Analytical Biochemistry 468 (2015) 75–82

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Group determination of 14-membered macrolide antibiotics and azithromycin using antibodies against common epitopes

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ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form 14 August 2014
Accepted 15 September 2014
Available online 22 September 2014

Keywords:
Erythromycin
Clarithromycin
Roxithromycin
Azithromycin
Group-specific immunoassay

ABSTRACT

Erythromycin (ERY), clarithromycin (CLA), roxithromycin (ROX), and azithromycin (AZI) are macrolide antibiotics widely used in livestock and human medicine. Therefore, they are frequently found as pollutants in environmental water. A method based on indirect competitive enzyme-linked immunosorbent assay (ELISA) for group determination of these macrolides in foodstuffs, human biofluids, and water was developed. Carboxymethyloxime of clarithromycin (CMO–CLA) was synthesized and conjugated to bovine serum albumin (BSA) and gelatin to prepare immunogen and coating antigen with advantageous presentation of target epitopes, L-cladinose and D-desosamine, common for these analytes. Antibodies generated in rabbits were capable of recognizing ERY, CLA, and ROX as a group (100–150%), and AZI (12%) and did not cross-react with ERY degradants, which lack antibiotic activity. Assay displayed sensitivity of determination of 14-membered macrolides (IC_{50} = 0.13–0.2 ng/ml) and low limit of detection (LOD) that was achieved at 0.02 to 0.03 ng/ml. It allowed performing analysis of milk, muscle, eggs, bovine serum, water, human serum and urine, and avoiding matrix effect without special pretreatment using simple dilution with assay buffer. For 15-membered macrolide AZI, the corresponding characteristics were IC_{50} = 1.6 ng/ml and LOD = 0.14 ng/ml. The recoveries of veterinary and human medicine macrolides from corresponding matrices were validated and found to be satisfactory.

A family of antimicrobial macrolides is represented by groups of compounds with 14-, 15-, and 16-membered lactone ring [1]. The first macrolide antibiotic, erythromycin (ERY),[1] was isolated in 1952 from a strain of *Streptomyces erythreus* later assigned to *Saccharopolyspora erythraea* [2]. ERY has a wide antimicrobial spectrum but is found to be instable in acid medium. A more stable related 14-membered structural analog, oleandomycin, was discovered in 1954. However, because of less activity in comparison with ERY, it is hardly used in practice at this time. The following attempts to create acid-resistant antibiotics resulted in synthetic ERY derivatives roxithromycin (ROX), clarithromycin (CLA), and azithromycin (AZI). The latter has a 15-membered ring that is a result of methyl-substituted nitrogen atom incorporation into the lactone ring of 14-membered macrolides between C9 and C10 (Fig. 1).

The mechanism of antimicrobial action of these macrolides is associated with reversible binding to the 50S subunit of the bacterial ribosome that results in the blockage of peptide chain elongation and, thus, inhibition of protein synthesis. They display bacteriostatic activity and bactericide at high doses against gram-positive microorganisms (e.g., *Staphylococcus* spp., *Streptococcus* spp., *Bacillus anthracis*, *Corynebacterium diphtheriae*) and gram-negative species (e.g., *Neisseria gonorrhoeae, Haemophilus influenzae, Campylobacter jejuni, Bordetella pertussis, Brucella* spp., *Legionella* spp.) as well as against *Mycoplasma* spp., *Chlamydia* spp., *Treponema* spp., *Rickettsia* spp., *Entamoeba histolytica,* and *Listeria monocytogenes.*

The macrolides are considered to be the least toxic among antibiotics and belong to the safest antibacterials, which are well tolerated by patients [3]. Allergic reactions are extremely rare; nevertheless, several cases are described in the literature [4–8]. Dyspeptic disorders are common after administration of macrolides and are due to their agonistic effect on motilin receptors [9,10]. Besides, the macrolides are known for their ability to modulate inflammation and immunity in humans by influencing the

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[1] *Abbreviations used:* ERY, erythromycin; ROX, roxithromycin; CLA, clarithromycin; AZI, azithromycin; ELISA, enzyme-linked immunosorbent assay; CMO–CLA, carboxymethyloxime of clarithromycin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; BSA, bovine serum albumin; Gel, gelatin; DMF, dimethylformamide; CBB, carbonate–bicarbonate buffer; PBS, phosphate-buffered saline; PBS-T, PBS containing Tween 20; LOD, limit of detection; MRL, maximum residue limit.

http://dx.doi.org/10.1016/j.ab.2014.09.009
0003-2697/

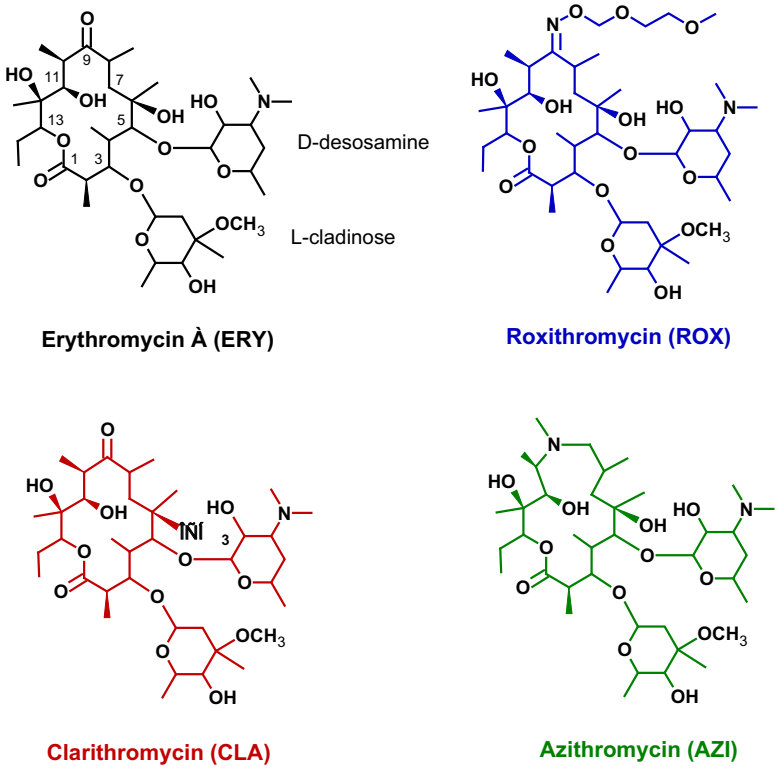

Fig.1. Structural formulas of 14-membered macrolides and azithromycin.

Table 1
Maximum residue limits (μg/kg) of ERY in livestock products established in the Russian Federation, the European Community, and the United States and by the Codex Alimentarius Commission.

	RF, EC	USA	CAC
Muscle	200	100	100
Fat	200	–	100
Liver	200	100	100
Kidney	200	100	100
Milk	40	–	–
Eggs	150	25	50

Note: RF, Russian Federation; EC, European Community; USA, United States; CAC, Codex Alimentarius Commission.

production of cytokines, decreasing mucus hypersecretion, inhibiting chemotaxis of neutrophils, accelerating apoptosis, and owing to many other effects on cellular functions [11,12].

ERY is widely used in human medicine and in veterinary practice for treatment of infection diseases in cattle, sheep, swine, and poultry. To avoid possible adverse effects and allergic sensitization and to prevent expansion of resistant microorganisms, the limitations for ERY content in edible products of animal origin are established in many countries [13–16]. Table 1 shows the levels of tolerance that were established for ERY residues in foodstuffs by authorities of several countries. Moreover, a number of articles provide evidence of high occurrence of macrolides in environmental specimens [17–19].

Antibiotics may be detected by microbioassay using the test strains of microorganisms that are susceptible to macrolides. However, it lacks specificity and is not sufficiently sensitive. Various physicochemical methods, such as different chromatographic, spectrophotometric, and electrochemical methods, have been described and used for both separate and simultaneous determination of macrolides [20]. Among immunoassays as inexpensive and convenient screening methods, a radioimmunoassay of erythromycin and derivatives was developed [21]. These derivatives, including anhydroerythromycin (a product of acid degradation of ERY), showed a high extent of cross-reactivity. They displayed a motilin-like effect but had no antimicrobial activity. Palleschi's group presented two works concerning the detection of macrolide antibiotic residues in bovine meat using electrochemical immunosensor based on enzyme-linked immunosorbent assay (ELISA) [22,23]. The commercial antibodies applied in this research were reported to be able cross-react only to ROX (145%). Methods for determination of macrolide antibiotics such as CLA, ROX, and AZI using specific antibodies have not been reported before. The current study is an attempt to prepare antibody for group recognition of macrolides and develop an ELISA that could serve to determine antibiotics employed in human medicine (ERY, ROX, CLA, and AZI) as well as in veterinary practice (ERY).

Materials and methods

Chemicals

9-(Carboxymethyloxime)-clarithromycin (CMO–CLA) was obtained from the Gause Institute of New Antibiotics (Moscow, Russia). Erythromycin, clarithromycin, roxithromycin, azithromycin, Freund's complete adjuvant, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), *o*-phenylenediamine, goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase, bovine serum albumin (BSA), and gelatin (Gel) were purchased from Chimmed (Moscow, Russia). Dimethylformamide (DMF) was obtained from Serva (Heidelberg, Germany). Bovine serum was obtained from Biolot (St. Petersburg, Russia). Samples of human blood serum and urine were obtained from the polyclinic department of the Mechnikov Research Institute for Vaccines and Sera (Moscow, Russia). Foodstuffs were purchased from regional supermarkets.