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Short communication

Rapid determination of amoxicillin in premixes by HPLC

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Abstract

A rapid analytical procedure for routine identification and quantification of amoxicillin in premixes by high performance liquid chromatography was developed and tested. The ground premix samples were extracted for 10 min using 100 ml extraction mixture water–methanol (800:200, v/v). The extract was analyzed by reversed-phase on Agilent Zorbax SB-C18 column (4.6 mm × 150 mm, i.d., 5 μm particle size) with water–methanol–phosphoric acid–triethylamine (842:150:4:4) containing 10 mM hexane-1-sulfonic acid sodium salt (pH 3.5) as mobile phase. UV detection was carried out at 230 nm. The method was validated for specificity, linearity, solution stability, accuracy, precision, limit of detection, and limit of determination. The detector response for amoxicillin was linear over the selected concentration range from 2.0 to 40.0 mg ml^{-1} with a correlation coefficient 0.9999. The mean accuracy was 100.1% with a standard deviation of 0.6%. The limit of detection and the limit of determination are 0.1 and 0.3 mg ml^{-1}, respectively, which corresponds to 10 and 30 mg kg^{-1}, respectively, in real premix sample. The sample and standard solutions were stable for 4 h. The method is selective and can be used in routine analysis.

Keywords: HPLC; Amoxicillin; Premix; Validation

1. Introduction

Amoxicilin is β-lactam antibiotic that belongs to the group of penicillins. The basic structure of penicillins, 6-aminopenicillanic acid, consists of a thiazolidine ring fused to β-lactam ring with a side chain. Amoxicillin presents in side chain a primary amine group, that does not exist in any other penicillins except epicillin and bacampicillin. Amoxicilin is extremely active against both Gram-positive and Gram-negative organisms, including several pathogenic enteric organisms. Amoxicillin is widely used in veterinary practice for the treatment of gastro-intestinal and systemic infections. Amoxicillin and ampicillin are know penicillins which is added to medicated feeds at the level of 250–500 mg kg^{-1}, because of its resistance to gastric juice.

The importance of a regular control of premixes for amoxicillin content is quite clear. Traditionally, penicillins are determined by microbiological assay [1]. However, it is very difficult to distinguish amoxicillin from ampicillin and other penicillins using microbiological methods. The method of thin layer chromatography for determination of aminoglycosides was used for manufacturing process and quality control in pure product batches [2]. The final choice of the technique depends upon criteria such as sensitivity, accuracy and selectivity. Separation technique of HPLC is better in all this factors than microbiological assay and thin layer chromatography. Capillary electrophoresis [3–6] or micellar electrokinetic capillary chromatography [7] is one of the techniques used for the determination of penicillin in feeds and pharmaceutical dosage forms. The method for determining amoxicillin in pharmaceuticals using sequential injection analysis (SIA) with a diode-array spectrophotometric detector was reported [8].

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In the literature, there are many chromatographic methods with diode array and fluorimetric detection for the determination of penicillins in food of animal origin [9–14] and in feeds [15] or pharmaceutical dosage forms [16–18]. The UV detection is carried out directly and after pre-column derivatization with imidazole and mercuric chloride [19]. The fluorescence detection is carried out using post-column derivatization with fluorescamine [20,21] or pre-column derivatization with formaldehyde in acidic solution [15].

The purpose of this study was to develop a rapid and sensitive quantitative chromatographic method for determination of amoxicillin in premixes.

2. Experimental

2.1. Materials

Solvents: methanol, phosphoric acid and triethylamine were of HPLC grade (J.T. Baker, USA). Water purified on Milli-Q system (Millipore, USA) was used. Other chemicals were of analytical grade. Extraction solvent I was made by combining 800 ml water and 200 ml methanol. Extraction solvent II was made by combining 790 ml water, 200 ml methanol and 10 ml phosphoric acid.

2.2. Instrumentation

Sample extraction was performed on laboratory horizontal shaker. Centrifugation of extract was made on laboratory centrifuge Hermle Z 230 MR (Hermle, Germany). All chromatographic experiments were carried out using a liquid chromatograph system consisting of pump W515, autosampler W717 Plus and UV detector W486 (all Waters, USA). The system was controlled by data station PC Compaq using Millennium software (Waters, USA).

2.3. Chromatographic conditions

HPLC separations were performed on a 150 mm × 4.6 mm, 5 μm Agilent Zorbax SB-C18 column (Agilent, USA). The mobile phase was 842:150:4:4 (v/v) water–methanol–phosphoric acid–triethylamine. In this solution was dissolved 1.881 g hexane-1-sulfonic acid sodium salt and then pH was adjusted to pH 3.5 with phosphoric acid. Mobile phase was prepared by mixing volume to volume of the components. The flow rate was 0.8 ml min^{-1}, the wavelength was 230 nm, the injection volume was 25 μl, the column temperature was thermostatted at 40 °C and the run time was 10 min.

2.4. Standard preparation

The standard of amoxicillin (Fluka, Germany) was dissolved in methanol at a concentration of 200 mg l^{-1} to obtain the standard stock solution. The working standard solutions of amoxicillin in extraction solvent I was prepared from stock standard solution on the day of use.

2.5. Sample preparation

The real samples of premixes were homogenized and grinded to particles of 0.5 mm and less. A portion (from 0.5 to 1.0 g of premix sample) was weighed into a 250 ml Erlen-Mayer flask, added 100 ml extraction solvent I, and this mixture was shortly shaken by hand. The ground premix sample was extracted for 10 min on a horizontal shaker and then for 1 min in ultrasonic bath. The extract after filtration was diluted with extraction solvent I. If necessary the extract was centrifuged before injection.

3. Results and discussion

3.1. Development and optimization of the HPLC method

The mobile phase composition allowed separation of amoxicillin from matrix components in a short analysis time (below 10 min, Fig. 1).

The final mobile phase was optimized so as to the capacity factor was $k \geq 1.5$, plate count $N \geq 5000$ and asymmetry factor $t_a \leq 1.3$. The following parameter (linearity) of optimized chromatographic method was determined using calibration solutions of standard.

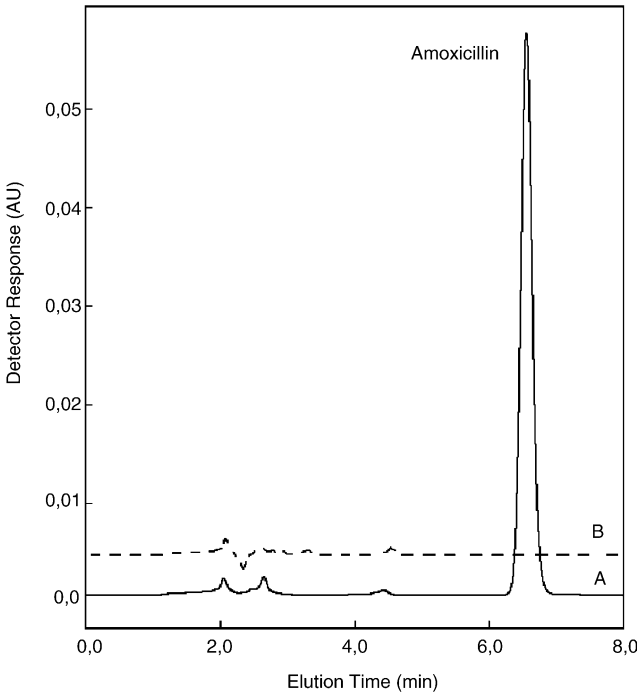

Fig. 1. Chromatograms of amoxicillin in real premix sample (content 200,000 mg kg^{-1}); (A) extract of real premix sample, dilution = 10; (B) blank. Capacity factor $k = 2.55$, plate count $N = 10{,}800$, asymmetry factor $t_a = 1.01$.

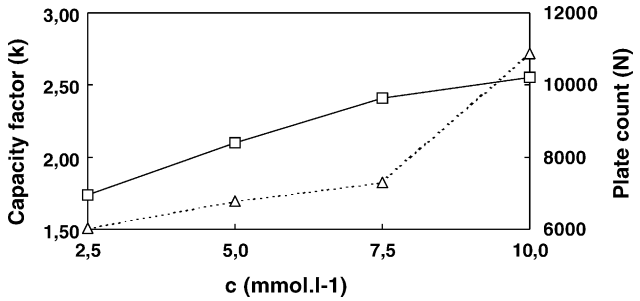

Fig. 2. The influence of concentration of ion-pair reagent (*c*) on capacity factor (*k*) and efficiency (plate count *N*).

Amoxicillin is an amphoteric compound with $pK_1 = 2.4$ and $pK_2 = 7.2$. Amoxicillin is a zwitterion at intermediate pH (3–6) and is mainly cationic at pH 2, and anionic at pH 7.2. The mobile phase was kept at pH 3.5 in this study, so that amoxicillin had a cationic character during the chromatography. The influence of concentration of ion-pair reagent on capacity factor was observed for concentration 2.5, 5.0, 7.5 and 10 mM hexane-1-sulfonic acid sodium salt in mobile phase. The retention of amoxicillin increases with increasing concentration of ion-pair reagent in mobile phase. As the optimum of concentration of ion-pair reagent was selected 10.0 mM. The change of retention is the least at this concentration and the efficiency (plate count *N*) is the greatest (Fig. 2).

Different chromatographic columns were tested for the mobile phase. The results are summarized in Table 1.

3.2. Linearity and limit of detection and limit of determination

A set of five standards at the following concentrations was prepared: 2.0, 4.0, 8.0, 16.0 and 40.0 mg ml^{-1}. Each of the five standards was analyzed in duplicate. The calibration curve was constructed by plotting the peak area against the concentration using linear regression analysis. It showed that the slope was 35166.8 with a *y*-intercept 1007.5 and correlation coefficient of 0.9999 and that indicates an excellent linearity. The calibration curve was prepared in ranging from 2 to 40 mg l^{-1}, which is satisfactory with regard to actual content of amoxicillin in premixes.

Table 1
Comparison of different chromatographic columns

Column	*k*	t_a	*N*
Agilent Zorbax SB-C18 (150 mm × 3.9 mm; i.d.; 5 μm)	2.55	1.01	10,800
Symmetry shield (150 mm × 3.9 mm; i.d.; 5 μm)	3.89	1.43	1060
Xterra (150 mm × 3.9 mm; i.d.; 5 μm)	2.81	1.76	950
Nova Pak (150 mm × 3.9 mm; i.d.; 4 μm)	1.48	1.52	4950
Supelco ABZ + (150 mm × 3.0 mm; i.d.; 5 μm)	2.42	1.52	1270

Notes: t_a: asymmetry factor; there is calculated by USP Tailing; *N*: plate count; *k*: capacity factor.

Table 2
Results and statistical parameters for analyses of model premix samples

Statistical parameters				
Expected value (mg kg^{-1})	20,000	50,000	100,000	200,000
HPLC assay-average (mg kg^{-1})	19,963	49,990	100,563	199,793
Relative standard deviation (%)	0.5	0.3	0.8	0.6
Recovery (%)	99.8	100.0	100.6	99.9

The average limit of detection of amoxicillin (based on a detector signal-to-noise ratio of 3:1) calculated from four different chromatograms of the blank feed extracts, was 0.1 mg ml^{-1}; the average limit of determination of amoxicillin (based on a detector signal-to-noise ratio of 10:1) was 0.3 mg ml^{-1}. The limit of detection and limit of determination correspond to 10 and 30 mg kg^{-1}, respectively, in a real feed sample using the treatment described in the experimental section. The baseline noise was measured in a blank experiment in the region of retention time of amoxicillin using chromatographic software.

3.3. Accuracy and precision

Model samples of premix were prepared to test the accuracy of the developed method. To the mixture of subsequent components 40% wheat and 60% calcite was added different amounts of amoxicillin to prepare samples with different concentration levels. For each level, six analyses were performed. The results and statistical parameters are summarized in Table 2. The average overall recovery at the 20,000, 50,000, 100,000 and 200,000 mg kg^{-1} levels was 100.1% with a standard deviation of 0.6%. Determined contents (c_d) were compared with expected ones (c_e) using linear regression. The regression equation (significance level $P = 0.95$) was $c_d = (57.96 \pm 208.8) + (0.9975 \pm 0.0042) \times c_e$ and $R^2 = 0.9998$. The first and second constants were no statistically different from zero and one, respectively. It can be concluded that analytical method gives accurate results.

Further repeatability of the developed method was assessed. Sixteen real samples of pre-mixes were analyzed in two parallel determinations. Value of the repeatability limit for content of analyte ranging from 200,000 to 500,000 mg kg^{-1} after elimination of one outlier (applying Cochran's test) was 6%.

3.4. Optimization of sample preparation

The influence of extraction time and the influence of size of sample weight on determined content of amoxicillin were tested. Excellent results were reached with extraction of analyte by the extraction solvent II containing phosphoric acid (98–102%). However, we found that the amoxicillin dissolved in the extraction solvent containing phosphoric acid, decomposed over time and therefore, this solvent is not acceptable for sample storing and manipulation (Fig. 3). This

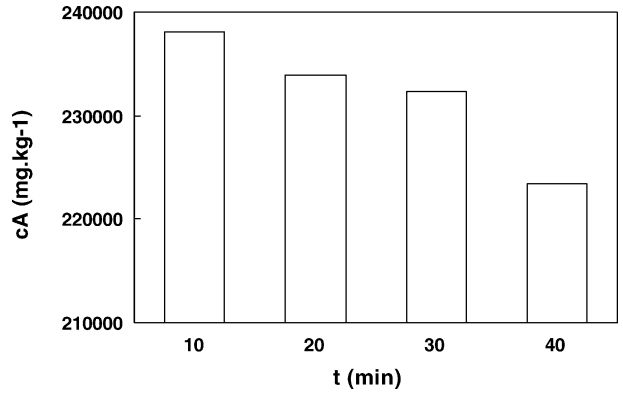

Fig. 3. The influence of extraction time (t) on content of amoxicillin (c_A). Real sample of premix of amoxicillin. Declared value of amoxicillin is 200,000 mg kg^{-1}.

conclusion is the same as in reported method [22]. The authors would find out that amoxicillin decomposed and the concentration of amoxicillin in the acidic solution was decreased. In the end, an extraction solvent I (without phosphoric acid) was found to be a reasonable compromise.

By reason of solubility of amoxicillin in extraction solvent, the content of extracted amoxicillin decreases with increase of sample weight. The optimum of sample weight ranged from 0.5 to 1.0 g for 100 ml volume of extraction solvent (Fig. 4).

3.5. Solution stability

In this study, the stability of amoxicillin in working standard solution and sample preparation solution were studied. In case of an unexpected delay during analysis, it is important to have information about the stability of all solutions. The working standard solutions and sample preparation solution was stored at ambient temperature in the dark over the period of 24 h. The responses for the aged solutions were evaluated using a freshly prepared standard. The sample preparation

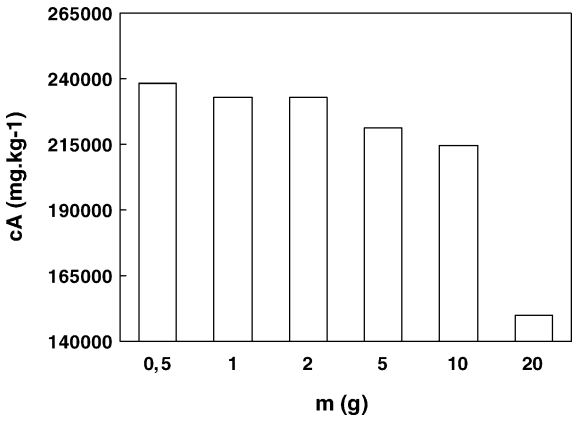

Fig. 4. The influence of size of sample weight (m) on content of amoxicillin (c_A). Real sample of premix of amoxicillin. Declared value of amoxicillin is 200,000 mg kg^{-1}.

Table 3
Solution stability results

Interval (h)	Dark/ambient (% peak area relative to initial)	
	Standard solution	Sample solution
0	100.0	100.0
4	99.0	99.9
12	98.2	99.9
24	97.1	98.7

solution was diluted before assay. The evaluation was done in relative percentage where $t = 0$ result was taken as 100%. The results are summarized in Table 3.

The working standard solution is stable for 4 h, when stored under dark/ambient temperature conditions. The sample preparation extract is stable for 12 h, when stored under dark/ambient temperature conditions.

4. Conclusion

The developed HPLC procedure allows short analysis time (below 10 min) with sensitive UV detection and it is convenient for determination of amoxicillin in premixes in content ranging from 20,000 to 500,000 mg kg^{-1}. In comparison with described method for determination of amoxicillin in feeds [15], developed HPLC method is very simple, rapid and enough sensitive for determination in premixes without derivatization step. Elimination of interfering compounds, without loss of target analyte, is achieved. Evaluation of method demonstrates satisfactory statistical parameters for its application of amoxicillin determination in studied matrices.

Preparation of samples in series and short chromatographic run also offers use of developed method in routine laboratory assays.

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