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Characterization of impurities in josamycin using dual liquid chromatography combined with mass spectrometry

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ABSTRACT

The European Pharmacopoeia (Ph. Eur.) prescribes a selective and sensitive liquid chromatography/ultraviolet (LC–UV) method for the separation of the 16-membered ring macrolide josamycin and its related compounds. Since josamycin is obtained by fermentation, several closely related substances can be found in the sample. Several impurities have already been identified using reference substances. However, many peaks in the chromatogram cannot be correlated with known compounds or correspond to structures which were not described previously.

The hyphenation of LC to mass spectrometry (MS) is a very useful tool for the characterization of impurities. The existing LC–UV method however uses non-volatile buffers, while for LC/MS a volatile mobile phase is required. In this study, each peak from the non-volatile system was collected separately and reinjected into a LC system using volatile mobile phase constituents. This way, the analyte could be separated from the buffer salts.

Mass spectral data of this macrolide antibiotic were acquired on a LCQ ion trap mass spectrometer, equipped with an electrospray ionization (ESI) probe operating in the positive ion mode. The identity of the unknown compounds was deduced using the MS/MS and MS^n collision-induced dissociation spectra of reference substances, combined with knowledge about the nature of functional group fragmentation behavior. The impurity profiling was done on 30 peaks in a josamycin bulk sample. This way, 12 compounds reported in the literature and Ph. Eur. were found in the bulk sample. Furthermore, 12 novel related substances were characterized and 18 compounds were partially characterized.

1. Introduction

Josamycin or leucomycin A_3, a 16-membered ring macrolide, is produced by *Streptomyces narbonensis* subsp. *josamyceticus* [1]. It has a spectrum of activity similar to erythromycin against Gram-positive bacteria. However, unlike erythromycin, it does not induce resistance in susceptible micro-organisms. Josamycin is particularly indicated for the treatment of infections of the skin, respiratory tract, ear, nose and throat. The leucomycin complex was isolated from the fermentation broth of *Streptomyces kitasatoensis* [2,3]. As a result several closely related substances can be found in a josamycin bulk sample.

Due to their chemical relationship to the main component (leucomycin A_3), it is difficult to remove these substances. The ICH guidelines for new drug substances prescribe that impurities should be identified above a 0.1% level. Even though antibiotics are not subject to this ICH guideline in view of the fermentation origin, the concept of purity control remains very useful for considering and evaluating impurities.

Structural studies of the leucomycins were initiated by Watanabe et al. [4,5] and his co-workers. Afterwards, Omura et al. [6] made numerous contributions to the structure and stereochemistry of leucomycins, their structural relationship and microbiological activities as well as their biosynthesis. Fig. 1 shows the structures of leucomycin A_3 and related substances. They are characterized by a mycaminosylmycarose disaccharide attached to the C-5 of the chromophoric lactone and have a common diene in positions α, β, γ, δ to the secondary hydroxyl group at C-9 and a formylmethyl group at C-6 [7,8]. The most potent pair is A_1/A_3 (R_2 = isovaleryl), followed by A_4/A_5 (R_2 = butyryl). Moreover, the free hydroxyl member of each pair is more active than its acylated counterpart [9].

An LC–UV method, for the separation of josamycin and its related substances is described in the European Pharmacopoeia (Ph. Eur.) [10] and is based on the method developed by Daidone et al. [11]. This method uses an Hypersil ODS C_{18} column and non-volatile buffer additives in the mobile phase to separate josamycin from the fourteen related substances of known identity and several unknown impurities, present in a commercial sample. The

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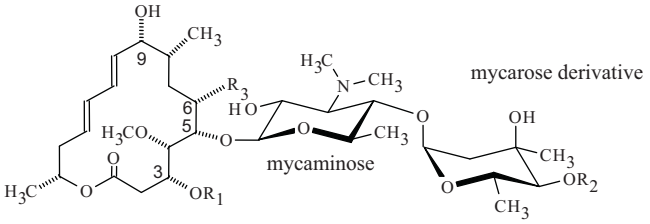
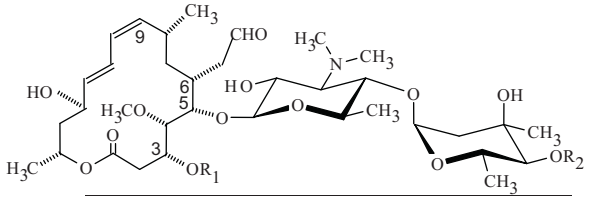


Leucomycin	R_1	R_2	R_3
A_1 or Imp H*	H	$COCH_2CH(CH_3)_2$	CH_2CHO
A_3 = josamycin	$COCH_3$	$COCH_2CH(CH_3)_2$	CH_2CHO
A_4 or Imp A*	$COCH_3$	$COCH_2CH_2CH_3$	CH_2CHO
A_5	H	$COCH_2CH_2CH_3$	CH_2CHO
A_6 or Imp K*	$COCH_3$	$COCH_2CH_3$	CH_2CHO
A_7 or Imp I*	H	$COCH_2CH_3$	CH_2CHO
A_8	$COCH_3$	$COCH_3$	CH_2CHO
A_9 or Imp G*	H	$COCH_3$	CH_2CHO
U (A10)	$COCH_3$	H	CH_2CHO
dHM_1 or Imp B*	$COCH_3$	$COCH_2CH(CH_3)_2$	CH_2CH_2OH
V (A_{11}) or Imp F*	H	H	CH_2CHO
X_2 or Imp J*	$COCH_3$	$CO(CH_2)_4CH_3$	CH_2CHO
X_3 or Imp E*	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$	CH_2CHO



Leucomycin	R_1	R_2
Isojosamycin or Imp D*	$COCH_3$	$COCH_2CH(CH_3)_2$

*according to the Ph. Eur.

Fig. 1. Chemical structures of josamycin and already described related substances.

Ph. Eur. reports five impurities as specified (Imp A, B, C, D and E) and six as other detectable impurities (Imp F, G, H, I, J, K). Furthermore, three additional known related substances are mentioned in the literature, namely A_5, A_8 and U or A_{10}. The aim of this work was to investigate and, if possible, to identify the unknown impurities present in josamycin bulk sample. Moreover, the confirmation of the structure of the known impurities in josamycin bulk sample was performed.

LC coupled with ESI-MS is a powerful and widely used analytical platform for separating and identifying large numbers of species from complex mixtures. However, the hyphenation cannot be done without carefully choosing suitable buffers. Buffers composed of non-volatile salts such as phosphates and sulphates are not compatible with ESI-MS. These species become deposited on the components of the MS, leading to a loss in signal transmission and the formation of clusters with analytes and solvents that suppress and dilute the analyte signal. As alternatives to such buffers, volatile additives such as formic acid and acetic acid are used.

The LCQ ion trap is ideally suited for the identification of related substances because it provides on-line LC/MS^n capability. It has been used before already for the characterization of josamycin propionate, a semi-synthetic derivative of josamycin [12].

Replacing the non-volatile additive by a volatile additive in the mobile phase system is not the best option, because the use of non-volatile additives yields superior chromatographic separation compared to the use of volatile additives. Therefore, a desalting procedure was developed to investigate each peak in the method of the Ph. Eur., which uses non-volatile additives in the mobile phase system. The exact procedure is explained in Section 2.5.

2. Material and methods

2.1. Chemicals

Acetonitrile (ACN) HPLC grade was supplied by Fisher Scientific (Loughborough, United Kingdom). Acetonitrile (LC/MS grade) and formic acid (99% ULC/MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Phosphoric acid 85% m/m, tetrabutylammonium hydrogensulphate 98% (TBA) and sodium dihydrogen phosphate dihydrate were obtained from Acros Organics (Geel, Belgium). A Milli-Q purification system (Millipore, Bedford, MA, USA) was used to further purify demineralized water.

2.2. Samples and sample preparation

The following reference substances were available in the laboratory: A_4, dHM_1, isojosamycin, X_3, V, A_9, A_1, A_7, X_2 and A_6. All reference substances were dissolved separately in acetonitrile/water (30:70, v/v) at a concentration of 0.05 mg/mL. Josamycin bulk powder was obtained from Biochemie (Kundl, Austria). The sample was dissolved at a concentration of 2.5 mg/mL in a mixture of ACN and water (30:70, v/v).

2.3. LC instrumentation and chromatographic conditions

2.3.1. Non-volatile system for LC–UV

The non-volatile mobile phase system consisted of an UltiMate 3000 pump, an ASI-100 automated sample injector from Dionex (Sunnyvale, CA, USA) and a variable-wavelength TSP Spectra 100 UV–VIS detector set at 232 nm (San Jose, CA, USA). The UV data were acquired with Chromeleon software Version 6.60. The Hypersil ODS column (5 μm, 250 mm × 4.6 mm i.d.), was obtained from Thermoquest (Bremen, Germany). The injection volume was 10 μL. The temperature of the column was maintained at 45 °C by immersion in a water bath with a heating circulator (Julabo EM, Seelbach, Germany). The following mobile phases were used for separation: Mobile phase A (MP A): ACN – 27.6 g/L sodium dihydrogen phosphate monohydrate pH 3.0 – 67.9 g/L TBA – water (21:5:3:up to 100 mL, v/v/v/v) and mobile phase B (MP B): ACN – 27.6 g/L sodium dihydrogen phosphate monohydrate pH 3.0 – water (50:5:up to 100 mL, v/v/v) at a flow rate of 2.0 mL/min. The phosphate buffer solution was adjusted to the required pH by adding a 10% solution of phosphoric acid. Strongly retained impurities eluting after the main peak require gradient elution, which is obtained by increasing linearly the acetonitrile concentration (from 21% to 50%, v/v) and decreasing the TBA concentration (from 3% in mobile phase A to 0% in mobile phase B, v/v) in the mobile phase. Gradient elution was performed as follows: 0–38 min, 100% A; 38–55 min, 100% A to 100% B; 55–65 min, 100% A. The mobile phase was degassed before use by sparging with helium.

2.3.2. Volatile system for LC/MS

The volatile mobile phase system consisted of a P680 HPLC pump from Dionex (Sunnyvale, CA, USA), a switching valve (VICI AG International, Schenkon, Switzerland) equipped with a 500 μL loop and a variable-wavelength TSP Spectra 100 UV–VIS detector