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Determination of xanomeline (LY246708 tartrate), an investigational agent for the treatment of Alzheimer's disease, in rat and monkey plasma by capillary gas chromatography with nitrogen–phosphorus detection

Thomas J. Brown*, Lisa A. Shipley

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, 46285, USA

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Abstract

A GC method is described for the determination of xanomeline (LY246708 tartrate) and selected metabolites in rat and monkey plasma. The analytes, including an internal standard, were extracted from plasma at basic pH with hexane. The organic extract was evaporated to dryness and the residue was reconstituted in hexane. The analytes were separated from metabolites and endogenous substances using a DB1701 capillary column. The analytes were detected using nitrogen–phosphorus detection (NPD). The limit of quantitation was determined to be 8 ng/ml, and the response was linear from 8 to 800 ng/ml. The method has been successfully applied to rat and monkey samples pursuant to the development of xanomeline as an agent for the symptomatic treatment of Alzheimer's disease.

1. Introduction

It has been demonstrated that in Alzheimer's disease, there is a loss of the cholinergic projections of the basal forebrain to the cerebral cortex and hippocampus [1], regions suggested to be important in processing information and in memory function [2–4]. Muscarinic receptors are situated at various critical points of control along these pathways [5,6]. On the basis of current in-vitro information regarding their coupling mechanisms in nervous tissue, compounds that selectively affect subtypes of muscarinic receptors could enhance cholinergic function. The possibility then exists that these compounds could ameliorate certain cognitive impairments associated with Alzheimer's disease [7]. Xanomeline, LY246708 tartrate, 3-[4-(hexyloxy)-1,2,5 - thiadiazol - 3 - yl] - 1,2,5,6 - tetrahydro - 1 - methylpyridine tartrate (I, Fig. 1), is a potent and selective muscarinic cholinergic M1 receptor agonist which, in animals, is absorbed orally and crosses the blood–brain barrier. As such, xanomeline may have therapeutic utility for treating Alzheimer's disease.

In order to facilitate pharmacokinetic and pharmacodynamic studies of xanomeline, a sensitive and selective method for its determination in plasma was required. Previously reported work by Hamilton et al. [8] describes a more sensitive HPLC method but an assay which addressed the simultaneous analysis of selected

* Corresponding author.

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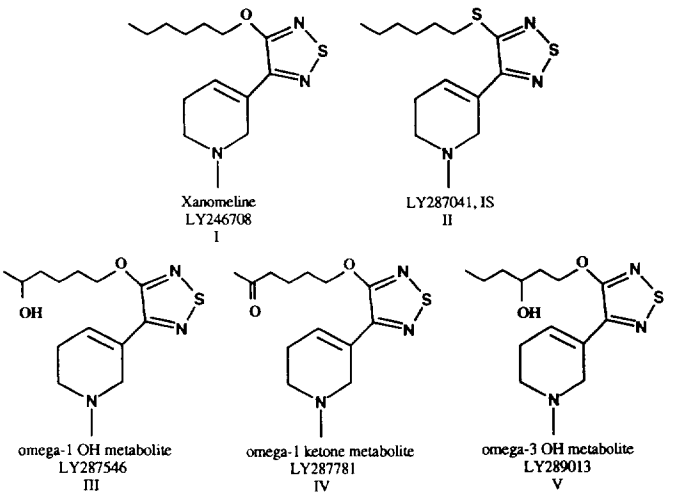

Fig. 1. Structure of analytes.

metabolites was required. Various solid-phase and liquid–liquid extraction techniques were explored before the superiority of the hexane liquid extraction was demonstrated. With alterations in ramp programming and temperature settings, this method has also proven successful in evaluating other potential drug candidates in this series of compounds.

This report describes a sensitive and selective method for the determination of xanomeline in rat and monkey plasma. Preliminary microsomal metabolism studies indicated the formation of both the ω-1-hydroxy (LY287546, III) and ω-3-hydroxy (LY289013, IV) as well as the ω-1-keto (LY287781, V) metabolites (Fig. 1). These metabolites were synthesized and considered in the development of this assay. Although not validated, these suspected metabolites should be detectable and quantifiable by this method if formed in vivo.

2. Experimental

2.1. Chemicals and reagents

Xanomeline tartrate, the ω-1-hydroxy metabolite (LY287546, III), ω-3-hydroxy metabolite (289013, IV) and ω-1-keto metabolite (287782, V), as well as LY287041, the internal standard (3-[(3-hexylthio)-1,2,5-thiadiazol-4-yl]-1,2,5,6-tetrahydro-1-methylpyridine hydrochloride, II Fig. 1), were obtained from Lilly Research Laboratories (Eli Lilly and Company, Indianapolis, IN, USA). High purity grade methanol and hexane were purchased from Burdick & Jackson (Division of Baxter Healthcare, Muskegon, MI, USA). Purified water (Milli-Q System, Millipore) was used in all aqueous solutions. All other chemicals were of analytical reagent grade. Control (blank) plasma was obtained from Fisher 344 rats and Rhesus monkeys at Lilly Research Laboratories.

2.2. Gas chromatography

The GC consisted of a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard Company, Palo Alto, CA, USA), an HP7673 automated sampler, an HP3396A integrator and a nitrogen–phosphorus detector (NPD) and splitless injection. The column used was a DB1701, 30 m $\times$ 0.25 mm I.D., 0.25 μm film thickness (J & W Scientific, Folsom, CA, USA). Gas (Air Products, Middletown, OH, USA) flow-rates, as measured at the detector, for hydrogen, helium and air were 4, 14 and 100 ml/min, respectively. The helium source (zero grade) was protected by an oxy-trap (ALLTECH Associates, Deerfield, IL, USA) while the air

supply was equipped with an indicating moisture trap and a hydrocarbon trap (J & W Scientific). The injector and detector temperatures were both set at 250°C with the temperature gradient as follows: with an equilibration time of 0.5 min, the first ramp ran from 90°C to 240°C at a rate of 20°/min; the second ramp then continued from 240°C to 270°C at a rate of 50°/min; the temperature then returned to 90°C at a rate of 70°/min with a total run time of 26.1 min. The purge value was set to go "on" at 2 min and "off" at 10 min with the initial bead response set to 25 pA.

2.3. Preparation of glassware

Preliminary data (unreported) indicated that this series of compounds have a tendency to bind to glassware. For this reason, all glassware that was to come into contact with samples or standards during the course of the assay was first treated by vapor-phase silylation [9].

2.4. Preparation of standard solutions

Individual stock solutions were prepared for each of the five compounds at a concentration of 100 μg/ml (free base) in methanol, sonicating each for 5 min to ensure dissolution. Working standard solutions were prepared as follows: the stock solution of xanomeline was further diluted in methanol to yield a concentration of 8000 ng/ml or 400 ng/50 μl methanol as the high standard; aliquots from each of the three suspected metabolites were combined and diluted in methanol to yield a concentration of each at 8000 ng/ml or 400 ng/50 μl methanol as the high standard; an aliquot of the LY287041 internal standard stock was diluted in methanol to yield a concentration of 1000 ng/ml or 100 ng/100 μl methanol.

Standard solutions of xanomeline were prepared at analyte concentrations of 800, 400, 200, 100, 40, 20, 8 and 0 ng/ml plasma. These standards were prepared by serial dilutions of working standard from 400 ng/50 μl methanol. In addition, 50 μl of serially diluted metabolite standards were added to the parent spiked control plasma to yield metabolite concentrations of 800, 600, 400, 200, 100, 50, 20 and 0 ng/ml plasma, respectively. Representative chromatograms of standards from control rat and monkey plasma can be found in Figs. 2 and 3.

2.5. Sample preparation procedures

Aliquots of plasma or standards (0.5 ml for analyte concentrations of 8–800 ng/ml of parent and 20–800 ng/ml of selected metabolites) were dispensed into 15-ml duplicate, clean and dry

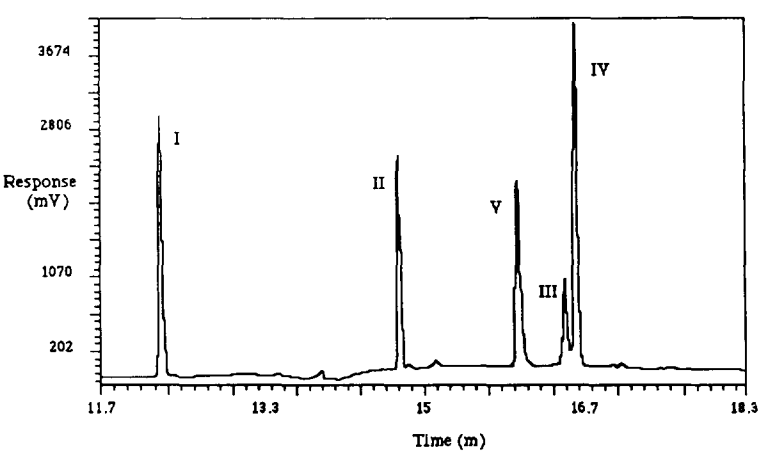

Fig. 2. Chromatogram of xanomeline (LY246708 tartrate) and metabolites spiked into control rat plasma. The concentration of each analyte was 400 ng/ml plasma.

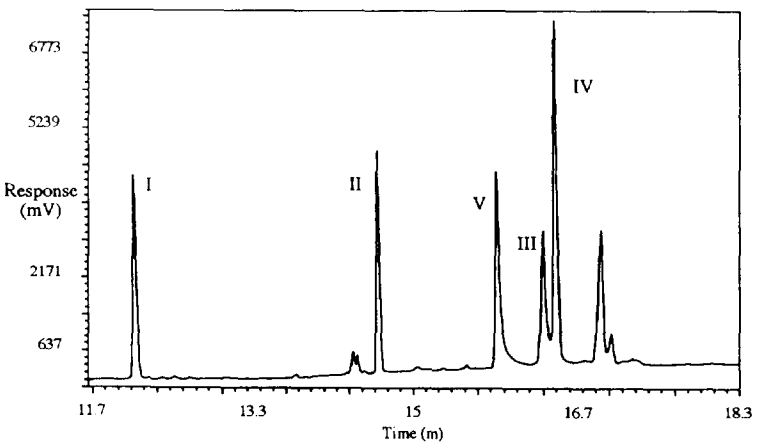

Fig. 3. Chromatogram of xanomeline (LY246708 tartrate) and metabolites spiked into monkey plasma. The concentration of each analyte was 400 ng/ml plasma.

culture tubes with PTFE-lined screw caps. In order to balance the addition to the standard tubes, 100 μl of methanol was added to the sample tubes and all were vortex-mixed. Following the addition of internal standard (100 μl), samples were vortex-mixed and then made basic by the addition of 100 μl of 5 *M* sodium hydroxide. After vortex-mixing, 5 ml of hexane was added to each sample. The samples were mixed on a rotary mixer, rotating at a speed of approximately 15 rpm, for 30 min and then centrifuged at approximately 2500 *g* for 15 min at 4°C. Robust mixing will cause emulsions to form which can be broken with repeated freezing and thawing. The lower, aqueous layer was immediately frozen by immersion of the tubes in a dry ice–acetone bath; the upper, organic layer was decanted into 13 × 100 disposable culture tubes. The supernatant was dried at 37°C under a stream of nitrogen. The samples were reconstituted with 100 μl of hexane, vortex-mixed twice and transferred to a 1-ml crimp-top GC autosample vial containing a glass 200-μl limited volume insert and the vial was sealed.

2.6. Calculations

A least-squares calibration curve was obtained by plotting the concentrations of the plasma standards vs. the peak-area ratios. The peak-area ratios were calculated by dividing the peak areas of the analytes by the peak areas of the internal standard. The concentration of each compound was determined from the peak-area ratio relative to the calibration curve.

2.7. Determination of precision and accuracy

The precision and accuracy of the method were determined by performing replicate analyses of four pools of plasma spiked with known concentrations of the compound. The pool concentrations were chosen to cover the range of the standard curve. These concentrations included the limit of quantitation. Three replicates of each pool were analyzed on three different days by the same analyst on the same instrument. Two standard curves were included each day, one at the beginning and one at the end of the run.

2.8. Analysis of samples from pharmacokinetic studies

Plasma samples from pharmacokinetic studies involving rats and monkeys were frozen and maintained at −70°C prior to analysis. On thawing, aliquots of each sample were dispensed into 15-ml screw-capped culture types and processed as stated above in section 2.5. Quality control samples (medium-low and medium-high concen-

trations), prepared from a separate weighing of standards, were included in duplicate in each run.

3. Results and discussion

3.1. Method development and chromatography

The assay conditions described in this report are a result of the evaluation and optimization of the sample preparation, chromatography, and detection of xanomeline, three of its suspected metabolites and an internal standard. In a continuous effort to maximize sensitivity, the nitrogen–phosphorus detector was the obvious choice as nitrogen atoms figure prominently in the ring structures of compounds throughout this series.

Both solid-phase and liquid–liquid extractions were explored when developing the sample preparation scheme. Analytichem Bond Elut (Varian, Harbor City, CA, USA) sorbents that were tried included CN, C_2, C_8 and C_{18}, all of which yielded less than satisfactory recoveries. Several solvents were investigated for use in liquid–liquid extraction. Toluene extraction resulted in insufficient recovery. Benzene and chloroform provided adequate recovery (data not shown) but were passed over due to safety concerns. Hexane was selected because the recovery of parent xanomeline out of plasma was high, 94% (tested by comparing peak areas obtained from chromatography of neat standards with those of extracted plasma samples that had been spiked with the analytes), providing good sensitivity and a relatively clean baseline.

Good resolution was obtained between the five analytes with no apparent interference from endogenous substances. Xanomeline and the internal standard, compound LY287041, had no interfering peaks and retention times were 12.2 and 14.5 min, respectively. Suggested metabolites III, IV and V were co-chromatographed and had retention times of 15.9, 16.4, and 16.5 min, respectively. Sufficient reproducibility in the estimation of metabolite (III, IV and V) plasma concentration could not be obtained for validation, however this method proved useful in the qualitative determination of suspected metabolite pharmacokinetic profiles. This method has also been utilized for the preliminary analysis of xanomeline in brain tissue, however, at the time of this writing, the assay has not yet been validated in this matrix.

3.2. Precision and accuracy

The determination of xanomeline was evaluated for precision and accuracy by replicate analyses of plasma pools spiked with analytes 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 estimated. Overall, the precision and accuracy of the method was very good. The intra-assay accuracy ranged from 82 to 118% for the rat and from 89 to 95% for the monkey and the intra-assay precision from 0.3 to 7.0% for the rat and from 0.4 to 5.8% for the monkey. The inter-assay (only rat data available) accuracy ranged from 98 to 105% and the inter-assay precision from 5.4 to 19% (see Table 1 for a complete listing).

3.3. Limits of detection and quantitation

The lower limit of detection (defined as the concentration which produces a signal-to-noise ratio of 3) for this assay was approximately 2–4 ng/ml with a lower limit of quantitation (defined to be equivalent to the lowest validation pool which, under the given conditions, yielded a value of $\leq$20% R.S.D. and $\leq$20% relative error, both within- and between-day) of 8 ng/ml. This limit could be lowered to 4 ng/ml by reconstituting the samples in 50 μl of hexane.

3.4. Linearity

The linearity of the responses with 100 μl sample volumes was established over the concentration range 8–800 ng/ml (xanomeline). Typical standard curves had correlation coefficients greater than 0.999. Without dilution, the upper limit of linearity is approximately 800 ng/ml. No attempt was made to establish the upper

Table 1
Precision and accuracy data and xanomeline

	Determined values for validation samples			
	4 ng/0.5 ml	20 ng/0.5 ml	100 ng/0.5 ml	400 ng/0.5 ml
Rat plasma				
Day 1				
Replicate 1	3.5	21.6	108.7	400.9
2	3.2	20.8	109.0	394.6
3	3.1	21.2	108.3	421.6
Intra-assay average	3.3	21.2	108.7	405.7
Intra-assay accuracy (%)	82	106	109	101
Intra-assay precision (%)	6.4	1.9	0.3	3.5
Day 2				
Replicate 1	3.6	18.7	96.3	364.6
2	4.1	19.4	96.8	369.6
3	3.7	19.5	92.0	366.8
Intra-assay average	3.8	19.2	95.0	367.0
Intra-assay accuracy (%)	95	96	95	92
Intra-assay precision (%)	7.0	2.3	2.8	0.7
Day 3				
Replicate 1	4.8	23.1	106.2	380.4
2	4.5	21.9	108.6	380.5
3	4.8	23.2	109.5	384.2
Intra-assay average	4.7	22.7	108.1	381.7
Intra-assay accuracy (%)	118	114	108	95
Intra-assay precision (%)	3.7	3.2	1.6	0.6
Summary				
Inter-assay average	3.9	21.0	103.9	384.8
Inter-assay accuracy (%)	98	105	104	96
Inter-assay precision (%)	19	8.7	7.6	5.4
Monkey plasma				
Day 1				
Replicate 1	3.8	18.2	89.9	379.4
2	3.6	17.4	94.8	381.9
3	3.4	17.7	89.8	381.6
Intra-assay average	3.6	17.8	91.5	381.0
Intra-assay accuracy (%)	90	89	92	95
Intra-assay precision (%)	5.8	2.3	3.1	0.4

Table 2
Plasma concentrations of xanomeline and metabolites (III, IV, V) following the oral administration of xanomeline tartrate (60 mg/kg, free base) to F344 rats (mean ng/ml)

Time (h)	Xanomeline	Xanomeline (S.D.)	V	III	IV
0	BLD[a]		BLD	BLD	BLD
0.25	407	344	BLD	BLQ[b]	252
0.5	1471	908	BLQ	BLQ	348
0.75	1933	518	BLQ	BLQ	391
1	1090	484	BLQ	BLQ	124
1.5	551	182	BLQ	BLD	54
2	382	92	BLQ	BLQ	40
3	79	32	BLQ	BLQ	BLQ
4	25	6	BLD	BLQ	BLQ
6	4	2	BLD	BLQ	BLD
8	BLQ		BLD	BLD	BLD
12	BLD		BLD	BLQ	BLD
24	BLD		BLD	BLD	BLD

[a] BLD = below the limit of detection.
[b] BLQ = below the limit of reliable quantitation (values less than 8 ng/ml for the parent and 100 ng/ml for the metabolites).

limit of linearity with dilution and the lower limit of the standard curve was not extrapolated to zero. Standard curves for individual metabolites had correlation coefficients of ≥ 0.984 and sensitivity to 40 ng/ml.

3.5. Application of the method in pharmacokinetic studies

This validated procedure was used to provide pharmacokinetic data for xanomeline in rats and monkeys following oral, intravenous and transdermal administration. Plasma samples were obtained at defined time intervals post-administration, extracted and analyzed by GC. Analysis of control plasma collected prior to xanomeline administration demonstrated that interferences from endogenous plasma were not compromising the quality of the results. Plasma concentrations of xanomeline as a function of time following oral administration to the rat can be found in Table 2.

This assay was also used in support of the selection of a non-rodent species for the preclinical development of xanomeline. Microsomes obtained from human, monkey, dog and rat livers were incubated in the presence of xanomeline (100 μM). After a prescribed period of time, the incubates were extracted and analyzed as described previously. Upon review of the resulting chromatograms (Fig. 4), it became obvious that in-vitro metabolism using monkey microsomes was more representative of the human in-vitro situation in the case of xanomeline. It was recommended that the monkey serve as the non-rodent toxicology species.

4. Conclusions

Concentrations of xanomeline may be accurately determined from rat and monkey plasma by the described procedure. This method was useful in the analysis of xanomeline pharmacokinetic studies with the rat and monkey designed to aid in the development of xanomeline for the treatment Alzheimer's disease. While this method proved useful in the analysis of toxicology samples, extensive metabolism of xanomeline in humans [10] will limit this assay's utility in the analysis of clinical samples.

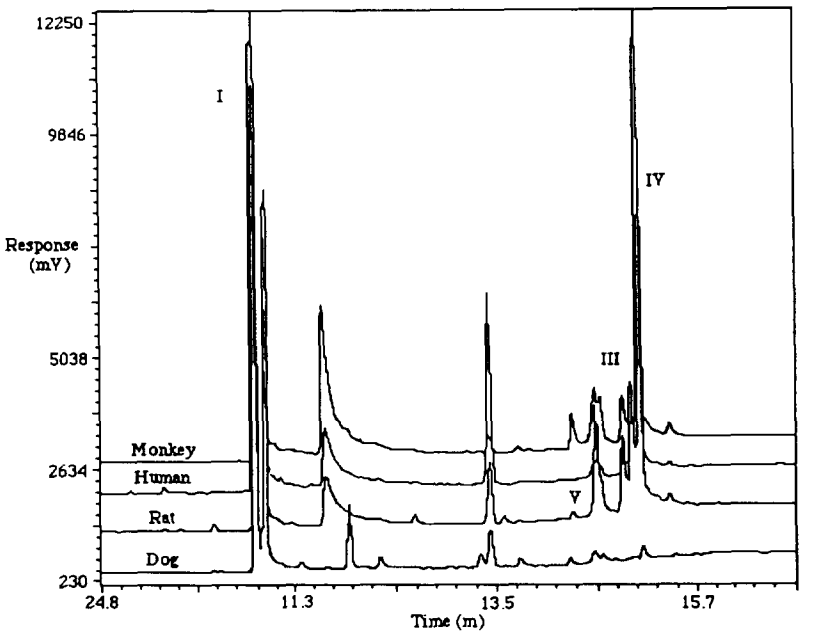

Fig. 4. Chromatogram of the comparative microsomal metabolism of xanomeline (LY246708 hydrochloride). Extraction of liver microsomes from selected species following incubation with xanomeline (100 μM).

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