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A fast and simple method for the determination of clavulanic acid in human plasma using derivatisation reaction kinetics

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Abstract

A stopped-flow mixing technique is used to determine clavulanic acid in human plasma after plasma deproteinisation with acetonitrile and removal of the organic solvent by extraction with dichloromethane. The reaction kinetic profiles for the reaction between clavulanic acid and imidazole are determined by measuring the absorbance at 312 nm of the imidazole derivative. The reaction rates are proportional to the concentration of the clavulanic acid and by plotting reaction rates against clavulanic acid concentrations linear calibration curves could be constructed over the range 0.30–10.0 μg/ml.

Keywords: Clavulanic acid; Plasma; Reaction kinetics

1. Introduction

Clavulanic acid (3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid) is a powerful inhibitor of β-lactamase enzymes, which protect micro-organisms against β-lactam antibiotics such as penicillins and cephalosporins. Consequently it enhances the activity of these antibiotics against many resistant bacterial strains and is most often formulated in combination with antibiotics such as amoxycillin [1].

Clavulanic acid is reported not to be stable for more than 3–4 h in solution after extraction that places a limit on the number of samples that can be analysed per day. The clavulanic acid molecule is also not stable in plasma and samples can be stored at −80°C for up to 3 months only [2]. Since the clavulanic acid molecule is so labile, correct storage and handling of samples are essential for accurate results [3]. A number of HPLC methods for the determination of clavulanic acid have been reported including precolumn derivati-

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sation [4,5] after ultrafiltration of plasma [6], derivatisation followed by solid phase extraction [7], ultrafiltration of plasma samples [3] followed by postcolumn alkaline degradation of clavulanic acid [8–11] and ion-pairing technology [2,12]. The instability of clavulanic acid in the injection medium and long chromatography times made a high sample throughput by HPLC impossible.

The challenge was to develop an alternative assay method that was sensitive enough, and fast enough that the stability of the analyte was ensured during analysis of batches of large numbers of plasma samples. A stopped-flow mixing technique that requires no sample pretreatment [1] was investigated. Although the method is fast and data can be obtained within a few seconds, we needed more sensitivity that could be acquired by sample pretreatment. Two HPLC methods reported [2,5] do not lend themselves to the automation required for multiple sample analysis due to long chromatography times. An adaptation of the sample preparation method could however be used. This paper describes a simple and fast method for the determination of clavulanic acid in human plasma using a combination of published methods [1,2,5].

Clavulanic acid is very water soluble and therefore difficult to extract from plasma. Sample preparation was accomplished by deproteinisation of the plasma with acetonitrile and removal of the acetonitrile by extraction with dichloromethane [2,5]. A stopped-flow mixing technique in which imidazole produces a reaction product with clavulanic acid that has a chromophore with strong absorbance at 312 nm, was used [1]. The reaction product is unstable at high pH values where the formation rate is high, but since dynamic measurements do not require that a stable final product be formed, pH values resulting in higher reaction rates could be used. Analytical data was obtained within minutes, which allowed for large numbers of samples to be analysed in batch mode. The method was successfully validated and applied for the determination of clavulanic acid in thousands of plasma samples.

2. Experimental

2.1. Reagents and materials

Clavulanic acid manufactured by Gist-Brocades AB, Sweden was obtained as the 1:1 combination of the potassium salt and Avicel containing 417.3 μg free acid per mg. Imidazole (Fluka, Buchs, Switzerland) and 32% hydrochloric acid (Merck, Darmstadt, Germany) were used without further purification. Acetonitrile and dichloromethane (Burdick & Jackson, High Purity) were obtained from Baxter, USA. All water used was purified by RO 20SA reverse osmosis system and Milli-Q® polishing system (Millipore, Bedford, MA, USA)

2.2. Instrumentation

A Cobas Fara II (Hoffmann–La Roche & Co., Switzerland), centrifugal analyser equipped with a 30 sample, temperature controlled (37°C) rotor fitted with disposable 30 sample circular cuvettes was used to process the samples by spectrophotometric monitoring of absorbance at 312 nm. All recording of data was done on the internal printer of the Cobas Fara II.

2.3. Preparation of calibration standards

Solutions of clavulanic acid were prepared in water, and calibration standards were prepared daily by spiking 0.5 ml aliquots of drug free plasma, spanning a concentration range of 0.30–10.0 μg/ml. Quality control standards, spanning the same range, were made using the same methodology, and used to verify the intra-day and inter-day assay method performance. These spiking solutions were kept for a maximum of 10 days and were stored at approximately 4°C.

2.4. Determination of clavulanic acid in plasma

To 0.5 ml plasma in a 1.2 ml microfuge tube was added 0.5 ml acetonitrile. The sample was vortexed for 15 s and centrifuged at $6000 \times g$ for 5 min. The supernatant was transferred to a 5-ml ampoule and the acetonitrile removed by extraction with 3 ml dichloromethane. The sample was

centrifuged at $500 \times g$ for 5 min, the aqueous supernatant transferred to a sample tube and the tube placed in the centrifugal analyser.

Supernatant (190 μl) from the sample tube is automatically transferred by the multi-access pipettor of the COBAS FARA II to the sample compartment of the rotor cuvette, while 200 μl of the imidazole derivatising reagent (1.2 M imidazole in 0.1 M HCl) is transferred to the reagent position. The rotor is accelerated and mixing occurs by centrifugal force and braking. The reaction kinetic profiles for the reaction between clavulanic acid and imidazole are determined by measuring the absorbance at 312 nm, 15 s after mixing and then every 5 s for a total of 12 readings.

The rates of change in absorbance for the different standard concentrations processed are plotted against the relevant concentrations to form a calibration curve and the concentrations of the unknown samples interpolated from this calibration curve.

2.5. Validation

The method was validated by analysing plasma quality control samples in fivefold at five different concentrations to determine the accuracy and precision of the method. The calibration curve was found to be linear (weighted 1/concentration^2) over the concentration range 0.30–10.0 μg/ml.

Specificity was determined by analysing drug free plasma from five different sources. These assays were inspected for reactions that could cause interference with the reaction of the analyte or in the measurement of absorbance.

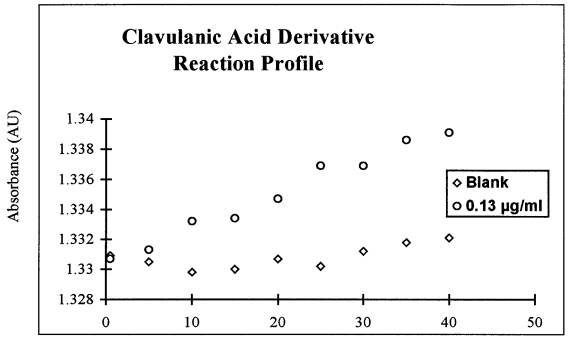

Fig. 1. Representative reaction profiles demonstrating the specificity and sensitivity of the assay method.

Absolute recovery of the analyte was determined in triplicate at three different plasma concentrations. Recoveries were calculated by comparison of the mean rate of change in absorbance for the extracted plasma samples with that of aqueous solutions representing 100% recovery.

3. Results and discussion

The mean absolute recoveries of analyte determined in triplicate at 0.30, 1.00 and 3.00 μg/ml were 111% (R.S.D. = 10.4%), 98% (R.S.D. = 9.6%) and 90% (R.S.D. = 2.1%) respectively.

Although the imidazole does react with endogenous plasma components, the difference in reaction rates between blank plasma and a spiked plasma sample at low concentration (0.13 μg/ml) could readily be distinguished (Fig. 1). The specificity with regards to possible clavulanic acid metabolites and antibiotics was not investigated. It has been reported that various antibiotics did not interfere with the selectivity of the kinetic method for the determination of clavulanic acid [1].

The intra-day accuracy and precision of the assay procedure were assessed and results from the intra-day validation assays indicated a valid calibration range of 0.30–10.0 μg/ml using a linear regression curve (weighted 1/concentration^2) with a $r^2 = 0.9919$. Fig. 2 represents examples of clavulanic acid derivative reaction profiles at three different concentrations of clavulanic acid. Fig. 3 is a representative calibration curve constructed from the rates of change in absorbance as calculated by the COBAS FARA II. The lower limit of quantification (LLOQ) was set at 0.30 μg/ml, at the concentration of the lowest acceptable calibration standard. For the assignment of a valid calibration range bias is taken as measure of accuracy and coefficient of variation (CV%) is taken as measure of precision. Table 1 shows the quality control data obtained during the validation of the method.

This method has been successfully applied to the analysis of plasma samples generated during six clinical bioavailability studies (a total of ap-

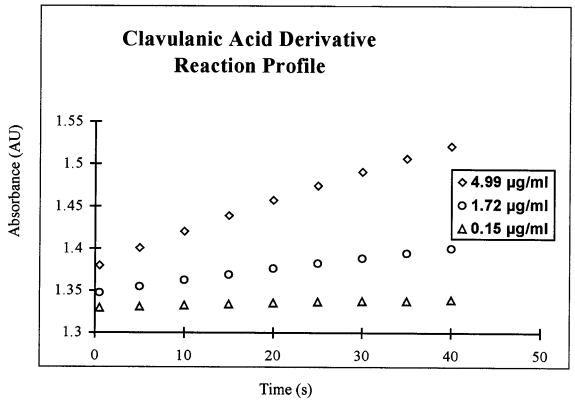

Fig. 2. Representative reaction profiles.

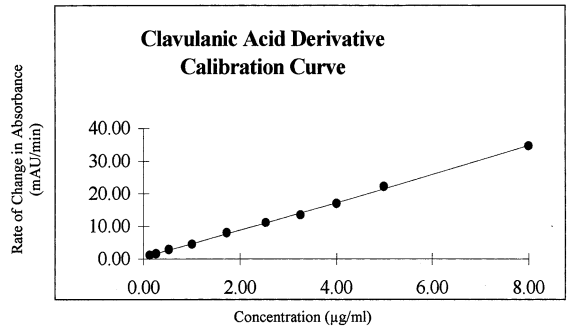

Fig. 3. Representative calibration curve.

proximately 8000 samples) over a period of 4 years. Due to the instability of the analyte in plasma and the imidazole derivative, the study samples were analysed immediately after collection and preferably within 24 h after collection. Samples were assayed in small batches (up to 30 samples per batch) for a total of up to 140 samples per day including calibration standards and quality controls. The small batches ensured that the plasma extracts were on instrument for a maximum of 20 min before measurement. The total processing time per batch of 30 samples, from the thawing of the plasma samples up to the printing of the reaction kinetics by the instrument took approximately 1 h. No sample was on the instrument for more than 20 min. A comparable batch of samples processed by HPLC means that the difference of the on-instrument time between sample 1 and 30 could be 3 h. Table 2 represents the accumulated inter-day quality control data for six studies performed using this assay procedure over a period of 4 years (note that calibration standards and quality control standards were not always made up to exactly the same concentrations over the six studies, thus the nominal concentrations are given as ranges). A linear regression curve (weighted 1/concentration^2) was used throughout and the relative standard deviations of the calibration curve slope for the calibration curves constructed over a period of 4 years (R.S.D. = 8.3%, mean: 3.7993, range: 3.2876–4.4112, $N = 63$) and intercept (R.S.D. = 63.6%, mean: 0.3660, range: −0.0794–0.7885, $N = 63$) reflect the inter-day reproducibility of the assay method. These data are considered to give a more

Table 2
Inter-day accuracy and precision of plasma quality controls

Nominal concentration range (µg/ml)	Mean %nom	Mean CV (%)	*N*
0.300–0.361	99.7	16.1	122
0.450–0.601	99.0	12.3	122
0.900–0.910	100.5	7.7	106
2.00–2.25	99.0	5.6	188
4.00–4.50	99.6	4.1	187

Table 1
Intra-day accuracy and precision of plasma quality controls

Nominal concentration (µg/ml)	Mean concentration found (µg/ml)	CV (%)	*N*
4.50	4.37	1.6	5
2.25	2.15	3.4	5
0.900	0.861	9.5	5
0.600	0.532	8.3	5
0.359	0.365	14.0	3

objective reflection of the assay performance and robustness under clinical study conditions than the validation data alone.

4. Conclusion

This method for the determination of clavulanic acid in plasma is sensitive and rapid enough to ensure the analysis of a large number of plasma samples generated during bioavailability studies in the shortest possible time, thus overcoming the constraints of the analyte's instability.

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