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High-performance liquid chromatographic assay for amiodarone *N*-deethylation activity in human liver microsomes using solid-phase extraction

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Abstract

A selective and sensitive assay for amiodarone *N*-deethylation activity in human liver microsomes by high-performance liquid chromatography (HPLC) with UV detection is reported. The extraction of desethylamiodarone from incubation samples was performed by means of an original solid-phase extraction (SPE) procedure using a polymeric reversed-phase sorbent (Oasis HLB). The method was validated for the determination of desethylamiodarone with respect to specificity, linearity, precision, accuracy, recovery, limit of quantitation and stability. Amiodarone *N*-deethylation activity from low to high substrate concentrations using human liver microsomes was precisely determined without a concentration step. This method is applicable to the study in vitro of the metabolism of amiodarone.

Keywords: Amiodarone; Desethylamiodarone; Cytochrome P450

1. Introduction

Amiodarone (Fig. 1) is an iodinated benzofuran derivative with predominantly Class III (Vaughan Williams' classification) antiarrhythmic effects [1]. It is used to treat supraventricular and ventricular arrhythmias either alone or in combination with other agents to gain additional arrhythmic efficacy [2,3]. Being a highly hydrophobic compound, amiodarone has a broad distribution and a long elimination half-life [4,5]. Amiodarone metabolism is not yet fully documented. It is suspected that in mammals, amiodarone would be biotransformed by *N*-deethylation, hydroxylation, *O*-dealkylation, deiodination and glucuronidation [5]. Further studies are needed to find the corresponding metabolites or to explain why they were not found in the blood circulation. The primary and principal metabolite of amiodarone is desethylamiodarone (Fig. 1), the result of *N*-deethylation, which is catalyzed by hepatic cytochrome

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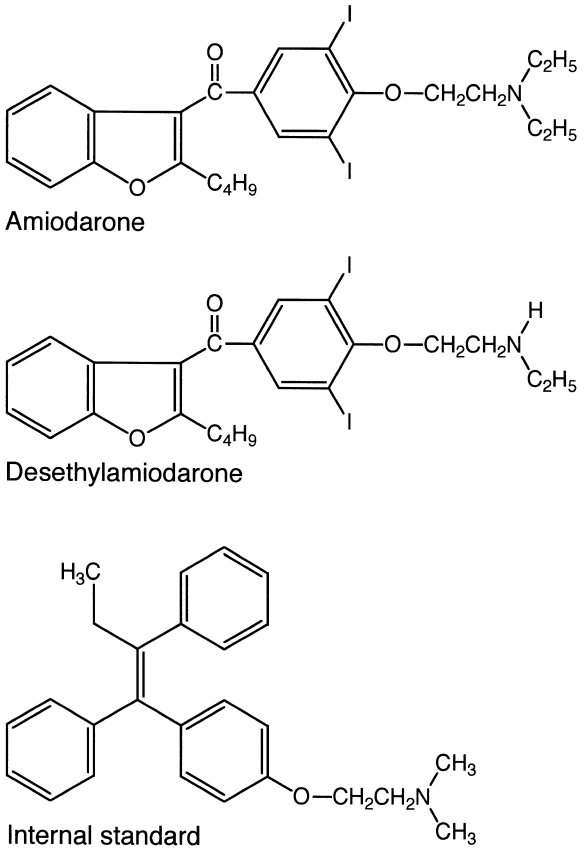

Fig. 1. Chemical structures of amiodarone, desethylamiodarone and internal standard.

P450 (P450) enzymes [6–8]. This metabolite is pharmacologically active and contributes to the antiarrhythmic effects along with the parent compound [9,10].

The P450 isoforms involved in amiodarone *N*-deethylation have been broadly identified using human liver microsomes and recombinant human P450s, and CYP3A4 and CYP2C8 have been suggested to play an important role clinically in the metabolism [6–8]. Although a number of methods for measuring amiodarone and its major metabolite desethylamiodarone in human plasma and urine (in vivo system) by high-performance liquid chromatography (HPLC) after preliminary liquid–liquid or solid-phase extraction (SPE) and concentration have been developed [11–14], reports on assays in vitro are limited. Trivier et al. [15] reported an HPLC assay for amiodarone *N*-deethylation activity in rat liver microsomes by protein precipitation with acetonitrile. The advantage of this method is that sample preparation is simple, however, peaks originating from the incubation medium interfere with the determination of desethylamiodarone. On the other hand, Ohyama et al. [8] employed a liquid–liquid extraction method with dichloromethane to clean up the incubation sample before HPLC for amiodarone *N*-deethylation activity in human liver microsomes, but it requires a concentration step to detect desethylamiodarone. Since these HPLC methods for in vitro systems also suffer from low sensitivity, they do not allow for the determination of low activity samples. Furthermore, the limit of quantitation and optimum incubation conditions for the in vitro assay of amiodarone *N*-deethylation activity in human liver microsomes have not been reported. As genetic polymorphisms of CYP3A4 and CYP2C8 have been recently identified [16–20], it is important to develop a quantitative assay for P450 activity toward amiodarone using small amounts of biological sample for individualized drug therapy.

The aim of this study is the implementation of a selective and sensitive HPLC method for the in vitro assay of amiodarone *N*-deethylation activity in human liver microsomes. The sample preparation comprises a careful SPE procedure using a polymeric reversed-phase sorbent. This method was validated with respect to specificity, linearity, precision, accuracy, recovery, limit of quantitation and stability.

2. Experimental

2.1. Chemicals and biological materials

Amiodarone (>98% pure) was obtained from Sigma (St. Louis, MO, USA). Desethylamiodarone (>99% pure) was kindly supplied by Sanofi-Synthelabo (Paris, France). Tamoxifen (internal standard) (>98% pure) was obtained from Wako Pure Chemical Industries (Osaka, Japan). NADPH was purchased from Oriental Yeast (Tokyo, Japan). Diethylamine was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals and solvents were of analytical-reagent or HPLC grade. Pooled human liver microsomes (H161) from 11 donors and human liver microsomes of single donors (H064 and

H112) were purchased from Gentest (Woburn, MA, USA). The microsomal protein contents were as described in the data sheets provided by the manufacturer. The microsomal samples were stored at −80 °C until use.

2.2. Standards

A stock solution (5000 nmol/ml) in methanol was prepared for desethylamiodarone. Stock solutions (1–200 nmol/ml) for calibration curves and quality control samples were prepared by the serial dilution of the 5000 nmol/ml stock solution with methanol. A stock solution of internal standard was prepared at 5000 nmol/ml in methanol–dimethyl sulfoxide (90:10, v/v) for solubility reasons. This solution was diluted 100-fold extemporaneously in distilled water before use. All stock solutions were stored at −20 °C protected from light and were stable for at least 2 months. Working solutions (200 μl) were freshly prepared each day and obtained by dilution from the stock solutions with 50 m*M* potassium phosphate buffer (pH 7.4) containing human liver microsomes (200 μg protein/ml) in a 1.5-ml polypropylene tubes. Then 120 μl of methanol–3 *M* acetic acid (80:20, v/v), 20 μl of internal standard (50 pmol/μl) and 800 μl of 3 *M* acetic acid were added, and the mixture was vortex-mixed. After centrifugation at 6000 *g* for 20 min at 4 °C, the supernatant was applied to the extraction cartridge.

2.3. Incubation conditions

Amiodarone *N*-deethylation activity was evaluated by quantitation of the desethylamiodarone production in human liver microsomes. Incubations were performed in 5-ml glass test tubes. The standard incubation mixture contained amiodarone (2.5–200 μ*M*) as substrate, human liver microsomes (0–600 μg protein/ml) and 1 m*M* NADPH in 50 m*M* potassium phosphate buffer (pH 7.4) in a final volume of 200 μl. Amiodarone was dissolved in methanol (final concentration in the reaction medium, 0.5%, v/v). After preincubation at 37 °C for 1 min, the reaction was started by the addition of amiodarone. The mixture was incubated at 37 °C for 0–30 min and the reaction terminated with 120 μl of methanol–3 *M* acetic acid (80:20, v/v) with vortex-mixing. Then 20 μl of internal standard (50 pmol/μl) and 800 μl of 3 *M* acetic acid were added, and the mixture was vortex-mixed. The contents of the tubes were transferred to 1.5-ml polypropylene tubes and centrifuged at 6000 *g* for 20 min at 4 °C. The supernatant was applied to the extraction cartridge. Blank samples contained all components except the NADPH, which was added after termination of the reaction.

2.4. Extraction procedure

Oasis HLB cartridges (10 mg/1 ml) (Waters, Milford, MA, USA) were used for the SPE procedure. The cartridges were conditioned with 750 μl of methanol, 750 μl of distilled water and 750 μl of 3 *M* acetic acid, then loaded with 1000 μl of the pretreated samples. After washing with 600 μl of methanol–1 *M* acetic acid (5:95, v/v), the cartridges were dried under vacuum and analytes were eluted with 180 μl of methanol. The eluate was diluted 2-fold in 100 m*M* acetic acid containing 15 m*M* diethylamine (pH 3.8) and analyzed by HPLC within 8 h.

2.5. Apparatus and HPLC conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of an SCL-10A system controller, two LC-10AD pumps, an SIL-10A auto injector with sample cooler, an SPD-10AV UV–Vis detector, a CTO-10A column oven, a DGU-14A degasser and a C-R7A chromatopac integrator. The samples were cooled at 4 °C, and 40-μl aliquots were injected into an Inertsil ODS-80A column (5 μm, 150×4.6 mm I.D., GL Sciences, Tokyo, Japan) with a 10×4.0 mm I.D. guard column containing the same materials. The column was kept at 40 °C. The elution was performed isocratically with 100 m*M* acetic acid containing 15 m*M* diethylamine (pH 3.8)–acetonitrile (45:55, v/v) at a flow-rate of 1.2 ml/min. The UV wavelength was fixed at 242 nm.

2.6. Method validation

A calibration curve was prepared at eight concentrations of desethylamiodarone (50, 100, 250, 500, 1000, 2500, 5000 and 10 000 pmol/ml), and a linear regression line ($y=ax+b$) was constructed by

plotting the peak area ratio of the desethylamiodarone/internal standard (y) against the nominal concentration of desethylamiodarone (x) to determine the slope (a), intercept (b) and correlate coefficient (r). The intra- and inter-day precision and accuracy of the method were assessed by performing repeated analyses of desethylamiodarone in quality control samples of three concentrations (80, 800 and 8000 pmol/ml). Concentrations in quality samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve. The intra-day precision and accuracy were determined by analyzing five times in duplicate on the same day. The inter-day precision and accuracy were determined by analyzing once in duplicate daily for 5 days. The precision was given by relative standard deviation (RSD), while the accuracy was calculated as relative mean error (RME) of back-calculated concentrations from nominal concentrations. The mean values found should be within ±15% of the nominal values, except for the lower limit of quantitation, at which the deviation should be within ±20% [20]. The limit of quantitation was defined as the lowest concentration of desethylamiodarone determined with acceptable precision and accuracy under the stated experimental conditions. The intra- and inter-day precision of the enzyme assay for amiodarone *N*-deethylation activity in human liver microsomes was also assessed at three substrate concentrations (5, 20 and 80 μ*M*). Two samples were analyzed five times for the intra-day precision and once in duplicate daily for 10 days for the inter-day precision. The specificity of the method was assessed to evaluate the influence of the matrix and non-enzymatic reactions in blank samples. The recovery of desethylamiodarone from human liver microsomes was evaluated by comparing the areas of pure standards with those of quality control samples containing the same concentrations of standards (80, 800 and 8000 pmol/ml) in pentuplicate. The stability of desethylamiodarone in quality control samples at concentrations of 80, 800 and 8000 pmol/ml containing human liver microsomes was assessed after storage in a freezer (−20 °C), a refrigerator (4 °C) and at room temperature (25 °C) for various intervals (24 h, 3 days and 1 week) in pentuplicate, by comparing peak areas from initial and subsequent determinations.

2.7. Kinetic analysis

Incubation conditions were chosen such that product formation was linear with respect to both microsomal protein concentration and incubation time for the determination of amiodarone *N*-deethylation activity in human liver microsomes (H161). The microsomal protein concentration and incubation time were 200 μg/ml and 10 min, respectively. The substrate concentration was 2.5–200 μ*M*. Kinetic parameters such as K_m and V_{max} were estimated using the computer program Prism v.3.0a (GraphPad Software, San Diego, CA, USA) designed for non-linear regression analysis of a hyperbolic Michaelis–Menten equation. Three separate experiments were performed in duplicate.

3. Results and discussion

3.1. Validation

Chromatograms of blank human liver microsomes and those spiked with amiodarone, desethylamiodarone and internal standard are shown in Fig. 2.

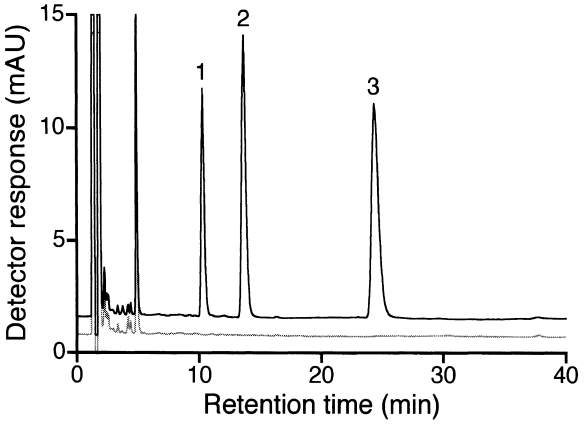

Fig. 2. Chromatogram of amiodarone, desethylamiodarone and internal standard. Amiodarone (1000 pmol), desethylamiodarone (800 pmol) and internal standard (1000 pmol) were added to 50 m*M* potassium phosphate buffer (pH 7.4) containing human liver microsomes (H161, 200 μg protein/ml) in a final volume of 200 μl. The method of sample preparation and the HPLC conditions are described in Section 2. Peaks: (1) internal standard; (2) desethylamiodarone; (3) amiodarone. The light line indicates the result for blank sample.

Amiodarone, desethylamiodarone and internal standard were separated by HPLC, and their retention times were 24.4, 13.7 and 10.3 min, respectively. Fig. 3 shows chromatograms of human liver microsomes incubated with 20 μ*M* amiodarone in the absence or presence of NADPH. The peaks interfering with the determination of desethylamiodarone and internal standard were not detected in blank samples from human liver microsomes (Figs. 2 and 3).

A calibration curve was prepared from a solution to which desethylamiodarone had been added in the range of 50–10 000 pmol/ml. A high correlation was found between the peak area ratio and the concentration of desethylamiodarone. The regression equations were $y=0.409x+0.006$ ($r>0.999$) for intra-day assay ($n=5$) and $y=0.407x+0.004$ ($r>0.999$) for inter-day assay ($n=5$). The means of the difference between the true and back-calculated concentrations of the calibration standards for intra- and inter-assay were 4.1 and 2.9% (range 0.3–17 and 0.1–9.6%), respectively (Table 1). The quality control samples of desethylamiodarone were prepared independently, and were analyzed with the calibration standards. The precision was better than 6% and the accuracy did not exceed 13% at all concentrations of quality control samples (Table 1). These results satisfy the validation criteria for biological samples [20], and indicate the suitability of the calibration model. The limit of detection for desethylamiodarone (signal-to-noise ratio of 3) was back-calculated to be 10 pmol/ml using the 50 pmol/ml standard solution. The value was lower than previous results obtained using the HPLC assay for amiodarone *N*-deethylation activity in mammalian liver microsomes (50–100 pmol/ml) [8,15]. The criteria used to estimate the limit of quantitation were maximal intra- and inter-day variation in precision and accuracy of 15 or 20%. The limit of quantitation for desethylamiodarone was estimated to be 50 pmol/ml.

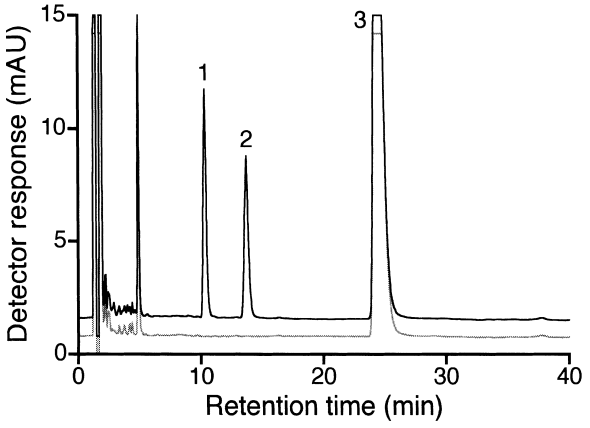

Fig. 3. Chromatogram obtained from the incubation of amiodarone with human liver microsomes. Reactions were performed in the presence of amiodarone (20 μ*M*), human liver microsomes (H161, 200 μg protein/ml) and NADPH (1 m*M*) in 50 m*M* potassium phosphate buffer (pH 7.4) in a final volume of 200 μl for 10 min. The method of sample preparation and the HPLC conditions are described in Section 2. Peaks: (1) internal standard; (2) desethylamiodarone; (3) amiodarone. The light line indicates the result for blank sample.

The extraction efficiency of SPE technique using Oasis HLB cartridge for desethylamiodarone and internal standard were studied. In preliminary studies, we found that acetic acid is required in all the steps of sample preparation except the elution from extraction cartridges to fully recover these compounds. The recoveries from human liver microsomes spiked with desethylamiodarone at concentrations of 80, 800 and 8000 pmol/ml were 98.9±2.6, 102.3±1.5 and 99.6±1.7%, respectively ($n=5$ at each concentration). The recovery of the internal standard (1000 pmol) was 100.7±2.0% ($n=5$). The extraction recoveries of desethylamiodarone and internal standard are not only high, but also similar at all concentrations studied.

To check the stability of the analytes, humans liver microsomes were spiked separately with desethylamiodarone at three different concentrations and internal standard (1000 pmol), and the HPLC samples were prepared as described above. The results for desethylamiodarone are shown in Table 2. Desethylamiodarone was stable for 1 week at −20, 4 and 25 °C at all concentrations. The internal standard was also stable for 1 week in all storage conditions (98.0–100.5%).

3.2. Amiodarone N-deethylation by human liver microsomes

To determine the optimum incubation conditions for the assay of amiodarone *N*-deethylation activity in human liver microsomes, the dependence of the

Table 1
Precision and accuracy of the determination of desethylamiodarone in human liver microsomes

Nominal concentration (pmol/ml)	Analyzed concentration (mean±SD) (pmol/ml)	RSD (%)	RME (%)
Intra-day ($n = 5$)			
Calibration standards			
50	41.55±1.10	2.65	−16.9
100	92.12±2.08	2.26	−7.88
250	250.7±10.9	4.35	0.28
500	502.8±19.7	3.92	0.56
1000	1023±30	2.93	2.30
2500	2541±73	2.87	1.64
5000	5111±74	1.45	2.22
10 000	9962±35	0.35	−0.38
Quality control samples			
80	69.85±1.00	1.43	−12.7
800	796.0±3.0	0.38	−0.50
8000	7786±68	0.87	−2.68
Inter-day ($n = 5$)			
Calibration standards			
50	45.20±3.64	8.05	−9.60
100	93.38±2.94	3.15	−6.62
250	244.4±8.5	3.48	−2.24
500	499.4±11.2	2.24	−0.12
1000	1033±20	1.94	3.30
2500	2512±35	1.39	0.48
5000	5019±105	2.09	0.38
10 000	9994±51	0.51	−0.06
Quality control samples			
80	73.43±3.87	5.27	−8.21
800	804.8±13.0	1.62	0.60
8000	7750±101	1.30	−3.13

Desethylamiodarone was added to 50 m*M* potassium phosphate buffer (pH 7.4) containing human liver microsomes (H161, 200 μg protein/ml) in a final volume of 200 μl. The method of sample preparation and the HPLC conditions are described in Section 2.

enzymatic activity on microsomal protein concentration and incubation time was studied. Substrate concentrations of 5, 20 and 80 μ*M* were used. Figs. 4 and 5 show the effect of microsomal protein concentration and incubation time on desethylamiodarone formation in human liver microsomes, respectively. Desethylamiodarone formation was almost linear for microsomal protein concentrations up to 200 μg protein/ml and for incubation times up to 10 min at all substrate concentrations ($r > 0.991$). The limit of quantitation for desethylamiodarone is 50 pmol/ml (10 pmol/incubation tube), and allows amiodarone *N*-deethylation activity as low as 25 pmol/min/mg protein to be determined in human liver microsomes (incubation of 40 μg protein/incubation mixture (200 μg protein/ml) for 10 min incubation). The reproducibility of the assay for the enzymatic reaction was also assessed at amiodarone concentrations of 5, 20 and 80 μ*M*. As shown in Table 3, the intra- and inter-day precision of the assay for amiodarone *N*-deethylation activity in human liver microsomes was less than 7%.

To characterize the enzymology of P450s toward amiodarone in human liver microsomes, kinetic analysis was performed by measuring twenty concentrations of substrate between 2.5 and 200 μ*M*. The non-linear regression curves and kinetic parameters for different microsomes from human livers are

Table 2
Stability of desethylamiodarone in human liver microsomes

Concentration (pmol/ml)	Recovery (mean±SD) (%)		
	−20 °C	4 °C	25 °C
24 h (n=5)			
80	99.5±2.1	99.2±1.5	98.7±2.7
800	99.6±1.8	99.7±1.0	100.3±0.4
8000	100.6±1.1	99.1±1.4	99.9±0.2
3 days (n=5)			
80	99.1±3.0	98.6±1.1	101.2±1.7
800	98.9±1.5	100.4±0.7	99.8±0.8
8000	99.8±0.6	99.9±0.7	100.9±0.3
1 week (n=5)			
80	96.8±2.7	97.9±2.2	97.1±3.1
800	100.3±1.1	98.4±2.4	99.9±1.0
8000	97.5±1.6	99.3±0.2	96.8±1.0

Desethylamiodarone was added to 50 m*M* potassium phosphate buffer (pH 7.4) containing human liver microsomes (H161, 200 μg protein/ml) in a final volume of 200 μl. The method of sample preparation and the HPLC conditions are described in Section 2.

shown in Fig. 6 and Table 4, respectively. The V_{max} and V_{max}/K_m values varied among the liver microsomes, although the K_m value was similar. These

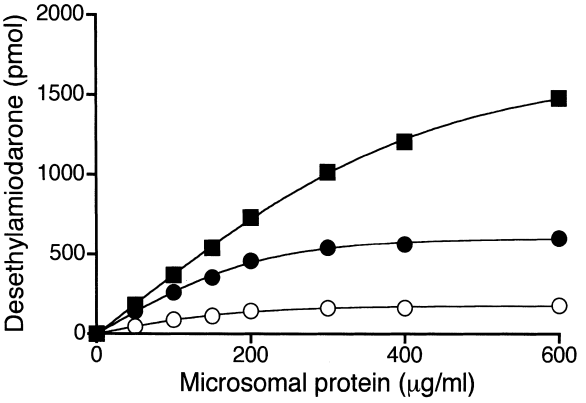

Fig. 4. Dependence on microsomal protein concentration of amiodarone *N*-deethylation activity in human liver microsomes. Reactions were performed in the presence of amiodarone (5, 20 or 80 μ*M*), human liver microsomes (H161, 0–600 μg protein/ml) and NADPH (1 m*M*) in 50 m*M* potassium phosphate buffer (pH 7.4) in a final volume of 200 μl for 10 min. The method of sample preparation and the HPLC conditions are described in Section 2. Symbols: (○) 5 μ*M* amiodarone; (●) 20 μ*M* amiodarone; (■) 80 μ*M* amiodarone. Each point represents the mean of three separate experiments performed in duplicate.

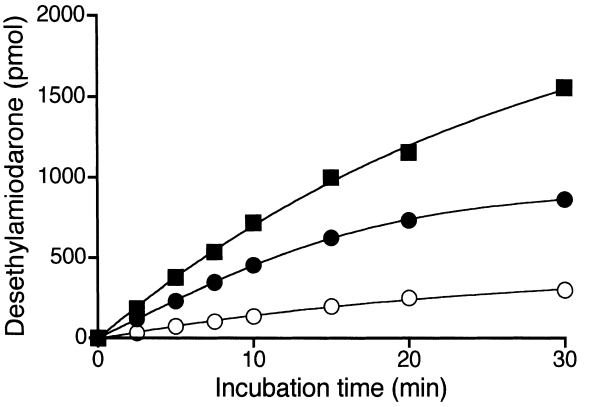

Fig. 5. Dependence on incubation time of amiodarone *N*-deethylation activity in human liver microsomes. Reactions were performed in the presence of amiodarone (5, 20 or 80 μ*M*), human liver microsomes (H161, 200 μg protein/ml) and NADPH (1 m*M*) in 50 m*M* potassium phosphate buffer (pH 7.4) in a final volume of 200 μl for 0–30 min. The method of sample preparation and the HPLC conditions are described in Section 2. Symbols: (○) 5 μ*M* amiodarone; (●) 20 μ*M* amiodarone; (■) 80 μ*M* amiodarone. Each point represents the mean of three separate experiments performed in duplicate.

kinetic constants were comparable to those in other reports [7,8]. The individual difference may depend on the ratios and expression levels of CYP3A4 and CYP2C8 in each liver microsomal fraction. Further study is required to identify the contribution rates of

Table 3
Precision of the assay for amiodarone *N*-deethylation activity in human liver microsomes

Substrate concentration (μ*M*)	Activity (mean±SD) (pmol/min mg protein)	RSD (%)
Intra-day (n=5)		
5	352±23	6.53
20	1139±18	1.58
80	1783±31	1.74
Inter-day (n=10)		
5	345±20	5.80
20	1146±27	2.36
80	1807±59	3.27

Reactions were performed in the presence of amiodarone (5, 20 or 80 μ*M*), human liver microsomes (H161, 200 μg protein/ml) and NADPH (1 m*M*) in 50 m*M* potassium phosphate buffer (pH 7.4) in a final volume of 200 μl for 10 min. The method of sample preparation and the HPLC conditions are described in Section 2.

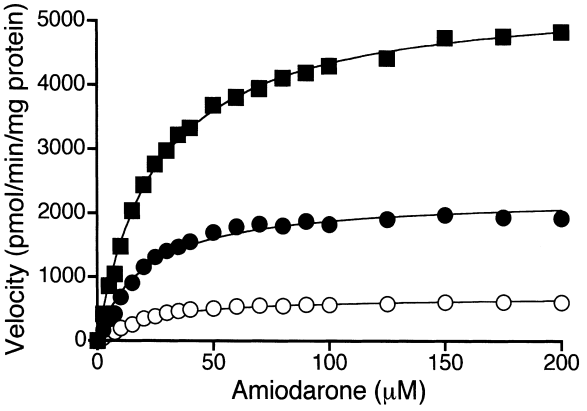

Fig. 6. Dependence on substrate concentration of amiodarone *N*-deethylation activity in human liver microsomes. Reactions were performed in the presence of amiodarone (2.5–200 μ*M*), human liver microsomes (200 μg protein/ml) and NADPH (1 m*M*) in 50 m*M* potassium phosphate buffer (pH 7.4) in a final volume of 200 μl for 10 min. The method of sample preparation and the HPLC conditions are described in Section 2. Symbols: (○) H064; (●) H161; (■) H112. Each point represents the mean of three separate experiments performed in duplicate.

P450 isoforms involved in amiodarone *N*-deethylation in humans.

4. Conclusion

Although amiodarone is one of the most effective antiarrhythmic drugs, it often causes severe side effects due to an accumulation in the body tissues or drug interactions [1,4,5]. P450 enzymes clinically play an important role in the *N*-deethylation of amiodarone, and information about the activity of hepatic P450 toward amiodarone in humans is beneficial to medicine. However, the range of metabolic activity toward drugs is generally varied among individuals, at least partly because of genetic polymorphisms in the P450 family (http://www.imm.ki.se/CYPalleles/), and high selectivity and sensitivity is needed to assay amiodarone *N*-deethylation activity from small amounts of biological samples. With the method using SPE described here, amiodarone *N*-deethylation activity from low to high substrate concentrations using human liver microsomes was precisely determined without a concentration step. Therefore, this method for the assay of amiodarone *N*-deethylation activity is useful for the study in vitro of the metabolism of amiodarone using small amounts of biological sample.

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Table 4
Michaelis–Menten parameters for amiodarone *N*-deethylation activity in human liver microsomes

	H064	H161	H112
K_m (μ*M*)	21.5±0.8	20.1±1.1	25.5±0.6
V_{max} (pmol/min/mg protein)	702±13	2254±95	5439±107
V_{max}/K_m (μl/min/mg protein)	32.7±1.9	112±2	213±6

Reactions were performed in the presence of amiodarone (2.5–200 μ*M*), human liver microsomes (200 μg protein/ml) and NADPH (1 m*M*) in 50 m*M* potassium phosphate buffer (pH 7.4) in a final volume of 200 μl for 10 min. The method of sample preparation and the HPLC conditions are described in Section 2. Each value represents the mean±SD of three separate experiments performed in duplicate.

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