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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TETRACYCLINES

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SUMMARY

The possibilities of two non-ionogenic resins (XAD-2 and PRP-1) in the chromatography of tetracyclines and their degradation products are described. It appears that, in contrast to silica-based materials, this type of column material produces linear calibration curves, even in the low nanogram range. Two different methods of sample pretreatment in bioanalysis of tetracyclines and their degradation products are compared with respect to selectivity towards the matrix background and recovery.

INTRODUCTION

During the last decade, a number of papers dealing with the liquid chromatographic determination of the tetracycline group and their degradation products have been published. Tetracyclines are widely prescribed, since they are highly active as antimicrobial agents. Although the concentration of tetracyclines in biological specimens can be determined by means of microbiological or spectrophotometric methods, liquid chromatography is often preferred because of its higher selectivity and/or sensitivity of detection. The microbiological as well as the spectrometric methods do not differentiate between the tetracyclines themselves and their degradation products.

Although several liquid chromatographic assays have been described, those reports deal mainly with the determination of tetracyclines in pharmaceutical preparations[1–9], where relatively high concentrations are involved. Most papers describe the use of reversed-phase systems, based on modified silica as stationary phase. However, with the use of silica-based materials, two severe problems are encountered: poor chromatographic efficiency and an apparently irreversible adsorption of small amounts of tetracyclines, presumably onto residual silanol groups of the column material[11–13]. The efficiency can be improved by adding EDTA to the mobile phase in order to suppress the complex formation of tetracyclines with metal ions, present in the chromatographic system. The strong adsorption onto the silanol groups is more difficult to eliminate. Some investigators apply "capped" reversed-phase materials by trialkylsilylation[13], while others use systems based on ion-pair formation[12,14,15] or based on the use of ion-exchange materials[1–2]. However, the efficiency remains rather poor. Also, different batches of the same type of packing material

give different results[11]. The strong adsorption of tetracyclines onto the packing material observed with silica-based materials, results in non-linear calibration curves. Apparently, certain amounts are captured by the residual silanol groups. Therefore, some investigators add another member of the tetracycline group to the mobile phase in order to "deactivate" the packing material[11,16]. However, this leads in an increase of the baseline noise of the system and, consequently, to higher detection limits.

We decided to investigate the possibilities of packing materials that do not possess active sites, such as the free silanol groups of silica-based materials, but limited ourselves to Amberlite XAD-2 and PRP-1 resins. Both materials are strongly hydrophobic non-ionogenic materials, prepared as copolymers of divinylbenzene and styrene. The former is available only in batches with large particle size, while the latter can be obtained as high-performance packing material. With both packing materials, columns are obtained that show linear calibration curves and high selectivities towards the compounds of interest.

Phase systems for the separation (and determination) of tetracyclines and their degradation products are characterized by investigating the pH and the modifier content. Tetracycline and its analogues (see Table I) have several functional groups resulting in strongly complexing properties. Between pH 2 and 9, the tetracyclines exist as zwitter ions[1,10].

It is important to determine not only the tetracycline itself but also the degradation products in biological and pharmaceutical materials. Next to a decreased potency, degradation can lead to toxic degradation products. This is already proven for epi-anhydrotetracycline[17,18]. It must be realized that degradation of tetracyclines can also occur in the stomach.

For the isolation of tetracyclines from biological materials, different methods are described: (i) dilution of the sample, followed by direct injection[19-21], (ii) simple deproteinization[11,22,23], (iii) liquid–solid isolation on modified silica[24,25] or (iv) liquid–liquid extraction[26]. The first two methods are only applicable to high concentrations, while the third method is not our method of choice for reasons mentioned above. We compared the possibilities of a simple deproteinization of plasma samples by perchloric acid with a liquid–liquid extraction. The former method is fast and allows a reliable determination of higher concentrations, while for lower concentrations (sub-microgram levels) liquid–liquid extraction is needed. This paper describes the separation of a number of tetracyclines and some of their degradation products. The chromatographic system is applied to the determination of tetracycline and its degradation products in plasma samples.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a microprocessor-controlled system (Model SP 8000B, Spectra Physics, Santa Clara, CA, U.S.A.), equipped with a 20-μl pneumatic sample injector (Rheodyne 7126, Berkeley, CA, U.S.A.) and a variable-wavelength detector (LC-UV 3, Pye Unicam, Cambridge, U.K.) operated at 268 or 357 nm. Chromatography was performed with laboratory-prepared columns (100 × 3.0 mm I.D.), thermostatted at 25°C by means of a water-jacket. The columns were packed by means of a high-pressure air-amplifier booster pump (DSHF 302, Haskell, Burbank, CA, U.S.A.).

Chemicals and materials

All organic solvents were of analytical grade (J. T. Baker, Phillipsburg, NJ, U.S.A.); water was purified by means of a Milli Q Water Purification System (Millipore, Bedford, MA, U.S.A.). The buffers used in the mobile phases were prepared from 0.05 or 0.2 mol/l solutions of acetic acid and sodium acetate. Tetracycline and its degradation products were obtained from The European Pharmacopoeia Committee (Strassbourg, France) and the other tetracycline analogues and their degradation products were kindly donated by Pfizer (Brussels, Belgium), Lederle (Haarlem, The Netherlands) and Hoechst (Amsterdam, The Netherlands). The 2 mol/l phosphate–sulphite buffer was prepared from disodium hydrogen phosphate and sodium sulphite and adjusted to pH 6.1 by adding concentrated phosphoric acid.

Preparation of micronized XAD-2 resin

A coarse grade of Amberlite XAD-2 resin (16–50 mesh, Rohm & Haas, Philadelphia, PA, U.S.A.) was washed with water, 0.1 M hydrochloric acid, ethanol and then acetone in order to remove soluble additives and impurities. After drying, the material was ground in a rotating mortar. The ground material was classified by means of an air classifier (Alpine MZR, Augsburg, F.R.G.) to a particle size range of 6–7 μm.

Column packing

The columns were packed with the pressurized-slurry technique: 500 mg of the packing material were suspended in 2 ml of methanol and, after vigorous shaking, 2 ml of water were added. This aqueous suspension was poured into a pre-column (25 cm × 4.6 mm I.D.), which was connected with the empty column, provided with a bottom frit. Then the column was packed by pumping through methanol at a pressure of 75 MPa.
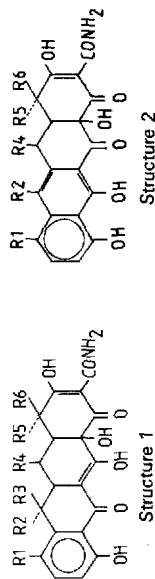
Chromatography

Capacity ratios were calculated from the retention times of the tetracyclines and of an unretained compound, for which potassium bichromate was used. The selectivity coefficients were calculated as the ratio of the corresponding capacity ratios. The theoretical plate height for a compound was calculated from its retention time and half the peak width at 0.6 of the peak height. The porosity of the column was calculated from the flow-rate, the retention time of the unretained compound and the volume of the column.
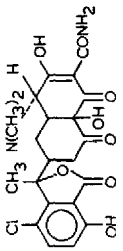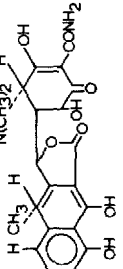
Sample pretreatment

Precipitation method. To 0.5 ml of plasma in a polyethylene cup (1.5 ml), 0.2 ml of perchloric acid solution (1.25 M) was added. After mixing (60 s on a Whirlmixer), the cup was centrifuged (10 min at 1000 g); 100 μl of the supernatant were injected into the column.

Liquid–liquid extraction. To 0.5 ml of plasma in a polypropylene centrifuge tube (12 ml), 1 ml of phosphate–sulphite (2 M, pH 6.1) was added. After mixing in a Whirlmixer for 60 s, 6 ml of ethyl acetate were added and gently mixed on a laboratory-built rotating-disk mixer (50 rpm) for 15 min. After centrifugation (10 min at 1500 g), the organic layer was decanted and evaporated under nitrogen at

TABLE I

ABBREVIATIONS AND STRUCTURES OF THE TETRACYCLINES

Structure 1

Structure 2

Compound	Abbreviation	R1	R2	R3	R4	R5	R6	Structure
Tetracycline	TC	-H	-CH$_3$	-OH	-H	-N(CH$_3$)$_2$	-H	1
Epi-tetracycline	ETC	-H	-CH$_3$	-OH	-H	-H	-N(CH$_3$)$_2$	1
Epi-anhydro-tetracycline	EATC	-H	-CH$_3$	—	-H	-H	-N(CH$_3$)$_2$	2
Anhydro-tetracycline	ATC	-H	-CH$_3$	—	-H	-N(CH$_3$)$_2$	-H	2
Oxytetracycline	OTC	-H	-CH$_3$	-OH	-OH	-N(CH$_3$)$_2$	-H	1
Chlortetracycline	CTC	-Cl	-CH$_3$	-OH	-H	-N(CH$_3$)$_2$	-H	1
Demethylchlortetracycline	DMCTC	-Cl	-H	-OH	-H	-N(CH$_3$)$_2$	-H	1
Methacycline	MeC	-H	—	=CH$_2$	-OH	-N(CH$_3$)$_2$	-H	1
Minocycline	MiC	-N(CH$_3$)$_2$	-H	-H	-H	-N(CH$_3$)$_2$	-H	1
Doxycycline	DC	-H	-CH$_3$	-OH	-OH	-N(CH$_3$)$_2$	-H	1
Rolitetracycline	RTC	CO-NH-CH-N						

instead of -CONH$_2$

apo OTC

Isochlortetracycline, apo-Oxytetracycline   ICTC

ambient temperature. The residue was dissolved in 200 μl of the mobile phase; aliquots of 100 μl were analysed by high-performance liquid chromatography (HPLC).

RESULTS AND DISCUSSION

Tetracyclines are compounds with different functional groups in the molecule. The presence of these groups complicates the mechanisms involved in the retention in chromatographic systems. As a consequence of the high polarity of tetracyclines, mobile phases with a high content of water are applied. In such phase systems, ion-pair formation, complex formation and strong interaction with residual silanol groups play an important role, in addition to the normal distribution, based on reversed-phase partition. Especially in trace analysis of tetracyclines, this creates a severe problem with respect to quantification. For these reasons, the possibilities of non-silica column materials was investigated. We compared Amberlite XAD-2 that was micronized in our laboratory with the more expensive commercially available PRP-1. Both materials are copolymers of divinylbenzene with styrene and do not contain any functional groups. Due to its relatively high specific area, combined with the strongly hydrophobic surface, considerable retention is obtained with these materials.

As reported by many authors[10,19,22,23,27-29], the addition of EDTA to the mobile phase is important in order to eliminate complex formation with metal ions in the chromatographic system. It appeared that, in particular, the walls of the stainless-steel column played an important role, because in PTFE-lined columns the peak shape was almost symmetrical when no EDTA was present in the mobile phase. Still, reasonable plate numbers were obtained. On the other hand, addition of ferric ions to the mobile phase resulted in a tremendous increase in the capacity ratio of the tetracyclines. In all other experiments, we applied a concentration of 0.025 M EDTA, which appeared optimal with respect to peak shape and solubility in the mobile phase.

Influence of dichloromethane on efficiency

In spite of the high pressure applied for packing the column, both packing materials are sensitive to pressure during chromatography. At pressures up to about 10 MPa there were no complications, but at higher pressures the XAD-2 bed in particular shrank to such an extent that the efficiency suffered considerably and even double peaks were observed. This problem could be overcome easily by adding a small amount of dichloromethane (1%, v/v) to the mobile phase. This probably makes the resin particles swell, because under these conditions the packed bed was fairly resistant towards pressure (up to 40 MPa). The efficiency of both XAD-2 and PRP-1 was increased 2- to 3-fold by the addition of 1% (v/v) dichloromethane, while the porosity of the column was not influenced.

Phase system selectivity and the role of pH

Each tetracycline mentioned in this study has three pK_a values, which are *ca.* 3.3, 7.9 and 9.7, respectively. Only MiC possesses a fourth pK_a of *ca.* 5.0. In Table II, the capacity ratios are listed as a function of the pH of the mobile phase for all tetracyclines and derivatives investigated. This table shows that the capacity ratio is

TABLE II

CAPACITY RATIOS OF THE TETRACYCLINES AS A FUNCTION OF THE pH OF THE MOBILE PHASE

Mobile phase: acetonitrile–dichloromethane–0.05 M acetate buffer (10:1:90, v/v) containing 0.025 M EDTA. ACTC = Anhydro-chlortetracycline; ADMCTC = anhydro-demethylchlortetracycline; EACTC = epi-anhydro-chlortetracycline; EADMCTC = epi-anhydro-demethylchlortetracyucline; ECTC = epi-chlor-tetracycline; EDMCTC = epi-demethylchlortetracycline; EOTC = epi-oxytetracycline; other abbreviations as in Table I.

Compound	pH = 3.4		pH = 3.6		pH = 3.8		pH = 4.8		pH = 5.5	
	PRP-1	XAD-2	PRP-1	XAD-2	PRP-1	XAD-2	PRP-1	XAD-2	PRP-1	XAD-2
ETC	1.99	1.54	2.10	1.78	1.93	1.68	1.72	1.33	1.72	0.63
TC	2.63	2.07	2.78	2.30	2.60	2.19	2.39	1.84	2.58	1.68
EATC	27.4	21.0	28.6	22.6	28.0	21.1	17.9	13.8	13.3	10.1
ATC	47.4	34.9	53.9	40.9	57.7	40.4	51.2	37.2	46.6	31.8
EOTC	1.13	0.95	1.24	0.98	1.00	0.94	0.86	0.69	0.90	0.71
OTC	1.63	1.24	1.75	1.35	1.56	1.28	1.46	1.15	1.59	1.20
α-apo OTC	3.09	2.48	3.51	2.95	3.88	2.81	2.93	2.44	3.00	2.45
β-apo OTC	32.7	22.3	36.7	26.4	37.9	25.6	15.5	12.0	10.5	8.10
ICTC	5.93	4.25	6.62	4.77	6.24	4.34	4.75	3.73	5.10	3.73
ECTC	5.79	4.25	6.65	4.89	6.51	4.57	5.01	3.96	5.05	3.82
CTC	9.00	6.59	10.1	7.77	9.91	7.01	7.62	6.20	8.25	6.27
EACTC	99.9	71.7	104	74.6	102	67.0	41.8	30.9	20.8	16.8
ACTC	216	159	248	165	212	156	156	100	71.9	53.5
EDMCTC	3.78	2.77	4.33	3.13	3.95	3.12	3.12	2.56	3.13	2.45
DMCTC	4.54	3.85	5.90	4.38	5.54	4.35	4.32	3.63	4.69	3.76
EADMCTC	51.2	35.7	51.8	38.5	44.9	35.0	20.1	16.5	12.0	10.0
ADMCTC	87.1	57.7	96.1	66.8	88.3	63.6	51.1	37.0	26.2	19.8
RTC	2.51	1.74	2.80	2.02	2.58	1.95	2.71	2.03	3.00	2.39
MiC	2.86	1.90	4.07	2.75	4.50	3.27	12.9	9.47	21.1	16.2
MeC	12.2	8.83	12.7	9.02	11.1	8.62	7.37	6.13	8.00	6.29
DC	14.7	10.3	14.4	10.2	10.1	9.56	9.18	6.88	9.19	7.09

maximal at pH 3.3. At this pH, EDTA exists in the doubly charged anionic form and the tetracyclines either as a zwitter-ion or as a cation[10]. Table II shows that the order of elution within one class of tetracyclines is always the same. The epimer of a certain tetracycline is always eluted first, while its anhydro-compound is most retained. Nevertheless, the influence of the pH of the mobile phase on the selectivity is of only minor importance.

The acetonitrile concentration plays a more important role in the retention of tetracyclines than the pH. However, the selectivity is hardly influenced and all the compounds of interest are affected in the same way. The main difference between the two packing materials is the extent of retention. This is mainly due to the difference in the specific area, which amounts to 450 and 330 m^2/g for PRP-1 and XAD-2, respectively. Linear regression analysis of the data, summarized in Table II, shows a correlation coefficient of 0.998 and a slope of 0.70, when the capacity ratios on XAD-2 are correlated with the capacity ratios on PRP-1. The calculated slope agrees well with the ratio of the specific areas (0.73).

Fig. 1 shows the separation of some tetracyclines and their degradation products, obtained with XAD-2 and with PRP-1. It can be seen that all compounds are separated, although no baseline resolution is achieved between EOTC and OTC and ICTC and ECTC, respectively. For the separation of these compounds, the phase system will have to be modified.

Extraction of plasma samples

In the determination of therapeutic levels in patient samples, high concentrations are generally encountered, allowing a simple pretreatment of plasma samples. Often this pretreatment consists only in the precipitation of proteins, followed by centrifugation and direct injection of the supernatant. But when the degradation products of a certain tetracycline must also be determined, this procedure is not adequate. This is due to the great differences in physicochemical properties and concentrations. We compared two methods of sample pretreatment: the above-mentioned precipitation method and a liquid–liquid extraction. The latter method is especially suited for the determination of lower levels, because a concentration step is built in, which is combined with the selectivity of the liquid extraction.

The precipitation method is based on the denaturation of proteins by perchloric acid. Unfortunately, under acidic conditions, the epimeric degradation product is easily formed. This implies that even when the supernatant is injected immediately after centrifugation, a certain amount of degradation is always observed. We therefore apply this method only for screening of unknown samples. In Fig. 2, a typical example of such a procedure is presented. In the liquid–liquid extraction, the tetracyclines are salted out by the phosphate–sulphite buffer and extracted into the ethyl acetate phase. Under these mild conditions (pH 6.1, ambient temperature), epimerization is limited. The addition of ascorbic acid, as suggested in the literature[26], in our hands gave low recoveries of the degradation products. These compounds could not be detected after addition of ascorbic acid to the decanted extraction phase. We obtained the best results by evaporation of the extraction solvent under nitrogen. It is clear that the precipitation method is less time-consuming and is more suited for relatively high concentrations, while liquid–liquid extraction allows the determination of lower levels. In Fig. 3 the chromatogram of an extract of plasma, spiked with
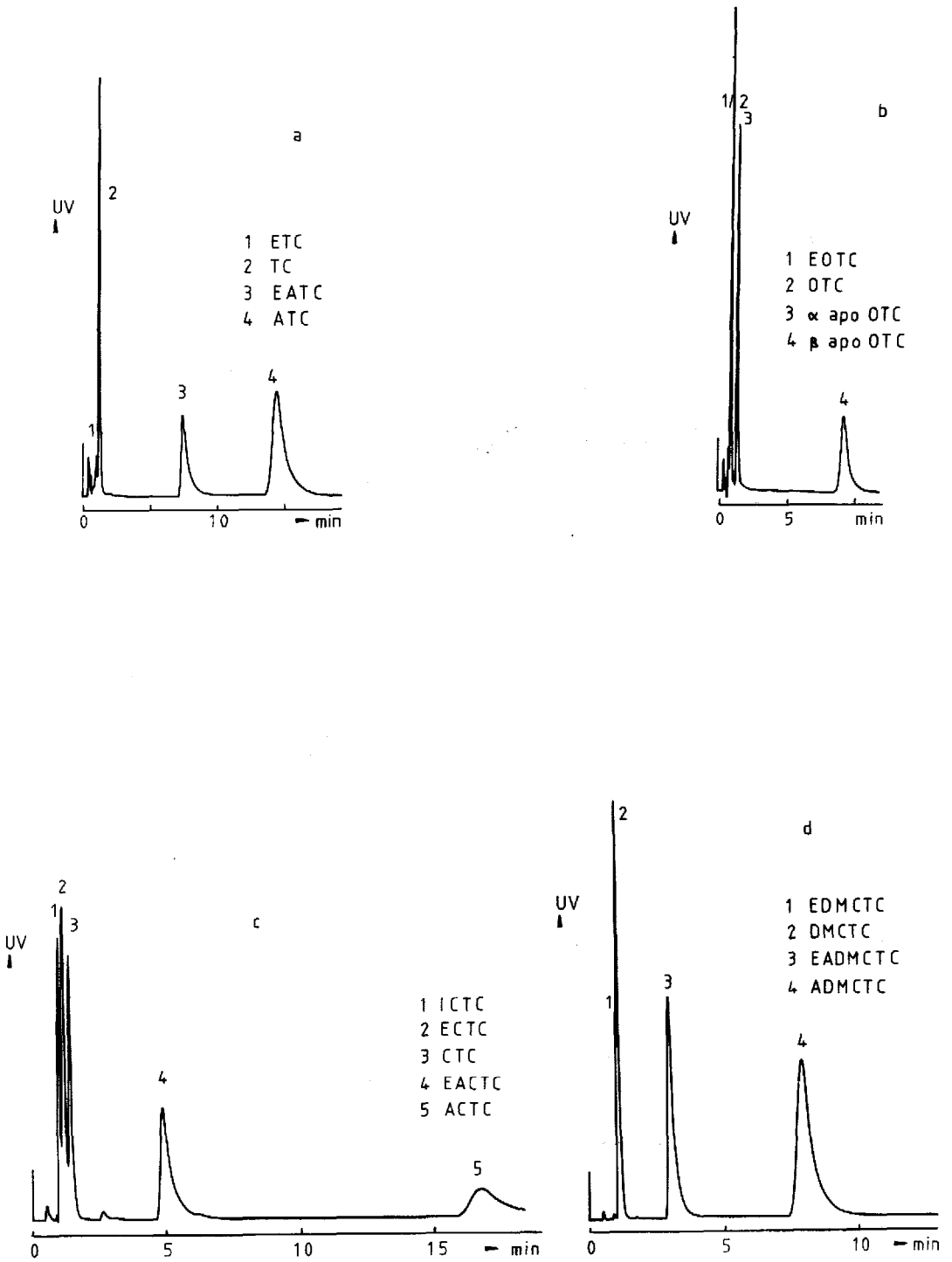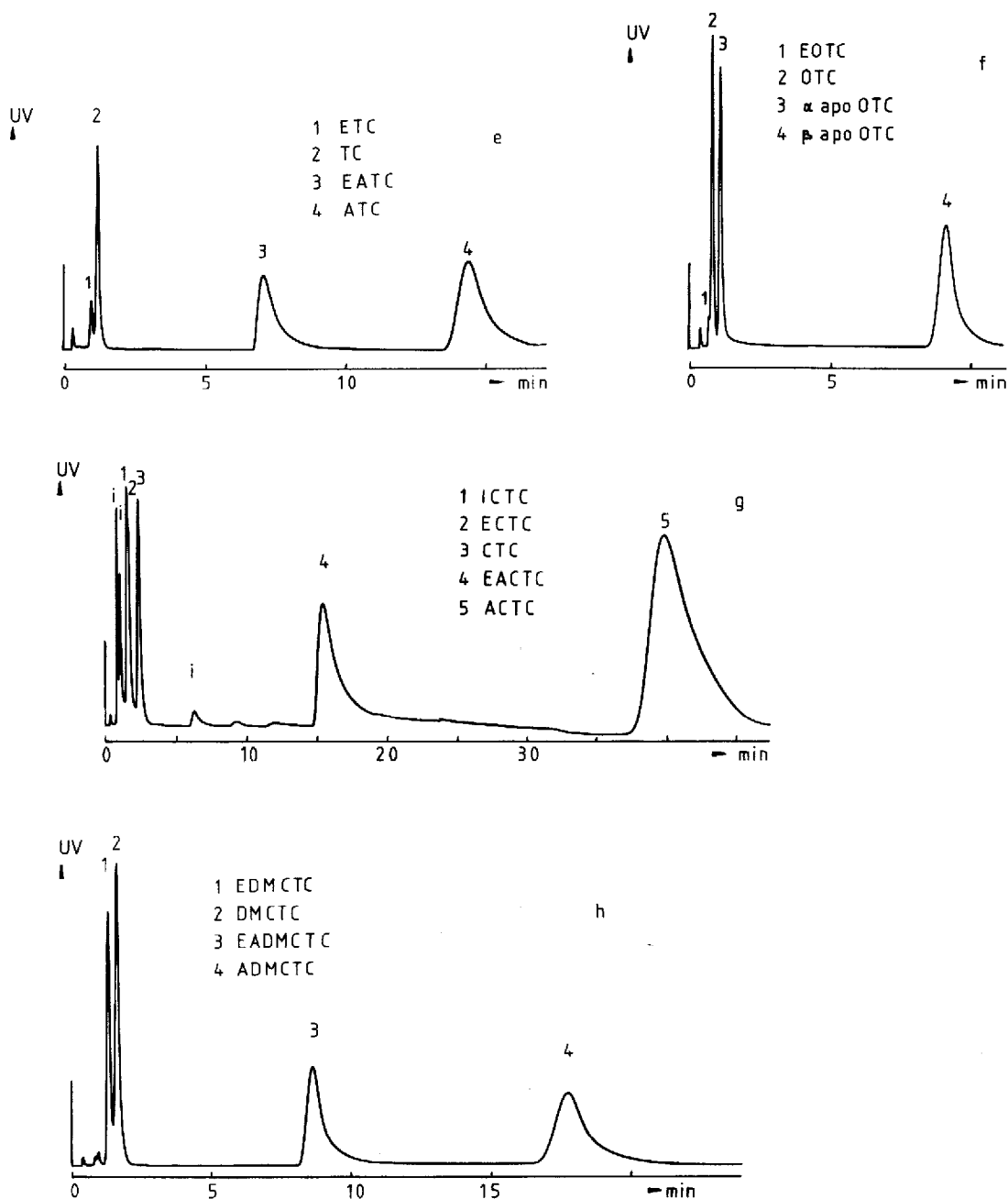
Fig. 1.

UV

2

1 ETC
2 TC
3 EATC
4 ATC

e

3

1

4

0   5   10   ← min

UV

2
3

1 EOTC
2 OTC
3 α apo OTC
4 β apo OTC

f

1

4

0   5   ← min

UV

1
2 3

1 ICTC
2 ECTC
3 CTC
4 EACTC
5 ACTC

g

5

4

i

i

0   10   20   30   ← min

UV

2
1

1 EDMCTC
2 DMCTC
3 EADMCTC
4 ADMCTC

h

3

4

0   5   10   15   ← min

Fig. 1. Chromatograms of test mixtures of some tetracyclines and degradation products. Conditions: column XAD-2 (6–7 μm) (a–d), PRP-1 (10 μm) (e–h); eluent: (a,b) acetonitrile–dichloromethane–0.2 M acetate buffer (pH 3.6) + 0.025 M EDTA (10:1:90, v/v), flow-rate 0.8 ml/min; (c,d) acetonitrile–dichloromethane–0.2 M acetate buffer (pH 3.6) + 0.025 M EDTA (15:1:85, v/v), flow-rate 0.6 ml/min; (e,f,g,h) acetonitrile–dichloromethane–0.05 M acetate buffer (pH 3.6) + 0.025 M EDTA (12.5:1:87.5, v/v). For abbreviations see Table I.

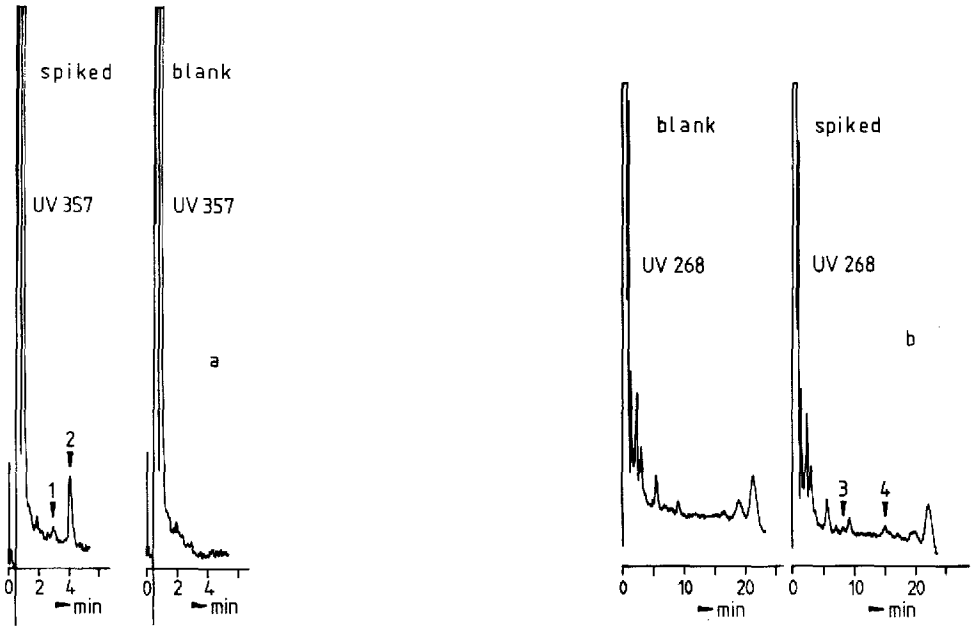Fig. 2. Chromatograms of a deproteinized plasma sample, spiked with tetracycline, and its degradation products. Conditions: column PRP-1 (10 μm); eluent: (a) acetonitrile–dichloromethane–0.2 M acetate buffer (pH 3.6) + 0.025 M EDTA (6:1:94, v/v); (b) acetonitrile–dichloromethane–0.2 M acetate buffer (pH 3.6) + 0.025 M EDTA (10:1:90, v/v). Peaks: 1 = ETC; 2 = TC; 3 = EATC; 4 = ATC. TC peak corresponds to 172 ng/ml, EATC peak to 97 ng/ml and ATC peak to 363 ng/ml.
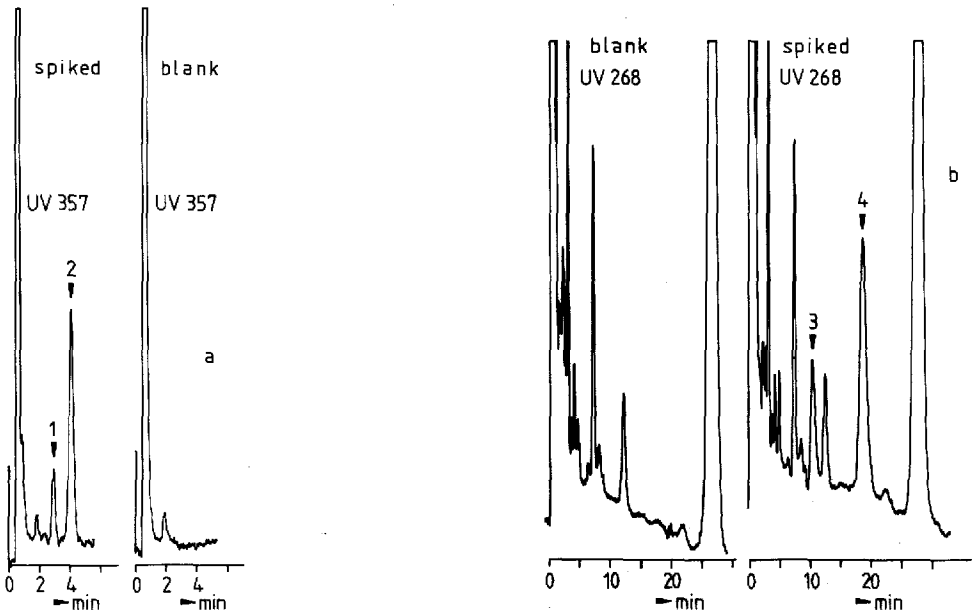
Fig. 3. Chromatogram of an extract of a plasma sample, spiked with tetracycline, and its degradation products. For conditions see Fig. 2.

TC and its degradation products, is given. It must be noted that the determination of ETC is hampered by the epimerization of TC in the sample. Therefore, the results for ETC are less accurate, even when the extraction residue is injected as soon as possible. Combination of both methods is not yet possible. Addition of potassium hydroxide to the perchloric acid containing the supernatant from the precipitation procedure resulted in dark-brown residues of the ethyl acetate phase, presumably due to some perchloric acid being present in the organic layer.

Quantitative aspects

The linearity of the method was investigated by determining different concentrations in test mixtures as well as in spiked plasma samples. The recovery was calculated as the ratio of the slopes of the calibration curves, obtained by injection of standard solutions (100%) and extracts, respectively. For the 100% curve it appeared that even at low concentrations (4 ng/ml TC), linear calibration curves with a linear dynamic range of at least four decades were obtained. The recovery for TC amounted to 74.2% [coefficient of variation (C.V.) = 1.3%] in the precipitation method and 76.3% (C.V. = 2.0%) in the extraction. For EATC, the corresponding data were 12.0% and 61.7%, respectively, while for ATC recoveries of 13% and 78.0% were found. From these results it may be concluded that the precipitation method is suited only for TC and that all compounds can be determined with the more laborious liquid–liquid extraction. Only the determination of ETC is cumbersome because it is formed during sample pretreatment.

CONCLUSIONS

Tetracyclines and their degradation products can be separated efficiently on non-ionogenic resins as Amberlite XAD-2 and PRP-1. On both column materials, linear calibration curves were obtained in a range of at least four decades. In the analysis of biological materials, concentrations down to 50 ng/ml can be measured by applying liquid–liquid extraction. Patient monitoring for the tetracycline itself is easily accomplished by the precipitation procedure.

The accurate determination of epimerization products demands further investigations.

REFERENCES

1 A. G. Butterfield, D. W. Hughes, N. J. Pound and W. L. Wilson, *Antimicrob. Agents Chemother.*, 4 (1973) 11.
2 A. G. Butterfield, D. W. Hughes, W. L. Wilson and N. J. Pound, *J. Pharm. Sci.*, 64 (1975) 316.
3 K. Dihuidi, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 246 (1982) 350.
4 A. A. Fernandez, V. T. Noceda and E. S. Carrera, *J. Pharm. Sci.*, 58 (1969) 443.
5 J. Hermansson and M. Andersson, *J. Pharm. Sci.*, 71 (1982) 222.
6 S. Laik Ali and T. Strittmaker, *Int. J. Pharm.*, 1 (1978) 185.
7 R. F. Lindauer, D. M. Cohen and K. P. Munnelly, *Anal. Chem.*, 48 (1976) 1731.
8 K. Tsuji, J. H. Robertson and W. F. Beyer, *Anal. Chem.*, 46 (1974) 539.
9 K. Tsuji and J. H. Robertson, *J. Pharm. Sci.*, 65 (1976) 400.
10 J. H. Knox and J. Jurand, *J. Chromatogr.*, 186 (1979) 763.
11 R. Bocker, *J. Chromatogr.*, 187 (1980) 439.
12 S. Eksborg, *J. Chromatogr.*, 208 (1981) 78.

13 J. H. Knox and J. Jurand, *J. Chromatogr.*, 110 (1975) 103.
14 D. Mourot, B. Delépine, J. Boisseau and G. Gayot, *J. Chromatogr.*, 190 (1980) 486.
15 B. Vej-Hansen, H. Bundgaard and B. Kreilgard, *Arch. Pharm. Chem. Sci. Ed.*, 6 (1978) 151.
16 J. P. Sharma and R. P. Bevill, *J. Chromatogr.*, 166 (1978) 213.
17 H. Oka and M. Suzuki, *J. Chromatogr.*, 314 (1984) 303.
18 A. Aszalos, *Chromatographia*, 10 (1985) 313.
19 J. Hermansson, *J. Chromatogr.*, 232 (1982) 385.
20 B. G. Charles, J. J. Cole and P. J. Ravenscroft, *J. Chromatogr.*, 222 (1981) 152.
21 S. Eksborg, H. Ehrsson and U. Lönroth, *J. Chromatogr.*, 185 (1979) 583.
22 C. v.d. Bogert and A. H. Kroon, *J. Am. Sci.*, 70 (1981) 186.
23 I. Nilsson-Ehle, T. T. Yoshikawa, M. C. Schotz and L. B. Guze, *Antimicrob. Agents Chemother.*, 9 (1976) 754.
24 H. Oka, K. Uno, K.-I. Harada, M. Hayashi and M. Suzuki, *J. Chromatogr.*, 295 (1984) 129.
25 H. Oka, H. Matsumoto, K. Uno, K.-I. Harada, S. Kadowaki and M. Suzuki, *J. Chromatogr.*, 325 (1985) 265.
26 A. P. de Leenheer and H. J. C. F. Nelis, *J. Pharm. Sci.*, 68 (1979) 999.
27 H. J. C. F. Nelis and A. P. de Leenheer, *J. Chromatogr.*, 195 (1980) 35.
28 H. Poiger and C. H. Schlatter, *Analyst*, 101 (1976) 808.
29 E. R. White, M. A. Carroll and J. E. Zarembo, *J. Antibiot.*, 30 (1977) 811.