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Note

Simple, rapid and sensitive high-performance liquid chromatographic procedure for the quantitation of itazigrel in human plasma or serum

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Itazigrel, 2-trifluoromethyl-4,5-bis(*p*-methoxyphenyl)thiazole (Fig. 1), is a drug candidate being investigated by The Upjohn Company for its effects on peripheral vascular disease. Itazigrel is known to be a potent in vitro inhibitor of collagen and arachidonic acid-induced aggregation in human platelet-rich plasma [1]. Human clinical studies to examine the effects of itazigrel on leukotriene B_4 synthesis and cyclooxygenase inhibition were planned and utilized single oral doses as low as 5 mg. Doses this low required analytical methodology with a limit of quantitation at least as low as 0.5 ng/ml.

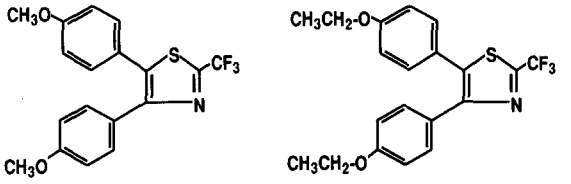

Fig. 1. Structures of itazigrel and the internal standard.

No analytical procedures for the quantitation of itazigrel in human biofluids have been reported in the open literature. We required a precise, sensitive and rapid procedure for the analysis of human biomatrix specimens during the clinical development of itazigrel. Ideally, the procedure would be rugged and easily automated. The method described in this paper met these criteria.

EXPERIMENTAL

Reagents

Itazigrel, 4,5-bis(*p*-methoxyphenyl)-2-(trifluoromethyl)thiazole, and the internal standard, 4,5-bis(4-ethoxyphenyl)-2-(trifluoromethyl)thiazole (Fig. 1), were prepared by The Upjohn Company (Kalamazoo, MI, U.S.A.). Solvents were UV grade from Burdick and Jackson (Muskegon, MI, U.S.A.). Deionized water was further purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.) prior to use in the chromatography system.

Apparatus

The chromatographic system consisted of a Constametric Model III solvent metering pump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), pumping the mobile phase at 1.5 ml/min. The high-performance liquid chromatography (HPLC) detector was a Model 650 10-S fluorescent detector (Perkin-Elmer, Norwalk, CT, U.S.A.) operated with 10-nm slit widths at 320 nm excitation and 430 nm emission. The detector output was monitored with a Model 585 chart recorder (Linear Instruments, Reno, NV, U.S.A.) and a Model 1000 superminicomputer (Harris, Fort Lauderdale, FL, U.S.A.). Data were collected and reduced on the Harris 1000 using custom chromatography software [2]. A Model ISS-100 autosampler (Perkin-Elmer) introduced 300-μl aliquots of sample into the system for chromatography on a Spherisorb RP-8, 250 mm $\times$ 4.2 mm I.D., 5-μm column (Brownlee, Santa Clara, CA, U.S.A.) with a Spherisorb RP-8, 10 mm $\times$ 4.2 mm I.D., 5-μm precolumn (Brownlee) using a mobile phase of methanol–water (80:20, v/v).

Analytical procedure

Approximately 1.5 mg of the internal standard, 4,5-bis(4-ethoxyphenyl)-2-(trifluoromethyl)thiazole, was accurately weighed into a polypropylene 100-ml flask (Nalgene, Rochester, NY, U.S.A.) and dissolved with acetonitrile. Appropriate dilutions were made in acetonitrile to yield a final concentration of about 75 ng/ml.

The calibration curve stock solution was prepared by accurately weighing 1.0 mg of itazigrel into a 100-ml polypropylene volumetric flask and diluting with methanol. Itazigrel was known to have some instability toward light; therefore, all stock solutions in organic solvents were protected from light during storage and use. Calibration curve standards were prepared by making ap-

propriate dilutions of the stock solution with methanol to achieve final concentrations of 5–3000 ng/ml. Plasma or serum standards were prepared by evaporating aliquots (at 20°C) of each standard in methanol in polypropylene tubes (Corning No. 25319, Corning Glass Works, Corning, NY, U.S.A.) and reconstituting with 0.5 ml of blank, normal human plasma or serum (Plasma Alliance, Knoxville, TN, U.S.A.) in order to achieve final concentrations of 0.5–600 ng/ml.

Plasma- or serum-fortified controls were prepared by evaporating aliquots of calibration curve solutions in polypropylene volumetric flasks and reconstituting with blank, normal human plasma or serum to yield final concentrations of about 7, 20, 60, 180 and 400 ng/ml.

Sample preparation consisted of pipetting 0.5 ml of each plasma/serum calibration curve standard, 0.5 ml of each plasma/serum control and 0.5 ml of each clinical specimen into individual polypropylene tubes, adding 100 μl of methanol to each tube and vortexing briefly to mix the contents. The internal standard (1 ml) was then pipetted into each tube, vortex-mixed briefly, then centrifuged at 600 *g* (Sorvall RT6000, Dupont, Kennett Square, PA, U.S.A.) for 5 min. Supernatants were transferred to plastic injector vials (No. 12964, Alltech Assoc., Deerfield, IL, U.S.A.) and 300-μl aliquots were directly injected into the HPLC system by the autosampler.

RESULTS

Chromatography from pre- and post-dose subject plasma specimens is demonstrated in Fig. 2. The post-dose chromatogram shows the itazigrel peak eluting at about 6.4 min with the internal standard peak at about 8.8 min. The

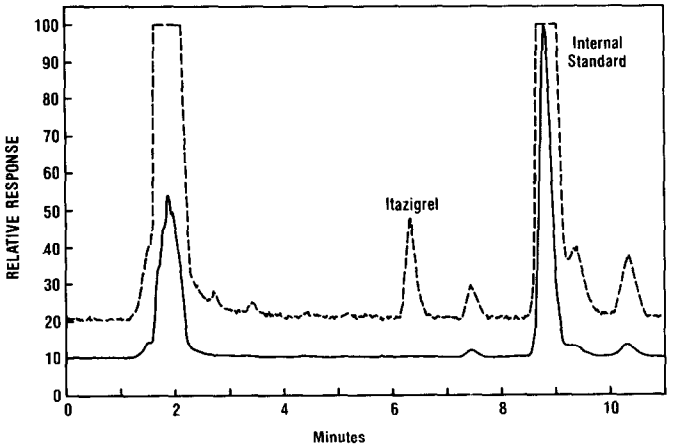

Fig. 2. Chromatogram of pre-dose (solid line) subject specimen and post-dose (dotted line) subject specimen. The scale of the post-dose specimen has been expanded five times.

itazigrel peak in this specimen was calculated to be equivalent to 22 ng/ml. At the retention time for itazigrel in the pre-dose sample a flat baseline was obtained. A small interference peak on the trailing edge of the internal standard was present in all samples. This peak was small in proportion to the internal standard, and because quantitation was with peak heights, no bias was introduced into the calculations. The three small peaks prior to and trailing the internal standard peak were matrix-related and remained constant during changes in itazigrel peak heights, verifying that these were not drug-related.

The recovery of itazigrel from glass versus plastic was investigated by analyzing calibration curves of itazigrel prepared in both glass and plastic with water, plasma and serum as the sample matrix. Comparison of calibration curve regression slopes indicated that with itazigrel in water, recovery from glass tubes was 75% that recovered from plastic tubes. We therefore assumed that itazigrel had some affinity for glass and carried out all experiments using plas-

TABLE I

CONTROL RECOVERY FROM HUMAN PLASMA

Added (ng/ml)	*n*	Found (ng/ml)	Recovery (%)	R.S.D. (%)
7	14	7.73 ± 0.2	110	3.0
20	32	19.5 ± 1.0	97.5	5.2
60	32	61.0 ± 2.2	102	3.5
180	32	184 ± 8.7	102	4.7
400	32	404 ± 8.7	101	4.6

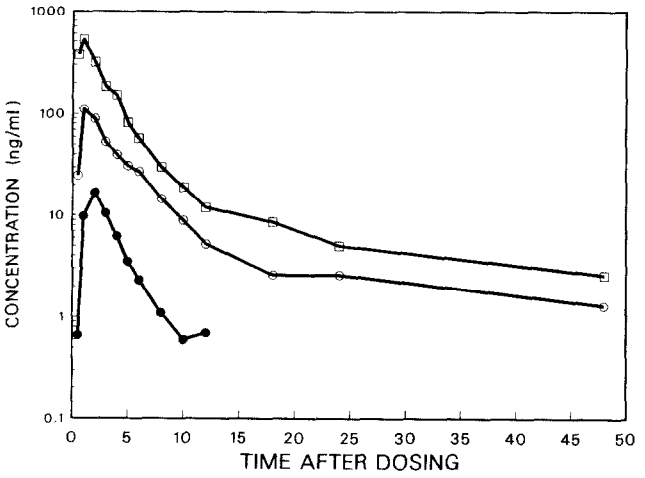

Fig. 3. Concentration-time profile (0–50 h) of subjects dosed with 5 mg (closed circles), 25 mg (open circles) and 100 mg (open squares) of itazigrel.

tic utensils. The absolute recovery of itazigrel from normal human serum and normal human plasma was determined by comparing calibration slopes from these two matrices (in plastic) with slopes from standards in water. The recovery of itazigrel from serum was 100.3% while that from plasma was 99.7%.

Over the 0.5–600 ng/ml calibration curve range correlation coefficients ranged from 0.9960 to 0.9999 for system analytical runs with a mean, through-the-origin slope of 0.0068 ± 0.00037 and a coefficient of variation (C.V.) of 5.4%. The between-run C.V. for back-calculated concentrations was 21.6, 20.5, 15.8, 8.5, 8.1, 8.0, 4.1, 2.9, 8.2, 2.6 and 1.5% for standards of 0.5, 1.0, 2.0, 5.0, 10, 20, 40, 100, 200, 400 and 600 ng/ml, respectively. The limit of quantitation was determined to be 0.5 ng/ml (47 pg on-column) using a between-run precision upper limit of $\pm 25\%$. In all cases the intercept of the slope was not significant; therefore, the through origin model was used for all calculations.

Control recoveries are given in Table I. Excellent recoveries and between-run precisions were obtained at all concentrations.

The utility of the method was demonstrated by the analysis of plasma specimens from subjects dosed with soft elastic capsules containing 5, 25 and 100 mg of itazigrel. Fig. 3 shows representative concentration–time profiles for subjects dosed with 5, 25 and 100 mg of itazigrel.

No metabolites of itazigrel in man have been identified in the blood; therefore, this procedure is highly specific for the intact drug. Furthermore, the use of fluorescence detection enhances the specificity of this method for itazigrel, when other drugs may be present in the blood. However, other drugs have not been tested with this method; therefore, it may not be appropriate as a forensic procedure.

The method described in this report has been shown to be precise and sensitive enough for use in pharmacokinetic studies where the minimum quantifiable level required will be 0.5 ng/ml or greater.

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