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Monitoring of ampicillin and its related substances by NMR

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Abstract

A ^{1}H NMR procedure for the monitoring of ampicillin (Amp) and its main related substance in different media, has been developed. The characteristics peak of Amp, 6-aminopenicillanic acid (6-APA), phenylglycine (PhG), and penicilloic acid in the range of 0.5–0.9 and 3.0–4.5 ppm were used for their identification in drugs and serum samples. The quantitative works were performed based on the intensity of protons of the methyl group link to the β-lactam cyclic of Amp and 6-APA and the aromatic protons of PhG relative to the protons of methylene group of maleic acid, as internal standard, at constant temperature. The resulting data are compared with those obtained with an HPLC method proposed by British Pharmacopoeia. Statistical studies show that, at a confidence limit of %95, there is no significant difference between the two methods. In comparison with the HPLC method, the proposed NMR method does not require any sample pretreatment, standard solution preparation, long analysis time and use of any carcinogenic solvent. The method was applied to the determination of Amp and its related substances in synthetic mixtures, drug powders and serum samples.

Keywords: Ampicillin; Related products; Determination; ^{1}H NMR

1. Introduction

β-Lactam antibiotics are the most widely used class of antimicrobial agents. These materials comprise several classes of compounds, among which penicillins and cephalosporins are the most important. These classes contain bulky side chains attached to the 6-aminopenicillanic acid (6-APA) or 7-aminocephalosporinic acid nuclei, respectively, [1–3]. The penicillins are of wide usage for their antimicrobial activity against both gram-positive and gram-negative organisms [4,5]. However, the pencillins have limited stability, especially in organic solvents, and produce different degradation products [6,7]. In addition, because of their use in veterinary medicine for the treatment of bacterial infections and potential for allergic reac-

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tions in certain individuals, the residue analysis in animal food products and milk is an important topic [8–11].

Ampicillin (Amp) is a widely used semi-synthetic penicillin-like drug. The most important related substances of Amp are 6-APA, phenylglycine (PhG), penicilloic acid (PA), penilloic acid and ampicillinyl-D-phenylglycine [12]. Since the presence of such degradation and related substances such as 6-APA and PhG may cause the decreased activity of Amp as well as some side effects and allergic reactions in human body, their simultaneous determinations are of critical importance Fig. 1 shows the structures of Amp, 6-APA and PhG [13–15].

Commonly applied methods for the determination of Amp and its related substances and metabolites use various extraction and deproteinization procedures [16] followed by HPLC separation techniques employing a variety of stationary and mobile phases [17–22]. Ultra violet and fluorescence detectors with derivitization in the pre- or post-columns have been the most routinely used methods [23–28]. However, in the presence of interferences, the confirmation of analyte identity has been demonstrated through thermospray LC-MS that is usually employed to analyze nonvolatile and thermally labile compounds [29–31] including several β-lactams [32–35]. This method is suitable for the simultaneous identification and determination of Amp and its related substances; however, the expensive price and long analysis time are its limitations. In addition, the chromatographic methods require large sample volumes and are time-consuming and tedious because of the liquid–liquid extraction and deproteinization steps needed for sample clean up [36]. Moreover, the determination of different impurities requires several different experimental conditions for analysis.

In a contrary, the NMR technique, as the most important form of spectroscopic methods, has been very successful and powerful in both academic and industrial chemical research. In most cases, this technique takes advantage of differences in the chemical shifts and direct proportionally of the signal intensity to the number of resonating nuclei for qualitative and quantitative analysis, respectively, [37–43]. In the past, the development

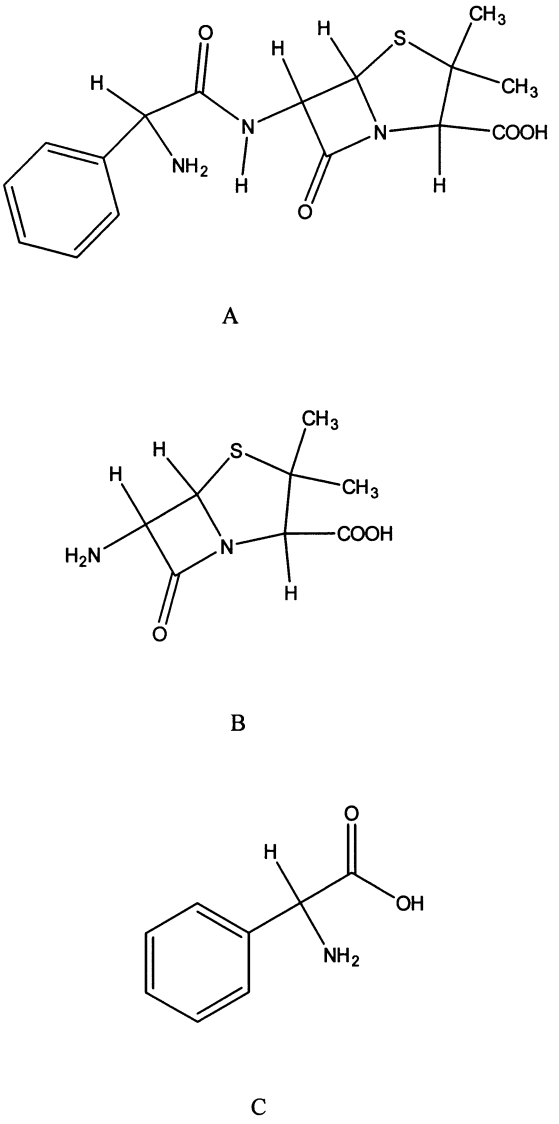

Fig. 1. Structure of Amp (B), PhG (B) and 6-APA (C).

of routine NMR spectroscopy procedures were limited by the high cost of the instrumentation, rather poor sensitivity and difficulty of quantitative reproducibility in comparison with other analytical techniques. However, nowadays, improvements in the automation of measurements and the increase in acquisition speed make the proton NMR spectroscopy very suitable for assessing routine methods for the analysis of complex mixtures [44–47]. Furthermore, the analysis can

take advantage of very fast, easily performed, and nondestructive procedures and do not usually require elaborate sample preparation or the use of sophisticated reagents. Quantitative NMR is more complicated for ^{13}C NMR, when it compared with ^{1}H NMR. Here, two phenomena are more severe, namely unequal T_1 relaxation times and NOE effect. However, ^{1}H NMR accounts for the most quantitative NMR applications, because the response of protons is nearly equal regardless of their chemical shifts or coupling to other nuclei with the important exception of exchangeable protons. This near unity response means that the ratios of the areas of proton resonance are usually accurate enough to determine the relative numbers of protons belonging to different functional groups. One of the most brightly applications of proton NMR is its use in the identification and simultaneous determination of drugs in the authentic mixtures or tablets [48–53].

The purpose of the present study is to develop a rapid, specific and selective NMR method for the simultaneous qualitative and quantitative analysis of Amp and its main related substances such as 6-APA and PhG in synthetic, drug and biological samples.

2. Experimental

2.1. Materials

Pure reference standards of ampicillin trihydrate, 6-APA and PhG of chemical purity of 99.1, 99.2 and 99.7%, respectively, were supplied by USP standards of Aldrich. These drug standards were stored in a dry atmosphere at 4 °C and were not dried or purified further before use. Zakaria Pharmaceutical Co. (Tabriz, Iran) kindly supplied real samples of tablets. Analytical-grade formic acid and maleic acid (both from Merck) were used as received. Reagent grade D_2O (> 99.8%) and HPLC grade methanol (both from Fluka) and 4,4-dimethyl-4-silapetance sodium sulfonate (DSS) from ARMAR were used as received.

2.2. Apparatus

Proton NMR experiments were carried out using a Bruker DRX 500 AVANCE spectrometer operating at 500.13 MHz and equipped with a dedicated 5-mm proton probe. The spectra were recorded in acidic D_2O medium, with all chemical shifts (δ) in the range 0–10 ppm referred to an internal DSS. Typical acquisition parameters employed in proton NMR experiments were as follows: acquisition time 1.6 s, spectral width 10330.578 Hz, mutation angle 30°, 16–64 K data points, relaxation delay 2 s and temperature 298 K. The 90° pulse widths were 6.9 μs for the ^{1}H NMR dedicated probe, of DRX 500 spectrometer. The digital resolution was 18. In addition, the water suppression using a 3–9–19 pulse sequence with gradients was used for serum analysis. NMR processing for all samples included phase correction, (performed manually for each replicate), and baseline correction over the entire spectral range. In some instances, the baseline was additionally corrected over the integrated regions.

The liquid chromatographic equipment was a Waters system equipped with a model 616 pump. A LC photodiode-array detector simultaneously reads the absorbance at 400 different wavelengths between 200 and 600 nm. The column used was a μ-bondapack C_{18}-column of Waters Co., working at a flow rate of 1.5 ml min^{-1}. The absorbance measurements were carried out on a Perkin-Elmer UV–Vis–NIR spectrophotometer model Lambada Bio 20. A Mettler model AT-20 electronic balance was used for all sample weighings.

The HPLC analysis of Amp, 6-APA and PhG were based on the British Pharmacopoeia (BP) method [12]. The mobile phase consisted of phosphate buffer at pH 5 and methanol: water (75:25). The flow rate was 1.5 ml min^{-1} and the photometric detection was performed at 210 nm. The column temperature was adjusted at 40 °C and a 20 μl sample loop was used.

2.3. Sample preparation

A 56% D_2O–44% formic acid (w/w) mixture was selected as solvent to properly dissolve the solid samples. Known standard mixtures of Amp, 6-

APA and PhG were prepared by direct weighing of the reagents to an accuracy of ±0.1 mg. Analyte weight was determined by its transfers into a NMR sample tube using a Teflon-coated micro-spatula based on actual weight gain of the tube. This avoids weight errors due to spillage or sample adherence to the micro-spatula. Weight ranges of the analytes were about 10–50 mg Amp and 0.5–10 mg 6-APA and PhG. Weight ranging of maleic acid, as an internal standard, was from 10 to 12 mg. The samples were then dissolved in about 0.5 ml of the stock solvent.

In order to determine the Amp and PhG content of Amp capsules, an accurate weight of five capsules equivalent to 30 mg was transferred into a NMR tube and completely dissolved in 0.5 ml of stock solvent containing maleic acid. For the preparation of serum sample, the serum and plasma of blood were separated first by centrifugation at 3000 rpm for 10 min. Then 0.4 ml of the serum sample was placed in the NMR tube, maleic acid was weighted in the tube and 0.1 ml D_2O was added as a locking solvent. Then Amp was spiked at different concentration levels to the serum. The strong water's peak was appeared in the spectrum of human serum, which was eliminated using the WATERGATE spectrum for correction.

NMR processing for all samples included phase correction (performed manually for each replicate) and baseline correction over the entire spectral range. In some instances, the baseline was additionally corrected over the integrated regions.

The HPLC assay of Amp was based on the BP method [12]. The mobile phase consisted of a phosphate buffer at pH 5 and methanol–water (75:25). The flow rate was 1.5 ml min^{-1} and the photometric detection was performed at 210 nm.

3. Results and discussions

Since the presence of PhG and, especially, 6-APA influence the Amp assay (e.g. by autocatalysis of Amp degradation) and show some side effects [13], we were interested to carry out both the identification and determination of Amp in synthetic and drug samples in the presence of 6-APA and PhG by NMR spectroscopy. For qualitative analyses of these compounds, both ^{13}C and ^{1}H NMR can be used. However, in the case of ^{13}C NMR, the Amp assay suffers from long analysis time of and NOE effects. Thus, we used ^{1}H NMR for the quantitative analyses.

3.1. Qualitative analysis

The biological medium is usually in an aqueous form, and for this reason, D_2O was selected for all of the NMR analyses performed in this work. However, the solubility of β-lactam antibiotics is low in pure water, so that an acidic modifier as a co-solvent is needed for their complete dissolution. Since the signal of formic acid dose not overlap with the characteristics peaks of Amp, 6-APA and PhG in the NMR spectrum, it was used as a suitable co-solvent.

However, the varying concentration of formic acid was found to affect the chemical shift of Amp and its related substances significantly. For this reason, a carefully prepared binary mixture of deuterium oxide/formic acid (56/44 w/w) was used throughout as a proper solvent.

The ^{1}H NMR spectra of Amp, 6-APA and PhG are shown in Fig. 2. The spectrum of Amp possesses two characteristic sharp singlet peaks at 0.640 and 0.675 ppm that are belong to the two methyl protons of Amp. The other peak around 3.64 ppm corresponds to the C–H proton next to the acid group. The spectrum also reveals a peak at 6.76, which is due to the protons of aromatic group. The spectrum of 6-APA is similar to the upfield spectrum of Amp and indicates two sharp singlets at 0.824 and 0.940 ppm due to the two methyl protons. A weak singlet peak at 3.87 ppm corresponds to the proton next to the acid group. Downfield spectrum of 6-APA does not indicate any other signal. These results agree well with other works [12,55], considering the fact that a constant shifts is observed in all peaks due to the presence of formic acid as co-solvent. The spectrum of PhG shows two peaks at 4.18 and 6.51 ppm correspond to the proton next to the amine group and phenyl protons, respectively. In all spectra, were observed two sharp singlet peaks at 4.78 and 7.63 ppm corresponding to H_2O and formic acid, respectively.

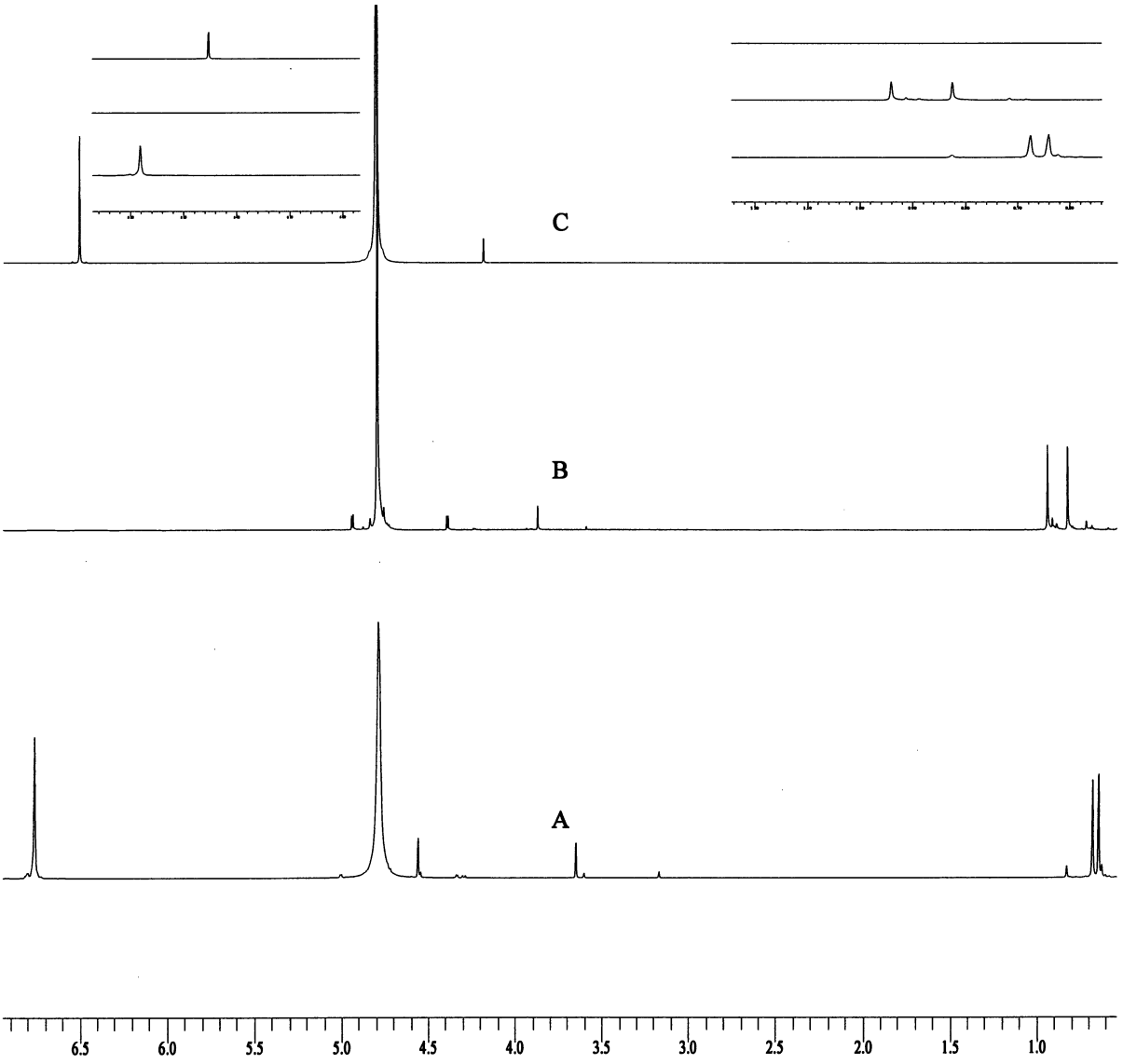

Fig. 2. NMR spectra of pure Amp (7.1 mg, A), 6-APA (6.5 mg, B) and PhG (5.1 Mg, C) in 0.5 ml solution of D_2O/formic acid (56/44 w/w) and their expanded parts.

Fig. 3 shows the ^1H NMR spectrum of ternary mixtures of Amp, 6-APA and PhG. It can be readily seen that the NMR spectrum of the ternary mixture contains the characteristic spectrum of each of three species, being easily distinguishable form each other, except for some minor overlapping between the phenyl protons of Amp and PhG. As it is quite obvious, in a chemical shift range of about 0.7–1.1 ppm, the spectroscopic information contained in the NMR spectrum are relevant for the complete resolution of these two compounds in the ternary mixture. On the other hand, for the quantitative analysis of the PhG present, its phenyl protons' signal at aromatic region (located at $\sim$6.51 ppm) can be safely employed.

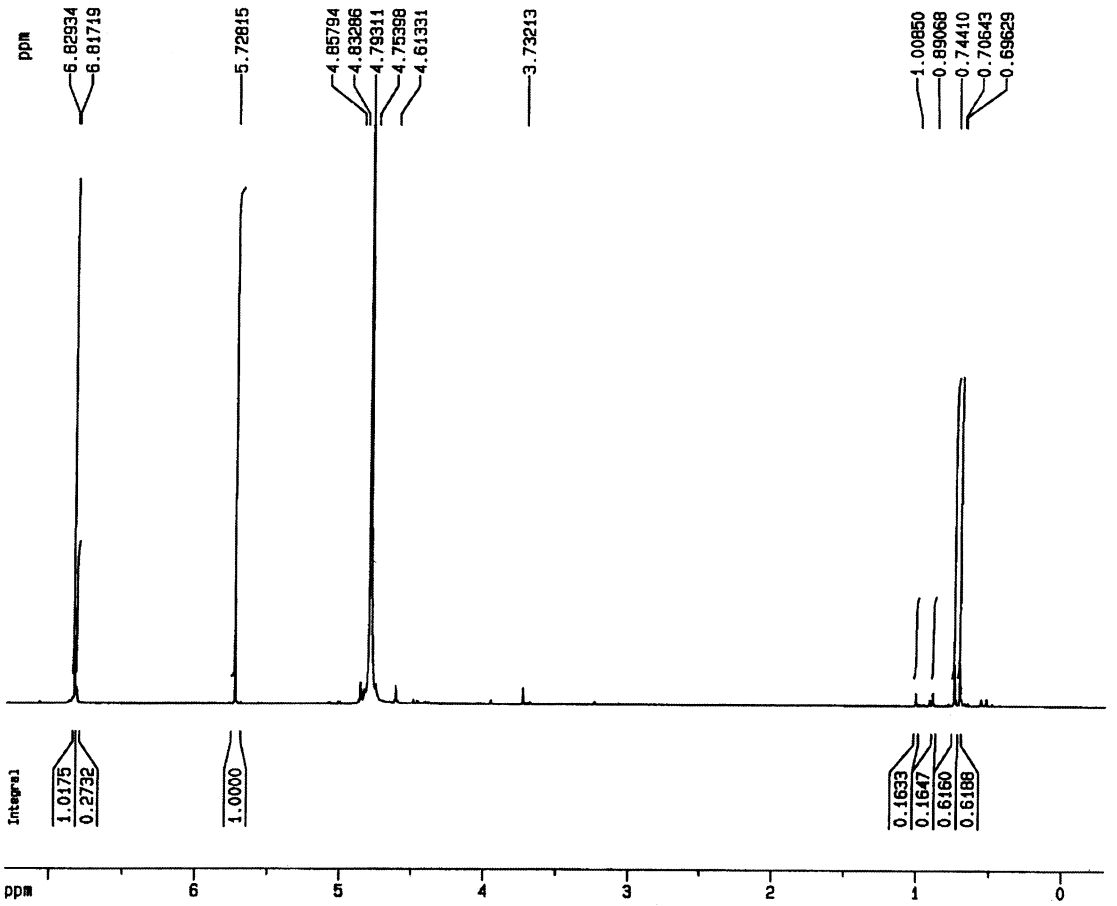

Fig. 3. NMR spectra of a ternary mixture containing of Amp (12.3 mg), 6-APA (4.0 mg) and PhG (1.9 mg) in 0.5 ml solution of D_2O/formic acid (56/44 w/w).

The characteristics peaks of Amp, 6-APA and PhG in the range of 3.0–4.5 ppm can be used for their monitoring in drug and serum samples. It should be noted that, after several mounts (>6 months), the spectrum of Amp shows new peaks in the range of 0.5–0.9 and 3.0–4.5 ppm (Fig. 4) that are belong to PA. The moisture at acidic and basic conditions, temperature and sunlight would cause to increase the degradation of Amp resulting in the appearance of the PA, penilloic acid and ampicillinyl-D-phenylglycine NMR peaks.

3.2. Quantitative analysis

In quantitative analysis of organic compounds, the desirable signal is usually a singlet, not subject to exchange phenomena and well resolved from any signal arising from the sample. In addition, intensity of the selected peak should be larger than other signals of the compound to achieve improved detection limit and sensitivity. Therefore, in this work, the quantitative calculations were based on the sum of integrals of the two upfield CH_3 singlets of Amp (0.640 and 0.675 ppm), two CH_3 singlets of 6-APA (0.824 and 0.940 ppm) and the integral of the phenyl peak of PhG (6.51 ppm). The use of sum of integrals of the two methylene groups of Amp and 6-APA provided a larger integral intensity for the sake of increased precision and sensitivity over the integration of a singlet CH_3 [56]. The other peak of PhG at 4.18 ppm corresponds to the proton next to the amine

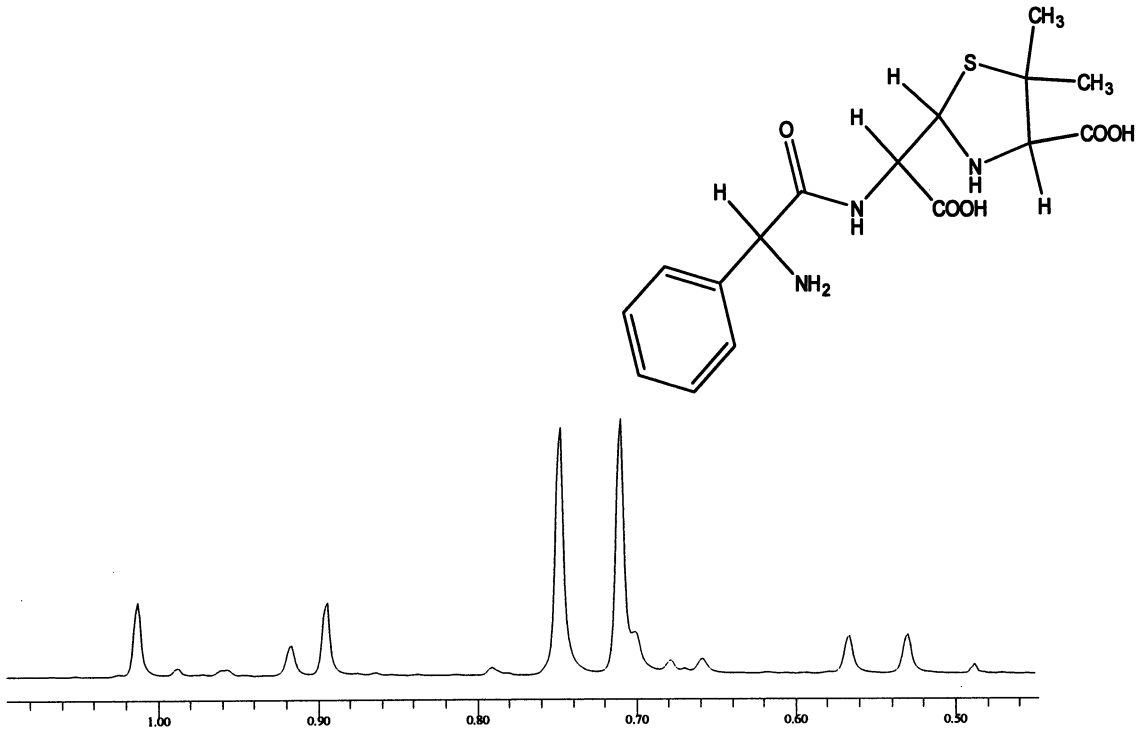

Fig. 4. PA structure and its NMR peaks.

group, and can be exchangeable with the D_2O signal and, thus, it is not suitable for quantitative purposes.

Most quantitative NMR assay methods are based on using an internal standard, for particular analyses and improvement of the results. In this endeavor, maleic acid was used as an internal standard, because it was soluble and stable in sample media, did not shown any interaction with Amp, 6-APA and PhG and was available in high purity. The chemical shift of methylene group of maleic acid located at 5.72 ppm (belong to the two equivalent methylene protons of molecule) was adequately removed from the nearest water signal near 4.80 ppm. The integral of selected peaks of Amp, 6-APA and PhG related to that of the sharp signal of maleic acid was employed for the quantitative analyses.

The procedure for quantitative evaluation of Amp, 6-APA and PhG, was validated by several experiments carried out on synthetic mixtures before being applied to real samples. The mixture standard solutions for the three compounds were prepared based on their range of concentration in the commercially available pharmaceutical preparations, as mentioned in Section 2.3. The accuracy of the quantitative procedure was checked by comparing the amounts of Amp, 6-APA and PhG at different concentrations present and measured by NMR.

Table 1 described the results of the ^1H NMR technique applied to the determination of Amp, 6-APA and PhG in synthetic mixtures. The concentration ranges, linear regression equations, correlation coefficients, estimated errors and limit of detection (LOD) obtained for each compound are summarized in Table 1. LOD of Amp, 6-APA and PhG in the mixtures are 80, 160 and 90 μg $m1^{-1}$, respectively.

In Table 2, the results obtained by HPLC are compared with those by ^1H NMR using the internal standard method. Obviously, the agreement in the compound concentration between the NMR and HPLC methods is excellent. According

Table 1
The linear regression equations for Amp, 6-APA and PhG obtained by the internal standard method in the synthetic mixtures

Compound	Concentration range (mg ml^{-1})	Regression equation[a]	Correlation coefficient ($n=5$)	Error estimated	LOD[b] (μg ml^{-1})
Amp	1.0–50	$I=1.72C+0.002$	0.997	0.028	80
6-APA	0.5–10	$I=0.480C+0.011$	0.995	0.022	160
PhG	0.5–10	$I=0.836C+0.007$	0.995	0.051	90

[a] I, relative integral (arbitrary unit); C, concentration of analytes (mg ml^{-1}).
[b] Limit of detection.

to the variance ratio test (F test) the calculated 'F' value was 2.2 for Amp, while the theoretical value was 6.34. Statistical comparison of the NMR method proposed with the HPLC method revealed that there is no significant difference at a confidence limit %95. Furthermore the proposed method is more selective and simple as it compared by the HPLC method. In addition, the analysis time with the ^{1}H NMR method ($\sim$0.5 min) is much shorter than that of the HPLC method ($\sim$ 45 min). This is very important, considering the fact that Amp and its degradation products are labile compounds that decompose longer analysis times and can nor identified by regular HPLC method.

3.3. Applications

Obviously, the results obtained on synthetic mixtures indicated that qualitative and quantitative analysis of Amp in the presence of its degradation products (i.e. 6-APA and PhG) can be carried out with the aid of the ^{1}H NMR spectra acquired with the selected parameters. Therefore, we applied the proposed same method to real samples.

The assay of Amp and its degradation products in five pharmaceutical capsules (from Zakaria Pharmaceutical Company) was performed by the internal standard method. A sample NMR spectrum is shown in Fig. 5. As is obvious, the resulting NMR spectrum indicated the main characteristic peaks of Amp, 6-APA and PhG, the integrals of which relative to that of maleic acid were employed for the quantitative analysis. In Table 3 are compared the results of the ^{1}H NMR analysis with the corresponding results of HPLC determinations according to the BP method [54]. As can be seen, correlation between the two sets of results is very good, and recovery of each sample is high.

The other application of the proposed NMR method was the direct determination of Amp in the human serum. The spectrum of human serum

Table 2
Comparison between internal standard of NMR and HPLC results in synthetic mixtures

Sample	Amount taken (mg)			% Recovery by NMR			% Recovery by HPLC		
	Amp	6-APA	PhG	Amp	6-APA	PhG	Amp	6-APA	PhG
1	30.1	1.5	1.2	96.0	104.9	102.3	100.4	99.5	99.1
2	29.3	1.1	1.3	99.1	101.5	100.9	98.1	98.1	97.8
3	31.2	1.4	1.2	98.7	102.2	101.8	99.4	98.9	98.9
4	29.5	1.2	1.1	97.9	103.4	102.6	99.0	97.8	98.2
5	30.4	1.3	1.4	99.0	101.7	101.5	99.8	99.1	99.2
Mean recovery				98.1	102.7	101.8	99.3	98.7	98.6
SD				1.3	1.4	0.67	0.87	0.71	0.61
F-test at $P=0.05$[a]				2.2	3.0	1.2			

[a] F-value in table at $P=0.05$ is 6.34.

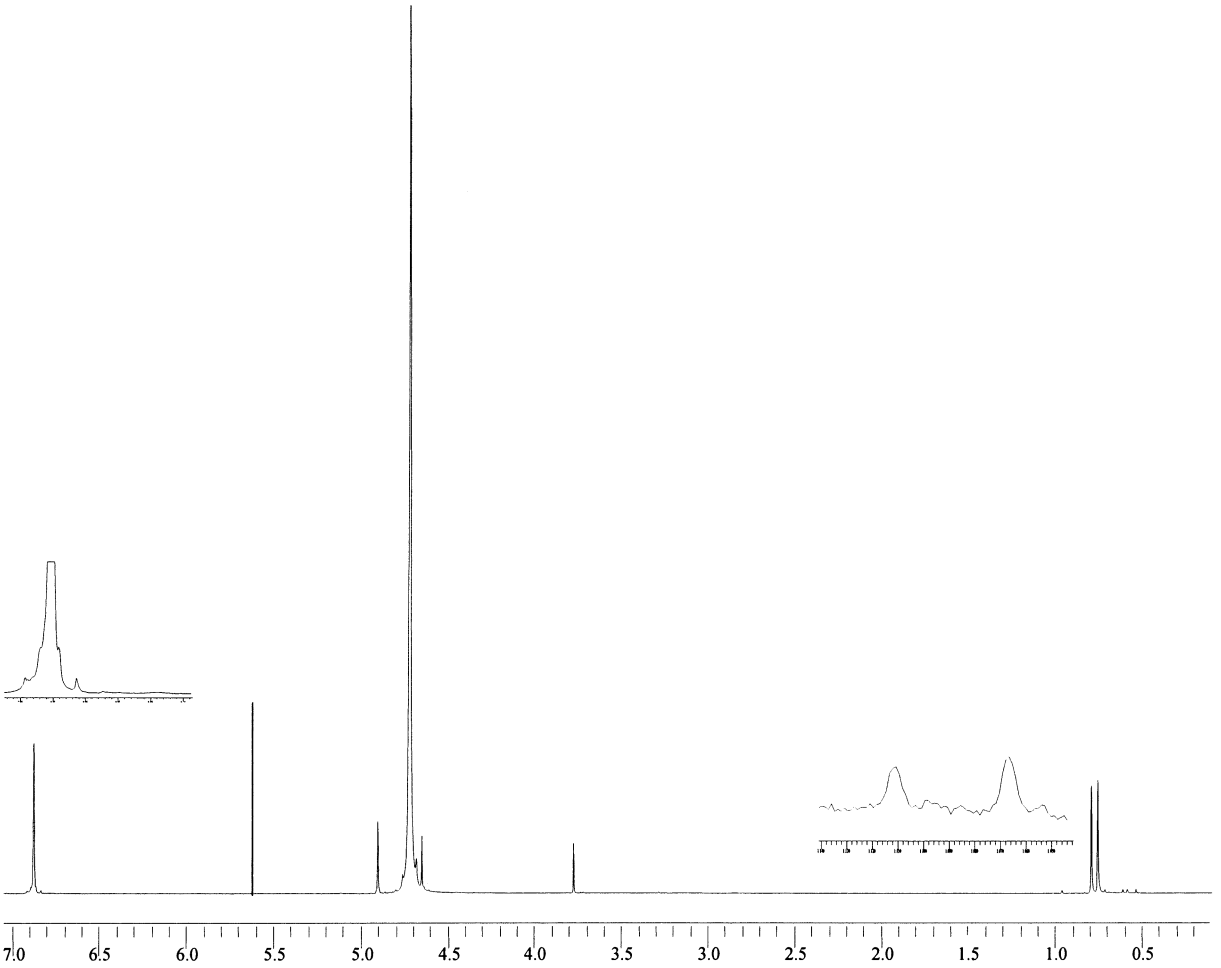

Fig. 5. NMR spectrum of 0.5 ml sample containing 30 mg of pharmaceutical capsule and 11 mg of maleic acid.

Table 3
Results of assay of five pharmaceutical capsules by the proposed NMR and the HPLC method

Sample	Taken (mg)	Found (mg) by NMR[a]			Found (mg) by HPLC[a]		
		Amp. $3H_2O$	6-APA	PhG	Amp. $3H_2O$	6-APA	PhG
1	30	29.02	0.62	0.30	29.15	0.59	0.26
2	30	28.85	0.69	0.34	28.93	0.67	0.31
3	30	28.96	0.59	0.34	29.05	0.57	0.32
4	30	28.87	0.73	0.35	29.00	0.68	0.33
5	30	28.79	0.67	0.33	28.92	0.64	0.30

[a] Average of three replicate measurements.

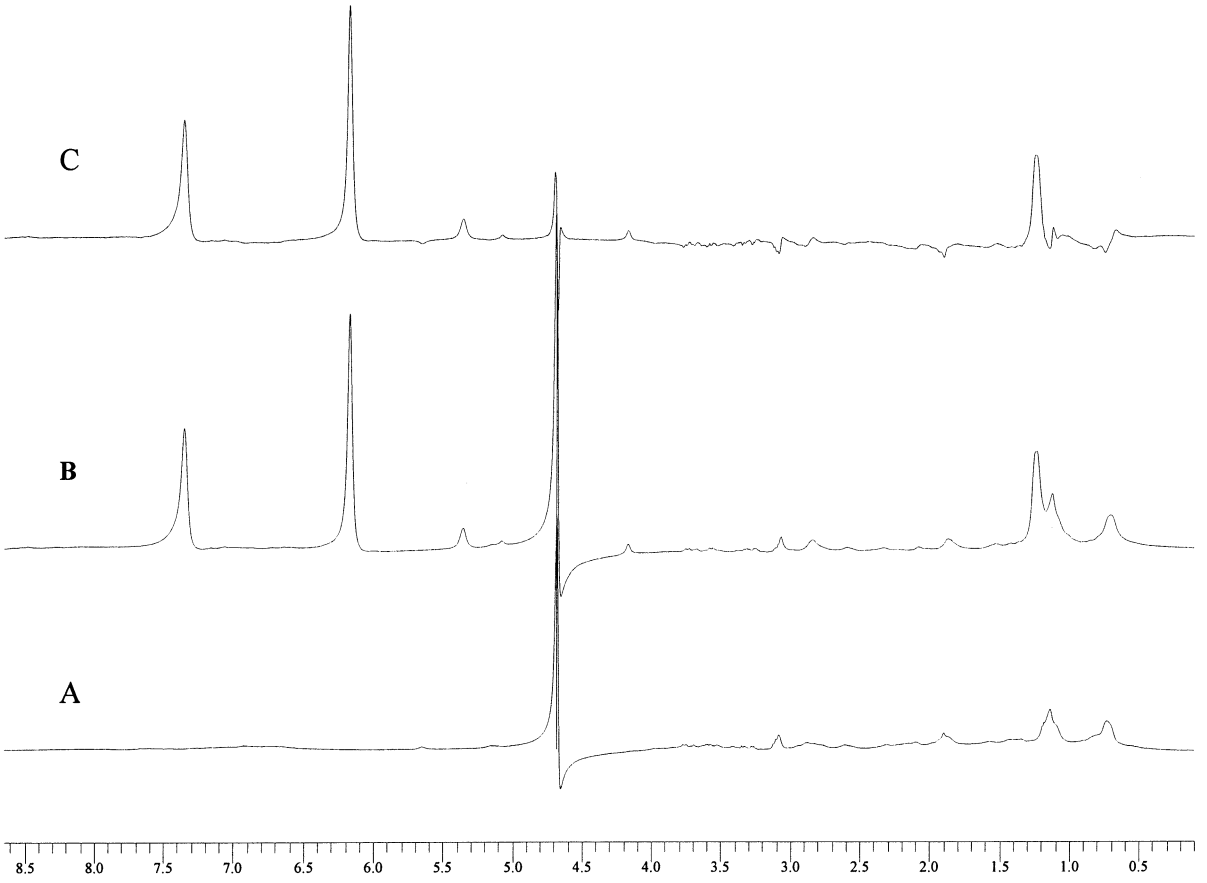

Fig. 6. WATERGATE spectrum of human serum before (A) and after (B) spiking with 6.5 mg of Amp and 10 mg of maleic acid, and the spectrum obtained after subtraction of A and B (C).

was obtained using a certain pulse sequence such as WATERGATE method (water suppression gradient tailored excitation), because the intense water signal in the biological fluid should be suppressed in the ^{1}H NMR spectra. Fig. 6 shows the WATERGATE spectrum of serum before (A) and after (B) spiking with known amounts of Amp and maleic acid. Subtracting the spectra 6A and 6B resulted in a clean NMR spectrum (C). In order to evaluate the recovery of Amp from the serum sample, different amounts of Amp assay were added to 0.4 ml sample containing 10 mg of maleic acid and the Amp assay as carried out. The results revealed a mean recovery of $(81.8 \pm 4.5)\%$. The LOD of Amp in serum sample, as determined from a signal-to-noise ratio of 3, was 105 $\mu g\ ml^{-1}$. It is worth mentioning that the characteristic peaks of 6-APA, PhG and PA appeared in the NMR detection range when the preconcentration of serum samples was performed by solid phase extraction discs [57].

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