Development of radioimmunoassay
I. Preparation of radiolabeled tracers theophylline

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Therapeutic monitoring of theophylline can be accurately performed by radioimmunoassay (RIA). It is radioactive tracer as an essential reagent for the development of very sensitive RIA. Direct radiolabeling of theophylline with 125I is very difficult due to the absence of appropriate functional groups. Hence carboxylic acid of theophylline was tagged to tyrosine methyl ester and then radiolabeled. The derivatives of theophylline, bearing a propionic acid and butyric acid side chains at seventh and eight position of theophylline, were synthesised and coupled to tyrosine methyl ester. Theophylline-tyrosine methyl ester conjugates were labeled with 125I using chloramine-T. Radiolabeled theophylline was purified by solvent extraction followed by thin layer chromatography. The purified radiolabeled compound were assessed for their radiochemical purity, specific activity and immunoreactivity. Stability studies of radiolabeled compounds were performed with different solvents at different temperatures. Thenophylline serum samples analysed using developed and commercial kits showed the correlation coefficient of 0.961 (n = 9).

Introduction

Theophylline is commonly used in the management of chronic asthma. This drug has low therapeutic index (10-20 mcg/ml) and hence this requires monitoring of plasma concentration of drug during treatment. The levels above 20 mcg/ml can cause serious side effects like increase in heart rate, cardiac arrhythmias, cerebral seizures or cardiorespiratory arrest and death. 1-3 It is therefore important to design a dose schedule to produce theophylline concentration which are less likely to be associated with adverse effect. Radioimmunoassay of theophylline using tritium labelled tracer were reported. 4-6 For counting of tritium labelled compounds cumbersome liquid scintillating counter and sample preparation is required. Isotopes of iodine are almost universally employed at present for the preparation of tracers for heptens such as steroids and drugs. 7-10 In this paper we present details of a preparation and evaluation of radioiodinated theophylline of moderate specific activity and immunoreactivity.

Experimental

Preparation of acid derivatives of theophylline

Two carboxylic acid derivatives of theophylline were synthesised.

Theophylline-7-propionic acid (7-PAT) was synthesised from anhydrous theophylline using a method reported by Takashi et al. 4 Theophylline (5 g) was allowed to react with 3-bromo-propionic acid (5 g) in a 250 ml round bottom flask containing 120 ml of alkaline solution (pH 10.5) at 60 °C for 16 hours. The pH was then adjusted to 6.5 with 6N hydrochloric acid and extracted twice with 15 ml of ethyl acetate. The pH of aqueous solution was adjusted to 1.5 with 6.5N hydrochloric acid and extracted repeatedly with ethyl acetate. The organic solvent was distilled out and the residue obtained was dried under desiccator at room temperature. The residue of 7-PAT was recrystallised from water : methanol (5 : 1 mixture).

Theophylline-8-butyric acid (8-BAT) was prepared by using a method of Cook et al. 5 Butyric anhydride (1.3 g) and 4,5-diamino-1,3-dimethyl pyrimidine-2,6-dione (1 g) in 10 ml of N,N-dimethyl aniline were refluxed for 2.5 hours under Dean Stark trap. The reaction mixture was filtered after adding 5 ml of solvent. The residue obtained was washed with benzene (25 ml) and recrystallised from water (50 ml/g) to yield crystals of Theophylline-8-butyric acid.

These derivatives were characterised by their melting point, elemental analysis, ultraviolet spectra infra red spectra and NMR spectra.

Preparation of drug-TME conjugates

(a) Preparation of Theophylline-7-propionic acid tyrosine methyl ester (T-PA-TME): A mixture of 34 mg (1 mole) of 7-PAT and 30.8 mg (1 mole) of tyrosine methyl ester hydrochloride in 14 ml of dimethyl formamide was kept at 0 °C for 20 minutes in a round bottomed flask. N-ethyl-N'(3,3-dimethylaminopropyl)-carbodimide (22 mg) was added to the reaction mixture and the solution was kept at 0 °C for 1 hour. The reaction mixture was stirred for 24 hours in darkness at room temperature (25 ± 2 °C). The 7-PAT-TME conjugate was purified by preparative TLC using ethyl acetate : chloroform : acetic acid (50 : 10 : 1) solvent system.

(b) Preparation of Theophylline-8-butyric-acid-tyrosine methyl ester (T-BA-TME): To a solution of 133 mg (1

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mole) of Theophylline-8-butyric acid (8-BAT) in 8 ml of dimethyl formamide, 98 mg (1 mole) of tyrosine methyl ester hydrochloride was added. The mixture was kept at 0 °C for 15 minutes. N-ethyl-N'(3,3-dimethyl amino-propyl)-carbodimide (49 mg) was added to the reaction mixture and kept at 0 °C for 1 hour. The reaction mixture was then stirred for 24 hours at room temperature. The conjugate (T-BA-TME) was separated by preparative TLC using solvent system given in the procedure for T-PA-TME.

Radioiodination of Theophylline-TME conjugates

Radioiodelling of TME-drug conjugates was performed by using GREENWOOD and HUNTER procedure. T-PA-TME conjugate [(1 mg/ml), 0.01 ml in methanol] and T-BA-TME conjugate [(0.1 mg/ml), 0.025 ml in methanol] was taken separately into glass tubes A and B, respectively. 0.02 ml of phosphate buffer (0.4M, pH 7.5) along with 600 μCi of sodium iodide (Na 125I) was added in tube A. 1 mCi of sodium iodide (Na 125I) along with 0.02 ml of phosphate buffer (0.4M, pH 7.5) was added in tube B. Chloramine-T solution 0.02 ml (1 mg/ml in 0.04M phosphate buffer, pH 7.5) was transferred in each tube. Sodium metabisulphite solution [0.02 ml (1 mg/ml in 0.04M phosphate buffer, pH 7.5)] was added in tube A after 90 seconds whereas in tube B after 120 seconds to terminate the reaction. The reaction mixture was finally diluted with 0.4 ml of 0.1M phosphate buffer.

Evaluation of purified tracers

Tracers were evaluated for radiochemical purity, radiolabeling yield, specific activity and immunoreactivity. The radiochemical purity of tracers after TLC purification were evaluated by paper electrophoresis using 0.02M phosphate buffer (pH 7.4) as electrolyte. Electrophoresis was carried out for 90 minutes at 8 V/cm at room temperature. Specific activity of purified tracers was determined by using self displacement analysis.

Immunoactivity of purified T-PA-TME 125I and T-BA-TME 125I were determined by their binding with excess antibodies raised in rabbits against T-PA-BSA and T-BA-BSA immunogens respectively at BARC, Bombay, India.

Stability of radiolabeled theophylline

The radiolabeled tracers were stored, in 0.2% BSA phosphate buffer (0.04M, pH 7.5), at -20, 4 and 25 °C. They were checked for binding with antibody at different time intervals for 2 months.

Estimation of theophylline in serum samples

Serum samples of patient taking theophylline orally were collected, samples were analysed by developed RIA method using T-BA-TME 125I as tracer as well as using marketed RIA kit of theophylline (Gamma-dab, Baxter Healthcare Corporation, USA).

Results

The chemical purity of carboxylic acid derivatives of theophylline were found to be 95%. Melting point, molar extinction and elemental analysis data (Table 1) correlate well with expected values. The presence of peak at 1600–1750 cm⁻¹ in IR spectra in both the acid derivatives confirms the presence of carboxylic groups. Absence of a peak for N–H group in NMR spectra confirms the presence of a propionic acid group at the C-7 position, whereas absence of a peak for N–CH–C proton confirms the presence of a butyric acid group at the C-8 position.

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<th>Parameter</th>
<th>Expected result</th>
<th>Observed result</th>
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<tr>
<td>Molar extinction</td>
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