

## Development of an Enzyme-Linked Immunosorbent Assay for the Quantification of Amiodarone

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**A sensitive and specific enzyme-linked immunosorbent assay for an antiarrhythmic drug, amiodarone (AMI), was developed, which is capable of measuring levels as low as 16 ng/ml. Anti-AMI antibody was obtained by immunizing rabbits with an antigen conjugated with bovine serum albumin using diazotized 4-amino-1-(2-diethylaminoethoxy)-2,6-diiodobenzene. Enzyme labeling of AMI with  $\beta$ -D-galactosidase was similarly performed using a diazotized 4-amino-1-(2-diethylaminoethoxy)-2,6-diiodobenzene. This enzyme-linked immunosorbent assay was specific for AMI and showed a very slight cross-reactivity (1.25%) with its major metabolite, mono-*N*-desethylamiodarone. The values of the AMI concentrations measured by this assay were in good correlation to those by HPLC. Its analytical applicability was demonstrated by a kinetic study with human liver microsomes. The enzyme-linked immunosorbent assay should be a valuable tool in therapeutic drug monitoring and pharmacokinetic studies of AMI.**

**Key words** amiodarone; ELISA; antiarrhythmic drug

Amiodarone (AMI) [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxybenzoyl)-benzofuran] is a potent class III antiarrhythmic drug.<sup>1)</sup> It is used to treat ventricular and supraventricular arrhythmias, especially when they are resistant to other conventional antiarrhythmic drugs.<sup>2,3)</sup> However, its use is sometimes complicated by serious adverse effects, including occasionally life threatening pulmonary fibrosis and hepatitis.<sup>4)</sup> AMI has a long serum elimination of 40 to 50 d, which was attributed to its huge distribution.<sup>5,6)</sup> Therefore, the AMI concentrations in patients treated with therapeutic doses of the drug vary considerably and therapeutic drug monitoring (TDM) of AMI may assist in individualizing the dosage regimen.

In humans, mono-*N*-desethylamiodarone (MDEA) is known as the major metabolite (Fig. 1).<sup>7)</sup> 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. Therefore, to carry out TDM or a pharmacokinetic study of AMI, it is necessary to develop an analytical method specific to both AMI and MDEA. Previous pharmacokinetic studies of AMI were undertaken using HPLC methods.<sup>8,9)</sup> However, pretreatments such as extraction of samples are necessary and the procedure is complex. Thus, the development of a simple quantification method of AMI is needed. ELISA appeared to be a suitable analytical method for this purpose. However, an ELISA system for AMI or MDEA has not previously been developed.

This paper reports the methodology for the antibody production, the labeling of AMI with  $\beta$ -D-galactosidase ( $\beta$ -Gal) to act as a tracer, the characterization of antibody specificity, and the technique developed for the measurement of AMI by ELISA. The initial analytical applicability is demonstrated by a kinetic study with human liver microsomes.

### MATERIALS AND METHODS

**Equipment** The IR spectrum was recorded using a Hitachi 270-30 spectrometer (Tokyo, Japan). A proton nuclear magnetic resonance (<sup>1</sup>H-NMR) spectrum was taken with a JEOL-MY60FT spectrometer (Tokyo, Japan) at 60 MHz

using tetramethylsilane as an internal standard. Enzymic activity was measured using a fluorescence microplate reader (Fluoroskan Ascent, Labsystems, Helsinki, Finland).

**Reagents** AMI hydrochloride and MDEA hydrochloride were supplied by Taisho Pharmaceutical (Tokyo, Japan). 2,6-Diiodo-4-nitrophenol was obtained from Aldrich Chem. Co. (St. Louis, MO, U.S.A.). 2-Chlorotriethylamine hydrochloride was obtained from Katayama Chemical (Osaka, Japan).  $\beta$ -D-Galactosidase ( $\beta$ -Gal; EC 3.2.1.23) from *Escherichia coli*, 4-methylumbelliferyl- $\beta$ -D-galactopyranoside and reduced  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Boehringer Mannheim (Mannheim, Germany). Human liver microsomes (single female donor microsomes) were obtained from In Vitro Technologies, Inc. (Baltimore, Maryland, U.S.A.).

**Preparation of the Immunogen for AMI** 2,6-Diiodo-4-nitrophenol (200 mg, 0.51 mmol) in toluene (10 ml) was added to a solution of 2-chlorotriethylamine hydrochloride (130 mg, 0.76 mmol) in 4.2% K<sub>2</sub>CO<sub>3</sub> (5 ml). The mixture was heated under reflux for 8 h. After cooling to room temperature, the toluene layer was washed with saturated NaCl and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The toluene solution was treated with HCl gas. The resulting precipitates were collected by filtration, washed with cold ethanol (5 ml) and dried. A total of 90 mg (33%) of 1-(2-diethylaminoethoxy)-2,6-diiodo-4-nitrobenzene (EINB) hydrochloride as a pale yellow powder was obtained. mp: 168—170 °C. IR (KBr) cm<sup>-1</sup>: 2636, 1514, 1338. <sup>1</sup>H-NMR (CDCl<sub>3</sub>)  $\delta$ : 1.19 (6H, t, *J*=7.2 Hz, N(CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), 2.70—3.32 (6H, m, CH<sub>2</sub>N(CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), 4.24 (2H, t, *J*=6.2 Hz, OCH<sub>2</sub>CH<sub>2</sub>N), 8.64 (2H, s, ArH).

A solution of 10 mg of EINB hydrochloride was dissolved in 1.5 ml of 60% ethanol and alkaline with 40 mg of MgO.

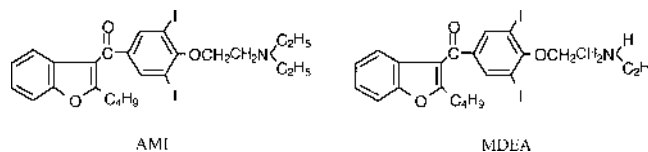


Fig. 1. Chemical Structures of AMI and Its Major Metabolite

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To this mixture was added 80 mg of  $\text{Na}_2\text{S}_2\text{O}_3$  which was vigorously shaken, and the solution was heated in a water—both at  $50^\circ\text{C}$  for 2 h. After cooling, ethanol was distilled off by evaporation *in vacuo*, and the aqueous solution was washed with AcOEt to remove unreacted EINB. The aqueous layer was evaporated, and the residue was dissolved in ethanol. The resulting suspension was filtered and the filtrate evaporated *in vacuo* to give 4-amino-1-(2-diethylaminoethoxy)-2,6-diiodobenzene (AEIB) (5 mg, 50%) as a yellow solid.

The resulting AEIB was conjugated with bovine serum albumin (BSA) and  $\beta$ -Gal, respectively, as an AMI immunogen and a tracer.

AEIB (approximately 5 mg,  $10.2\ \mu\text{mol}$ ) in  $40\ \mu\text{l}$  *N,N*-dimethylformamide (DMF) was acidified by the addition of  $100\ \mu\text{l}$  of 1 M acetic acid and then diazotized with sodium nitrite (1.4 mg,  $20.3\ \mu\text{mol}$ ) in  $50\ \mu\text{l}$  distilled water at  $0^\circ\text{C}$  for 10 min. The solution was immediately mixed with BSA (10 mg) in 1 ml of 0.5 M borate buffer (pH 9.5), followed by 1 h incubation at room temperature. The mixture was chromatographed on a Sephadex G-100 column ( $2.8 \times 42\ \text{cm}$ ) with 0.1 M phosphate buffer (pH 7.0) containing 3 M urea. The degree of coupling of diazotized AEIB with BSA was determined by amino acid analysis after hydrolysis with 6 M HCl at  $110^\circ\text{C}$  for 24 h.<sup>10</sup> Seven moles of the hapten were found to be coupled with one mole of BSA on the basis of the decrease in moles of histidine and tyrosine. The amount of protein was determined by Lowry's method.<sup>11</sup>

**Preparation of AMI Antibody** An aliquot of a solution containing 1 mg AEIB-BSA conjugate (1.5 ml) was emulsified with an equal volume of Freund's complete adjuvant. Two white female rabbits were administered multiple subcutaneous injections at sites along both sides of their backs. Booster injections were then administered three times at bi-weekly intervals, using one-half the amount of the dose of the first immunization. The rabbits were bled from an ear vein 10 weeks after immunization began. The sera (10 ml) were separated by centrifugation and heated at  $55^\circ\text{C}$  for 30 min. Fractions of IgG were extracted from the sera with 50% saturated ammonium sulfate and chromatographed on a column of DEAE-Sephacel ( $2.1 \times 23\ \text{cm}$ ) using 17.5 mM phosphate buffer (pH 6.8) as an eluant.<sup>12</sup> The fraction passed through the column was lyophilized and used as anti-AMI IgG for ELISA.

**Preparation of the AMI- $\beta$ -Gal Conjugate** AMI was labeled by binding to  $\beta$ -Gal, essentially by the same method used for the preparation of AMI immunogen.

AEIB (approximately 2.5 mg,  $5.1\ \mu\text{mol}$ ) in  $20\ \mu\text{l}$  DMF was acidified by the addition of  $100\ \mu\text{l}$  of 1 M acetic acid and then diazotized with sodium nitrite (0.7 mg,  $10.1\ \mu\text{mol}$ ) in  $50\ \mu\text{l}$  distilled water at  $0^\circ\text{C}$  for 10 min. Next,  $50\ \mu\text{l}$  portion of the above reaction mixture containing diazotized AEIB (*ca.*  $1.5\ \mu\text{mol}$ ) was added directly to  $\beta$ -Gal (156  $\mu\text{g}$ , 0.28 nmol) in 0.5 ml of 0.5 M phosphate buffer (pH 7.5), followed by 1 h incubation at room temperature. The mixture was chromatographed on a column of Sepharose 6B ( $2.0 \times 40\ \text{cm}$ ) using 20 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM  $\text{MgCl}_2$ , 0.1% BSA and 0.1%  $\text{NaN}_3$  (buffer A) to remove the remaining small molecules. Four-milliliter fractions were collected, and fractions 14 to 16, representing the main peak showing enzyme activity, were combined and used as a label in the ELISA.

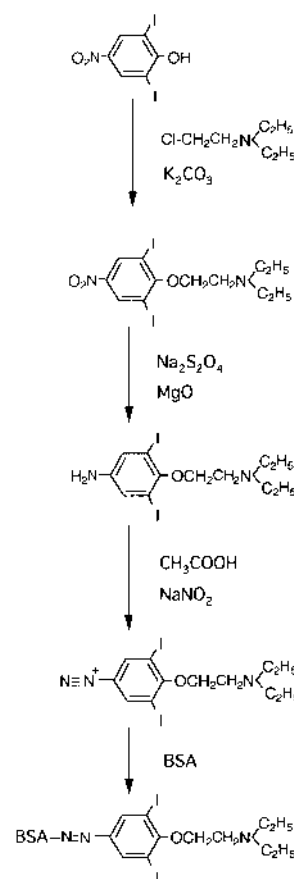


Fig. 2. Preparation of the Immunogen for AMI

**ELISA for AMI** ELISA is based on the principle of competition between enzyme-labeled and unlabeled drugs for an immobilized antibody, followed by measurement of the marker enzyme activity of the immunocomplex bound to the solid phase. Briefly, the wells in microtiter plates (Nunc F Immunoplates I; Nunc, Reskilde, Denmark) were coated by loading  $150\ \mu\text{l}$  of anti-AMI IgG (2.0  $\mu\text{g/ml}$ ) in 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM  $\text{NaN}_3$  and allowed to stand for 1 h at  $37^\circ\text{C}$ . After the plates had been washed twice with 60 mM phosphate buffer (pH 7.4) containing 10 mM ethylenediaminetetraacetate, 0.1% BSA and 0.1%  $\text{NaN}_3$  (buffer B), they were incubated with  $200\ \mu\text{l}$  of 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM  $\text{NaN}_3$  containing 2% BSA for 20 min at  $37^\circ\text{C}$  to prevent non-specific adsorption. The anti-AMI IgG-coated wells were then filled with  $50\ \mu\text{l}$  of either AMI-treated samples, or buffer B as a control, followed immediately by  $50\ \mu\text{l}$  of the pooled AMI- $\beta$ -Gal conjugate (diluted 1 : 500 in buffer B for AMI). The wells were then incubated overnight at room temperature and once again washed thoroughly with buffer B.

The activity of the enzyme conjugate bound to each well was then measured by the addition of  $125\ \mu\text{l}$  of 0.1 mM 4-methylumbelliferyl- $\beta$ -D-galactopyranoside in buffer A, followed by incubation of the wells at  $37^\circ\text{C}$  for 60 min. The enzyme reaction was stopped by the addition of  $75\ \mu\text{l}$  of 0.5 M glycine-NaOH buffer (pH 10.3) to each well, and the resulting 4-methylumbelliferone was determined by spectrofluorometry at wavelengths of 355 nm for excitation and 460 nm

for emission using a fluorescence microplate reader.

**HPLC Method** The HPLC system consisted of a Shimadzu Model LC-10AT liquid chromatograph equipped with a spectrophotometric detector SPD-10AV and a 4×125 mm Lichrospher 100 RP-18 endcapped (5 μm) column (Merck, Darmstadt, Germany). The column was eluted with methanol: water: 28% ammonium hydroxide (90:9.8:0.2, v/v/v) at a flow rate of 1.5 ml/min, and the elute was monitored at 254 nm. The retention time and peak height were measured with a Shimadzu C-R7A Chromatopac.

Thirty microliters of 1.2 M HCl was added to 1 ml of serum in a 15×10 mm glass test tube. After the addition of 100 μl of mexiletine (5 μg/ml) as the internal standard, the mixture was extracted using 5 ml of diethyl ether and shaken for 10 min. After centrifugation for 5 min at 2270×g, the organic layer (4 ml) was transferred to another glass tube. The extracted solvent was evaporated to dryness at 40 °C under N<sub>2</sub> gas. The residue was reconstituted in 50 μl of methanol, and 40 μl was injected. The peak height ratios of AMI to the internal standard mexiletine showed a linear relation to the serum concentration within the range 0.5 to 8.0 μg/ml.

#### Metabolism of AMI by Human Liver Microsomes

The metabolism of AMI was investigated on a human liver microsomes. Briefly, an incubation mixture (1 ml of total volume) contained 100 mM sodium phosphate buffer (pH 7.4) containing 5% BSA, 1 mM NADPH, 50 μM AMI, and 1 mg/ml human liver microsomes. The reaction was initiated by the addition of NADPH and incubated at 37 °C for various periods after 30 min preincubation. The enzymatic reaction was stopped by the addition of a 20-fold volume of buffer A containing 1 mM SKF 525A, a nonspecific cytochrome P-450 inhibitor. After 1 min centrifugation at 10000×g, the supernatant fluid was recovered and stored at -20 °C until assay for the AMI concentration. The solution was analyzed directly by ELISA.

## RESULTS

**ELISA for AMI** The optimal quantities and optimal incubation time for each reaction were established. The dose-response standard curves of AMI obtained in the serum system are shown in Fig. 3. The detection ranges by ELISA were between 3.2 ng/ml and 10 μg/ml of AMI. For practical purposes, the working range was arbitrarily set between 16 and 2000 ng/ml based on the precision and accuracy findings for the ELISA (Table 1), which showed this ELISA to be a reproducible technique. The recoveries of four different levels of AMI ranging from 16 to 2000 ng/ml were satisfactory, 95.0 to 104.0% (*n*=5). The coefficients of variation (CV) for intra- and inter-assays between AMI concentrations of 16 to 2000 ng/ml at four different levels were 5.5 to 12.5% and 4.0 to 9.6% (*n*=5 for each), respectively.

The standard curve in the serum system was similar to that in the buffer system.

**Specificity of the Anti-AMI Antibody** The antibody specificity was determined by the displacement of bound AMI-β-Gal by similar compounds. Values of the cross-reactivity were defined as the ratio of each compound to AMI in the concentrations required for 50% inhibition of AMI-β-Gal binding to the antibody. The anti-AMI antibody showed 594.6% cross-reaction with the AEIB used as a hapten anti-

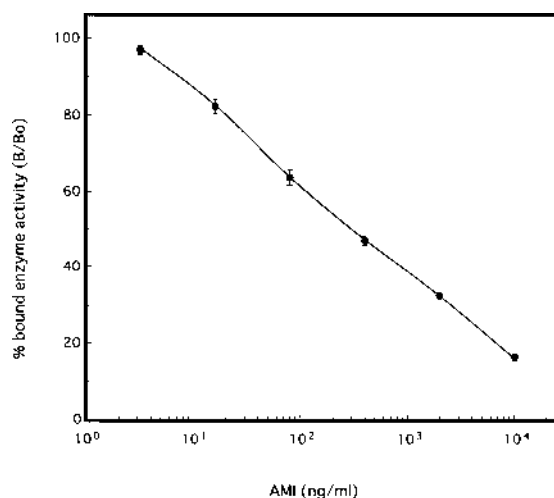


Fig. 3. Standard Curves for AMI in Serum

The curve shows the amount (%) of bound enzyme activity for various doses of AMI (B) as a ratio of that bound using AMI-β-Gal alone (B<sub>0</sub>). Each point represents the mean±S.D. of 3 replicates.

Table 1. Precision and Recovery (%) of ELISA for AMI in Serum

	Added (ng/ml)	Estimated (ng/ml)	Recovery (%)	CV (%)
Intra-assay	16.0	15.2±1.9	95.0	12.5
	80.0	83.2±7.7	104.0	9.3
	400.0	397.0±21.7	99.3	5.5
	2000.0	2050.0±180.3	102.5	8.8
Inter-assay	16.0	15.7±1.5	98.1	9.6
	80.0	78.6±5.6	98.3	7.1
	400.0	400.0±15.8	100.0	4.0
	2000.0	2080.0±192.4	104.0	9.3

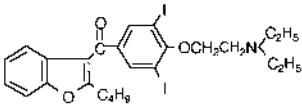
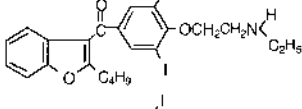
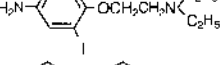
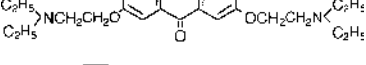
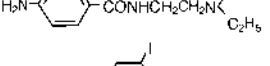
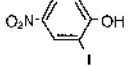
Values represent the means±S.D. of 5 experiments.

gen, 1.25% with the major AMI metabolite, MDEA, 4.4% with tilorone. No detectable cross-reaction, however, was found in procainamide and 2,6-diiodo-4-nitrophenol (Table 2).

**Comparison of ELISA and HPLC** Using 11 human serum samples of various concentrations ranging from 0.3 to 10 μg/ml, which contained AMI and MDEA (AMI:MDEA concentration ratio was 1), the ELISA method was compared with an HPLC method using specific quantities of AMI. ELISA determination was carried out using these AMI samples, properly diluted to the drug-concentration range detectable by ELISA. Figure 4 shows that there was a good correlation between the values determined by the two methods, and the plot was linear as predicted by the equation  $Y=0.995X-0.048$ , where *Y* is the concentration determined by HPLC and *X* is that determined by ELISA; the correlation coefficient was 0.993 (*n*=11).

**Kinetic of AMI in Human Liver Microsomes** This ELISA was applied to the kinetic study of AMI in human liver microsomes. As shown in Fig. 5, immediately after the start of the reaction, a rapid decrease in AMI was observed. From 30 min after the initiation of the reaction, the reaction rate was lowered. However, in the presence of SKF 525A, the reaction was inhibited.

Table 2. Specificity of Anti-AMI IgG

Compounds		% cross-reaction (50%)
	AMI	100.0
	MDEA	1.25
	AEIB	594.6
	Tilorone	4.4
	Procainamide	<0.1
	2,6-Diiodo-4-nitrophenol	<0.1

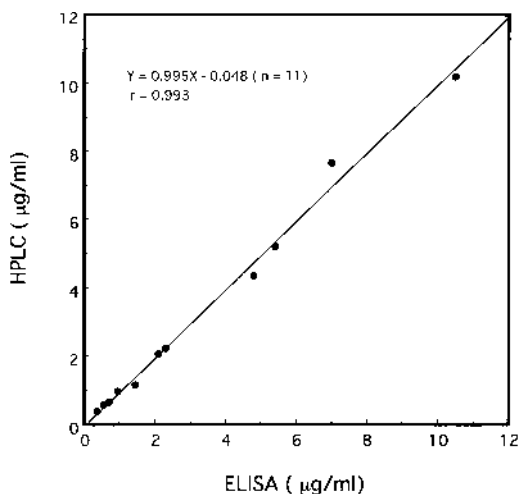


Fig. 4. Correlation of AMI Quantification by the Present ELISA and HPLC Methods

DISCUSSION

To develop an ELISA for AMI, which can be applied to TDM and the pharmacokinetic study of AMI, it is necessary to produce an anti-AMI antibody that does not show a cross-reaction with the major metabolite MDEA. In general, the antibody specificity on the hapten appears to be towards the group furthest away from the region of conjugation to the carrier protein in the immunogen structure.<sup>13,14</sup> Therefore, to produce an antibody specific for the diethylamino moiety of AMI, AMI immunogen was prepared using a partial structure of AMI (AEIB). The AEIB was coupled by diazotization to tyrosine and histidine residues on the carrier protein. The AEIB-BSA conjugate, with 7.0 mol of AEIB per mol of BSA, induced the formation of specific antibodies in each of two rabbits immunized. AMI-β-Gal conjugate was also prepared following essentially the same procedure. The conju-

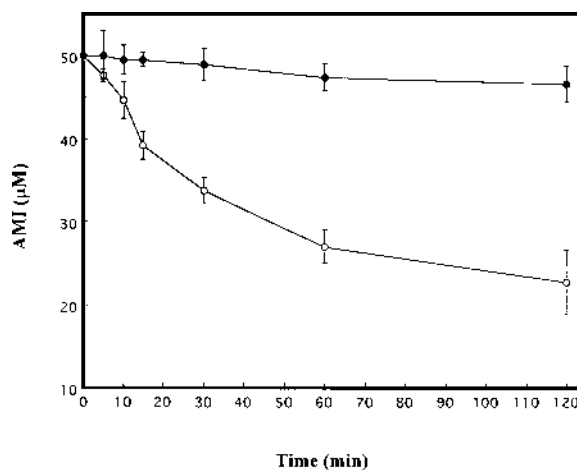


Fig. 5. Kinetic of AMI in Human Liver Microsomes

Human liver microsomes (1 mg/ml) was incubated at 37 °C in a total volume of 1 ml of 100 mM sodium phosphate buffer (pH 7.4) containing 5% BSA, with 50 mM AMI, in the absence (○) or the presence (●) of 1 mM SKF 525A. Each point represents the mean ± S.D. of 3 replicates.

gate thus obtained was stable for more than 6 months in eluted buffer (pH 7.0) at 4 °C without any loss of the enzyme and immunoreactive enzyme activity.

The present ELISA was sensitive enough that AMI concentrations less than 16 ng/ml could be measured reproducibly (Fig. 3, Table 1). The therapeutic range of AMI was reported to be 0.5—2.5 µg/ml.<sup>15</sup> Therefore, this ELISA may be sensitive enough to quantify AMI for TDM.

The specificity of this ELISA is shown in Fig. 4 and Table 2. The anti-AMI antibody showed high affinity for AMI and the AEIB used as a hapten antigen. However, the antibody showed very limited reactivity with the major metabolite MDEA and an AMI analog (tilorone). In addition, the antibody showed no cross-reaction with the other tested AMI analogs. These findings suggested that the antibody recog-

nizes almost the whole structure of the AEIB, and thus is specific enough to the structure of AMI. During long-term therapy, the plasma level of MDEA is comparable with that of AMI. Therefore, using the human serum samples which contain AMI and MDEA (AMI : MDEA concentration ratio was 1), the ELISA method was compared with the HPLC method by using specific quantities of AMI. There was a good correlation; the correlation coefficient was 0.993 between the two (Fig. 4). This finding also supports the high specificity of ELISA.

As a demonstration of the potential of this ELISA, a preliminary metabolic study of AMI in human liver microsomes was performed (Fig. 5). In human liver microsomes, a rapid decrease in AMI was observed. However, in the presence of SKF 525A, nonspecific cytochrome P-450 inhibitor, the reaction was inhibited. These results suggest that AMI was metabolized by cytochrome P-450 isozyme(s) in human liver microsomes. In previous studies, MDEA is known as the major metabolite of AMI in mammals, and cytochrome P-450 3A isoforms are involved in this dealkylation.<sup>16)</sup> Recently, it was observed that MDEA was further hydroxylated to *n*-3'-hydroxybutyl-MDEA in mammals.<sup>17)</sup> The cross-reactivity of the *n*-3'-hydroxybutyl-MDEA has not yet been confirmed. However, it is estimated that this metabolite shows a similar cross-reaction to MDEA judging from the specificity of the anti-AMI antibody. Therefore, this ELISA may be specific enough to quantify AMI for pharmacokinetic studies in animals and humans.

The ELISA procedure for AMI here is sensitive, specific, reproducible, simple and adaptable for analyses of a large number of samples. This ELISA will be a valuable tool in studies of the TDM and in pharmacokinetic studies.

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