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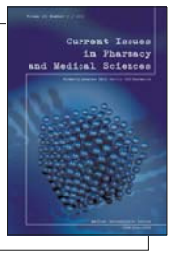

Theoretical and experimental analytical studies on potassium clavulanate

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ABSTRACT

The spectral and chromatographic procedures (FT-IR, D-UV, TLC, DSC, HPLC-DAD) were developed as alternative tools, to these which are recommended by pharmacopeial guidelines, for identification of potassium clavulanate. As a support for the studies quantum chemical calculations based on the density functional theory (DFT) were used. For a determination of potassium clavulanate in the presence of degradation products two analytical methods were determined: HPLC-DAD and derivative spectroscopy. In the HPLC-DAD method, C-18 stationary phase and 12 mM ammonium acetate-acetonitrile (96:4 *V/V*) were used. A quantitative determination of potassium clavulanate was carried out by using PDA detector at 220 nm, with a flow rate of 1.0 mL min^{-1}. In derivative spectroscopy the change of values of amplitudes of potassium clavulanate and the first-derivative absorption spectra were used (λ=228 nm) for its determination.

Keywords: potassium clavulanate, identification, determination, FT-IR, D-UV, DSC,TLC, HPLC-DAD

INTRODUCTION

Clavulanic acid is a bicyclic β-lactam inhibitor (Fig. 1), which shows inhibition of many â-lactamase enzymes, which are responsible for the resistance of some bacteria to â-lactam antibiotics [15]. Clavulanic acid is more stable than some β-lactam antibiotics. Moreover it is stable against gastric acids, what results in improvement of antibacterial effect of β-lactam antibiotics co-administered together with it and in overcoming the bacterial resistance [11,18]. The penam analog-clavulanic acid combination is frequently used as the antibacterial preparation for the treatment of many infections caused by G(+) and G(-) bacteria [1,2]. Moreover, recent studies have reported that the combination of clavulanic acid with meropenem demonstrates high antimycobacterial activity against extensively drug-resistant *Mycobacterium tuberculosis* strains [5,7,8].

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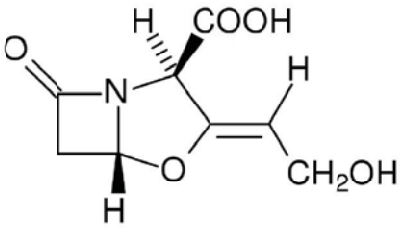

Fig. 1. Chemical structure of clavulanic acid

Research reports devoted to analytical studies on clavulanic acid, similarly to pharmacopeial guidelines (FP IX, EP X and USP 25) [14,3,17], focus on the application of chromatographic procedure in its identification. The determination of clavulanic acid in biological matrix is based on the application of an HPLC method [10,16]. The literature sources provide HPLC methods and derivative spectroscopy, as the recommended ones for determination of clavulanic acid in pharmaceutical matrix, in the presence other active pharmaceutical substances [4,12,13].

The aim of the work was two-fold: to develop short and inexpensive analytical methods for identification (UV, FT-IR, DSC, TLC, HPLC) and determine potassium cla-

vulanate (D-UV, HPLC-DAD) in the presence of degradation products.

MATERIAL AND APPARATUS

Potassium clavulanate, diluted was obtained from CHEMOS, Germany. It is white or light yellow crystalline powder containing 50% potassium clavulanate, diluted, 50% silicon dioxide (<2% impurities).

All other chemicals and solvents were obtained from Merck KGaA (Germany) and were of analytical grade. High quality pure water was prepared by using the Millipore purification system (Millipore, Molsheim, France, model Exil SA 67120).

The ultraviolet measurements were performed by using an UV-VIS Lambda 20 spectrophotometer equipped with 1.0 cm-in-width quarts cells and controlled by the UV WinLab software. The infrared spectrometric measurements were developed by using an FT-IR Bruker Equinox 55 spectrometer equipped with a Bruker Hyperion 1000 microscope. The different scanning calorimetry was performed on a Mettler Toledo 822e. In a TLC method, the detection of potassium clavulanate spots was performed by UV 254 nm Spectroline ENF-260C/F lamp. A silica gel $G_{60}F_{254}$ (Merck, Darmstadt, Germany) was used as the stationary phase. The chromatographic determinations were performed using a high performance liquid chromatograph containing a Shimadzu pump, model LC-6A, an UV-VIS detector SPD-6AV (Shimadzu), a Rheodyne 7120 with a 50 μL loop.

EXPERIMNETAL AND THEORETICAL STUDIES

Derivative spectroscopy (*D-UV*). The D-UV method was developed for identification studies and determination of concentration changes of potassium clavulanate in the presence of related products, including degradation products. The first-derivative of adsorption spectra ($\Delta A/\Delta\lambda$) with $\Delta\lambda$ = 4 nm and a scaling factor = 10 were obtained. The amplitudes of the first-derivative spectra of potassium clavulanate were measured at 228 nm. The position of maxima of the first derivative spectra of solution of potassium clavulanate (200 μg mL^{-1}) were used for its identification. The changes in values of the first-derivative spectra were based on the difference between maximum of amplitude and the base line. The method was validated with regards to selectivity, linearity, precision, accuracy, according to International Conference on Harmonization Guidelines [9]. Detection limit of and quantitative limit were calculated. The selectivity of determination was examined for the determination of potassium clavulanate in the presence of degradation products formed during stress conditions of hydrolysis (acid, base) at 323K, oxidation (H_2O_2) and thermal degradation (323K). Precision of the assay was determined in relation to repeatability (intra-day) and intermediate precision (inter-day). In order to evaluate the repeatability of the methods, six samples were determined during the same day for three concentrations of potassium clavulanate. Intermediate precision was studied comparing the assays performed on two different days. The accuracy of the method was determined by recovering potassium clavulanate from the placebo. The recovery test was performed at three levels 80%, 100%, and 120% of the nominal concentration of potassium clavulanate during degradation studies. Three samples were prepared for each recovery level. The solutions were analyzed and the percentage of recoveries was calculated from the calibration curves. The LOD and LOQ parameters were determined from the regression equation for potassium clavulanate: LOD = 3.3 S_y/a, LOQ = 10 S_y/a; where S_y is a standard error and a is the slope of the corresponding calibration curve.

Infrared spectroscopy (*FT-IR*). The vibrational infrared spectra of potassium clavulanate were recorded between 400 and 7000 cm^{-1} in polycrystalline powder, at room temperature. A combination of experimental and theoretical studies was performed for analyzing the vibrational FT-IR spectra of potassium clavulanate. Intensity and positions of bands for potassium clavulanate were estimated. By employing the theoretical approach, it was possible to eliminate necessity of using reference standards.

Different scaning calorimetry (DSC). The analysis was carried out for about 2.5 mg of dry samples of potassium clavulanate directly weighed on the platinum pans at a heating rate of 10°C min^{-1}. The measurements were performed under nitrogen continuous flow (100 mL min^{-1}) in the temperature range between 0 and 350°C.

Thin layer chromatography. Potassium clavulanate solutions were prepared by dissolving 10 mg in methanol to get the concentration of 1.0 mg mL^{-1}.The identification of potassium clavulanate was performed by using a mobile phase consisting of 90 volumes of acetonitrile and 10 volumes of water. As the stationary phase, silica gel TLC plates were used. The wavelength of the DAD detector was set at 254 nm. A ninhidrin reagent was used as indicator of spots of potassium clavulanate.

High performance liquid chromatography. The determination of potassium clavulanate in the presence of its degradation products was possible when a Lichrospher RP-18 column, 5 μm particle size, 250 mm × 4 mm (Merck, Darmstadt, Germany) was used **as** the stationary phase. The mobile phase consisted of 4 volumes of acetonitrile and 96 volumes of ammonium acetate, 12 mmol L^{-1}. The flow rate of the mobile phase was 1.2 mL min^{-1}. The wavelength of the DAD detector was set at 220 nm. The method was validated with regard to selectivity, line-

arity, precision, accuracy. Limit of detection and quantitative limit were calculated. The procedure of preparation of samples of potassium clavulanate and validation studies was the same as the one used during determination of potassium clavulanate by derivative spectroscopy [9].

Theoretical studies. In order to interpret the experimental results of vibrational spectroscopy, quantum chemical calculations were also performed. The molecular geometry of clavulanic acid was optimized by means of a density functional theory (DFT) method with the B3LYP hybrid functional and 6-31G(d,p) basis set. All the calculations were made using the Gaussian 03 package [6].

RESULTS AND DISCUSSION

For identification of potassium clavulanate, the following analytical metods were developed: D-UV, FT-IR, TLC, DSC, HPLC.

As the first analytical method for identification of potassium clavulanate, the first-derivative spectrophotometry of absorptive spectra was proposed. The first-derivative spectra of potassium clavulanate are shown in Fig 2. First-derivatives spectra have characteristic shapes of curves and maxima of amplitude at λ=228 nm.

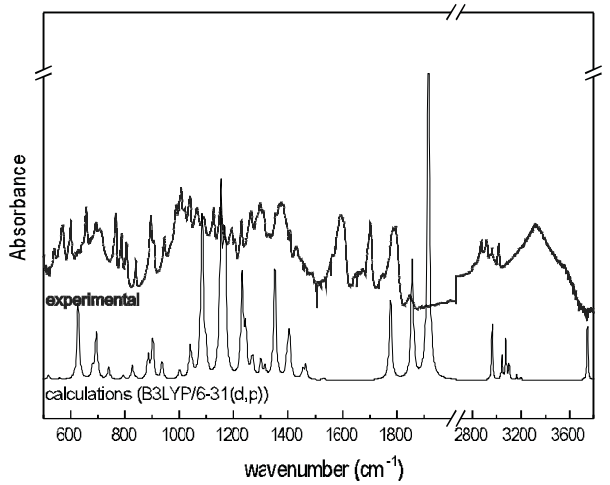

Fig. 2. Experimental and calculated FT-IR spectra of potassium clavulanate

For identification studies of potassium clavulanate by using FT-IR spectra, the normal mode assignment for acid is based on the comparison of calculated and experimental IR absorption spectra (Fig. 3). Calculated spectra correspond quite well with the spectra obtained in the experimental way. Band position shifts and the disappearance of some of them in the theoretical spectra are the results of the fact that the calculation was made on the isolated molecules within the harmonic approximation while the real molecule is not isolated and vibrates anharmonically. The most characteristic bands were associated with vibrations of bands occurring in 4:5 bicyclic ring and 2-hydroxyethylidene substituent. These positions of bands and their intensities can be used during identification of doripenem studies. The most important bands are also collected in Table 1.

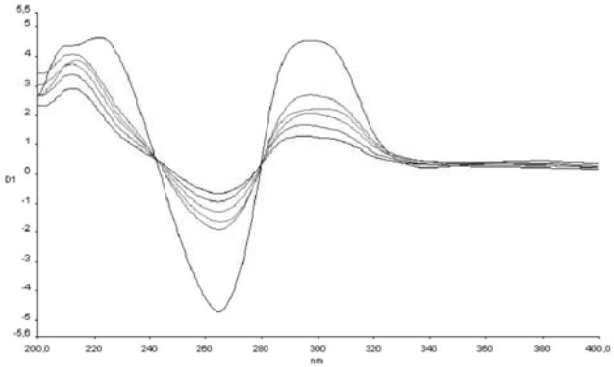

Fig. 3. The first-derivative spectrum of potassium clavulanate (c = 200 μg mL^{-1})

Table 1. Main characteristic of vibrational modes of acid before and after degradation from experimental and calculated spectra

Wavenumber (cm^{-1})		Band assignment	Wavenumber (cm^{-1})		Band assignment
Theor. data	Exper. data		Theor. data	Exper. data	
628	658	O-H b in COOH group + C-N-C b in penem ring	1351	1370	C-N s in penem ring + C-H w/b
740	766	C-C s between penem ring and COOH group + CH2 t at penem + breathing penem ring	1777	1593	C=C s
885	896	C-C s + C-O s in penem ring + C-C-C b in penem ring and COOH group	1857	1702	C=O s in COOH group
936	944	C-N s in penem ring + C-C s between penem ring and COOH group	1916	1793	C=O s at penem ring
1040	1040	C-O s in acetaldehyde group + C-O-C b in penem ring	2961	2870	C-H s in acetaldeh yde group
1084	1091	C-C s in acetaldehyde group + C-O s in acetaldehyde group + CH2 t at penem ring	3042	2911	C-H s in acetaldeh yde group
1153	1151	C-O s in COOH group + CH2 t	3074	2961	C-H s at penem ring
1164	1151	C-N s in penem ring + C-C s in penem ring + CH2 t + O-H b	3101	3015	C-H s at penem ring
1230	1229	C-N s in penem ring + CH2 w/t			

Vibrational modes: s – stretching, b – bending, w – wagging, t – twisting.

The DSC studies were applied to characterize the potassium clavulanate considering its behaviour under the influence of heat factor. The thermogravimetric curves for the potassium clavulanate, obtained with a heating rate of 10°C min^{-1}in nitrile atmosphere showed the possible to verify three degradation processes at 190°C and 210°C (Fig. 4).

The identification of potassium clavulanate in dilutions was studied by using a TLC method. The identification of spot of potassium clavulanate was performed on silica gel TLC plates with acetonitrile and water as the mobile phase. Under developed conditions, it was possible to ob-

serve the spots of potassium clavulanate diluted at R_f = 0.6, similar to the standard reference (Fig. 5).

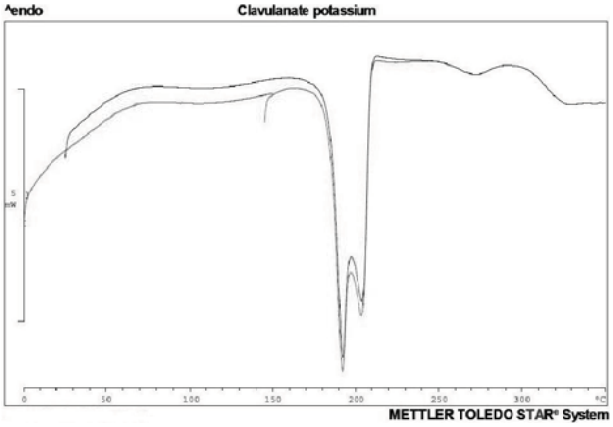

Fig. 4. Derivative thermogravimetric curves of potassium clavulanate

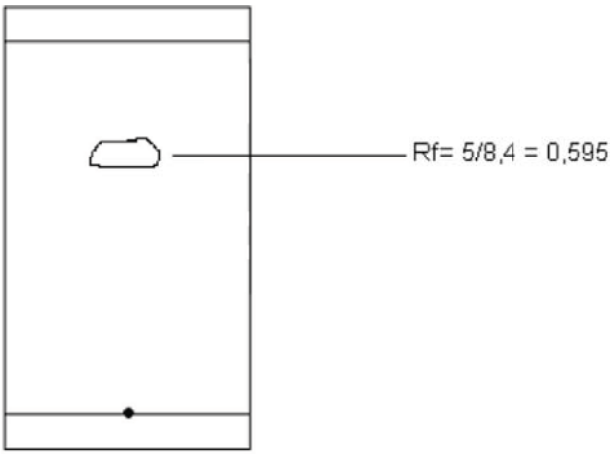

Fig. 5. The chromatograms of TLC of potassium clavulanate (c = 5.0 mg mL^{-1})

The basis of application of the HPLC method for studies of identity of potassium clavulanate was the comparison of its peak elution time with the time of reference standard (t_R=3.8 min). The determination of potassium clavulanate was obtained in the results of application of a Lichrospher RP-18 column (5 µm, 250 mm × 4 mm) as stationary phase and acetonitrile and of ammonium acetate (12 mmol L^{-1}) as the mobile phase mixture.

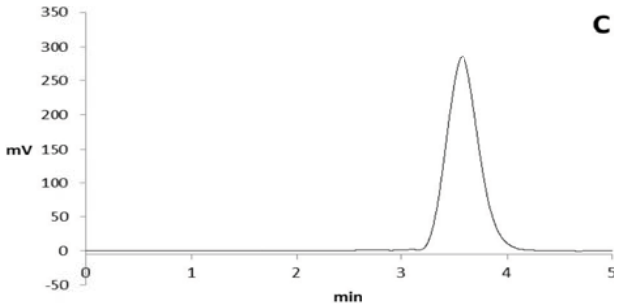

Fig. 6. The chromatograms of HPLC of potassium clavulanate C (c=500 µg mL^{-1})

The wavelength of the DAD detector of potassium clavulanate was set at 220 nm (Fig. 6).

Table 2. Validation parameters for determination of potassium clavulanate by using d-UV and HPLC-DAD

Validation parameter	D-UV method	HPLC-DAD method
Linearity	18.8–156.2 µg mL^{-1} (n = 23, r = 0.9990)	(0.50–60) 10^2 µg mL^{-1} (n = 23, r = 0.9990)
Level of concentration	Intra-day precision, RDS %	
80% 100% 120%	25.0 [µg mL^{-1}], 1.21% 62.5 [µg mL^{-1}], 0.24% 100.0 [µg mL^{-1}], 0.16%	400.0 [µg mL^{-1}], 0.81% 500.0 [µg mL^{-1}], 2.02% 600.0 [µg mL^{-1}], 1.77%
Level of concentration		
100.0 [µg mL^{-1}] 100%	0.6975 0.3870 0.2525	500.0 [µg mL^{-1}] 100% 2.3361
Recovery studies	RDS %	
25.0 [µg mL^{-1}] 80% 62.5 [µg mL^{-1}] 100% 100.0 [µg mL^{-1}] 120%		400.0 [µg mL^{-1}] 80% 0.6732 500.0 [µg mL^{-1}] 100% 1.0145 600.0 [µg mL^{-1}] 120% 0. 6839
LOD [µg mL^{-1}]	6.064 [µg mL^{-1}]	28.841[µg mL^{-1}]
LOQ [µg mL^{-1}]	18.321 [µg mL^{-1}]	87.397[µg mL^{-1}]

For determination of potassium clavulanate, the following analytical methods were developed: D-UV and HPLC-DAD. Both methods were validated according to International Conference on Harmonization Guidelines [9]. The validation parameters (range of linearity, precision, accuracy, LOD, LOQ) were collected in Table I. Selectivity of determinations of D-UV method was studied by carrying out determination of potassium clavulanate in the presence of degradation products formed during acidic, basic hydrolysis, oxidation, and hemolysis in solid state. The determination of potassium clavulanate by using HPLC-DAD was checked by evaluation of spetrophotometric purity peak of potassium clavulanate in degraded samples.

CONCLUSIONS

The different analytical techniques were developed to identify and determine potassium clavulanate in dilutions. In comparable to recommended previously analytical procedures based on the application of HPLC method, they meet additional requirements and improve some criteria: short time of analysis, inexpensive analytical solutions and greener approach to pharmaceutical analysis. The application of theoretical standard reference substance is the new solution permitting elimination of using reference standard during analytical studies.

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