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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CLAVULANIC ACID IN HUMAN SERUM

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A convenient, rapid and sensitive method by UV detection has been developed for routine analysis of clavulanic acid in human serum. The method involved a pre-column derivatization with imidazole and precipitation of serum protein with 0.6M perchloric acid. The analysis utilized a Nucleosil 100-5 C_{18} 5 μm column (125x4 mm i.d.) together with a mobile phase consisting of acetate buffer–methanol-acetonitrile (91:5:4, v/v/v). The eluent was adjusted to pH 3.0 with glacial acetic acid and pumped isocratically at ambient temperature with a flow rate of 1.0 ml min^{-1}. the UV detection was set at 311 nm. The retention time of analyse was 2.4 min and the minimum detectable amount was. 0.1 μg ml^{-1} of serum.

The intra and inter-day coefficients of variation for the concentrations 0.3 to 3.5 μg.ml^{-1} ranged from 2.09-3.82% and 2.84-6.39%, respectively. The response of the assay over the concentration range stated was linear with a correlation coefficient (r) = 0.9998 ± 3.12. Stability tests showed that clavulanic acid was stable in serum for at least four weeks at –70 ± 5°C.

The method was validated and applied successfully in a bioassay study of two drugs (a test versus a reference) administered orally to 24 healthy male subjects.

Keywords:*Augmentin®; Clavulanic acid; Imidazole; HPLC; Human serum*

Introduction

Clavulanic acid (CA) is a natural penicillic acid with the formula of $C_8H_9NO_5$, know as 3–(2-hydroxyethylidene) –7-oxo-4-oxa-1-azabicyclo [3, 2, 0] heptane –2-carboxylic acid, Fig. 1 (1-4).

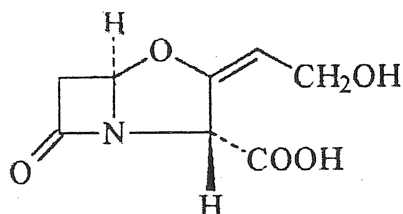

Fig. 1 Clavulanic acid

CA is a potent inhibitor of β-lactamases, and is used in combination with synthetic penicillin antibiotics to protect them against hydrolysis and hence extend their antibacterial activities (2-4). Augmentin® containing amoxicillin trihydrate an: potassium clavulanate is a broad spectrum bactericidal antibiotic prescribed for the treatment of urinary and respiratory tract infections.

Drug level determination is increasingly significant and represents a demanding analytical challenge particularly in clinical and pharmacokinetic studies. In this context, several methods have been described to determine CA in various matrices. Among those reported are biochemical (5), microbiological (6), spectrophotometric (4,7) and fluorometric (8)

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methods. Liquid chromatographic methods that employ pre or post-column reactions with imidazole or 1,2,4-triazole and mercuric chloride have also been reported (9-17). However, these methods have some disadvantages, as some of them are time consuming (10,16), or subject to limitations on sample throughout (13-15), others were developed for in vitro use (5,6,12,16) or demanded special additives or detectors such as diode array or MS which are not normally or commonly available (10,12,16,17). Such kind of limitations put restrictions to its use or adoption for routine analysis. It was therefore deemed relevant to develop and validate a new method to enhance to quality of routine analysis in terms of efficiency, sensitivity, reproducibility and also to shorten the time of analysis. Moreover, to utilise it in a bioassay study of two products of clavulanic acid administered orally to 24 healthy male volunteers.

Materials and Methods

CA, Augmenting® and Amoclane Forte drug powders were kindly supplied by Hikma Pharmaceuticals (Jordan). The drugs were labelled to contain 500 mg amoxicillin trihydrate and 125 mg potassium clavulanate per 10 ml of the reconstituted powder. Acetonitrile and methanol were HPLC grade (Merck, Germany). All other chemicals and reagents used were of analytical grade. Water was glass distilled or deionized.

Imidazole derivatization reagent was prepared at a final concentration of 15 mM in deionized water and adjusted to pH 6.8 with 5M HCl.

Apparatus and chromatographic conditions: Chromatographic analyses were performed using Shimadzu-Japan HPLC system which consisted of a solvent delivery system (LC-10AD pump), a UV-visible spectrophotometric detector (SPD-10A), a communication bus module (CBM-10A) and a manual injector (Rheodyne). The column used was Nucleosil 100-5 C_{18} 5 µm (124x4 mm id), Macherey – Nafel, Germany. The mobile phase consisted of acetonitrile, methanol, 4 mM sodium acetate (4:5:91%, v/v/v) and was adjusted to pH 3.0 with glacial acetic acid. It was also degassed daily by filtration through 0.45 µm membrane filter (Gelman Sciences, Michigan- USA), and pumped isocratically at a flow rate of 1.0 ml min^{-1} at ambient temperature. CA xas monitored by UV set at 311 nm (0.016 AUFS).

Preparation and derivatization of standards: Two working solutions of potassium clavulanate at concentrations of 40 $µg.ml^{-1}$ (Solution A), and 80 $µg.ml^{-1}$ (Solution B) in deionized water were prepared from a stock of 1 mg.ml. Aliquots of 0 (no drug), 12,24,60,120 µl (Solution A) and 120,180 and 300 µl (Solution B) were separately added to a total of 4.8 ml of drug-free serum, giving a series of eight standards at concentrations of 0 (blank), 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 $µg.ml^{-1}$ 400 µl of each standard was then transferred to a 1.5 ml disposable polyethylene microcentrifuge tube and stored at –70 ± 5°C until assayed.

Procedure and derivatization: Prior to analysis, thawed samples of the standards in serum were treated with 100 µl of imidazole derivatizing reagent, vortex-mixed for 30 sec and left at ambient temperature for five minutes. 100 µl of 0.6M perchloric acid was then added. The mixture was vortexed for 30 sec and centrifuged at 13000 rpm for 1 min, allowing serum protein to precipitate. Finally, portions of 50 µl of in replicates of eight from the supernatant of each standard were injected directly into the HPLC system. Peak area of the chromatograms were recorded and plotted versus their respective concentrations. The method of the external calibration graph and/or linear regression equation was followed to estimate the unknown concentrations of the analysed samples.

Eventually, and prior to its application in a bioassay study, the proposed method was validated for linearity, efficiency and precision-accuracy as follows:

Linearity: CA standards in serum at seven concentrations over the range of 0.10 to 5 $µg.ml^{-1}$ were analysed in eight replicates. Seven calibration graphs were constructed and studied by the method of least square analysis. The mean correlation coefficient, slope and intercept were estimated. The sensitivity of detection was assayed as the minimum detectable amount that could be measured without interference from the base line noise.

Precision and accuracy: The precision of the method was evaluated using eight replicate analyses of the pooled serum samples containing CA at three different levels, from 0.30 to 3.50 $µg.ml^{-1}$.

The intra and inter-day runs were deduced and presented as coefficients of variation (CV). Accuracy was estimated as percent bias [100 (measured conc.-added conc.)/Added conc.] of the measured quantity from the initial added amount (Table 1).

Table 1. Intra and inter-day precision and accuracy of the HPLC assay of CV

	Intra-day runs		Inter-day runs	
Added $\mu g.ml^{-1}$	Measured* $\mu g.ml^{-1}$	%Bias	Measured* $\mu g.ml^{-1}$	%Bias
0.300	0.314	+4.76	0.313	+4.33
SD	0.012		0.020	
CV%	3.82		6.39	
1.500	1.548	+0.32	1.545	+0.30
SD	0.037		0.079	
CV%	2.39		5.11	
3.500	3.589	+2.54	3.560	+1.71
SD	0.075		0.101	
CV%	2.09		2.84	

*Mean values of eight serum replicates for each concentration

Recovery: To determine the overall recovery of CA from serum by this method known concentrations of CA were mixed with drug free serum and worked up as previously described. Eight replicate analyses of three different concentrations (0.3, 1.5, 3.5 $\mu g.ml^{-1}$) were performed separately in serum and the mobile phase. Peak areas were compared and the mean absolute recovery was estimated.

Stability: Stability of CA in serum from 0.3 to 3.5 $\mu g.ml^{-1}$ has been studied over four weeks through five freeze-thaw cycles (-70 ± 5°C to room temp.). In each cycle, samples were removed from the freezer and left to thaw on the bench under room lightning for half an hour prior to its analysis (Table 2).

Table 2. Freeze-thaw stability of CA

Added ($\mu g.ml^{-1}$)	0.30	1.50	3.50
Found ($\mu g.ml^{-1}$)	0.33	1.63	3.67
	0.31	1.58	3.63
	0.31	1.55	3.56
	0.28	1.43	3.42
	0.27	1.41	3.40
Mean*	0.30	1.52	3.54
SD±	0.024	0.096	0.122
CV%	8.00	6.32	3.45

Applicability: As part of a bioassay study to determine and compare the bioavailability of two products, a test (Amoclane Forte) was produced by Hikma Pharmaceuticals (Jordan), against a reference (Augmentin®) produced by Beecham Pharmaceuticals Research (UK). Each in the form of a suspension containing a mixture of amoxicillin trihydrate (500 mg) and potassium clavulanate (125 mg) per 10 ml. Twenty four healthy male subjects were participated in the study. Non of the subjects had any medical abnormality as revealed from their medical history, clinical and lab investigations. Each volunteer was given a single oral dose of (10 ml) suspension containing 125 mg potassium clavulanate in two treatment days, separated by a 7 day wash out interval. The order of drug administration was done according to a randomised crossover design following an overnight fasting.

Blood samples (5 ml) were withdrawn from forearm veins of the subjects into vaccutainers at 0 (pre-dose) and at various intervals afterwards over eight hours (post-dose). Serum was separated following centrifugation and stored immediately at –70°C until assayed.

The bioavailability was deduced by comparing the extent of drug absorption of the two products as revealed from the profiles of the mean serum concentration versus time (Fig. 3).

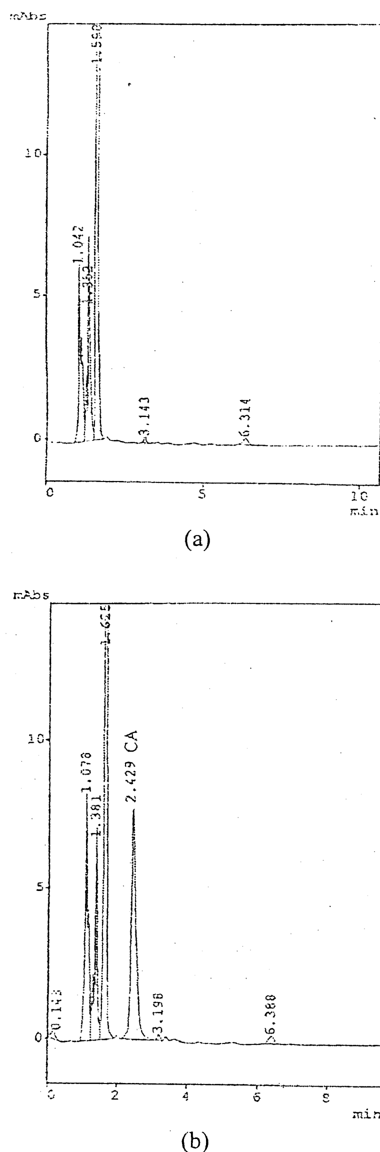
Results and Discussion

CA belongs to the penicillic acid group, which has no specific chromophore; accordingly direct UV-measurement of the acid is not suitable, as potential interference from endogenous components prom serum would be inevitable. However, Bird et al (7) and Kenig (4) reported that derivatization of CA with imidazole resulted in β- lactam cleavage of the drug and formation of a product which absorbs radiation strongly at 311 nm. Accordingly, this was taken as the bases for CA quantitation in this method. Derivatization conditions were chosen as optimal in terms of imidazole and drug concentrations, incubation time, pH and temperature. This was achieved by incubating CA in serum at levels corresponded to those encountered in patient samples, with imidazole at a final concentration of 2.5 mM (pH 6.8) for 5 min at ambient

temperature. The pH of the mobile phase was adjusted to be slightly acidic in order to enhance the solubility of the CA derivative and lower its retention capacity. The validity of the proposed method was established through confirmatory studies of linearity, sensitivity, recovery and precision–accuracy assay.

Representative chromatograms of blank serum and serum sample containing CA (1 μg ml^{-1}) are shown in Fig. 2; from where it can be seen a good resolution of CA at 2.429 min without any interference from serum components. The linearity of the method was assayed on the bases of regression analysis. The plots were linear over the range of 0.10 to 5.0 μg ml^{-1}, $y=(86.121 \pm 0.876)\,x + (0.0574 \pm 0.341)$ and a correlation coefficient (n=56) $r=0.9998 \pm 0.0002$. The minimum detectable amount under the condition employed was 0.1 μg ml^{-1} serum. The intra and inter day coefficients of variation of serum samples containing CA from 0.30 to 3.50 μg ml^{-1} were evaluated and were found to range from 2.09 –3.82% and 2.84 – 6.39%, respectively (Table 1). Recovery determinations were found to have a high value with an average of 95.79% ± 3.12. clavulanic acid in serum at –70 ± 5°C was found to be stable for not less than four weeks (Table 2).

Eventually, the applicability of the method for routine analysis has been evaluated and found to be very convenient, as hundreds of serum samples containing CA were analysed successfully. The bioassay of the two products (Amoclane Forte and Augmentin®) was assayed by comparing the extent of drug absorption of both as indicated by the mean serum concentration-time profiles Fig. 3. Examination of respective profiles revealed that there is no significant difference in the extent of absorption of the two products.



(a)

(b)

Fig. 2. Liquid chromatogram of (a) drug-free serum, (b) serum sample spiked with clavulanic–acid (1.00 μg/ml)

In view of the stated results and findings, it seems reasonable to conclude to the exceptional value of the proposed HPLC method for the determination of clavulanic acid in human serum. The method is very suitable for routine analysis with large numbers of samples. Yet, it can be adopted for various clinical and pharmacokinetic studies where precision,

sensitivity, time and cost effectiveness of analytical methods are important.

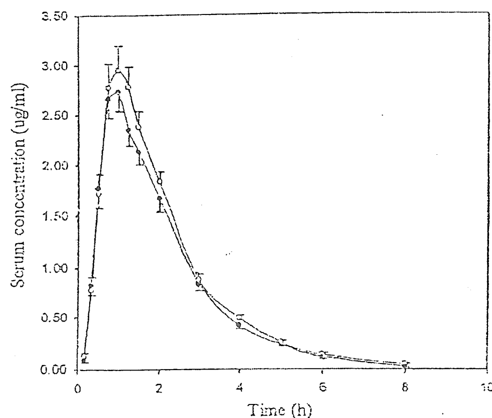


Fig. 3. Mean serum concentration-time profiles (linear scale) of clavulanic acid for 24 healthy male subjects following oral administration of *Amoclan Forte* (Test-black circles) versus augmentin® (Reference-open circles) suspensions. Each containing 500 mg of amoxicillin and 125 mcg of –clavulanic acid. Vertical bars represent the standard error of the mean

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