Development and validation of a HPLC-UV method for 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), a toxic metabolite of haloperidol, in humans: providing in vivo evidence of CYP3A4-mediated CPHP formation

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We developed a high-performance liquid chromatographic procedure for the determination of 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), a toxic metabolite of haloperidol, in human. Chromatographic analysis was performed on a reverse-phase C₁₈ column with a mobile phase containing 50 mM potassium phosphate buffer/acetonitrile (75:25, vol/vol) using UV detection with a wavelength of 220 nm. The limits of detection for CPHP were 1 ng/ml in urine and the assay was linear over the concentration range of 2-500 ng/ml for urine. This analytical method was applied to measure CPHP in human. Nineteen healthy subjects were enrolled and all subjects received a single oral dose of 5 mg haloperidol following a treatment of placebo or itraconazole at 200 mg/day for 10 days in a randomized crossover manner. CPHP was detected in urine samples and average recovered amount of CPHP was 81.31 μg/24 hr in the placebo phase and it was significantly reduced to 30.34 μg/24 hr after itraconazole treatment. The finding provides in vivo evidence that CPHP is an in vivo metabolite of haloperidol in human and its formation is mediated by CYP3A4.

Introduction

Haloperidol, the prototypical butyrophenone antipsychotic drug, has been commonly prescribed over decades to treat both acute and chronic schizophrenia. The metabolic pathway of haloperidol in human includes oxidative N-dealkylation, oxidation to pyridinium metabolites, glucuronidation, and carbonyl reduction (Fig. 1).[1,2] According to previous in vitro and in vivo studies, these metabolic pathways of haloperidol appear to be mediated mainly by cytochrome P450 (CYP), especially, CYP2D6 and CYP3A4, and carbonyl reductase.[1-3] It has been reported that some of haloperidol metabolites may be responsible for some of the pharmacological and toxicological effects of haloperidol. For instance, the pyridinium metabolite of haloperidol, HPP⁺ (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-pyridinium),[4] an analogue of neurotoxic N-methyl-4-phenylpyridinium (MPP⁺) known to cause parkinsonism, was suggested to be involved in the neurotoxic side effects of haloperidol.[5-7] HPTP (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridine), an intermediate metabolite of haloperidol, was also suggested to be involved in the development of neurotoxicity in vivo and in vitro.[5,8]

Few studies have assessed the biological significance of CPHP [4-(4-chlorophenyl)-4-hydroxypiperidine]), a major oxidative metabolite formed by CYP3A4 catalyzed N-dealkylation.[9] CPHP has a moderate affinity to the sigma receptor, suggesting that it may mediate neurological side effects and a potent inhibition of dopamine uptake in the brain.[10,11] Structurally, CPHP resembles 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which induces severe neurotoxicity, including Parkinson-like disease and dyskinesia.[12,13] Therefore, these symptoms
CYP3A4-mediated CPHP formation in humans

from patients treated with haloperidol might result from CPHP accumulation. Even though in vitro studies revealed that CPHP is formed from haloperidol by CYP450-mediated metabolism,[1,9] there is no available data on the metabolic formation of CPHP in human.

Gas chromatographic (GC) and mass spectrometric (MS) methods have been developed to measure CPHP in human biological sample.[14,15] However, these methods showed a poor sensitivity. Fang et al. developed an assay method of CPHP using GC in biological samples of rats and revealed that CPHP was a major metabolite of haloperidol in rats.[15] However, their GC method is difficult to use in clinical laboratory because of the complicated derivatization procedures involved and extraction using harmful toluene. Higashi et al. identified CPHP with a HPLC method with fluorescence detection but this method also used a complicated derivatization step.[16]

We therefore developed a convenient and more sensitive HPLC method to determine CPHP levels in the human biological samples. Additionally, we assessed whether CPHP was formed from haloperidol in human and CPHP formation was related to CYP3A4-mediated metabolism.

Methods

Materials and reagents

CPHP was obtained from Research Biochemical International (Natick, MA, USA). Metoprolol, potassium phosphate, phosphoric acid, hydrogen chloride, and sodium hydroxide were purchased from Sigma Co. (St. Louis, MO, USA). Acetonitrile and hexane were obtained from Merck (Darmstadt, Germany). All chemicals and solvents were of analytical grade or the equivalent.

Preparation of stock solutions

A standard stock (1.0 mg/ml) was prepared by dissolving CPHP in 0.1 N HCl initially, and then diluting it further with the same solution. Working CPHP concentrations ranging from 2 to 500 ng/ml were prepared in blank urine samples. A stock solution of metoprolol, an internal standard, was prepared by dissolving 10 mg of the drug in 10 ml of 0.1 N HCl. All preparations were stored at 4°C until use.

Study protocol and urine sample collection

A randomized 2-way crossover study was performed, and the study phases were separated by a 4-week washout period.[17] The general study design was identical in both phases. The volunteers were given an oral administration of either 200 mg itraconazole or a matched placebo twice daily for 10 days. On day 7, a single oral dose of 5 mg dose of haloperidol was administered with 240 mL of water. Urine samples were collected over the 24 hour period following the administration of haloperidol. After measuring urine volumes, 50 ml of urine aliquots were stored at -80°C until the assay. Nineteen healthy Korean male subjects were enrolled in the study. The participants provided informed, written consent to the protocol, which was approved by the Institutional Review Board (IRB) of Inje University, Busan Paik Hospital, Busan, Korea.

Sample Preparations

An 80 μl volume of internal standard solution (1 μg/ml in 0.1 N HCl) solution, 75 μl of 8 M sodium hydroxide, and 6 ml of hexane-isooamyl alcohol (98:2, vol/vol) were added to 1 ml of urine samples in a glass tube. The mixtures were vigorously mixed for 2 min and then centrifuged at 3,500 rpm for 15 min at 4°C. The upper organic layer was transferred to a new glass tube and 130 μl of 0.1 N HCl were added. These samples were back-extracted by vigorous mixing for 2 min and then centrifugation at 3,500 rpm for 15 min at 4°C. The organic layer was removed and the remaining aqueous layer was washed with 1 ml of diethyl ether to ensure removal of all remaining hexane; 100 μl of the aqueous layer was injected onto the HPLC system.

Instrumental analysis

The HPLC system consisted of a Gilson 307 pump, 118 UV detector, and 234 Autoinjector (Gilson Co., France) with a 100 μl sample loop (Rheodyne®, Sigma Co., St. Louis, MO, USA). A Unipoint© analysis system (Gilson Co., France) was used for the storage and integration of chromatograms in addition to controlling the HPLC systems. The HPLC system was operated at ambient room temperature. The CPHP and internal standard were separated using μ-Bondapak C8 column (300 x 4.6 mm I.D., 10 μm particle size, Waters, USA) and the mobile phase composed of 50 mM potassium phosphate buffer and acetonitrile (75:25, vol/vol) with the pH 3.2 adjusted with 85% phosphoric acid. The flow rate of mobile phase was 0.5 ml/min and the chromatograms were obtained from the UV detector set at

Figure 1. Main metabolic pathways of haloperidol.
220 nm wavelength.

**Assay validation**

Samples for CPHP were quantified using the peak area ratio of CPHP over the internal standard. To prepare the standards used to construct the calibration curve, an appropriate volume of working standard solution was added to 1 ml aliquots of blank human urine samples. For calibration, we used drug-free urine samples spiked CPHP ranging from 2 to 500 ng/ml.

To evaluate the extraction recovery, the same human urine samples were spiked with 5, 50, and 500 ng/ml in urine. The recovery of CPHP and the internal standard were calculated by comparing the peak area obtained after extraction with that of aqueous drug solution at the corresponding concentration without extraction.

Intra-day and inter-day precision and accuracy were determined by analyzing samples spiked with CPHP at concentrations of 5, 50, and 500 ng/ml in urine. Determinations were performed with four replicates on either the same day or separate days. The accuracy of the CPHP determination in the urine was determined by calculating the mean percentage difference between the nominal and measured concentrations. The assay precision was characterized by the mean value and relative standard deviation (RSD, %).

**Statistical analysis**

Data are expressed as mean values ± SD, and P-values < 0.05 were considered statistically significant. Statistical comparisons of recovered CPHP between placebo and itraconazole phases were made using paired t-test. Statistical analyses were performed using the statistical software package, SAS version 9.2 (SAS Institute, Cary, NC, USA).

**Results**

**Chromatographic separation**

A representative chromatogram of CPHP and the internal standard from urine samples is shown in Figure 2A. Following extraction under the conditions described above, the retention times for CPHP and the internal standard were 12.0 and 14.5 min, respectively. Both CPHP and the internal standard were eluted as sharp symmetrical peaks, and no significant interfering peaks overlapped the CPHP or internal standard were observed in testing of several batches of human urine samples. Figure 2B shows the chromatograms of extracts prepared from a blank urine sample.

**Calibration curves and limit of detection**

The standard CPHP curve was constructed from the peak area ratio of CPHP and the internal standard. The standard curve showed good linearity over the range of 2 to 500 ng/ml in urine samples and the equation describing the calibration curve was $y = 0.0083 \times x - 0.0016$, where $y$ was the peak area ratio and $x$ was the drug concentration. The mean correlation coefficient was 0.9994. The limit of detection for CPHP in urine samples was 1 ng/ml.

**Recovery and intra-day and inter-day assay variability**

The recovery of CPHP following the extraction was determined by comparing the peak area of stock solutions directly injected onto the HPLC system, with those of samples extracted from human urine samples. The average efficacy of extraction from urine samples was 86.9% (Table 1). In urine samples spiked with 5.0, 50.0 and 500 ng/ml of CPHP, the mean RSD of intra-day and inter-day variability was 6.4% and 3.9%, respectively.

**Table 1.** Extraction recovery for the assay of CPHP in urine samples (n=4)

<table>
<thead>
<tr>
<th>CPHP Concentration (ng/ml)</th>
<th>Recovery rate (%)</th>
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<tbody>
<tr>
<td>5</td>
<td>82.6 ± 5.7</td>
</tr>
<tr>
<td>50</td>
<td>87.6 ± 9.4</td>
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<tr>
<td>500</td>
<td>90.4 ± 6.5</td>
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Figure 2. Representative chromatogram of urine samples spiked with 100 ng/ml of CPHP and internal standard (A) of blank urine samples, no spiked blank urine samples (B), and collected urine sample from a healthy subject who administered with 5 mg of haloperidol (C). The labeled chromatographic peaks indicate CPHP (I) and the internal standard metoprolol (II), respectively.
Effect of itraconazole treatment on the formation of CPHP

The described method was applied for the determination of CPHP in urine samples obtained from healthy subjects taking a single oral dose of 5 mg haloperidol. No endogenous peaks to interfere with CPHP and internal standard were found from the samples (Fig. 2C). Additionally, the itraconazole peak was not found and did not interfere with both CPHP and internal standard peaks. The average recovery of CPHP in collected in urine for 24 hours was 81.31±37.13 μg/24 hr after placebo treatment. After itraconazole treatment, recovered CHPH amount was significantly reduced to 30.34±12.53 μg/24 hr (P<0.001) (Fig. 3).

Discussion

We developed a convenient and sensitive HPLC method for the determination of CPHP in human samples and identified CPHP is an in vivo metabolite of haloperidol in human.[9,18] Even though the pharmacological role of CPHP is not understood fully, CPHP showed an affinity to sigma receptor,[2,10] and a potent inhibition on dopamine uptake in rat brain slices.[5] Haloperidol is used to treat psychiatric diseases for long-periods of time, but there has been no available data to show that CPHP is an in vivo metabolite of haloperidol in human.

In vitro studies revealed that CYP3A4 involves in the metabolism of haloperidol into CPHP using human liver microsomes and cDNA-expressed CYP450s.[1,9,10] Consistently, our results showed, itraconazole, a CYP3A4 inhibitor,[19] reduced the urinary recovery of CPHP, suggesting the involvement of CYP3A4 in the formation of CPHP from haloperidol in human.

In the developed HPLC method, the recoveries of the extraction for blank urine and plasma samples spiked with CPHP were between 82 and 90%. Intra-day and inter-day precision were less than 13.2%. These values were relatively lower than or comparable to those of a previously reported method using GC.[4] These findings suggest that our analytical procedure is both sensitive and reproducible. In addition, the limit of detection was 1 ng/ml. Unfortunately, we failed to determine CPHP in plasma samples with the current method. It might be from far lower levels of CPHP in the blood samples compared with those in urine samples. In this study, we collected the biological samples from healthy subjects after a single oral dose administration of 5 mg haloperidol. Considering the fact that psychiatric patients are generally medicated with multiple and higher doses, CPHP levels in patients would be higher. Therefore CPHP could be measured in patients’ samples with the current method.

Compared with LC-MS and GC-MS methods to determine CPHP, the present HPLC method needs more analytical time and large volumes of samples. However, previous studies[14,20] using LC-MS exhibited rather higher detection limits of CPHP (75~300 ng/mL). Assumed that the LOQ of this study (1 ng/mL) was much lower than the previous reports, the current method might be more appropriate to analyze CPHP in human biological samples.

In conclusion, a convenient and sensitive HPLC method with UV detection was developed for separation and analysis of CPHP. This method reaches optimum performance in terms of sensitivity, selectivity, precision, and accuracy for determination
of CPHP in the urine samples. Additionally, we provided unequivocal evidence that CPHP is an in vivo metabolite of haloperidol and that CPHP is formed from haloperidol by CYP3A4-mediated disposition in human.

**Conflict of interest**
The authors declared no conflict of interest.

**References**