Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 347–355
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development of a liquid chromatography method for the analysis of josamycin

F. Daidone, R. Heuvelmans, L. Aerden, J. Hoogmartens, E. Adams *

Laboratory for Pharmaceutical Analysis, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, O&N 2, Herestraat 49 (PB 923), B-3000 Leuven, Belgium

Received 7 September 2007; received in revised form 1 February 2008; accepted 5 February 2008
Available online 12 February 2008

Abstract

Out of three methods for the analysis of josamycin, the best one was selected and used as starting point for further development. A central composite design was applied to find the most influencing parameters and to optimize the chromatographic conditions and a full factorial design was used to perform a robustness study. The final method uses a Hypersil ODS column 5 μm, 250 mm × 4.6 mm i.d. maintained at 45 °C. The mobile phase is composed of acetonitrile–phosphate buffer (pH 3, 0.2 mol l^{-1})–tetrabutylammonium hydrogen sulphate 0.2 mol l^{-1}–water (21:5:3:71, v/v/v/v). Strongly retained impurities 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% to 0%, v/v) in the mobile phase. The total run time was 65 min. UV detection is performed at 232 nm and the flow rate is 1 ml/min. The method shows good selectivity, precision, linearity and sensitivity. Five commercial bulk samples were analyzed.
© 2008 Elsevier B.V. All rights reserved.

Keywords: Josamycin; Liquid chromatography; Analysis; Development; Validation

1. Introduction

Josamycin is a 16-membered macrolide antibiotic effective against mycoplasma, Gram-positive *cocci*, *bacilli* and certain Gram-negative organisms [1]. It is therefore used in human and veterinary practice. Josamycin is identical to leucomycin A3 and is a part of the leucomycin complex. This complex was isolated from the broth of *Streptomyces kitasatoensis* [2,3] and contains up to 14 components with different activity. The most active pair is A1/A3, followed by A4/A5. Leucomycins, like other macrolides have a mechanism of action that is based on protein synthesis inhibition: they bind to the 50 S ribosomal subunit, hence blocking the entrance to the ribosomal tunnel. This eventually leads to the arrest of the growth of the peptide, due to steric hindrance, and the dissociation of the peptidyl-tRNA from the ribosome [4].

Commercial grade josamycin is produced through fermentation by *Streptomyces narbonensis* var. *josamyceticus* [5]. However, the process is not entirely selective and results in the production of a mixture of numerous structurally related compounds. These metabolites are produced through the same biosynthetic pathway, which explains the structural resemblance and the similar therapeutic activity [6]. The extraction process from the fermentation broth, the manufacturing of the final product and the storage can lead to an increase in the quantity of impurities, which can be found in a josamycin commercial sample.

Therefore for quality control, a selective and sensitive method is needed, able to separate and quantify josamycin and its impurities.

The European Pharmacopoeia (Ph. Eur.) prescribes thin-layer chromatography for the purity control of josamycin [5]. This method does not show the necessary selectivity and accuracy required for the determination of josamycin's purity. The Ph. Eur. also prescribes a microbiological assay for the determination of josamycin's activity. As mentioned previously, some metabolites of josamycin are also active, which makes this type of analysis non-selective for the main compound. In the past years, the Ph. Eur. is replacing these tests with more selective methods like liquid chromatography (LC) to perform related substances testing and assay.

* Corresponding author. Tel.: +32 16323443; fax: +32 16323448.
E-mail address: Erwin.Adams@pharm.kuleuven.be (E. Adams).

0731-7085/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2008.02.002

A variety of methods can be found in literature, such as LC coupled with UV, mass spectrometry (MS) and spectrofluorimetric detection. However, most of these methods describe the analysis of josamycin in biological fluids [7–17]. Only a few of them deal with the analysis of josamycin as bulk drug [18,19]. The latter were considered in this article to choose the initial chromatographic system, on which the final method is based.

The aim of this study is to develop a simple, selective and sensitive analytical LC method for the separation of josamycin from its related substances.

2. Experimental

2.1. Reagents and samples

Sodium dihydrogen phosphate monohydrate was purchased from Merck (Darmstadt, Germany). Phosphoric acid 85% and tetrabutylammonium hydrogen sulphate 98% (TBA) were obtained from Acros Organics (Geel, Belgium). Acetonitrile HPLC grade was supplied by Fisher Scientific (Southborough, United Kingdom). A MilliQ-water purification system (Millipore, Milford, MA, USA) was used to purify demineralized water. The phosphate buffer pH 3 was prepared by dissolving the appropriate amount of sodium dihydrogen phosphate monohydrate in water to obtain a 0.2 mol l^{-1} solution and adjusting the pH with phosphoric acid 0.2 mol l^{-1}.

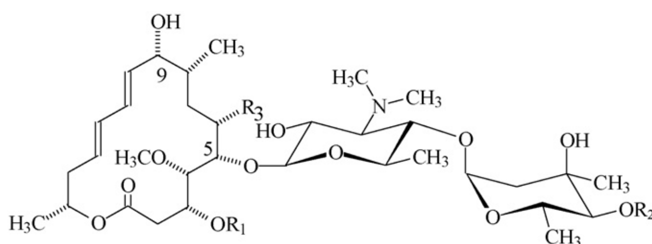
Different commercial josamycin bulk samples were obtained from Astellas (Tokyo, Japan), Yamanouchi (Leiderdorp, Netherlands) and the Ph. Eur. The commercial sample with the highest amount of impurities was chosen for method development and was used to obtain all chromatograms shown. The sample from the Ph. Eur. was used for determination of the quantitative aspects of the method, because a relatively large amount was available. Solutions containing 2.5 mg/ml were analyzed. A 3% (v/v) dilution was used to assess the impurity content. The samples were dissolved in acetonitrile–water (3:7, v/v).

Small amounts of the following josamycin components were isolated from commercial samples and identified: V, A9, A7, A6, A4, A1, isojosamycin, X3 and X2. Reference substance dHM1 was furnished by Astellas. They were used as 0.05 mg/ml solutions to identify the peaks.

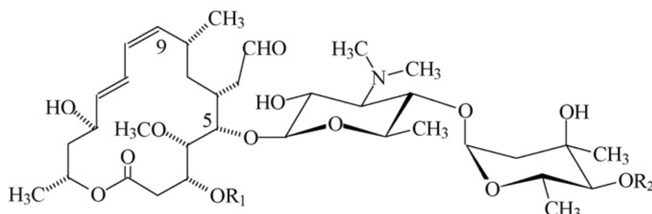
The structure of josamycin and its related substances is shown in Fig. 1.

2.2. Instrumentation and chromatographic conditions

The LC apparatus consisted of an L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany), an autosampler Elite LaChrom Hitachi L2200 and a UV detector Elite LaChrom Hitachi L2400 set at 232 nm. The temperature of the column was maintained by using a water bath (Julabo EM heating circulator, Seelbach, Germany). Chromeleon software (Dionex, Sunnyvale



	R1	R2	R3
Josamycin	$COCH_3$	$COCH_2CH(CH_3)_2$	CH_2CHO
A1	H	$COCH_2CH(CH_3)_2$	CH_2CHO
A4	$COCH_3$	$COCH_2CH_2CH_3$	CH_2CHO
A6	$COCH_3$	$COCH_2CH_3$	CH_2CHO
A7	H	$COCH_2CH_3$	CH_2CHO
A9	H	$COCH_3$	CH_2CHO
dHM1	$COCH_3$	$COCH_2CH(CH3)_2$	CH_2CH_2OH
V	H	H	CH_2CHO
X2	$COCH_3$	$CO(CH_2)_4CH_3$	CH_2CHO
X3	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$	CH_2CHO



	R1	R2
Isojosamycin	$COCH_3$	$COCH_2CH(CH_3)_2$

Fig. 1. Structure of josamycin and some of its related substances.

CA, USA) was used for data acquisition. The developed method made use of a Hypersil column, ODS 5 μm, 250 mm × 4.6 mm i.d. (Thermoquest, Bremen, Germany) and the injection volume was 10 μl.

The chromatographic conditions of the final method are summarized in Table 1.

The final pH of the mobile phase obtained was not the same as that of the phosphate buffer due to the presence of the hydrogen sulphate ion in the TBA solution, of which the pH was not adapted.

2.3. Experimental design

The robustness study and optimization of the method were performed by means of an experimental design and multivariate analysis using Modde 4.0 statistical graphic software (Umetrics, Umea, Sweden). A central composite design and a two-level full factorial design were used for optimization and robustness study, respectively.

The central composite design permits to model surface responses by fitting a second order polynomial model with a number of experiments equal to 2^k+2k+n with k is the number of variables and n the number of extra points at the centre of the design. These central values are important to estimate the experimental error. This design is derived from a two-level full factorial design in which there are 2^k+n points, which are raised with $2k$ points in order to allow the model to evaluate the curvature response.

The parameter variations and consequent responses can be correlated through a second order polynomial model ($Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 + \beta_{ii} X_j^2 + E$), in which the βs are the regression coefficients. The linear coefficients, β_i and β_j, express quantitatively the effect of varying the respective variables; the interaction coefficients, β_{ij}, the interaction between two parameters and the squared terms, $\beta_{ii} X_i^2$ and $\beta_{ii} X_j^2$, quantify the non-linear effect of the variables on the response, β_0 the intercept and E is the experimental error.

The ranges examined in the robustness study were smaller and therefore it can be assumed that the effects of the parameter variations are linear. For this reason a two-level full factorial design, which is a linear model, was chosen.

Table 1
Chromatographic conditions of the three initial methods and the final gradient method

Initial method I		
Stationary phase	Zorbax extend C_{18} 5 μm, 150 mm × 4.6 mm i.d. at 25 °C	
Mobile phase	Acetonitrile	50% (v/v)
	Ammoniumacetate (0.01 mol l^{-1})	50% (v/v)
Initial method II		
Stationary phase	PLRP-S 8 μm, 1000 A, 250 mm × 4.6 mm i.d. at 65 °C	
Mobile phase	2-Methyl-propanol	24.5% (v/v)
	Phosphate buffer (pH 10.5, 0.2 mol l^{-1})	5% (v/v)
	Water	70.5% (v/v)
Initial method III		
Stationary phase	Hypersil® ODS 5 μm, 250 mm × 4.6 mm i.d. at 30 °C	
Mobile phase	Acetonitrile	25% (v/v)
	Phosphoric acid (0.2 mol l^{-1})	5% (v/v)
	TBA (0.2 mol l^{-1})	4% (v/v)
	Water	66% (v/v)
Final gradient method		
Sample	2.5 mg/ml josamycin in acetonitrile–water (3:7, v/v)	
Stationary phase	Hypersil® ODS, 250 mm × 4.6 mm i.d.	
Mobile phase A	Acetonitrile	21% (v/v)
	Tetrabutylammonium hydrogen sulphate (0.2 mol l^{-1})	3% (v/v)
	Phosphate buffer (pH 3, 0.2 mol l^{-1})	5% (v/v)
	Water	71% (v/v)
Mobile phase B	Acetonitrile	50% (v/v)
	Phosphate buffer (pH 3, 0.2 mol l^{-1})	5% (v/v)
	Water	45% (v/v)
Injection volume		10 μl
Column temperature		45 °C
UV detection		232 nm
Flow rate		2 ml/min
Gradient program	0–38 min	100% A
	38–55 min	100 to 0% A
	55–65 min	100% A

This design permits to model surface responses by fitting a first order polynomial with a number of experiments equal to $2^k + n$ with k the number of variables and n the number of extra points at the centre of the design.

The parameter variations and consequent responses can be correlated through a first order polynomial model ($Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + E$), which can be interpreted similarly to the second order polynomial discussed above.

3. Results and discussion

3.1. Method development

3.1.1. Comparison of different methods

Three chromatographic methods for the analysis of josamycin as bulk drug were evaluated to choose the best, which was then further developed. The three initial chromatographic systems were: the method developed by Hu and Hu (method I) [18], the method developed by Paesen et al. (method II) [19] and one that was developed in our lab (method III). The slightly adapted chromatographic parameters of the three methods are summarized in Table 1. The flow rate was always 1 ml/min and the UV detector was set at 232 nm. Method III gave the best overall separation and was retained for further development.

3.1.2. Preliminary method development

Method III was further developed. The following organic modifiers were examined: methanol, 2-methyl-2-propanol and acetonitrile. The latter gave the better separation, lower backpressure and the highest sensitivity. So, it was chosen for further investigation. Besides the 10 known components, seven unknown components were monitored.

A rough optimization of the acetonitrile concentration between 20% and 30% learned that 23% was a good compromise between the overall selectivity and total analysis time.

The phosphoric acid was replaced by a phosphate buffer (0.2 mol l^{-1}, pH 2). This change improved the separation: two additional impurities were separated and therefore it was retained for further investigation. Different concentrations (0.1 M, 0.2 M and 0.5 M) of the phosphate buffer were examined, but the buffer concentration had no significant influence on the selectivity.

The influence of the temperature was roughly checked at 30 °C, 40 °C and 50 °C and 40 °C was found to give the best result in terms of overall selectivity, peak symmetry and total analysis time.

So, the retained conditions are 23% (v/v) of acetonitrile, 4% of TBA (v/v), a column temperature of 40 °C and phosphate buffer pH 2. These conditions allowed the separation or partial separation of 10 known josamycin components and several unknown impurities of which only the critical ones that were coeluted close to the josamycin components were considered for further optimization and are shown in Fig. 2. From further experiments it was observed that under these preliminary conditions A4 was coeluted with an unknown impurity named UNK 1.

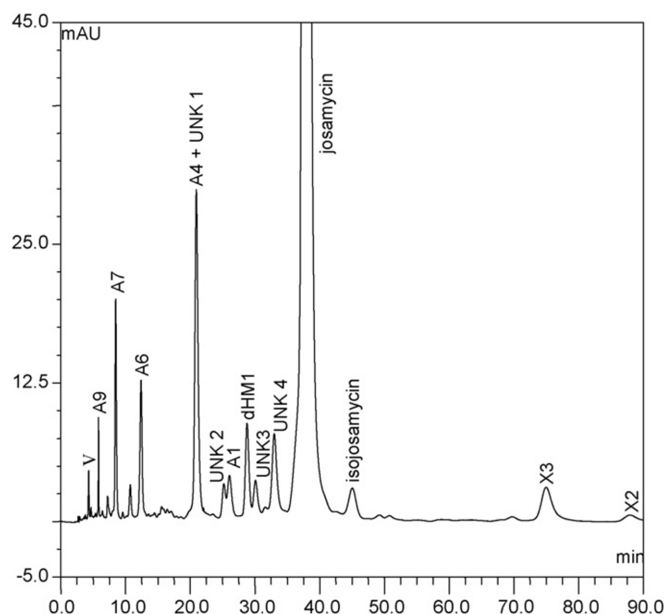


Fig. 2. Typical chromatogram of a real commercial josamycin bulk sample obtained with the preliminary conditions. *Chromatographic conditions*: column: Hypersil® ODS 5 μm, 250 mm × 4.6 mm i.d.; mobile phase: acetonitrile–phosphate buffer (0.2 mol l^{-1}, pH 2)–0.2 mol l^{-1} TBA–water (23:5:4 up to 100, v/v/v/v); flow rate: 1 ml/min; temperature: 40 °C; detection: UV at 232 nm.

3.1.3. Optimization

A central composite design was used to analyze the influence of the chromatographic parameters and to optimize the most important ones.

Four parameters were examined: the amount of acetonitrile and TBA in the mobile phase, the temperature of the column and the buffer pH. No buffer pH below 2 was used to protect the column, so pH 2–4 were examined. The central values were repeated three times. So, the number of experiments equals $2^4 + 2 \times 4 + 3 = 27$. The settings of the parameters in the experimental design are shown in Table 2.

The following responses were considered: the peak to valley ratio (p/v ratio) of critical peak pair A4–UNK 1, resolutions (Rs) between critical peak pairs UNK 2–A1, dHM1–UNK 3 and the retention time (t_r) of josamycin. These values were calculated by applying the formulas of the Ph. Eur. [5]. All the other peak pairs were always separated under the conditions applied in the experimental design and were therefore ignored. The data were collected and processed by using Modde.

The linear and quadratic effects of the parameters and the interactions between two parameters on the responses are sum-

Table 2
Chromatographic parameter settings applied in the central composite design, corresponding to low (−), central (0) and high (+) levels

Parameter	Low value	Central value	High value
pH of phosphate buffer	2	3	4
Temperature (°C)	35	40	45
Acetonitrile (%, v/v)	21	23	25
TBA (%, v/v)	3	4	5

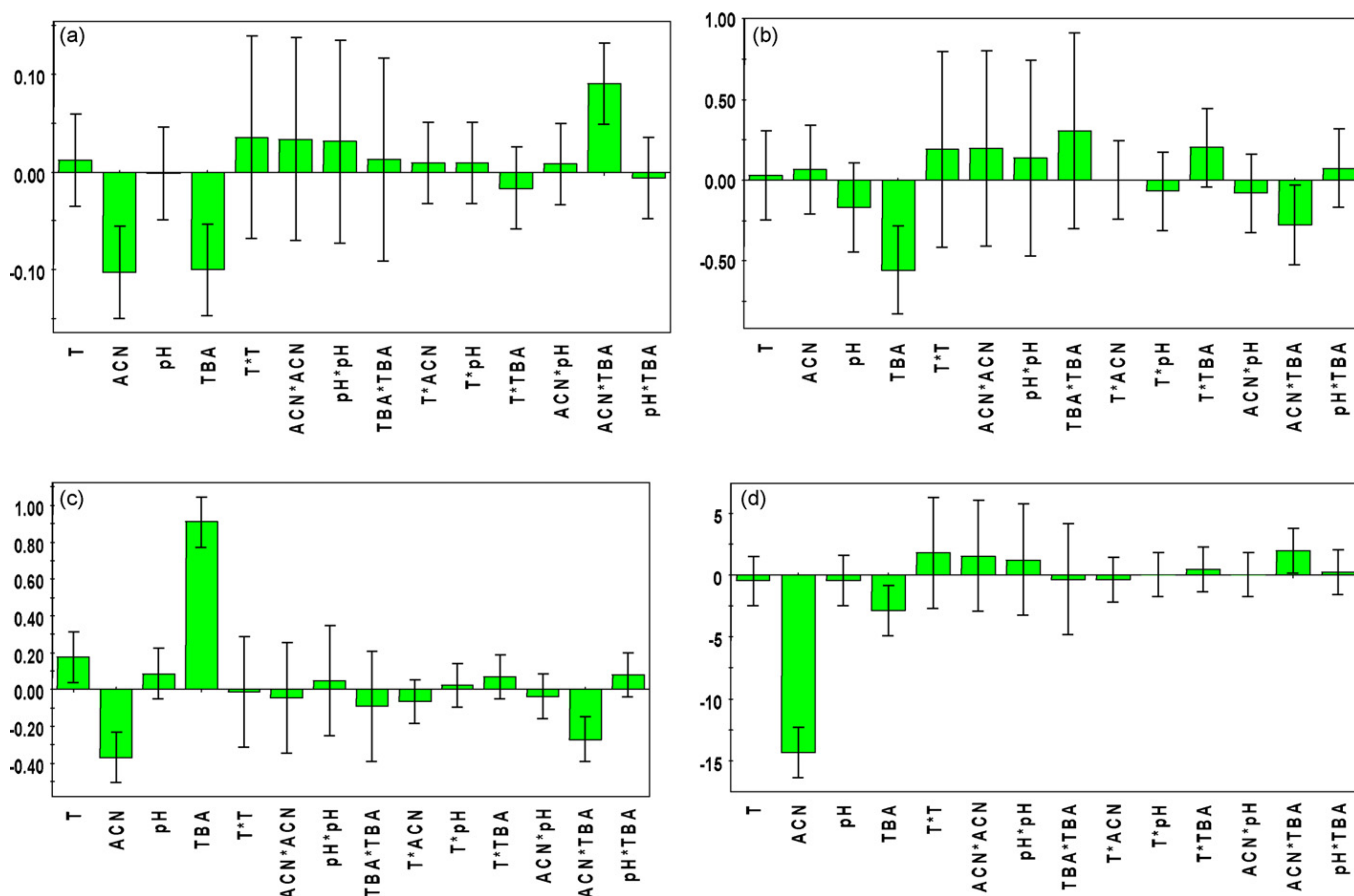


Fig. 3. Regression coefficient plots for the responses of peak couples (a) p/v of A4–UNK 1, (b) Rs of UNK 2–A1, (c) Rs of dHM1–UNK 3 and (d) retention time of josamycin.

marized in the diagrams in Fig. 3. In these diagrams the bars are proportional to the respective effects of the factors and their interactions. The 95% confidence limits are symbolized by lines. If the 95% limit is larger than the regression coefficient, this parameter can be considered as not significant in the system, because the variation of the response caused by the modification of the parameter is smaller than the experimental error.

The coefficients of the terms in the model were estimated by the partial least squares (PLS) method. The reliability of the model is reflected by the R^2 and Q^2 values. R^2 represents the fraction of the response variation that can be explained by the model and Q^2 the fraction of the response variation that can be predicted by the model. The more these values approach 1 the better the experimental values can be explained and predicted by the model. The R^2 and Q^2 values for the responses are shown in Table 3.

Table 3
Summary of the R^2 and Q^2 values for the responses

Response	R^2	Q^2
p/v A4–UNK 1	0.872	0.505
Rs UNK 2–A1	0.904	0.515
Rs UNK 3–UNK 4	0.957	0.758
t_r josamycin	0.960	0.801

Acetonitrile has a negative significant effect on the p/v of A4–UNK 1, the Rs of dHM1–UNK 3 and the t_r of josamycin. TBA has a negative significant effect on the p/v of A4–UNK 1, Rs of UNK 2–A1 and on t_r of josamycin. On the other hand, TBA has a significant large positive effect on Rs of dHM1–UNK 3.

The temperature has only a little positive significant effect on Rs of dHM1–UNK 3.

Response surfaces representing the variation of the response as a function of two of the studied parameters are represented in Fig. 4. The other parameters were kept at their central values.

Response surfaces indicate that the optimal conditions, proposed by the model, for the separation of the different peak pairs were not always the same, and therefore a compromise had to be found to obtain the best overall separation.

The pH had no significant effect on the separation, but it was observed that pH 3 and 4 gave a better symmetry for the main peak and pH 3 was retained.

Increasing the acetonitrile concentration reduced the analysis time, but also reduced the separation of A4–UNK 1, and dHM1–UNK 3. Peak pair A4–UNK 1 could be partially separated with 21% acetonitrile and therefore this concentration was chosen in the optimized mobile phase.

Increasing the TBA concentration reduced the total run time and improved the separation between dHM1 and UNK 3. How-

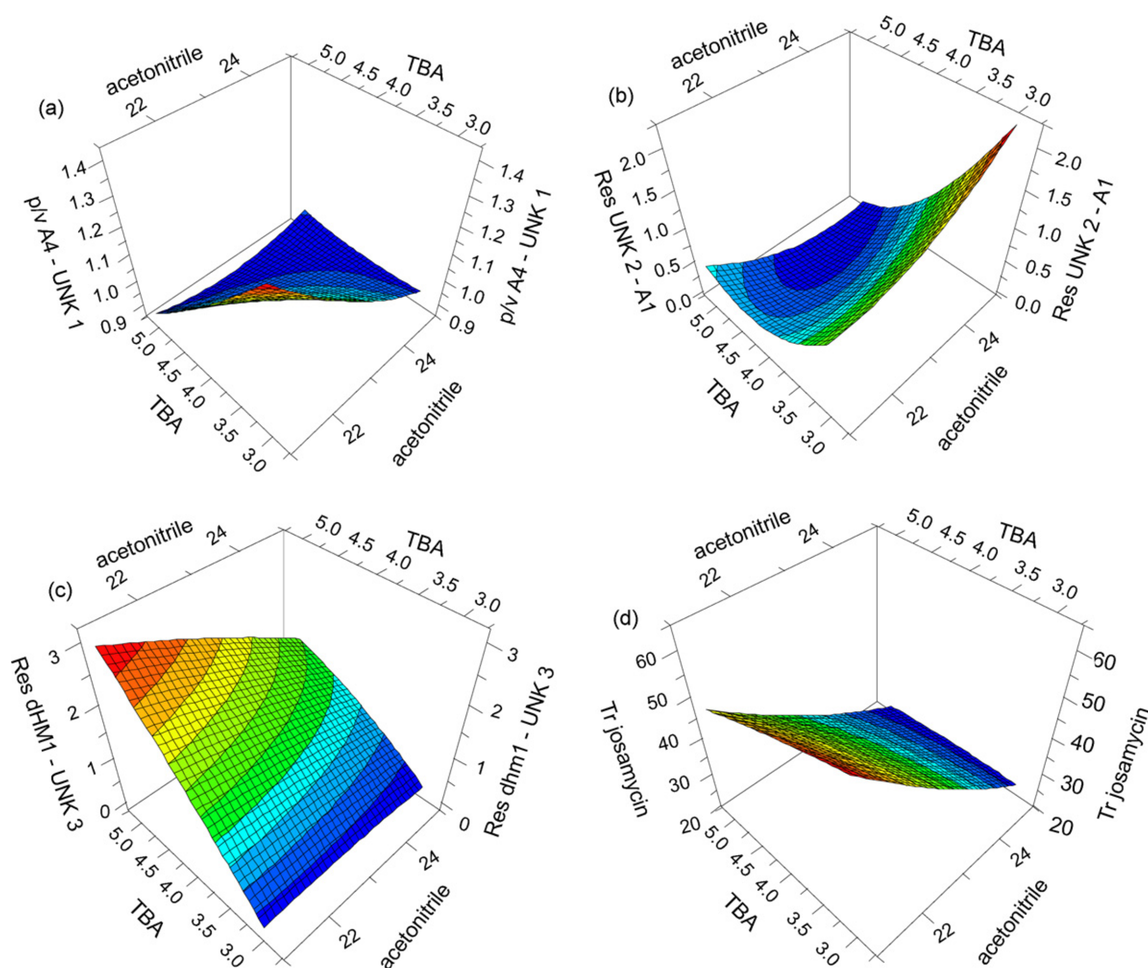


Fig. 4. Response surface plots for the separation of the peak pairs (a) p/v of A4–UNK 1, (b) Rs of A1–UNK 2, (c) Rs of dHM1–UNK 3 and (d) the retention time of josamycin in function of the concentration of acetonitrile, TBA and the temperature.

ever, it decreased the separation between A4–UNK 1 and UNK 2–A1. Since peak pair A4–UNK 1 was only separated at a 3% concentration of TBA, this value was selected.

Rising the temperature improved somewhat the separation of dHM1–UNK 3, hence 45 °C was chosen.

So the optimized conditions are 21% (v/v) of acetonitrile, 3% of TBA (v/v), a column temperature of 45 °C and phosphate buffer pH 3. With the optimized method 10 known impurities and four unknown impurities were separated or partially separated.

The results point out that the most critical factors for the separation of josamycin and its related substances are acetonitrile and TBA, which should both be carefully monitored.

A typical chromatogram of a real commercial bulk sample of josamycin using the optimized method is shown in Fig. 5. X3 and X2 were eluted respectively around 135 min and 160 min and are not shown in Fig. 5.

3.1.4. Gradient development

To reduce the run time and to improve the sensitivity of late eluted peaks, higher flow rates were tried: 1.5 ml/min and 2 ml/min.

Although the analysis time was much shorter using a flow rate of 2 ml/min, it was still insufficient to elute the strongly retained compounds within a reasonable time. So, a gradient was added by increasing the concentration of acetonitrile after the main peak was eluted.

Three major impurities were detected after X2: UNK 5, UNK 6 and UNK 7 (Fig. 6).

In further experiments, a positive effect of TBA reduction was observed on peak pair UNK 5–UNK 6. It was therefore decided to increase ACN (0–50%) and decrease TBA (3–0%) during gradient elution. The total run time decreased to 65 min and the sensitivity of late eluting peaks increased consistently.

The final chromatographic conditions are summarized in Table 1 and a chromatogram of a real commercial josamycin bulk sample obtained under these conditions is depicted in Fig. 6. With the final method 10 known impurities, the seven monitored unknown impurities (UNK 1–7) and in total more than 30 components were partially or totally separated.

3.2. Robustness study

A robustness study was carried out: significant parameters governing the separation were studied in a smaller experimental domain by means of an experimental design.

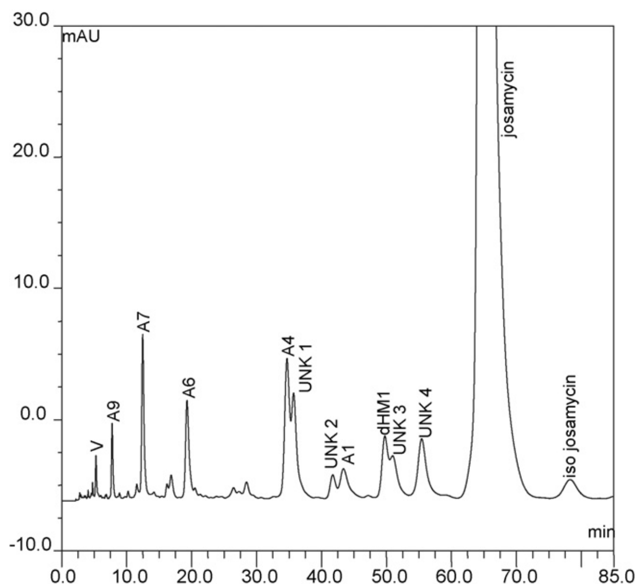


Fig. 5. Typical chromatogram of a real commercial josamycin bulk sample obtained with the optimized method. *Chromatographic conditions*: column: Hypersil® ODS 5 μm, 250 mm × 4.6 mm i.d.; mobile phase: acetonitrile–phosphate buffer (0.2 mol l^{-1}, pH 3)–0.2 mol l^{-1} TBA–water (21:5:3 up to 100, v/v/v/v); flow rate: 1 ml/min; temperature: 45 °C; 25 μg of josamycin injected, detection: UV at 232 nm.

A two-level full factorial design was applied and the factors considered were: the temperature of the column, the TBA and the acetonitrile concentration in the mobile phase. The central values were repeated three times. So, the number of experiments was $2^3 + 3 = 11$.

The settings of the parameters in the experimental design are summarized in Table 4.

The following responses were monitored: p/v ratio of A4–UNK 1, the Rs of peak pairs UNK 2–A1, dHM1–UNK 3 and UNK 6–UNK 7 and the t_r and theoretical plates of josamycin. The responses were calculated according to the formulas of the Ph. Eur. and were processed using Modde as described in Section 2.3.

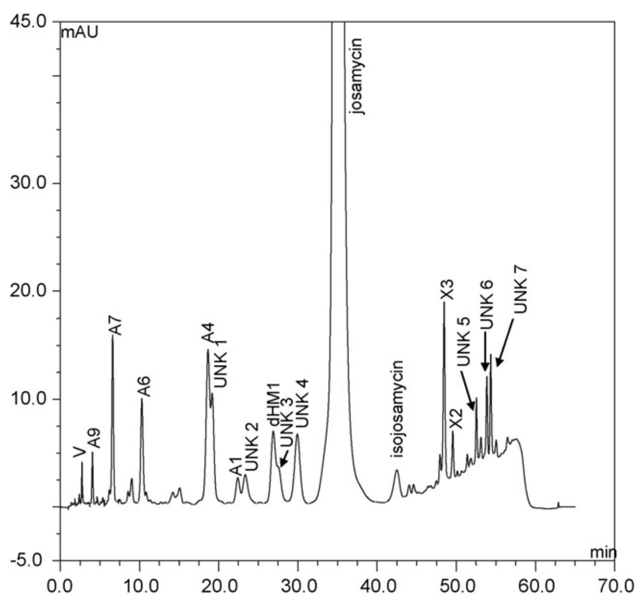


Fig. 6. Typical chromatogram of a real commercial bulk sample of josamycin obtained with the final method. *Chromatographic conditions*: as described in Table 1.

Table 4
Chromatographic parameter settings applied in the robustness study, corresponding to low (−), central (0) and high (+) levels

Parameter	Low value	Central value	High value
Temperature (°C)	42	45	48
Acetonitrile (%, v/v)	20	21	22
TBA (%, v/v)	2.8	3	3.2

The coefficients of the terms in the model were estimated by the multiple linear regression (MLR) method. The reliability of the model is reflected by the R^2 and Q^2 values, which can be interpreted similarly to the values discussed in the experimental design in Section 3.1.3. The R^2 values of all responses were above 0.90. Q^2 values for t_r and theoretical plates of josamycin were both above 0.90, but for other responses they were very small. This means that the model was not able to fully predict the experimental values. This is not exceptional for a robustness test with small ranges since the contribution of the experimental error (which can not be predicted) to the responses measured is considerable.

The results confirmed that in this range also acetonitrile has a negative significant effect on the p/v ratio of A4–UNK 1, on the Rs of dHM1–UNK 3 and on the retention time and theoretical plates of josamycin. On the other hand, acetonitrile has a large positive significant effect on the Rs of UNK 6–UNK 7. TBA was confirmed to have a negative significant effect on Rs UNK 2–A1, but a positive significant effect on the Rs of dHM1–UNK 3. It was observed that it also had a positive effect on the Rs of UNK 6–UNK 7. So, the most critical factors for the separation of josamycin and its related substances are acetonitrile and TBA, which should both be carefully monitored.

3.3. Quantitative aspects

3.3.1. Sensitivity and linearity

The signal-to-noise ratio (S/N) was used to estimate the LOQ (S/N = 10) and the LOD (S/N = 3), which were 0.025% (6.3 ng on column, R.S.D. = 3.2%, n = 6) and 0.0083% (2.1 ng on column), respectively. The concentrations were calculated with respect to the nominal value (100% = 25 μg).

The linearity was evaluated performing repeated analyses in the range LOQ: 125%. Eleven experimental concentrations were considered and three injections were effectued for every value. The results obtained were: $y = 2.8964$, $x + 0.0277$; $R^2 = 0.9999$ and $S_{y,x} = 0.7061$ with y the peak area and x the concentration of the josamycin solution expressed as a percentage (100% = 25 μg), R^2 the coefficient of determination and $S_{y,x}$ the standard error of estimate. Good linearity was observed in the range studied.

3.3.2. Precision

The precision was assessed using multiple preparations of the same josamycin sample. Two different josamycin solutions

Table 5
Precision data (%R.S.D.) for josamycin components

Component	V	A9	A7	A6	A4	UNK 1	UNK 2	A1	dHM1	UNK 3	UNK 4	Isojosamycin	X3	X2	UNK 5	UNK 6	UNK 7
Component level (%)	0.1	0.1	0.4	0.6	1.6	1.1	0.4	0.6	1.5	0.5	1.7	0.7	2.0	0.2	0.3	0.4	0.5
Day 1 (%R.S.D., $n=6$)	2.4	9.4	2.1	1.8	2.1	1.6	2.7	1.7	1.9	3.7	3.7	6.9	2.2	1.7	5.1	1.2	1.3
Day 2 (%R.S.D., $n=6$)	2.4	1.3	1.8	1.9	2.4	5.8	2.9	1.5	2.1	6.5	1.8	3.1	2.3	1.8	7.2	1.1	1.5
Day 3 (%R.S.D., $n=6$)	2.0	9.0	3.6	2.6	2.8	3.0	1.9	2.6	2.2	4.7	2.9	2.0	2.5	2.8	1.7	3.6	2.0
Days 1–3 (%R.S.D., $n=18$)	2.8	10.9	3.9	4.7	5.3	8.2	3.0	2.1	3.5	8.4	6.8	4.7	2.5	2.1	5.2	2.7	1.8

Table 6
Impurities found in commercial samples (%m/m)

RRT	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
0.08 (V)	<D.L.	<D.L.	<D.L.	0.16 (2.2)	0.25 (5.1)
0.12 (A9)	<D.L.	0.16 (6.7)	<D.L.	0.29 (6.6)	0.38 (2.1)
0.19 (A7)	0.18 (4.5)	0.77 (1.80)	0.36 (3.6)	1.74 (1.3)	1.48 (4.3)
0.25	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.
0.26	<D.L.	0.15 (3.4)	0.17 (4.0)	0.29 (1.9)	0.37 (1.8)
0.30 (A6)	0.32 (1.7)	1.24 (1.26)	0.54 (2.6)	1.58 (1.2)	1.38 (0.9)
0.41	<D.L.	0.17 (2.3)	<D.L.	0.25 (2.0)	<D.L.
0.43	<D.L.	0.20 (2.0)	0.17	0.31 (1.5)	0.40 (1.5)
0.54 (A4)	0.86 (1.9)	3.60 (1.7)	1.50 (2.7)	4.07 (1.0)	4.19 (1.7)
0.55 (UNK 1)	0.22 (6.0)	0.94 (5.1)	1.16 (3.0)	1.48 (2.4)	1.99 (3.1)
0.64 (UNK 2)	<D.L.	0.35 (1.0)	0.41 (1.9)	0.52 (0.9)	0.60 (0.8)
0.67 (A1)	0.42 (3.3)	0.85 (1.4)	0.55 (2.6)	0.74 (0.9)	0.57 (1.0)
0.77 (dHM1)	1.00 (2.6)	1.82 (1.3)	1.47 (2.2)	2.05 (2.5)	0.49 (3.7)
0.79 (UNK 3)	0.20 (2.9)	0.47 (2.4)	0.49 (4.7)	0.61 (7.7)	0.87 (1.8)
0.86 (UNK 4)	1.93 (3.5)	1.81 (1.1)	1.69 (2.9)	1.88 (0.7)	1.98 (1.5)
1.22 (isojosamycin)	1.05 (1.7)	0.81 (1.4)	0.68 (2.0)	0.81 (1.0)	0.94 (1.0)
1.27	<D.L.	0.18 (1.6)	<D.L.	< D.L.	0.15 (1.8)
1.29	< D.L.	<D.L.	<D.L.	<D.L.	0.24 (1.2)
1.39	<D.L.	0.18 (3.1)	0.18 (8.1)	0.23 (5.4)	0.27 (5.1)
1.40 (X3)	3.55 (0.9)	1.97 (1.2)	2.02 (2.5)	1.79 (0.4)	2.17 (0.8)
1.43 (X2)	<D.L.	0.40 (1.4)	0.19 (2.8)	0.46 (1.9)	0.44 (2.3)
1.52 (UNK 5)	<D.L.	0.34 (2.9)	0.28 (1.7)	0.43 (1.8)	0.64 (0.7)
1.53	<D.L.	0.2 (8.9)	<D.L.	0.19 (11)	0.22 (1.4)
1.56 (UNK 6)	0.18 (2.0)	0.81 (3.4)	0.43 (3.6)	0.72 (2.1)	<D.L.
1.57 (UNK 7)	<D.L.	0.48 (2.5)	0.48 (2.1)	0.76 (4.8)	1.09 (1.4)
Total of impurities	9.91	17.9	12.77	21.36	21.11

RRT: relative retention time; D.L.: disregard limit. The R.S.D. values ($n=6$) are given in parentheses. Total of impurities is the sum of all impurities above the disregard limit.

(2.5 mg/ml) were prepared and analyzed in triplicate on a single day. New preparations were made and analyzed the same way on 2 other days. The impurity content was determined using a 3% dilution of the sample as reference. R.S.D. values for the impurities were calculated for the first, second and third day separately ($n=6$) and also for 3 days together ($n=18$). The data obtained are summarized in Table 5.

Since no josamycin reference substance with certified content was available in the laboratory, no assay could be performed. The repeatability of the method was assessed by six replicate analyses of a 2.5 mg/ml josamycin solution. The R.S.D. on the areas of the main peak was 0.45% ($n=6$).

3.3.3. Analysis of commercial samples

Five commercial samples were analyzed. For each sample two fresh 2.5 mg/ml solutions were prepared and analyzed in triplicate. The impurity content was assessed using a 3% dilution of each sample. The impurities are expressed as josamycin.

Over 30 impurities were detected in the samples analyzed. Several were very small, just above the limit of quantification. A 0.15% disregard limit was set to reduce the number of impurities shown in Table 6. The results show that A7, A6, A4, UNK 1, dHM1, UNK 4, isojosamycin and X3 were present in all analyzed samples and that they were the impurities with the highest content.

4. Conclusion

The gradient method developed is suitable for the separation of josamycin and its related substances in bulk samples. It can separate josamycin from more than 30 impurities, which is much better than obtained with previous methods. The method

shows good sensitivity, linearity and repeatability. The results of the experimental design showed that the acetonitrile and TBA content in the mobile phase have the greatest influence on the selectivity.

Acknowledgement

E. Adams is a postdoctoral fellow of the Fund for Scientific Research (FWO) Flanders, Belgium.

References

[1] Y. Kikuchi, T. Teramura, J. Sekino, T. Nishimura, H. Miura, T. Watanabe, S. Higuchi, J. Chromatogr. B: Biomed. Sci. Appl. 720 (1998) 81–87.
[2] T. Hata, Y. Sano, N. Ohki, Y. Yokoyama, A. Matsumae, S. Ito, J. Antibiot. (Tokyo) 6 (1953) 87–89.
[3] S. Omura, Y. Hironaka, T. Hata, J. Antibiot. (Tokyo) 23 (1970) 511–513.
[4] T. Tenson, M. Lovmar, M. Ehrenberg, J. Mol. Biol. 330 (2003) 1005–1014.
[5] European Pharmacopoeia, Monograph 1983, 5th edition, Council of Europe, Strasbourg, France, 2005.
[6] C. Vezina, C. Bolduc, A. Kudelski, P. Audet, Antimicrob. Agents Chemother. 15 (1979) 738–746.
[7] M. Ducci, V. Scalori, Int. J. Clin. Pharmacol. Res. 4 (1984) 195–199.
[8] K. Rader, A. Wildfeuer, A. Schwedass, H. Laufen, J. Chromatogr. 344 (1985) 416–421.
[9] M. Skinner, I. Kanfer, J. Chromatogr. 459 (1988) 261–267.
[10] P. Leroy, D. Decolin, A. Nicolas, P. Anhimbault, Analyst 119 (1994) 743–747.
[11] M. Horie, K. Saito, R. Ishii, T. Yoshida, Y. Haramaki, H. Nakazawa, J. Chromatogr. A 812 (1998) 295–302.
[12] Y. Kikuchi, T. Teramura, J. Sekino, T. Nishimura, H. Miura, T. Watanabe, S. Higuchi, J. Chromatogr. B 720 (1998) 81–87.
[13] C. Leal, R. Codony, R. Compano, M. Granados, M.D. Prat, J. Chromatogr. A 910 (2001) 285–290.
[14] R. Codony, R. Compañó, M. Granados, J.A. García-Regueiro, M.D. Prat, J. Chromatogr. A 959 (2002) 131–141.
[15] M. Horie, H. Takegami, K. Toya, H. Nakazawa, Anal. Chim. Acta 492 (2003) 187–197.
[16] M.J. Gonzalez de la Huebra, G. Bordin, A.R. Rodriguez, Anal. Chim. Acta 517 (2004) 53–63.
[17] M.J. Gonzalez de la Huebra, U. Vincent, J. Pharm. Biomed. Anal. 39 (2005) 376–398.
[18] M. Hu, C.-Q. Hu, Anal. Chem. Acta 535 (2005) 89–99.
[19] J. Paesen, A. Solie, E. Roets, J. Hoogmartens, Fresenius J. Anal. Chem. 352 (1995) 797–800.