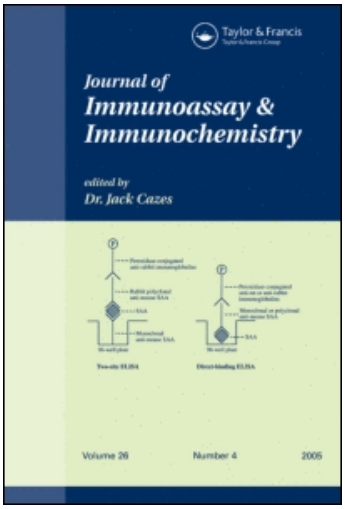

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Specific Antibodies to Theophylline for Use in a Homogeneous: Enzyme Immunoassay

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SPECIFIC ANTIBODIES TO THEOPHYLLINE FOR USE IN A HOMOGENEOUS ENZYME IMMUNOASSAY

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Contribution No. 85

ABSTRACT

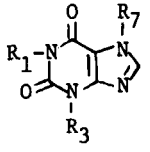
Bovine serum albumin conjugate of 1-methyl-3-(3'-carboxypropyl)xanthine elicits highly specific anti-theophylline antibodies when injected into sheep. When used in a homogeneous enzyme immunoassay for theophylline these antibodies show insignificant cross-reactivity (< 1%) to 1-methyl- and 1,3-dimethyluric acid, 3-methylxanthine, caffeine, and theobromine. In contrast, immunogens prepared from the C-8 functionalized drug afford antibodies which show more serious cross-reactivity to these compounds. Plausible rationale for attachment of the drug to carrier proteins through its N-3 position which furnishes specific antibodies are given.

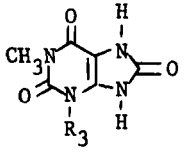
INTRODUCTION

Theophylline, 1,3-dimethylxanthine, I, is one of the most widely used broncodialators for treatment of apnea and asthma (1,2). It has a narrow therapeutic window, and its optimum therapeutic blood level has been established as 10-20 μg/ml (3). Serious toxic effects such as nausea, headache, vomiting, and in

extreme cases death have been reported when its serum level is greater than 20 μg/ml (4,5). A variety of non-immunochemical methods, namely ultraviolet spectroscopy (6,7), gas liquid chromatography (8,9) and high pressure liquid chromatography (10) have been described to monitor serum theophylline levels.

As a prerequisite for the development of a homogeneous enzyme immunoassay for this drug (11) it was necessary to obtain highly specific antibodies. Of particular concern was potential cross-reactivity of anti-theophylline antibodies to major metabolites of the drug (Fig. 1), namely 3-methylxanthine, IV, 1-methyl and 1,3-dimethyl uric acids, VI and VII (12), as well as to the structurally similar compounds, caffeine, II, and theobromine, III, which are widely consumed in the form of coffee, cocoa, tea and certain soft drinks.





I, $R_1 = R_3 = CH_3$ $R_7 = H$
II, $R_1 = R_3 = R_7 = CH_3$
III, $R_1 = H$, $R_3 = R_7 = CH_3$
IV, $R_1 = R_7 = H$, $R_3 = CH_3$
V, $R_1 = CH_3$, $R_3 = R_7 = H$

VI, $R_3 = H$
VII, $R_3 = CH_3$

Figure 1. Structure of theophylline and major potential cross-reactants.

We report here the preparation of highly specific anti-theophylline antibodies. During the course of this work Cook et al (13), and more recently Neese and Soyka (14) reported anti-theophylline antibodies for use in radioimmunoassays (RIA) for theophylline. However, they observed substantial cross-reactivity to some of the more important xanthine analogs.

MATERIALS AND METHODS

Materials

Bovine gamma globulin (BGG) and bovine serum albumin (BSA) were procured from Miles Laboratories; N-hydroxysuccinimide (NHS), aminoacetaldehyde diethyl acetal, and 2-(2'-ethoxyethoxy)ethanol (Carbitol®) were purchased from Aldrich Chemical Co. 1-Ethyl 3-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), glucose-6-phosphate dehydrogenase (G6PDH), glucose-6-phosphate monosodium salt (G6P), and β-nicotinamide adenine dinucleotide (NAD) were supplied by Sigma Chemical Co. Dialysis tubing with a 6000-8000 Dalton cut off was obtained from Spectrum Medical Industries.

Synthesis of Haptens

(a) N-3 Theophylline Acid, VIII. 3-(3'-Carboxypropyl-1-methylxanthine, VIII, mp. 220-222° C, used for conjugation to BGG and glucose-6-phosphate dehydrogenase was synthesized from 1-methylxanthine, V by a procedure described elsewhere (15,16).

(b) C-8 Theophylline Aldehyde, XII. A solution of 8-carboxymethyl-1,3-dimethylxanthine (2.00 g) (17) NHS (0.99 g), and EDCI (1.89 g) in dry N,N-dimethylformamide (DMF) was kept overnight at 4° C. Aminoacetaldehyde diethyl acetal (1.20 g) was added and the reaction mixture stirred at 4-6° C. After 6 hr the reaction mixture was poured into water (40 ml) and extracted with chloroform. The organic extract was washed with saturated, aqueous sodium chloride, dried over sodium sulfate, evaporated and the residue treated with hexane (100 ml). The precipitate was filtered, recrystallized from ethanol/chloroform to furnish (1.2 g) pure theophylline diethylacetal derivative XI, mp. 240-243° C.

Anal. Calcd for $C_{15}H_{23}N_2O_5$: C, 50.98; H, 6.56; N, 19.82. Found: C, 50.65; H, 6.48; N, 19.83.

The diacetal derivative XI (1 g) was stirred under nitrogen at room temperature with 27 ml of 50% aqueous acetic acid. After 90 hr the solution was dried under vacuum and the residue recrystallized from acetic acid/water to give the pure C-8 theophylline aldehyde, XII, mp. 257-264° C dec.

Anal. Calcd for $C_{11}H_{13}N_5O_4.H_2O$: C, 44.40; H, 5.04; N, 23.55; Mol wt. 279. Found: C, 44.10; H, 5.13; N, 23.31; Mol wt. (mass spectrum), 279.

(c) C-8 Theophylline Acid, XIV. A solution of 8-carboxymethyl-1,3-dimethylxanthine (1.67 g), NHS (0.81 g) and EDCI (1.54 g) in dry N,N-dimethylformamide (48.7 ml) was kept overnight at 4°. Ethyl glycinate hydrochloride (0.97 g) and triethylamine

(0.70 g) were then added and the reaction mixture stirred at 5°. After 6 hrs distilled water (50 ml) was added and the aqueous solution extracted with chloroform. A portion of the product (379 mg), ethyl N-(8-theophylline acetyl)glycinate, XIV, precipitated out of chloroform and was collected by filtration. Evaporation of the solvent from the filtrate afforded additional crop of the product (904 mg). Recrystallization from ethanol/chloroform (3:7) gave pure ethyl N-(8-theophylline acetyl)glycinate, XIII, mp. 268-269° C.

Anal. Calcd for $C_{13}H_{17}N_5O_5$: C, 48.29; H, 5.30; N, 21.66; Mol wt. 323. Found: C, 47.99; H, 5.27; N, 21.63; Mol wt. (mass spectrum), 323.

The ethyl N-(8-theophylline acetyl)glycinate, XIII, (886 mg) was stirred with 8% aqueous sodium hydroxide (10 ml) at room temperature and under nitrogen. After one hour the solution was filtered through Norit A, and the resulting filtrate cooled to 4° C and acidified with hydrochloric acid (3 ml). The white precipitate (826 mg) thus formed was collected and recrystallized from 25% methanol-water to provide pure N-(8-theophylline acetyl)-glycine, XIV, (637 mg), mp. > 270° C.

Anal. Calcd for $C_{11}H_{13}N_5O_5$: C, 44.75; H, 4.41; N, 23.73. Found: C, 44.37; H, 4.44; N, 23.20.

Preparation of Immunogens and Antisera

(a) To a solution of 3-carboxyproply-1-methylxanthine, VIII, (45 mg) in DMF (1.5 ml) was added NHS (20.5 mg) and EDCI (39.1 mg) at 0° C under nitrogen. The solution was stirred at 5° C for

18 hrs after which it was added slowly (1.5 h) to a stirring, ice cold solution of BGG (550 mg) in DMF (3.5 ml) and sodium carbonate buffer (27 ml, pH 9, 0.05 M). The pH of the reaction mixture was maintained between 8.5 and 9.0 by the addition of 1 N sodium hydroxide. The reaction mixture was stirred at 5° C overnight. The resulting conjugate was dialyzed thoroughly against water containing a small amount of ammonium hydroxide (pH 9.0). Lyophilization yielded 470 mg of a conjugate bearing approximately 15 theophyllines per BGG as determined by ultraviolet spectral analysis.

(b) To a mixture of BSA (600 mg) and C-8 theophylline aldehyde, XII, (306 mg) in phosphate buffer (0.025 M, pH 6.51, 50 ml) at 0° C was added a suspension of sodium cyanoborohydride (138 mg) in 2 ml of water. The pH of the reaction mixture was carefully kept at 6.3 for an hour by adding 1 N hydrochloric acid, and the resulting suspension was then stirred in cold room (5° C). After 90 hr the conjugation mixture was stirred with Norit A (ca 500 mg) and then centrifuged (10K, 15 minutes). The pale yellow supernatant was passed through a Millipore filter (0.22 mμ), and the filtrate was dialyzed against water (10 x 4ℓ) and ammonia-water (pH 9.0, 2 x 4ℓ). Lyophilization yielded 434 mg of the conjugate which was shown by ultraviolet analysis to have a theophylline-BSA molar ratio of 25.

Sheep were immunized monthly with an emulsion of 1 mg of the antigen and incomplete Freund's adjuvant except for the initial injection which carried out with complete Freund's adjuvant.

The animals were bled once a month and the antisera were processed in the usual manner. All sheep immunized with either antigen produced antisera.

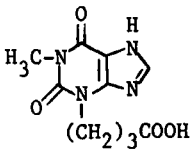
Preparation of Glucose-6-Phosphate Dehydrogenase Drug Conjugates

A solution of N-(8-theophylline acetyl)glycine, XIV, (15.5 mg), NHS (6 mg) and EDCI (11.5 mg) in dry N,N-dimethylformamide (500 µl) was kept overnight at 4° C. The resulting activated ester solution (70 µl) was added in 10 µl portions to a solution containing G6PDH (2.8 mg, 2.7×10^{-5} mmol), G6P (10 mg), disodium NADH.$3H_2O$ (20 mg), carbitol (300 µl) in sodium carbonate buffer (1 ml, 0.1 M, pH 9) at 0° C. During conjugation the pH of the solution had dropped at 8.4. The solution, containing the enzyme conjugate, was then dialyzed five times against tris buffer (0.055 M, pH 8.1) at 4° C. The drug enzyme conjugate was found to have retained 27% of the original enzyme activity and the activity was 82% inhibited upon treatment with antibodies prepared from the C-8 theophylline-BSA immunogen. The enzyme conjugate was diluted 75 folds and 50 µl of the diluted conjugate was used in the assay (18).

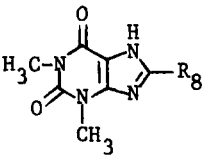
A G6PDH conjugate of 3-(3'-carboxypropyl)-1-methylxanthine, VIII, was similarly prepared by an analogous method.

RESULTS

Anti-theophylline antibodies were used to develop homogeneous enzyme immunoassays (19). For this purpose the NHS ester of



VIII



IX, R_8 = $(CH_2)_3COOH$

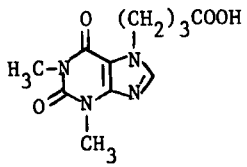
X, R_8 = $COOH$

XI, R_8 = $CH_2CONHCH_2CH(OC_2H_5)_2$

XII, R_8 = $CH_2CONHCH_2CHO$

XIII, R_8 = $CH_2CONHCH_2COOC_2H_5$

XIV, R_8 = $CH_2CONHCH_2COOH$



XV

Figure 2. Structures of theophylline derivatives

theophylline derivatives, VIII and XIV, were conjugated to glucose-6-phosphate dehydrogenase (G6PDH) by reaction at pH 8.1. When the enzyme conjugates were mixed with antibody against the homologous antigens the enzyme activity was reduced. In the assay, theophylline in the sample competes with the enzyme-labelled drug for binding sites on the antibody. Provided a limited amount of antisera is used, increasing the amount of drug in the sample increases the concentration of the unbound drug-G6PDH conjugate. The enzyme activity is monitored spectroscopically by the appearance of NADH at 340 nm.

Using this technique sensitive assays capable of quantitating theophylline at less the 1 μg/ml of serum in less than a minute could be set up. The detailed assay procedure has been described (18,20).

Specificities of the anti-theophylline antisera were determined by studying the cross-reactivity of a number of potentially interfering purine derivatives. After calibration of the assay with known concentrations of theophylline, the assay response was determined using varying amounts of potential cross-reactants. The amount of the cross-reactant giving a signal equal to 1 μg of theophylline was determined. The data are given in Table 1.

DISCUSSION

Xanthines, of which theophylline is an important member, are a class of compounds which occur widely in nature. The large variety of alkylated xanthines and hypoxanthines present in food and as metabolites present a substantial challenge in the development of a specific immunoassay of theophylline. Figure 1 has some of the more important potential cross-reacting compounds among which 1-methyluric acid, VI, 1,3-dimethyluric acid, VII, 3-methylxanthine, IV, caffeine, II, and theobromine, III, are of particular concern. The first three compounds are important metabolites of the drug in man (12) while the last two are commonly consumed stimulants. Since the only common substituent of these compounds is the methyl at position 3 of the xanthine

TABLE 1

Percent Cross-reactivity of Theophylline Antisera to Structurally Related Compounds

Cross-reactant	Derivative Used for Immunogen				
	VIII (N-3)[a] adsorbed[b]	VIII (N-3)[a] unadsorbed	XII (C-8)[a]	IX (C-8)[c,d]	X (C-8)[c,e]
Theophylline (I)	100%	100%	100%	100%	100%
Caffeine (II)	0.6	< 1	6.7	4.5	16.6
Theobromine (III)	< 0.1	< 1	1.1	0.5	1.1
3-Methylxanthine (IV)	0.1	< 1	0.3	2.0	2.0
1-Methyluric acid (VI)	< 0.1	--	--	--	0.1
1,3-Dimethyluric acid (VII)	< 0.1	< 1	7.7	5.1	2.8
1-Methylxanthine (V)	4.5	10	1.2	< 0.1	0.9
1,7-Dimethylxanthine	0.2	--	--	0.21	--
7-Methylxanthine	0.2	--	--	< 0.1	--
Xanthine	< 0.1	--	--	< 0.1	< 0.1
Hypoxanthine	< 0.1	--	--	--	< 0.1
Uric acid	< 0.1	--	--	--	< 0.1
8-Chlorotheophylline	1.2	--	10	--	--

a) Determined by homogeneous enzyme immunoassay and expressed as [theophylline] to [cross-reactant] ratio in percent needed to give a response equivalent to 1.0 μ/ml theophylline, present work.
b) Anti-theophylline antibody solutions contained 2×10^{-8} M 1-methylxanthine (~0.2 μg/ml in the assay medium).
c) Determined by RIA as a ratio in percent of [theophylline] to [cross-reactant] at 50% displacement of initially bound label.
d) Reference 13.
e) Reference 14.

nucleus precise molecular recognition at this site should be of limited importance. Accordingly, we concluded that an immunogen prepared by attaching the drug to carrier proteins at the 3 position might elicit the most specific immune response. By using a 3-carboxyalkyl linkage the electronic and steric characteristics at this site would be retained while providing maximum accessibility of protein binding sites to the variably substituted 1,7, and 8 positions. Similar considerations were given in the selection of attaching sites of morphine to proteins to provide specific antibodies to this drug (21).

3-(3'-Carboxypropyl)-1-methylxanthine, VIII, was synthesized from 1-methylxanthine and conjugated to BGG through its N-hydroxysuccinimide ester derivative. This conjugate elicited anti-theophylline antibodies in sheep which not surprisingly showed strong immuno reactivity not only to theophylline but also to 1-methylxanthine. These antibodies, when employed in an homogeneous enzyme immunoassay, showed clinically insignificant cross-reactivity when challenged with a number of other xanthine derivatives. The cross-reactivities are compared in Table I with those of antisera raised from the C-8 derivatized drug, XII, using a homogeneous enzyme immunoassay and with data reported for radioimmunoassays in which antisera prepared from 8-(3'-carboxypropyl)-theophylline, IX, (13) and 8-carboxytheophylline, X, (14) were used. While the assay based on 3-carboxypropylxanthine, VIII, derived antibodies did not show significant cross-reactivity with any of the major metabolites IV, VI, VII, or caffeine (< 1%),

significant cross-reactivity was observed in assays based on the 8-substituted derivatives. Theobromine was found practically non cross-reactive in all of the assays.

The addition of 1-methylxanthine, V, to antibodies prepared from 3-substituted theophylline reduced cross-reactivity to this compound (4.5%) without adversely affecting other cross-reactivities (Table 1). The adsorbed antibodies were nevertheless highly cross-reactive (200%) with 3-isobutyl-1-methylxanthine due to its close structural resemblance to the hapten, VIII. However, the cross-reactivities to 1-methylxanthine and 3-isobutyl-1-methylxanthine are clinically unimportant since the former is a minor, inactive metabolite of the drug and the latter is employed only as an internal standard for glc assays. The specificity of the adsorbed antibodies which are used in commercial EMIT® homogeneous enzyme immunoassays have been confirmed by Koup and Brodsky (18).

Antibodies produced against 7-(3'-carboxypropyl)theophylline, XV, have recently been reported for use in a nephelometric assay (22) and a radioimmunoassay (23) of theophylline. Although no cross-reactivity data was given in the former assay, the latter is reported to show serious cross-reactivity (200%) with caffeine.

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