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PACKED-COLUMN SUPERCRITICAL FLUID CHROMATOGRAPHY OF BENZODIAZEPINES

ROGER M. SMITH* and M. MARSIN SANAGI[a]

Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire LE11 3TU (U.K.)

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SUMMARY

Eleven benzodiazepines were separated using supercritical fluid chromatography on columns packed with polystyrene–divinylbenzene and ODS- and cyano-bonded silica. The effect of the addition of methanol as a modifier to the mobile phase was examined and the selectivities of the separations were compared using retention indices based on the alkyl aryl ketone scale.

INTRODUCTION

The basic methods and equipment for supercritical fluid chromatography (SFC) using packed or capillary columns are now well established[1-3], but work is still in progress to determine the scope of the technique, with particular interest in its potential application for the separation of pharmaceutical compounds. A number of typical drugs have been examined[1,4-6] and a detailed study of the separation of barbiturates on polystyrene-divinylbenzene (PS–DVB) and ODS-bonded silica columns has been recently reported[7,8].

The benzodiazepines are widely used drugs[9] and the chromatographic determination of individual compounds has been frequently reported[10]. There is also considerable interest in their identification in therapeutic monitoring and toxicology. Gas–liquid chromatography (GLC) can be used but some members of this group are thermally unstable and give multiple peaks[11]. High-performance liquid chromatography (HPLC) is generally applicable and the separations of 21 widely used benzodiazepines and their metabolites have been compared on ODS-bonded silica and silica columns using different mobile phases to cover the range of drug polarities[12]. The normal-phase separation of sixteen benzodiazepines on silica and amino- and cyano-bonded silica columns have also been examined[13]. A collection of the retention data of separations by GLC, HPLC and thin-layer chromatography (TLC) has been compiled for benzodiazepines and benzophenones controlled by the Misuse of Drugs Act in the U.K.[11].

[a] Present address: Department of Chemistry, University of Technology Malaysia, Johor Bahru, Malaysia.

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In this paper, the separation of benzodiazepines by supercritical fluid chromatography on ODS- and cyano-bonded silica and PS–DVB columns is reported and the influence of the proportion of modifier on the retention and selectivity is discussed. It was expected that these methods might offer an alternative to conventional gas or liquid chromatography with different selectivity and that any changes in the order of elution could be used to aid identification or to improve the resolution in complex mixtures.

EXPERIMENTAL

Chemicals

Alkyl aryl ketones from butyrophenone to octadecanophenone were of laboratory grade from a range of suppliers. Benzodiazepines were obtained from the reference collection of the Central Research Establishment, Home Office Forensic Science Service (U.K.). Carbon dioxide was of industrial grade (99.98 %) from British Oxygen (U.K.).

Equipment

The separations were carried out using a packed column chromatograph[1] consisting of a Jasco BIP-1 pump, with cooled check valves and pumping head, operating under constant-pressure conditions, a Pye Unicam LC-XPS pump for the addition of modifier through a stirred mixing chamber, a Rheodyne 7125 injection valve with a 20-μl loop and a Pye 104 gas chromatographic oven (Pye Unicam, Cambridge, U.K.). The analytes were detected at 254 nm using an ACS 750/12 variable-wavelength UV spectrophotometric detector (Applied Chromatography Systems, Macclesfield, U.K.) fitted with a high-pressure flow cell.

The samples were separated on either a 150 × 4.6 mm I.D. column packed with PLRP-S (PS–DVB, 5-μm particles) (Polymer Labs., Church Stretton U.K.) or a 250 × 4.6 mm I.D. column packed with either Ultrasphere-ODS (5-μm particles) or Ultrasphere cyano (5-μm particles) (Beckman) and peaks were recorded using a Hewlett-Packard 3390 integrator.

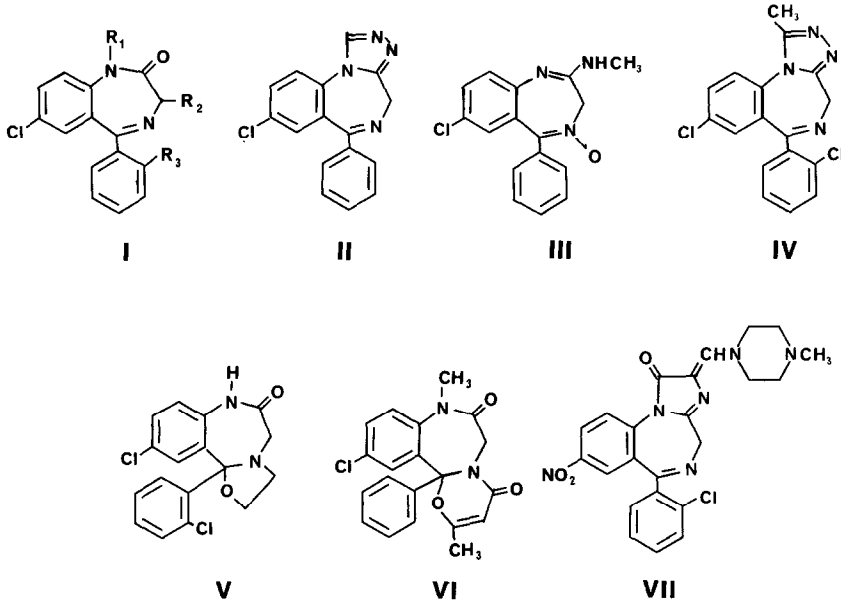
Method

Samples of the benzodiazepines and alkyl aryl ketones retention index reference compounds in methanol were injected onto the column and eluted with supercritical carbon dioxide. Methanol was added to the eluent as a proportion by mass. A sample of acetone was used as the column void volume marker.

Capacity factors were calculated as $k' = (t_R - t_0)/t_0$ and the means of triplicate injections were used in calculations. Retention indices based on the alkyl aryl ketones, butyrophenone–octadecanophenone for the PS–DVB column or valerophenone–octadecanophenone for the ODS-bonded silica column, were calculated as described for HPLC[14] by fitting log k' against carbon number × 100 for the standards to a linear correlation using a least-squares routine and then interpolating the log k' values for the test compounds.

RESULTS AND DISCUSSION

In an initial study, a number of benzodiazepines were found to be totally retained on a Spherisorb ODS-2 column using SFC with unmodified carbon dioxide as the mobile phase and conditions of temperature and pressure that would satisfactorily elute many aromatic compounds. As it was thought that interaction of the drugs with free silanols on the surface of the silica might be causing the retention, the benzodiazepines were then examined on a PLRP-S (PS–DVB) column. In this instance most of the analytes were again totally retained, but lorazepam, temazepam and diazepam were eluted as highly retained broad peaks. In previous studies with the PS–DVB column, the addition of methanol or acetonitrile as a modifier to the carbon dioxide eluent reduced the retention times and considerably improved the peak shapes of barbiturates[8] and polar test compounds[15]. Both modifiers also markedly improved the separation of diazepam.



A detailed study was therefore carried out of the effect of the addition of methanol on the capacity factors of eleven benzodiazepines (Table I). With 4.3% methanol all the compounds except loprazolam were eluted within a reasonable time with symmetrical peak shapes (Table II). Increasing the proportion of methanol further reduced the retention times and at 15.3% methanol loprazolam was eluted with a retention time of less than 12 min (Fig. 1).

The order of elution for closely related compounds, such as nordazepam and diazepam, depended on their relative molecular sizes. The presence of a 2'-chloro group appeared to cause a small reduction in relative retention as with lormetazepam and its parent compound temazepam. Although compounds containing imino and amino groups, such as estazolam and triazolam, were strongly retained relative to the other compounds with 4.3% methanol, increasing the proportion of methanol had a

TABLE I

BENZODIAZEPINES USED

Compound	*No.*	R_1	R_2	R_3
Diazepam	I	CH_3	H	H
Lorazepam	I	H	OH	Cl
Lormetazepam	I	CH_3	OH	Cl
Nordazepam	I	H	H	H
Temazepam	I	CH_3	OH	H
Estazolam	II			
Chlordiazepoxide	III			
Triazolam	IV			
Cloxazolam	V			
Ketazolam	VI			
Loprazolam	VII			

greater effect than with less polar compounds (Fig. 2). Significant changes in the elution order, particularly for compounds containing hydroxyl, phenolic and amide groups, compared with less polar compounds were also observed in an earlier study of model compounds[15]. Related changes have also been observed by McNally *et al.*[16] with substituted pesticides on addition of an range of modifiers.

In studies of the HPLC separation of drugs, retention indices based on a series of alkyl aryl ketones have been used to improve the reproducibility of recording and reporting retentions[17]. The retention index values are more independent than capacity factors of small changes in the eluent composition and column temperature, such

TABLE II

SEPARATION OF BENZODIAZEPINES ON A POLYSTYRENE–DIVINYLBENZENE COLUMN

Conditions: column, PLRP-S; eluent, carbon dioxide–methanol; mean pressure, 2515 p.s.i.; temperature, 60°C; UV detection at 254 nm.

Compound	*Capacity factor*		
	Methanol (%, w/w)		
	4.3	*9.7*	*15.3*
Nordazepam	15.61	4.46	1.82
Lormetazepam	16.24	5.57	2.20
Lorazepam	16.48	4.10	1.40
Temazepam	16.84	5.62	2.28
Chlordiazepoxide	18.45	4.81	2.39
Diazepam	18.68	6.11	2.80
Ketazolam	20.44	6.22	2.25
Cloxazolam	24.70	6.70	3.03
Triazolam	28.57	5.78	1.94
Estazolam	32.82	6.83	2.23
Loprazolam	–	26.24	7.67

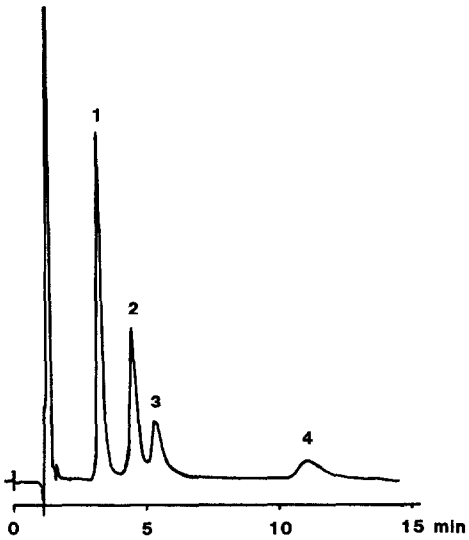


Fig. 1. Separation of benzodiazepines on a polystyrene–divinylbenzene column using carbon dioxide with 15.3% methanol as the mobile phase. Conditions as in Table II. Compounds: 1 = lorazepam; 2 = temazepam; 3 = cloxazolam; 4 = loprazolam.

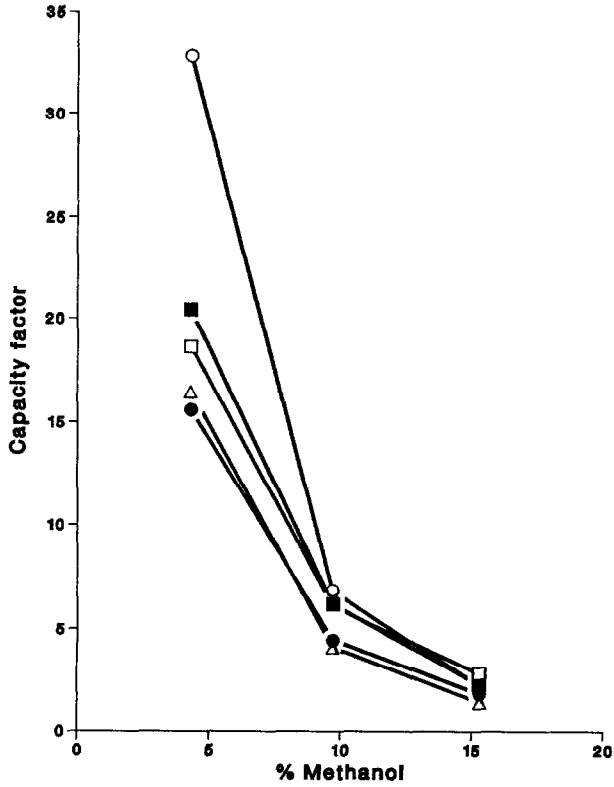


Fig. 2. Changes in capacity factors of selected benzodiazepines with proportion of methanol on a PLRP-S column. Conditions as in Table II. Compounds: ● = nordazepam; □ = diazepam; △ = lorazepam; ■ = ketazolam; ○ = estazolam.

TABLE III

RETENTION INDICES OF BENZODIAZEPINES ON A POLYSTYRENE–DIVINYLBENZENE COLUMN

Conditions as in Table II.

Compound	*Retention index*		
	Methanol (%, w/w)		
	4.3	*9.7*	*15.3*
Nordazepam	2401	2037	1677
Lormetazepam	2425	2193	1822
Lorazepam	2434	1978	1472
Temazepam	2448	2200	1850
Chlordiazepoxide	2503	2090	1887
Diazepam	2510	2258	2007
Ketazolam	2565	2270	1840
Cloxazolam	2679	2323	2068
Triazolam	2768	2219	1727
Estazolam	2852	2334	1834
Loprazolam	-	3283	2741

as might occur on repeating separations or transferring methods to different laboratories. It has been shown that they can be used in SFC to compensate for small differences in column pressure, temperature and density on PS–DVB[15] and ODS-bonded silica columns[18], but the retention indices of compounds containing different functional groups changed markedly with changes in the proportion of organic modifiers in the eluent.

As part of this study, the retention indices of the benzodiazepines on the PS–DVB column were determined relative to the alkyl aryl ketones, butyrophenone to octadecanophenone (Table III, Fig. 3). There were large variations of up to 100 units with changes in modifier, similar to those observed with very polar model compounds such as benzamide and benzoic acid[15]. In addition, the retentions of the individual benzodiazepines changed to different extents, and consequently neither retention indices nor relative retentions compared with an internal standard would be able to compensate for variations in the mobile phase composition. This emphasizes the major changes that can occur in the selectivity of the separation of compounds containing different functional groups as a result of changing the proportion of modifier. Therefore, in defining an SFC separation method for use for identification purposes, it will be necessary to define closely the proportion of modifier in the eluent.

The effect of adding methanol as a modifier was also examined on an Ultrasphere ODS (ODS-silica) column. The capacity factors of the benzodiazepines decreased markedly on increasing the proportion of methanol and were usually smaller than on the PS–DVB column (Table IV). The three hydroxyl-containing analytes, lormetazepam, lorazepam and temazepam, suffered considerable tailing with low proportions of methanol, but the peak shape improved as the proportion of methanol increased, suggesting that these compounds were interacting with the surface silanols

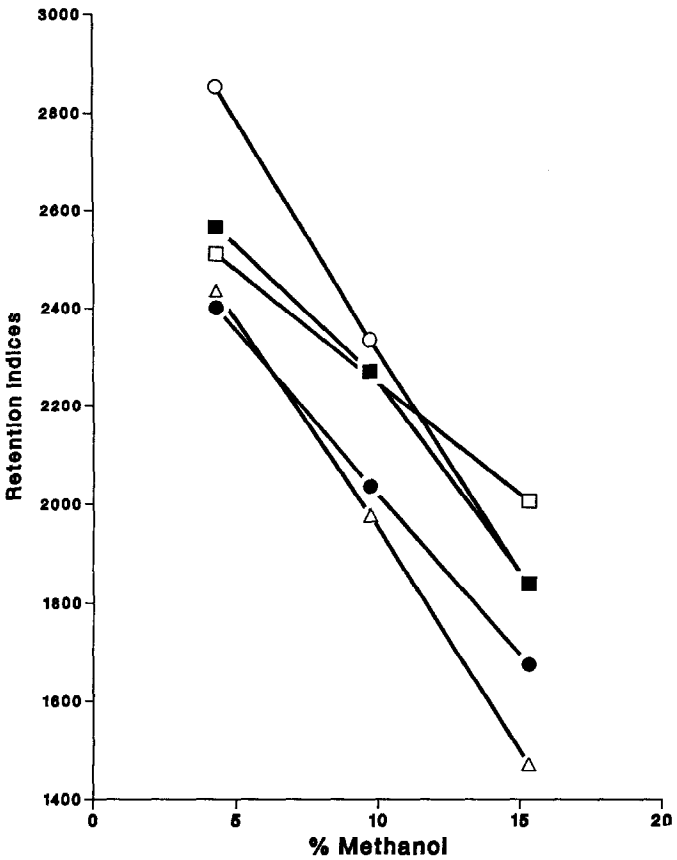


Fig. 3. Changes in the retention indices of benzodiazepines on a PLRP-S column. Analytes and conditions as in Fig. 2.

on the silica. The elution order was very different from that on the PS–DVB column and at low proportions of methanol there was a much reduced retention for ketazolam, diazepam and nordazepam compared with the other compounds. At higher proportions of methanol the differences were smaller and the relative retentions often changed. When the separation with a low (4.0%) proportion of methanol in the carbon dioxide was compared with the separation by HPLC[11] (see Table IV), with the exception of cloxazolam and loprazolam the order of elution was almost completely reversed emphasizing that there are major differences in selectivity between the two modes of separation. In HPLC ketazolam and diazepam were among the most highly retained benzodiazepines and a higher proportion of methanol was needed for their rapid elution[11,12], whereas in SFC they were the most rapidly eluted. In contrast, the compounds highly retained in SFC, triazolam and lorazepam, were readily eluted by HPLC.

The retention indices on the Ultrasphere ODS column were calculated compared with the alkyl aryl ketones, valerophenone to octadecanophenone (Table V).

TABLE IV

SEPARATION OF BENZODIAZEPINES ON AN ODS-BONDED SILICA COLUMN

Conditions: column, Ultrasphere ODS; eluent, carbon dioxide–methanol; mean pressure, 2470 p.s.i.; temperature, 60°C; UV detection at 254 nm.

Compound	*Capacity factor*				*HLPC*[a]
	Methanol (%, w/w)				
	4.0	*8.3*	*12.7*	*16.4*	
Ketazolam	1.54	0.76	0.51	0.31	13.02
Diazepam	1.56	0.75	0.50	0.31	9.47
Nordazepam	1.64	0.65	0.40	0.23	8.00
Cloxazolam	2.88	1.15	0.73	0.58	14.27
Chlordiazepoxide	5.12	1.19	0.66	0.46	6.41
Lormetazepam	5.15	1.39	0.79	0.44	6.39
Estazolam	5.63	1.05	0.52	0.28	3.80
Temazepam	5.86	1.54	0.83	0.48	5.76
Triazolam	6.19	1.08	0.53	0.26	4.38
Lorazepam	7.91	1.44	0.61	0.35	4.60
Loprazolam	–	–	–	3.17	6.09

[a] Conditions: column, ODS-Hypersil; eluent, methanol–phosphate buffer, pH 7.25 (55:45)[11].

Again, the retention indices changed with the proportion of methanol, with some compounds, estrazolam and lorazolam, showing particularly marked changes compared with diazepam and ketozalam (Fig. 4), again emphasizing the marked selectivity alterations with the proportion of modifier. Similar differences in selectivity have also been observed with model compounds on this column[18].

TABLE V

RETENTION INDICES OF BENZODIAZEPINES ON AN ODS-BONDED SILICA COLUMN

Conditions as in Table IV.

Compound	*Retention index*			
	Methanol (%, w/w)			
	4.0	*8.3*	*12.7*	*16.4*
Ketazolam	1934	1765	1554	1350
Diazepam	1939	1710	1545	1342
Nordazepam	1976	1599	1378	1115
Cloxazolam	2365	2025	1834	1831
Chlordiazepoxide	2761	2050	1760	1656
Lormetazepam	2766	2165	1897	1612
Estazolam	2828	1957	1569	1258
Temazepam	2856	2245	1938	1690
Triazolam	2893	1979	1582	1203
Lorazepam	3063	2195	1700	1447
Loprazolam	–	–	–	3127

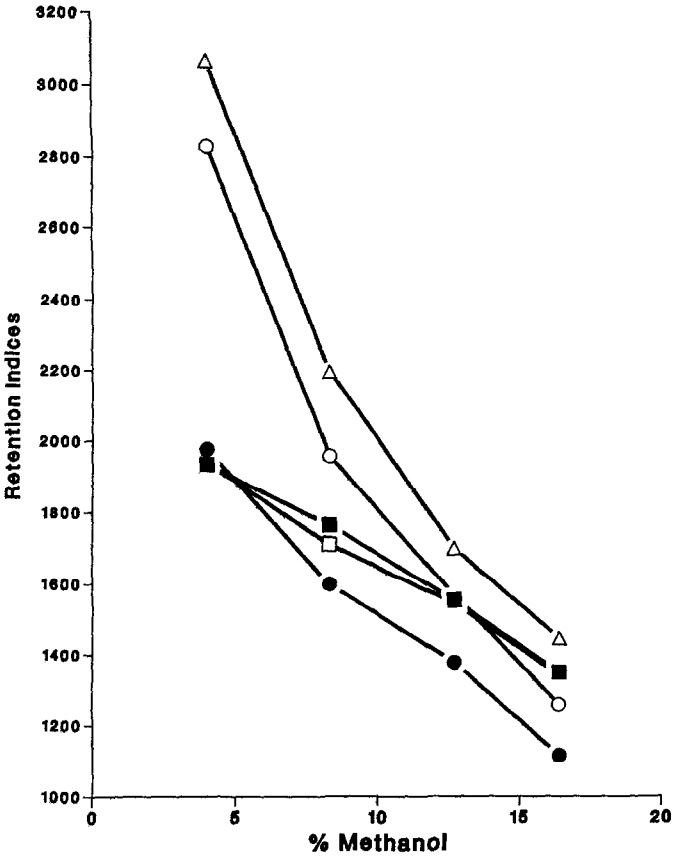


Fig. 4. Changes in retention indices with proportion of methanol on an ODS-bonded silica column. Conditions as in Table IV. Analytes as in Fig. 2.

Pietrogrande *et al.*[13] found that the benzodiazepines can be readily separated on cyano-bonded silica columns by using normal-phase HPLC. A preliminary study was therefore carried out using an Ultrasphere cyano column. In the absence of modifier none of the benzodiazepines were eluted but, with the exception of loprazolam, they were all eluted in the presence of 12.8% methanol (Table VI, Fig. 5). Except for cloxazolam, all the analytes gave good peak shapes, even the hydroxyl-containing analytes tempazepam and lorazepam. Although only a few benzodiazepines were chromatographed using both systems, the longer retentions of temazepam and lorazepam compared with diazepam and nordazepam correspond to the HPLC separation on the cyano column with hexane–isopropanol (90:10) as the mobile phase, which was considered to be dominated by the hydrogen bonding and polar interactions with the stationary phase[13]. However, chlordiazepoxide was relatively unretained in HPLC whereas in SFC it was well retained. At lower proportions of modifier diazepam was readily eluted, but loprazolam, triazolam and cloxazolam were highly retained. The retention indices were not calculated on the cyano-silica column because the retentions of the alkyl aryl ketone standards appeared to be abnormal; this is being further investigated.

TABLE VI

CAPACITY FACTORS OF BENZODIAZEPINES ON A CYANO-BONDED SILICA COLUMN

Conditions: column, Ultrasphere CN; eluent, carbon dioxide containing 12.8% (w/w) methanol; mean pressure, 2470 p.s.i.; temperature, 60°C; UV detection at 254 nm.

Compound	*Capacity factor*	*Compound*	*Capacity factor*
Diazepam	1.25	Chlordiazepoxide	2.83
Ketazolam	1.30	Cloxazolam	4.76
Temazepam	1.58	Estazolam	5.19
Nordazepam	1.63	Triazolam	6.42
Lormetazepam	1.70	Loprazolam	>19
Lorazepam	2.39		

Even at a similar proportion of organic modifier (12–15%), the relative retentions of the benzodiazepines on the three stationary phases are very different, showing the effect of the different stationary phases on the separations.

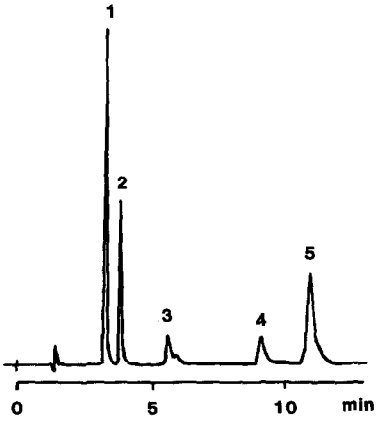


Fig. 5. Separation of selected benzodiazepines on an Ultrasphere cyano column. Conditions as in Table VI. Compounds: 1 = diazepam; 2 = nordazepam; 3 = chlordiazepoxide; 4 = estazolam; 5 = triazolam.

CONCLUSION

SFC offers a range of selectivities for the separation of these benzodiazepines which differs from that obtained by HPLC, but the selectivity of the separations and the relative retentions are very dependent on the proportion of modifier in the mobile phase.

These results emphasize that because of the marked effect of the proportion of the modifier in SFC on the relative retentions of compounds containing different functional groups, particular care will be needed to reproduce these and similar separations in different laboratories. It will be necessary to specify closely the proportion of methanol by mass in the eluent. However, the reproducible achievement of the

defined composition poses severe practical problems on different instruments because, owing to the compressibility of carbon dioxide, its density in the pump heads will depend on their temperature and the column back-pressure. Hence different mass flows may be obtained on different instruments even with pumps set to the same nominal constant flow-rates. Normally the modifier will be added from a second pump at a constant flow-rate and so will represent a different proportion in the column eluent in each instance. Different problems will be encountered with pumps working at constant pressure against a restrictor, as these will give different mass flow-rates if the back-pressure alters. An alternative method could be to use cylinders of premixed eluents, but it has been reported that the composition delivered from the cylinders can change as the contents are consumed[19].

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