386.9	12.53	75.85	481.8
%C.V.	11.78	9.26	10.32	11.75	10.09	14.59
% of Target	107.9	98.91	107.1	106.6	93.94	94.37
%RE	7.9	1.1	7.1	6.6	−6.1	−5.6

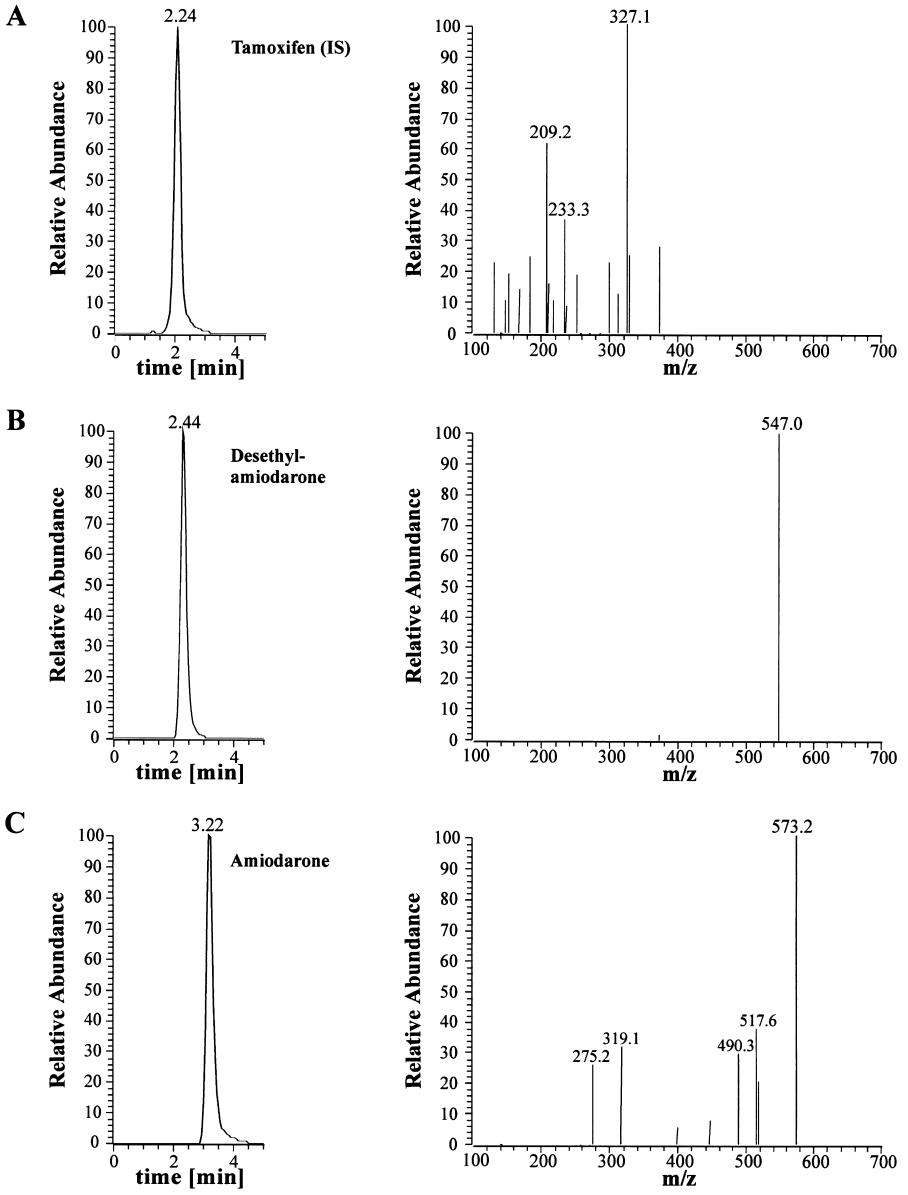

Fig. 3. Product ion chromatograms and spectra of an extracted patient plasma sample containing 1703 μg/l amiodarone and 705 μg/l desethylaminodarone. (A) Tamoxifen as the internal standard, (B) desethylaminodarone, and (C) aminodarone.

termination and quantitation of amiodarone and desethylamiodarone in human plasma. The simple mixed mode SPE procedure provides a highly efficient sample clean up with excellent recoveries from samples with analyte concentrations ranging from subtherapeutic to toxic values. The combination of HPLC and MS–MS with an ESI interface leads to specificity and sensitivity of drug identification, which is crucial in both clinical and forensic applications.

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