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Short communication

High-performance liquid chromatography with peroxyoxalate chemiluminescence determination of propentofylline concentrations in rat brain microdialysate

Yozo Hamachi[a,*], Mihoko N. Nakashima[b], Kenichiro Nakashima[b]

[a]*Hoechst Marion Roussel Ltd, Pharmaceutical Development Dept., Laboratory for Analytics, 1-3-2 Minamidai, Kawagoe, Saitama 350-1165, Japan*

[b]*School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyou-machi, Nagasaki 852-8131, Japan*

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Abstract

A high-performance liquid chromatographic determination of a neuronal cell protective compound, propentofylline [3-methyl-1-(5-oxohexyl)-7-propyl-7*H*-purine-2(3*H*),6(1*H*)-dione] was performed combining a microdialysis technique with peroxyoxalate chemiluminescence (PO-CL) detection. The microdialysate was subjected to a fluorescent derivatization of propentofylline with 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) without further cleanup, because the membrane used in the microdialysis excluded high-molecular-mass proteins. The proposed method showed a good linearity in the determination range of 0.031 to 1.25 ng/injection; y (μV)=4234 x (ng)−13.43, r=0.9993 (y=peak height and x=amount of propentofylline). The very low determination limit of 0.031 ng/injection was ca. 200 times more sensitive than that of HPLC–UV determination. The HPLC–PO-CL method was applied for the first time to determine propentofylline concentration in the dialysate obtained from the rat hippocampus after a single oral administration (25 mg/kg). Propentofylline showed its maximum extracellular fluid (ECF) concentration of 125.1±15.1 ng/ml (mean±SD, n=3) at 0.33 h after administration.

Keywords: Propentofylline

1. Introduction

Excitatory amino acids (EAAs) such as glutamate and asparate were reported to be released at the early stage of a neuronal damage followed by delayed neuronal cell death. Propentofylline was reported to reduce the ischemia-induced build-up of extracellular glutamate and asparate [1–3]. Propentofylline was also reported to inhibit the release of dopamine during transient cerebral ischemia when propentofylline was perfused in the rat striatum [4]. Although an in vivo microdialysis has been widely used in monitoring medicinal substances and neurotransmitters, the sample amount of a brain dialysate is restricted and small. Generally, HPLC analysis of propentofylline in biological studies require a relatively large amount of sample because of the extraction procedures [5]. Alternatively, using a highly sensitive chemiluminescence detector can minimize the sample amount for HPLC analysis. The aim of the present work is to develop a selective and sensitive method for propentofylline determination.

*Corresponding author.

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A driving force of microdialysis is a concentration gradient which controls the diffusion of solute through a microdialysis membrane from ECF surrounding the membrane to dialysate. Since the membrane excludes high molecular weight proteins in the biological fluid, the dialysate is suitable for a fluorescent labeling without further cleanup. Therefore, a direct derivatization of propentofylline in a microdialysate sample was attempted with a fluorogenic labeling reagent, DBD-H [4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole] [6].

In this paper, we describe the derivatization of propentofylline with DBD-H under mild reaction conditions, and, the HPLC–PO-CL determination of hydrazone. As it applies to the determination of extracellular propentofylline in rat hippocampus.

2. Experimental

2.1. Chemicals

Propentofylline was synthesized by Hoechst Marion Roussel (Frankfurt, Germany). DBD-H and fluorometric-grade imidazole were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO), hydrogen peroxide and nitric acid were purchased from Wako Pure Chemical (Osaka, Japan). HPLC-grade acetonitrile and distilled water were purchased from Kanto Merck (Tokyo, Japan). Other reagents used were analytical grade.

2.2. Apparatus

The HPLC system is shown in Fig. 1. The system consisted of a Waters Automated Gradient Controller with two Model 510 pumps (Japan Waters, Tokyo, Japan) for gradient elution, a Rheodyne 7125 injector with a 100 μl loop (Rheodyne, Cotati, CA, USA); a Model 510 pump, and a JASCO BIP-I pump (Japan Spectroscopic Co., Tokyo, Japan) for delivering a pH conditioning buffer solution and a PO-CL reagent solution, respectively. The system was equipped with two mixing tees and stainless-steel coils (C1, 150×0.25 mm I.D.; C2, 300×0.25 mm I.D.) and a 825CL Intelligent chemiluminescence detector (Japan Spectroscopic Co.) with a noise cleaner UNI-1 (UNION, Tokyo, Japan). A Vydac C_{18} column (150×4.6 mm, 5 μm, The Separation Group, Hesperia, CA, USA) was used for separation, and two Inertsil ODS columns (150×4.6 mm I.D., GL Science, Tokyo, Japan) were used as a damper. The mixing tees and coils were maintained at a constant temperature (30°C) with a Model 860CO column oven (Japan spectroscopic Co.). A Hamilton Model 705-SNR syringe (Hamilton Co., Reno, Nevada, USA) was to facilitate loading.

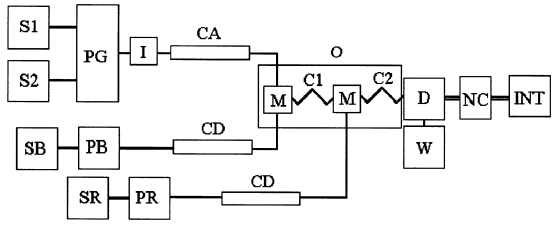

Fig. 1. Diagram of HPLC-PO-CL. S1, solvent A; S2, solvent B; PG, Waters Automated Gradient Controller with two Model 510 pumps; I, Rheodyne 7125 loop injector; CA, column for analytical separation; SB, Buffer solution; PB, Model 510 pump; CD, column for pulse-damper; M, mixing tee; SR, PO-CL reagent; PR, JASCO BIP-I pump; C1 and C2, stainless-steel mixing coils; O, Oven; D, CL detector; NC, noise cleaner; INT., integrator; W, waste bottle.

The microdialysis system was composed with a CMA/100 Microinjection pump, a CMA/111 Syringe Selector and a CMA/150 Temperature Controller (CMA/Microdialysis, Stockholm, Sweden) with a stereotaxic folding apparatus NARISHIGE type SR-5 (Narishige Scientific Instrument Lab., Tokyo, Japan). The microdialysis probe (PC-10; membrane length, 2 mm) was purchased from BSA (Tokyo, Japan).

2.3. Synthesis of DBD-H-Pro derivative

Propentofylline (0.1 mmol) and DBD-H (0.1 mmol) were dissolved with 6 ml of acetonitrile, and 100 μl of trifluoroacetic acid (TFA) was added to the solution. The mixture was stirred in the dark at 5°C for 6 h and evaporated by an EYELA rotary vacuum evaporator (Tokyo Rikakikai, Tokyo, Japan) at 30°C. The residue was dissolved with 500 μl of chloroform, applied to a TLC plate (Silica gel 60, 0.25 mm, Merck) and developed with a mixture of

chloroform and ethanol (97/3, v/v). The main spot, exhibiting orange fluorescence (λ_{ex}=366 nm), was scrapped off, and extracted with acetonitrile. The solvent was evaporated and the resultant residue was applied again to a TLC plate to obtain DBD-H-Propentofylline derivative (DBD-H-Pro) (Fig. 2). On recrystallization from ethanol, DBD-H-Pro precipitated as orange, fine needles; DBD-H-Pro: m.p. 166.0–168.5°C, ^{1}H NMR ($CDCl_3$) δ: 0.947 (t, 3H, J=7.3 Hz, n), 1.6–2.0 (m, 6H, g, h, m), 2.042 (s, 3H, e), 2.480 (t, 2H, J=6.7 Hz, f), 2.880 (s, 6H, c), 3.584(s, 3H, j), 4.070 (t, 2H, J=6.8 Hz, i), 4.249 (t, 2H, J=7.1, l), 7.071 (d, 1H, J=7.8 Hz, b), 7.542 (s, 1H, k), 7.962 (d, 1H, J=7.8, a), 8.264 (s, 1H, d); UV λ_{max} (in ethanol) 205.0, 270.0, 443.0 nm; FAB-MS (m/z) 546 (MH^+).

2.4. HPLC–PO-CL system for propentofylline

The analysis of the DBD-H-Pro derivative was achieved by HPLC with a two-step mixing system set under a controlled temperature. Chromatography was performed using the linear gradient program at a flow rate of 1.0 ml/min. A mobile phase started with a 100:0 mixture of solvent A=acetonitrile:water (1:3, v/v) and solvent B=acetonitrile:water (3:1, v/v), was allowed to run for 10 min and changed to 75:25, then changed again to 50:50 at 15 and 30 min, respectively. DBD-H-Pro was analyzed within 30 min. The pH conditioning was attained with a mixed solution consisting of a 100 m*M* imidazole solution, whose pH was adjusted with nitric acid to pH 6.0, and acetonitrile (2:3, v/v) at a flow rate of 1.0 ml/min. The PO-CL reagent of 0.5 m*M* TDPO/acetonitrile solution containing 100 m*M* hydrogen peroxide was pumped at a flow rate of 1.2 ml/min.

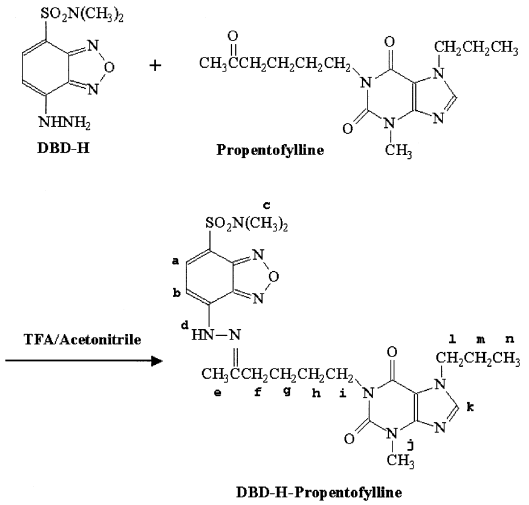

Fig. 2. Derivatization reaction of Propentofylline with DBD-H.

2.5. Preparation of standard solution for method validation

Propentofylline (ca. 10 mg) was weighed accurately and dissolved with a 0.5% TFA/acetonitrile solution to prepare a 100 ml stock solution. This solution was diluted serially with a 0.5% TFA/acetonitrile solution down to 2.5 ng/ml. Standard sample solutions were prepared to the following concentrations: 2.5, 5, 10, 25, 50, and 100 ng/ml. A 25 μl portion of 2.5 m*M* DBD-H/acetonitrile solution was added to 25 μl of each standard sample solution in a screw capped polypropylene reaction tube and allowed to stand at room temperature for 30 min. A 25 μl portion of the reaction mixture was analyzed, utilizing the HPLC–PO-CL method.

2.6. Rat brain microdyalisis of propentofylline

Wister strain rats (male, 7 week-old), weighing from 250 to 290 g, were anaesthetized with urethane (1.5 g/kg). Each rat was inserted with a feeding tube into its stomach for the administration of a propentofylline solution. The rat head was placed in a stereotaxic folding apparatus, NARISHIGE type SR-5. A microdialysis probe (PC-10, 20 000 Dalton cutoff probe) was implanted into the hippocampus (A:−4.0, L:+2.2, H:−5.0 from the skull) according to the atlas of Paxinos and Watson [7]. Using a CMA/150 temperature controller, the rat's body temperature was monitored and kept constant (37°C) during anaesthetisation.

The perfusion of Ringer's solution was started immediately after inserting a microdialysis tube into the hippocampus. The flow rate was maintained at 2 μl/min by a CMA/100 microinjection pump. After 120 min of perfusion for stabilization, a propentofylline solution was administered (25 mg/kg) through the feeding tube. Brain dialysates fractions were collected into polypropylene tubes (1.5 ml,

ASSIST, Tokyo, Japan) at −30, −25, −20, −15, −10, −5, 0 (administration), 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 120, and 180 min. Samples collected before administration were used as a propentofylline-free control. Sampling duration was 5-min (corresponding to 10 μl) for 30 to 60 min, and 10-minute (corresponding to 20 μl) for 120 and 180 min. The collection tubes contained initially 20 μl of 2.5% TFA acetonitrile solution to stabilize the propentofylline collected. The obtained dialysates were freeze-dried in vaccuo and stored at −30°C until HPLC–PO-CL analysis. A standard solution was prepared using a freeze-dried control sample dissolved in 25μl of 10 ng propentofylline/ml of 0.5% TFA/acetonitrile solution.

2.7. Derivatization of propentofylline dialysed with DBD-H for HPLC analysis

Each freeze-dried dialysate sample was reconstituted with 25 μl of 0.5% TFA/acetonitrile solution, added to 25 μl of a 2.5 m*M* DBD-H/acetonitrile solution and allowed to stand at room temperature for 30 min. The reaction mixture was centrifuged at 9000 rpm for 3 min, and 40 μl of the resultant supernatant solution was subjected to HPLC–PO-CL analysis.

3. Results and discussions

3.1. HPLC–PO-CL detection of DBD-H-Pro

Non-fluorescent propentofylline was fluorescent-labelled with DBD-H to obtain fluorescent DBD-H-Pro. Instrumental analyses by ^{1}H–NMR and FAB–MS spectrometry of synthetic DBD-H-Pro indicated that the carbonyl group of the 5-oxohexyl substituent of propentofylline reacted with the hydrazino group of DBD-H to yield the monohydrazone.

The analytical system used in this study consisted of three functions; separation, pH conditioning and chemiluminescence reaction. DBD-H-Pro was well separated from the reaction mixtures using the gradient system, and sufficiently detected by PO-CL. To minimize noise and signal drift, the HPLC system was temperature controlled. The mixing tees and reaction coils were kept at a constant temperature (30°C) using a oven.

The optimization of fluorescent labeling reaction was studied using 100 ng/ml of propentofylline acetonitrile solution. The maximum chemiluminescence intensity of DBD-H-Pro remained constant from 10 to 50 min. Since a rapid and easy treatment of samples was important for the application of this system to actual biological samples, the derivatization was performed for 30 min at room temperature.

The effects of acidity of the reaction mixtures and the concentration of DBD-H on the labeling reaction were also investigated. The chemiluminescence intensity maximized and formed a plateau at 0.25~0.5% of TFA. The intensity also increased with the concentration of DBD-H and then reached a plateau at 2.5~25 m*M*. As a result, 2.5m*M* DBD-H/acetonitrile solution and a 0.5% TFA/acetonitrile solution were finally selected for the fluorescent labeling reaction.

Under the above experimental conditions, a good linearity was obtained between the amount injected and the peak height of propentofylline in the determination range of 0.031 to 1.25 ng/injection (y (μV)=4234 x (ng)−13.43, r=0.9993), and thus this method was applied to determine propentofylline concentrations in biological fluid obtained by microdialysis. The estimated values for the determination limit and the detection limit [8] were obtained by calculation as 0.029 ng/injection (10×SD/S) and 0.009 ng/injection (3.3×SD/S), respectively, where SD (12.16 μV) was the standard deviation of the response obtained from the analysis of six samples at the lowest concentration, and the S [4234 μV/ng] was the slope of the calibration curve. As the result of calculation, it is reasonable that the limit of determination of this HPLC method is 0.031 ng/injection.

3.2. Application of HPLC–PO-CL to microdialysis of propentofylline

Since microdialysis sampling is performed through a membrane, the analyte concentration in dialysate differs from that in the brain ECF. The ratio between both concentrations across the membrane is important and is defined as a relative recovery [9]. To calibrate in vivo microdialysis recovery, zero net

flux, slow perfusion and retrodialysis methods are commonly applied [9,11]. Although it is known that the recovery decreases with increasing perfusion flow rate [9,10], an appropriate flow rate is necessary to obtain enough dialysate to measure the concentration of a target compound by a pre-column derivatization. In the retrodialysis method, it is assumed that the delivery is equal to the recovery of the compound [12].

The probe relative recovery, *R*, is defined by the following equation:

$$R = C_{\text{dialysate}}/C_{\text{sample}} \quad (1)$$

The delivery, D, is defined by the equation of

$$D = (C_{\text{in}} - C_{\text{out}}) \times 100/(C_{\text{in}}) \quad (2)$$

where C_{in} is the concentation in the perfusate, and C_{out} is the concentration in the dialysate. The relative recovery and delivery of the probe used at a flow rate of 2 μl/min is shown in Table 1.

Although retrodialysis requires in vitro relative recovery and delivery of the probes are equal, there was a slight difference between recovery and delivery. Therefore, in this study, the in vivo relative recovery of the prove was calculated by the delivery as following; A PC-10 probe was inserted into the hippocampus of rat brain, a propentofylline/Ringer's solution (C_{in}, 3 μg/ml) was used to determine C_{out}. Then the relative recovery (%) of the probe was calculated by Eq. (2) as 14.2% and used for the in vivo calibration of PC-10.

Typical chromatograms of the dialysates are shown in Fig. 3. DBD-H-Pro was successfully separated from the blank peaks. A time-dependent propentofylline concentration in the brain hippocampus is shown in Fig. 4. After a single oral administration (25 mg/kg), propentofylline reached its maximum ECF concentration of 125.1±15.1 ng/ml (mean±SD, $n=3$) at 0.33 h after administration.

This work presents, for the first time, a combination of HPLC–PO-CL and microdialysis for a highly selective and sensitive determination of propentofylline concentration. The proposed method showed the very low determination limit of 0.031 ng/injection being ca. 200 times more sensitive than that of 6 ng/injection limit reported for HPLC–UV [13]. This method was successfully applied to the analysis of propentofylline in rat brain ECF without further cleanup.

Table 1
Recovery and delivery of propentofylline with PC-10 ($n=3$)

	Recovery (%)	Delivery(%)
In vitro	15.2±0.8	11.6±0.2
In vivo	–	14.1±4.8

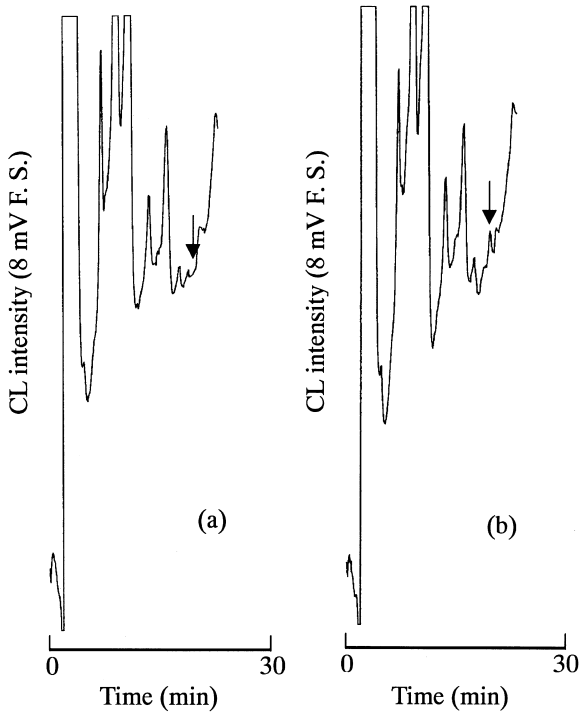

Fig. 3. Chromatograms of Rat's hippocampus dialysate. (a) Control, (b) 30 min after oral administration of propentofylline (25 mg/kg)

4. Conclusion

HPLC–PO-CL determination is one of the highly sensitive analytical methods. However, its high sensitivity causes unexpected background peaks which interfere with the analysis. On the other hand, because microdialysis uses a membrane which can exclude high molecular weight proteins, the number of interfering background peaks are reduced. The proposed HPLC–PO-CL with microdialysis sam-

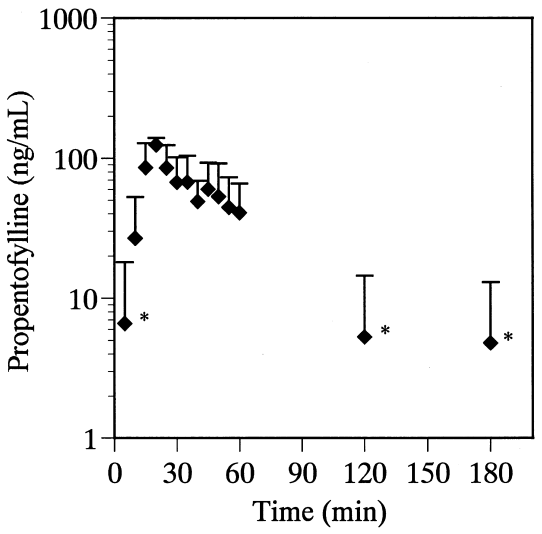

Fig. 4. Microdialysis measurement of propentofylline in rat hippocampus. After 2 h pre-perfusion for stabilization, propentofylline solution was administered (25 mg/kg in Ringer) through the feeding tube. The asterisk represents the value under the lower limit of determination.

pling should be a very useful method in clinical studies involving microdialysis.

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