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Direct enantiomeric purity determination of the chiral anesthetic drug bupivacaine by ^{1}H NMR spectroscopy

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The direct determination of the enantiomeric purity of the chiral anaesthetic drug bupivacaine has been developed using ^{1}H NMR (400 MHz) spectroscopy with a chiral solvating agent. Optimization of experimental conditions in terms of temperature, substrate concentration and solvating agent to substrate molar ratio provided two significant signal splittings for chiral recognition resulting from diastereomeric solvation. Based on the relative intensities of the aliphatic methyl resonances assigned to (*S*)-(−)- and (*R*)-(+)-bupivacaine, the analysis of synthetic mixtures of the enantiomers by the proposed NMR method resulted in assay values which agreed closely with the known quantities of each enantiomer in the mixtures tested. The mean ± SD recovery values for the (*R*)-(+)-enantiomer was 100.0 ± 0.6% of added antipode (n = 7). The optically pure enantiomers were used to establish the minimum sensitivity of the NMR spectroscopic method of chiral analysis.

1. Introduction

Bupivacaine, 1-butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide, is a local anesthetic that produces reversible impulses by preventing the propagation of action potentials in the axons of autonomic, sensory, and motor nerve fibers. Bupivacaine is composed of a lipophilic benzene ring connected to a hydrophilic tertiary amine by means of a hydrocarbon chain and an amide linkage. It is used for infiltration, nerve block, spinal and epidural anesthesia [1, 2].

Bupivacaine is a chiral compound because the carbon atom bonded to the tertiary amine is chiral. The enantiomers of a chiral drug generally differ in pharmacodynamic and/or pharmacokinetic properties as a consequence of stereoselective interaction with optically active biological macromolecules [3–13]. Recently, the medical disciplines have developed a renewed interest in the implication of stereoisomerism to drug action and disposition. In bupivacaine both enantiomers are local anesthetics but only the (*S*)-(−)-enantiomer has an additional vasoconstrictor action. Accordingly, the efficacy of the (*S*)-(−)-enantiomer is greater than that of the non-vasoconstrictor (*R*)-(+)-enantiomer [14]. This increases the local anaesthetic efficacy of the racemate by prolonging its action. It has been reported also that the systemic disposition of bupivacaine is enantioselective [15]. The enantioselectivity was due to a difference in the degree of plasma binding of the enantiomers. The development of facile enantiospecific and accurate analytical methods capable of identifying and quantifying the enantiomers for purity assessment is pivotal.

Separation of bupivacaine enantiomers on alpha-acid glycoprotein HPLC columns has been reported [16–21]. Since several parameters had to be optimized, the method development takes a long time. In addition, chiral columns, highly purified solvents and other chemicals can be very costly. NMR approaches have been found as appropriate for enantiomeric purity determination [22–26]. Pharmacopoeia commissions in the US and Europe have recently introduced NMR spectroscopic methods for the determination of isomeric composition and enantiomeric purity of drugs, because these methods were found superior to the methods of specific optical rotation used in all pharmacopoeias [27]. The NMR methods are robust and the time of analysis is short because the NMR instruments are always ready for measurement, without any proceeding conditioning or calibration procedure being necessary [27]. The purpose of this report is to describe a ^{1}H NMR spectroscopic method for the direct determination of the enantiomeric composition of bupivacaine. The required resolution of the enantiomeric resonance lines is accomplished through the use of a chiral solvating agent.

2. Investigations, results and discussion

Due to the close proximity of the trifluoromethyl group, the carbinol group of TFAE is a sufficiently acidic function to interact strongly with a hydrogen bond receptor in the bupivacaine enantiomers. The trifluoromethyl, hydroxyl and anthryl groups of TFEA are directing an electric dipole roughly along the methine C-H bond axis to render the methine hydrogen slightly acidic and, thus, amenable to additional interaction with a secondary basic site in the bupivacaine molecule. The primary and secondary basic sites in bupivacaine are the carbonyl oxygen and amide nitrogen atoms, respectively. Consequently, two such points of interactions will engender greater populations of specific, short-lived, chelate-like, diastereomeric solvated conformers. The anthryl moiety with its high diamagnetic anisotropy in the vicinity of its stereogenic center is able to translate different average spatial environments around the enantiomeric solute nuclei into spectral lines with different chemical shifts. This effect is expected to be substantial in view of the two possible spatial orientations the anthryl moiety can adopt relative to the functional groups under its influence.

Fig. 1a shows the upfield region of a 400 MHz ^{1}H NMR spectrum of a mixture of (*S*)-(−)- and (*R*)-(+)-bupivacaine enriched in the (*R*)-(+)-enantiomer. The triplet centered at ca 0.91 ppm and the singlet at ca 2.24 ppm represent the unresolved enantiomeric signals of the aliphatic chain methyl and the methyls on the phenyl group, respectively. Fig. 1b shows the corresponding upfield region of the diastereomeric solvates of bupivacaine enantiomers formed upon the fast interactions with (*R*)-(−)- TFAE in $CDCl_3$. The signals for the enantiomeric protons of the methyl of the aliphatic chain and those of the two methyls on the phenyl group are clearly resolved into two triplets centered at 0.89 ppm and 0.81 ppm and two singlets at 2.08 ppm and 2.02 ppm, respectively. The upfield signals

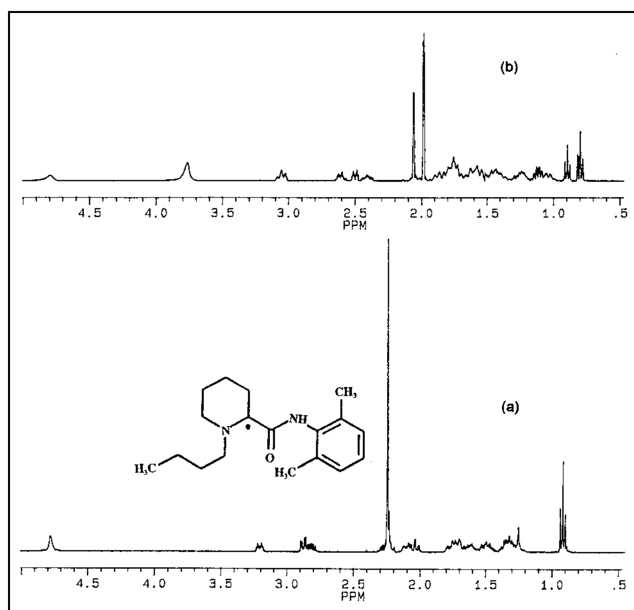

Fig. 1: The 400.13 MHz ^{1}H NMR spectrum (upfield region) of a mixture of (*R*)-(+)- and (*S*)-(−)-bupivacaine (combined concentration of ca. 0.02 M) in $CDCl_3$: (a) without chiral solvating agent; and (b) with (*R*)-(−)-TFAE (ca. 0.1 M)

were assigned to (*R*)-(+)- and the downfield signals were assigned to the corresponding (*S*)-(−)-enantiomer.

The effect of varying the chiral solvating agent to substrate molar ratio on the separation of enantiomeric signals was studied systematically with a mixture of (*S*)-(−)- and (*R*)-(+)-enantiomers, total drug content 0.02 M in $CDCl_3$. The solvation with the (*R*)-(+)-TFEA caused the resonance of the protons of bupivacaine aliphatic methyl and the methyls on the phenyl group to undergo an upfield shift from their original position in the unsolvated spectrum. The induced upfield shift increased with increasing solvating agent to substrate molar ratio up to a point, and then tended to level out at higher ratios. Table 1 shows that the induced enantiomeric shift and the induced enantiomeric shift differences. These shifts were observed for the resonance of the protons of bupivacaine aliphatic methyl and the methyls on the phenyl group of (*S*)-(−)- and (*R*)-(+)-bupivacaine short-lived diastereomeric solvates. The shifts were increased in parallel fashion to the increase in solvating agent-substrate molar ratio until a molar ratio of 5.0, after which a smaller increase is observed. Fig. 2a and 2b show the plot of induced enantiomeric shift ($\Delta\delta$) and the induced enantiomeric shift difference ($\Delta\Delta\delta$) for resonance's of the protons of the aliphatic methyl and the methyls on the phenyl group of (*S*)-(−)- and (*R*)-(+)-bupivacaine diastereomeric solvates versus (*R*)-(−)-TFAE to substrate molar ratio.

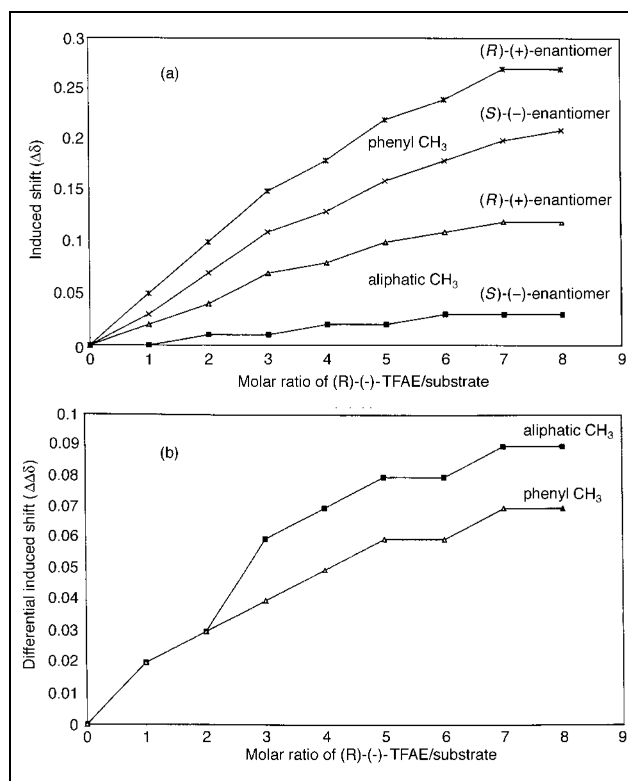

Fig. 2: Plot of molar ratio of (*R*)-(−)-TFAE/substrate (mixture of (*S*)-(−)- and (*S*)-(+)-bupivacaine (combined concentration of ca. 0.02 M) versus: (a) induced shift ($\Delta\delta$); and (b) differential induced shift ($\Delta\Delta\delta$) for the aliphatic chain methyl protons and the phenyl methyl protons

Enantiomeric spectral resolution was found also to increase by increasing sample dilution as a result of diminishing sample viscosity. However, nonequivalence was found to decrease with increased dilution of chiral solvating agent at constant molar ratio. Nonequivalence arises under these conditions only from spectral differences in the diastereomeric solvates, not from different degrees of association of solute enantiomers with chiral solvating agent, since no diminution of nonequivalence is observed even at high chiral solvating agent concentrations. Obviously, this occurs when the solute is completely solvated by the chiral solvating agent, and solvent appears not to

Table 1: Shift data for the aliphatic CH_3 and the phenyl $(CH_3)_2$ signals of a mixture of (*S*)-(−)- and (*R*)-(+)-bupivacaine after solvation with various molar ratios of (*R*)-(−)TFAE

Solvating agent/ substrate	Aliphatic CH_3					Phenyl $(CH_3)_2$				
	(*S*)-(−)		(*R*)-(+)			(*S*)-(−)		(*R*)-(+)		
	δ	Δδ	δ	Δδ	ΔΔδ	δ	Δδ	δ	Δδ	ΔΔδ
0.00	0.91	0.00	0.91	0.00	0.00	2.24	0.00	2.24	0.00	0.00
1.00	0.91	0.00	0.89	0.02	0.02	2.21	0.03	2.19	0.05	0.02
2.00	0.90	0.01	0.87	0.04	0.03	2.17	0.07	2.14	0.10	0.03
3.00	0.90	0.01	0.84	0.07	0.06	2.13	0.11	2.09	0.15	0.04
4.00	0.89	0.02	0.82	0.08	0.07	2.11	0.13	2.06	0.18	0.05
5.00	0.89	0.02	0.81	0.10	0.08	2.08	0.16	2.02	0.22	0.06
6.00	0.88	0.03	0.80	0.11	0.08	2.06	0.18	2.00	0.24	0.06
7.00	0.88	0.03	0.79	0.12	0.09	2.04	0.20	1.97	0.27	0.07
8.00	0.88	0.03	0.79	0.12	0.09	2.03	0.21	1.96	0.28	0.08

interfere with solute-chiral solvating agent interactions. In these instances, solute-solute interactions were kept to a minimum by the combined use of an excess of solvating agent and a concentration of solute that was just enough to produce adequate strength. At molar ratio of 5, and at concentrations of 0.015 M, spectral nonequivalence among the enantiomeric signals were found to reflect structural differences inherent to each diastereomeric solvate and not the result of different degrees of association of solute enantiomers with chiral solvating agent. This is supported by the fact that no diminution of nonequivalence was observed even at high chiral solvating agent concentrations. Apparently, this occurs when the solute is completely solvated by the chiral solvating agent. The absence of line broadening at all molar ratios examined also indicated that self-associations among solute or chiral solvating molecules are at best, negligible.

Because NMR spectroscopy is a sensitive probe for the occurrence of solvent-solute and solute-solute interactions it can provide detailed information concerning the nature of these interactions. A diamagnetic chiral solvating agent with certain structural features and solute-complementary functionality are the minimum requirements to dissimilarly perturb the NMR spectra of enantiomeric solutes.

Since NMR spectroscopy provides a weighed time-average view of a dynamic process, then, a less than enantiomerical pure solvating agent will only affect the position but not the relative size of the bands stemming from the particular enantiomer. Nonequivalence magnitude is apparently dependent on the enantiomeric purity of the solvating agent. Accordingly, using more enantiomerically pure chiral solvating agent than the one used, >98%, will contribute only a negligible effect to the magnitude induced shift and the induced shift differences.

The effect of varying the temperature on the enantiomeric separation was evaluated with a mixture of (*S*)-(−)- and (*R*)-(+)-enantiomers, total drug content 0.02 M and (*R*)-(+)-TFAE to substrate molar ratio of *ca.* 5.0 in $CDCl_3$. The degree of non-equivalence of the enantiomeric methyl proton signals increased in a proportional manner by decreasing the temperature. However, at lower temperatures than 15 °C these signals started to broaden and overlap with other less shifted signals. Sufficiently large $\Delta\delta\delta$ values were obtained in the temperature range between 15 °C and 45 °C.

Nonequivalence sense for the chiral solvating agent-solute combination depends mainly on the configuration of each component; reversed senses of nonequivalence were observed for the combination of (*S*)-(+)-TFAE and (*R*)-(+)- or (*S*)-(+)-bupivacaine. The chemical shifts were of the same magnitude, but their assignments were exchanged when going from one TFAE enantiomer to the other. As could be seen from three-dimensional models, it is the spatial orientation of the methyl groups of each of solvates that will determine if these groups will be shielded or less shielded by the anthryl group of the chiral solvating agent. Enantiomeric purity determination by this method is absolute in the sense that no reference to a standard of known optical purity is required.

The advantages of using ^{1}H NMR spectroscopy for measuring enantiomeric composition lie in the ubiquity and high sensitivity of the ^{1}H nucleus and in the fact that relative signal intensities directly reflect the relative number of resonating nuclei and hence relative enantiomeric populations. Seven mixtures of (*S*)-(−)- and (*R*)-(+)-bupivacaine were made up in proportions shown in Table 2. Mixed with the specific amounts of chiral solvating agent, and dissolved in $CDCl_3$ to yield solutions with *ca* 0.02 M solute concentration and a TFAE/solute molar ratio of *ca* 5.0. Enantiomeric composition or purity was calculated from the intensities of the resonance for the aliphatic methyl protons as illustrated in Fig. 3. The assay values agreed very well with the known values. Average recovery ±SD for the (*R*)-(+)-enantiomer was 100.0 ± 0.6%. The optically pure (*R*)-(+)-bupivacaine was found to contain 0.1% of (*S*)-(−)-enantiomer.

Table 2: Results of assay of synthetic mixtures of (*R*)-(+)- and (*S*)-(−)-bupivacaine by ^{1}H NMR spectroscopy with chiral solvating agent

Mixture* No.	(*S*)-(−)-isomer, mg	(*R*)-(+)-isomer, mg	Amount of (*R*)-(+)-bupivacaine, %		
			Added	Found	Recovery**
1	0	5.095	100.0	99.9	99.9
2	0.125	4.968	97.5	97.8	100.3
3	0.251	4.840	95.1	95.4	100.3
4	0.376	4.713	92.6	92.2	99.6
5	0.502	4.586	90.1	90.4	100.3
6	0.627	4.458	87.7	87.2	99.4
7	0.753	4.331	85.2	84.8	99.5
Av.					100.0
SD					0.6

* The total concentration of drug was *ca* 0.02 M in $CDCl_3$; the concentration of TFAE was ca 0.1 M.
** Amounts recovered were calculated from: (amount found × 100)/amount added; where amount found, mg(*R*)-(+)-enantiomer, was calculated from: $[A_{(R)\text{-}(+)} \times \text{mg taken}/[A_{(R)\text{-}(+)} + A_{(S)\text{-}(-)}]$.

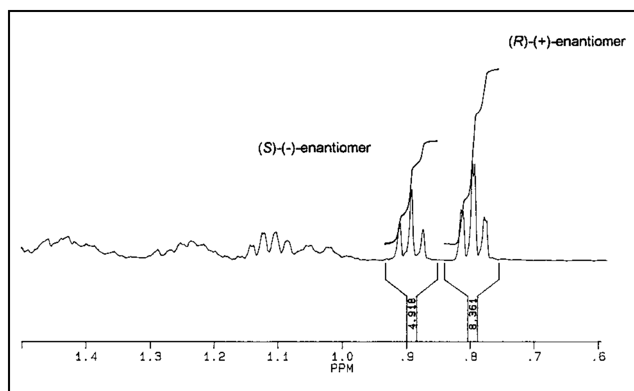

Fig. 3: The 400.13 MHz ^{1}H NMR spectrum of the methyl protons of the aliphatic chain of the diastereomeric solvates of (*R*)-(+)- and (*S*)-(−)-bupivacaine (combined concentration of ca. 0.02 M) with (*R*)-(−)-TFAE (ca. 0.1 M) in $CDCl_3$

3. Experimental

3.1. Apparatus

^{1}H NMR spectra were obtained on an AM-400 spectrometer (Bruker Instruments, Inc., Billerica, MA, USA). The ^{1}H NMR spectra were obtained under the following conditions: acquisition time, 2.03 s; data point resolution, 0.492 Hz/point; pulse width, 7.0; relaxation delay, 2.0 s; number of scans, 32. Chemical shifts were referred to $CHCl_3$ (7.26 ppm).

3.2. Chemicals

Deuterochloroform ($CDCl_3$, 99.8 atom% D, stabilized with Ag foil), and (*R*)-(−)- and (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (TFAE >98%) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

3.3. Samples

The samples of (*R*)-(+)-, (*S*)-(−)-, and (±)-1-butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide hydrochloride were obtained from Astra Pharmaceutical Products Inc. (Westboro, MA, USA). Polarimetry and the proposed NMR method checked the samples.

3.4. Sample preparation

The hydrochloride salt of bupivacaine was converted to the base form as follows: a quantity of the drug, accurately weighed, was dissolved to the extent possible in 1.5 ml of D_2O. One drop of 1.5 M sodium deuteroxide was added to $CDCl_3$ (0.75 ml). Both solutions were bubbled with N_2 and then combined. The $CDCl_3$ layer was removed using a separator funnel. A second extraction was performed and $CDCl_3$ fractions were combined and evaporated to dryness. The sample was dried *in vacuo* at 50 °C for approximately 45 min and weighed.

3.5. Optimization

Practical optimum experimental conditions for the determination of the enantiomeric composition were explored by observing (a) the effect of varying the chiral solvating agent to substrate molar ratio, and (b) the effect of temperature on the chemical shifts of the aliphatic chain methyl protons.
The required changes in chiral solvating agent to substrate molar ratios were obtained by first preparing stock solutions of bupivacaine in $CDCl_3$ (50 mg/ml) and (*R*)-(−)-TFAE (165 mg/ml). A total of 0.1 ml of bupivacaine (5 mg) solution and the appropriate amount of (*R*)-(−)-TFAE solution were added to a 5 mm NMR tube. The final volume was adjusted with $CDCl_3$ to 0.75 ml. The NMR tube was capped with a Teflon cap; its contents were mixed by inversion, allowed to stand for 10 min, and then placed in the spectrometer to obtain the ^{1}H NMR spectrum.
The additions and spectral recording were repeated until an appropriate number of spectra were available for properly defining the effects of molar ratio of chiral solvating agent/substrate on the enantiomeric spectral lines.

3.6. Determination of enantiomeric purity

A quantity of bupivacaine hydrochloride sample (approximately 5.0 mg) was converted to the base as described in section 2.4. The dry residue was dissolved in 0.5 ml $CDCl_3$, and the solution was transferred to a dry NMR tube containing approximately 20.0 mg of (*R*)-(−)-TFAE. The final volume was adjusted to 0.75 ml and then the tube was capped, inverted several times to effect solution, allowed to stand for 10 min, and then used to obtain the ^{1}H NMR spectrum. The intensities of enantiomeric aliphatic methyl proton signals centered at *ca* 0.89 ppm and *ca* 0.81 ppm corresponding to (*S*)-(−)- and (*R*)-(+)-bupivacaine, respectively, were measured and the percentage of each enantiomer was calculated based on the contribution of each resonance to the sum of both resonance as follows:

$$\%\ (S)\text{-}(-)\text{-enantiomer} = \frac{100 \times A_{(-)}}{A_{(-)} + A_{(+)}}$$

$$\%\ (R)\text{-}(-)\text{-enantiomer} = \frac{100 \times A_{(+)}}{A_{(+)} + A_{(-)}}$$

Where $A_{(+)}$ = area of the resonance signal for the methyl protons of the (*R*)-(+)-enantiomer, and $A_{(-)}$ = area of the resonance signal for the methyl protons of the (*S*)-(−)-enantiomer.

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