Anal. Chem. **1999,** *71,* 2838−2843

Directly Coupled Chiral HPLC−NMR and HPLC−CD Spectroscopy as Complementary Methods for Structural and Enantiomeric Isomer Identification: Application to Atracurium Besylate

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Directly coupled HPLC−NMR spectroscopy has now become a standard, commercially available technique for mixture characterization. Here the extension of the technique to chiral HPLC separation is reported and it is shown that HPLC−NMR together with HPLC−CD provide complementary approaches for the identification of structural isomers and enantiomers. The general approach has been exemplified using the neuromuscular blocking agent atracurium besylate, which comprises a mixture of 10 isomers in various proportions as four racemic pairs and two meso compounds. Diagnostic reporter resonances in the ^{1}H NMR spectrum of atracurium besylate were assigned using a combination of one-dimensional and two-dimensional NMR experiments at 750 MHz. Stop-flow 750-MHz ^{1}H NMR spectroscopy was used on-line after chiral column HPLC separation to identify the enantiomeric pairs, to distinguish the meso compounds, and to identify key configurational features of the isomers. The parallel HPLC−CD experiments served to assign the enantiomers based upon the known CD and absolute stereochemistry of (*R*)-laudanosine hydrochloride, an analogue with the same tetrahydroisoquinoline structural unit as atracurium. It is thereby demonstrated that high-field HPLC−NMR and HPLC−CD is a powerful combination of techniques which could be combined on-line for mixture characterization.

Many pharmaceutical products are chiral molecules either as single isomers or more commonly as racemic mixtures. The pressure to develop single isomer forms as therapeutics in preference to racemic mixtures arises from the fact that one enantiomer is usually more biologically active than the other and that enantiomers can have very different toxicity profiles. Consequently, it is becoming common for drug substances to be registered as single-isomer materials.

Chromatographic techniques used to investigate the enantiomeric proportions of substances include diastereoisomer formation, use of chiral mobile-phase additives such as cyclodextrins, and chiral stationary phases. There are many different types of chiral columns available and chiral HPLC is a very sensitive technique that can detect low levels of isomers depending on the chromatographic resolution.[1,2] Chiral HPLC is often carried out using low flow rates to improve the resolution since high column loading will often affect the separation achieved.

HPLC−NMR spectroscopic approaches have been reviewed[3,4] and have been applied to the characterization of individual components in mixtures including drug metabolites,[3,5] drug substances,[6,7] chemical mixtures,[8] and natural products.[9] Conventional reversed-phase HPLC has been employed to separate and characterize chemically and structurally different components. In this study, chiral HPLC is combined on-line with NMR to demonstrate the application of chiral HPLC−NMR spectroscopy to the separation and characterization of different isomers present in a drug substance using, as an example, atracurium besylate (**1**), a neuromuscular blocking agent used widely in surgery.

Atracurium besylate (2,2′-(3,11-dioxo-4,10-dioxatridecamethylene)bis(2-methyl-1,2,3,4-tetrahydropapaverinium benzenesulfonate) is prepared from racemic 1,2,3,4-tetrahydropapaverine and has four

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10.1021/ac990132e CCC: $18.00
Published on Web 05/26/1999

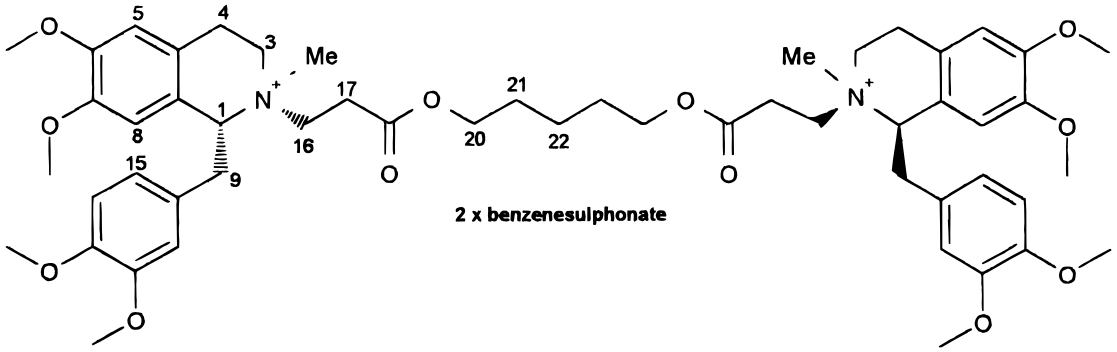

Figure 1. Structure of atracurium besylate (**1**). Only the *R*-cis/*R*-cis isomer is shown. The material is formulated as a mixture of cis and trans isomers (see text for definition) with (*R*)- and (*S*)-tetrahydroisoquinoline residues.

chiral centers. However, because of the symmetry of the molecule, atracurium has 10 distinct species. The structure of **1** is given in Figure 1 where the configuration at C1 can be *R* or *S*. Since the final stage of synthesis is quaternization at N2, the isomers have been distinguished, simply for convenience, by the configuration of the substituents at the C1−N2 bond, such that when a tetrahydroisoquinoline residue has the benzyl group at C1 and, arbitrarily, the 3,11-dioxo-4,10-dioxatridecamethylene chain in a cis configuration, this is called a "cis" residue.[10] Thus Figure 1 shows **1** in the *R*-cis/*R*-cis isomer form only.

In achiral media, different NMR spectra are expected for each of the four types of enantiomeric pairs and for the two meso compounds. In general, because of the synthetic approach,[10] the ratio of cis to trans residues is ∼3.07 which, assuming that quaternization at one tetrahydroisoquinoline residue does not affect quaternization at the other, leads to proportions of cis−cis, cis−trans, and trans−trans isomers in the ratio of 10.5:6.2:1. The preference for cis residues has been proved using NOE NMR measurements on similar compounds[11] and also by X-ray crystallography on related substances.[12] It has also been found[10] that in nonpolar solvents the two ends of the atracurium molecule can interact such that the ^{1}H NMR chemical shifts, principally of the H8 proton, can be affected not only by whether the C1−N2 configuration in the residue is cis or trans but also by the configuration of the remote tetrahydroisoquinoline unit.

In summary, therefore, after on-line chiral HPLC separation, NMR spectroscopy is used to characterize compounds in terms of the cis and trans isomers and to identify the racemic pairs on the basis of their identical NMR spectra. In addition, HPLC−CD is used to identify the absolute configuration of the enantiomers on the basis of the known CD spectrum of (*R*)-laudanosine hydrochloride, a closely related molecule. The application of HPLC separation with on-line CD detection to pharmaceutical analysis has been the subject of one publication.[13]

EXPERIMENTAL SECTION

Materials. Deuterium oxide (Fluorochem Ltd., Glossop, U.K.), acetonitrile (ACN) Pestanal grade (Riedel de Haan, Seelze, Germany) and sodium perchlorate (Aldrich, Gillingham, U.K.) were obtained commercially. The sample of atracurium besylate was obtained from GlaxoWellcome (Dartford, U.K.), and a reference sample of (*R*)-laudanosine hydrochloride was from Aldrich, UK.

Analytical Chromatography. Chiral HPLC was performed using a Varian 9012 HPLC pump and a Varian 9060 diode array UV detector operated at 280 nm and controlled using Varian STAR chromatography. The outlet of the UV detector was connected to the HPLC−NMR flow probe via an inert polyether(ether)ketone (PEEK) capillary. A column oven was used to enable separation at 60 °C. It was not possible to get chromatographic baseline resolution of all 10 isomers of atracurium, but sufficient resolution was achieved to carry out HPLC−NMR. Analysis was performed using two 250 × 4.6 mm, 5-μm Chiracel OD-H columns connected to a Chiracel OD guard column of 50 × 4.6 mm i.d. (Daicel Chemical Industries, Ltd.). This column is based on a cellulose tris(3,5-dimethylphenyl carbamate) phase coated on a 5-μm silica gel substrate that contains chiral cavities having a high affinity for aromatic groups. For the chiral HPLC−NMR analysis, 10 mg of atracurium was dissolved in 1 mL of ACN/$NaClO_4$ buffer (2:5). The buffer was 0.5 M sodium perchlorate, adjusted to pH 2.0 with perchloric acid. Typically 20 μL of sample was injected using a flow rate of 0.3 mL/min. Isocratic elution was performed using 60:40 (v/v) 0.5 M $NaClO_4$ buffer (pH 2.0)/ACN.

NMR Spectroscopy. A full assignment of the ^{1}H NMR spectrum of the atracurium besylate mixture sample was achieved using one- and two-dimensional NMR methods including ^{1}H−^{1}H total correlation spectra (TOCSY), ^{1}H−^{13}C heteronuclear single quantum coherence spectra (HSQC), and gradient-enhanced ^{1}H−^{13}C heteronuclear multiple-quantum coherence spectra based on long-range couplings (GHMBC) using a Varian INOVA-750 NMR spectrometer with ^{1}H observation at 750 MHz.

The TOCSY spectrum was acquired with a solvent preirradiation time of 1.5 s and a spin-lock mixing time of 40 ms using 400 increments of 8 transients each. Free induction decays (FIDs) were collected into 4K data points with a spectral width of 10 kHz and an acquisition time of 0.2 s. The HSQC experiment was acquired with a 1-s preirradiation time using 256 increments of 64 transients each. FIDs were collected into 2K data points with a spectral width of 10 kHz and an acquisition time of 0.1 s. The GHMBC experiment was acquired using 256 increments of 256 transients after suppression of the solvent signals using a 1-s preirradiation. FIDs were collected into 4K data points with a spectral width of 10 kHz and an acquisition time of 0.25 s.

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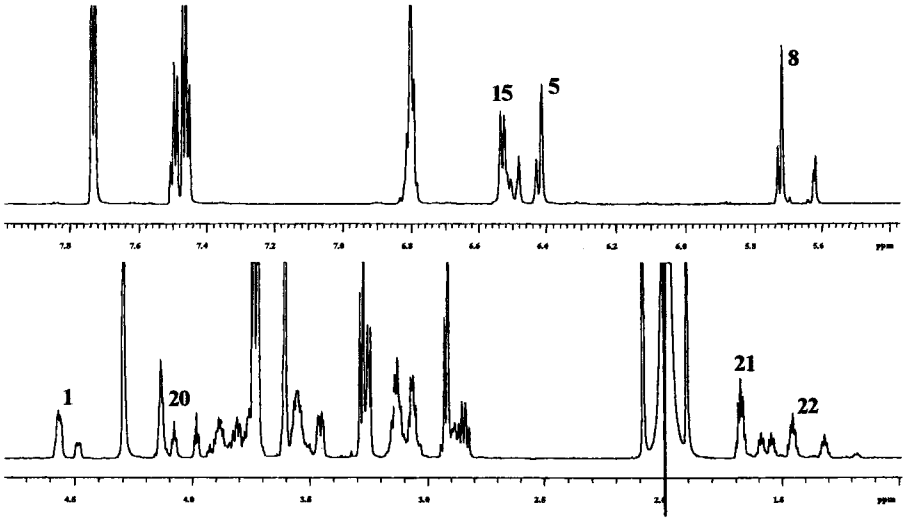

Figure 2. ^{1}H NMR spectrum of atracurium besylate isomer mixture at 750 MHz.

HPLC−NMR Spectroscopy. The HPLC−NMR spectroscopic data were acquired using a Varian Inova-750 NMR spectrometer equipped with an indirect detection z-gradient ^{1}H flow probe (4-mm-i.d. cell of 65-μL volume). ^{1}H NMR spectra were obtained at 750 MHz in the stop-flow mode. Suppression of the solvent signals was achieved using a pulse sequence based on the first increment of a two-dimensional NOESY experiment (Varian Instruments Ltd.) with dual-frequency irradiation for suppression of both the residual water and acetonitrile signals for 1.5 s before the first 90° pulse. FIDs were collected into 64K data points with a spectral width of 15 kHz and an acquisition time of 2 s. NMR spectra were acquired using between 512 and 2048 transients depending on the peak intensity. Prior to Fourier transformation, an exponential apodization function was applied to the FID corresponding to a line broadening of 1 Hz.

HPLC−CD Spectroscopy. The separation was performed using a Hewlett-Packard 1050 HPLC system with a Jones column oven/chiller. As for the HPLC−NMR experiments, the chiral stationary phase, Chiracel OD-H, was contained in two 25 cm × 4.6 mm analytical columns closely coupled in series and preceded by a similarly packed 5-cm guard column, all held at 60 °C. An isocratic mobile phase of 40:60 (v/v) acetonitrile/aqueous sodium perchlorate (0.5 M, pH 2) was employed at a flow rate of 0.3 mL/min and prewarmed to a temperature of 60 °C, using a 20-μL injection of a 10 mg/mL aqueous solution of atracurium besylate. The CD and UV detection were achieved using a Jasco J-600 CD spectrometer with a specially constructed Jasco HPLC cell (5-mm path length, 2-mm aperture, 16-μL volume), including quartz doublet focusing and defocusing optics, connected to the HPLC column via a PEEK capillary. Spectroscopic detection was performed at 236 nm (at which the atracurium isomers have a CD peak), using an effective spectral bandwidth of 3 nm achieved through the use of manual slit widths, a time constant of 8 s, a data interval of 5 s, and sensitivity of 10 mdeg. The instrument was continually flushed with at least 15 L/min evaporated dry nitrogen to ensure optimal spectroscopic performance. CD spectra of the individual components were likewise acquired using the Jasco J-600 spectrometer by stopping the flow at chromatographic peaks. Instrumental parameters for such spectra were 320−200-nm wavelength range, 1-nm spectral bandwidth, 50 nm/min scan speed, 2-s time constant, 0.1-nm data interval, and with a single accumulation. All spectra were baseline corrected by subtraction of a corresponding spectrum for the mobile phase alone acquired under equivalent stop-flow conditions.

RESULTS

Assignment of the ^{1}H NMR Spectrum of Atracurium Besylate. The ^{1}H NMR spectrum of atracurium besylate was fully assigned and this is shown in Figure 2 with key assignments marked. Initial assignments were made by inspection of the ^{1}H NMR spectrum as far as possible. This was further supplemented by TOCSY, HSQC, and GHMBC data.

From the ^{1}H NMR spectrum it was possible to resolve and assign different chemical shifts for the cis−cis, cis−trans, trans−cis, and trans−trans isomers for various protons in the molecule. The HSQC spectrum, given in Figure 3, was particularly useful in making these assignments since the ^{13}C NMR chemical shifts of atracurium besylate were less sensitive to the different configurations, often resulting in only a single peak for each of the carbon nuclei.

A ^{1}H−^{1}H TOCSY spectrum was especially useful in confirming the assignments of H20, H21, H22, and H9 from the distinctive correlations observed. The GHMBC spectrum was used to confirm the assignment of H16 and H17, which gave correlations to the carbonyl carbon at position 18. The full assignment of the ^{1}H NMR spectrum of atracurium besylate at 750 MHz is given in Table 1, and these results are consistent with the partial assignment made previously at a much lower ^{1}H NMR observation frequency.[10]

HPLC−NMR Spectroscopy. The isocratic chiral HPLC method was developed in order to separate the 10 different enantiomers in atracurium besylate. Figure 4a shows the resulting chromatogram giving good resolution for of 9 out of the 10 isomers. These did not elute until after a retention time of ∼60 min. All 10 of the isomers then eluted in the next 40 min. This chromatogram also shows some early-eluting substances in the atracurium besylate mixture which were seen at retention times between 20 and 35 min. These peaks are minor impurities or degradation products related to atracurium.

Expansions of key reporter resonances in the 750-MHz ^{1}H NMR spectra obtained in stop-flow mode are given in Figure 5. It

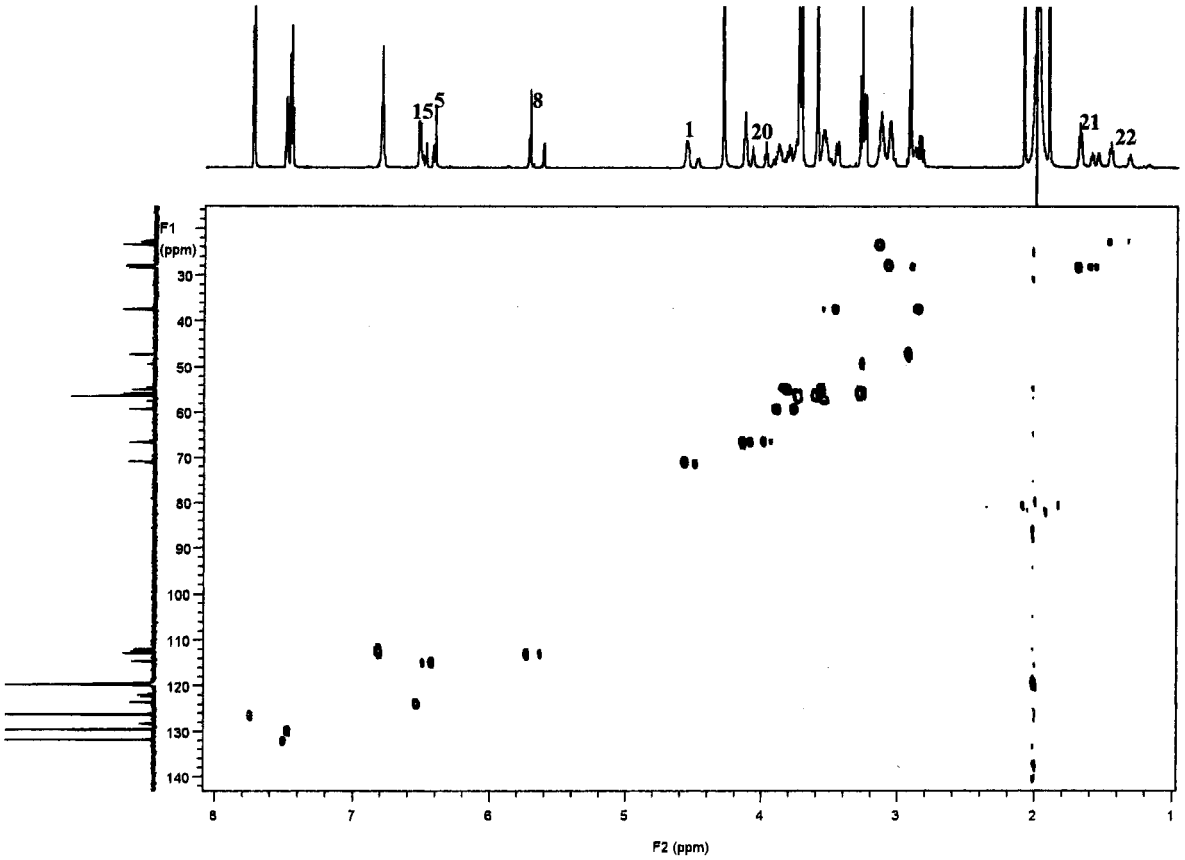

Figure 3. Two-dimensional $^{1}H-^{13}C$ HSQC NMR spectrum, contour plot, of atracurium besylate with corresponding ^{1}H and ^{13}C NMR spectra on the horizontal and vertical axes. This demonstrates that the ^{13}C NMR chemical shifts are not as sensitive to changes in configuration as the ^{1}H NMR chemical shifts.

Table 1. Assignments of the Chemical Shifts (δ, ppm) and Representative Spin Coupling Constants (J, Hz) in the ^{1}H NMR Spectrum of Atracurium Besylate at 750 MHz[a]

		cis−trans			
assign-ment	cis−cis	cis residues	trans residues	trans−trans	multiplicity
H1	4.57			4.49	m
H3	3.81			3.55	m
H4	3.13			3.13	m
H5	6.41	6.43	6.48	6.51	s
H8	5.72	5.73	5.62	5.63	s
H9, H9′	3.45, 2.85			3.55, 2.89	m
H11	6.80 (8.1)			6.80 (8.1)	s
H14	6.80 (8.1)			6.80 (8.1)	d
H15	6.53			6.53	d
H16, H16′	3.76, 3.89			3.76, 3.89	
H17	3.07 (6.8)			2.89	t
H20	4.14 (6.6)	4.08 (6.9)	3.98 (6.3)	3.93 (6.2)	t
H21	1.68	1.55	1.59	1.47	m
H22	1.46		1.32	1.18	m

[a] The cis−trans isomer shifts are given only in the instances where they are resolved from the cis−cis and trans−trans isomer peaks. These are not resolved for all the peaks in the spectrum. Besylate aromatic ^{1}H NMR resonances appear at δ 7.46, 7.50, and 7.73.

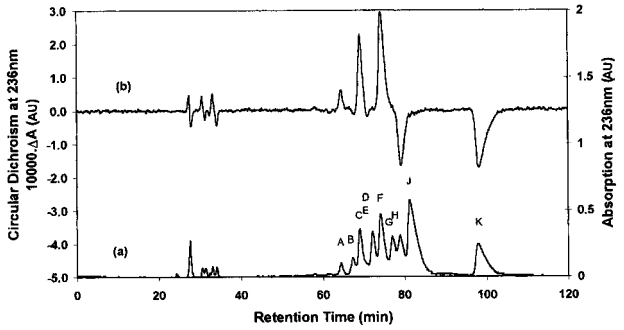

Figure 4. (a) HPLC separation of the atracurium mixture, using UV detection at 280 nm. Elution was isocratic using 60:40 $NaClO_4$ buffer in D_2O adjusted to pH 2.0 with perchloric acid/acetonitrile at 60 °C and using two 250 × 4.6 mm, 5-μm Daicel Chiracel OD-H columns connected to a Daicel Chiracel OD guard column (50 × 4.6 mm i.d.). (b) HPLC separation of the atracurium mixture, using CD detection at 236 nm and with the same chromatographic conditions as for UV detection. The left-hand vertical scale refers to the CD detection, and the right-hand vertical scale refers to the UV detection.

is clear from the chiral HPLC chromatogram that although some resolution of 9 out of the 10 peaks has been achieved, most of the peaks show considerable overlap. It might therefore have been expected that NMR spectra produced from the chiral HPLC−NMR experiments would not show clean single isomers. However, it has been found that these NMR spectra were in general remarkably pure. Although the HPLC peaks were in some cases as much as 8 min wide, the NMR flow probe used in this work only had a 65-μL flow cell. Hence, only a small slice from the center of each HPLC peak was collected in the NMR flow cell for spectral data acquisition, giving maximum probability of a spectrum uncontaminated by other components.

Peaks A, B, and D are identified as the trans−trans isomers by NMR. This is because not only are they the smallest peaks in

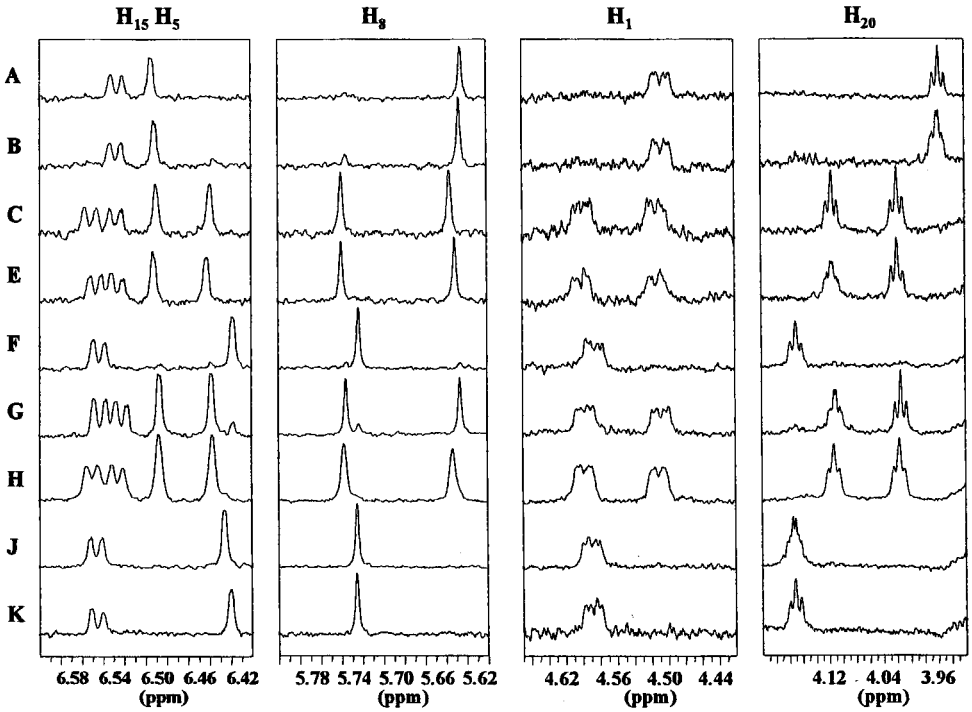

Figure 5. Expansions of ^{1}H NMR spectra for key peaks in the HPLC−NMR spectra for the different isomers.

the chromatogram but they also give a high-field shift for a single H8 proton at δ ~5.62 which is consistent with the assignments of the trans isomers from the whole mixture.[10] Peaks F, J, and K are identified as the cis isomers. These peaks are the largest in the HPLC chromatogram and give a single lower field ^{1}H chemical shift for the H8 proton at δ 5.72 which is also consistent with the assignments of the cis isomers from the whole mixture.[10] This leaves peaks C, E, G, and H, which have been identified as the cis−trans isomers because they each give two H8 resonances in their spectra for the different cis and trans parts of the isomer. Other key resonances in the ^{1}H NMR spectrum including H5, H1, H20, H21, and H22 also show differences which are consistent with the assignments made on the whole mixture to confirm the stereochemistry. The identification of the enantiomeric pair from the three cis−cis isomers was possible from the NMR spectra in that the spectra from components F and K were essentially identical and that from component J showed distinct chemical shift differences (e.g., as indicated by resonances for H20 in particular; see Figure 5). From this it can be deduced that peaks F and K were the *R*/cis−*R*/cis, *S*/cis−*S*/cis enantiomeric pair and that peak J was the *R*/cis−*S*/cis meso compound. Similarly, it could be shown that peaks C and H formed a cis-trans enantiomeric pair as did peaks E and G (as indicated by resonances for H5 and H20, in particular, as seen in Figure 5).

Finally, although good NMR spectra of only two out of the three trans−trans isomers were obtained (peaks A and B), these were clearly different and thus one is from the meso compound and the other is one of the enantiomeric pair isomers.

HPLC−CD Spectroscopy. The results from this experiment are given in Figure 4b. This shows a HPLC−CD trace on the same time scale as the UV chromatogram. The result was a series of CD peaks, some of which were positive, others negative, and some of which gave virtually no CD spectrum. An additional CD spectrum was also collected on a reference compound (*R*)-laudanosine hydrochloride, which gave a negative CD spectrum. From this it was therefore possible to confirm that, for the previously identified cis−cis isomers, peak F had *S*/*S* configuration, peak K was *R*/*R*, and peak J was the *R*/*S*-meso compound. Similar arguments could be applied to the peaks from the trans−trans isomers in the chromatogram in that peak A was *S*/*S*, peak D was *R*/*R*, and peak B was the *R*/*S*-meso compound. Finally, for the cis−trans isomers, again in agreement with the NMR results, peak C was *S*/*S*, peak E was *R*/*S*, peak G was *R*/*S*, and peak H was *R*/*R*. The CD spectra were insensitive to the nature of the configuration of the isomers at the C1−N2 bond. The identity of each isomer is given in Table 2 together with HPLC retention times and HPLC UV peak areas.

Table 2. Chromatographic Retention Times and Identities Confirmed by Chiral HPLC−NMR and HPLC−CD[a]

peak	retention time (min)	HPLC peak area (%)	isomer by NMR	sign of CD spectrum	identity
A	61.95	2.09	trans−trans	+	*S*-trans/*S*-trans
B	64.72	2.89	trans−trans	0	*R*-trans/*S*-trans
C	66.08	9.61	cis−trans	+	*S*-cis/*S*-trans
D[a]	68.39	1.30	trans−trans	−	*R*-trans/*R*-trans
E	69.22	8.75	cis−trans	0	*R*-cis/*S*-trans
F	70.99	14.81	cis−cis	+	*S*-cis/*S*-cis
G	74.09	8.49	trans−cis	0	*R*-trans/*S*-cis
H	76.02	9.40	cis−trans	−	*R*-cis/*R*-trans
J	78.00	28.29	cis−cis	0	*R*-cis/*S*-cis
K	94.90	14.37	cis−cis	−	*R*-cis/*R*-cis

[a] The identity of this peak was assigned by elimination since insufficient chromatographic resolution precluded acquisition of a clean HPLC−NMR spectrum. Isomer peaks giving a negative CD spectrum were assigned the *R*/*R* configuration by comparison with a closely related model compound, (*R*)-laudanosine hydrochloride.

Table 3. Table of Assignments for Key Protons in the Chiral HPLC−NMR Spectra of the Different Isomer Peaks

	^{1}H chemical shifts[a] (δ, ppm)				
isomer	H15	H5	H8	H1	H20
A	6.51 d	6.46 s	5.61 s	4.50 dd	3.93 t
B	6.51 d	6.46 s	5.61 s	4.50 dd	3.93 td
C	6.51 d	6.46 s	5.71 s	4.56 dd	4.10 t
	6.55 d	6.41 s	5.61 s	4.50 dd	4.00 t
E	6.51 d	6.46 s	5.71 s	4.56 dd	4.10 td
	6.55 d	6.41 s	5.61 s	4.50 dd	4.00 t
F	6.55 d	6.41 s	5.71 s	4.56 dd	4.15 t
G	6.51 d	6.46 s	5.71 s	4.56 dd	4.10 td
	6.55 d	6.41 s	5.61 s	4.50 dd	4.00 t
H	6.51 d	6.46 s	5.71 s	4.56 dd	4.10 t
	6.55 d	6.41 s	5.61 s	4.50 dd	4.00 t
J	6.55 d	6.41 s	5.71 s	4.56 dd	4.15 m
K	6.55 d	6.41 s	5.71 s	4.56 dd	4.15 t

[a] s, d, dd, td, and m, singlet, doublet, doublet of doublets, triplet of doublet,s and multiplet, respectively.

DISCUSSION

This study demonstrates the feasibility for the first time of directly coupled chiral HPLC−NMR spectroscopy, which has been applied to the separation and identification of the 10 isomers of the neuromuscular blocking agent atracurium besylate. The HPLC−NMR spectroscopy was useful for identifying the isomeric configuration at the C1−N2 bond, for identifying the enantiomeric pairs of compounds, and for distinguishing them from the meso forms. The HPLC−CD experiments were complementary in that, while unable to distinguish the C1−N2 isomers (cis or trans), it was possible to determine the absolute stereochemistry at C1 at each tetrahydroisoquinoline residue as *R*/*R*, *S*/*S*, or *R*/*S* on the basis of the sign of the CD response at a chosen wavelength. A consistent finding was that the *S* isomers eluted before the *R* isomers and the trans forms eluted before the cis forms. By these means, a full characterization of all of the 10 isomers of atracurium has been achieved. This approach offers a new method for identification of components of complex pharmaceutical and natural product mixtures and may also be important in studies of racemization of drug metabolites, a process that often occurs in vivo.

Received for review February 10, 1999. Accepted February 20, 1999.

AC990132E