

Anaphylaxis to excipient mannitol: evidence for an immunoglobulin E-mediated mechanism

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Summary

Background Anaphylaxis to mannitol present naturally in pomegranate and cultivated mushroom in a sensitized subject has been described recently, and an IgE-mediated mechanism to this sugar alcohol has been proposed. The same subject also experienced severe allergic reactions to a chewable pharmaceutical (cisapride drug).

Objective The purpose of the study was to identify allergenic component in the pharmaceutical preparation, and also, to understand the mechanism of immediate hypersensitivity to mannitol.

Methods Methodology involved skin prick tests (SPTs), high-performance liquid chromatographic (HPLC) analysis of pharmaceutical preparations, separation of mannitol by Ca^{++} -ion-moderated cation-exchange chromatography, preparation of alditol–protein conjugates by reductive amination, SPT using the conjugates, hapten affinity purification of the allergic serum on D-mannitol–keyhole limpet haemocyanin (KLH)–Sepharose CL-6B, and detection of serum mannitol-specific IgE by ELISA.

Results Component testing by SPT, and HPLC analysis of various pharmaceuticals indicated that the excipient mannitol is the causative allergen. Mannitol separated from Cisapride MPS showed allergenic activity by SPT. Among the several conjugates tested by SPT, D-mannitol–bovine serum albumin and D-mannitol–KLH showed positive weal/flare reaction, demonstrating the presence of cell-bound mannitol-specific IgE *in vivo*. Negative results with D-glucitol, D-galactitol, meso-erythritol, and L-mannitol protein conjugates clearly showed that the mannitol-specific human IgE is very specific to the D-isomer of mannitol. ELISA using the hapten affinity-purified allergic serum was positive, demonstrating the presence of mannitol-specific serum IgE in the allergic subject.

Conclusion Mannitol, which is widely used as a food and drug additive (excipient), can rarely cause IgE-mediated anaphylaxis. This study is the first one to demonstrate the presence of mannitol-specific human IgE in a sensitized allergic subject to validate an IgE-mediated hypersensitivity mechanism for mannitol.

Keywords alditol–protein conjugates, anaphylaxis, chemical allergy, cisapride, excipient, food additive, hapten, mannitol, mannitol–BSA, mannitol-specific IgE

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Introduction

Anaphylaxis is a severe, life-threatening allergic reaction that affects both children and adults, and may be caused by classical IgE-mediated hypersensitivity. Agents that most commonly cause anaphylaxis are foods, drugs, insect stings, and latex. Severe allergic reactions to several chemicals and drugs have been reported, and well studied in many cases with respect to their mechanism [1–4]. A drug usually contains one or more active ingredients, which form the principal components responsible for its action, and other inactive ingredients such as dyes and excipients. Excipients are usually inert substances added to a pharmaceutical preparation to

preserve and stabilize active components. Excipients also provide suitable consistency, acting as a binder, matrix, base, or diluent in pills, tablets, creams, salves, etc. An allergic reaction to a medication might be because of any component of the preparation, including dyes and excipients [5–9].

Mannitol, a non-toxic and non-metabolizable osmotically active compound is administered as a therapeutic agent in many clinical situations such as drug intoxication, oliguric renal failure, glaucoma, and increased intracranial pressure. Mannitol is widely used as a food additive in processed foods, and as an excipient in pharmaceutical preparations. Several cases of hypersensitivity reactions to 10% or 20% (w/v) mannitol IV infusion have been reported [10–20]. These appear to be anaphylactoid reactions caused by direct action of mannitol at hyperosmolar (>100 mM equivalent to 1.8% w/v) concentrations on mast cells/basophils [13, 21] in a limited number of atopic subjects.

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We reported a case of anaphylaxis to mannitol present naturally in pomegranate (*Punica granatum*) [22] and cultivated mushroom (*Agaricus bisporus*) [23]. A hypothesis has been proposed recently to explain the mechanism of immediate hypersensitivity to this low-molecular weight allergen [24]. As mannitol is an inert hapten not having any reactive groups, it cannot form conjugates with macromolecular carriers by itself. It was proposed [24] that the Schiff base intermediate of D-mannose with proteins (formed by Maillard reaction *in vivo*) bearing D-mannitoyl groups might be responsible for eliciting a mannitol-specific IgE response. The allergic subject also had a history of anaphylaxis to a chewable cisapride tablet. The present work was carried out to find out if the allergic reaction to the cisapride drug is because of the active ingredient itself or because of any other component among the excipients. Since the subject had mannitol hypersensitivity, we suspected mannitol as the excipient allergen in cisapride drug, and if so, to understand whether the anaphylaxis to mannitol is because of an IgE-mediated mechanism as proposed earlier in our hypothesis [24].

Materials and methods

Allergic subject

A 32-year-old woman, described before [22, 23], had a history of several episodes of anaphylaxis to pomegranate fruit [22], mushroom curry [23], and a chewable tablet Cisapid MPS. The allergic subject experienced severe angioedema, generalized urticaria, difficulty in breathing, and loss of consciousness, within 5–10 min of consuming a Cisapid MPS chewable tablet, needing emergency treatment to return to normalcy.

Control subjects were 12 normal individuals without any history of allergy to food, pollen, or drugs. The present study was carried out after obtaining Institutional Ethics Committee clearance, and informed consent from the allergic and normal subjects. Skin prick tests (SPTs) were carried out at the Health Centre (CFTRI, Mysore).

Preparation of aqueous extracts from drugs

One tablet of Cisapid MPS or other pharmaceutical preparations (listed in Table 1) was powdered and dissolved separately in 5 mL of phosphate-buffered saline (PBS, pH 7.4), and filtered through Whatman 1 filter paper to obtain a clear filtrate. This 'aqueous extract' was used for SPTs. The extracts were further filtered using 0.45 µm membrane filter (Millipore Corp., Bedford, MA, USA) and were used for high-performance liquid chromatographic (HPLC) analysis. Listerine Cool Mint being a liquid was used as such for both SPT and HPLC analyses.

Skin prick test

SPT was performed as per the standard procedure [25]. Briefly, a drop of each sample was placed on the volar side of the forearm, and pricked using a sterile prick lancetter (Bayer Pharmaceutical Division, Spokane, WA, USA). Histamine dihydrochloride (10 mg/mL) in 50% glycerol-PBS was the positive control. Glycerinated PBS was used as the negative control. Weal and flare diameters were measured after 20 min. A mean weal diameter of 3 mm greater than the negative control was considered as a positive skin test response.

Alditol-protein conjugates, after extensive dialysis against PBS at 4 °C, were sterile filtered and used for SPT. All the conjugates were tested at 1% w/v (10 mg/mL) concentration.

High-performance liquid chromatography analysis

HPLC analyses were carried out using Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) on Supelcosil LC-NH₂ column (4.6 × 250 mm, 5 µm aminopropyl-bonded silica) with acetonitrile:water (85:15, v/v) as the mobile phase. The column was run at 25 °C, at a flow rate of 1 mL/min. Sugars and sugar derivatives were detected using a refractive index (RI) detector (Shimadzu RID-6A, Kyoto, Japan). Sugar standards were run separately to determine elution profile, and a mixture of sugar standards (such as D-glucose, D-fructose, and D-mannitol) was run to find out the chromatographic resolution.

Table 1. Composition of Cisapid MPS and other pharmaceutical preparations tested in this study

Name of drug	Manufacturer	Composition as declared on the label
Cisapid MPS (chewable tablet)	Kopran Ltd, Mumbai, India	Cisapride monohydrate equivalent to cisapride (10 mg), Simethicone IP (125 mg), colour: brilliant blue and tartrazine
Sysapride MPS (chewable tablet)	Systopic Lab., New Delhi, India	Cisapride BP (10 mg), activated methyl polysiloxane IP (125 mg), colour: erythrosine
CENZ-10	Crescent Pharma, Bangalore, India	Cisapride BP (10 mg), excipients (QS), colour: brilliant blue FCF and tartrazine
Moticare MPS (chewable tablet)	Modi-Mundi Pharma, Chennai, India	Cisapride BP equivalent to cisapride anhydrous (10 mg), Simethicone IP (125 mg)
Cisapid	Kopran, Khalapur, India	Cisapride monohydrate BP equivalent to Cisapride (10 mg)
Gelusil MPS (chewable tablet)	Parke-Davis (I) Ltd, Hyderabad, India	Activated dimethicone IP (50 mg), magnesium hydroxide IP (250 mg), dried aluminium hydroxide gel IP (250 mg), magnesium aluminium silicate hydrate (50 mg)
Paractol (chewable tablet)	German Remedies Ltd, Mumbai, India	Activated dimethicone IP (250 mg), dried aluminium hydroxide gel IP (500 mg)
PH4 800 (chewable tablet)	Biological Evans, Mumbai, India	Magaldrate IP (800 mg), activated Simethicone IP (50 mg)
Listerine Cool Mint (antiseptic mouth wash)	Warner-Lambert Pharmaceutical Co., Morris Plains, NJ, USA	Thymol (0.064%), eucalyptol (0.092%), methyl salicylate (0.06%), menthol (0.042%), Sorbitol solution, alcohol (21.6%), Poloxamer 407, Benzoic acid, flavoring, sodium saccharin, sodium citrate, citric acid, and FD&C green # 3, water

Ion-moderated cation-exchange chromatography

This was carried out as described earlier for determination of polyols in chewing gums and confections [26], using a cation-exchange resin (H^+ form) after converting it to Ca^{2+} form.

Aqueous extract of Cisapid MPS was subjected to ultrafiltration in an Amicon stirred-cell using DIAFLO YM3 disc membrane (Millipore Corp.) having molecular weight cut-off of 3000 to obtain 3K-filtrate. This 3K-filtrate was subjected to ion-moderated cation-exchange chromatography on a calcium form of Dowex-50W column (1×60 cm) equilibrated with water, and was run at $25^\circ C$ at a flow rate of 15 mL/h. One milliliter of standard mixture containing 2 mg each of D-glucose, D-fructose, D-mannitol, and sorbitol (D-glucitol) was chromatographed under identical conditions. All these standards were also chromatographed separately. Reducing sugars were detected by phenol-sulphuric acid reagent [27]. Fructose was detected using cold anthrone reagent [28]. Sugar alcohols were detected by polyol assay [29], which involves periodate oxidation followed by estimation of the formaldehyde formed upon condensation with chromotropic acid.

Preparation of hapten-protein conjugates containing mannitol epitopes

D-Mannose was conjugated to a carrier protein, bovine serum albumin (BSA), or keyhole limpet haemocyanin (KLH), by reductive amination (also termed reductive alkylation) in the presence of a mild reducing agent, sodium cyanoborohydride, as described in the literature for other sugars [30, 31] to obtain stable mannitol epitopes on the conjugated carrier protein (as outlined earlier in the hypothesis [24]). BSA (68 mg), D-mannose (100 mg), and sodium cyanoborohydride (100 mg) were dissolved in 5.0 mL of 0.2 M borate buffer (pH 8.0) and incubated at $37^\circ C$. Aliquots of the reaction mixture were withdrawn at various time intervals; the reaction was stopped by adjusting the pH to ~ 4 using dilute acetic acid. The aliquots were dialysed against PBS at $4^\circ C$, and analysed by trinitrobenzenesulphonic acid (TNBS) assay [32] to determine the degree of substitution. As a control, BSA was incubated as above, excluding D-mannose (control BSA). Other conjugates (D-glucitol-BSA, D-galactitol-BSA, *meso*-erythritol-BSA, L-mannitol-BSA, and D-mannitol-KLH) were prepared similarly using the appropriate aldose sugar (monosaccharide). Protein estimation was carried out by the method of Bradford [33].

Trinitrobenzenesulfonic acid assay for amino groups [32]

The number of ϵ -amino groups remaining in conjugated BSA samples was determined using TNBS reagent. To 1 mL of control or BSA conjugate (100 μ g) solution, 1 mL of 4% $NaHCO_3$ (pH 8.5) and 1 mL of 0.01% aqueous TNBS solution (prepared freshly) were added. The reaction was carried out at $42 \pm 2^\circ C$ for 2 h, followed by the addition of 1 mL of 10% SDS and 0.5 mL of 1 N HCl. The absorbance of the solution was monitored at 335 nm in a spectrophotometer, after appropriate blank corrections. A standard curve was obtained with unconjugated BSA (50–200 μ g range). The percent conjugation for a conjugate was calculated from decrease in absorbance, when compared with that of an

identical concentration of standard BSA in TNBS reaction. Percent conjugation was converted to degree of substitution in terms of moles of alditol per mole of carrier, assuming that BSA has 61 TNBS-reactive amino groups [32].

Preparation of D-mannitol-keyhole limpet haemocyanin-Sephacrose CL-6B affinity matrix

Mannitol-KLH conjugate with 50% modification of amino groups was used for immobilization onto Sepharose CL-6B according to the method of Stults et al. [34]. Sepharose CL-6B gel was first washed thoroughly with water to remove preservative, and then suspended in water to obtain 0.2 g moist gel/mL (total 10 mL). The washed gel was then oxidized by adding solid sodium periodate ($NaIO_4$) to a final concentration of 25 mM. The mixture was swirled at $25^\circ C$ for 30 min; unreacted $NaIO_4$ was consumed by the addition of an equimolar amount of ethylene glycol. After 15 min, the gel was thoroughly washed with water followed by 0.2 M phosphate buffer, pH 7.0. The moist gel (0.2 g/mL suspension) in phosphate buffer was mixed with D-mannitol-KLH (2 mg) followed by direct addition of borane-pyridine complex (Fluka, St Louis, MO, USA) to a final concentration of 25 mM. The reaction mixture was mildly agitated, first at $25^\circ C$ for 3 h, and then at $4^\circ C$ for 10 h. Next, gel beads were washed extensively with water to remove unreacted borane-pyridine and the ligand. The gel was finally packed into a glass column (0.8 cm id $\times$ 4 cm; 2 mL bed volume), and equilibrated with 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM NaCl, and stored at $4^\circ C$ in the same buffer with 0.01% merthiolate as a preservative.

Hapten-affinity chromatography

This was carried out at $4^\circ C$. Serum (4 mL) from the subject allergic to mannitol was diluted 1:1 in PBS (pH 7.4), and passed through the D-mannitol-KLH-Sephacrose CL-6B column (2 mL bed volume), pre-equilibrated with PBS. The flow-through was recycled twice through the column. After washing with 50 mL PBS, the bound antibody was eluted in two portions of 0.5 mL of 0.1 M glycine-HCl buffer, pH 2.9. The eluate was neutralized immediately using 0.5 M Tris base. The eluate was aliquoted in 200 μ L portions and stored below $-20^\circ C$.

Enzyme linked immunosorbent assay for mannitol-specific human immunoglobulin E

This was done by non-competitive ELISA [35] using coated antigen format. D-Mannitol-KLH conjugate with 50% modification of amino groups was coated (50 μ g/well) onto flat-bottom polystyrene microtitre wells (Maxisorp; NUNC, Roskilde, Denmark) in 0.1 M carbonate-bicarbonate buffer, pH 9.6. The plate was incubated at $4^\circ C$ for 16 h. The wells were washed thrice between steps using PBS/0.05% Tween-20 (PBS-T). Blocking was carried out using 0.5% gelatin in PBS-T at $37^\circ C$ for 30 min. The serum from the subject allergic to mannitol, as well as the eluate from the mannitol-KLH-Sephacrose CL-6B affinity column, was used as a source of primary antibody (diluted 1:1 in blocking buffer, 100 μ L/well) and the plate was incubated again at $4^\circ C$ for 16 h. After washing, 100 μ L of 1:1000 diluted mouse monoclonal

anti-human IgE-alkaline phosphatase conjugate (Sigma-Aldrich, St Louis, MO, USA) was added and incubated at 37 °C for 2 h. Colour development was carried out using *p*-nitrophenyl phosphate (1 mg/mL, 100 µL/well) in 1% diethanolamine buffer, pH 9.8, at 37 °C for 30 min. The reaction was stopped by adding 3 M NaOH (40 µL/well) and the absorbance at 405 nm was read in an ELISA reader.

Results

Detection of excipient mannitol as the allergen

Since the allergic subject had been shown to be sensitized to mannitol present naturally in pomegranate [22] and cultivated mushroom [23], we suspected mannitol (commonly present as an excipient in drugs/pharmaceuticals) to be the causative allergen. However, Cisapid MPS and other pharmaceutical preparations used in the study (Table 1) did not mention mannitol on their labels. Cisapid MPS, to which the present subject had severe allergic reaction, contained cisapride monohydrate, simethicone, brilliant blue, and tartrazine as mentioned on the label. This preparation being a chewable tablet may have other excipients (sweeteners, stabilizers), which are not declared on the label. To detect or rule out one or more components of this drug as a causative agent responsible for allergy in the present case, a 'component testing' (SPT) was carried out using aqueous extracts of several pharmaceutical preparations, and common excipients (Tables 1 and 2). The results of SPT are presented in Table 2, and were analysed in light of the composition.

Cisapid MPS as well as Moticare MPS gave a strong weal/flare reaction. The negative SPT reactions given by Sysapride MPS, Cenx-10, and Cisapid indicate that neither cisapride nor methyl polysiloxane is an allergen. Positive SPT given by Gelusil MPS and PH4 800 indicates that activated dimethicone or simethicone can be ruled out as an allergen. Paractol, which contained only dimethicone, also gave a negative SPT. Based on all these results, it appeared likely that one of the excipients (not mentioned on the label) could be the allergenic substance.

Common dyes used in pharmaceutical preparations gave negative SPT (Table 2). Aqueous extracts of pharmaceutical preparations were analysed by HPLC on a Supelcosil LC-NH₂ column (Fig. 1). Only those pharmaceutical preparations, that had mannitol as shown by HPLC analysis were positive in SPT. Both mannitol and sorbitol elute at the same position under the conditions of HPLC analysis (data not shown). However, 1% (w/v) solution of authentic D-mannitol produced a positive weal/flare reaction in SPT, while 1% pure sorbitol was negative. Surprisingly, food-grade sorbitol (1% w/v) gave a positive SPT. Among foods, pumpkin (*Cucurbita pepo* L.) is known to contain high amounts (15–20%) of mannitol [36]. When pumpkin extract was tested by SPT on the allergic subject, it produced a positive reaction with a weal/flare diameter of 8/>30 mm (data not shown).

In order to separate mannitol from sorbitol, an aqueous extract of Cisapid MPS was subjected to Ca²⁺-ion-moderated cation-exchange chromatography on Dowex-50W column. The chromatographic profile showed several peaks when assayed using a polyol-specific reagent, chromotropic acid

Table 2. SPT (component testing) in the subject allergic to Cisapid MPS using drugs and commonly used excipients

Sample	Weal/flare diameter (mm)*
Glycerinated PBS (negative control)	1/0
Histamine.2HCl (positive control)	8/>30
(a) Cisapid MPS	9/25
(b) Sysapride MPS	2/0
(c) Cenx-10	2/0
(d) Moticare MPS	5/25
(e) Cisapid	2/0
(f) Gelusil MPS	5.5/25
(g) Paractol	2/0
(h) PH4 800	6/30
(i) Listerine Cool Mint	6/25
Azo dyes	
Tartrazine† (1%)	2/0
Sunset yellow‡ (1%)	1/0
Non-azo dyes	
Brilliant Blue FCF§ (1%)	1/0
Erythrosine¶ (1%)	2/0
D-Mannitol, A.R. (1%)	7/>30
Sorbitol, food-grade (1%)	4/30
Sorbitol, pure (1%)	2/0

*Drugs (a), (d), (f), (h), (i), D-mannitol and food-grade sorbitol were tested on 12 control subjects, and gave values similar to negative control. †FD&C Yellow # 5; E102. ‡FD&C Yellow # 6; orange yellow S; E110. §FD&C Blue # 1; 133 (not approved by EC). ¶FD&C Red # 3; Erythrosine BS; E127. PBS, phosphate-buffered saline; SPT, skin prick test.

(Fig. 2a). One of the peaks (eluting around 43rd fraction) corresponded to the elution position of authentic D-mannitol, confirming the presence of this polyol in Cisapid MPS. When the peak fractions were tested on the allergic subject by SPT, the allergenic activity was found to be associated only with the peak fraction (fraction 43) corresponding to D-mannitol (weal/flare diameter, 5/25 mm).

Food-grade sorbitol, which gave positive SPT (Table 2), was also subjected to cation-exchange chromatography on Dowex-50W (Fig. 2b). When the peak fractions corresponding to the two components were tested by SPT, allergenic activity was found to be associated only with the peak corresponding to mannitol (weal/flare diameter, 4/25 mm). Based on the peak area, this sample of food-grade sorbitol was found to contain 6.3% of mannitol.

Analyses of alditol-protein conjugates

In order to test the proposed hypothesis [24] for the mechanism of IgE-mediated immediate hypersensitivity to mannitol, D-mannose was coupled to BSA by reductive amination as described under Materials and methods. The time course of reductive amination reaction for D-mannose and D-glucose is shown in Fig. 3a. The figure represents the degree of substitution calculated from decrease in number of amino groups, determined by TNBS assay, as a function of time.

When these conjugates were tested using chromotropic acid reagent, which is specific for polyols, they gave a positive colour reaction indicating the formation of respective polyol epitopes during conjugation; control BSA and D-mannose (or

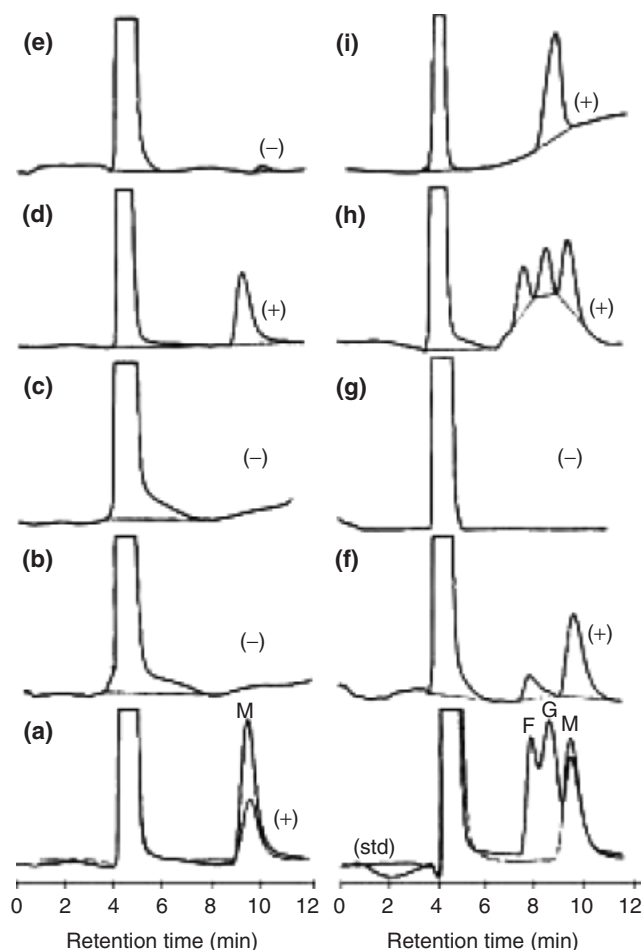


Fig. 1. High-performance liquid chromatography analysis of pharmaceuticals on a Supelcosil LC-NH₂ column to detect the presence of mannitol. (a) Cisapride MPS profile overlaid with that of standard mannitol, (b) Sysapride MPS, (c) Ceniz-10, (d) Moticare MPS, (e) Cisapride, (f) Gelusil MPS, (g) Paractol, (h) PH4 800, (i) Listerine Cool Mint, (std) Standard mannitol profile overlaid with that of standard mixture (D-fructose, D-glucose, and D-mannitol). Retention time of standards: D-fructose (F, 7.94 min), D-glucose (G, 8.69 min), and D-mannitol (M, 9.53 min). Based on retention time, samples (a), (d), (f), (h), and (i) showed the presence of mannitol. The allergenic activity, as shown by skin prick test response [positive (+), negative (-)], for each sample is indicated in the figure.

other aldoses used in this study) did not give any colour reaction in this test (data not shown). The conjugates were also analysed by SDS-PAGE (Fig. 3b), where an increase in molecular mass was evident in the conjugates when compared with control BSA. The increase in the relative molecular masses (M_r) of hexitol-BSA conjugates when compared with BSA control were 8.9 kDa (for D-mannitol-BSA), 8.4 kDa (for D-glucitol-BSA), and 8 kDa (for D-galactitol-BSA), which transforms to 54, 51, and 49 mol of hexitol coupled per mole of BSA, respectively. Other hapten-protein conjugates used in the study (*meso*-erythritol-BSA, D-mannitol-KLH, L-mannitol-BSA) were prepared similarly under identical conditions and were analysed (data not shown).

Detection of mannitol-specific human immunoglobulin E

Cell-bound mannitol-specific immunoglobulin E by skin prick test The results of SPT with various conjugates tested along with free haptens are presented in Table 3. Among the

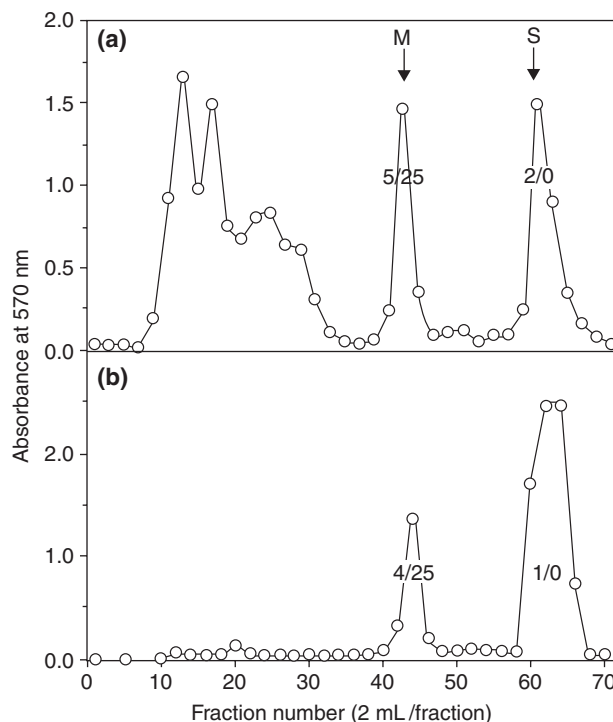


Fig. 2. Ca²⁺-ion-moderated cation-exchange chromatography of (a) aqueous extract of Cisapride MPS, and (b) 1% v/v food-grade sorbitol. Flow rate: 15 mL/h; detection: polyol assay. Arrows indicate the elution position of authentic D-mannitol (M) and sorbitol (S). The numbers on the peaks indicate skin prick test result using the peak fraction, represented as weal/flare diameter (mm).

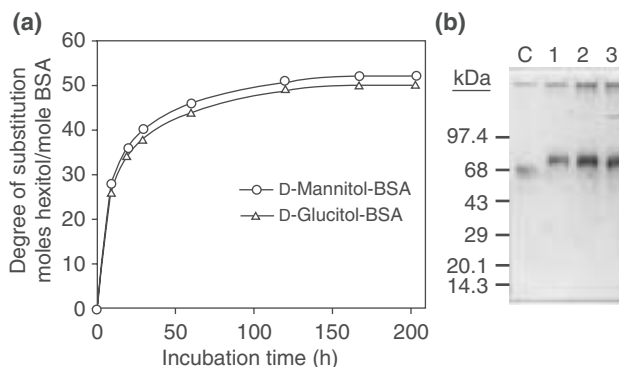


Fig. 3. Preparation of alditol-bovine serum albumin (BSA) conjugates. (a) Time course of reductive amination reaction for D-mannitol-BSA and D-glucitol (sorbitol)-BSA conjugates. D-Galactose was also conjugated similarly to obtain D-galactitol-BSA conjugate (data not shown). (b) SDS-PAGE (12% reducing) analysis of alditol-BSA samples from the 204 h reductive amination reaction (lane C, control-BSA; lane 1, D-mannitol-BSA; lane 2, D-glucitol-BSA; lane 3, D-galactitol-BSA). Molecular weight markers (in kilodalton) are shown on the left side of the photograph.

conjugates tested, only D-mannitol-BSA and D-mannitol-KLH produced a positive weal/flare reaction in the allergic subject. The table also shows the degree of substitution for each alditol-protein conjugate sample used for SPT. There appear to be no perceptible difference in the magnitude of the SPT response with respect to the degree of substitution of mannitol groups on BSA. One percent mannitol-BSA conjugate bearing ~38 mannitol groups is equivalent to 0.1% (w/v) free D-mannitol. Increasing the number of

Table 3. SPT using alditol-protein conjugates

Sample	Degree of substitution (moles alditol/mole protein)	Weal/flare diameter (mm)
Glycerinated PBS	NA	1/0
Histamine.2HCl	NA	7/25
D-Mannose, 0.1%	NA	0/0
D-Mannitol, 0.1%	NA	5/25
D-Mannitol-BSA	25	4/25
	33	4.5/25
	50	4/25
	54	5/30
L-Mannitol-BSA	29	1/0
D-Mannitol-KLH	(50% conjugation)	4/25
D-Glucitol-BSA	51	1/0
D-Galactitol-BSA	48	0/0
meso-Erythritol-BSA	39	1/0
Control BSA	0	1/0
Control KLH	0	1/0

NA, not applicable; PBS, phosphate-buffered saline; BSA, bovine serum albumin; KLH, keyhole limpet haemocyanin.

Table 4. ELISA for detection of mannitol-specific IgE

IgE source	ELISA units (A_{405})*
Normal serum control†	0.220
Eluate from mannitol-affinity step	0.135
Allergic serum‡	0.389
Eluate from mannitol-affinity step	0.994

*Mean of triplicate determinations. †Pooled serum from three normal subjects.

‡Serum from the subject allergic to mannitol

mannitol groups from 25 to 54 on BSA is equivalent to increasing the concentration of mannitol by approximately twofold, which is smaller than a log increase. It may be recalled here that negative response was obtained with 0.001% mannitol, while 0.01%, 0.1%, and 1% mannitol produced a weal/flare diameter of 4/0, 5/25, and 6/30, respectively [23]. SPTs of the conjugates on 12 healthy volunteers were negative (data not shown).

Serum mannitol-specific immunoglobulin E by enzyme-linked immunosorbent assay The result of IgE ELISA is presented in Table 4. The allergic serum showed slightly higher ELISA units (0.169 U) compared with normal serum. The result was more pronounced and markedly higher with mannitol-specific antibody obtained from the allergic serum by mannitol-affinity adsorption. This appears to be because of a fourfold enrichment of mannitol-specific IgE in the eluate from mannitol-affinity column. As a control, normal serum was subjected to affinity adsorption under similar conditions, and showed a negative result in ELISA.

Discussion

Cisapid MPS contains cisapride, a peristaltic stimulant, and methyl polysiloxane, an antifoaming active ingredients. It

may have other inactive components such as dyes and excipients, which are generally not declared completely on the label. One of the objectives of the present study was to investigate if the severe drug allergy (anaphylaxis) caused by Cisapid MPS in the allergic subject is because of the active ingredient(s) or because of any other component present in the drug. Analyses of various pharmaceutical preparations by SPT and HPLC, and of Cisapid MPS by Dowex-50W chromatography, in addition, showed that the subject was not allergic to any active drug components or dyes, but to excipient mannitol. Analysis of food-grade sorbitol by Dowex-50W chromatography, and SPT, further showed that the subject was specifically allergic to mannitol without any cross-reactivity to pure sorbitol. Food-grade sorbitol is commercially supplied as an 70% syrup to which a small amount of mannitol is added to prevent sorbitol from crystallizing. Also, the subject was not allergic to apple, which is known to contain sorbitol (0.51% w/w) [36], but not mannitol. It may be recalled here that when pure apple juice was used as a placebo in double-blind placebo-controlled food challenge [22], it did not elicit an allergic reaction in the sensitized subject.

Earlier, a hypothesis had been proposed by us to explain the mechanism of sensitization as well as hypersensitivity to mannitol [24]. In this hypothesis, D-mannose acts as a *prosensitizer*, the Schiff base conjugate with proteins acts as a *sensitizer*, and D-mannitol acts as a *non-sensitizing elicitor*. The reductive amination reaction proposed in the hypothesis [24] was used to prepare stable mannitol-protein conjugates possessing mannitol epitopes. These conjugates were prepared and used in the present study for SPT, and as coating antigens in ELISA to demonstrate the presence of cell-bound and free mannitol-specific IgE, respectively. Hapten-protein conjugates have been used earlier by other investigators to demonstrate hapten-specific IgE by SPT and ELISA, and to delineate a haptenic epitope responsible for hypersensitivity [37–48]. Some examples of low-molecular-weight chemicals (including drugs and vitamins) that have been shown to act as haptens are chlorogenic acid [37, 38], penicillin [39–41], sulphur drugs [42–44], chlorhexidine [45], thiamine [46], folic acid [47], and pyridoxal [48]. Hypersensitivity reactions to erythritol, a four-carbon polyol used as a non-caloric sweetener in foods and beverages, have been described recently [49, 50]. Although an IgE-mediated anaphylactic reaction to haptenic erythritol has been suggested [50], erythritol-specific IgE has not been demonstrated in that case report.

In the present study, positive SPT with D-mannitol-BSA and D-mannitol-KLH, and negative SPT with other conjugates (D-glucitol-BSA, D-galactitol-BSA, meso-erythritol-BSA) clearly show the presence of highly specific cell-bound IgE directed against mannitol epitope. As L-mannitol is not available commercially, L-mannitol-BSA conjugate was prepared using L-mannose; negative SPT with L-mannitol-BSA shows that the IgE is very specific to the D-isomer of mannitol.

The allergic serum was subjected to hapten-affinity chromatography, and the eluate, which must contain mannitol-specific antibodies enriched in it, was used in IgE ELISA. The ELISA for mannitol-specific IgE was marginally positive with the allergic serum when compared with that of control serum. This may be because of very low titre of free,

circulating mannitol-specific IgE in serum. However, the result was markedly positive with the affinity-purified mannitol antibody from the allergic serum. All these results clearly demonstrate the presence of cell-bound, and serum mannitol-specific IgE in the allergic subject. Although Ackland and Hillcoat [15] reported hypersensitivity to mannitol secondary to cisplatin, this is the first study to report mannitol-specific human IgE in an allergic subject specifically sensitized to mannitol, and thus validates our hypothesis published previously [24]. This study also stresses the importance of labelling of all the ingredients among the excipients in drugs and pharmaceuticals.

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