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Short Communication

Sensitive liquid chromatographic method for the determination of a specific M1 agonist, LY246708, an investigational agent with potential for the treatment of Alzheimer's disease, in human plasma

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ABSTRACT

A reversed-phase HPLC method is reported for the determination of a new M1 agonist, LY246708, in human plasma. The compound and an internal standard were extracted from plasma with hexane at basic pH. The organic extract was evaporated to dryness and the residue was reconstituted with mobile phase [0.5% diethylamine (pH 3, adjusted with phosphoric acid)–acetonitrile (70:30, v/v)]. The analytes were separated from endogenous substances on a Zorbax CN column; the effluent was monitored by measuring its absorbance at 296 nm. The limit of quantification was determined as 1.5 ng/ml and the response was linear from 1.5 to 20 ng/ml. Validation studies showed the method to be both repeatable and reproducible. Its robustness was demonstrated by transfer between analytical laboratories and continued use in support of pharmacokinetic studies and therapeutic monitoring of the compound.

INTRODUCTION

Alzheimer's disease, a dementing disorder of insidious onset and progressive deterioration, remains one of the greatest challenges in medical research. The disease is thought to involve specific loss of cholinergic function as opposed to a diffuse and non-specific cell death throughout the brain [1–3]. To date, potential symptomatic treatments have included administration of choline, cholinesterase inhibitors and cholinergic agonists. The clinical utility of these agonists has been limited by their relative selectivity for non-M1 receptors and prominent side-effects. A specific M1 agonist may be beneficial in targeting post-synaptic M1 receptors while avoiding the non-M1 receptor-mediated side-effects.

3-[4-(Hexyloxy)-1,2,5-thiadiazol-3-yl]-1,2,5,6-

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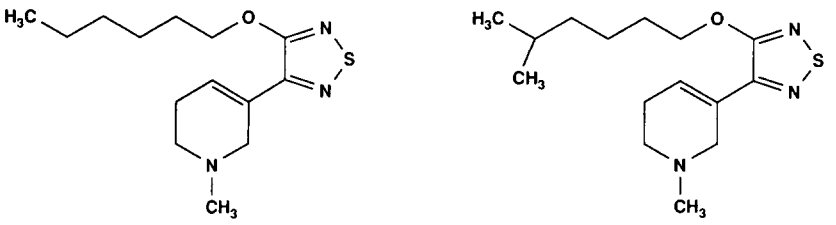

LY246708 (I) **Internal Standard (II)**

Fig. 1. Structures of LY246708 (I) and the internal standard (II).

tetrahydro-1-methylpyridine [LY246708 (NNC 11-0232; hexyloxy-TZTP)] (I, Fig. 1) has been characterized as one of the most selective M1 agonists known and is currently undergoing early clinical trials [4]. In support of these trials, a method has been developed and validated for the determination of the parent drug in human plasma. This paper presents the scientific rationale for the development of the method together with its validation prior to its use in support of a phase I clinical study.

EXPERIMENTAL

Chemicals and reagents

I (tartrate salt) and the internal standard, 3-(4-(5-methylhexyloxy-1,2,5-thiadiazol-3-yl)-1,2,5,6-tetrahydro-1-methylpyridine oxalate (II, Fig. 1) were obtained from Lilly Research Labs. (Eli Lilly and Company, Indianapolis, IN, USA) and from Novo Nordisk (Malov, Denmark). HPLC-grade methanol, hexane and acetonitrile were purchased from Burdick & Jackson or Fisons. HPLC-grade water [*e.g.*, prepared using a Milli-Q system (Millipore) or purchased from Fisons] was used to prepare all aqueous solutions. All other chemicals were of analytical-reagent grade. Control (blank) human plasma was obtained from healthy volunteers.

Liquid chromatography

The HPLC system consisted of an isocratic pump, a Spectroflow 783 variable-wavelength UV detector (Kratos Division, ABI Analytical, Ramsey, NJ, USA) and an autosampler. The analytical column was a Zorbax CN column (15 cm × 4.6 mm I.D., 5 μm (DuPont, Wilmington, DE, USA). An in-line filter (2 μm) (Upchurch Scientific, Oak Harbor, WA, USA) was positioned directly in front of the analytical column. A Hewlett-Packard Model 1000 computer was used for on-line data acquisition and subsequent calculations.

The mobile phase was 0.5% diethylamine (adjusted to pH 3.0 with concentrated phosphoric acid)–acetonitrile (70:30, v/v). Chromatography was performed at ambient temperature at a flow-rate of 1.0 ml/min. A total chromatography time of 25 min per sample was required. The analytes were detected by measuring their absorbance at 296 nm.

Preparation of standard solutions

Silanized glassware was used throughout for the preparation of standards and samples. A stock standard solution containing I was prepared in water at a concentration of 1 μg/ml (free base). A stock standard solution of the internal standard was prepared in methanol at a concentration of 1 μg/ml.

Plasma standards were prepared at analyte concentrations of 1.50, 3.00, 5.00, 7.50, 12.5 and 20.0 ng/ml by diluting appropriate aliquots of the stock standard solution with blank plasma.

Sample preparation procedures

Aliquots of plasma samples or standards (1 ml) were dispensed into silanized 15-ml disposable glass tubes with PTFE-lined screw-caps. Following the addition of internal standard solution (25

μl), the samples were vortex-mixed and then made basic by the addition of 1 ml of 125 m*M* KCl–NaOH buffer (pH 12.5). After mixing, hexane (5 ml) was added to each sample. The samples were mixed on a rotary mixer at 2–20 rpm for 20 min and then centrifuged at *ca.* 2900 *g* for 10 min at 5°C. Following centrifugation, the lower aqueous layer was immediately frozen by immersing the tubes in a dry ice–acetone bath; the upper organic layer was decanted into silanized culture tubes. The supernatant was dried at 40°C under nitrogen. The samples were reconstituted in 200 μl of mobile phase, vortex-mixed and transferred into silanized glass HPLC autosampler vials.

Calculations

A least-squares calibration graph was obtained by plotting the concentrations of the plasma standards against the peak-height ratios. The peak-height ratios were obtained by dividing the peak heights of I by the peak heights of the internal standard. The concentration of I in each sample was determined from the peak-height ratio relative to the calibration graph.

Determination of recovery, precision and accuracy

The extraction efficiency (recovery) of the sample preparation procedure was tested on three days by comparing the peak heights obtained from chromatography of aqueous standards with those of extracted plasma samples that had been spiked with the analytes. The recovery was determined at 15 ng/ml I.

The precision and accuracy of the method were determined by performing replicate analyses of six pools of plasma spiked with known concentrations of the compounds. The pool concentrations were selected to cover the range of the calibration graph and included the limit of quantification. Five replicates of each pool were analyzed on three different days by the same analyst on the same instrument. All samples were analyzed in random order. Two calibration graphs were included each day, one at the end and one at the beginning of the run.

Determination of stability

The stability of I in plasma was determined by preparing pooled plasma with known amounts of I (2, 10 and 18 ng/ml). The pools were aliquoted (1 ml) into silanized glass tubes, sealed and stored at −20°C. Six replicates of each pool were assayed after storage for four months. More extensive plasma stability studies (room temperature, 4°C, −20°C, −70°C and freeze–thaw) are in progress.

Processed sample stability was ensured by including calibration standards at the beginning and end of each set of samples analyzed.

Analysis of samples from a clinical study

Plasma samples from volunteers in a clinical study were frozen and maintained at −20°C prior to analysis. On thawing, samples were centrifuged (2900 *g* for 10 min at 5°C) and a 1-ml aliquot was pipetted into a 15-ml disposable glass tube. The samples were then processed as described under *Sample preparation procedures.* High, medium and low quality control samples, prepared from a separate weighing of standard, were included with each batch of clinical samples analyzed.

RESULTS AND DISCUSSION

Method development and chromatography

The assay conditions described in this paper evolved from the evaluation and optimization of the sample preparation, chromatography and detection of I and the internal standard. This process began with the selection of an appropriate detection scheme. The options available for the detection of I were restricted by the required sensitivity of the assay (less than 5 ng/ml) and the physical characteristics of the compound. I has minimal inherent fluorescence and it is not electroactive at an analytically useful potential. The compound is not detected by electron-capture GC and the sensitivity offered by flame ionization detection (*ca.* 100 ng/ml) is insufficient. Although I has no functional sites suitable for derivatization that could be used to enhance its detection, it does possess a moderate chromophore

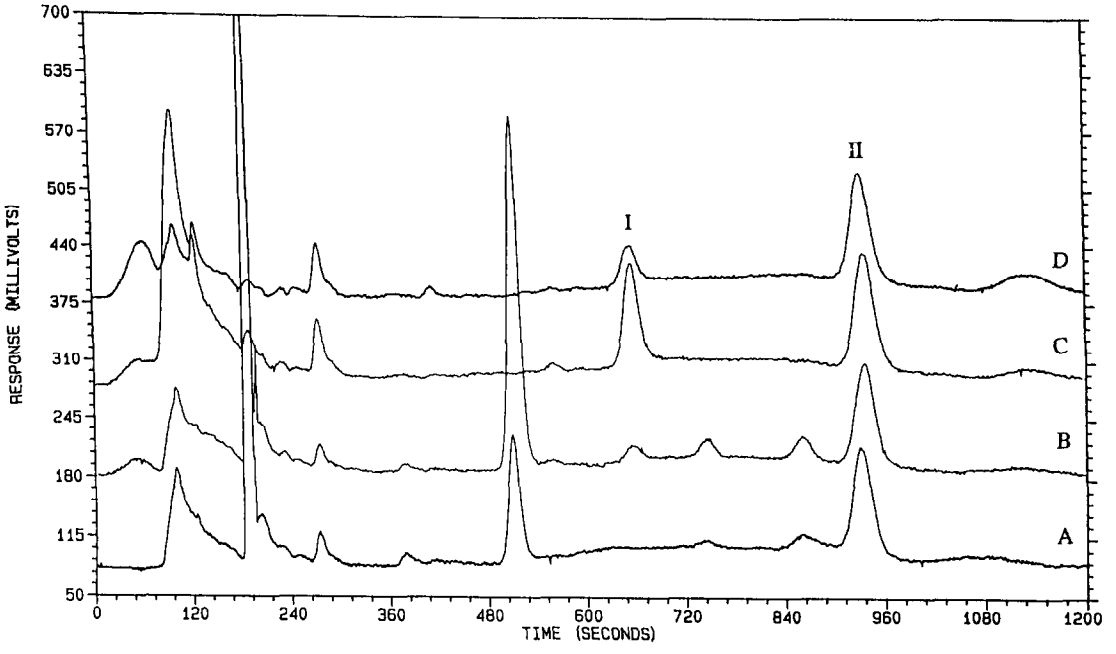

Fig. 2. Representative chromatograms from a subject administered a single oral dose of I. Subject samples: (A) pre-administration of drug; (B) 1 h post-administration; (C) 4 h post-administration; (D) 8 h post-administration. Peaks: I = LY246708; II = internal standard.

that absorbs light at 296 nm. I can also be detected using a nitrogen–phosphorus detector on a gas chromatograph. The choice of liquid over gas chromatography was dictated by ruggedness concerns. The performance of the nitrogen–phosphorus detector was variable from day to day, making it difficult to generate high-quality data consistently.

In evaluating the chromatographic separation, several columns were screened for optimum peak shape and sensitivity. Base-deactivated columns, including YMC-basic, Zorbax RX-C_{18}, Vydac C_{18} (end-capped) and Zorbax CN, were selected owing to the basicity of I. The Zorbax CN column yielded superior peak shapes, which were further optimized by using an acidic buffer in the mobile phase.

Hexane extraction was chosen for sample preparation because it yielded a clean extract with acceptable recovery. The extraction was facilitated by adjusting the plasma samples to basic pH prior to extraction, thereby deprotonating the amine groups on I.

Representative chromatograms of extracted samples are shown in Fig. 2. The chromatogram obtained from the pre-dose sample indicates the absence of interferences at the retention times of the analytes (Fig. 2).

Sample recovery

The mean recoveries of I and the internal standard were determined as 76 and 69% at 7 and 25 ng/ml, respectively.

Precision, accuracy and limit of quantification

The determination of I in plasma was evaluated for precision and accuracy by replicate analyses of plasma pools spiked with the analyte at various concentrations. Replicates of the same pools were evaluated on three different days so that both within-day and between-day precision and accuracy could be determined. Overall, the precision and accuracy of the method are excellent. The within-day and between-day relative standard deviations and accuracies were within 10% at all levels except the lowest level (1.5 ng/

TABLE I

PRECISION AND ACCURACY DATA FOR I

Day	Parameter	Theoretical concentration (ng/ml)					
		1.50	3.00	6.00	9.00	16.0	24.0
1	Mean result (n = 5) (ng/ml)	1.63	2.91	5.71	8.90	14.4[a]	22.2
	R.S.D. (%)	9.8	9.4	11.6	6.7	4.2	2.5
	% of theory	109	97.0	95.2	98.9	90.0	92.5
2	Mean result (n = 5) (ng/ml)	1.72	2.84	6.54[a]	9.41	15.6	23.3
	R.S.D. (%)	11.6	5.4	2.6	4.4	3.2	4.3
	% of theory	115	94.7	109	105	97.5	97.1
3	Mean result (n = 5) (ng/ml)	1.73	3.07	5.79	8.59[a]	14.7	22.8[a]
	R.S.D. (%)	12.8	6.4	2.5	0.8	4.4	3.8
	% of theory	115	102	96.5	95.4	91.9	95.0
Overall	Mean result (n = 15) (ng/ml)	1.69	2.94	5.93	9.00	15.0	22.8
	R.S.D. (%)	11.0	7.5	8.9	5.9	5.1	3.9
	% of theory	113	98.0	98.8	100	93.8	95.0

[a] A sample was lost owing to poor chromatography, resulting in n = 4. There were therefore only fourteen samples of this pool overall.

ml) (Table I). The precision and accuracy of the 1.5 ng/ml plasma pool were $\leqslant$15%, which, for the purposes of the study, was considered satisfactory; this level was therefore taken as the limit of quantification.

Linearity

Linearity of the method with 1-ml sample volumes was established over the concentration range 1.5–20 ng/ml. Typical correlation coefficients were greater than 0.99.

TABLE II

STABILITY OF I IN HUMAN PLASMA STORED AT −20°C FOR FOUR MONTHS

Theoretical concentration (ng/ml)	Mean (n = 6) assay result (ng/ml)	R.S.D. (%)	% of theory
2.00	1.92	17.8	95.8
10.0	8.05	2.9	80.5
18.0	16.8	3.9	93.3

Stability

Analyses of pooled plasma samples, with concentrations of I in the range 2–18 ng/ml, showed no appreciable decrease in drug content after four months of storage at −20°C (Table II). This indicates that I is essentially stable for prolonged periods when stored at −20°C.

As each batch of clinical samples took *ca.* 50–60 h to process using the HPLC system and calibration standards were included at the start and end of every batch, it may be concluded that processed samples are stable over this period at room temperature.

Application of the method in pharmacokinetic studies

The validated procedure was used to provide pharmacokinetic data for I in man following administration of single oral doses in a safety study. Plasma samples were obtained at defined time intervals post-administration, extracted and analyzed by HPLC. In addition, analysis of plasma samples collected before drug was administered demonstrated that interferences from endogenous plasma components did not compromise

the quality of the results obtained. Representative chromatograms showing the variation of plasma levels of I with time are given in Fig. 2.

CONCLUSIONS

The method reported here is sensitive and selective for the determination of I in human plasma. This procedure is simple and has undergone cross-site validation, verifying its ruggedness. The assay was shown to be sufficiently sensitive for use in pharmacokinetic studies following single oral doses of I.

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