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Note

Separation of tetracycline and related substances by high-performance liquid chromatography on poly(styrene–divinylbenzene)

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The separation of tetracycline (TC) and its related compounds 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) by high-performance liquid chromatography (HPLC) has been the subject of many scientific communications. Early work up to 1980 has been briefly reviewed previously[1]. At that time, separation on reversed-phase materials was preferred to chromatography on ion-exchange materials, and relatively low pH values (1.5–3) were used. At these pH values, the main problem consists of an important difference in retention time between the groups ETC–TC and EATC–ATC. When the mobile phase is suitable for separation of ETC and TC, EATC and ATC are eluted much later and small amounts are not detectable. On the other hand, when ATC and EATC are eluted fast enough to be detected at limit levels, ETC and TC are not sufficiently separated. Many authors have minimized this problem by showing separations of almost equal amounts of ETC and TC, mixed with substantial amounts of EATC and ATC. In reality, very small amounts of EATC and ATC and small amounts of ETC must be separated from TC. The *European Pharmacopoeia* limits ETC at 5% and EATC and ATC at 0.5%[2]. Generally, this chromatographic problem was solved by using gradient chromatography, which was also successfully applied in our laboratory[3–5]. Adaptations of the classical system at low pH on reversed-phase materials were also published more recently[6–8]. Systems using mobile phases at moderate pH do not give much better results[9]. ETC and TC are not better separated. EATC is eluted very close to TC and ATC is still strongly retained.

Since 1980, methods on reversed-phase materials with mobile phases at relatively high pH (8–8.5) have been published, although it is known that silica-based reversed-phase materials are less stable at higher pH[10,11]. The higher pH is obtained by addition of an amine to the mobile phase and the quality of the separation is reported to be influenced by the nature of the amine; N,N-dimethyloctylamine seems to give the best separations[11]. An adaptation of this method will probably be introduced in the *United States Pharmacopeia*[12]. The chromatograms obtained under these conditions are quite comparable with those obtained at neutral pH, but the separation of TC–EATC is better and ATC is less strongly retained. The main disadvantages seem to be some tailing of the TC peak and instability of the packing material due to the high pH of the mobile phase. However, the stability of the packing material, used under such conditions, has been mentioned to be satisfactory[13].

For kinetic studies, the use of mobile phases at pH 2.0 containing sodium lauryl sulphate, or at pH 7.0 containing cetrimonium bromide, have been described very recently[14]. These systems do not seem to present special advantages for application in the purity control of tetracycline.

Very recently, we described the separation of doxycycline and related substances by HPLC on poly(styrene–divinylbenzene) copolymer (PSDVB)[15]. It was observed that this stationary phase was very useful for the separation of various tetracycline epimers. In this paper, a method using PSDVB is described which allows the separation of ETC, TC, EATC and ATC in about 15 min.

EXPERIMENTAL

Samples and chemicals

Reference samples of ETC, EATC and ATC, *tert.*-butanol (99.5%) and organic reagents were obtained from Janssen Chimica (Beerse, Belgium). Potassium phosphates of *pro analysi* quality were from Merck (Darmstadt, F.R.G.). Water was distilled twice.

HPLC apparatus and operating conditions

A Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was equipped with a pulse dampener and a Bourdon manometer as described previously[16]. The flow-rate was 1.0 ml/min. The HPLC apparatus further consisted of an injector, Model CV-6-UHPa-N60, equipped with a 20-μl loop (Valco, Houston, TX, U.S.A.), a Model LC 3 UV variable-wavelength detector (Pye Unicam, Cambridge, U.K.) set at 254 nm and 0.01 a.u.f.s., and a recording integrator Model 3390 A (Hewlett-Packard, Avondale, PA, U.S.A.). The column is kept at 60°C by immersion in a waterbath.

Column, mobile phase and preparation of samples

The column (100 mm × 4.6 mm I.D.) was packed in the laboratory with PLRP-S, 8 μm, 100 Å (Polymer Labs., Church Stretton, U.K.). The packing material (2.0 g) was wetted with 2.5 ml of acetone and slurried in 10 ml of an aqueous solution containing 2.5% (w/v) sodium chloride and 10% glycerol. The slurry was sonicated for 4 min and quickly packed in the column, as described previously[17].

The mobile phase was prepared by weighing 55.0 g of *tert.*-butanol, which was rinsed in a 1000-ml flask with water, and 100 ml of 0.2 *M* phosphate buffer (pH 9.0), 50 ml 0.02 *M* tetrabutylammonium hydrogen sulphate (TBA) solution and 10 ml of 0.1 *M* sodium ethylenediaminetetraacetate (EDTA) were added. During preparation of the latter two solutions, the pH was brought to pH 9.0 by the addition of sodium hydroxide solution. The mixture was finally diluted to the mark with water. The mobile phase was degassed by sonication.

Tetracycline samples to be examined were dissolved in the mobile phase at a concentration of 1 mg/ml. Reference samples, used for identification purposes, were dissolved in the mobile phase at a concentration of 0.05 mg/ml.

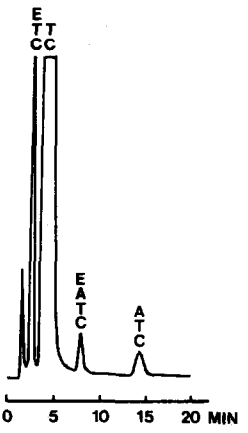

Fig. 1. Chromatogram of a commercial sample of tetracycline. See experimental for chromatographic conditions. ETC = 4-Epitetracycline (4%), TC = tetracycline, EATC = 4-epianhydrotetracycline (0.5%), ATC = anhydrotetracycline (0.5%).

RESULTS AND DISCUSSION

As already mentioned, the present HPLC method is derived from a method previously published for doxycycline[15], where tetrahydrofuran (THF) was mentioned as being the most suitable organic modifier. THF was also found to be very suitable for the analysis of tetracycline. However, the use of THF has several disadvantages. This solvent is susceptible to the formation of peroxides and therefore a stabilizer, often butylated hydroxytoluene, is added to the commercial product. Because of its strong UV absorbance, the stabilizer has to be removed by distillation after the control of absence of peroxides. Another drawback is that mobile phases containing THF easily form gas bubbles in certain types of UV detectors. Further efforts were therefore made to find another organic modifier giving sufficient separation. *tert.*-Butanol was found to be satisfactory for the analysis of doxycycline or tetracycline. With *tert.*-butanol it was possible to reduce the analysis time for doxycycline from about 50 min, as previously published, to about 20 min.

For tetracycline, it was observed that the main impurities, ETC, EATC and ATC, could be sufficiently separated on a 10-cm column. A chromatogram obtained with a commercial sample is shown in Fig. 1. A complete analysis takes about 15 min. The quantitation of small amounts of impurities can be realized thanks to the good separation. It is necessary to immerse the column in a water bath; the use of a water jacket does not allow adequate heating of the top of the column and causes peak distortion. Although the column is kept at 60°C, it can be used over several months. The only maintenance required is refilling with a suspension of packing material in methanol if a small gap forms at the top of the column bed after several weeks of continuous use. This completely restores the original separating power of the column. With a 25-cm column it is possible to perform an even more complete analysis of tetracycline. Details of the analysis of tetracycline standards and of a number of commercial samples will be dealt with later.

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