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Sensitive determination of a new antiarrhythmic agent, trecetilide, in plasma by high-performance liquid chromatography with fluorescence detection[1]

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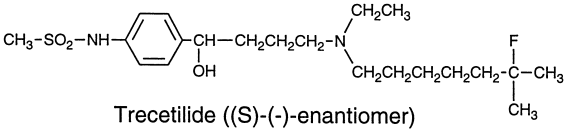
Abstract

Two high-performance liquid chromatography (HPLC) methods were developed for the determination of trecetilide in plasma samples. Differing only in the addition of a derivatization step and different detection wavelengths, the two methods encompassed a wide concentration range. In both methods, plasma samples (0.1 ml) with added internal standard were applied to solid-phase extraction discs containing a non-polar/strong cation mixed-phase, washed and eluted with an acetone-acetonitrile–triethylamine mixture. The eluate was evaporated to dryness, and either reconstituted and directly injected onto an HPLC column or first derivatized with 1-naphthyl isocyanate before HPLC analysis. In both methods, the separation was performed isocratically on a cyano analytical column utilizing a mobile phase composed of acetonitrile–pH 7.9 phosphate buffer (70:30, v/v). The column effluent was monitored by fluorescence detection at 290/345 nm (with derivatization) or 235/320 nm (without derivatization). The limits of detection and quantitation of the assay were 0.57 and 1.9 ng/ml, respectively, when derivatization was used, or 4.3 and 14 ng/ml, respectively, without derivatization.

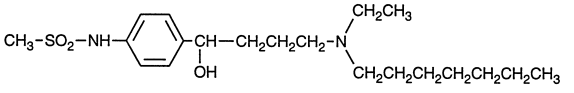
Keywords: Trecetilide; Ibutilide

1. Introduction

Trecetilide fumarate (Fig. 1) is a class III antiarrhythmic agent under development for the treatment of atrial flutter and fibrillation [1]. It contains one chiral center and was synthesized as the (*S*)-(−)-enantiomer. A plasma assay was needed that could cover a wide concentration range to accommodate low dose pharmacology studies (maximum plasma concentrations of approximately 10 ng/ml) and high-dose toxicology studies (maximum plasma concen-



Trecetilide ((S)-(-)-enantiomer)



Ibutilide (racemate)

Fig. 1. Chemical structures of trecetilide and ibutilide (internal standard). The fumarate salt moiety is not shown.

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[1]This paper is dedicated to the memory of Chang-Yuan (Sharon) L. Hsu, who died on March 18, 1998, at age 40.

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trations of approximately 10 000 ng/ml) in various species. In this work, we describe two methods developed for this purpose. The simpler method utilized solid-phase extraction (SPE) followed by isocratic high-performance liquid chromatography (HPLC), with detection using the weak native fluorescence of the trecetilide molecule. In the second method, trecetilide was derivatized with 1-naphthyl isocyanate (NIC) after extraction and the strongly fluorescent product was chromatographed under identical conditions except for detector wavelength.

2. Experimental

2.1. Chemicals and reagents

Trecetilide fumarate and ibutilide fumarate (the internal standard, Fig. 1) were synthesized at Pharmacia and Upjohn (Kalamazoo, MI, USA). HPLC-grade high purity acetonitrile, methanol, heptane and acetone were purchased from EM Science (Gibbstown, NJ, USA). NIC was obtained from Regis (Morton Grove, IL, USA). Blank dog plasma with EDTA as anticoagulant was obtained from dogs housed at Pharmacia and Upjohn.

Acetonitrile–ammonium acetate buffer: acetonitrile–0.01 *M* ammonium acetate (30:70, v/v). Sodium phosphate–TEA buffer, pH 7.0: 0.05 *M* NaH_2PO_4 containing 1% (v/v) triethylamine (TEA) adjusted to pH 7.0 with sodium hydroxide. Potassium phosphate buffer (0.024 *M*), pH 7.9, for mobile phase: water–KH_2PO_4–phosphoric acid (1000:4.0:0.07, v/w/v). Mobile phase: acetonitrile–pH 7.9 potassium phosphate buffer (70:30, v/v).

2.2. SPE instrumentation

Spec Plus 3ML MP1 columns with 15 mg of sorbent were from Ansys (Irvine, CA, USA) and contained a non-polar/strong cation mixed phase in a disc format. The extraction procedure was performed using a Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA). A Zymark TurboVap LV evaporator (Hopkinton, MA, USA) was used for drying collected eluates.

2.3. Chromatographic instrumentation

The chromatographic system consisted of a pump (127S solvent delivery module, Beckman, Fullerton, CA, USA), an autosampler (507e, Beckman) equipped with a 100-μl loop and Peltier cooling, a guard column (MetaGuard MetaSil 5 CN, 10×4.3 mm, 5 μm particle size, Metachem, Torrance, CA, USA), an analytical column (5 CN/E cyano column, 250×4.6 mm, 5 μm particle size, Metachem), and a fluorescence detector with 16-μl flow cell (Model 474, Waters, Milford, MA, USA) set at 235 nm excitation wavelength and 320 emission wavelength (no derivatization) or at 290/345 nm (derivatization method). The system was operated at room temperature (22–24°C), with the sample tray nominally cooled to 5°C (actual measured temperature in autosampler vials was 9°C). The mobile phase was pumped isocratically at 1.6 ml/min. Samples were injected using a full loop injection volume of 100 μl. The wash solution for the autosampler was acetonitrile–water–trifluoroacetic acid (TFA)–TEA (70:30:0.1:0.1, v/v/v/v). One sample was injected every 12 min (non-derivatization method) or 14 min (derivatization method). Data were collected using a PE-Nelson 900 Series interface (Norwalk, CT, USA) connected to a Concurrent Computer Nighthawk 5800 series computer (Fort Lauderdale, FL, USA) and analyzed using custom software (Pharmacia and Upjohn).

2.4. Standard solutions and quality controls

The standard stock solution was prepared from trecetilide fumarate at a concentration of 0.10 mg/ml (trecetilide free base equivalents) in acetonitrile–ammonium acetate buffer and serially diluted with the same buffer to prepare 14 working standard solutions ranging from 0.5–10 000 ng/ml trecetilide. Quality control stock trecetilide solutions were similarly prepared and then diluted with blank plasma to concentrations of 5000, 875, 35 and 1.4 ng/ml. Internal standard (ibutilide fumarate) solutions were prepared in acetonitrile–ammonium acetate buffer and diluted to concentrations of 100 and 1000 ng/ml for use in assays with and without derivatization, respectively. All solutions were stored at ≤ -10°C,

except the internal standard solutions were stored at 2–5°C.

2.5. Sample preparation

Plasma unknowns or quality control samples (100 μl) were transferred to 75×12 mm disposable glass test tubes containing 100 μl of internal standard solution and 100 μl of acetonitrile–ammonium acetate buffer. Standard samples were prepared by adding 100 μl of the appropriate standard solution to a 75×12 mm disposable glass test tube containing 100 μl of internal standard and 100 μl of blank dog plasma. Standards, controls and unknowns were diluted with 1 ml of sodium phosphate–TEA buffer. SPE columns were activated by sequential washings with 0.3 ml of acetone–acetonitrile–TEA (50:50:0.2, v/v/v) and 1 ml of sodium phosphate–TEA buffer. The prepared samples were applied to the activated SPE columns and allowed to drain to the top of the sorbent bed by gravity. Full vacuum was then applied to remove the final bed volume. Next, the SPE columns were washed with 3.75 ml of water, drained under maximum vacuum, washed with 0.5 ml of methanol, dried under maximum vacuum for 1 min, washed with 0.3 ml heptane, and dried under maximum vacuum for 3 min. The SPE columns were eluted by gravity with 0.6 ml of acetone–acetonitrile–TEA (50:50:0.2, v/v/v) into clean 75×12 mm disposable glass test tubes. Positive pressure was used to remove the final bed volume. The samples were transferred to an evaporator and evaporated to dryness at room temperature for approximately 10 min under nitrogen flow. The dried samples were treated according to one of the following procedures:

Derivatization method: 10 μl of 0.1% (v/v) acetic acid in acetonitrile and 20 μl of 0.01% (v/v) NIC in acetonitrile were added to the sample tubes, which were then placed in a heated block (Vortex-Evaporator, Searle, Fort Lee, NJ, USA) and incubated at 30°C for 30 min. The derivatization reaction was terminated by adding 0.2 ml of acetonitrile–water–TFA (50:50:0.1, v/v/v) to each sample. The samples were transferred to autosampler vials, capped, and placed in the HPLC autosampler tray. Chromatographic conditions are outlined in Section 2.3.

Non-derivatization method: each dried sample was reconstituted with 0.4 ml of acetonitrile–water–TFA (50:50:0.1, v/v/v), then the samples were transferred to autosampler vials, capped, and placed in the HPLC autosampler tray. Chromatographic conditions are outlined in Section 2.3.

2.6. Quantitative determination

The peak-height ratios of trecetilide to internal standard were used to generate the standard curve. Regression analysis was utilized to fit the standard curve data, weighted by 1/concentration, to a straight line with intercept. The concentrations of trecetilide in the unknown samples were obtained by back-calculation from the standard curve. Concentrations of trecetilide are reported as ng/ml (in free base equivalents).

3. Results and discussion

3.1. Selectivity

The analytical peaks of trecetilide and ibutilide (internal standard) were well resolved, with retention times of approximately 8.2 and 9.6 min, respectively, without derivatization. When analyzed with derivatization, the retention times, using the same chromatographic conditions, increased to approximately 8.9 min (trecetilide) and 10.7 min (internal standard). In both methods, the peaks were well resolved from known degradation products and metabolites (Fig. 2). Retention time shifts were observed from column to column (compare Fig. 2 with Figs. 3 and 4). Blank samples tested from three different sources or lots of dog, rat, mouse and rabbit plasma using the derivatized and non-derivatized methods did not exhibit endogenous peaks at positions corresponding to the drug or internal standard. A peak, well resolved from both the drug and internal standard, was observed in both the non-derivatized and derivatized assays eluting at the same retention time as degradation product ''D1'' in Fig. 2. Investigation into the source of this peak suggested that it was a residual from the MP1 SPE discs. When quantitation of degradation products and metabolites was necessary, an alternative extraction process using a C_{18} phase was employed. The procedure was the same except that

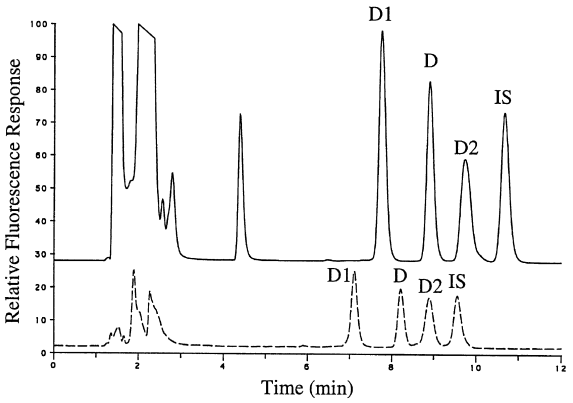

Fig. 2. Chromatograms comparing derivatized and non-derivatized samples. All concentrations are 500 ng/ml and all chromatograms are shown at the same scale. Top: A derivatized sample with fluorescence detection at excitation/emission wavelengths of 290/345 nm. Bottom: A non-derivatized sample with detection at 235/320 nm. Peak identification: D1 and D2 are metabolites and/or degradation products of the drug; D is trecetilide; and I.S. is the internal standard, ibutilide.

the SPE column was washed with acetonitrile–methanol–water (15:15:70, v/v/v) rather than pure methanol.

3.2. Extraction efficiency

Absolute recovery was investigated by analysis of extracted samples prepared in plasma versus directly injected standards at concentrations of 10, 100 and 1000 ng/ml trecetilide ($n=6$). Trecetilide recoveries ranged from 88–95% with relative standard deviations (R.S.D.s) of 2–19%. Recovery of the internal standard (1000 ng/ml) was 96% with a R.S.D. of 6%.

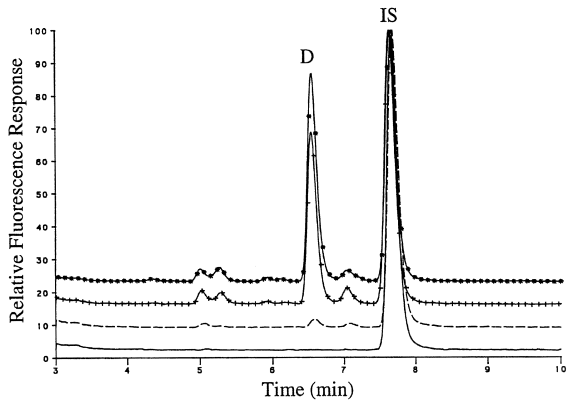

Fig. 3. Chromatograms of extracts of dog plasma samples without derivatization. From top to bottom: samples collected at 2, 12, 32 and 72 h after dosing, with concentrations up to 722 ng/ml. D=Trecetilide; I.S.=internal standard.

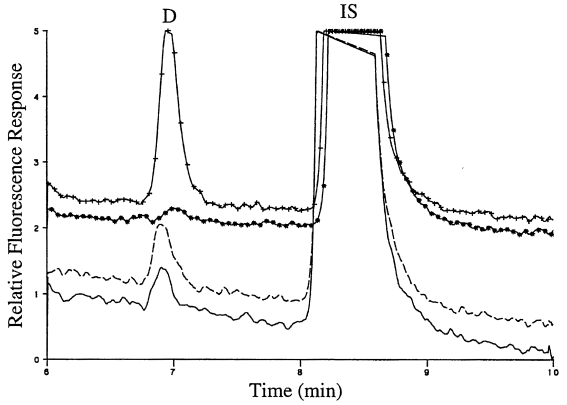

Fig. 4. Expanded chromatograms of extracts of low-concentration dog plasma samples using the derivatization method. From top to bottom: samples drawn at 48 h (4.3 ng/ml) and 72 h (not quantifiable) after dosing and calibration standards (1.0 and 0.5 ng/ml). D=Trecetilide; I.S.=internal standard.

3.3. Linearity

Linearity of the assay was determined by generation of a standard curve from peak height ratios of trecetilide to internal standard in spiked plasma samples. The data were weighted by 1/concentration and fitted to a straight line with intercept using linear regression analysis. Summaries of non-derivatized and derivatized standard curve data for trecetilide in dog plasma are shown in Table 1.

Without derivatization, standard curves were found to be linear on three separate days between the range of 5–10 000 ng/ml trecetilide using 11 standard levels. Mean accuracies (±S.D.) at 5 and 10 000 ng/ml were 98±13% and 100±0.4%, respectively. The correlation coefficient (r) was 1.000 on all three days. Average values of the slope and y-intercept were 1.053±0.021 and 2.2±0.6, respectively.

With derivatization, standard curves were also

Table 1
Standard curve data for trecetilide in dog plasma (mean of three assay runs; two lowest standards assayed in duplicate)

Theoretical concentration (ng/ml)	Without derivatization		With derivatization	
	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)
0.5			98	9.0
1			95	6.0
2			101	6.0
5	98	13	105	2.4
10	98	9.0	99	7.0
20	105	5.0	109	1.5
50	100	2.3	100	4.0
100	99	2.7	98	2.9
200	103	1.1	103	2.9
500	100	1.0	97	4.0
1000	101	1.4	101	1.7
2000	99	0.4		
5000	99	0.5		
10 000	100	0.4		

found to be linear on three separate days between the range of 0.5–1000 ng/ml trecetilide using 11 standard levels. Mean accuracies at 0.5 and 1000 ng/ml were 98±9% and 101±1.7%, respectively. The average correlation coefficient (r) over three days was 0.9995±0.0005. Average values of the slope and y-intercept were 1.13±0.04 and 0.02±0.06, respectively.

3.4. Limits of detection and quantitation

The limits of detection (LODs, concentration at a signal-to-noise ratio of 3) and quantitation (LOQs, concentration at a signal-to-noise ratio of 10) were determined by measuring background noise in blank plasma samples in the trecetilide retention window. The LOD was 0.57 and 4.3 ng/ml for the derivatized and non-derivatized methods, respectively. The LOQ was 1.9 and 14 ng/ml for the derivatized and non-derivatized methods, respectively. Thus, the assay was 7.5-fold more sensitive when derivatization was utilized. Further analysis demonstrated that the improved signal-to-noise ratio was partially due to higher fluorescence after addition of the naphthyl fluorophore and partially due to lower noise at the longer detection wavelength used in the derivatization method. Chromatograms of low concentration calibration standards and unknowns are shown in Fig. 4 for the derivatization method.

3.5. Precision and accuracy

Accuracy and precision were evaluated using quality control samples at low, mid and high concentrations within the standard curve range. Intra-day determinations were evaluated by assaying quality controls in triplicate at each level. Inter-day evaluations were made from the data collected over three days of intra-day precision and accuracy trials. Accuracy was determined by calculating the mean recovery at each level, while precision was assessed from the R.S.D.s of the mean concentrations.

Without derivatization, quality control samples were assayed at concentrations of 35, 875 and 5000 ng/ml trecetilide. Intra-day mean accuracy ranged from 99.6–105% with precision of 0.4–7%. The mean inter-day accuracy ranged from 101–104% with mean precision of 1.0–4%. Overall accuracy and precision for the non-derivatized assay was 102±2.8%.

With derivatization, quality controls samples were assayed at concentrations of 1.4, 35 and 875 ng/ml trecetilide. Intra-day mean accuracy ranged from 90.0–105% with precision of 0.6–7%. The mean inter-day accuracy ranged from 95.5–102% with

mean precision of 3–9%. Overall accuracy and precision for the derivatized assay was 98±6%.

3.6. Stability

Stability of trecetilide working standard solutions stored at ≤−10°C was demonstrated for 10 months. The standard stock solution was stable for at least six months under the same storage conditions, and the internal standard was stable for at least eight months at 2–5°C. Stability of trecetilide in dog plasma was proven for two months when stored at ≤−10°C. At room temperature (20°C), trecetilide in plasma was stable for at least one day. Quality control samples in dog plasma were found to be stable when subjected to six freeze–thaw cycles.

The NIC solution was stable for at least seven days when stored at room temperature. Care was taken to avoid contamination by water or acetic acid as these may inactivate the solution. Prepared samples (derivatized or non-derivatized) were stable in the autosampler (cooled to ≤9°C) for at least four days.

3.7. Robustness

A number of lots of analytical columns, guard columns and SPE columns were successfully used. The cyano analytical column, operated at pH 7.9, needed to be monitored closely because it had a lifetime of only a few hundred injections. The conditions used for derivatization (NIC concentration, temperature and reaction time) were well above the minimum required for complete derivatization. Substantially longer derivatization times (60 min) at elevated temperature (40°C) were avoided as this resulted in some sample degradation and formation of interferences.

3.8. Application

The reported method was used for the determination of trecetilide in dog, rat, rabbit and mouse plasma samples for a variety of toxicokinetic and bioavailability studies. By assaying most of the samples in a study with the simpler non-derivatization method and the lowest concentration samples with the derivatization method, we were able to quantitate over a very wide concentration range. For example, in a study in dogs, the peak concentrations were 7600 ng/ml, and we followed the concentrations out to 72 h after dosing, when the concentrations had decreased to 2 ng/ml in some animals.

3.9. Comparison with previous methods

We previously described a rather elaborate enantiospecific column-switching method for ibutilide which also utilized NIC for derivatization [2]. The previous method utilized five pumps, four columns, two detectors and three column-switching valves. Although the method reported here is not enantiospecific, it did accomplish with one pump what the previous method required four pumps and three columns to accomplish. The improvements made in the new method were primarily (1) the use of a cyano analytical column, which separated the drug and internal standard from excess derivatization agent much better than the previously-used C_8 column, and (2) a five-fold decrease in the amount of NIC used for derivatization, which reduced background interferences. The method reported here could also be used for quantitation of ibutilide, using trecetilide as the internal standard.

Another difference between the previous ibutilide method [2] and the method reported here was the use of the MP1 (mixed phase) SPE columns rather than a C_{18} phase. The MP1 phase retained the drug and internal standard during washing with pure methanol. This helped remove water from the column, which allowed for shorter drying times. Thorough drying was especially important when derivatization was utilized.

4. Conclusions

Derivatization and non-derivatization methods were described for the quantitation of trecetilide in plasma. A rather unique aspect of the methods was that both utilized the same extraction scheme and the same chromatographic conditions, except for detection wavelength. We preferred the use of the simpler and faster non-derivatization method whenever high sensitivity was not necessary. Since the

methods were so similar, we frequently utilized the non-derivatization method to assay most of the samples from a study, then assayed only the low concentration samples with the derivatization method. Most aspects of both assay methods were quite rugged, but an improvement could be made if a more durable cyano analytical column could be found.

References

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[2] C.L. Hsu, R.R. Walters, J. Chromatogr. B 667 (1995) 115.