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Characterization of impurities in tylosin using dual liquid chromatography combined with ion trap mass spectrometry

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ABSTRACT

Investigation of unknown impurities in a tylosin sample was performed using liquid chromatography coupled to mass spectrometry (LC/MS). Separation was performed according to the recently described LC-UV method of Ashenafi et al. (2011) [14]. This method was reported to have a good selectivity as it was able to separate the four main components of tylosin from the already known and 23 unknown impurities. However, as this method uses a mobile phase with non-volatile constituents, direct characterization of these impurities using LC/MS was not possible. The impurity fractions were therefore first collected and then desalted before sending them to the MS. Identification of the impurities in the tylosin sample was performed with a quadruple ion trap (IT) MS, with an electrospray ionization (ESI) source in the positive ion mode. The structure of the impurities was deduced by comparing their fragmentation pattern with those of the main components of tylosin. As several peaks in the LC-UV method contained multiple compounds, using this method in total 41 new impurities were (partly) characterized.

1. Introduction

Tylosin is a macrolide antibiotic produced by *Streptomyces fradiae* [1]. It is a medium spectrum antibiotic used in veterinary medicine for the treatment of infections caused by most Gram-positive bacteria, mycoplasmas, some Gram-negative bacteria and *Chlamydia* species [2]. It is also used as a feed additive to promote livestock growth [3].

Tylosin (Fig. 1) is composed of a 16-membered polyketide lactone ring (produced as tylactone and converted to tylonolide after its oxidation at C-20 and C-23) and three 6-deoxyhexose sugars: mycaminose, mycinose and mycarose [4,5]. Tylosin is a complex mixture of four main components: tylosin A (TA), tylosin B (TB) or desmycosin, tylosin C (TC) or macrocin and tylosin D (TD) or relomycin of which TA is the major component. All the main components contribute to the potency of tylosin, but TA is the most active [6]. Several related substances are also formed during the production of tylosin by fermentation. A few of these, 5-*O*-mycaminosyltylonolide (OMT), demycinosyltylosin (DMT), lactenocin (LACT) and 20-dihydrotylosin B (DHTB) have been described in literature. Other impurities like tylosin A aldol (TAD) and isotylosin A (IsoTA), have also been reported. TAD is formed from TA in neutral and alkaline solutions whereas IsoTA is a photodegradation product formed on exposure of a TA solution to light [7–9].

A stringent control of the quality of veterinary medicines is required to ensure the safety when these animals are used in food production. So, these drugs must also comply with regulatory guidelines related to quality, safety and efficacy. Content determination, identification of the unknown (UNK) impurities and establishing proper limits for them are important parameters that help to evaluate the quality of a drug.

Several LC methods have been reported for the purity control of tylosin. Fish and Carr described an LC-UV method [10], which is also recommended by the European Pharmacopoeia (Ph. Eur.) to determine the composition of tylosin [11]. Later, Roets et al. [12] described an LC-UV method which was suitable for the determination of TA, TB, TC, TD, DMT and three minor degradation products. In 1995, Paesen et al. could separate TA from its known and two additional unknown impurities [13]. All these methods either had limited selectivity for the related substances or were only useful for the determination of the main components. Recently, a method with good selectivity for the main components and related substances of tylosin was developed by Ashenafi et al. This method was able to separate the four main components of tylosin from its known and over 20 unknown impurities [14].

It is evident from the literature that not many efforts have been spent to identify unknown impurities in tylosin samples. However, impurity profiling of a drug is an integral part of establishing drug

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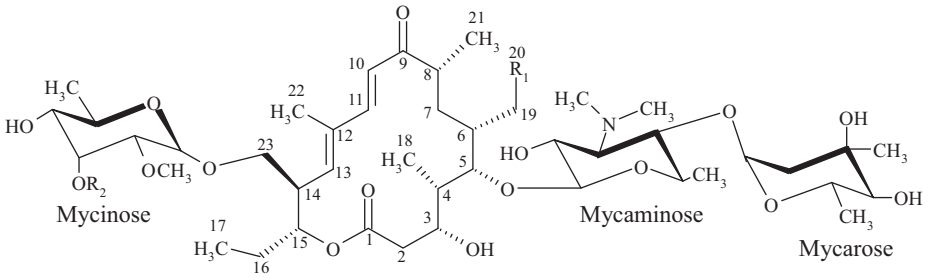

	R_1	R_2	Mycarose	Mycinose
Tylosin A	CHO	CH_3	+	+
Tylosin B (desmycosin)	CHO	CH_3	-	+
Tylosin C (macrocin)	CHO	H	+	+
Tylosin D (relomycin)	CH_2OH	CH_3	+	+
Lactenocin (LACT)	CHO	H	-	+
5-O-mycaminosyltylonolide (OMT)	CHO		-	-
Demycinosyltylosin (DMT)	CHO		+	-
20-dihydrotylosin B (DHTB)	CH_2OH	CH_3	-	+

+ = sugar present; - = sugar not present

Tylosin A Aldol (TAD) Isotylosin A (IsoTA)

Fig. 1. Chemical structures of tylosin components and some of the related substances.

quality standards. In routine practice, veterinary drugs must comply with the guidelines of regulatory authorities like the International Cooperation on Harmonization on technical requirements for registration of Veterinary products (VICH), which states that all impurities above a threshold of 0.2% should be characterized [15]. An important remark is that antibiotics produced by fermentation have been kept out of this framework. So, to control their quality, the European Medicines Agency (EMA) published a guideline with thresholds for reporting, identification and qualification of related substances in them. The identification threshold is 0.20% for the related substances in antibiotics which are produced by fermentation, contain one or more than one active component and are used exclusively in veterinary medicine [16]. Impurities in these antibiotics are mostly structurally similar compounds present in low concentrations.

The aim of this work was to characterize the unknown impurities in a tylosin sample, separated with the method of Ashenafi et al. [14]. As the mobile phase of the latter method contains non-volatile constituents, direct coupling with MS was not possible. So, peaks were collected in fractions which were desalted using a second LC system before introduction into the MS. This approach has been successfully applied to other macrolide antibiotics [17,18]. Our approach in this work was to investigate as many as possible impurities, even if their content was lower than the ICH and EMA threshold of 0.2%. The reason behind this approach was that as a result of fermentation, the amounts of impurities in antibiotics can vary depending on the origin.

2. Experimental

2.1. Reagents and samples

Acetonitrile (ACN) MS grade and formic acid (FA) 99% ULC/MS grade were purchased from Biosolve LTD (Valkenswaard, the Netherlands). LC gradient grade ACN was purchased from Fischer Scientific (Leicester, United Kingdom). Dipotassium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Acros (Geel, Belgium). Nitrogen supplied by Air Liquide (Liège, Belgium) was used as sheath and auxiliary gas for MS. Helium gas was purchased from Air Products (Brussels, Belgium). Water was produced in-house by further purifying demineralized water using a Milli-Q Gradient water purification system (Millipore, Bedford, MA, USA). The tylosin sample was obtained from Shandong Lukang Pharmaceutical (Jining, Shandong, China). A house standard of TA and tylosin that could be used for peak identification containing known amounts of TA, TB, TC, TD and other impurities were available in the laboratory. TB, TC, TD, LACT, OMT, DMT, DHTB, TAD and isoTA were also available in the laboratory as reference substances.

2.2. Liquid chromatographic instrumentation and conditions

2.2.1. LC-UV for collecting the impurity peaks

An LC-UV system from Dionex (Germering, Germany) was used for the separation of the impurity peaks from the main