METABOLISM OF AMIODARONE (PART I): IDENTIFICATION OF A NEW HYDROXYLATED METABOLITE OF AMIODARONE

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ABSTRACT:

Amiodarone (AMI) is a potent antiarrhythmic drug, but its metabolism has not yet been fully documented. Mono-N-desethylamiodarone (MDEA) is its only known metabolite. Our preliminary investigations using rabbit liver microsomes had shown that in vitro AMI was biotransformed to MDEA, and the latter was rapidly further biodegraded to other unknown products. The aim of the present study was to investigate the chemical structure of the biotransformed compound of MDEA. Upon incubation of MDEA with rabbit liver microsomes and NADPH as cofactor, MDEA was biotransformed into three unknown products: X1, X2, and X3. The products were purified using chromatography. The chemical structure of the major product, X1, was investigated in detail. HPLC-ESI-MS revealed that MDEA had been oxygenated. Hydrogen-deuterium exchange experiments showed that the X1 molecule contained one exchangeable hydrogen atom more than its precursor MDEA, indicating that MDEA had been hydroxylated. Further results from ESI-MS/MS analysis indicated that the site of hydroxylation was the n-butyl side chain. NMR analysis (1H NMR, one-dimensional-total correlation spectroscopy, and heteronuclear multiple-bond correlation spectroscopy) established the 3-position (ω-1) of the butyl moiety as the specific carbon atom that is hydroxylated. Rat liver microsomes were also able to catalyze MDEA hydroxylation. Compound X1, as analyzed by HPLC-ESI-MS and ESI-MS/MS, was detected in the liver, heart, lung, and kidney tissue of four rats receiving AMI, suggesting that the hydroxylated MDEA was a secondary metabolite of AMI. Conclusion: in mammals, MDEA is hydroxylated to the secondary metabolite of AMI (2-(3-hydroxybutyl)-3-[4-(3-ethylamino-1-oxapropyl)-3,5-diiodobenzoyl]-benzofuran).

Amiodarone (AMI) {2-butyl-3-[4-(3-diethylamino-1-oxapropyl)-3,5-diiodobenzoyl]-benzofuran} is a potent antiarrhythmic and anti-anginal drug. In humans, mono-N-desethylamiodarone (MDEA) is the only known metabolite of AMI (Flanagan et al., 1982), and cytochrome P450 3A isoforms are involved in this dealkylation (Ha et al., 2000).

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1 Abbreviations used are: AMI, amiodarone; PB7.4, 0.1 M sodium phosphate buffer, pH 7.4; ESI-MS, electrospray ionization mass spectrometry; GC, gas chromatography; HPLC, high-performance liquid chromatography; MDEA, mono-N-desethylamiodarone; MS/MS, tandem mass spectrometry; HMBC, heteronuclear multiple-bond correlation spectroscopy; H/D, hydrogen-deuterium; 1D and 2D, one- and two-dimensional, respectively; TOCSY, total correlation spectroscopy; DQF-COSY, double quantum-filtered correlation spectroscopy; HSQC, heteronuclear single quantum correlation; gs, gradient-selected; aq., aqueous; amu, atomic mass units.

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1992, 1996; Fabre et al., 1993). During long-term therapy, the plasma level of MDEA is comparable with that of AMI, and this metabolite may contribute to the therapeutic effect of AMI.

Metabolically, it is possible that MDEA is further dealkylated to di-N-desethylamiodarone. The latter has been positively identified in plasma and myocardial tissue of dogs but not in the plasma of humans (Latini et al., 1984). In previous studies, Storey et al. (1969) reported that MDEA concentration in serum and organ tissue of rabbits receiving AMI (peritoneally 40 mg/kg/day for 4 weeks) was very low. Kannan et al. (1985) analyzed the bile of rabbit receiving AMI (20 mg/kg/day for 6 weeks) and found two chromatographic signals other than those corresponding to AMI and MDEA. The identity of these compounds was not investigated. These data suggested strongly that 1) other metabolic pathways of AMI may exist; 2) AMI metabolism is species-dependent; and 3) in rabbits, the clearance of AMI is particularly high, and in Storey’s experiments, MDEA is further biotransformed to other unknown products.

These data seem to be in agreement with our own recent observations. Indeed, when AMI was incubated with mammal (e.g., rabbit, rat) liver microsomes in the presence of NADPH, the drug was biotransformed to MDEA and more than three other high-performance liquid chromatography (HPLC)-detectable compounds (submitted for publication). Moreover, the peak height of one signal was directly related to the MDEA concentration in the incubation, sug-
gesting that MDEA was its precursor. Thus, MDEA was selected as the substrate since it provided the most efficient means of generating, isolating, and characterizing the structure of this material. Finally, formation of this new compound was investigated both in vitro and in vivo in the rat.

**Experimental Procedures**

**Chemicals.** MDEA was a gift from SANOFI Research Center (Montpellier, France). The chemicals, such as tri-isocitric acid, NADPH, MgCl₂•6H₂O, Na₂HPO₄, and isotropic dehydrogenase (EC 1.1.1.42) from porcine heart, were purchased from Sigma and Fluka (Buchs, Switzerland). All solvents used for solid phase extraction, HPLC, and HPLC-ESI-MS were of HPLC grade (Merck, Darmstadt, Germany).

**Biological Materials.** Rat liver microsomes were prepared as described by Meier et al. (1981). Rabbit liver microsomes were isolated from untreated male New Zealand White rabbits (3–4 kg) using the same method. Protein concentrations were measured using the method of Bensadoun and Weinstein (1976).

**Animals and Application of Amiodarone.** Two groups of four male Sprague-Dawley rats, weighing 210 ± 20 g, were used to study the detection of new metabolites of AMI after i.p. administration of the drug. The animals were housed in cages and had free access to standard diet and tap water. For 5 days, the commercially available i.v. injection solution (containing 50 mg of amiodarone hydrochloride, 20 mg of benzyl alcohol, and 100 mg of polysorbate 80 per milliliter of solution) was injected daily into four rats (100 mg/kg i.p.). The rats (n = 4) of the control group received (also i.p. injection) distilled water mixed with 20 mg of benzyl alcohol and 100 mg of polysorbate 80 per milliliter.

Twenty-four hours after the last injection, the animal was kept under general anesthesia (i.p. sodium pentobarbital 0.5 mg/kg), and the bile duct was cannulated for bile sampling (for 3 h). Thereafter, the animal was killed by overdose of anesthetic, and the organs (liver, heart, lung, and kidney) were removed, rinsed with ice-cooled 0.9% sodium chloride solution, dried between Kleenex tissues, and stored at −20°C until time of analysis.

**Biosynthesis of the Biotransformed Products of MDEA.** Empirical conditions were used to generate the MDEA biodegraded products. Rabbit liver microsomes (8 mg of protein) were suspended in 20 ml of 0.1 M sodium phosphate buffer, pH 7.4 (PB7.4). An NADPH-regenerating system (19 ml) consisting of 150 mg of tri-isocitric acid, 0.8 ml of isotropic dehydrogenase, and 0.1 g of MgCl₂•6H₂O was added. MDEA hydrochloride (0.25 mg in 0.2 ml of ethanol) was added, and the mixture was maintained at 37°C for 2 min. The reaction was then started by addition of 80 mg of NADPH dissolved in 1 ml of ice-cooled PB7.4. To monitor the reaction, every 15 min, 50 μl of the incubation was pipetted into 0.5 ml of methanol, vortexed for 30 s, and centrifuged at 1000g for 5 min. An aliquot of 50 μl was injected into the chromatograph (system A; see Instrumentation). The production of the unknown substances was reactivated after 4 h by adding 50 mg of NADP⁺ dissolved in 0.5 ml of PB7.4 and prolonging the incubation time up to 8 h. The final volume of the incubation was 40 ml.

**Isolation and Purification of the Major Unknown Product.** NMR analysis required at least 0.1 mg of the unknown compound; therefore, liquid extraction and solid phase extraction were used for isolation and purification of products. The incubation medium (40 ml) which had been previously conditioned successively with 5 ml of methanol, 3 ml of ethanol, 3 ml of n-hexane, and 3 ml of chloroform. The extraction device was then washed with 6 ml of n-hexane and 5 ml of acetic acid (2%) in diethyl ether. The unknown products were then eluted with three portions (2 ml) of chloroform:2-propanol (2:1 v/v). The fractions were pooled and evaporated to dryness under reduced pressure and stored under nitrogen at −20°C.

The residue was dissolved in 0.5 ml of methanol and purified by means of semipreparative HPLC (system B; see Instrumentation). The retention times of the major unknown product and MDEA were 12.6 min and 19.8 min, respectively. Between 12 min and 14 min, the eluent was collected, pooled, and evaporated to dryness under the reduced pressure at 22°C. Finally, the residue was suspended in 2 ml of PB7.4, and the unknown products were extracted three times with 3 ml of diethyl ether. The hydrochloride salt was obtained by adding 1 ml of 1% HCl in methanol and evaporating to dryness.

**Experiments Performed with Rats.** In vitro. Rat liver microsomes were also able to catalyze MDEA hydroxylation. However, to produce HPLC signals comparable with those observed with rabbit liver microsomes, 2 mg/ml microsomal protein and 30 μM MDEA must be used. To characterize the unknown products generated from rat liver microsomes, the incubation medium was extracted with diethyl ether as described above and analyzed using HPLC systems A and C (see Instrumentation).

**HPLC system A.** The analytical assay was operated under the following conditions: Nucleosil 100-5 Protect-1 250-×-4 mm column (Macherey-Nagel) maintained at 45°C; mobile phase consisting of methanol:water:aq.NH₃ 25% (300:80:0.25 w/w); flow rate of 1.2 ml/min, and pressure of 190 bar.

**HPLC system B.** For the semipreparative HPLC assay, system A was modified as follows: Nucleosil 100-7 C₁₈, 125-×-10 mm column maintained at 22°C; mobile phase consisting of methanol:water:aq.NH₃ 25% (300:65:0.50 w/w); flow rate of 2 ml/min; and fractions collector (LKB Superac 2211) setting at 5-s intervals between 12 and 14 min. At 15 min, the flow rate was increased to 3 ml/min, and the column was washed with 12 ml of methanol containing 5%aq.NH₃ and re-equilibrated with the mobile phase for 5 min.

**HPLC system C.** HPLC-ESI-MS experiments were performed on an HP 1100 HPLC system (Hewlett Packard, Palo Alto, CA). The assay was operated under the following conditions: HPLC column (RP-C₄ Waters Symmetry, 150 × 2 mm) maintained at 40°C; variable-wavelength detector setting at 242 nm. The same mobile phase as that used in HPLC system A was pumped through the column at a flow rate of 0.2 ml/min, generating a pressure of 70 bar. The ESI-MS detector was interfaced directly to the output of the UV.

**ESI mass spectra were obtained using a Bruker ESQUIRE-100 quadrupole ion-trap instrument (Bruker-Franzen GmbH, Bremen, Germany) connected to an orthogonal electrospray ion source (Hewlett Packard). The MS detector was operated under the following conditions: nitrogen nebulizer gas, 20 psi; nitrogen dry gas, 6 l/min; dry temperature, 300°C; capillary voltage, 4000 V; end-plate, 3500 V; capillary exit, 132 V; and skimmer 1, 42 V. The MS acquisitions were performed at normal resolution (0.6 amu at the half-peak height), under ion charge control conditions (10,000), and in the mass range from m/z 100 to 1000 with a m/z 60 cut-off value. To get a representative mass spectra, eight scans were averaged.

**Hydrogen-deuterium (HD) exchange.** The HD exchange experiments were performed by dissolving MDEA and unknown product in 99.96% CD₃OD.
(Cambridge Isotope Laboratories, Woburn, MA) and waiting for 10 min to achieve a complete exchange of hydrogen atoms.

**HPLC-ESI-MS/MS and ESI-MS/MS analysis using direct flow injection technique.** The HPLC eluent or the solutions were introduced continuously through the electrospray interface at a rate of 0.24 ml/h. The MS instrument operated under the following conditions: nitrogen, 15 psi; nitrogen dry gas, 6 ml/min; dry temperature, 250°C; capillary current, 4200 V; endplate, 3700 V; capillary exit, 100 V; and skimmer 1, 35 V. The MS/MS acquisitions were performed at normal resolution (0.6 amu at the half-peak height) in the mass range from m/z 100 to 600 with a m/z 60 cut-off value. The isolation width was monoisotopic (m/z 0.7), the fragmentation cut-off set by “fast calc”, and the fragmentation amplitude set at 0.9 V.

**Experimental Procedures**

**Biosynthesis and Purification of the Major Unknown Product X1.** Upon incubation of MDEA with rabbit liver microsomes in the presence of the NADPH-generating system at pH 7.4, at least three unknown compounds, X1, X2, and X3, with respective retention times of 4.26, 5.67, and 6.07 min, were observed (Fig. 1). The substrate MDEA eluted at 7.23 min. The signal at 4.26 min had the highest intensity and may correspond to the major degraded product. The reaction reached steady state after 4 h. After one reactivation cycle (see Experimental Procedures), 5 to 7% of the initial MDEA present was biotransformed into unknown products, as judged by HPLC system A.

When the incubation medium was centrifuged at high speed, MDEA and its derivatives were mainly located in the protein precipitate. Therefore, they may be isolated efficiently by washing the precipitate with methanol (five times). However, this operation was not selective for the drug as lipids were also coextracted. Separation of the drug from contaminants required considerable effort.

The first purification step was performed using a silica cartridge. Under basic conditions, MDEA was retained less than its degraded products on the silica; thus, the unreacted MDEA may be washed out using a mixture of hexane:2-propanol:aq.NH3 25%. Thereafter, mixtures of hexane:diethyl ether:acetic acid and chloroform:methanol:acetic acid:water were applied successively to the silica cartridge for eluting cholesterol, glycerides, and phospholipids (Cairns and Peters, 1983). HPLC system A was used for assaying the benzofuran derivatives in the eluents and revealed that they coeluted with phospholipids. Thus, a further purification step using NH2-sorbent and chloroform:2-propanol as eluent was necessary. Under these conditions, phospholipids were retained on the sorbent (Kaluzny et al., 1985), whereas MDEA and its derivatives were not. Thereafter, semipreparative HPLC was used to separate X1 from X2, X3, and a trace of MDEA. The fractions corresponding to the major signal were collected and evaporated to dryness. Rejected into the HPLC system A, X1 was detected as one signal at 4.26 min. To accumulate the required amount of X1 for NMR analysis, X1 was isolated from 10 incubations, and the residues were combined.

**UV Spectrum of X1.** The UV spectrum of the purified X1 in methanol showed the following characteristics: 208 nm, 242 nm (maxima), 223 nm (minimum), and 275 nm, 282 nm (shoulders) and was comparable with that of MDEA-HCl in methanol. Thus, its concentration may be calculated using the molar extinction coefficient of MDEA-HCl at 241 nm (E = 440.000 l/mmol/cm) (Plomp, 1991), and the total amount of X1 was estimated to be 0.15 mg. The compound was stable (as assayed by UV spectra measurement) in methanol at 22°C or dry-stored at −20°C for at least 10 days.

**MS Results.** Since HPLC-ESI-MS was operated using a low flow rate (0.2 ml/min), HPLC system A was modified to system C. When the incubation medium was extracted with diethyl ether and immediately analyzed, the same chromatogram as that shown in Fig. 1 was obtained. The UV signals of X1, X2, X3, and MDEA displaced to 4.4, 5.4, 6.0, and 7.45 min, respectively (data not shown). ESI-MS spectra...
revealed the quasi-molecular ions ([M + H]^+) at m/z 618 and 634 for MDEA and X1, respectively (Fig. 2, a and b). Compared with MDEA, the molecular ion of X1 showed a mass shift of +16 amu, suggesting that the MDEA molecule may have been oxygenated. The mass spectra of the minor products X2 and X3 revealed that their quasi-molecular ions were at m/z 591 and 590, respectively (submitted for publication).

In this study, only the chemical structure of X1 was investigated. For this, MDEA and off-line purified X1 were dissolved separately in methanol (unlabeled) and d_4-methanol (labeled), and analyzed using ESI-MS and ESI-MS/MS successively. These H/D exchange experiments allowed us to investigate the number and the position of the exchangeable hydrogen atoms in the structures of MDEA and X1.

ESI-MS analysis revealed a mass shift of +2 amu for labeled MDEA (m/z 618–620, Fig. 2a insert), suggesting that, as expected, the quasi-molecular ions of MDEA contained an exchangeable hydrogen atom in addition to its site of protonation (deuteration), whereas X1 contained two exchangeable hydrogen atoms in addition to its site of protonation (deuteration) (mass shift m/z 634–637, Fig. 2b insert). Thus, based on the observations that X1 contained an additional oxygen and an additional exchangeable hydrogen atom, one may infer that its structure could have a hydroxy (OH) group.

The combination of ESI-MS/MS data obtained from unlabeled and labeled MDEA and X1 may allow the position of the OH function in the X1 structure to be determined. In fact, the ESI-MS/MS spectrum of unlabeled X1 showed the presence of fragments at m/z 563, 545 (base peak), 503, 417, 376, 373, 291, and 249 (Fig. 2d), whereas under identical analysis conditions, the MDEA molecule gave only two fragment ions at m/z values of 547 and 373 (Fig. 2c). However, analyzing [d_2-X1 + D]^+ showed three major daughter ions at m/z 566, 546, and 374 and other low-intensity signals at m/z 504, 418, 377, 292, and 250 (Fig. 2f), whereas [d_1-MDEA + D]^+ gave two major fragments at m/z 549 and 374 (Fig. 2e).

Plausible fragmentation pathways leading to the observed fragments in Fig. 2f are summarized in Fig. 3. The origin of the fragment ions at m/z 549 (Fig. 2e) and 566 (Fig. 2f) may be explained by the loss of 71 amu from the quasi-molecular ions [d_1-MDEA + D]^+ and [d_2-X1 + D]^+ (Fig. 3a), respectively. This indicated that the hydroxylation of MDEA did not take place on the -CH_2-CH_2-NH-ethyl chain. Interestingly, the fragment ions b at m/z 374 (Fig. 3b) were found for both structures of MDEA and X1 (Fig. 2, c and f). This indicated that the diiodobenzene ring of X1 was unchanged.

In ESI-MS/MS analysis, X1 gave more signals than its precursor MDEA. This was probably due to the instability of the ions a at m/z 566 (Fig. 3). In fact, they may lose D_2O (~20 amu) to give the fragment ions c at m/z 546 (Fig. 2f). In turn, ions c may be further deiodinated to fragment ions at m/z 418 and 292 (Fig. 3c). These data suggested strongly that the benzofuran moiety of X1 was not hydroxylated, because this type of “dehydration” cannot occur on an aromatic group. Additionally, a careful examination of the low-intensity signals of X1 at m/z 504, 377, and 250 (Fig. 2f) supported the hypothesis that, due to the presence of an additional OH group, the ions a may lose their n-hydroxybutyl moiety to give the fragment ions d at m/z 504. In turn, the latter were deiodinated to fragment ions at m/z 377 and 250 (Fig. 3d). These data supported the hypothesis that the OH function of X1 may be found on the -n-butyl side chain. Nevertheless, the exact position of the OH function on this part of the molecule could not be deduced from the MS data. Thus, further NMR analyses were necessary.
NMR Spectrometric Results. NMR experiments were exclusively performed with the off-line purified X1 isolated from the rabbit liver microsome experiments. The comparison of the $^1$H NMR spectra of MDEA and X1 revealed that the chemical shifts and coupling constants of the signals corresponding to protons of the 3,5-diiodo-4-ethyl-aminoethoxybenzoyl moiety and those of the benzene ring of the benzofuran group were unchanged (Table 1, the carbon atoms of the X1 molecule were arbitrarily numerated). However, the proton signals of the n-butyl group of X1 were shifted and split (Fig. 4b). The $^1$H NMR data for MDEA and X1 is in preparation.

TABLE 1

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* Chemical shifts were obtained from gs-HMBC and gs-HSQC experiments.

In vitro experiments. Methanolic extracts of organ (liver, heart, lung, and kidney) tissues obtained from rats receiving AMI were analyzed using HPLC system A. Under our HPLC conditions, AMI (retention time 8.9 min) was resolved from MDEA (retention time 7.3 min), and the UV (242 nm) signals at 4.26 and 4.84 min were found.
Eluent between 4.1 min and 4.3 min was collected, concentrated, and reanalyzed by means of HPLC-ESI-MS and ESI-MS/MS using the direct flow injection mode. ESI-MS detection revealed signals corresponding to the quasi-molecular ions \([M + H]^+\) at \(m/z\) 634 and 632. As estimated by peak area measurements, they were 85 and 15%, respectively. The fragmentation of the ions at \(m/z\) 634 using the ESI-MS/MS technique was identical to that of X1. These data confirmed that 3OH-MDEA was found in organ tissue of rats receiving AMI. Thus, it may be considered as a secondary metabolite of AMI, at least in rats. Twenty-four hours after the last injection, the concentrations of 3OH-MDEA in all organs studied were 3 to 8.5% of AMI concentrations (based on the integration of peak areas). Using the same techniques, the compounds eluting at 4.6 to 5.2 min showed quasi-molecular ions \([M + H]^+\) at \(m/z\) between 560 and 662, and might correspond to oxygenated products of AMI (submitted for publication).

**Discussion**

There are probably two main reasons why data on the metabolism of AMI are scarce. First, there is a great difference in the lipophilicity between AMI and its metabolites. Second, the appropriate analytical techniques simply were not available. The available HPLC-UV assays are not selective and sensitive enough to quantify metabolites other than MDEA. While classic gas chromatography GC-MS has excellent sensitivity, it cannot be used for AMI derivatives because the diiodobenzofuran derivatives are thermolabile. In contrast, the new HPLC-ESI-MS and ESI-MS/MS seem to be the analytical methods of choice for studying AMI metabolism: the drug and its derivatives give interpretable and high intensity MS signals. Therefore, in the current studies, our first efforts were designed to find an analytical assay capable of detecting compounds more polar than the known MDEA. This was achieved by chromatographing the AMI analogs synthesized for our previous studies (Ha et al., 2000). Then, HPLC interfaced to an ESI-MS/MS was used to detect and, in combination with NMR, identify metabolites other than MDEA. However, the method was complicated by lipid contamination emanating from the liver microsomes and led to an unstable baseline for the MS detector (ion source must be cleaned after 3–5 injections) or, in the case of NMR, interference with signals in the range of 0.8 to 2.5 ppm (Fig. 4b).

In the literature, the biotransformation of MDEA (and AMI) in rabbit liver microsomes has already been investigated by Young and Mehendale (1986). The authors reported that MDEA was deiodinated to di-deiodinated MDEA. Using HPLC-ESI-MS as an analytical tool, our detector did not reveal any ions that could be related to the presence of di-deiodinated MDEA in the incubation; in place of it, the hydroxylated MDEA was found. This disagreement is understandable because, in Young’s investigations, the identity of product was based exclusively on the comparison of retention time of the unknown product with that of the reference substance L32790 obtained from the AMI manufacturer.

Since rabbit liver microsomes were found to metabolize MDEA more rapidly than rat liver microsomes, they were used to produce X1. About 0.15 mg of X1 was obtained, and its structure was characterized as 3OH-MDEA. For drug metabolism investigations, the rat model is more often used than the rabbit; thus, efforts were made to demonstrate that the newly discovered 3OH-MDEA is also found in vitro and in vivo experiments using rats. HPLC-ESI-MS and ESI-MS/MS techniques confirmed the presence of 3OH-MDEA in the rat model.
In conclusion, the previous results (Flanagan et al., 1982) and the observations in the present study suggest that, in mammals, AMI is dealkylated to MDEA. Thereafter, MDEA (probably together with AMI) is further degraded to other compounds by hydroxylation (Fig. 5). Other studies are being planned to investigate the quantitative aspect of the hydroxylation of AMI and MDEA in laboratory animals and in humans. Only after these evaluations will the importance of the hydroxylation pathway in the metabolism of AMI be known.

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References


**Fig. 5.** Metabolism pathways of amiodarone.