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Journal of Chromatography B, 667 (1995) 115–128

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

Assay of the enantiomers of ibutilide and artilide using solid-phase extraction, derivatization, and achiral–chiral column-switching high-performance liquid chromatography

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Received 15 December 1994; accepted 29 December 1994

Abstract

Ibutilide fumarate and artilide fumarate, new drugs for the treatment of cardiac arrhythmias, each contain a stereogenic center bearing a secondary alcohol group. Reversed-phase achiral–chiral column-switching HPLC separations of the enantiomers of each compound were developed and validated for quantitation in plasma and other biofluids. The key component of the method was derivatization with 1-naphthyl isocyanate, which enhanced the sensitivity of fluorescence detection and enabled the enantiomers to be separated on a Pirkle column (covalent 3,5-dinitrobenzoyl-D-phenylglycine stationary phase). The lower limit of quantitation of ibutilide fumarate was typically 0.1 ng/ml or less of each enantiomer in 1 ml of plasma. Two of the special features of the column-switching system included operation with two samples in the system at one time, which reduced analysis time to 16 min/sample for ibutilide and 19 min/sample for artilide, and a relay-operated switching of detector outputs, which allowed achiral and chiral column chromatographic data to be gathered from two detectors into a single data acquisition channel.

1. Introduction

Ibutilide fumarate, N-[4-[4-(ethylheptylamino)-1-hydroxybutyl]phenyl]methanesulfonamide, (E)-2-butenedioate (2:1 drug/salt) is a class III antiarrhythmic agent in clinical development for the treatment of atrial flutter and fibrillation [1–3]. It is intravenously administered as a racemate. The drug is very potent, as demonstrated by its low therapeutic dosage of 0.01–0.03 mg/kg, with peak plasma concentrations frequently below 20 ng/ml [4]. Artilide fumarate, (+)-(*R*)-N-[4-[4-(dibutylamino)-1-hydroxybutyl]phenyl]methanesulfonamide, (*E*)-2-butenedioate (2:1 drug/salt) is one enantiomer of a similar compound in an earlier stage of development [1,5]. Both ibutilide and artilide contain a single stereogenic center bearing a secondary alcohol group. To study racemization and enantioselective pharmacokinetics, a chiral assay with a lower limit of quantitation of approximately 0.1 ng/ml was needed.

The biofluid assays for ibutilide and artilide were based on a previously described formulation assay for the enantiomers of ibutilide, in which the alcohol moiety at the stereogenic center was derivatized with 1-naphthyl isocyanate (NIC), as shown in Fig. 1 [6]. Although

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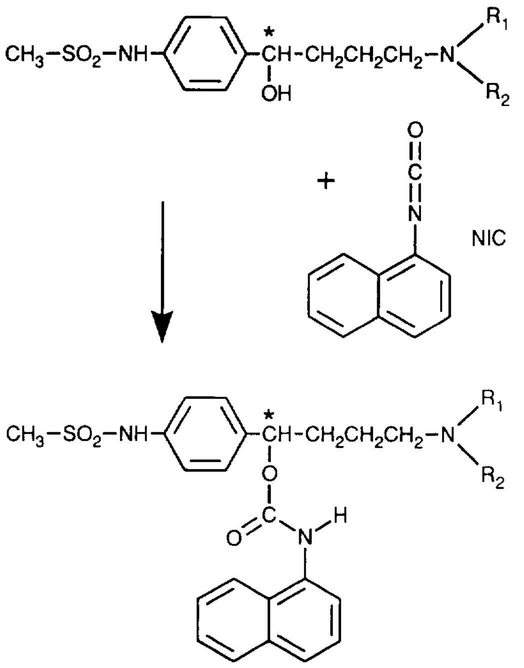
SSDI 0378-4347(95)00005-4



	R_1	R_2
Ibutilide	$-CH_2CH_3$	$-(CH_2)_6CH_3$
Artilide	$-(CH_2)_3CH_3$	$-(CH_2)_3CH_3$

Fig. 1. Structures of ibutilide, artilide, and the derivatives formed by reaction with 1-naphthyl isocyanate (NIC). The chiral center is marked with an asterisk.

NIC is achiral, it introduces a pi-electron donating group to the drug molecule which, along with the polarity of the carbamate functionality, interacts stereoselectively with a chiral stationary phase, covalently-bonded 3,5-dinitrobenzoyl-D-phenylglycine [7]. This method was chosen as the nucleus of a biofluid assay method since it coupled the fluorescent naphthyl group to the drug molecule and since the chiral Pirkle columns were chromatographically efficient and commercially available containing 3-μm particles, thereby allowing both rapid and sensitive chiral analysis. Because NIC is an achiral derivatizing agent, the derivatives of ibutilide and artilide were enantiomeric and thus the physical and chemical properties, including reaction rate and optical properties, of the derivatives of each pair of enantiomers were identical.

Column-switching is widely used to provide multidimensional sample cleanup and has been applied in a number of chiral analytical methods in which the drug peak is typically switched from an achiral column onto a chiral column for enantiomer separation [8–10]. In the assay described here, several steps of column-switching were utilized in order to remove a 10^6-fold excess of derivatizing agent, then to separate drug from internal standard, and finally to separate the enantiomers of the drug.

Most of the validation data presented here will focus on the ibutilide assay, which has been the most extensively tested. The artilide assay was very similar except for a few adjustments because of the greater polarity of the artilide molecule.

2. Experimental

2.1. Reagents

The fumarate salts of racemic ibutilide (Ibu), racemic artilide (Art), (+)-ibutilide [(+)-Ibu], (−)-ibutilide [(−)-Ibu], (+)-(*R*)-artilide [(+)-Art], and (−)-(*S*)-artilide [(−)-Art] were provided by the Upjohn Co. (Kalamazoo, MI, USA). The internal standard for the ibutilide assay (U-74747) was another analog, N-[4-[4-(ethyloctylamino) - 1 - hydroxybutyl]phenyl]-methanesulfonamide, and was also provided by Upjohn. NIC was obtained from Regis (Morton Grove, IL, USA) and the HPLC/spectro grade trifluoroacetic acid (TFA) was from Pierce (Rockford, IL, USA). Other reagents were of analytical grade and solvents were of HPLC or UV grade.

2.2. Apparatus

Manual solid-phase extraction (SPE) was performed using 1-ml Bond-Elut C_{18} SPE columns containing 100 mg of stationary phase and a Vac-Elut extraction manifold (Varian, Harbor City, CA, USA). Extracted samples were evaporated with a Zymark TurboVap LV Evaporator (Hopkinton, MA, USA). The HPLC system is

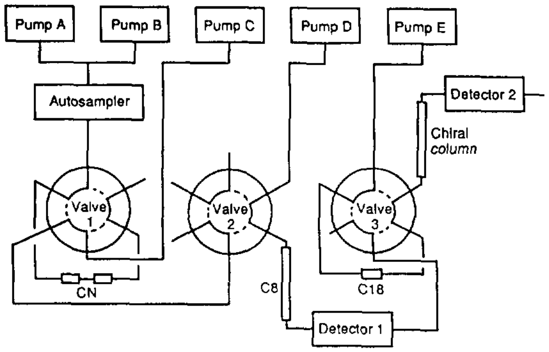


Fig. 2. Diagram of the column-switching system. Not shown is a C_{18} guard column located between valve 2 and the C_8 analytical column.

diagrammed in Fig. 2. Five Model 110B, 112 or 114M pumps were utilized (Beckman, Berkeley, CA, USA). Other components included a Model 421A controller (Beckman), a System Gold NEC controller with Model 406 analog interface module (Beckman), a Perkin-Elmer ISS 100 autosampler with a 200-μl loop and refrigerated tray (Norwalk, CT, USA), a Lauda RM6 refrigerated circulating bath (Brinkman, Westbury, NY, USA), an Applied Biosystems Spectroflow 980 fluorescence detector (Ramsey, NJ, USA) with the excitation wavelength set at 224 nm, a 340-nm emission cut-off filter, a high voltage of 800 V, and a rise time of 2 s), a Waters 470 fluorescence detector (Millipore, Milford, MA, USA), with excitation/emission wavelengths of 290/345 nm, a gain of 1000 and a 1.5 s filter), and three column-switching valves (Rheodyne 7000 switching valves, Rheodyne 5701 pneumatic actuators, and Autochrom Solenoid interface, all from Rainin, Woburn, MA, USA). Data collection was performed using a PE-Nelson 900 Series interface (Norwalk, CT, USA) connected to a Harris Nighthawk 4402 computer (Melbourne, FL, USA) using custom software (Upjohn). A custom computer-actuated relay device (Upjohn) allowed data from both detectors to be sequentially collected in a single channel of the data acquisition system.

The chromatographic columns were two Brownlee cyano Newguard cartridges, 15 × 3.2 mm I.D., 7 μm (Rainin), a Sentry Nova-Pak C_{18} guard column, 20 × 4 mm I.D., 4 μm (Waters), a Phenomenex Ultremex 3 C_8 column, 75 × 4.6 mm I.D., 3 μm (Torrance, CA, USA), a Brownlee Spheri-5 RP-18 cartridge, 30 × 4.6 mm I.D., 5 μm (Rainin), and a Pirkle covalent dinitrophenyl-D-phenylglycine column, 100 × 4.6 mm I.D., 3 μm (Regis).

2.3. Sample preparation

A 1-ml volume of buffer (0.05 *M* sodium dihydrogen phosphate, 0.1% triethylamine, pH 7.0) and 100 μl of internal standard (I.S.) solution (100–250 ng/ml U-74747 for the ibutilide assay or ibutilide for the artilide assay in 30:70 (v/v) acetonitrile–0.01 *M* ammonium acetate) were mixed with 0.1–1 ml of plasma. The mixture was slowly applied to a C_{18} SPE which had been activated, in sequence, with 1 ml of 50:50:0.2 (v/v) acetone–acetonitrile–triethylamine (TEA), 1 ml of 0.1% TEA in water, and 1 ml of water. After sample application, the SPE was washed with 1 ml of water, 2 ml of 25:25:50 (v/v) acetonitrile–methanol–water (1 ml in the artilide assay), and 1 ml of water, then dried under vacuum air flow for 10 min, washed with 300 μl of hexane, and dried again for 5 min. The drug and internal standard were eluted with 0.5 ml of 50:50:0.2 (v/v) acetone–acetonitrile–TEA and evaporated to dryness at 30°C using nitrogen flow.

The samples were derivatized by adding 10 μl of 0.1% acetic acid in acetonitrile and 100 μl (75 μl for the artilide assay) of 0.1% NIC in acetonitrile to the dried extract, mixing, and reacting for 10 min at 30°C. The reaction was terminated by addition of 600 μl (800 μl for the artilide assay) of 10:90:0.1 (v/v) methanol–water–TFA. The solutions were transferred to autosampler vials and placed in the autosampler tray at 2–4°C.

2.4. Chromatographic procedures

The HPLC system shown in Fig. 2 was programmed to operate automatically. Pump A delivered mobile phase A [40:60 (v/v) methanol–water for the ibutilide assay, 35:65 (v/v) methanol–water for the artilide assay] and was used during part of the cycle for sample application and washing of the cyano columns. Pump B delivered mobile phase B [40:40:20:0.1:0.1 (v/v) methanol–acetonitrile–water–TFA–TEA] and was used for cleanup of the cyano columns between injections. Pump C delivered mobile phase C [5:25:70:0.1:0.1 (v/v) methanol–acetonitrile–water–TFA–TEA], which was an intermediate strength solvent for elution of the derivatives of the drug and internal standard from the cyano columns and reconcentration of the peaks on the head of a Sentry C_{18} guard column preceding the C_8 reversed-phase analytical column. Pumps A, B, and C operated alternately, each at 1 ml/min. Pump D delivered mobile phase D [52:48:0.1:0.1 (v/v) acetonitrile–water–TFA–TEA for the ibutilide assay or 50:50:0.1:0.1 (v/v) acetonitrile–water–TFA–TEA for the artilide assay] and was used to separate the derivatives of drug and internal standard on the C_8 analytical column. Mobile phase E was 100:0.3:0.3 (v/v) methanol–TFA–TEA and was used to separate the enantiomers of the derivatized drug on the Pirkle column. Mobile phases D and E were pumped continuously at a flow-rate of 1 ml/min.

Prior to starting the automated operation of the column-switching HPLC system, the system was set up with mobile phases B, D, and E pumping, the valves in the 'resting' positions shown in Fig. 2, the autosampler programmed to inject three 200-μl volumes of each sample at approximately 1 min intervals upon receiving a signal from the controller, and the data acquisition system set to acquire data for 15 min upon receiving a signal from the controller.

The programmed sequence of events for operation of the system is listed in Table 1 for both assay methods. Because two samples were in the system at the same time, the times for some of the events refer to the previously-injected sample. To avoid confusion, the time when that event actually takes place for a single sample is given first, followed by the programmed event time in parentheses (Table 1).

To summarize the operation of the column-switching system, 600 μl of the sample were injected onto the short cyano columns and washed with mobile phase A to remove excess washed with mobile phase A to remove excess derivatizing agent. An intermediate strength mobile phase (C) was then applied to elute the derivatized drug and internal standard from the cyano columns and reconcentrate them on the C_{18} guard column preceding the C_8 analytical column. Mobile phase D was used to separate drug and internal standard on this column. When the drug peak eluted from the C_8 column, it was temporarily switched onto a short C_{18} column using mobile phase D, then eluted onto the chiral Pirkle column and the enantiomers separated using mobile E. In the meantime, the cyano columns were cleaned with mobile phase B, then reequilibrated with mobile phase A prior to the next injection.

2.5. Validation studies

Standard solutions were prepared containing racemic ibutilide or artilide fumarate in 30:70 (v/v) acetonitrile–0.01 *M* ammonium acetate. A 100-μl volume of standard solution was added to 0.1–1 ml of blank plasma and assayed as described above. Control samples were prepared from separate weighings of racemic ibutilide or artilide fumarate. Small volumes of the drug solutions in the same buffer as the standards were diluted with blank plasma and stored at −20°C. Control samples were sometimes prepared from the individual enantiomers, usually in a 1:10 ratio, to check for racemization. Control samples were typically prepared at three concentration levels.

3. Results and discussion

3.1. Solid-phase extraction

The simple C_{18} solid-phase extraction procedure described previously for formulation samples containing ibutilide fumarate [6] was modified for plasma samples to include a wash with 2 ml of an aqueous solution containing 25% each of methanol and acetonitrile. This removed most interferences from the plasma extract but main-

Table 1
Column-switching programs for ibutilide and artilide chiral assays

Time of event for ibutilide assay (min)	Time of event for artilide assay (min)	Event
Initial	Initial	Pump B on to clean cyano columns.
2.0	1.0	Pump B off, pump A on to reequilibrate cyano columns.
4.0	4.0	Inject sample (3 injections of 200 μl each) onto the cyano columns.
12.0	11.5	Switch cyano columns in-line with Sentry C_{18} guard column and C_8 analytical column for 3.1 min using valve 1. Send mobile phase D to waste using valve 2. Pump A off, pump C on to elute drug and internal standard from the cyano columns and onto the head of the Sentry C_{18} guard column.
15.1	14.6	Valves 1 and 2 returned to their resting positions. Pump C off, pump B on to wash the cyano columns to waste. Elute the Sentry C_{18} guard column and C_8 analytical column with mobile phase D.
20.2 (4.2)	20.2 (1.2)	Begin data acquisition from achiral column detector for 7 min.
21.2 (5.2)	21.2 (2.2)	Switch valve 3 for 2 min to collect the drug peak eluting from the C_8 analytical column onto the Spheri-5 C_{18} column.
23.2 (7.2)	23.2 (4.2)	Return valve 3 to its resting position to switch the Spheri-5 C_{18} column in-line with the chiral column.
27.2 (11.2)	27.2 (8.2)	Switch relay to begin data acquisition from the chiral column detector for 8 min.
32.0 (16.0)	38.0 (19.0)	Loopback to time zero.
35.2 (19.2)	35.2 (16.2)	Complete data acquisition.

tained a quantitative recovery of ibutilide and internal standard. Artilide fumarate was more weakly retained on the C_{18} phase and consequently the wash volume was reduced to 1 ml. The drying steps proved to be critical. Insufficient drying resulted in decreased recoveries and/or dirtier chromatograms. Other factors relating to recovery have been discussed separately [11].

3.2. Derivatization

In previous formulation assay work, it was shown that the reaction rate of ibutilide with

NIC increased with temperature and that reaction was complete within 5–15 min at room temperature, as judged both by appearance of product and disappearance of unreacted drug [6]. The reaction yield was high when the concentration of added NIC exceeded 0.03%, and 0.1% added NIC was chosen for the final assay [6]. Addition of a small amount of acetic acid in the reaction mixture did not affect the kinetics of the reaction, but it did help to stabilize the products [6]. However, the biofluid assay, which was run at much lower drug concentrations, was different in this latter respect. As shown in Table 2, the reaction yield was only about 20% unless a small amount of acetic acid, 10 μl of 0.01–1.0% acetic acid, was added to the reaction mixture. Too much acid prevented reaction from taking place. Thus, 10 μl of 0.1% acetic acid was chosen for routine use.

After reaction was complete in the assay for ibutilide, the samples were diluted with 600 μl of 10:90:0.1 (v/v) methanol–water–TFA. Although it would have been desirable to use a small volume such that the entire sample could have been injected in a single 200-μl injection, this was not possible since retention on the cyano columns was weak and thus a fairly large volume of water was needed to decrease the solvent strength (the derivatization was performed in acetonitrile). Too much water was also a problem since the excess NIC precipitated. Thus, diluting the sample to a large volume and then injecting 600 μl onto the HPLC system was a compromise between needs of sensitivity, retention, and solubility.

Because artilide was slightly more polar than ibutilide, a few changes were made to decrease the organic solvent content of the sample. The volume of NIC solution was decreased from 100 μl to 75 μl, and the reaction mixture was diluted with 800 μl of 10:90:0.1 (v/v) methanol–water–TFA rather than 600 μl.

3.3. Chromatography and validation

In the initial version of the assay, data from the two detectors were collected independently. Data analysis of unknown samples was based on the calculation of total drug concentrations using data from the achiral part of the assay, and a separate calculation of the enantiomer percentages using the relative peak areas from the chiral part of the assay. Individual enantiomer concentrations were calculated by merging the data. Examples of chromatograms from each part of the assay are shown in Fig. 3 for the ibutilide assay and Fig. 4 for the artilide assay. Validation data from three ibutilide assay runs (using duplicate standards in each run) are shown in Table 3 and indicated that the linearity, accuracy, and precision of the achiral chromatographic data were excellent over the range 0.35–69 ng/ml in 1 ml of dog plasma (bias and coefficient of variation 4%) and acceptable (bias and coefficient of

Table 2
Effect of added acetic acid solution on the derivatization of ibutilide and internal standard with 1-naphthyl isocyanate

Concentration of added acetic acid[a] (%)	Normalized mean ibutilide peak height	Peak-height ratio of ibutilide/U-74747
100	0	–
10	0.01	1.27
1	0.93	1.47
0.1	1.00[b]	1.49
0.01	1.03	1.47
0.001	0.64	1.49
0	0.22	1.48

[a] 10 μl of this solution (volume percent) added to 50 μl of 100 ng/ml ibutilide and U-74747 and reacted with 100 μL of 0.1% NIC. All solutions were prepared in acetonitrile.

[b] Data normalized to this sample.

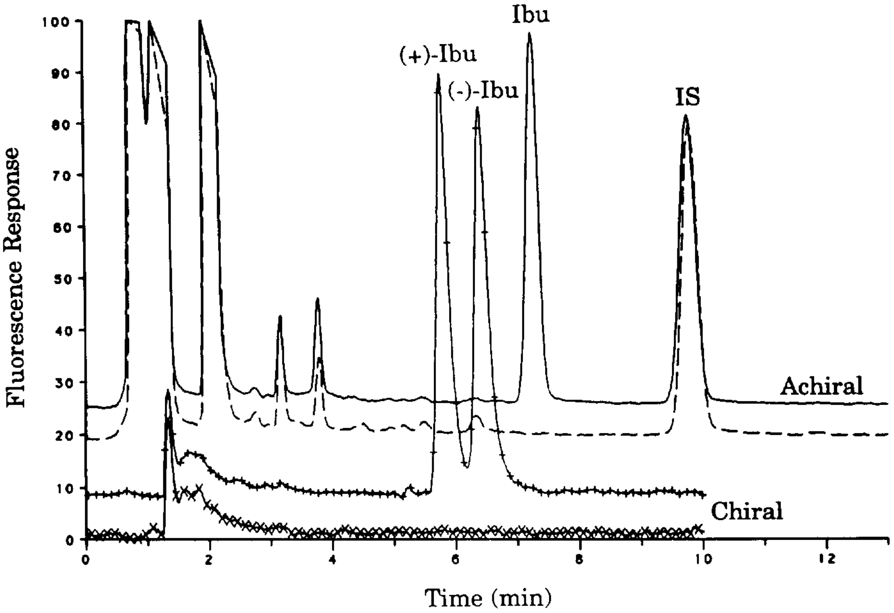


Fig. 3. Chromatograms from an achiral–chiral column-switching method for assay of ibutilide. The top two chromatograms show the achiral separation taking place on a reversed-phase column. The lower two chromatograms show the chiral separation taking place on the Pirkle column after column switching. Relative to the upper chromatograms, the lower chromatograms were recorded approximately 7 min later than the time indicated on the x-axis. The upper chromatogram of each pair was a dog plasma sample (1 ml) containing approximately 7 ng/ml racemic ibutilide, and the lower chromatogram of each pair was a pre-dose (blank) dog plasma sample.

variation <20%) down to 0.069 ng/ml. A linear fit of the calibration data with intercept and 1/concentration weighting was used. Table 4 likewise demonstrates the accuracy and precision achieved in determining the percentage of each ibutilide enantiomer based on relative peak areas from the second detector. The precision and accuracy were both excellent down to concentrations of 0.17 ng/ml of each enantiomer and acceptable to as low as 0.017 ng/ml.

Note that the range of the chiral portion of the ibutilide assay corresponded to a lower limit of quantitation, for the racemate, of 0.0346 ng/ml, which was slightly lower than that achieved in the achiral part of the assay. This was a result of an extremely clean background in the chiral part of the assay after so many stages of purification by column-switching. The good sensitivity of the chiral portion of the assay combined with the inconvenience of merging achiral and chiral data from separate data files to calculate individual enantiomer concentrations led us to consider means of directly measuring the enantiomer concentrations. One limitation was that the internal standard could not easily be switched to the chiral column because the void peak interfered with the previously-switched drug peak. A simple electronic trick to achieve almost the same result was to send the outputs from the two detectors to a single data acquisition channel using an electrical relay which selected between the outputs. By timing the switching between the two outputs, it was possible to store the important parts of both detector outputs in the same channel of the same data file. The peak-height ratios of each enantiomer peak from the chiral portion of the chromatogram to the internal standard in the achiral portion were used to establish separate standard curves for the enantiomers. Examples of the chromatograms using this method are shown in Fig. 5 for the ibutilide assay and Fig. 6 for the artilide assay.

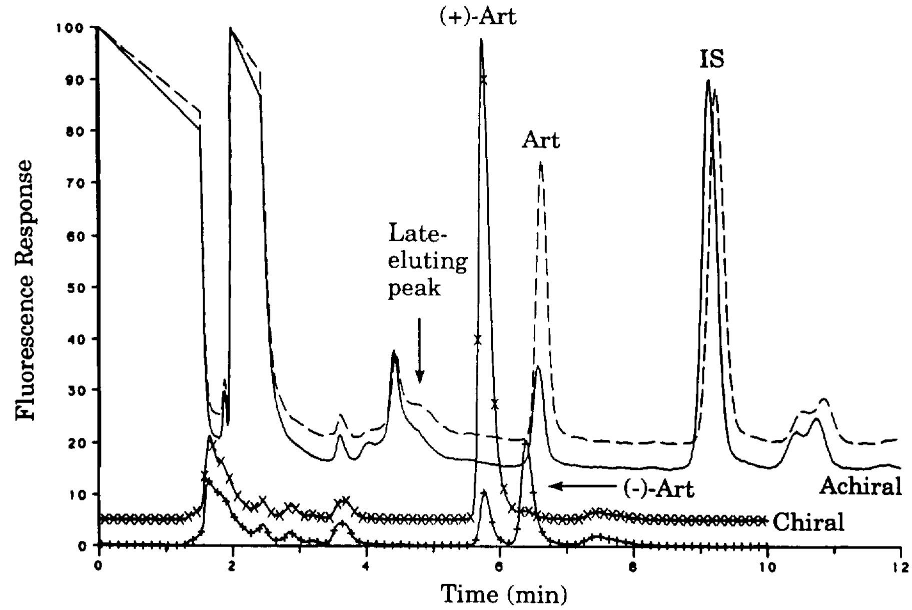


Fig. 4. Chromatograms from an achiral–chiral column-switching method for assay of artilide. The top two chromatograms show the achiral separation taking place on a reversed-phase column. A late-eluting peak from the previous sample, as discussed in the text, can be seen as a broad tail on the unidentified background peak at approximately 4.5–5 min. If the time between samples had been reduced to 16 min (as in the ibutilide assay) rather than 19 min, this peak would have eluted near the drug peak at approximately 7.5–8 min. The lower two chromatograms show the chiral separation taking place on the Pirkle column after column-switching. Relative to the upper chromatograms, the lower chromatograms were recorded approximately 7 min later than the time indicated on the *x*-axis. The upper chromatogram of each pair was from a plasma sample (1 ml) obtained at 24 h after dosing from a dog intravenously administered (+)-(*R*)-artilide at a dose of 0.3 mg/kg. The lower chromatogram of each pair was from a dog administered (−)-(*S*)-artilide. The chromatograms from the chiral separations suggest that partial racemization of (−)-(*S*)-artilide, but not (+)-(*R*)-artilide, took place in vivo.

Table 3
Summary of ibutilide achiral calibration curve data from three assay runs using 1 ml of dog plasma

Theor. conc. (ng/ml)	Mean data from peak height ratios				
	n	Mean conc. (ng/ml)	Bias (%)	S.D. (ng/ml)	C.V. (%)
0.0346	5	0.0429	24	0.0094	22
0.0692	6	0.0707	2.2	0.0122	17
0.138	6	0.149	7.6	0.012	8
0.346	6	0.359	3.6	0.014	4
0.692	6	0.718	3.8	0.012	2
1.38	4	1.41	2.0	0.02	2
3.46	6	3.59	3.8	0.06	2
6.92	6	6.93	0.1	0.06	1
13.8	6	13.9	0.2	0.2	1
34.6	6	35.0	1.2	0.5	1
69.2	6	68.5	−0.9	0.4	1

n = number of replicates; S.D. = standard deviation; C.V. = coefficient of variation.

The modified method, in addition to allowing direct calculation of the enantiomer concentrations, exhibited similar accuracy and precision compared to the original version of the assay. Validation data from three ibutilide assay runs (using duplicates of the standards in each run) are shown in Table 5 and indicated that the linearity, accuracy, and precision of the chiral chromatographic data were excellent over the range 0.070–18 ng/ml of each enantiomer of ibutilide in 1 ml of human plasma (bias and coefficient of variation <6%) and acceptable down to 0.018 ng/ml (bias <7%, coefficient of variation <17%). A linear fit of the calibration data with intercept and 1/concentration weighting was used. Racemic drug was used to prepare the standard curves. Control samples, prepared at three concentration levels from the individual enantiomers in ratios of approximately 10:1 and

Table 4
Summary of ibutilide chiral control data from three assay runs using 1 ml of dog plasma

Theoretical			Measured based on relative peak areas		
(+)-Ibutilide conc. (ng/ml)	(−)-Ibutilide conc. (ng/ml)	(+)-Ibutilide (%)	(+)-Ibutilide (%)	Bias (%)	S.D. (%)
0.0173	0.0173	50.0	53.7	3.7	4.1
0.0346	0.0346	50.0	48.5	−1.5	12.7
0.0692	0.0692	50.0	47.2	−2.8	6.8
0.173	0.173	50.0	51.2	1.2	1.5
0.346	0.346	50.0	48.5	−1.5	0.7
0.692	0.692	50.0	49.5	−0.5	0.7
0.865	0.865	50.0	49.9	−0.1	0.5
1.92	0.196	90.7	91.3	0.4	1.1
0.192	1.96	8.9	9.6	0.7	0.6
1.73	1.73	50.0	49.7	−0.3	0.5
3.46	3.46	50.0	49.9	−0.1	0.2
6.92	6.92	50.0	49.6	−0.4	0.2
8.65	8.65	50.0	49.6	−0.4	0.2
17.3	17.3	50.0	49.7	−0.3	0.1
38.4	3.92	90.7	93.3	2.5	0.1
3.84	39.2	8.9	9.0	0.1	0.1
34.6	34.6	50.0	49.7	−0.3	0.2

S.D. = standard deviation.

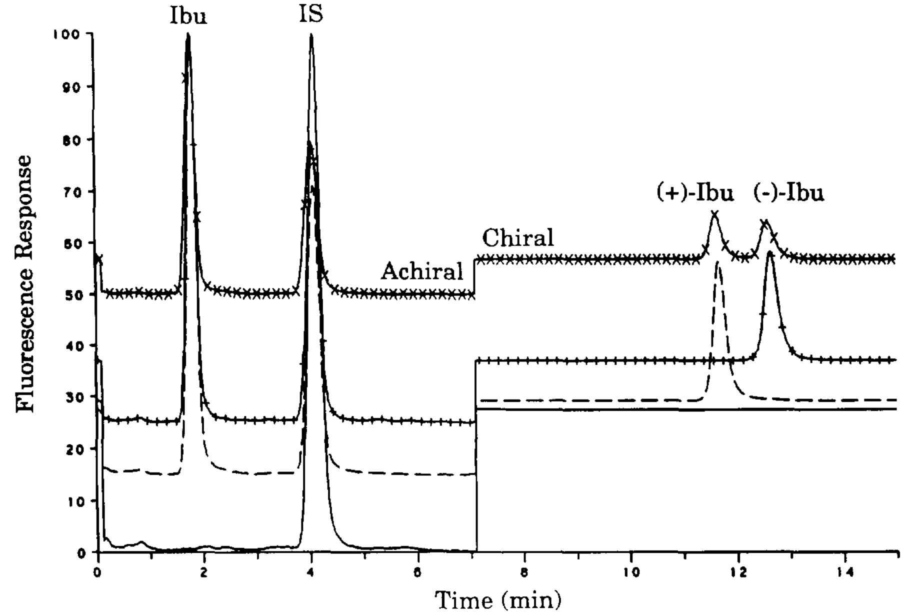


Fig. 5. Combined achiral–chiral chromatograms of ibutilide from the plasma (1 ml) of a patient shortly after administration of a 0.01 mg/kg intravenous dose of drug. The bottom curve shows a pre-dose sample. The other curves, from top to bottom, were for administration of the racemate, (−)-ibutilide, and (+)-ibutilide. The total drug concentrations were approximately 12 ng/ml. The very clean and flat baseline in the chiral portion of the chromatogram compared to the achiral portion is apparent in the pre-dose chromatogram.

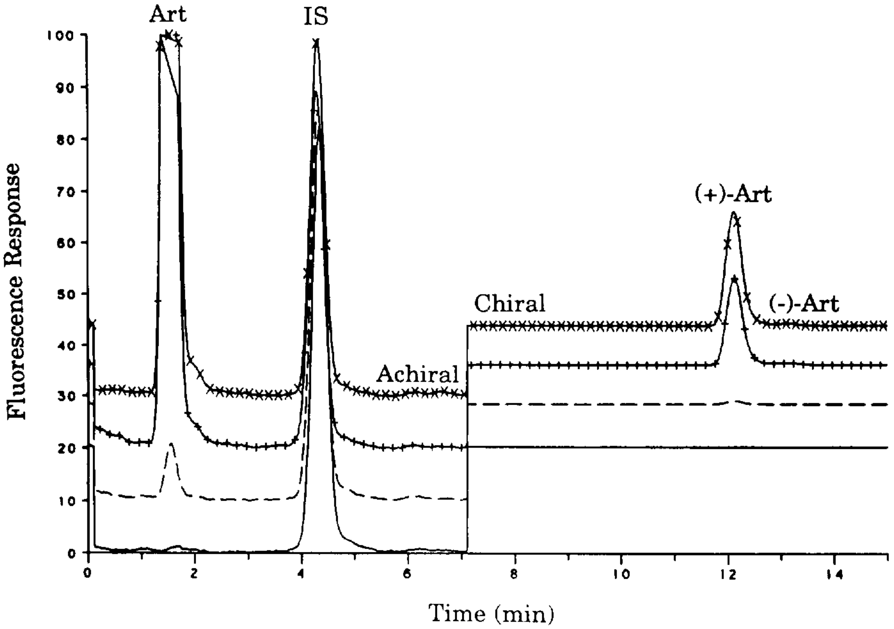


Fig. 6. Combined achiral–chiral chromatogram of artilide from the plasma (0.05 ml) of a rat following oral administration of a 30 mg/kg solution dose of (+)-(*R*)-artilide. From top to bottom, the chromatograms were from plasma samples obtained at 1.5 h (880 ng/ml), 6 h (630 ng/ml), 15 h (24 ng/ml), and 24 h (no measurable drug) after dosing. A trace of the other enantiomer can be seen at approximately 13 min in the chromatograms.

1:10 and assayed in triplicate in each run, exhibited average inter-day recoveries of 97 ± 6% for (+)-ibutilide and 96 ± 9% for (−)-ibutilide over a concentration range 0.13–24 ng/ml. In a similar validation study using dog plasma, the mean inter-day recoveries for control samples prepared from the racemate (4 concentrations ranging from 0.1 to 1000 ng/ml of each enantiomer, assayed in triplicate in each run) averaged 96 ± 4% for both enantiomers.

In a separate study in which 2 ml of human plasma were assayed, as little as 9 pg/ml of each enantiomer of ibutilide could be quantitated with good accuracy and precision using a narrower calibration range (0.009–1.8 ng/ml).

3.4. Factors in assay development

Cyano column chromatography

Direct injection of the derivatized samples onto a reversed-phase column was unsuccessful because the NIC reagent, present in approximately a million-fold excess relative to drug and containing many impurity peaks, dominated the chromatograms. Therefore, several 'orthogonal' column types were tested in an effort to separate excess reagent from derivatized drug. Short cyano columns were found to be optimal. The derivatized drug showed good retention in methanol–water mixtures, while the excess reagent was easily washed off. The derivatized drug was then sharply eluted by mobile phases containing 0.1% TFA and TEA. In the final procedure, the cyano columns were washed to waste with 40% methanol (35% methanol for the artilide assay) for 5 min after completion of sample injection to remove the excess reagent, then eluted for 3 min with 5% methanol–25% acetonitrile–0.1% TFA and TEA onto the C_8 analytical column. A stronger mobile phase (B) was used during the remainder of the cycle to wash any strongly retained material from the cyano columns to waste and to prevent carryover from one sample to the next.

Table 5
Summary of standard curve data for the ibutilide enantiomers (combined achiral–chiral assay method) from three assay runs using 1 ml of human plasma

Theor. conc. (ng/ml)	Mean data from peak-height ratios				
	n	Mean conc. (ng/ml)	Bias (%)	S.D. (ng/ml)	C.V. (%)
(+)-*Ibutilide*					
0.0175	6	0.0187	6.6	0.0031	17
0.0351	6	0.0358	2.0	0.0038	11
0.0702	6	0.0695	−1.0	0.0024	3
0.175	6	0.174	−1.1	0.010	6
0.351	6	0.347	−1.2	0.017	5
0.702	6	0.699	−0.4	0.032	5
1.75	6	1.70	−2.9	0.10	6
3.51	6	3.39	−3.3	0.16	5
7.02	6	7.06	0.5	0.26	4
17.5	6	17.7	0.8	0.8	4
(−)-*Ibutilide*					
0.0175	6	0.0186	6.5	0.0013	7
0.0351	6	0.0357	1.6	0.0044	12
0.0702	6	0.0693	−1.2	0.0039	6
0.175	6	0.176	0.2	0.010	6
0.351	6	0.347	−1.2	0.016	5
0.702	6	0.696	−0.9	0.032	5
1.75	6	1.70	−3.1	0.10	6
3.51	6	3.40	−3.2	0.16	5
7.02	6	7.05	0.5	0.25	4
17.5	6	17.7	0.8	0.8	4

n = number of replicates; S.D. = standard deviation; C.V. = coefficient of variation.

Recoveries of the drug and internal standard derivatives from the cyano columns were tested by comparing peak heights of 4-ng samples directly injected onto the C_8 column vs. those obtained by injection onto the cyano column first. Quantitative recovery was obtained using the wash and elution method described above: 103 ± 2% recovery of ibutilide and 101 ± 3% recovery of U-74747.

C_8 column chromatography

A 3-μm particle size reversed-phase column was chosen for the achiral analytical column to minimize analysis time and maximize sensitivity. The peak widths were minimized by the use of the intermediate strength mobile phase C to concentrate the sample on the head of guard column preceding the C_8 column, then separating drug and internal standard with the stronger mobile phase D. The assay could be run with one less valve and pump by using mobile phase D directly to elute the cyano columns onto the C_8 column, but with some loss of sensitivity.

C_{18} column chromatography

A 3-cm C_{18} column was used as a temporary holding column for the drug peak after it was switched off of the C_8 column. Direct switching onto the chiral column could have been performed, but the back-pressure might have damaged the flowcell of the first detector. Switching the peak into a 2-ml loop was also tried, but band-broadening was unacceptably large. The short C_{18} column allowed the peak to be switched with very little band-broadening.

Pirkle column chromatography

Retention of derivatized ibutilide and artilide on the dinitrophenyl-D-phenylglycine column was found to be very strong, probably due to the tertiary amine group of the drugs [6]. Pure methanol with a small amount of TFA and TEA was used as the mobile phase. This was very useful in the column-switching assay since the chiral column mobile phase was compatible with aqueous mobile phases. The methanol readily eluted the drug from the short C_{18} column onto the chiral column. Nearly baseline resolution of the enantiomers was observed, as shown in Figs. 3–5.

Overlap of samples

As described in the Experimental section, the design of the column-switching system allowed two samples to be in the system at one time. While one sample was being injected and washed on the cyano columns, the previous sample was being eluted from the C_8 column and switched to the chiral column. This resulted in a considerable saving in analysis time. Although each sample required approximately 35 min from the beginning of the cycle until data acquisition was completed (Table 1), the analysis time per sample was only 16 min for ibutilide because of the overlap. The analysis time was slightly greater for artilide (19 min) because of a late-eluting peak (Fig. 4).

Stability

NIC-derivatized high concentration ibutilide samples were stabilized by acidification with 0.1% TFA in methanol such that runs of 24 h could be performed [6]. However, in the biofluid assay described here, water as well as methanol and TFA were added to the samples (see section on Derivatization). Since the biofluid assay demanded even better sample stability because very low levels of drug needed to be measured, sample stability was further examined. In one experiment, samples containing 4 ng/ml each of ibutilide and U-74747 were derivatized, then assayed fresh and after 4 days at room temperature or 4°C. The drug and internal standard peak heights declined by about one-half after storage at room temperature, but at 4°C were as large as when freshly prepared. Most importantly, the peak-height ratios of drug to internal standard did not change at 4°C: 1.19 ± 0.01 (fresh); 1.18 ± 0.01 (4°C); and 1.45 ± 0.03 (room temperature). Thus, an autosampler tray refrigerated to 2–4°C was used in the assay to maintain sample stability for long runs. The stability was further examined by comparing blank dog plasma chromatograms from freshly derivatized samples or samples stored for 17 h at room temperature or 4°C. The achiral chromatographic baseline in blank plasma was substantially cleaner in the vicinity of the ibutilide-NIC peak when the samples were freshly prepared or stored at 4°C compared to those stored at room temperature.

The reagents, mobile phases, and standard solutions used in this assay were stable for weeks, with the exception of the NIC solution. NIC solutions in acetonitrile were prepared fresh every other day.

3.5. *Ruggedness*

An assay of this complexity might be expected to fail quite often. In fact, this assay proved to be quite rugged over more than four years of routine use. One reason for this is that the samples were quite clean after solid-phase extraction and the mobile phases did not contain any non-volatile salts. Therefore, very few problems of back-pressure increases or pump seal or check valve failure were encountered. Most mechanical failures were due to autosampler malfunctions.

The cyano columns were the least robust element of the column-switching system. Over time, they appeared to lose retention, resulting in poor recovery of the drug relative to the more strongly retained internal standard. Problems related to this were solved by using two cyano columns in series. They were alternately replaced after approximately 250 samples, i.e. each column was used for about 500 samples. This simple approach resulted in a much more robust procedure.

For chiral analysis following the achiral separation, the drug peak, with a peak width of approximately 1 min, was column-switched after eluting from the C_8 analytical column. Using a 2-min window for the switch, it was critical that the retention time of the drug peak on the C_8 column remained constant within runs and between runs. The assay was very rugged in this respect and no problems of this type were encountered.

Ruggedness of the guard column preceding the C_8 analytical column did prove to be a problem in the assay. Although the samples were very clean by the time they reached the guard column, a broadening of the peaks eluting from the guard column–C_8 analytical column combination was observed, sometimes over the course of a single run. This broadening was apparently due to the pressure fluctuations in the column-switching system, which rapidly damaged the structure of the packed bed in the guard column. Since quantitation of the enantiomers was performed using calibration curves based on peak-height ratios of drug eluting from the chiral column vs. internal standard eluting from the achiral column, a change in broadening on one column but not the other over the course of a single run was detrimental to the accuracy of the assay. A number of C_8 and C_{18} guard columns from various manufacturers were tested to remedy this problem. Of those tested, the Waters Sentry guard column was found to be the most durable under the conditions of this assay.

The C_8, C_{18}, and Pirkle columns were very rugged and each was used for thousands of samples.

To ensure the quality of the assay results, system suitability samples containing a fixed ratio of drug to internal standard in acetonitrile solution were derivatized and injected at the beginning and end of each run. The height of the peaks was a general indicator that the derivatization procedure was working. The peak-height ratio of drug/internal standard on the C_8 analytical column was an indicator of whether recoveries from the cyano columns were satisfactory. The ratio of the enantiomer peak height on the chiral column/internal standard peak height on the achiral column from the beginning to the end of the run indicated whether the guard column preceding the C_8 analytical column needed to be replaced.

Plasma samples from rats, rabbits, dogs, and humans have been assayed using this method. Urine has also been assayed, but drug recoveries tended to be low unless blank plasma was added to each sample prior to solid-phase extraction [11].

4. Conclusions

Through the use of derivatization, efficient HPLC columns, and column switching, it was possible to design an extremely sensitive achiral–chiral assay system for ibutilide and artilide in plasma. In spite of its complexity, the system was quite rugged and was used for the assay of several thousand samples from animal and human pharmacokinetic studies.

Acknowledgements

The authors thank D.A. Naugle for building the detector output switching relay.

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