Establishment of Enzyme Immunoassay for Measuring β-Methyldigoxin Levels in Human Serum by Specific Antiserum

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We investigated the specificity of obtained antiserum to β-methyldigoxin by the enzyme immunoassay. Three types of hapten-bovine serum albumin (BSA) conjugates were synthesized to obtain high specific antiserum to β-methyldigoxin. The hapten was linked to the carrier protein through hemisuccinate at C-3′ and C-3″ positions in the digitoxose chain and at C-12 position in the aglycone. Anti-β-methyldigoxin 3′-hemisuccinate–BSA anti-serum showed a low detection limit (0.2 ng/ml) and possessed high specificity for β-methyldigoxin, exhibiting low cross-reactions with digoxigenin bisdigitoxoside (8.3%), dihydrodigoxin (4.8%), digitoxin (1.5%), and digoxigenin monodigitoxoside (0.95%), except for cross-reaction with digoxin (43%). Compared with commercial anti-digoxin antiserum, clinically used to monitor β-methyldigoxin concentration in human serum, cross-reaction data of anti-β-methyldigoxin 3′-hemisuccinate–BSA antiserum showed higher specificity for β-methyldigoxin. The intra-assay and inter-assay variation using this antiserum were less than 6.9% and 8.1%, respectively. The recovery tests were good, within the range of 96.2–104.3%. Phenyl boric acid (PBA) column treatment was effective to rapidly and selectively separate β-methyldigoxin from the mixture of β-methyldigoxin and its metabolites in human serum. The recovery tests of β-methyldigoxin with PBA column in human serum were about 110% and interference of metabolites of β-methyldigoxin was negligible. These results suggest that anti-β-methyldigoxin 3′-hemisuccinate–BSA antiserum and PBA column treatment are useful to more precisely monitor the unchanged type of β-methyldigoxin concentration in human serum.

Key words β-methyldigoxin; anti-β-methyldigoxin 3′-hemisuccinate–BSA antiserum; phenyl boric acid column; anti-digoxin antiserum; enzyme immunoassay; cross-reactivity

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

Immunoassay procedures have been used to monitor cardiac glycosides concentrations in serum. Recently, anti-Dx3 antibody has been clinically used to monitor MDx3 levels in human serum.1–3) However, because administered MDx3 is metabolized to mainly Dx3, digoxigenin bisdigitoxoside (Dx2), digoxigenin monodigitoxoside (Dx1), digoxigenin (Dx0), and dihydrodigoxin (DihDx3) in the body,4–6) low specificity of used antibodies is one of the most severe problems for measuring MDx3. To obtain a highly specific antibody, the binding position of hapten to 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. Previously, we reported preparation and antigenic properties of Dx3–bovine serum albumin (BSA) and digoxigenin–BSA conjugates linked at the digitoxose C-3′ and C-3″ positions using radioimmunoassay (RIA).9–11) Our previous results suggested that antiserum to these conjugates linked at the digitoxose C-3′ position showed higher specificity to metabolites. However, a more convenient and simple method is needed, because RIA uses radioactive substances and a special facility.

In this paper, we prepared MDx3–BSA conjugates possessing bridges at the hydroxyl groups of C-3′, C-3″, or C-12 as binding positions in synthesizing antigens, and show specific properties to MDx3 of three obtained antiserum and commercial anti-Dx3 antiserum in enzyme immunoassay (EIA). Then, using a phenyl boric acid (PBA) column, which has the ability to adsorb compounds possessing cis-diol group in the chemical structure,12,13) we carried out the pretreatment to separate MDx3 from mixtures of MDx3 and its metabolites in human serum and investigated the usefulness of a PBA column to monitor the unchanged type of MDx3.

MATERIALS AND METHODS

Materials MDx3 and DihDx3 were obtained from Boehringer Mannheim (Mannheim, Germany), spironolactone, BSA (fraction V), anti-rabbit IgG antiserum developed in goat, and anti-Dx3 antiserum from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Dx3 from Aldrich (Milwaukee, WI, U.S.A.). Sephadex LH-20 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Reversed-phase KC18 plates (5 × 10 cm) for TLC from Whatman (Clifton, NJ, U.S.A.), Kiesel-gel 60 for column chromatography and high performance thin-layer chromatography (HPTLC) plates (5 × 10 cm) from E. Merck (Darmstadt, Germany). Digi-toxin, β-D-galactosidase (EC 3.2.1.23) from Escherichia coli, and 4-methylumbelliferyl-β-D-galactopyranoside, and other general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Dx0, Dx1, and DihDx3 were prepared by hydrolysis of Dx3 according to the methods by Kaiser and co-workers.14) DihDx3 12-hemisuccinate was synthesized by the method of Ikeda and Fujii.9) PBA column and VacElut Vacuum Manifold were purchased from Varian (Harbor City, U.S.A.) and GL Science (Tokyo, Japan), respectively.

Apparatus All melting points were determined with a Yanagimoto micro hot-stage apparatus and are uncorrected.

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Optical rotations were measured with a JASCO DIP-370 digital polarimeter. FAB-MS measurements were made on a JEOL HX-100 instrument equipped with a FAB ion source using glycerol and NaCl as the matrix agents. UV spectra were obtained with a Shimadzu UV-3000 recording spectrophotometer. 1H-NMR spectra were recorded using tetramethylsilane as an internal standard on a JEOL EX-90A spectrometer at 90 MHz. Abbreviations used: s = singlet, d = doublet, and m = multiplet.

**MDX3 3'-Hemisuccinate (1)** To a solution of MDX3 (1.2 g, 1.5 mmol) in pyridine (70 ml), succinic anhydride (1.2 g, 12 mmol) was added, and the mixture was allowed to stand at 70°C for 73.5 h. The reaction mixture was extracted with AcOEt, and the organic layer was washed with 1% HCl, 1% NaHCO₃, and H₂O, then dried over anhydrous Na₂SO₄. The aqueous layer was percolated through an Amberlite XAD-2 column (80×1.6 cm i.d.). The column was washed with H₂O, then the desired material, which could not be extracted with AcOEt, was eluted with MeOH. The AcOEt extract and the MeOH eluate were combined and submitted to silica-gel column (133×2.5 cm i.d.) chromatography using CHCl₃–MeOH–H₂O (9:2:8:0.5, v/v) as a mobile phase. The eluate corresponding to 1 was further purified on a Sephadex LH-20 column (47×1.5 cm i.d.) using MeOH as an eluent. The eluate was recrystallized from acetone–hexane (1:2, v/v) to give 1 (75 mg, 13.6%) as a colorless amorphous solid. mp 103.4° (c=0.20, MeOH). Anal. Caled for C₄₆H₇₀O₁₇·0.5H₂O: C, 61.73; H, 7.88. Found: C, 61.37; H, 8.12.

**MDX3 12-Hemisuccinate (2)** To a solution of MDX3 (1.52 g, 1.7 mmol) in dioxane (20 ml), and the mixture was stirred at room temperature for 8 h. After evaporation of the solvent, the residue was spotted on TLC plates.

**MDX3 3'-Hemisuccinate p-Nitrophenyl Ester (4)** Di-cyclohexylcarbodiimide (DCC, 43 mg, 0.21 mmol) was added to a solution of 1 (80 mg, 0.089 mmol) and p-nitrophenol (410 mg, 2.9 mmol) in dioxane (13 ml), and the mixture was stirred at room temperature for 8 h. After evaporation of the solvent, the crude product obtained was submitted to silica-gel column (47×1.5 cm i.d.) chromatography using hexane–AcOEt (1:2, v/v) as a mobile phase, and 4 (61 mg, 67.2%) was obtained as a yellow oil. 1H-NMR (CDCl₃) δ: 0.79 (3H, s, 18-CH₃), 0.93 (3H, s, 19-CH₃), 0.93 (3H, s, 19-CH₃), 1.21—1.27 (9H, m, sugar-CH₃), 2.67 (4H, brs, –CO(CH₂)₂CO–), 3.41 (3H, s, –OCH₃), 5.38 (1H, m, 3'-H), 5.95 (1H, s, 22-H).
stirred at room temperature for 7 h. After evaporation of the solvent, the crude product obtained was submitted to silica-gel column (47×1.5 cm i.d.) chromatography using hexane–AcOEt (1:2, v/v) as a mobile phase, and 6 (83 mg, 48.1%) was obtained as a yellow oil. 1H-NMR (CDCl3) δ: 0.90 and 0.91 (6H, s, 18-CH3 or 19-CH3), 1.19—1.30 (9H, m, sugar-CH3), 2.71—2.81 (4H, m, –CO(CH2)2CO–), 3.42 (3H, s, –OCH3), 5.98 (1H, s, 22-H), 7.15, 8.27 (each 2H, d, J=9 Hz, aromatic H).

MDx3 3′-Hemisuccinate-BSA (7), MDx3 3′-Hemisuccinate–BSA (8), and MDx3 12′-Hemisuccinate–BSA (9) A solution of BSA (50 mg) in 0.05 M phosphate buffer (pH 7.4, 0.5 ml) was added to a solution of 5 (40 mg, 0.039 mmol), 6 (20 mg, 0.020 mmol), or 7 (52 mg 0.051 mmol) in pyridine (0.5 ml), and then these mixtures were stirred at room temperature for 8 h to synthesize 7 or 8 and for 5 h to synthesize 9. The resulting solutions were dialyzed against a constant flow of cold water (201) at room temperature overnight. Lyophilization of these solutions afforded 7, 8, or 9 as a fluffy powder.

Preparation of β-d-Galactosidase-Labeled Antigens A solution of 4 (5.8 mg), 5 (6.0 mg), or 6 (2.0 mg) in dioxan (0.5 ml) were incubated with a solution of β-d-galactosidase (2.1 mg) in 1 ml of 0.05 M phosphate buffer (pH 7) for 4 h at 4 °C. Then, the reaction mixtures were submitted to Sephadex G-25 column (45×1.5 cm i.d.) chromatography using 0.05 M phosphate buffer (pH 7.0) as a mobile phase. 2.7 ml of fractions were collected, and fractions representing the main peak of the enzyme activity were further submitted to Sephadex G-100 column (45×1.5 cm i.d.) chromatography using 0.05 M phosphate buffer (pH 7.0) as a mobile phase. Fractions representing the main peak of the enzyme activity were chosen as a labeled antigen in EIA. On the other hand, each fraction was spotted on TLC plates using CH3Cl–MeOH–AcOH (90 : 10 : 0.8, v/v) as a developing solvent, and coloration for steroid compounds at origin was observed by spraying with concentrated H2SO4. From these results, three enzyme-labeled antigens (MDx3 3′-g, 3′-h, and 12′-hemisuccinate–β-d-galactosidase) were obtained.

Immunoassay Procedure Three domestic strains of male albino rabbits were used for immunization with each hapten–BSA conjugate. The antigen (3 mg) was dissolved in sterile isotonic saline (0.9 ml) and emulsified with complete Freund’s adjuvant (2.1 ml). The emulsion was injected into sterile isotonic saline (0.9 ml) and emulsified with complete Freund’s adjuvant (2.1 ml). The emulsion was injected into each rabbit. The procedure was repeated at intervals of two weeks for five months and then the rabbits were boosted twice a month. After confirmation of the increase in the antibody titer, blood was collected from the marginal ear veins. Sera were separated by centrifugation at 1400×g for 10 min and stored at −18°C in small aliquots. The antisera were thawed, diluted with phosphate saline buffer (pH 7.4) and used in the assay.

EIA Method EIA is 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 adjusted to pH 7.3 by addition of a solution containing NaH2PO4 (3.9 g), NaN3 (4.5 g), MgCl2 (0.005 g), and BSA (0.5 g) in H2O (500 ml) to a solution containing K2HPO4 (8.7 g), NaCl (9.0 g), NaN3 (1.0 g), MgCl2 (0.01 g), and BSA (1.0 g) in H2O (1000 ml). Standard samples (0.2 ml) of MDx3 (0.2–20 ng/ml) and diluted antisera (0.1 ml) were mixed and incubated for 2.5 h at 4°C. Then, 0.1 ml of synthetic enzyme–labeled antigens (diluted 1:1200 in phosphate saline buffer) was added and incubated for 0.5 h at 4°C. 0.2 ml of goat anti-rabbit IgG antisera (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×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-methylumbelliferol-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 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 spectrophotometry at wavelengths of 362 nm for excitation and 446 nm for emission using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan).

Cross-Reaction Study The antisera raised against 7, 8, and 9, and commercial anti-Dx3 antiserum were abbreviated to Antiserum-A, Antiserum-B, Antiserum-C, and Antiserum-D, respectively. The specificities of these antisera were tested by calculating the percentage cross-reactivity with various compounds. Cross-reactivity was determined by the above-mentioned assay procedure, by comparing the concentrations of MDx3 and test compounds necessary for a 50% displacement of the antibody-bound enzyme-labeled MDx3.

Extraction of MDx3 and Its Metabolites in Human Serum 0.2 ml of human serum containing MDx3, Dx3, Dx2, Dx1, Dx0, and DihDx3, 0.1 ml of boric acid (10 mxt), and 0.1 ml of NH4OH (0.3%) were mixed and the mixtures were extracted into 2 ml of AcOEt at two times. Then, each 1.8 ml of AcOEt phase was combined, evaporated with a
Separation of MDx3 Using PBA Column

Separation procedures were as follows. 1 ml of HCl (0.1 M) was added to the column, which was allowed to completely run through with VacElut (GL Science, Tokyo, Japan). Then, 4 ml of NH$_4$OH (1.0%), 2 ml of (NH$_4$)$_2$SO$_4$ (0.01 M, pH 8.5), and 2 ml of NH$_4$OH (0.3%) were passed through the column, successively. The above CH$_3$CN solution was added to the column, allowed to completely run at gravity, and the column was extracted four times with 1 ml of CH$_3$CN. About 4 ml of collected CH$_3$CN solution was evaporated with a concentrator (TAITEC, Saitama, Japan), and 1 ml of CH$_3$CN was added to the residue.

RESULTS AND DISCUSSION

Initially, the synthesis of MDx3 3′-, 3″-, and 12-hemisuccinate–BSA conjugates (7–9) was carried out. In the previous papers of this series, we reported the preparation of digitoxin 3′- and 3″-hemisuccinates from digitoxin and Dx3 3′- and 3″-hemisuccinates from Dx3 by utilizing the reactivities of the hydroxyl groups in the digitoxoside moiety. In a similar way, MDx3 3′-, 3″-, and 12-hemisuccinates (1–3) could be prepared from MDx3 with succinic anhydride in pyridine. The resulting crude product was submitted to silicagel column chromatography using a CHCl$_3$–MeOH–AcOH (95:5:0.2, v/v) or CHCl$_3$–MeOH–H$_2$O (92:8:0.5, v/v) as an eluent, and the chromatography provided a satisfactory separation of three isomeric hemisuccinates. In the $^1$H-NMR spectra of 1, 2, and 3, the four-methylene protons appeared as a broad singlet, showing that one succinyl moiety had been introduced.

The position of the succinyl group was elucidated by partial hydrolysis of these compounds. Reversed-phase TLC of their hydrolyzate is shown in Fig. 1. When MDx3 was treated with 0.05 M HCl under mild conditions, the glycosidic bond was partially hydrolyzed to give a mixture of Dx0, Dx1, Dx2, and the intact starting material. When 1 and 2 were treated in the same way, Dx0 12-hemisuccinate was not produced from 1 and 3. Dx0 was only produced from 1 and both Dx0 and Dx1 from 2. Therefore, 1 and 2 were designated as the 3′-hemisuccinate and 3″-hemisuccinate of MDx3, respectively. When 3 was treated in a similar fashion, Dx0 12-hemisuccinate was only produced from 3. Therefore, 3 was designated as the 12-hemisuccinate of MDx3. The results of FAB-MS of three compounds also supported these structures.

To couple the hapten with BSA, 1, 2, and 3 were transformed into their p-nitrophenyl esters (4–6) by treatment with p-nitrophenol and DCC in dioxane. These p-nitrophenyl esters were linked with BSA, followed by dialysis of reaction mixture against cold water to give the desired BSA conjugates (7–9).

Each immunogen thus obtained was administered to three rabbits to produce the antibody. Among the antisera elicited in rabbits, the most specific antiserum to MDx3 was selected for characterization in each group. The properties of antisera were investigated by ELISA with MDx3 hemisuccinate–β-D-galactosidase. The separation of bound and free fractions was performed using formation of an immunoprecipitate.

The standard curves of MDx3 using homologous assay by the three obtained antisera are presented in Fig. 2. Antiserum-A, Antiserum-B, and Antiserum-C bound approximately 50% of MDx3 at final dilutions of 1:1500, 1:15000, 1:2000, respectively. The plots of percent bound fluorescence intensity vs. logarithm of the concentration of non-labeled MDx3 showed a linear relationship at range 0.2 to 20 ng/ml using Antiserum-A. However, Antiserum-B and Antiserum-C showed a linear relationship over range 0.5 to 10 ng/ml. It was shown that the detection limit of Antiserum-A was the best of three obtained antisera.

The specificities of Antiserum-A, Antiserum-B, and Antiserum-C were assessed by cross-reaction tests with various related compounds. The percentage cross-reactivity was calculated at 50% displacement of the antibody-bound labeled MDx3, and the results are listed in Table 1. Antiserum-A possessed high specificity, exhibiting low cross-reactions with Dx2 (8.3%), DihDx3 (4.8%), and digitoxin (1.5%), except for cross-reaction with Dx3 (43%). Also, there was no significant cross-reaction with Dx1 (0.95%). All other compounds tested showed negligible values of <0.05%. In contrast, Antiserum-B exhibited considerable cross-reactivity with Dx3 (82%), Dx2 (65%), Dx1 (55%), Dx0 (58%), and digitoxin (36%) and low cross-reaction with DihDx3 (4.7%). Antiserum-C showed considerable cross-reactivity with Dx3.
The standard curves of MDx3 using Antiserum-A and Antiserum-D are presented in Fig. 3. The standard curve using Antiserum-D which bound approximately 50% of MDx3 at a final dilution of 1:500 showed a linear relationship in the range 0.5 to 5 ng/ml, while Antiserum-A showed a linear relationship in the range 0.2 to 20 ng/ml. These results show that Antiserum-D has poorer sensitivity and measurable range to MDx3 as compared with those of Antiserum-A.

The percentage of cross-reactivity was calculated at 50% displacement of the antibody-bound labeled MDx3, and the results are listed in Table 1. Antiserum-D possessed much lower specificity to MDx3 compared with antiserum-A, exhibiting high cross-reactions with Dx3 (81%), Dx2 (103%), Dx1 (95%), and Dx0 (63%), and low cross-reaction with DihDx3 (3.7%). All other compounds tested showed negligible values of <0.05%. Antiserum-D showed high cross-reactivity with metabolites formed by the successive cleavage of the digitoxose residues, indicating that Antiserum-D is much inferior to Antiserum-A in monitoring of unchanged type of MDx3, because Antiserum-D is antiserum produced by peroxide-oxidized Dx3–BSA conjugate as an antigen at the position of terminal digitoxose.

As shown in Table 2, we investigated the interference of MDx3 metabolites in human serum by using Antiserum-A and Antiserum-D. Each metabolite (Dx3, Dx2, Dx1, Dx0, and DihDx3) at ratios of 10%, 30% and 100% to MDx3 concentration were added to the MDx3 in human serum, and the recovery test was performed. The recovery ratios using Antiserum-A at 10%, 30%, and 100% were in the range of 103—110%, 128—146%, and 175—291%, respectively. In contrast, those using Antiserum-D at 10%, 30%, and 100% were >146%, >226%, and >493%, respectively. These results indicate that the interference of MDx3 metabolites by using Antiserum-A in human serum was much lower than that of Antiserum-D, because of its low specificity to MDx3.

Antiserum-A showed much lower cross-reactivity in comparison with Antiserum-D, indicating that Antiserum-A may be used for more precise determination of intact MDx3 in human serum. However, it is considered that these low cross-reactivities may result in error in the monitoring of MDx3. Removal of cross-reactive compounds in human serum is a good method. Recently, the PBA column has been used for the rapid and selective isolation of urinary catecholamine. Antiserum-A showed much lower cross-reactivity in comparison with Antiserum-D, indicating that Antiserum-A may be used for more precise determination of intact MDx3 in human serum. However, it is considered that these low cross-reactivities may result in error in the monitoring of MDx3. Removal of cross-reactive compounds in human serum is a good method. Recently, the PBA column has been used for the rapid and selective isolation of urinary catecholamine.

Table 1. Cross-reaction Data for EIA of Antiserum-A, Antiserum-B, Antiserum-C, and Antiserum-D

<table>
<thead>
<tr>
<th>% Cross-reactivity (50%)</th>
<th>Antiserum-A</th>
<th>Antiserum-B</th>
<th>Antiserum-C</th>
<th>Antiserum-D</th>
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<tr>
<td>MDx3</td>
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<td>100</td>
<td>100</td>
<td>100</td>
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<td>58</td>
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<tr>
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Values are calculated on a molar basis.
The above text refers to the separation and detection of MDx3 and its metabolites in human serum using a PBA column. After extraction of the human serum with AcOEt, evaporation of the extracted organic layer, and addition of 1 ml of CH3CN to a test tube, MDx3 could be detected only on HPTLC as shown in Fig. 4. By the second addition of 1 ml of CH3CN to PBA column, MDx3 and Dx0 could be detected on HPTLC. However, using the same procedure 4 times, nothing could be detected on HPTLC. Dx3, Dx2, Dx1, and DihDx3 could be detected on HPTLC by washing the column with MeOH after eluent of CH3CN solution at 4 times, indicating that these compounds interacted with boric acids in the PBA column because they possess a cis-diol group.

These results suggest that MDx3 and Dx0, which did not possess a cis-diol group in the molecular structure, can be selectively separated from the mixture of MDx3 and its metabolites in human serum by PBA column treatment.

About 4 ml of CH3CN solution developed in the PBA column were collected and evaporated in vacuo. Then, MDx3 was measured by homologous EIA using Antiserum-A after PBA column treatment in phosphate buffer and human serum was investigated. The data are listed in Table 3. Recovery rates in phosphate buffer and human serum were about 100% and about 110%, respectively. As shown in Table 4, we investigated the effect of PBA column treatment on the interference of metabolites. Each metabolite (Dx3, Dx2, Dx1, Dx0, and DihDx3) at an equal concentration with the MDx3 concentration was added to the MDx3 in human serum, and the recovery test was performed. The recovery ratios at non-PBA column treatment and PBA column treatment were in the range of 175—291% and 107—114%, respectively. These data indicated that no significant interference by metabolites of MDx3 in our EIA was confirmed.

When PBA column was used for the pretreatment of human serum sample containing Dx3, Dx2, Dx1, Dx0, and
DihDx3, MDx3 and Dx0 were separated (Fig. 4). Antiserum-D showed high cross-reactivity with Dx0 (63%), while Antiserum-A showed negligible value of 0.05%. It is considered that EIA by Antiserum-D using PBA column shows the significant measurement error. In addition, the measurement range of EIA by Antiserum-A (0.2—20 ng/ml) is much sensitive compared with that of Antiserum-D (0.5—5 ng/ml). In these points, Antiserum-A of our novel specific antiserum is superior to Antiserum-D.

Consequently, our results suggest that unchanged type of MDx3 concentration in human serum after administration will be more precisely monitored using both Antiserum-A and PBA column treatment. We expect that our newly developed assay system for MDx3 will be clinically popularized in the world in the near future.

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