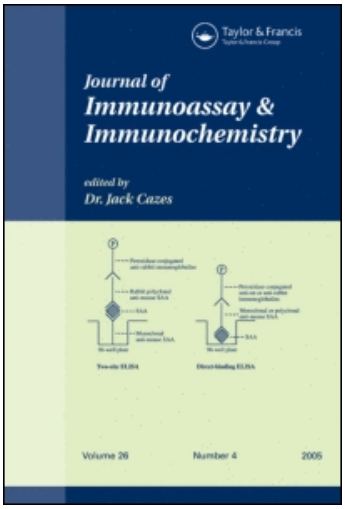

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An Enzyme Immunoassay for a Metabolite of a New Sleep Inducer (450191-S), A Ring-Opened Derivative of 1,4-Benzodiazepine, in Human serum

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AN ENZYME IMMUNOASSAY FOR A METABOLITE OF A NEW SLEEP INDUCER (450191-S), A RING-OPENED DERIVATIVE OF 1,4-BENZODIAZEPINE, IN HUMAN SERUM

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ABSTRACT

A competitive enzyme immunoassay was developed for metabolites of 450191-S, a ring-opened derivative of triazolobenzodiazepines, in human serum. Three kinds of labelled antigens, β-D-galactosidase conjugates, and antisera from three kinds of immunogens, bovine serum albumin conjugates, were tested and the hapten heterologous assay was selected. B/F separation was performed using immobilized second antibody and the enzyme activity was measured using fluorescent substrate. One of the serum metabolites, the carboxyl form, was extracted from acidified serum and measured by the assay. The minimum detectable concentration was 1.5 ng/ml in serum, 15 pg/tube. The intra-assay and inter-assay variances were 5.5% and 7.7% at 6 ng/ml of serum, respectively. Standard curves of other related metabolites were assessed with various combinations of labelled antigens and antibodies.

(KEY WORDS: Enzyme immunoassay, Hapten heterologous assay, Benzodiazepines, in Human serum, β-Galactosidase, Fluorescent substrate)

INTRODUCTION

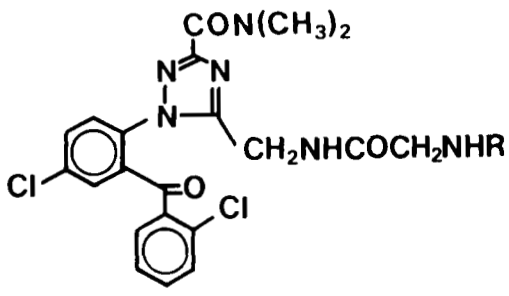
Blood concentrations of drugs and/or their metabolites need to be monitored (1), but for some, especially those of receptor binding drugs, such as benzodiazepines, the dose administered is so small that the serum concentration

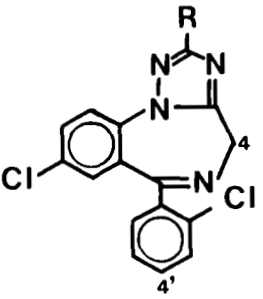
of the drug and its metabolites is very low in human blood, less than 10 ng/ml in some cases. To enable the assay of such minute quantities, we developed a sensitive enzyme immunoassay (EIA) for metabolites of benzodiazepines. The parent drug, a sleep inducer, is a new ring-opened derivative,5-{[(aminoacetyl)-amino]methyl}-1-[4-chloro-2-(2-chlorobenzoyl)phenyl]-*N,N*-dimethyl-1*H*-1,2,4-triazole-3-carboxamide hydrochloride (450191-S, cpd 1 in Fig. 1), which changes to various metabolites (cpd 2-6) in the human body (2, 3). The dose for a human is so small, 1-2 mg, that the serum concentration of its metabolites can not be determined by conventional assay methods. In this paper, we describe an EIA for the metabolites and the determination of the final metabolite, the carboxyl form (cpd 6), which could be separated by extraction from other metabolites in serum. We also discuss the effects of heterologous combinations of enzyme-labelled antigen and antiserum on the characteristics of the EIA.

MATERIALS AND METHODS

Materials

Authentic compounds of cpd 1-8 and some other derivatives for the estimation of cross-reactivities were gifts from Drs. K. Hirai and S. Takahashi of our laboratories. For the determination of extraction efficiencies of metabolites, ^{14}C-labelled cpd 2, 3, 5, and 6 from Mr. T. Nagasaki and Mr. Y. Katsuyama of our laboratories were used. β-D-Galactosidase (β-Gal) from E. coli (EC. 3.2.1.23) was purchased from Boehringer Mannheim GmbH. m-Maleimidobenzoyl *N*-hydroxy-succinimdate (MBS) was synthesized according to ref. 4. EIA buffer was 0.01 mol/l phosphate-buffered saline, pH 7.3, containing 0.001 mol/l $MgCl_2$, 0.05% (w/v)





1, R = H

7, R = $COCH_2CH_2COOH$

2, R = $CON(CH_3)_2$

3, R = $CONHCH_3$

4, R = $CONHCH_2OH$

5, R = $CONH_2$

6, R = COOH

8, R = $CONHCH_2CH_2NH_2$

FIGURE 1. Structures of parent drug (450191-S) (cpd 1), active metabolites (cpd 2-6), and related compounds (cpd 7, 8).

NaN_3, and 1% (w/v) bovine serum albumin (BSA) (Sigma, recrystallized and lyophilized). Immobilized anti-rabbit second antibody, Immunobead®, was obtained from Bio-Rad Lab. and was diluted to 1 mg/ml suspension with the EIA buffer. Other chemicals were of analytical grade.

Extraction

A sample of human serum or plasma, 500 µl, was extracted with 3.0 ml of dichloromethane by shaking for 10 min to eliminate all the other metabolites except cpd 6. Hydrochloric acid (7.5 mol/l), 37.5 µl, was added to 150 µl of the residual serum or plasma (final concentration 1.5 mol/l) and was re-extracted with

1.0 ml of dichloromethane. The organic phase was dried with *ca.* 0.7 g of anhydrous sodium sulfate. The extract, 100 µl, was evaporated in an EIA tube at 25°C. A 500-µl portion of the EIA buffer was added to the EIA tube.

Preparation of Antisera

An antigen, a BSA conjugate of cpd 6 [6-BSA (MA)], was prepared by the mixed anhydride (MA) method. The procedure was similar to that described in ref. 5, except that cpd 6 was dissolved in *N,N*-dimethylformamide. BSA conjugates of cpd 8 [8-BSA (EDC) and 8-BSA (MBS)] were prepared by the carbodiimide (EDC) method (6) and the MBS method (4), respectively.

Reaction mixtures were dialyzed against distilled water and then lyophilized. The number of hapten molecules bound to a BSA molecule of 6-BSA (MA) was determined by the trinitrobenzenesulfonic acid method (7) to be 39.5 ± 1.5. That of 8-BSA (EDC) was 12 to 15 and that of 8-BSA (MBS) was less than 1.0 by the EIA method and weight analysis (weight difference between the BSA conjugate and control BSA). New Zealand white rabbits were immunized with these immunogens in the usual way (5).

Preparation of Enzyme-labelled Antigens for EIA

Labelled antigens, β-Gal conjugate of cpd 6 [6-Gal (MA)] and that of the hemisuccinate derivative of cpd 1 [7-Gal (MA)], were prepared by the mixed anhydride reaction (5). A β-Gal conjugate of cpd 8 [8-Gal (MBS)] was by the MBS method (4). These conjugates were dialyzed against the EIA buffer (without BSA) and stored at 4°C until use. A precipitate appeared in the 6-Gal (MA) conjugate and was used after centrifugation.

EIA

A 100-µl portion of enzyme-labelled antigen [7-Gal (MA), diluted to 1: 40,000, *ca.* 1 x 10^{-5} µU/100 µl] was pipetted into each assay tube which contained 500 µl of one of the samples described above or various concentrations of standard solutions of cpd 6 in the EIA buffer, the amount varying from 12.6 to 400 pg/500 µl/assay tube. Next, 100 µl of the antiserum (K-im, diluted to 1:100,000) was added to each tube, and the mixture was incubated for 16 h at 22°C. Immunobead® suspension, 100 µl, was added to each tube and incubation was continued for 1.5 h at 22°C. After centrifugation for 10 min at 2,000 x *g* at 22°C, the supernatant was aspirated off and the residual Immunobead® was suspended with 100 µl of the EIA buffer.

Measurement of Enzyme Activity

The suspension was incubated with 500 µl of the fluorescent substrate solution (4-methylumbelliferyl β-D-galactoside, 80 µg/ml in the assay buffer without BSA) for 30 min at 37°C. The enzyme reaction was stopped by the addition of 2.5 ml of 0.1 mol/l glycine-NaOH buffer (pH 10.5). The fluorescent intensity of the slightly suspended solution was measured at an excitation wavelength of 360 nm and an emission wavelength of 445 nm using a Hitachi MPF-4 fluorescence spectrophotometer.

RESULTS

All metabolites except cpd 6 were extracted by the first dichloromethane extraction. The extraction efficiencies for cpd 2, 3, and 5 were 100.4 ± 2.5% (n =

7), 101.1 ± 2.2% (n = 7), and 98.6 ± 1.7% (n = 8), respectively, and 96.8 ± 3..6% (n = 8) of cpd 6 remained in the residual serum. After the second extraction from acidified serum, 73.2 ± 5.7% of cpd 6 was extracted. A large amount of a precipitate of proteins appeared when the residual serum was acidified and the presence of cpd 6 in the precipitate may have decreased the extraction efficiency and precision.

We prepared BSA conjugates for immunogens and β-Gal conjugates for enzyme-labelled antigens in which the carrier proteins were coupled to the 3-position of the triazole ring (where "R" of metabolite in Fig. 1 is located). The determinant for the antibody would thus be expected to be far from the triazole ring. Three good antisera were produced [K-im from 6-BSA (MA), K-10 from 8-BSA (EDC), and K-13 from 8-BSA (MBS)]. We compared the standard displacement curves of these antisera under suitable antiserum dilutions (B_0/T: *ca.* 60%) using three enzyme-labelled antigens [6-Gal (MA), 8-Gal (MBS), and 7-Gal (MA)] diluted to the equal enzyme activities (*ca.* 1 x 10^{-5} μU/100 μl).

Fig. 2 shows that the sensitivity on the homologous EIA system with anti [6-BSA (MA)] and the supernatant of 6-Gal (MA) was very low. This enzyme-labelled antigen was not suitable because of its low solubility and very low competitiveness. The 8-Gal (MBS) conjugate which had a longer bridge than 6-Gal (MA) reacted to anti [6-BSA (MA)], anti [8-BSA (EDC)], and anti [8-BSA (MBS)] competitively with cold cpd 6. Less sensitivity was observed for the homologous combination of labelled compounds by the MBS method, 8-Gal (MBS), and the antiserum from the BSA conjugate by the same method, 8-BSA (MBS).

Surprisingly, the β-Gal conjugate (MA) of cpd 7, which is very different from cpd 6 in structure, bound anti [6-BSA (MA)] and displaced competitively by the

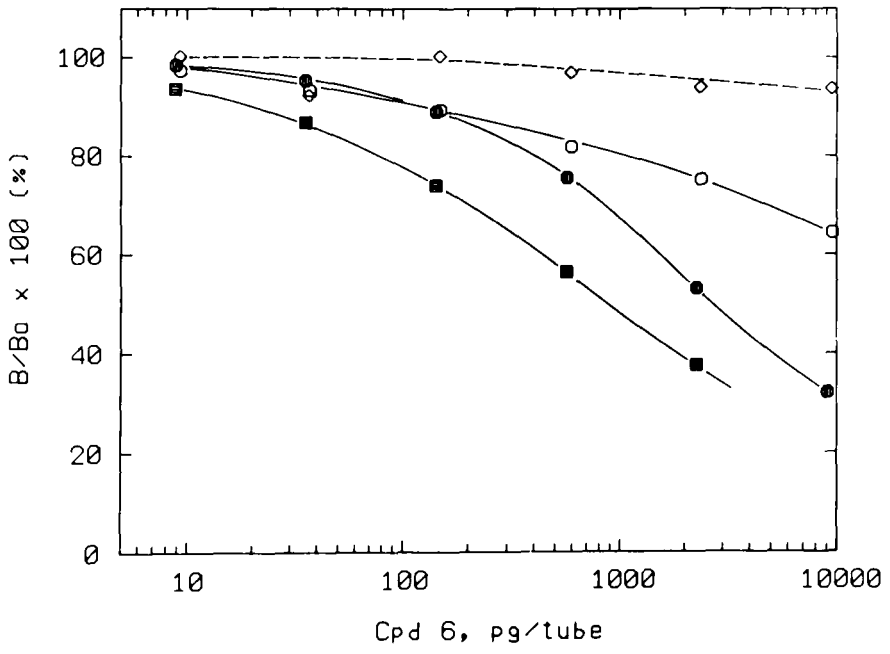


FIGURE 2. Standard curves of various combinations of antiserum and enzyme-labelled antigen. Anti [6-BSA (MA)] and 6-Gal (MA), --◇--; anti [6-BSA (MA)] and 8-Gal (MBS), —●—; anti [8-BSA (EDC)] and 8-Gal (MBS), —■—; and anti [8-BSA (MBS)] and 8-Gal (MBS), —○—.

metabolites in this heterologous system (Fig. 3). The sensitivities on the standard curves using 7-Gal (MA) were about ten times better than those of 8-Gal (MBS). For the determination of the metabolites in serum, we selected 7-Gal (MA) for labelled antigen and routinely used anti [6-BSA (MA)], K-im. This combination gave an assay range of about 15-1,000 pg/tube and the minimum detectable concentration in serum was 1.5 ng/ml. The EIA reaction conditions were set up according to ref. 8. The BSA concentration of the assay buffer, 1%, was high to aid the solubility of the metabolites after the second extraction.

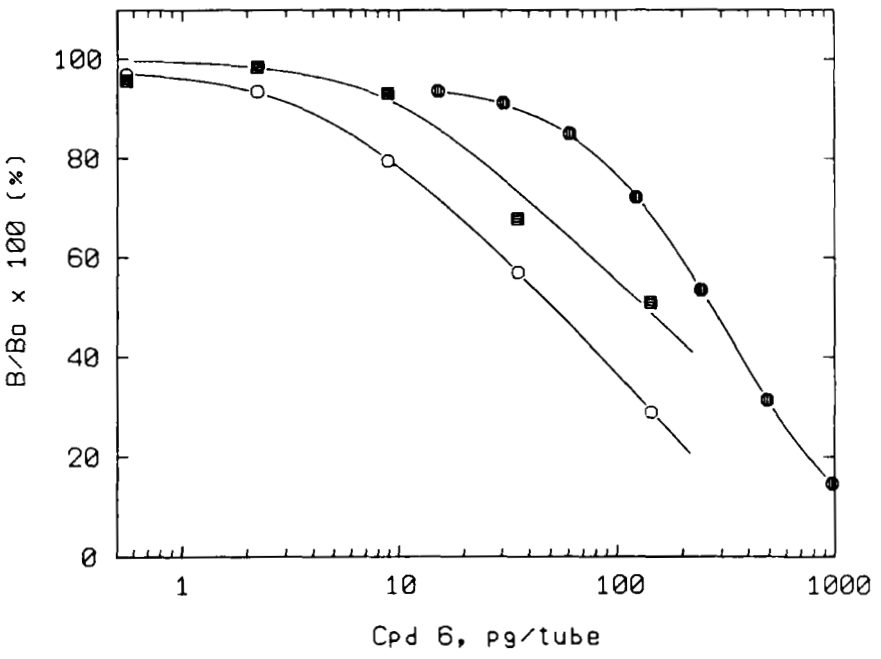


FIGURE 3. Standard curves of various combinations of antiserum and enzyme-labelled antigen. Anti [6-BSA (MA)] and 7-Gal (MA), —●—; anti [8-BSA (EDC) and 7-Gal (MA), —■—; and anti [8-BSA (MBS) and 7-Gal (MA), —○—.

The displacement curves of cpd 1-6 and some other derivatives are shown in Fig. 4, which suggests that the difference between C(4')-OH and C(4')-H is significant but that between C(4)-OH and C(4)-H is not. Thus, the antigenic determinant of these compounds is centered at the 2-chlorophenyl group as predicted from the coupling position to carrier protein. The gentler slope of the displacement curve of cpd 1 suggests that it weakly binds with the anti [6-BSA (MA)] and competes for 7-Gal (MA) by some different mode from the other metabolites. We assessed some cross-reactivities for other combinations of the labelled antigens [7-Gal (MA) and 8-Gal (MBS)] and antisera {anti [6-BSA (MA)],

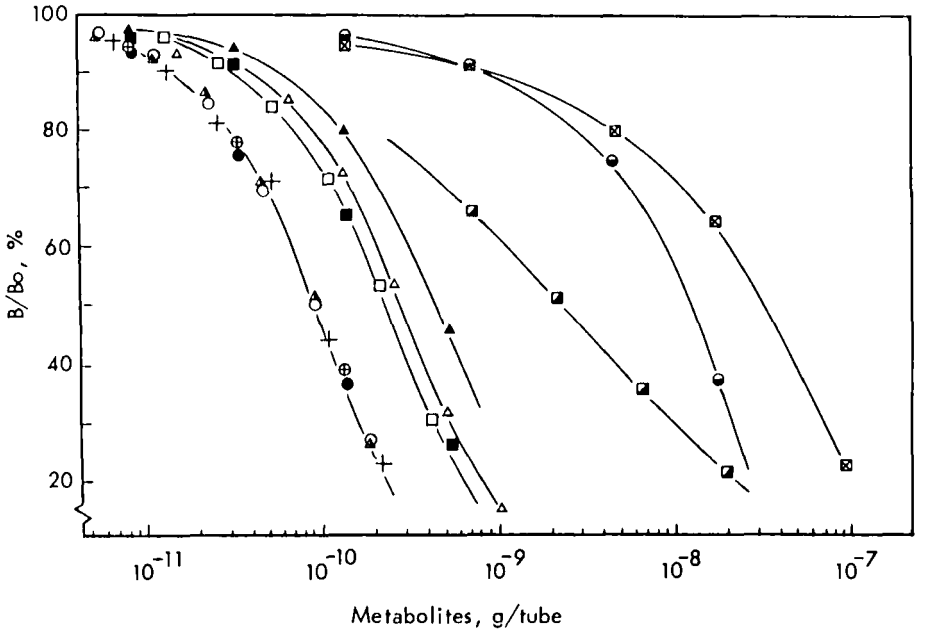


FIGURE 4. EIA standard displacement curves for various compounds. Cpd 1 (parent drug), —◪— (13.6); cpd 2, —□— (128); cpd 3, —○— (328); cpd 4, —◭— (280); cpd 5, —+— (339); cpd 6, —△— (100); 4-hydroxy derivative of cpd 2, —■— (126); 4-hdyroxy derivative of cpd 3, —●— (329); 4-hydroxy derivative of cpd 5, —⊕— (323); 4-hydroxy derivative of cpd 6, —▲— (62.0); 4'-hydroxy derivative of cpd 2, —⊠— (1.05); and 4'-hydroxy derivative of cpd 5, —◒— (2.75). ( ), % Cross reactivity regarding cpd 6 as 100 on a weight basis.

anti [8-BSA (EDC)], and anti [8-BSA (MBS)]} (Table 1). The cross-reactivity for each compound showed a similar tendency to those at other combinations though the concentrations of the 50% intercept on standard curves were different.

These standard curves did not vary during the course of one year since the enzyme-labelled antigens were very stable.

For the determination of cpd 6 in serum, we investigated the effect of serum on the EIA. Some inhibition was observed and the pipetting volume of the

TABLE 1

Cross-reactivities at various combinations of labelled antigens and antibodies. Concentrations of the 50% intercept on the standard curves

Labelled antigen: 7-Gal (MA)

Immunogen Antiserum	6-BSA (MA) K-im		8-BSA (EDC) K-10		8-BSA (MBS) K-13	
	pg/tube	(%)	pg/tube	(%)	pg/tube	(%)
Cpd 1	2040	(13.5)	7280	(2.0)	2450	(2.5)
Cpd 2	214	(128.5)	134	(106.7)	16.9	(358.6)
Cpd 3	83.8	(328.2)	17.1	(836.3)	4.0	(1511.2)
Cpd 4	97.9	(280.9)	18.9	(756.6)	3.1	(1942.3)
Cpd 5	81.0	(339.5)	21.8	(656.0)	4.4	(1371.0)
Cpd 6	275	(100)	143	(100)	60.6	(100)

Labelled antigen: 8-Gal (MBS)

Immunogen Antiserum	6-BSA (MA) K-im		8-BSA (EDC) K-10	
	pg/tube	(%)	pg/tube	(%)
Cpd 1	---	(<0.5)	---	(<0.5)
Cpd 2	4020	(71.4)	723	(126.1)
Cpd 3	558	(514.3)	94.1	(969.2)
Cpd 4	814	(352.6)	159	(573.6)
Cpd 5	953	(301.1)	188	(485.1)
Cpd 6	2870	(100)	912	(100)

second extract had to be decreased to 100 µl. No differences were found using serum instead of plasma. Various concentrations of cpd 6 were added to normal human serum and these samples were subjected to the EIA. The recovery of the added cpd 6 was determined as shown in Fig. 5. The relationship between added and measured cpd 6 was linear but the slope was 0.70 because of its low extraction efficiency from the acidified serum. Serum samples including cpd 6 were measured repeatedly and intra-assay and inter-assay variances were estimated

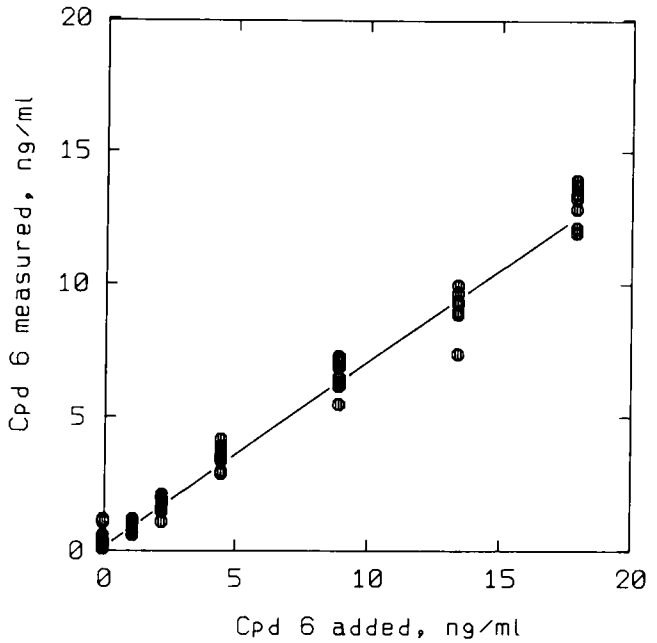


FIGURE 5. Recovery of cpd 6 added to human serum. The relationship between added (x ng/ml) and measured (y ng/ml) was y = 0.70 X + 0.20, CV = 12.8%, n = 70.

(Table 2). The precisions were good enough to enable measurement of cpd 6 in serum.

DISCUSSION

We developed a sensitive EIA for the metabolites of 1,4-benzodiazepines. It can measure the concentration of cpd 6 in human serum, as shown in Fig. 6. This EIA should be very useful for pharmacological assays not only because it requires no radio isotopes but also because it is very sensitive and its labelled compounds

TABLE 2

Precision of the EIA for cpd 6

	Intra-assay			Inter-assay		
	Low	Medium	High	Low	Medium	High
n	5	5	6	5	4	4
Mean (ng/ml)	3.1	6.1	20.3	2.3	6.2	18.9
SD	0.36	0.33	2.2	0.77	0.48	2.5
CV (%)	11.8	5.5	11.6	32.8	7.7	13.1

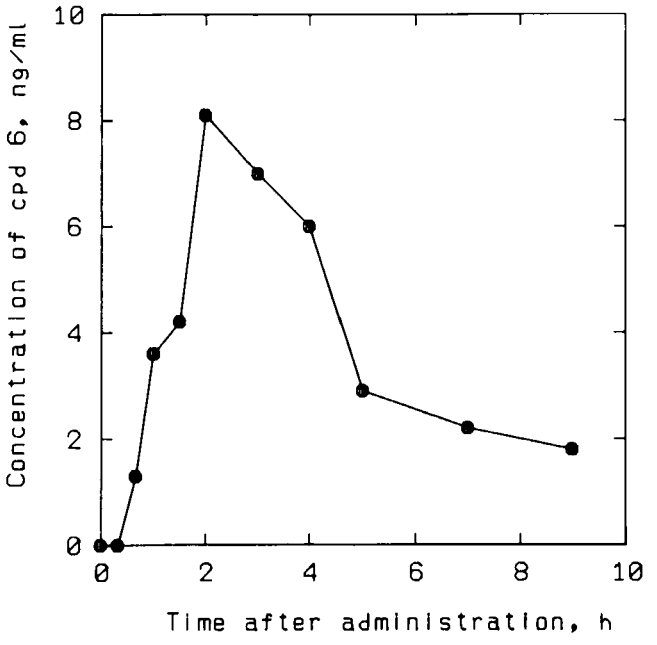


FIGURE 6. Determination of cpd 6 in human serum after p.o. administration of cpd 1 (450191-S), 1 mg.

are very stable. One drawback, however, is that its sensitivity for the serum sample was not as good as we had expected due to interference from something in the serum extract. The labelled antigen of EIA, the β-Gal conjugate, is a very large molecule and the EIA may be affected more easily than RIA by other substances in serum.

Our results with anti [6-BSA (MA)] and 6-Gal (MA), compared with those for other antisera and enzyme-labelled antigens, suggest that both immunogens and labelled antigens need some spacer bridge between the hapten and the proteins. Those conjugates had no spacers and therefore might be less reactive both in the immune system and the EIA system.

Standard curves of EIA were changed by the heterology of the assay system as shown in Fig. 2 and 3. The labelled antigen made from cpd 7 competed with the sample antigen, cpd 6, much better than with that from cpd 8. Cpd 7 is very different in structure from cpd 8 or 6, from which the antibodies were made. Increased heterology leads to a more sensitive EIA (9). Weaker affinity of labelled antigen than that of sample antigen is preferable but both of them must bind competitively to the antibody (9). Labelled antigen 7-Gal (MA) might have an appropriate affinity to the antibody since the slope of cpd 1 in Fig. 4 was gentle and its reactivity was not high.

The fact that the EIA system with 8-Gal (MBS) and anti [8-BSA (MBS)] was less sensitive suggests that 8-BSA (MBS) produced the antibodies not only for the hapten but also for the bridging agent, MBS. The labelled antigen binding to anti MBS antibody cannot be inhibited by unlabelled cpd 6 (10). Cpd 6 was less sensitive at all combinations of labelled antigen and antiserum than cpd 2-5 as shown in Table 1. It has a negative charge and have difficulty in approaching the

binding sites on the antibody molecule which have an affinity for the hydrophobic group, the 2-chlorophenyl group. The cross-reactivities of each compound observed at different combinations of labelled antigens and antibodies were similar (Table 1). Many kinds of antibodies in an antiserum can be produced depending on the immunogen but the antibody reacting with the determinant on the hapten, 2-chlorophenyl group, have characteristics akin to those from other immunogens.

We are now developing a simultaneous assay for the other metabolites after their separation by HPLC using this EIA system.

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