Journal of Chromatography, 526 (1990) 282–288
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5105

Note

High-performance liquid chromatographic determination of *cis*-N-(1-benzyl-2-methylpyrrolidin-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide in rat blood and brain using electrochemical detection

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(First received July 13th, 1989; revised manuscript received November 6th, 1989)

cis-N-(1-Benzyl-2-methylpyrrolidin-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide (YM-09151-2, **I**) is a novel benzamide derivative with the structure shown in Fig. 1. This compound is an analogue of sulpiride, and has been suggested to possess antipsychotic activity based on behavioural pharmacological investigations [1,2]. In a recent study, we demonstrated biochemically that **I** is a specific D-2 rather than a D-1 dopamine receptor blocking agent [3]. As its antipsychotic activity has been reported to parallel the D-2 blocking activity [4], it is expected to prove a potent therapeutic drug in psychiatric clinics. With recent therapeutics, it has become important to monitor

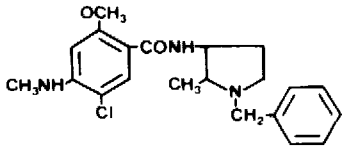

Fig. 1. Structure of **I**.

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0378-4347/90/$03.50

the fate of the administered drugs in the body and for this purpose a simple and sensitive procedure for the determination of the drugs is required. Although **I** is detectable by gas chromatography combined with mass spectrometry, the gas chromatography is complicated by the requirement for a volatile derivative.

High-performance liquid chromatography (HPLC) with electrochemical detection has been employed for the determination of substances metabolically related to monoamine transmitters in the brain [5,6] and biological fluids [7,8]. Electrochemical detection has also been reported to be useful for the determination of therapeutic drugs possessing phenolic hydroxy groups such as *p*-hydroxyamphetamine [9] and morphine [10]. In addition, we have demonstrated that electrochemical detection could be applicable to drugs with a heterocyclic structure [11,12], and in this study we applied electrochemical detection to the determination of **I**.

EXPERIMENTAL

Chemicals

I was a generous donation from Yamanouchi Pharmaceuticals (Tokyo, Japan). Desmethylimipramine hydrochloride (DMI), the chromatographic internal standard, was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals for extraction and chromatography were purchased from Wako (Osaka, Japan).

Apparatus

A Model 510 liquid chromatographic system (Waters Assoc., Milford, MA, U.S.A.) was used with a Model 7125 six-port injector (Rheodyne, Cotati, CA, U.S.A.) and an EC-100 glassy carbon amperometric detector (Eicom, Kyoto, Japan). The analytical column consisted of an Eicom-Pack ODS reversed-phase column (average particle size 5 μm; 150×4.0 mm I.D.). The detector potential was set at +0.95 V versus an Ag/AgCl reference electrode. The mobile phase consisted of 0.1 *M* acetate buffer (pH 4.5) containing 40% (v/v) acetonitrile. The flow-rate was set at 1.2 ml/min.

Sample preparation

Wistar rats weighing about 250 g were used. All animals were kept in a room with a controlled temperature (23 ± 0.5°C), humidity (55 ± 5%) and light cycle (12 h illumination with the light turned on at 07.00). They were injected intravenously with 1 mg/kg **I** and killed by decapitation at different times after the injection. The brain was quickly removed and stored at −80°C until assay. Blood samples were collected from the carotid artery when the animals were decapitated. The serum was also stored at −80°C after separation.

Extraction procedure

The brain samples were transferred to a glass-stoppered tube containing 200 ng of DMI (internal standard), 500 μl of 1 *M* sodium hydroxide and 10 ml of heptane–isoamyl alcohol (99:1, v/v). The sample was homogenized with a Polytron (Kinematica, Lucerne, Switzerland) and centrifuged at 1000 *g* for 1 min. The organic layer was transferred to another tube. After adding 100 μl of 0.1 *M* hydrochloric acid to the tube, the mixture was mixed on a vortex mixer for 1 min. Brief centrifugation was carried out to separate the layers and a portion of the hydrochloric acid layer was injected into the chromatographic system.

To a tube containing 100 μl of the serum sample were added 0.1 ml of 1 *M* sodium hydroxide, 5 ml of heptane–isoamyl alcohol (99:1, v/v) and DMI. The tube was then mixed vigorously and the same extraction procedure as above was applied.

RESULTS AND DISCUSSION

I is of interest both in clinical research as an antipsychotic drug and in experimental research as a dopamine D-2 antagonist. Assay of the prototype benzamide drug sulpiride by HPLC with fluorescence detection has been reported [13]. The therapeutic dosage of this drug is expected to be 10 mg or less per person, compared with 200 mg for sulpiride. If the volume of blood for sampling from patients without discomfort is 1 ml, ultraviolet detection may be insufficient for the precise determination of this new drug.

In electrochemical detection, the electrode response depends on the applied voltage. Although a higher potential yields a greater response, the background noise is also increased. The hydrodynamic voltammograms of **I** and DMI are illustrated in Fig. 2. Both substances gave their initial electrochemical response at an applied potential of +0.6 V. The electrochemical response of **I** increased steadily until the applied voltage reached +1.1 V, and then reached a plateau. On the other hand, the response of DMI was maximum at +0.95 V. We decided to set the applied voltage at +0.95 V because it gave the best signal-to-noise ratio.

The recoveries in the extraction procedure were calculated, after adjusting for solvent loss, to be $92 \pm 5\%$ and $80 \pm 4\%$ for **I** and DMI, respectively. The intra- and inter-assay coefficients of variation were also calculated to be less than 4 and 6%, respectively. Quantitative determination was based on the peak heights in the chromatograms. The ratios of the peak heights for **I** and DMI were compared for samples and standards taken through the entire extraction procedure. In the range 1 ng–1 μg, the relationship between the peak-height ratio of **I** and DMI and concentration was linear ($r=0.998$, $P<0.001$). This made it possible to calculate the concentration from a simple measurement of the ratio. The detection limit of **I** was 1 ng at a signal-to-noise ratio of 5.

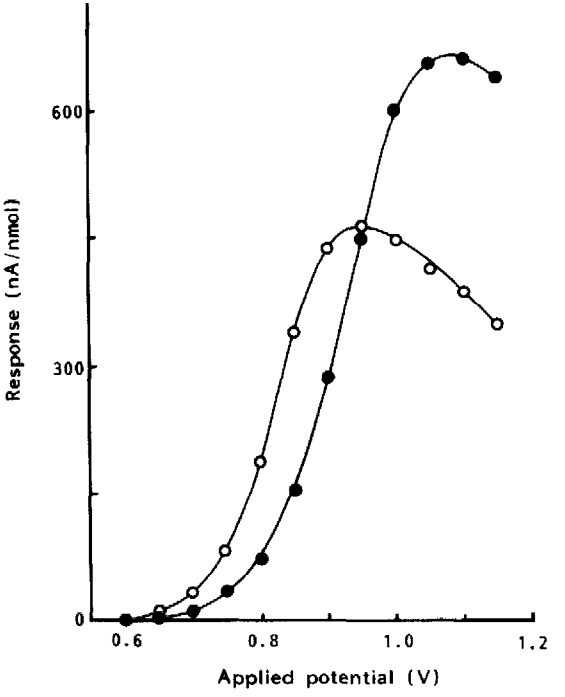

Fig. 2. Hydrodynamic voltammograms of (●) I and (○) DMI.

The retention times of substances in a reversed-phase column are influenced by the concentrations of organic solvents in the mobile phase. However, as the electrochemical reaction occurs only in aqueous solution, the choice of organic solvent is restricted. Acetonitrile is known to promote reactions involving ionization, and we therefore used it here to shorten the retention times of hydrophobic substances in the reversed-phase column. The effects of acetonitrile on the retention times of **I** and DMI were examined to determine the optimum concentration of acetonitrile (Fig. 3). An increase in concentration of the organic solvent shortened the retention times of both substances by almost the same amount. A concentration of 40% (v/v) was the optimum for separating the two substances in one chromatographic run. This concentration did not interfere with the electrochemical reactions.

Typical chromatograms for blood and brain samples are shown in Fig. 4. In this instance, a rat was injected intravenously with 1 mg/kg **I**. The animal was killed 1 h after the injection and a blood sample was obtained from the carotid artery. Following extraction, 20 μl of the final aqueous layer were injected into the chromatograph. When a relatively high voltage was applied to the electrode, no biological substances such as monoamine transmitters and aromatic

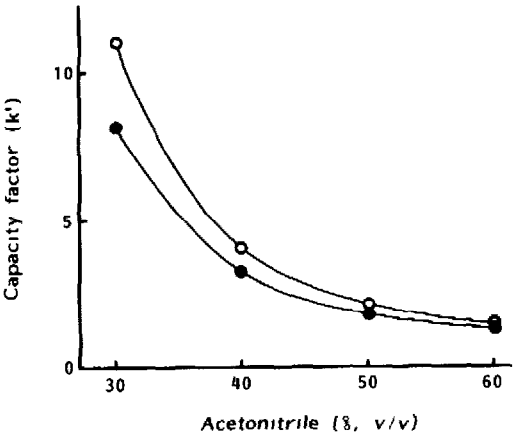

Fig. 3. Effects of acetonitrile on the retention times of (●) **I** and (○) DMI.

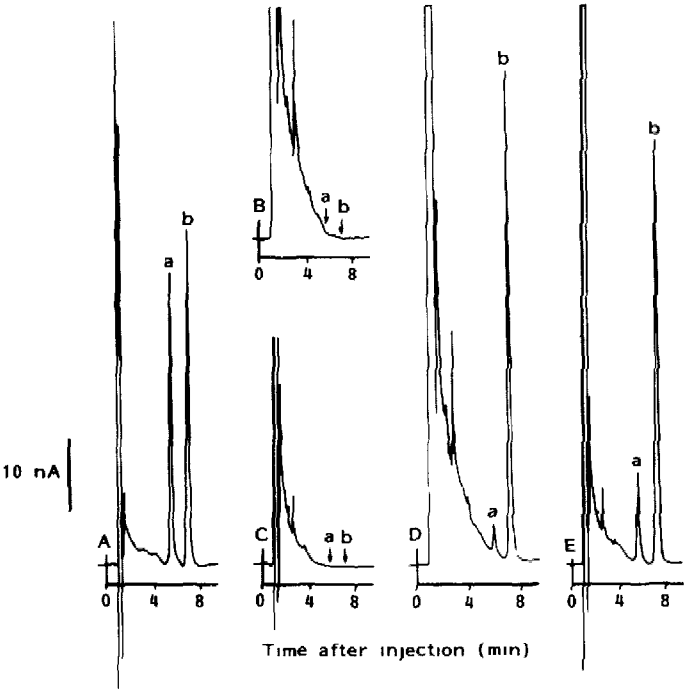

Fig. 4. Typical chromatograms of (a) **I** and (b) DMI. (A) Chromatogram obtained by the injection of an authentic substance mixture. (B) Blood and (C) brain chromatograms of samples obtained from an animal injected with physiological saline (blank control). (D) Blood and (E) brain chromatograms of samples from an animal injected intravenously with 1 mg/kg **I** 1 h prior to decapitation. Note that no interfering peaks appeared in the chromatograms for both **I** and DMI, for which the retention times are indicated by arrows. For details of the chromatographic conditions, see *Experimental*.

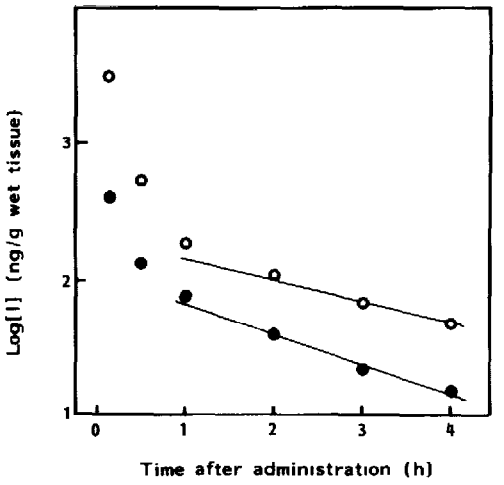

Fig 5. Disappearance curves of **I** from the (●) blood and (○) brain samples of rats. Regression curves (elimination phase) were simulated by the least-squares fitting method employing a computer (PC-9801 VM) with commercial software The biological half-lives were calculated to be 158 ± 23 and 233 ± 18 min for the blood and brain, respectively.

amino acids interfered with the determination of **I** (Fig. 4B and D). No interference was observed in the sample obtained from the brain (Fig. 4C and E). One chromatographic run was completed within 8 min, and 60 samples could be assayed in 8 h of routine work. The chromatographic sample, which was redissolved in 0.1 *M* hydrochloric acid, was stable overnight. This means that, if available, an automatic sample processor can be used for the assay of **I**.

The time course of the disappearance of **I** was examined in rats after a single intravenous injection at a dose of 1 mg/kg (Fig. 5). The concentrations of the drug were always higher in the brain tissue than in the blood. For example, the concentrations in the brain and blood were 1.8 and 1.2 μg/g wet tissue, respectively, 10 min after intravenous administration. These pharmacokinetic results meant that **I** readily penetrated into the brain tissue. Determination was possible in the blood, using 0.1 ml, up to 4 h later (13 ng/ml of blood). The detector sensitivity was sufficiently good to apply the proposed method to pharmacokinetic studies of the new benzamide derivative in clinical pharmacology. The biological half-lives of the elimination phase of **I** were calculated to be 160 and 230 min in the blood and brain, respectively.

In conclusion, the procedure described here was found to be useful for monitoring the concentration of **I**. The detection limit of 1 ng with a signal-to-noise ratio of 5 may be adequate for monitoring the concentration of **I** in blood samples from humans administered a dose of 1 mg.

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