Journal of Pharmaceutical & Biomedical Analysis
Vol. 3, No. 4, pp. 381–384, 1985
Printed in Great Britain

0731–7085/85 $3.00 + 0.00

Short Communication

Automated enzyme-inhibition assay for clavulanic acid in biological samples

BRIAN LEGG[1], JOHN G. N. CLARKE[3], BRIAN E. DAVIES* and GEOFFREY A. WADDS[2]

Drug Metabolism Unit, Beecham Pharmaceuticals Research Division, The Pinnacles, Harlow, Essex, UK

Keywords: *Clavulanic acid; β-lactamase inhibition; automated analysis.*

Introduction

Clavulanic acid is a β-lactamase inhibitor [1, 2] with low intrinsic antibiotic activity. The conventional cup–plate assay is unsuitable for its determination in biological fluids because of the low sensitivity to clavulanic acid of test organisms such as *Staphylococcus aureus* and *Bacillus subtilis*.

HPLC methods for the determination of clavulanic acid in plasma and urine have been reported [3–5], but these methods are unsuitable for the measurement of low nanogram concentrations of clavulanic acid which are encountered in single-dose pharmacokinetic studies.

A synergistic plate assay has been reported [6] in which bacterial growth is inhibited by penicillin G in regions where β-lactamase is inhibited by clavulanic acid. The measurement of residual penicillin G resulting from β-lactamase inhibition offers potential for a selective and sensitive chemical assay for clavulanic acid in biological fluids. Penicillin G is hydrolysed by β-lactamase and residual intact penicillin is measured chemically using the Bundgaard reaction [7]. If a β-lactamase inhibitor is introduced into the system, less penicillin G is hydrolysed resulting in a higher measured penicillin concentration. The measured increase in penicillin concentration is a function of the inhibitor concentration.

An autoanalyser method based on this principle has been developed for the determination of clavulanic acid in plasma and urine. The continuous-flow incubation manifold is combined with a penicillin autoanalyser (Wadds and Legg, unpublished observations) for measurement of the substrate concentration.

* To whom correspondence should be addressed.
[1] Present address: Department of Pharmacy, University of Manchester, UK.
[2] Present address: Britvic Ltd, Chelmsford, UK.
[3] Present address: Beecham Pharmaceuticals, Product Division, Irvine, UK.

Materials and Methods

Reagents

Penicillin G (0.58 mM) in isotonic saline (pH 6.5, 0.1 M citrate buffer) contained 0.05% Brij 35 (BDH Chemicals Ltd). R-factor mediated β-lactamase derived from *Escherichia coli* W3310 [8] (Microbiological Research Establishment, Porton, UK) in isotonic saline (pH 6.5, 0.1 M citrate buffer) contained 0.05% Brij 35. The wash solution and sample diluent were isotonic saline (pH 7.4, 0.05 M phosphate buffer) containing 0.05% Brij 35. The imidazole reagent contained imidazole (1.21 M, low UV absorbance, Hopkin & Williams Ltd) dissolved in mercuric chloride solution (0.1 mM, Fisons Ltd) with the pH adjusted to pH 6.8.

Apparatus

Standard autoanalyser glassware, transmission and pump tubing (Acculab) were used. The vertical action autosampler (CS40), dialyser bath with Cuprophane C membrane (Acculab) and the high temperature oil baths were obtained from Chemlab. Two variable speed peristaltic pumps (Gilson HP-8, Anachem Ltd) were used independently and the streams were monitored by two spectrophotometers (Cecil CE 212) set at 325 nm with a sensitivity of 0.5 a.u.f.s. Peak characteristics were improved using a dual channel differentiating and summating amplifier [9] and the outputs were displayed on a dual pen chart recorder (PM 8221, Philips).

Procedure

The autoanalyser configuration shown in Fig. 1 was used. The incubation and reaction baths both contained 20 ft coils (1.6 mm i.d.). The manifolds for the incubation loop and the penicillin autoanalyser loop were driven by separate pumps with pump speed potentiometer settings of 325 and 400, respectively. Manifold tubing sizes (internal diameter in inches) were as specified on the flow diagram; at pump potentiometer settings of 325 and 400, a manifold tube with i.d. 0.06 inch gave flows of 1.67 and 1.95 ml/min, respectively. Both spectrophotometers of the penicillin autoanalyser were backed-off to a base line of 10% FSD. Under these conditions, 95% of the initial content of penicillin G was inactivated before reaching spectrophotometer A; the residual level of penicillin G corresponded to zero inhibition of β-lactamase activity. The addition of clavulanic acid to the system led to a rise in the residual penicillin G substrate concentration as a consequence of inhibition of the β-lactamase. Changes in the residual substrate concentration resulting from the presence of varying concentrations of clavulanic acid in samples were observed as peaks on the chart recorder.

To overcome the problem of variable background interference from the biological samples a second spectrophotometer was incorporated. By this arrangement, the absorbance of the sample was measured directly after incubation and dialysis into the reagent stream, and then again after reaction had occurred between the penicillin and the reagent. The difference between the two spectrophotometer readings was directly proportional to the residual penicillin concentration in the stream so that the method accounted for variations in the background absorbance of the sample. The parameters of the autoanalyser were optimized to give maximum sensitivity of response consistent with good peak resolution and base-line stability.

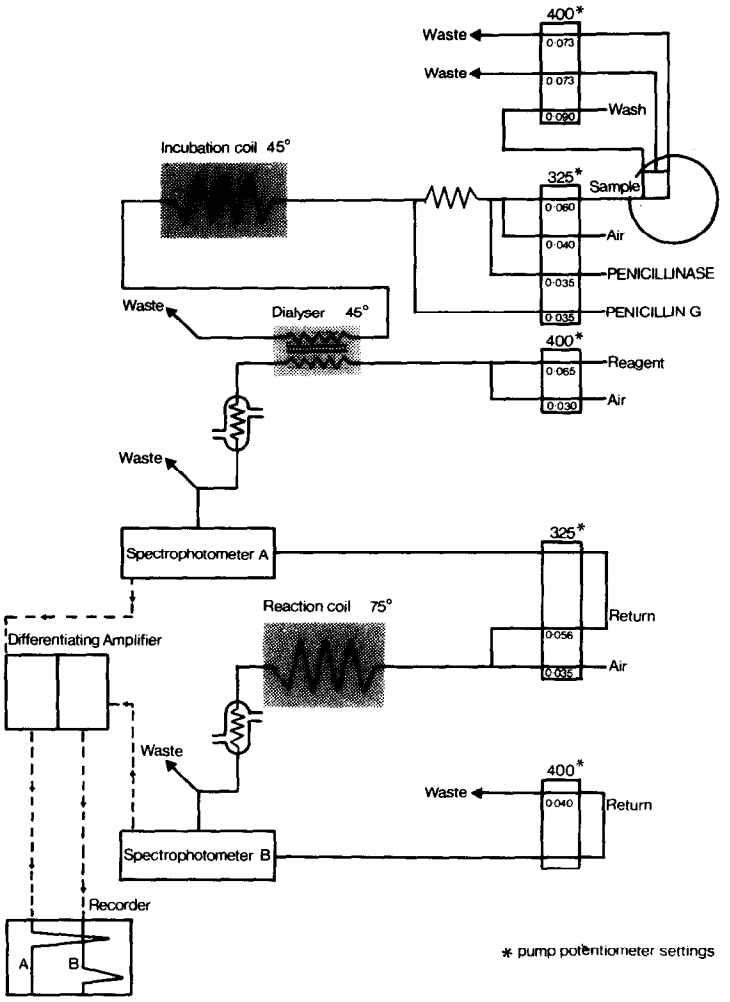

Figure 1
Flow diagram for automated determination of clavulanic acid in biological samples.

Results and Discussion

The response of the equipment was best for determination of concentrations of clavulanic acid in the range 0–2 μg/ml. The calibration curve of peak height difference against clavulanic acid concentration was non-linear; above 1 μg/ml large changes in clavulanic acid concentrations produced only small changes in peak height difference. Samples which were thought to contain less than 1 μg/ml of clavulanic acid were run undiluted and compared with standards prepared in blank plasma or urine. Samples which were anticipated to contain more than 1 μg/ml were diluted, as appropriate, with isotonic saline in pH 7.4 buffer and compared with standards diluted accordingly. Single calibration samples in the range 0–1 μg/ml were assayed and the unweighted calibration data obtained were fitted to a quadratic regression line using a 'least squares' routine.

The method demonstrated good within-run precision with a relative standard deviation of 2.5% and a lower limit of reliable determination of 26 ng/ml [10] for a typical calibration curve for the drug in (Fig. 2) human plasma. Advantages of the autoanalyser method over the reported HPLC methods are the minimal sample preparation necessary before analysis and the capability to assay 100 samples together with standards and quality control samples in a working day. The limit of reliable determination, which is consistently <50 ng/ml, is two-fold lower than that reported for the most sensitive HPLC procedure [5]. The method has been used successfully for some years to assay plasma and urine samples from toxicology studies and human volunteer studies, for the assay of dose solutions and for potency determinations on batches of radiolabelled material.

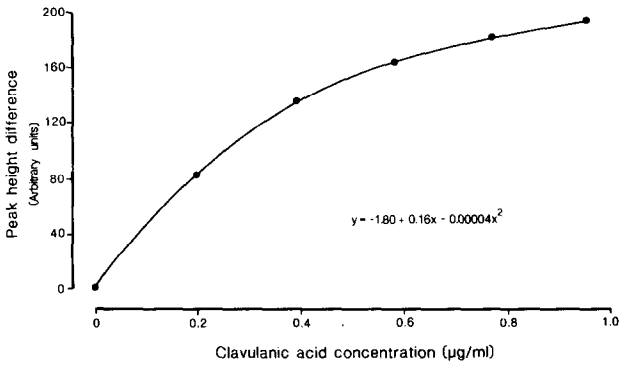

Figure 2
Typical calibration curve for clavulanic acid in human plasma.

In samples containing both penicillin and clavulanic acid where the ratio of penicillin to clavulanic acid is high, there would be interference by the penicillin in the clavulanic acid assay. Therefore, the method should not be used to assay samples obtained following administration of co-formulations of penicillin and clavulanic acid.

Acknowledgements: The authors thank Mrs J. Pennick and Mr J. Pratt for skilled technical assistance and Mr C. David for artwork.

References

[1] C. Reading and M. Cole, *Antimicrob. Agents Chemother.* **11**, 852–857 (1977).
[2] H. C. Neu and K. P. Fu, *Antimicrob. Agents Chemother.* **14**, 650–655 (1978).
[3] J. Haginaka, T. Nakagawa, T. Hoshino, Y. Yamaoka and T. Uno, *Chem. Pharm. Bull.* **29**, 3342–3349 (1981).
[4] J. Haginaka, T. Nakagawa, Y. Nishino and T. Uno, *J. Antibiot.* **34**, 1189–1194 (1981).
[5] M. Foulstone and C. Reading, *Antimicrob. Agents Chemother.* **22**, 753–762 (1982).
[6] A. G. Brown, D. Butterworth, M. Cole *et al.*, *J. Antibiot.* **29**, 668–669 (1976).
[7] H. Bundgaard and K. Ilver, *J. Pharm. Pharmacol.* **24**, 790 (1972).
[8] J. Melling and G. K. Scott, *Biochem. J.* **130**, 55–62 (1972).
[9] W. H. Walker, J. Townsend and P. M. Keane, *Clin. Chim. Acta* **36**, 119 (1972).
[10] C. I. Bliss, *The Statistics of Bioassay*. Academic Press, New York (1952).

[*First received for review 24 February 1984; revised manuscript received 10 May 1984*]