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Simultaneous RP-HPLC-DAD quantification of bromocriptine, haloperidol and its diazepam structural analog in rat plasma with droperidol as internal standard for application to drug-interaction pharmacokinetics

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Abstract

A simple and rapid RP-HPLC-DAD method was developed and validated for simultaneous determination of the dopamine antagonists haloperidol, its diazepam analog, and the dopamine agonist bromocriptine in rat plasma, to perform pharmacokinetic drug-interaction studies. Samples were prepared for analysis by acetonitrile (22.0 µg/mL) plasma protein precipitation with droperidol as an internal standard, followed by a double-step liquid-liquid extraction with hexane:chloroform (70:30) prior to C-18 separation. Isocratic elution was achieved using a 0.1% (v/v) trifluoroacetic acid in deionized water, methanol and acetonitrile (45/27.5/27.5, v/v/v). Triple-wavelength diode-array detection at the λ_{max} of 245 nm for haloperidol, 254 nm for the diazepam analog and droperidol, and 240 nm for bromocriptine was carried out. The LLOQ of DAL, HAL, and BCT were 45.0, 56.1, and 150 ng/mL, respectively. In rats, the estimated pharmacokinetic parameters (i.e., $t_{1/2}$, CL , and V_{ss}) of HAL when administered with DAL and BCT were $t_{1/2} = 16.4$ min, $V_{ss} = 0.541$ L/kg for HAL, $t_{1/2} = 28.0$ min, $V_{ss} = 2.00$ L/kg for DAL, and $t_{1/2} = 24.0$ min, $V_{ss} = 0.106$ L/kg for BCT. The PK parameters for HAL differed significantly from those previously reported, which may be an indication of a drug-drug interaction.

Keywords

bromocriptine; diazepam analog; droperidol; haloperidol; HPLC-DAD

Introduction

Haloperidol is a well-known dopamine antagonist and is a treatment alternative in schizophrenia and ergot-derivative (e.g. bromocriptine) induced psychosis. The therapeutic use of haloperidol (HAL) is limited by extrapyramidal side-effects and thus a diazepam analog of haloperidol (DAL) was designed and synthesized to be safer and equally effective in the

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treatment of schizophrenia (Lyles-Eggleston *et al.*, 2004). The 4-hydroxypiperidine in HAL was replaced with 4-azepane (homopiperazine) and now lacks structural features required to oxidatively form quaternary pyridinium metabolites (MPP⁺), thought to cause Parkinsonian-like symptoms (Rollema *et al.*, 1994). Dopamine agonist therapy in Parkinson's disease, however, can lead to psychotic symptoms; thus investigation of dual therapy of antagonistic and/or synergistic drugs acting on dopamine receptors and their potential drug–drug interactions is needed.

The purpose of this study was to develop a rapid and selective, reversed-phase HPLC method with diode-array detection (DAD) useful for plasma monitoring of DAL and HAL in the presence of the dopamine agonist bromocriptine (BCT) with droperidol (DRO) as an internal standard (IS). Chemical structures are shown in Fig. 1. The pharmacology of DAL as an atypical antipsychotic agent has been reported previously (Ablordepey *et al.*, 2008). Current published methods for the analysis of HAL using HPLC with UV detection (Jatlow *et al.*, 1982; Wilhelm and Kemper, 1990; Fang and Gorrod, 1993; Boehme and Strobel, 1998; Trabelsi *et al.*, 2002; Yasui-Furukori *et al.*, 2004; Zhang *et al.*, 2007a) and MS/MS detection (Kirchherr and Kuhn-Velten, 2006; Zhang *et al.*, 2007b; Kumazama *et al.*, 2009) did not include a dopamine agonist such as bromocriptine for pharmacokinetic monitoring of agonist–antagonist drug interactions. Methods have also been previously published for HPLC with UV analysis of BCT (Phelan *et al.*, 1990; Foda and El Shafie, 1996; Yun-Jeong *et al.*, 1997) and DRO (Lee *et al.*, 2005; Ali *et al.*, 2006; Higashi *et al.*, 2006), but none were acceptable for simultaneous analysis of compounds of interest in rat plasma. Here, we report simultaneous analysis of all three dopamine receptor ligands out of a plasma matrix, where preparative and chromatographic conditions require no adjustments depending on which compound is being analyzed. Additionally, this method is fairly rapid, as previously published methods require longer run times. The method was validated according to FDA Guidance (US Food and Drug Administration, 2001) and applied to single-dose *in vivo* pharmacokinetic determination of HAL and our novel compound DAL in the presence of BCT in rats. For pharmacokinetic analysis, an HPLC method with UV detection can be very robust, and has the advantage of being more economical than methods with higher resolving power, such as mass spectrometry.

Experimental

Chemicals and Reagents

{4-[4-(4-Chlorophenyl)-1,4-diazepan-1-yl]-1-(4-fluorophenyl) butan-1-one} was synthesized according to a previously published method (Ablordepey *et al.*, 2008). Haloperidol, droperidol, bromocriptine, ethyl-enediaminetetraacetic acid disodium salt dihydrate (EDTA), citric acid and glycerol were all purchased from Sigma (St Louis, MO, USA) and trifluoroacetic acid (TFA) was purchased from Mallinckrodt (Paris, KY, USA). Methanol and acetonitrile were of HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Pooled blood samples were obtained from adult male Sprague–Dawley (Charles-River, Wilmington, MA, USA) rats *post mortem*. Samples were centrifuged at 3950g for 10 min in the presence of EDTA as an anticoagulant. The plasma supernatant was removed and stored at –20°C until use.

Instrumentation

The Shimadzu (Kyoto, Japan) HPLC system comprised an SIL-10ADVP auto injector fitted with a 50 µL fixed loop, DGU-14A inline degasser, dual LC-10ADVP binary pumps, a CTO-10ASVP column oven and the SPD-M10AVP diode array detector. Data collection and integration were accomplished using Shimadzu EZ-start[®] 7.2.1 SP1 program software running on Windows XP. Least-squares regression analysis (analyte/IS peak area ratios vs concentration) was carried out using MSEXcel[®] (Microsoft, Seattle, WA, USA).

Chromatographic Conditions

DAL, HAL, BCT and DRO were quantitatively analyzed using reversed-phase chromatographic separation with UV detection. The mobile phase consisted of 55% organic 50:50 mixture of methanol and acetonitrile (v/v) and 45% aqueous containing 0.1% (v/v) TFA in deionized water, pumped at a flow rate of 1.0 mL/min through a Zorbax 300SB-C₁₈ StableBond[®] analytical, 4.6 × 250 mm, 5 µm column (Agilent, Santa Clara, CA, USA) at ambient temperature. The injection volume was 20 µL with UV monitoring at 254 nm for DAL and DRO, 245 nm for HAL and 240 nm for BCT. Triple wavelength analysis was chosen to enhance the sensitivity of the method by observing each analyte at its λ_{max} . DRO exhibits adequate absorbance at 254 nm and is co-monitored at that wavelength along with DAL.

Standard Solutions

Stock solutions containing DAL, HAL and BCT were prepared by dissolving a weighted amount of pure drug standard in methanol. Working standard solutions with concentrations between 0.057 and 12.8 µg/mL of each analyte were obtained by sequential dilutions of the appropriate stock solutions with methanol. Solutions were kept in capped test tubes and stored at 4°C. Blank plasma was spiked with analytes for the preparation of calibration curves.

Sample Preparation

To prepare spiked plasma samples, 100 µL of each standard solution was evaporated to dryness under a stream of filtered air and 100 µL of rat plasma added. A 200 µL aliquot of internal standard (22.0 µg/mL) in acetonitrile solution, stored in the refrigerator at 4°C, was added to denature plasma proteins and each sample was vortexed for 30 s. A 0.50 mL aliquot of hexane : chloroform (70:30, v/v) was then added and the tubes vortexed for an additional 30 s. After centrifugation at 3950g for 10 min, the organic phase was transferred into clean, glass 5 mL tubes. A second extraction was performed where the organic layer was combined with the first and evaporated to dryness at ambient temperature under a gentle stream of filtered air. The residue was dissolved in 100 µL of methanol and transferred into 200 µL screw-cap polypropylene vials for chromatographic analysis.

Method Validation

Linearity—Spiked plasma samples were assayed using the ratio of peak-area of analytes to internal standard as the assay response. Linearity was evaluated by plotting assay response as a function of analyte concentration for the assay range. Least sum-of-squares linear regression analysis (without weighting) was used to determine the calibration equation and correlation coefficients. Plasma calibration samples were prepared in the mean concentration range of 0.060–12.1 µg/mL for DAL and HAL and 0.212–10.6 µg/mL for BCT and each of three samples assayed in triplicate, and the mean used to construct the calibration curve.

Precision, accuracy and recovery—Precision and accuracy were assessed by replicate analysis of three quality control plasma samples (QC) containing the analytes at low, middle, and high average concentrations of 0.12, 1.21 and 6.03 µg/mL for DAL and HAL and 0.530, 5.30 and 10.6 µg/mL for BCT. Precision was expressed by coefficient of variation (%CV) and accuracy by percentage error in the analysis of reference samples. Intra-day precision and accuracy were calculated from three separate runs throughout a single day, and inter-day was determined over five different days.

$$\%CV = \frac{SD \times 100}{\text{mean}} \quad (1)$$

$$\%error = \text{abs} \left[\left(\frac{\text{observed concentration} \times 100}{\text{referenced concentration}} \right) - 100 \right] \quad (2)$$

The lower limit of quantitation (LLOQ) was determined from three replicate spiked plasma samples based on a signal-to-noise ratio of 5 : 1 and the limit of detection (LOD) was determined at a signal-to-noise ratio of 3 : 1 (US Food and Drug Administration, 2001). Extraction efficiency of DAL, HAL and BCT were obtained by comparing peak area ratios of analytes extracted from QC samples to peak area ratios from referenced solutions and expressed as extraction recovery.

$$\text{Extraction recovery}(\%) = \frac{\text{peak area in spiked plasma}}{\text{peak area in std solution}} \quad (3)$$

Selectivity was evaluated using blank plasma samples and inspected for interference from endogenous compounds for DAL, HAL, BCT and IS.

Stability—The stability of all analytes was determined under five different conditions using quality control standards (1.13 µg/mL for DAL, 1.28 µg/mL for HAL and 1.06 µg/mL for BCT). Stability was quantified in stock solutions maintained at 4°C and in rat plasma kept at room temperature (22 ± 1°C) and at 4°C for up to 7 days. Freeze–thaw stability was determined by first freezing reconstituted plasma samples, storing at –20°C and thawing at room temperature for three cycles. The stability of analytes post extraction and reconstituted in methanol was evaluated for short-term stability on the bench-top and autosampler under ambient conditions for 24 h. Three replicates from each group were analyzed daily for concentration by HPLC quantitation.

Application to Pharmacokinetics

To validate the method *in vivo*, the method was applied to three adult male Sprague–Dawley rats with an average weight of 270 g. The rats were housed under controlled environmental conditions (temperature, 22 ± 1°C; humidity, 50 ± 5%) with water freely available and a diet of Purina laboratory rodent chow (Purina Mills, St Louis, MO, USA). The animals were dosed intravenously via the tail vein with DAL (1.7 mg/kg), HAL (0.7 mg/kg) and BCT (0.1 mg/kg) in a 0.9% (w/v) saline solution with pH adjusted to 4.71 with citric acid. Blood was withdrawn from the tail vein of each rat at 5, 15, 30, 60, 90, 120, 240 and 360 min, into a centrifuge tube containing EDTA as an anticoagulant. Samples were centrifuged for 10 min at 3950g. Plasma supernatant was collected and stored at –20°C until analysis. The experimental animal protocols described were approved by the Institutional Animal Care and Use Committee at the Authors' institution. Pharmacokinetic parameters were estimated using WinNonlin® software (Pharsight Inc., Cary, NC, USA), for log–linear regression of the terminal portion of plasma–concentration vs time data and noncompartmental area and moment analysis by linear trapezoidal method.

Results and Discussion

Method Development and Optimization

During method development, a variety of chromatography conditions were assessed and optimized to achieve the best resolution and separation of DAL, HAL, BCT and DRO. The mobile phase initially consisted of phosphate buffer at pH 2.50 and acetonitrile (65 : 35, v/v)

adapted from a previously reported method for HAL and its degradation products (Trabelsi *et al.*, 2002). Sufficient separation between DAL and BCT was not attained and the aqueous phase concentration was increased to 45%, which increased retention times but did not improve separation. Methanol was introduced into the organic phase to improve separation and different ratios of acetonitrile and methanol were investigated. High concentrations of methanol (e.g. 75%) caused significant increases in pressure whereas low concentrations (e.g. 25%) provided inadequate separation. An organic phase ratio of 50 : 50 (v/v) acetonitrile to methanol provided the best separation with adequate column pressure. Finally, phosphate buffer was replaced with a 0.1% (v/v) TFA aqueous solution in the same pH region, as it was found to give quicker stabilizing baselines to enhance the speed of the method. The change to TFA did not significantly effect the retention times or separation of the analytes.

Liquid-liquid extraction with 70 : 30 (v/v) hexane : chloroform provided the highest recovery for simultaneous analysis of all three compounds from previously reported extraction methods (Larsen *et al.*, 1979; Walter *et al.*, 1998; Titier *et al.*, 2003; Yasui-Furukori *et al.*, 2004; Singh and Sharma, 2005). It was also determined that a second extraction step recovered more of the compounds from the plasma than a single, organic extraction.

Linearity and Sensitivity

Linearity for each analyte was determined in the mean concentration range used: 0.060–12.1 µg/mL for DAL and HAL and 0.212–10.6 µg/mL for BCT. The regression equations and correlation coefficients for each compound are in Table 1. The LOD in plasma was estimated to be 13 ng/mL for DAL, 33 ng/mL for HAL and 90 ng/mL for BCT.

Selectivity and Lower Limit of Quantitation

There were no interfering peaks co-eluted with the analytes of interest and the mobile phase used for the assay provided a well defined separation ($R_s > 1.50$) between all analytes and internal standard. The LLOQ was 45, 56 and 150 ng/mL for DAL, HAL and BCT, respectively. The mean retention times of DAL, BCT, HAL and DRO were 6.72, 5.85, 4.55 and 3.76 min, respectively. Chromatograms are shown in Fig. 2 for blank plasma (a), high and middle range concentrations of analytes in spiked plasma with mean concentrations of 1.16 and 5.80 µg/mL, (b and c) respectively, and a chromatogram from a dosed rat (1.7 mg/kg DAL, 0.7 mg/kg DAL, 0.1 mg/kg BCT), revealing no interfering compounds.

Precision, Accuracy and Recovery

The %CV for intra-day precision (repeatability) ranged from 0.954 to 13.9% and inter-day precision (reproducibility) from 0.480 to 10.1% of QC standards (Table 2). The intra-day percentage error (accuracy) ranged from 2.98 to 12.5% and the inter-day percentage error from 0.645 to 12.1% of the reference samples (Table 3). These results were within acceptable limits as the inter-and intra-day precisions and accuracies for the LLOQ should be within $\pm 20\%$ with higher concentrations within $\pm 15\%$. The percentage extraction recoveries (mean $\pm$ SD) for DAL, HAL, and BCT were 87.0 ± 3.32 , 91.3 ± 4.75 and 90.7 ± 4.09 , respectively (Table 4).

Stability

The stock solutions of DAL, HAL and DRO stored at 4°C showed no degradation after 7 days. BCT in methanol stored at 4°C began to degrade significantly after 3 days. In plasma at room temperature and refrigerated (4°C), all compounds showed less than 5% degradation after 7 days. After three cycles of freezing and thawing spiked plasma samples, all compounds showed less than 5% degradation after recovery procedure. DAL, HAL and DRO had no degradation reconstituted in methanol after recovery and kept at room temperature, but BCT began to

degrade after 3 days; therefore, plasma samples from dosed rats were analyzed within 12 h of recovery to avoid any possible BCT degradation due to its instability in methanol.

Drug-interaction Pharmacokinetics

The method was successfully applied to determine the pharmacokinetic profiles of both HAL and DAL in the presence of BCT after single-dose intravenous administration in adult male Sprague–Dawley rats. The mean plasma concentration–time profiles of DAL, HAL and BCT are presented in Fig. 3. Estimated noncompartmental parameters of DAL, HAL and BCT are given in Table 5.

Pharmacokinetic parameters of HAL in Sprague–Dawley rats following i.v. administration have been previously published (Wurzbacher *et al.*, 1981; Cheng and Paalzow, 1992) as HAL exhibits linear disposition kinetics over a dosing range of 0.5–2.5 mg/kg (Cheng and Paalzow, 1992), which was consistent with the dose given in this preliminary PK study. The estimated parameters (i.e. $t_{1/2}$, CL and V_{ss}) for HAL when administered with DAL and BCT differed significantly from those parameters previously reported, which may be an indication of a drug–drug interaction between the two dopamine antagonists, the antagonist and agonist, or between all three compounds. When dosed together, DAL has a longer half-life and greater volume of distribution than its predecessor. These preliminary results suggest we can successfully evaluate the pharmacokinetics dopamine antagonists HAL and our novel development compound DAL in the presence an ergot-derivative dopamine agonist BCT. More comprehensive experiments would be needed to interpret these initial results.

Conclusions

The method presented is simple, fast, reproducible and validated for the simultaneous recovery and quantification of DAL, HAL and BCT in rat plasma with DRO as an internal standard. The assay utilizes triple wavelength DAD monitoring, double-step extraction and reversed-phase separation with sufficient selectivity and sensitivity. Our procedure has a relatively short run time at 10 min and is fully validated for the assay range. The method was successfully applied to study drug-interaction pharmacokinetics of DAL and HAL in rats. Future pharmacokinetic studies will be performed utilizing this method to fully understand the behavior of DAL in the presence of other butyrophenone dopamine antagonists and dopamine agonists such as BCT.

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Abbreviations used

BCT	bromocriptine
DAL	diazepane analog
DRO	droperidol
HAL	haloperidol

Figure 1. Chemical structures of (a) haloperidol, (b) diazepane analog of haloperidol, (c) bromocriptine and (d) droperidol (internal standard).

Figure 2.

Representative chromatograms at 254 nm of (a) blank plasma, DAL, BCT, HAL and DRO (IS) in (b) spiked plasma at mean concentration of 5.80 $\mu\text{g/mL}$, (c) spiked plasma at mean concentration of 1.16 $\mu\text{g/mL}$, and (d) plasma from a dosed rat (0.7 mg/kg HAL, 1.7 mg/kg DAL, 0.1 mg/kg BCT), with retention times of 6.72, 5.58, 4.55 and 3.76 min for DAL, BCT, HAL and DRO, respectively.

Figure 3. Mean plasma concentration–time profiles of (■) DAL (1.7 mg/kg), (▲) HAL (0.7 mg/kg) and (◆) BCT (0.1 mg/kg) after i.v. dose in Sprague–Dawley rats ($n = 3$).

Table 1

Linear regression and statistical analysis

Analyte	Concentration range (µg/mL)	Slope (value ± SD)	Intercept (value ± SD)	Determination coefficient (r^2)
DAL	0.057–11.3	0.111 ± 0.003	−0.003 ± 0.014	0.997
HAL	0.064–12.8	0.062 ± 0.002	0.008 ± 0.009	0.997
BCT	0.212–10.6	0.044 ± 0.001	0.001 ± 0.007	0.998

Table 2

Precision

Analyte	Concentration level of QC standards (µg/mL)	Intra-day (repeatability), mean response ±SD	CV % ^a	Inter-day (reproducibility), mean response ±SD	CV % ^b
DAL	5.65	0.585 ± 0.006	0.954	0.602 ± 0.014	2.24
	1.13	0.122 ± 0.009	6.19	0.124 ± 0.012	8.84
	0.113	0.011 ± 0.005	8.94	0.014 ± 0.003	9.37
HAL	6.40	0.378 ± 0.021	4.80	0.414 ± 0.014	3.27
	1.28	0.083 ± 0.008	7.81	0.087 ± 0.001	0.213
BCT	0.128	0.009 ± 0.002	13.9	0.009 ± 0.002	10.1
	10.6	0.408 ± 0.013	3.12	0.471 ± 0.002	0.48
	5.30	0.207 ± 0.023	10.2	0.227 ± 0.005	2.17
	1.06	0.044 ± 0.006	12.8	0.047 ± 0.004	8.28

Degrees of freedom (d.f.) for between/within-day %CV calculation:

^a d.f. = 2;

^b d.f. = 4.

Table 3

Accuracy

Analyte	Concentration level of QC standards (µg/mL)	Analyzed concentration (µg/mL)	Intra-day % error (n = 3)	Analyzed concentration (µg/mL)	Inter-day % error (n = 5)
DAL	5.65	5.27 ± 0.137	6.66	5.42 ± 0.043	4.13
	1.13	1.10 ± 0.157	2.98	1.12 ± 0.045	1.00
	0.113	0.100 ± 0.018	11.5	0.126 ± 0.003	12.1
HAL	6.40	6.10 ± 0.146	4.64	6.68 ± 0.290	4.45
	1.28	1.34 ± 0.013	4.58	1.40 ± 0.019	9.73
	0.128	0.139 ± 0.023	8.22	0.138 ± 0.023	7.81
BCT	10.6	9.27 ± 0.321	12.5	10.7 ± 0.645	0.645
	5.30	4.71 ± 0.420	11.1	5.09 ± 0.301	3.87
	1.06	1.01 ± 0.130	4.01	1.07 ± 0.020	0.434

Table 4

Extraction recovery from rat plasma

Analyte	Concentration level of QC standards (µg/mL)	Extraction recovery (%), (n = 3)
DAL	5.65	90.0 ± 1.33
	1.13	87.5 ± 3.66
	0.113	83.4 ± 5.91
HAL	6.4	95.5 ± 1.87
	1.28	92.2 ± 1.48
	0.128	86.1 ± 9.06
BCT	10.6	86.2 ± 4.23
	5.3	94.2 ± 4.48
	1.06	91.7 ± 5.42

Table 5

Noncompartmental pharmacokinetic parameters of DAL, HAL, and BCT

Parameter	Analyte		
	DAL	HAL	BCT
$t_{1/2}$ (min)	28.04 ± 8.55	16.39 ± 3.20	24.05 ± 4.70
λ_z (1/min)	0.025 ± 0.008	0.042 ± 0.008	0.029 ± 0.006
AUC _{0-∞} (μg h/L)	579 ± 130	497 ± 65.8	595 ± 83.2
CL (L/h/kg)	2.97 ± 0.670	1.37 ± 0.182	0.183 ± 0.026
AUMC (μg/L/h ²)	5063 ± 2597	2536 ± 800	4461 ± 1456
MRT (h)	0.674 ± 0.205	0.394 ± 0.077	0.578 ± 0.113
V_{ss} (L/kg)	2.00 ± 0.030	0.541 ± 0.059	0.106 ± 0.010