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Extending the use of "Inverted Chirality Columns Approach" for enantiomeric excess determination in absence of reference samples: Application to a water-soluble camptothecin derivative

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ABSTRACT

The aim of the present study was to extend the use of the "Inverted Chirality Columns Approach (ICCA)" previously developed for the identification and quantitation of the trace enantiomer in highly enriched samples of the camptothecin (CPT) family of drugs to a novel water-soluble CPT derivative, namely namitecan (ST1968), currently undergoing phase I clinical trials as anticancer agent. Namitecan, identified from a series of hydrophilic 7-oxyiminomethyl derivatives, contains a free terminal amino group, which traditionally hampers the analysis under normal-phase HPLC conditions. Nevertheless, commercially available Pirkle-type chiral stationary phases (CSPs) available in both the enantiomeric forms (i.e., having the same bound selector with opposite configuration) mainly operate under normal-phase HPLC conditions. For this reason, namitecan was pre-column *N*-protected with isocyanates A–D and their sulfur analogues E–H to reduce its polarity by converting the amino group into a fragment compatible with the chiral recognition mechanism (i.e., ureido and thioureido groups). Once the optimal columns system and derivatizing agents were selected, an original enantioselective HPLC–MS/MS technique was developed on the Whelk-O1 CSPs.

1. Introduction

Two years ago we described an original enantioselective HPLC–MS/MS technique for the identification and quantitation of the trace enantiomer in highly enriched samples of the camptothecin (CPT) family of drugs, even in absence of reference samples [1]. The approach we developed, designated as "Inverted Chirality Columns Approach (ICCA)", is based on the reversal of the elution order of a given enantiomeric pair [2] as a result of the columns switching, under identical chemical conditions. This technique, which is not available when naturally occurring selectors such as polysaccharides or proteins are employed, is very useful for enantiomeric trace analysis when the minor enantiomer follows the major one and is partially hidden by the tailing of the leading enantiomer: on the chiral stationary phase (CSP) with opposite configuration the trace enantiomer is eluted first, thus enabling a more precise and accurate quantitation by peak area integration [3]. ICCA was developed to respond to important incoming needs in the field of pharmaceutical analysis: (i) the complete assignment of the stereoisomeric composition of chiral drugs [4,5], including natural products and synthetic intermediates, as established by the International Conference on Harmonization (ICH) guidelines [6]; in such cases, however, only one enantiomer is available as reference, and the racemate could be prepared by means of expensive and multistep, time-consuming total syntheses; (ii) extreme enantiomeric excesses (ee > 99%) must be estimated with large accuracy, which is a critical point in any of the available techniques [4]; (iii) low limits of detection (LODs) and quantitation (LOQs) in highly enriched samples contained in complex mixtures are strongly required to yield an unequivocal peak identification. Application of the ICCA to semi-synthetic derivatives of CPT endowed with anticancer activity [1] clearly showed its selectivity and specificity in the accurate determination of extreme ee, even when only one enantiomer was available. The success of the approach was assured by the combination of Pirkle-type CSPs with multistage mass spectrometry (APCI-MS/MS) detection.

The accomplished familiarity in the ICCA technique prompted us to approach a novel water-soluble CPT derivative, namely namitecan (ST1968) [7–10], currently undergoing phase I clinical trials as anticancer agent [11]. The CPT family of drugs appear to have a unique mechanism of action: they inhibit the nuclear enzyme topoisomerase I (topo I), by forming a ternary complex with topo

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I and DNA [12]. As topo I was found to be over expressed in colorectal, prostate and other tumours compared to non-malignant tissues [13], this has led to a resurgence of studies on this drug family. Both semi- and totally synthetic CPT derivatives have been prepared in recent years [14], and a number of CPT analogues are currently undergoing clinical evaluation. From structure-activity (SAR) studies [15], it appears that the lactone ring and the natural 20*S*-configuration of CPTs are essential for anticancer activity. In particular, researchers have focused on modifying CPT at the 7-position to synthesize agents with improved pharmacological and toxicological profiles. Namitecan, identified from a series of hydrophilic semi-synthetic 7-oxyiminomethyl derivatives, exhibited a remarkable ability to stabilize the cleavable topo I–DNA complex and its *in vivo* activity was found promising [8].

2. Experimental

2.1. Instrumentation

Liquid chromatography was performed using a Thermo HPLC separation module (San José, CA, USA) consisting of a Surveyor MS micro pump, a Surveyor AS autosampler equipped with a Rheodyne Model 7725i-20 μl injector, and a Surveyor PDA Photodiode Array Detector, coupled to a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer, equipped with an orthogonal APCI ion source. Chromatographic data were collected and processed using the Thermo Xcalibur Chromatography Manager software, version 1.2. A Jasco PU-980 Intelligent HPLC pump coupled with a Jasco 995 UV/CD detector was also used, and data were collected using the Borwin software (Jasco, Europe). The (*S*,*S*)- and (*R*,*R*)-DACH-DNB (250 mm × 4.6 mm I.D.), 5 μm particle size chromatographic columns used were purchased by Regis Technologies (Morton Grove, IL, USA). (*S*,*S*)- and (*R*,*R*)-Whelk-O1 (5 μm particle size) CSPs, obtained from Regis Technologies, were packed into 250 mm × 4.6 mm I.D. stainless steel columns using a slurry packing procedure already described [16], with *n*-hexane as pressurizing agent (700 bar for 20 min). Notably, although sold as the (*S*,*S*)-Whelk-O1, the true configuration is (3*R*,4*S*). The incorrect assignment is a remnant of the original Whelk-O CSP wherein the selector was bound to silica via an undecyl linkage and thus correctly assigned the (*S*,*S*)-configuration [17].

2.2. Chemicals and reagents

Purified samples of (*S*)-CPT and (*R*,*S*)-CPT were purchased from Boehringer Ingelheim Pharma KG, Germany. 7-(*E*)-(2-Aminoethyloxyimino)methyl camptothecin hydrochloride or namitecan (ST1968) was supplied by sigma-tau S.p.A. (Pomezia, Italy) [7–10].

HPLC-grade *n*-hexane, methanol, dichloromethane, triethylamine (Et_3N), dry tetrahydrofuran (THF), dry toluene, isocyanates A–D, as well as phenyl isothiocyanate (F) and pentafluorophenyl isothiocyanate (H) were purchased from Sigma–Aldrich (St. Louis, MO, USA), whereas *tert*-butyl isothiocyanate (E) and 3,5-dimethylphenyl isothiocyanate (G) were from Fluorochem (Derbyshire, UK).

2.3. Derivatization procedures

All the solutions of ST1968 were prepared in amber glass volumetric flasks, since the *E* and *Z* diastereoisomers of the oxime group in position 7 undergo light-induced interconversion at room temperature. Special care was taken in the handling of CPT and derivatives because of the carcinogenic nature of the compounds. All samples were sequestered and disposed of according to the material safety data sheet (MSDS) and sigma-tau S.p.A. hazardous handling and waste policy.

The various pre-column *N*-terminal derivatizations of ST1968 were carried out as follows: approximately 100 μl aliquots of the proper isocyanate (A, B, and D) or isothiocyanate (E–H) in dry toluene (19.7–27.6 mmol in 10 ml) were added to a solution (500 μl) of ST1968 in dry and degassed THF (2.1×10^{-3} mmol in 5 ml) containing Et_3N (2.4×10^{-2} mmol) in an amber screw-cap vial, resulting in analyte concentration of approximately 0.42 mmol/l. For isocyanate C, a more diluted dry toluene solution was prepared (7.1 mmol in 10 ml), due to lower solubility. The mixture was vortexed for 5 min at room temperature, and directly injected into the HPLC system (10–20 μl). ST1968 derivatives from isocyanates A–C were stable for at least 3 h, whereas the reaction mixture with D proved instable within a few minutes. Isothiocyanates F–H gave also stable derivatives, while derivatization reaction with E yielded a mixture of side-products, and was discarded.

2.4. Chromatographic conditions

HPLC separations were carried out under normal-phase conditions on both DACH-DNB and Whelk-O1 columns family. In the first case, the mobile phase was made up of 40% *n*-hexane and 60% dichloromethane (stabilized with ethanol ~0.25%), plus 3% methanol added (v/v). For Whelk-O1 columns family, the polar modifier was increased to 5%, since amylene stabilized dichloromethane was employed. The flow-rate was set to 1.00 ml/min and the columns were thermostated at 25 °C. All derivatization reaction mixtures were directly injected without further dilution. Aliquots of 10–20 μl were injected. The chromatograms were recorded by monitoring the UV trace at 370 nm (200–400 nm PDA range). Columns hold-up time (t_0) was determined from the elution time of an unretained marker (1,3,5-tri-*t*-butylbenzene) using as eluent a mixture made up of 95% dichloromethane (amylene stabilized) and 5% methanol, at $T = 25$ °C, flow-rate 1.00 ml/min and UV detection at 254 nm. Hold-up times, obtained as mean value of three injections, were 2.75 and 2.55 min on the DACH-DNB and Whelk-O1 columns family, respectively.

2.5. APCI-MS/MS detection

MS detection was performed by an Atmospheric Pressure Chemical Ionization (APCI) interface, the sheath and auxiliary gases (high purity nitrogen) being 80 and 10 (arbitrary units), respectively. MS parameters were optimized as follows: APCI vaporizer $T = 450$ °C, corona discharge current 5.00 mA, tube lens offset 30.0 V, source voltage 4.5 kV and current 80 μA, capillary voltage 15 V at $T = 250$ °C.

The acquisition was operated in positive ion mode and identification and quantitation were based on selected reaction monitoring (SRM) detection; in particular the scan range for the parent scan was 250–650 atomic mass units (amu), each scan consisting of three microscans with a maximum ion injection time of 50 ms while the SRM scan consisted of one microscan and a 50 ms injection time; the precursor isolation window was set at 2 amu and collision energy at 35% (arbitrary units).

2.6. Method validation for the DMP-thioureido derivative of ST1968

Method validation based on sensitivity, linearity, accuracy, and precision was performed only for the DMP-thioureido derivative of ST1968. Sensitivity was evaluated by determining the limit of detection (LOD) and quantitation (LOQ) according to the signal-to-noise ratio approach. Signal-to-noise ratios of at least 3:1 for LOD

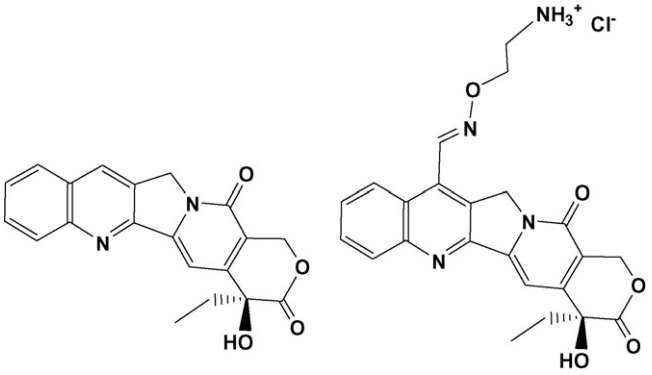

Fig. 1. Chemical structures of camptothecin (left) and namitecan (ST1968) (right).

and 9:1 for LOQ were considered acceptable. Linearity was investigated by calculation of the regression line by the least squares method and expressed by the correlation coefficient (R^2). In particular, it was checked on different (*S*)-enantiomer solutions at nine concentration levels in a range from 0.0075 to 250 μg/ml (five orders of magnitude). Accuracy and precision were assessed at the LOQ value on six replicated injections ($n = 6$). Accuracy was expressed as the percent recovery of the analyte with respect to the nominal concentration of the (*S*)-enantiomer solutions which were considered as the theoretical value. Precision was expressed as RSD%.

3. Results and discussion

3.1. Extending the use of the "Inverted Chirality Columns Approach (ICCA)"

The main limitation of the ICCA method previously developed [1] is that it can be exploited only with stationary phases based on totally synthetic selectors, since complex natural selectors such as polysaccharides, proteins or glycopeptides are not suitable to yield CSPs in both the enantiomeric versions. On the contrary, Pirkle-concept CSPs are ideally suited to these purposes, since some of them have been developed and commercialized in both the enantiomeric forms. This is the case of the DACH-DNB columns family [18], that we indeed applied in the analysis of CPT and some lipophylic derivatives endowed with anticancer activity [1], and of the Whelk-O1 columns, introduced by the Pirkle group a few years after the DACH-DNB [19]. A distinctive feature of the above columns is that they have been designed to undergo simultaneous

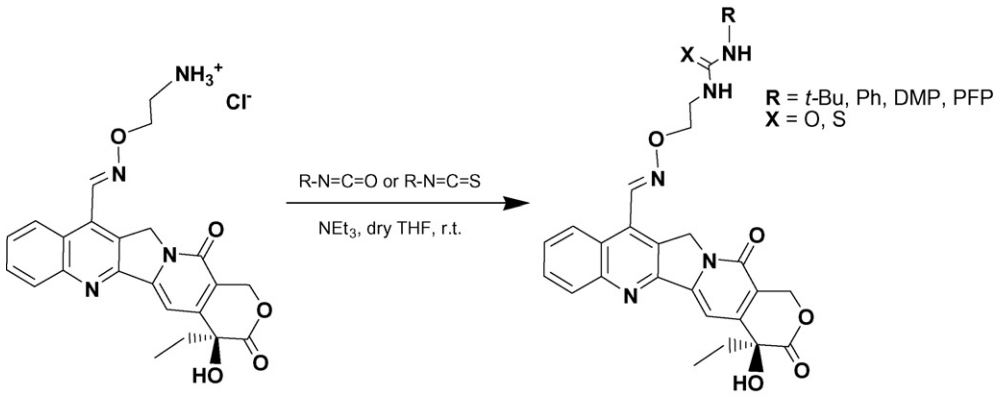

Fig. 2. Synthetic scheme for the *N*-terminal derivatization of ST1968.

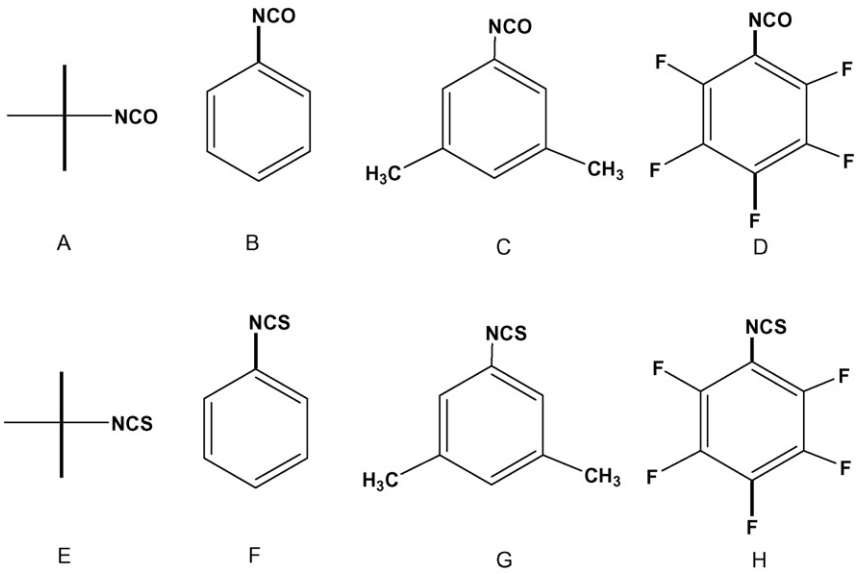

Fig. 3. Chemical structures of isocyanates and their sulfur analogues used as *N*-protecting reagents. (A) *tert*-Butyl isocyanate (*t*-BuI); (B) phenyl isocyanate (PhI); (C) 3,5-dimethylphenyl isocyanate (DMPI); (D) pentafluorophenyl isocyanate (PFPI); (E) *tert*-butyl isothiocyanate (*t*-BuIS); (F) phenyl isothiocyanate (PhIS); (G) 3,5-dimethylphenyl isothiocyanate (DMPIS); (H) pentafluorophenyl isothiocyanate (PFPIS).

H-bonding, face-to-face, and face-to-edge aromatic interactions with the analytes, and mainly operate under normal-phase HPLC conditions. With the aim of extending the use of the ICCA method to the novel water-soluble CPT derivative namitecan (ST1968; Fig. 1), we faced the problem of the presence of a free terminal amino group, which traditionally hampers the analysis under normal-phase conditions and in our case would certainly decrease the enantiorecognition ability of the CSPs. It is known, in fact, that polar sites where non specific (i.e., non enantioselective) interactions may occur are detrimental to chiral recognition, and the amino group in our CPT derivatives, remote from the stereogenic centre at C20 (see Figs. 1 and 2), is such a site. For this reason, namitecan was pre-column *N*-protected with a set of isocyanates (A–D in Fig. 3) in alkaline medium, to reduce its polarity by converting the amino group into a fragment compatible with the chiral recognition mechanism (i.e., ureido groups). We also decided to study isothiocyanates E–H as derivatizing agents (Fig. 3) because we expected to improve the overall performances of the HPLC separation in terms of enantioselectivity and peak shapes. Compared to the urea derivatives, the thiourea analogues have reduced superfluous interactions with the chiral selector of the stationary phase and diminished interactions with the underlying silica support: indeed, the sulfur atom is less electronegative and larger than oxygen, and the thiourea function has decreased H-bonding ability compared to the corresponding urea, leading to an overall improvement of the solute-stationary phase adsorption equilibria.

3.2. Choice of the proper chiral columns system

Initially, we decided to begin our investigation by referring to the previous work [1], where the DACH-DNB columns family was employed. Thus, we pre-column derivatized ST1968 with isocyanate A (Fig. 3) by a standard procedure (see Fig. 2 and Section 2.3 for details) [20]. For ST1968, neither the racemate nor the (*R*)-enantiomer were easily available as reference, since total synthesis is a complex and expensive task. Thus, the position of the minor enantiomer peak was assessed by computer reprocessing of chromatograms obtained by running the single (*S*)-enantiomer on (*R*,*R*)- and (*S*,*S*)-DACH-DNB CSPs alternatively (Fig. 4A and B, respectively), to give the "virtual racemate" shown in Fig. 4C (obtained by ElabChrom, a lab-made software that merges two independent chromatograms) [1]. As expected, elution order was the same as already found for the whole CPT series and confirmed by CD spectra [1], i.e., (*S*)-enantiomers (leading peaks) were better retained by the stationary phase with (*S*,*S*)-configuration. For this reason, detection of the trace (*R*)-enantiomer was achievable only on such CSP, where the trace was eluted first. The results of the HPLC separations of the *N*-protected-ST1968 derivatives on the (*R*,*R*)- and (*S*,*S*)-DACH-DNB CSPs are presented in Table 1. Unfortunately, enantioselectivity (α) values between the two ST1968 enantiomers as *t*-butyl-ureido derivatives were quite low ($\alpha = 1.10$), when compared with the previous results obtained for lipophylic CPT derivatives ($\alpha = 1.22$–1.50) [1]; no significant increase of such value was obtained by introducing in the analyte an aromatic moiety (DMP) close to the ureido functionality (i.e., by pre-derivatizing ST1968 with isocyanate C), whereas a sizeable increase in retention was observed, with capacity factors (k') growing up from 1.83 to 3.20 for the trace (*R*)-enantiomer (see Table 1). These unhelpful preliminary results prompted us to explore the second typology of Pirkle-type columns system, i.e., the Whelk-O1 family, containing the 4-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydro-phenanthrene as selector [17,19]. First of all, we analyzed CPT samples for which the racemate was available as reference. The CSP gave easy access to the two enantiomers, with attractive enantioselectivity ($\alpha = 1.50$) and time of analysis (10–15 min), under the same chromatographic conditions (see

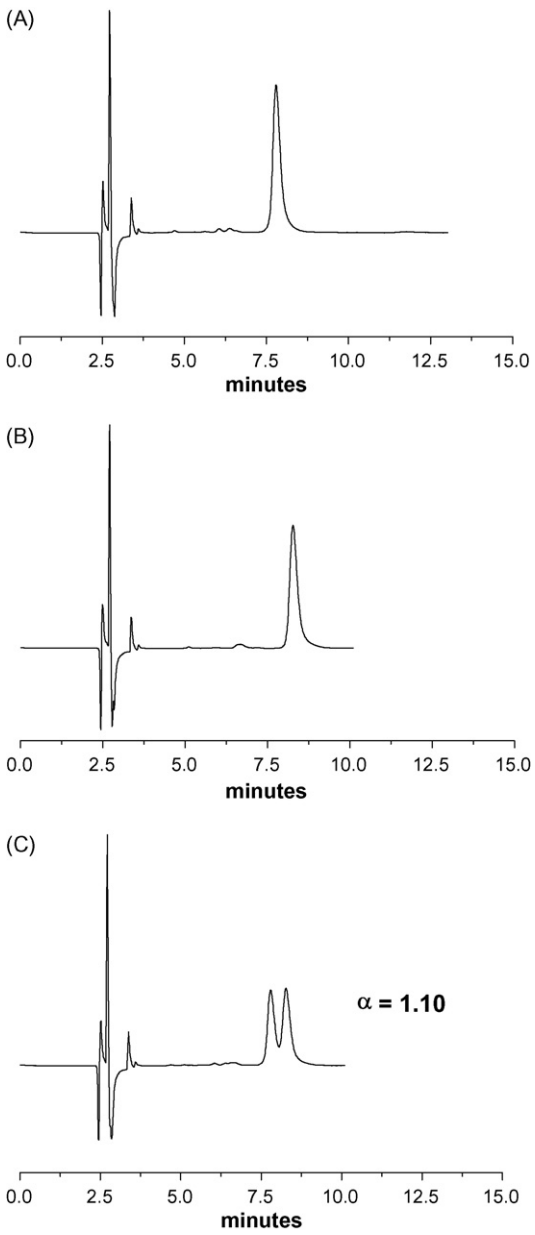

Fig. 4. (A) Chromatogram of (*S*)-ST1968 as NH–CO–NH–*t*-Bu derivative on (*R*,*R*)-DACH-DNB. (B) Chromatogram of (*S*)-ST1968 as NH–CO–NH–*t*-Bu on (*S*,*S*)-DACH-DNB. (C) Computer-generated chromatogram of the "virtual racemate". Eluent: *n*-hexane-dichloromethane (EtOH stabilized) (40:60, v/v) + 3% methanol; flow-rate: 1.00 ml/min; $T = 25\,^{\circ}C$; UV detection at 370 nm.

Fig. 5). Notably, we found the opposite elution order with respect to the DACH-DNB columns family, i.e., (*S*)-CPT was better retained by the stationary phase with (*R*,*R*)-configuration. For this reason, detection of the trace (*R*)-enantiomer was achievable only on such CSP, where the trace was eluted first. Since preliminary experiments with ST1968 as *t*-butyl- and DMP-ureido derivatives showed larger retention and enantioselectivity values (see Table 2 and Fig. 5), the Whelk-O1 columns family was selected to perform a more in-depth investigation.

3.3. Check of the chromatographic equivalence of columns with opposite configuration

As already stated in our previous work [1], the two columns with opposite selector configuration used in the ICCA technique

Table 1
Chromatographic data for the analysis of ST1968 as ureido derivatives on (*R*,*R*)- and (*S*,*S*)-DACH-DNB columns.

CSP configuration	Sample	Chromatographic peak	k'^{a}	α^{b}	R_s^{c}
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–*t*-Bu	1.83	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–*t*-Bu	2.01	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CO–NH–*t*-Bu (*S*)-ST1968 as NH–CO–NH–*t*-Bu	1.83 2.01	1.10	1.08
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–DMP	3.20	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–DMP	3.49	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CO–NH–DMP (*S*)-ST1968 as NH–CO–NH–DMP	3.20 3.49	1.09	1.15

[a] Retention factor.
[b] Enantioselectivity factor.
[c] Resolution factor.

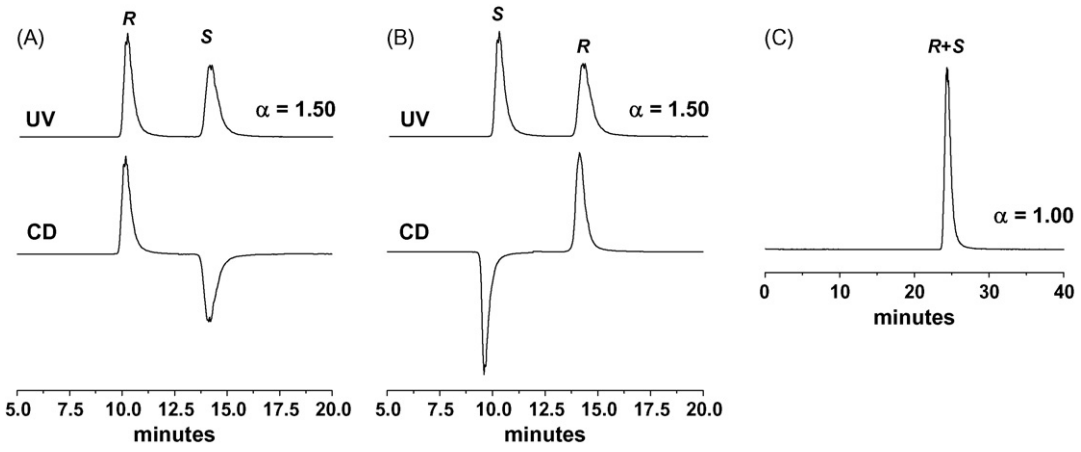

Fig. 5. (A) Chromatogram of (*R*,*S*)-CPT on (*R*,*R*)-Whelk-O1 column ($k'_R = 3.41$; $k'_S = 5.11$; $\alpha = 1.50$). (B) Chromatogram of (*R*,*S*)-CPT on (*S*,*S*)-Whelk-O1 column ($k'_S = 3.43$; $k'_R = 5.14$; $\alpha = 1.50$). (C) Chromatogram of (*R*,*S*)-CPT on tandem (*R*,*R*)-Whelk-O1 plus (*S*,*S*)-Whelk-O1 columns arrangement ($k'_R = k'_S = 4.17$; $\alpha = 1.00$). Eluent: *n*-hexane-dichloromethane (amylene stabilized) (40:60, v/v) + 5% methanol; flow-rate: 1.00 ml/min; $T = 25\,^{\circ}C$; UV and CD detection at 370 nm.

must be perfectly equivalent both chemically (i.e., same retention and selectivity) and geometrically (i.e., identical dimensions, particle size, packing efficiency, etc.). (*R*,*R*)- and (*S*,*S*)-Whelk-O1 columns equivalence was demonstrated by running the (*R*,*S*)-CPT sample on the two columns: chromatograms were found to be superimposable (see Fig. 5A and 5B). Afterwards, the same sample was analyzed on a tandem-columns arrangement, i.e., on the two columns connected in series via a zero dead-volume column coupler. In such case, the two CPT enantiomers were coeluted ($\alpha = 1.00$), yielding a single peak with double area and retention times which were the sum of the averaged values obtained on the single columns (Fig. 5C).

Table 2
Chromatographic data for the analysis of ST1968 as ureido derivatives on (*R*,*R*)- and (*S*,*S*)-Whelk-O1 columns.

CSP configuration	Sample	Chromatographic peak	k'^{a}	α^{b}	R_s^{c}
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–*t*-Bu	3.93	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–*t*-Bu	2.82	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CO–NH–*t*-Bu (*S*)-ST1968 as NH–CO–NH–*t*-Bu	3.93 2.82	1.39	3.59
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–Ph	6.86	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–Ph	5.64	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CO–NH–Ph (*S*)-ST1968 as NH–CO–NH–Ph	6.86 5.64	1.22	2.97
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–DMP	5.72	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–DMP	4.43	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CO–NH–DMP (*S*)-ST1968 as NH–CO–NH–DMP	5.72 4.43	1.29	3.75
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–PFP	1.87	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CO–NH–PFP	1.31	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CO–NH–PFP (*S*)-ST1968 as NH–CO–NH–PFP	1.87 1.31	1.43	3.08

[a] Retention factor.
[b] Enantioselectivity factor.
[c] Resolution factor.

Table 3
Chromatographic data for the analysis of ST1968 as thioureido derivatives on (*R*,*R*)- and (*S*,*S*)-Whelk-O1 columns.

CSP configuration	Sample	Chromatographic peak	k'[a]	α[b]	R_s[c]
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CS–NH–Ph	5.19	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CS–NH–Ph	4.06	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CS–NH–Ph (*S*)-ST1968 as NH–CS–NH–Ph	5.19 4.06	1.28	3.13
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CS–NH–DMP	4.79	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CS–NH–DMP	3.67	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CS–NH–DMP (*S*)-ST1968 as NH–CS–NH–DMP	4.79 3.67	1.31	3.13
(*R*,*R*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CS–NH–PFP	2.15	–	–
(*S*,*S*)	(*S*)-ST1968	(*S*)-ST1968 as NH–CS–NH–PFP	1.61	–	–
(*S*,*S*)	Virtual (*R*,*S*)-ST1968	(*R*)-ST1968 as NH–CS–NH–PFP (*S*)-ST1968 as NH–CS–NH–PFP	2.15 1.61	1.34	2.72

[a] Retention factor.
[b] Enantioselectivity factor.
[c] Resolution factor.

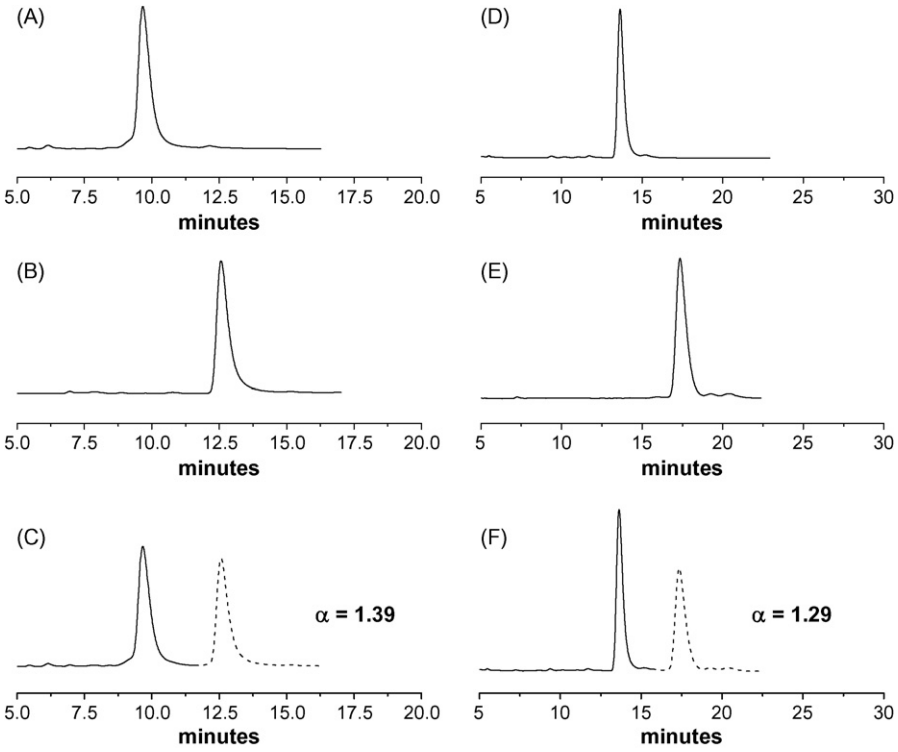

Fig. 6. (A) Chromatogram of (*S*)-ST1968 as NH–CO–NH–*t*-Bu derivative on (*R*,*R*)-Whelk-O1 column. (B) Chromatogram of (*S*)-ST1968 as NH–CO–NH–*t*-Bu on (*S*,*S*)-Whelk-O1 column. (C) Computer-generated chromatogram of the "virtual racemate". (D) Chromatogram of (*S*)-ST1968 as NH–CO–NH–DMP derivative on (*R*,*R*)-Whelk-O1 column. (E) Chromatogram of (*S*)-ST1968 as NH–CO–NH–DMP on (*S*,*S*)-Whelk-O1 column. (F) Computer-generated chromatogram of the "virtual racemate". Eluent: *n*-hexane-dichloromethane (amylene stabilized) (40:60, v/v) + 5% methanol; flow-rate: 1.00 ml/min; $T = 25\,^{\circ}C$; UV detection at 370 nm.

Table 4
Precursor and product ions obtained by APCI-MS/MS detection for the four ST1968 derivatives.

	Precursor ions $[M+H]^+$	Product ions	
	m/*z*	*m*/*z*	Attribution
ST1968 as NH–CO–NH–*t*-Bu	534	435	$[ST1968+H]^+$
ST1968 as NH–CO–NH–DMP	582	435	$[ST1968+H]^+$
ST1968 as NH–CS–NH–PFP	660	435 477	$[ST1968+H]^+$ $[M+H-C_6F_5NH_2]^+$
ST1968 as NH–CS–NH–DMP	598	435 477	$[ST1968+H]^+$ $[M+H-C_6H_3Me_2NH_2]^+$

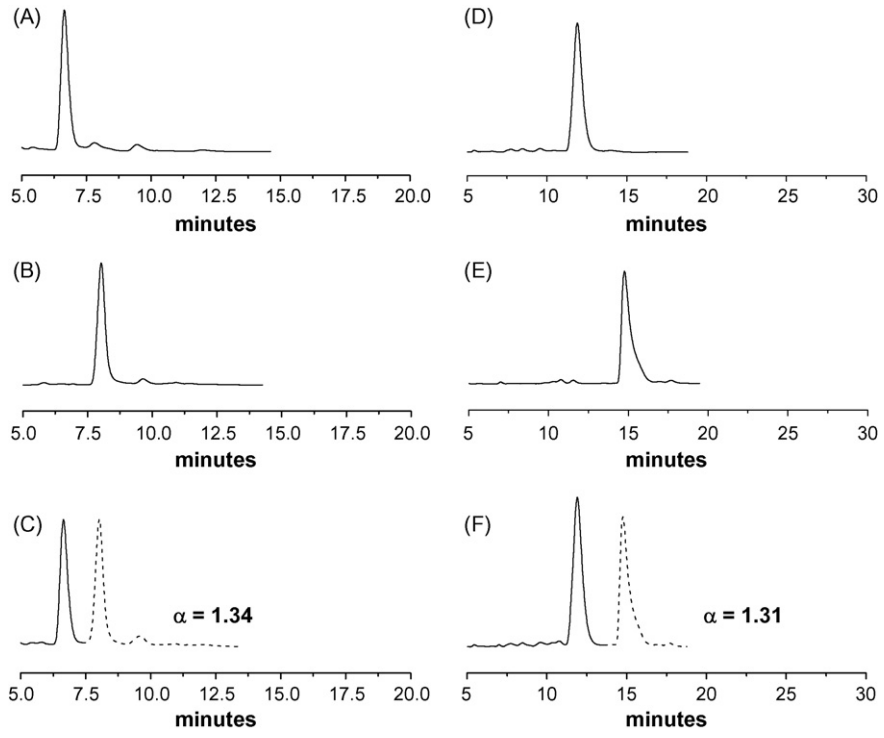

Fig. 7. (A) Chromatogram of (*S*)-ST1968 as NH–CS–NH–PFP derivative on (*R*,*R*)-Whelk-O1 column. (B) Chromatogram of (*S*)-ST1968 as NH–CS–NH–PFP on (*S*,*S*)-Whelk-O1 column. (C) Computer-generated chromatogram of the "virtual racemate". (D) Chromatogram of (*S*)-ST1968 as NH–CS–NH–DMP derivative on (*R*,*R*)-Whelk-O1 column. (E) Chromatogram of (*S*)-ST1968 as NH–CS–NH–DMP on (*S*,*S*)-Whelk-O1 column. (F) Computer-generated chromatogram of the "virtual racemate". Eluent: *n*-hexane-dichloromethane (amylene stabilized) (40:60, v/v) + 5% methanol; flow-rate: 1.00 ml/min; $T = 25\,^{\circ}C$; UV detection at 370 nm.

3.4. Choice of N-protecting groups of ST1968

In the present study four isocyanates (A–D in Fig. 3) were at first investigated as potential *N*-protecting groups of the free terminal amine of ST1968, ranging from alkyl- (A) to aryl-substituted (B), in the latter case containing electron-rich (C) or electron-poor (D) moieties. Later, we included in the study the corresponding isothiocyanates (E–H in Fig. 3), in order to explore the role of sulfur in terms of intermolecular H-bonding interaction, polarity and steric hindrance with respect to oxygen. In general, both alkyl- and aryl-derivatizing agents reacted rapidly with ST1968; however, isothiocyanate E gave an excess of impurities and was discarded. Derivatization solutions were stable for at least 3 h, with the exception of isocyanate D, which worked in the proper way only if freshly prepared.

With regard to the chromatographic behaviour of the diversely *N*-protected analytes on the Whelk-O1 columns family (see Tables 2 and 3), the presence of an aromatic moiety bonded to the ureido function (Table 2 and Fig. 6) yielded much higher capacity factors ($k' = 5.64$ for the trace (*R*)-enantiomer), with respect to the *t*-butyl substituent ($k' = 2.82$). This is likely due to the more efficient π-stacking interactions between analytes and the chiral selector.

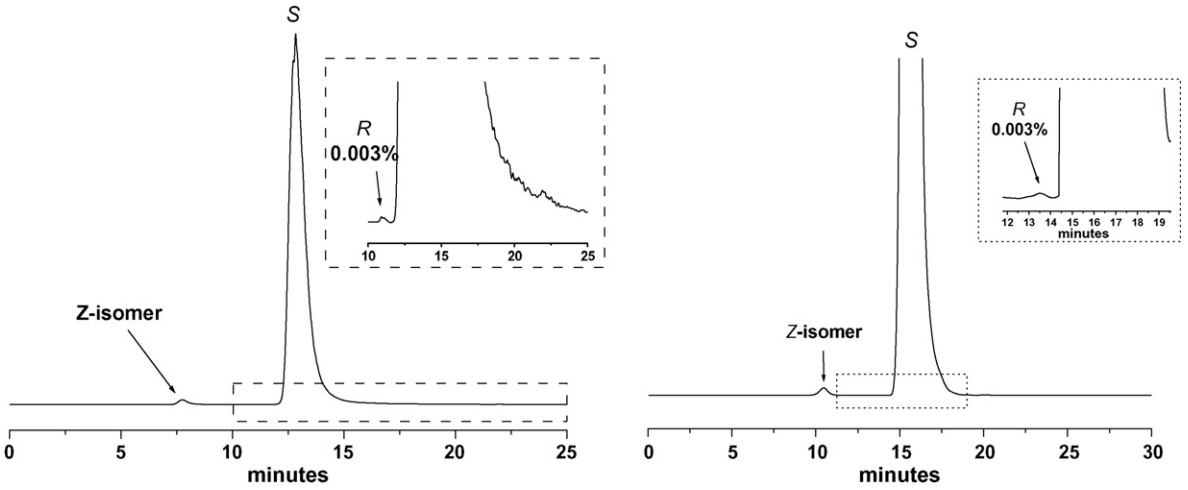

Fig. 8. (Left) Chromatogram of (*S*)-ST1968 as NH–CO–NH–*t*-Bu derivative on (*R*,*R*)-Whelk-O1 column. The ion transition 534 → 435 was chosen for the SRM acquisition. (Right) Chromatogram of (*S*)-ST1968 as NH–CS–NH–DMP derivative on (*R*,*R*)-Whelk-O1 column. The ion transitions 598 → 435 and 598 → 477 were chosen for the SRM acquisition. Eluent: *n*-hexane-dichloromethane (amylene stabilized) (40:60, v/v) + 5% methanol; flow-rate: 1.00 ml/min; $T = 25\,^{\circ}C$; APCI-MS/MS detection (for conditions, see Section 2.5).

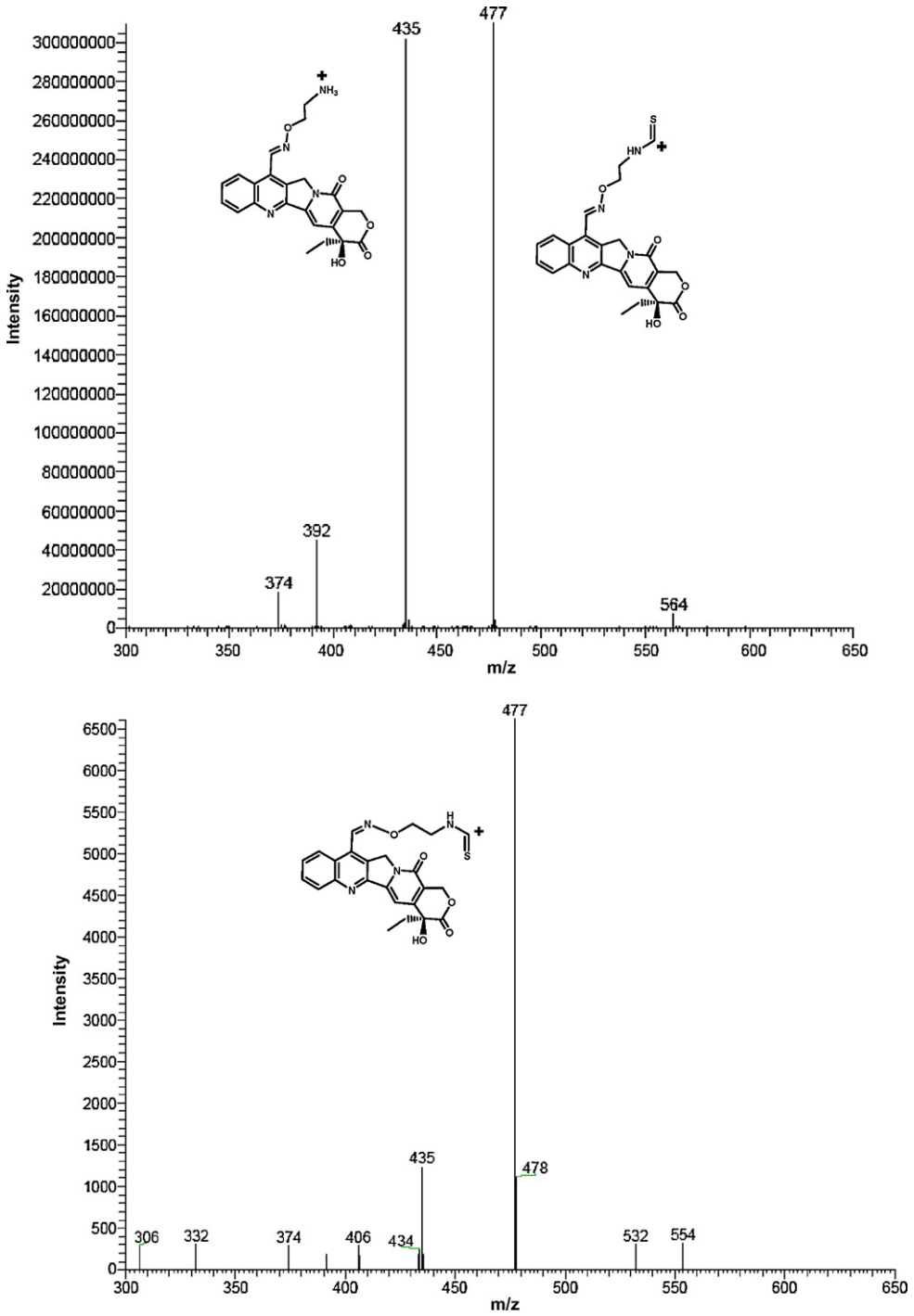

Fig. 9. APCI-MS/MS spectra of (*S*)-ST1968 as NH–CS–NH–DMP derivative. Fragmentation was obtained by isolation of the $[M+H]^+$ ion at 598 *m*/*z*, with a collision energy of 35% (arbitrary units), corresponding to chromatographic peak of (*S*)-ST1968 (top) and to its (*Z*)-isomer (bottom).

Notably, with electron-donor substituents (such as 3,5-DMP) on the aromatic ring retention slightly decreased to 4.43 (probably for steric hindrance), whereas with electron-withdrawing groups (i.e., PFP) it drastically dropped to 1.31.

Enantioselectivity approximately showed the opposite trend, i.e., it increased with the lowering of retention: α changed from 1.22 to 1.43 going from the most retained derivative, i.e., –NH–CO–NH–Ph ($k' = 5.64$) to the less retained, i.e., –NH–CO–NH–PFP ($k' = 1.31$).

As expected, with isothiocyanate derivatives (Table 3 and Fig. 7) we observed the same trend, i.e., the less the retention factor, the highest the enantioselectivity: in fact, α changed from 1.28 to 1.34 going from the most retained derivative, i.e., –NH–CS–NH–Ph ($k' = 4.06$) to the less retained, i.e., –NH–CS–NH–PFP ($k' = 1.61$). In general, the change from oxygen to sulfur on the derivatizing agent yielded to lower retentivity, whereas enantioselectivity was almost insensitive to the O → S displacement.

This preliminary screening of potential *N*-protecting groups of ST1968 allowed us to select four amenable candidates to submit to APCI-MS/MS detection: two ureido derivatives, namely NH–CO–NH–*t*-Bu ($\alpha = 1.39$) and NH–CO–NH–DMP ($\alpha = 1.29$), and two thioureido derivatives, i.e., NH–CS–NH–PFP ($\alpha = 1.34$) and NH–CS–NH–DMP ($\alpha = 1.31$). The selection was made on the grounds of both enantiodiscrimination ability and chemical sta-

bility: although the ST1968 derivative prepared from isocyanate D exhibited the highest enantioselectivity values of all the series of derivatizing agents ($\alpha = 1.43$), its elevated chemical instability was considered as a detrimental factor; on the contrary, its sulfur analogue, which indeed gave the highest α amongst the sulfur series, was shown to be an easy to handle analyte.

3.5. Chromatographic APCI-MS/MS method development

An APCI interface was chosen since it is known to be well suited to detect non polar and medium polarity molecules with masses between 100 and 1000 Da, and to be compatible with analytical flow-rates, offering a wide linear dynamic range for quantitative purposes [21]. The precursor and product ions for each analyte of interest were determined by direct infusion of single analyte solutions. The mass spectra recorded in full scan acquisition mode showed the protonated molecular ions $[M+H]^+$ as base peaks, with mass-to-charge ratios (m/z) as reported in Table 4. The fragmentation pattern obtained by isolation of the different $[M+H]^+$ ions from isocyanate derivatives gave an MS/MS spectrum containing a major fragment at $m/z = 435$, arising from the loss of the *N*-protecting groups. The following ion transitions were chosen for the SRM acquisition: $534 \rightarrow 435$ and $582 \rightarrow 435$, for *t*-butyl-ureido and DMP-ureido derivatives, respectively. Fig. 8 (left) shows a typical chromatogram of (*S*)-ST1968 as NH–CO–NH–*t*-Bu derivative obtained on (*R*,*R*)-Whelk-O1 column with APCI-MS/MS detection: as it can be easily seen, the trace (*R*)-enantiomer was easily identified and quantified (0.003%). Notably, the fragmentation pattern obtained by isolation of the different $[M+H]^+$ ions from isothiocyanate derivatives gave an MS/MS spectrum containing, beside the above mentioned peak at $m/z = 435$, another major fragment at $m/z = 477$, corresponding to the loss of an aniline-substituted moiety, i.e., $[M+H-C_6F_5NH_2]^+$ and $[M+H-C_6H_3Me_2NH_2]^+$ for PFP-thioureido and DMP-thioureido derivatives, respectively. The following ion transitions were chosen for the SRM acquisition: $660 \rightarrow 435$ and $660 \rightarrow 477$ for the PFP-thioureido derivative, and $598 \rightarrow 435$ and $598 \rightarrow 477$ for the DMP-thioureido derivative. Fig. 8 (right) also shows a typical chromatogram obtained on (*R*,*R*)-Whelk-O1 column by MS detection in SRM mode for ST1968 as DMP-thioureido derivative. An interesting benefit of the thioureido derivatives with respect to the ureido is that the (*Z*)-impurity of ST1968 can be easily detected with high selectivity and sensitivity since it gave a fragmentation pattern where the peak at $m/z = 477$ was the major one (Fig. 9). This behaviour was not found with the oxygen derivatives.

3.6. Chromatographic APCI-MS/MS method validation

The method was evaluated according to the criteria described in Section 2.6. LOD was determined at 50 pg injected, whereas LOQ at 150 pg. The accuracy and precision ($n = 6$) obtained at the LOQ level were 70.6% and 16.1%, respectively. Linearity was verified throughout the tested range with the following linear relationship and correlation coefficient (R^2): $y = 6.45 \times 10^8 x - 5.49 \times 10^4$, $R^2 = 0.9991$. As it can be observed, the wide dynamic linearity range obtained (five orders of magnitude) enabled the quantitation of the trace (*R*)-enantiomer down to 0.003% of the main compound, corresponding to enantiomeric excess values up to 99.994%.

4. Conclusions

A set of alkyl- and aryl-substituted isocyanates and isothiocyanates were investigated as potential *N*-protecting groups of the free terminal amine of ST1968, a novel water-soluble CPT derivative currently undergoing phase I clinical trials as anticancer agent. Successful separations on the Whelk-O1 columns could be obtained with all protecting groups (α ranging from 1.22 to 1.43), although those including pentafluoroaryl moieties were found to yield higher enantioselectivities ($\alpha = 1.43$) than those with aliphatic residues ($\alpha = 1.39$). However, pentafluoroaryl derivatives gave the lowest retention values, and hence did not show chemoselectivity towards eventual impurities, such as the (*Z*)-diastereoisomer of ST1968. As expected, the change from oxygen to sulfur on the derivatizing agent yielded to lower retentivity, whereas enantioselectivity was almost insensitive to the O → S displacement.

The screening of potential *N*-protecting groups of ST1968 allowed us to identify an interesting candidate for further investigations on ST1968: the NH–CS–NH–DMP derivative indeed exhibited satisfying enantioselectivity ($\alpha = 1.30$) and chemoselectivity towards the (*Z*)-diastereoisomer of ST1968, as well as a good chemical stability. Moreover, it showed a good response for the protonated molecule in the positive ion mode APCI-MS/MS detection. The successful application of the ICCA technique combined with multistage mass spectrometry (APCI-MS/MS) detection enabled us to detect the trace (*R*)-enantiomer of ST1968 on the (*R*,*R*)-Whelk-O1 CSP at 0.003% level, corresponding to enantiomeric excess values up to 99.994%.

It is worthy of note that the procedure we developed is not limited to the CPT class of compounds but can be easily applied to other chiral amines, allowing their separation on commercially available Pirkle-type CSPs under normal-phase HPLC conditions.

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