Journal of Chromatography, 631 (1993) 227–232
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2602

Determination of oxiracetam in human plasma by reversed-phase high-performance liquid chromatography with fluorimetric detection

Richard C. Simpson, Venkata K. Boppana, Bruce Y.-H. Hwang and Gerald R. Rhodes

Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406 (USA)

ABSTRACT

Reversed-phase HPLC methodology utilizing pre-column derivatization and post-column reaction fluorimetric detection has been developed and applied to the determination of oxiracetam in human plasma. The method involves preliminary isolation of oxiracetam and internal standard from plasma by solid-phase extraction prior to the formation of their *n*-propyl carbamate derivatives. The carbamate derivatives were subsequently isolated by solid-phase extraction and subjected to a gradient liquid chromatographic separation on an octadecylsilica column prior to on-line post-column alkaline hydrolysis to produce the corresponding primary amine, which was in turn derivatized with *o*-phthalaldehyde and 3-mercaptopropionic acid to yield a fluorescent isoindole. The isoindole was then quantified using a fluorescence detector. The method provided an on-column detection limit of 0.5 ng of oxiracetam and was sufficiently sensitive, accurate, and precise to support pre-clinical or clinical pharmacokinetic studies.

INTRODUCTION

Oxiracetam (4-hydroxy-2-oxo-1-pyrrolidine acetamide) is a highly polar achromophoric monohydroxy investigational nootropic agent, which has shown potential for the treatment of various cognitive disorders [1–3]. Oxiracetam has also been shown to improve both learning and memory processes [4,5]. In order to suport pharmacokinetic characterization of the drug, a sensitive and specific assay was required for the determination of oxiracetam in human plasma. Previous analytical methods for the determination of oxiracetam in physiological matrices have utilized normal-phase HPLC [6–10], many of which employed column-switching techniques [7–10]. Due to a lack of native fluorophoric or suitable chromophoric properties, detection of oxiracetam was typically performed at low wavelength in the UV region. Major limitations of these methods include lack of sensitivity and/or specificity encountered with low-wavelength UV detection and long run times required by the column-switching techniques. The goal of our study was to develop a sensitive and specific assay for oxiracetam in human plasma which would eliminate or minimize such limitations. We recently reported an approach for the pre-column derivatization and post-column reaction detection of monohydroxy compounds [11]. The technique involved the pre-column conversion of the monohydroxy compound to an *n*-alkyl carbamate, which was then subjected to reversed-phase HPLC separation, followed by post-column alkaline hydrolysis to the corresponding free primary amine. The primary amine was subsequently derivatized with *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid (3-MPA) to yield a fluorescent isoindole which was quantified by fluorescence detection. The current paper describes the utilization of this methodology to develop a sensentive and specific assay for oxiracetam in

Correspondence to: R. C. Simpson, Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406, USA.

0021-9673/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

human plasma in order to permit pharmacokinetic evaluation of the drug. Although oxiracetam was used as its racemate, the assay described here is not enantio-specific. Thus no differentiation between the two enantiomeric forms of oxiracetam was achieved.

EXPERIMENTAL

Materials

Materials and reagents were obtained from the sources previously described [11].

o-Phthalaldehyde reagent solution

The post-column OPA reagent solution was prepared as previously described [11].

Standards solutions

Working aqueous standard solutions of oxiracetam at concentrations of 40, 4, and 0.4 μg/ml were prepared by appropriate dilutions of a 500 μg/ml aqueous stock solution. These solutions were stable for 5 weeks when stored at 5°C. Similarly, a working aqueous solution of internal standard at a concentration of 25 μg/ml was prepared by appropriate dilution of a 1000 μg/ml aqueous stock solution. The working internal standard solution was stable at ambient temperatures for 2 weeks.

Sample preparation

Solid-phase extraction from plasma. A 200-μl aliquot of human plasma, 50 μl of water (or aqueous standard solutions when preparing calibration standards), and 50 μl of working internal standard solution were mixed in a 1.5-ml polypropylene microcentrifuge tube. Plasma proteins were precipitated by the addition of 500 μl of acetonitrile. Following vortex-mixing and centrifugation for 10 min at 8800 *g*, the resulting supernatant was transferred to a borosilicate tube containing 500 μl of 0.1 *M* potassium phosphate buffer (pH 8.5). After brief vortex-mixing, the sample was applied to a phenylboronic acid solid-phase extraction cartridge which had been conditioned with one column volume of 0.1 *M* potassium phosphate buffer (pH 8.5). Using vacuum, the sample was slowly passed through the cartridge and collected in a 75 × 12 mm polypropylene tube. The sample was evaporated to dryness at 50°C under a nitrogen flow. The resulting residue was reconstituted with 1 ml of methanol, followed by brief sonication to break up phosphate salt residue and vortex-mixing to aid the dissolution of the drug and internal standard. The tube was centrifuged for 5 min at 1500 *g*. The resulting supernatant, free of insoluble potassium phosphate, was transferred to a borosilicate tube and evaporated to dryness at 50°C under a nitrogen flow.

Pre-column formation and extraction of carbamate derivatives. To the dry residue from the previous step, 200 μl of anhydrous pyridine and 100 μl of *n*-propyl isocyanate were added followed by vortex-mixing. The tube was sealed with Parafilm and immersed in a water bath at 50°C for 1 h to achieve formation of the *n*-propyl carbamate derivatives of the drug and internal standard. After the reaction period, the crude derivatization solution was evaporated to dryness under a nitrogen flow. The dry residue was dissolved in two 0.5-ml aliquots of acetonitrile and the resulting solutions were applied to a silica solid-phase extraction cartridge which had been conditioned with one column volume of acetonitrile. The sample was passed through the cartridge and discarded. The cartridge was washed with 1 ml of acetonitrile, followed by elution of the carbamate derivatives with 1 ml of 50% (v/v) methanol in acetonitrile. The eluate was collected in a 75 × 10 mm borosilicate tube and evaporated to dryness at 50°C under a nitrogen flow. The residue was reconstituted with 200 μl of HPLC-grade water and transferred to an autosampler via. Volumes of 10–25 μl were injected for HPLC analysis. The *n*-propyl carbamate derivatives were stable in aqueous solution for a period of 4 days at ambient temperatures.

High-performance liquid chromatography

The HPLC system used has been described elsewhere [11]. In addition, a 500-p.s.i. pump pre-load cartridge (Upchurch Scientific, Oak Harbor, WA, USA) and a pulse damper (Scientific Systems, State College, PA, USA) were used in conjunction with the post-column reagent pump in order to provide a more stable baseline.

Standard curves

Using the oxiracetam and internal standard working solutions described above, a series of 200-μl plasma samples were prepared at drug concentrations of 40, 60, 100, 200, 600, 1000, 2000,

6000 and 10 000 ng/ml. These samples were subjected to the solid-phase extractions and derivatization procedure described above to generate a nine-point standard curve. The peak-height ratios of drug to internal standard were obtained, weighted by a factor of $1/y$ (based on analysis of residuals) and plotted *versus* plasma drug concentrations. Linear regression analysis gave a calibration curve that was used to calculate plasma oxiracetam concentrations.

RESULTS AND DISCUSSION

Derivatization

The general reactions involved in the derivatization and detection scheme have previously been described [11]. The scheme essentially consists of two steps. The first step is the pre-column derivatization of oxiracetam (and internal standard) with *n*-propyl isocyanate to form the corresponding *n*-propyl carbamate derivative. A study to determine the optimal reaction conditions indicated a 1-h reaction time at 50°C maximized the formation of the *n*-propyl carbamate derivative of oxiracetam.

In addition to *n*-propyl isocyanate, the use of phenyl isocyanate was also investigated. Although formation of the phenyl carbamate derivative would permit the use of UV detection, phenyl isocyanate does not possess the high volatility of propyl isocyanate, which prohibited a simple evaporation of excess reagent. Consequently it was difficult to completely remove excess phenyl isocyanate and a large reagent peak was encountered which interfered in the HPLC resolution of the drug and internal standard carbamate derivatives. Therefore, propyl isocyanate was the reagent of choice.

The second step in the derivatization scheme is the on-line alkaline hydrolysis of the carbamate derivative to the corresponding primary amine and subsequent derivatization of the primary amine (*n*-propylamine) with OPA and 3-MPA to yield the isoindole fluorophore, which is the species fluorimetrically monitored and quantified.

The effects of several variables on the post-column reaction were examined. Variation of sodium hydroxide concentration in the post-column reagent from 0.01 to 0.3 *M* indicated a concentration of 0.05 *M* provided optimal response. Similarly, varying the reaction coil temperature from 40 to 110°C showed a temperature of 90°C to provide maximal fluorimetric response. Additionally, use of a 0.5-ml reaction coil (providing a reaction time of approximately 1 min) yielded optimal detector response. Use of larger-volume reaction coils to increase the reaction time did not result in increased detector response, but did begin to introduce significant extra-column band broadening.

Recovery

Known amounts of oxiracetam (80, 800, and 8000 ng/ml) and internal standard (6250 ng/ml) were dissolved in drug-free human plasma and processed according to the described method. Recovery was determined as a percentage relative to the results obtained for aqueous reference standards at the same concentrations. The results of the recovery study are summarized in Table I. The recovery of oxiracetam and the internal standard were high and consistent at the concentrations examined.

Sensitivity, linearity, and selectivity

Typical chromatograms of extracts of drug-free human plasma and plasma spiked with both oxiracetam (800 ng/ml) and internal standard are shown in Fig. 1. Retention times for the *n*-propyl

TABLE I

RECOVERY OF OXIRACETAM AND INTERNAL STANDARD FROM HUMAN PLASMA

Compound	Concentration (ng/ml)	Recovery (mean ± S.D.) (%)	R.S.D. (%)	*n*
Oxiracetam	80	91.19 ± 7.95	8.72	6
Oxiracetam	800	113.21 ± 10.26	9.06	4
Oxiracetam	8000	99.35 ± 2.90	2.92	5
Internal standard	6250	81.06 ± 6.72	8.30	15

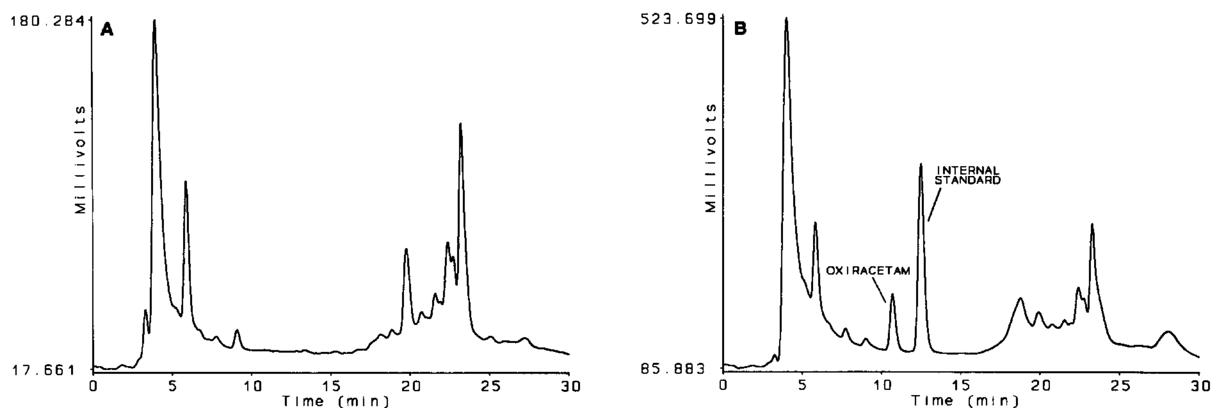

Fig. 1. Chromatograms of extracts of (A) drug-free plasma and (B) plasma spiked with both oxiracetam (800 ng/ml) and internal standard. See text for chromatographic conditions.

TABLE II

SUMMARY OF ACCURACY AND PRECISION STUDY

	Plasma concentration of oxiracetam (ng/ml) (n = 6)		
	80	800	8000
Day 1 statistics			
Mean found concentration (ng/ml)	80.83	825.50	8217.21
S.D. (ng/ml)	7.54	34.61	392.20
R.S.D. (%)	9.33	4.19	4.77
Mean accuracy (%)	101.04	103.19	102.72
Day 2 statistics			
Mean found concentration (ng/ml)	94.21	804.53	8004.15
S.D. (ng/ml)	5.16	42.46	171.92
R.S.D. (%)	5.47	5.28	2.15
Mean accuracy (%)	117.76	100.57	100.05
Day 3 statistics			
Mean found concentration (ng/ml)	78.40	739.96	8638.86
S.D. (ng/ml)	6.86	52.12	88.34
R.S.D. (%)	8.75	7.04	1.02
Mean accuracy (%)	98.00	92.49	107.99
Within-day R.S.D. (%) [a]	7.85	5.50	2.65
Between-day statistics (all three days)			
Mean found concentration (ng/ml)[b]	84.48	789.99	8286.74
S.D. (ng/ml)[c]	6.95	36.40	263.74
R.S.D. (%)[d]	8.23	4.61	3.18
Mean accuracy (%)[e]	105.60	98.75	103.58

[a] Mean of daily R.S.D.s.
[b] Mean of daily means.
[c] S.D. of daily means.
[d] R.S.D. of daily means.
[e] Mean of daily accuracies.

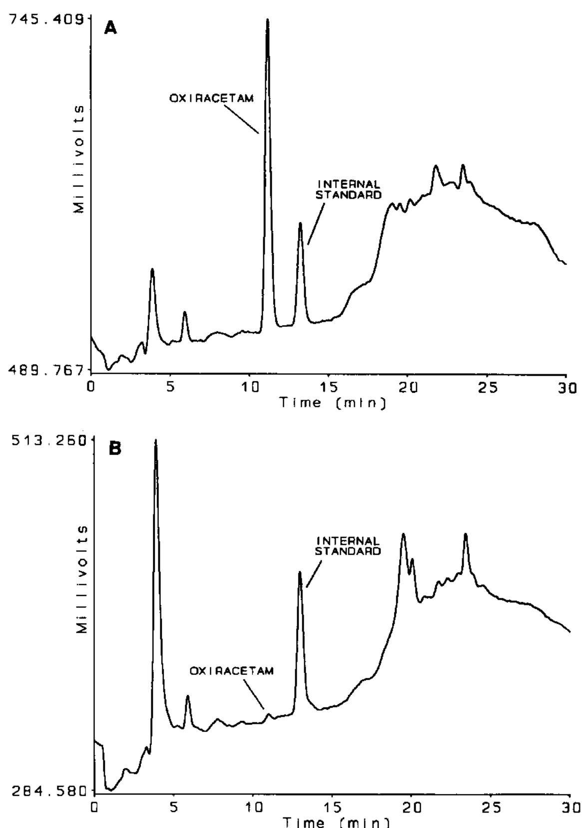

Fig. 2. Chromatograms of authentic human plasma samples obtained (A) 2.5 h (8658 ng/ml) and (B) 97.3 h (136 ng/ml) after an 800-mg oral dose of oxiracetam. See text for chromatographic conditions.

carbamate derivatives of oxiracetam and the internal standard were 10.9 and 12.9 min, respectively. As can be observed, endogenous plasma components did not interfere with the measurement of oxiracetam or internal standard over the concentration range described here.

Utilizing a 2.1 mm I.D. HPLC column and optimizing the derivatization conditions, the on-column limit of detection (at a signal-to-noise ratio of 3) for oxiracetam was 0.5 ng (3.2 pmol). Based on a 200-μl plasma sample volume, linear detector response for the peak-height ratio of drug to the internal standard was observed for plasma oxiracetam concentrations ranging from 40 to 10 000 ng/ml. Correlation coefficients obtained using weighted ($1/y$) linear regression analysis data were typically 0.996 or better.

Accuracy and precision

Accuracy and precision results obtained in a three-day validation study are presented in Table II. Replicate plasma samples (n = 6) at oxiracetam concentrations of 80, 800, and 8000 ng/ml were assayed each day by the described method. The between-day mean accuracy of the assay ranged from 98.8 to 105.6%. The within-day precision of the method, indicated by the mean of the daily relative standard deviation (R.S.D.) at each concentration, ranged from 2.6 to 7.8%. The between-day precision, calculated as the R.S.D. of the daily means at each concentration, ranged from 3.2 to 8.2%.

Authentic plasma samples

The method has also been applied to human plasma samples obtained following oral administration of oxiracetam. Fig. 2 presents the chromatograms for human plasma samples obtained 2.5 and 97.3 h following an 800-mg oral dose of oxiracetam. These chromatograms illustrate the utility of the assay to determine plasma oxiracetam concentrations over a wide concentration range, for use in pharmacokinetic evaluation in clinical studies.

CONCLUSIONS

This paper described a sensitive and selective HPLC assay for oxiracetam in human plasma. The method involves pre-column derivatization prior to post-column reaction fluorimetric detection. The assay is sensitive, with an on-column detection limit of 0.5 ng for oxiracetam, and provides a linear detector response range of 40–10 000 ng/ml based on a 200-μl plasma sample volume. Employing manual sample preparation procedures and an autoinjector, a reasonable estimate of sample throughput is in the range of 60–70 per day. The method has successfully been applied to human plasma samples for orally dosed patients and provides sufficient sensitivity, accuracy, and precision to support clinical pharmacokinetic studies.

REFERENCES

1 G. Pifferi and M. Pinza, *Farmaco, Ed. Sci.*, 32 (1977) 602.
2 T. M. Itil, C. Soldatos, M. Bozak, E. Ramadanoglu, G. Dayican, V. Morgan and G. N. Menon, *Curr. Ther. Res.*, 26 (1979) 525.
3 B. Saletu, L. Linzmayer, J. Grünberger and H. Pietschmann, *Neuropsychobiology*, 13 (1985) 44.

4 S. Benfi, L. Dorigotti, M. P. Abbracchio, W. Bolduini, E. Coen, C. Ragusa and F. Cottabeni, *Pharma. Res. Commun.*, 16 (1984) 69.
5 G. Spignoli and G. Pepeu, *Eur. J. Pharm.*, 126 (1986) 253.
6 E. Perucca, A. Albrici, G. Gatti, R. Spalluto, M. Visconti and A. Crema, *Eur. J. Drug Metab. Pharmacokin.*, 3 (1984) 267.
7 J. B. Lecaillon, N. Febvre and C. Souppart, *J. Chromatogr.*, 317 (1984) 493.
8 M. Visconti, R. Spalluto, T. Crolla, G. Pifferi and M. Pinza, *J. Chromatogr.*, 416 (1987) 433.
9 J. B. Lecaillon, C. Souppart, J. P. Dubois and A. Delacroix, *Methodol. Surv. Biochem. Anal.*, 18 (1988) 225.
10 J. B. Lecaillon, C. Souppart, F. LeDuigou and J. P. Dubois, *J. Chromatogr.*, 497 (1989) 223.
11 V. K. Boppana, R. C. Simpson, K. Anderson, C. Miller-Stein, T. J. A. Blake, B. Y.-H. Hwang and G. R. Rhodes, *J. Chromatogr.*, 593 (1992) 29.