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[Chem. Pharm. Bull. 29(2) 577—580 (1981)]

Synthesis and Evaluation *in Vitro* of 4-Acetamidophenyl Phosphate[1)]

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(Received September 10, 1980)

4-Acetamidophenyl phosphate was synthesized by a phosphorylation procedure with polyphosphoric acid to examine its properties as a prodrug of acetaminophen. Separation of the ester from the phosphorylation mixture was carried out by precipitation with organic solvents. 4-Acetamidophenyl phosphate was soluble in water, was practically tasteless, and was quickly hydrolyzed to acetaminophen by alkaline phosphatase at 37°. The ester was stable in aqueous solution at neutral and acidic pH at 37°.

Keywords——4-acetamidophenyl phosphate; phosphate ester; prodrug; synthesis; acetaminophen; polyphosphoric acid; hydrolysis; high performance liquid chromatography; gas chromatography; gas chromatography-mass spectrometry

Although acetaminophen lacks the anti-inflammatory effect of the salicylates, it is probably the analgesic-antipyretic of choice as an alternative to aspirin, particularly in patients allergic to aspirin or with a history of peptic ulcer.[2)] Although drops, elixir, and syrup are available[2)] for pediatric patients, the drug has a bitter taste.

In the present study, we synthesized a phosphate ester of acetaminophen, employing the phosphorylation procedures used for the synthesis of uridine-5′-phosphate.[3)] The ester was found to be very soluble in water, practically tasteless, and stable as a solid or in solution. Further, it was hydrolyzed quickly in the presence of alkaline phosphatase. Thus, the ester may be administered orally in the form of an aqueous solution to pediatric patients. Injections may also be prepared because of the high water-solubility and chemical stability of the ester in solution.

Experimental

Materials——Acetaminophen was purchased from Yamanouchi Pharmaceutical Co., Tokyo, while orthophosphoric acid (*ca.* 90%) and phosphorus pentoxide were from Wako Pure Chemical Industries, Osaka, *p*-nitrophenyl phosphate dipotassium salt hexahydrate and alkaline phosphatase were from Sigma Chemical Co., St. Louis, and tetrabutylammonium phosphate (PIC$^{T.M.}$ reagent A) was from Waters Associates, Milford, Mass. All other chemicals were of reagent grade.

Apparatus——Gas chromatography (GC) was carried out with a Shimadzu GC-6A gas chromatograph equipped with a flame ionization detector. The GLC column, 2 m×3 mm i.d., was packed with 2% SE-30 on 100—120 mesh Chromosorb G, HP. The chromatographic conditions were: column temp., 190°; inj. and det. temp., 300°; N_2 flow rate, 57 ml/min.

High performance liquid chromatography (HPLC) was carried out with a TRI ROTAR (Japan Spectroscopic Co.) equipped with a variable wavelength detector (Jasco UVIDEC-100), under the following conditions[4]: column, reversed phase type, Jasco ss-10-ODS-B (particle size 10 μm, 250×4.6 mm i.d.); flow rate, 1.5 ml/min; detector wavelength, 250 nm; mobile phase, water–methanol–acetic acid (85:13.5:1.5 v/v/v) containing 0.5 g/l potassium nitrate and 0.1 mM tetrabutylammonium phosphate.

Paper electrophoresis was performed for 20 min at 700 V on Toyo filter paper 51A (6×40 cm) using 0.05 M triethylammonium bicarbonate buffer solution, pH 8.0.

Gas chromatography–mass spectrometry (GC–MS) was carried out with a Hitachi RMU-6MG mass spectrometer coupled with a Hitachi 002B Datalizer (Hitac 10 II/model A). The GLC column, 1.5 m×3 mm i.d., was packed with 1% OV-1 on 60—80 mesh Chromosorb W, AW, DMCS. The chromatographic conditions were: column temp., 190°; inj. and separator temp., 300°. Typical ion source settings were; ionization chamber temp., 210°; ionization potential, 20 eV; the electron beam total emission was regulated at 100 μA.

Ultraviolet (UV) spectra were recorded on Beckman Acta MIV machine. Nuclear magnetic resonance (NMR) spectra were obtained with a JEOL FX-200 FT-NMR spectrometer in DMSO-d_6. Elemental analyses of carbon, hydrogen, and nitrogen were carried out in the Analytical Center, Hokkaido University, and phosphorus was analyzed in the Japan Food Analytical Center, Tokyo.

Synthesis and Isolation of 4-Acetamidophenyl Phosphate Monosodium Salt——Acetaminophen (16.5 g) was added to a mixture of 90 g of orthophosphoric acid and 67.5 g of phosphorous pentoxide, and the solution was kept at 70° with stirring for 6 hr. The mixture was cooled to room temperature, poured into an ice-water mixture, and subsequently kept in the refrigerator overnight in order to hydrolyze any polyphosphoric compounds. Hydrolysis of polyphosphoric compounds was carried out at low temperature to avoid hydrolysis of the acetamido group. Ammonia water (25%) was added to the solution to adjust the pH of the mixture to neutral in the ice-bath. The mixture was concentrated to about 500 ml on a water bath (30°) *in vacuo*, then an approximately equal volume of ethanol was added. A clear viscous liquid was removed. The supernatant was concentrated to about 200 ml, and then about 800 ml of ethanol was added. The precipitate was collected by filtration. The filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of water, then about 1 liter of acetone was added to obtain a white precipitate. The precipitate was redissolved in water, and the solution was poured onto a column of Dowex 50W×4 [Na^+] resin and eluted with water–methanol (7:3 v/v) in order to exchange ammonium ions for sodium ions. The amount of the resin corresponded to about a 20-fold excess of sodium ions over ammonium ions in both acetamidophenyl phosphate and inorganic phosphates. The eluate was evaporated to dryness *in vacuo* at 30° on a water bath, and then the residue was dissolved in a minimum amount of water. A white crystalline precipitate was recovered from the aqueous solution following the addition of acetone; yield, about 4 g of solid. This corresponds to about 14% overall yield. The solid was recrystallized from acetone-water.

Examination of Aqueous Solubility, Taste, and Chemical Stability——4-Acetamidophenyl phosphate (11 mg) was added to 30 μl of water at room temperature to test the water solubility. The taste of the crystalline solid ester was examined by two healthy men. To test for hydrolysis, 10 ml of 100 μg/ml 4-acetamidophenyl phosphate was incubated in 0.1 N H_2SO_4 and 0.27 M Tris-HCl buffer solution, pH 7.4 at 37° for 24 hr.

Enzymatic Hydrolysis of 4-Acetamidophenyl Phosphate with Alkaline Phosphatase——Ten milliliters of 100 μg/ml 4-acetamidophenyl phosphate was hydrolyzed with 10 mg of alkaline phosphatase in 0.27 M Tris-HCl buffer solution at pH 7.4 and 37° overnight. The hydrolytic product was extracted with 5 ml of ethyl acetate[5] from 1 ml of hydrolysate, and the mixture was centrifuged. A 4 ml portion of the organic layer was evaporated to dryness under a nitrogen stream. The residue was dissolved in 20 μl of ethyl acetate and 2 μl of the solution was injected into the GC–MS and GC columns. One milliliter of 10% perchloric acid was added to another 1 ml of the hydrolysate solution, and proteins were precipitated by centrifugation, then 2 μl of the supernatant was injected into the high performance liquid chromatographic column.

Results and Discussion

Synthesis of 4-Acetamidophenyl Phosphate

Three spots which had UV absorption and one spot of inorganic phosphate were detected

after paper electrophoresis of the hydrolytic products obtained at low temperature from the phosphorylated mixture. One of the spots which absorbed UV had a mobility equal to that of authentic acetaminophen and others had lower mobility than *p*-nitrophenyl phosphate, which was employed as a representative monophosphate ester, but higher mobility than acetaminophen. These two spots gave positive reactions with Hanes's reagent.[6] This indicates that these two compounds each contain a phosphate group. One of the two compounds was identified as a monophosphate ester of acetaminophen. Another could not be identified because it could not be separated from inorganic phosphate by the precipitation procedures. The compound showed higher mobility than the monophosphate ester and slightly lower mobility than *p*-nitrophenyl phosphate. 4-Acetamidophenyl phosphate was hygroscopic and melted with decomposition at 225°.

The analytical data for 4-acetamidophenyl phosphate were as follows: NMR (DMSO-d_6), δ 9.76 (s, 1H, =NH), 7.38—7.00 (m, 4H, ArH), 1.99 (s, 3H, –CO–CH_3); *Anal.* Calc for $C_8H_9NO_5PNa \cdot 1/2H_2O$: C, 36.65; H, 3.84; N, 5.34; P, 11.8. Found; C, 36.46; H, 3.80; N, 5.18; P, 12.2; UV, λ_{max}, 239 nm (H_2O), 243.5 nm (CH_3OH).

Water Solubility, Taste, and Chemical Stability

4-Acetamidophenyl phosphate (11 mg) quickly dissolved in 30 μl of water. Its water solubility was thus more than 367 mg/ml at room temperature. 4-Acetamidophenyl phosphate was practically tasteless.

After incubation for 24 hr in 0.1 N H_2SO_4 and 0.27 M Tris-HCl buffer solution, pH 7.4, at 37°, there was no peak in the chromatogram other than the peak of the ester upon HPLC. Thus, the ester was sufficiently stable in water.

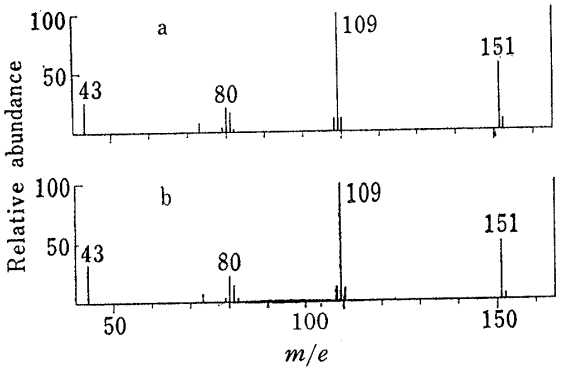

Fig. 1. a) Mass Spectrum of Authentic Acetaminophen, b) Mass Spectrum of the Hydrolytic Product of 4-Acetamidophenyl Phosphate with Alkaline Phosphatase

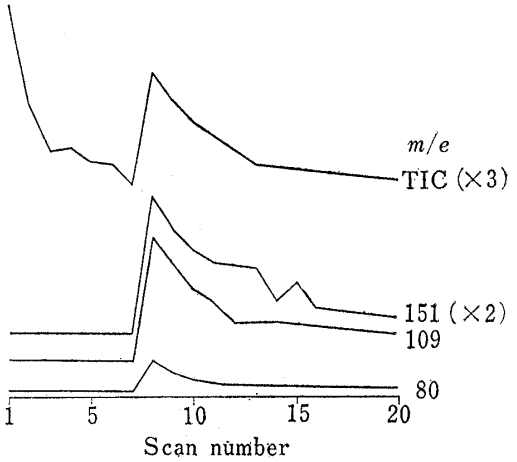

Fig. 2. Typical Ion Chromatogram of the Hydrolytic Product of 4-Acetamidophenyl Phosphate with Alkaline Phosphatase

The solvent was precut when gas chromatography was carried out. TIC=total ion chromatogram.

Identification of an Enzymatic Hydrolytic Product of 4-Acetamidophenyl Phosphate

4-Acetamidophenyl phosphate has to be hydrolyzed to acetaminophen *in vivo* in order to exhibit analgesic-antipyretic action. Alkaline phosphatase, which is abundant in the lumen of the small intestine, was used.

Following treatment with the enzyme for 15 hr, no 4-acetamidophenyl phosphate was detected and a new peak which had the same retention time as authentic acetaminophen was detected by HPLC.

The extract of the hydrolysate with ethyl acetate had a peak which showed the same

retention time as acetaminophen in GC. The hydrolytic product was identified as acetaminophen by GC–MS (Figs. 1 and 2).

Acknowledgement The authors are grateful to Drs. T. Ueda and M. Saneyoshi, and Mr. C. Nakayama for advice on phosphorylation procedures, and to Dr. K. Miyazaki and Mr. K. Umeniwa for GC-MS. The authors are also grateful to Drs. Awadzu and Hayashi for making their HPLC assay procedures available prior to publication.

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[Chem. Pharm. Bull. 29(2) 580—584 (1981)]

Toxicological Approaches to Streptothricin Antibiotics. IV.[1] Toxicity of Streptothricin Antibiotics to the Blood

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(Received June 18, 1980)

The hematoxicity of a streptothricin antibiotic was investigated in rats by blood morphological examination, by scanning electron microscopic observation of changes in the erythrocytic membrane and by means of the coil planet centrifuge (CPC) technique to detect alterations in erythrocytic membrane dysfunction. The administration of the antibiotic caused no appreciable hematological change, nor any alteration in the morphology or function of the red blood cell membrane. The results indicate that the streptothricin antibiotic has no hematotoxic potential.

Keywords——racemomycin-D; coil planet centrifuge; erythrocyte; erythrocyte membrane; hemolysis; delayed toxicity; scanning electron microscopic observation

In previous reports from this laboratory, the cause of toxicity of streptothricin antibiotics in mice and rats was investigated by assessments of antibiotic distribution in various organs and tissues,[3] histopathological studies[4,5] and serum biochemical examinations,[3] and a marked nephrotoxic potential of this group of compounds was demonstrated. The animals dosed with the antibiotics showed no significant adverse histopathological changes in the spleen or liver, though the organs showed a marked progressive decrease in weight. These two organs, as well as the kidneys, which exhibited conspicuous pathologic changes, are closely related to the blood. This report describes a study of the toxicological effect of a streptothricin anti-