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Supercritical fluid chromatography coupled to electrospray mass spectrometry: a powerful tool for the analysis of chiral mixtures

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Abstract

Supercritical fluid chromatography coupled to a hybrid mass spectrometer (Q-Tof2) equipped with electrospray ion source has been used to separate and characterise a wide range of pharmaceutical racemates. We have chosen diverse molecular structures to demonstrate the potential of such experimental arrangement for high throughput analyses. The use of three different chiral stationary phases and different pressure/temperature working conditions provided clear indications on how such a high throughput method can be developed. The use of mass spectrometry was found to be essential for an unambiguous assignment of the eluting components particularly in the case of complex mixtures. The direct coupling of both systems without the need for a special interface resulted in similar peak shapes and peak widths in the UV and total ion current (TIC) chromatograms.

Keywords: Chiral stationary phases, SFC; Enantiomer separation

1. Introduction

Over the past two decades, the development of analytical methods that can separate and quantify enantiomers has attracted great interest, since it became evident that enantiomers of pharmaceutical products may display different pharmacological behaviours, and that the desired biological activity of enantiomers is mostly restricted to one of the enantiomeric structures. Frequently, one of the enantiomers will be biologically active (and is called ''eutomer'') while, the other (called ''distomer'') can exhibit unexpected adverse reactions, antagonistic activities, or toxic effects [1]. This interest has been intensified since the US Food and Drug Administration (FDA) has set more stringent guidelines for marketing chiral drugs. Optical resolution of racemic compounds has advanced rapidly in the last years with the development of various chiral stationary phases (CSPs). A number of publications [2,3] reported that many chiral pharmaceutical compounds can be efficiently resolved by liquid chromatography (LC) using either Chiralpak or Chiralcel CSPs. It also has been demonstrated that these CSPs can be used under supercritical fluid chromatography (SFC) conditions, without any damage to the chromatographic support. In recent years SFC has assumed a relevant role in pharmaceutical research and the technique has shown distinct advantages over traditional liquid chromatography (LC) both on the analytical scale for purity assessment as well as on a

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larger preparative scale for the separation of racemic mixtures and isolation of large quantities of the individual enantiomers. Because of the lower viscosity and higher diffusivity in the mobile phase, packed SFC columns typically provide a 3- to 5-fold reduction in analysis time over LC [4,5]. In this paper a number of chiral pharmaceutical compounds were analysed by SFC using three different CSPs (Chiralcel OD, OJ and Chiralpak AD) under different analysis conditions (pressure and temperature). As in the case of other separation techniques such as LC and capillary electrophoresis (CE), the on-line coupling of SFC to mass spectrometry renders the technique more specific and more informative particularly when we are dealing with mixtures containing fully unknown or UV poor absorption components. Furthermore, UV assessment of enantiomeric purity of novel chiral drugs can be hindered by the presence of low level impurities, which render the identification and quantification of the minor enantiomer (lower than 5%) more challenging. Such interference can be bypassed by on-line MS detection, which allows reliable monitoring of the molecular masses of both minor and major enantiomer. Several types of interfaces have been reported in the literature, some being modified LC–MS interfaces and some being specially developed for SFC–MS. The ionisation techniques used for such interfaces have included particle beam [6], thermospray [7], FAB [8], APCI [9] and more recently ES [10]. The authors have recently reported a novel arrangement in which SFC was directly coupled to a hybrid mass spectrometer (Q-Tof 2) which provided accurate mass positive electrospray mass spectra for a number of compounds [11]. In the present work this arrangement was used to examine several commercial drugs of various pharmaceutical classes and with different molecular structures, features which were deliberately chosen to give the deductions based on the present work a wider future applicability.

2. Experimental

2.1. Instruments

SFC separations were performed on a Berger supercritical fluid chromatography system (Newark, DE, USA) equipped with an Alcott 718AL autosampler (Norcross, GA, USA) (96 samples tray) and a Hewlett-Packard UV-HP100 variable wavelength detector (Milan, Italy). Mass spectrometric measurements were performed using a Micromass Q-Tof 2 hybrid mass spectrometer (Manchester, UK), operating in positive electrospray ionisation mode. The coupling of the SFC system to the mass spectrometer is depicted in Fig. 1, which shows that the coupling between the two systems is simply achieved by the use of two T-pieces and a length of commercial silica capillary (100 μm I.D.).

2.2. Mobile phases

The materials used in the present work were: Carbon dioxide (CO_2) 99.99% grade (Rivoira, Milan, Italy) as the main mobile phase component. Methanol, Ethanol and Isopropanol HPLC grade (JT Backer, Deventer, Holland), and Isopropylamine (IPA) (Sigma–Aldrich, St. Louise, MO, USA) as modifiers.

2.3. Racemates

Commercial names, formulae, relative molecular masses (M_r) and structures of the investigated racemates are given in Table 1, all of which were purchased from Sigma–Aldrich, except Clenbuterol, Homatropine, Nicardipine and Verapamil, purchased from ICN Biomedicals Inc. (Aurora, OH, USA).

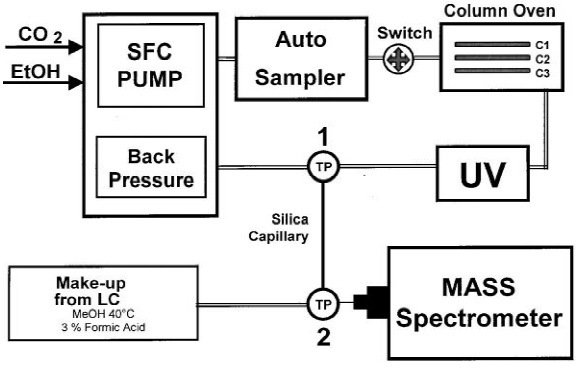


Fig. 1. Direct coupling of an SFC system to a hybrid mass spectrometer Q-Tof 2, without the need for a special interface.

Table 1
Commercial names, formulae, relative molecular masses (M_r) and structures of the investigated racemates

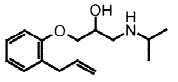
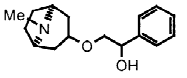
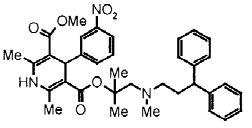
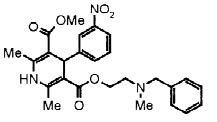
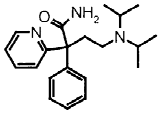
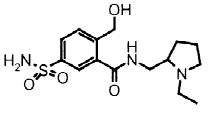
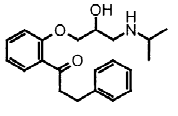
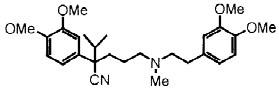
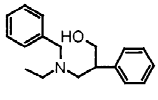
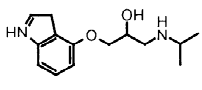
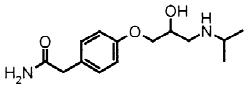
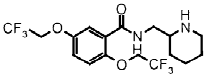
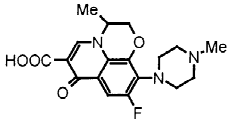
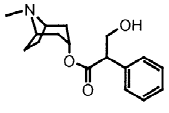
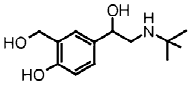
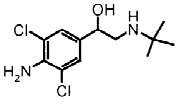
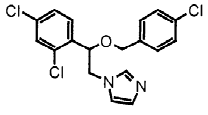
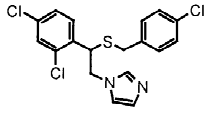
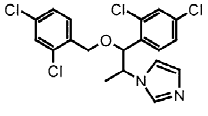
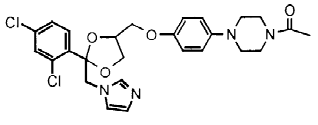
Compound	Structure	Compound	Structure
Alprenolol $C_{15}H_{23}NO_2$ Mr = 249		Homatropine $C_{16}H_{21}NO_3$ Mr = 275	
Lercanidipine $C_{36}H_{41}N_3O_6$ Mr = 611		Nicardipine $C_{26}H_{29}N_3O_6$ Mr = 479	
Disopyramide $C_{21}H_{29}N_3O$ Mr = 339		Sulpiride $C_{15}H_{23}N_3O_4S$ Mr = 341	
Propafenone $C_{21}H_{27}NO_3$ Mr = 341		Verapamil $C_{27}H_{38}N_2O_4$ Mr = 454	
Tropicamide $C_{17}H_{20}N_2O_2$ Mr = 284		Pindolol $C_{14}H_{20}N_2O_2$ Mr = 248	
Atenolol $C_{14}H_{22}N_2O_3$ Mr = 266		Flecainide $C_{28}H_{40}N_2O_5$ Mr = 484	
Ofloxacin $C_{18}H_{20}FN_3O_4$ Mr = 361		Atropine $C_{17}H_{23}NO_3$ Mr = 289	
Salbutamol $C_{13}H_{21}NO_3$ Mr = 239		Clenbuterol $C_{12}H_{18}N_2OCl_2$ Mr = 276	
Econazole $C_{18}H_{15}Cl_3N_2O$ Mr = 380		Sulconazole $C_{18}H_{15}Cl_3N_2S$ Mr = 396	
Miconazole $C_{18}H_{14}Cl_4N_2O$ Mr = 414		Ketoconazole $C_{26}H_{28}Cl_2N_4O_4$ Mr = 530	

Table 2
Analysis conditions of: (a) SFC and (b) MS

(a) SFC	
Column	Chiralpak AD, Chiralcel OD and OJ 25×0.46 cm, 5 μ
Column oven temperature	35 °C
Mobile phase	Carbon dioxide (CO_2) (99.99% grade)
Modifier	EtOH with 0.1% v/v Isopropylamine (IPA)
	t_0 = 20%, t_1(5 min) = 20%, t_2(10 min) = 35%, t_3(15 min) = 35%
Dissolving medium	EtOH
Sample concentration	150 μg/ml
Injection volume	10 μl
Flow	2.5 ml/min
Column pressure	18 MPa
UV detection	220 nm
(b) Mass spectrometer	
Make-up	MeOH–H_2O (80:20) with 3% formic acid (55 °C)
Make-up flow	150 μl/min
Source block temperature	150 °C
Desolvation temperature	175 °C
Capillary voltage	3750 V
Cone voltage	55 V
Collision energy	10 eV
Collision gas	Research Grade Argon
Collision cell pressure	4×10^{-3} Pa

2.4. SFC/MS

All measurements were performed using the analysis conditions listed in Table 2(a,b). The elution times reported in this study refer to the total ion current chromatograms (TIC) acquired by the mass spectrometer; the measured delay time between the UV and MS detection was about 10 s; the t_0 value (dead volume or hold-up time) (1.36 min) was measured from the initial disturbance of the baseline following injection and in accordance with the calculations of the column volume, while the retention times, t_r of the enantiomeric solutes were measured from the time of injection. The retention factors ($k = [t_r - t_0]/t_0$), the selectivity factors ($\alpha = k_2/k_1$, where 1 and 2 refer to the first and the second-eluting enantiomers, respectively) and resolution ($R = 2[t_2 - t_1]/[\omega_2 + \omega_1]$) were mean value from duplicate injections.

2.5. Chiral stationary phases

Three polysaccaride-based chiral stationary phases were tested: Chiralcel OD (cellulose tris[3,5-dimethylphenylcarbamate]), Chiralcel OJ (cellulose tris[4-methylbenzoate]) and Chiralpak AD (amylose tris[3,5-dimethylphenylcarbamate]), the structures of wich are shown in Fig. 2. All these CSPs phases (25 cm×4.6 mm I.D., particle size 5 μm) were purchased from Diacel (France).

3. Results

To optimise the separation conditions and to identify a general method which can be used for the initial screening of diverse structures, a number of racemates in Table 1 were examined under different column pressure and temperature. These initial measurements were conducted on two chiral stationary phases, Chiralpak AD and Chiralcel OD. Relevant information regarding the chromatographic behaviour of the investigated racemates can be drawn from two sets of graphs in Figs. 3 and 4. In any chiral separation the stereoselectivity is always considered a central parameter in the overall assessment of such separation. Having this in mind we have used the initial measurements to assess the influence of both

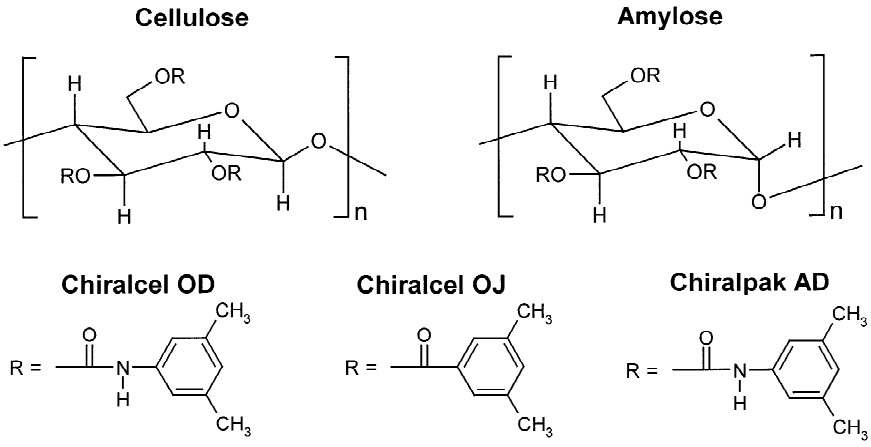


Fig. 2. Structure of polysaccaride-based chiral stationary phases used: Chiralcel OD, Chiralcel OJ and Chiralpak AD.

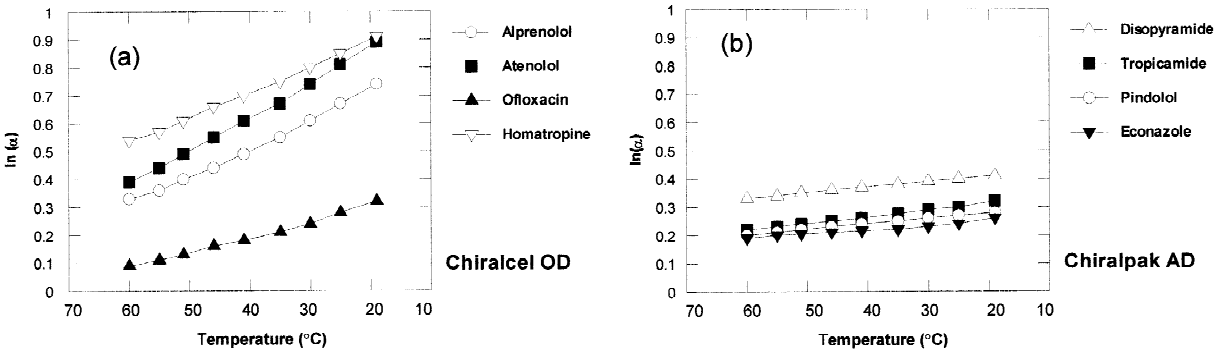


Fig. 3. Plot of ln(α) vs. temperature in the enantioseparations of various standard racemates using Chiralcel OD (a) and Chiralcel AD (b) CSPs. Mobile phase: CO_2–EtOH (73/27). Flow: 2.5 ml/min. Pressure: 18 MPa.

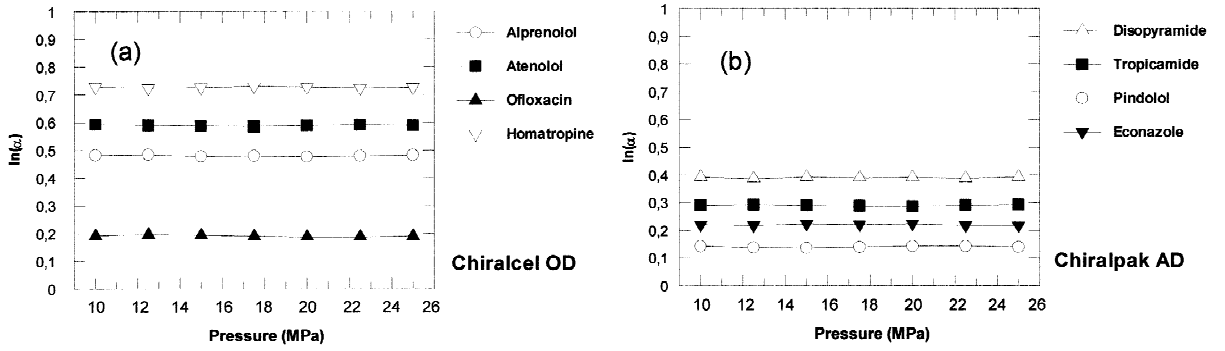


Fig. 4. Plot of ln(α) vs. pressure in the enantioseparations of various standard racemates using Chiralcel OD (a) and Chiralcel AD (b) CSPs. Mobile phase: CO_2–EtOH (73/27). Flow: 2.5 ml/min. Temperature: 35 °C.

temperature and pressure on such parameter. The two sets of measurements performed at different column temperature in Chiralcel OD and Chiralpak AD yielded the graphs in Fig. 3(a and b), respectively. The two graphs show the variation of the natural log of the stereoselectivity factor ($\ln(\alpha)$) as a function of temperature, this approach of data presentation has been adapted from the work by Svensson et al. [12]. The racemates in Fig. 3(a) exhibit unmistakable increase in $\ln(\alpha)$ over the investigated temperature range; the same effect albeit to a lesser extent is also observed in Fig. 3(b). The influence of column pressure on $\ln(\alpha)$ for the two CSPs is shown in Fig. 4(a,b). These data show insignificant influence of the pressure on $\ln(\alpha)$ of each individual racemate, however the data in (a) indicate higher values than those in (b). The authors are aware that the two sets of graphs in (a) and (b) refer to two different sets of racemates and therefore the observed qualitative differences can not be fully attributed to the identity of the chiral phase. However, some of these observations can be compared with the deductions reported in existing literature. Bargmann-Leyder et al. [4] investigated the influence of pressure on SFC stereoselectivity and reported that, when Chiralpak AD was used, column pressure had significant effect on the stereoselectivity factor while only slight variations were observed for Chiralcel OD. The authors tentatively suggested that such difference might be related to the helical chain conformation of the cellulose-derived phase, which are more rigid than their amylose-derived counterpart. The present data do not support such deduction, which implies that the measured selectivity factor is also analyte dependent. We have pointed out that, the main objective of the present work is to develop an SFC–MS method which can be used to screen the structures listed in Table 1. The results of our various attempts to develop such method are summarised in Table 3.

These data refer to three different polysaccharide based CSPs, which were used under the analysis conditions in Table 2. Based on the molecular structures in Table 1 and the data in Table 3 the following observations can be made. (i) In terms of stereoselectivity and resolution, Chiralpak AD was

Table 3
Capacity factor (k_2), selectivity (α) and resolution (R) obtained on polysaccaride-based CSPs, injecting in the SFC–MS system 10 μl of each standard racemate in Table 1 using the analysis conditions in Table 2

Compound name	Chiracepak AD			Chiralcel OD			Chiralcel OJ		
	k_2	α	R	k_2	α	R	k_2	α	R
Alprenolol	0.82	1.43	1.19	3.07	1.45	2.45	0.58	1.00	
Lercanidipine	7.19	1.03	0.83	9.19	1.00		8.17	1.00	
Disopyramide	4.11	1.48	2.59	1.99	1.00		2.50	1.00	
Propafenone	5.89	1.04	1.24	6.15	1.03	0.93	3.11	1.00	
Tropicamide	3.23	1.30	2.38	3.17	1.07	0.86	4.14	2.31	4.27
Atenolol	6.35	1.00		9.97	1.22	3.97	5.48	1.00	
Flecainide	0.79	1.00		1.34	1.00		1.82	1.18	1.01
Ofloxacin	5.46	1.00		9.33	1.07	1.66	6.41	1.04	0.87
Pindolol	5.86	1.10	1.54	8.96	1.00		6.70	1.03	0.72
Salbutamol	3.78	1.15	0.58	8.33	1.00		5.05	1.00	
Sulpiride	8.30	1.04	0.97	8.60	1.03	0.80	6.73	1.03	0.86
Atropine	3.44	1.21	1.19	5.70	1.04	0.94	3.22	1.00	
Homatropine	3.42	1.14	1.01	6.96	1.34	4.84	3.15	1.16	0.91
Nicardipine	1.98	1.00		6.15	1.00		6.77	1.04	1.24
Clenbuterol	2.23	1.29	1.53	4.63	1.06	0.57	2.09	1.14	1.00
Verapamil	1.09	1.30	0.85	4.00	1.00		2.84	1.00	
Miconazole	4.93	1.22	4.82	6.85	1.00		6.82	1.13	3.63
Ketoconazole	15.71	1.18	4.27	72.26	1.00		13.99	1.00	
Econazole	6.83	1.11	2.43	6.92	1.02	0.60	8.41	1.22	3.18
Sulconazole	8.72	1.05	1.81	9.40	1.00		8.58	1.02	0.93

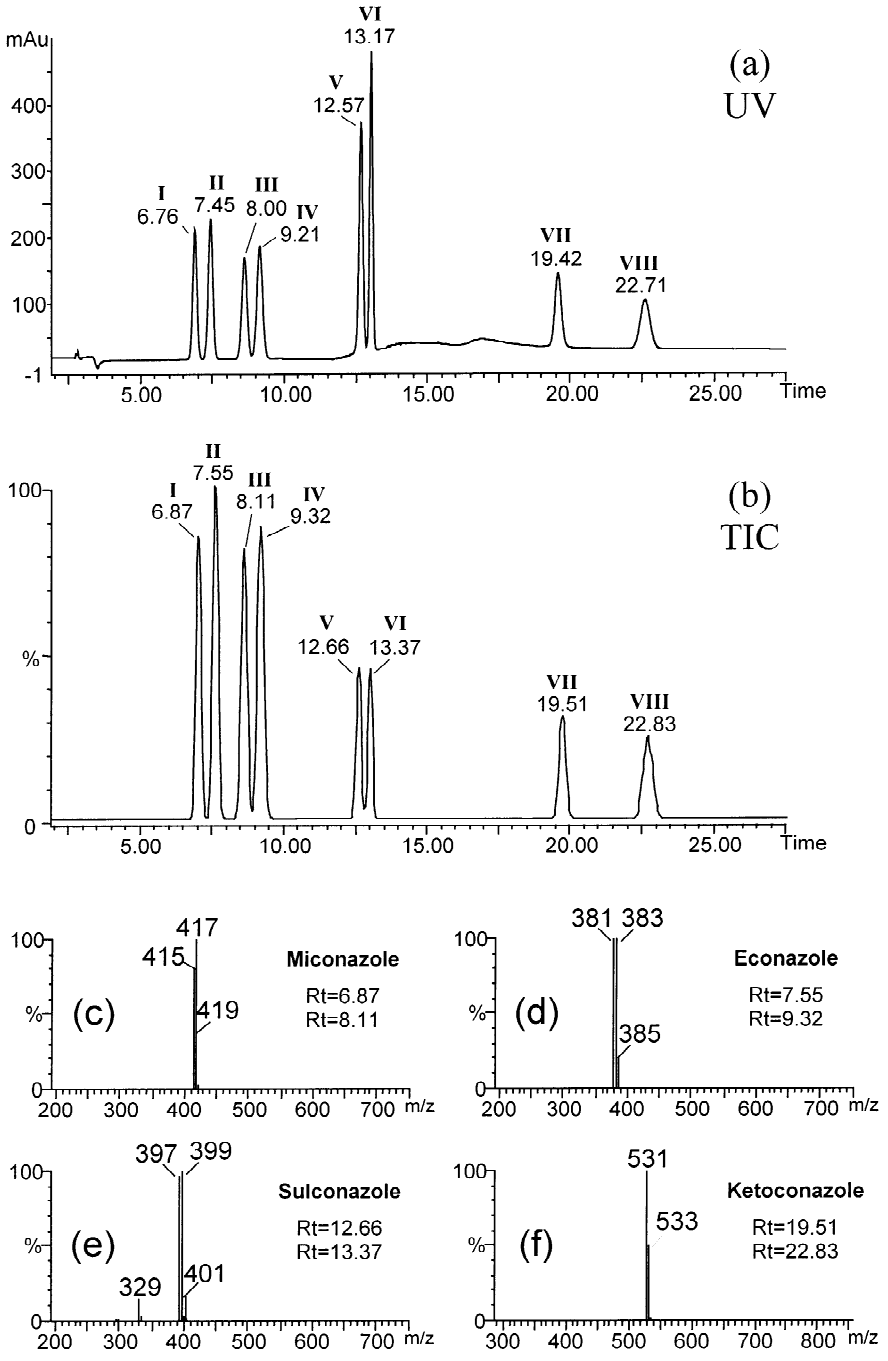


Fig. 5. UV (a) and TIC (b) chromatograms of a solution containing four antimycotic drugs. (d–f) Mass spectra associated with the peaks present in the TIC chromatogram.

found to be suitable for about 80% of the investigated structures, compared to 45% in the case of Chiralcel OD and 50% in the case of Chiralcel OJ. (ii) The influence of the analyte on the outcome of the separation is clearly evident in Table 3. Such influence on the selectivity factor can be derived by considering the data associated with sulconazole (1H-Imidazole, 1-[2-[[(4-chlorophenyl)methyl]thio]-2-(2,4-dichlorophenyl)ethyl]) and econazole (1H-Imidazole, 1-[2-[(4-chlorophenyl) methoxy]-2-(2,4-dichlorophenyl)ethyl]), which have very similar structures except the latter has an ''O'' atom instead of ''S'' at the benzilic position. The first structure was resolved using both Chiralpak AD and Chiralcel OJ with similar retention (k_2~8.6) and selectivity (α~1.02) factors; the second structure on the other hand, was resolved by all three CSPs, where the measured values of the stereoselectivity factor were found higher than the values measured for sulconazole. Furthermore, the retention factor obtained on Chiralpak AD and Chiralcel OJ are respectively 5.83 and 8.41, which indicates a considerable difference in the elution time, and also in the selectivity factor, which is 1.31 for Chiralpak AD and 1.22 for Chiralcel OJ. In the separation of sulconazole the values of k_2 and α on the investigated CSPs were quite constant. These observations clearly indicate that the simple substitution of a sulphur atom with an oxygen has substantially influenced the interaction of the analyte with the chiral stationary phases. Such difference in terms of interaction with the CSP is tentatively attributed to a higher steric hindrance of the sulphur atom compared to the oxygen, which can substantially modify the 3D aspect of the molecule. (iii) The data in Table 3 indicate that despite what can be considered a limited degree of variation in the investigated structures, an SFC method which can separate each of the listed racemates has to take into account the combined stereospecificity of more than one chiral stationary phase. In other word, the real challenge in setting up a high throughput SFC–MS method is how to exploit such combined stereospecificity without creating a bottleneck in the course of testing one compound by different chiral phases.

To test the capability of our SFC–MS arrangement for the investigation of chiral mixtures, 10 μl of a solution containing four antimycotic drugs (miconazole, econazole, sulconazole and ketoconazole) was injected into the system under the experimental conditions in Table 2, which resulted in the UV and TIC chromatograms in Fig. 5(a,b); Fig. 5(d–e) report the mass spectra associated with the eight peaks present in the TIC chromatogram.

The chromatograms in Fig. 5 show eight well separated peaks, due to the various enaniomers of the mixture, the mass spectra associated with each single TIC peak give a fast correlation between the chromatographic peaks with related retention times and the investigated structures. Considering the chromatograms in Fig. 5(a,b) some observations can be made. Comparison of the UV and the TIC chromatograms reveals no significant differences in peak shape and peak width. Although the mass spectrometer was connected to the SFC system by 10 m of capillary silica, the low viscosity of the supercritical fluid and the high flow-rate resulted in the same quality of signal in both UV and MS chromatograms, with a very short delay time (about 10 s). The eight enantiomers of the mixture elute in three groups. The first four elute within 9 min, while the remaining four elute in two groups within 13 and 23 min, respectively. These latter two pairs of peaks are not difficult to assign to two different racemates, sulconazole and ketoconazole. The question of peak assignments becomes more complicated when the group of peaks below 9 min is considered. Based on the UV trace it would appear that each pair of adjacent peaks is due to a specific racemate. This deduction is fully dismissed by the MS data in Fig. 5(c–f) which shows that the alternate and not adjacent peaks belong to the same racemate. In other words, the two peaks at Rt = 6.87 and 8.11 min are due to miconazole enantiomers, while the peaks at Rt = 7.55 and 9.32 min are due to econazole enantiomers. Such assignments would not have been possible without the use of mass spectrometry. Clearly in a more complex mixture the role of MS for reliable detection becomes more decisive.

4. Conclusions

A newly commissioned SFC/Q-Tof2 experimental arrangement has been used to examine a substantial number of pharmaceutical chiral compounds. The present study had two main objectives: to demon-

strate the potential of such an experimental arrangement in high throughput chiral analysis and to underline the role of mass spectrometry for more reliable identification of the eluting components. Considering the experimental approach used inhere and the SFC–MS data it can be argued that both objectives have been demonstrated.

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