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Direct and indirect approaches to enantiomeric separation of benzodiazepines using micro column techniques

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Abstract

The ability of gas chromatography, supercritical fluid chromatography (SFC) and subcritical fluid chromatography to separate ten benzodiazepine racemates, using both direct and indirect methods, has been investigated. Racemic lorazepam, oxazepam, ethyl loflazepate and dihydrodiazepam were baseline separated in open tubular column SFC using a permethylated β-cyclodextrin stationary phase. Racemic oxazepam, lorazepam, temazepam and ethyl loflazepate, derivatized with (*S*)-trolox methyl ether, were separated on a non-chiral packed capillary column with diol functionality, employing a subcritical fluid as mobile phase. The temazepam derivatives were also baseline separated using an open tubular biphenyl column in SFC.

1. Introduction

A racemic mixture should be considered as a mixture of two different substances since optical isomers may differ in biological activity, toxicity and metabolism [1]. Enantiomeric separation can be achieved using either direct or indirect methods. The direct methods include the use of a chiral stationary (or mobile) phase, while the indirect methods are based on chiral derivatizations, i.e. conversion of the enantiomers into diastereomers, and separations using non-chiral stationary phases. Chiral derivatization is unfortunately not a straightforward procedure as the optical purity of the reagent must be known and high, the yield of the reaction must be carefully controlled and racemisation of the sample during reaction must be prevented. On the other hand, with the indirect method the elution order of the diastereomers can be altered by using either the (*R*)- or the (*S*)-form of the chiral reagent. A broad range of non-chiral columns is also commercially available, for both gas chromatography (GC) and supercritical fluid chromatography (SFC).

The most widely used chiral stationary phases in both GC and SFC are based on permethylated β-cyclodextrin (β-CD-M) [2–6]. It is believed that the cavity of the cyclodextrin-bucket empowers chiral recognition through the formation of inclusion complexes [6,7]. Due to the solvating power of supercritical carbon dioxide, the stationary phases used in open tubular column SFC must be carefully immobilised [8]. Several reports have been published on the synthesis of stable chiral open tubular columns for SFC [2–

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4,9,10], but yet there are no such columns commercially available. In packed-column SFC, as in liquid chromatography (LC), this becomes a minor problem since the chiral selector is covalently bound to the packing material. Most chiral LC columns can also be used in enantiomeric separations carried out by SFC.

Benzodiazepines are tranquillising and anticonvulsive drugs and can be classified as compounds of medium to high polarity. According to the literature most separations of benzodiazepines have been carried out using LC and in a recent article the advantages of using normal-phase LC (NPLC) instead of reversed-phase LC (RPLC) were pointed out [11]. Also the direct enantiomeric separation of these types of analytes is favoured by NPLC [12]. Wännman et al. [13] reported of the difficulties using RPLC in enantiomeric separation of oxazepam, which undergoes racemisation in aqueous media leading to the formation of a diffuse zone between the two enantiomeric peaks. In a comparative study between NPLC and subcritical fluid chromatography (SubFC) using chiral stationary phases, Macaudière et al. [6] showed that SubFC can be used with advantage for the resolution of various classes of racemates. Racemic oxazepam was, for example, separated both in SubFC and NPLC with a resolution of 3.5. The separation took less than 5 min employing SubFC but more than 20 min in LC [6]. Separations of racemic benzodiazepines using a supercritical fluid as mobile phase and a cyclodextrin-based open tubular column have been reported [3].

The use of GC for separation of benzodiazepine-like compounds is often hindered by thermolability [7], high molecular mass and high polarity. Some of the benzodiazepines have, nevertheless, been routinely analysed by GC [14–16], but to our knowledge no enantiomeric separation has been reported. The efficiency is generally higher in GC than in SFC and SubFC. On the other hand, the lower temperatures used in SFC and SubFC are advantageous as the chiral selectivity generally increases with decreasing temperature [17–19]. Additional advantages with a supercritical fluid mobile phase is the minimal consumption of organic solvents and the possibility to use lower UV wavelengths for detection.

In this work both direct and indirect microcolumn techniques (SFC, SubFC and GC) for enantiomeric separations of several benzodiazepines are compared and discussed.

2. Experimental

2.1. Instrumentation

The studies with open tubular columns and unmodified carbon dioxide as mobile phase were carried out using a series 600 SFC system equipped with a flame ionisation detection (FID) system (Dionex, Salt Lake City, UT, USA). Time-split injections were performed with a Valco C14W high-pressure four-port valve (Valco Instruments, Houston, TX, USA) equipped with a 0.2-μl sample loop. Both the 7.5 m $\times$ 50 μm I.D. (originally 10 m) SB-Biphenyl-30 (30% biphenyl methylpolysiloxane, film thickness d_f = 0.25 μm) column, and the fused-silica frit restrictor were obtained from Dionex. Chromatograms were recorded with a W + W 1100 recorder (Kontron Instruments, Zurich, Switzerland).

Separations on the packed capillary column, using methanol-modified carbon dioxide as mobile phase were carried out using a Carlo Erba SFC 3000 series (Fison Instruments, Milan, Italy) connected to a Phoenix 20 CU pump equipped with a Phoenix 20 slave pump (Fison Instruments). The SFC system was programmed using an IBM PC XT Model 286 computer and software from Fison Instruments. Time-split injections were, also in this set-up, carried out using a Valco C14W high-pressure four-port valve equipped with a 0.2-μl sample loop. UV detection was performed at 254 nm using a capillary detector 433 with a 0.2-μl fused-silica Z-cell (Kontron Instruments). The 30 cm $\times$ 320 μm I.D. packed capillary column, with LiChrosorb Diol as stationary phase (5 μm), was purchased from LC Packings (Zurich, Switzerland). A fused-silica frit restrictor 50 μm I.D. (Dionex) was mounted after the UV detector.

Chromatograms were recorded with a W + W 1100 recorder (Kontron Instruments)

GC separations were carried out using a Carlo Erba GC 6000 Vega Series 2, equipped with an FID system (Fison Instruments). The injections were performed with split flow (ca. 1:40) and the injection temperature was 250°C. Chromatograms were recorded with a Chrom Jet integrator (Spectra-Physics, Houston, TX, USA)

The chiral stationary phases that were used in open tubular column SFC and in GC were based on permethylated β-cyclodextrin. In the GC experiments, 25 m × 320 μm I.D. and 17 m × 320 μm I.D. deactivated fused-silica capillaries coated with β-CD-M dissolved in OV-61 (33% diphenyl dimethylpolysiloxane) were used. The separations carried out with SFC were obtained using a 5 m × 50 μm I.D. deactivated fused-silica capillary coated with approximately 0.25 μm side-arm substituted methyloctylsiloxane, having β-CD-M as chiral selector [20].

2.2. Chemicals

SFC-grade carbon dioxide was obtained from Scott Speciality Gases (Plumsteadville, PA, USA). Oxazepam, temazepam, lorazepam, ethyl loflazepate, dihydrodiazepam, camazepam, flutazolam, cloxazolam, tofisopam and otazolam were all kindly supplied by Hoffmann-La Roche (Basle, Switzerland). The derivatization reagents, acetic anhydride (AA), trifluoroacetic anhydride (TFAA), dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were obtained from Fluka (Buchs, Switzerland). (*S*)-Trolox methyl ether was prepared at Hoffmann-La Roche but can also be obtained from Fluka. All solvents were of analytical grade.

2.3. Derivatization procedures

Derivatization using acetic anhydride and DMAP

The analyte was dissolved in water-free pyridine and derivatized with AA using DMAP as a catalyst. The reaction time was 2 h at 70°C. The reaction mixture was first dried under a stream of nitrogen and was then dissolved in methylene chloride.

Chiral derivatization using (S)-trolox methyl ether

The analyte was first dissolved in methylene chloride (*S*)-trolox methyl ether, DCC and DMAP were then added. The reaction time was 1 h at room temperature. Because of the formation of dicyclohexyl urea, the mixture was filtered trough a 0.45-μm filter prior to injection. Ethyl loflazepate, otazolam and cloxazolam were derivatized, although not quantitatively, using higher amounts of DCC and DMAP and a reaction time of 10 h at room temperature.

3. Results and discussion

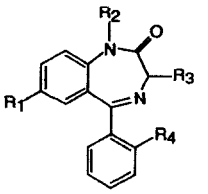
3.1. Direct separation of benzodiazepine enantiomers

The underivatized benzodiazepine standards, shown in Table 1, were chromatographed by chiral open tubular column SFC, using a slow density program (starting at 0.20 g ml^{-1}, with 0.007 g ml^{-1} min^{-1} up to 0.750 g ml^{-1}) under isothermal conditions (60°C). The enantiomers having the longest retention times and thus the highest elution densities, i.e. dihydrodiazepam, oxazepam, lorazepam and ethyl loflazepate were separated. To obtain high resolution (i.e. R_s = 1.5) in a minimum amount of time, the separation of dihydrodiazepam, oxazepam, lorazepam and ethyl loflazepate was optimised according to the procedure described in Refs. 21 and 22. The optimised separations (Table 2) were performed using a short focusing step, i.e. a low density (e.g. 0.20 g ml^{-1}) was maintained for a short initial period (e.g. 2 to 3 min) as part of the injection procedure, after which the density was rapidly increased (e.g. 0.20–0.50 g ml^{-1} min^{-1}) to the calculated optimal density. This density was then maintained until the analyte had eluted.

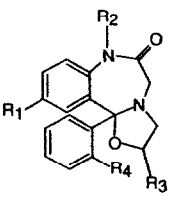
In a recent work [23] little or no enantio-

Table 1
Structures and names of the ten racemic benzodiazepines used in this study

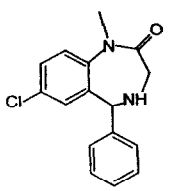
Name



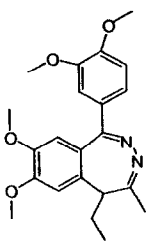
	R_1	R_2	R_3	R_4
Temazepam	Cl	CH_3	OH	H
Oxazepam	Cl	H	OH	H
Lorazepam	Cl	H	OH	Cl
Ethyl loflazepate	Cl	H	$COOCH_2CH_3$	F
Camazepam	Cl	CH_3	$OCON(CH_3)_2$	H



	R_1	R_2	R_3	R_4
Flutazolam	Cl	CH_2CH_2OH	H	F
Otazolam	Cl	H	CH_3	H
Cloxazolam	Cl	H	H	Cl



Dihydrodiazepam



Tofisopam

selectivity was observed for oxazepam and lorazepam using a cyclodextrin-based open tubular column in SFC. As shown in Table 2 and Fig. 1a, separation of these analytes is indeed possible, although the optimised separations of oxazepam and ethyl loflazepate gave R_s values below 1.5, due to a slight tailing of the peaks. As can be seen in Fig. 1b, baseline-separated peaks in less than 4 min were obtained for dihydrodiazepam.

A general advantage with open tubular columns in SFC is the ability to obtain well-deactivated columns and thereby the possibility to use a non-modified mobile phase for elution. This enables the use of the "universal" FID as detection method. However, all test compounds in this study, except dihydrodiazepam gave poor FID responses. The smaller signal-to-noise ratio for e.g. lorazepam compared to dihydrodiazepam can be seen in Fig. 1. Poor FID sensitivity for these analytes has earlier been reported by Chiarotti et al. [24].

None of the underivatized benzodiazepines could be eluted using a chiral open tubular column (β-CD-M/OV-61) in GC, using a temperature program from 100 to 220°C.

3.2. *"Direct" separation of benzodiazepine enantiomers after a non-chiral derivatization*

Oxazepam, lorazepam and ethyl loflazepate were derivatized with AA and TFAA according to the method described in the Experimental section. While the derivatization of lorazepam and oxazepam, that contains a hydroxyl group, gave a quantitative yield, ethyl loflazepate was not quantitatively derivatized.

In chiral open tubular column SFC, this approach resulted in decreased retention times, improved peak shapes and an almost unchanged resolution for the acetylated analytes compared to that for the underivatized analytes. This is illustrated by underivatized and AA-derivatized oxazepam in Fig. 2. Under the same SFC conditions for the acetylated compounds as for the underivatized, the FID response was still not sufficient although a small increase in the signal was observed for the compounds derivatized with AA (Fig. 2). UV detection or electron-capture detection could be an alternative for this type of analytes, the latter especially after a derivatization with TFAA.

None of the AA or TFAA derivatives could be eluted in GC using the β-CD-M/OV-61 column, and a temperature program from 170 to 215°C.

Table 2
Resolution of racemic benzodiazepine standards using a β-CD-M open tubular column in SFC

Compound	Density ($g\ ml^{-1}$)	Temperature (°C)	t_R (min)	R_s
Dihydrodiazepam	0.520	85	3.6	1.6
Oxazepam	0.445	55	44.9	1.2
Lorazepam	0.635	60	17.1	1.7
Ethyl loflazepate	0.400	50	23.1	1.3

Open tubular column SFC. Column 5 m × 50 μm I.D. fused-silica capillary, coated with side-arm substituted methyloctylsiloxane having β-CD-M as chiral selector, $d_f = 0.25$ μm. Supercritical carbon dioxide as mobile phase. t_R = Retention time for the last-eluting peak. The densities shown in Table 2 are the calculated optimal densities.

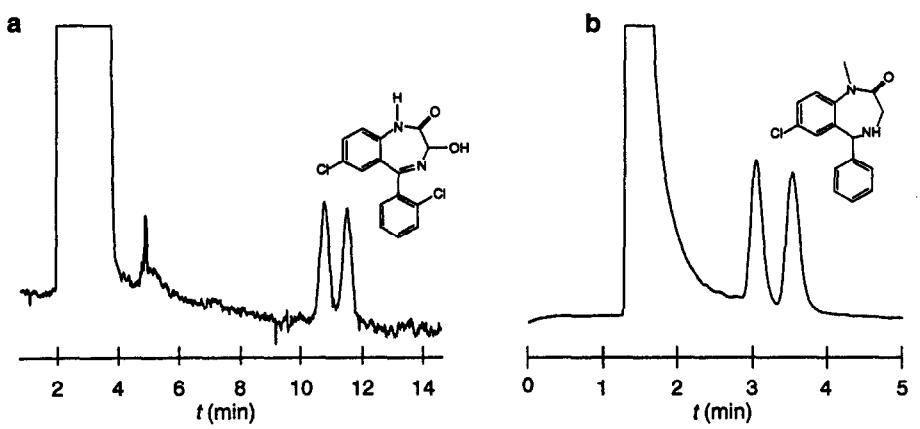


Fig. 1. Enantiomeric separation of (a) lorazepam (5 mg ml^{-1}), CO_2 at 0.79 g ml^{-1}, 60°C and (b) dihydrodiazepam (5 mg ml^{-1}), CO_2 at 0.52 g ml^{-1}, 85°C. Using an open tubular column (5 m × 50 μm), coated with side-arm substituted methyloctylsiloxane, having β-CD-M as a chiral selector, $d_f \approx 0.25$ μm.

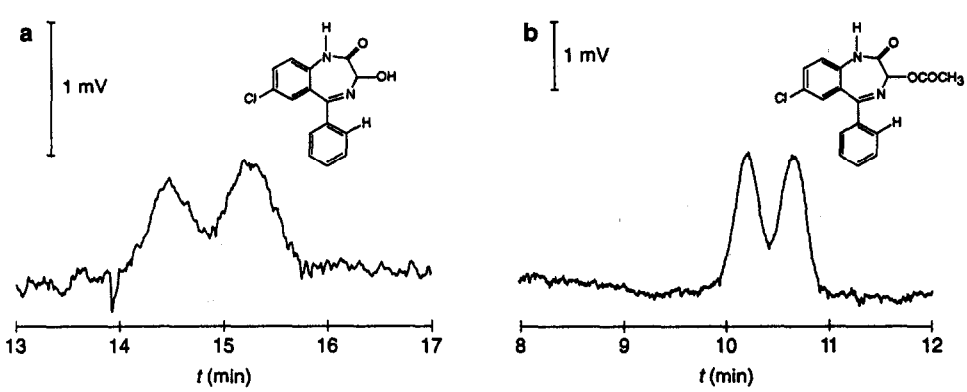


Fig. 2. Enantiomeric separation of (a) oxazepam (5 mg ml^{-1}) and (b) oxazepam derivatized with acetic anhydride (5 mg ml^{-1}). Using an open tubular column (5 m × 50 μm), coated with side-arm substituted methyloctylsiloxane having β-CD-M as a chiral selector, $d_f \approx 0.25$ μm. CO_2 at 0.79 g ml^{-1}, 60°C.

3.3. Indirect separation of benzodiazepine as diastereomers

Chiral derivatization of enantiomers into diastereomers is an alternative to the separation on chiral stationary phases. Today there are more than 20 chiral reagents commercially available and most of them have been tested for a broad range of racemic compounds. Esterification using (*S*)-trolox methyl ether (Fig. 3) as chiral reagent has proved to be a versatile method for preparing diastereomers from enantiomers [25,26]. As the indirect separation approach requires that the analyte contains a functional group, derivatization is not possible for tofisopam and camazepam.

The racemic benzodiazepine standards, with exception for dihydrodiazepam, were derivatized with (*S*)-trolox methyl ether. While the derivatization of flutazolam, oxazepam, lorazepam and ethyl loflazepate gave a quantitatively yield, ethyl loflazepate, otazolam and cloxazolam could not be quantitatively derivatized. Using an open tubular column coated with biphenyl methylpolysiloxane (7.5 m × 50 μm I.D.) and supercritical carbon dioxide as mobile phase all derivatives eluted. While the temazepam derivatives were baseline separated (Fig. 4), the separations of the derivatives of oxazepam, lorazepam, otazolam, cloxazolam and ethyl loflazepate were poor due to peak tailing. No separation was obtained for the flutazolam derivatives, presumably because the derivatization takes place too far away from the chiral centre.

When tested in GC the (*S*)-trolox methyl ether derivatives did not elute, even from a short (5 m) open tubular column coated with SE-30 (methylpolysiloxane, $d_f = 0.4$ μm) at temperatures up to 350°C.

The (*S*)-trolox methyl ether derivatives were also chromatographed using a packed fused-silica capillary column with diol functionality, and subcritical methanol-modified carbon dioxide as mobile phase. The large surface area of packed columns contains residual silanol groups that can cause adsorption of polar solutes, an effect that is not seen in deactivated open tubular columns. Addition of a polar modifier decreases adsorption in packed columns via competition for these active sites. The change of the mobile phase density (at constant temperature and pressure) and polarity when adding a modifier, will also facilitate the solubility of more polar solutes [27]. Elution of the (*S*)-trolox methyl ether derivatized analytes in approximately 1 h was observed at a methanol concentration of 8% (as defined in the Carlo Erba software) or higher. All separations were carried out in the subcritical region, at isothermal conditions (80°C), with methanol concentrations between 8.5 and 10%, and at isopycnic conditions. Fig. 5 shows the separation of racemic oxazepam derivatized with (*S*)-trolox methyl ether, using the above-mentioned conditions. The small rise in background signal before the peaks, is believed to be due to a minor change in flow-rate, leading to a change in the amount of added modifier. As shown in Table 3, the (*S*)-trolox methyl ether derivatives of temazepam, lorazepam, oxazepam and ethyl loflazepate were separated with a resolution

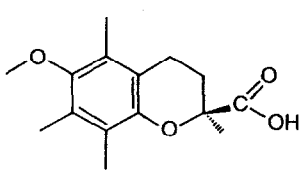


Fig. 3. Structure of (*S*)-trolox methyl ether.

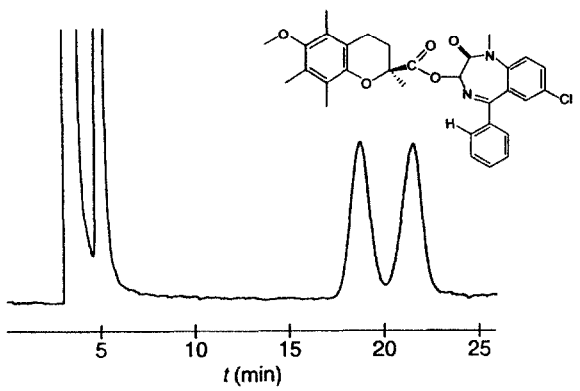


Fig. 4. Separation of (*S*)-trolox methyl ether derivatives of racemic temazepam using an open tubular column (5 m × 50 μm) coated with biphenyl methylpolysiloxane, $d_f \approx 0.25$ μm. CO_2 at 0.75 g ml^{-1}, 60°C.

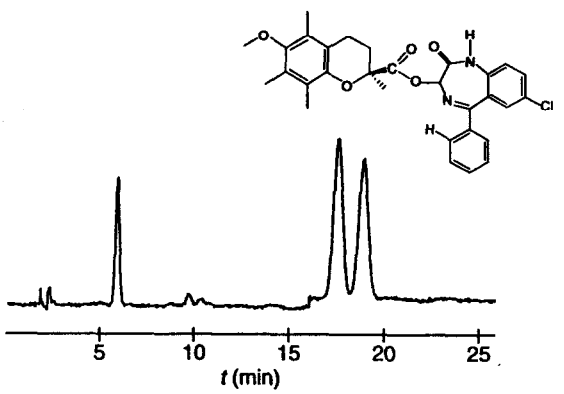


Fig. 5. Separation of (*S*)-trolox methyl ether derivatives of racemic oxazepam using a packed capillary column (30 cm × 320 μm), LiChrosorb Diol (5 μm). CO_2–MeOH (90:10) at 0.65 g ml^{-1}, 80°C.

higher than 1.0 when using the packed capillary column.

In conclusion, racemic lorazepam, oxazepam, ethyl loflazepate and dihydrodiazepam were separated using a chiral β-cyclodextrin column in open tubular SFC. The peak shapes and FID responses were enhanced by a derivatization with AA. The racemates of temazepam, oxazepam, lorazepam and ethyl loflazepate were, after a chiral derivatization with (*S*)-trolox methyl ether, separated using a packed capillary column in SubFC. The temazepam derivatives were also separated using a non-chiral open tubular column in SFC. Flutazolam that was quantitatively derivatized both using an anhydride and (*S*)-trolox methyl ether, could not be separated using either of the discussed approaches, possibly as the derivatization takes place too far away from the chiral centre. Otazolam, cloxazolam, tofisopam and camazepam, could not be separated using the direct method and the two latter could not be derivatized due to the lack of a functional group. Although derivatization was possible for cloxazolam and otazolam, no separation was obtained for the (*S*)-trolox methyl ether derivatives.

The direct separation method is recommended since additional steps in the analysis are excluded. A disadvantage with the direct approach employing SFC is, at present, the lack of commercially available chiral open tubular columns.

GC and SFC are complementary methods in this area, but when analysing thermolabile and highly polar compounds like the benzodiazepines it must be concluded that SFC or SubFC is usually preferable over GC. Compared to LC, packed-column SFC and SubFC are techniques well worth considering since the analysis times often can be shortened.

Acknowledgements

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Table 3
Resolution of (*S*)-trolox methyl ether derivatives of racemic benzodiazepines using a packed capillary column, LiChrosorb Diol, in SubFC

Compound	Density (g ml^{-1})	Mobile phase: carbon dioxide–methanol	t_R (min)	R_s
Temazepam	0.510	91.5:8.5	64.2	1.1
Oxazepam	0.650	90:10	19.1	1.4
Lorazepam	0.620	91.5:8.5	50.2	1.2
Ethyl loflazepate	0.700	90:10	11.8	1.2

Packed capillary column SubFC. Column 30 cm × 320 μm I.D. fused-silica packed capillary column, LiChrosorb Diol, 5 μm. Subcritical methanol-modified carbon dioxide as mobile phase. The compounds are derivatized with (*S*)-trolox methyl ether. All separations were carried out at 80°C. t_R = Retention time for the last-eluting peak.

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