ELSEVIER

Journal of Chromatography A, 705 (1995) 229–245

JOURNAL OF CHROMATOGRAPHY A

Investigations into the epimerisation of tipredane ethylsulphoxide diastereoisomers during chromatographic analysis on reversed-phase silica
II. The involvement of metals in commercially available C_{18} silicas

Melvin R. Euerby[a,*], Christopher M. Johnson[a], Ian D. Rushin[a], D.A.S. Sakunthala Tennekoon[b]

[a]*Analytical Chemistry Department, Research and Development Laboratories, Fisons Pharmaceuticals PLC, Bakewell Road, Loughborough, Leicestershire LE11 0RH, UK*

[b]*Department of Pharmaceutical Sciences, University of Strathclyde, Royal College, 204 George Street, Glasgow G11 XW, UK*

First received 27 September 1994; revised manuscript received 24 February 1995; accepted 24 February 1995

Abstract

The degree of sulphoxide epimerisation (and concomitant ethylsulphenic acid elimination) of the α-ethylsulphoxide epimers of tipredane showed no direct correlation with measured chromatographic parameters for a wide range of commercially available octadecylsilyl stationary phases. Correlation was however established with the metal content (i.e. iron(II) and titanium) of these phases. Low metal content stationary phases have been shown to cause epimerisation (in addition to poor chromatography for analytes with chelating properties) via contamination of the top of the column with metal ions extracted from the stainless-steel or titanium frits when the column had been shipped in neat acetonitrile or methanol. The total metal content of packed columns could be conveniently assessed using the highly sensitive 2,3- and 2,7-dihydroxynaphthalene efficiency ratio test.

1. Introduction

It has previously been shown that the ethylsulphoxide diastereoisomers of tipredane (I, the steroidal sulphoxide diastereoisomers will be referred to as epimers) can undergo epimerisation of the sulphoxide moiety when exposed to certain chromatographic conditions (see preceding paper [1]). The epimerisation (and concomitant elimination of ethylsulphenic acid) occurs when the ethylsulphoxides are adsorbed onto the top of the octadecylsilyl stationary phase prior to the chromatographic separation. The degree of epimerisation was shown to be related to the oven temperature, the pH of the mobile phase, stationary-phase chemistry and the duration that the ethylsulphoxides were adsorbed to the stationary phase prior to separation.

This paper presents a detailed investigation into the involvement of a wide range of commercially available octadecylsilyl stationary-phase

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00273-1

packing materials in the epimerisation and elimination reactions and attempts to correlate numerous chromatographic parameters of the columns (such as hydrophobicity, steric selectivity, hydrogen bonding capacity, ion-exchange capacity at pH > 7 and < 3 and the quantity of alkyl chains) to the degree of epimerisation that the stationary phase promotes. These investigations have additionally shown a direct involvement of metal ions (present in the native silica or introduced as contaminants from stainless-steel frits) in the epimerisation and elimination reactions.

Stainless-steel and titanium frits in the presence of organic shipping solvents have been shown to leach metals including iron(II) and titanium which are then bound to the geminal silanols of the high-purity deactivated silicas. These metals promote the epimerisation and elimination reactions in addition to being deleterious to the chromatography of analytes possessing the capacity for chelation.

The ethylsulphoxides and the 2,3- and 2,7-dihydroxynaphthalenes (IV, V) have been used

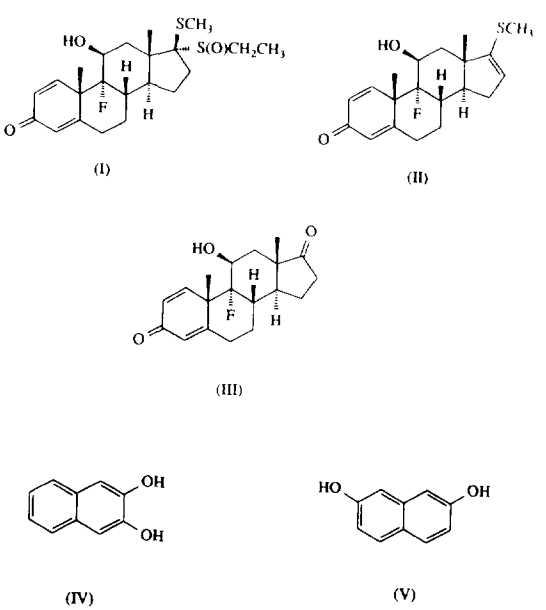

Fig. 1. Structure of compounds mentioned in the present paper. Sulphoxides are chiral because of the stable pyramidal configuration about sulphur, bringing about the formation of steroidal epimers. For simplicity the stereochemistry of the sulphoxide moiety has not been shown.

subsequently to evaluate the metal content/contamination of a range of new octadecylsilyl stationary-phase chemistries and to evaluate the efficiency of procedures used to remove unwanted metals from such stationary phases. The implications of these findings in relation to the chromatography of compounds possessing chelating properties will be discussed.

The structures of the compounds mentioned in this paper are shown in Fig. 1.

2. Experimental

2.1. Chemicals

All buffer chemicals and solvents used were of HPLC grade, other chemicals were of AR grade (Fisons Scientific Apparatus, Loughborough, UK). Water was purified by means of an Elgastat Spectrum RO and ion-exchange/carbon filter system (High Wycombe, UK). The ethylsulphoxide epimers of tipredane (I) were prepared according to the method of Euerby and co-workers [2,3] and were stored at −18°C for up to 48 h. 2,3- and 2,7-Dihydroxynaphthalene (IV, V) were obtained from Sigma (Dorset, UK)

2.2. Chromatography

HPLC was performed using a Hewlett-Packard 1090M HPLC system equipped with a Model 1040 linear photodiode-array UV detector. Data acquisition and integration was controlled by a Hewlett-Packard 79994A Chem Station (Hewlett-Packard, Stockport, UK). Chromatography was performed on a variety of columns shown in Table 1. Detection was at 240 nm, based on the λ_{max} of tipredane.

The dead time (t_o) was determined from the first deviation of the baseline after injection.

2.3. Investigations into the effect of commercially available octadecylsilyl stationary phases on the epimerisation/elimination reactions (HPLC conditions 1)

Chromatography was performed on a wide range of commercially available ODS columns

Table 1
Dimensions, particle size and storage solvents of columns investigated

Stationary phase/column	Dimensions (mm)	Particle size (μm)	Storage solvent (v/v)
Hypersil ODS	100 × 4.9	5	MeOH
	150 × 4.9	3	
RP-18 Lichrospher guard column[a]	4 × 4	5	na
RP-18 Lichrospher guard column endcapped[a]	4 × 4	5	na
RP-8 Lichrospher guard column[a]	4 × 4	5	na
Pellicular ODS guard column[a]	75 × 2	37–53	na
Resolve C_{18}	150 × 3.9	5	MeCN/H_2O 60:40
Nova-pak C_{18}	150 × 3.9	4	MeCN/H_2O 50:50
YMC Basic	250 × 4.6	5	MeCN/H_2O 60:40[b]
Lichrosphere 60 RP Select B	125 × 4.0	5	MeCN/H_2O 75:25[b]
SynChropak SCD-100	250 × 4.6	5	MeOH/H_2O 75:25
Spherisorb ODS1	150 × 4.6	5	MeOH/H_2O 65:35
Spherisorb ODS2	150 × 4.6	5	MeOH/H_2O 70:30
Astec C_{18} polymer	150 × 4.6	5	MeCN/H_2O 65:35
Ultrasphere ODS	150 × 4.6	5	MeOH/H_2O 70:30
μBondapak C_{18}	300 × 3.9	10	MeCN/H_2O 60:40
Zorbax R_x C_{18}	150 × 4.6	5	MeOH/H_2O 80:20[b]
Hypersil BDS C_{18}	150 × 4.6	5	MeOH/H_2O 70:30
Suplex pKb-100 C_{18}	250 × 4.6	5	MeCN
Supelcosil LC-ABZ C_{18}	150 × 4.6	5	MeCN
Kromasil C_{18}	150 × 4.6	5	MeOH
Hichrom RPB C_{18}	150 × 4.6	5	MeOH
Spherisorb ODSB	150 × 4.6	5	MeOH/H_2O 70:30
Purospher C_{18}	250 × 4.6	5	MeCN
Symmetry C_{18}	150 × 3.9	5	MeCN
Zorbax SB C_{18}	150 × 4.6	5	MeCN/H_2O 90:10

[a] Guard column material used in conjunction with Hypersil ODS Excel column.
[b] Test evaluation mobile-phase composition.
na = Not applicable.

shown in Table 1. The eluent consisted of mobile phases A and B which were 0.025 *M* NH_4OAc (pH 7.2) and 0.025 *M* NH_4OAc (pH 7.2) in acetonitrile–water (65:35, v/v), respectively. The flow-rate was 1.5 ml/min and the oven temperature was thermostatically held at 40°C. An initial 20-min isocratic mobile phase composition of 10% B followed by a linear gradient run over 5 min to 45% B, 10 min from 45 to 50% and then 50 to 95% mobile phase B over 10 min was employed; the final eluent composition was then held for a further 5 min.

Reaction products were identified by comparison of retention times and spiking experiments with those of authentic materials and UV diode-array spectroscopy.

All calculations were based on the relative response factor for each compound being 1. This assumption relied on the fact that the absorbance at 240 nm was due to the A ring of the steroid and is present in all of the compounds investigated.

2.4. 2,3- and 2,7-Dihydroxynaphthalene efficiency ratio test (DERT)

Chromatography was performed on a range of octadecylsilyl stationary phases. The eluent consisted of 0.025 *M* NH_4OAc (pH 7.2) in acetonitrile–water (25:75, v/v). The flow-rate was 1.5 ml/min and the oven temperature was thermostatically held at 40°C. Detection at 230 nm and a run time of 60 min were employed. A 10-μl amount of the test mixture in acetonitrile was injected (on-column loading of 334 and 157 ng of 2,3- and 2,7-dihydroxynaphthalene IV and V, respectively). The base peak efficiency for each analyte was calculated by Multichrom software (VG Data Systems, Altrincham, Cheshire, UK).

The dihydroxynaphthalene efficiency ratio test (DERT) value was calculated as follows:

$$\text{DERT} = \frac{\text{Base peak efficiency 2,7-dihydroxynaphthalene}}{\text{Base peak efficiency 2,3-dihydroxynaphthalene}}$$

2.5. Investigations into the effect of metal ions on epimerisation/elimination reactions

The effect of loading methanolic solutions of metal ions onto the column was investigated by first equilibrating the column at 40°C in neat methanol at 1.5 ml/min for 20 min and then injecting the methanolic metal solution (100 μl, 500 nmol). The column was then rinsed with pure water to remove the methanol before re-equilibration with the HPLC conditions. The column was then evaluated chromatographically for metal content by the epimerisation/elimination and DERT determinations.

2.6. Atomic absorption spectroscopy

Total iron content was determined using a Perkin Elmer 703 atomic absorption spectrometer (Beaconsfield, UK). Methanolic samples were analysed against external methanolic standards at 248.3 nm using an air/acetylene flame.

2.7. Inductive coupled plasma spectroscopy (ICP)

Elemental analysis was performed using an ARL 3580 optical emission ICP argon plasma spectrometer (Fisons Instruments, Crawley, UK) by external calibration against aqueous standards. Methanolic extracts of frits were evaporated to dryness and then re-dissolved in dilute hydrochloric acid prior to analysis.

2.8. Column characterisation HPLC conditions [4]

The flow-rate was 1.5 ml/min and the oven temperature was thermostatically held at 40°C. Detection at 254 nm and a run time of 20 min were employed.

Amount of alkyl chains (k'_{AB})

The eluent consisted of methanol–water (80:20, v/v); 10 μl of the test solution which contained amylbenzene dissolved in mobile phase, was injected (on-column loading of 6 μg). The capacity factor and the column efficiency at 50% peak height were calculated.

Hydrophobicity [$\alpha(CH_2)$]

The eluent consisted of methanol–water (80:20, v/v); 10 μl of the test solutions which contained amylbenzene dissolved in mobile phase and butylbenzene dissolved in methanol, were injected (on-column loading of 6 and 4 μg, respectively). The quotient of the capacity factor of amylbenzene to that of butylbenzene was calculated.

Steric selectivity ($\alpha_{T,O}$)

The eluent consisted of methanol–water (80:20, v/v); 10 μl of the test solution which contained triphenylene and *o*-terphenyl dissolved in methanol, was injected (on-column loading of 5 μg of each). The quotient of the capacity factor of triphenylene to that of *o*-terphenyl was calculated.

Hydrogen bonding capacity ($\alpha_{C,P}$)

The eluent consisted of methanol–water (30:70, v/v); 10 μl of the test solutions which contained caffeine and phenol dissolved in methanol, were injected (on-column loading of 5 and 10 μg, respectively). The quotient of the capacity factor of caffeine to that of phenol was calculated.

Ion-exchange capacity at pH > 7 ($\alpha_{A,P}$)

The eluent consisted of 0.02 *M* KH_2PO_4 (pH 7.6) in methanol–water (30:70, v/v), 20 μl of the test solutions which contained benzylamine and phenol dissolved in mobile phase, were injected (on-column loading of 10 μg of each). The quotient of the capacity factor of benzylamine to that of phenol was calculated.

Ion-exchange capacity at pH < 3 ($\alpha_{A,P}$)

The eluent consisted of 0.02 *M* KH_2PO_4 (pH 2.7) in methanol–water (30:70, v/v), 20 μl of the test solutions which contained benzylamine and phenol dissolved in mobile phase, were injected (on-column loading of 10 μg of each). The quotient of the capacity factor of benzylamine to that of phenol was calculated.

3. Results and discussion

The preceding paper (Part I, [1]) described the sulphoxide epimerisation and concomitant ethylsulphenic acid elimination from the ethylsulphoxide diastereoisomers of tipredane (I) adsorbed onto a Pellicular ODS guard column prior to the chromatographic separation (see Fig. 2).

The sulphoxide epimerisation and elimination are thought to arise via a common intermediate—an immobilised ethylsulphoxide–metal–geminal silanol complex (see preceding paper, Part I [1]). Therefore, for the sake of simplicity, this paper will initially focus on the epimerisation process and subsequently discuss the elimination process in terms of what has been shown for the former.

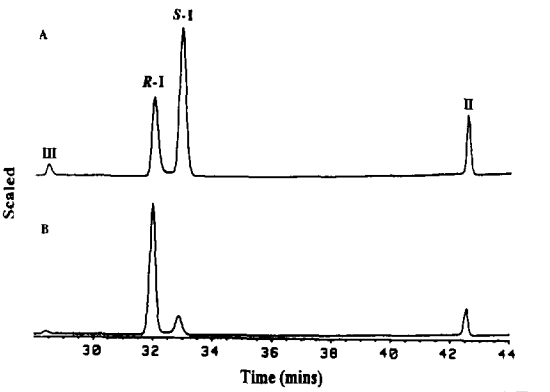

Fig. 2. HPLC analysis of ethylsulphoxide epimers (I) on a Pellicular ODS guard column and a Hypersil ODS column. Chromatographic conditions 1 as in Section 2. (A) *S*-epimer, (B) *R*-epimer.

3.1. Effect of stationary-phase chemistry on the epimerisation reaction

The type of octadecylsilyl (ODS, C_{18}) stationary-phase material appeared to influence the degree of epimerisation observed. For example, the Pellicular ODS guard column resulted in a significantly greater amount of epimerisation compared with the effect of the Hypersil column alone (see Table 2).

Therefore, it was decided to evaluate a wide range of commercially available C_{18} stationary-phase materials (see Table 1) with special emphasis on whether the column had been end-capped or not and whether the column had been deactivated. Preliminary investigations indicated that the previous history of the column could have a pronounced effect on the degree of epimerisation, therefore, for consistency, where possible a new 150 × 4.6 mm I.D., 5-μm column was investigated (where a longer column had to be employed the flow-rate was adjusted accordingly to elute the epimers at approximately the

Table 2
Effect of stationary phases on the epimerisation and elimination of ethylsulphoxide epimers (I) and their resolution using HPLC conditions 1

Stationary phase column	*R*-epimer (I)		*S*-epimer (I)		Resolution *R*- and *S*-epimer (I)
	% epi	% eli[b]	% epi	% eli[b]	
Hypersil ODS	5.4	7.19	9.4	3.7	2.8
Pellicular ODS[a]	12.5	8.7	26.2	9.2	nd
RP-18 Lichrospher[a]	nd	nd	4.8	3.8	nd
RP-18 Lichrospher[a] end-capped	nd	nd	2.8	2.8	nd
RP-8 Lichrospher[a]	nd	nd	4.5	6.9	nd
Resolve C_{18}	13.2	14.3	29.2	20.9	1.3
Nova-pak C_{18}	1.8	4.5	3.5	0.6	2.7
YMC Basic Lichrosphere 60	3.5	5.2	6.6	4.3	3.8
RP Select B	0.8	13.5	2.6	8.1	2.3
SynChropak SCD-100	2.9	12.5	5.8	13.4	2.6
Astec C-18 Polymer	0.0	11.6	0.0	6.6	1.1
Spherisorb ODS1	14.2	33.7	44.7	41.3	0.9
Spherisorb ODS2	16.8	9.7	33.7	13.6	2.5
Ultrasphere ODS	1.5	6.3	3.2	4.4	2.6
μBondapak C_{18}	3.3	7.4	7.5	6.6	nd
Zorbax R_x C_{18}	8.0	8.2	17.0	9.3	2.6
Hypersil BDS C_{18}	0.4	6.5	0.2	2.5	3.2
Suplex pKb-100 C_{18}	0.0	5.3	0.8	3.8	1.6
Supelcosil LC-ABZ C_{18}	nd	7.4	nd	1.1	co-elution
Kromasil C_{18}	24.8	8.3	30.6	45.3	2.6
Hichrom RPB C_{18}	8.7	8.4	24.2	8.7	3.2

[a] Guard column material used in conjunction with Hypersil ODS Excel column.
[b] Summation of elimination products (II and III).
nd = Not determined.

same retention times). Each column was washed with 15 column volumes of mobile phase B and 15 column volumes of initial mobile-phase composition, from HPLC conditions 1, prior to starting the experiment.

Most of the stationary phases showed excellent separation of ethylsulphoxide epimers (I) (see Table 2) in addition to separating the products arising from the elimination process, i.e. the C-17 keto (III) and methylthio (II) derivatives (see Fig. 2).

The Resolve and the Spherisorb materials produced a pronounced "saddle" between the peaks corresponding to the ethylsulphoxides epimers, which was indicative of epimerisation occurring during the chromatographic process (see Fig. 4) [5–8]. This was in contrast to all the other column materials examined which resulted in baseline resolution with no evidence of any true "on-column" epimerisation (see Fig. 3). It was therefore deduced that the epimerisation reaction on the majority of column materials occurred when the ethylsulphoxides were adsorbed onto the stationary phase prior to the gradient commencing and hence prior to the separation process.

3.2. Evidence to implicate the silanol moieties in the epimerisation reactions

Epimerisation of the ethylsulphoxide epimers (I) was not observed with the C_{18} polymer-based column (see Table 2). However, thermal elimi-

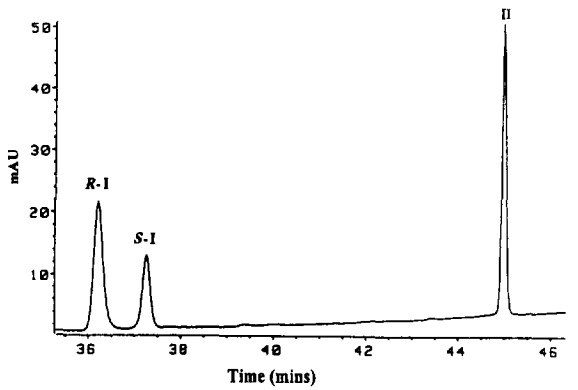

Fig. 3. HPLC analysis of the *S*-epimer of the ethylsulphoxide (I) on a Kromasil C_{18} column (column-packing company A). Chromatographic conditions 1 as in Section 2.

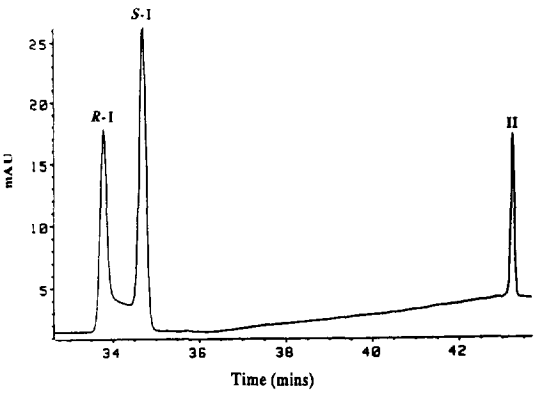

Fig. 4. HPLC analysis of the *S*-epimer of the ethylsulphoxide (I) on a Resolve C_{18} column. Chromatographic conditions 1 as in Section 2.

nation of ethylsulphenic acid still occurred (for results of the *R*-epimer see Table 3).

This observation implicated the involvement of the silanol groups present on the silica-based ODS stationary phase in the epimerisation reaction. Further evidence for this hypothesis came from the fact that the end-capped column, Novapak C_{18}, resulted in lower total epimerisation and elimination than the Resolve C_{18} column which had been prepared from the same base silica but was not end-capped (see Table 2). In addition, the new generation of columns for use with bases, which use nucleophilic shielding groups to protect silanol groups from interactions with basic compounds, e.g. the Suplex pKb-100, showed very little epimerisation.

The deactivated columns, i.e. Lichrosphere 60 RP Select B and Hypersil BDS C_{18}, in general gave lower amounts of epimerisation than traditional columns; however, there appeared to be many anomalies (i.e. the Kromasil C_{18}, Hichrom RPB and Zorbax R_x C_{18} columns).

Table 3
Elimination of ethylsulphenic acid from the *R*-epimer of the ethylsulphoxide (I) as a function of temperature on the Polymer C_{18} column using HPLC conditions 1

Temperature (°C)	Elimination[a] (%)
26	1.5
40	11.6
44	17.2

[a] Summation of the elimination products (II and III).

3.3. Correlation of epimerisation with chromatographic parameters of the various stationary phases

In an attempt to correlate the epimerisation and elimination with specific stationary-phase properties, the columns shown in Table 4 were characterised in accordance with the methodology described by Kimata et al. [4]. This approach provided information regarding the amount of alkyl chains (k'_{AB}), hydrophobicity [$\alpha(CH_2)$], steric selectivity ($\alpha_{T/O}$), hydrogen bonding capacity ($\alpha_{C/P}$) and ion-exchange capacity ($\alpha_{A/P}$) at pH > 7 and pH < 3 for the stationary phase. The efficiency of each column was additionally calculated for amylbenzene (see Table 4).

After extensive examination of the data no direct correlation could be established between the extent of epimerisation and/or elimination with any of the parameters studied. However, the evidence still strongly indicated the involvement of silanol groups in the epimerisation reaction. Although no correlation with total silanol content could be established, silanol groups have been characterised into five subclasses, all with differing acidities and therefore,

Table 4
Characterisation of RP HPLC columns

Columns	k'_{AB}	$\alpha(CH_2)$	$\alpha_{T,O}$	$\alpha_{C,P}$	$\alpha_{A/P}$		N	N/m
					pH 7.6	pH 2.7		
Zorbax R_x C_{18}	5.68	1.57	1.61	0.54	0.55	0.11	13210	88065
Spherisorb ODS	1.78	1.47	1.64	1.57	2.84	2.55	12871	85805
Kromasil C_{18}	7.01	1.48	1.53	0.40	0.31	0.11	12739	84925
Spherisorb ODS2	3.00	1.51	1.56	0.59	0.76	0.23	12390	82601
Hypersil ODS	4.44	1.45	1.28	0.48	1.04	0.64	11412	76078
Hypersil BDS C_{18}	4.50	1.47	1.49	0.39	0.19	0.17	11186	74576
Hichrom RPB C_{18}	4.56	1.40	1.21	0.36	0.18	0.11	10787	71916
Nova-pak C_{18}	4.49	1.49	1.44	0.48	0.27	0.14	10532	70216
Supelcosil LC-ABZ C_{18}	3.14	1.37	2.23	0.24	0.20	0.03	10120	67464
Ultrasphere ODS	6.41	1.52	1.42	0.48	0.31	0.16	9946	66305
YMC Basic	2.33	1.26	0.98	0.57	0.51	0.27	13034	52136
Resolve C_{18}	2.40	1.46	1.59	1.29	4.06	1.23	7161	47741
Suplex pKb 100 C_{18}	2.07	1.35	2.84	0.34	0.29	0.00	10289	41158
Astec C_{18} Polymer	4.92	1.35	4.09	0.15	0.04	0.01	4695	31302

Stationary phase properties and HPLC experimental conditions are as described in Section 2; k'_{AB} number of alkyl chains; $\alpha(CH_2)$ surface density of alkyl groups or hydrophobicity; $\alpha_{T,O}$ steric selectivity; $\alpha_{C,P}$ hydrogen-bonding capacity; $\alpha_{A/P}$ pH 7.6 ion-exchange capacity at pH 7.6; $\alpha_{A,P}$ pH 2.7 ion-exchange capacity at pH 2.7, N (number of theoretical plates) and N/m of amylbenzene.

reactivities [9,10]. It was, therefore, thought that the epimerisation may be attributed to a particular class or classes of silanol groups.

3.4. Evidence for the involvement of a specific class of silanol in the epimerisation reaction

The degree of epimerisation resulting from the use of the Kromasil C_{18} column was of particular interest because of the manufacturer's claims for this column. These include an extremely low metal content in the silica support material and a uniform silanol surface rather than the heterogeneous silanol population of more traditional columns (theoretically producing a stationary-phase material with very low numbers of acidic silanol groups). This was borne out in practice as the column was shown to have low hydrogen bonding and ion-exchange capacities both at pH > 7 and pH < 3, suggesting a low number of acidic silanol sites through the column (see Table 4). However, this column resulted in an extremely high degree of epimerisation for the ethylsulphoxide epimers (I); see Table 2 and Fig. 3 for the chromatography of the *S*-epimer. This further suggested that epimerisation may be facilitated by an active site which was not at present being quantified.

Various manufacturers have made claims that their deactivation processes result in stationary phases with increased numbers of less acidic types of silanols, e.g. geminal silanols. However, due to the highly secret nature of the deactivation procedure there has been an absence of published research into this field. (A ^{29}Si NMR spectroscopic survey of commercially available stationary phases is planned to address this lack of detailed knowledge.) Iron(III) has been reported to selectively block geminal silanols [11], hence it was suggested that if the increased number of geminal silanol groups on the Kromasil material were involved in the epimerisation reaction then loading the column with iron(III) should retard the reaction. However, investigations proved that no reduction in the degree of epimerisation occurred on addition of iron(III) to a Kromasil C_{18} column; in fact a

slight increase in both elimination ($\Delta 2\%$)[1] and epimerisation ($\Delta 5\%$) was observed on chromatography of the *S*-epimer of the ethylsulphoxide (I). It was subsequently found that after an EDTA or 0.1% v/v phosphoric acid wash was used to remove the iron(III) from the column, levels of both epimerisation ($\Delta 11$ and $\Delta 19\%$ reductions for EDTA and acid washes, respectively) and elimination ($\Delta 9$ and $\Delta 12\%$ reductions for EDTA and acid washes, respectively) were reduced, implicating the involvement of metal ions in these reactions.

3.5. Evidence of metal ion involvement in the epimerisation reaction

Investigations of four different batches of new Kromasil C_{18} and a C_8 column, from the same highly reputable column packing company (A), all produced epimerisations; however, the degree of epimerisation and elimination did vary (ranges for epimerisation 30.6–37.3% and elimination 18.7–48.0%, $n = 5$). Examination of the stationary-phase material at the inlet and outlet ends of these columns showed a wide variation in colour ranging from white to dark orange. The dark orange coloration seen with some columns was attributed to hydrated iron(III) oxide. This strongly suggested the presence of metal contamination arising from the stainless-steel frits. It has previously been reported that stainless-steel frits are responsible for poor chromatographic performance [12–15] and on-column reactions [7,16,17].

Atomic absorption spectroscopy of silica from the top few mm of a coloured column revealed levels of approximately 250 ppm total iron, whereas the Kromasil packing material manufacturers "Eka Nobel" claimed < 10 ppm for the native silica.

3.6. Identification of metal ion(s) responsible for the epimerisation reaction

The most probable causative metals which could have been leached from the "316 stainless-steel" frits and were present in any abundance, were nickel (12%), chromium (17%) and iron (69%) [18]. However, the latter in its most stable oxidation state of iron(III) had previously been shown not to cause significant epimerisation. Further investigations of loading methanolic solutions of chromium(III) and nickel(II) chlorides onto the Kromasil C_{18} guard column packing material via injection or stirring overnight at ambient temperature failed to cause any significant increase in epimerisation. However, the adsorption of these metal species onto the silica surface was in doubt as the dihydroxynaphthalene test (see later) suggested that little, if any, metal had been adsorbed presumably due to the slow and/or weak complexation of chromium(III) and nickel(II).

In comparison, when a methanolic solution of the less stable oxidative state of iron [iron(II) chloride] was loaded, significant epimerisation (34.0%) and elimination (15.8%) was observed. It is important to note that although an injection of 5 μmol iron(II) was made, the majority of this passed straight through the column without chelation, as detected by the large UV absorption at the void volume, suggesting that the epimerisation was caused by the much smaller fraction of iron(II) held by the geminal silanols. The dihydroxynaphthalene efficiency ratio test (DERT, see below) indicated that iron(II) had been successfully adsorbed onto the column, which gave a DERT value of 16.7.

3.7. Dihydroxynaphthalene efficiency ratio test (DERT)

The relative concentration of metal ions throughout the column was estimated by an independent method, the DERT value, which compares the peak efficiency, measured at the base, of the two regioisomers 2,3- (IV) and 2,7-dihydroxynaphthalene (V). The former possesses the ability to chelate with metals while the latter does not. Therefore, the nearer the ratio of peak efficiency at base for the 2,7/2,3-dihydroxynaphthalene (DERT value) to unity, the lower the metal content; conversely a value of infinity means that the 2,3-dihydroxynaphthalene

[1] Δ Refers to absolute change (%).

(IV) analyte elutes very slowly over a long time, effectively losing the peak in the baseline noise or that the analyte binds strongly to the metal and fails to be eluted [19]. This test has been shown to be a much better diagnostic probe for metal content than more traditional tests such as salicylaldehyde and the phenol/benzylamine ratio [20]. It has subsequently been shown that the metal probe acetylacetone failed to detect metal contamination on the Waters Symmetry and Merck Purospher columns, whereas the DERT value did.

3.8. Identification of the source of metal contamination

Investigation of an identical batch of Kromasil C_{18} material packed as guard columns "in-house" and also packed as an analytical column by another packing company (B) resulted in only trace levels of epimerisation and minimal elimination. For example, only 0.7 and 3.1% epimerisation and elimination, respectively, were seen with the analytical column for the *S*-epimer (see Table 5, entry 8).

The source of the metal contamination on the Kromasil column batches was suspected to be related to the stainless-steel inlet and outlet frits used by the column packing company (A).

There appeared to be little difference between columns packed with passivated and non-passivated stainless-steel frits (see Table 5, entries 1 and 2); however, the duration of time for which the columns were left prior to testing was directly related to the degree of epimerisation observed (see Table 5, entries 2, 4 and 5).

The epimerisation of the ethylsulphoxides (I) observed with the Kromasil columns under investigation resulted in baseline separation of the epimers indicating that the epimerisation occurred only at the top of the column (see Fig. 3). However, after loading a methanolic solution of iron(II) chloride onto the Kromasil column a saddle was seen between the two epimer peaks illustrating that iron(II) was bound throughout the column resulting in true "on-column" epimerisation (see Fig. 4 for a chromatogram typifying this phenomenon).

The fact that the PEEK alloyed with Teflon (PAT) frits failed to generate significant epimerisation (see Table 5, entries 6 and 7) showed that metals [e.g. iron(II)] were being leached out of the stainless-steel frit. Contrary to our expectations, titanium frits still gave a considerable degree of epimerisation (see Table 5, entry 3), indicating that a unspecified oxidation state of titanium was also able to facilitate the epimerisation.

Substitution of acetonitrile in place of methanol as the storage solvent failed to reduce the extent of epimerisation (compare entries 5 and 12, Table 5). In contrast, the Kromasil C_{18} column supplied by company (B), which caused negligible epimerisation, had been stored in 6:4 v/v acetonitrile–water prior to testing (see Table 5, entry 8). To test the hypothesis that it was the percentage of organic in the shipping solvent which leached metals from the stainless-steel frits, the column packing company (A) stored a newly packed Kromasil C_{18} column in the same shipping solvent as company (B). As anticipated, this column failed to cause epimerisation (see Table 5, entry 13).

(Another potential source of metal contamination lay in the fact that company (A) supplied columns with stainless-steel end stops (epimerisation noted) whereas company (B) used plastic (no epimerisation). This potential source was ruled out as storage of the column with PAT frits supplied with stainless-steel end stops, from company (A), failed to cause epimerisation.)

The most probable explanation was that the leaching of metal ions from the stainless-steel frits was exacerbated by the use of pure organic solvents. Support for this hypothesis comes from the fact that it has previously been shown that various 316 stainless-steel HPLC components are extremely susceptible to corrosion in the presence of "neat" acetonitrile or methanol, iron(II) being generated by the anodic reaction of the corrosion process [21]. "Neat" acetonitrile and methanol are used by certain column manufacturers for packed column storage. It was subsequently shown by ICP that the amount of iron extracted from the passivated stainless-steel frits (1 frit per ml of methanol) at ambient tempera-

Table 5
Effect of column storage solvents and time on the degree of epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (I) on Kromasil C_{18} columns using HPLC conditions 1

Entry number	Frit composition (column storage conditions)	Epimerisation (%)	Elimination (%)[a]	DERT	Batch number of packing material
1	Non-passivated stainless steel (4 months MeOH)	32.4	33.4	∞	0078
2	Passivated stainless steel (4 months MeOH)	39.4	26.2	∞	0078
3	Titanium (4 months MeOH)	25.0	18.0	∞	0078
4	Passivated stainless steel (1 day MeOH)	3.4	4.2	16.9	0078
5	Passivated stainless steel (6 days MeOH)	16.3	11.9	30.9	0078
6	PEEK and Teflon (PAT) (1 day MeOH)	0.3	3.7	16.8	0078
7	PEEK and Teflon (PAT) (12 days MeCN)	0.5	5.5	nd	0033
8	Passivated stainless steel (company B, 7 days MeCN–H_2O, 6:4)	0.7	3.1	0.8	0033
9	Passivated stainless steel (company A, MeOH)	35.4	48.0	nd	0033
10	Passivated stainless steel (company A, MeOH)	37.3	35.2	nd	0077
11	Passivated stainless steel (company A, MeOH)	36.1	44.0	nd	0065
12	Passivated stainless steel (company A, 12 days MeCN)	10.7	10.8	5.1	0078
13	Passivated stainless steel (7 days MeCN–H_2O, 6:4, company A)	0.3	3.9	nd	0078

nd = Not determined.
[a] Summation of elimination products (II and III).

ture was related to the length of exposure of the frits to methanol (see Table 6, entries 1 and 2, and Figs. 5 and 6).

In addition, the incorporation of water into the extraction solvent mixture resulted in lower quantities of iron being extracted (see Table 6, entries 1, 3 and 4).

ICP spectroscopy showed that methanol in contact with 1 passivated frit for 35 days at ambient temperature can extract in the order of

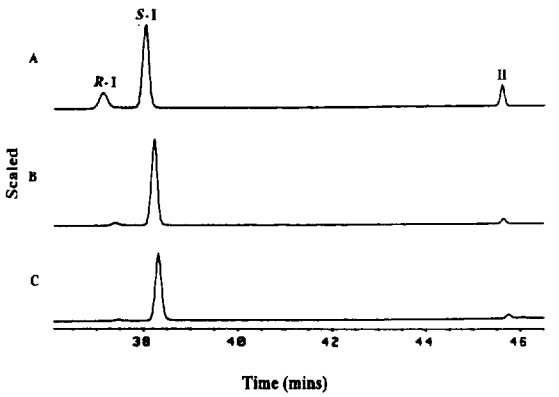

Fig. 5. HPLC analysis of the *S*-epimer of the ethylsulphoxide (I) on a Kromasil C_{18} column (column-packing company A). Chromatographic conditions 1 as in Section 2. Storage of columns in (A) methanol for 6 days, (B) methanol for 1 day, and (C) acetonitrile–water (60:40, v/v) for 7 days at ambient temperatures.

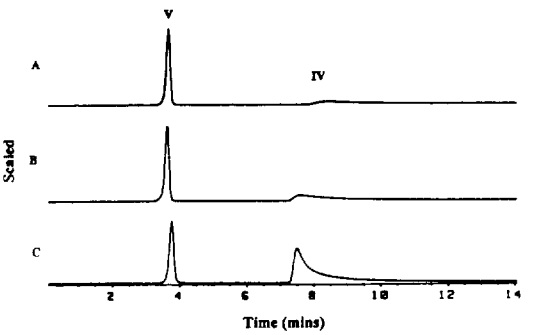

Fig. 6. HPLC analysis of 2,3- and 2,7-dihydroxynaphthalene (IV, V) on a Kromasil C_{18} column (column packing company A). Chromatographic conditions as in Section 2. Storage of columns in (A) methanol for 6 days, (B) methanol for 1 day, and (C) acetonitrile–water (60:40, v/v) for 7 days at ambient temperatures (see Table 5 for DERT values obtained).

1 μg of total iron (see Table 6, entry 1). Typically, $1.8 \cdot 10^{-9}$ mol of the ethylsulphoxide of tipredane was loaded onto the column. Assuming that the total amount of iron was adsorbed onto the top of the column (and available to react), the iron would have been in a molar excess of 10:1 over the ethylsulphoxide (I) and therefore could have a profound effect.

Table 6
Quantity of metals extracted from frits as a function of extraction solvent composition and time as assessed by ICP

Entry number	Frit composition and storage conditions	Quantity extracted (ppm)			
		Iron	Nickel	Chromium	Titanium
1	Passivated stainless steel, 35 days, MeOH	1.0	1.2	0.8	0.0
2	Passivated stainless steel, 4 days, MeOH	0.1	0.8	0.1	0.0
3	Passivated stainless steel, 23 days, 80:20 MeOH–H_2O	0.3	1.0	0.2	0.0
4	Passivated stainless steel, 23 days, 60:40 MeOH–H_2O	0.2	0.9	0.2	0.0
5	Titanium, 35 days, MeOH	0.1	0.0	0.0	1.8

The equivalent of one frit was exposed to 1 ml of extraction solvent at ambient temperature.

The facile nature of extraction of titanium from the titanium frits in methanol was exemplified by entry 5 (see Table 6). The complexed titanium (oxidation state undefined), in addition to iron(II), can promote the epimerisation reaction as titanium frits in contact with methanol have been shown to cause significant epimerisation (see Table 5, entry 3).

Nickel was additionally extracted in reasonable quantities from the passivated stainless-steel frits irrespective of the extraction composition. However, as can be seen from Table 6 (entries 3 and 4), nickel can be ruled out as the causative agent since high levels are extracted with solvent combinations not associated with epimerisation. High levels of chromium were extracted from the passivated stainless-steel frit in the presence of methanol and may be partly responsible for the epimerisation reaction; however, chromium(III) has been shown not to be loaded onto the C_{18} material, therefore, further work is planned to examine the effect of chromium(II).

Neat methanol has been favoured as the storage solvent for reversed-phase columns as it has been previously reported that aqueous methanol storage solvents result in poor stationary-phase stability [22]; no such data is currently available in the open literature for corresponding acetonitrile–aqueous storage solvents.

3.9. The involvement of metal contamination of high-purity deactivated silicas on the degree of epimerisation

Metal ions, extracted from the frits by the use of neat organic shipping solvents, were apparently complexed near the top of the column (in the first few mm) by geminal silanols on the surface of the silica support material. This effect was particularly noticeable for high-purity silica-based C_{18} columns where the intrinsic metal contents are low (<10 ppm iron) and the number of geminal silanols are believed to be high; for example, Kromasil and RPB columns (packed by the same column-packing company) and Zorbax R_x (direct from the manufacturer) produce high levels of epimerisation compared to the Hypersil ODS material (column also stored in neat methanol) which is known to contain a high level of metal in the native silica (i.e. iron >200 ppm, manufacturer's literature).

Evidence to support this comes from the [$\alpha_{A/P}$ pH 7.6] and [$\alpha_{A/P}$ pH 2.7] results of the high-purity silica stationary phases shown above, which indicated a significant difference between the amount of relatively high-acidic silanols (i.e. value at $\alpha_{A/P}$ pH 2.7) and the low-acidic silanols (i.e. value at $\alpha_{A/P}$ pH 7.6) (see Table 4). A large difference between the two values represents a large proportion of low-acidic silanols, such as geminal ones, over that of high-acidity types. It is of interest to note that the difference between the silanols at pH 2.7 and 7.6 for the Hypersil BDS column is very small, suggesting that the few silanols which are present are high-acidity ones and not geminals, hence a low epimerisation result was obtained. In addition, this column was stored in 7:3 methanol–water which causes less iron(II) extraction, and Hypersil materials are known to contain a higher metal content in the native silica than materials such as Kromasil C_{18}. In this case other metals, e.g. iron(III), may already be bound to the geminals, hence preventing access of metal ions which can promote epimerisation to the geminal silanols.

The smaller and more variable degree of epimerisation observed when iron(III) was loaded onto the Kromasil C_{18} material may be caused by a small percentage of the iron(II) species being present in methanolic iron(III) solution or the acidic methanolic injection solution leaching off iron(II) from the stainless-steel frit.

3.10. Implications

The ability of certain stationary phases to enhance the rate of epimerisation of individual tipredane ethylsulphoxide epimers (I) may be attributed to their iron(II) (or titanium) content.

The Zorbax R_x C_{18} column was also shown to cause significant epimerisation (see Table 2), far more than would have been expected given its low metal content (manufacturers claim). Given the fact that the degree of epimerisation and

elimination of the ethylsulphoxide epimer (I) dramatically reduced (epimerisation 0.9 and elimination 5.1%) after the column had been washed with 0.1% v/v phosphoric acid and then an EDTA overnight at 60°C, it was postulated that in common with the Kromasil column and presumably the RPB column, from the same source, inadvertent metal ion contamination from the stainless-steel frits had occurred. This could have been due to the high methanol content used to store the columns (see Table 1) and that the columns appeared to contain a large proportion of non-acidic silanols, e.g. geminals (see Table 4). This result was quite significant as it illustrated that this problem was not confined to only one supplier/manufacturer (see later for further examples).

Columns such as Resolve and Spherisorb ODS1 and 2 (obtained from sources which were not suspected as being involved with inadvertent contamination, i.e. the three columns had not been stored in high-organic solvents) are manufactured from native silica which contains a high metal content and, after the octadecylsilane has been bound, a large proportion of free silanols remain, many will be geminal in nature and possess the ability to complex with metal ions such as iron(II) and titanium. Therefore, the epimerisation can occur throughout the column; this is substantiated by the saddle which is seen between the two ethylsulphoxide peaks (see Fig. 4).

Organic/aqueous based column storage packing solvents such as methanol or acetonitrile–water may, in fact, extract iron(II) from the frits, but the iron(II) may be rapidly converted to iron(III) in the aqueous environment. Alternatively, iron(II) may only be extracted in very high-percentage organic–water mixtures; further investigations in this area are at present being pursued. Our preliminary results indicate that the total iron extracted in aqueous methanol solutions is substantially lower than that extracted in pure methanol (0.3 and 0.2 μg/frit extracted after 23 days exposure to 80:20 and 60:40 methanol–water, respectively, compared to 1 μg/frit with neat methanol; see Table 6, entries 1, 3 and 4).

There may be other metal ions which can facilitate this reaction; e.g. titanium frits have been shown to promote the epimerisation/elimination reaction.

Iron (and possibly other cations) may be present in the initial silica or may be introduced via metal components of the column when there are sufficient geminal silanols to allow significant complexation of iron(II).

3.11. Investigations of further commercially available stationary phases

Subsequent to these findings we have evaluated a range of recently introduced C_{18} stationary-phase materials (see Table 7). It was anticipated that the Symmetry C_{18} and Purospher C_{18} columns may promote the epimerisation reaction owing to the fact that both are formed via polymerisation of silyl ethers which produces a "low acidity" silanol surface (presumably increased geminal population, manufac-

Table 7
Comparison of the epimerisation/elimination reactions and that of the DERT value before and after an EDTA wash

Column material	*S*-epimer (I)		Resolution (I) epimers	DERT value	
	Epimerisation (%)	% Elimination[a] (%)		Before	After EDTA wash
Spherisorb ODSB	3.3	5.8	1.5	∞	nd
Waters Symmetry C_{18}	15.2	11.2	3.3	5.6	0.5
Merck Purospher C_{18}	1.2	0.0	2.1	41.6	0.6
Zorbax SB C_{18}	4.8	15.9	2.4	0.8	nd

nd = Not determined.
[a] Summation of elimination products (II and III).

turer's claim) and are stored in neat organic solvent (acetonitrile) (see Table 1). The latter column's chemistry also possessed a nucleophilic shielding moiety. Due to the cartridge design of the Purospher column, the packing material probably dried out during storage, therefore the cartridge system was assembled and the packing material left in neat acetonitrile for 7 days at ambient temperature.

Contrary to expectation, the Purospher material produced a low level of epimerisation, whereas the DERT value indicated surface metal contamination (see Table 7 and Fig. 7). These results can be rationalised as follows. After the cartridge had been shipped out in pure acetonitrile, the cartridge design allowed the column bed to dry out. Hence, the extracted iron(II) would have been converted to its more stable oxidation state iron(III) which does not promote the epimerisation reaction but its presence was detected in the DERT determination. The refilling and storage of the column in acetonitrile which we performed, did not influence the metal content of the column as most of the "extractable metal" had already been deposited onto the column from the frit.

An EDTA wash successfully removed the surface metal as assessed by the DERT determination (see Table 7 and Fig. 7). The success of this procedure is indicative of surface-bound metal derived from contamination via the frit rather than in the native silica.

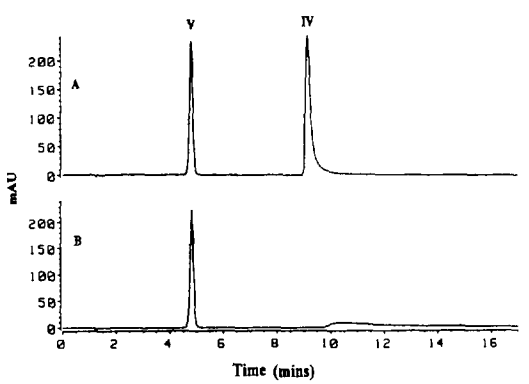

Fig. 7. HPLC analysis of 2,3- and 2,7-dihydroxynaphthalene (IV, V) on a Purospher C_{18} column. Chromatographic conditions as in Section 2. (A) After a subsequent 0.05 *M* Na_2 EDTA wash (1 h at 1.5 ml/min, 40°C), and (B) initial conditions of the column after storage for 1 week at ambient temperature in acetonitrile.

The Spherisorb ODSB column gave a low epimerisation result, indicating low iron(II) content on the column which is in keeping as the column was stored in 7:3 methanol–water; however, the DERT value indicated a high metal content of the silica which was expected (see Table 7).

In contrast to the Zorbax R_x C_{18} column (non-end-capped), the corresponding Stable Bond version in which the silanols are sterically protected by the bulky di-isopropyl silyl side chains gave a low epimerisation result and DERT value, indicative of a low metal content silica (see Table 7). A possible explanation for this may be that metal ions or the probes are prevented access to the surface geminal silanols via the steric protecting groups.

The Symmetry C_{18} column gave a high epimerisation result (see Table 7), indicating the presence of iron(II) adsorbed onto the top of the column (sharp peaks and baseline resolution observed), leached after storage of the column in pure acetonitrile (Coulometric Karl Fischer determination indicated the presence of only 0.1% water content in the storage solvent). This was substantiated by the DERT value which indicated the presence of surface metal on the material which could be easily removed by washing the column for 1 h with 0.05 *M* Na_2 EDTA solution.

3.12. Elimination process

The same overall trends have been observed for the elimination process as for the epimerisation. However, the degree of elimination appears to be more variable than the epimerisation process. This may have been expected as elimination products can arise via another mechanism: that of thermal elimination of ethylsulphenic acid. The wide differences in the degree of elimination (especially seen with different Kromasil columns and batches) may additionally be attributed to the effect of additional metals [other than iron(II)] on the column and/or

metals bound to additional "active sites" on the stationary phase.

4. Summary

The use of the ethylsulphoxide epimers of tipredane and the DERT determination has highlighted vast chromatographic differences between identical packing materials from different packing companies. The source of these differences has been attributed to contamination of the otherwise "metal-free" high-purity stationary-phase material, with metals such as iron(II), by storage of these types of columns with stainless-steel frits in high-organic content solvents. This additionally highlights the possibility of further interactions of analytes possessing chelating potential with metals bound to the surface of these types of stationary phases [7,14,19,23–26].

This research has also indicated the possible need for additional quality control procedures to be introduced by packing companies to monitor for metal content not only in the base silica but at each stage of manufacture through to the final packed column.

Research is presently in progress to assess the feasibility of using the ethylsulphoxide epimers (I) and 2,3- (IV) and 2,7-dihydroxynaphthalene (V) regioisomers before and after various EDTA pre-treatments as probes to chromatographically characterise the proportion of various metal ion(s) bound to differing classes of silanol groups. This may also allow one to determine the proportion of differing classes of silanols such as geminal to non-geminal silanols present on various stationary phases. Preliminary investigations suggest that the two probes exhibit different selectivities (see Table 5, entries 4, 6 and 8). In these three examples the degree of epimerisation is quite similar, whereas with the dihydroxynaphthalene test only entries 4 and 6 gave results suggestive of high metal contamination. One can, therefore, assume that the two tests are measuring two distinct parameters and at present insufficient data are available to allow one to correlate them to one another.

The epimerisation of tipredane ethylsulphoxide (I) has been shown to be an extremely sensitive probe for iron(II) speciation with detection limits below 5 μmol. Further work is presently in progress to assess the feasibility of using this approach to determine trace levels of iron(II) in the presence of iron(III), and to assess the specificity of the epimerisation/elimination reactions to other metals of varying oxidation states.

Acknowledgements

We are grateful to Dr. D. Mallern, Messrs. R. Shaw, D. Stanley (Hichrom, Reading, UK), J. Plant, Dr. H. Ritchie (Shandon HPLC, Runcorn, UK), D. Collis, A. Holmes (Waters Chromatography Division, Watford, UK), M. Clemance (Merck, Poole, UK) and A. Dams (Rockland Technologies, Nuenen, Netherlands) for support and helpful advice. Mr. U. Misty (Fisons Scientific Apparatus, Loughborough, UK) for performing the ICP determinations, Dr. J. Crew for critical appraisal of the manuscript, Miss A. Stockley for typographical advice and the British Council for financial support (DASST).

References

[1] M.R. Euerby, C.M. Johnson, I.D. Rushin and D.A.S.S. Tennekoon, J. Chromatogr. A, XXX (1995) XXX.
[2] M.R. Euerby, J. Hare and S.C. Nichols, J. Pharm. Biomed. Anal., 10 (1992) 269–277.
[3] M.R. Euerby, C.M. Johnson, R.J. Lewis and S.C. Nichols, J. Pharm. Biomed. Anal., 12 (1994) 761–769.
[4] K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosoya, M. Araki and N. Tanaka, J. Chromatogr. Sci., 27 (1989) 721–728.
[5] R.A. Keller and J.C. Giddings, J. Chromatogr., 3 (1960) 205–220.
[6] M. Van Swaay, in R.A. Keller and J.C. Giddings (Editors), Advances in Chromatography, Dekker, New York, NY, Vol. 8, 1969, pp. 363–385.
[7] J.-X. Huang, J.D. Stuart, W.R. Melander and Cs. Horvàth, J. Chromatogr., 316 (1984) 151–161.
[8] W.R. Melander, H.-J. Lin, J. Jacobson and Cs. Horvàth, J. Phys. Chem., 88 (1984) 4527–4536.

[9] M.A. Stadalius, J.S. Berus and L.R. Snyder, LC · GC, 6 (1988) 494–500.
[10] A. Berthod, J. Chromatogr., 549 (1991) 1–28.
[11] B. Pfleiderer and E. Bayer, J. Chromatogr., 468 (1989) 67–71.
[12] S.M. Cramer, B. Nathanael and Cs. Horváth, J. Chromatogr., 295 (1984) 405–411.
[13] C.-Y. Jeng and S.H. Langer, J. Chromatogr. Sci., 27 (1989) 549–552.
[14] P.C. Sadek, C.J. Koester and L.D. Bowers, J. Chromatogr. Sci., 25 (1987) 489–493.
[15] P.C. Sadek, P.W. Carr, L.D. Bowers and L.C. Haddad, Anal. Biochem., 144 (1985) 128–131.
[16] Z. Deng, J.Z. Zhang, A.B. Ellis and S.H. Langer, J. Liq. Chromatogr., 16 (1993) 1083–1103.
[17] B.S. Ludolph, C.-Y. Jeng, A.H.T. Chu and S.H. Langer, J. Chromatogr. A, 660 (1994) 3–15.
[18] R.C. Weast (Editor), CRC Handbook of Chemistry and Physics, 64th ed., 1983–1984, p. F-112.
[19] R. Vespalec and J. Neca, J. Chromatogr., 281 (1983) 35–47.
[20] M.R. Euerby, C.M. Johnson and D.A.S.S. Tennekoon, Paper presented at the 20th International Symposium on Chromatography, Bournemouth, 19–24 June 1994.
[21] R.A. Mowery Jr., J. Chromatogr. Sci., 23 (1985) 22–29.
[22] H. Engelhardt and M. Jungheim, Chromatographia, 29 (1990) 59–68.
[23] J. Nawrocki, D.L. Moir and W. Szczepanik, Chromatographia, 28 (1989) 143–147.
[24] B. Law and P.F. Chan, J. Chromatogr., 552 (1991) 429–438.
[25] J.W. Dolan, Liq. Chromatogr. HPLC Mag., 4 (1986) 222–226.
[26] G.B. Cox, J. Chromatogr. A, 656 (1993) 353–367.