Pharmacokinetic Study of β -Methyldigoxin by Enzyme Immunoassay Using a Novel Specific Antiserum in Rats

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We previously showed that enzyme immunoassay (EIA) of β -methyldigoxin (MDx3) using anti-MDx3 3'-hemisuccinate-bovine serum albumin antiserum (Antiserum-I) was superior to that using commercial anti-digoxin antiserum (Antiserum-II) in terms of specificity and that pretreatment of human serum with phenyl boric acid (PBA) column was effective. In the present study, we examined the precision of EIA using Antiserum-I and the recovery of MDx3 after PBA column treatment in rat serum, and also investigated pharmacokinetic changes of MDx3 in rats. The intra- and inter-assay variations and recovery tests using Antiserum-I were good. The PBA column was effective in selectively separating MDx3 from rat serum containing MDx3 and its metabolites. The recovery tests using Antiserum-I with PBA column showed about 110% and the interference of metabolites of MDx3 was negligible. Serum concentration–time courses of MDx3 by EIA using Antiserum-I with PBA column and Antiserum-I were lower than that using Antiserum-II. The distribution volume at steady state and total body clearance values of MDx3 in these conditions were significantly higher than those using Antiserum-II. The usefulness of PBA column was ascertained, while effects of PBA column on these parameters were not significant. In addition, rapid absorption of MDx3 was observed by EIA using Antiserum-I with PBA column. These results suggest that EIA using Antiserum-I with PBA column for the pretreatment of serum samples should be a more useful and valuable system in therapeutic drug monitoring and pharmacokinetic studies of the unchanged type of MDx3 than Antiserum-II.

Key words enzyme immunoassay; β -methyldigoxin; pharmacokinetic study; anti- β -methyldigoxin 3'-hemisuccinate-bovine serum albumin antiserum; commercial anti-digoxin antiserum; phenyl boric acid column

Cardiac glycosides are clinically used as an important drug for the treatment of congestive heart failure and atrial fibrillation. Their therapeutic monitoring has been performed because their effective concentration in serum is very low and narrow. β -Methyldigoxin (MDx3) is a compound conjugating methyl group at the C-4''' position of digoxin (Dx3), and it is a more absorptive drug as compared with Dx3 after *p.o.* administration.

Immunoassay procedures have been used to monitor cardiac glycoside concentrations in serum. In general, commercial anti-Dx3 antiserum has been clinically used to monitor serum MDx3 levels in humans.^{1–3)} However, because orally administered MDx3 was mainly metabolized to Dx3, digoxigenin bisdigitoxoside (Dx2), digoxigenin monodigitoxoside (Dx1), digoxigenin (Dx0), and dihydrodigoxin (DihDx3) in the body,^{4–6)} the low specificity of used antibody is one of the most severe problems for measuring MDx3 (Chart 1).

To obtain a high specific antibody, the binding position of hapten to the carrier protein is a key point. Shimada *et al.*⁷⁾ and Thong *et al.*⁸⁾ used conjugates where the hapten was linked to the carrier protein at the C-12 (or C-17) and C-22 positions, respectively. These antisera exhibited remarkable cross-reactivity with DihDx3, one of the metabolites. Recently, we have reported on the preparation and antigenic properties of digitoxin-bovine serum albumin (BSA), Dx3–BSA, and MDx3–BSA conjugates linked at the digitoxose C-3' and C-3'' positions using radio or enzyme immunoassay (EIA).^{9–12)} Our previous results have suggested that antisera to these conjugates linked at the digitoxose C-3' position showed higher specificity to metabolites, while commercial anti-digitoxin and Dx3 antisera exhibited remarkable cross-reactivities.^{9–12)} In addition, we have investigated a

method to rapidly and selectively separate MDx3 from mixtures of MDx3 and its metabolites in human serum using phenyl boric acid (PBA) column. However, there have not yet been reports the evaluating pharmacokinetic change of MDx3 using newly prepared antiserum.

In the present study, we describe comparisons among the pharmacokinetic parameters of MDx3 by EIA using anti-MDx3 3'-hemisuccinate–BSA antiserum (Antiserum-I) with PBA column, Antiserum-I, and commercial anti-Dx3 antiserum (Antiserum-II) in rats.

MATERIALS AND METHODS

Materials MDx3 and DihDx3 were obtained from Boehringer Mannheim (Mannheim, Germany), BSA (fraction V), anti-rabbit IgG antiserum developed in goats, and anti-Dx3 antiserum from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and Dx3 from Aldrich (Milwaukee, WI, U.S.A.). Reversed-phase KC₁₈ plates (5×10 cm) for TLC were purchased from Whatman (Clifton, NJ, U.S.A.). Digitoxin, 4-methylumbelliferyl- β -D-galactopyranoside, β -D-galactosidase (EC 3.2.1.23) from *Escherichia coli*, and other general reagents were supplied by Wako Pure Chemical Industries (Osaka, Japan). Dx0, Dx1, and Dx2 were prepared by hydrolysis of Dx3 according to the method of Haack and co-workers.¹³⁾ PBA column and VacElut Vacuum Manifold were purchased from Varian (Harbor City, CA, U.S.A.) and GL Science (Tokyo, Japan), respectively.

EIA Method EIA was based on the principle of competition between enzyme-labeled and unlabeled drugs for an antibody, followed by measurement of the marker enzyme activity of the immune precipitate. EIA was performed in phosphate saline buffer (pH 7.3). Phosphate saline buffer was

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Chart 1. Molecular Structures of MDx3 and Its Metabolites

1, MDx3; 2, Dx3; 3, Dx2; 4, Dx1; 5, Dx0; 6, DihDx3.

adjusted to pH 7.3 by addition of a solution containing NaH_2PO_4 (3.9 g), NaCl (4.5 g), NaN_3 (0.5 g), MgCl_2 (0.005 g), and BSA (0.5 g) in H_2O (500 ml) to a solution containing K_2HPO_4 (8.7 g), NaCl (9.0 g), NaN_3 (1.0 g), MgCl_2 (0.01 g), and BSA (1.0 g) in H_2O (1000 ml). Standard samples (0.2 ml) of MDx3 (0.2–20 ng/ml) and diluted antiserum (0.1 ml) were mixed and incubated for 2.5 h at 4°C. Then, a 0.1 ml volume of synthetic enzyme-labeled antigens¹²⁾ was added and incubated for 0.5 h at 4°C. A 0.2 ml volume of goat anti-rabbit IgG antiserum (1.67%) and 0.1 ml of normal rabbit serum (1%) were added and allowed to stand for 12 h at 4°C. Mixtures were centrifuged at $1600\times g$ for 20 min, the supernatant was aspirated, and the immune precipitate was twice washed by 1 ml of phosphate saline buffer. The activity of enzyme conjugate bound to each tube was then measured by the addition of 0.5 ml of 4-methylumbelliferyl- β -D-galactopyranoside (2.0×10^{-5} M), followed by incubation of the tubes at 30°C for 3 h after pre-incubation of 0.5 ml of phosphate saline buffer at 30°C for 3 min. The enzyme reaction was stopped by the addition of 2.0 ml of glycine-NaOH buffer (0.1 M, pH 10.3) to each tube, and the resulting 4-methylumbelliferone was measured by spectrofluorometry at wavelengths of 362 nm for excitation and 446 nm for emission using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan).

Extraction of MDx3 and Its Metabolites in Rat Serum

A 0.2 ml volume of rat serum containing MDx3, Dx3, Dx2, Dx1, Dx0, and DihDx3, 0.1 ml of boric acid (10 mM), and 0.1 ml of NH_4OH (0.3%) were mixed and the mixtures were extracted into 2 ml of AcOEt twice. Then, each 3.6 ml of AcOEt phase was combined, evaporated with a concentrator (TAITEC, Saitama, Japan), and 1 ml of CH_3CN was added to the residue.

Separation of MDx3 Using PBA Column Separation procedures were as follows. A 1 ml volume of HCl (0.1 M) was added to the column, which was allowed to completely run through with VacElut. Then, a 4 ml volume of NH_4OH (1.0%), 2 ml of $(\text{NH}_4)_2\text{SO}_4$ (0.01 M, pH 8.5), and 2 ml of NH_4OH (0.3%) were passed through the column, succes-

sively. The above CH_3CN solution was added to the column, allowed to completely run at gravity, and the column was extracted four times with 1 ml of CH_3CN . About 4 ml of collected CH_3CN solution was evaporated with a concentrator, and was used in the assay.

Serum Concentration–Time Courses of MDx3 in Rats

Wistar male rats (8–10 weeks, 267 ± 21 g; mean $\pm$ S.D., Sankyo Laboratory Animal, Toyama, Japan) were used in the pharmacokinetic study. A 250 μl volume (18 $\mu\text{g}/\text{ml}$) of 5% ethanol solution containing MDx3 was prepared. Under the light anesthetization by diethyl ether, blood samples (0.2–1.5 ml) were withdrawn from the carotid vein at designated time intervals (0.033, 0.25, 0.5, 0.75, 1, 2.5, 6, 10, and 24 h for intravenous administration; 0.033, 0.25, 0.5, 0.75, 1, 2.5, 6, 10, and 24 h for oral administration) after a single oral or intravenous administration of MDx3 (0.017 mg/kg, about 4.5 $\mu\text{g}/\text{head}$) to rats, and collected in tubes. Serum samples were separated by centrifugation ($1600\times g$, 10 min) after blood samples were allowed to stand for 3 h at 4°C and clotting. Because the measurement by EIA using Antiserum-II at 24 h after administration of MDx3 was below the quantitation limit, MDx3 levels in rat serum were determined after the addition of 0.2 ml of phosphate saline buffer to a residue of 0.6 ml of serum sample evaporated with a concentrator. And, because serum MDx3 levels at 0.033, 0.25, and 0.5 h after intravenous administration were over 5 ng/ml, MDx3 levels were determined after the dilution of the serum samples by phosphate saline buffer.

Data Analysis Pharmacokinetic parameters were estimated according to model-independent moment analysis as described by Yamaoka *et al.*¹⁴⁾ The data were analyzed using the Student's *t*-test to compare the unpaired mean values of the two sets of data. The number of determinations (*n*) is noted in a table and figure. A value of *p*-values < 0.05 was taken to indicate a significant difference between sets of data.

RESULTS

It is very important to determine the concentration of

MDx3 in serum in order to carry out pharmacokinetic studies of MDx3. In the previous study, we showed that the standard curve of MDx3 using Antiserum-I at a final dilution of 1 : 1500 was a linear relationship at a range of 0.2 to 20 ng/ml and, in contrast, that using Antiserum-II at a final dilution of 1 : 500 was a linear relationship at a range of 0.5 to 5 ng/ml.¹²⁾ Antiserum-I possessed high specificity, exhibiting considerable cross-reaction with Dx3 (43%) and low cross-reactions with Dx2 (8.3%), DihDx3 (4.8%), and digitoxin (1.5%). Also, there was no significant cross-reaction with Dx1 (0.95%) and Dx0 (<0.05%). In contrast, Antiserum-II possessed much lower specificity to MDx3 compared with antiserum-I, exhibiting high cross-reactions with Dx3 (81%), Dx2 (103%), Dx1 (95%), and Dx0 (63%). Also, there was low cross-reaction with DihDx3 (3.7%) and digitoxin (0.55%).¹²⁾ These results suggested that Antiserum-II was inferior to Antiserum-I in term of the detection limit, measurable range, and specificity.

To test the precision and accuracy of EIA in rat serum, intra- and inter-assays ($n=7$) were performed between 0.5 and 5 ng/ml. In intra-assay, the range of standard deviation to average was within 4.8 to 8.2%, and the recovery was within

98.0 to 104.0%. In inter-assay, the range of standard deviation to average was within 6.7 to 8.9%, and the recovery was within 98.0 to 105.0%. These data show that the precision of EIA is satisfactory.

As shown in Table 1, we investigated the interference of MDx3 metabolites in rat serum by using Antiserum-I and Antiserum-II. Each metabolite (Dx3, Dx2, Dx1, Dx0, and DihDx3) at ratios of 10%, 30% and 100% to MDx3 concentration was added to the MDx3 in rat serum, and a recovery test was performed. The recovery ratios using Antiserum-I at 10%, 30%, and 100% were within ranges of 109–114%, 135–144%, and 176–264%, respectively. In contrast, those using Antiserum-II at 10%, 30%, and 100% were within ranges of >148%, >264%, and >510%, respectively. These results indicate that the interference of MDx3 metabolites by using Antiserum-I in rat serum was much lower than that of Antiserum-II and that the pharmacokinetic studies of MDx3 using Antiserum-I in rat serum may be more useful than those using Antiserum-II because of the low specificity of Antiserum-I to MDx3.

Removal of cross-reactive compounds from mixtures of MDx3 and its metabolites in rat serum is one of the best methods to eliminate interference of EIA. Recently, PBA column has been used for the rapid and selective isolation of urinary catecholamine.^{15,16)} Also, our previous results have shown that MDx3 was separated from mixture of MDx3 and its metabolites in human serum using PBA column.¹²⁾ When we tried rapid and selective separation of MDx3 from the mixtures in rat serum using PBA column according to our previous methods, MDx3 and Dx0 exhibiting no cross-reactivity (<0.05%) were separated. As shown in Tables 2 and 3, recoveries of MDx3 by EIA using PBA column in rat serum were satisfactory (about 110%, $n=9$), and no significant interference of metabolites was observed. These data were consistent with our previous results in human serum.¹²⁾ These results suggest that PBA column is a useful tool to separate

Table 1. Interference of MDx3 Metabolites on the Assay of MDx3 in Rat Serum

Added	Antiserum-I		Antiserum-II	
	Measured (ng/ml) Mean $\pm$ S.D. ($n=7$)	Recovery (%)	Measured (ng/ml) Mean $\pm$ S.D. ($n=7$)	Recovery (%)
MDx3 (0.5 ng/ml)				
+Metabolites (each 0.05 ng/ml)	0.57 $\pm$ 0.04	114	0.74 $\pm$ 0.07	148
+Metabolites (each 0.15 ng/ml)	0.70 $\pm$ 0.07	140	1.32 $\pm$ 0.14	264
+Metabolites (each 0.5 ng/ml)	0.88 $\pm$ 0.09	176	2.55 $\pm$ 0.24	510
MDx3 (1 ng/ml)				
+Metabolites (each 0.1 ng/ml)	1.09 $\pm$ 0.10	109	1.80 $\pm$ 0.16	180
+Metabolites (each 0.3 ng/ml)	1.43 $\pm$ 0.14	143	2.68 $\pm$ 0.23	268
+Metabolites (each 1 ng/ml)	2.11 $\pm$ 0.20	211	>5.0	(-)
MDx3 (2 ng/ml)				
+Metabolites (each 0.2 ng/ml)	2.20 $\pm$ 0.22	110	3.56 $\pm$ 0.36	178
+Metabolites (each 0.6 ng/ml)	2.88 $\pm$ 0.24	144	>5.0	(-)
+Metabolites (each 2 ng/ml)	4.61 $\pm$ 0.46	231	>5.0	(-)
MDx3 (5 ng/ml)				
+Metabolites (each 0.5 ng/ml)	5.52 $\pm$ 0.52	110	>5.0	(-)
+Metabolites (each 1.5 ng/ml)	6.74 $\pm$ 0.78	135	>5.0	(-)
+Metabolites (each 5 ng/ml)	13.2 $\pm$ 1.45	264	>5.0	(-)

Metabolites: Dx3, Dx2, Dx1, Dx0, and DihDx3.

Table 3. Recovery of MDx3 in Rat Serum by Homologous Assay Using Antiserum-I after PBA Column Treatment

Added (ng/ml)	Measured (ng/ml) Mean $\pm$ S.D. ($n=9$)	C.V. (%)	Recovery (%)
0.5	0.56 $\pm$ 0.05	8.9	112
1	1.14 $\pm$ 0.12	10.5	114
2	2.27 $\pm$ 0.22	9.7	114
5	5.45 $\pm$ 0.58	10.6	109

Table 2. Interference of MDx3 Metabolites on the Assay of MDx3 by Homologous Assay Using Antiserum-I in Rat Serum after PBA Column Treatment

Added	Non-PBA treatment			PBA treatment		
	Measured (ng/ml) Mean $\pm$ S.D. ($n=7$)	C.V. (%)	Recovery (%)	Measured (ng/ml) Mean $\pm$ S.D. ($n=9$)	C.V. (%)	Recovery (%)
MDx3 (0.5 ng/ml)						
+Metabolites (each 0.5 ng/ml)	0.88 $\pm$ 0.09	10.2	176	0.55 $\pm$ 0.06	10.9	110
MDx3 (1 ng/ml)						
+Metabolites (each 1 ng/ml)	2.11 $\pm$ 0.20	9.5	211	1.11 $\pm$ 0.11	9.9	111
MDx3 (2 ng/ml)						
+Metabolites (each 2 ng/ml)	4.61 $\pm$ 0.46	10.0	231	2.29 $\pm$ 0.22	9.6	115
MDx3 (5 ng/ml)						
+Metabolites (each 5 ng/ml)	13.2 $\pm$ 1.45	11.0	264	5.65 $\pm$ 0.57	10.1	113

Metabolites: Dx3, Dx2, Dx1, Dx0, and DihDx3.

Fig. 1. Serum Concentration–Time Courses of MDx3 after a Single Intravenous or Oral Administration of MDx3 (0.017 mg/kg) in Wistar Male Rats (A) Intravenous administration; (B) oral administration; ● EIA-I; ○ EIA-II; △ EIA-III. Each point with the bar represents the mean $\pm$ S.D. of seven rats.

Table 4. Pharmacokinetic Parameters of MDx3 after a Single Intravenous or Oral Administration of MDx3 (0.017 mg/kg) in Rats

	Conditions		
	Antiserum-I with PBA column	Antiserum-I	Antiserum-II
Intravenous administration			
$AUC_{0 \rightarrow \infty}$ (ng $\cdot$ h/ml)	32.0 $\pm$ 6.5*	38.9 $\pm$ 4.9*	49.2 $\pm$ 6.0
MRT (h)	12.4 $\pm$ 2.5	11.6 $\pm$ 1.8	10.7 $\pm$ 1.2
V_{dss} (l/kg)	6.57 $\pm$ 1.48*	5.09 $\pm$ 0.77*	3.71 $\pm$ 0.44
CL_{tot} (l/h/kg)	0.531 $\pm$ 0.112*	0.437 $\pm$ 0.062*	0.346 $\pm$ 0.051
k_e (h $^{-1}$)	0.0808 $\pm$ 0.0165	0.0859 $\pm$ 0.0101	0.0933 $\pm$ 0.0109
Oral administration			
$AUC_{0 \rightarrow \infty}$ (ng $\cdot$ h/ml)	25.1 $\pm$ 5.5*	29.1 $\pm$ 3.8*	36.1 $\pm$ 5.5
BA (%)	78.4	74.7	73.4
MRT (h)	12.9 $\pm$ 2.7	12.4 $\pm$ 1.5	11.7 $\pm$ 1.4
k_a (h $^{-1}$)	1.92	1.38	1.00
T_{max} (h)	1.72	2.14	2.61
C_{max} (ng/ml)	1.77	2.08	2.64

Pharmacokinetic parameters were estimated according to model-independent moment analysis. Each value represents the mean $\pm$ S.D. of seven rats. * Significantly different from values using Antiserum-II at $p < 0.05$. Abbreviations: $AUC_{0 \rightarrow \infty}$, the areas under the serum concentration–time curves up to infinity; MRT , mean residence time; V_{dss} , distribution volume at steady state; CL_{tot} , total body clearance; k_e , elimination rate constant; BA , bioavailability; k_a , absorption rate constant; T_{max} , time taken to reach to maximum serum concentration; C_{max} , maximum serum concentration.

MDx3 and our EIA by Antiserum-I with PBA column for pretreatment of serum is cable to precisely monitor the unchanged type of MDx3 in rat serum.

In the next step, serum concentration–time courses of MDx3 after a single intravenous or oral administration of MDx3 (0.017 mg/kg) in rats were investigated by EIA using Antiserum-I with PBA column for pretreatment of rat serum samples (EIA-I), EIA using Antiserum-I without PBA column for pretreatment of rat serum samples (EIA-II), and EIA using Antiserum-II (EIA-III) as shown in Fig. 1. The time courses of MDx3 by EIA-I after a single intravenous or oral administration of MDx3 were significantly lower than those by EIA-III, and nearly consistent with values measured by EIA-II. Pharmacokinetic parameters of MDx3 estimated by EIA-I, EIA-II, and EIA-III are listed in Table 4. Judging from the area under the serum concentration–time curves up to infinity ($AUC_{0 \rightarrow \infty}$), the values by EIA-I in both administrations were significantly lower than those by EIA-III and were not significantly different when compared with those by EIA-II. The distribution volume at steady state (V_{dss}) and the total

body clearance (CL_{tot}) values of MDx3 by EIA-I and EIA-II were significantly higher than those by EIA-III. Significant difference between EIA-I and EIA-II was not observed in V_{dss} and CL_{tot} values. The bioavailability (BA) of MDx3 by EIA-I, EIA-II, and EIA-III were 78.4, 74.7, and 73.4%, respectively. Maximum serum concentrations (C_{max}) of MDx3 by EIA-I and EIA-II were reduced to 75 and 80% compared with that by EIA-III. In addition, time taken to reach to maximum serum concentration (T_{max}) of MDx3 by EIA-I and EIA-II was reduced to 66 and 82% compared with that by EIA-III. Moreover, the absorption rate constant (k_a) value of MDx3 by EIA-I was the highest among EIA-I, EIA-II, and EIA-III. Significant changes in other parameters of MDx3 were not observed.

DISCUSSION

In the present study, we compared the utility of a novel specific antiserum of anti-MDx3 3'-hemisuccinate–BSA antiserum (Antiserum-I) and PBA column for pretreatment of rat serum samples in EIA with commercial anti-Dx3 antiserum (Antiserum-II). Obvious interference of metabolites was observed in EIA using Antiserum-II (Table 1). These data show that significant measurement errors to metabolites generated after administration of MDx3 are more evident in EIA using Antiserum-II compared with Antiserum-I.

However, it is speculated that the high cross-reactivity of Dx3 using Antiserum-I may result in a significant measurement error. Removal of Dx3 in assay samples is a good method to delete measurement error. When a PBA column was used for the pretreatment of rat serum samples containing Dx3, Dx2, Dx1, Dx0, and DihDx3, MDx3 and Dx0 were separated.¹²⁾ Recovery of MDx3 using PBA column was satisfactory as shown in Table 1. Antiserum-II showed high cross-reactivity with Dx0 (63%), while Antiserum-I showed a negligible value of $<0.05\%$. It is considered that EIA by Antiserum-II using PBA column shows a significant measurement error. On these points, Antiserum-I of our novel specific antiserum is superior to Antiserum-II and PBA column is a very useful tool to separate MDx3 in rat serum samples.

When MDx3 was intravenously or orally administered to rats, serum concentration–time courses of MDx3 by EIA-I, EIA-II, and EIA-III were investigated (Fig. 1). As shown in Table 4, significant changes of pharmacokinetic parameters

(increasing of V_{dss} and CL_{tot} and reducing of $AUC_{0 \rightarrow \infty}$ and C_{max}) of MDx3 by EIA-II were observed compared with EIA-III. Because the specificity of Antiserum-I to MDx3 is superior to Antiserum-II, it is considered that obvious changes of these parameters may be mainly involved in higher specificity to MDx3 than Antiserum-II. Accordingly, it is supposed that V_{dss} and CL_{tot} of MDx3 by EIA-I show the highest level and $AUC_{0 \rightarrow \infty}$ shows the lowest level, because measurement of MDx3 by EIA-II and EIA-III contains MDx3 and its metabolites. As expected, the present results showed that $AUC_{0 \rightarrow \infty}$ of MDx3 by EIA-I was reduced by 17% compared with EIA-II, while V_{dss} and CL_{tot} were increased by 29 and 22%, respectively. In addition, the rapid absorption of MDx3 after oral administration to rats was observed by EIA-I. However, the effects of PBA column on pharmacokinetic parameters of MDx3 were not significantly observed. Here, a question arises as to whether PBA column must be utilized in monitoring of MDx3. Hinderling *et al.* have reported that Dx3 produced in the body after MDx3 administration could be detected at a ratio of about 10% to the dosage.^{5,6)} Our results and their reports indicate that Dx3 metabolized after MDx3 administration should bring about measurement errors at low level. Therefore, it is estimated that serum levels monitored by EIA-II after MDx3 administration provide MDx3 concentration containing Dx3. Then, so far, it has also been shown that several diseases such as hepatic and renal failures and administration of drugs such as corticosteroids resulted in producing strong abilities to increase endogenous Dx3-like compound levels and induce metabolic enzyme activity.^{17–21)} Rameis *et al.* have suggested that demethylation at C-4''' of MDx3 was remarkably reduced in patients with cirrhosis.¹⁷⁾ Due to changes of hepatic and renal function and/or metabolic enzyme activity in the body, monitoring of MDx3 by EIA-II may not be successfully carried out after administration of MDx3. While further studies are needed on factors and mechanism changing serum concentration of MDx3 by several diseases and combined drugs, it is considered that EIA-I should be required to precisely monitor the unchanged type of MDx3 in digitalized patients.

Consequently, our present results suggest that the EIA method using Antiserum-I with PBA column for pretreatment of serum samples is successfully applied to the deter-

mination of the unchanged type of MDx3 in serum after administration of MDx3 to rats. We expect that our newly developed assay system to MDx3 will be clinically popularized in the world in the near future.

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