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Short communication

High-performance liquid chromatographic assay for xanomeline, a specific M-1 agonist, and its metabolite in human plasma

Stephen C. Kasper*, Peter L. Bonate, Allyn F. DeLong

Lilly Research Laboratories, Lilly Laboratory for Clinical Research, Eli Lilly and Company, Indianapolis, IN 46202, USA

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Abstract

A reversed-phase high-performance liquid chromatographic assay (HPLC) was utilized for monitoring xanomeline (LY246708/NNC 11-0232) and a metabolite, desmethylxanomeline, in human plasma. Xanomeline, desmethylxanomeline and internal standard were extracted from plasma with hexane at basic pH. The organic solvent extract was evaporated to dryness with nitrogen and the dried residue was reconstituted with 0.2 *M* HCl–methanol (50:50, v/v). A Zorbax CN 150 × 4.6 mm I.D., 5-μm column and mobile phase consisting of 0.5% (5 ml/l) triethylamine (TEA) adjusted to pH 3.0 with concentrated orthophosphoric acid–tetrahydrofuran (THF) (70:30, v/v) produced consistent resolution of analytes from endogenous co-extracted plasma components. Column effluent was monitored at 296 nm/0.008 a.u.f.s. and the assay limit of quantification was 1.5 ng/ml. A linear response of 1.5 to 20 ng/ml was sufficient to monitor plasma drug/metabolite concentrations during clinical trials. HPLC assay validation as well as routine assay quality control (QC) samples indicated assay precision/accuracy was better than ±15%.

1. Introduction

Increasing scientific evidence indicates that drug-induced alteration of the M-1 receptor in the brain may produce beneficial effects in patients with Alzheimer's disease [1,2]. Xanomeline (I) is believed to directly stimulate the post synaptic receptor site(s) in brain thus providing replacement therapy of acetylcholine which facilitates neurotransmission. Since xanomeline acts directly on receptor sites, replacement therapy of acetylcholine is not dependent on intact neurons as is the case with acetylcholine replacement therapy using acetylcholinesterase inhibitors. Xanomeline (LY246708/NNC 11-0232 or 3-[4-(hexyloxy)-1,2,5-thiadiazol-3-yl]-1,2,5,6-tetrahydro-1-methylpyridine) (Fig. 1) has been shown to be the most selective partial M-1 agonist discovered to date [3–5]. With the initiation of clinical trials, analytical methods to quantify drug/metabolite(s) of interest have been developed. A HPLC–MS–MS method has been used in pharmacokinetic studies which require quantification of plasma xanomeline below concentrations of

* Corresponding author. Address for correspondence: Eli Lilly Laboratory for Clinical Research, Wishard Memorial Hospital, 1001 West 10th Street, Indianapolis, IN 46202, USA.

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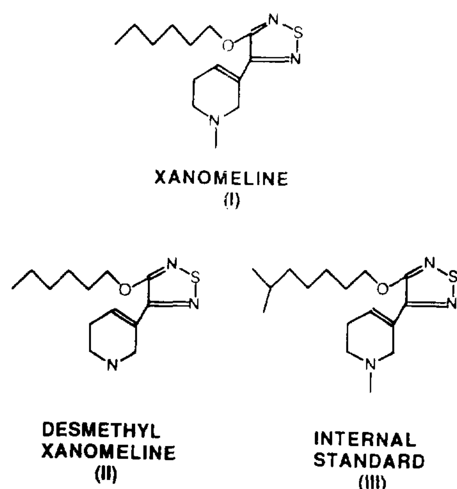

Fig. 1. Structures (base form) of xanomeline (I), desmethylxanomeline metabolite (II), and internal standard (III).

1.5 ng/ml [6]. In human plasma, the detection of low levels of a metabolite, desmethylxanomeline (II), has necessitated the modification of a previously published method [7]. Desmethylxanomeline has the potential to contribute to the overall clinical pharmacology of xanomeline. This communication describes the modification, validation and use of the HPLC–UV method to quantify xanomeline and desmethylxanomeline metabolite during clinical trials in Alzheimer's patients.

2. Experimental

2.1. Chemicals and reagents

Xanomeline (I), desmethylxanomeline (II) and internal standard (III) were provided by C.H. Mitch and S.J. Quimby of Lilly Research Laboratories (Eli Lilly and Company, Indianapolis, IN, USA) (Fig. 1, structures, base forms) and from Novo Nordisk (Malov, Denmark). HPLC quality water was prepared from a Milli-Q System (Millipore Marlborough, MA, USA), and methanol, hexane, tetrahydrofuran, were purchased from Burdick and Jackson division of Baxter Diagnostics (Muskegon, MI, USA). Triethylamine (TEA) HPLC grade was purchased from Fisher Chemical Division of Fisher Scientific (Itasca, IL, USA). All other chemicals were of analytical reagent grade. Drug-free control (blank) plasma was purchased from Biological Specialty (Lansdale, PA, USA) or obtained from healthy human subjects.

2.2. High-performance liquid chromatography

The HPLC system consisted of a Waters Model 715 Ultra WISP Millipore (Waters Chromatography Division, Milford, MA, USA), Beckman Model 126 isocratic pump (Beckman Instruments, Fullerton, CA, USA), Beckman Model 166 UV detector and Perkin Elmer Access*Chrom data reduction system. The guard column–column combination was a Zorbax CN, 12.5 × 4.0 mm, 5 μm–Zorbax CN, 150 × 4.6 mm, 5 μm (Dupont, Wilmington DE, USA). The mobile phase consisted of an aqueous solution of 0.5% (5 ml/l) triethylamine adjusted to pH 3.0 with concentrated orthophosphoric acid (approximately 2.5 ml). This solution was mixed with tetrahydrofuran in a ratio of (70:30, v/v). The flow-rate was 1.0 ml/min and the column was maintained at a constant 35°C temperature using a TC-50 (FIAtron Systems) column heater. After injecting approximately 180 μl, the chromatographic run required 25 min for completion. The analytes were detected and quantified by UV absorption at 296 nm at a sensitivity setting of 0.008 a.u.f.s. UV.

2.3. Preparation of standard solutions

The use of silylated glassware and selected plastic ware throughout the preparation of samples and standards was required due to the characteristic nonspecific absorption of analytes to glassware. Individual stock solutions of I, II, and III were prepared in 0.1 *M* HCl–methanol (50:50, v/v) at concentrations of approximately 10 μg/ml. Appropriate amounts of I and II were added to drug-free, blank plasma to obtain a plasma solution of 100 ng (free base)/ml. The plasma calibrators with concentrations of 1.5, 3.0, 7.5, 12.5, 15, and 20 ng/ml were prepared by addition of appropriate amounts of the 100-

ng/ml plasma solution to blank plasma. Quality-control samples at concentrations of 1.5, 3, 7.5 and 20 ng/ml were prepared as described above but from separate, duplicate stock solutions of I and II. The final maximum concentration of 0.1 *M* HCl–methanol drug (I and II) added was no greater than 0.25% in plasma. A duplicate 6-point standard curve was prepared for each daily assay run. Duplicate QC samples at 4 different concentration levels were stored under conditions identical to the unknown samples and were analyzed with the unknowns.

2.4. Sample preparation procedures

Aliquots (1 ml) of plasma unknowns, quality-assurance samples and freshly prepared calibrators were placed in individual silanized 12-ml glass tubes with Teflon-lined screw caps (Kimble No. 348656). Following the addition of approximately 25 ng of internal standard in 100 μl of (50:50, v/v) 0.1 *M* HCl–methanol and 1 ml of 125 m*M* KCl-NaOH, pH 12.5 buffer, the samples were briefly mixed with a mechanical mixer. Hexane (5 ml) was added and the tubes were tightly sealed with the screw cap. The samples were placed on a horizontal shaker (Eberback, Ann Arbor, MI, USA) at about 170 rpm for 30 min. The samples were centrifuged at 1500 *g* for 10 min. After freezing the lower aqueous layer, the upper organic layer was decanted into a 5-ml, silanized conical glass tube (Kimble 73785). The tubes containing the organic solvent layer were placed in a Savant Speed Vac Concentrator (Savant Instruments, Farmingdale, NY, USA) for approximately 40 min at about 40°C or until dry. A Multivap (Organomation Associates, Berlin, MA, USA) sample concentrator can be used in place of the Savant Speed Vac Concentrator. The dried residue was dissolved in 200 μl of 0.2 *M* HCl–methanol (50:50, v/v) and about 180 μl was injected into the HPLC system. Compared to reconstituting the dried residue in mobile phase as previously recommended [7] the acid–methanol diluent was found to reduce late eluting (endogenous) peaks which sometimes interfered with assay performance. A typical chromatographic separation of xanomeline, desmethylxanomeline and internal standard extracted from a patient's plasma and a plasma calibrator is presented in Fig. 2.

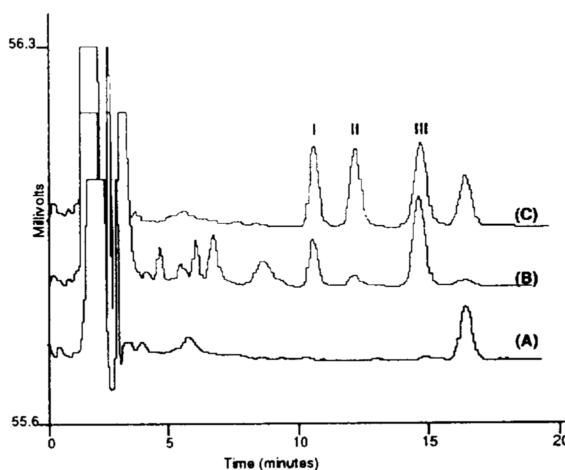

Fig. 2. Representative chromatograms of blank plasma (A), a patient sample with 8.0(I) and 1.8(II) (B), and a calibrator with 15.0(I) and 15.0(II) (C). Peaks: I, xanomeline; II, desmethylxanomeline; III, internal standard. All concentrations are ng/ml.

2.5. Data reduction/data acceptability criteria

Chromatographic data reduction via Perkin-Elmer Access*Chrom produced a least-squares calibration graph by plotting the plasma calibrator concentrations against the peak-height ratios. The peak-height ratios were obtained by mathematically dividing the peak heights of I and II, respectively, by the peak height of the internal standard (III). The concentration of I in each sample was determined from the peak-height ratio relative to the calibration graph. Each calibration curve had a minimum of 4 calibrators and a coefficient of variation of 0.98 in order to be accepted. The calibration curves were not forced through the origin and were not weighted. The control samples may have a relative error no greater than 15% of the nominal theoretical value. The control sample at 1.5 ng/ml may have an error of no greater than 20%.

2.6. Determination of standard curve characteristics, precision and accuracy

Linear regression analysis of the xanomeline and desmethylxanomeline standard curves produced regression coefficients which were greater than 0.99. Back calculation of the standard curve with the regression parameters demonstrated the precision and accuracy of the standard curves (Table 1). HPLC assay precision and accuracy were evaluated by performing statistical analysis of repeated daily replicate assays of plasma samples which contained known concentrations of xanomeline and the desmethylxanomeline metabolite (Table 2). All assays were conducted by a single analyst using a single chromatographic HPLC system. Samples were assayed in random order and calibration samples were assayed both at the beginning and end of the assay run. The assay precision and accuracy obtained indicated that the HPLC assay was suitable for monitoring concentrations of drug/metabolite during clinical trials and/or pharmacokinetic studies, providing plasma drug/metabolite concentrations were greater than 1.5 ng/ml.

2.7. Determination of drug/metabolite stability in plasma

The stability of I and II in plasma was determined by analysis of replicate plasma samples which contained known amounts of xanomeline and desmethylxanomeline and which were stored under controlled conditions. Conditions studied included storage at room temperature, −20°C, −70°C, and freeze–thaw cycles. Preliminary studies show that xanomeline and desmethylxanomeline are stable in plasma at concentrations of 1.5 ng/ml to 20 ng/ml when stored at −20°C for at least 1 month and −70°C for at least 3 months. Xanomeline and desmethylxanomeline are unaffected by freeze–thaw 3 × at either −20°C or −70°C and are stable in plasma for up to 12 h at room temperature. In addition, plasma extracts of xanomeline and desmethylxanomeline prepared for chromato-

Table 1
Standard curve characteristics

Target value (ng/ml)	Xanomeline (I)			Desmethylxanomeline (II)		
	Mean[a] (ng/ml)	C.V. (%)	Target (%)	Mean[a] (ng/ml)	C.V. (%)	Target (%)
1.5	1.5	6.6	100.6	1.5	8.0	101.0
3	3.0	1.7	99.3	3.1	3.6	102.4
5	5.0[b]	1.4	99.3	5.2[b]	7.5	103.5
7.5	7.5	2.3	99.9	7.3	3.5	97.6
12.5	12.7	3.2	101.7	12.9	3.9	102.9
15	14.8	3.3	98.4	14.7	2.9	97.7
20	20.0	1.1	100.1	20.1	2.0	100.5
	Linear regression[c] Standard curve characteristics					
Day	K_0	K_1	R^2	K_0	K_1	R^2
1	0.0161	0.0924	0.9973	0.0092	0.0909	0.9933
2	−0.0003	0.0815	0.9988	−0.0204	0.0779	0.9986
3	0.0039	0.0808	0.9995	−0.0305	0.0845	0.9985

[a] $n = 6$, derived from duplicate determinations over 3 days.
[b] $n = 5$ determinations.
[c] K_0 = intercept of standard curve; K_1 = slope of curve; R^2 = coefficient of determination.

Table 2
Precision and accuracy for I and II

Day		Target concentration (ng/ml)			
		1.5	3	7.5	20
Xanomeline I					
1	Mean[a] (ng/ml)	1.5	2.9	7.6	21.1
	C.V. (%)	6.4	6.3	7.5	3.4
	Target (%)	97.7	97.3	100.9	105.7
2	Mean[a] (ng/ml)	1.5	2.9	7.6	20.8
	C.V. (%)	7.8	11.7	2.3	2.2
	Target (%)	97.3	95.8	101.5	104.1
3	Mean[a] (ng/ml)	1.4	2.9	7.6	21.1
	C.V. (%)	5.7	3.9	3.5	3.4
	Target (%)	95.2	97.4	101.8	105.7
Overall[b]	Mean (ng/ml)	1.5	2.9	7.6	21.0
	C.V. (%)	6.6	7.3	4.4	3.0
	Target (%)	96.8	96.8	101.4	105.2
Desmethylxanomeline II					
1	Mean[a] (ng/ml)	1.5	2.9	7.2	19.7
	C.V. (%)	14.4	6.5	5.7	5.9
	Target (%)	96.5	95.1	96.2	98.6
2	Mean[a] (ng/ml)	1.4	3.0	7.1	19.9
	C.V. (%)	8.9	1.3	2.6	2.4
	Target (%)	95.7	99.7	95.2	99.5
3	Mean[a] (ng/ml)	1.6	3.0	7.4	20.2
	C.V. (%)	6.7	1.5	2.1	1.4
	Target (%)	107.7	101.2	98.5	100.9
Overall[b]	Mean (ng/ml)	1.5	3.0	7.3	19.9
	C.V. (%)	10.0	3.1	3.5	3.3
	Target (%)	100.0	98.7	96.6	99.7

[a] $n = 5$ determinations.
[b] $n = 15$ determinations.

graphic injection are stable for at least 48 h at 5°C.

2.8. Analysis of plasma samples from Alzheimer's patients

Selected plasma samples were obtained from Alzheimer's patients who were enrolled in a clinical trial to evaluate the effectiveness of xanomeline tartrate in the treatment of the disease. Patients in the clinical trial received selected TID (three times a day) doses of xanomeline for up to 6 months. The patient samples obtained from the clinical trial were used to demonstrate the feasibility of the HPLC assay to quantify both xanomeline and desmethylxanomeline simultaneously in plasma.

3. Results and discussion

3.1. HPLC–UV method modification

After oral administration, xanomeline is rapidly and extensively biotransformed to a number of metabolites [8,9]. The desmethylxanomeline metabolite was potentially of interest, so the HPLC–UV assay procedure was modified in order to quantify both parent drug and the desmethylxanomeline metabolite.

3.2. Overall analyte recovery and assay linearity

Over the concentration range of 1.5 to 20 ng/ml, the overall recovery of I, II and III was greater than 74%. The recovery was determined by comparison of peak heights of various sample concentrations prepared by the assay procedure vs. the peak height produced by injecting a correspondingly equivalent amount of analyte. Linear regression analysis of calibration curves over the range of 1.5 to 20 ng/ml demonstrated correlation coefficients which were greater than 0.99 (Table 1).

3.3. Precision, accuracy and limit of quantification

The precision and accuracy of the HPLC determination of I and II in plasma was evaluated by replicate analysis of plasma samples with known concentrations of xanomeline and desmethylxanomeline (Table 2). Within-day and between-day precision (C.V. %) and accuracy (Target %) were found to be within the acceptable guidelines [10] for bioanalytical HPLC assays. Plasma samples with xanomeline and desmethylxanomeline concentrations of 1.5 ng/ml were the lowest concentration evaluated and the concentration which had the greatest amount of variation in precision and accuracy. Therefore, the assay lower limit of quantification (LOQ) was defined as 1.5 ng/ml, the lowest plasma concentration that could routinely be determined within the data acceptance guidelines.

3.4. Use of the method during patient clinical trials

The method was used to detect steady state levels of xanomeline and desmethylxanomeline during clinical trials. Of the patients evaluated, plasma concentrations of desmethylxanomeline were usually lower than the corresponding concentration of xanomeline (Table 3). The data indicate that the HPLC–UV assay is capable of detecting and quantifying plasma concentrations of xanomeline and desmethylxanomeline during clinical trials.

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Table 3
Plasma concentration of xanomeline (I) and desmethylxanomeline (II) in Alzheimer's patients

Time (weeks)	Concentration (ng/ml)													
	Patient A		Patient B		Patient C		Patient D		Patient E		Patient F		Patient G	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
4	–[a]	–	–	–	25.1	2.4	4.6	0[b]	7.0	1.8	8.0	1.8	4.4	0
6	–	–	–	–	26.5	2.4	5.2	1.7	19.8	2.4	14.7	0	4.8	0
8	–	–	16.1	2.5	24.1	2.5	5.7	0	22.7	2.1	6.2	0	–	–
12	5.1	0	15.4	2.1	28.7	2.3	6.7	0	2.8	0	14.3	2.0	–	–
16	6.4	0	19.0	2.1	–	–	3.6	0	11.0	2.1	–	–	–	–
20	9.1	4.0	–	–	–	–			–	–	–	–	3.2	0

Time = weeks of xanomeline TID (dose given three times a day) therapy.
[a] – = Sample not available for analysis.
[b] 0 = Concentration < LOQ.

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References

[1] M. McKinney and J.T. Coyle, Mayo Clin. Proc., 66 (1991) 1225.

[2] A. Fisher, Y. Karton, E. Heldman, D. Gurwitz, R. Haring, H. Meshulam, R. Brandeis, Z. Pittel, Y. Segall and D. Marciano, Ann. N.Y. Acad. Sci., 695 (1993) 300.

[3] P. Sauerberg, P.H. Olesen, S. Nielson, S. Treppendahl, M.J. Sheardown, T. Honore, C.H. Mitch, J.S. Ward, A.J. Pike, F.F. Bymaster, B.D. Sawyer and H.E. Shannon, J. Med. Chem., 35 (1992) 2275.

[4] H.E. Shannon, F.P. Bymaster, D.O. Calligaro, B. Greenwood, C.H. Mitch, B.D. Sawyer, D.T. Wong, P.H. Olesen, M.J. Sheardown, M.D.B. Swedberg, P.D. Suzdak and P. Sauerberg, J. Pharmacol. Exp. Ther., 269 (1994) 271.

[5] F.P. Bymaster, D.T. Wong, C.H. Mitch, J.S. Ward, D.O. Calligaro, D.D. Schoepp, H.E. Shannon, M.J. Sheardown, P.H. Oesen, P.D. Sudak, M.D.B. Swedberg and P. Sauerberg, J. Pharm. Exp. Ther., 269 (1994) 282.

[6] A.T. Murphy, P.L. Bonate, S.C. Kasper, T.A. Gillespie and A.F. DeLong, J. Biol. Mass Spectrom., 23 (1994) 621.

[7] C.L. Hamilton, J.A. Kirkwood, G. Carter and R.S. Williams, J. Chromatogr., 613 (1993) 365.

[8] A.F. DeLong, P.L. Bonate, T.A. Gillespie, J.H. Satterwhite and D.P. Henry, in 3rd Int. Conf., Alzheimer's and Parkinson's Diseases, Recent Developments, Chicago, IL. November 1–6, 1993, Plenum Press, in press.

[9] T.A. Gillespie, P.L. Bonate, J.D. Cornprobst, A.F. DeLong, and L.A. Shipley, Proceedings 41st ASMS Conference on Mass Spectrometry and Allied Topics, May 31–June 4, San Francisco, CA, 1993, J. Am. Soc. Mass Spectrom., 5 (1994) 36a and 36b.

[10] H.T. Karnes, G. Shiu and V.S. Shah, Pharm. Res., 8 (1991) 421.