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Chiral bioanalysis by normal phase high-performance liquid chromatography–atmospheric pressure ionization tandem mass spectrometry

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Abstract

A highly sensitive and specific chiral high-performance liquid chromatographic tandem mass spectrometric (HPLC–MS–MS) technique has been developed for the determination of several chiral drugs and their metabolites. Examples include enantiomers of verapamil and its most abundant active metabolite, norverapamil, enantiomers of oxybutynin and its metabolite, N-desethyloxybutynin, enantiomers of doxazosin and those of sotalol. These drugs are marketed as racemates although their optical isomers possess different pharmacological activities. Drug enantiomers and their respective chiral metabolites were separated by isocratic normal-phase chiral chromatography on a small-bore (100×2.1 mm I.D.) Chiralpak AD column. Post-column reagent addition of an aqueous mobile phase was introduced to enable direct coupling of hexane-based mobile phases chiral chromatography with the heated nebulizer interface of the SCIEX API 300 tandem mass spectrometer. Quantification of individual drug and metabolite enantiomers was obtained by multiple reaction monitoring. This novel approach was developed and used in bioanalytical studies. It successfully combines the convenience of normal-phase chiral separations on small-bore HPLC columns with high specificity and low limits of quantification achieved by atmospheric pressure ionization MS–MS detection.

Keywords: Enantiomer separation; Detection, LC; Verapamil; Norverapamil; Sotalol; Doxazosin; Oxybutynin; Desethyl-oxybutynin; Prazosin

1. Introduction

The need for highly sensitive and specific bioanalytical methods has become even more acute after the "FDA's Policy Statement for the Development of New Stereoisomeric Drugs" [1] was issued in May 1992: "The stereoisomeric composition of a drug with a chiral centre should be known and the quantitative isomeric composition of the material used in pharmacological, toxicological and clinical studies known.... To evaluate the pharmacokinetics of a single enantiomer or mixture of enantiomers, manufacturers should develop quantitative assays for individual enantiomers in in vivo samples early in drug development."

Chiral compounds cannot be directly separated by conventional HPLC stationary phases without prior derivatization of the enantiomers into diastereomers. Over the past fifteen years, increasingly powerful chiral stationary phases (CSPs) have become avail-

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able (Pirkle-type, protein-based, cyclodextrins, cellulose/amylose etc. [2]) that allow direct separation and quantitation of enantiomers without the need to convert them to diastereomers. Each type of CSP has specific advantages and no single CSP can claim universal application. In the separation of enantiomers of verapamil and its metabolite norverapamil, protein-based CSPs could separate the optical isomers of each compound but were unsuccessful in separating the parent drug from the metabolite. These shortcomings of the available column technology had been previously addressed by using derivatization [3] or by coupled achiral–chiral HPLC [4] on an α_1-acid glycoprotein CSP.

However, one of the CSPs that seem to have demonstrated success with a number of compounds is Chiralpak AD, Daicel's amylose Tris(3,5-dimethylphenyl carbamate) coated on a 10-μm silica gel. This CSP has an additional advantage in that, for many drugs, the enantiomers of the prevailing metabolite can also be separated in the same chromatographic run, which makes Chiralpak AD particularly attractive in bioanalysis. This CSP is used in the normal-phase mode and as such presents a mobile phase incompatibility when attempting to interface it to an atmospheric pressure ionization (API) mass spectrometer. Under the conditions used with the heated nebulizer source on most API mass spectrometers, a mobile phase composed largely of hexane would be considered to be an explosion hazard.

In the past, a variety of mobile phase incompatibilities were overcome and allowed MS analysis in LC. A recent review on the general principles and instrumentation in LC–MS [5] discusses the major difficulties met while trying to reconcile the high liquid flow-rates (1 ml min^{-1}) from conventional analytical HPLC columns into the high-vacuum environment of the mass spectrometer, as well as dealing with non-volatile additives in conventional mobile phases. The SCIEX tandem mass spectrometer with the heated nebulizer source used in this work operates well with 1 ml min^{-1} flows of volatile eluates. The main challenge was to find a set of conditions that would allow heated nebulizer operation with hexane as the mobile phase. This work is not the first to use HPLC–MS with CSPs [6–9] nor is it the first to overcome mobile phase incompatibilities [6,10–18]. It provides, however, a simple and elegant manner of linking an API-MS–MS instrument with a normal-phase CSP that has been particularly useful for a wide range of chiral bioanalytical work. This highly sensitive and specific analytical approach has been developed in our laboratory and has been tested on enantiomers of the drugs and some of their metabolites that are mentioned below.

The chiral calcium channel blocker, verapamil, is presently marketed as a racemate. Its enantiomers have different pharmacokinetic and pharmacodynamic properties and are present in plasma in different enantiomeric ratios (*R*:*S*), which is determined by a combination of factors. Different degrees of plasma protein binding, volumes of distribution and clearances of the verapamil enantiomers are caused by external factors such as the administration route (intravenous or oral) and the rate of adsorption [19]. Moreover, the type of the dosage form influences the adsorption rate of verapamil from the gastrointestinal tract by controlling the dissolution of the drug. Mean (*R*)-verapamil–(*S*)-verapamil ratios for sustained release and immediate release oral racemic formulations were shown to be 4:1 and 3:1, respectively [19]. Norverapamil, verapamil's major active metabolite, is also chiral. Two papers published almost at the same time [3,20] described direct simultaneous quantitative chiral analysis of these compounds on a Chiralpak AD stationary phase. The limits of quantification by fluorescence detection ranged from 5 to 20 ng ml^{-1} for verapamil and from 10 to 50 ng ml^{-1} for norverapamil at the *S*/*N* ratio of 5:1 [3,20].

Sotalol is a chiral β-adrenergic blocking drug used in therapy as a racemic mixture. The (*R*)-enantiomer has both β-blocking (class II antiarrhythmic) activity and potassium channel-blocking (class III antiarrhytmic) properties. The (*S*)-enantiomer has class III properties similar to those of (*R*)-sotalol. However, the affinity of (*S*)-sotalol for β-adrenergic receptors is 30 to 60 times lower than the affinity of (*R*)-sotalol [21,22]. The detection limit of an published HPLC assay for the determination of sotalol enantiomers by fluorescence detection after derivatization with (−)-methyl chloroformate was 12.5 ng ml^{-1} per enantiomer, using 1 ml of plasma or urine [23].

Another chiral drug marketed as a racemate is doxazosin, a new long acting α_1-adrenoceptor an-

tagonist that is structurally related to prazosin and terazosin [24]. Doxazosin enantiomers have been previously separated by HPLC on an α_1-acid glycoprotein column using UV detection; at 254 nm, the limit of detection for the first and second eluting enantiomers were 1.3 and 1.7 ng on-column, respectively [25]. In another validated achiral HPLC assay using solid-phase extraction and fluorescence detection, the limit of quantification from 1 ml of plasma for *rac*-doxazosin was 1 ng ml^{-1} [26].

Oxybutynin is an anti-muscarinic drug that is widely used as a racemate in the treatment of urinary incontinence due to neurogenic bladder. The two enantiomers of oxybutynin showed different pharmacological properties when tested both in vivo and in vitro [27,28]. Oxybutynin enantiomers were resolved on a preparative (250×10 mm I.D.) Chiralpak AD column [29], but no chiral chromatographic method for the simultaneous determination of enantiomers of oxybutynin and its metabolite, N-desethyloxybutynin, has been published to our knowledge. Minimal detectable concentrations (at a *S/N* ratio of 3:1) of oxybutynin and N-desethyloxybutynin from 2 ml of plasma in an achiral assay using electrochemical detection were 0.5 and 5 ng ml^{-1}, respectively [30]. While developing the chiral assay, we estimated the limit of quantification, using UV detection, to be in the range of 5 and 25 ng ml^{-1} for oxybutynin and N-desethyloxybutynin, respectively. This is not satisfactory for most bioanalytical assays. The need to obtain a lower limit of quantification prompted us to consider tandem mass spectrometry for detection in bioanalytical studies.

2. Experimental

2.1. Chemicals and solutions

rac-Oxybutynin chloride and prazosin hydrochloride were USP standards, *rac*-sotalol hydrochloride was a BP standard. The enantiomers of doxazosin mesylate, (*R*)- and (*S*)-oxybutynin hydrochloride and (*R*)- and (*S*)-N-desethyloxybutynin hydrochloride were a generous gift from Sepracor (Marlborough, MA, USA). *rac*-Verapamil hydrochloride was purchased from Sigma (St. Louis, MO, USA). *rac*-Norverapamil hydrochloride and the enantiomers of verapamil were obtained from RBI (Natick, MA, USA).

All solvents and water were of HPLC grade. Chemicals were of the highest purity available and were obtained from local commercial sources. Mobile phases were filtered through a Millipore 0.45-μm nylon filter.

Stock solutions were prepared in reagent alcohol (ethanol denatured with 5% 2-propanol) obtained from Sigma. For Q1 scan and for parent–daughter ion spectra (obtained by infusion using an ion-spray interface), 0.1 ml of the respective stock solution was diluted with 9.9 ml of a 1:1 (v/v) mixture of 0.05 *M* ammonium acetate and acetonitrile. The final concentration of each compound in the infusion solution was 1 μg ml^{-1}.

2.2. Equipment

2.2.1. HPLC systems and columns

Two HPLC systems were used. The first was used to develop chiral separations on analytical and small-bore chiral HPLC columns. It consisted of a Hewlett-Packard series 1050 solvent delivery system with autosampler (Hewlett-Packard, Waldbronn, Germany). The detector was a Perkin-Elmer LS 40 fluorescence detector (Perkin-Elmer, Beaconsfield, UK) with a 7-μl flow cell. The mobile phase was degassed by sparging with UHP/Zero purity helium (Air Products, Toronto, Canada). The integrator used was Chrom Jet (Spectra Physics Analytical, San Jose, CA, USA).

The second system (see Fig. 1) comprised a Waters 616 pump, a Waters 600S controller, a Waters 717plus autosampler and a Waters in-line degasser. Another HPLC pump, Waters Model 510 (Waters, Milford, MA, USA), was used to deliver post-column the "make-up" mobile phase (see Fig. 1). On both systems, the temperature of chiral chromatographic columns was maintained with Keystone Cool Pocket (Keystone Scientific, Bellefonte, PA, USA).

Compounds were separated on a Chiralpak AD CSP. The analytical Chiralpak AD columns (250×4.6 mm I.D., 10 μm) were purchased from Chiral Technologies (Exton, PA, USA), while the small-bore (100×2.1 mm I.D., 10 μm) Chiralpak AD columns used for HPLC–MS–MS assays were custom-packed.

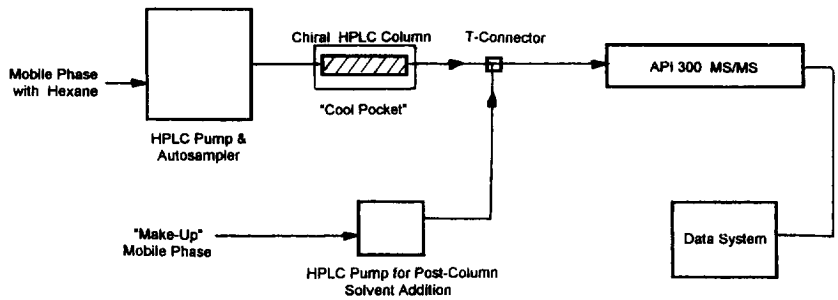

Fig. 1. HPLC–MS–MS equipment set-up with post-column reagent addition.

The hold-up time (t_0) was determined by baseline disturbance upon injecting the mobile phase.

The infusion pump (model 11) used to determine the m/z of ionized molecules and perform the parent–daughter scans was purchased from Harvard Apparatus (South Natick, MA, USA).

2.2.2. Atmospheric pressure ionization tandem mass spectrometer

The tandem mass spectrometer used was the Perkin-Elmer SCIEX API 300 (SCIEX, Toronto, Canada) equipped with a heated nebulizer interface. All work was performed in the positive-ion mode. The spectrometer mass axis was calibrated against polypropylene glycol (PPG) standards provided by SCIEX. Resolution was adjusted to unit mass resolution, as demonstrated with the PPG reference calibrator.

The heated nebulizer used nitrogen as both the nebulizer and the auxiliary gas, with nebulizer pressure at 5.5 bar and the auxiliary flow set to ten, in arbitrary units. The nebulizer temperature was set at 440°C. Corona discharge was operated in constant current mode at 4 μA. The nebulizer was adjusted to point to the interface plate at 1 o'clock position, 0.5 cm from the orifice center.

2.3. Extraction from plasma

The bioanalytical assays for the chiral determination of verapamil and norverapamil, oxybutynin and N-desethyloxybutynin, and for doxazosin were first developed to be used in HPLC with fluorescence or UV detection and were transferred later to HPLC–MS–MS. In all cases, 1 ml of plasma was used. All the extractions used were liquid–liquid.

Verapamil and norverapamil were extracted along with the internal standard (+)-glaucine from plasma by simple liquid–liquid extraction with a mixture of pentane–dichloromethane (2:1, v/v, 5 ml). Prior to extraction, each plasma sample was made basic by the addition of 0.1 ml of 2 *M* sodium hydroxide. The organic extract was separated and evaporated to dryness in a gentle stream of nitrogen. The dry residue was reconstituted in mobile phase and injected into the HPLC system. An identical extraction procedure was used for the determination of doxazosin enantiomers in plasma, with the internal standard being prazosin. For the determination of oxybutynin and N-desethyloxybutynin, after addition of 0.1 ml of 0.1 *M* sodium hydroxide to 1 ml of plasma, the compounds of interest were extracted by liquid–liquid extraction with 5 ml of 1-chlorobutane containing 5% (v/v) ethyl acetate. Again, the organic layer was evaporated to dryness under a stream of nitrogen, the residue was reconstituted in the mobile phase and injected into the HPLC system.

3. Results and discussion

3.1. Verapamil and norverapamil assay

Replacing the 250×4.6 mm I.D. Chiralpak AD analytical column with a small-bore 100×2.1 mm I.D. Chiralpak AD column and replacing the fluorescence detector by the API tandem mass spectrometer resulted in shortened chromatographic run times and lower limits of detection for both verapamil and norverapamil enantiomers. For example, Fig. 2A shows the chromatogram obtained on injection of 1 μl of a 1 μg ml^{-1} solution of commercially obtained

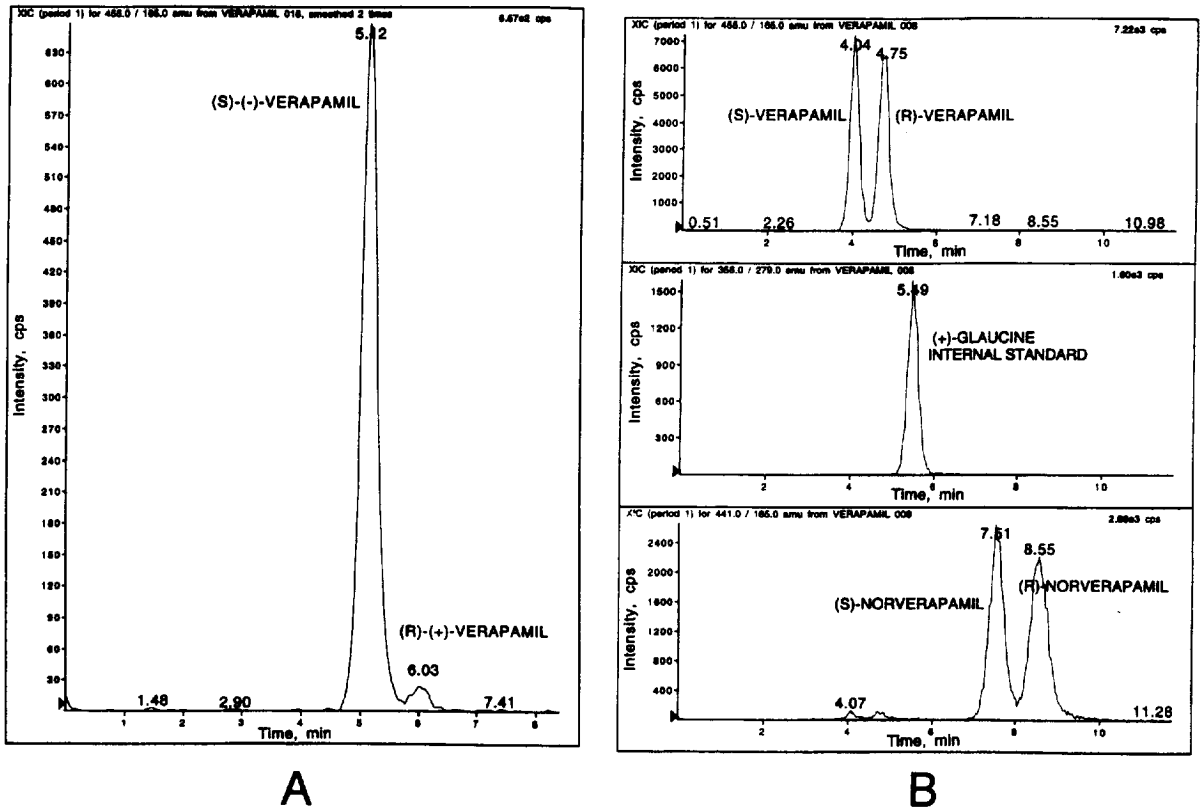

Fig. 2. (A) Determination of enantiomeric purity of commercially available (*S*)-(−)-verapamil by chiral HPLC–MS–MS on SCIEX API 300. A 1-μl volume of solution containing 1 μg ml^{-1} of (*S*)-(−)-verapamil was injected into a 100×2.1 mm I.D. Chiralpak AD column. The column was kept at 25°C. The mobile phase used was *n*-hexane–2-propanol–diethylamine (92.5:7.5:0.1, v/v/v) at a flow-rate of 0.22 ml min^{-1}. The parent–daughter ion pair monitored was 455.0/165.0. (B) Extracted ion chromatogram for chiral verapamil and norverapamil assay. A 1-μl volume of reference solution containing *rac*-verapamil and *rac*-norverapamil, each compound at 2 μg ml^{-1}, and the internal standard at 1 μg ml^{-1}, were injected. Chromatographic conditions: The mobile phase was *n*-hexane–2-propanol–diethylamine (92.5:7.5:0.1, v/v/v) at 0.2 ml min^{-1}, the column temperature was 32°C. The parent–daughter ion pairs monitored were 455.0/165.0 for verapamil, 441.0/165.0 for norverapamil and 356.0/279.0 for the internal standard, (+)-glaucine. Collision cell off-set voltage, −45 V. Abscissa, retention time (in min); ordinate, intensity in counts per second (cps).

(*S*)-(−)-verapamil. This represents a total mass on-column of 1 ng at 96% purity and at an enantiomeric excess of 92%. The amount of the impurity is approximately 40 pg of (*R*)-(+)-verapamil (4%, w/w) on-column and is easily identified and quantified.

For verapamil and norverapamil, small changes in column temperature and mobile phase composition have a considerable effect on separation, resolution and chromatographic run time (Table 1). However, (+)-glaucine, which is an ideal internal standard in the verapamil–norverapamil chiral assay using fluorescence detection, does not follow the typical chromatographic behavioural pattern of the other two compounds. Thus, conditions A and B in Table 1 represent the optimized chromatographic conditions for fluorescence detection and MS–MS detection, respectively, on the same small-bore column. In both cases, it was necessary to optimize for baseline separation of the enantiomers, but, unlike MS–MS, fluorescence detection requires additional separation of the (+)-glaucine peak from the analytes, resulting in longer run times. When using MS–MS detection, complete separation of different chemical species in time is not required because they are also most often separated in either parent ion and/or daughter ion mass. Consequently, there can be an advantage with

Table 1
Chiral HPLC–MS–MS assay for verapamil and norverapamil: chromatographic parameters and conditions

Compound	Condition A				Condition B			
	t_R (min)	k'	α	R_s	t_R (min)	k'	α	R_s
Verapamil	6.10	3.52	1.28	5.40	4.04	1.99	1.27	2.99
Norverapamil	13.71	9.15	1.21	4.88	7.54	6.19	1.16	2.48
(+)-Glaucine	7.82	4.79	–	–	5.49	3.07	–	–
Mobile phase	*n*-hexane–2-propanol–diethylamine (95:5:0.1, v/v/v)				*n*-hexane–2-propanol–diethylamine (92.5:7.5:0.1, v/v/v)			
Mobile phase flow	0.2 ml min^{-1}				0.2 ml min^{-1}			
Column temperature	28°C				32°C			
Run time	18 min				10 min			

t_R=retention time of first eluted enantiomer, k'=capacity factor of first eluted enantiomer, α=separation factor, R_s=stereochemical resolution. Injected: 1 μl of reference solution containing *rac*-verapamil (12 μg ml^{-1}), *rac*-norverapamil (2 μg ml^{-1}) and (+)-glaucine (internal standard) (1 μg ml^{-1}), i.e. 1 ng of each enantiomer on-column. Chromatographic column: Chiralpak AD, 100×2.1 mm I.D., 10 μm.

MS–MS detection of shorter run times. Obviously, optical isomers of the same compound must still be separated in time because the tandem mass spectrometer gives little chiral discrimination.

The extracted ion chromatogram for the simultaneous determination of verapamil and norverapamil enantiomers is presented in Fig. 2B and the chromatographic parameters for this example are listed in Table 1 (condition B). The daughter-ion spectra and the abundance vs. ion energy plots for verapamil, norverapamil and (+)-glaucine are shown in Fig. 3.

3.2. Sotalol

The abundance vs. ion energy plot for sotalol is presented in Fig. 4A, while Fig. 4B shows the HPLC–MS–MS determination of sotalol enantiomers following separation on a Chiralpak AD small-bore column. This extracted ion chromatogram was obtained upon injecting 10 μl of solution containing only 10 ng ml^{-1} of *rac*-sotalol and represents 100 pg of *rac*-sotalol on-column. The mobile phase was composed of reagent alcohol–*n*-hexane–2-propanol–diethylamine (30:63:7:0.17, v/v). The flow-rate was 0.1 ml min^{-1} and the column temperature was kept at 20°C. The separation factor, α, and the stereochemical resolution, R_s, for the sotalol enantiomers were 1.52 and 1.96, respectively, with a total run time of less than 8 min.

3.3. Doxazosin and prazosin assay

The chromatographic parameters for a highly sensitive chiral HPLC–MS–MS assay for doxazosin are presented in Table 2. The extracted ion chromatogram (Fig. 5C) shows the result on injection (10 μl) of a solution containing 100 ng ml^{-1} of both *rac*-doxazosin and prazosin (internal standard in the assay), representing 500 pg of each doxazosin enantiomer on-column. The abundance vs. ion energy plots for doxazosin and prazosin that were used to optimize the MS–MS detection are shown in Fig. 5A,B.

3.4. Oxybutynin and N-desethyloxybutynin assay

The simultaneous separation of both oxybutynin and N-desethyloxybutynin enantiomers on a Chiralpak AD column is presented in Fig. 6. Extracted ion chromatograms for an extracted spiked plasma sample (A1) and its corresponding blank (A2) are shown. Plasma was spiked with *rac*-oxybutynin at the level of 150 pg ml^{-1} per enentiomer and with *rac*-N-desethyloxybutynin at the level of 750 pg ml^{-1} per enantiomer. The mobile phase used was *n*-hexane–2-propanol–diethylamine (90:10:0.1, v/v/v) at 0.22 ml min^{-1}. The column temperature was kept at 22°C. The parent–daughter ion pairs monitored were 358.4/142.2 for oxybutynin and 330.4/94.0 for N-desethyloxybutynin. Fig. 6B shows ex-

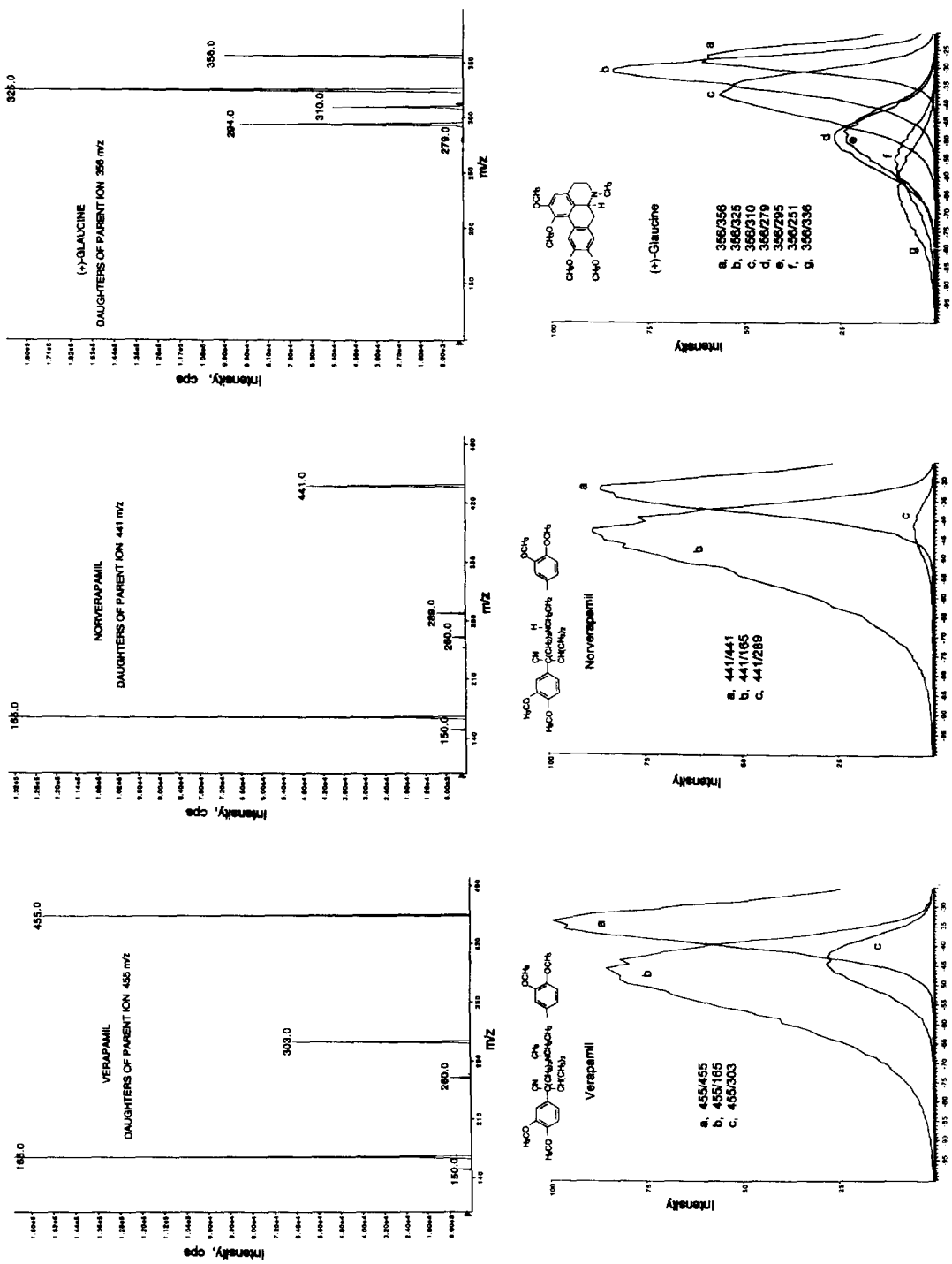

Fig. 3. MS–MS parameters for chiral verapamil assay on a SCIEX API 300 system. Top panels show respective MS–MS scans for daughter ions of verapamil, norverapamil and (+)-glaucine $[M+H]^+$ ions. Bottom panels depict MS–MS plots of abundance vs. RO2 ion energy of selected parent–daughter ion pairs for the compounds under investigation.

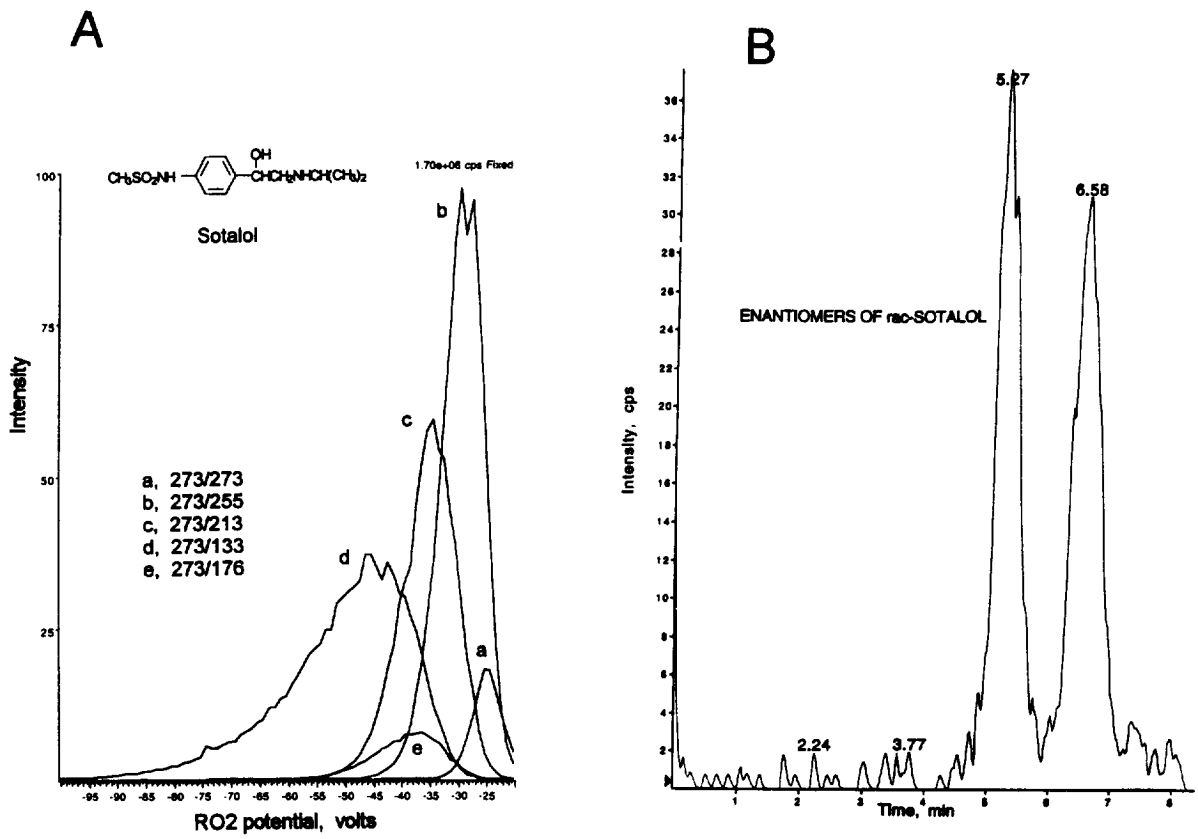

Fig. 4. Determination of sotalol enantiomers by chiral HPLC–MS–MS on a SCIEX API 300 system. (A) MS–MS plot of abundance vs. RO2 ion energy for sotalol. Abscissa, ramped RO2 potential (V); ordinate, intensity in cps (fixed for 100 ordinate units to equal 1700 000 cps). (B) Extracted ion chromatogram for sotalol enantiomers obtained after injection of 10 μl of solution containing 10 ng ml^{-1} of *rac*-sotalol, i.e., 50 pg of each sotalol enantiomer on a 100×2.1 mm I.D. Chiralpak AD column. The parent–daughter ion pair monitored was 273.0/213.0; collision cell off-set voltage, −35 V. Abscissa, retention time (in min); ordinate, intensity (in cps).

Table 2
Chiral HPLC–MS–MS assay for doxazosin: chromatographic parameters and conditions

Compound	t_R (min)	k'	α	R_s
Doxazosin	5.59	3.14	1.50	4.65
Prazosin (internal standard)	4.47	2.31	–	–

Mobile phase: *n*-hexane–2-propanol–diethylamine (70:30:0.1, v/v/v) at a flow-rate of 0.2 ml min^{-1}. Column temperature: 30°C. t_R=retention time of first eluted enantiomer, k'=capacity factor of first eluted enantiomer, α=separation factor, R_s=stereochemical resolution. Injected: 10 μl of a reference solution containing *rac*-doxazosin (100 ng ml^{-1}) and prazosin (100 ng ml^{-1}), i.e. 500 pg of both doxazosin enantiomers on column and 1 ng of prazosin (internal standard) on-column. Chromatographic column: Chiralpak AD, 100×2.1 mm I.D., 10 μm.

tracted ion chromatograms obtained upon injecting 10 μl of reference solution containing *rac*-oxybutynin (100 ng ml^{-1}) and *rac*-N-desethyloxybutynin (500 ng ml^{-1}), i.e. 0.5 ng of each oxybutynin enantiomer and 2.5 ng of each N-desethyloxybutynin enantiomer on-column, respectively. The mobile phase used was the same as that used in Fig. 6A, the flow-rate was 0.15 ml min^{-1} and the column temperature was kept at 20°C. The parent–daughter ion pairs monitored were 358.0/142.0 for oxybutynin and 330.0/230.0 for N-desethyloxybutynin. The same mobile phase, but different flow-rates, column temperatures and parent–daughter pairs were used in Fig. 6A,B. Short run times, complete resolution of the main metabolite from the parent,

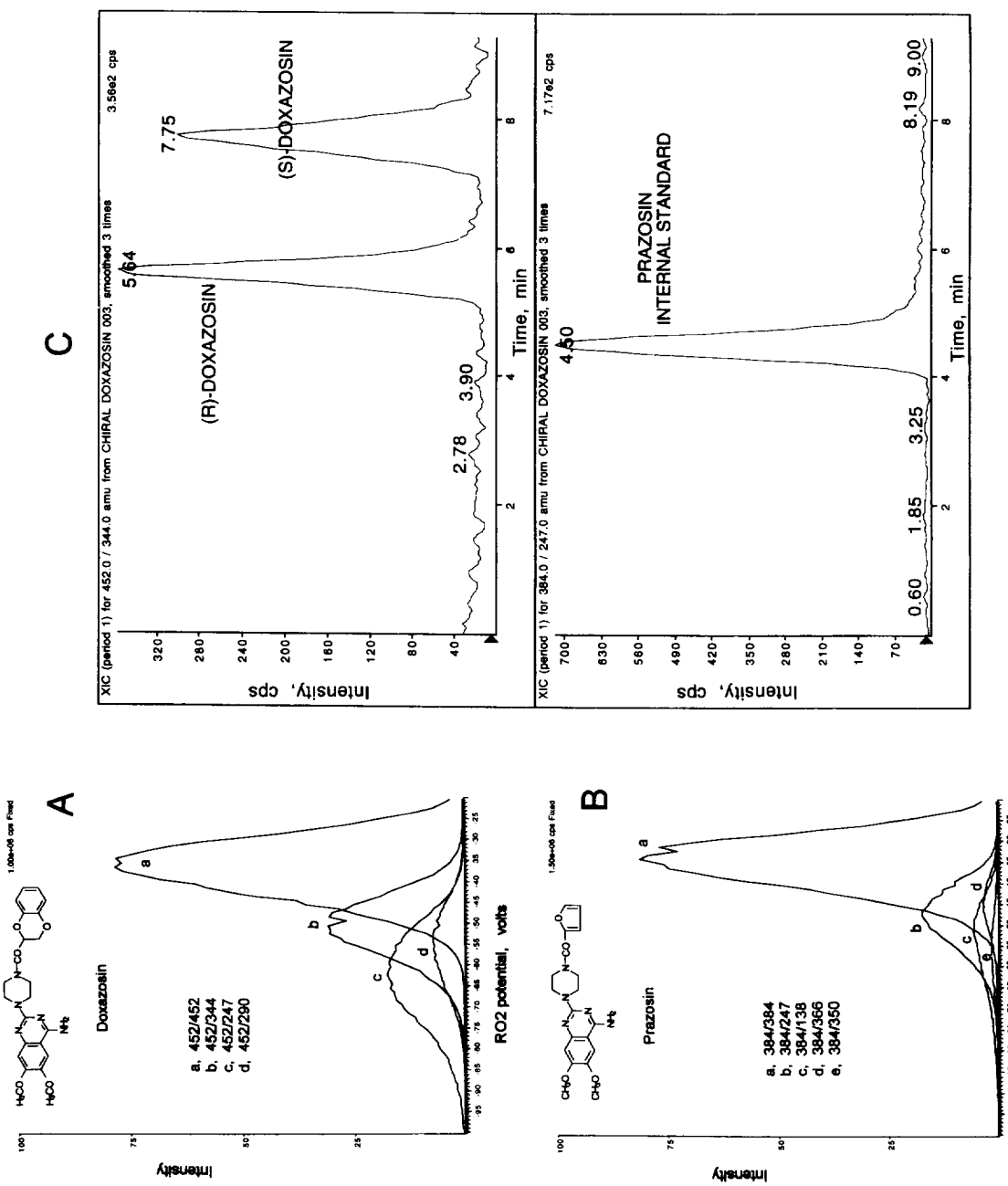

Fig. 5. MS–MS plots of abundance vs. RO2 ion energy for doxazosin (A) and prazosin (B) on an API 300 system. Abscissa, ramped RO2 potential (in V); ordinate, intensity in cps (fixed for 100 ordinate units to equal 1000 000 cps for doxazosin and 1500 000 cps for prazosin). (C) Extracted ion chromatograms for the doxazosin chiral assay upon injection of 10 μl of reference solution containing both *rac*-doxazosin and prazosin (internal standard) at a concentration of 100 ng ml^{-1}. The mobile phase used was *n*-hexane–2-propanol–diethylamine (70:30:0.1, v/v/v) at 0.2 ml min^{-1} and the column temperature was 30°C. The parent–daughter ion pairs monitored were 452.0/344.0 for doxazosin and 384.0/247.0 for prazosin. Collision cell off-set voltage, −50 V. Abscissa, retention time (in min); ordinate, intensity (in cps).

separation of the enantiomers from each other (see Table 3) and the level of sensitivity required for bioavailability studies make this chiral HPLC–MS–MS method ideal for simultaneous, direct determination of oxybutynin and N-desethyloxybutynin optical isomers.

3.5. The interfacing of normal-phase HPLC to a heated nebulizer atmospheric pressure chemical ionization MS–MS

Although chiral HPLC–MS–MS was utilized earlier in bioanalysis of both metoprolol and terbutaline as examples [6,7], an effective but rather sophisticated column-switching instrumental set-up was required to address the problem of mobile phase incompatibility. These previous works, as well as the efforts of other groups [8,9], used either protein- or cyclodextrin-based CSPs for separation and either thermospray (TSP) or direct liquid interface (DLI) as the MS interface. Difficulties with either the flow-rate of the mobile phase or its composition were central to problems in the early LC–MS work and the work with CSPs was no exception. The widespread application of API-MS–MS has reduced the problems with mobile phase flow-rates but mobile phase salts must still be of the volatile variety, hence the continued difficulties with the use of protein columns in LC–MS–MS, even when the API mode is employed for ionization. Moreover, while protein-based CSPs can separate enantiomers of a given compound, they can have difficulty with the simultaneous chiral separation of the parent compound and its metabolites. The advantage of Chiralpak AD is that, unlike most of the protein-based CSPs, it can often separate the enantiomers of both drug and metabolite in a single chromatographic run and without the use of non-volatile additives.

The compounds tested were sensitive to front-end collision-induced dissociation; therefore, the orifice voltage was set to 0 V, relative to the skimmer, which is set at ground. The difference between the offset voltages of the first quadrupole (Q0) and the quadrupole in the collision cell (RO2) is related to the ion energy of the parent ion in eV. For example, a Q0 setting of 10 V and an RO2 setting of 40 V would result in an ion energy for the parent ion of 30 eV. The appearance of a specific daughter ion, as well as its abundance, is greatly dependent on the ion energy before collision with the buffer gas in the collision cell. In the design of an HPLC–MS–MS method, it is important, as a guide to daughter-ion selection, to study the appearance and abundance of daughter ions from the parent as a function of the parent ion's energy. In the mass spectrometer used in this work, such an experiment is performed by ramping the offset voltage (RO2) applied to the quadrupole in the collision cell. This experiment will be referred to as an RO2 ramp.

Initially, the RO2 ramp is conducted in the scan mode and spectra are acquired at a number of ion energies. A note is taken of the major daughter ions produced at all ion energies. A subsequent RO2 ramp is performed in the multiple reaction monitoring (MRM) mode for the daughter ions recorded above. This process is repeated for all compounds under study, and parent ion–daughter ion pairs were established based on investigation of the daughter ion spectra and the RO2 ramp for all major observed daughter ions. These data are presented as a plot of abundace vs. RO2 volts (ion energy). MRM chromatograms for each compound were subsequently recorded.

API-MS–MS is usually performed with water as one of the mobile phase constituents. The direct introduction of a mobile phase consisting predomi-

Fig. 6. Simultaneous determination of enantiomers of oxybutynin and N-desethyloxybutynin by chiral HPLC–MS–MS. Extracted ion chromatograms for blank plasma (A2) and extracted spiked plasma sample (A1). Plasma was spiked with *rac*-oxybutynin at the level of 150 pg ml^{-1} per enantiomer and with *rac*-N-desethyloxybutynin at the level of 750 pg ml^{-1} per enantiomer. The mobile phase used was *n*-hexane–2-propanol–diethylamine (90:10:0.1, v/v/v) at a flow-rate of 0.2 ml min^{-1} and a column temperature of 22°C. The parent–daughter ion pairs monitored were 358.4/142.2 for oxybutynin and 330.4/94.0 for N-desethyloxybutynin. (B) Extracted ion chromatogram after injection of 10 μl of reference solution containing *rac*-oxybutynin (100 ng ml^{-1}) and *rac*-N-desethyloxybutynin (500 ng ml^{-1}), i.e. 0.5 ng of each oxybutynin enantiomer and 2.5 ng of each N-desethyloxybutynin enantiomer on column, respectively. The mobile phase used was *n*-hexane–2-propanol–diethylamine (90:10:0.1, v/v/v) at 0.15 ml min^{-1} and the column temperature was kept at 20°C. The parent–daughter ion pairs monitored were 358.0/142.0 for oxybutynin and 330.0/230.0 for N-desethyloxybutynin. The collision cell offset voltage was −33 V. Abscissa, retention time (in min); ordinate, intensity (in cps).

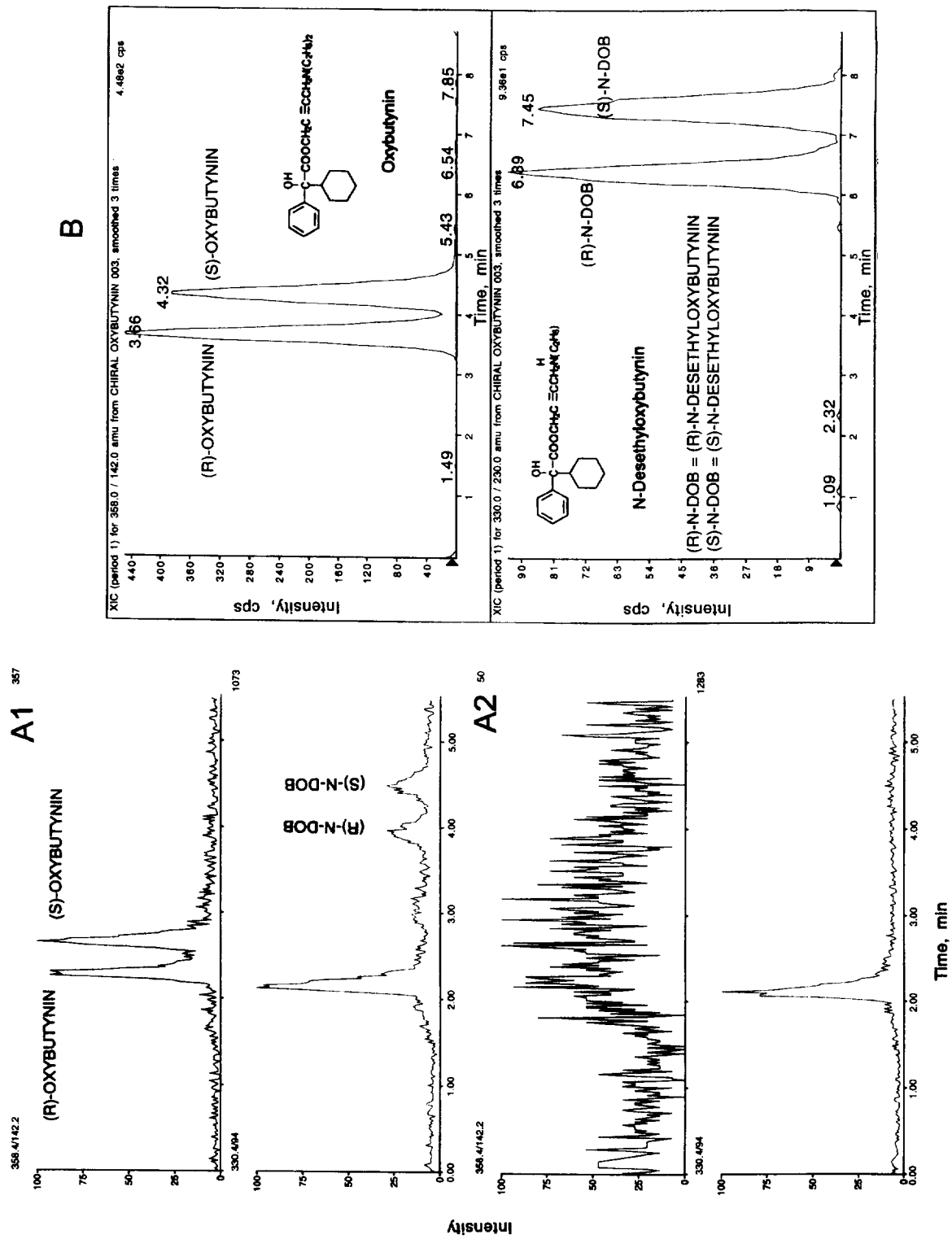
A1
(R)-OXYBUTYNIN
(S)-OXYBUTYNIN
(R)-N-DOB
(S)-N-DOB
A2
Intensity
Time, min
B
XIC (period 1) for 358.0 / 142.0 amu from CHIRAL OXYBUTYNIN 003, smoothed 3 times
(R)-OXYBUTYNIN
(S)-OXYBUTYNIN
Oxybutynin
XIC (period 1) for 330.0 / 230.0 amu from CHIRAL OXYBUTYNIN 003, smoothed 3 times
(R)-N-DOB
(S)-N-DOB
N-Desethyloxybutynin
(R)-N-DOB = (R)-N-DESETHYLOXYBUTYNIN
(S)-N-DOB = (S)-N-DESETHYLOXYBUTYNIN
Intensity, cps
Time, min

Table 3
Chiral HPLC–MS–MS assay for oxybutynin and N-desethyloxybutynin: chromatographic parameters and conditions

Compound	t_R (min)	k'	α	R_s
Oxybutynin	3.66	0.83	1.43	1.54
N-Desethyloxybutynin	6.49	2.25	1.26	1.92

Mobile phase: *n*-hexane–2-propanol–diethylamine (90:10:0.1, v/v/v) at a flow-rate of 0.15 ml min^{-1}. Column temperature: 20°C. t_R = retention time of first eluted enantiomer, k' = capacity factor of first eluted enantiomer, α = separation factor, R_s = stereochemical resolution. Injected: 10 μl of reference solution containing *rac*-oxybutynin (100 ng ml^{-1}) and *rac*-N-desethyloxybutynin (500 ng ml^{-1}), i.e. 1 ng of *rac*-oxybutynin and 5 ng of *rac*-N-desethyloxybutynin on-column. Chromatographic column: Chiralpak AD, 100×2.1 mm I.D., 10 μm.

nantly of hexane into a heated high-voltage corona discharge environment can present an explosion hazard, especially if air is used as an auxiliary gas. In order to take advantage of the unique chiral separation properties of the Chiralpak AD stationary phase, chromatography must be carried out in the normal-phase mode. However, it is possible to couple a normal-phase chromatographic system to an API-MS–MS system and to remove the explosion hazard by using nitrogen as an auxiliary and nebulizer gas and by the post-column addition of a solution containing significant proportions of water with a second organic solvent added to overcome the problem with miscibility. In addition, normal-phase systems do not easily incorporate the ion concentrations conventionally required to sustain ionization in the API source. The post-column reagent addition scheme described in this paper (Fig. 1) can address this issue satisfactorily.

A mixture containing 25% 0.025 *M* ammonium acetate in water and 75% 2-propanol as a post-column addition reagent provides both miscibility with mobile phases consisting of up to 95% hexane at the flow-rates described and also provides the ion concentrations often needed to promote smooth ionisation in API [31]. The mixture was arrived at by mixing 1 ml of hexane with 4 ml of various mixtures of 2-propanol–0.025 *M* aqueous ammonium acetate and observing the degree of miscibility. The system that was chosen allowed the inclusion of the greatest percentage of water, while not effecting complete miscibility.

Since the API source for the SCIEX API 300 performs optimally at flow-rates between 0.2 and 1 ml min^{-1} total flow into the nebulizer, it becomes essential that column flow-rates are not much higher than about 0.2 ml min^{-1} with the post-column reagent added at a ratio of 4:1 to the mobile phase. This flow regime is consistent with optimum efficiencies in 2 mm I.D. column geometries, as a result, Chiralpak AD was custom-packed into a 2-mm I.D. format. When the 0.2 ml min^{-1} flow-rate from the column was added to the 0.8 ml min^{-1} rate of the post-column addition pump, the total flow-rate and composition of the resulting effluent was entirely compatible with HPLC–API-MS–MS, as performed on the SCIEX API 300. The combined use of nitrogen as both the auxiliary and the nebulizer gas as well as the inclusion of a significant portion of water in the mobile phase has eliminated the occurrence of any explosions in the source housing. In addition, the ionization of analytes proceeded smoothly, as evidenced by relatively low noise throughout the chromatograms.

The validation process for the above-mentioned chiral assays is underway in our laboratory and the results of the bioavailability studies will be published separately.

4. Conclusions

Normal-phase chiral HPLC was successfully interfaced to API-MS–MS by the use of custom-packed small-bore (100×2.1 mm I.D.) Chiralpak AD chromatographic columns and post-column reagent addition.

The explosion hazard of introducing a mobile phase of hexane through a heated nebulizer at ca. 400°C into the MS–MS system was overcome by introducing post-column the make-up mobile phase. This reagent, consisting of 2-propanol–0.025 m*M* ammonium acetate (75:25, v/v) at a flow-rate of 0.8 ml min^{-1} was completely miscible with the eluate, made up predominantly of hexane, at a flow-rate of 0.2 ml min^{-1} from the chiral column.

This approach enabled the development of several simple and elegant chiral bioanalytical assays without the need for derivatization, without column and/or mobile phase switching, with chromatographic run times that were shorter than in conventional LC and

at detection levels that were below those achieved by conventional chromatography with UV or fluorescence detection.

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