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Characterization of amiodarone metabolites and impurities using liquid chromatography/ atmospheric pressure chemical ionization mass spectrometry

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Using the high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) technique, together with established trends from the literature, the structures of metabolites and impurities of amiodarone, an anti-arhythmic drug, have been assigned. By comparing analyses of products of incubation with rat liver microsomes with controls in which glucose 6-phosphate dehydrogenase was omitted, metabolites could be distinguished from impurities. Structures for the two proposed metabolites and four impurities are proposed. Copyright © 2000 John Wiley & Sons, Ltd.

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Amiodarone is a powerful anti-arrhythmic drug for the treatment of supraventricular and ventricular tachyarrhythmias. Amiodarone, 2-butyl-3-[3,5-diiodo-4-b-diethylaminoethoxybenzoyl]benzofuran, is a diiodinated benzofuran derivative, structurally distinct from the other classes of anti-arrhythmic agents. Previous studies have shown that the major metabolites identified in plasma from experimental animals and human are desethylamiodarone and didesethylamiodarone.[1–3]

The biotransformations that result from the metabolic pathways usually produce less lipophilic and less toxic entities, but there are many recorded examples where the metabolism of a xenobiotic results in bioactivation to a toxic metabolite. In addition, sometimes it is difficult to differentiate the metabolites from drug impurities. A critical role of any analytical group which supports the process development of a drug substance is the ability to provide purity information to process chemists in a timely manner. Accurate data are needed to evaluate the effects of changes in synthetic chemistry on the purity of the synthesized product. In order to meet these needs, the analyst must develop analytical methods which provide high quality results in the shortest amount of time possible.

The application of mass spectrometry (MS) in the field of drug metabolism and impurity profiles has been accelerated by the development of atmospheric pressure ionization techniques, including atmospheric pressure chemical ionization (APCI), which have allowed the routine coupling of high performance liquid chromatography (HPLC) and mass spectrometry (LC/MS).[4] Recently, tandem mass spectrometry (MS/MS) has been increasingly used in the characterization of metabolites and impurities of drugs. In this paper, to investigate *in vitro* metabolism and impurities for amiodarone, LC/APCI-MS and MS/MS techniques were utilized.

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EXPERIMENTAL

Materials

Amiodarone and desethylamiodarone (**M4**) were generously donated by Sanofi Recherche (Montpellier, France). Methanol and acetonitrile were HPLC grade from Merck Ltd (Poole, UK). Glucose 6-phosphate, ß-$NADP^+$ and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

In vitro incubation

Rat liver microsomal preparations (0.5 mg) were preincubated with 10 μL of amiodarone (10 mM) in 50 μL of potassium phosphate buffer (pH 7.4, 0.1 M) at 37 °C for 10 min. Incubation was started by the addition of 1 unit of glucose 6-phosphate dehydrogenase with a NADPH generating system (50 μL of glucose 6-phosphate (0.1 M), 25 μL of β-$NADP^+$ (10 mg/mL)), and continued for 2 h. Reaction was terminated by the addition of 2 mL of methylene chloride. The mixture was shaken mechanically for 10 min and centrifuged at 2000 rpm for 5 min and the organic layer was transferred to other vials and analyzed by LC/MS.

Instrumentation

HPLC separation was carried out using a Hewlett-Packard 1100 HPLC system (Palo Alto, CA, USA). Amiodarone, its metabolites and impurities were separated isocratically on a

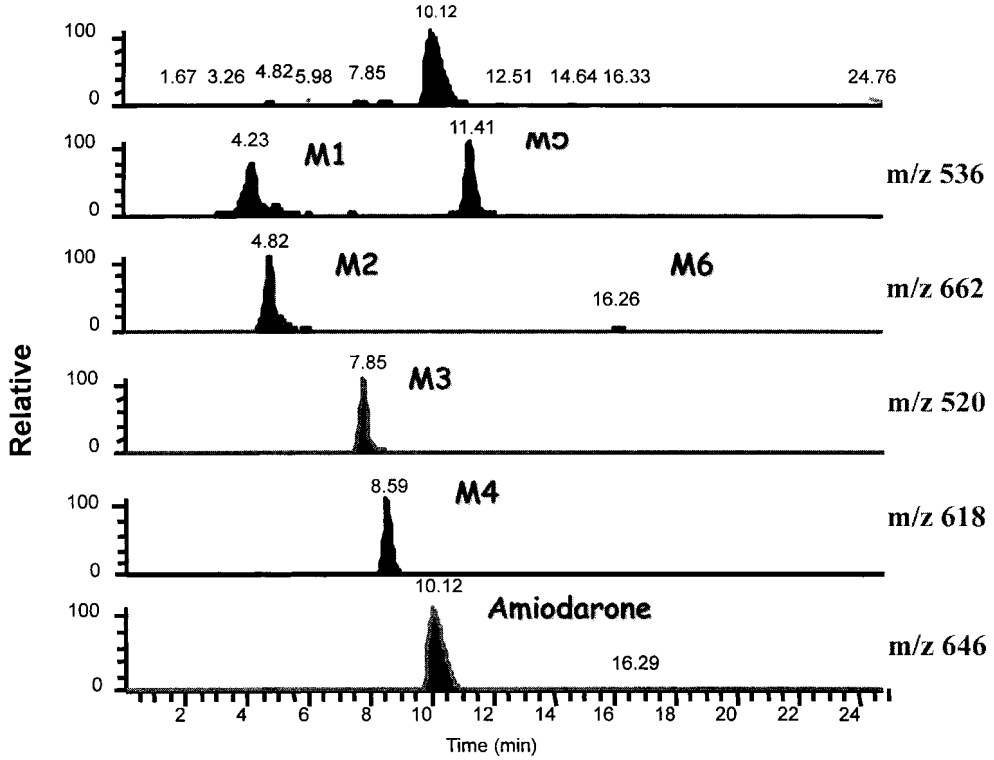

Figure 1. Extracted mass chromatograms from LC/MS data obtained under isocratic chromatographic conditions. Top panel is the total ion chromatogram, and the others are the extracted ion chromatograms indicated.

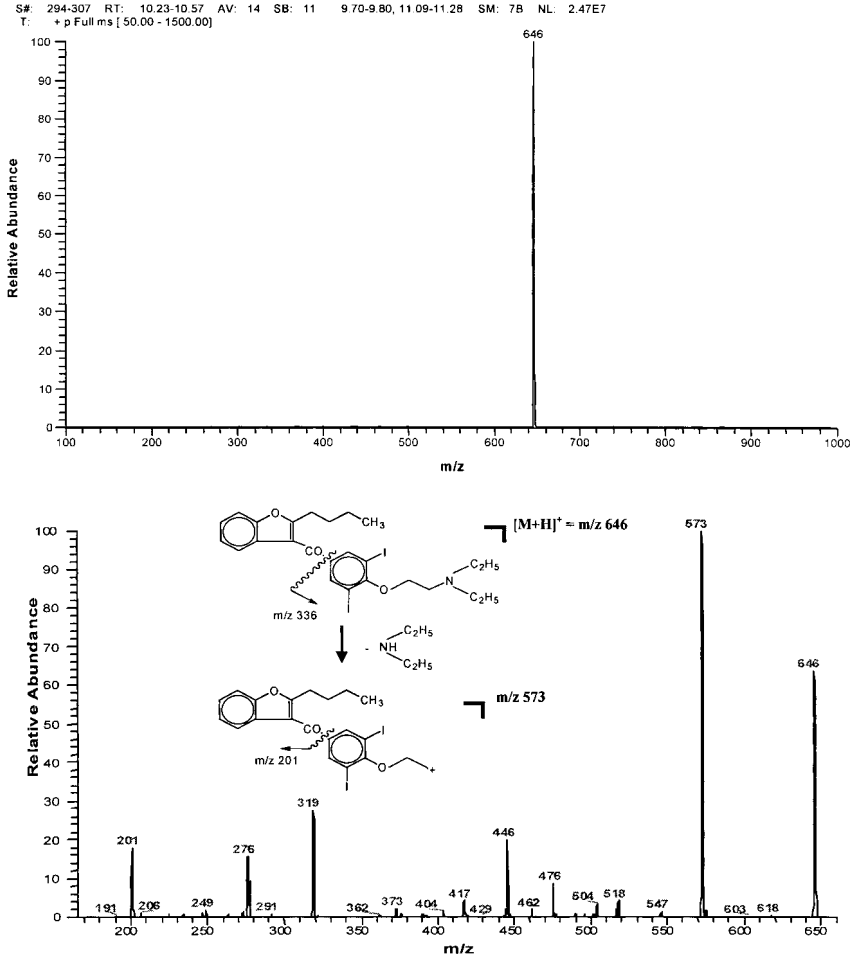

Figure 2. HPLC/APCI-MS spectrum (upper panel) and MS/MS spectrum (lower panel) of the MH^+ ion at *m/z* 646 for amiodarone.

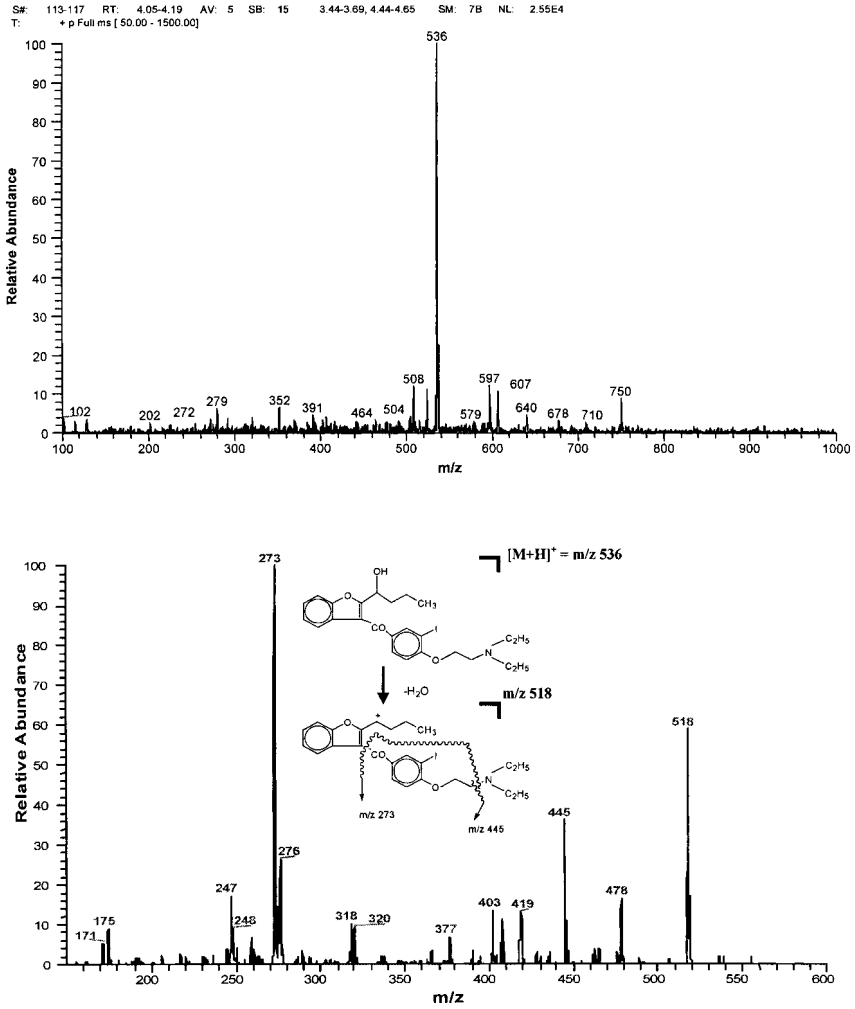

Figure 3. Mass spectrum (upper panel of **M1**) and MS/MS spectrum (lower panel) of the MH^+ precursor ion at *m/z* 536. The precursor ion is not shown in the MS/MS spectrum.

RP-18 GP (Kanto Chemical Co., Japan) column, 4.6 × 250 mm, 5 μm particle size. Methanol/acetonitrile/acetic acid buffer (40:35:25, pH 3.5) was used as mobile phase with a flow rate of 0.8 mL/min. Samples were analyzed using a LCQ mass spectrometer (Finnigan, USA) fitted with an atmospheric pressure chemical ionization (APCI) source. The flow leaving the conventional HPLC column was split in a ratio of 50:1, such that only 40 μL/min entered the mass spectrometer. The temperature of vaporizer and capillary were 350 and 150 °C, respectively. The potential on the corona discharge needle was 6 kV, and the MS/MS precursor selection width and collision energy were 2 Da and 30% of the full excitation voltage (5 V), respectively.

RESULTS AND DISCUSSION

Identification of the metabolites and impurities

The characterization of amiodarone metabolites and impurities was performed by HPLC/APCI-MS/MS. Comparisons among authentic standards, control samples (without glucose 6-phosphate dehydrogenase), and *in vitro* incubation samples (with the NADPH generating system) showed that **M4** and **M6** are the major metabolites of amiodarone, while the others (**M1, M2, M3** and **M5**) are identified as trace impurities from the synthesis of authentic compounds. The six metabolites and impurities were identified in *in vitro* incubation samples, based on both the presence or absence of peaks in the chromatograms compared with those in the corresponding *in vitro* control sample, and on their characteristic MS/MS spectra. The total ion chromatogram (TIC) and extracted mass chromatograms from LC/MS experiments on an incubation sample scan are shown in Fig. 1. Most of the metabolites and impurities except **M5** and **M6** were eluted prior to amiodarone in the reversed phase HPLC system. In positive ion mode, amiodarone is observed as its protonated molecule (MH^+) at *m/z* 646 (10.1 min), whilst the other metabolites were detected as their MH^+ ions at *m/z* 536 (**M1**), 662 (**M2**), 520 (**M3**), 618 (**M4**), 536 (**M5**) and 662 (**M6**) with retention times of 4.2, 4.8, 7.8, 8.5, 11.4 and 16.2 min, respectively.

Figure 2 shows the mass spectrum for the drug (amiodarone, upper panel), and the product ion mass spectrum (lower panel) from the MH^+ precursor at *m/z* 646. The product ion spectrum shows a predominant fragment ion at *m/z* 573, which corresponds to loss of diethylamine. The product ions at *m/z* 201 and 446 are produced by cleavage between the benzofuran and diio-

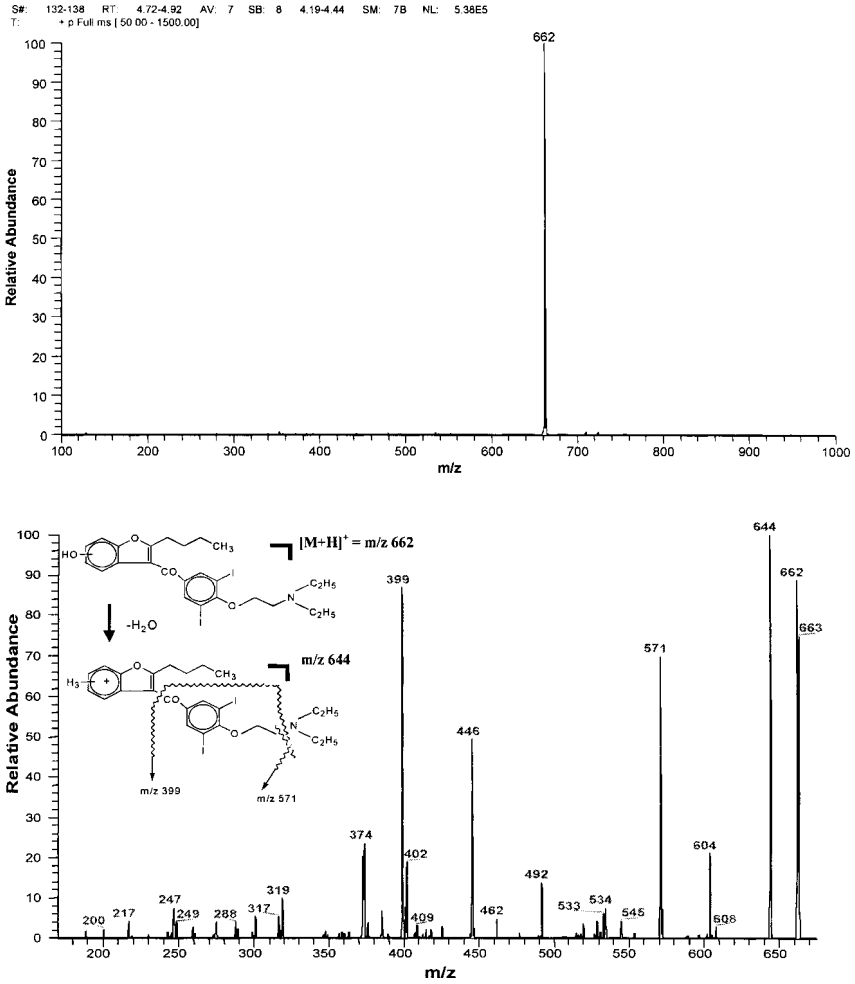

Figure 4. HPLC/APCI-MS full-scan spectrum (upper panel) and MS/MS spectrum (lower panel) of the MH^+ precursor ion at *m/z* 662 for **M2**. The precursor ion and the $[MH - H_2O]^+$ fragment are shown at almost the same intensity.

doethylaminoethoxybenzoyl moieties. These ions provide important clues for the characterization of the changes in the metabolites and impurities, relative to the parent drug, by interpretation of the fragment ion spectra.

The molecular weight of **M1** is 535 u (MH^+ at *m/z* 536; see the mass spectrum in Fig. 3, upper panel). The mass difference of 110 Da relative to the molecular mass of the parent drug indicates that this compound corresponds to amiodarone that has undergone aliphatic hydroxylation together with loss of one iodine atom (Fig. 3). The product ion spectrum of *m/z* 536 (Fig. 3, lower panel) shows a predominant loss of water (*m/z* 518) and no surviving precursor ion. Generally the loss of water from a phenolic position is difficult and not common,[6] strongly suggesting the loss of water from aliphatic hydroxylation. The fragment ions at *m/z* 273 and 445 are 126 u lighter relative to the fragment at *m/z* 399 and 571 of the product ion spectrum of **M2**, respectively. This suggests that **M1** and **M2** are related by H/I substitution on the diiodoethylaminoethoxybenzoyl moiety, which is intact in **M2**.

Based on its molecular mass (MH^+ at *m/z* 662) and elution time, **M2** can be rationalized as a hydroxylated metabolite. The likely site for hydroxyl incorporation is the benzene ring within the benzofuran ring system. In phase I metabolism, aromatic hydroxylation is a very common reaction for drugs and xenobiotics containing a benzene ring.[5] As shown in Fig. 4, the product ion spectrum gives rise to a fragment at *m/z* 644, which corresponds to loss of H_2O from the MH^+ ion, and to other fragments at *m/z* 571 and 399. Although under LC/APCI-MS conditions the loss of water is not favored when hydroxylation occurs at the phenolic position, because in this case the MH^+ ion (*m/z* 662) shows almost the same intensity as the base peak (*m/z* 644) under APCI-MS/MS conditions, this assumption seems reasonable. The fragment ion at *m/z* 571 results from the loss of water from MH^+ followed by subsequent loss of diethylamine. The expected fragment ion corresponding to the unchanged benzofuran ring at *m/z* 201 was not found, but an ion was observed at *m/z* 446, which can be interpreted to mean that the diiodoethylaminoethoxybenzoyl moiety is unchanged.

The **M3** compound was found to give rise to an MH^+ ion at *m/z* 520 with a retention time of 7.8 min (Fig. 5, upper panel). The mass difference of 126 u relative to the parent drug indicates that amiodarone has undergone H/I substitution. From the MS/MS spectrum of the MH^+ ion (Fig. 5, lower panel), the base peak at *m/z* 447 is analogous to the ion at *m/z* 573 formed from the parent drug through the loss of

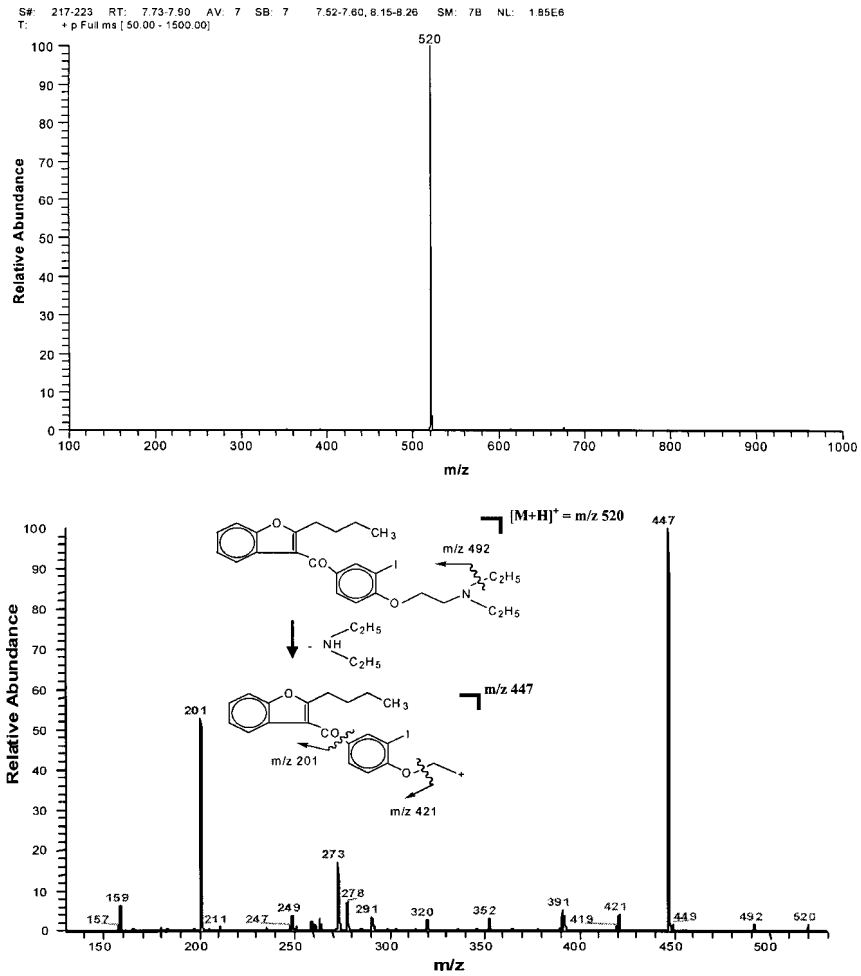

Figure 5. HPLC/APCI-MS spectrum (upper panel) and MS/MS spectrum (lower panel) of the MH^+ precursor ion at *m/z* 520 for **M3**. The ion corresponding to loss of diethylamine is detected at *m/z* 447.

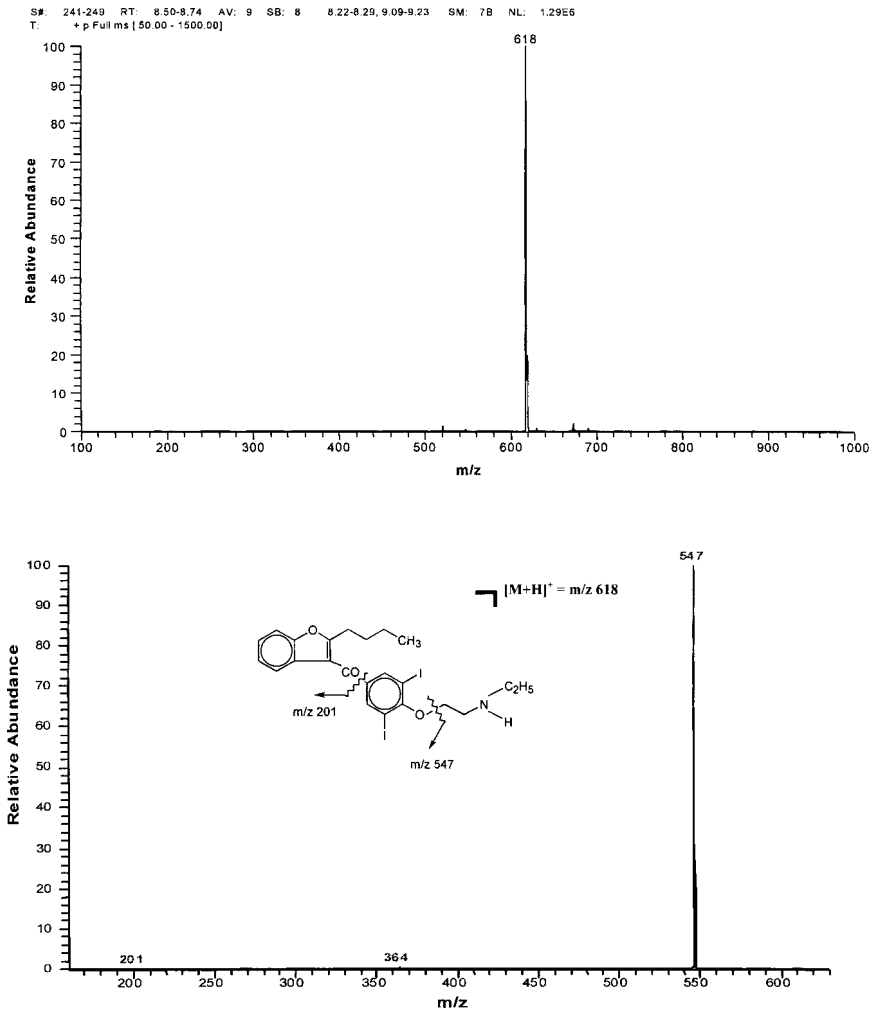

Figure 6. Mass spectrum (upper panel) and MS/MS spectrum (lower panel) of the MH^+ ion of **M4**.

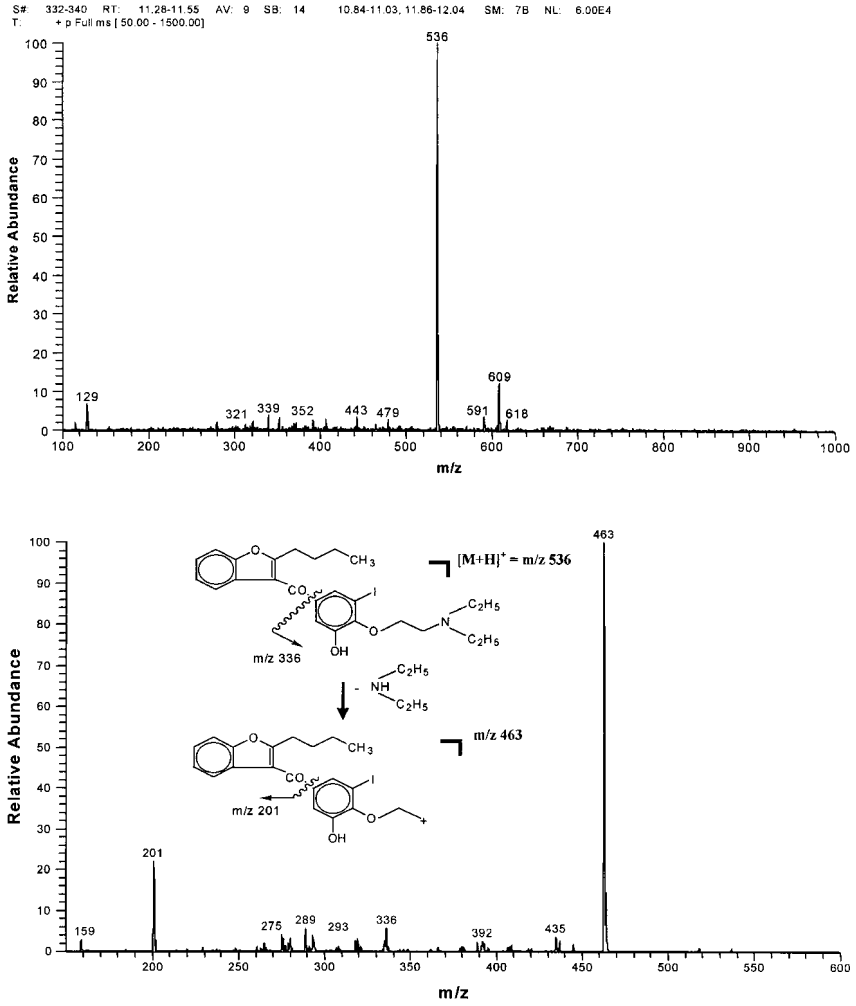

Figure 7. HPLC/APCI-MS full-scan spectrum (upper panel) and MS/MS spectrum (lower panel) of the MH^+ ion at *m/z* 536 for **M5**. The base peak results from the loss of the *N*-diethyl group as diethylamine.

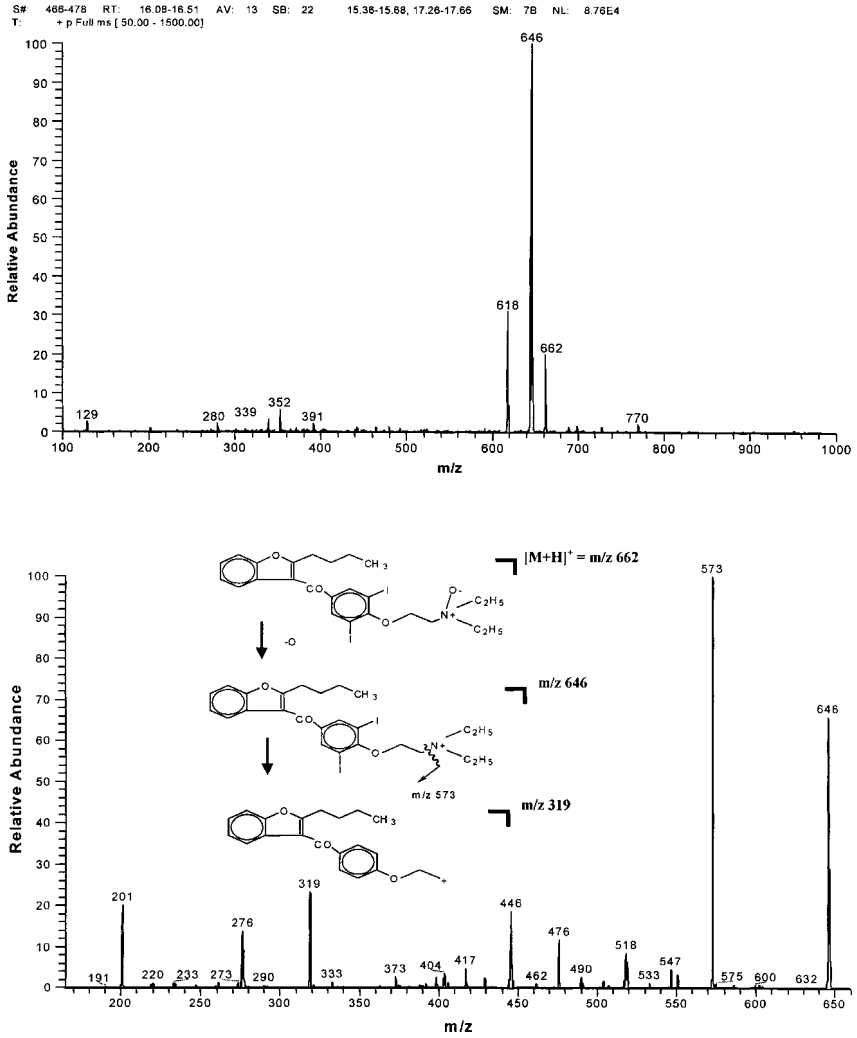

Figure 8. Mass spectrum (upper panel) and MS/MS spectrum (lower panel) of the MH^+ precursor ion at *m/z* 646 for **M6**. The $[MH - O]^+$ ion was detected at *m/z* 662.

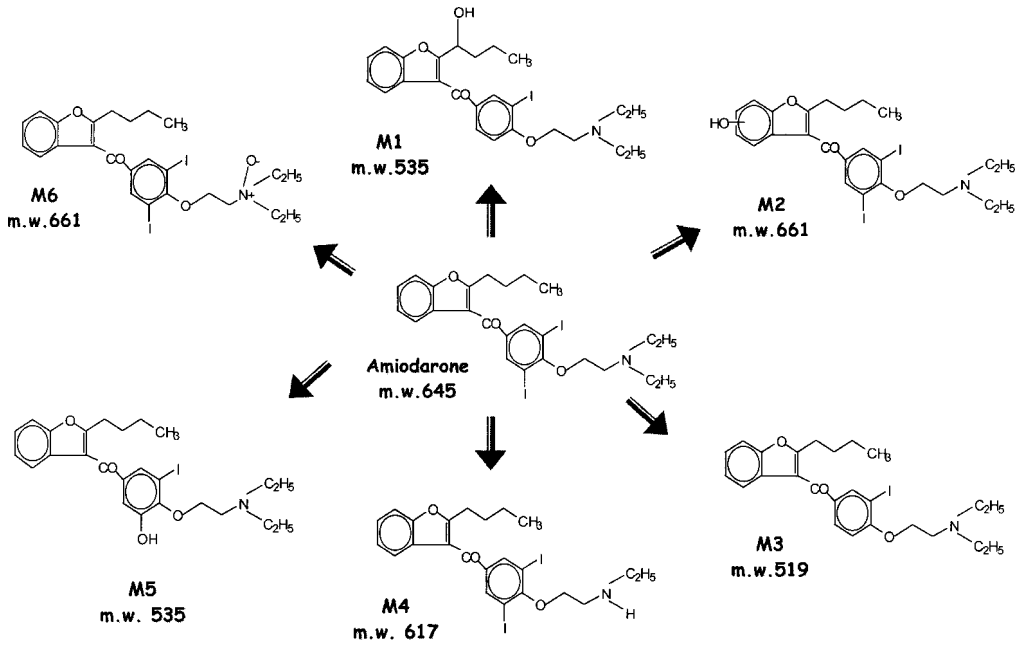

Figure 9. Proposed *in vitro* metabolites and impurities of amiodarone.

diethylamine. The characteristic ion at *m/z* 201, characteristic of the benzofuran ring, is observed. The fragment at *m/z* 492 corresponds to loss of 28 u from the MH^+ ion, possibly ethylene from the diethylamine moiety, and *m/z* 421 results from the cleavage of the O-C (ethoxy) bond with H-transfer.

The MS and MS/MS spectra of the MH^+ ion of desethylamiodarone (**M4**), already known to be a major metabolite of amiodarone, are shown in Fig. 6. The MS/MS spectrum of the MH^+ ion of the metabolite **M4** (precursor ion *m/z* 618) exhibits a predominant product ion at *m/z* 547, corresponding to the cleavage of the O-C (ethoxy) bond, and there is little or no fragmentation other than the base peak. The identification of the metabolite **M4** was confirmed by comparison with the authentic compound.

The MH^+ ion of **M5** was detected at *m/z* 536 (Fig. 7, upper panel), and **M5** was characterized as an impurity that had substituted a hydroxyl for an iodine in the parent drug. The product ion spectrum of the MH^+ ion (Fig. 7, lower panel) shows that *m/z* 463, expected from the loss of an *N*-diethyl group as diethylamine, is the base peak, and *m/z* 201, which is the ion characteristic of the unchanged benzofuran moiety, is observed as an abundant ion.

The metabolite **M6** (MH^+ at *m/z* 662) is assigned as the *N*-oxide of amiodarone (Fig. 8). The base peak of the mass spectrum is *m/z* 646, which is the $[M+ H - O]^+$ ion. As observed previously, *N*-oxides produce abundant $[M + H - O]^+$ ions under LC/APCI-MS conditions.[6] The loss of the *N*-diethyl group leads to the fragment at *m/z* 573, and the characteristic ion at *m/z* 201 is again observed.

CONCLUSIONS

Using the HPLC/APCI-MS/MS technique, together with established trends from the literature, the structures of metabolites and impurities of amiodarone have been assigned. The summarized results for the two proposed metabolites and four impurities are shown in Fig. 9. Knowledge of these metabolites and impurities will be helpful in future xenobiotic studies and in providing purity information for synthetic processes.

Acknowledgement

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