ELSEVIER

Journal of Chromatography A, 895 (2000) 43–49

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Analysis of doxycycline by capillary electrophoresis Method development and validation

E. Castellanos Gil[a], A. Van Schepdael[b,*], E. Roets[b], J. Hoogmartens[b]

[a]*Centro de Química Farmacéutica, Calle 200 y 21, Atabey, Playa, P.O. Box 16042, Havana, Cuba*

[b]*Laboratory for Pharmaceutical Chemistry and Drug Analysis, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium*

Abstract

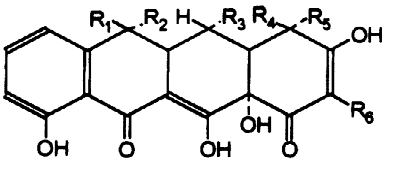
An optimized capillary electrophoresis method for the analysis of doxycycline is described. The influence of methanol as organic modifier, buffer pH, buffer concentration, capillary length, column temperature, Triton X-100 and methyl-β-cyclodextrin was systematically investigated. A central composite design was performed in order to optimize the method. The optimal separation conditions were: capillary, uncoated fused-silica [40 cm (32 cm effective length)×50 μm I.D.]; background electrolyte, a solution of 145 m*M* sodium carbonate and 1 m*M* EDTA brought to pH 10.3–methanol (89:11, v/v); temperature, 15°C; voltage, 12 kV. The method showed good selectivity, repeatability, linearity and sensitivity. Six commercial samples were quantitatively analyzed. The results were compared with those established by the liquid chromatography method from the European Pharmacopoeia.

Keywords: Validation; Doxycycline; Antibiotics

1. Introduction

Doxycycline (DOX) is a semi-synthetic broad-spectrum antibiotic with improved serum half-life. Potential impurities are oxytetracycline (OTC), metacycline (MTC), 2-acetyl-2-decarboxamido-doxycycline (ADDOX), 6-epidoxycycline (6-EDOX), 4-epidoxycycline (4-EDOX) and 4,6-epi-doxycycline (4,6-EDOX). The structures of these compounds are shown in Fig. 1.

Several methods have been used for the analysis of DOX and the United States Pharmacopeia (USP) as well as the European Pharmacopoeia (Ph. Eur.)



	R_1	R_2	R_3	R_4	R_5	R_6
DOX	H	CH_3	OH	H	$N(CH_3)_2$	$CONH_2$
OTC	OH	CH_3	OH	H	$N(CH_3)_2$	$CONH_2$
MTC	$=CH_2$		OH	H	$N(CH_3)_2$	$CONH_2$
ADDOX	H	CH_3	OH	H	$N(CH_3)_2$	$COCH_3$
6-EDOX	CH_3	H	OH	H	$N(CH_3)_2$	$CONH_2$
4-EDOX	H	CH_3	OH	$N(CH_3)_2$	H	$CONH_2$
4,6-EDOX	CH_3	H	OH	$N(CH_3)_2$	H	$CONH_2$

Fig. 1. Structures of doxycycline and related substances.

*Corresponding author. Fax: +32-16-323-448.

E-mail address: ann.vanschepdael@farm.kuleuven.ac.be (A. Van Schepdael).

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00523-9

prescribe a liquid chromatography (LC) method for purity control of DOX [1–4].

Only one capillary electrophoresis (CE) method has been described for the separation of DOX from its potential impurities which may be formed during synthesis. In this previously published CE method ADDOX was not separated from DOX [5]. In the current study, a selective CE method capable of resolving DOX from ADDOX and other related substances is described.

2. Experimental

2.1. Apparatus

CE was performed on Spectraphoresis 500 equipment (Thermo Separation Products, Fremont, CA, USA), coupled to a 3396 series III integrator (Hewlett-Packard, Avondale, PA, USA). Tetracyclines were detected by UV absorption at 254 nm. Injection was performed hydrodynamically for 4 s (0.75 p.s.i.; 1 p.s.i.=6894.76 Pa). The untreated fused-silica capillary (50 μm I.D.) with a 46 cm or 40 cm length (38 or 32 cm to detection window) was from Polymicro Technologies (Phoenix, AZ, USA). Buffer pH was measured with a Consort C-831 pH meter (Turnhout, Belgium).

2.2. Chemicals, reagents, samples and solutions

Methanol (HPLC-grade) was from Rathburn (Walkerburn, UK). Methyl-β-cyclodextrin (M-β-CD) was purchased from Aldrich (Bornem, Belgium). Triton X-100 was from Acros Organics (Geel, Belgium). Phosphoric acid (analytical grade) was from Riedel-de Haën (Seelze, Germany). Sodium carbonate and EDTA were from Acros Organics. When necessary, the pH of running buffers was adjusted using either hydrochloric acid (HCl) or sodium hydroxide (NaOH) before diluting to volume. NaOH was from BDH (Poole, UK) and HCl was obtained from Merck Eurolab (Haasrobe, Belgium). DOX from Gist-Brocades (Delft, The Netherlands) (86% DOX, 0.08% 4-EDOX, 0.38% 6-EDOX and 0.15% MTC) was used as a standard. Six commercial samples were analyzed: sample 1 (Merck Eurolab, Belgium), sample 2 (Pfizer, Belgium), sample 3 (V.M.D. Chemie, Belgium), sample 4 (Certa, Belgium), sample 5 (V.M.D. Chemie) and sample 6 (V.M.D. Chemie). Sample 2 was a monohydrate and the other samples were hyclates. European Pharmacopoeia Chemical Reference Substances for OTC, MTC, 6-EDOX and 4-EDOX were used. 4,6-EDOX was obtained by epimerization of 6-EDOX, by boiling in water for 1 h. ADDOX was isolated by preparative thin-layer chromatography from a commercial sample [6]. All solutions were filtered through 0.2 μm nylon filters (Alltech, Laarne, Belgium).

2.3. Procedure

Before use, a new capillary was treated with 1 *M* NaOH for 10 min. The capillary was conditioned at the beginning of each day with 0.1 *M* NaOH for 5 min, followed by 0.1 *M* phosphoric acid for 5 min and 20 m*M* EDTA for 5 min. Before every analysis, the capillary was washed for 1 min with 0.1 *M* H_3PO_4 and 3 min with running buffer. The parameters were determined using mixtures containing approximately equal amounts of OTC, MTC, 4-EDOX and 6-EDOX (20% with respect to DOX 2.5 mg/ml) and small amounts of 4,6-EDOX and ADDOX.

2.4. Software

Experimental design and optimization were performed using Modde 4.0 software (Umetri, Umeå, Sweden).

3. Results and discussion

3.1. Method development

In preliminary work sodium carbonate (120 m*M*) was used as the background electrolyte and 1 m*M* EDTA was added to prevent interaction of the tetracyclines with metals through complexation. The solution was adjusted to pH 10.5. All development experiments were performed on an uncoated fused-silica capillary of 38 cm effective length×50 μm I.D. Non-ionic surfactant Triton X-100 (0.1–0.6%, v/v) and M-β-CD (5–15 m*M*) were tested as

additives. The cyclodextrin was dissolved in this buffer to give the run buffer for the inlet buffer vial, while the outlet buffer vial contained no cyclodextrin. However, the improvement of selectivity was rather limited because the peaks of ADDOX and DOX still overlapped. The separation of OTC and DOX became critical when the concentration of M-β-CD was increased.

The selectivity between DOX and ADDOX was improved dramatically when methanol was added to the buffer (0–12%) before making up to volume. Methanol is used in CE to change the physico–chemical nature of the separations system. It alters the polarity and the viscosity of the mobile phase. As a consequence both the electroosmotic flow and the electrophoretic mobility of the analytes are affected.

The effect of buffer concentration ranging from 40 to 150 m*M* (keeping the buffer pH at 10.5 and the methanol concentration at 10%, v/v) and buffer pH from 10 to 11 (keeping the buffer concentration at 120 m*M* and the methanol concentration at 10%, v/v) was investigated.

Increasing the column temperature (15–25°C) the resolution between DOX and ADDOX decreased. The lowest point (15°C) of the range was preferred. Furthermore, the effect of capillary length and applied voltage (range examined: 30–38 cm and 10–14 kV, respectively) on separation was also investigated. It was found that a capillary with an effective length of 32 cm combined with an applied voltage of 12 kV could give a satisfactory separation in a reasonable analysis time.

3.2. Optimization and robustness

When more than one variable is potentially important, it is difficult to obtain optimal conditions through the commonly used step-by-step optimization procedure. In this case a large number of independent runs are involved. The experimental design offers an efficient route for identifying the conditions yielding the best resolution [7–9]. Many factors can be screened simultaneously to determine which has a significant effect on the separation. The interactions between factors are obtained and with a low number of experiments the factors can be optimized. Robustness is an important feature of analytical method development, which has to be verified. Experimental design can also be used for this purpose [10,11].

Three variables and three responses were involved in the experimental design. Variables and their ranges studied are summarized in Table 1. The high and low values of each variable were defined based on preliminary experiments. The responses (S_1, S_2 and S_3), corresponding to the separation selectivity between critical peak pairs 6-EDOX–4-EDOX, 4-EDOX–MTC and DOX–ADDOX, respectively, were calculated by the equation: $S=t_2/t_1$ (t_1 and t_2 are the migration times of the peaks selected).

A central composite design was used for the purpose of this study. This experimental design needed 17 experiments in total (2^k+2k+3, k is the number of variables) including three center points. The center points are very important because they yield the information concerning repeatability of the design. The collected experimental data were fitted by a partial least-square (PLS) model with which several responses (three or more) can be dealt with simultaneously, to provide an overview of how all the factors affect all the responses. The response of the model, R^2 and Q^2 values were over 0.97 and 0.81, respectively, implying that the data fitted well with the model. Here, R^2 is the fraction of the variation of the response that can be modeled and Q^2 is the fraction of the variation of the response that can be predicted by the model. R^2 and Q^2 values close to 1 indicate an excellent model. The relationship between a response y and the variables x_i, x_j ... can be described by Taylor expansion:

Table 1
Nominal values corresponding to −1, 0 and +1 levels

Variable	Low level (−1)	Central value (0)	High level (+1)
Methanol (%, v/v)	8	10	12
Buffer concentration (m*M*)	130	140	150
Buffer pH	10.2	10.35	10.5

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \beta_{ii} x_i^2 + \beta_{jj} x_j^2 + \ldots + E$$

where, β is the regression coefficient and E is the overall experimental error. The square term of each variable describes the non-linear effect on the response, and the cross term of the two different variables describes the effect of their interaction on the response [12]. Fig. 2 shows the regression coefficient plot for three responses. The 95% confidence interval was expressed in terms of error bar over the coefficient. If the coefficient is smaller than the interval, the variation of the response caused by changing the variable is smaller than the experimental error. Therefore the variable is considered not to be significant.

The buffer pH has a significant positive effect on S_1 and a negative effect on S_2. The pK_a values for DOX have been reported: 3.4 (β-tricarbonyl system), 7.7 (protonated dimethylamino function), 9.7 (β-dicarbonyl system) and around 11 (phenol function) [13,14]. The pK_a for OTC: 3.27 (β-tricarbonyl system), 7.32 (protonated dimethylamino function), 9.11 (β-dicarbonyl system) and 10.7 (phenol function) [15]. All the compounds investigated in this study will most probably behave in the same way and will thus carry two to three negative charges in this pH range, and their electrophoretic mobility is opposite to electroosmosis. The selectivity is dependent on the sum of electrophoretic mobility of each solute and electroosmotic mobility. For every substance, migration time was longer with increasing pH, which shows that an increase in electrophoretic movement of the substances has overcome the increase in electroosmosis. However, some substances (especially 4-EDOX) were affected more than others due to structural differences, which results in a selectivity change.

Methanol has a significant positive effect on S_1 and S_3, and a negative effect on S_2. Buffer concentration has a significant non-linear effect on the separation selectivities S_1 (positive) and S_2 (negative) in the range studied. On the other hand, the interaction between pH–methanol has a significant positive effect on S_2, and the interaction pH–buffer has a negative effect on selectivity S_1 and S_3.

The response surface plots constructed by plotting separation selectivity as a function of important

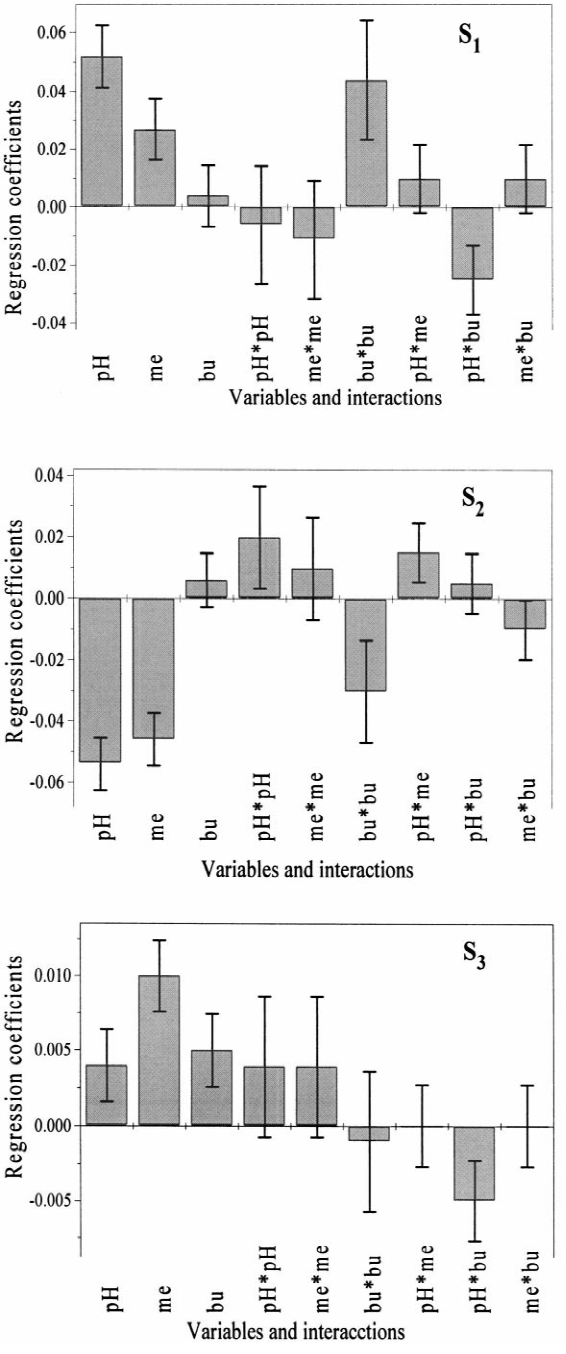


Fig. 2. Regression coefficient plot for the separation selectivity. S_1 = selectivity between 6-EDOX and 4-EDOX; S_2 = selectivity between 4-EDOX and MTC; S_3 = selectivity between DOX and ADDOX.

variables are shown in Fig. 3. In this figure the optimal conditions for S_1 (12% methanol and pH 10.5), S_2 (8% methanol and pH 10.2) and S_3 (12% methanol and pH 10.5) can be seen. Since the optimal conditions predicted by the model for each peak pair were not completely the same, the overall one was obtained by balancing. Thus in order to find the best compromise between selectivity and analysis time, methanol 11%, pH 10.3 and 145 m*M* buffer concentration were selected as the optimal conditions (I~111 μA). Fig. 4 shows a typical electropherogram. In the ranges for methanol from 9 to 12%, pH from 10.25 to 10.40 and buffer concentration from 140 to 150 m*M*, the selectivity remains good. It means that the method is robust in this range.

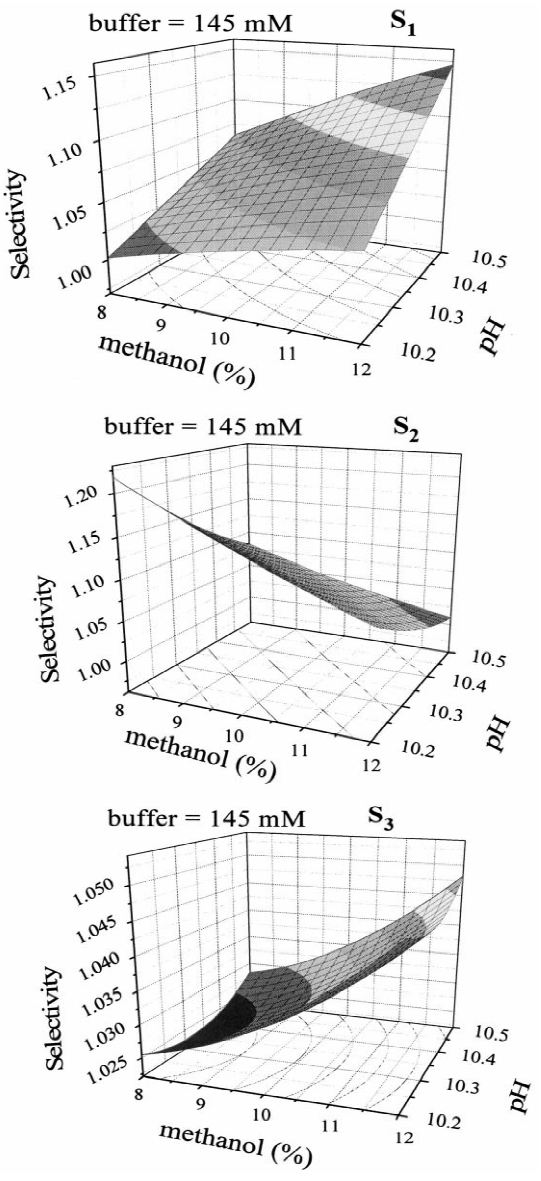


Fig. 3. Response surface plot showing the selectivity as a function of significant separation parameters. S_1 = selectivity between 6-EDOX and 4-EDOX; S_2 = selectivity between 4-EDOX and MTC; S_3 = selectivity between DOX and ADDOX.

3.3. Quantitative analysis

The quantitative features of this method were examined and the results are shown in Table 2. The calibration curve obtained by replicate analysis (n = 3) of a series of analyte concentrations corresponding to 0.25, 0.5, 1.5, 2.5 and 3 mg/ml was subjected to linear regression analysis. The calibration curve was not used to calculate the content of the samples but only to check the linearity. The calculations for the content of the main component were based on the results obtained for DOX standard in each series of analyses. Repeatability studies were performed with a 2.5 mg/ml solution, which is suitable for assay of DOX. In limit of quantitation (LOQ) and limit of detection (LOD) tests, this solution was diluted gradually. LOQ corresponded to 0.05%, relative to 2.5 mg/ml.

3.4. Analysis of commercial samples

The method was applied to the assay of different commercial samples. DOX from Gist-Brocades (86% DOX as base) was used as a standard. Replicate sampling (n = 3) was carried out in each case. Results of the assay are presented in Table 3. The content of MTC and 6-EDOX was determined using a diluted solution of MTC and 6-EDOX corresponding to 2% of the nominal content of DOX (2.5 mg/ml). The content of the other related substances was determined using the diluted solution of 6-EDOX. All the results were expressed as the base.

Samples 1, 2 and 3 were analyzed only by CE. Samples 4, 5 and 6 were analyzed by CE and also by LC according to the Ph. Eur. An impurity of unknown identity (0.12%) was detected in sample 2.

Results obtained by this CE method are found to be similar to those found with the LC method from

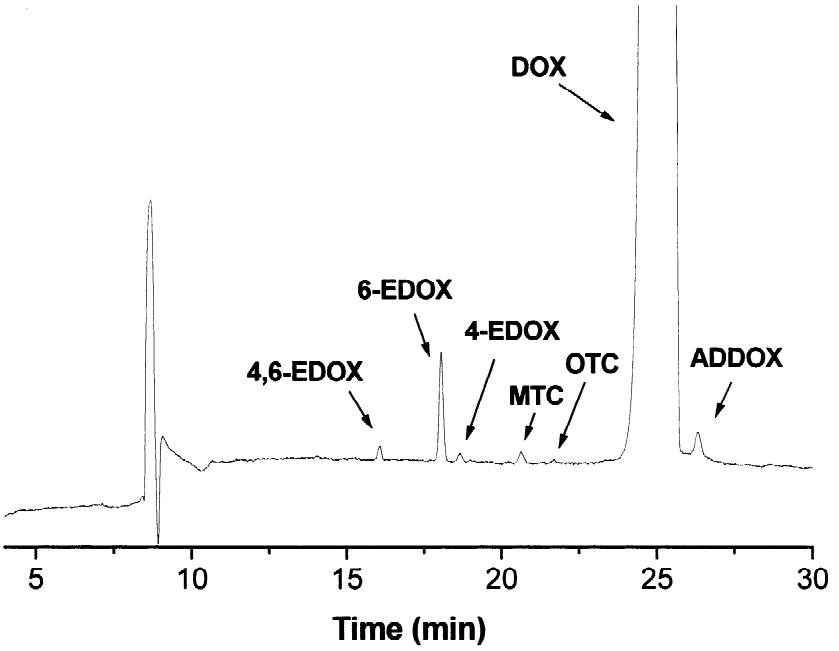


Fig. 4. A typical electropherogram of a commercial sample of DOX (2.5 mg/ml). Capillary: uncoated fused-silica [40 cm (32 cm effective length)×50 μm I.D.]; background electrolyte, a solution of 145 m*M* sodium carbonate and 1 m*M* EDTA brought to pH 10.3–methanol (89:11, v/v); column temperature, 15°C; applied voltage, 12 kV; detection wavelength, 254 nm; hydrodynamic injection, 4 s.

the Ph. Eur. The ADDOX content by LC is only reported for sample 6. The low amount of ADDOX in samples 4 and 5 and lower efficiency obtained by LC make quantitative determination difficult in these cases. CE offers several advantages over LC in that it possesses high resolution and efficiency for ADDOX. At the other hand CE shows higher relative standard deviation (RSD). Compared to the LC and the previous CE method, this method, washing procedure included, has a somewhat longer analysis time.

4. Conclusion

The CE method presented here is suitable for the

Table 2
Quantitative features for DOX[a]

Parameter	DOX
Within-day repeatability ($n=6$)	
Migration time	RSD 1.3%
Corrected area	RSD 0.9%
Day-to-day repeatability (6 days, $n=18$)	
Migration time	RSD 3.5%
Corrected area	RSD 2.4%
Linearity: range (0.25–3 mg/ml)	$y=233\,193x+21\,653$
y=corrected area, x=DOX concentration (mg/ml),	$r=0.999$
number of concentrations=5, total number of analyses=15	$S_{y,x}=9099$
LOD ($S/N=3$)	0.0005 mg/ml (0.02%, relative to 2.5 mg/ml)
LOQ ($S/N=10$), RSD=12.6% ($n=7$)	0.0012 mg/ml (0.05%, relative to 2.5 mg/ml)

[a] Hydrodynamic injection 4 s.

Table 3
Content (%) of DOX and related substances in commercial samples

Component	Contents (RSD, %)[d]								
	Sample 1,	Sample 2,	Sample 3,	Sample 4		Sample 5		Sample 6	
	CE	CE	CE	CE	LC	CE	LC	CE	LC
4,6-EDOX	–[b]	–[b]	–[b]	0.12 (3.3)	0.09 (7.2)	–[b]	–[b]	–[b]	–[b]
6-EDOX	1.11 (3.9)	0.44 (2.6)	1.21 (5.2)	0.89 (6.2)	0.90 (1.7)	1.29 (2.6)	1.30 (1.3)	1.77 (4.7)	1.73 (3.5)
4-EDOX	0.18 (5.7)	0.16 (8.2)	0.12 (18.0)	0.08 (18.4)	0.04 (15.4)	0.26 (6.3)	0.30 (8.1)	<LOQ	–[b]
MTC	0.11 (4.7)	0.73 (4.4)	0.61 (2.8)	0.08 (15.5)	0.06 (20.3)	0.37 (16.2)	0.48 (11.8)	0.19 (5.1)	0.20 (8.9)
OTC	–[b]	<LOQ	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]
DOX[a]	77.59 (2.7)	85.91 (1.7)	84.74 (1.9)	85.90 (1.5)	85.24 (1.0)	84.93 (2.2)	84.75 (1.4)	88.30 (2.7)	88.05 (1.7)
ADDOX	0.27 (2.8)	0.27 (9.5)	0.79 (6.1)	0.21 (4.7)	–[b]	0.23 (8.5)	–[b]	0.64 (3.4)	0.49 (0.5)
Unknown[c]	–[b]	0.11 (9.1)	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]

[a] The values of DOX are expressed as DOX base.
[b] Not detected.
[c] Unknown: peak was eluted between 4-EDOX and MTC.
[d] RSDs are given in parentheses. Each sample was injected in triplicate and the average value was listed. CE: Capillary electrophoresis; LC: liquid chromatography.

separation of doxycycline from its potential impurities. It was established that methanol was necessary for selectivity improvement between DOX and ADDOX. The overall optimal separation conditions were obtained through a central composite experimental design. Under the optimal conditions, this robust method shows good selectivity, repeatability and linearity. It was applied satisfactorily to quantitative analysis of commercial DOX samples.

References

[1] K. Dihuidi, M.J. Kucharski, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Chromatogr. 325 (1985) 413.
[2] J. Hoogmartens, N.H. Khan, H. Vanderhaeghe, A.L. Van Der Leeden, M. Oosterbaan, G.L. Veld-Tulp, W. Plugge, C. Van Der Vlies, D. Mialanne, R. Melamed, J.H.McB. Miller, J. Pharm. Biomed. Anal. 7 (1989) 601.
[3] European Pharmacopoeia, 3rd ed., Council of Europe, Strasbourg, 1997, p. 786.
[4] United States Pharmacopeia 23, United States Pharmacopeial Convention, Rockville, MD, 1995, p. 558.
[5] A. Van Schepdael, R. Kibaya, E. Roets, J. Hoogmartens, Chromatographia 41 (1995) 367.
[6] W. Naidong, S. Hua, E. Roets, J. Hoogmartens, J. Planar Chromatogr. 7 (1994) 297.
[7] E. Morgan, K.W. Burton, P.A. Church, in: D.L. Massart, R.G. Brereton, R.E. Dessy, P.K. Hopke, C.H. Spiegelman, W. Wegscheider (Eds.), Chemometrics Tutorials, Elsevier, Amsterdam, 1990, p. 104.
[8] M. Thorsteinsdottir, D. Westerlund, G. Andersson, P. Kaufmann, Chromatographia 46 (1997) 545.
[9] M. Jimidar, P.F. De Aguiar, S. Pintelon, D.L. Massart, J. Pharm. Biomed. Anal. 15 (1997) 709.
[10] K.D. Altria, S.D. Fildey, Chromatographia 39 (1994) 306.
[11] S. Fanali, S. Furlanetto, Z. Aturki, S. Pinzauti, Chromatographia 48 (1998) 395.
[12] R. Carlson, Design and Optimization in Organic Synthesis, Elsevier, Amsterdam, 1992.
[13] C.R. Stephens, K. Murai, H.R. Rennhard, L.R. Conover, K.J. Brunings, J. Am. Chem. Soc. 80 (1958) 5324.
[14] W. Naidong, S. Hua, E. Roets, R. Busson, J. Hoogmartens, Int. J. Pharm. 96 (1993) 13.
[15] L.A. Mitscher, in: The Chemistry of the Tetracycline Antibiotics, Medicinal Research Series, Vol. 9, Marcel Dekker, New York, 1978, p. 103.