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Determination of an arylacetamide antiarrhythmic in rat blood and tissues using reversed-phase high-performance liquid chromatography

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Abstract

A method was developed for quantification of (+)-*trans*-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzo[*b*-]thiophene-4-acetamide (compound I), an antiarrhythmic drug, in rat whole blood, heart, brain, liver and skeletal muscle. Blood and tissue samples were homogenized and purified by chemical extraction. Chromatographic separations were achieved using reversed-phase high-performance liquid chromatography (HPLC) coupled with UV detection (215 nm). Drug recoveries from the extraction procedure ranged from 77 to 90%. Within- and between-day reproducibility of peak area (coefficient of variation) ranged from 1.1 to 15.7%. The detection limit was 80–200 ng/ml (in a 500-μl extracted solution) depending on the type of biological sample. This method was used in a pharmacokinetic study of compound I disposition in rats after a bolus intravenous dose of 3.1 mg/kg.

Keywords: Arylacetamide antiarrhythmic; (+)-*trans*-N-Methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzo[*b*]thiophene-4-acetamide

1. Introduction

Compound I (Fig. 1) is the (+)-enantiomer of a kappa-opioid receptor agonist [1–3] and possesses antiarrhythmic activity [4]. We report here a method for the determination of blood and tissue concentration of compound I in rats. To our knowledge, this is the first quantitative method described for measurement of an arylacetamide of this type in biological matrices. Reversed-phase HPLC was used because it is highly efficient, able to handle biological samples containing many polar impurities, suitable for evaluation of metabolism, and convenient for the analysis of basic drugs such as this one [5–8].

An extraction procedure involving liquid–liquid extraction and back extraction was necessary due to the large amount of particulate impurities in blood and tissue homogenates. After this procedure, chromatograms of purified samples were clear of contaminating peaks and sample peaks were easily resolved.

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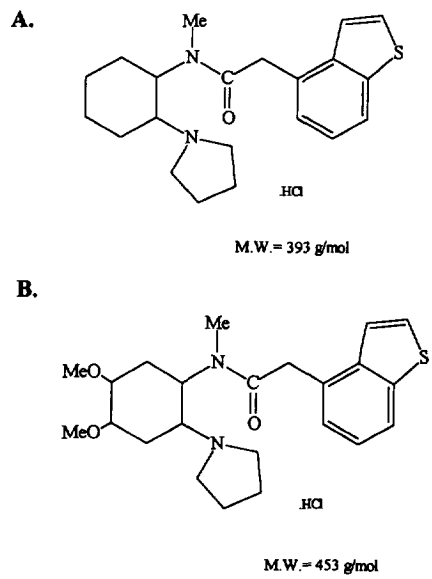

Fig. 1. Structures of (A) compound I, and (B) internal standard.

2. Experimental

2.1. Reagents

Compound I and the internal standard (a structural analogue of compound I—see Fig. 1) were synthesized and supplied by Rhythm Search Developments (Vancouver, Canada). HPLC-grade acetonitrile (MeCN), methanol (MeOH) and acetone were obtained from Fisher Scientific. HPLC-grade methyl *tert.*-butyl ether (MtBE) was obtained from BDH. Various ACS standard, non-organic reagents were used and obtained from the usual laboratory supply firms. Deionized water was used throughout.

2.2. Apparatus

The HPLC system consisted of a WISP 710B automated injector (Waters, Milford, MA, USA), a twin-piston Beckman 100A pump (Beckman Instruments, CA, USA), a Model 441 Waters 215 nm fixed-wavelength UV detector, a Rikadenki Model B104 chart recorder (Rikadenki Kogyo, Tokyo, Japan), an SGE cyanophase 10GLC4 pre-column (10 mm×4 mm I.D., 5 μm particle size, 80 Å pore size; Fisher Scientific, Nepean, Canada), and an SGE cyanophase column (100 mm×4 mm I.D., otherwise same as pre-column). Data was analysed on an Apple IIe computer using the Chromatochart program (Interactive Microware State College, PA, USA).

2.3. Drug solutions

Stock solutions of 1 mg/ml compound I and internal standard were prepared by dissolving the appropriate amount of the drug, accurately weighed, in 10% aqueous ethanol. Aliquots of stock solutions were diluted with 10% aqueous ethanol to give working solutions of 0.25–2.0 μg/ml. All solutions were stored at −20°C when not in use.

2.4. Extraction procedure

Frozen tissues were thawed, weighed, and homogenized in saline. Frozen blood homogenates were thawed and stirred prior to extraction. Each homogenate was aliquotted into three 2.5-ml samples. To each sample was added 1 μg/ml internal standard; samples were lightly vortexed, then 3 ml acetone was added to each. After vigorous vortexing to a uniform texture, samples were let stand for 15–30 min then centrifuged at 4000 *g* for 15 min. A fixed volume of supernatant was removed and the pellet discarded. To the supernatant was added 0.1 volume of NaH_2PO_4 (0.1 *M*, pH 9 with NaOH), which acted as a weak base to alkalinize the extract, then 5 ml of MtBE was added. Tubes were mixed on a Labquake mixer (LabIndustries, Berkeley, CA, USA) for 10 min. The organic fraction was collected and two more washes with 2 ml MtBE were performed on the aqueous fraction. These volumes were used for all subsequent washings as they provided maximal recovery of drug into the desired fraction and small enough volumes such that a change of vessel size was not needed during the procedure. After the last wash the aqueous fractions were discarded. A back extraction was performed on the organic fractions using 0.4 *M* sulfuric acid: three washes of 5, 2, and 2 ml, respectively. The aqueous fraction was collected this time and after the final wash the organic fractions were discarded. The aqueous fractions were then neutralized by adding

solid sodium carbonate until pH paper indicated a pH between 8 and 9. The MtBE washes were repeated as above and the aqueous fractions discarded. The organic fractions were evaporated under nitrogen gas in a Reacti-Therm heating module (Pierce Chemical, Rockford, IL, USA) set on low heat (40°C). Dry samples were reconstituted by first dissolving in 50 μl ethanol, sonicating, then adding 450 μl distilled water so that total sample volume was 500 μl.

2.5. Chromatography

Chromatographic analysis was performed at ambient temperature with a mobile phase of 45% MeCN, 30.5% water, 16% MeOH, and 8.5% ammonium acetate buffer (0.15 *M*, pH 7). This solution was degassed in situ and flow-rate was maintained at 1 ml/min. Injection volume was 20 μl, samples were eluted isocratically, and absorbance was monitored at 215 nm.

2.6. Standard (calibration) curves

Whole blood, brain, heart, liver, and skeletal muscle calibrations were constructed using four concentrations of spiked samples (0.25, 0.5, 1.0, and 2.0 μg/ml). The samples were extracted and analysed using the method described above. Standard curves were constructed using peak-area ratios (compound I: internal standard) versus concentration of compound I. The linearity of the curves was verified by means of linear regression analysis and variability was also determined.

2.7. Validation

The recovery of compound I and internal standard from whole blood and tissue was calculated by comparing observed concentrations in spiked extracted samples with those in unextracted aqueous standard solutions over a range of 0.25–2.0 μg/ml. Within- and between-day reproducibility of chromatographic data was determined by repeated measurement of a 1 μg/ml aqueous solution of internal standard and 0.25, 0.5, 1.0 and 2.0 μg/ml solutions of compound I.

2.8. Pharmacokinetic study in rats

Male Sprague–Dawley rats were acclimatized to the laboratory environment in the animal room for approximately one week before study. Animals received a bolus dose of 3.1 mg/kg via the tail vein and were decapitated at 15, 30, 60, 120, 300, and 600 s after dose. Blood (approx. 8 ml) was collected from the neck area, mixed with variable amounts of saline (0.9%, w/v) and a few drops of heparin (1000 i.u./ml) then homogenized with an Ultra-Turrax T25 high speed grinder (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) before storage at −20°C. Heart, brain, liver, and a sample of skeletal muscle from the right hind leg were removed, rinsed in cold saline, and stored frozen at −20°C.

3. Results and discussion

3.1. Sample pre-treatment

Purification of samples from whole blood and tissue proved to be very difficult. Several methods were tried before settling on the one described above. Precipitation of proteins using 0.8 *M* perchloric acid did not clear up the chromatogram sufficiently to see the analyte peaks, even when

Table 1
Comparison of recoveries using various liquid extraction reagents

Reagents	Recovery (%)	
	Compound I	I.S.
Organic extraction		
Na_2CO_3, MtBE	46	36
Na_2CO_3, toluene	7	nd
NaH_2PO_4, MtBE	77	74
NaH_2PO_4, toluene	53	49
Back extraction		
Sulfuric acid, Na_2CO_3	98	68
Sulfuric acid, NaOH	96	68
Phosphoric acid, Na_2CO_3	85	63
Phosphoric acid, NaOH	52	60

In the organic extraction step Na_2PO_4 and MtBE yielded the highest recovery of both compounds and so were used to test the results of adding a subsequent back-extraction step. Recoveries were improved even further when the organic extraction was repeated after the back extraction (see Table 3). nd=Not detectable.

precipitation and centrifugation steps were repeated. Neutralizing the sample with potassium carbonate cleaned up the chromatogram but resulted in very low recoveries. Variations on this method were attempted with different reagents with the same unsatisfactory results. Other methods, including homogenization in sodium acetate and several filtration processes all resulted in low recoveries. Solid-phase extraction on cyanophase columns gave good recoveries but poor reproducibility.

Liquid–liquid extraction coupled with back extraction proved to be the best method. Several combinations of reagents were tried. One organic extraction step alone did not provide adequate recoveries. Coupled with a back-extraction step the recoveries increased, and were maximal when a second organic extraction was performed. Table 1 compares the recoveries of compound I and internal standard using various reagents for the extraction steps. A combination of MtBE, NaH_2PO_4, sulfuric

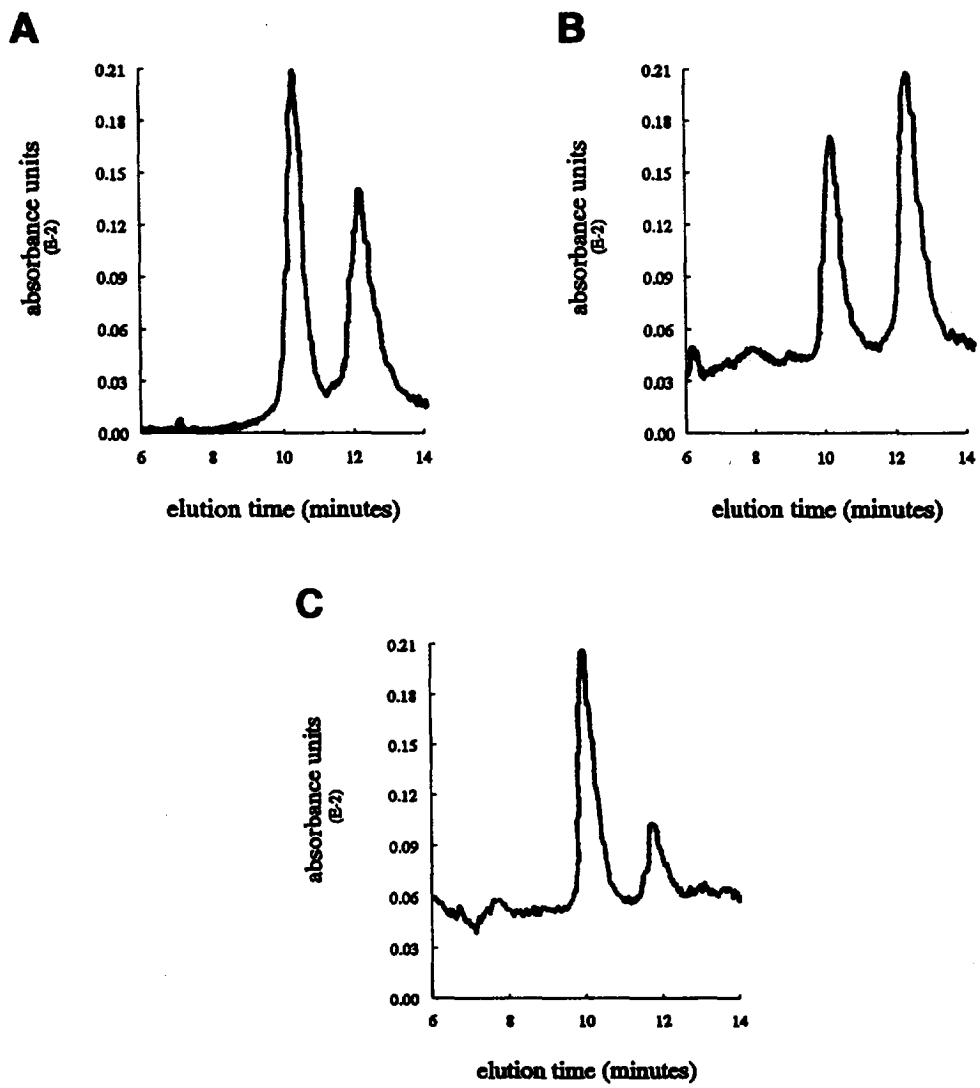

Fig. 2. Chromatograms of compound I and internal standard: (A) standard solution of 1 μg/ml internal standard and 1.0 μg/ml compound I; (B) extracted liver sample that had been spiked with 2.0 μg/ml compound I; (C) extracted brain sample from a rat given 3.1 mg/kg compound I (bolus i.v.) and sacrificed 30 s later.

acid, and Na_2CO_3 gave the best results. Since the molecules of interest have pK_a values of approximately 8, this sequence allowed for elimination of both polar and non-polar endogenous impurities, and resulted in acceptable recoveries and reproducibility.

3.2. Chromatography

Fig. 2 shows chromatograms for standard solutions of compound I and internal standard (A), spiked extracted samples from various tissues and blood (B), and samples from a rat given a bolus i.v. dose of compound I (C). Retention times were 12 and 10 min for drug and internal standard, respectively. Detection limits, defined as a signal-to-noise ratio of 3, ranged from 80 ng/ml for liver to 200 ng/ml for brain. The detection limits for different samples are shown in Table 2

3.3. Recoveries from extraction procedure

The recovery was defined as the peak area of an extracted sample divided by the peak area of an unextracted aqueous solution of the same concentration. Ratios of compound I to internal standard could not be compared since the two compounds were recovered in different quantities and these numbers varied between blood and the different tissues. These differences were, however, consistent within a single tissue type and therefore the internal standard served its purpose. The highest recovery was that of internal standard in the brain (90%) and the lowest was that of compound I in the brain (77%). Data for all the tissues and blood are shown in Table 3. There were no significant variations in recovery at different concentrations in a single tissue as evidenced by the low S.E.M. values.

Table 2
Detection limits of the chromatographic method for blood and tissues

Sample type	Detection limit (ng/ml extracted solution)
Brain	200
Liver	80
Skeletal muscle	180
Blood	140
Heart	99

Minimum concentrations were calculated on the basis of a peak-area ratio where the compound I peak had a 3:1 signal-to-noise ratio. Because the calibration curves differ for each tissue, so does the corresponding concentration of the minimum peak-area ratio.

Table 3
Recovery of compound I and internal standard by chemical extraction

Sample type	Recovery (mean±S.E.M.) (%)	
	Compound I	I.S.
Liver	78±3.8	87±1.1
Heart	85±2.1	88±2.5
Skeletal muscle	79±2.6	88±1.7
Blood	86±2.0	87±1.4
Brain	77±0.7	90±0.9

Each number represents the mean and S.E.M. of the recoveries for twelve samples ($n=3$ at each of four concentrations of compound I).

3.4. Standard curves

Regression analysis was performed on all calibration curves. The results are shown in Table 4. All calibration curves had $r>0.996$ and showed low variability in the calculated slopes. The fact that the y-intercepts were very close to zero indicates that there was little interference from endogenous contaminants.

3.5. Reproducibility of chromatograms

The variability in peak height was determined by repeated measures of solutions of compound I and internal standard (see Table 5). Within-day variability was calculated by determining the mean coefficient of variation over several days (duplicate measurements each day). Between-day values represent the coefficient of variance of the mean peak area over a period of several days. Within-day values ranged from 1.1% to 3.9%. Between-day values range from 3.4 to 15.7%.

3.6. Pharmacokinetic experiment

The applicability of the method to pharmacokinetic studies was demonstrated by an experiment to

Table 4
Linearity and variability of calibration curves

Sample type	Slope (mean±S.E.M.)	y-Intercept (mean±S.E.M.)	r
Blood	0.693±0.012	0.020±0.013	0.999
Brain	0.757±0.019	−0.030±0.022	0.998
Heart	0.787±0.008	0.042±0.009	1.000
Liver	0.668±0.017	0.064±0.020	0.998
Skeletal muscle	0.903±0.034	−0.039±0.039	0.996

Slope and y-intercept values represent the mean of three trials for each sample (±S.E.M.). Regression coefficients are calculated from a line of the mean slope and mean y-intercept.

Table 5
Variability of peak area

Concentration (μg/ml)	n	Within-day C.V. (mean±S.E.M.) (%)	Between-day C.V. (%)
Internal standard			
1	5	2.0±0.4	3.4
Compound I			
0.25	6	3.9±0.7	15.7
0.5	7	1.6±0.4	15.6
1.0	7	1.1±0.4	3.8
2.0	6	1.4±0.4	4.0

Duplicate measurements were made on each of n days. Within-day values represent the mean C.V. over n days. Between-day values represent the variance of the mean peak area ratio over n days.

determine blood, brain, heart and skeletal muscle concentration of compound I in rats following a bolus i.v. dose. The concentration-versus-time curves are shown in Fig. 3.

4. Conclusion

The HPLC assay described here for compound I in rat whole blood and tissues, pre-treated with liquid–

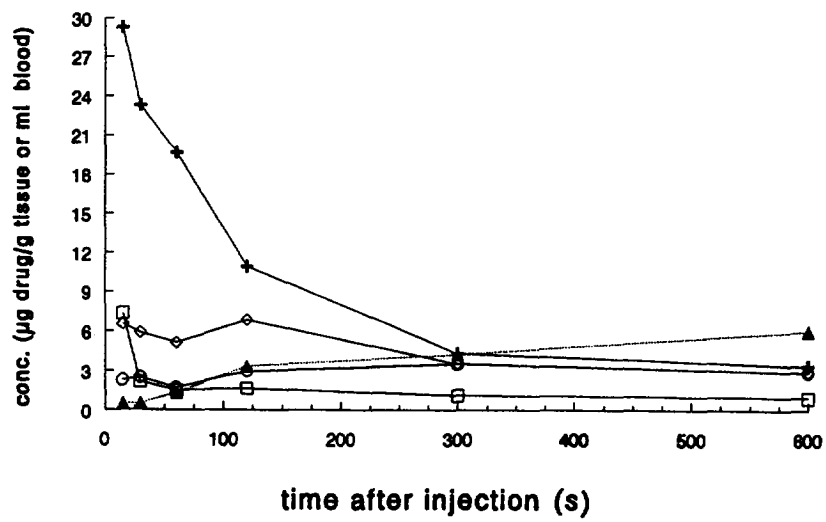

Fig. 3. Disposition of compound I in rat blood and tissue following a bolus i.v. dose of 3.1 mg/kg. ▲=Liver; ◇=brain; +=heart; ○=skeletal muscle; □=blood.

liquid extraction and back extraction, is a sensitive, validated and efficient method for the determination of this drug in a variety of biological matrices. This assay was used for pharmacokinetic studies of compound I in the rat, and is almost certainly applicable to analyses of primate samples.

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