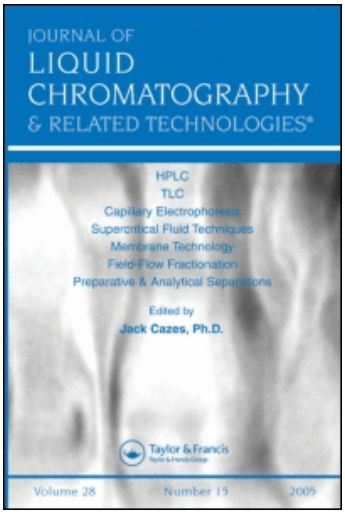

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Simultaneous Determination of the *N*-Dealkylated Metabolites of Four Butyrophenone-Type Agents in Rat Plasma by HPLC with Fluorescence Detection after Precolumn Derivatization with 4-Fluoro-7-Nitro-2,1,3-Benzoxadiazole

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Abstract: The basic metabolites of butyrophenone type agents are sometimes more neurotoxic than the parent compounds. We have developed a high performance liquid chromatographic method with fluorescence detection (HPLC-FL) to quantify the *N*-dealkylated basic metabolites, i.e., 1,3-dihydro-1-(1,2,3,6-tetrahydro-4-pyridinyl) -2*H*-benzimidazole-2-one, 1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, 4-(4-chlorophenyl)-4hydroxypiperidine, and 4-(4-bromophenyl)-4-hydroxypiperidine, of droperidol, spiperone, haloperidol, and bromperidol, respectively, in rat plasma after precolumn derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Mexiletine was carried through the procedure as an internal standard (IS). After liquid-liquid extraction with benzene and evaporation, derivatization with NBD-F was conducted in borate buffer (pH 8.5) at 60°C for 3 min. HPLC was conducted with a reversed-phase (C_{18}) column, eluted with a mixture of methanol-water-trifluoroacetic acid (600:400:0.4, v/v/v) at a flow rate of 1.0 mL/min at 25°C. The derivatives of four basic metabolites and the IS were well separated from each other in less than 48 min. The calibration curves were linear up to 0.5 μg/mL, and the lower limits of detection were 0.002 to 0.03 μg/mL. The coefficients of variation were less than 13.3%. These results confirm that HPLC-FL after precolumn derivatization with

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NBD-F is sensitive and satisfactory for the simultaneous assay of basic metabolites of four butyrophenone type agents in rat plasma.

Keywords: Butyrophenone-type agent, Basic metabolite, 4-fluoro-7-nitro-2,1,3-benzoxadiazole, Derivatization

INTRODUCTION

Droperidol, spiperone, haloperidol, and bromperidol are butyrophenone type agents used clinically as pretreatments for systemic anesthetization, and to treat schizophrenia. They are blockers of the dopamine D1 or D2 receptor, and are metabolized to 1,3-dihydro-1-(1,2,3,6-tetrahydro-4-pyridinyl)-2*H*-benzimidazole-2-one (DTP), 1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (PTS), 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), and 4-(4-bromophenyl)-4-hydroxypiperidine (BPHP), respectively, see Figure 1.[1,2] Ablordeppey *et al.* reported that CPHP induces a delayed and persistent freezing action, which may involve sigma receptors,[3] rather than the dopamine D2 receptor.[4] Similarly, BPHP may be at least partly responsible for the acute dystonia that can be induced by bromperidol treatment.[5,6] These four basic metabolites structurally resemble 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine,

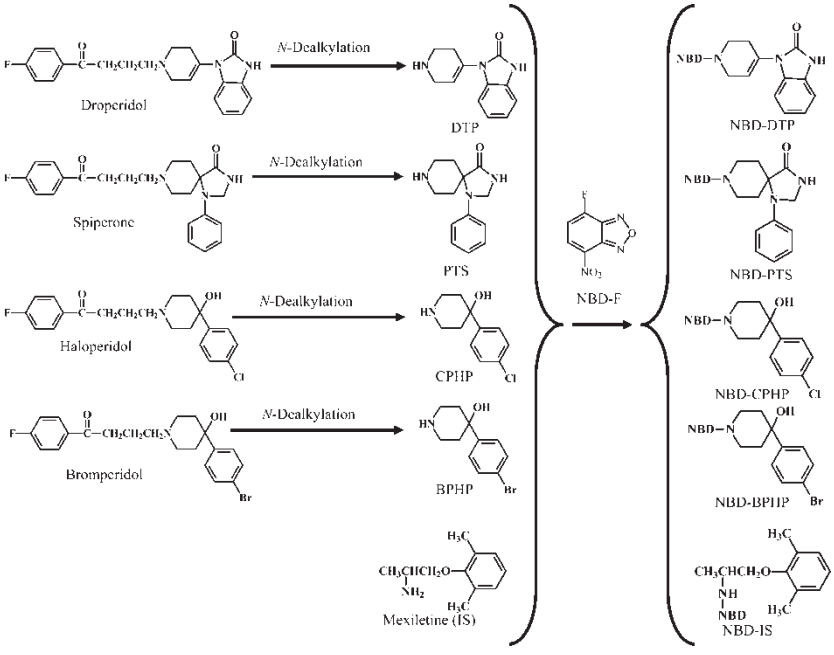

Figure 1. *N*-Dealkylation of butyrophenone-type agents, chemical structures, and scheme of derivatization with NBD-F.

which is a potent neurotoxin capable of causing Parkinson like disease and dyskinesia.[5,7] and may induce neurotoxicity, so it is important to establish a convenient assay system for their determination.

Several methods have been reported for CPHP determination, using gas chromatography with nitrogen selective detection[3] or with electron capture detection,[8], high performance liquid chromatography (HPLC)-mass spectrometry,[9–11] and HPLC with UV detection[12,13] or with fluorescence detection (FL) using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F).[14] Very recently, we developed a procedure for the simultaneous determination of four basic metabolites by HPLC-dual UV detection.[15]

Here, in order to improve the sensitivity of our system[14] and to avoid the use of acetonitrile, which was required in our previous method, we present an HPLC-FL procedure for the simultaneous separation and quantitation of DTP, PTS, CPHP, and BPHP in rat plasma (200 μL) after precolumn derivatization with NBD-F in methanol. The reaction scheme is presented in Figure 1. We also utilized a more environmentally friendly methanol-water system as the mobile phase, in place of the acetonitrile-water mobile phase used in the previous procedure.[13–15]

EXPERIMENTAL

Equipment

The HPLC system consisted of a model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne injection valve (Cotati, CA, USA) with a 50 μL loop, and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 470 nm and an emission wavelength of 540 nm. The HPLC column (150 × 4.6 mm i.d., Mightysil RP-18 GP, Kanto Chemical, Tokyo, Japan) was packed with 5 μm particles of C_{18} packing material.

Reagents

PTS, CPHP, BPHP, and mexiletine hydrochloride as an internal standard (IS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). DTP was purchased from Acros Organics (Geel, Belgium). NBD-F and other general reagents were supplied by Wako Pure Chemical Industries (Osaka, Japan).

Chromatographic Conditions

Quantification of the peaks was performed with a Chromatopac Model C-R3A integrator (Shimadzu). The mobile phase was prepared by addition of methanol (600 mL) and trifluoroacetic acid (0.4 mL) to water (400 mL). The samples were eluted from the column at a flow rate of 1.0 mL/min at 25°C.

Extraction from Rat Plasma

Control plasma was prepared from rats. An aliquot of 200 μL of sample was rendered alkaline by the addition of NaOH (0.1 M, 100 μL). Mexiletine hydrochloride solution in water (0.5 μg/mL, 100 μL) was added as an IS to prepare standard curves for the four basic metabolites. Then, the mixture was vortex-mixed for 1 min and extracted with benzene (3 mL, twice). The pooled benzene phase was evaporated to dryness, and the derivatization was performed as follows.

Derivatization

Borate buffer (0.1 M) containing ethylenediaminetetraacetic acid disodium salt (1 mM) was adjusted to pH 8.5 by the addition of NaOH (1 M). Borate buffer (300 μL) was added to the extract. NBD-F solution in methanol (20 mM, 100 μL) was added and vortex-mixed. The mixture was allowed to stand for 3 min at 60°C. Then, it was kept on ice for 3 min to stop the derivatization reaction before addition of HCl (0.05 M, 400 μL). The derivatives (50 μL) were injected into the HPLC system.

Plasma from Rats

Under light anesthesia induced with diethyl ether, blood was withdrawn from the right jugular vein of 9- to 10-week-old rats (280 ± 15 g, mean ± S.D., Sankyo Laboratory Animal, Toyama, Japan) into heparinized syringes and centrifuged (1,600 × *g*, 5 min) to obtain the plasma. The drug free plasma was immediately frozen and stored at -18°C until assayed.

RESULTS AND DISCUSSION

Derivatization

For the time course study, the reaction time was set at 2, 3, 6, 10, or 20 min. The four basic metabolites (each 0.5 μg/mL) and IS (0.5 μg/mL) in borate buffer (pH 8.5) were derivatized as described under Experimental. The ratio of derivatization of the metabolites reached a maximum at 3 min (data not shown), and tended to decrease after 6 min. Therefore, the derivatization time of 3 min was selected. Among borate buffers of pH 8.0 to 9.5, no significant difference of peak area was observed, so borate buffer of pH 8.5 was selected.

Chromatograms

Typical chromatograms of drug free rat plasma (A), rat plasma spiked with IS (B), and rat plasma spiked with the standard mixture of DTP, PTS, CPHP, BPHP, and IS (C) are shown in Figure 2. The retention times of the derivatives of DTP, PTS, CPHP, BPHP, and IS were 11.2, 14.1, 30.2, 36.1, and 44.3 min, respectively. Chromatographic running time was 48 min.

Linearity and Lower Limit of Detection

Standard curves of DTP, PTS, CPHP, and BPHP were constructed by plotting integrated peak area to IS peak area ratio *vs.* concentration for each metabolite. Table 1 gives the slopes, intercept, concentrations range, and squared regression coefficients (r^2). The lower limits of detection of DTP, PTS, CPHP, and BPHP were 0.003, 0.03, 0.002, and 0.003 μg/mL (signal-to-noise ratio of 3:1), respectively.

Fang et al. determined CPHP by means of GC-electron capture detection, which provided a detection limit of 0.005 μg/mL.[12] Our previous assays of CPHP by means of HPLC-FL using NBD-F and HPLC dual UV detection

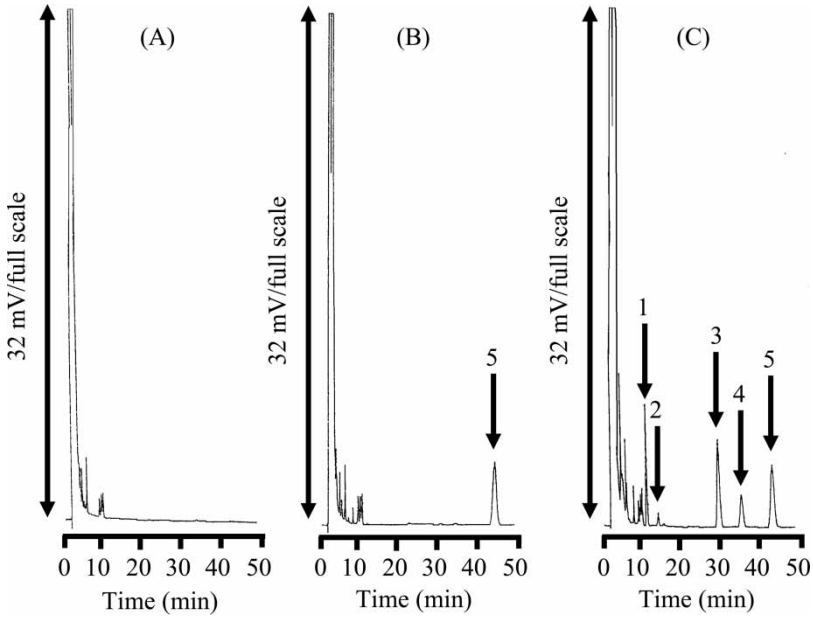

Figure 2. Chromatograms of DTP, PTS, CPHP, BPHP, and IS derivatives. (A), Drug-free rat plasma; (B), Rat plasma spiked with IS (0.5 μg/mL); (C), Rat plasma spiked with DTP, PTS, CPHP, BPHP, and IS (each 0.5 μg/mL). Peaks: 1 = DTP derivative, 2 = PTS derivative, 3 = CPHP derivative, 4 = BPHP derivative, 5 = IS derivative.

Table 1. Linear correlation parameters

Four basic metabolites	Slope	Intercept	Concentration range	r^2
DTP	1.0682	0.0086	0.01 to 0.5 μg/mL	0.9999
PTS	0.1919	0.0007	0.05 to 0.5 μg/mL	0.9836
CPHP	2.0034	0.0197	0.01 to 0.5 μg/mL	0.9998
BPHP	1.2955	0.0063	0.01 to 0.5 μg/mL	0.9998

showed detection limits of 0.008 and 0.012 μg/mL, respectively.[13,14] The detection limits of CPHP using the HPLC-mass spectrometric procedures by Arinobu et al. were relatively poor (0.075 to 0.3 μg/mL).[9,10] Our previous assay of BPHP by means of HPLC dual UV detection had a detection limit of 0.008 μg/mL.[15] Thus, the method presented here was found to be more sensitive for CPHP and BPHP detection, compared with the previous methods. The sensitivity for DTP was improved 1.7-fold over our previous results, but that of PTS was 2.5-fold poorer.[15]

Precision and Accuracy

Precision and accuracy for intra- and inter-day assays of the compounds are shown in Tables 2 and 3. In the intra-day assay, the coefficients of deviation for DTP, PTS, CPHP, and BPHP were in the ranges of 6.8 to 7.8, 6.0 to 7.2, 5.8 to 6.9, and 5.6 to

Table 2. Intra-day assay reproducibility for determination of DTP, PTS, CPHP, and BPHP

Concentration (μg/mL)	Measured (μg/mL) (Mean ± S.D., $n = 5$)	C.V. (%)	Recovery (%)
DTP			
0.01	0.0102 ± 0.0008	7.8	102.0
0.05	0.0497 ± 0.0034	6.8	99.4
0.5	0.505 ± 0.037	7.3	101.0
PTS			
0.05	0.0498 ± 0.0036	7.2	99.6
0.5	0.504 ± 0.030	6.0	100.8
CPHP			
0.01	0.0101 ± 0.0007	6.9	101.0
0.05	0.0497 ± 0.0034	6.8	99.4
0.5	0.502 ± 0.029	5.8	100.4
BPHP			
0.01	0.00989 ± 0.00058	5.9	98.9
0.05	0.0505 ± 0.0029	5.7	101.0
0.5	0.501 ± 0.028	5.6	100.2

Table 3. Inter-day assay reproducibility for determination of DTP, PTS, CPHP, and BPHP

Concentration (μg/mL)	Measured (μg/mL) (Mean ± S.D., $n = 5$)	C.V. (%)	Recovery (%)
DTP			
0.01	0.0103 ± 0.0012	11.7	103.0
0.05	0.0505 ± 0.0042	8.3	101.0
0.5	0.502 ± 0.042	8.4	100.4
PTS			
0.05	0.0502 ± 0.0067	13.3	100.4
0.5	0.501 ± 0.049	9.8	100.2
CPHP			
0.01	0.00989 ± 0.00118	11.9	98.9
0.05	0.0499 ± 0.0049	9.8	99.8
0.5	0.500 ± 0.048	9.6	100.0
BPHP			
0.01	0.0102 ± 0.0013	12.7	102.0
0.05	0.0496 ± 0.0062	12.5	99.2
0.5	0.503 ± 0.058	11.5	100.6

5.9%, respectively. The recoveries were in the range of 98.9 to 102.0%. In the inter-day assay, the corresponding values were 8.3 to 11.7, 9.8 to 13.3, 9.6 to 11.9, and 11.5 to 12.7%, respectively, with recoveries of 98.9 to 103.0%.

CONCLUSION

We have established an HPLC-FL method for the simultaneous determination of DTP, PTS, CPHP, and BPHP, which are metabolites of droperidol, spiperone, haloperidol, and bromperidol, respectively, in rat plasma after precolumn derivatization with NBD-F in methanol. The mobile phase was a methanol-water system. The lower limits of detection of DTP, CPHP, and BPHP in rat plasma were in the range of 0.002 to 0.003 μg/mL, while that of PTS was relatively inferior (0.03 μg/mL). Our assay system is expected to be useful for toxicological analysis and monitoring the levels of the metabolites in patients and in experimental animals.

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