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Separation of erythromycin and related substances on base-deactivated reversed-phase silica gel columns

H.K. Chepkwony, I. Vanderriest, J.M. Nguyo, E. Roets, J. Hoogmartens*

Katholieke Universiteit Leuven, Faculteit Farmaceutische Wetenschappen, Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Van Evenstraat 4, B-3000 Leuven, Belgium

Abstract

An official liquid chromatographic method for the analysis of erythromycin and related substances, which is based on a polymer reversed-phase, is described in the European Pharmacopoeia and in the United States Pharmacopeia. The pH of the mobile phase used in this system is 9.0. Recent advanced technology has led to the introduction of a new generation of silica-based reversed-phase column packings, which are claimed to be much more stable towards bases. They are useful for the analysis of basic compounds. Studies to verify the separation of erythromycin and related substances on Hypersil BDS C_{18}, Luna C_{18}(2), Inertsil ODS-2 and Supelcosil ABZ+ have been performed and the results are presented. It is shown that these base-deactivated phases give a better sensitivity and selectivity towards erythromycins than the polymer phase, provided that an adapted mobile phase is used. This is the first liquid chromatographic method described for the separation of erythromycin D from erythromycin A. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Pharmaceutical analysis; Erythromycins; Antibiotics

1. Introduction

Erythromycin is a complex macrolide antibiotic consisting mainly of erythromycin A, a 14-membered lactone ring with a 9-keto group, carrying a neutral and an amino sugar [1]. The structures of erythromycin, and its related substances and degradation products are shown in Figs. 1 and 2, together with abbreviations used throughout the text.

Liquid chromatography (LC) has been applied to the determination of erythromycin, its related substances and degradation products in bulk samples and preparations [2–11]. Reversed-phase packings based on silica gel are the most common stationary phases in liquid chromatography. However, poor stability limits the use of classical reversed-phase silica gels with mobile phases at extreme pH. Mobile phases at low pH cannot be used for erythromycin analysis due to its instability in acidic media. A number of publications using silica reversed-phase materials and mobile phases at a pH between 4.9 and 7.0 do not describe the separation from EA of components such as EF [2–5,11], EC [2], ED [2–7,11], EE [2–7], EANO [2–5,11], NdMeEA [2–4,6], AEA [2,11], PsEAEN [2–4,11] and EAEN [2].

Chromatographic selectivity for erythromycin components was found to be best on a polystyrene–divinylbenzene (PS–DVB) reversed phase at 70°C and a mobile phase at pH 9.0 [8,10]. This method is capable of separating all the known components of erythromycin, except ED. The European Phar-

*Corresponding author. Tel.: +32-16-323-442; fax: +32-16-323-448.

E-mail address: jos.hoogmartens@farm.kuleuven.ac.be (J. Hoogmartens)

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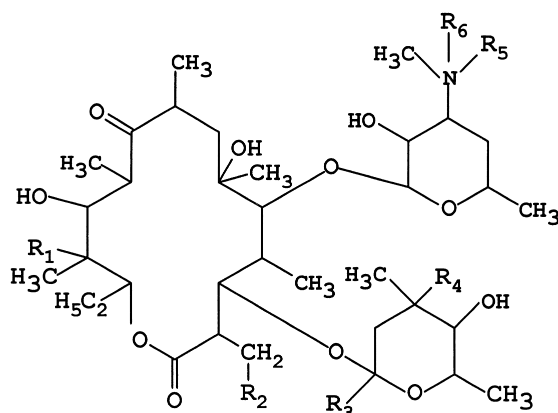


	R_1	R_2	R_3	R_4	R_5	R_6
Erythromycin A (EA)	OH	H	H	OCH_3	CH_3	-
Erythromycin B (EB)	H	H	H	OCH_3	CH_3	-
Erythromycin C (EC)	OH	H	H	OH	CH_3	-
Erythromycin D (ED)	H	H	H	OH	CH_3	-
Erythromycin E (EE)	OH	- O -		OCH_3	CH_3	-
Erythromycin F (EF)	OH	OH	H	OCH_3	CH_3	-
N-demethylerythromycin A (NdMeEA)	OH	H	H	OCH_3	-	-
Erythromycin A N-oxide (EANO)	OH	H	H	OCH_3	CH_3	O

Fig. 1. Structure of erythromycins.

macopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) [12,13] now prescribe it as the official liquid chromatographic method. Besides coelution of ED and EA, this system suffers from the drawback that it exhibits low efficiency, leading to poor sensitivity compared with silica reversed phases.

Advanced reversed-phase technology has led to the introduction of a new generation of silica gel packings, which are claimed to be much more stable towards high-pH mobile phases. Therefore, it was the intention of this work to utilise base-deactivated (BDS) reversed-phase silica gel columns to find a LC method with a better selectivity and sensitivity than the official method described in the Ph. Eur. and USP.

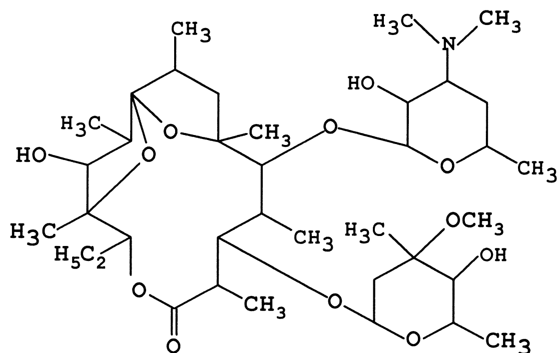
In this paper a gradient elution LC method utilising BDS reversed phases is described which allows better separation and quantitation of 11 known components of erythromycin at levels down to 0.1% (m/m).

2. Experimental

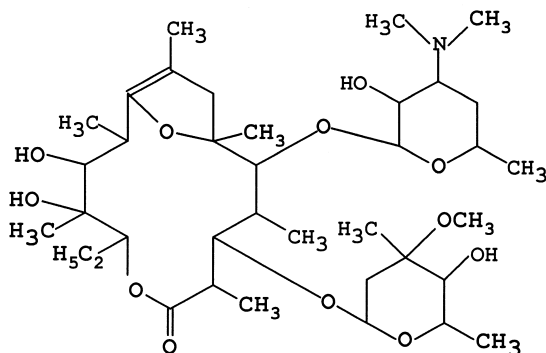
2.1. Reagents and samples

Water was distilled twice from glass apparatus. Acetonitrile, HPLC grade 'S' and methanol, HPLC grade, were from Rathburn Chemicals (Walkerburn, UK). 2-Methyl-2-propanol (Vel, Leuven, Belgium) was distilled before use. Dipotassium hydrogenphosphate (Merck, Darmstadt, Germany) 0.2 *M* solution was brought to the required pH by adding 0.2 *M* phosphoric acid (Merck).

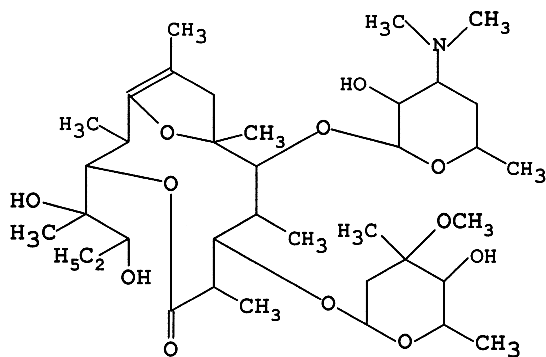
Reference substances EB and EC were obtained from the Ph. Eur. A commercial erythromycin sample which contained the impurities EANO, EF,



Anhydroerythromycin A enol ether (AEA)



Erythromycin A enol ether (EAEN)



Pseudoerythromycin A enol ether (PsEAEN)

Fig. 2. Structures of degradation products of erythromycin.

NdMeEA, EC, EE, AEA and EB was obtained from Phenix (Belgium). This sample was used as a house standard. Pure EA [14], PsEAEN [15,16], EANO [17], AEA [18], EAEN [19], NdMeEA [20], EE [21] and EF [22] were prepared according to procedures found in the literature. A commercial sample, known to contain ED, as verified by thin-layer chromatography, was also available [23].

2.2. LC instrumentation and chromatographic conditions

The LC apparatus consisted of a SpectraSystem P1000XR quaternary pump (Thermo Separation Products, Fremont, CA, USA), an Autosampler SpectraSeries AS100 equipped with a 100 μl loop, a variable-wavelength Spectra 100 UV–Vis detector set at 215 nm and a Hewlett-Packard integrator Model HP 3396 series II (Avondale, PA, USA). The columns were immersed in a waterbath at 30°C. The following BDS reversed-phase stationary phases were used: Hypersil BDS C_{18}, 5 μm (250×4.6 mm I.D.) (Shandon, Runcorn, UK); Luna C_{18}(2), 3 μm (100×4.6 mm I.D.) (Phenomenex, Torrance, CA, USA); Inertsil ODS-2, 5 μm (250×4.6 mm I.D.) (Alltech Associates, Deerfield, IL, USA); and Supelcosil ABZ+, 5 μm (250×4.6 mm I.D.) (Supelco, Bellefonte, PA, USA). A flow-rate of 1.0 ml/min was used.

2.3. Mobile phase

The mobile phases finally used with the Hypersil BDS C_{18} 5 μm column were: (A) 2-methyl-2-propanol–2-propanol–0.2 *M* phosphate buffer pH 7.5–water (8.5:8.5:5:78); (B) 2-methyl-2-propanol–acetonitrile–0.2 *M* phosphate buffer pH 7.5–water (22:5:5:68). The organic solvents were first mixed and the buffer–water mixture added. The mixture was degassed by sparging helium.

2.4. Sample preparation

Methanol–water (3:7) was always used as the sample solvent. For identification purposes, EA, EB, EC, EE, EF, NdMeEA, EANO and AEA were dissolved separately at a concentration of 0.2 mg/ml and PsEAEN and EAEN at a concentration of 0.005 mg/ml. A test mixture with all the components at the concentrations mentioned above was also prepared.

3. Results and discussion

3.1. Method development

Previous experiments on BDS columns with mobile phases at pH 6.0 had not shown good selectivity [9]. Therefore, it was decided to start the method development with a mobile phase at pH 9.0, which is also used with the PS–DVB column in the Ph. Eur. and USP method. This mobile phase contains acetonitrile–2-methyl-2-propanol–0.2 *M* K_2HPO_4 pH 9.0–water (3:16.5:5:75.5, v/v) and was used on the BDS columns at 45°C. Preliminary results obtained with Hypersil BDS showed a similar elution order of erythromycin components as on PS–DVB: EANO, EF, NdMeEA, EC, EE, EA, but with better overall selectivity. The separation between EE and EA was significantly improved on these columns versus the PS–DVB, but the resolution between EC and NdMeEA was reduced.

Experiments to verify further the separation of erythromycin and its related substances were carried out at lower mobile phase pH and a column temperature at 30°C. Better results were obtained when a mobile phase at pH 7.5 was used. The elution sequence changed: EF, EC, EE, NdMeEA, EANO, EA. However, the resolution between EC, EE and NdMeEA remained poor. The composition of the organic modifier in the mobile phase was then investigated. The effect of 2-methyl-2-propanol and acetonitrile in the mobile phase was studied independently. Fast elution of erythromycins with good peak symmetries was observed when using 2-methyl-2-propanol without acetonitrile in the mobile phase, however separation of EC, EE and NdMeEA was not achieved. Consequently, when acetonitrile was used alone as the organic modifier, delayed elution of erythromycins and poor separation of EC, EE and NdMeEA, coupled with poor peak symmetries, was observed.

Therefore, it became obvious that 2-methyl-2-propanol required another comodifier to improve its selectivity. Other organic modifiers, such as methanol and 2-propanol, were studied. Based on the

results obtained, it was concluded that a mixture of alcohols is necessary in order to obtain good separation between EC and EE. The system was optimized using Drylab software (LC Resources, Berlin, Germany). A mobile phase A containing 2-methyl-2-propanol–2-propanol–0.2 *M* phosphate buffer pH 7.5–water (8.5:8.5:5:78) was finally chosen and used isocratically to show the repeatability of the separation on different BDS C_{18} stationary phases: Luna, Inertsil ODS-2 and Supelcosil ABZ+. However, the selectivity between EC and EE, and EE and NdMeEA on Luna was reduced, whereas it remained unchanged on the other two phases. It was necessary to reduce the concentration of organic modifier in order to obtain approximately the same selectivity on Luna as on the other three columns used. When a commercial sample, containing ED, was analyzed a small peak was observed between the major peak (EA) and AEA. This peak was shown to coelute with a reference sample of ED. This is the first time that ED has been reported to be separated from all other erythromycin components by LC.

Isocratic elution with this mobile phase A was insufficient for the apolar components to be eluted. Therefore, a gradient elution method was developed on the Hypersil BDS column, using as mobile phase B: 2-methyl-2-propanol–acetonitrile–0.2 *M* K_2HPO_4 pH 7.5–water (22:5:5:68, v/v). 2-Propanol was now replaced by acetonitrile, being more UV transparent. A three-step gradient was introduced, after isocratic elution with mobile phase A (0–33 min), the second step (A–B, 50:50) was started after the small ED peak (33–48 min) and the third (100% B) after elution of EB (48–63 min) in order to elute EAEN within about 1 h. The time programme of the gradient had to be adapted as a function of the brand of the BDS stationary phase. Fig. 3 shows a typical chromatogram of the erythromycin laboratory standard obtained on Hypersil BDS. The unknown peaks labeled 1, 2 and 10 are degradation products which are formed as a function of time from either EA or related substances as erythromycin is unstable in aqueous solutions [24].

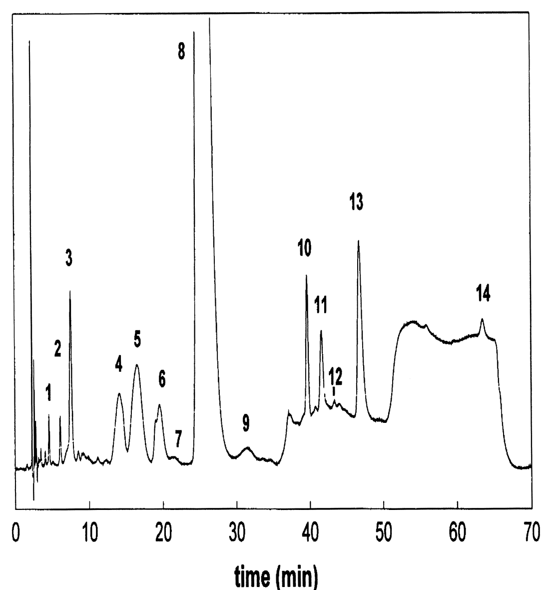


Fig. 3. Typical chromatogram of erythromycin laboratory standard. Stationary phase, Hypersil BDS C_{18}, 5 μm (250×4.6 mm I.D.); column temperature, 30°C; mobile phase, (A) 2-methyl-2-propanol–2-propanol–0.2 *M* K_2HPO_4 pH 7.5–water (8.5:8.5:5:78), (B) 2-methyl-2-propanol–acetonitrile–0.2 *M* K_2HPO_4 pH 7.5–water (22:5:5:68); gradient elution time, 0–33 min (100% A), 33–48 min (A–B, 50:50), 48–63 min (100% B) and 63–73 min (100% A); flow-rate, 1.0 ml/min; sample concentration, 4 mg/ml; injection volume, 100 μl; detection, UV at 215 nm. Peaks: 1, 2 and 10=unknown, 3=EF (1.4%), 4=EC (2.2%), 5=EE (2.4%), 6=NdMeEA (0.6%), 7=EANO (0.5%), 8=EA (81.8%), 9=ED (0.5%), 11=AEA (2.5%), 12=PsEAEN (<0.1%), 13=EB (3.3%) and 14=EAEN (<0.1%).

3.2. Robustness

Robustness is an important aspect of method validation. One evaluates the influence of small changes in the operating conditions (variables) of the analytical procedure on measured or calculated responses. The changes introduced when performing a robustness test reflect the changes that can occur when a method is transferred between different laboratories.

Using the Hypersil BDS column a full-factorial design experiment was used to evaluate the robustness of the method for the first step of the gradient programme, with the help of the statistical graphic software system Statgraphics version 6.0 (Manugistics, Rockville, MD, USA). The three variables investigated were the concentration of the 1:1 mixture of organic modifiers in the mobile phase, the pH of the mobile phase and the temperature. The values of the variables in the design are listed in Table 1.

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

Chromatographic variable	Low value (−1)	Central value (0)	High value (+1)
(A) Temperature (°C)	25.0	30.0	35.0
(B) pH	7.0	7.5	8.0
(C) 1:1 Mixture of 2-methyl-2-propanol–2-propanol (%)	16.5	17.0	17.5

For these three variables, 2^3 different experiments, one central level experiment plus duplicate experiments were performed and 18 chromatograms obtained. The estimated effects of the three variables with their second-order interactions on the selectivity between EC and EE, EE and NdMeEA, NdMeEA and EANO, and EANO and EA are presented on the standardized pareto charts in Fig. 4. 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 the critical t-value for $\alpha = 0.05$. Effects for which the bars are smaller than the critical t-value are considered as not significant.

The charts clearly show that the selectivity EC–EE and EANO–EA is significantly improved by a pH increase of 1.0, but the selectivity EE–NdMeEA is negatively influenced. A temperature increase has a positive effect on the selectivity EC–EE, NdMeEA–EANO and EANO–EA, but an insignificant, negative effect on EE–NdMeEA. A slight increase in the organic modifier concentration slightly reduces the selectivity between EC and EE and EE and NdMeEA, however this effect is insignificant for the other selectivities. The most important interaction is AB. This means that a small increase in the pH at low temperature may lower the selectivities EE–NdMeEA, NdMeEA–EANO and EANO–EA.

Response surface plots were constructed for EC, EE, NdMeEA, EANO and EA (Fig. 5) with retention times as a function of the most important chromatographic variables, the mobile phase pH and the column temperature. The response surface plots corresponding to EC–EE and EE–NdMeEA are nearly parallel to each other. The response surface plots for NdMeEA–EANO and EANO–EA cut across each other, whereas the surface plots corresponding to NdMeEA and EA remain parallel under all conditions. EANO is a neutral compound. It was observed that the retention of this compound is less dependent on pH and on all the BDS columns examined. A pH of 7.5±0.2 and a temperature of 30±2°C are necessary in order to achieve the separation of EC, EE and NdMeEA.

It was also observed that, upon ageing of the BDS columns, the relative retention time of the minor component (EANO) was shifted from a position before EA to after EE, which might lead to coelution with NdMeEA.

3.3. Repeatability, linearity and detection limits

The repeatability of the method was assessed using five solutions corresponding to 50, 75, 100, 120 and 150% concentrations of a 4.0 mg/ml solution of EA each injected three times. After normalisation of the peak areas of EA to 100%, the relative standard deviation (RSD) was 1.3%. The calibration curve obtained by analysis ($n = 7$) of a series of analyte concentrations corresponding to 0.1, 1, 5, 25, 75, 100 and 120% of 4 mg/ml of EA was subjected to linear regression analysis: $y = 864\,317 + 24\,945\,885x$, where y is the peak area and x the concentration (mg/ml); correlation coefficient $r = 0.999$, standard error of estimate $S_{y,x} = 1\,572\,534$. For an injection of 400 μg, the limit of quantitation (LOQ) (in %, m/m) was 0.1%, except for PsEAEN and EAEN: 0.01%, and the limit of detection (LOD) with a signal-to-noise ratio of 3 was 0.03%, except for PsEAEN and EAEN: 0.003%.

4. Conclusion

It can be concluded that this gradient elution LC

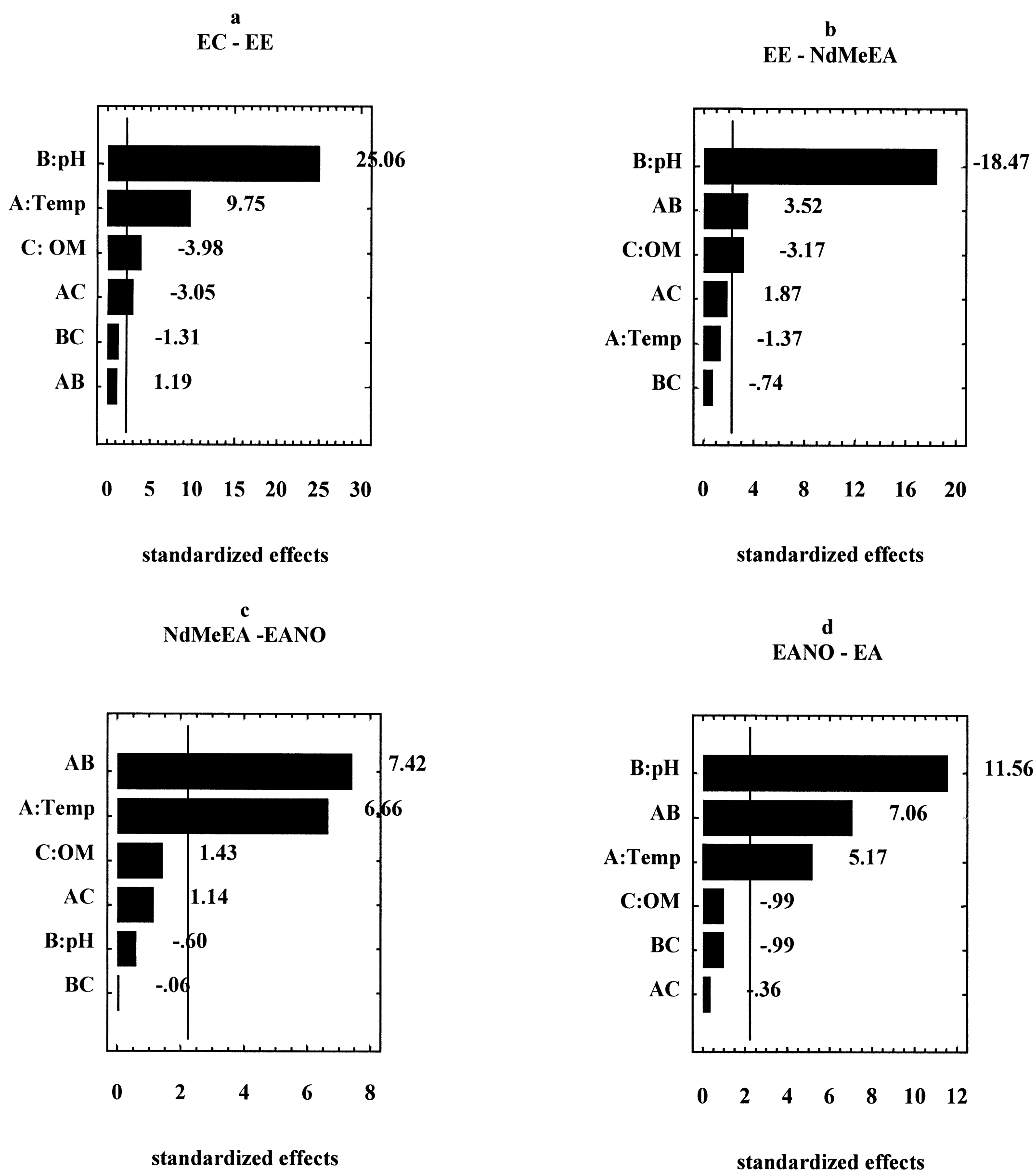


Fig. 4. Standardized pareto charts representing the estimated effects of parameters (A, B, C) and parameter interactions (AB, BC and AC) on the selectivity between (a) EC and EE, (b) EE and NdMeEA, (c) NdMeEA and EANO and (d) EANO and EA. A, column temperature; B, mobile phase pH; C, concentration of organic modifier (1:1 mixture of 2-methyl-2-propanol–2-propanol).

method utilising Hypersil BDS separates all known components of erythromycin from the main component EA. This method is linear, repeatable and more sensitive than the official LC method described in the Ph. Eur. and USP. This LC method is the first described for the separation of ED from EA. Other BDS columns may also be used successfully, pending slight adaptations of the gradient elution. The

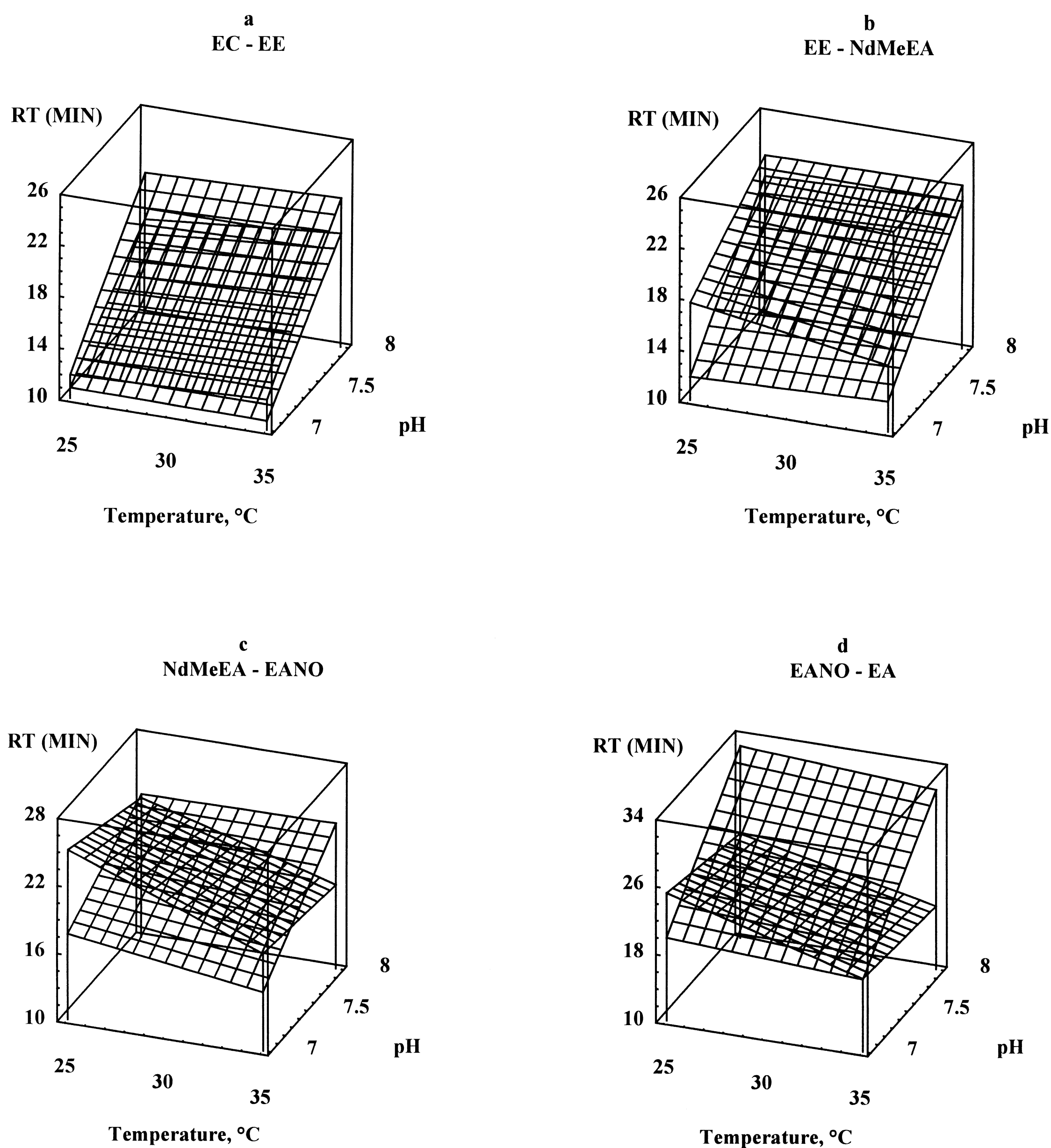


Fig. 5. Estimated response surface plots for (a) EC–EE, (b) EE–NdMeEA, (c) NdMeEA–EANO and (d) EANO–EA (lower planes to upper planes, respectively) constructed with the retention times as a function of pH and temperature.

elution order of EANO may change as a function of the type and age of the BDS column. The method described here is less robust than the official method in the Ph. Eur. and USP.

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References

[1] A. Bryskier, C. Agouridas, J.-C. Gasc, in: A.J. Bryskier, J.-P. Butzler, H.C. Neu, P.M. Tulkens (Eds.), Macrolides: Chemistry, Pharmacology and Clinical Uses, Arnette Blackwell, Paris, 1993, p. 10.
[2] S. Omura, Y. Suzuki, A. Nakagawa, T. Hata, J. Antibiot. 26 (1973) 794–795.
[3] K. Tsuji, J.F. Goetz, J. Chromatogr. 147 (1978) 359–367.
[4] K. Tsuji, M.P. Kane, J. Pharm. Sci. 71 (1982) 160–1164.
[5] Th. Cachet, I.O. Kibwage, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Liq. Chromatogr. 409 (1987) 91–100.
[6] Th. Cachet, K. De Turck, E. Roets, J. Hoogmartens, J. Pharm. Biomed. Anal. 9 (1991) 547–555.
[7] Th. Cachet, I. Quintens, J. Paesen, E. Roets, J. Hoogmartens, J. Liq. Chromatogr. 14 (1991) 1203–1218.
[8] J. Paesen, E. Roets, J. Hoogmartens, Chromatographia 32 (1991) 162–166.
[9] J. Paesen, P. Claeys, E. Roets, J. Hoogmartens, J. Liq. Chromatogr. 630 (1993) 117–122.
[10] J. Paesen, D.H. Calam, J.H.McB. Miller, G. Raiola, A. Rozanski, B. Silver, J. Hoogmartens, J. Liq. Chromatogr. 16 (1993) 1529–1544.
[11] M.M. Nasr, T.J. Tschappler, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 2329–2348.
[12] European Pharmacopoeia, 3rd ed., Council of Europe, Strasbourg, 1997:0179, pp. 807–808.
[13] United States Pharmacopeia, 23rd ed., United States Pharmacopeia Convention, Rockville, MD, Suppl. 7, 1997, pp. 3884–3885.
[14] I.O. Kibwage, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Chromatogr. 330 (1985) 275–286.
[15] I.O. Kibwage, R. Busson, G. Janssen, J. Hoogmartens, H. Vanderhaeghe, J. Org. Chem. 52 (1987) 991–996.
[16] H.A. Kirst, J.A. Wind, J.W. Paschal, J. Org. Chem. 52 (1987) 4359–4362.
[17] E.H. Flynn, M.V. Sigal, P.F. Wiley, K. Gerzon, J. Am. Chem. Soc. 76 (1954) 3121–3130.
[18] P.F. Wiley, K. Gerzon, E.H. Flynn, M.V. Sigal, O. Weaver, U.C. Quarck, R.R. Chauvette, R. Monahan, J. Am. Chem. Soc. 79 (1957) 6062.
[19] P. Kurath, P.H. Jones, R.S. Egan, T.J. Perun, Experientia 27 (1971) 362.
[20] L.A. Freiberg, US Pat., 3 725 385 (1972); C.A. 77 (1972) 19965e.
[21] J.R. Martin, R.S. Egan, A.W. Goldstein, P. Collum, Tetrahedron 31 (1975) 1985–1989.
[22] J.R. Martin, L. De Vault, A.C. Sinclair, R.S. Stanaszek, P. Johnson, J. Antibiot. 35 (1982) 426–430.
[23] I.O. Kibwage, E. Roets, J. Hoogmartens, J. Chromatogr. 256 (1983) 164–171.
[24] J. Paesen, K. Khan, E. Roets, J. Hoogmartens, Int. J. Pharm. 113 (1994) 215–222.