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Chiral separation of ibutilide enantiomers by derivatization with 1-naphthyl isocyanate and high-performance liquid chromatography on a Pirkle column

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ABSTRACT

The enantiomers of ibutilide fumarate, a new antiarrhythmic agent with a single secondary alcohol chiral center, were solid-phase extracted, derivatized with 1-naphthylisocyanate, and separated by high-performance liquid chromatography on a chiral Pirkle column (covalent 3,5-dinitrobenzoyl-D-phenylglycine stationary phase) with UV detection. Due to the strong interaction between the ibutilide derivatives and the stationary phase, a mobile phase containing 0.05% trifluoroacetic acid and 0.05% triethylamine in methanol was used. The assay was accurate to within 0.2% absolute error for samples ranging from primarily one enantiomer to racemic mixtures, measured as the percentage of peak area of one enantiomer relative to the total peak area of the two enantiomer peaks. The day-to-day reproducibility (standard deviation) ranged from ± 0.03% relative peak area for samples containing primarily one enantiomer to ± 0.2% for racemic mixtures. Accurate results over a wide range of enantiomer ratios were dependent on the method used to draw high-performance liquid chromatography peak baselines. The method was applied to racemization studies of ibutilide enantiomers in formulations and gastric fluid.

INTRODUCTION

Ibutilide fumarate, N-{4-[4-(ethylheptylamino)-1-hydroxybutyl]phenyl}methanesulfonamide, (*E*)-2-butenedioate (2:1 drug : salt, drug shown in Fig. 1), is a class III antiarrhythmic agent in clinical development. Previously, an achiral assay was developed for quantitating the racemate in formulations using reversed-phase high-performance liquid chromatography (HPLC) [1]. The method described here was developed for quantitating the enantiomers, (+)-ibutilide fumarate and (−)-ibutilide fumarate, in formulations. It was based on the reaction of the chiral hydroxy moiety of ibutilide with 1-naphthylisocyanate (NIC) (Fig. 1). The achiral NIC introduces a π-electron donating group which, along with the polarity of the carbamate functionality, interacts stereoselectively with a chiral stationary phase, covalent 3,5-dinitrobenzoyl-D-phenylglycine [2]. Other chiral alcohols and amines have been resolved by similar procedures or reciprocal procedures in which the compounds were derivatized with the 3,5-dinitrobenzoyl moiety and separated on naphthylene-derived stationary phases [3–8].

The important advantages of a procedure utilizing an achiral derivatizing agent include the identical physical properties of the derivatives of the two enantiomers,

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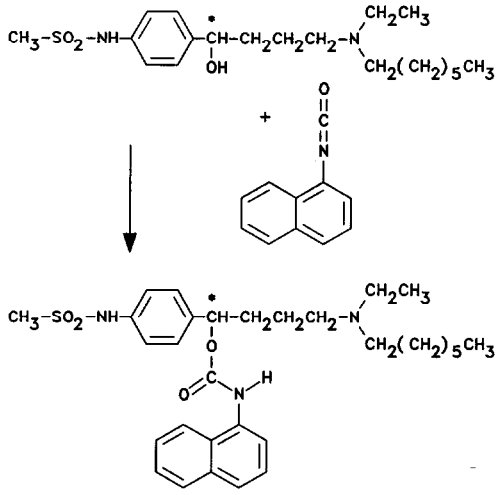

Fig. 1. Reaction of ibutilide with 1-naphthylisocyanate. The chiral center is marked with an asterisk.

which means that they will react at the same rate in an achiral medium and have identical optical properties, and elimination of any need to determine the chiral purity of the derivatizing agent. Therefore, relative enantiomer concentrations can be determined directly from the relative peak areas of the enantiomer derivatives after chromatographic separation and detection. The method was applied to *in vitro* racemization studies of ibutilide enantiomers in formulations and simulated gastric fluid.

EXPERIMENTAL

Materials

Ibutilide fumarate (racemic mixture and individual enantiomers) was synthesied by Upjohn (Kalamazoo, MI, USA). The NIC was from Regis (Morton Grove, IL, USA) and the sequanal quality trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). Other reagents were of analytical grade and solvents were of HPLC or UV grade. The samples prepared for racemization testing were 0.25 mg/ml solutions of the individual enantiomers in 0.1% sorbic acid, 0.9% sodium chloride, or simulated gastric fluid (0.034 *M* sodium chloride adjusted to pH 1.8 with 1 *M* hydrochloric acid).

Apparatus

Solid-phase extraction (SPE) was performed using Bond-Elute C_{18} SPE columns containing 100 mg of stationary phase and a Vac-Elute solid-phase extraction manifold (Analytichem, Harbor City, CA, USA). The HPLC equipment consisted of a Beckman 114M solvent delivery module (San Ramon, CA, USA), a Perkin-Elmer ISS 100 autosampler with 100 μl loop (Norwalk, CT, USA), a Rexchrom covalent D-phenylglycine column, 25 cm × 4.6 mm I.D. with 10 μm packing (Regis), an ABI Spectroflow 783 UV detector (Ramsey, NJ, USA), and a Harris H1000 computer (Ft. Lauderdale, FL, USA) with custom interface and software (Upjohn) for data acquisition and analysis.

Procedure

A volume of sample containing approximately 0.25 mg of ibutilide was pipeted into a glass culture tube. If necessary, water was added to bring the volume to 0.5–1.0 ml. A 1-ml volume of buffer, 0.05 *M* NaH_2PO_4 containing 0.1% triethylamine (TEA), adjusted to pH 7.0 with sodium hydroxide, was added to each tube. Control samples containing variable ratios of (+)- and (−)-ibutilide were prepared in water with a total drug concentration of 0.25 mg/ml and similarly treated.

C_{18} SPE columns were attached to the vacuum manifold and activated with 1 ml of an acetone–acetonitrile–TEA solution (50:50:0.2, v/v/v), followed by 1 ml of water containing 0.1% TEA. The diluted samples were applied to the SPE columns, washed with 1 ml of water, dried for 3 min under maximum vacuum, washed with 0.3 ml hexane, and dried for an additional 10 min. Each SPE column was removed from the manifold and placed in a 75 × 10 mm glass culture tube. The ibutilide was eluted under gravity using 0.5 ml of the acetone–acetonitrile–TEA solution, with the last bed volume forced out with pressurized nitrogen.

A 50-μl aliquot of each solution was transferred to a glass autosampler vial. A 10-μl volume of 1.0% acetic acid in acetonitrile and 100 μl of 0.1% NIC in acetonitrile were added, and the reaction allowed to proceed for 10–15 min at room temperature. A 500-μl volume of mobile phase (methanol containing 0.05% TFA and 0.05% TEA by volume) was added to each vial to terminate the reaction.

The samples (100 μl) were injected and chromatographed isocratically at ambient temperature at a flow-rate of 1 ml/min and UV detection at 230 nm. Injections were typically performed at 32 min intervals. Peak area ratios were directly used as a measure of enantiomer ratios.

RESULTS AND DISCUSSION

Optimization of solid-phase extraction conditions

This inital step was used to extract the drug from an aqueous solution into an organic solvent so that derivitization could take place under anhydrous conditions. The samples were applied to C_{18} SPE columns after dilution of approximately 0.25 mg of ibutilide fumarate in an aqueous solution with a pH 7 buffer (0.05 *M* sodium phosphate) containing 0.1% TEA. The columns were washed with water to remove non-retained components, then dried briefly. Quantitative recoveries of ibutilide could be obtained after elution with either acetonitrile or acetone–acetonitrile–TEA (50:50:0.2, v/v). However, recovery was only 80% in the first 0.5 ml of eluent when acetonitrile was used, but quantitative when the latter solution was used. The TEA apparently served to elute the drug from polar sites on the column. An aliquot (50 μl) of the eluted solution of drug reacted quantitatively with NIC when either eluent was used. Varying the drying time prior to elution from 10 to 3 min of vacuum did not affect recovery nor the extent of reaction with NIC.

Derivatization conditions

Reactions of isocyanates are often catalyzed by acids or bases [9–12]. Therefore, catalysis by acetic acid or TEA was examined. The drug-containing extract consisted of 50 μl of acetone–acetonitrile–TEA (50:50:0.2). Experiments were performed in which 10 μl aliquots of acetic acid or TEA in acetonitrile were added, followed by 100

μl of 0.1% NIC in acetonitrile. Addition of 10 μl of 1% acetic acid was slightly more than what was required to neutralize the TEA in the extract. After reaction at room temperature for 15 min, the samples were diluted and chromatographed. There was a wide range (0–1% added acetic acid and 0–0.1% added TEA) over which the reaction appeared to give high yields (>96%). It was only in the presence of very high concentrations of acid or base that the reaction was diminished. No catalytic effect was observed compared to a reaction performed without any TEA or acetic acid present. Addition of 1% acetic acid was chosen for the final assay, in part because it had a slightly higher reaction yield than the other conditions and in part for stability reasons indicated below.

The effect of the concentration of NIC on the reaction yield was examined by derivatizing 50 μl eluates containing the enantiomers of ibutilide with 10 μl of 1% acetic acid in acetonitrile and 100 μl of various concentrations of NIC in acetonitrile (0.003–0.3%, v/v) for 15 min at room temperature. The reaction yield was high at or above 0.03% added NIC. Since NIC slowly degrades, 0.1% NIC (0.06% or 4.3 m*M* NIC in the final reaction mixture) was chosen for the final assay to ensure an adequate excess of the drivatizing agent.

The time course of the reaction of NIC with ibutilide was followed at temperatures of 0, 25 and 40°C using the standard conditions described above. After 15 min at 25 and 40°C the concentration of derivative reached a plateau and no unreacted drug remained. Reaction was much slower at 0°C and not complete even after 1 h. Therefore, room temperature derivatization for 15 min provided convenient conditions for the final assay procedure and the mild conditions minimized the possibility of racemization.

The conditions reported for the reaction of isocyanates with alcohols have varied from extreme, such as reaction in toluene at 80°C for 36 h, to mild, such as reaction in N,N-dimethylformamide at room temperature for 15 min [9–16] and the similar conditions reported here using acetonitrile. It has been suggested that polar organic solvents catalyze the reaction of phenyl isocyanate with amines in N,N-dimethylformamide [17]. Aliphatic alcohols were reported to react rapidly with phenyl isocyanate in N,N-dimethylformamide, acetonitrile, and dioxane [12].

The structure of the derivative (Fig. 1) was confirmed by fast atom bombardment mass spectrometry of collected chromatographic fractions. The protonated molecular ion at *m/z* 554 corresponded to the addition of one equivalent of NIC. An analogue containing the sulfonamide moiety but no hydroxyl group did not react with NIC, confirming that the site of derivatization of ibutilide was the hydroxyl group.

Chromatographic conditions

Pirkle columns are generally operated with mobile phases containing mixtures of hexane and isopropanol, sometimes with small amounts of other polar organic solvents as modifiers [2,7,8,18]. However, using isopropanol–hexane mixtures or even pure isopropanol as the mobile phase, the ibutilide–NIC derivative did not elute, probably because of strong interactions between the tertiary amine and the stationary phase. However, addition of TEA to the mobile phase did result in elution and chiral resolution, but the UV detector baseline drifted upward, suggesting a loss of stationary phase. Adding an excess of TFA in addition to the TEA appeared to stabilize the baseline and columns used under these conditions worked well for more than one year.

Various mobile phases were tested in order to optimize resolution and analysis time. The alcohols (methanol, ethanol and isopropanol) were the weakest solvents. Modifying retention with TFA–TEA (0.05–0.2% of each) worked best because the separation factor, α, remained constant (approximately 1.16) as the capacity factor, k', decreased, whereas with acetonitrile or tetrahydrofuran α always declined with k'. Methanol containing 0.05% TFA (6.7 m*M*) and 0.05% TEA (3.6 m*M*) was chosen as the mobile phase for the routine assay. Resolution was excellent (Fig. 2), although run times were moderately long (approximately 30 min). The elution order on a 3,5-dinitrobenzoyl-D-phenylglycine column was the (+)-ibutilide derivative followed by the (−)-ibutilide derivative. The absolute configuration of each enantiomer was not known.

Stability of derivatized samples

After reaction, the samples were diluted with 500 μl of mobile phase. Since analytical runs sometimes proceeded for 24 h or more, good stability of the derivatives in autosampler vials at room temperature was necessary. Initially, derivatization was performed without any added acetic acid, and the product was diluted with pure methanol. Thus, the final mixture was somewhat basic due to the TEA present in the eluate from the initial extraction. There was significant degradation of the product within a few hours and little remained after 26 h at room temperature (Fig. 3). Stability was better when the derivatization reaction was quenched with mobile phase containing an excess of TFA (middle tracing), and best (same chromatogram as when freshly prepared) when the reaction mixture contained an excess of acetic acid and the methanol used for dilution contained TFA (upper tracing). Samples prepared with acetic acid in the reaction mixture but diluted only with methanol also showed a slow loss of derivative. These results suggest that acetic acid in the reaction mixture prevented degradation from ocurring during the derivatization, while the TFA in the final sample solution enhanced the long-term stability of the derivative. Degradation of phenyl isocyanate derivatives of aliphatic alcohols has been observed in the presence of TEA [12].

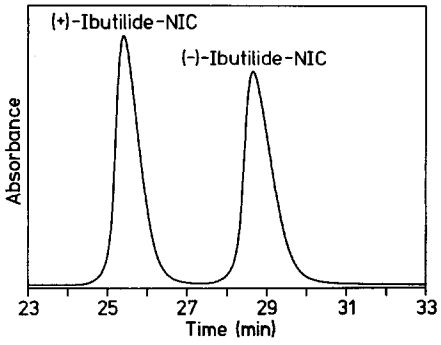

Fig. 2. Resolution of the enantiomers of ibutilide on a Pirkle column after derivatization with 1-naphthylisocyanate. Mobile phase: methanol containing 0.05% TFA and 0.05% TEA. Flow-rate: 1 ml/min. Detection: UV absorbance at 230 nm.

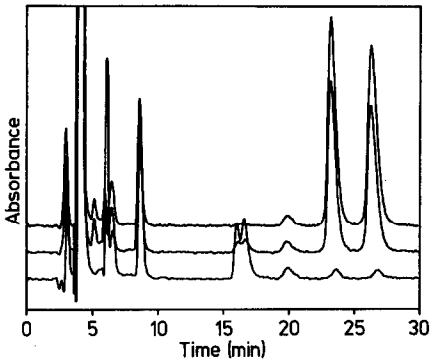

Fig. 3. Chromatograms of the NIC derivatives of ibutilide, approximately 26 h after preparation (stored at room temperature). Lower curve: a 50 μl extract containing ibutilide in acetone–acetonitrile–TEA (50:50:0.2, v/v/v) derivatized with 10 μl of acetonitrile and 100 μl of 0.1% NIC in acetonitrile, then diluted with 500 μl of methanol. Middle curve: same except for dilution with 0.05% TFA and 0.05% TEA in methanol. Upper curve: same as middle curve except that the 10 μl of acetonitrile contained 1% acetic acid. The enantiomer peaks eluted at 23–27 min. An unknown pair of peaks characteristic of degradation can be seen at a retention time of 16–17 min. The peaks at 3–9 and 20 min are from NIC and its impurities.

Effect of baseline drawing method

Although the resolution and peak shape of the derivatized enantiomers were good, difficulties were encountered when trying to quantitate a trace of one enantiomer in the presence of an excess of the other enantiomer. This common problem of trace analysis [19] is illustrated in Fig. 4 and was particularly acute when the (−)-enantiomer was present in trace quantities and eluted on the tail of the (+)-enantiomer. To ameliorate this problem, several different baseline drawing techniques were used (Fig. 4). Samples containing approximately 0.4% of the minor enantiomer or exactly 50% of each enantiomer were tested.

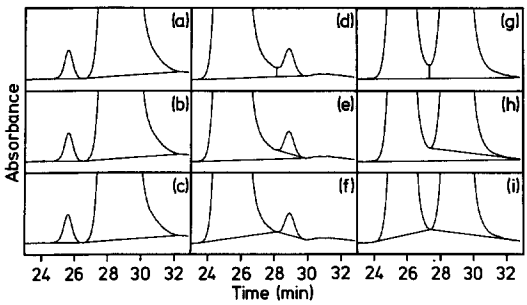

Fig. 4. Chromatograms showing baseline drawing methods for ibutilide enantiomer peaks ranging from 0.4% (+)-ibutilide (a–c) to racemic ibutilide (g–i) to 0.4% (−)-ibutilide (d–f). The perpendicular drop method was used for a, d and g. A tangent-skim of the smaller peak was used for b, e and h. A tangent-skim of both peaks was used for c, f and i. The large truncated peaks had peak heights of 0.27–0.59 absorbance units, while the small peaks had heights of 0.002–0.004 absorbance units.

All methods worked well when the (+)-enantiomer was present in trace amounts since it was totally resolved from the large (−)-enantiomer peak (Fig. 4a–c).

When the (−)-enantiomer was present in trace amount, the perpendicular drop method biased the results high by 0.2% for the (−)-enantiomer because part of the tail of the large peak was included in the small peak (Fig. 4d). Tangent-skimming both peaks or only the small peak worked well for trace amounts of the (−)-enantiomer (Fig. 4e and f). Another possibility would have been to use an L-phenylglycine column to reverse the elution order of the enantiomers.

The perpendicular drop method worked poorly for the racemic mixture since it truncated the tail of the first peak but not the second peak, giving a low result (0.3% error) for the percentage of the (+)-enantiomer (Fig. 4g). Tangent-skimming the second peak resulted in a 0.9% error in the opposite direction since the baseline of the first peak was extended too far (Fig. 4h). But by tangent-skimming both peaks (Fig. 4i), the error was only 0.1% (the second peak being slightly high). This appeared to be the best accuracy that could be obtained without using more sophisticated baseline drawing methods. Since tangent-skimming of both peaks worked well over a wide range of enantiomer ratios, it was chosen for routine use.

Accuracy and precision

The sample size (0.25 mg) chosen for the initial extraction gave peaks that were in the range of 0.3–1.0 absorbance unit full-scale, thus maximizing the signal-to-noise for samples containing only a trace of one enantiomer (Fig. 4).

Day-to-day reproducibility and accuracy were estimated from repeated analysis of control samples. The racemic control sample averaged 49.95% ± 0.20% (+)-ibutilide based on 15 assays on 5 days. Thus, the average error for this sample was 0.05%. The (+)-ibutilide control solution averaged 99.86% ± 0.03% (+)-ibutilide while the (−)-ibutilide control sample averaged 99.72% ± 0.03% (−)-ibutilide. The errors for these samples containing predominantly one enantiomer can not be stated exactly since they were not independently assayed, but were probably less than 0.05%, based on the purest enantiomer sample available. Errors for control samples prepared by mixing the enantiomers in varying ratios were typically less than 0.2% on any single

TABLE I

RACEMIZATION OF (+)- AND (−)-IBUTILIDE FUMARATE IN GASTRIC FLUID, SORBIC ACID AND SALINE

Sample	Conditions	Mean (%) (+)-ibutilide formed from (−)-ibutilide fumarate	Mean (%) (−)-ibutilide formed from (+)-ibutilide fumarate	Mean (%) increase in minor component
Gastric fluid	37°C, 24 h	9.73	9.30	9.5
0.1% Sorbic acid	4°C, 30 days	0.02	0.01	0.0
	30°C, 30 days	1.00	0.91	1.0
	40°C, 30 days	4.09	3.75	3.9
0.9% Sodium chloride	4°C, 30 days	−0.03	0.01	0.0
	30°C, 30 days	0.03	0.02	0.0
	40°C, 30 days	0.11	0.11	0.1

assay day. This accuracy and precision should be sufficient for most practical applications, such as the racemication studies described below.

Application to racemization studies

To evaluate possible racemization in formulations of various pHs and in the stomach prior to absorption, solutions of the ibutilide fumarate enantiomers (0.25 mg/ml) were prepared in the following matrices: simulated gastric fluid (0.034 *M* sodium chloride, adjusted to pH 1.8 with 1 *M* hydrochloric acid); 0.1% sorbic acid (pH 3.5); and 0.9% sodium chloride (pH 6.2.). The solutions were stored at controlled temperature. There was good agreement between the results from the individual enantiomers, as expected since the matrices were achiral (Table I). Ibutilide racemized slowly under most conditions but more rapidly in an acidic environment and at elevated temperature. The results from simulated gastric fluid suggest that only a small amount of racemization should occur in the stomach *in vivo* after oral dosing of the drug, assuming the gastric residence time is short.

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REFERENCES

1 C. L. Hsu and R. R. Walters, Upjohn Co., unpublished results, 1988.
2 W. H. Pirkle, D. W. House and J. M. Finn, *J. Chromatogr.*, 192 (1980) 143–158.
3 W. H. Pirkle and J. E. McCune, *J. Liq. Chromatogr.*, 11 (1988) 2165–2173.
4 W. H. Pirkle and M. H. Hyun, *J. Org. Chem.*, 49 (1984) 3043–3046.
5 W. H. Pirkle, G. S. Mahler, T. C. Pochapsky and M. H. Hyun, *J. Chromatogr.*, 388 (1987) 307–314.
6 N. Oi and H. Kitahara, *J. Chromatogr.*, 265 (1983) 117–120.
7 Q. Yang, Z.-P. Sun and D.-K. Ling, *J. Chromatogr.*, 447 (1988) 208–211.
8 A. M. Dyas, A. F. Fell and M. L. Robinson, *Chirality*, 2 (1990) 120–123.
9 R. Wintersteiger and G. Wenninger-Weinzierl, *Fresenius' Z. Anal. Chem.*, 309 (1981) 201–208.
10 T. Ibuka, G.-N. Chu, T. Aoyagi, K. Kitada, T. Tsukida and F. Yoneda, *Chem. Pharm. Bull.*, 33 (1985) 451–453.
11 Y. Yamazaki and H. Maeda, *Agric. Biol. Chem.*, 50 (1986) 79–87.
12 B. Bjorkqvist and H. Tiovonen, *J. Chromatogr.*, 153 (1978) 265–270.
13 P. Michelsen, E. Aronsson, G. Odham and B. Akesson, *J. Chromatogr.*, 350 (1985) 417–426.
14 R. Wintersteiger, G. Wenninger-Weinzierl and W. Pacha, *J. Chromatogr.*, 237 (1982) 399–406.
15 K. M. Williams, *Clin. Pharmacol. Ther.*, 36 (1984) 817–823.
16 J. Gal, D. DeVito and T. W. Harper, *Drug Metab. Dispos.*, 9 1981) 557–560.
17 B. Bjorkqvist, *J. Chromatogr.*, 204 (1981) 109–114.
18 W. H. Pirkle and J. M. Finn, *J. Org. Chem.*, 46 (1981) 2935–2938.
19 J. A. Perry, J. D. Rateike and T. J. Szczerba, *J. Chromatogr.*, 389 (1987) 57–64.