Journal of Chromatography, 619 (1993) 172–176
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7015

Short Communication

High-performance liquid chromatographic determination of concentrations of the reversible H^+/K^+ ATPase inhibitor SK&F 97574 in plasma

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(First received March 5th, 1993; revised manuscript received June 2nd, 1993)

ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) assay using ultraviolet spectrophotometric detection has been developed for the determination of the concentration of 3-butyryl-4-(2-methylphenylamino)-8-(2-hydroxyethoxy)quinoline (I) in rat, dog and human plasma. Prior to analysis, the protein in plasma samples was precipitated with acetonitrile containing 3-butyryl-4-(2-methylphenylamino)-8-methoxyquinoline to act as an internal standard. The supernatant layer was injected onto the HPLC column with no further clean-up. The assay requires 200 μl of plasma and is precise and accurate within the range 25–1000 ng/ml. The mean within-run and between-run coefficients of variation were $<6\%$ at 25 ng/ml and greater concentrations. The mean accuracy of quality control standards was generally within $\pm 5\%$ of the nominal concentration. Recovery of I and internal standard from plasma was approximately 100% over the entire assay range irrespective of species.

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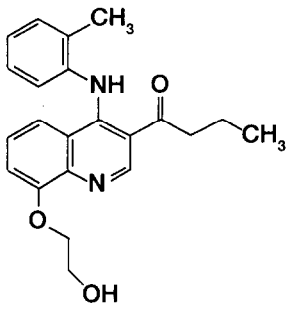
0378-4347/93/$06.00

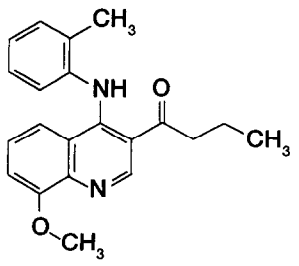
INTRODUCTION

Clinical trial evaluation has started on the hydrochloride salt of 3-butyryl-4-(2-methylphenylamino)-8-(2-hydroxyethoxy)quinoline (SK&F 97574) (I, Fig. 1). This compound is a reversible proton pump (H^+/K^+ ATPase) inhibitor intended for use as an oral treatment for acid-related gastrointestinal disorders. The H^+/K^+ ATPase is the enzyme involved in the final stage of the production of hydrochloric acid by the gastric parietal cell.

Irreversible proton pump inhibitors such as omeprazole [1] are already available and have been shown to be effective in healing upper gastrointestinal ulceration. Compounds in this class cause a profound and long lasting inhibition of acid secretion by covalently binding as an acid-activated intermediate to one or more thiol groups on the enzyme [2]. Profound suppression of gastric secretion is a very effective way of rapidly healing acid-related upper gastrointestinal ulceration. The extent and rate of healing has been correlated with the degree and duration of acid suppression during treatment [3,4].



SK&F 97574



SK&F 96067

Fig. 1. Structures of I (SK&F 97574) and I.S. (SK&F 96067).

Like omeprazole, I accumulates in the acidic compartments of the parietal cell adjacent to the H^+/K^+ ATPase. However, unlike omeprazole, I reversibly inhibits the proton pump by competing with luminal potassium. Thus, profound inhibition of acid secretion due to all stimuli should be achievable but the duration of action will be dependent on the pharmacokinetics of the compound rather than enzyme turnover. Both the degree and duration of inhibition should therefore be controllable by choosing an appropriate dosing regimen.

An HPLC method has been developed for the quantification of concentrations of I in rat, dog and human plasma. The method consists of the precipitation of protein in plasma samples with acetonitrile and the injection of the centrifuged supernatant for isocratic reversed-phase HPLC with UV detection. Consequently the analytical procedure is simple and the method has a high sample throughput. This uncomplicated approach is possible because I has a large absorption coefficient at 256 nm which allows accurate measurement at levels as low as 25 ng/ml with minimal sample clean-up.

EXPERIMENTAL

All chemicals used were at least reagent grade and solvents of HPLC grade. They were purchased from J. T. Baker (Phillipsburg, NJ, USA), Fisher (Fair Lawn, NJ, USA) and Aldrich (Milwaukee, WI, USA). Pure water was obtained using a 661A water purification system (Hewlett Packard, Palo Alto, CA, USA).

Compound I and 3-butyryl-4-(2-methylphenylamino-8-methoxyquinoline (free base) (internal standard, I.S., Fig. 1) were synthesised by Smith-Kline Beecham Pharmaceuticals (Welwyn, UK) and were >99% pure by HPLC analysis.

Preparation of drug standard solutions

Approximately 10 mg of I were weighed out and dissolved in acetonitrile (10 ml) to give a stock standard solution of approximately 1.0 mg/ml. This stock solution was stored at −80°C and was stable for at least two months at this temperature. Separate weighings were made for preparing standard curves and samples used for the estimation of precision.

Preparation of internal standard solution

Approximately 10 mg of I.S. were weighed out and dissolved in acetonitrile (10 ml) to give a stock standard solution of approximately 1.0 mg/ml. This stock solution was stored at −80°C and was stable for at least two months at this temperature. The stock solution was diluted 1:100 in acetonitrile, mixed and stored at −20°C. It was stable for one week at this temperature. On the day of assay the 10 μg/ml solution was diluted to 500 ng/ml with acetonitrile and used in the precipitation of plasma proteins in samples and standards.

Preparation of plasma standards

Blood samples were taken from rat, dog and human subjects who had not received any medication in the previous 48 h, into heparinised tubes, mixed and centrifuged. The plasma was then frozen immediately and stored at −20°C until required for assay. Standards were prepared at concentrations of 1000 and 800 ng/ml in plasma by adding appropriate amounts of I as a standard solution (approximately 100 or 10 μg/ml). to 6 ml of control plasma collected as described above. These plasma standards were thoroughly mixed and diluted with control plasma to provide calibration standards over the range 25–1000 ng/ml. Standards used for the estimation of precision were prepared in the same way, but from a different stock solution.

Apparatus

We used HPLC equipment consisting of a WISP Model 710B (Millipore, Milford, MA, USA), a Model 114M pump (Beckman Instruments, Fullerton, CA, USA) and Model 773 UV detector (Kratos Analytical, Ramsey, NJ, USA). A Model LC 22A column heater (BAS, West Lafayette, IN, USA) was used to maintain the column (Beckman Ultrasphere C_8, 5 μm, 250 mm × 4.6 mm I.D.) at a constant temperature of 40°C. The mobile phase flow-rate was 1 ml/min and the detector was set to monitor at 256 nm. A PE/Nelson ACCESS CHROM data system (Nelson Analytical, Cupertino, CA, USA) was used to acquire chromatographic data.

Mobile phase

Acetonitrile (1200 ml) containing triethylamine (1.5 ml) was mixed with ammonium formate solution (800 ml; 0.07 *M*) containing 0.1% triethylamine, and the mixture was adjusted to pH 6.8 by dropwise addition of dilute formic acid (*ca.* 22%). The mobile phase was filtered and degassed using a solvent filtering apparatus (Millipore) and 0.45-μm filter. Once prepared the ammonium formate solution can be stored in a stoppered glass bottle at room temperatures for at least two months.

Sample preparation

Samples and standards were prepared for direct injection onto the HPLC column after precipitation of the plasma protein with acetonitrile containing I.S. Plasma (0.2 ml) and I.S. solution (0.4 ml) were pipetted into a polypropylene tube (3.6 ml). The tube was capped and contents mixed on a vortex mixer for approximately 1 min (3 × 20 s). The tube was allowed to stand for approximately 20 min to reach equilibrium and then centrifuged at approximately 1600 *g* for about 20 min. The supernatant was then carefully pipetted into an autoinjector vial containing a low-volume insert and the vial was capped. Finally, 100 μl of the extract were injected onto the HPLC column.

Calibration and calculation

A calibration curve containing eleven points and a blank were prepared in the plasma of each species, rat, dog and human. Standard and I.S. peak-height ratios were determined and plotted against concentration of I to construct the line.

The slope and intercept were determined by a weighted ($1/y^2$) least-squares linear regression. This weighting factor was chosen because it gave the best individual residual values.

Determination of precision and accuracy

Five replicate standards were prepared in rat, dog and human plasma at each of three concentrations (25, 400 and 900 ng/ml), and assayed on three consecutive days. The mean of the daily coefficients of variation was calculated to estimate the within-day precision. The between-day precision was estimated by calculating the ratio of the standard deviation of the daily mean concentration to the mean of the daily mean concentration, and expressed as a percentage. Accuracy was estimated as the ratio of the mean concentration by analysis to the nominal concentration, and expressed as a percentage. Mean accuracy was reported as the mean of the daily accuracy estimations.

Determination of recovery

The recovery of I was determined at concentrations of 50 and 500 ng/ml of I in plasma by comparison of plasma samples with separately prepared standard solutions. Triplicate standards in rat, dog and human plasma were prepared. Triplicate solution standards were prepared by adding an appropriate amount of I in solution (in 100 μl) to I.S. solution (10 ml). Water was then added to equal the volume of plasma supernatant obtained for a plasma standard. Aliquots (100 μl) were then injected onto the HPLC system, and the peak heights from plasma standards and directly injected solution samples were compared.

RESULTS AND DISCUSSION

Since the method was required to support toxicological and clinical pharmacokinetic studies with I, the assay had to be precise to a coefficient of variation within 20% at the lower limit of quantification and $<15\%$ throughout the required concentration range. Accuracy ($\pm 15\%$ bias) and linearity had also to be demonstrated throughout the required concentration range. In addition, the assay had to have a high rate of sample throughput to be able to support a large development programme. These conditions were met because of the simplistic approach to sample preparation that yielded a clean extract with essentially quantitative recovery of I and I.S.

Chromatography

Similar chromatograms were obtained from rat, dog and human plasma extracts. No significant interfering peasks that co-eluted with I or I.S. were observed. These results were good evidence that the assay was selective for I over en-

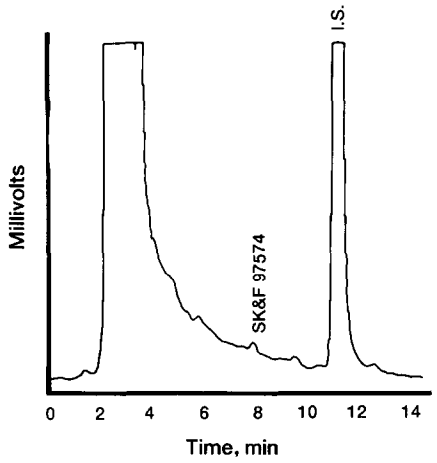


Fig. 2. Representative chromatograms for I in human plasma.

dogenous compounds. No information is available on the metabolism of I, but no peaks have been observed other than I and I.S. in the relevant region of the chromatogram when post-dose samples have been run. Typical chromatography is presented in Fig. 2, which shows chromatograms of human control and control plasma spiked at the lower limit of quantification (25 ng/ml).

Linearity

Back-calculated concentrations for the calibration curves for each species are within 5%. Linearity was demonstrated over the range 25–1000 ng/ml for I extracted from 200 μl of plasma. Smaller volumes of plasma (*e.g.* 50 μl for a rat plasma assay) may be used either by adapting the extraction conditions or by adding control plasma to make the total equal to 200 μl. This will change the lower and upper limits of quantification of the assay depending upon the final amount of I injected onto the HPLC column.

Precision and accuracy

The within-day and between-day precision values for the assay were better than 6% at all concentrations in all three species studied. Mean accuracy [calculated as the mean (over three days) of the daily ratio of mean concentration by analysis to nominal concentration, and expressed as a percentage] was generally within 5% of nominal at the concentrations studies in these three species.

Recovery

The sample was prepared for introduction into the HPLC column by protein precipitation. Consequently, there was no traditional phase separation step. However, the peak heights from treated plasma standards were compared with those from known amounts injected onto the HPLC system as separately prepared solutions. Comparison of the peak height for treated plasma samples with those for equivalent standard solutions injected directly onto the HPLC column showed that recovery of I and I.S. was essentially complete.

REFERENCES

1 A. Brandstrom, P. Lindberg, U. Junggren and B. Wallmark, *Scand. J. Gastoenterol.*, 21 (Suppl. 118) (1986) 54.
2 D. J. Keeling, C. Fallowfield, K. J. Milliner, S. K. Tinoley, R. J. Ife and A. H. Underwood, *Biochem. Pharmacol.*, 34 (1985) 2967.
3 D. W. Burget, S. G. Civerton and R. H. Hunt, *Gastroenterology*, 99 (1990) 345.
4 C. H. Howden and R. H. Hunt, *Aliment. Pharmacol. Ther.*, 4 (1990) 25.