The Use of Complimentary Stationary Phases and 2-Dimensional HPLC for the Separation of the Synthesis and Degradants of Tipredane (INN)

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The article describes how stationary phase characterisation can be successfully employed to identify RP-LC columns of complimentary chromatographic selectivity for use in a 2-dimensional LC 'heart cutting technique' to separate a complex mixture of a pharmaceutically relevant steroid and its degradants/synthetic impurities including the intractable separation of two C17 diastereoisomers (epimers). The separation mechanism of the C17 epimers appears to be controlled by the spacial orientation of the bonded ligands which preferentially restricts access of one of the epimers into the RP. The technique has been able to detect as low as 0.1% w/w of the unwanted C17 epimer.

The steroidal active pharmaceutical ingredient (API) Tipredane (INN, I) presents the chromatographer with an interesting and complex separation challenge in that its C17 dithioketal moiety is extremely susceptible to hydrolytic, thermal and oxidative degradation, hence the drug substance may contain a plethora of potential impurities *(see Figures 1 and 2)*. Many of these putative degradants can then undergo further secondary degradation via another degradative pathway to form further degradants - for example the monoalkylthio thermal degradants (III & IV) can then undergo further oxidation to the diastereoisomeric sulphoxides (VII 1 & 2 and VIII 1 & 2). Given that the six chiral centres in Tipredane are fixed (discounting the C17 position) and that the sulphoxides are chiral in nature, each sulphoxide degradant of Tipredane will exist as two diastereoisomers (in the field of steroids these are referred to as epimers) hence further increasing the complexity of the separation. In the mid-1990s when this API was in development this challenging separation of Tipredane and all of its potential degradants was successfully achieved and validated using a conventional C18 phase [1], see *Figure 2* for a typical separation on a new generation C18 column. The HPLC method was originally developed using early computer method development software such as Interactive Computer Optimization Software (ICOS) from Hewlett Packard and HIPAC from Phase Separations. The synthesis of Tipredane however can potentially give rise to its corresponding C17 epimer (IX). Being diastereoisomers, they should possess differing physico/chemical properties and hence should be separable on conventional RP columns – surprisingly however, over 30 RP columns were tested at the time and all failed to resolve Tipredane from its C17 epimer. In fact it was necessary to resort to the use of chiral stationary phases to resolve these diastereoisomers [3].

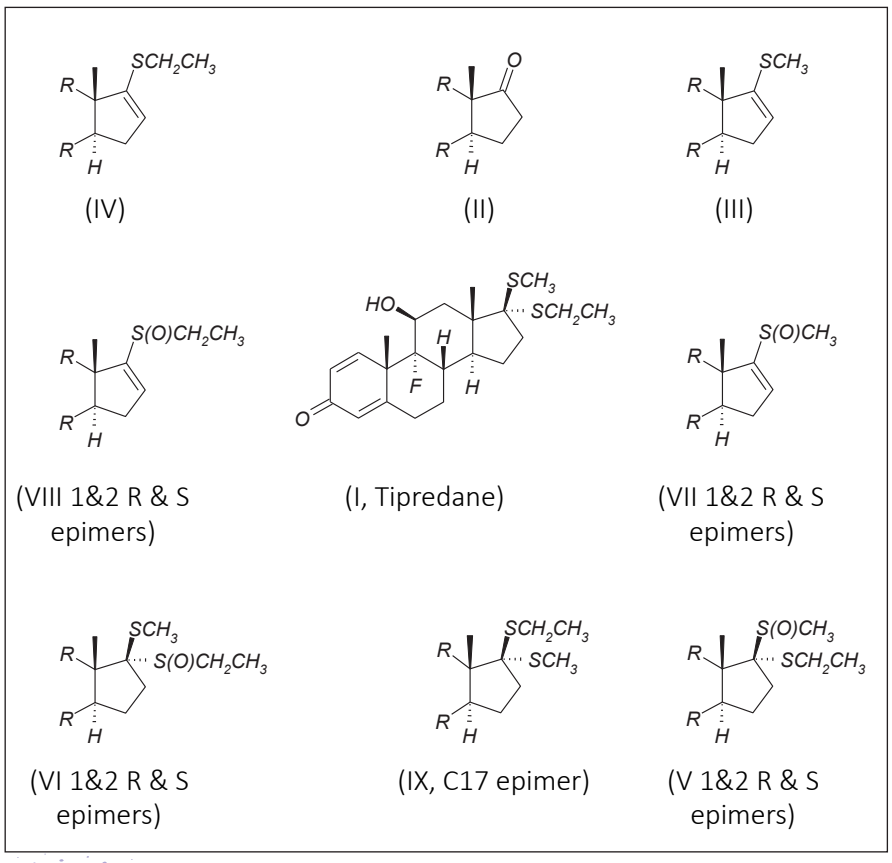

Figure 1. Structure of Tipredane and its potential synthetic and degradative impurities

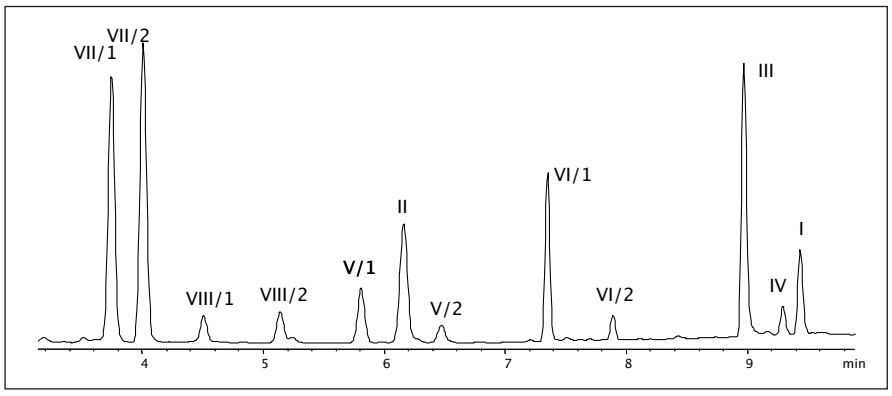

Figure 2. Optimised RP-LC separation of Tipredane and its related impurities on an ACE 3 C18 column with an MeCN / water gradient (see Experimental section for details and Figure 1 for peak assignment)

Given the wide diversity of currently available stationary phases with respect to chromatographic selectivity it was decided to re-evaluate the separation of Tipredane and its C17 epimer. Stationary phases of complimentary chromatographic selectivity were selected and identified using the chemometric tool of Principal Component Analysis (PCA). Once a successful column that separated Tipredane from its C17 epimer had been identified it was decided to assess the scope and applicability of performing 2-dimensional HPLC (2-D LC) on one of the recently introduced commercial systems using the technique of 'heart-cutting' to assess the C17 epimer content in degraded Tipredane solutions.

Experimental

Column characterisation work and evaluations for the separation of Tipredane from its C17 epimer were performed on an Agilent 1100 series HPLC while 2-D 'heart cutting' was performed on an Agilent 1290 Infinity 2-D LC system. 2-D LC conditions were optimised for analyte loading, peak sampling, column dimensions, flow rate and peak focusing onto the 2nd domain.

Each phase was tested using isocratic conditions on an Agilent 1100 series to assess the effect of % MeOH and MeCN (i.e. 90 – 60% organic in the mobile phase) on the separation of the Tipredane C17 epimers. Where there was any separation the effect of temperature (range 10 – 70°C) was then evaluated.

The optimised separation of Tipredane and its degradants was performed on an ACE 3 C18 150 x 4.6 mm column at 1.5 ml/min, 20°C, detection at 240 nm and a 5 µl sample injection, mobile phase A = water and B = MeCN, gradient conditions 30%B at 0 min, 40%B at 5 min and 100% at 8 min. The hydrolytic, thermal and oxidative degradants were prepared as described in reference 1, 2 and 10.

2-Dimensional LC

The first dimension separation was performed on an ACE 3 C18 150 x 1 mm column at a flow rate of 0.21 ml/min, 20°C, detection at 240 nm and a 0.2 µl sample injection, mobile phase A = water and B = MeCN, gradient conditions 30%B at 0 min, 100%B at 5.06 min and held for 0.68 min. The injector programme was used to drop the sample after the gradient had started to maintain the chromatographic selectivity. Heart cutting was performed after 4.1 min with a 0.1 min collection into a 40 µl loop, a delay of 15 µl was used to compensate for the volume between the detector and the loop.

The second dimension separation was performed on a Fortis Phenyl column 150 x 3 mm column at a flow rate of 0.43 ml/min, 40°C, detection at 240 nm, mobile phase water / MeOH (30:70 v/v).

Results and discussion

Separation of Tipredane C17 epimers

A range of RP materials possessing complimentary chromatographic selectivity to that of conventional C-alkyl phases was selected based on their modified Tanaka column characterisation parameters [4,6]. The selected range of phases, including new generation C18, polar embedded C18, extended polar selectivity C18, pentafluorophenyl, cyano, aromatic (i.e. phenyl with differing alkyl spacers, diphenyl, biphenyl, pyrenyl and naphthalyl) and a cholesteryl phase, for evaluating the separation of Tipredane and its C17 epimer was shown by a PCA score plot to be very different from each other with respect to their phase classification *(See Table 1 and Figure 3)*. From the corresponding PCA loading plot (data not shown) the X-axis represents hydrophobic retention (i.e. right = high and left = low hydrophobic retention) while the Y-axis represented aromatic / shape selectivity of the phases (i.e. top = high, bottom = low aromatic / shape selectivity). Most of the new generation C18 phases were located along the X-axis and were discriminated only by their differences in hydrophobicity, the exception to this was for the extended polar selectivity C18 and cholesteryl phases which both possessed higher apparent shape selectivity and hydrogen bonding capability compared to conventional C18 phases.

Table 1. Stationary phases evaluated, their modified Tanaka column characterisation parameters (see Reference 6 for descriptions), manufacturers' description and their ability to separate the C17 epimers

		Tanaka column characterization parameter [6]								
Column no.	Stationary phase	k_{PB}	α_{CH2}	$\alpha_{T/O}$	$\alpha_{C/P}$	$\alpha_{B/P\ pH\ 7.6}$	$\alpha_{B/P\ pH\ 2.7}$	$\alpha_{TNB/NB}$	Type	$R_s > 1.5$ C17 epimers[a]
1	Ascentis C18	8.01	1.50	1.57	0.40	0.35	0.10	0.64	C18	nd
2	XBridge 5 C18	3.81	1.45	1.38	0.34	0.27	0.14	0.59	C18	No
3	HyPURITY C18	3.20	1.47	1.60	0.37	0.29	0.10	0.63	C18	nd
4	Zorbax SB-C18	6.00	1.49	1.20	0.65	1.46	0.13	0.65	C18	nd
5	Prodigy ODS3	7.27	1.49	1.26	0.42	0.27	0.09	0.66	C18	nd
6	XBridge Shield RP 18	2.85	1.38	2.22	0.33	0.26	0.12	0.84	Polar embedded	No
7	HyPURITY 3 AQUASTAR	1.32	1.39	2.65	1.25	2.66	0.13	0.60	Enhanced Polar selectivity	Yes
8	Asahipak ODP-50	nd	nd	nd	nd	nd	nd	nd	Polymeric C18	No
9	Hypersil Gold PFP	0.70	1.25	2.55	1.02	0.94	0.17	0.71	PFP	No
10	Fluophase PFP	1.60	1.23	2.50	0.63	0.70	0.30	0.66	PFP	nd
11	BDS Hypersil Phenyl	0.52	1.24	1.00	0.85	0.56	0.25	1.00	Phenyl	No
12	Betabasic 3 Phenyl	0.39	1.21	0.92	1.00	0.64	0.19	0.87	Phenyl	Yes
13	Nucelodur 5 Sphinx	4.74	1.43	0.95	0.67	0.45	0.09	1.18	Mixed phenyl / alkyl	No
14	Betasil 3 Phenyl Hexyl	1.78	1.32	0.75	0.42	0.39	0.12	0.69	Phenyl hexyl	Yes
15	Ascentis 3 Phenyl	2.32	1.31	1.00	0.96	0.44	0.14	2.19	Phenyl butyl	No
16	Luna 3 Phenyl Hexyl	2.82	1.33	1.10	0.91	0.33	0.11	1.97	Phenyl hexyl	No
17	XBridge 3.5 Phenyl	1.45	1.31	1.00	0.86	0.41	0.19	1.91	Phenyl hexyl	No
18	Allure 5 Biphenyl	3.73	1.33	1.55	2.21	0.81	0.05	2.52	Biphenyl	No
19	Fortis 5 Phenyl	1.22	1.27	0.87	0.88	0.46	0.14	0.86	Diphenyl	Yes
20	Pursuit 3 Diphenyl	0.52	1.21	0.89	0.77	0.44	0.23	0.90	Diphenyl	Yes
21	Cosmosil 5 PYE	1.95	1.37	3.02	24.10	0.58	0.20	10.87	Pyrenylethyl	No
22	Cosmosil 5 Pi NAP	1.74	1.35	1.51	4.08	0.34	0.10	13.40	Naphthalethyl	No
23	Cosmosil 5 Cholester	5.24	1.49	3.26	0.39	0.23	0.03	0.60	Cholesterylpropyl	No
24	Discovery CN	0.29	1.00	1.00	1.00	nd	0.55	nd	Cyano	No
25	Hypersil Gold CN	nd	nd	nd	nd	nd	nd	nd	Cyano	No

[a] Based on the maximum obtainable resolution in differing proportions of MeOH / water and MeCN / water

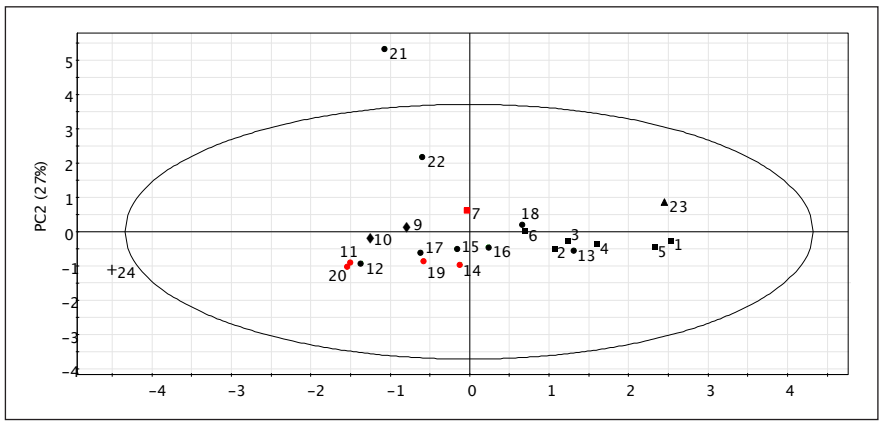

Figure 3. PCA Score plot of the modified Tanaka column characterisation parameters of the phases investigated (● = aromatic, ♦ = PFP, ■ = C18, ▲= cholesteryl and + = cyano). Phases which separated the C17 epimers are marked in red

A significant number of aromatic based phases were included in the investigation as they have been recently shown to be successful in the separation of steroidal diastereoisomers [5]. This class of phases, as can be seen in the PCA score plot, are very diverse in their chromatographic properties [6], especially with respect to their hydrophobicity, aromatic interaction capability, hydrogen bonding and shape selectivity – this is presumably due to their differences in base silica, endcapping, monomeric / polymeric bonding and the alkyl spacer length in addition to differences in the aromatic ligand attached.

The selected new generation C18 and its corresponding polar embedded C18 phase, pentafluorophenyl, cyano or polymeric based phases failed to separate Tipredene from its C17 epimer – even when differing proportions of either MeOH or MeCN in the mobile phase were evaluated or as a function of temperature. In contrast, many of the phenyl phases exhibited resolution (Rs > 1.5) of Tipredane from its C17 epimer. It would appear that the aromatic nature of the phase is not solely responsible for the C17 epimer separations, as phases with exceptionally high aromatic character (i.e. Cosmosil PYE, π-NAP and the biphenyl phases) failed to resolve them. Unfortunately, there is little information supplied by the manufacturers relating to the exact nature of the ligand which is attached or the bonding technology employed hence it is difficult to make firm conclusions. However, it would appear from the PCA score plot, that the Betabasic phenyl and Pursuit diphenyl are very similar chromatographically (i.e. very close together in space in the PCA score plot) and both resolve the C17 epimers which strongly suggests that they possess similarly bonded ligands. The only other aromatic phases to separate the C17 epimers were the Fortis phenyl and the Betasil phenyl hexyl phase (the latter has previously been shown not to behave like conventional phenylhexyl phases where the phenyl moiety is attached to a hexyl chain). In comparison, the BDS Hypersil phenyl and the XBridge phenyl failed to resolve the C17 epimers while being close to other phases that did *(see Figure 3 and Table 1)*. The XBridge phenyl (phenyl ring attached to a hexyl chain) and other phases with the phenyl ring attached via a butyl (Ascentis) or hexyl (Luna) chain also failed to separate the C17 epimers suggesting that the Nucleodur and BDS Hypersil phenyl phases may possess the phenyl group attached via an alkyl chain. These type of phases have been shown previously to possess higher aromatic character than phenyl groups attached directly to the surface of the silica [6] – it should be noted that separation of the C17 epimers was only achieved on phenyl phases which possess an α TNB/NB value < 0.9 (i.e. low aromatic character).

The HyPURITY Aquastar (Extended polar C18 selectivity phase) and the Cosmosil Cholester phases exhibited good to partial resolution of the C17 epimer despite them not possessing a phenyl grouping (as evidenced from chromatographic and degradative studies), giving further evidence that the presence of the phenyl groups per se is not responsible for the separation. It is possible that the way the various ligands are bonded onto the silica controls the accessibility of the C17 epimers into the phase and their interaction with silanol groups near the silica surface as suggested by the higher shape and silanophilic activity of these phases.

The use of methanol in the mobile phase, combined with lower temperatures in general, afforded improved separation of C17 epimers *(see Figure 4)*. It was concluded that the decreased temperature resulted in an increased rigidity of the bonded ligands on the phase which disfavoured the accessibility of one of the epimers more than the other in accessing the silanol groups close to the silica surface [7].

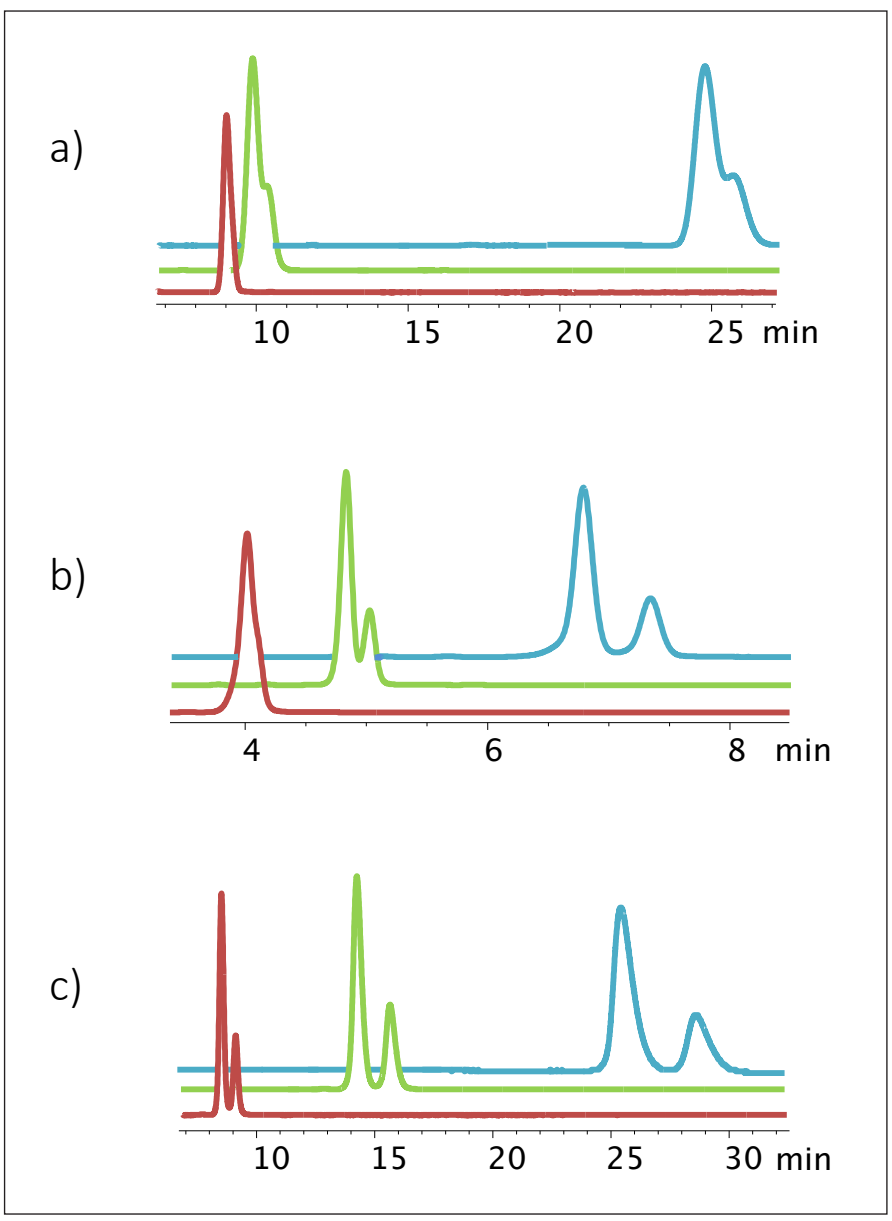

Figure 4. Effect of temperature on the resolution of Tipredane C17 epimers on selected phases (150 x 4.6 mm, 1 ml/min, 5 µl, 240 nm, mobile phase MeOH/water 70:30 v/v). Red = 70°C, Green = 40°C, Blue = 10°C, a) new generation C18, b) extended polar selectivity phase and c) phenyl

2-Dimensional LC

Despite the success of the newer phases which separate the C17 epimer, none of the phases afforded the separation of the C17 epimers as well as all of the other putative impurities of Tipredane. Given the fact that several commercial 2-dimensional LC (2-D LC) systems have recently been launched, it was decided to evaluate whether a more elegant solution to this challenging chromatographic problem was possible using 2-D LC.

A new generation C18 column (150 x 1mm, 0.21 ml/min) and an optimised MeCN / water gradient at reduced temperature (i.e. 20°C) to maintain the integrity of these unstable degradants [10]), was used in the first dimension to separate the Tipredane/ C17 epimer peak (which co-eluted) from their degradants. In order to switch the entire volume of the Tipredane/C17 epimer peak onto the second dimension column (150 x 3 mm, 0.43 ml/min) run at 40°C it was necessary to reduce the peak volume on the first dimension by employing a reduced internal diameter column (i.e. 150 x 1 mm), flow rate (0.21 ml/min) and loop size (40 µl) to collect the peak (it was also necessary to adjust the gradient conditions to maintain the desired selectivity as highlighted in references 8 and 9). Once this had been optimised, the entirety of the Tipredane / C17 epimer peak volume was switched onto the second dimension which utilised an optimised isocratic MeOH / water (70:30 v/v) mobile phase and a diphenyl phase at an elevated temperature to afford baseline separation of the C17 epimers *(see Figure 5)*. The column dimension was increased (i.e. 150 x 3 mm) in order to minimise any peak distortion resulting from the injection of a relatively high volume of mobile phase containing a high proportion of acetonitrile from the first dimension mobile phase. By employing this novel 2-D LC approach we were able to demonstrate an acceptable limit of detection (LOD) of the C17 epimer (i.e. 0.1%) in Tipredane *(see Figure 5)* in a reduced analysis time frame. The method was demonstrated to be reproducible in terms of retention time and % peak areas obtained.

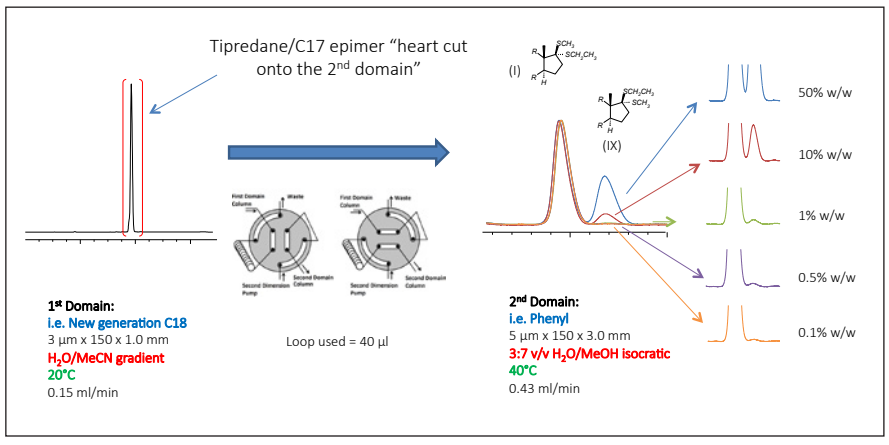

Figure 5. 2-D LC heart cutting from the 1st domain onto the 2nd domain and LOD of the C17 epimer in Tipredane

Conclusions

The usefulness of a chemometric characterisation approach (i.e. Principal Component Analysis – 'PCA') to identify and select HPLC stationary phases of complimentary chromatographic selectivity has been highlighted. The modified Tanaka column characterisation parameters coupled with the PCA score and loading plots suggested that the phases which successfully resolved the C17 epimers of Tipredane possess high shape / steric discrimination and high silanophilic (i.e. hydrogen bonding) capacity. The results suggest that aromatic interaction of the phenyl phases and the C17 epimers is not directly responsible for the C17 epimer resolution as phases with high aromatic activity (i.e. large αTNB/NB) failed to separate the C17 epimers. It is believed that the way the differing ligands are bonded onto the silica imposes differing degrees of accessibility of the structurally rigid steroidal epimer into the phase which then interact with the silanol groups close to the silica surface – the spacial orientation of one of the C17 epimers permits greater penetration into the phase than the other and hence separation [7].

The paper has highlighted the usefulness of 2-dimensional LC in solving intractable chromatographic challenges. The original LC methodology can be simply employed in the first dimension and the desired / suspect peak can then be transferred to the second dimension which employs LC conditions of complementary chromatographic selectivity to afford separation of any impurities that co-eluted with the drug substance or to confirm that the major peak is pure. The only practical restrictions to this 2-D LC 'heart cutting' technique, is that it requires the peak that is transferred to the second dimension to be 'focussed' on top of the second domain column so that peak distortion is not a problem. This can be readily achieved on this commercial 2-D LC system by optimisation of analyte loading, peak sampling and the appropriate selection of column dimension and flow rate in both domains. The application of this technology on a commercial 2D-LC instrumentation has been successfully highlighted, permitting a LOD for the C17-epimer of 0.1% w/w to be obtained.

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