Fluorescent TLC Analysis of Traces of Penicillin

Keyphrases ☐ Penicillin, trace amounts—analysis ☐ 9-Isothiocyanatoacridine—fluorescent labeling ☐ TLC—analysis ☐ Fluorometry—analysis, TLC plates

Sir:

We would like to report a specific and sensitive (nanogram range) quantitative chemical determination of penicillins which should have application to biological materials and for monitoring penicillin contamination in pharmaceutical manufacturing procedures. This method was developed as part of our program in the synthesis and applications of fluorescent labeling agents. The general approach in this determination is to hydrolyze trace amounts of penicillin with either base or more specifically with *B*-lactamase and to react the secondary amino function of the resulting penicilloic acid with a fluorescent isothiocyanate to form the corresponding fluorescent thiourea.

The commercially available amine labeling agents, fluorescein isothiocyanate and 1-dimethylaminonaphthalene-5-sulfonyl chloride, were applied initially for fluorescent labeling but they proved unsatisfactory. That is, a suitable separation of the labeled penicilloic acid from excess acidic reagent and by-products was not developed. Separation of excess labeling agent from product was simplified by employing a nonacidic fluorescent labeling reagent, 9-isothiocyanatoacridine. This reagent was synthesized essentially by the procedure reported by Kristian (1) from the reaction of 9-chloroacridine with silver thiocyanate.

The procedure for analysis of benzylpenicillin was carried out as follows: to each of a series of 5-ml. glass-stoppered pear-shaped boiling flasks was added 50 μl. of a 0.50 mg./ml. buffered[1] aqueous solution of penicillinase (Calbiochem) and 20 to 400 μl. of a 15 mcg./ml. buffered[1] solution of benzylpenicillin (sterile potassium penicillin G, USP, Lilly). Hydrolysis was allowed to occur for 30 min. and then 50 μl. of a 0.18 mg./ml. absolute alcohol solution of 9-isothiocyanatoacridine was added to each flask. Corresponding controls containing equal amounts of penicillin but without enzyme were prepared, as well as a blank containing only the reagent and enzyme. The solutions were shaken in a water bath at 60 ± 1° for 2 hr. and then lyophilized. The residue was redissolved in 100 μl. of a 1:1 v/v mixture of absolute alcohol and pH 7 buffer,[1] and 1-μl. aliquots of each sample and its control were spotted in alternate 0.5-cm. scored channels of Silica Gel G thin-layer plates (250-mμ thick). Development was for a distance of 13 cm. with a DMF–$CHCl_3$–28% NH_4OH (10:5:4) solvent system. Plates were dried at 106° for 15 min. and the fluorescent spot at R_f 0.50 was examined with a scanner (Aminco No. 4-8221A) with the silica surface of the TLC plates placed toward the activation light. The scanner was connected to a spectrophotofluorometer (Aminco-Bowman) and recorder (Heath No. EU 20B) equipped with expanded range attenuation.[2] An activation wavelength setting of 410 mμ and a fluorescence setting of 510 mμ were employed.

Phenoxymethylpenicillin was determined in a similar manner with the spot at R_f 0.88 being scanned at an activation setting of 260 mμ and a fluorescence setting of 460 mμ. Penicillinase-resistant methicillin was hydrolyzed with 50 μl. of 0.1 *N* NaOH before a similar determination. Its fluorescent spot at R_f 0.73 was activated at 350 mμ and fluorescence measured at 480 mμ.

While a blank of 9-isothiocyanatoacridine reagent and penicillinase was routinely employed, it was not critical as all fluorescent material moved with the solvent front. However, a control of penicillin and reagent is essential. A contribution at the same R_f value as great as a third, in some instances, was subtracted from the corresponding sample determined after hydrolysis. The contribution was assigned to the amount of hydrolyzed penicillin and amines in the sample originally as well as that produced in the control reaction process *per se*.

The difference in fluorescence between reacted hydrolyzed samples of benzylpenicillin and their corresponding controls increased in a linear manner when compared to an increase in original sample concentration in the range from 3–30 ng. There is an expected loss in linearity at higher concentrations probably due to a quenching effect.

An indication of the sensitivity[3] of the procedure is given by the ability to detect 3.0 ng. of benzylpenicillin, 0.76 ng. of phenoxymethylpenicillin, and 0.82 ng. of methicillin. Of particular interest is the fact that this sensitivity exceeds that needed to meet the Food and Drug Administration's requirement of less than 0.05 unit (30 ng.) of penicillin cross-contamination per maximum single dose for parenteral drugs.

As applications of this procedure to cross-contamination problems may vary with dosage forms and be dependent upon preliminary separation methods, penicillin recovery studies should be included with each material examined. The sensitivity of the method, together with specificity presented by use of penicillinase hydrolysis and TLC R_f values, is suggested as being

[1] The buffer solution employed was 0.1 *M* K_2HPO_4 adjusted with 5% H_3PO_4 to pH 7.0.

[2] The expanded range attenuator was specially manufactured by Johnson Instrumentation Specialties, Ann Arbor, Mich. utilizing circuit No. 3 (shifting zero circuit) from Manual of Beckman model 76 Expanded Scale pH Meter, p. 36, catalog No. 80694, Beckman Instruments, Inc., Fullerton, Calif.

[3] Sensitivity is based upon the weights of buffered commercial penicillin samples.

potentially advantageous in the determination of trace amounts of penicillin.

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Rapid Peptide Synthesis: Synthesis of the Heptapeptide A_{65}-A_{71} of Abnormal Human α-Hemoglobin

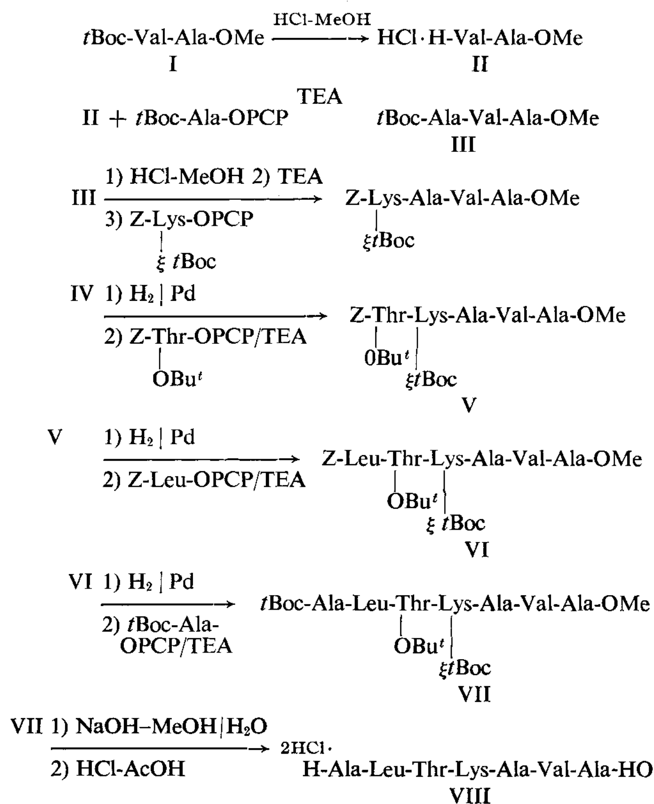
Keyphrases ☐ Peptide synthesis ☐ Heptapeptide A_{65}-A_{71} synthesis—abnormal α-hemoglobin component ☐ IR spectrophotometry—reaction monitoring

Sir:

Recently the *N*-carbobenzoxy and *N-t*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters have been described.[1,2] We wish to report the utility of these intermediates for extremely rapid peptide synthesis. For this purpose the synthesis of the heptapeptide, L-alanyl-L-leucyl-L-threonyl-L-lysyl-L-alanyl-L-valyl-L-alanine corresponding to the sequence A_{65}–A_{71} of α-hemoglobin having a point motation at position 68[3–6] is described. The synthesis is shown in Scheme I, its rapidity being due to the minimal necessity to purify the intermediate peptides which is a result of the ease of purification of the starting amino acid pentachlorophenyl esters and their high activity toward aminolysis.

The *N-t*-butyloxycarbonyl protecting group was removed from the Dipeptide I using HCl in methanol. Evaporation of the solvent gave the Dipeptide Hydrochloride II, which was coupled to *N-t*-butyloxycarbonyl-L-alanine pentachlorophenyl ester in methylene chloride containing one equivalent of triethylamine. By following the IR absorption spectrum of the pentachlorophenyl ester peak at 1775 cm.$^{-1}$ it was observed that the coupling reaction was essentially over after 4 hr. at room temperature. Removal of the solvent gave the crude Tripeptide III. The Tetrapeptide IV was obtained by repeating the cycle of deprotection and coupling with *N*-carbobenzoxy-ξ-*N-t*-butyloxycarbonyl-L-lysine pentachlorophenyl ester.

The *N*-carbobenzoxy protecting group was removed from the Tetrapeptide IV by catalytic hydrogenolysis in



Scheme I

dimethylformamide until no further evolution of carbon dioxide was observed. Addition of *N*-carbobenzoxy-L-threonine pentachlorophenyl ester and triethylamine yielded the Pentapeptide V in solution. This cycle of hydrogenolysis and coupling was continued until the protected Heptapeptide VII was obtained. The methyl ester was hydrolysed from VII by use of 1 *N* NaOH in methanol and the remaining protecting groups were removed by HCl in glacial acetic acid, to give the crude free Heptapeptide VIII. Purification of this material was obtained by passage through a column of synthetic polysaccharide (Sephadex G-25) (100 × 2.5 cm.) using water as eluent at a flow rate of 8 ml./hr. The pure heptapeptide $[\alpha]^{26}$ − 34.0° (c 2.2 in water) was eluted as the first major fraction (30% overall yield) as shown by amino acid analysis: Ala, 3.01; Leu, 1.02; Lys, 0.98; Thr, 0.95; Val, 1.01.

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