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Journal of Chromatography A, 1031 (2004) 303–305
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

Isocratic reversed-phase high-performance liquid chromatographic separation of tetracyclines and flumequine controlled by a chaotropic effect

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Abstract

Fast isocratic reversed-phase high-performance liquid charomatographic (RP-HPLC) separation of four tetracyclines and flumequine was obtained using a Zorbax SB-C_{18} column. Baseline resolution was achieved in 11 min. The peaks were narrow, well separated and without any tails although there was no chelating agents added to the mobile phase. Due to the chaotropic effect, the addition of potassium perchlorate allowed controlling the tetracyclines retention while the retention of flumequine was almost constant.

Keywords: Chaotropic effect; Tetracyclines; Flumequine; Antibiotics

1. Introduction

Antibiotics are used not only in humans but also in prevention and treatment of animals as well as growth promoters in husbandry. That can result in appearance of potentially harmful residues of antibiotics in food. Monitoring such residues should be an important task for government authorities. The analytical methods for determining antibiotics in food are based on microbiological, immunochemical and physicochemical principles. High-performance liquid chromatography (HPLC), belonging to the latter group, offers good separation and reliable methods of antibiotics detection at low levels of concentration. However, the main advantage of HPLC, i.e. effective separation, can be reached only when appropriate conditions of analysis are established.

Reversed-phase (RP)-HPLC separation of tetracyclines (oxytetracycline, OTC; tetracycline, TC; chlorotetracycline, CTC; doxycyline, DC) and fluoroquinolones (flumequine, FL) can be complicated because of mixed retention mechanism. Since both groups of drugs have propensity to form chelate complexes, it is necessary not only to control hydrophobic interactions but also to reduce chelation of metal impurities in chromatographic packings. For old columns, it is necessary to control [1]:

(i) dissociation of analytes–pH value of the mobile phase,
(ii) interaction with metal impurities in the support–addition of chelating agents to the mobile phase,
(iii) ion exchange on silanols–pH value of the mobile phase,
(iv) strong adsorption–addition of amines to the mobile phase.

For the modern column, it is possible to obtain good separation of tetracyclines and fluoroquinolones with simple mobile phases. In the present paper, such results are demonstrated.

2. Experimental

2.1. Reagents

Analytical grade oxalic acid was obtained from P.O.Ch. (Gliwice, Poland), flumequine and uracil from Sigma (St. Louis, MO, USA), doxycycline hydrochloride from Fluka (Buchs, Switzerland), HPLC-grade methanol from E. Merck (Darmstadt, Germany) and acetonitrile from P.O.Ch. The

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doi:10.1016/j.chroma.2003.12.024

other tetracyclines were supplied by Polfa (Tarchomin, Poland). Trifluoroacetic acid (TFA), was purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Water was redistilled and purified in a Milli-Q system (Millipore, Milford, MA, USA). 0.1 g portions of FL and tetracyclines were weighed accurately into a 10 ml volumetric flask. FL was dissolved in 9 ml of 1% Na_2CO_3 and then diluted to the volume with water, tetracyclines were dissolved in 10 ml of water. The working solutions were the mixtures, prepared by the dilution with water of the stock solutions.

2.2. HPLC equipment

The HPLC system consisted of 110B pump (Beckman Instruments, San Ramon, USA) with UV/VIS–155 detector (Gilson, Middleton, USA), a sample injector model 7125 with 20 μl loop, (Rheodyne, Cotati, USA), CSW32 acquisition system (DataApex, Prague, Czech Republic). A 250 mm × 4.6 mm Zorbax SB-C_{18}, 5 μm column (Agilent Technologies, USA) was used.

2.3. HPLC mobile phases

Mobile phases were prepared by mixing appropriate volumes of acetonitrile, methanol and an aqueous phase. The composition of the aqueous solution of a given pH value was calculated using the dissociation constants of oxalic acid ($pK_{A1} = 1.23$ and $pK_{A2} = 4.19$) and trifluoroacetic acid ($pK_A = 0.5$). The calculations were performed using Microsoft Excel'97 spreadsheet.

2.4. Chromatographic conditions

Dead volume of the column were estimated from peak of uracil using MeOH–water (85:15, v/v) (the column producer test mobile phase). All measurements were performed at room temperature. Flow rate of the mobile phase was 1 ml min^{-1}.

3. Results and discussion

For modern column, like Zorbax SB-C_{18}, it is possible to perform chromatographic separation at low pH (close to 1), which should completely suppress dissociation of analytes acidic groups. Due to full deactivation of the support surface, there is no need to add chelating agents to the mobile phase. In Fig. 1, it is demonstrated that tetracyclines peaks have no tails even for the mobile phase containing only hydrochloric acid to set the pH value. The replacing hydrochloric acid by TFA results in increase of tetracyclines retention while flumequine retention is slightly reduced (the retention coefficient of FL decreased from 3.33 to 3.19). This behavior can be explained by the chaotropic effect [2,3]. The higher concentration of chaotropic anions in

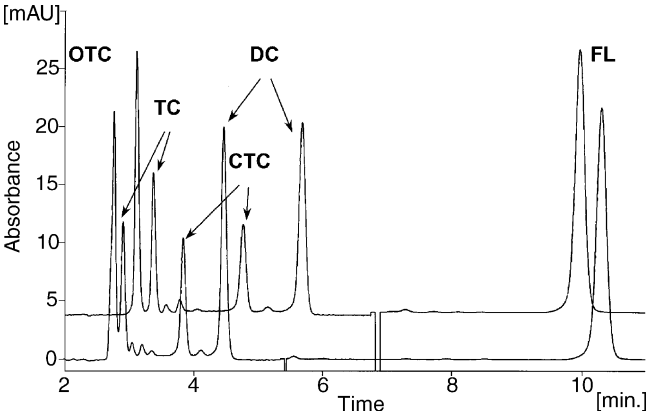

Fig. 1. Example chromatograms of the antibiotics mixture. OTC, TC, CTC, DC detected at 350 nm and FL at 315 nm. Antibiotic concentrations were 0.01 mg/ml in water, 20 μl injected, flow rate 1 ml/min. Mobile phase composition was ACN–MeOH–aqueous phase of pH 1.3 (30:15:55, v/v). The aqueous phase consists of 0.1 M HCl or 0.058 M TFA. The chromatogram obtained with mobile phase containing TFA is shifted up by 4 mAU.

the mobile phase of low pH value the stronger retention of basic analytes is obtained. The disadvantage of TFA at very low pH could be its partial dissociation resulting in lower anion concentration comparing to the total acid concentration. Another common chaotrop is perchlorate anion. Perchlorate acid is considered to be a strong acid and it should be fully dissociated independently of pH value of its solution. The influence of perchlorate anion concentration on tetracyclines retention is demonstrated in Figs. 2 and 3. Potassium perchlorate was used as an additive to the mobile phase containing oxalic acid as pH value regulator. Significant increase in the retention of tetracyclines is observed. It is possible to obtain good separation of tetracyclines and flumequine by adjusting the concentration of perchlorate.

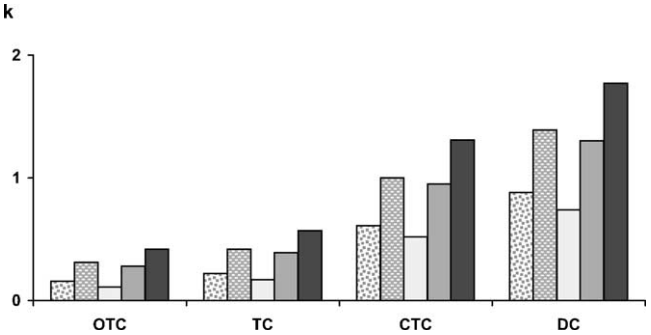

Fig. 2. Tetracycline retention coefficient for different mobile phases. Organic modifier contents (30%, v/v, of ACN and 15%, v/v, of MeOH) and the pH value of aqueous phase (1.3) are the same in all cases. The aqueous phase contains HCl (the first bar), TFA (the second bar), oxalic acid (0.0925 M) (the others bars). The fourth and fifth bars represent mobile phases with addition of potassium perchlorate, 10 and 30 mM in the mobile phase, respectively.

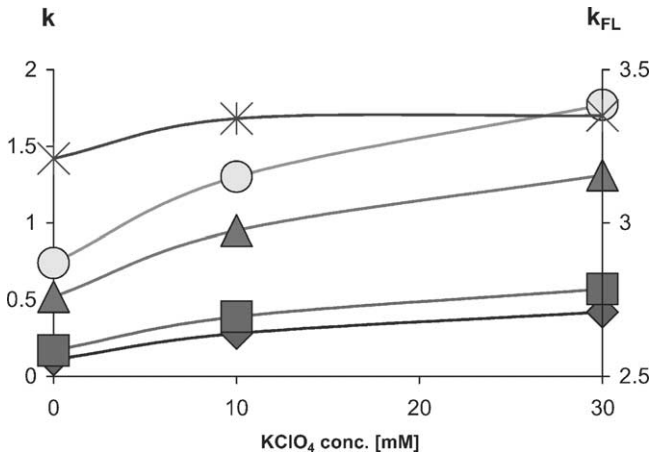

Fig. 3. Dependence of tetracyclines and flumequine retention coefficient on potassium perchlorate concentration in the mobile phase containing oxalic acid. Symbols: (◆) OTC, (■) TC, (▲) CTC, (●) DC, and (✕) FL.

Although the chromatograms were pretty good, some problems still remained. The peaks of tetracyclines were too wide. The number of theoretical plates (peak width measured at half height) for DC was less then 14000 while 23000 was obtained for phenol and almost 20000 for FL. The reproducibility of peak area for DC was slightly worse then for FL. We address the problem in our current investigations.

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