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Note

Simple determination of *p*-hydroxyamphetamine by high-performance liquid chromatography with electrochemical detection

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p-Hydroxyamphetamine (pOH-AMPH) is a principal metabolite of amphetamine [1, 2]. This substance has also been suggested to manifest some of the effect of amphetamine [3—5], leading to an interest in the intracerebral distribution of pOH-AMPH. The main procedure for the determination of pOH-AMPH is gas chromatography with electron-capture detection [6]. The substance, however, is not volatile and has to be derivatized prior to injection into the gas chromatographic system. It is generally considered that liquid chromatography is more suitable for the assay of substances that are water-soluble and non-volatile.

The electrochemical reaction has been introduced as a means of detection in liquid chromatography. This reaction is specifically sensitive for a substance having a phenolic hydroxy group in its molecular structure [7]. pOH-AMPH is such a substance and is expected to be detectable with an electrochemical detector. The present report outlines a simple procedure for the determination of pOH-AMPH using high-performance liquid chromatography combined with electrochemical detection.

MATERIALS AND METHODS

Apparatus

The chromatographic system was constructed using commercially available components including a thin-layer voltammetric detector with a glassy carbon electrode (Yanagimoto VMD-101, Kyoto, Japan). The chromatographic

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column consisted of an Ultrasphere-ODS (average particle size 5 μm) prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column (Altex Scientific, Berkeley, CA, U.S.A.). The detector potential was set at +750 mV versus the Ag/AgCl reference electrode.

Reagents

pOH-AMPH was a generous gift from Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.). Isoproterenol, the internal standard for chromatography, was obtained from Sigma (St. Louis, MO, U.S.A.). Reagent grade chemicals, *n*-butanol, *n*-heptane, hydrochloric acid, sodium chloride, ethylenediaminetetraacetate sodium salt (EDTA), methanol and tetrahydrofuran (THF), were all purchased from a single source (Mallinkrodt, Paris, KY, U.S.A.). These chemicals were used as obtained commercially without further purification.

The buffer for chromatography was prepared as follows: 14.7 g of sodium citrate were dissolved in about 400 ml of distilled water. After addition of 50 ml of methanol and 5 ml of THF, the pH was adjusted to 4.5 with citric acid. The volume was finally made up to 500 ml by adding distilled water. The buffer was sonicated under vacuum to eliminate air.

Extraction procedure

Swiss-Webster mice were injected with 10 mg/kg pOH-AMPH. The animals were sacrificed by decapitation 1 h after the injection. The brain was removed as quickly as possible and homogenized in a tube containing 0.2 ml of 0.1 *M* hydrochloric acid, 50 μl of 0.2 *M* EDTA and the internal standard (isoproterenol, 100 ng). The homogenate was mixed with 12 ml of butanol and 4 g of solid sodium chloride and then shaken on a reciprocal shaker for 60 min. Centrifugation was carried out at 1000 *g* for 10 min, and the butanol layer (10 ml) was transferred to another tube. After addition of 0.1 ml of 0.1 *M* hydrochloric acid and 20 ml of heptane to the tube, the mixture was again shaken on the reciprocal shaker for 10 min. The tube was centrifuged at 1000 *g* for 5 min to separate aqueous and organic phases. A portion of the aqueous phase (in general, 50 μl) was injected into the chromatographic column via a six-port injector (Rheodyne, Berkeley, CA, U.S.A.).

RESULTS AND DISCUSSION

In electrochemical detection, the electrode responses vary with the applied voltage [7]. The current—voltage curve for steady-state voltammetry of pOH-AMPH is shown in Fig. 1. pOH-AMPH initiated its electrochemical response at an applied voltage of +600 mV, whereas the parent substance, amphetamine, which has no hydroxy group in its molecule, did not. The applied voltage was higher than that for diphenolic compounds (catechols; for example, noradrenaline and dopamine) and was almost the same as that for the monophenolic amino acid, tyrosine [8]. This means that the response of pOH-AMPH is due to one hydroxy moiety on the benzene ring. The electrochemical response of this substance reached a plateau at about 850 mV, which is also similar to tyrosine. The applied voltage was decided as +750 mV in accordance

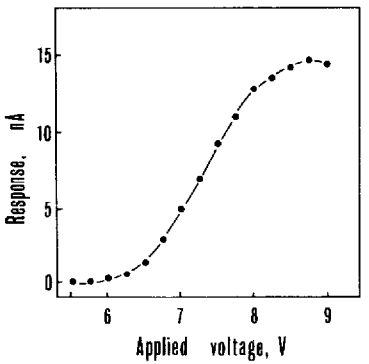

Fig. 1. Current—potential curve for pOH-AMPH.

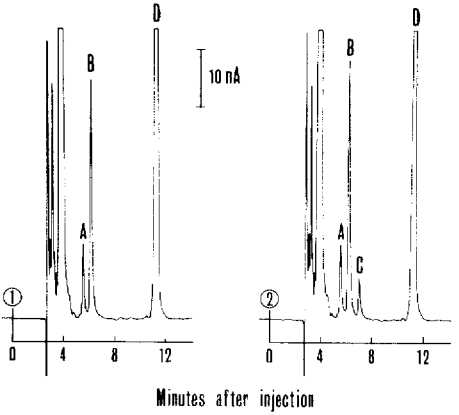

Fig. 2. Typical chromatograms obtained from the brain of animals injected intraperitoneally with 10 mg/kg pOH-AMPH 1 h before sacrifice (2) and with saline (1). Chromatographic conditions: stationary phase, Ultrasphere-ODS (average particle size, 5 μm); mobile phase, 0.1 *M* sodium citrate—citric acid buffer (pH 4.5) containing 1% THF and 10% methanol; detector applied voltage, +750 mV versus Ag/AgCl reference electrode. Peak identifications: A, isoproterenol (internal standard); B, 5-hydroxytryptamine; C, pOH-AMPH; D, tryptophan.

with the following criteria: (1) a voltage which is adequate to obtain a sufficient response and (2) a voltage which does not show significant background noise [9].

pOH-AMPH had a similar retention time to 5-hydroxytryptamine when chromatography was carried out with the buffer utilized for routine assay of monoamines in our department [8]. Thus, the most important aspect of the present study was the selection of a mobile phase which could effectively separate pOH-AMPH from biogenic substances. Some organic solvents are available to separate substances by HPLC with a reversed-phase column. We have employed THF in the assay of monoamine-related substances [8]. However, the organic solvent was not effective in the present separation of pOH-AMPH from 5-hydroxytryptamine. Methanol is also a solvent which can be used for this purpose [8]. While addition of the solvent decreased the retention time, it altered the relative retentions of pOH-AMPH and 5-hydroxytryptamine. Separation and resolution in HPLC were optimized by varying the methanol/buffer ratio of the mobile phase. Addition of 10% methanol was most effective for separating both substances. Fig. 2 shows typical chromatograms obtained for the brain of mice with and without injection of pOH-AMPH, which appeared just after the peak of 5-hydroxytryptamine and was not interfered with by other biogenic substances. Since a combination of butanol extraction and a higher applied voltage meant that the peak of tryptophan was detected [8], one run of the present chromatography required about 12 min to complete. The identification of pOH-AMPH and other biogenic substances was performed on the basis of the chromatographic behaviours and hydrodynamic voltammograms [9].

The recovery and reproducibility were studied by adding known quantities

of pOH-AMPH to blank samples of whole brain and analysing the samples according to the procedure described. The recovery rates by the present butanol extraction from brain samples, after adjusting for solvent loss, were estimated as 85 ± 2% and 75 ± 4% for pOH-AMPH and isoproterenol, the internal standard, respectively. The coefficient of variation was also estimated to be about 4% for both substances. The quantitation was based on peak heights of the resulting chromatogram. Ratios of the peak heights for the pOH-AMPH and the internal standard were compared for samples and standards taken through the entire extraction procedure. By this procedure, ratios of pOH-AMPH and the internal standard varied linearly with the amounts of the drug added in a range between 500 pg and 100 ng. This made it possible to calculate the concentration from simple measurement of the ratio.

The intracerebral concentration of the pOH-AMPH was estimated at 38 ± 9 ng per g wet tissue at 1 h after intraperitoneal injection of 10 mg/kg. This value was lower than that of other central-acting drugs (for example, morphine: 205 ng/g) [10]. This may be because the substance is interfered with in penetrating into the central nervous system at blood–brain barrier. However, even these low concentrations may be sufficient to contribute to the pharmacological effect of the drug [11].

The present study offers a simple and sensitive procedure for measuring the intracerebral concentration of pOH-AMPH. The method involves butanol extraction and quantification by HPLC with electrochemical detection. The sensitivity of the proposed procedure is comparable to that of gas chromatography [6].

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