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Journal of Chromatography B, 757 (2001) 309–315

JOURNAL OF CHROMATOGRAPHY B

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Metabolism of amiodarone
II. High-performance liquid chromatographic assay for mono-*N*-desethylamiodarone hydroxylation in liver microsomes

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Received 9 November 2000; received in revised form 8 March 2001; accepted 8 March 2001

Abstract

Amiodarone (AMI) is a potent antiarrhythmic drug. In vivo and in vitro, AMI is biotransformed to mono-*N*-desethylamiodarone (MDEA). Recently, it was observed that MDEA was further hydroxylated to *n*-3′-hydroxybutyl-MDEA (3′OH-MDEA). The performance of a HPLC–UV assay being developed for the quantification of the new compound was investigated. Liver microsomes isolated from rabbit, rat and human biotransformed MDEA to 3′OH-MDEA. Their estimates of Michaelis–Menten parameters were K_m=6.39, 25.2, 19.4 μ*M*; V_{max}=560, 54, 17.3 pmol/mg protein/min), respectively. Thus, hydroxylase activity in mammals may be the origin of the species dependence observed in the AMI metabolism.

Keywords: Hydroxylation; Amiodarone; Mono-*N*-desethylamiodarone

1. Introduction

Amiodarone (AMI) [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran] is a potent antiarrhythmic drug. Data on the metabolism of AMI are scarce. In mammals, only mono-*N*-desethylamiodarone (MDEA) is known as the major metabolite [1] and cytochrome P450 3A isoforms are involved in this dealkylation [2–4]. During long-term therapy, serum concentration of this pharmacologically active metabolite is comparable with that of AMI. Previous observations have suggested that other metabolites of AMI may exist but they were not documented [5–7]. Interestingly, Storey et al. [5] reported that, in contrast to rats and humans, the MDEA concentration in blood and in organ tissue of rabbits receiving AMI (intraperitoneally 40 mg/kg/day for 4 weeks) was very low. It is suspected, therefore, that in rabbits this metabolite is further biotransformed to unknown product(s). This observation prompted us to use rabbit liver microsomes to investigate the biotransformation of MDEA. It was found that, in rabbit and rat liver microsomes, MDEA is biotransformed to *n*-3′-

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00165-7

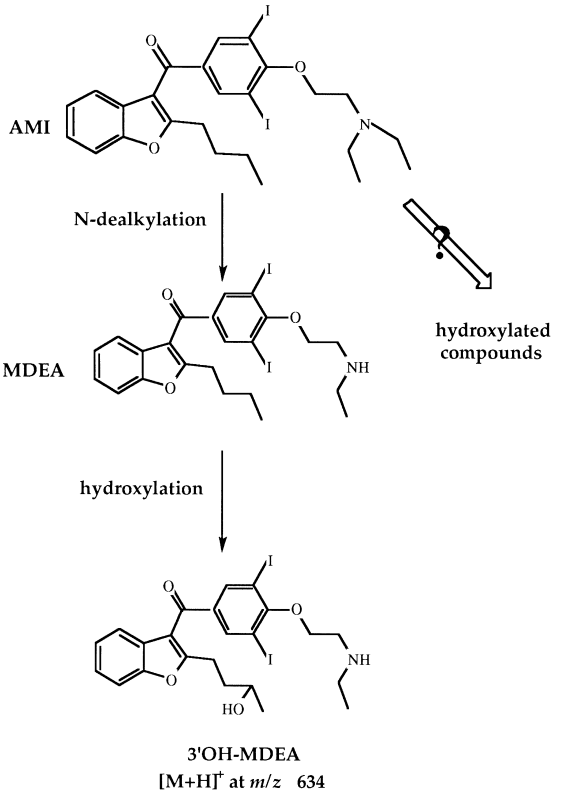

Fig. 1. Metabolism of amiodarone. Amiodarone (AMI), mono-*N*-desethylamiodarone (MDEA), *n*-3-hydroxybutyl-desethylamiodarone (3′OH-MDEA). Note: 3OH-MDEA was used in the previous paper (Ref. [8]).

hydroxybutyl-MDEA (3′OH-MDEA) [8] (Fig. 1). Using high-performance liquid chromatography (HPLC) interfaced to electrospray ionization tandem mass spectrometry (ESI-MS–MS), this compound is also found in organs tissue of rats receiving AMI·HCl (intraperitoneally 100 mg/kg for 5 days). Thus, 3′OH-MDEA may be considered as a secondary metabolite of AMI. In order to investigate the in vitro formation kinetics of this new metabolite, a quantitative assay is needed. We report here a simple HPLC–UV assay to quantify 3′OH-MDEA.

2. Experimental

2.1. Chemicals

MDEA·HCl was a gift from Sanofi (Montpellier, France). Hydroxylated MDEA (3′OH-MDEA) was isolated from the bioreaction using MDEA·HCl and rabbit liver microsomes, as described previously [8]. Its purity was 97% as assayed by HPLC–ESI-MS. The chemicals such as D,L-isocitric acid, NADPH, $MgCl_2 \cdot 6H_2O$, Na_2HPO_4 and isocitric dehydrogenase (EC 1.1.1.42) from porcine heart were purchased from Sigma and Fluka (Buchs, Switzerland). All solvents used were of HPLC grade (Merck, Darmstadt, Germany).

2.2. Biological materials

Rat liver microsomes were prepared as described by Meier et al. [9]. The rabbit liver microsome fraction was isolated from untreated male New Zealand White rabbits (3–4 kg) using the same method. Human liver microsomes were prepared from liver of three donors as described by Dayer et al. [10]. Protein concentrations were measured using the method of Bensadoun and Weinstein [11].

2.3. Instrumentation

HPLC separations of compounds were achieved using an LKB liquid chromatograph (Bromma, Sweden) consisting of two HPLC 2150 pumps, a 2152 HPLC controller, and a 2152 UV detector set at 242 nm. Signals were managed by WinChrom software version 1.3 (Dandenong, Australia). The assay was operated under the following conditions: Nucleosil 100-5 Protect1 250×4 mm (Macherey-Nagel, Oensingen, Switzerland) maintained at 45°C; mobile phase methanol–water–25% NH_3 (300:90:0.25, w/w); flow 1.2 ml/min; pressure 190 bar.

2.4. Incubation conditions

In an Eppendorf tube of 1.5 ml (Hamburg, Germany), rabbit liver microsomal protein (200 μg/ml) was incubated in 0.2 ml (final volume) of 0.1 *M* sodium phosphate buffer, pH 7.4 (PB7.4) at 37°C in the presence of an NADPH generating system (1 IU isocitrate dehydrogenase per ml, 5 m*M* isocitrate, 5 m*M* $MgCl_2$). Substrate MDEA was dissolved in

ethanol, diluted with PB7.4 to desired concentrations, and then added as a twofold concentrated solution in PB7.4. After 5 min incubation in a water bath, the reaction was started by addition of 40 μl 0.5 m*M* NADPH. In the negative control incubation, deactivated rabbit liver microsomes (heated at 90°C for 5 min) were used.

After the incubation (15 min at 37°C), the reaction was stopped by addition of 1 ml ice-cooled diethyl ether and vortex-mixed. The tube was then capped and shaken horizontally for 10 min (400 rpm). The organic phase was separated by centrifugation, pipetted into another glass test tube. To ensure a quantitative measurement, the extraction was repeated three times. The organic phases were pooled and evaporated to dryness at 21°C. The residue was then dissolved in 0.15 ml of the mobile phase, and 0.1 ml of the aliquot was injected onto the HPLC column.

Rat liver microsomes were also able to hydroxylate MDEA. However, for the same incubation time (15 min), many more rat liver microsomes (2 mg protein/ml) and substrate MDEA (30 μ*M*) are needed to generate a comparable amount of 3′OH-MDEA to that observed in experiments using rabbit liver microsomes.

Human liver microsomes biotransformed also MDEA to 3′OH-MDEA. In order to be able to measure 3′OH-MDEA by HPLC–UV assay, the incubation conditions was modified to: incubation time (20 min) and 1 mg microsomal protein in a final volume of 0.4 ml.

2.5. Standards

The purified 3′OH-MDEA was dissolved in methanol and measured in an UV spectrophotometer (Ultropec 3000; Pharmacia Biotech, Cambridge, UK). Its concentration was deduced using the molar extinction coefficient at 241 nm of MDEA·HCl ($E=$ 440 000 M^{-1} cm^{-1}) [12]. The working standards were then prepared by diluting this stock solution with the incubation medium containing 200 μg/ml deactivated proteins to give the final concentrations of the base form of 3′OH-MDEA in the range of 0.25–2.0 μg/ml. Stored in aliquots of 2 ml at −20°C, the standards were stable for at least 1 month.

2.6. Validation tests

The validation tests were performed exclusively with the rabbit liver microsomes.

The recovery test for the diethyl ether extraction was investigated using two methanolic solutions containing 0.75 and 1.5 μg/ml 3′OH-MDEA. On different occasions, 50 μl of these solutions was injected in duplicate directly into the chromatograph. The same volumes were placed into the glass tubes and evaporated to dryness. The residue was then dissolved in 0.2 ml of the incubation medium containing 200 μg/ml deactivated microsomes and extracted as described above. The ratio of peak heights was used for calculating the extraction recovery.

The pH effect on the extraction recovery using diethyl ether as solvent was investigated by dissolving 0.5 μg 3′OH-MDEA in 0.5 ml samples of various aqueous solutions with pH values ranging from 1.0 to 10.0. The solutions with pH values ranging from 3.0 to 10.0 were prepared from 0.1*M* Na_3PO_4, and pH was adjusted to desired values using 2 *M* HCl. The pH 2 solution was 0.01 *M* HCl.

The limit of detection was determined by diluting successively a standard solution containing 0.5 μg/ml 3′OH-MDEA with the incubation medium containing 200 μg/ml deactivated microsomes. Diluted samples were then extracted and analyzed. The detection limit of the assay was defined as a signal-to-baseline ratio of 3:1.

The stability of 3′OH-MDEA was studied by extracting 2 μg of the compound from 0.5 ml of the incubation medium containing 200 μg/ml deactivated microsomes into 6 ml diethyl ether. The organic phase was then divided in two parts (by weighing) and evaporated to dryness. The residue in one tube was dissolved in 600 μl of mobile phase, and 50 μl of the aliquot was injected in duplicate onto the HPLC column. The rest of the aliquot was tightly capped, stored at 22°C, and re-analyzed in duplicate after 6, 24 and 48 h. The residue of the second tube was dry stored at 4°C. Two days later, it was dissolved in 600 μl mobile phase and analyzed. The peak-heights from different occasions were compared.

Precision and accuracy of the assay were investigated by measuring seven standard samples con-

taining different amounts of 3′OH-MDEA fives times on the same day and in duplicates on 5 different days.

3. Results

Upon incubation of MDEA with rabbit liver microsomes and NADPH for 15 min, the drug was biodegraded to three unknown products X1, X2 and X3. Under the described HPLC conditions, their retention times were 4.26, 5.67 and 6.07 min, respectively (Fig. 2a). MDEA eluted at 7.23 min. The data of our previous studies demonstrated that the major compound X1 corresponds to 3′OH-MDEA [8]. The identity of the minor compounds X2 ($[M+H]^+$ at m/z 591) and X3 ($[M+H]^+$ at m/z 590) is currently being investigated.

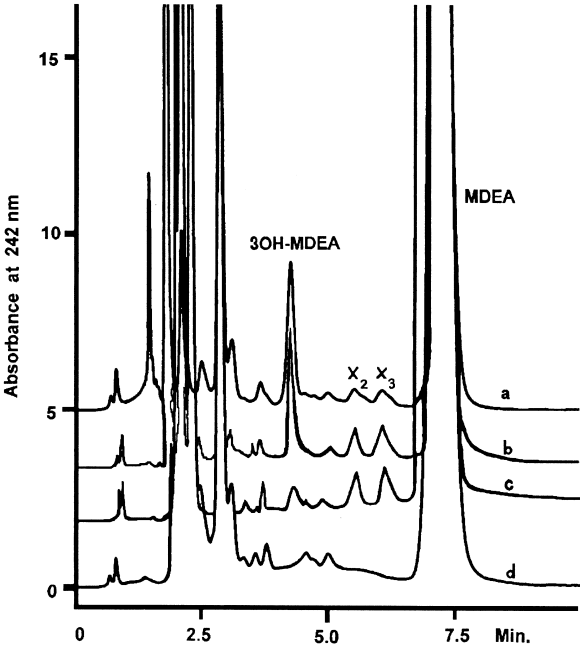

Fig. 2. Chromatogram (UV, 242 nm) obtained from the incubation of mono-*N*-desethylamiodarone (MDEA; 10 μ*M*) with rabbit liver microsomes (200 μg/ml) after 15 min (a). In order to generate the same amount of 3OH-MDEA for the same time as found for rabbit microsomes, many more rat liver microsomes (2 mg protein/ml) and MDEA (30 m*M*) must be used (b). Human liver microsomes (500 μg/ml) have also ability to hydroxylate MDEA (30 m*M*) and the incubation time was 20 min (c). Blank control (d). The retention times of 3′OH-MDEA, X2, and X3 were 4.26, 5.67, and 6.07 min, respectively. MDEA eluted at 7.23 min.

The UV spectrum of 3′OH-MDEA·HCl in methanol showed the following characteristics: 208 nm, 242 nm (maxima), 223 nm (minimum), 275 nm, 282 nm (shoulders) and comparable to that of MDEA·HCl [12]. Thus, using the molar extinction coefficient E_{241}=440 000 M^{-1} cm^{-1} of MDEA·HCl, the total amount of 3′OH-MDEA isolated in our previous studies was estimated to be 0.15 mg.

The new 3′OH-MDEA derivative was stable. Within 2 days, neither the stored temperature (from 4°C to 22°C) nor the storing conditions (dry or in methanol) seemed to have any influence on its stability. In the concentration range of 0.75–1.5 μg/ml and in the pH range of 4–9, 3′OH-MDEA may be quantitatively extracted (recovery 95±8%; n=3) by diethyl ether. This recovery decreased rapidly to 10% at pH 2. The calibration graph (peak height vs. added 3′OH-MDEA concentrations) was linear in the range 0.2–5.0 μg/ml, and the intercept was not different from zero. The correlation coefficient for the regression line was 0.999. Using 0.2 ml for extraction, the detection limit at a signal to noise of 3:1 was 0.1 μg/ml.

The inter- and intra-assay was investigated by analyzing seven standards containing 3′OH-MDEA. The spiked solutions were analyzed five times on the same day and in duplicate on 5 different days. In the concentration range of 0.5–2.0 μg/ml, the relative standard deviations (standard deviation×100/found) for the determination of 3′OH-MDEA were below 7%. At lower concentrations, e.g., twice the detection limit, it was 11.5%. The performance of the assay is summarized in Table 1.

In order to investigate the possible species dependence of AMI metabolism, rabbit liver microsomes

Table 1
Precision (RSD) and accuracy (R.E.) of 3′OH-MDEA measurement

	Added (μg/ml)	Found (μg/ml)	RSD (%)	R.E. (%)
Within-day (n=5)	0.25	0.262	11.5	+4.8
	0.50	0.493	6.96	−1.4
	1.0	1.044	3.68	+4.4
	2.0	2.120	4.26	+6.0
Between-day (n=5)	1.5	1.558	5.56	+3.86
	0.75	0.728	2.66	−2.93

RSD: Relative standard deviation=(standard deviation×100)/found. R.E.: Relative error=(found−added)×100/added.

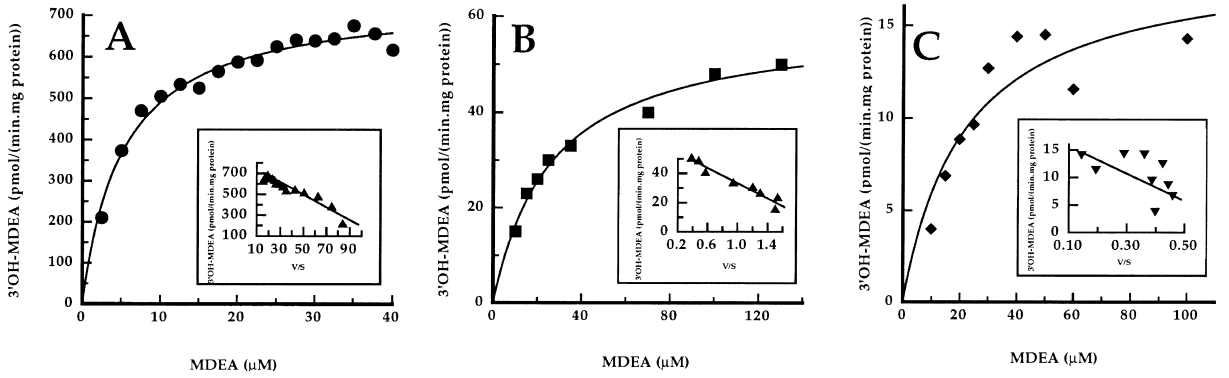

Fig. 3. Enzymatic hydroxylation of mono-*N*-desethylamiodarone (MDEA) investigated in the range of 2–50 μ*M* in rabbit liver microsomes (A), 5–100 μ*M* in rat (B) and human liver microsomes (C). The Eadie–Hoftee plot (A, B, C insert) suggests that the hydroxylation of MDEA is monophasic.

were replaced by those of humans and untreated rats. Rat liver microsomes also possess the ability to hydroxylate MDEA and the production of X2 and X3 is more abundant than that of rabbits (Fig. 2b). Quantitatively, the production of 3′OH-MDEA in rat liver microsomes required higher concentrations of microsomal protein and substrate. In human liver microsomes samples ($n=3$), the production of 3′OH-MDEA was also weak but the formation of X2, X3 seemed to be dominant (Fig. 2c). These metabolic pathways will be the subject of a separate communication.

This assay was used to investigate the enzymatic hydroxylation of MDEA in rabbit, rat and human liver microsomes. Our own preliminary assays had shown that, in the absence of either microsomal protein or NADPH, the production of 3′OH-MDEA was totally abolished, suggesting that the MDEA 3′-hydroxylation is an enzymatic reaction. In the incubation containing 10 μ*M* MDEA, the reaction rates were linear (data not shown) with the incubation time (up to 45 min) and with the protein concentrations (between 0.05 and 0.5 mg/ml). Using standard conditions (0.2 mg protein/ml, 0.1 m*M* NADPH, and incubation time 15 min), the substrate dependence (2–50 μ*M* MDEA) formation kinetics of 3′OH-MDEA was investigated in duplicate. Fig. 3A indicated clearly that in vitro the hydroxylation of MDEA was enzymatically catalyzed by rabbit liver microsomes. Using Origine software (Microcal Software, Northampton, MA, USA), the estimates of K_m and V_{max} values were 6.39±1.07 μ*M* and 560±210 pmol/mg protein/min ($n=5$). In experiments performed with rat liver microsomes (Fig. 3B), the K_m and V_{max} values were 25.2±2.8 μ*M* and 54±15 pmol/mg protein/min ($n=5$). Comparable results were also observed with human liver microsomes. However, the 3′OH-MDEA concentrations in these experiments were low (2–6-fold of detection limit) and in the range of 50–100 μ*M* MDEA, they tended to decrease (Fig. 3C). It is possible that, under experimental conditions 3′OH-MDEA would be further biotransformed to the other product(s). The estimates of K_m and V_{max} were 19.4±2.6 μ*M* and 17.3±2.48 pmol/mg protein/min ($n=3$). The Eadie–Hoftee plots (Fig. 3A–C, inserts) suggested that the MDEA hydroxylation is monophasic. It is necessary to note that, as 3′OH-MDEA was not obtained in a weighable amount, the V_{max} values in the present report should be regarded as estimates only.

4. Discussion

Incubation of MDEA with rabbit liver microsomes and NADPH as a cofactor for short periods produces HPLC-detectable amounts of unknown products. Young and Mehendale have reported previously that MDEA was di-deiodinated in rabbit liver microsomes [13]. The identity of this compound was supported only by the comparison of HPLC retention time of the unknown product with that of the reference substance L32790 obtained from the AMI

manufacturer. Using milder incubation conditions than those of Young's experiments – shorter incubation time (15 min instead of 60 min) and a lower concentration of MDEA (10 μ*M* instead of 125 μ*M*) and HPLC–ESI-MS as analytical tools, our analysis reveals no fragment ions, which may be related to the presence of di-deiodinated MDEA in the incubation. In its place, the presence of the mono-hydroxylated MDEA ($[M+H]^+$ m/z at 634) is dominantly detected.

Reversed-phase HPLC assay is a very useful tool for the quantification of drugs, especially in in vitro experiments. After the incubation, protein can be precipitated by adding organic solvent (such as methanol, acetonitrile), separated by centrifugation, and the supernatant may be injected directly into the chromatograph. The use of an internal standard would facilitate the manual work and increase the precision of the assay. Several AMI derivatives, which have been synthesized in our laboratory [14], have been tested for this task, but none of them can be used. They co-eluted either with MDEA or with its degraded products X2 or X3. Therefore, in order to get consistent results in duplicate measurements, care must be taken to quantitatively isolate 3′OH-MDEA from the incubation medium and to precisely load the sample on the HPLC column.

Using our chromatographic conditions, 3′OH-MDEA concentrations can be assayed every 10 min. Endogenous substances in rabbit (or rat, human) liver microsomes do not interfere with the signal of the tested compound. The assay is selective enough for AMI, MDEA, and 3′OH-MDEA. The retention times of MDEA and AMI are 7.3 and 8.9 min, respectively, but the assay performance for quantifying DEA and MDEA has not been evaluated.

The present assay was used successfully to investigate the in vitro formation kinetics of 3′OH-MDEA in liver microsomes of rabbits, rats and humans. Data in Table 2 show that the enzymes in the liver of all investigated species are suitable to hydroxylate MDEA to 3′OH-MDEA. Their intrinsic clearance values ($Cl_{int}=V_{max}/K_m$) are $87\cdot10^{-3}$, $2\cdot10^{-3}$ and $0.9\cdot10^{-3}$ ml/min/mg protein, for rabbits, rats, and humans, respectively. These data suggest also that MDEA has the highest affinity to the hydroxylase in rabbit liver. Thus, species dependence of AMI metabolism [15] may be explained, at least a part, by the difference in their hydroxylase activities. Other studies are being conducted to find out the identity of the enzyme involved in the hydroxylation of MDEA and to know whether AMI is also hydroxylated.

Table 2
Michaelis–Menten parameters of the MDEA 3′-hydroxylation in untreated rabbit, rat and human liver microsomes

Species	$K_m \pm$SD (m*M*)	$V_{max} \pm$SD (pmol/mg protein/min)
Rabbits ($n=5$)	6.39±1.07	560±210
Rats ($n=5$)	25.2±2.8	54±15
Humans ($n=3$)	19.4±2.6	17.3±2.5

SD: Standard deviation.

In conclusion, our studies demonstrate that, in liver microsomes isolated from humans and untreated rabbits, rats, the major metabolite MDEA of AMI is enzymatically further hydroxylated. Rabbit liver microsomes are more active than those of rats and humans. Thus, the species dependence in AMI metabolism is provable and the fate of MDEA is now better documented.

Acknowledgements

Part of this work was supported by Swiss National Science Foundation (to L.B.). P.K. thanks Cardiovascular Therapy Research, Department of Internal Medicine, University Hospital Zurich, Switzerland for financial support. The authors also thank Dr. A. McAinsh, Nashville, TN, USA, for reviewing the manuscript. This publication represents in part the requirements of the Ph.D. Thesis of P.K. at the Swiss Federal Institute of Technology Zurich (ETHZ), Switzerland.

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