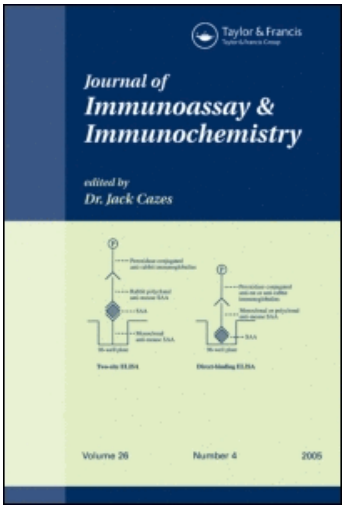

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IMMUNOASSAY OF THEOPHYLLINE BY LATEX PARTICLE COUNTING

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ABSTRACT

We set up an immunoassay by particle counting for theophylline. Theophylline concentration is assayed by its capacity to inhibit the agglutination of theophylline coated latex particles by a specific monoclonal antibody, the agglutination being enhanced by a rabbit anti-mouse IgG antiserum. The dose range is 2-64 mg/L.

The cross-reactions observed with caffeine (0.3%), theobromine (0.2%), 3-methylxanthine (0.7%) and 8-chlorotheophylline (2%) are very good when compared with other published methods.

Within and between-run precisions measured at low, medium and high level of the calibration curve show coefficients of variation ranging from 3.9% to 9.5%. Our assay was correlated with the Fluorescence Polarization Immunoassay (FPIA) and a correlation coefficient of 0.96 was determined for 89 samples.

KEY WORDS: Particle counting immunoassay, latex immunoassay, latex agglutination; theophylline.

INTRODUCTION

Theophylline is a drug used in the treatment of asthma and apnoea in premature infants (1). Its concentration must be serologically monitored because even at therapeutic dose, some toxic effects can occur.

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Usual methods of titration are U.V. spectrophotometric methods, liquid chromatography, radioimmunoassay (2), high pressure liquid chromatography (3,4,5,6), homogeneous enzyme immunoassay (5,7,8), fluorescence polarization immunoassay (FPIA) (9,10), homogeneous substrate-labelled fluorescent immunoassay (11), apo-enzyme reactivation immunoassay system (ARIS) (12), particle enhanced turbidimetry inhibition immunoassay (13) and ligand displacement immunoassay (14).

We describe here an immunoassay of theophylline based on latex agglutination where the extent of the reaction is measured by the optical counting of non-agglutinated latex particles. The basic principles of the technique and the instrumentation have already been described (15) and an application to the determination of digoxin reported elsewhere (16). In the latter assay, a double antibody system using a polyclonal anti-digoxin antibody and a human rheumatoid factor was used. The aim of our work here was to investigate the possibility of using a monoclonal anti-drug antibody and rabbit anti-mouse IgG as second antibody for latex agglutination. To set up the theophylline assay, we use latex particles coated with an albumin-theophylline conjugate. We titrate the drug by its capacity to inhibit the agglutination of the theophylline-coated particles by the mouse monoclonal antibody directed against theophylline and the rabbit anti-mouse IgG antiserum. To prevent serum interferences, samples are first digested by pepsin, and sodium dodecyl sulphate (SDS) is included in the reagent solution.

We present here recovery, precision and specificity of our technique and a correlation obtained with the FPIA method.

MATERIALS AND METHODS

Reagents

Caffeine, 8-chlorotheophylline, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), phenobarbital, phenytoin, theobromine, theophylline, xanthine and twice crystallized pepsin were obtained from Sigma Chemical Co., (St. Louis, MO 63178). Ethylene diamine tetraacetic acid (EDTA), ethanolamin, glycine, hypoxanthine, Tris, urea and uric acid were obtained from Merck (Darmstadt, F.R.G.). 1-methyluric

acid, 3-methyluric acid, 1,3-dimethyluric acid, 1-methylxanthine and 3-methylxanthine were provided by Fluka A.G. (Buchs, Switzerland). Bovine serum albumin (BSA) came from Calbiochem (Lucerne, Switzerland), SDS, from BDH Chemicals Ltd. (Poole, England), N-hydroxysuccinimide (NHSI) from Aldrich Chemie (Steinheim, F.R.G.), and Tween 20 from Technicon Instruments Corp. (Tarrytown, NY 10591). Estapor K150 0.8 μm carboxylated latex particles were obtained from Rhône-Poulenc (Courbevoie, France). HCl-pepsin was 0.3 mol HCl containing 10 g pepsin per litre.

Buffers

Glycine buffered saline (GBS) was 0.17 mol NaCl, 0.1 mol glycine, and 400 mg Na azide per litre, the pH being adjusted to 9.2 with NaOH. GBS-BSA was GBS containing 10 g BSA per litre.

Standards and Samples

A solution of 2 g of theophylline per litre of deionized water was stored at 2-8 °C. Standard solutions of 1-128 mg/L were prepared by diluting the stock solution in normal rabbit serum.

Samples were collected from the clinical department of Mont-Godinne University Hospital (Mont-Godinne, Belgium) and stored frozen before assays. In order to prevent chylomicron interferences in the latex particle counting, the sera were freon-treated before the assay by vortex mixing with an equal volume of Freon 113 (Serva, Heidelberg, F.R.G.) and centrifuged for 5 minutes at 5000 rpm (15).

Protein-Theophylline Conjugates

Theophylline was conjugated to BSA or to keyhole limpet hemocyanin (KLH) according to the method of Hinds *et al* (14). 8-(3-carboxypropyl)-1,3 dimethylxanthine was first synthesized and then coupled to the carrier by the mixed anhydride method.

Preparation of Latex

Carboxylated latex particles were covalently coated with BSA-theophylline conjugate as described previously (16), except that two coupling agents, EDC and NHSI, were used, ethanolamin was added in the buffer to stop the coupling reaction and the coated latex was treated with SDS; the protein/latex weight ratio was 1/20 before conjugation. Finally, the latex was suspended at a concentration of 0.5% (w/v) in GBS-BSA and kept at -20 °C for long-term storage. Before use, this suspension was diluted 10-fold in GBS-BSA containing 0.8% SDS and sonicated for a few seconds with a Model B-12 sonifier (Branson, Danbury, CT 06810).

Anti-Theophylline Monoclonal Antibody

BALB/c mice were immunized by one intraperitoneal injection of 100 μg of KLH-theophylline in complete Freund adjuvant and two subcutaneous injections at 10 days interval. The immune response was tested by enzyme linked immunosorbent assay (ELISA) with BSA-theophylline coated microplates as further described. Boosting before cellular fusion was carried out by intravenous injection of 100 μg of KLH-theophylline per day during four days. Following the method of Van Snick and Coulie (17), 80 million splenic cells were fused with 16 million NSO myeloma cells. Antibody secreting hybridomas were detected by ELISA. The hybridomas were subcloned by limiting dilution and tested for their specificity by inhibition with the various cross-reagents mentioned above.

Ascitic fluids were produced by intraperitoneal injection of one million cells in pristane-treated BALB/c mice.

ELISA for Anti-Theophylline Antibody Titration

Microtiter plate wells were coated with 10 mg/L of BSA-theophylline in fivefold diluted GBS: plates were first incubated for 10 min at 70 °C and then overnight at 37 °C. Fifty μL of sera or hybridoma culture medium diluted in GBS containing 50 mM EDTA and 0.1% Tween 80 were incubated in the wells for 1 hour at room temperature.

After five washings, goat anti-mouse IgG-peroxidase conjugate diluted in GBS-EDTA containing 0.1% Tween 80 and 2% foetal calf serum were added and incubated for 1 hour at room temperature. Bound peroxidase was revealed by 20 min incubation with o-phenylenediamine 0.4 g/L and hydrogen peroxide 0.006% in 0.05 M pH 5 citrate-phosphate buffer.

Rabbit Anti-Mouse IgG Antiserum

New Zealand white rabbits were submitted to intradermal multi-site injections of a total of 100 μg of purified IgG, isolated from NMRI mice. After three injections at 15 days interval, rabbits were bled. Each month, boosts of 100 μg were repeated and followed by bleeding ten days later. We use a pool of several bleedings from different rabbits in our assay.

Immunoassay by Particle Counting

Fifty μL of serum was mixed with 100 μL of HCl-pepsin solution and 100 μL of saline during 5 minutes. The digestion was stopped by adding 60 μL of 1.6 M Tris. After a short mixing, 30 μL of the digested serum was then sampled and mixed at 37 °C for 40 min with 30 μL of BSA-theophylline latex suspension containing 0.8% SDS and 30 μL of the rabbit anti-mouse IgG antiserum diluted 500-fold in GBS-BSA containing 5% Tween 20.

The agglutination reaction was then stopped by adding 1.2 mL of GBS containing 0.02% Tween 20. Diluted particles were then aspirated into the optical cell to count non-agglutinated particles. The suspended agglutinates were stable for several hours.

The concentration of non-agglutinated particles was expressed as peak height on a recorder.

Comparison Method

FPIA was performed with TDX kits (Abbott Laboratories, North Chicago, Il 60064) by the laboratory of Clinical Biology of Mont-Godinne University Hospital (Mont-Godinne, Belgium).

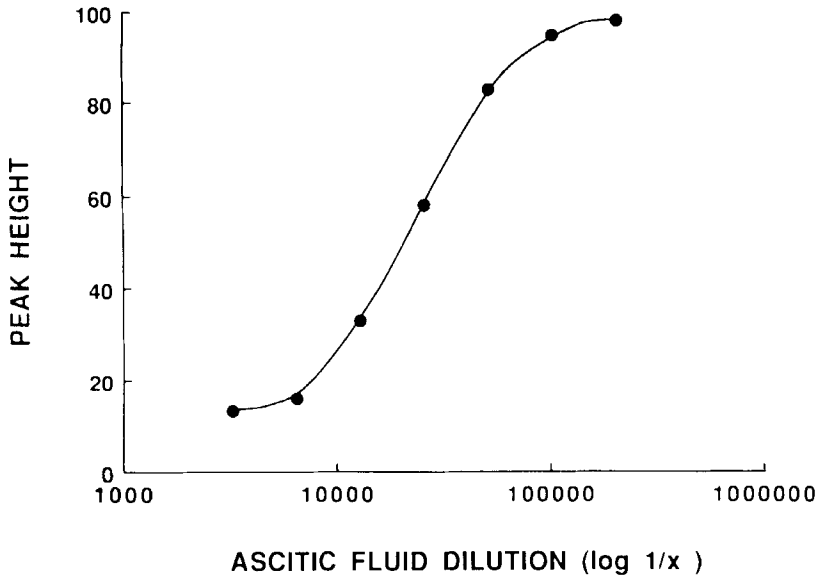

FIGURE 1. Agglutination Curve of Theophylline-latex Conjugate by Various Dilutions of Anti-theophylline Monoclonal Antibody.

RESULTS

Calibration Curve

To obtain optimum sensitivity, we used anti-theophylline monoclonal antibody and rabbit anti-mouse IgG antiserum dilutions that gave a decrease of the number of particles to about 13% of the total number of particles observed in absence of anti-theophylline antibody. Figure 1 illustrates the agglutinability of latex particles by various concentrations of anti-theophylline antibody, the rabbit anti-mouse IgG concentration being constant (1/500). Addition of increasing amounts of theophylline inhibited the particle agglutination causing an increase of the number of free-particles (Figure 2). As slight variation of the maximum agglutination particles number between runs might occur, the calibration curve was expressed as a percentage of the total

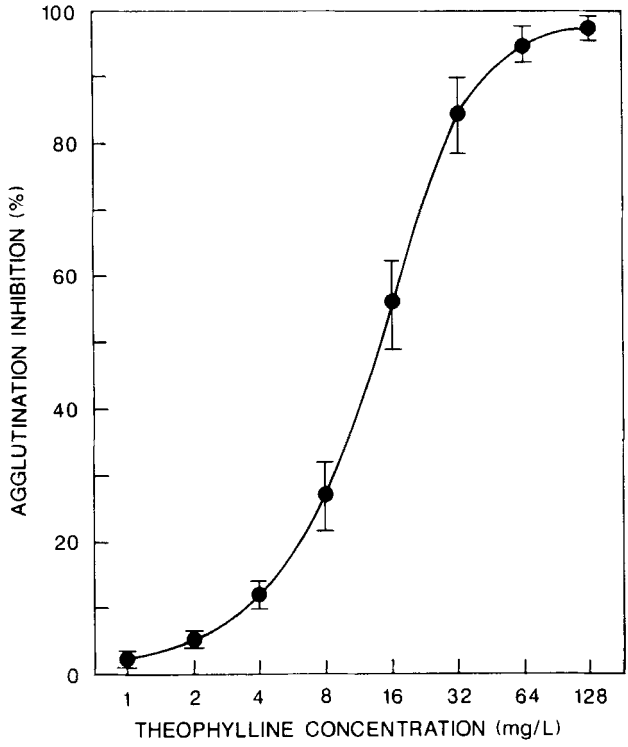

FIGURE 2. Calibration Curve for the Theophylline Determination by the Present Method. Vertical Bars represent the Standard Deviation Calculated on Seven Independent Determination.

inhibition corresponding to the differences between peak height obtained in the absence of anti-theophylline antibody and peak height in the absence of theophylline standard. The calibration curve covered the range from 2 to 64 mg/L with nearly total inhibition at 128 mg/L.

Analytical Recovery

Seventeen serum samples with theophylline concentration ranging from 1 to 15 mg/L were spiked with 10% (v/v) of a 80 mg/L theophylline solution in phosphate

TABLE 1

Analytical Recovery of Theophylline in the Assay
(a) : Serum Samples containing less than 10 mg/L Theophylline
(b) : Serum Samples containing 10-15 mg/L Theophylline

Number of samples	Added (mg/L)	Mean (and SD) recovered mg/L	Mean (and SD) % recovery
12(a)	8	7.86 (0.69)	98.3 (8.9)
5(b)	8	7.96 (0.35)	99.5 (4.4)

TABLE 2

Precision of the Assay as applied to Determination of Theophylline
(a) : ten Assays repeated within a Day with one Standard Curve
(b) : Assays repeated during 6 Days, with new Reagents taken every day

Mean (mg/L)	SD (mg/L)	CV %
within-run (a)		
2.09	0.09	4.4
15.93	0.75	4.7
63.70	4.47	7.0
between-run (b)		
1.89	0.18	9.5
15.97	0.63	3.9
62.50	4.8	7.7

TABLE 3

Drug Cross-reactivity of the Assay

Drugs or metabolites	Cross-reactivity (%)
caffeine	0.3
theobromine	0.2
1-methylxanthine	< 0.4
3-methylxanthine	0.7
1-methyluric acid	< 0.4
3-methyluric acid	< 0.4
1,3-dimethyluric acid	< 0.4
xanthine	< 0.3
hypoxanthine	< 0.3
uric acid	< 0.5
urea	< 0.4
phenobarbital	< 0.2
phenytoin	< 0.3
8-chlorotheophylline	2.0

buffered saline containing 1% BSA (Table 1). The mean recoveries were 98.3% (SD = 8.9%) for samples initially containing less than 10 mg/L of the drug and 99.5% (SD = 4.4%) for samples with original concentrations of 10-15 mg theophylline/L.

Precision

The precision study was done on 3 standard values, 2, 16 and 64 mg/L, which correspond to subtherapeutic, normal and toxic drug concentrations. The assays were repeated 10 times during the same day and once daily during 6 days (Table 2). Maximum within-assay and between assay C.V. were 7.0% and 9.5%, respectively.

Specificity

The specificity of the theophylline assay was assessed by the determination of the cross-reactivity towards metabolites and drugs structurally related to it (Table 3). These drugs diluted in the standard diluent at different concentrations were tested as serum samples. The percentages of theophylline concentration to cross-reagent concentration giving the same inhibition of the latex agglutination was calculated.

Correlation

Correlation on 89 serum samples, including samples with normal and toxic theophylline levels, was established with FPIA (TDX). The results of both methods were related by the regression line: $Y = 0.987x + 2.367$ where $r = 0.96$.

DISCUSSION

The method described here shows suitable performances for drug monitoring in the treatment of adult and newborn children. A good precision has been obtained both for therapeutic and subtherapeutic doses. We are able to titrate drug levels until 64 mg/L with a good precision and without predilution of the serum; this is of interest since theophylline may reach 70 mg/L concentration without provoking death of the patient (18).

A very good specificity of the assay has been obtained thanks to the monoclonal antibody used. No cross reaction of more than 1% was observed, except with 8-chlorotheophylline (2%). Whereas other methods exhibit important cross reactions, sometimes above 10% with some other drugs or theophylline metabolites, particularly caffeine, 8-chlorotheophylline, 1,3-dimethyluric acid and 1-methylxanthin (2,7,10,12,-14,19,20). Two of those assays, FPIA and ARIS, use monoclonal antibody as reagent. The lower cross reactivity displayed by our method may be due to the use of a more specific monoclonal antibody. Spike recovery experiments and a correlation with FPIA method on 89 clinical samples suggest that the present method using agglutination inhibition of theophylline-albumin coated particles with monoclonal anti-theophylline

antibody and rabbit anti-mouse IgG antiserum can be accurately performed with human serum.

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