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Short Communication

The determination of dimethindene in human serum by enzyme-linked immunosorbent assay (EIA)

MAURICE WERMEILLE,*† ETIENNE MORET,† JEAN-PASCAL SIEST,‡ SYLVIE GHRIBI,‡ ANNE-MARIE PETIT§ and MARIANNE WELLMAN§

†*ZYMA SA, P.O. Box, CH-1260-Nyon, Switzerland*
‡*Stabiligen SA, Nancy, France*
§*Université de Nancy, Nancy, France*

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Introduction

Dimethindene maleate is a potent histamine H1 antagonist and is therapeutically effective following oral daily doses of 3–6 mg. A sensitive GLC method is available for measuring blood serum and urine levels at concentrations of about 10 ng ml^{-1} [1, 2]. Recently, a fast sensitive HPLC method using solid-phase extraction was developed with a detection limit of 2 ng ml^{-1} [3]. Both methods can be used for the determination of dimethindene in urine. However, they are not sensitive enough for blood serum assay in pharmacokinetic studies.

This paper reports the development and validation of an enzyme-linked immunosorbent assay (EIA) for measuring dimethindene at concentrations ranging from 0.2 to 20 ng ml^{-1} in human serum.

Experimental

Chemicals

Dimethindene (Structure 1, Fig. 1) maleate and hapten (Structure II) were obtained from ZYMA (Nyon, Switzerland). Immunogen (Structure III) for the immunization was prepared at the University of Nancy (Nancy, France). The primary metabolite of dimethindene, 6-hydroxy-dimethindene (Structure IV) was obtained from the University of Liège (Liège, Belgium) [4]. Another primary metabolite recently described [5], *N*-desmethyl-dimethindene (Structure V) was synthesized at ZYMA. Other potential metabolites (Structures VI and VII) and intermediates of synthesis (Structures VIII and IX) were prepared at the University of Nancy.

Complete and incomplete Freund's adjuvant, bovine serum albumin (BSA), sodium cyanoborohydride ($NaBH_3CN$) and *o*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma (St Louis, MO). Horseradish peroxidase (HRP) was obtained from Pharmacia (Uppsala, Sweden). Other re-Protein-A-sepharose CL-4B were purchased from Pharmacia (Upsala, Sweden). Other reagents and drugs used throughout the study were of analytical grade and were commercially obtained from Merck (Darmstadt, Germany) and Aldrich (Steinheim, Germany) and Sigma.

Immunization schedule

Male rabbits (Fauve de Bourgogne, University of Nancy, France) were immunized with immunogen (dimethindene-BSA) in Freund's complete adjuvant administered at 20 different subcutaneous sites. The same procedure, but this time including the imcomplete adjuvant, was repeated at 30 day intervals; blood samples were taken from the marginal ear vein 8–10 days after the fourth booster challenge. Serum was separated by centrifugation at 3000 rpm and stored at −20°C directly for IgG purification.

* Author to whom correspondence should be addressed.

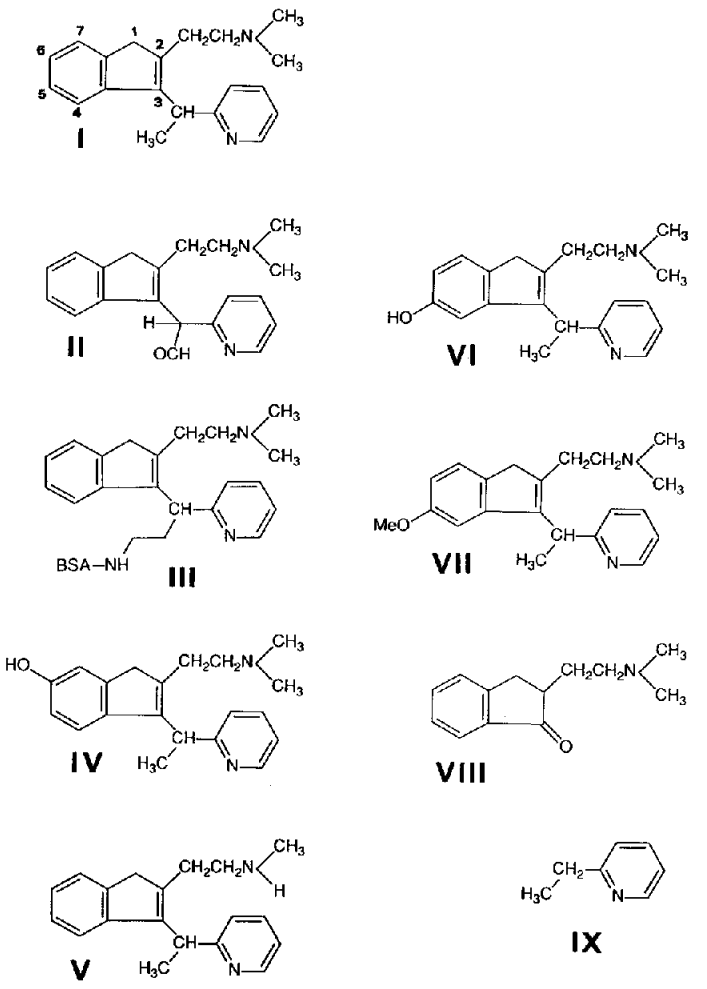

Figure 1
Chemical structures of dimethindene (I), haptene (II), antigen (III), metabolites (IV and V), potential metabolites (VI and VII) and intermediates of synthesis (VIII and IX).

Immunoglobulin G preparation

The immunoglobulin G (IgG) fraction of the antiserum was purified by immuno-affinity chromatography with Protein-A Sepharose. The IgG was eluted with a glycine–HCl buffer at pH 2.3 and dialysed against phosphate buffer 0.1 M at pH 7.4. The IgG concentration was measured by its absorption at 280 nm.

Preparation of dimethindene-HRP tracer

A 2 ml volume of 0.05 M phosphate buffer, pH 6.8, containing 10 mg of hapten was added to 50 mg of HRP. The solution was gently stirred at room temperature for 5 min and then added to 10 mg of $NaBH_3CN$. This mixture was allowed to react during 48 h. The separation of the free hapten from the tracer dimethindene-HRP was carried out by chromatography on a Sephadex G-25 column using phosphate buffer 0.1 M at pH 7.4.

Buffer solutions for EIA

Phosphate buffer 0.1 M (pH 7.4). A 2.67 g mass of KH_2PO_4 and 14.00 g of K_2HPO_4 were dissolved in 1 l of ultra-purified distilled water; the solution was filtered through a membrane filter of pore size 0.22 μm.

Citrate buffer solution (pH 5.8). A 2.08 g mass of citric acid monohydrate and 4.30 g of Na_2HPO_4 were dissolved in 250 ml of ultra-purified water; the solution was filtered through a membrane filter of pore size 0.22 μm and then 200 μl of H_2O_2 30% were added.

Washing solution. A 0.02% (w/v) solution of Tween 20 was prepared by using 0.1 M phosphate buffer.

BSA 1% solution. A 1% (w/v) solution of

BSA was prepared in 0.1 M phosphate buffer.

OPD solution. OPD (30.5 mg) was dissolved in 10 ml citrate buffer solution.

EIA procedure

The technique was generally carried out in accordance with the one described by Voller *et al.* [6]. A detailed protocol for the dimethindene-EIA method is given below.

Step 1. A 100 μl volume of IgG (3.4 μg ml^{-1}) in 0.1 M phosphate buffer, are allowed to adsorb onto the walls of well of polystyrene microtitration plates (96 wells plates, Immuno-Maxisorb F16, Nunc, Roskilde, Denmark) at 25°C overnight. The content of each plate is then discarded and the plate washed three times with the washing solution. All the washings are done with a manual immunowash apparatus (Nunc). Residual binding sites in the wells are saturated with BSA 1% solution at 25°C for 30 min and subsequently washed once with the washing solution.

Step 2. A 50 μl volume of serum to be assayed or pooled human serum with known concentrations of dimethindene (0–20 ng ml^{-1}) for the standard calibration curve are used. A 50 μl volume of the tracer solution (dimethindene-HRP in BSA 1%) are added subsequently to each well. After incubation at 25°C for 2 h, the washing procedure with the washing solution is repeated three times.

Step 3. A 100 μl volume of the enzyme substrate (OPD) solution is added to each well. After a 30 min reaction period at room temperature in the dark, the peroxidase activity is stopped by addition of 100 μl 2 N H_2SO_4. The absorbance at 492 nm was measured using the plate reader Dynatec MR 5000 (Dynatec, France).

Establishment of assay conditions

The sensitivity of an ELISA is dependant on both the concentration of the antibodies labelled antigen. Solutions of anti-dimethindene antibody of IgG fraction were therefore prepared in phosphate buffer solution at concentrations between 1.0 and 20.0 μg ml^{-1}. After the saturation and washing steps, the tracer (which had been diluted 1/15000 in BSA 1% solution) and a standard zero serum were added and incubated at 25°C for 2 h. After washing, the OPD solution was added. The reaction was stopped after 30 min incubation with 2 N H_2SO_4 and the absorbance measured at 492 nm. The absorbance reached a plateau at above 5 μg ml^{-1} IgG. For further experiments, the concentration of 3.4 μg ml^{-1} of IgG was used. Figure 2 shows the increase of the absorbance as a function of the IgG concentration and of the tracer dilution. A dilution of 1:15000 was found suitable to produce high, but not saturated absorbance, in the absence of dimethindene.

The influence of freezing the serum samples containing 0.5 and 10 ng ml^{-1} dimethindene maleate was examined. No statistical differences were obtained before and after freezing the samples.

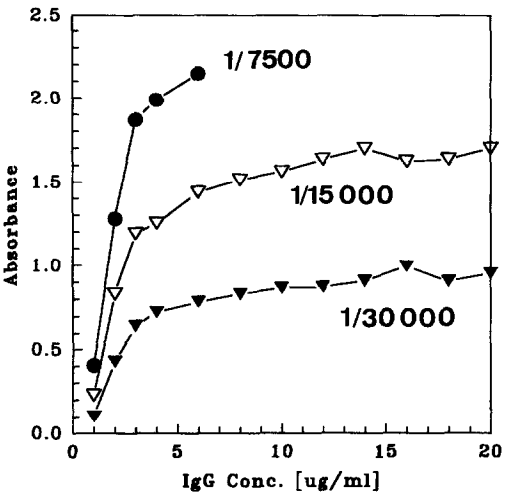

Figure 2
Absorbance as a function of immunoglobin (IgG) concentration and tracer dilution.

Calculations

Assays were performed in triplicates. Each plate contained: (a) blank reagents, i.e. wells without IgG (B_r); (b) blank serum samples of volunteer V_i, i.e. serum samples of volunteer V_i before treatment (B_{oi}); (c) serum samples to be assayed (B_i); and (d) standard serum samples, i.e. a pool of serum without dimethindene (B_o) and with known concentrations of dimethindene (B).

A standardization B/B_o curve was established for each plate by plotting the ratio $(B - B_r)/(B_o - B_r)$ as a function of the logarithm of the concentration of dimethindene maleate, in ng ml^{-1}. The concentration in the serum samples of volunteer V_i to be assayed were

directly obtained from this curve and the ratio $(B_i - B_r)/(B_{oi} - B_r)$.

Results and Discussion

Calibration curves

As illustrated in Fig. 3, the calibration curve for the enzyme immunoassay was obtained by plotting the B_i/B_{oi} (B/B_o) ratio values versus the corresponding dimethindene concentrations (ng ml^{-1}) on a semi-log scale. The minimal measurable concentration was set to 0.2 ng ml^{-1} at a ratio of approximately 0.8 (limit of quantification) and the linearity was observed between 0.2 and 20.0 ng ml^{-1}.

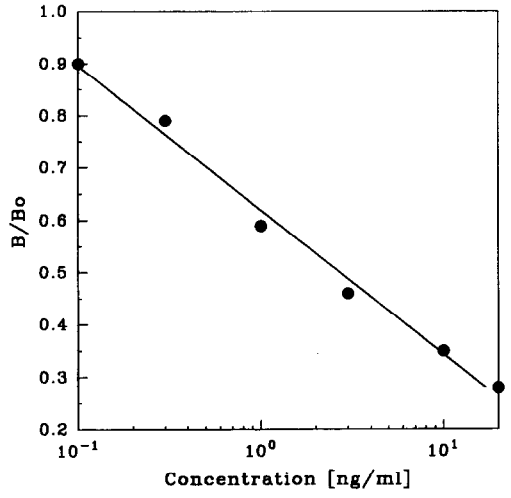

Figure 3
Representative calibration curve. B/B_o: ratio of the absorbance of the assay (B) over the absorbance of the blank (B_o).

Validation

Since the preliminary results with double blind samples indicated that (a) the precision was between 10 and 30% and (b) the accuracy was increased by using the volunteers' blank serum it was necessary to establish the calibration curves by adding known concentration of dimethindene to the blank serum of the volunteer and to use the same plate for the calibration curve and the assay of the samples of the pharmacokinetic study. The interplates' reproducibility at various concentrations was expressed as the relative standard deviation at three concentrations (0.2, 2.0 and 10.0 ng ml^{-1}) for each plate used. Typical results obtained from 31 plates throughout a pharmacokinetic study showed that the precision was 30, 14 and 17% and the accuracy −30, −10 and −6% at the three above-mentioned concentrations of 0.2, 2.0 and 10.0 ng ml^{-1}, respectively. The recovery was approximately 70% at the limit of quantification (0.2 ng ml^{-1}) and 95% at 2.0 and 10.0 ng ml^{-1}.

Cross reactions

The cross-reactivity of antigens other than dimethindene (Structures III–VIII in Fig. 1) was also examined. These compounds were tested at various concentrations in the serum. Assuming the cross-reactivity of dimethindene would occur at 100%, the cross-reactivity of the other substances was expressed as:

$$\% \text{ of cross-reactivity} = \frac{C_{50\%\,\text{dim}}}{C_{50\%\,\text{sub}}} \times 100,$$

where $C_{50\%\,\text{dim}}$ = concentration of dimethindene giving 50% of displacement and $C_{50\%\,\text{sub}}$ = concentration of the substance giving 50% of displacement.

The results obtained showed that the percentage of cross-reactivity never exceeded 0.1% for all the parent compounds and metabolites tested with exception of *N*-desmethyldimethindene which cross reacted to approx. 20%. Unpublished results from urinary excretion indicated however that this metabolite was excreted 10 times less than dimethindene. Therefore, the level of cross reaction measured in the serum should have a very minor effect on the dimethindene assay.

The cross-reactivity of 34 widely used drugs was also examined (Table 1). The results obtained showed that the percentage of cross-reactivity never exceeded 0.01% for all the drugs tested with exception of antidepressants which presented a minor cross-reactivity <0.5%.

Pharmacokinetic application

The pharmacokinetics of dimethindene (Dimethindene maleate, Fenistil®) was studied after its single intravenous and oral administration to eight healthy male volunteers. Serum concentrations were measured for 48 h post-dose using the EIA method. The related pharmacokinetic parameters were determined by the clearance approach [7]. Several new pharmacokinetic studies have recently been carried out using this technique for the assay of dimethindene.

Table 1
Cross reactions with widely used drugs

Pharmacological activity	Drug	% of cross reaction
Antibacterials	Amoxicillin	<0.01
	Cephalosporin	<0.01
	Gentamycin	<0.01
Antidepressants	Amitriptyline	0.33
	Imipramine	0.40
Anti-inflammatories	Diclophenac	<0.01
	Indomethacin	<0.01
	Paracetamol	<0.01
	Piroxicam	<0.01
	Salicylic Acid	<0.01
Beta-blocker	Propranolol	<0.01
Calcium channel blocker	Nifedipine	<0.01
Diuretic	Spironolactone	<0.01
H2 Antagonist	Cimetidine	<0.01
Tranquilizer	Chlordiazepoxide	<0.01
	Diazepam	<0.01
	Meprobamate	<0.01
Vitamin	Vitamin B1	<0.01
	Vitamin B2	<0.01
	Vitamin B6	<0.01
	Vitamin B12	<0.01
	Vitamin C	<0.01
	Vitamin H	<0.01
	Vitamin PP	<0.01
	Folic Acid	<0.01
	Pantothenic Acid	<0.01
Other drugs	Choline	<0.01
	Methionine	<0.01

Conclusions

The results obtained with the described EIA were suitable to measure the concentration of dimethindene in the serum samples. This method has allowed the calculation of the main pharmacokinetic parameters of this drug [7]. In addition, the results are in good agreement with previous information obtained from urinary excretion data [2].

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