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High-performance liquid chromatographic assay of bromocriptine in plasma and eye tissue of the rabbit

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Abstract

A reliable reversed-phase high-performance liquid chromatographic method has been developed for the determination of bromocriptine (BCT) in plasma and eye tissues. The BCT and propranolol, added as an internal standard (I.S.), were extracted by a liquid–liquid technique followed by an aqueous back-extraction, allowing injection of an aqueous solvent into a 4-μm Nova-Pak C_{18} column (150×3.9 mm I.D.). The mobile phase was a mixture of 30 parts of acetonitrile and 70 parts of 0.2% triethylamine (pH 3) at a flow-rate of 1 ml/min. Fluorescence detection was at an excitation wavelength of 330 nm and an emission wavelength of 405 nm. The retention times of I.S. and BCT were 4.1 and 11.6 min, respectively. The calibration curve was linear over the concentration range 0.2–10 μg/l for plasma (r>0.999) and vitreous humour (r>0.997) and 1–50 μg/l for aqueous humour (r>0.985). The limit of quantification was 0.2 μg/l for plasma and vitreous humour using a 1-ml sample and was 1 μg/l for aqueous humour using a 0.2-ml sample. The quality control samples were reproducible with acceptable accuracy and precision. The within-day recovery (n=3) was 100–102% for plasma, 91–106% for aqueous humour and 96–111% for vitreous humour. The between-day recovery (n=9) was 90–114% for plasma, 83–115% for aqueous humour and 90–105% for vitreous humour. The within-day precision (n=3) and the between-day precision (n=9) were 1.7–7.0% and 8.1–13.6%, respectively. No interferences from endogenous substances were observed. Taken together, the above simple, sensitive and reproducible high-performance liquid chromatography assay method was suitable for the determination of BCT in plasma and eye tissues following ocular application of BCT for the therapy of myopia.

Keywords: Bromocriptine

1. Introduction

Bromocriptine (BCT), a semi-synthetic derivative of the ergotoxin group of ergot alkaloids, is a dopamine receptor type II agonist and a prolactin inhibitor. BCT activates dopaminergic receptors in the neostriatum of the central nervous system, which may help in the treatment of Parkinsonism. BCT reduces the serum prolactin concentration by a direct effect on the pituitary and/or a complicated catecholamine pathway. BCT inhibits the release of prolactin from the anterior pituitary gland and/or induces release of prolactin-inhibitory factor by stimulating post-synaptic dopamine receptors on the hypothalamus [1]. Also, BCT reduces axial length growth in the eye, which suggests that it may be therapeutically useful in the treatment of human myopia (short sightedness). Axial growth was significantly

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reduced by the ocular instillation of 0.0125% BCT eye drops in an eyelid-sutured chick [2], and a similar result was also reported in the monkey, using apomorphine [3]. Evaluation of plasma pharmacokinetics of BCT has been thoroughly investigated for the therapy of hyperprolactinemia and Parkinsonism [4–6], whereas evaluation of the eye tissue pharmacokinetics of BCT, used for the treatment of myopia, has not been reported.

Several analytical procedures have been published for quantification of BCT levels. Radioimmunoassay revealed good sensitivity, but is not selective enough to measure BCT in rat tissue and human plasma [7,8]. Mass spectrometry revealed good sensitivity and selectivity in human plasma, but these were achieved by using a triple-stage-quadrupole spectrometer in combination with highly sophisticated spectrometric techniques [9]. Furthermore, gas chromatography with electron-capture detector [10] revealed high sensitivity in plasma but needed a complex extraction procedure, whereas the high-performance liquid chromatography (HPLC) method with UV detection [11] showed low sensitivity in plasma, although there was a simple extraction procedure using acetonitrile. Therefore, it was necessary to develop a simple, specific and sensitive assay method for the detection of low concentrations of BCT, by which the pharmacokinetics of BCT in the plasma and in eye tissues could be investigated following the ocular application of BCT.

2. Experimental

2.1. Chemicals and reagents

Bromocriptine mesylate (BCT mesylate), triethylamine hydrochloride and propranolol hydrochloride (I.S.) were obtained from Sigma (St. Louis, MO, USA), and 2-hydroxypropyl β-cyclodextrin (molecular mass, 1500; molar substitution, 0.8) was obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile and methanol (Burdick and Jackson, Muskegon, MI, USA) and diethyl ether (J.T. Baker, Phillipsburg, NJ, USA) were of HPLC grade, and all other reagents were of analytical grade. Deionized water was purified using a Milli-Q filter system (Millipore, Milford, MA, USA). Male, albino rabbits, weighing 2.0–2.5 kg, were purchased from Yonam College of Live Stock and Horticulture (Choongnam, Korea) and the dosing solution was 0.1% BCT mesylate prepared in 20% 2-hydroxypropyl β-cyclodextrin in water, adjusted to pH 5 with 0.1 *M* NaOH.

2.2. Preparation of standard solutions

A stock solution (1 g/l as a free form) of BCT was prepared by dissolving 11.479 mg of BCT mesylate (M_r of BCT, 654; M_r of BCT mesylate, 750.7) in 10 ml of methanol. Diluted working solutions of BCT (2, 3, 5, 10, 20, 50 and 100 μg/l) were made by serial dilution of the stock solution with methanol. The working solution of I.S. (2.5 mg/l as a free form) was prepared by dissolving 2.851 mg of propranolol hydrochloride (M_r of propranolol, 259.4) in 1 l of water. The stock and working solutions were stored at −20°C and were stable at least for six months. For the preparation of calibration standards, 100 μl of the corresponding working solution was evaporated to dryness under a nitrogen stream at room temperature, and blank plasma (1 ml), blank aqueous humour (0.2 ml) and blank vitreous humour (1 ml) were spiked to obtain BCT concentrations of 0.2–10 μg/l (1–50 μg/l for aqueous humour) in the samples. Thereafter, 200 μl of 0.05 *M* H_2SO_4 was added and the solutions were mixed. Calibration curves were constructed using BCT-to-I.S. peak area ratios: Values from calibration samples were plotted against nominal concentrations and linear regression analysis was used to determine the slope of the line (i.e. the response factor) that would best fit the data with the intercept fixed at zero. The limit of quantification (LOQ) was determined at a signal-to-noise ratio of approximately 3.

For the preparation of quality control (QC) samples, working solutions of BCT [3 (plasma only), 10 and 100 μg/l] were made by serial dilution of the stock solution (1 g/l as a free form) with methanol. Volumes (1 ml) of the corresponding working solutions were evaporated to dryness under a nitrogen stream at room temperature, and then 10 ml of blank plasma, 2 ml of blank aqueous humour and 10 ml of blank vitreous humour were added and mixed to generate 0.3, 1 and 10 μg/l plasma quality control samples; 5 and 50 μg/l aqueous humour quality

control samples and 1 and 10 μg/l vitreous humour quality control samples. Afterwards, a 1-ml volume (0.2 ml for aqueous humour) of each quality control sample was mixed with 200 μl of 0.05 *M* H_2SO_4 and was stored at −20°C for quality control analysis. The within-day variation for multiple assays ($n=3$) and the between-day variation over a three-day period ($n=9$) were determined from quality control samples. The accuracy and the precision of the assay were determined by measuring the concentration of BCT using calibration standards and comparing them with the nominal concentrations. The absolute recovery of BCT was determined by comparing the peak area of a treated quality control sample with that of an untreated standard (corresponding standard in 0.05 *M* H_2SO_4–MeOH, 2:1, v/v).

2.3. Sample preparation

At the time of assay, calibration standards, quality control samples and study samples were mixed in a 15-ml conical polypropylene tube (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 50 μl of I.S., 20 μl of 2.5 *M* K_2CO_3 and 2 ml of diethyl ether. The tubes were vortex-mixed for 5 min and centrifuged for 10 min at 1500 *g*. Following centrifugation, the organic supernatant was transferred to another 15-ml conical polypropylene tube containing 300 μl of 0.05 *M* H_2SO_4, the tubes were vortex-mixed for 3 min and centrifuged for 10 min at 1500 *g*. After discarding the upper organic layer, 150 μl of methanol were added to the aqueous phase in the tube. The addition of methanol was to prevent the adsorption of BCT onto the polypropylene tube or autoinjection glass vial. Finally, 300 μl of the aqueous phase, containing BCT and I.S., was injected onto the HPLC to obtain the BCT-to-I.S. peak area ratio. All procedures for sample handling and processing were carried out at ambient temperature.

2.4. HPLC analysis

BCT was quantified using reversed-phase HPLC on a Waters Nova-Pak C_{18} column (150×3.9 mm, 4 μm particle size) fitted with a Waters Nova-Pak C_{18} Guard-Pak precolumn. The HPLC system (Shimadzu, Tokyo, Japan) consisted of Class-LC10A system control software, a CBM-10A communication bus module, two LC-10AD pumps, an SIL-10A autoinjector with the sample cooler set at 4°C, an RF-10A spectrofluorometric detector and a GLP-2050+ laser printer (LG Elect, Seoul, Korea). The mobile phase was a mixture of 30 parts of acetonitrile and 70 parts of 0.2% triethylamine hydrochloride in water, adjusted to pH 3 with HCl, and the flow-rate was 1 ml/min. The peak areas of BCT and I.S. were monitored fluorometrically at an excitation wavelength of 330 nm (15 nm bandwidth) and an emission wavelength of 405 nm (15 nm bandwidth).

2.5. Plasma concentration

Fifteen minutes before solution instillation, each rabbit was cannulated in a central ear artery with polyethylene tubing (PE-50, Intramedic, Sparks, MP, USA), heparinized with 1000 IU/ml of sodium heparin (Choong Wae, Seoul, Korea). Thereafter, 25 μl of 0.1% BCT mesylate was instilled directly onto the cornea of both eyes, collecting in the cul-de-sac. At 1, 3, 6, 10, 15, 20, 30, 45, 60, 90 and 120 min, 2.5-ml blood samples were collected into heparinized tubes, centrifuged for 10 min at 1500 *g*; 1 ml of plasma was transferred to a 15-ml conical polypropylene tube containing 200 μl of 0.05 *M* H_2SO_4 and stored at −20°C until analysis, which took place within one week. BCT is known to be gradually isomerized into isoBCT at higher pH values [4], thus, the addition of H_2SO_4 was to prevent the in vitro change of BCT in biological samples. Four rabbits were used for the plasma concentration study and the rabbits were not reused.

2.6. Eye tissue concentration

A 25-μl volume of dosing solution was instilled directly onto the cornea of both eyes, collecting in the cul-de-sac, and the rabbits were euthanized with an overdose of a sodium pentobarbital solution (Entobar, Han Lim, Seoul, Korea) at 30, 60, 120, 240 and 360 min. Thereafter, the eye balls were immediately enucleated, aqueous humour was aspirated with a 1-ml tuberculin syringe through a 26G hypodermic needle and the vitreous humour was separated from the iris ciliary body. Then, 0.2 ml of aqueous humour and 1 ml of vitreous humour were transferred to a 15-ml conical polypropylene tube con-

taining 200 μl of 0.05 *M* H_2SO_4, and stored at −20°C until analysis, which occurred within one week. Four eyes were used per time point.

3. Results and discussion

The fluorescence response of BCT was maximal at an excitation wavelength of 330 nm and an emission wavelength of 405 nm. These wavelengths were therefore used for the HPLC analysis. Fig. 1 shows typical chromatograms of blanks containing I.S., drug standards containing 1 ng of BCT and I.S., and study samples 1 h after receiving BCT. The two-step extraction of the samples with fluorescence detection gave no interference from endogenous substances in the biological samples. The blanks without I.S. did not contain peaks at the retention time of the I.S. (not shown). The peaks of I.S. and BCT were symmetrical and eluted at 4.1 and 11.6 min, respectively, in a mobile phase containing acetonitrile and 0.2% tri-

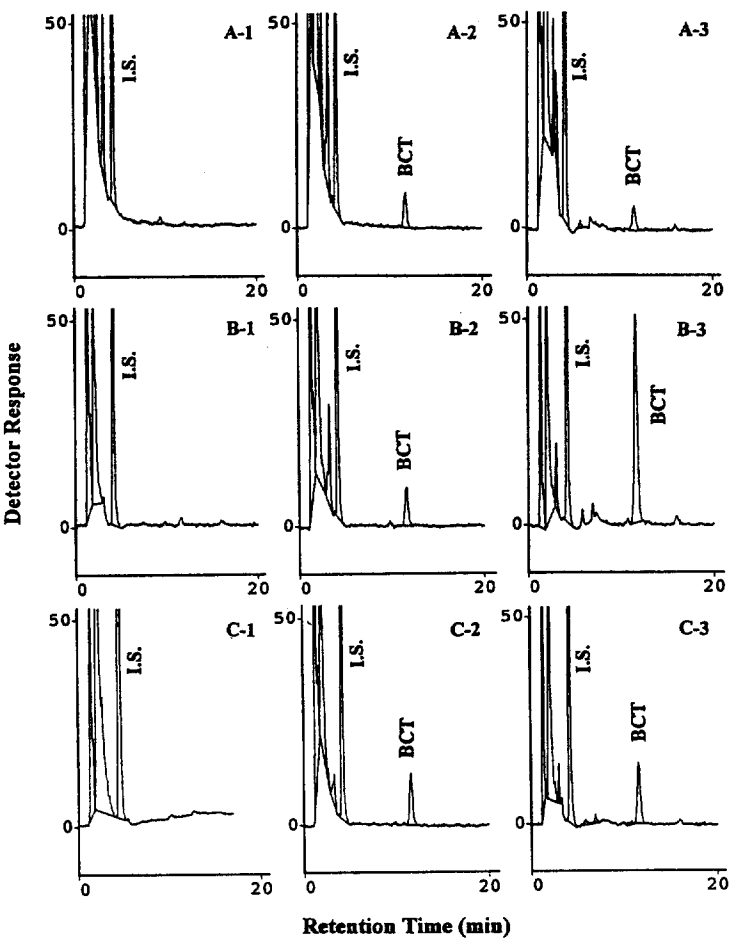

Fig. 1. HPLC chromatograms of blank samples containing I.S.: Plasma (A-1), aqueous humour (B-1) and vitreous humour (C-1); HPLC chromatograms of standard samples containing 1 ng of BCT and I.S.: Plasma (A-2), aqueous humour (B-2), and vitreous humour (C-2); HPLC chromatograms of study samples obtained from a rabbit 1 h after ocular instillation of 25 μl of 0.1% BCT mesylate onto both eyes: Plasma (A-3), aqueous humour (B-3) and vitreous humour (C-3)

ethylamine hydrochloride, pH 3. Mobile phases with a pH value higher than 4, however, resulted in low numbers of theoretical plates on a reversed-phase column with free silanol groups.

The limit of quantification was 0.2 μg/l for plasma and vitreous humour using a 1-ml sample and was 1 μg/l for aqueous humour using a 0.2-ml sample, based on a signal-to-noise ratio of approximately 3. The plot of BCT-to-I.S. peak area ratios versus nominal concentration was linear in the following ranges: For plasma, 0.2–10 μg/l [$r>0.999$; slope, 0.109±0.00958 (mean±SD); $n=6$]; for aqueous humour, 1–50 μg/l [$r>0.985$; slope, 0.114±0.00878 (mean±SD); $n=3$] and for vitreous humour, 0.2–10 μg/l [$r>0.997$; slope, 0.125±0.0111 (mean±SD); $n=3$].

Assay variability was calculated using quality control samples. The within-day and between-day recoveries are listed in Table 1. This assay method had a within-day recovery (within-day accuracy) of 100–102% for plasma, 91–106% for aqueous humour and 96–111% for vitreous humour, and between-day recovery (between-day accuracy) of 90–114% for plasma, 83–115% for aqueous humour and 90–105% for vitreous humour. The within-day precision and between-day precision were 1.7–7.0% and 8.1–13.6%, respectively. These values indicate that this method is acceptably reproducible: The criteria for a valid analytical assay were defined by Shah et al. [12] as an assay with acceptable accuracy (85–105%) and precision (within 15%). Under the conditions reported here, the absolute recovery of BCT was 83–89% for plasma, 66–71% for aqueous humour and 68–76% for vitreous humour, and the absolute recovery of I.S. was 69–88% (78±5.2, mean±SD, $n=12$).

In our ether extraction method, isoBCT (retention time, ca. 16 min) was not detected in either standard samples or study samples of plasma and eye tissues (Fig. 1). However, Schran et al. [6] reported that isoBCT could be isolated from rat bile and incubates of a rat liver cell preparation. It seems that BCT does not change into isoBCT during ocular and systemic absorption, by avoiding hepatic first-pass metabolism.

The time course of BCT in the plasma and eye tissues following ocular instillation is shown in Fig. 2. The plasma concentration showed a peak (1.63 μg/l) at 15 min, with a rapid decrease up to 90 min, and finally decreased to a level that was below the limit of quantification at 120 min. The aqueous humour concentration showed a peak (32.7 μg/l) at 120 min, with a slow decrease up to 360 min. However, the vitreous humour concentration showed a saturated profile in the range of 0.52–1.13 μg/l from the first sampling point (30 min) up to 360 min.

In summary, a simple, sensitive and reproducible HPLC assay method using a single ether extraction and fluorescence detection was developed for the determination of BCT in plasma and eye tissues following ocular instillation of a 0.1% BCT mesylate eye solution in the albino rabbit. This method showed a linear response over the concentration range of 0.2–10 μg/l for plasma and vitreous humour, and of 1–50 μg/l for aqueous humour. The quality control samples were reproducible with acceptable accuracy (85–105%) and precision (within 15%) at 0.3, 1 and 10 μg/l of plasma, 5 and 50 μg/l

Table 1
Recovery (mean±SD) of bromocriptine in tissues

Tissue	Concentration added (μg/l)	Within-day (%, $n=3$)	Between-day (%, $n=9$)	Absolute (%, $n=9$)
Plasma	0.3	100±7.0	90±9.7	89±7.9
	1	103±2.1	93±12.0	83±7.2
	10	102±2.2	114±13.6	87±9.2
Aqueous humour	5	91±7.0	83±8.1	66±7.6
	50	106±1.7	115±10.2	71±1.3
Vitreous humour	1	96±6.8	90±10.6	68±15.5
	10	111±4.3	105±9.2	76±2.3

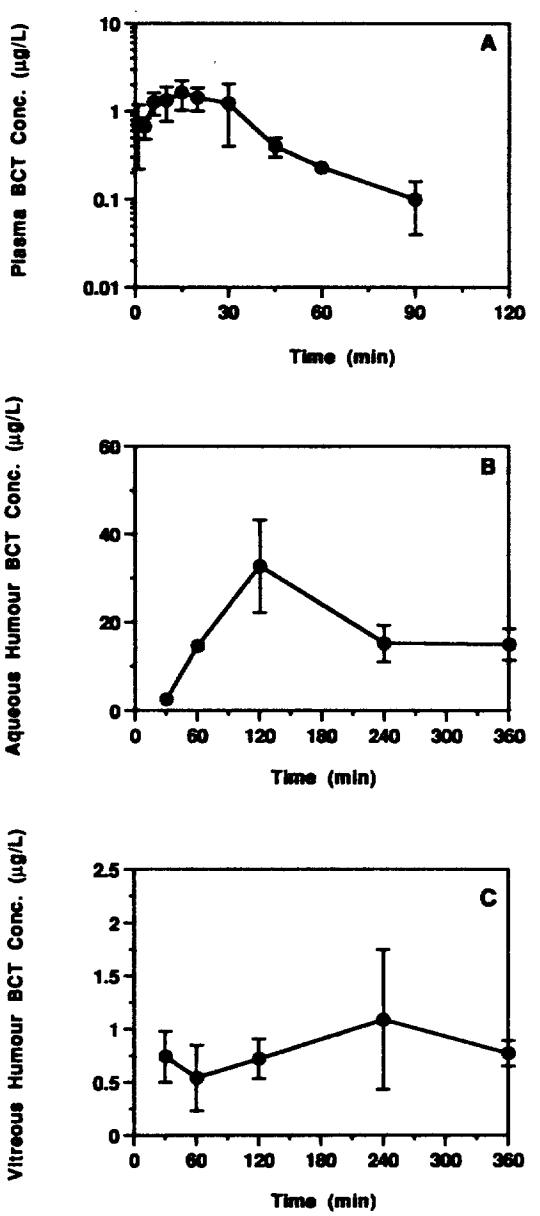

Fig. 2. Concentration–time profiles of BCT in plasma (A), aqueous humour (B) and vitreous humour (C) following ocular instillation of 25 µl of 0.1% BCT mesylate onto both eyes of an albino rabbit (mean±S.E.M.). Four rabbits were used for the plasma concentration–time profiles, whereas four eyeballs were used for each eye tissue concentration.

of aqueous humour, and 1 and 10 µg/l of vitreous humour. The limit of quantification was 0.2 µg/l for plasma and vitreous humour using a 1-ml sample, and was 1 µg/l for aqueous humour using a 0.2-ml sample. Thus, the above assay method was considered to be applicable to the various investigations of ocular and systemic absorption of bromocriptine.

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