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Short Communication

Enzymatic detection of β-lactam antibiotics in pharmaceutical products

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Introduction

The United States Food and Drug Administration has declared that contamination of drugs with penicillin be limited to 0.05 IU per single dose for parenteral preparations and to 0.5 IU per single dose for oral preparations [1]. Although there are no published limits for the contamination of medicinal products by penicillin antibiotics in the UK, it is understood that the levels first promulgated in the USA Code of Federal Regulations are accepted informally for practical purposes. A sensitive method for the detection of benzylpenicillin and other β-lactam antibiotics in pharmaceutical products is therefore required to meet these stringent requirements.

Thin-layer chromatographic techniques involving chemical and microbiological detection systems have been used to monitor β-lactam contaminants in pharmaceutical products [2, 3]. These methods, however, require several extraction steps and are time-consuming. High-performance liquid chromatographic methods [4, 5] are faster but are usually not sufficiently sensitive. Immunoassay techniques are sensitive but may require the preparation of antiserum [6].

The aim of this contribution was to investigate the application of an enzymatic method, recently described for the rapid determination of β-lactam antibiotics in milk [7, 8], to the monitoring of benzylpenicillin cross-contamination in pharmaceuticals. The reagents for the method are available as a test kit marketed under the trade name Penzym®.

Experimental

Reagents

Penzym® kit (Imperial Biotechnology Ltd, London, UK under licence from UCB Bioproducts s.a., Belgium) contained the following reagents in four individual vials:

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D-Ala–D-Ala carboxypeptidase (reagent 1); $(Acetyl)_2$–L-Lys–D-Ala–D-Ala and chromogen (part 1) (reagent 2); chromogen (part 2) (reagent 3); peroxidase, flavin adenine dinucleotide and D-amino acid oxidase (reagent 4).

The reagents in vials 1, 2 and 3 were supplied in lyophilized form and reconstituted with 1 ml of distilled water. Reagent 4 was supplied as a suspension. Benzylpenicillin was from Sigma Chemical Company Ltd, Poole, UK.

Sample preparation

Tablets (surface contamination). Wash the surface with 2 ml of water, and centrifuge the washings if necessary.

Extraction of tablets. Extract the powdered tablet, or a portion of the tablet, with 2 ml of water and centrifuge.

Capsule (surface contamination). Wash the surface with 2 ml of water.

Capsules (contents). Extract the capsule contents, or a portion of the contents, with 2 ml of water and centrifuge if necessary.

Injections. No prior treatment is required.

Procedure

To 10 µl of reagent 1 add 50 µl of sample solution, mix and incubate at 47°C for 5 min. Add 10 µl each of reagents 2, 3 and 4 (or 30 µl of the pre-mixed reagents), mix and incubate at 47°C for 10 min. Observe the colour produced. Prepare a negative control by using 50 µl of an uncontaminated sample solution instead of the sample under investigation. Prepare a positive control by using a penicillin solution of 0.02 IU/ml (12 ng/ml) in place of water during the sample preparation or, for injections, dilute the sample with an equal volume of a penicillin solution of 0.04 IU/ml. The control solutions should be treated in the same way as the test solution.

Results and Discussion

The enzymatic technique makes use of the ability of β-lactam antibiotics to inactivate quantitatively a D-Ala–D-Ala carboxypeptidase enzyme (EC 3.4.17.8). If β-lactam antibiotics are not present in the test solution, the carboxypeptidase enzyme releases D-alanine from the substrate during incubation. D-Alanine then acts as a substrate for D-amino acid oxidase, releasing pyruvic acid and hydrogen peroxide. In the presence of hydrogen peroxide the colourless chromogen is oxidized by a peroxidase enzyme, when the oxidized form of the chromogen imparts a deep pink colour to the test solution (λ_{max} at 513 nm). If, however, the test solution contains 0.02 IU per ml or more of penicillin, the carboxypeptidase enzyme will be completely inhibited during the first incubation step. Neither D-alanine nor oxidized chromogen will be formed during the subsequent incubation step, and the final solution will be colourless.

With levels of penicillin below 0.02 IU per ml partial inhibition of the carboxypeptidase enzyme occurs, and the intensity of the pink colour will depend on the concentration of pencillin. Figure 1 illustrates the relationship between the colour of the

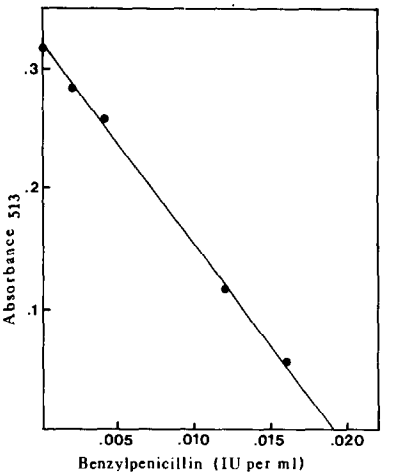

Figure 1
Plot of absorbance of benzylpenicillin standard solutions, after reaction with the Penzym® reagents, against concentration (absorbance at 513 nm and a path length of 1 mm). The intercept on the concentration axis indicates the amount of penicillin required to completely inhibit the carboxypeptidase enzyme under the test conditions employed. The regression equation is: $A = 0.321 - 16.7x$, where x is benzylpenicillin concentration (in IU/ml); the 95% confidence interval of the slope is ±1.2.

test solution (absorbance at 513 nm) and penicillin concentration for a series of penicillin standard solutions treated according to the procedure described. The theoretical background of the method and the kinetic parameters which govern the interaction between β-lactam antibiotics and carboxypeptidase have been fully described elsewhere [9].

In this study benzylpenicillin was added to a variety of pharmaceuticals at a level of 0.02 IU (12 ng) per ml of sample solution. Examples of products in which benzylpenicillin was successfully detected at this level are given in Table 1.

The Penzym® method was also used to test a pharmaceutical preparation which had previously been shown to have penicillin contamination by a microbiological (agar diffusion) technique [10]. The sample consisted of 25 batches of pemoline tablets, of which 12 had been shown to have penicillin contamination ranging from 40 to 190 ng per tablet. Re-examination of each batch by the Penzym® method* confirmed β-lactam contamination in the 12 contaminated batches, and also indicated slight contamination (about 10 ng per tablet) in a further two batches.

Table 1
Pharmaceuticals in which benzylpenicillin could be detected at 0.02 IU per ml of sample solution*

Capsules:	Chlordiazepoxide, Indomethacin, Quinalbarbitone
Injections:	Adenosine triphosphoric acid, Aminophylline, Atropine sulphate, Calcium chloride, Frusemide, Lignocaine hydrochloride, Neostigmine, Potassium chloride, Salbutamol, Sodium chloride
Tablets:	Aluminium hydroxide, Bendrofluazide, Codeine phosphate, Compound magnesium trisilicate, Co-trimoxazole, Diazepam, Diethylpropion hydrochloride, Digoxin, Ergometrine, Ethinyloestradiol, Frusemide, Glyceryl trinitrate, Nitrazepam, Ibuprofen, Pemoline, Phenobarbitone, Prednisolone, Propranolol, Thyroxine, Trimethoprim, Sulphaguanidine

* Prepared as described in text.

* This investigation was carried out using the version of the Penzym kit available in 1983–84.

Various chemicals can inhibit the amino acid oxidase reagent [11]. Inhibition of this enzyme prevents the formation of the chromogenic oxidized form of the chromogen and gives an apparent positive test result for β-lactams. For this reason it is necessary to check for interference in an uncontaminated sample of the same product as the test sample, if a positive test result occurs. On the other hand, potentiation of the amino acid oxidase may occur if amino acids are present in the sample, and penicillin contamination may then go undetected. Inclusion of a positive control will test for this possibility. However, once the validity of the Penzym® test for a particular pharmaceutical preparation has been established, it should not be necessary to include control samples with each subsequent assay.

In the experience of the present authors, the method has proved to be a rapid and sensitive screening test for the presence of β-lactam antibiotics in a wide range of products. It has been adopted in this laboratory as the primary method of detecting β-lactam antibiotics in pharmaceutical products when cross-contamination is suspected. Although intended as a qualitative test for the detection of β-lactam antibiotics, a semi-quantitative estimation of penicillin content can be made by adjusting the quantity of sample taken for the test. The technique can also be used to monitor cross-contamination in the pharmaceutical environment. Swabs taken of equipment and working surfaces can be readily tested for the presence of β-lactam antibiotics using this procedure.

References

[1] *The Federal Register*, title 21, part 211.58 (1976).
[2] P. J. O'Neil, *Proc. Anal. Div. Chem. Soc.* **11**, 30–32 (1974).
[3] E. M. May and G. V. Sefton, *Proc. Anal. Div. Chem. Soc.* **14**, 290–292 (1977).
[4] T. B. Vree, Y. A. Hekster, A. M. Baars and E. van der Kleijn, *J. Chromatogr.* **145**, 496–501 (1978).
[5] A. Alemanni and M. Rudmaan, *Chromatographia* **12**, 396–398 (1979).
[6] J. M. Wal and G. F. Bories, *Anal. Biochem.* **114**, 263–267 (1981).
[7] J. Degelaen, *Int. Labmate* **7**, 16–17 (1982).
[8] S. Thorogood and A. Ray, *J. Soc. Dairy Technol.* **37**, 38–40 (1984).
[9] J-M. Frere, D. Klein and J-M. Ghuysen, *Antimicrob. Agents Chemother.* **18**, 506–510 (1980).
[10] J. V. Bennet, J. L. Brodie, E. J. Benner and W. M. Kirby, *Appl. Microbiol.* **14**, 170–177 (1966).
[11] L. Hellerman, D. S. Coffey and A. H. Neims, *J. Biol. Chem.* **240**, 290–298 (1965).

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