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Journal of Chromatography A, 914 (2001) 53–58

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Liquid chromatography of troleandomycin

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Abstract

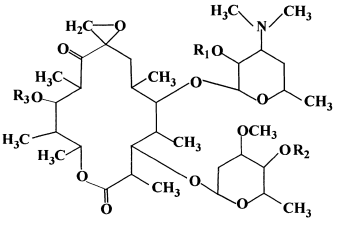
Until now no liquid chromatography (LC) method is described to determine the purity and content of troleandomycin and its related substances. A simple, robust, sensitive and selective isocratic liquid chromatographic method suitable for the determination of the antibiotic troleandomycin and its related substances is described. This method utilizes as a stationary phase: XTerra RP_{18} 5 μm (25 cm×4.6 mm I.D.) at 30°C and as mobile phase: acetonitrile–0.2 *M* ammonium acetate buffer (pH 6.0)–water (45:5:50, v/v), delivered at a flow-rate of 1.0 ml/min. UV detection is performed at 205 nm. Troleandomycin is separated from the partially acetylated related substances and from several unknown impurities present in commercial samples. The robustness of the method was evaluated by a full-factorial experimental design.

Keywords: Troleandomycin; Antibiotics

1. Introduction

Troleandomycin (TAO) is a semi-synthetic antibiotic obtained from oleandomycin after complete acetylation [1]. It is active against gram-positive organisms and has proved useful for treating staphylococcal infections resistant to erythromycin, penicillin and tetracycline [2]. The structures of troleandomycin and its related substances are shown in Fig. 1, together with abbreviations used throughout the text.

In the French Pharmacopoeia the purity of troleandomycin is checked by thin-layer chromatography (TLC) [3]. TLC is more useful for identification purposes but less for purity control as it lacks the selectivity required. The United States Pharmacopeia (USP) prescribes a chemical method to determine the acetyl content in troleandomycin [4]. The described procedure is quite lengthy as it involves hydrolysis, distillation and titration processes. The method does not distinguish troleandomycin and



	R_1	R_2	R_3
Triacetyloleandomycin (TAO)	CH_3CO	CH_3CO	CH_3CO
2,3-Diacetyloleandomycin (2,3-DAO)	H	CH_3CO	CH_3CO
1,3-Diacetyloleandomycin (1,3-DAO)	CH_3CO	H	CH_3CO
1,2-Diacetyloleandomycin (1,2-DAO)	CH_3CO	CH_3CO	H
3-Monoacetyloleandomycin (3-MAO)	H	H	CH_3CO
2-Monoacetyloleandomycin (2-MAO)	H	CH_3CO	H
1-Monoacetyloleandomycin (1-MAO)	CH_3CO	H	H

Fig. 1. Structures of troleandomycin and its related substances.

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0021-9673/01/$ – see front matter
PII: S0021-9673(00)01170-5

partially acetylated related substances. The French Pharmacopoeia and the USP further prescribe microbiological assay to determine the activity of troleandomycin [3,4]. Microbiological assay suffers from poor precision and poor selectivity, hence specific information about the composition of the sample cannot be obtained.

Since antibiotics are known to be complex products of natural origin, suitable analytical techniques are required to selectively and accurately determine their composition and content in drug substance or drug product. One such technique is liquid chromatography (LC), but until now no LC method is described to determine the purity and content of troleandomycin. However, LC methods for esters of other macrolides have been reported, such as erythromycin ethylsuccinate [5], erythromycin estolate [6] and josamycin propionate [7].

In order to develop an LC method that separates TAO from its related substances, a novel reversed-phase packing based on silica gel was preferred because of its improved selectivity, efficiency and its thermal and pH stability. Compared to polymer packings a better sensitivity can also be obtained, thanks to the better efficiency.

A selective, sensitive, robust, isocratic LC method utilizing XTerra RP_{18} reversed-phase is described which allows the separation of TAO from its related substances.

2. Experimental

2.1. Reagents and samples

Acetonitrile, HPLC-grade ''S'' was from Biosolve (Valkenswaard, The Netherlands). 2-Propanol, Chromasolv-grade was from Riedel-de Haën (Seelze, Germany). Dipotassium hydrogenphosphate (Merck, Darmstadt, Germany) 0.2 *M* solution was brought to the required pH by adding 0.2 *M* phosphoric acid (Merck). Ammonium carbonate, ammonium formate and ammonium acetate were from Acros Chimica (Geel, Belgium). Ammonium formate 0.2 *M* solution was brought to the required pH by adding dilute formic acid R (Merck) and ammonium acetate 0.2 *M* solution was brought to the required pH by adding dilute acetic acid R (Merck). Water was distilled twice from glass apparatus.

TAO commercial sample was obtained from Pharmachim (Sofia, Bulgaria). TAO used as a laboratory reference substance was obtained by crystallization from 2-propanol. 2,3-Diacetyloleandomycin (2,3-DAO) was obtained by methanolysis of TAO laboratory reference substance at room temperature. Other acetylated products were obtained by refluxing TAO laboratory reference substance in methanol or by partial acylation of oleandomycin base with acetic anhydride. Apart from 2,3-DAO, other acetylated products were obtained as impure substances. Oleandomycin (OLE) was obtained from Pfizer (Brussels, Belgium).

A solution of commercial-grade TAO at a concentration of 40 mg/ml in acetonitrile, spiked with 1.0 mg/ml of 2,3-DAO and with similar amounts of other partially acetylated derivatives was used in method development as well as in the robustness study. For quantification purposes, a solution of TAO laboratory reference substance was prepared at a concentration of 40 mg/ml in acetonitrile. The injected amount was 400 μg.

Acetonitrile was used as the solvent because it was observed that in aqueous solutions hydrolysis of esters occurred rapidly.

2.2. LC instrumentation and chromatographic conditions

The LC apparatus consisted of an L-6200 Intelligent pump (Merck–Hitachi, Darmstadt, Germany), an autosampler (Gilson 234 autoinjector, Villiers-le-Bel, France) equipped with a 10-μl loop, a variable-wavelength linear UV–Vis detector set at 215 nm and a Hewlett-Packard integrator Model HP 3396 series II (Avondale, PA, USA). XTerra (Waters, Milford, MA, USA) RP_{18} 5 μm (25 cm×4.6 mm I.D.) was used as a stationary phase at 30°C. Acetonitrile–0.2 *M* ammonium acetate, pH 6.0–water (45:5:50, v/v) at a flow-rate of 1.0 ml/min was used as the mobile phase. The mixture was purged by helium to degas.

3. Results and discussion

3.1. Method development

In preliminary experiments, the use of Waters

XTerra RP_{18} as the stationary phase at 30°C and acetonitrile–0.2 *M* phosphate buffer, pH 7.0–water (35:5:60, v/v) as the mobile phase showed excellent selectivity in the separation of TAO and its potential impurities present in the solution prepared for method development. The peaks corresponding to TAO and partially acetylated products were asymmetrical.

The use of volatile buffers was also examined, as they allow easier coupling to mass spectrometry. Ammonium formate, carbonate and acetate were investigated. With the latter the same number of components was separated as with the phosphate buffer. The peak symmetry for TAO (3.0) was also quite similar as with the phosphate buffer. However, the elution of components was fast and the selectivity was good. The 0.2 *M* phosphate buffer in the mobile phase was therefore replaced with an ammonium acetate buffer at the same concentration (5%, v/v).

UV detection at 205 nm resulted in an increase in sensitivity for TAO and partially acetylated products by a factor of 2.5 against detection at 215 nm. It was therefore decided to use 205 nm in all subsequent experiments.

The influence of the pH on the selectivity was investigated between 5.5 and 7.0. At pH 6.0 to 7.0 the selectivity for all components remained excellent, but at pH 5.5 the selectivities between the early-eluting components, TAO and an adjacent unknown component were reduced. The capacity factor of TAO in the pH range examined was within 8.1 and 11.3. The column efficiency as calculated with reference to the main component (TAO) was higher at pH 5.5 (9900) and 6.0 (8560), but lower at pH 7.0 (5000). The peak symmetry for TAO improved with decreasing pH in the range 7.0 (3.0) to 5.5 (1.2). pH 6.0 was finally chosen because the column efficiency, the symmetry factor (1.4) and the selectivity between the main component (TAO) and partially acetylated related products were optimal. Chromatographic parameters were calculated according to the European Pharmacopoeia [8].

2-Propanol (25%, v/v) as the organic modifier gave no better separation than acetonitrile (45%, v/v) and higher back-pressure was observed. Therefore, acetonitrile, also being more UV transparent, was preferred.

The influence of column temperature was investigated in the range from 0 to 50°C. The retention times increased with increasing temperature. Above 35°C the baseline rose immediately after the first eluted partially acetylated substance until after the elution of TAO. This chromatographic phenomenon is an indication for partial hydrolysis of TAO. It has been described that TAO undergoes rapid hydrolysis in aqueous solutions to form degradation products such as 2,3-DAO, 1,3-DAO, 1,2-DAO, 1-MAO, 2-MAO and 3-MAO [9]. This process is further enhanced by a temperature increase. Below 25°C the peak shapes became broad and the unknown component III (see Fig. 2) adjacent to TAO was no longer separated. Between 25 and 35°C, TAO was completely separated from all the components. It was therefore decided to use a temperature of 30°C as a compromise in further experimental work.

The chosen composition of the mobile phase was further optimized using Drylab software (LC Resources, Berlin, Germany) to obtain the final proportions as described in Section 2.2. A typical chromatogram of a commercial sample (labeled A) and a spiked sample (labeled B), mentioned in Section 2.1, is shown in Fig. 2. Only the peaks corresponding to TAO, oleandomycin, 2,3-DAO and 3-MAO are labeled because reference substances of other impurities were not available. 2,3-DAO and components III and IV were found to be present in commercial sample at levels of 0.2, 2.5 and 0.5%, respectively, calculated by a normalization procedure.

Analyses were also carried out with Hypersil BDS C_{18} 5 μm as the stationary phase. Under the described chromatographic conditions, the selectivity of components eluted up to the unknown component III was found to be similar as on XTerra RP_{18}. However, the selectivity of components eluted after the main component TAO is different as few components were observed. Hypersil exhibits higher retention properties than XTerra by a factor of about 1.6. Moreover, the main component TAO shows more significant peak tailing (4.0) compared to 1.4.

3.2. Robustness study

The importance of the individual chromatographic parameters and parameter interactions of this LC system was studied by means of a three-factorial design. The set-up of the applied factorial design was supported by a statistical graphics software system,

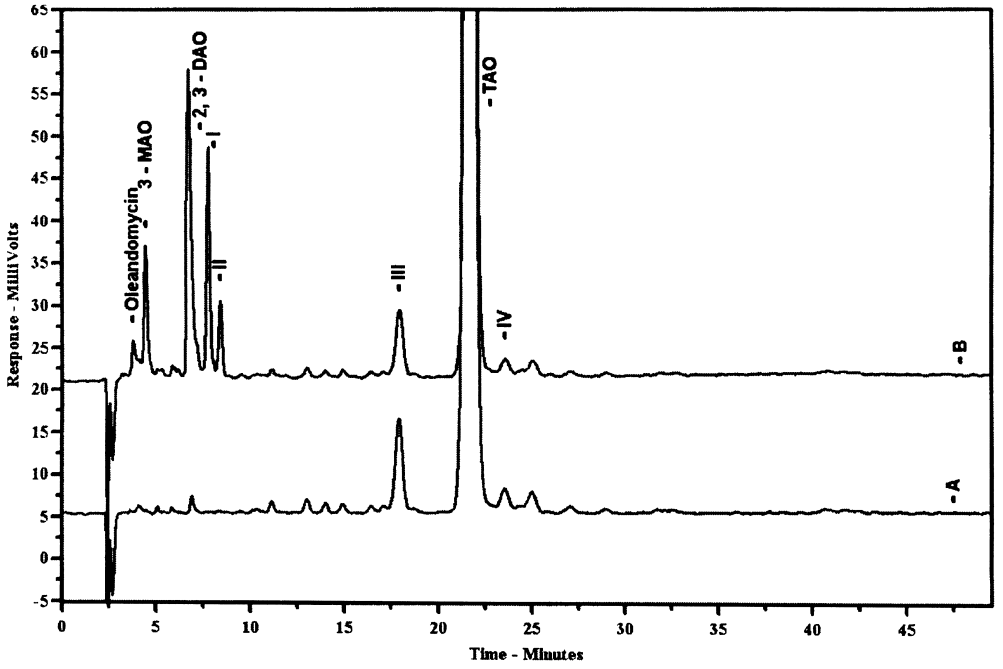

Fig. 2. Typical chromatogram of a troleandomycin commercial sample (A) and of a commercial sample spiked with 2,3-DAO and partially acetylated products of oleandomycin (B). Stationary phase: XTerra RP_{18} 5 μm (250×4.6 mm I.D.); column temperature: 30°C; mobile phase: acetonitrile–0.2 *M* ammonium acetate, pH 6.0–water (45:5:50); flow-rate: 1.0 ml/min; injection volume: 10 μl; detection: UV at 205 nm. The peaks labeled I, II, III (2.5%), IV (0.5%) and unlabeled peaks are of unknown identity. The amounts indicated are with respect to chromatogram A.

Statgraphics Plus standard edition, version 4.1 (Manugistics, Rockville, MD, USA). The chromatographic parameters examined as variables were the pH of the mobile phase buffer, the column temperature and the concentration of acetonitrile. The values used in the design are depicted in Table 1. A three-factorial design at two levels involves 2^3 experimental measurements. Duplicate experiments were carried out plus two central experiments. A total of 18 chromatograms were obtained. The measured response variables were the retention times of TAO, 2,3-DAO and some related substances labeled I, II, III and IV (see Fig. 2). 3-MAO was not included in the study because it is well separated from other partially acetylated products. For each experiment, the average selectivity of two analyses was calculated. The estimated effects of the three variables with their second-order interactions on the selectivity between 2,3-DAO–I, I–II, II–III, III–TAO and TAO–IV are presented on the standardized pareto charts in Fig. 3. The bars are displayed in order of the size of the effects, with the largest effects on top. The charts include a vertical line at a critical *t*-value for $\alpha = 0.05$. Effects for which the bars are smaller

Table 1
Robustness study[a]

Chromatographic variable	Low value (−1)	Central value (0)	High value (+1)
(A) pH	5.75	6.0	6.25
(B) Temperature (°C)	25.0	30.0	35.0
(C) Acetonitrile (%)	44.0	45.0	46.0

[a] Nominal values corresponding to −1, 0 and +1 levels.

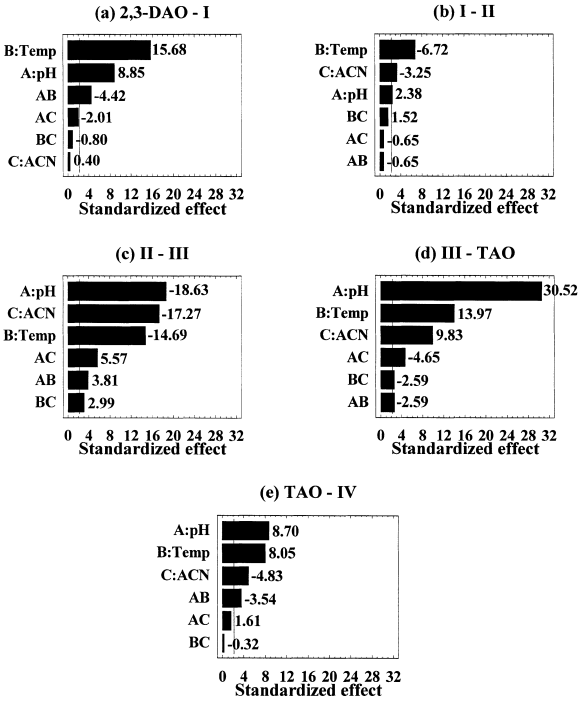

Fig. 3. Standardized pareto charts representing the estimated effects of parameters (A, B, C) and parameter interactions (AB, BC and AC) on the selectivity between (a) 2,3-DAO–I, (b) I–II, (c) II–III, (d) III–TAO; (e) TAO–IV. A, Mobile phase pH; B, column temperature; C, concentration of organic modifier (acetonitrile).

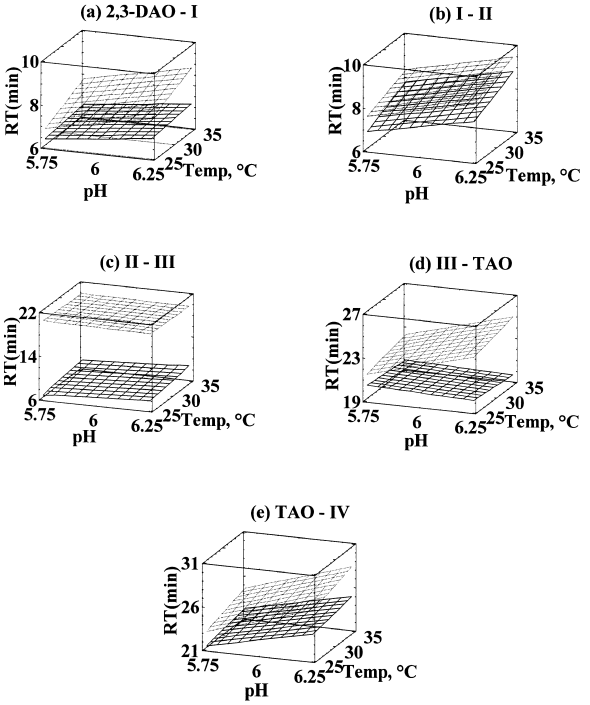

Fig. 4. Estimated response surface plots for (a) 2,3-DAO–I, (b) I–II, (c) II–III, (d) III–TAO (e) TAO–IV, constructed with retention times as a function of pH and temperature.

than the critical *t*-value are considered as not significant.

Under the conditions examined, temperature positively influences the selectivities 2,3-DAO–I, III–TAO and TAO–IV, but negatively the selectivities I–II and II–III. A pH rise improves the selectivities 2,3-DAO–I, III–TAO and TAO–IV, but significantly reduces the selectivity II–III. A slight increase in the concentration of acetonitrile slightly reduces the selectivities I–II, II–III and TAO–IV, but improves the selectivity III–TAO. Of the second-order interactions, AC, AB and BC, the major effect is shown by AC.

In view of the fact that the temperature and pH mainly influence the selectivity, response surface plots were constructed using these two parameters. Fig. 4 shows how the retention times (t_R, min) corresponding to 2,3-DAO, I, II, III, TAO and IV change with respect to the pH and column temperature. The plots clearly show that the method remains selective in the range of conditions examined, since the planes do not overlap. This means therefore, that the method is robust within the range of conditions examined.

3.3. Repeatability, linearity and detection limits

The repeatability of the method was assessed using a 40 mg/ml solution of TAO injected six times. The relative standard deviation (RSD) was 0.46%. The linearity was examined by analysis (n= 3) of solutions corresponding to 0.05, 1, 5, 25, 75, 100 and 120% of the theoretical value (40 mg/ml). The following relationships were found: y=80.48x+ 30.58, r=0.999, $S_{y,\,x}$=56, where y=peak area/ 100 000, x=concentration in mg/ml; r=correlation coefficient, $S_{y,\,x}$=standard error of estimate. For an injection of 400 μg, the limit of quantitation (LOQ) for TAO (in %, m/m) was 0.05% (n=6, RSD=

13.6%) and the limit of detection (LOD) with a signal-to-noise ratio of 3 was 0.02%.

4. Conclusion

The isocratic liquid chromatographic method described is suitable for the determination of the antibiotic troleandomycin and its related substances. The method is simple, selective, repeatable, linear and sensitive. Its compatibility with mass spectrometry will allow for identification of the unknowns.

Acknowledgements

H.K.C. thanks the Belgian Technical Co-Operation for financial support.

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