Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 10, pp. 927–931, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Stopped-flow photometric determination of clavulanic acid in pharmaceutical and serum samples

P. IZQUIERDO, A. GÓMEZ-HENS and D. PÉREZ-BENDITO*

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, E-14004, Córdoba, Spain

Abstract: The stopped-flow mixing technique was used to develop a simple, fast kinetic method for the determination of clavulanic acid by reaction with imidazole. Whereas the conventional method requires about 12–15 min for equilibrium to be reached, kinetic measurements can be made within a few seconds. The calibration graph was linear over the range 1–40 μg ml^{-1} of clavulanic acid and the detection limit achieved was 0.3 μg ml^{-1}. The precision and selectivity of the method are reported. The results obtained by applying the proposed method to the analysis of pharmaceutical and serum samples show how easily it can be adapted for routine analyses.

Keywords: *Clavulanic acid; stopped-flow; photometry; kinetics; automatic analysis; pharmaceutical and serum samples.*

Introduction

Clavulanic acid is a powerful inhibitor of β-lactamase enzymes, which protect microorganisms against β-lactam antibiotics such as penicillins and cephalosporins. Consequently it enhances the activity of these antibiotics against many resistant bacterial strains and it is commercially available in formulations containing amoxycillin.

Clavulanic acid in body fluids is usually determined by high-performance liquid chromatographic methods. Since clavulanic acid does not absorb above *ca* 225 nm, interference with direct UV detection is caused by other UV-absorbing components in the matrices; hence pre-column and post-column derivatization procedures have been developed to overcome this problem. Pre-column derivatization procedures involve pre-treatment including extraction with ethyl ether [1], deproteination with acetonitrile and addition of methylene chloride [2], or incubation with the reagent [3], all of which are time-consuming. Post-column derivatization procedures usually result in band broadening, dilution and noisy, drifting baselines which offset the sensitivity gains provided by derivatization. These drawbacks can be avoided by using a hollow-fibre post-column reactor suspended in an alkaline solution [4, 5] to convert clavulanic acid into a decay product which absorbs at 272 nm. Although the detection limits typically afforded by these chromatographic methods are very low, they feature poor throughputs and are difficult to adapt to routine determinations of clavulanic acid. Differential pulse polarography has also been used to determine clavulanic acid [6] in the presence of amoxycillin; however, no measurements can be made until *ca* 2 h after the samples have been prepared, which also hinders automation of the method.

The stopped-flow mixing technique has been used to develop a simple, fast kinetic photometric method for the determination of clavulanic acid in routine analyses. For this purpose various derivatization reagents have been used and imidazole was found to provide the best results. Imidazole is one of the most frequently used reagents for the direct photometric determination of clavulanic acid [7, 8] and for pre-column derivatization in liquid chromatography [1, 9]. The product formed absorbs intensely at 312 nm and has been identified as a mixture of isomers of 1-(4-aza-8-hydroxy-6-oxo)oct-2-en-1-oylimidazole [7]. The conventional equilibrium method [7, 8] is subject to two shortcomings which hinder its automation. Since the reaction product is unstable at pH 8, where the formation rate is very high, the method requires the use of a lower pH, where the time needed for equilibrium to be reached is about 12–15 min. On the other hand, the

* Author to whom correspondence should be addressed.

background absorbance of the samples and interfering substances requires absorbance measurements on three different blank solutions. As shown below, these shortcomings are avoided by kinetic methodology. In addition, the use of a simple stopped-flow module which can be readily coupled to any spectrophotometer facilitates automation of the method and application to routine analyses. A kinetic photometric method for clavulanic acid was described elsewhere [10] and was based on the ability of this analyte to inhibit the β-lactamase-facilitated degradation of penicillins. The measured parameter was the time required for the enzymatic degradation of penicillin G to penicilloic acid. The calibration graph of this photometric variable-time method was not linear, the time required for each assay was 1–2 h and no application to real samples was reported.

The lack of automatic methods for the determination of clavulanic acid in real samples led to development of a kinetic study of the imidazole–clavulanic acid system by using the stopped-flow mixing technique, which allows reactant handling to be minimized and analytical data to be obtained in only a few seconds. The method was validated by application to the determination of clavulanic acid in several pharmaceutical and serum samples, none of which required any pretreatment.

Experimental

Instrumentation

A Pye–Unicam 8625 UV–vis spectrophotometer fitted with a stopped-flow module [11] supplied by Quimi-Sur Instrumentation was used for reaction rate measurements. The module, furnished with an observation cell of 1-cm path length, was controlled by the associated electronics and a Netset microcomputer with a linear-regression program for application of the initial-rate method. The solutions in the stopped-flow module and the cell compartment were kept at a constant temperature (25°C) by circulating water from a thermostatically controlled tank.

Reagents

All chemicals were of analytical-reagent grade. Clavulanic acid (lithium salt) was kindly supplied by Beecham Pharmaceuticals. A stock solution was freshly prepared in distilled water before use; 2.4 M aqueous imidazole (Merck) was also used.

General procedure

One of the two 2-ml drive syringes of the stopped-flow module was filled with a previously prepared aqueous solution containing imidazole (1.2 M) and hydrochloric acid (0.1 M). The other syringe was filled with clavulanic acid standard or sample solution at a final concentration of 1–40 μg ml^{-1}. In each run, 0.15 ml of each solution was mixed in the mixing chamber. The variation of the absorbance at 312 nm was monitored throughout the reaction and data were processed by the microcomputer, with a linear regression program for application of the initial-rate method. The initial reaction rate was determined in about 15–20 s and each sample was assayed in triplicate. The blank signal was found to be negligible. All measurements were carried out at 25°C.

Determination of clavulanic acid in pharmaceuticals

No sample pretreatment was needed for these analyses other than appropriate dilution of the sample in order to obtain a concentration within the linear range of the calibration graph. An accurately weighed amount of sample (powder for suspension, *ca* 1.250 g) was transferred into a 1000-ml calibrated flask and diluted to volume with distilled water. The suspension was shaken for 5 min in an ultrasonic bath and then filtered. A measured volume of this solution was treated as described above.

Determination of clavulanic acid in serum

No sample pretreatment was required for these analyses. Each determination was carried out using 0.8 ml of human serum, spiked with an appropriate amount of clavulanic acid, which was diluted to 2 ml with distilled water and treated as described above.

Results and Discussion

The conventional method for the determination of clavulanic acid using imidazole as reagent [7, 8] involves calculating the net absorbance of the reaction product at 312 nm by subtracting the background absorbance of the reagent and the matrix when serum samples are analysed. Kinetic methodology

avoids this operation since only the rate of formation of the reaction product between clavulanic acid and imidazole is measured. Figure 1 shows the variation of the absorbance with time for solutions containing different concentrations of clavulanic acid at pH 8. The solution containing no clavulanic acid (curve 1) showed a low absorbance which remained constant, at least over the interval where the reaction rate was measured (15–20 s). It was confirmed that, when serum samples were analysed, the background absorbance of serum was higher but was constant while the reaction rate was being measured. These results show that kinetic measurements simplify and facilitate the determination of clavulanic acid since no sample blanks need to be prepared.

Optimization of variables

The variables affecting the performance of the proposed stopped-flow method for the determination of clavulanic acid were studied by the univariate method. All stated concentrations are initial concentrations in the syringes (twice the actual concentrations in the reaction mixture at time zero after mixing). Each kinetic result was the mean of three measurements.

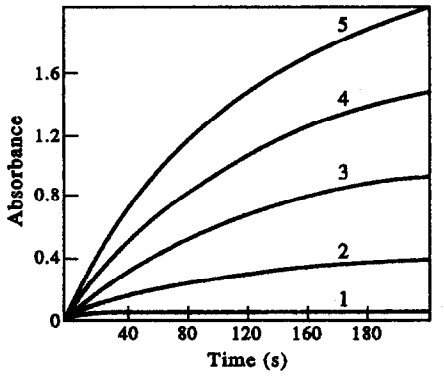

Figure 1
Kinetic curves obtained for the imidazole–clavulanic acid system. Clavulanic acid concentrations: (1) 0, (2) 5, (3) 15, (4) 25, (5) 35 μg ml^{-1} (λ = 312 nm). Imidazole conc. = 1.2 M, pH = 8, temp. = 25°C.

The effect of pH on the reaction rate of the imidazole–clavulanic acid system was studied in the range 6.7–10.0 by adding different volumes of 5 M hydrochloric acid to the syringe containing imidazole. As shown in Fig. 2(A), the reaction rate was maximal and independent of the pH over the range 7.8–8.3. In the equilibrium method developed by Bird *et al.* [7] a pH 6.8 is chosen because the product formed is slightly less stable at pH 8. Although the absorbances obtained at equilibrium at both pH values are similar, working at pH 6.8 entails waiting for about 12 min for equilibrium to be reached. This can be avoided by using kinetic methodology with the stopped-flow mixing technique since reaction-rate measurements can be made within a few seconds and no problem is posed by the instability of the reaction product. Thus pH 8 was chosen to implement the proposed kinetic method. At an adequate concentration, imidazole acts both as reagent and pH adjuster for the solution in the mixing chamber. The pH was also optimized by adjusting the pH of the solutions in both syringes with phosphate buffers of different pH values (6.75–9.9); the results were identical to those obtained in the absence of buffer.

Figure 2(B) shows the effect of the imidazole concentration on the reaction rate, which was not affected by this variable over the range 1–2 M imidazole. Temperature variations in the range 25–50°C had no significant effect on the reaction rate. The decreased dielectric constant of the solutions resulting from addition of ethanol was concomitant with a decrease in the reaction rate; the rate was virtually zero at an ethanol content of 40%.

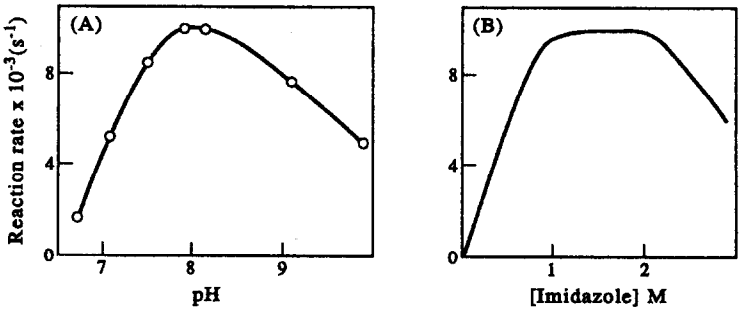

Figure 2
Effect of (A) pH and (B) imidazole concentration on the initial reaction rate.

Based on the slopes of the absorbance versus time curves obtained for solutions containing different amounts of clavulanic acid, the reaction is first-order with respect to this analyte. Under the working conditions used here, the reagents showed a pseudo-zero order dependence, for which the following kinetic equation is proposed: $v = k$[clavulanic acid], where v is the rate of formation of the product and k the conditional rate constant.

Features of the proposed method

The absorbance–time curves obtained at different concentrations of clavulanic acid under the optimum experimental conditions were processed by using the initial-rate method. The most salient feature of the proposed stopped-flow method is how rapidly the data required to determine the initial reaction rate for each kinetic curve can be acquired (15–20 s). The calibration graph was linear over the concentration range 1.0–40.0 μg ml^{-1}. The equation for the calibration graph is: $v = 5 \times 10^{-4}$ (s^{-1} μg^{-1} ml) [clavulanic acid] + 4×10^{-4} (s^{-1}), with a Pearson's correlation coefficient $r = 0.999$. The detection limit, as defined by IUPAC [12], was 0.3 μg ml^{-1}. The precision of the method was assessed at two concentrations of clavulanic acid, 2.5 and 10.0 μg ml^{-1} in aqueous solutions. The relative standard deviations (n = 10) were 2.1 and 1.3%, respectively. The precision was also evaluated by assaying replicates of two serum pools containing 6.25 and 25.0 μg ml^{-1} clavulanic acid, respectively. The within-day relative standard deviations (n = 10) were 2.2 and 2.1%, whereas the between-day relative standard deviations (n = 5) were 3.4 and 2.5%.

In order to study the selectivity of the proposed kinetic method, various antibiotics (penicillins, cephalosporins and tetracycline) were added to samples containing clavulanic acid. The tolerated limits found are listed in Table 1, which shows that none of the antibiotics interfered with the determination of clavulanic acid.

Table 1
Selectivity of the kinetic method proposed for the determination of clavulanic acid (5 μg ml^{-1})

Maximum tolerated conc. (μg ml^{-1})	Antibiotic
500*	amoxycillin, cephalexin, cephradine
375	cloxacillin, cephaloridin
250	ampicillin
125	dicloxacillin
10	tetracycline

*Maximum concentration tested.

Applications

In order to test its applicability, the proposed stopped-flow method was used to determine clavulanic acid in various commercially available pharmaceutical preparations and in human serum samples.

All the pharmaceutical samples assayed contained four times more amoxycillin than clavulanic acid. Table 2 summarizes the results. Recoveries were calculated by adding two different amounts of clavulanic acid standard to each sample suspension and subtracting the results obtained for similarly prepared unspiked drug samples. The recoveries obtained were between 92.0 and 106.0% (mean 100.2%).

Table 2
Determination of clavulanic acid in pharmaceutical preparations

	Clavulanic acid content (mg)		Recovery		
Sample*	Stated	Found†	Added (μg ml^{-1})	Found† (μg ml^{-1})	% Recovery
Clavumox (Antibioticos)	125	122.5	15	13.8	92.0
			30	30.6	102.0
Bigpen (Fides)	125	123.9	15	14.9	99.3
			30	30.3	101.0
Augmentinc (Beecham)	125	126.2	15	14.7	98.0
			30	29.4	98.0
Clavepen (Prodes)	125	123.7	15	15.3	102.0
			30	29.4	98.0
Burmicin (Cepa)	125	121.3	15	15.9	106.0
			30	31.8	106.0

*Trademark (manufacturer).
†Mean of three determinations.

Table 3
Recovery of clavulanic acid added to serum samples

Sample	Clavulanic acid ($\mu g\ ml^{-1}$) Added	Found*	% Recovery
1	10.0	9.7	97.0
	12.5	13.2	105.6
	15.0	15.3	102.0
2	10.0	10.2	102.0
	12.5	13.2	105.6
	15.0	15.2	101.3
3	10.0	10.2	102.0
	12.5	12.3	98.4
	15.0	14.8	98.7

*Mean of three determinations.

Three serum samples were spiked with different amounts of clavulanic acid and 0.8 ml of each was analysed as described above. Table 3 lists the concentrations and analytical recoveries obtained. The recoveries ranged from 97.0 to 105.6% (mean 101.4%). Although the absorbance at time zero after mixing of the reactants was relatively high owing to the background absorbance of serum at 312 nm, it was constant in the absence of clavulanic acid, so that no blank signal subtraction was required for kinetic measurements.

Conclusions

The results obtained by applying the stopped-flow mixing technique to the determination of clavulanic acid show the method to be a useful alternative to the automatic routine determination of this analyte in pharmaceutical and serum samples. The proposed method avoids preparation of any blank solutions of the reagent and also pretreatment of the samples, which simplifies the determination. Also, since dynamic measurements do not require that a stable final product be formed, pH values resulting in higher reaction rates than in the equilibrium method can be used. These are salient features of a very simple, fast method which allows analytical data to be obtained within a few seconds.

Acknowledgements — The authors are grateful to the CICyT for financial support (Grant No. PB91-0840) and to Beecham Pharmaceuticals for providing a lithium clavulanate sample.

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[Received for review 23 December 1992; revised manuscript received 8 March 1993]