SYNTHESES OF NEW DIOXIN HAPTENS AND DEVELOPMENT OF ENZYME IMMUNOASSAY FOR DIOXINS USING POLYCLONAL ANTIBODIES

Takako Anjo[1], Mitsunobu Okuyama[1], Masayuki Satoh[2], Akira Kambegawa[3] andYasuhiko Matsuki[1]

[1] Hatano Research Institute, Food and Drug Safety Center, 729-5, Ochiai, Hadano, Kanagawa 257-8523, Japan
[2] School of Pharmaceutical Sciences, University of Shizuoka, 52-1, Yata, Shizuoka, Shizuoka 422-8526, Japan
[3] Kambegawa Research Institute, 3-8-5, Izumi, Komae, Tokyo 201-0013, Japan

Introduction

Contaminations in environment and foods by dioxins and their resulting influences on human health have been taking growing concern in public. It therefore becomes urgent and important subject to evaluate human exposure and contamination by dioxins. On the other hand, dioxins in various kinds of samples are determined mainly by high resolution gas chromatography/mass spectrometry (GC/MS)[1,2,3]. However, GC/MS method requires a tedious clean-up procedure which is time-consuming to turn out to be extremely expensive. Therefore, it is strongly desired to develop the method of determining dioxins which would be simple and highly sensitive procedure and at lower cost. Immunoassay[4,5,6,7] could be one of the methods, but any applications to biological samples have been reported. From the above-mentioned standpoints, it was desired to develop a simple and highly sensitive enzyme immunoassay for determining dioxins in human biological samples such as milk and blood to monitor contamination in human.

In this study, dioxin haptens were initially synthesized which were necessary to develop an enzyme immunoassay. Those were new and known haptens with several kinds of side chains at C-1 or at C-2 of dioxin ring. Then, the haptens were coupled with bovine serum albumin (BSA) to serve as immunogen to prepare anti-dioxin antisera. Enzyme immunoassay was examined using the anti-dioxin antisera and horseradish peroxidase (HRP)-labelled haptens.

Experimental Methods

1. Sytheses of dioxin haptens with side chains at C-1

To synthesize dioxin haptens with side chains containing amide bond in spacers, two kinds of 1-amino-dioxins[8] were first synthesized by coupling of 4,5-dichlorocatechol[9] with chloronitrobenzenes followed by reduction of nitro group. 1-Amino-dioxins obtained were then reacted with acid anhydrides or acid chlorides, in latter the resulting esters were then hydrolyzed, to give the haptens with side chains containing amide bond in spacers. Seven kinds of haptens, -1,-2, -3[8], -4, -7, -8 and-9, were synthesized by this synthetic route. Secondly, the haptens with side chains containing double bond in spacers were synthesized. 2,3,6-Trichlorobenzaldehyde as the starting material was first nitrated, then submitted to the Horner-Emmons reaction to add an unsaturated esters and finally coupled with 4,5-dichlorocatechol to form dioxin ring. The esters obtained were hydrolyzed to give three kinds of haptens, -5[12], -6 and -10 (Table 1).

2. Syntheses of dioxin haptens with side chains at C-2

The dioxin haptens with side chains containing ether bond in spacers were initially synthesized. 2,5-Dichlorophenol as the starting material was first reacted with bromoacetic acid ethyl ester to add ether bond, then nitrated to give dichloronitro derivative[10,11] followed by coupling with 4,5-dichlorocatechol[9,12]. The compound obtained was 2-hydroxy derivative of dioxin, which was again added ether bond by reacting with bromoalkylcarboxylic acid ethyl esters. The esters were then hydrolyzed to give the haptens with side chains containing ether bond in spacers, -1, -2 and -3. On the other hand, 2,4-dichlorobenzaldehyde was the starting material to synthesize the haptens with side chains containing double bond in spacers. The aldehyde was first nitrated and then reacted with Wittig reagents to add olefinic moeties. The compounds obtained were coupled with 4,5-dichlorocatechol to form dioxin ring, followed by hydrolysis of esters to give -1 and -2[13]. 4-Chloro-3-nitrobenzaldehyde was another starting material to synthesize the haptens with side chains at C-2. After reaction of the benzaldehyde with Wittig reagents, the derivative obtained was coupled with 4-methyl- and 4-*tert*-butyl-5-chlorocatechol which were obtained by chlorination of 4-methyl- and 4-*tert*-butylcatechol, respectively. The esters obtained were hydrolyzed to give -6, -6M and -6B (Table 1).

3. Preparation of anti-dioxin antisera

Six kinds of haptens (-1, -2, -5, -1, -2 and -4) were selected to prepare immunogens. The haptens were coupled with BSA as carrier protein by the active ester method. Namely, the haptens were first converted to N-hydroxysuccinimide esters, then reacted with BSA, and the haptens coupled with BSA as the immunogens were obtained after removing unreacted haptens by dialysis. Antisera were prepared by injection of the immunogens repeatedly after emulsifying with complete Freund's adjuvant to male rabbits which were three for each immunogen.

4. Enzyme immunoassay for dioxins

Enzyme labelled haptens were first prepared using the selected eight kinds of haptens (-2, -3, -5, -6, -4, -6, -6M and -6B) and HRP as labeling enzyme by the same method as the above. Enzyme immunoassay was conducted by solid phase double antibody method using microplates. Namely, to each well coated with anti-rabbit IgG goat antibody was added anti-dioxin antiserum, HRP-labelled hapten and dioxin standard solution, then incubated at 4°C overnight. After washing each well, *o*-phenylenediamine as the enzyme substrate was added to each well to measure enzymic activity. Standard curves were prepared at the concentrations of 1-625 pg/well for 2,3,7,8-TCDD and 2,3,7-trichloro-8-methyldibenzo [1,4]dioxin (TMDD), and cross reactivity of dioxin isomers was calculated based on the amount of each isomer added and value obtained using standard curves.

Results and Discussion

1. Syntheses of dioxin haptens with side chains at C-1

Ten kinds of dioxin haptens were synthesized which were with side chains containing amide bond and double bond in spacers and carboxyl group at the end of side chains. The haptens synthesized were –1 - -10 in which eight compounds were new. These structures were confirmed by physical and spectral data of NMR, MS and others.

2. Syntheses of dioxin haptens with side chains at C-2

Eight kinds of dioxin haptens were synthesized which were with side chains containing ether bond and double bond in spacers and carboxyl group at the end of side chains. The haptens synthesized were –1 - -6, -6M and -6B in which five compounds were new. These structures were confirmed by physical and spectral data of NMR, MS and others.

3. Development of enzyme immunoassay for dioxins

Enzyme immunoassay was examined using polyclonal anti-dioxin antisera and HRP- labelled haptens. Solid phase double antibody method was employed as the assay system, and the highest sensitivity was obtained using the following heterologous combination, namely, the antiserum prepared by injection of -2 coupled with BSA as the immunogen and HRP-labelled -6. After further examinations of solvent and other factors in the assay system, standard curve could be prepared at the concentrations of 1-625 pg/well for 2,3,7,8-TCDD and TMDD with satisfactory precision and accuracy. In addition, cross-reactivities of 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HeCDD, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF with the high toxicity equivalent factors were relatively high, and it was therefore suggested that the assay systems could be applicable for monitoring the toxicity equivalents.

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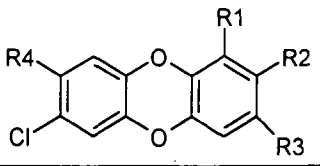
References

1. Letcher, R.J., Norstrom, R.J., Lin, S., Ramsey, M.A., Bandiera, S.M. (1996) *Toxicol. Appl. Pharmacol.* 137, 127-140
2. Elliott, E., Machmer, M.M., Henny, C.J., Wilson, L.K., Norstrom, R.J. (1998) *Arch. Environ. Contam. Toxicol.* 35, 620-631
3. Giesy, J.P., Kannan, K., Kubitz, J.A., Williams, L.L., Zabik, M.J. (1999) *Arch. Environ. Contam. Toxicol.* 36, 432-446
4. Luster, M.I., Albro, P.W., Chae, K., Lawson, L.D., Corbett, J.T., McKinney, J.D. (1980) *Anal. Chem.* 52, 1497-1500
5. Vanderlaan, M., Stanker, L.H., Watkins, B.E. (1988) *Environ. Toxicol. Chem.* 7, 859-870
6. Harrison, R.O., Carlson, R.E. (1997) *Chemosphere* 34, 915-928
7. Sugawara, Y., Gee, S.J., Sanborn, J.R., Gilman, S.D., Hammock, B.D. (1998) *Anal. Chem.* 70, 1092-1099
8. Chae, K., Cho, L.K., McKinny, J.D. (1977) *J. Agric. Food Chem.* 25, 1207-1209
9. Gray, A.P., Cepa, S.P., Solomon, I.J., Aniline, O. (1976) *J. Org. Chem.* 41, 2435-2437
10. Singh, S.K., Kumar, S. (1993) J. *Agric. Food Chem.* 41, 1511-1516
11. Oliver, J.E., Ruth, J.M. (1983) *Chemosphere* 12, 1497-1503
12. Sanborn, J.R., Gee, S.J., Gilman, S.D., Sugawara, Y., Jones, A.D., Rogers, J., Szurdoki, F., Stanker, L.H., Stoutamire, D.W., Hammock, B.D. (1998) *J. Agric. Food Chem.* 46, 2407-2416
13. Aldous, F.A., Barrass, B.C., Brewster, K., Buxton, D.A., Green, D.M., Pinder, R.M., Rich, P., Skeels, M., Tutt, K.J. (1974) *J. Med. Chem.* 17, 1100-1111

Table 1 Structures of Dioxin Haptens



Hapten	R1	R2	R3	R4
1	$MHCO(CH_2)_2COOH$	H	Cl	Cl
2	$MHCO(CH_2)_3COOH$	H	Cl	Cl
3	$MHCO(CH_2)_4COOH$	H	Cl	Cl
4	MHCOCH=CHCOOH	H	Cl	Cl
5	CH=CHCOOH	Cl	Cl	Cl
6	$CH=C(CH_3)COOH$	Cl	Cl	Cl
7	$NHCO(CH_2)_2COOH$	Cl	Cl	Cl
8	$NHCO(CH_2)_3COOH$	Cl	Cl	Cl
9	$MHCO(CH_2)_4COOH$	Cl	Cl	Cl
10	$(CH=CH)_2COOH$	Cl	Cl	Cl
1	H	OCH_2COOH	Cl	Cl
2	H	$O(CH_2)_3COOH$	Cl	Cl
3	H	$O(CH_2)_4COOH$	Cl	Cl
4	H	CH=CHCOOH	Cl	Cl
5	H	$(CH=CH)_2COOH$	Cl	Cl
6	H	CH=CHCOOH	H	Cl
6M	H	CH=CHCOOH	H	CH_3
6B	H	CH=CHCOOH	H	$C(CH_3)_3$