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Liquid chromatography of josamycin propionate on poly(styrene–divinylbenzene)

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Abstract

An isocratic liquid chromatographic method is described using as stationary phase the very stable poly(styrene–divinylbenzene) PLRP-S (8 μm, 1000 Å). The mobile phase was acetonitrile–0.2 *M* phosphate buffer, pH 10.0–water (52:20:up to 100, v/v/v), delivered at a flow-rate of 1.0 ml/min. UV detection was performed at 232 nm. The column was heated at 60°C. Josamycin propionate was separated from all impurities which were present in commercial samples. The main impurities were josamycin, leucomycin A4 propionate, josamycin 2′,9-dipropionate, josamycin 3″,9-dipropionate and platenomycin A1 propionate. Full factorial design was applied to evaluate the robustness of the method.

Keywords: Pharmaceutical analysis; Josamycin propionate; Antibiotics

1. Introduction

Josamycin, identical with leucomycin A3, is a macrolide antibiotic produced by *Streptomyces narbonensis* var. *josamyceticus* [1,2]. Soon after the introduction of this antibiotic a number of esters were prepared by acylation of the various hydroxyl groups present. Josamycin propionate, with the acyl group at position C9, is the most potent and pharmacologically and pharmaceutically suitable [3]. Structures of josamycin propionate and related substances are shown in Fig. 1.

At the present time the French pharmacopoeia prescribes thin-layer chromatography with silica gel as stationary phase to limit the related substances. The main impurity should be less than 10% and all other impurities less than 5%. Detection is performed visually after reaction with sulphuric acid and subsequent heating. Although this method is sensitive, it is not very selective [4]. A microbiological method is performed for the determination of the activity.

No reversed-phase liquid chromatographic (LC) method is available for the purity control of this compound. The method described here uses a wide-pore poly(styrene–divinylbenzene) (PS–DVB) stationary phase and a mobile phase at pH 10.0. This method is derived from those previously developed for the analysis of other macrolides i.e., erythromycin, tylosin, josamycin and spiramycin [5–8].

2. Experimental

2.1. LC apparatus and operating conditions

LC analyses were performed using a L-6200

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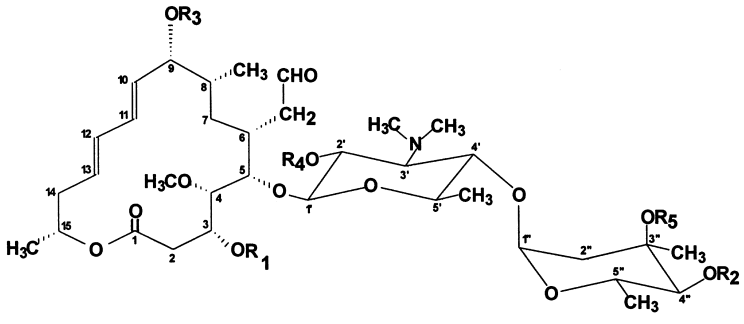

	R_1	R_2	R_3	R_4	R_5
Josamycin propionate (JP)	$COCH_3$	$COCH_2CH(CH_3)_2$	$COCH_2CH_3$	H	H
Josamycin (J) (=Leucomycin A3)	$COCH_3$	$COCH_2CH(CH_3)_2$	H	H	H
Leucomycin A4 propionate (LP)	$COCH_3$	$COCH_2CH_2CH_3$	$COCH_2CH_3$	H	H
Leucomycin A5 propionate	H	$COCH_2CH_2CH_3$	$COCH_2CH_3$	H	H
Josamycin 2', 9-dipropionate	$COCH_3$	$COCH_2CH(CH_3)_2$	$COCH_2CH_3$	$COCH_2CH_3$	H
Josamycin 3'', 9-dipropionate	$COCH_3$	$COCH_2CH(CH_3)_2$	$COCH_2CH_3$	H	$COCH_2CH_3$
Leucomycin A8 propionate	H	$COCH_3$	$COCH_2CH_3$	H	H
Platenomycin A1 propionate (PL)	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$	$COCH_2CH_3$	H	H

Fig. 1. Structures of josamycin propionate and related substances.

Intelligent pump (Merck–Hitachi, Darmstadt, Germany) equipped with a Valco injector Model CV-6-UHPa-N60 (Houston, TX, USA) or with a 655 A-40 autosampler (Merck–Hitachi), both with a 20-μl loop, a L-4200 UV–Vis detector (Merck–Hitachi) set at 232 nm and a 3396 A integrator (Hewlett-Packard, Avondale, PA, USA). The column was immersed in a water-bath, heated by a Julabo EM thermostat (Julabo, Seelbach, Germany). Columns (250×4.6 mm I.D.) were packed with PLRP-S 8 μm 1000 Å (Polymer Labs., Church Stretton, UK). The flow-rate was 1.0 ml/min. The column dead volume was determined by injecting potassium nitrate. All chromatographic parameters were calculated using formulas from the French Pharmacopoeia.

2.2. Reagents and mobile phase

Dipotassium hydrogenphosphate, 2-methyl-2-propanol and tetrahydrofuran were of analytical-reagent grade from Acros Organics (Geel, Belgium). Acetonitrile, diethyl ether and methanol were of chemi-

cal grade from the same source. Diethyl ether and methanol were distilled before use. Water was distilled twice before use. The following mobile phase was finally used: acetonitrile–0.2 *M* potassium phosphate buffer (pH 10.0)–water (52:20:up to 100, v/v/v). Mobile phases were degassed by purging with helium gas for 1 min before use. The phosphate buffer was prepared by mixing 0.2 *M* potassium phosphate solution and 0.2 *M* dipotassium hydrogenphosphate solution.

2.3. Reference substances and samples

Commercial samples were obtained from Yamanouchi (Netherlands), Biochemie (Austria) and UCB (Belgium).

Josamycin propionate house standard (JP-HS) was obtained by open column chromatography on silica gel (60 H, 15 μm) using *n*-hexane–diethyl ether–acetonitrile (10:89.5:0.5, v/v/v) as mobile phase. The collected fractions were evaporated and the residue was dried in vacuo.

Leucomycin A8 propionate, leucomycin A4 propionate (LP), leucomycin A5 propionate and platenomycin A1 propionate (PL) were isolated from a commercial sample. The same products were also obtained by propionylation of the corresponding leucomycin related substances with the free 9-hydroxy group [7]. The related substances josamycin 2,9′-dipropionate and 3″,9-dipropionate were obtained after propionylation starting from josamycin (J).

The structures of all the reference substances mentioned were confirmed by mass and nuclear magnetic resonance (NMR) spectrometry. The reference and test solutions were prepared by weighing 25.0 mg of sample and diluting with acetonitrile–water (1:1) to 25.0 ml. The content of related substances present in the samples was determined using dilutions of the JP-HS as a reference.

3. Results and discussion

3.1. Method development

Wide pore PS–DVB PLRP-S (8 μm 1000 Å) combined with alkaline mobile phases has shown excellent selectivity in the separation of other macrolide antibiotics [5–8]. This stationary phase, kept at 65°C, in combination with a mobile phase consisting of 2-methyl-2-propanol–0.2 *M* phosphate buffer (pH 10.5)–water (30:5:65, v/v/v) was chosen as the starting conditions for method development. Compared to the mobile phase described for josamycin, a higher amount of organic modifier, 30% versus 24.5%, was necessary to elute the less polar compounds within 60 min [7].

From a series of organic modifiers investigated, i.e., tetrahydrofuran, methanol, 2-methyl-2-propanol and acetonitrile, the latter showed the highest number of products separated. Tetrahydrofuran gave problems with baseline stability. For methanol the amount necessary to elute the compounds was as high as 77%. Most of the late eluting products were not separated. The number of theoretical plates and the symmetry factor for the main compound was similar for 2-methyl-2-propanol and acetonitrile. The latter showed the highest selectivity and the lower back pressure.

The influence of the pH on the selectivity was investigated between pH 7.0 and 10.5. At pH below 8.5 the separation was poorer and the column efficiency decreased by 20% relative to values above pH 8.5. The results indicated that in the range pH 8.5 to 10.5 the symmetry factor and the separation between josamycin propionate and its closest eluting peaks improves up to pH 10.0.

Upon increasing the concentration of 0.2 *M* phosphate buffer from 5% to 30% the number of theoretical plates increased by 20% and the separation between the main compound and PL improved up to 20% of buffer. At the same time the retention times decreased by 25%. The symmetry factor was affected only in a limited fashion. At buffer concentrations higher than 30% (v/v), acetonitrile became immiscible with the aqueous portion of the mobile phase. The intermediate value of 20% was retained for further work.

The influence of column temperature was examined in the range from 50 to 70°C. The retention times decreased regularly with increasing temperature. A temperature of 60°C was chosen because the number of theoretical plates, the symmetry factor and resolution of the main compound with its closely eluting related substances were optimal.

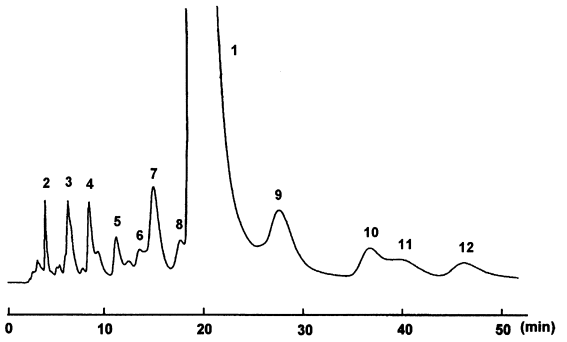

Fig. 2. Typical chromatogram of a commercial josamycin propionate sample (sample 6). Stationary phase PLRP-S 8 μm 1000 Å (250×4.6 mm I.D.); flow-rate: 1.0 ml/min; temperature 60°C; mobile phase acetonitrile–0.2 *M* phosphate buffer (pH 10.0)–water (52:20:up to 100, v/v/v); detection at 232 nm. Peaks: 1=Josamycin propionate (JP); 2, 6, 8 and 10=unknown; 3=josamycin (J); 4=leucomycin A8 propionate; 5=A5 propionate; 7=leucomycin A4 propionate (LP); 9=platenomycin A1 propionate (PL); 11=josamycin 2′,9-dipropionate; 12=josamycin 3″,9-dipropionate.

The final composition of the mobile phase chosen was acetonitrile–0.2 *M* phosphate buffer, pH 10.0–water (52:20:up to 100, v/v/v). A typical chromatogram of a commercial sample is shown in Fig. 2.

The robustness of the method was evaluated by performing a full factorial design experiment. The influence of each of four chromatographic parameters that most governed the separation was studied (low and higher values are mentioned in parentheses): the concentration of acetonitrile (50%, 54%); the pH of the buffer (pH 9.5, 10.5); the concentration of the buffer (15%, 25%) and the column temperature (55°C, 65°C). The response values of the experimental design were the symmetry factor and number of theoretical plates of JP, the retention times of J, LP, JP and PL and the resolutions between LP, JP and PL. The application of this factorial design, analysis of the measured response variables and multivariate regression calculation, were carried out using the statistical graphic software STATGRAPHIC (version 5.0, STSC, Rockville, MD, USA). This software package allows one to obtain estimated parameters for main effects, and to produce analysis of variance (ANOVA) tables, standardised Pareto charts and response surface plots.

Estimated effects, divided by the standard error, for the resolution between LP and JP, and between JP and PL, for the symmetry factor and for the number of theoretical plates of the main compound were obtained from the Pareto charts and are summarized in Table 1. The codes A, B, C and D correspond to the effects due to the acetonitrile (A), the pH (B), the amount of buffer (C) and the temperature (D). The two-code combinations correspond to the two-way interactions between the parameters. An effect is considered significant when its value exceeds the *t*-critical value. Results reveal that for the resolution between LP and JP the acetonitrile content (A) is the most important chro-

Table 1
Estimated effects (divided by its standard error) of parameters and parameter interactions on resolutions between leucomycin A4 popionate and josamycin propionate (R_{LP-JP}), josamycin propionate and platenomycin A1 propionate (R_{JP-PL}), on the symmetry factor of josamycin propionate (S_{JP}), and on the number of theoretical plates of josamycin propionate (N_{JP})

Parameter	Resolution		Symmetry factor, S_{JP}	No. of theoretical plates, N_{JP}
	R_{LP-JP}	R_{JP-PL}		
(A) MeCN	**−5.11**	−1.95	**−2.42**	**8.41**
(B) Buffer	−1.48	−0.74	−0.99	**3.55**
(C) pH	1.85	1.11	0.93	**7.02**
(D) Temperature	−0.31	**−3.71**	**2.30**	0.21
AB	−0.43	−1.95	0.62	0.70
AC	1.05	−0.74	0.93	−0.32
AD	0.74	0.46	0.50	−1.11
BC	0.00	−0.19	0.25	**2.41**
BD	0.68	−0.37	−1.99	1.39
CD	−0.80	−1.76	−0.07	−0.65
t-Critical (α=0.05)	2.44	2.44	2.07	2.07

Data in bold exceed the *t*-critical value.

matographic parameter. An increase of the amount has a negative effect (−5.11). For the resolution between JP and PL the temperature (D) is the most important parameter. Here also an increase leads to a diminution of the resolution. The symmetry factor of the JP peak decreases by increasing the acetonitrile content and increases by increasing the temperature. The number of theoretical plates for the JP peak increases with higher content of organic modifier or of buffer in the mobile phase and with higher pH. A slightly significant interaction on the number of theoretical plates between pH and buffer concentration is observed. In the other cases no significant interactions occurred between the parameters studied.

The estimated response surface plots (upper plane: PL; middle plane: JP; lower plane: LP) were also constructed (Fig. 3). They allowed the visualisation of the evolution of the retention times as a function of two variable parameters, acetonitrile concentration and buffer concentration. The column temperature 60°C and buffer pH 10.0 were kept constant. For all conditions examined there were no overlapping peaks. The results of this study show that the developed separation is robust: small changes of the parameters tested do not significantly alter the separation pattern of the josamycin derivatives.

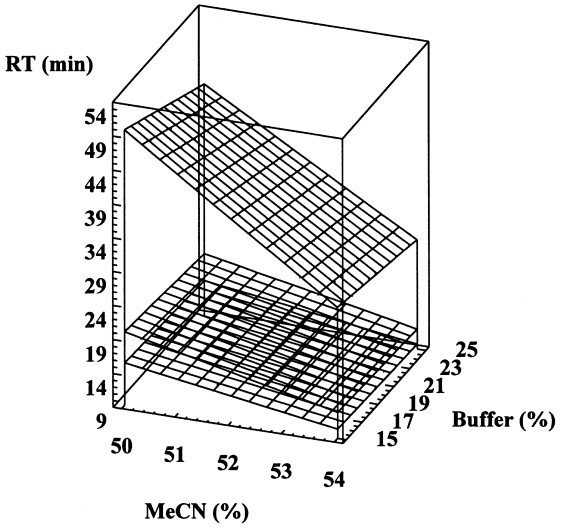

Fig. 3. Estimated response surface plots for josamycin propionate (middle plane), leucomycin A4 propionate (lower plane) and platenomycin A1 propionate (upper plane). RT=Retention time.

3.2. Quantitative aspects of the LC method

For six consecutive injections of a solution of JP-HS (20 μg injected on the column) the relative standard deviation (R.S.D.) on the peak area of the main peak was 0.5%. For the determination of the related substances, the linearity of the method was checked at four points, with a total of 12 analyses, corresponding to 0.5% to 4% of the normally injected mass. The following relationship was found by linear regression analysis $y=4.587x+0.128$, where y=peak area$\cdot 10^{-6}$, x=mass (μg) of sample injected; correlation coefficient $r=0.9988$; standard error of estimate $S_{y,x}=0.0815$. The limit of detection (LOD) with signal-to-noise ratio of 3 was 0.04% calculated for an injection of 20 μg of JP-HS. The limit of quantitation (LOQ) with signal-to-noise ratio of about 10 was 0.08% ($n=4$; R.S.D.=11%). For josamycin 2′,9- and 3″,9- dipropionates, with retention times more than twice that of JP the LOQ was about 0.2%.

3.3. Analysis of commercial samples

Bulk samples were analysed three times, using individually prepared solutions. The amount of the related substances was calculated with reference to a 1% dilution of JP-HS reference solution (Table 2). The composition of JP is rather complex. The so-called polar impurities, eluted before J, except UNK 1 were counted as a group. Old samples (e.g., sample 7 that is more than 10 years old) contain more of the polar impurities. The sum of the polar impurities ranges from 1.0 to 8.0%. The more recently produced samples contain smaller amounts of related substances. Leucomycin A4 propionate, with content ranging from 1.97 to 5.58% and PL, with content ranging from 0.75 to 2.29% are the most important related substances. This corresponds to what was observed for J [7]. The starting product J is a minor impurity, with a content of less than 1.5%. The sum of all related substances ranges from 11.7 to 24.3%.

4. Conclusions

The wide-pore PS–DVB stationary phase (PLRP-S, 8 μm, 1000 Å) shows good selectivity towards

Table 2
Amount of related substances (%, m/m) in bulk samples of josamycin propionate

Sample No.	1[a]	2	3	4	5	6	7	8	9
Polar impurities	1.88 (36)	3.03 (1.8)	6.68 (2.1)	3.0 (6.5)	1.45 (13)	2.12 (35)	7.95 (2.3)	2.59 (9.2)	1.04 (23)
UNK 1	ND	0.73 (5.5)	1.13 (2.6)	0.81 (1.8)	0.46 (15)	0.58 (35)	2.71 (4.0)	0.54 (26)	0.38 (3.0)
Josamycin (J)	ND	0.78 (0.70)	1.16 (3.7)	1.47 (3.3)	1.15 (2.5)	1.13 (20)	ND	1.39 (5.4)	0.82 (27)
Leucomycin A8 propionate	ND	1.53 (1.0)	1.12 (1.7)	1.65 (0.61)	1.07 (1.6)	1.57 (9.6)	0.19 (6.2)	1.6 (4.7)	0.80 (2.5)
Leucomycin A5 propionate	ND	1.54 (3.9)	1.63 (4.0)	1.77 (1.8)	0.89 (2.5)	1.53 (8.6)	0.20 (2.8)	1.30 (3.2)	0.54 (2.1)
UNK 2	ND	0.71 (0.8)	1.53 (2.4)	0.85 (7.5)	0.55 (3.8)	0.74 (7.6)	0.61 (4.3)	0.79 (6.5)	0.39 (1.5)
Leucomycin A4 propionate (LP)	0.66 (2.3)	5.58 (1.6)	5.03 (1.8)	4.56 (1.0)	3.47 (2.7)	4.38 (5.4)	2.91 (1.7)	4.17 (5.5)	1.97 (1.6)
UNK 3	ND	0.83 (1.8)	0.71 (2.4)	1.35 (6.4)	1.10 (6.8)	1.17 (12)	ND	0.74 (7.6)	0.44 (18)
Platenomycin A1 propionate (PL)	ND	1.79 (1.5)	1.73 (1.7)	2.06 (2.2)	2.11 (8.6)	2.09 (2.7)	0.78 (0.70)	2.29 (4.0)	2.0 (4.7)
UNK 4	0.73 (5.7)	0.39 (1.4)	1.18 (2.4)	1.35 (39)	1.93 (25)	1.69 (23)	0.23 (16)	2.55 (5.3)	1.26 (2.2)
Josamycin 2′,9-dipropionate	ND	0.57 (4.6)	1.64 (4.3)	ND	ND	ND	0.66 (4.6)	ND	1.22 (4.0)
Josamycin 3″,9-dipropionate	ND	0.72 (2.9)	0.74 (1.3)	0.66 (18)	0.71 (25)	0.66 (3.0)	0.29 (9.1)	0.60 (33)	0.82 (20)
Sum	3.3	18.2	24.3	19.5	14.9	17.7	16.5	18.6	11.7

R.S.D. (%) is given in parentheses; ND: not detected; No. of replicates: 3;
[a] Sample 1: house standard.

josamycin propionate and related substances. The isocratic method presented here is suitable to separate the main component from the potential impurities. This method is selective and robust.

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