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Research Article

Preparation and Physicochemical Characterization of Amoxicillin β-cyclodextrin Complexes

Catherine Bisson-Boutelliez,[1] Stephane Fontanay,[1,2] Chantal Finance,[1,2] and Francine Kedzierewicz[1,2,3]

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Abstract. Amoxicillin (AMOX), a penicillin A, belongs to the β-lactam family It is usually the drug of choice within the class because it is better absorbed, following oral administration, than other β-lactam antibiotics. Its β-lactamase degradation might be prevented by using a molecular [AMOX:β-CD] complex. The aim of this work was to prepare complexes using two methods and then characterize interactions between AMOX and the native β-CD. The extent of complexation in solution has been evaluated by high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and 2D rotating-frame Overhauser enhancement spectroscopy (2D ROESY). Mass changes (TG), calorimetric effects (DSC), and mass spectrometry (MS) were determined on the same sample under identical conditions using the Skimmer coupling system. Skimmer and infrared spectroscopy (FT-IR) were used to characterize the solid state of the binary system. Complexation of AMOX with β-CD was proven by FT-IR, NMR, DSC, and HPLC. The 2D ROESY spectra did not show any dipolar proton interaction of the AMOX with cyclodextrin. The 1:1 stoichiometry of the complex was obtained by HPLC. The stability constant for AMOX with β-CD was determined to be 1,878 M^{-1}. In the [AMOX:β-CD] complex, the phenyl group is included inside the β-CD, and the ionized carboxyl group on the penam ring forms hydrogen bonds with the secondary hydroxyl groups of another β-CD to keep the complex stable. Preparation methods allowed exactly the same complex.

KEY WORDS: amoxicillin; complex; β-cyclodextrin; DSC-TG-SM; FT-IR; HPLC stability constant; NMR.

INTRODUCTION

Amoxicillin (AMOX), a penicillin A, belongs to the β-lactam family and is often used for either prophylactic (1) or curative anti-biotherapy. Because of its wide spectrum, this antimicrobial agent is frequently used for the treatment of oral, digestive infections, and Lyme disease (2–4). Resistance of Gram-positive and -negative bacteria to β-lactam antibiotics is mostly associated with the production of β-lactamases and sometimes with decreased transport across the bacterial cell wall (5–9). Thus, aminopenicillins are used in combination with nitro-imidazole or clarithromycin, which may lead to an increase in the selection pressure on both pathogenic bacteria and other bacteria belonging to the commensal flora and may consequently induce the selection of multi-resistant strains (10–12). It has been suggested that the degradation of AMOX might be prevented using a molecular complexation with β-cyclodextrin (β-CD), which might therefore represent an interesting alternative therapy.

Cyclodextrins belong to a family of three well-known, industrially produced, major cyclic oligosaccharides and several minor, rare ones. The three most used cyclodextrins are built up from six, seven, and eight glucopyranose units known as α-, β-, and γ-CD, respectively. Cyclodextrins can be represented as toroïds, with the larger and the smaller openings of the toroïd exposing to the solvent secondary and primary hydroxyl groups, respectively. The interior of the cavity is less hydrophilic than the aqueous environment and thus able to host other hydrophobic molecules. In contrast, the exterior of the cavity is hydrophilic (13).

They are potential candidates for drug carriers (14–17) because of their ability to alter physicochemical and biological properties of guest molecules through the formulation of inclusion complexes. Natural cyclodextrins (α, β, and γ) have been widely used for complexation because of their principal advantages as drug carriers, namely the availability of cyclodextrins of different sizes, low toxicity, and low pharmacological activity and the protection of the included drug molecules from biodegradation. Recently, the existence of a specific transport system for cyclodextrins in the bacterial cell wall seems to have been revealed (cym transport system), initially evidenced in *Klebsiella oxytoca* (18,19). Apparently α- and β-cyclodextrins are taken up as intact entities via the components of this transport system; thus, the transport of antibiotics could be improved if they are complexed with cyclodextrin.

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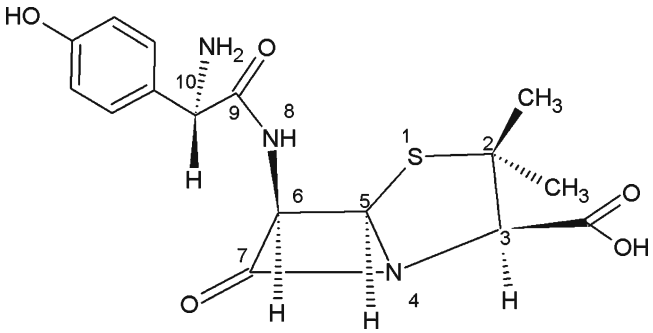

Fig. 1. Amoxicillin

Considering the above, cyclodextrins may be important for the protection of the β-lactam molecule from biodegradation, with all reserve of host–guest complex formation. Rare examples of AMOX studies by ^{1}H nuclear magnetic resonance (^{1}H NMR) and some other works concerning ampicillin complexation are reported in the literature (20–22).

The aim of the present study was to investigate an [AMOX:β-CD] complex in order to explore more deeply the physicochemical interactions between AMOX and β-cyclodextrin. The extent of complexation in solution has been evaluated by high-performance liquid chromatography (HPLC) and NMR. Infrared spectroscopy (FT-IR), thermogravimetry (TG), and differential scanning calorimetry (DSC) coupled with mass spectrometry (MS) were used to characterize the solid state of all binary systems.

MATERIALS AND METHODS

Materials

Amoxicillin anhydride (AMOX) is the 6-[D(−)-αamino-phydroxyphenyl acetamido] penicillanic acid (Fig. 1) (23). It was purchased from Sigma-Aldrich. (Saint Quentin Fallavier, France) and used as received. The native β-CD was given by Wacker-Chemie GmbH (Lyon, France). The deuterium oxide (D_2O, deuterium content 99.9%) was purchased from Eurisotop (Gif-sur-Yvette, France). All solutions were prepared using deionized water. Other chemicals and solvents were of analytical grade or HPLC grade and were used as received.

Methods

Preparation of the Complexes

The [AMOX:β-CD] complexes were obtained according to the conditions described by Higuchi and Connors (24). AMOX and β-CD molecules were dissolved in deionized water at different molar ratios: [AMOX:β-CD], 1:0.25, 1:0.5, 1:1, 1:3, 1:5, with a final pH of 5. The solutions were:

- In method 1, equilibrated for 24 h at 37°C under stirring at 250 rpm with a Certomat®M apparatus (B. Braun Biotech, Plaisance du Touch, France)
- In method 2, sonicated for 20 min before equilibration 24 h at 37°C under stirring at 250 rpm

In the two cases, solutions were then freeze-dried before the experiments.

Complex Characterisation

Fourier Transform Infrared

FT-IR spectra were obtained with a Bruker Vector 22 spectrophotometer (Wissembourg, France). They were registered in potassium bromide pellets (1%, *w*/*w*) between 4,000 and 450 cm^{-1}.

Nuclear Magnetic Resonance

β-CD and AMOX powders were dissolved in deuterated water or deuterium oxide (D_2O) at the different molar ratios. ^{1}H-NMR spectroscopic experiments were performed at 300 K

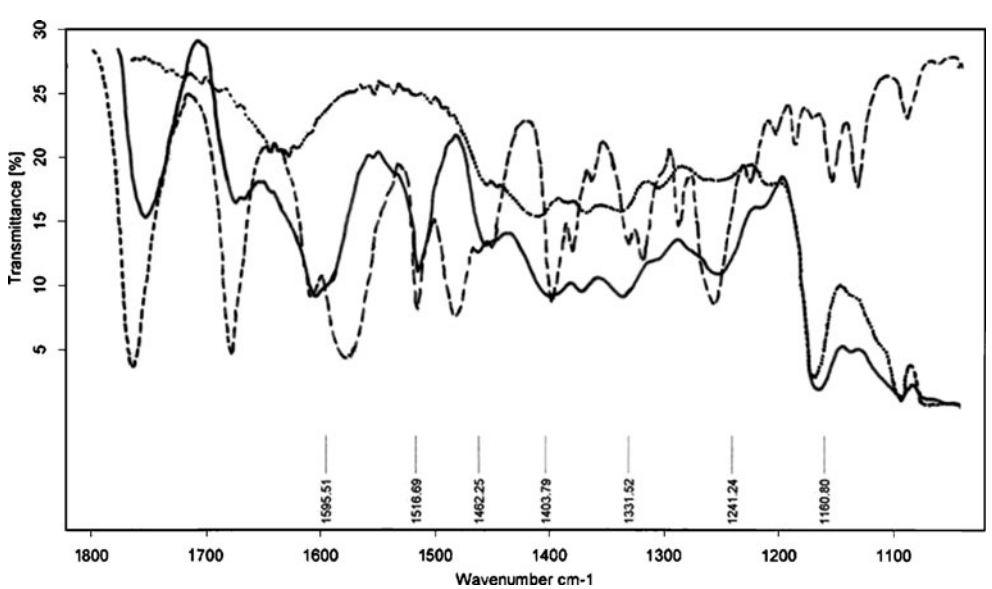

Fig. 2. FT-IR spectrum of amoxicillin (*dashed line*), β-CD (*dotted line*), and amoxicillin–β-CD (*solid line*) binary system of molar ratio 1:1 in the region 1,100–1,800 cm^{-1}

Table I. Assignment of Relevant IR Absorption Bands of Amoxicillin and Amoxicillin–β-CD Binary System

Assignment	AMOX	Complex: method 1 (cm^{-1})					Complex: method 2 (cm^{-1})				
	Pure (cm^{-1})	1:0.25	1:0.5	1:1.0	1:3.0	1:5.0	1:0.25	1:0.5	1:1.0	1:3.0	1:5.0
$\nu_{C=O}$ (β-lactamic)	1776	1777	1770	1769			1770	1770	1769		
$\nu_{C=O}$ (amide)	1,687	1,689	1,691	1,691	1,686		1,692	1,691	1,691	1,686	
C=C (aromatic)	1,616	1,616	1,614	1,614	1,613		1,615	1,614	1,614	1,613	
ν_{asCOO^-}	1,582	1,595	1,596	1,597			1,595	1,596	1,597		
δ_{NH} (amide)	1,519	1,517	1,517	1,517	1,516	1,515	1,516	1,517	1,517	1,516	1,515
ν_{sCOO^-}	1,397	1,401	1,403	1,404	1,406	1,405	1,403	1,403	1,404	1,406	1,405

on a Bruker DRX-400 spectrophotometer (Wissembourg, France). Induced changes in the chemical shifts for AMOX ($\Delta\delta$) by complexation were calculated using the following equation:

$$\Delta\delta = \delta_{complex} - \delta_{free}. \quad (1)$$

Spatial connectivity between cyclodextrin and drug was established by rotating-frame Overhauser enhancement spectroscopy (2D ROESY) experiments. Spectra were acquired with number of scans = 32, number of dummy scans = 16, recycle delay = 1.5 s, acquisition size 1k×512, processing size 2k×2k, and mixing time 400 ms (25).

Thermogravimetry, Differential Scanning Calorimetry, and Mass Spectrometry

Mass changes (TG), calorimetric effects (DSC), and MS were determined on the same sample under identical conditions using the Skimmer Coupling System (Netzsch, Gerätebau, Germany). This system combines the simultaneous thermal analysis instruments (STA 409C) for TG and DSC and quadrupole mass spectrometer for detection and analysis of the reaction gases. All instruments were calibrated before use.

The thermal behavior was studied by heating 5 mg of the sample in an aluminum open crucible in argon atmosphere (flow, 50 mL min^{-1}) with a heating rate of 10 K min^{-1} over the temperature range 30–350°C.

Determination of Stability Constant by HPLC

HPLC method was used to determine the stability constant between AMOX and native β-CD. HPLC experiments were carried out using a Thermo-Finnigan (San Jose, CA, USA) liquid chromatographic system equipped with a vacuum degasser SCM1000 and a narrow-bore quaternary Spectra System P1000XR gradient pump with a loop of 20 μL. A stainless steel column Hyperbond C18 (300 length×3.9 mm i.d., 10 μm particle size) Shandon was used and thermostated at 25°C with a column temperature controller 560-CIL (Cluzeau Info Labo, Puteaux-la-Defense, France). The effluents were monitored with a double-beam spectrophotometric detector (Spectra System UV1000) at 228 nm.

The mobile phase was constituted of increasing concentrations of β-CD (0, 0.15, 0.75, 1.5, 3.0, and 4.5 mM) dissolved in an aqueous monobasic potassium phosphate buffer (0.2 M, pH 5). It was then filtered through a Millipore membrane (0.45 μm). The flow rate of eluent was set at 0.5 mL min^{-1}. The retention times of drug final concentration (15×10^{-6} M) in the absence and in the presence of excess amounts of CDs in the mobile phase were measured.

The stability constant (K_C) was determined by the retention method as described by Uekama *et al.* (26):

$$\frac{(CD)_m}{(T_0' - T_{obs})} = \frac{1}{(T_0' - T_c)}(CD)_m + \frac{1}{K_c(T_0' - T_c)} \quad (2)$$

Table II. Induced Changes in the Amoxicillin ($\Delta\delta$) Chemical Shifts for Binary Systems Obtained by Method 1

Assignment	$\Delta\delta = \delta_{complex} - \delta_{free}$ (Hz)				
	1:0.25	1:0.5	1:1	1:3	1:5
Aromatic	0.000	−0.001	−0.004	−0.011	−0.015
Aromatic	−0.002	−0.002	−0.005	−0.011	−0.016
Aromatic	−0.001	−0.003	−0.005	−0.011	−0.015
Aromatic	−0.001	−0.002	−0.006	−0.011	−0.016
β-lactam	0.005	−0.004	−0.015	−0.036	−0.055
β-lactam	−0.004	−0.006	−0.017	−0.037	−0.054
β-lactam	0.004	0.006	0.016	0.033	0.049
β-lactam	−0.006	–	0.013	0.030	0.046
10H	0.001	0.002	0.000	0.000	0.000
3H thyazol	0.002	0.004	0.003	0.003	0.004
Methyl (thyazolidic)	0.000	−0.002	−0.004	−0.010	−0.015
Methyl (thyazolidic)	0.003	0.003	0.009	0.018	0.023

Table III. Induced Changes in the Amoxicillin ($\Delta\delta$) Chemical Shifts for Binary Systems Obtained by Method 2

	$\Delta\delta = \delta_{complex} - \delta_{free}$ (Hz)				
Assignment	1:0.25	1:0.5	1:1	1:3	1:5
Aromatic	−0.005	−0.001	−0.007	−0.013	−0.018
Aromatic	−0.004	−0.002	−0.007	−0.013	−0.018
Aromatic	−0.005	−0.003	−0.008	−0.014	−0.019
Aromatic	−0.005	−0.002	−0.008	−0.013	−0.019
β-lactam	−0.007	−0.004	−0.018	−0.038	
β-lactam	−0.007	−0.006	−0.018	−0.038	−0.057
β-lactam	0.001	0.006	0.013	0.028	0.044
β-lactam	0.000	–	0.011	0.027	
10H	−0.004	0.002	−0.002	−0.004	−0.003
3H thyazol	−0.001	0.004	0.001	0.000	0.001
Methyl (thyazolidic)	−0.005	−0.002	−0.006	−0.011	−0.017
Methyl (thyazolidic)	−0.001	0.003	0.006	0.015	0.021

where $(CD)_m$ was the concentration of CD in the mobile phase and T_0', T_c, and T_{obs} were retention times of AMOX, of [AMOX:CD] complex, and of AMOX at a given concentration of CD, respectively. A plot of the left hand term *versus* $(CD)_m$ gave both the K_c and T_c values from the linear relationship by slope on intercept.

RESULTS AND DISCUSSION

FT-IR Results

The complexation between AMOX and β-CD can be inferred by comparing the IR spectra of free AMOX, AMOX-β-CD physical mixture and [AMOX:β-CD] complex. FT-IR is not suitable for the detection of inclusion compounds if the resulting spectra present a superposition of host and guest bands. Fortunately, AMOX exhibits some characteristic IR absorption bands in the spectra region where β-CD has a weak one, making this region (1,775–1,240 cm^{-1} in this case) suitable for detecting host–guest interactions. The FT-IR spectra in this region of AMOX and AMOX-β-CD binary system, of molar ratio 1:1, are shown in Fig. 2.

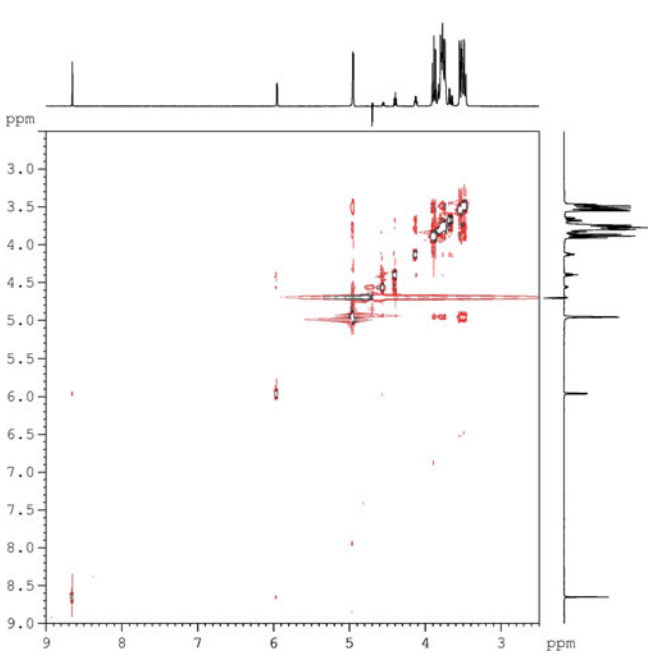

Fig. 3. 2D ROESY spectrum of [AMOX: β-CD] (1:1) mixture

Frequencies assigned to aromatic C=C, to ν_{NH}, to amidic $\nu_{C=O}$, to β-lactamic C=O ($\nu_{C=O}$), and to carboxylate (ν_{sCOO^-}) (ν_{asCOO^-}), have all been identified in free AMOX (zwitterion) and are reported in Table I. These results are in accordance with the literature (27,28). The more relevant bands of the IR spectra of the complexes (1:0.25 to 1:5.0) obtained by the two methods are compiled in Table I.

The infrared spectrum of the free AMOX showed strong absorption at 1,776 cm^{-1}, characteristic of the β-lactam ring. Same shifts have been observed whatever preparation method used. In all the complexes, the β-lactamic $\nu_{C=O}$ was shifted toward lower frequencies 1,769 cm^{-1} for 1:1 complexes. On the other hand, the carboxylate ν_{asCOO^-} (1,582 cm^{-1}) and ν_{sCOO^-} (1,397 cm^{-1}) were shifted toward higher frequencies (1,597 cm^{-1}) and (1,404 cm^{-1}), respectively, for 1:1 complexes as shown in Table I. The shift of the stretching vibration to higher frequency is the result of the formation of a strong hydrogen bond between the carboxylate group and the proton of an alcohol function on the CD host. As the spectral changes are due to atom group vibrations directly involved in interaction, these experimental findings clearly indicate both the β-lactamic carbonyl group and the carboxylate being involved in the complexation in all the binary systems AMOX-β-CD.

NMR Results

^{1}H-NMR analysis of AMOX, β-CD, and putative complexes of AMOX with β-CD were performed in D_2O due to the good solubility of the AMOX, native β-CD, and the binary AMOX-β-CD systems in this solvent. Induced changes in the chemical shifts for AMOX ($\Delta\delta$) were collected in Tables II and III for binary systems obtained by methods 1 and 2, respectively. Complexation of AMOX with β-CD, obtained with the two methods, resulted in little or no shift of the H-3 protons located in the CD cavity, which is in agreement with data reported by Maffeo *et al.* (22). An

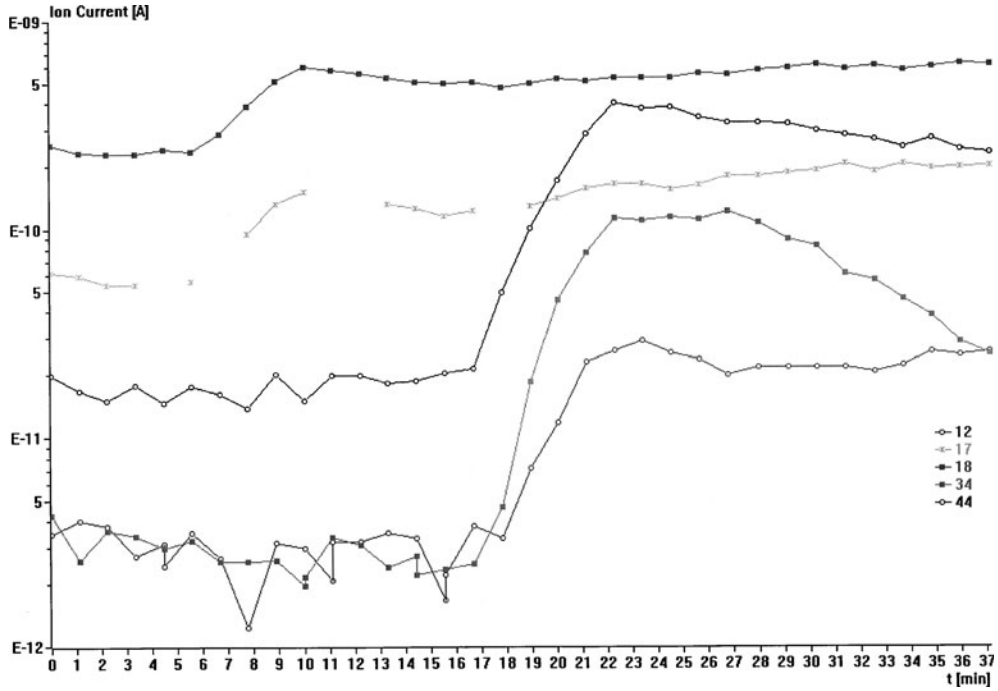

Fig. 4. Mass spectrum of amoxicillin selected molecular ions

absence of H-5 protons signal appeared after the first addition of the CD.

AMOX did not reveal any chemical variation of the phenol and the H-3 thiazol, whereas a little shift was found for aromatic, β-lactamic, and methylenic hydrogens from the 1:1 to the 1:5 [AMOX:β-CD] complex.

The H-5, H-6 resonance of AMOX took the form of an AB quartet in the 5.38- to 5.42-ppm range with doublet separations (^3J) of 3.8 Hz characteristic of *cis* protons in lactam (29). For the complex, they were in resonance also in the quartet form (5.39–5.40) with same ($^3J_{5,6}$) magnitude which confirms a closed β-lactam ring.

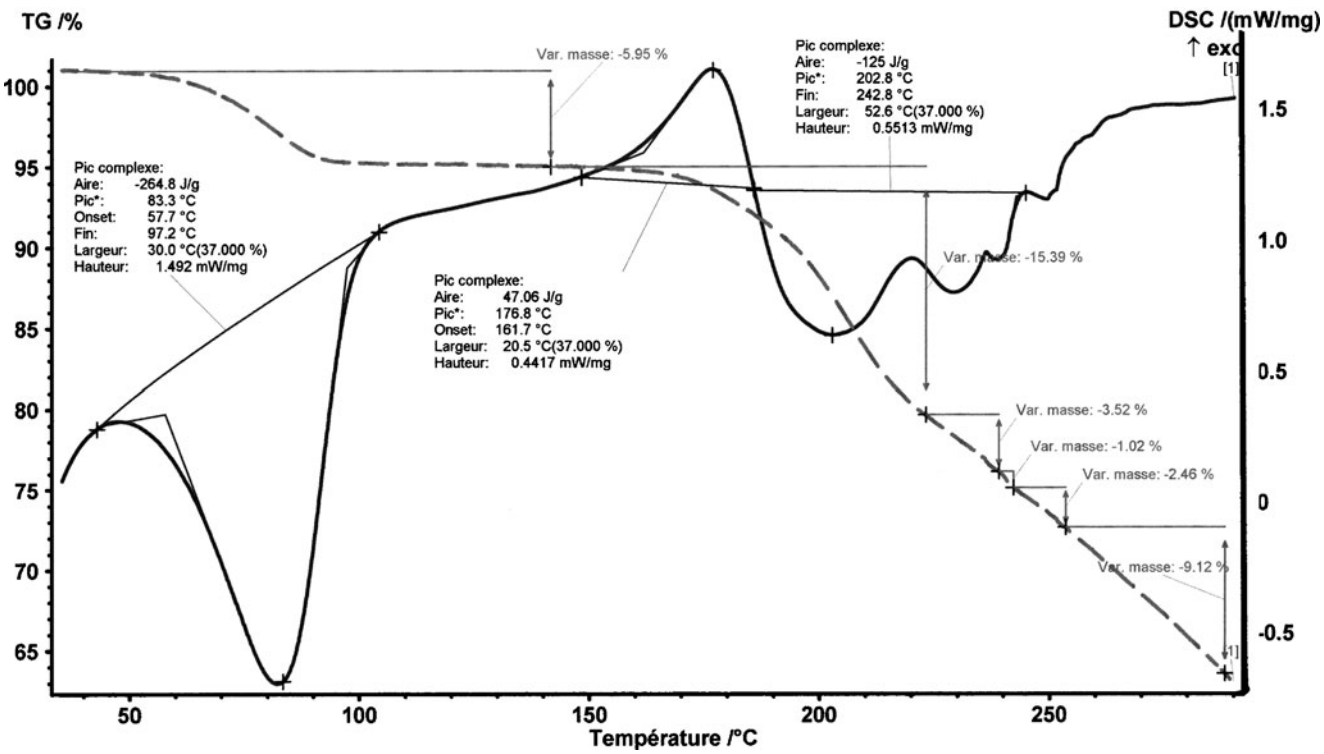

Fig. 5. Mass change (*dashed line*) and DSC *solid line*) thermogram of amoxicillin

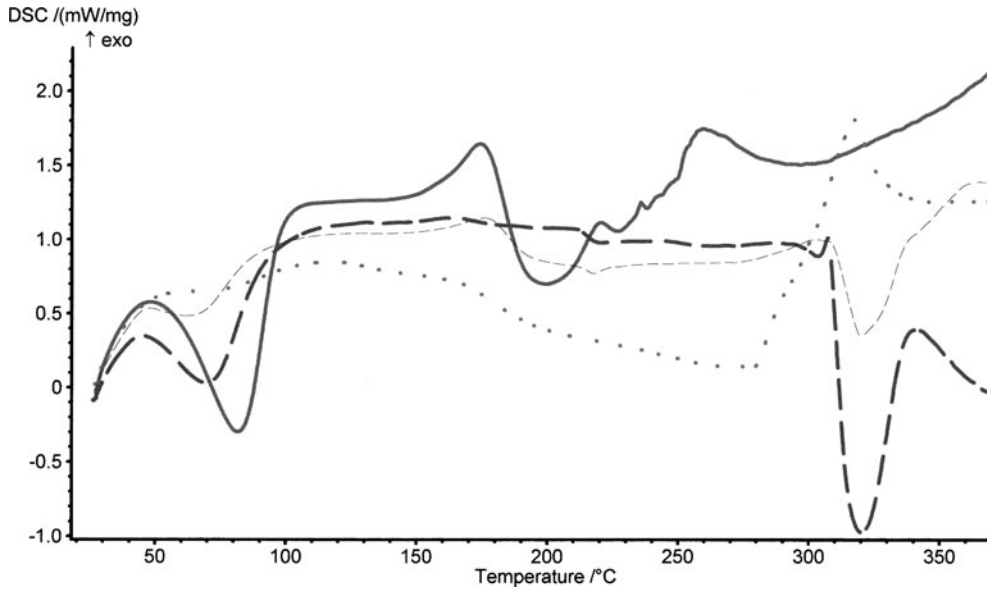

Fig. 6. DSC thermogram of AMOX (*solid line*), β-CD (*thick dashed line*), AMOXβ-CD physical mixture (*thin dashed line*) and [AMOX:β-CD] complex (*dotted line*)

No dipolar proton interactions between AMOX and CD were observed in the 2D ROESY spectrum (Fig. 3), which suggests the absence of any defined molecular association in solution. However, we cannot exclude that fast intermolecular exchanges have occurred and may have prevented the observation of such an association at the NMR scale. The coexistence of two different orientational isomers may also explain the absence of a chemical shift of the β-CD cavity protons (24,25,29).

DSC Results

TG and DSC analysis are widespread approaches in characterization of multicomponent systems such as inclusion compounds in the solid state. Therefore, the thermal properties of AMOX, β-CD, [AMOX:β-CD] complex, and physical mixture of AMOX with β-CD were investigated.

The AMOX mass spectrum (Fig. 4) presents five major molecular ions (m/z=12, 17, 18, 34, and 44). At 6 min, the 17 and 18 molecular ions in the mass spectrum correspond to the loss of water (5.95%). At 17 min, the m/z=44, 34, and 12 indicate AMOX degradation.

Figure 5 shows the TG and DSC data of AMOX from 30°C to 300°C. The AMOX DSC thermogram exhibits a first endothermic peak between 57.7°C and 97.2°C with a maximum at 83.3°C (ΔH=−264.8 J g^{-1}), which corresponds to the dehydration of AMOX. At this endothermic peak corresponds a mass loss of 5.95%. Then, AMOX is thermally decomposed at several stages with a total weight loss of 51.06%, leaving a residual mass at 350°C of 48.94%. The DSC confirms the thermal decomposition of AMOX. Moreover, AMOX DSC curve displays an exothermic peak with an onset at 161.7°C related to the AMOX transition into the crystal state (ΔH=47.06 J g^{-1}) and an endothermic broad peak at 185.5–242.8°C attributed to the AMOX fusion degradation event (ΔH=−125.0 J g^{-1}). Marciniec *et al.* (30) reported a melting point for AMOX trihydrate at 190.1°C in helium atmosphere and at a scanning rate of 5°C min^{-1}.

Figure 6 compares DSC measurements of AMOX, β-CD, [AMOX:β-CD] [1:1] complex, and AMOX-β-CD physical mixture. The [1:1] complex shows the complete disappearance of exothermic and endothermic peaks between 200.5°C and 242.3°C. This flattening profile may be regarded as a conclusive evidence of a molecular encapsulation of AMOX inside the β-CD cavity. A control of the inclusion complex formation was brought by registering the physical mixture DSC profile which shows the superimposition of the two components profiles.

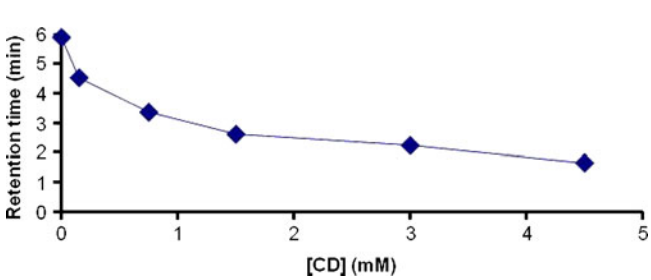

Fig. 7. Observed HPLC retention times for AMOX with varying concentrations of β-CD in the phosphate buffer mobile phase

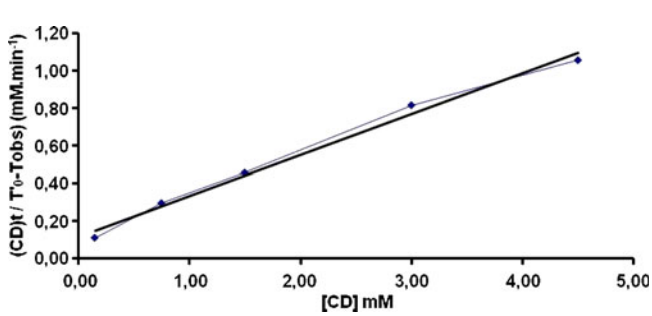

Fig. 8. Determination of K_c from the HPLC retention time data of [AMOX:β-CD] complexes

Stability Constant Determined by HPLC

In order to determine stability constant of [AMOX:β-CD] complex, we used HPLC. An adaptation of the European Pharmacopoeia technique on anion exchange support with an aqueous mobile phase was developed. Potassium dihydrogenophosphate buffer was used as mobile phase since phosphate anions cannot interfere with the complexation process. The column void volume was determined by elution of a thiourea solution (0.273 μg mL^{-1}).

It was found that increasing the concentration of β-CD in the phosphate buffer solution resulted in a shorter retention time for AMOX (Fig. 7), indicating an enhancement of the solubility of this compound by binding to β-CD. Figure 8 shows the determination of K_C from the retention time data of [AMOX:β-CD] complex according to Eq. 1. As shown in this figure, a linear plot (correlation coefficient r^2=0.99) was obtained with β-CD, indicating 1:1 stoichiometry of the [AMOX:β-CD] complex.

The equation of the line for AMOX with β-CD was:

$$y = 0.2179x + 116.02\left(R^2 = 0.9905\right). \quad (3)$$

A stability constant of 1,878 M^{-1} was found for the [AMOX:β-CD] complex, suggesting that this system might be suitable for pharmaceutical use (31). This association constant represents the proportions of free drug and free β-CD in solution *versus* the concentration of [AMOX:β-CD] complex.

In summary, complexation of AMOX anhydride with β-CD at pH 5 was provn by FT-IR, NMR, HPLC, and thermal analysis. The 1:1 stoichiometry for the complexes was obtained by HPLC experiments. It should be pointed out that β-CD shows good interaction with AMOX regards to its high stability constant. Preparation methods were conducted exactly to the formation of the same complexes. Sonication in solution of the two molecules before equilibration was unnecessary. The major spectral changes on FT-IR spectra of AMOX and β-CD complex were between 1,775 and 1,250 cm^{-1}. They showed an interaction of AMOX carbonyl group on β-lactam ring and of carboxylate with CD macrocycle. The shift of the carboxylate vibrational band characterized the establishment of hydrogen bonds with secondary hydroxyl groups of β-CD. As observed by NMR, complexation of AMOX with β-CD resulted in very little or no shift of the CD inner protons, whereas the aromatic, β-lactamic, and methylenic hydrogen of AMOX were little shifted and in the lack of any visible correlation in the ROESY spectrum. Considering the size and structure of AMOX, we could not consider that AMOX was totally included in the β-CD cavity which was too small. Then, we could imagine the coexistence of different complexes which could be orientational isomers (32); each complex should include an opposite part of AMOX. As suggested by Maffeo (22), apparently, the results in the net zero chemical shift displacement was possibly due to nearly equal shielding caused by phenyl ring inclusion and deshielding caused by the penam ring inclusion.

CONCLUSION

In the [AMOX:β-CD] complex, the phenyl group was included inside the β-CD, whereas the penam ring was gradually driven out of the β-CD cavity so that the ionized carboxyl group on the penam ring formed hydrogen bonds with the secondary hydroxyl groups of β-CD to keep the complex stable, as previously observed by Aki (20) for the ampicillin–HPCD complex by other methods.

ACKNOWLEDGMENTS

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