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Journal of Chromatography B, 653 (1994) 221–226

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Determination of a novel sigma receptor antagonist, DuP 734, in rat plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection

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(First received July 13th, 1993; revised manuscript received November 15th, 1993)

Abstract

A selective high-performance liquid chromatographic (HPLC) assay for a sigma receptor antagonist, DuP 734 (I), in rat plasma has been developed. Compound I and internal standard, XC031 (I.S.), were first extracted from plasma into an ethyl acetate–toluene mixture (3:7, v/v) and then back-extracted into freshly prepared phosphoric acid (0.03 *M*). Separation of I and I.S. with no interference from endogenous substances was achieved on a reversed-phase octyl column and detection was by UV at 229 nm. The mobile phase consisted of acetonitrile–glacial acetic acid–triethylamine–0.05 *M* ammonium acetate (670:4:2:2000, v/v). Using 0.5 ml of rat plasma for extraction, the limit of quantitation was 43 ng/ml and the assay was linear from 43 to 8536 ng/ml. The intra- and inter-day coefficients of variation ranged from 0.7 to 3.0%, and from 1.4 to 14.5%, respectively, over the entire concentration range. The accuracy was within 16.1% of the spiked concentrations. I was stable in frozen plasma at −20°C for at least 68 days.

1. Introduction

DuP 734, [1-(cyclopropylmethyl)-4-(2′(4″fluorophenyl)-2′-oxoethyl) piperidine hydrobromide](I), is a selective sigma receptor antagonist (Fig. 1) currently under investigation as a potential antipsychotic agent [1–5]. It is soluble in water (123 mg/ml at pH 6) with a pK_a of 9.9 [6]. To facilitate pharmacokinetic investigations of I, a specific reversed-phase HPLC assay with UV detection has been developed using XC031 [1-benzyl-4-(4′fluorobenzamido) piperidine hydrobromide] as internal standard (I.S.). This report describes the validation of the HPLC method in rat plasma.

2. Experimental

2.1. Chemicals and solutions

I (Lot XC-734-9) and I.S. were synthesized and characterized by The DuPont Merck Pharmaceutical Company and stored as dry powders at room temperature. Ammonium acetate, ethyl

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SSDI 0378-4347(93)E0416-N

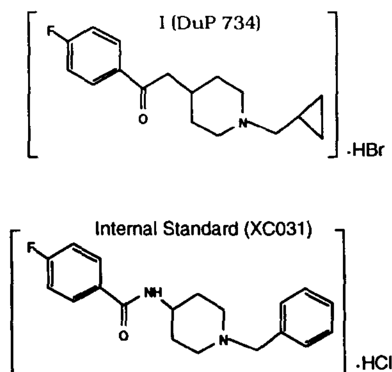

Fig. 1. Structures of I (DuP 734) and internal standard (XC031).

acetate, toluene, and acetonitrile were of HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). All other chemicals were of reagent grade (J.T. Baker). Blank rat plasma was obtained from male Sprague–Dawley rats (Charles River, Kingston, NY, USA) housed at the DuPont Stine-Haskell Research Center.

2.2. Instrumentation

The HPLC system consisted of an isocratic solvent delivery pump (Spectroflow 400, Applied BioSystems, Ramsey, NJ, USA), an autosampler (Waters WISP 710B, Milford, MA, USA), a reversed-phase column (Beckman Ultrasphere Octyl, 250 mm × 4.6 mm I.D., 5 μm particle size, Fullerton, CA, USA) with a similar pre-column (4.5 cm × 4.6 mm I.D.) and a variable wavelength UV detector (Spectroflow 783, Applied BioSystems). The data system consisted of an A/D converter (Nelson Analytical Model 760, Cupertino, CA, USA), a 16-bit desktop computer (Hewlett-Packard, HP 9200, Wilmington, DE, USA) with a hard disk drive (HP 9133), a thermal printer (HP 2671G) and Xtrachrome 4400 software (version 7.2, Nelson Analytical).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile–glacial acetic acid 0.05 *M* ammonium acetate–triethylamine (670:4:2000:2, v/v). The flow-rate was 1.0 ml/min. The detector wavelength was set at 229 nm.

2.4. Stock solutions

Primary stock solution

A primary stock solution (552 μg/ml) was prepared by dissolving 13.8 mg of I in 25 ml of methanol and stored in the refrigerator. This solution was periodically analyzed and compared to the peak-area results of freshly prepared methanolic solutions. I was stable for at least 4 months in methanol when stored refrigerated.

Working stock solutions A and B

A working stock solution A, equivalent to 4268 ng/ml free base concentration, was prepared by evaporating 1 ml of the primary stock solution under a gentle stream of nitrogen and reconstituting with 100 ml of distilled water. Aqueous solution B, 427 ng/ml concentration, was prepared by diluting solution A 10 times. These stock solutions were stored in the refrigerator and under these conditions I was stable for at least 4 months.

Internal standard stock solution (XC031)

A primary stock solution (188 μg/ml) was prepared by dissolving 4.7 mg of I.S. in 25 ml of methanol. A 5-ml aliquot was evaporated and reconstituted with 100 ml of distilled water. This solution was further diluted 20 times to obtain the working stock solution (465 ng/ml) of the I.S.

2.5. Preparation of plasma standards and samples, and extraction procedure

Plasma standards of I were prepared by adding 0.05, 0.1, 0.2, 0.4 and 1.0 ml of working stock solution B and 0.2, 0.4 and 1.0 ml of working stock solution A into 15-ml glass tubes (16 × 125 mm) containing 0.5 ml of blank plasma. The volume in each tube was adjusted to 1.5 ml with distilled water. The resultant plasma concentrations of I were 43, 85, 171, 341, 854, 1707, 3414 and 8536 ng/ml. To each tube, the following components were added: 0.5 ml of I.S. (465 ng/ml of I.S.), 1.0 ml of 1 *M* boric acid–potas-

sium chloride–sodium hydroxide buffer (pH 9.0) and 10 ml of ethyl acetate–toluene (3:7, v/v). The preparation of plasma samples was similar to that of standards with the exception of the following changes: distilled water (1 ml) was used instead of stock solution of I, and blank plasma (0.5 ml) was replaced with 0.5 ml plasma sample. Tubes were capped and shaken for 20 min on a rocking shaker (an in-house modification of a Labquake model). The tubes were then centrifuged for 5 min at 2100 *g* (Sorval RC-3B, DuPont Instruments, Wilmington, DE, USA). The upper organic layer (9 ml) was transferred to a clean 15-ml glass centrifuge tube containing 300 μl of 0.03 *M* phosphoric acid (freshly prepared) and shaken for 20 min. The ethyl acetate–toluene mixture was discarded after centrifugation for 5 min at 2100 *g*. Residual organic layer above the aqueous layer was evaporated under nitrogen for 12 min, and 150 μl of the aqueous layer was injected onto the HPLC column.

2.6. Sample preparation for precision, accuracy and stability evaluation

Precision

Quadruplicate plasma samples of I were prepared in the concentration range 43–8536 ng/ml for intra- and inter-day reproducibility. The samples were extracted and analyzed on the same day on different occasions.

Accuracy

One-ml aqueous samples of varying concentrations of I were prepared and coded by a co-worker of the analyst. The assay of unknown I samples was then carried out by the analyst in the aforementioned manner.

Stability

Twenty-ml samples of rat plasma containing 171, 854 and 8536 ng/ml of I were prepared and 0.5-ml aliquots were transferred into 15-ml glass tubes and stored frozen at −20°C. The samples were thawed and analyzed at various times to assess stability.

2.7. Calibration and calculation of plasma concentrations

The peak-area ratios (PAR) of I free base to the I.S. of the extracted plasma samples were evaluated against the plasma standards. Two unweighted linear regression curves were used to determine slopes, intercepts and correlation coefficients of the standard curves. One regression curve was used to evaluate standard concentrations ranging from 43 to 854 ng/ml (low standard curve) and the second regression curve was used to evaluate standard concentrations ranging from 854 to 8536 ng/ml (high standard curve). For unknown plasma samples whose PAR was less than or equal to the PAR of 854 ng/ml standard, the low standard curve was used for the calculations. For samples whose PAR was higher than 854 ng/ml, the high standard curve was used. Interpolation in this manner yielded most satisfactory results. All plasma concentrations and standard solutions are expressed as the free base of I.

2.8. Rat pharmacokinetics

Sprague–Dawley Rats (CD rats, Charles River, Kingston, NY, USA) were used for the experiment. One day prior to dosing, one of the two jugular veins of each animal was cannulated with polyethylene tubing (PE 50, 0.58 mm I.D., Clay Adams, Division of Becton Dickinson, Tarsippany, NJ, USA) with a 1-cm tip of silastic tubing (0.64 mm I.D., Dow Corning Corporation, Midland, MI, USA) for dosing and blood sampling. The animals were fasted overnight and during the sampling period. Three male rats received I as a single intravenous (i.v.) bolus dose at 3.7 mg/kg (equivalents of free base) in normal saline (3 ml/kg) via the jugular vein cannulas. The cannulas were primed before and flushed after the injection with plain dosing vehicle. Blood samples (1 ml) were collected from the jugular vein cannulas at predose and at 2, 5, 10, 20, 30, 60, 90, 120 and 180 min in heparinized microcentrifuge tubes (Eppendorf, VWR Scientific, Bridgeport, NJ, USA). An equal volume of donor blood was transfused into the animals after each blood sample collection.

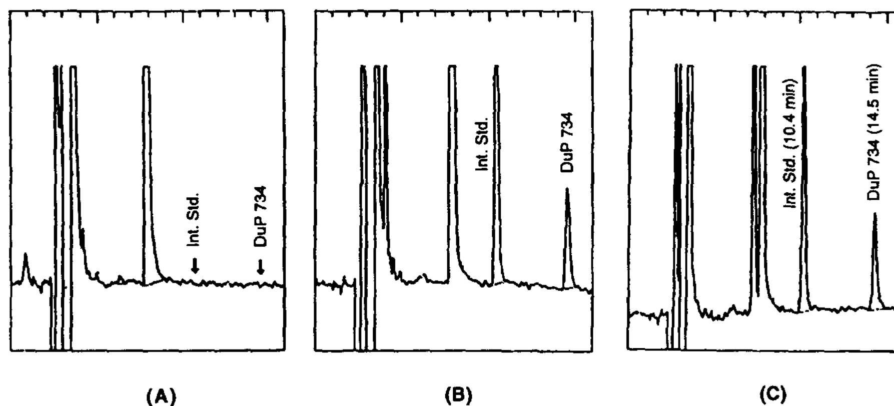

Fig. 2. Representative chromatograms of (A) extracted rat plasma blank, (B) rat plasma spiked with I (DuP 734) (170 ng/ml, and (C) rat plasma at 5 min after receiving a single 50 mg/kg oral dose of I. The found concentration of I was 180 ng/ml.

Plasma was harvested following centrifugation for 15 min at 2100 *g*. Plasma samples were stored frozen at −20°C prior to analysis.

3. Results and discussion

3.1. Selectivity

Figs. 2A and B show representative chromatograms obtained from rat plasma blank and rat plasma blank spiked with I and I.S. No interference from endogenous substances in plasma was detected at the retention times (t_R) of I (t_R = 14.5 min) or I.S. (t_R = 10.4 min). Fig. 3C depicts a chromatogram from a 5-min sample after a 50

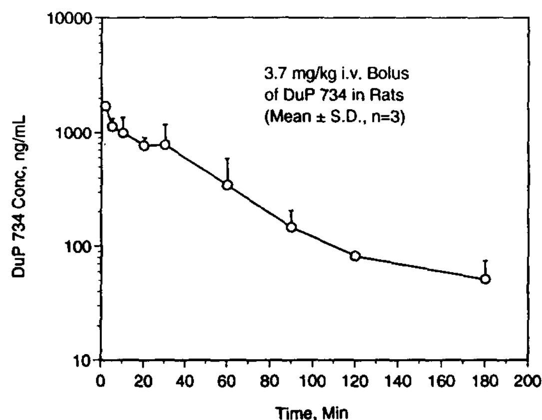

Fig. 3. Plasma concentration *versus* time profile of I (DuP 734) in rats following a single 3.7 mg/kg intravenous dose of I.

Table 1
Precision for the quantitation of I in rat plasma

Spiked concentration (ng/ml)	Intra-day		Inter-day	
	Found concentration[a] (ng/ml)	C.V. (%)	Found concentration[a] (ng/ml)	C.V. (%)
43	43 ± 1	1.4	46 ± 7	14.5
85	83 ± 2	2.1	87 ± 10	11.9
171	175 ± 15	8.8	180 ± 16	8.8
341	330 ± 6	1.7	347 ± 30	8.7
854	851 ± 21	2.5	862 ± 356	4.2
1707	1738 ± 38	2.2	1673 ± 34	2.0
3414	3465 ± 86	2.5	3447 ± 115	3.3
8536	8891 ± 63	0.7	8562 ± 116	1.4

[a] Values are mean ± S.D., $n = 4$.

Table 2
Extraction recovery of I from rat plasma

I free base concentration (ng/ml)	Area counts[a]		Recovery (%)
	Unextracted I	Extracted I	
43	10 519 ± 46	11 260 ± 678	107.1
85	22 217 ± 780	22 610 ± 971	101.8
171	46 132 ± 944	45 373 ± 1167	98.4
341	90 192 ± 3603	81 711 ± 4739	90.6
854	238 993 ± 2143	207 812 ± 25 422	87.6
1707	436 476 ± 62 488	455 122 ± 17 791	104.3
3414	900 789 ± 66 676	961 429 ± 81 191	106.7
8536	2 283 544 ± 143 646	2 452 413 ± 148 088	107.4

[a] Values are mean ± S.D., $n = 4$.
Recovery of I over the entire concentration range = 100.4 ± 7.8%.
Recovery of I.S. at 465 ng/ml was 74%.

mg/kg oral dose of I in a rat. The chromatographic peaks of the two compounds were well resolved.

The standard curve for the assay of I in rat plasma was linear between 43 to 8536 ng/ml using 0.5 ml of rat plasma. The correlation coefficients of standard curves for the rat plasma assay usually exceeded 0.99.

3.2. Intra-day reproducibility

The results are summarized in Table 1. The coefficients of variation (C.V.) for the intra-day results ranged from 0.7 to 8.8%.

3.3. Inter-day reproducibility

The results obtained from similar extraction of I on different occasions are summarized in Table 1. The C.V.s for the inter-day results ranged from 1.4 to 14.5%.

3.4. Extraction recovery

The extraction recovery was determined by comparing the peak areas of extracted and unextracted standards of I over the entire concentration range of 43 to 8536 ng/ml. The results are summarized in Table 2. The recovery of I from 0.5 ml rat plasma during the extraction ranged from 88 to 107% and was apparently linear over the entire concentration range. The recovery of I.S. (465 ng/ml) was 74%.

Table 3
Accuracy of the HPLC-UV assay for I in rat plasma

Spiked concentration (ng/ml)	Found concentration (ng/ml)	Difference (%)
43	43	1.1
	43	0.3
	43	0.2
	42	−2.1
51	45	−13.0
	43	−16.1
	51	−1.1
1260	1280	1.6
	1329	5.5
	1308	3.8
	1327	5.3
3780	3701	−2.1
	3623	−4.2
	3710	−1.9
	3827	1.2
6300	6625	5.2
	6138	−2.6
	6513	3.4

Table 4
Stability of I at −20°C in rat plasma

Spiked concentration (ng/ml)	Stability day	Found concentration[a] (ng/ml)
170	1	180[b]
	2	189 ± 14
	13	205 ± 5[c]
	68	191 ± 8
854	1	857 ± 53
	2	1032 ± 48[c]
	28	965[b]
	68	948 ± 54
8536	1	9091[b]
	13	8297 ± 113
	25	8568 ± 540
	68	9278 ± 132

[a] Values are mean ± S.D., $n = 3$.
[b] One sample.
[c] Two samples.

3.5. Accuracy

The results from the accuracy studies in which unknown concentrations of I in rat plasma were measured are shown in Table 3. The absolute percent difference between the spiked and measured (found) concentrations of I ranged from 0.2 to 16%.

3.6. Stability

Triplicate stability samples were analyzed on four separate occasions during 68 days. The results are summarized in Table 4. I appears to be stable in rat plasma, stored frozen at −20°C, for at least 68 days.

3.7. Rat pharmacokinetic study

The mean plasma concentration–time curve of I in male rats following a single i.v. bolus dose of 3.7 mg/kg is illustrated in Fig. 3. The terminal elimination half-life of I was *ca.* 20 min. Plasma concentrations of I (>43 ng/ml) could be quantitated 3 h (approx. 9 half-lives) after dosing. These results indicate that the assay is sufficiently sensitive and specific. The assay has been successfully utilized in preclinical investigations of I.

4. References

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