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# Investigations into the epimerisation of tipredane ethylsulphoxide diastereoisomers during chromatographic analysis on reversed-phase silica
# I. Investigations into the reaction mechanism

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**Abstract**

The epimeric α-ethylsulphoxides of tipredane have been shown to undergo sulphoxide epimerisation and elimination of ethylsulphenic acid during reversed-phase (RP) HPLC, prior to the chromatographic separation. This has been shown to be promoted by increased oven temperature, acidic mobile phase, extended analysis time, column chemistry (i.e. silanol population and distribution) and increased metal content (i.e. iron(II) and titanium) of the RP stationary material. Their propensity to undergo such reactions is related to the facile C-17α C-S(O)Et bond breakage. Epimerisation and elimination have been postulated to arise via a common intermediate: an immobilised ethylsulphoxide–metal–geminal silanol complex.

## 1. Introduction

It has previously been shown that tipredane (I) can undergone S-oxidation in a site- and stereo-selective manner to yield both the methyl (II) and ethylsulphoxide (III) diastereoisomers (β- and α-plane oxidations, respectively) [1,2]. The ethylsulphoxide diastereoisomers of tipredane (III, the steroidal sulphoxide diastereoisomers will be referred to as epimers) are extremely labile compounds undergoing elimination of ethylsulphenic acid at neutral pH at temperatures above 0°C to yield the corresponding C-17 methylthiovinyl derivative (IV).

During chromatographic analysis of the epimers of the ethylsulphoxide (III), elimination of ethylsulphenic acid and epimerisation of the sulphoxide moiety was observed. The degree of elimination and epimerisation was found to be related to the actual chromatographic conditions and the type of octadecylsilyl stationary phase

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employed. This paper presents a detailed investigation into the mechanism of the unusual phenomena of epimerisation and elimination arising from a common intermediate. The prerequisites which predispose the ethylsulphoxide (III) to epimerisation and elimination are the labile carbon–sulphur bond of the C-17$\alpha$ substituent, metal ions (present in the native silica or introduced as contaminants from stainless-steel frits) and geminal silanol groups.

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 tipredane epimeric sulphoxides (II, III) were prepared according to the method of Euerby and co-workers [1,2].

The epimeric ethylsulphoxides (III) were stored at −18°C for up to 48 h. The analogous $\alpha$- and $\beta$-sulphoxides of the C-17 epimer of tipredane (V, VI) were prepared and separated in a similar manner and characterised by LC–MS.

### 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). Detection was at 240 nm, based on the $\lambda_{max}$ of tipredane.

The purity of the separated fractions (75 $\mu$l) was established by analysis using HPLC conditions 1 and 2.

### 2.3. HPLC analysis of the tipredane sulphoxides

*HPLC conditions 1 [2].*

Chromatography was performed on a Hypersil ODS "Excel" column (5 $\mu$m, 100 × 4.6 mm I.D., Hichrom, Reading, UK). 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 26°C. A linear gradient was run during 10 min from 45 to 50% and then from 50 to 95% mobile phase B during 10 min; the final eluent composition was then held for a further 5 min.

*HPLC conditions 2*

The conditions as described in HPLC conditions 1 were followed with the exception that a Pellicular ODS guard column (37–53 $\mu$m, 75 × 2 mm, Whatman, NJ, USA) was placed in front of the Hypersil ODS column (5 $\mu$m, 100 × 4.6 mm I.D.), that the oven temperature was thermostatically held at 40°C and that an initial 20 min isocratic mobile-phase composition of 10% B followed by a linear gradient run for 5 min to 45% B were employed prior to that described in HPLC conditions 1. 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 assumption that the relative response factor for each compound was 1. This assumption is based on the fact that the absorbance at 240 nm is due to the A ring of the steroid and is present in all of the compounds investigated.

### 2.4. Investigations of the effect of metal ions on epimerisation and elimination

Solutions of the chloride salts of iron(II) and iron(III) in methanol were prepared. To study the effect of these metals in solution, 1 $\mu$l (5 $\mu$mol) was added to a solution of the tipredane *S*-ethylsulphoxide (III, 90 $\mu$l, 90 $\mu$mol) in a plastic vial. The contents of the vial were mixed and then incubated at 40°C for 40 min. The

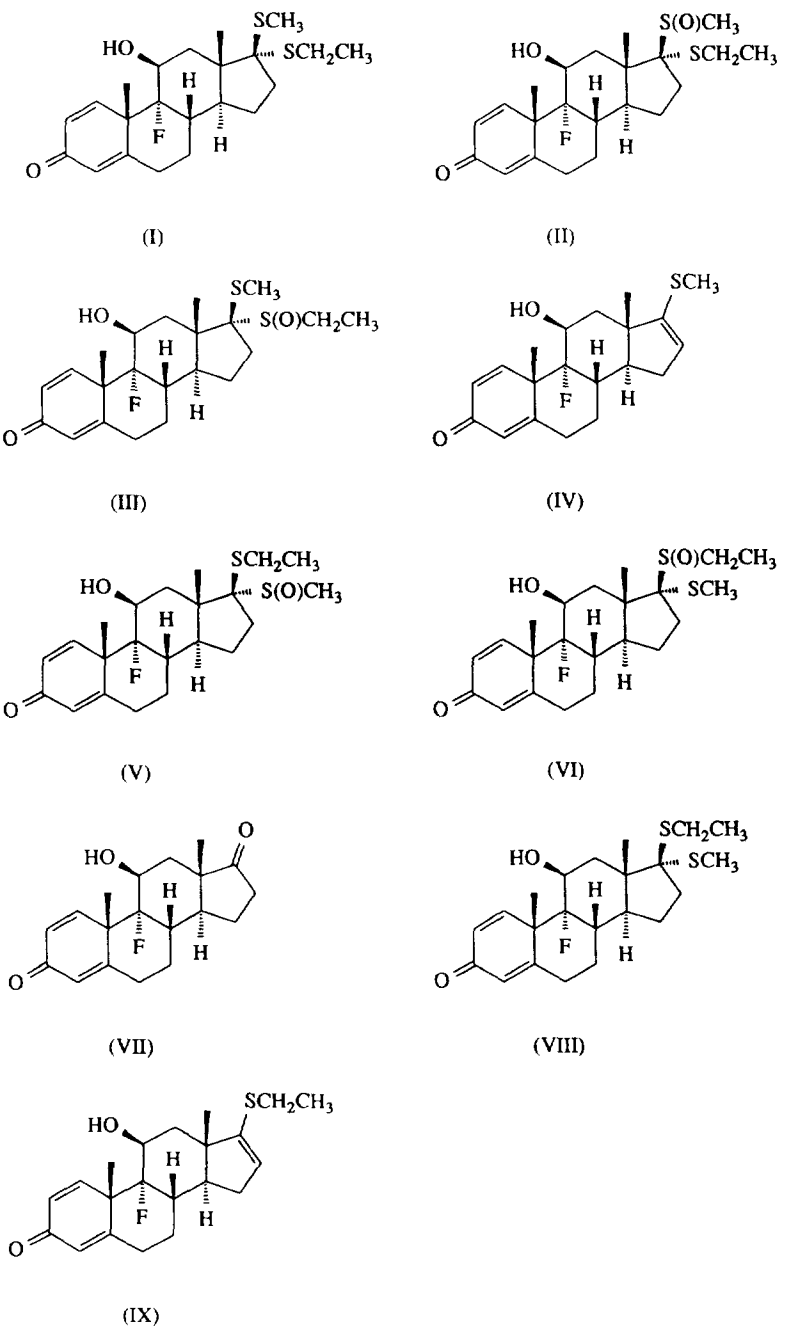


Fig. 1. Structure of compounds mentioned in the present paper. Sulphoxides are chiral because of the stable pyramidal configuration about sulphur, bringing about the formation of steroidal epimers. For simplicity the stereochemistry of the sulphoxide moiety has not been shown.

resulting solution was then analysed using HPLC conditions 1.

## 3. Results and discussion

### 3.1. Chromatography of the ethylsulphoxide epimers of tipredane (III)

It has previously been shown that the thermally labile ethylsulphoxide epimers of tipredane (III) can be successfully chromatographed without elimination of ethylsulphenic acid provided that the column temperature is maintained at 26°C or lower, the residency time on the column is minimal and the mobile phase pH is near neutral (see HPLC conditions 1) [2].

In the course of our research into the reaction kinetics of the disulphoxidation of tipredane it was necessary to chromatograph the ethylsulphoxide epimers (III) using a modification of HPLC conditions 1, i.e. the oven temperature was increased from 26 to 40°C and an initial isocratic eluent composition of 10% mobile B for 20 min was substituted prior to commencement of the gradient (see HPLC conditions 2). In addition, a Pellicular ODS guard column was employed.

As expected, due to the elevated column temperature, significant elimination of ethylsulphenic acid occurred yielding the C-17 methylthiovinyl derivative (IV) due to the thermal instability of the ethylsulphoxides (III) (see Table 1). Additionally, a percentage of the C-17 methylthio derivative (IV) underwent hydrolysis to the C-17 keto derivative (VII) (see Fig. 2).

In addition to elimination which occurred, both ethylsulphoxide epimers (III) exhibited epimerisation (see Fig. 2). The epimerisation could be deduced as occurring prior to separation as the peak shape was sharp with no "saddle or reaction zone" between peaks (typically observed if interconversion occurs during the chromatographic process). The phenomenon of "reaction zones" between peaks was first described by Keller and Giddings [3] and a theoretical basis developed by Melander et al. [4]. This phenomenon has subsequently been observed for the *cis–trans* interconversion of peptides [5,6] and various reactions occurring during the chromatographic separation process [7–10]. The *S*-sulphoxide epimer showed a greater tendency towards epimerisation than its corresponding *R*-epimer. This may suggest that in an equilibrium mixture one of the epimers is favoured. This was not verified experimentally as other competing elimination reactions were concomitantly in operation.

Table 1
Effect of chromatographic temperature on the epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (III) using HPLC conditions 2 with a Hypersil ODS column and Pellicular ODS guard column

| Temperature (°C) | Epimerisation (%) | Elimination[a] (%) |
|---|---|---|
| 26 | 5.8 | 1.9 |
| 40 | 17.0 | 8.3 |
| 50 | 36.6 | 26.0 |
| 60 | 25.9 | 57.6 |

[a] Summation of elimination products (IV, VII); the C-17 keto derivative (VII) is classed as an elimination product as it can only arise from subsequent hydrolysis of the elimination product (IV).

The epimerisation observed is quite remarkable, as previously sulphoxides have only been shown to undergo chiral inversion when exposed to harsh conditions such as photolysis or ther-

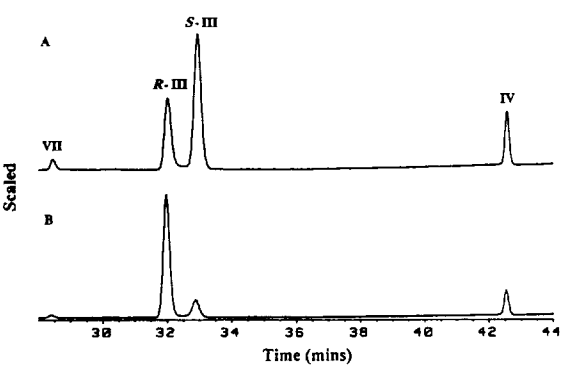


Fig. 2. HPLC analysis of ethylsulphoxide epimers (III) on a Pellicular ODS guard column and a Hypersil ODS column. Chromatographic conditions 2 as in Section 2. (A) *S*-epimer, (B) *R*-epimer.

molysis [11]. The sulphoxides under investigation are known to be extremely labile undergoing elimination at temperatures far below those necessary for thermolytic epimerisation [2].

In contrast to the ethylsulphoxide epimers (III), both methylsulphoxide epimers (II) failed to undergo elimination or epimerisation reactions during chromatography using HPLC conditions 2 with a Pellicular ODS guard column.

### 3.2. Structural and stereochemical requirements for the epimerisation reaction

Using the same experimental approach as that described previously [2], the C-17 epimer of tipredane (VIII) (in which the position of the ethyl and methyl groups have been interchanged) was oxidised with monomagnesium peroxyphthalic acid to yield the corresponding $\alpha$-methylsulphoxide (V) and $\beta$-ethylsulphoxide (VI) epimers of tipredane. These were subsequently separated using an analytical over-load technique (using HPLC conditions 1, in purities in an excess of 96% and concentration of approximately 50–100 $\mu$g/ml) and chromatographed using the epimerisation conditions (HPLC conditions 2). In an analogous manner to the $\alpha$-ethylsulphoxides of tipredane (III), the $\alpha$-methylsulphoxides of the C-17 epimer of tipredane (V) showed the same tendency to epimerise and eliminate. The $\beta$-methylsulphoxides of tipredane (II) and the $\beta$-ethylsulphoxides of the C-17 epimer of tipredane (VI) remained resistant to epimerisation and elimination under the conditions investigated (see Table 2 for results arising from the C-17 epimer of tipredane).

This indicated that it is not the sulphoxide substituent that predisposes it to these anomalous reactions but instead it is the site of the sulphoxide substituent, i.e. epimerisation and the extreme thermal instability are associated with the $\alpha$-sulphoxides (III, V). This raises the possibility that these processes proceed by a common mechanism. Supporting evidence for this comes from the fact that increasing the column temperature resulted in increased levels of both epimerisation and elimination of the

Table 2
Effect of sulphoxide substituent position on the degree of elimination and epimerisation using HPLC conditions 2 with a Hypersil ODS column and Pellicular ODS guard column

| Epimer | Epimerisation (%) | Elimination (%)[a] |
|---|---|---|
| $\alpha$-MeS(O)EtS more polar[b] (V) | 3.9 | 2.3 |
| $\alpha$-MeS(O)EtS less polar[b] (V) | 16.6 | 5.0 |
| $\beta$-EtS(O)MeS more polar[b] (VI) | 0.9 | 0.0 |
| $\beta$-EtS(O)MeS less polar[b] (VI) | 0.0 | 0.0 |

[a] Summation of elimination products (VII and the corresponding vinyl thioether).
[b] Expressed in terms of retention behaviour only.

*S*-ethylsulphoxide epimer (III) up to a temperature of 50°C, whereas at higher column temperatures, elimination of ethylsulphenic acid became more favoured (see Table 1).

### 3.3. Chromatographic parameters effecting the degree of epimerisation

The residence time on the column per se was shown not to be crucial to the epimerisation reaction. It was shown that use of a 25-cm column in place of the 10-cm Hypersil ODS column under HPLC conditions 1 resulted in a longer residence time on the column; however, there was only a slight increase in the elimination of ethylsulphenic acid and no epimerisation was noted. This is further evidence to support the conclusion that the reaction does not occur during the separation process.

The duration of the initial isocratic phase, in which the *R*-ethylsulphoxide epimer (III) was strongly adsorbed onto the top of the $C_{18}$ stationary phase, was found to dictate the amount of epimerisation and elimination observed. Exposure of the *R*-ethylsulphoxide epimer (III) to 10% B mobile phase for 40 min rather than 20 min resulted in retention times 20 min longer, and concomitantly more epimerisation and elimination (see Table 3).

The effect of mobile phase pH on the degree

Table 3
Effect of duration of exposure to the initial chromatographic conditions on the elimination and epimerisation of the *R*-epimer of the ethylsulphoxide (III) using HPLC conditions 2 with a Hypersil ODS column and Pellicular ODS guard column

| Duration at 10% B (min) | Epimerisation (%) | Elimination[a] (%) |
|---|---|---|
| 0 | 0.0 | 1.0 |
| 20 | 6.3 | 6.5 |
| 40 | 8.7 | 12.5 |

[a] Summation of the products of elimination (IV, VII).

of epimerisation and elimination produced by exposure to chromatography conditions 2 can be seen in Table 4. The results suggest that the *S*-ethylsulphoxide epimer (III) is extremely prone to acid-promoted elimination of ethylsulphenic acid yielding the C-17 methylthiovinyl derivative (IV) which can subsequently undergo hydrolysis to the C-17 keto derivative (VII). In addition, the epimerisation reaction appears to be promoted by acidic conditions; however, at pH 5.3 the elimination reaction is more favoured than epimerisation.

The nature of the stationary phase in these reactions was shown to be important in that when the *S*-ethylsulphoxide epimer (III) was chromatographed using HPLC conditions 2 (reaction on the Pellicular ODS guard column and separation on the Hypersil ODS column), 26.2 and 9.2% epimerisation and elimination,

Table 4
Effect of pH of mobile phases defined in HPLC conditions 2 on the epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (III), using a Hypersil ODS column with a Pellicular ODS guard column

| pH of mobile phase A | Epimerisation (%) | Elimination[a] (%) |
|---|---|---|
| 7.51 | 21.9 | 7.6 |
| 6.73 | 27.8 | 9.5 |
| 6.11 | 34.6 | 17.1 |
| 5.30 | 35.0 | 44.2 |

[a] Summation of the products of elimination (IV, VII).

respectively, were observed, whereas without the Pellicular ODS guard column only 9.4 and 3.7% epimerisation and elimination, respectively, were observed. The involvement of the stationary-phase chemistry in these reactions is described in a subsequent paper [12].

### 3.4. Solution reactions of the ethylsulphoxide epimers of tipredane (III)

Storage of the *R*-ethylsulphoxide epimer (III) in mobile phase (A–B, 45:55, v/v) at 45°C for 32 min and subsequent analysis using HPLC conditions 1 (which do not cause elimination or epimerisation) indicated that only 0.6% and 27% epimerisation and elimination, respectively, had occurred. In comparison, chromatography using HPLC conditions 2 indicated that a significant level of epimerisation had occurred. This confirmed the involvement of the stationary-phase chemistry in the epimerisation reaction.

In the subsequent paper [12] the involvement of the stationary-phase chemistry in the epimerisation and elimination reactions is investigated. It has been shown that the prerequisites for these reactions are the presence of a high proportion of geminal silanols and metals, either present in the native silica or inadvertently added via contamination of the stationary phase with metals leached from the stainless-steel frits when exposed to neat organic solvents.

Only negligible amounts of epimerisations were observed with either iron(II) or (III) in solution; however, elimination was accelerated with both oxidative states of iron. In the case of iron(III) this presumably reflected the acidic pH of the reaction mixture (see Table 5). Whereas for iron(II) the degree of elimination increased with the concentration of iron(II) in the solution, while the pH of the solution did not change significantly, suggesting that iron(II) was directly facilitating the elimination. Hung et al. [10] have also observed that *p*-hydroquinones were oxidised faster during chromatography than in free solution and postulated a heavy metal catalysed reaction at the stationary-phase surface. In this case the metal responsible was iron(III).

Table 5
Effect of iron(II) and (III) concentrations on the epimerisation and elimination of the *S*-epimer of the ethylsulphoxide (III) separated fraction at 40°C for 30 min; HPLC conditions 1 using a 75-μl injection volume (equivalent to approximately 75 μg loaded on-column)

| Iron (nmol) | | pH* | Epimerisation (%) | Elimination[a] |
|---|---|---|---|---|
| Fe(II) | Fe(III) | | | |
| 50 | | 6.9 | 0.4 | 40.6 |
| 100 | | 6.8 | 0.1 | 52.9 |
| 250 | | 6.7 | 0.0 | 78.7 |
| 0 | 0 | 6.9 | 0.5 | 13.3 |
| | 0 | 5.0[b] | 0.0 | 99.2 |
| | 50 | 5.9 | 0.2 | 41.3 |
| | 100 | 5.6 | 0.1 | 67.3 |
| | 250 | 5.0 | 0.0 | 94.4 |

[a] Summation of elimination products (IV, VII).
[b] pH adjusted manually with dilute HCl.
pH* Apparent pH.

### 3.5. Postulated mechanism of the epimerisation/elimination reaction

The reaction of tipredane *S*-ethylsulphoxide (III) with a bound metal can be rationalised by the formation of an immobilised sulphoxide–metal–geminal silanol complex (using the sulphur of the methylthio group and the oxygen of the ethylsulphoxide group to complex the metal in a five-membered ring (see Fig. 3).

The formation of a similar complex in solution

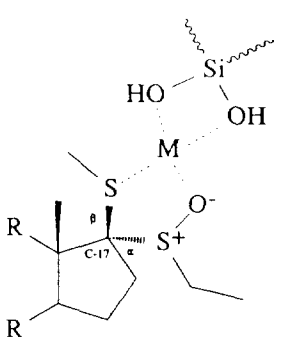


Fig. 3. Schematic representation of the interaction of tipredane ethylsulphoxide epimers (III) and bound metal to geminal silanols. M corresponds to cationic species such as iron(II) and titanium.

with iron(II) may explain the enhanced elimination of ethylsulphenic acid observed. In this case the iron(II) increases the capacity of the ethylsulphoxide moiety to act as a leaving group and, as the complex is not immobilised once the C-17α C-S(O)Et bond breaks, the metal is free to form the ethylsulphenic acid salt and hence epimerisation does not occur. In comparison, when the complex is immobilised, after the facile breaking of the C-17α C-S(O)Et bond has occurred, the liberated ethylsulphenic acid is held firmly by the metal complex in close proximity to the C-17 position. However, sufficient rotation of the carbon–sulphur bond of the ethylsulphoxide moiety is possible to allow either of the lone pairs of electrons of the sulphur to reform the five-membered ring complex resulting in epimerisation, statistically producing a 1:1 epimeric mixture. This in practice may not be the case as steric effects and the stability of the metal complex may favour formation of one of the epimers or preferential elimination of ethylsulphenic acid.

The ease with which this reaction occurs depends on the relative instability of the C-17α carbon–sulphur bond of the ethylsulphoxide epimers (III). The increased stability of the *R*-epimer towards epimerisation may be attributed to the small but possibly significant greater thermal stability of the *R*-epimer over the *S*-epimer [2].

### 3.6. Epimerisation of the methylsulphoxide epimers of tipredane (II) at elevated chromatographic temperatures

The methylsulphoxide epimers (II) failed to undergo any significant epimerisation and/or elimination with either the Pellicular ODS and Kromasil ODS stationary-phase material, using HPLC conditions 2. However, when the column temperature was increased to 80°C, using the Kromasil ODS column, the energy barrier to the C-17β C-S(O)Me bond breakage was overcome, hence, facilitating epimerisation and elimination (see Table 6 and Fig. 4). This implied that the methylsulphoxide epimers (II) possess the ability to complex with immobilised iron(II) under

Table 6
Effect of column temperature on the degree of epimerisation and elimination of the *S*- and *R*-epimers of the methylsulphoxide (II) on a metal-contaminated Kromasil ODS column using HPLC conditions 2

| Configuration of methylsulphoxide epimer (II) | Epimerisation (%) | | Elimination (%)[a] | |
|---|---|---|---|---|
| | 40°C | 80°C | 40°C | 80°C |
| *S* | 0.3 | 10.8 | 0.4 | 13.0 |
| *R* | 0.0 | 4.5 | 0.0 | 2.9 |

[a] Summation of elimination products (VII, IX).

these conditions, in a similar manner to that depicted in Fig. 3 for the corresponding ethylsulphoxide epimers (III). However, at lower temperatures (40°C), the lack of epimerisation observed with the methylsulphoxide epimers may be attributed to either lack of complex formation or, more likely, the increased stability of the carbon–sulphur bond of the C-17$\beta$ C-S(O)Me moiety. The *R*-epimer has previously been shown to be more stable to thermolysis than its corresponding *S*-epimer [1] and this is reflected in the *R*-epimer's reduced tendency to undergo epimerisation and elimination reactions (see Fig. 4).

## 4. Conclusions

In conclusion, the tipredane sulphoxides have been shown to undergo sulphoxide epimerisation and concomitant elimination of alkylsulphenic acid prior to chromatographic separation. These reactions are directly related to the stability of the C-17 carbon–sulphoxide bond. The extent of these reactions is related to certain chromatographic parameters (such as mobile-phase pH, column temperature, stationary-phase chemistry and metal content and time bound to the stationary phase). These results can be explained by the formation of an immobilised sulphoxide–metal–geminal silanol 5-membered ring complex, prior to the chromatographic separation process.

The subsequent paper [12] describes and examines the use of the ethylsulphoxide epimerisation reaction to probe the metal content and silanol surface chemistry of a wide range of commercially available RP stationary phases.

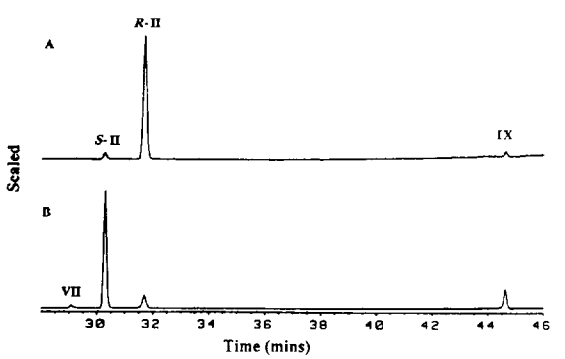


Fig. 4. HPLC analysis of methylsulphoxide epimers (II) on a Kromasil $C_{18}$ column (inadvertently contaminated with metals), no guard column employed. Chromatographic conditions 2 as in Section 2 with the exception that the analysis was performed at 80°C. (A) *R*-epimer, (B) *S*-epimer.

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