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BIOMEDICAL APPLICATIONS

Determination of the muscarinic agent [(3-(3-1-butylthio)-1,2,5-thiadiazol-4-yl)-1-azabicyclo[2.2.2]octane], in rat, rabbit, and monkey plasma, using high-performance liquid chromatography in conjunction with tandem mass spectrometry

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Abstract

A method for determining a selective muscarinic agent, LY297802 (compound I), [(3-(3-1-butylthio)-1,2,5-thiadiazol-4-yl)-1-azabicyclo[2.2.2]octane], indicated in the treatment of pain, in rat, rabbit, and monkey plasma is described. The analytes, including an internal standard, were extracted from plasma at basic pH with hexane. The organic fraction was evaporated to dryness and the residue reconstituted with mobile phase. The analytes were detected utilizing HPLC in conjunction with electrospray (ES) tandem mass spectrometry (MS–MS). The limit of quantitation was 0.25 ng/ml, and the response was linear to at least 100 ng/ml.

1. Introduction

Compound I (Fig. 1) is a selective muscarinic agent indicated in the treatment of pain. In vivo, compound I is equiefficacious to and more potent than morphine in several animal models of analgesia [1,2].

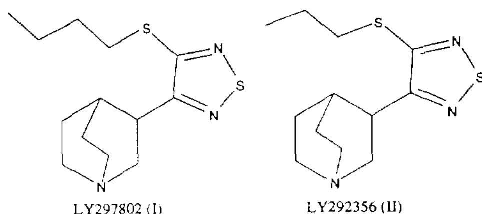

Fig. 1. Structures of LY297802 (I) and internal standard (II).

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In order to facilitate the pharmacokinetic studies of compound I in rats, rabbits, and monkeys, a selective method for the determination of compound I in plasma was required. In addition, observed concentrations of compound I in ongoing rat studies and anticipated dosages for future studies suggested that quantitation of compound I in plasma would require a very sensitive assay as well. A liquid–liquid extraction was used to effect sample clean-up. To fulfil selectivity and sensitivity requirements, as well as improve the sample analysis time, an electrospray tandem mass spectrometric (ES-MS–MS) apparatus interfaced with an HPLC system was employed [3]. This report describes the methodology used for determining compound I in rat, rabbit, and monkey plasma.

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2. Experimental

2.1. Chemicals and reagents

Compound I and the internal standard LY292356 [compound II, 3-(4-propylthio-1,2,5-thiadiazol-3-yl)-1-azabicyclo[2.2.2]octane ethanedioate] (1:1) (Fig. 1), were obtained from Novo Nordisk, Malob, Denmark. HPLC-grade methanol (MeOH), acetonitrile (ACN), and hexane were purchased from Burdick and 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. Blank rat, rabbit, and monkey plasma were obtained from naive animals.

2.2. Liquid chromatography–electrospray mass spectrometry

LC–MS–MS was performed on a Finnigan MAT (San Jose, CA, USA) TSQ700 triple quadrupole mass spectrometer interfaced via a Finnigan "high-flow" electrospray to an HPLC system composed of a Model 600MS solvent delivery system (Waters Chromatography, Division of Millipore Corporation, Milford, MA, USA), and a Model 717 autoinjector (Waters). The chromatography was performed on a YMC cyano analytical column (5 cm × 4.0 mm I.D., 3 or 5 μm, YMC, Wilmington, NC, USA) with an in-line filter (2 μm, Upchurch Scientific, Oak Harbor, WA, USA) positioned directly in front of the analytical column. The column temperature was ambient. The mobile phase was 33 mM ammonium acetate in 0.33% acetic acid–acetonitrile (30:70, v/v) at a flow-rate of 0.8 ml/min which was directly pumped into the electrospray interface of the mass spectrometer. The heated capillary temperature setting was 270°C. The sheath (nebulizing) gas pressure and auxiliary flow of nitrogen were set at 70 p.s.i. (ca. $4.8 \cdot 10^5$ Pa) and 20 ml/min, respectively. Electrospray ionization was effected by a spray voltage of +4.5 kV. The mass spectrometer was set to admit the positively charged protonated molecules $[M+H]^+$ at m/z 284 (compound I) and m/z 270 (internal standard, compound II) via the first quadrupole filter (Q1) with collision-induced fragmentation in Q2 [collision gas argon, −25 eV, 1.5 mTorr (ca. 0.20 Pa)] and monitoring, via Q3, the product ions of m/z 228 for both compound I and its internal standard, compound II. The ion at m/z 228 was selected based on the relative abundance in the daughter ion mass spectra obtained (Fig. 2) from direct infusion at 5 μl/min with a syringe pump (Harvard Apparatus, South Natick, MA, USA) of 100 ng/μl solution of each standard in methanol–water (50:50, v/v). Each selected reaction was monitored at a dwell time of 0.2 s. Prior to analysis the instrumental performance was evaluated by injecting a 0.25 ng/ml absolute standard solution utilizing the above conditions. A response of ≥3 times the average background signal for at least two con-

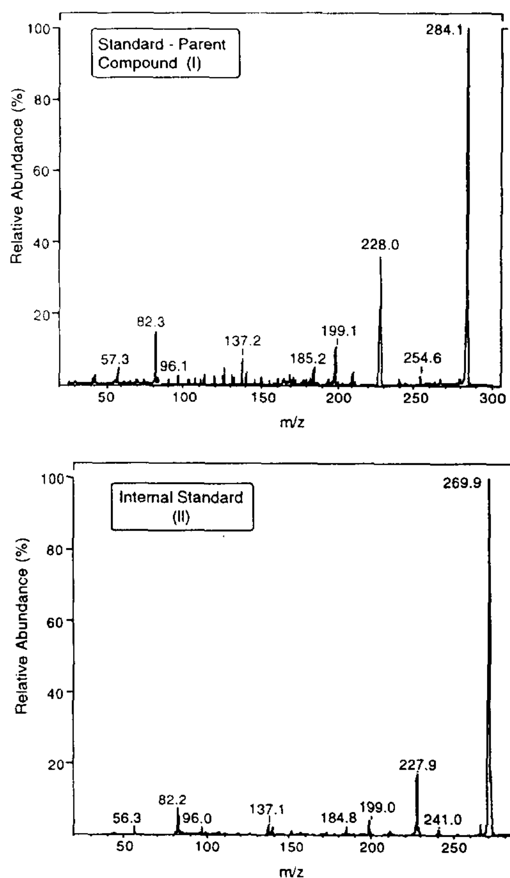

Fig. 2. Daughter ion mass spectra of compounds I and II.

secutive injections indicated adequate performance.

2.3. Preparation of standard solutions

A standard stock solution containing compound I was prepared in 0.9% sodium chloride irrigation solution (saline) at a concentration of 100 μg/ml (free base). The internal standard stock solution containing compound II was prepared in methanol at a concentration of 100 μg/ml (free base).

Solution standards were prepared at analyte concentrations of 2.5, 5.0, 10.0, 50.0, 100, 250, and 500 ng/ml by diluting appropriate aliquots of the standard stock solution with saline. Plasma standards were prepared at analyte concentrations of 0.25, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, and 100 ng/ml by transferring 50-μl amounts of appropriate stock solution into 0.5-ml amounts of blank plasma. A 10.0 ng/ml working internal standard solution was prepared by diluting appropriate aliquots of the internal standard stock solution with methanol.

2.4. Sample preparation procedures

Aliquots of plasma samples or standards were dispensed into 15-ml disposable glass tubes with PTFE-lined screw caps. After aliquoting 100 μl of the working internal standard solution, samples were vortex-mixed and then made basic with 100 μl of 5 *M* NaOH. After another vortex-mix, 5 ml of hexane was added to each sample and then capped. The samples were mixed on a rotary mixer for 25 min at approximately 15 rpm and then centrifuged at approximately 2200 *g* for 20 min at ambient temperature. 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 glass culture tubes. The supernatant fraction was dried at 42°C under a stream of nitrogen gas. The samples were reconstituted with 125 μl of mobile phase (30:70, 33.3 m*M* NH_4OAc in 0.33% acetic acid–ACN).

2.5. Calculations

A least-squares calibration curve was obtained by plotting the concentrations of the plasma standards vs. the peak-area ratios (peak area of compound I/peak area of compound II). The concentration for each sample was determined from the peak-area ratio relative to the calibration curve.

2.6. Determination of recovery, precision, and accuracy

The extraction efficiency (recovery) of the sample preparation procedure was tested by comparing the peak heights obtained from the chromatograms of absolute standards (not extracted) to those of extracted rat plasma samples containing spiked amounts of compound I.

The precision and accuracy of the method were determined by performing replicate analysis of plasma spiked with known concentrations of compound I. The pool concentrations were selected to cover the range of the standard curve. Multiple replicates of each pool were analyzed by the same analyst on the same instrument. All samples were analyzed in random order.

2.7. Analysis of samples from pharmacokinetic studies

Rat, rabbit, and monkey plasma samples from three different pharmacokinetic studies were analyzed utilizing the process described in Section 2.4. Quality control samples (low, medium, and high concentrations), prepared from a separate weighing of compound I, were included in each run.

3. Results

3.1. Precision, accuracy, and sensitivity

The determination of compound I was evaluated for precision, accuracy, and sensitivity by replicate analyses of plasma pools spiked with compound I at various concentrations. Sensitivity

Table 1
Precision and accuracy data for compound I in rat plasma, 3 day validation

Day		Theoretical concentration			
		0.25 ng/ml	1.00 ng/ml	5.00 ng/ml	10.0 ng/ml
1	Mean ($n = 5$)	0.28[a]	1.11	4.98	9.78
	R.S.D. (%)	4.5	1.8	3.9	5.3
	% of theory	109	112	99.6	97.8
2	Mean ($n = 5$)	0.29	1.06	4.76	10
	R.S.D. (%)	3.1	2.1	1.7	2.9
	% of theory	114	106	95.3	100
3	Mean ($n = 5$)	0.20	0.91	4.55[a]	9.69
	R.S.D. (%)	8.5	3.3	0.96	5.8
	% of theory	81.7	91.3	91.0	96.9
Overall	Mean ($n = 15$)	0.25[b]	1.03	4.78[b]	9.83
	R.S.D. (%)	19.3	10.2	4.98	4.78
	% of theory	101	103	95.7	98.3

[a] $n = 4$.
[b] $n = 14$.

(LOQ) was defined to be equivalent to the lowest validation pool which, under the given conditions yielded a value of ≤ 20% total relative standard deviation and ≤ 20% overall accuracy. Due to the various species involved and anticipated concentrations from the pharmacokinetic studies to be assayed, a three-day validation was performed in rat plasma with subsequent species or changes in concentration ranges being validated through a one-day protocol.

The precision and accuracy of the method over the three-day validation in rat plasma were acceptable, with overall precision (total relative standard deviation) at each concentration (including LOQ concentration) within 20% and overall accuracies ranging from 95.7 to 103% (Table 1). The LOQ concentration for this three-day validation was determined to be 0.25 ng/ml with an upper limit of quantitation of at least 10.0 ng/ml. Increasing the concentration range (to 100 ng/ml) and performing a one-day validation in rat plasma also yielded acceptable results with overall precision at each concentration within 13% and overall accuracies ranging from 101 to 118% (Table 2). The upper limit of quantitation for this one-day validation was determined to be 100 ng/ml.

The precision and accuracy of the method over the one-day validation in rabbit plasma were acceptable, with overall precision at each concentration (including LOQ concentration) within

Table 2
Precision and accuracy data for compound I in rat plasma, 1 day validation

Day		Theoretical concentration		
		1.00 ng/ml	50.0 ng/ml	100.0 ng/ml
1	Mean ($n = 5$)	1.18[a]	52.6	101.1
	R.S.D. (%)	12.1	0.98	1.3
	% of theory	118	105	101

[a] n = 4.

Table 3
Precision and accuracy data for compound I in rabbit plasma, 1 day validation

Day		Theoretical concentration			
		0.25 ng/ml	1.00 ng/ml	5.00 ng/ml	10.0 ng/ml
1	Mean (n = 4)	0.24	0.97	4.73	9.48[a]
	R.S.D. (%)	18.9	8.09	5.35	10.6
	% of theory	94.4	96.6	94.6	94.9

[a] n = 5.

19% and overall accuracies ranging from 94.4 to 96.6% (Table 3). The LOQ concentration for this one-day validation was determined to be 0.25 ng/ml with an upper limit of quantitation of at least 10.0 ng/ml. Plasma blanks in this matrix indicated that a nonspecific interference peak was present at intensity and area levels below our limit of detection (Fig. 3).

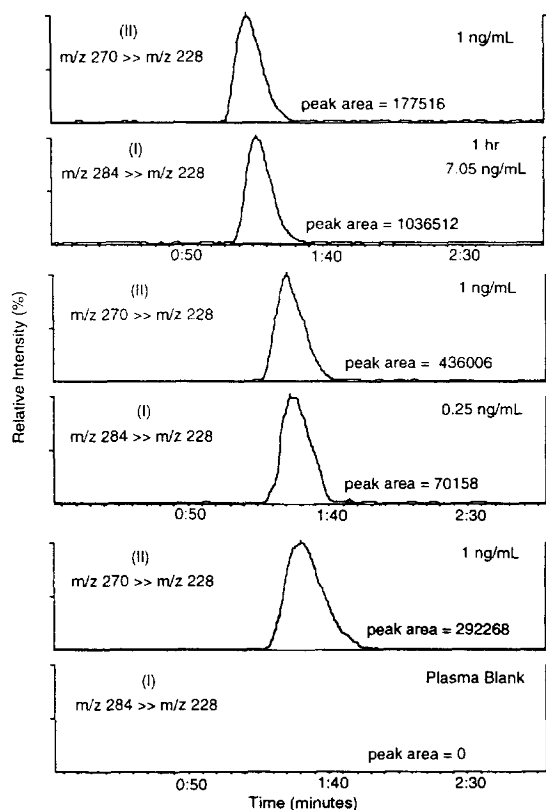

Fig. 3. Representative rat plasma chromatogram following subchronic doses of 60 mg/kg per day administered orally, a 0.25 ng/ml plasma standard, and a control rat plasma chromatogram.

The precision and accuracy of the method over the one-day validation in monkey plasma were acceptable, with overall precision at each concentration (including LOQ concentration) within 14% and overall accuracies ranging from 84.5 to 99.2% (Table 4). The LOQ concentration for this one-day validation was determined to be 0.25 ng/ml with an upper limit of quantitation of at least 10.0 ng/ml. Plasma blanks in this matrix indicated that a nonspecific interference peak was present at intensity and area levels below our limit of detection (Fig. 4).

3.2. Linearity

The linearity of the responses with 0.5-ml sample volumes was established over the concentration range of 0.25 to 100 ng/ml. Typical correlation coefficients were greater than 0.98. Concentration ranges in excess of 100 ng/ml were not examined.

4. Discussion

4.1. Method development and chromatography

The assay conditions described in this report evolved from the evaluation and optimization of the sample preparation, chromatography, and detection of compound I and its internal standard in plasma. This evolutionary process was driven more from sensitivity issues rather than selectivity issues.

Table 4
Precision and accuracy data for compound I in monkey plasma, 1 day validation

Day		Theoretical concentration			
		0.25 ng/ml	1.00 ng/ml	5.00 ng/ml	10.0 ng/ml
1	Mean ($n = 5$)	0.21	0.94	4.64	9.92
	R.S.D. (%)	13.2	5.85	1.50	3.44
	% of theory	84.5	93.8	92.9	99.2

Initially, a liquid–liquid (hexane–aqueous) extraction utilizing gas chromatography (GC) with a nitrogen–phosphorus detection (NPD) was developed [4]. Previous work on structurally similar compounds [5,6] had suggested that a hexane extraction would provide adequate analyte recovery while minimizing plasma interferences. This approach led to the development of a very reliable, rugged method with good analyte recoveries (approximately 88%) and worked well for the initial animal pilot studies conducted. However, as additional animal studies were conducted, sensitivity issues began to arise.

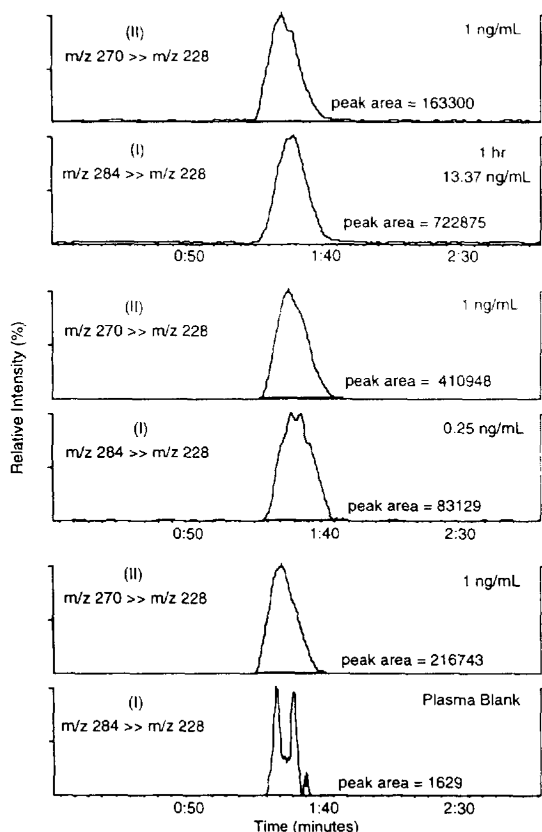

Fig. 4. Representative rabbit plasma chromatogram following a single 5 mg/kg dose administered orally, a 0.25 ng/ml plasma standard, and a control rabbit plasma chromatogram.

The initial hexane extraction utilizing GC–NPD had a limit of quantitation (LOQ) of 5.0 ng/ml with a linear response range to at least 200 ng/ml. Observed concentrations of compound I in ongoing rat studies and anticipated dosages for future studies, suggested that at least a ten-fold or possibly a twenty-fold increase in sensitivity would be required. Based upon recovery data from the hexane extraction, changing the sample preparation did not seem to be a viable alternative to improving the sensitivity to within the sub-ng/ml range. This led to the decision to utilize a different mode of detection, namely HPLC–ES-MS–MS.

Changing detection schemes to HPLC–ES-MS–MS offered several benefits over the initially developed GC–NPD method. These benefits included improved sensitivity, selectivity, and reduction of sample analysis time.

4.2. Application of the method in pharmacokinetic studies

The validated procedure was used to provide pharmacokinetic data for compound I in rat, rabbit, and monkey studies. Plasma samples were obtained at defined time intervals post administration, extracted, and analyzed by HPLC–ES-MS–MS. Representative chromatograms of rat, rabbit, and monkey plasma are shown in Figs. 3–5. Representative plasma profiles of com-

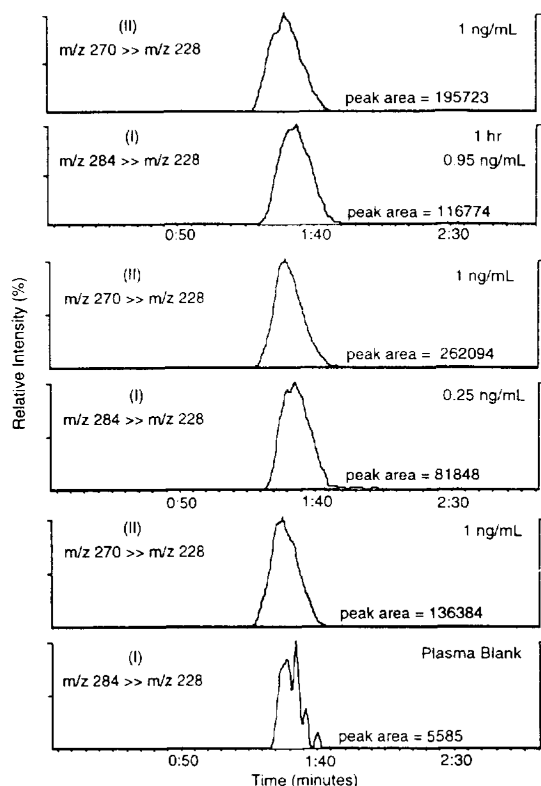

Fig. 5. Representative monkey plasma chromatogram following a single 2.5 mg/kg dose administered orally, a 0.25 ng/ml plasma standard, and a control monkey plasma chromatogram.

pound I as a function of time for three different pharmacokinetic studies (one for each species) are shown in Figs. 6–8.

The representative plasma profile shown for the monkey (Fig. 8) clearly illustrates the impact this quantitative procedure had on sample analysis. Compound I plasma levels from this study yielded concentrations ranging from below limit of quantitation to approximately 1.4 ng/ml. Analyzing the monkey plasma via the GC–NPD method originally developed would have resulted in no quantifiable values being obtained. Additionally, our total sample analysis time was substantially reduced. The original GC–NPD methodology required an instrument analysis time of 25 min per sample. This can be compared to a instrument analysis time of 3 min per sample for the HPLC–ES-MS–MS method.

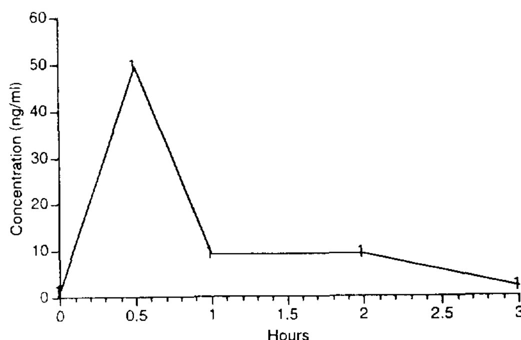

Fig. 6. Representative plasma profile of compound I as a function of time for rats subchronically dosed with 60 mg/kg per day of compound I.

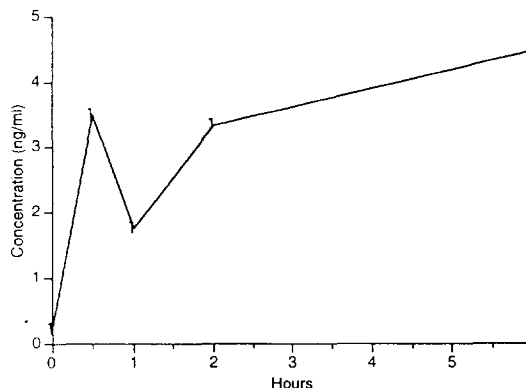

Fig. 7. Representative plasma profile of compound I as a function of time for rabbits dosed with 5 mg/kg of compound I.

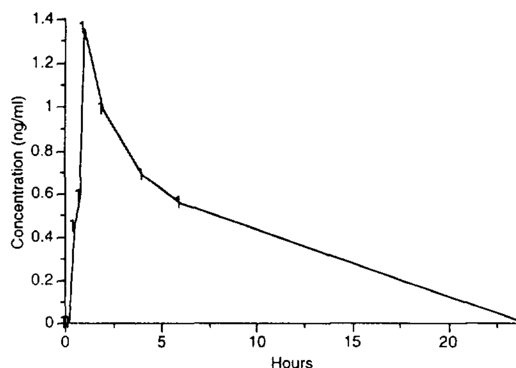

Fig. 8. Representative plasma profile of compound I as a function of time for monkeys dosed with 2.5 mg/kg of compound I.

5. Conclusions

Compound I may be accurately determined in plasma by the described procedure. The use of an HPLC–ES-MS–MS detection scheme improved the selectivity, greatly enhanced the sensitivity, and significantly reduced sample analysis time. This method was used to analyze plasma samples from various pharmacokinetic studies conducted in rats, rabbits, and monkeys.

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