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Determination of the fluorescent drugs dipyridamole and benzydamine in rat plasma by liquid chromatography with peroxyoxalate chemiluminescence detection

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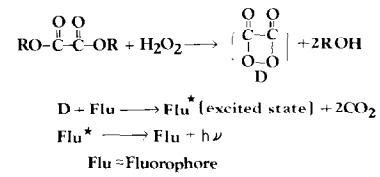
Abstract

A facile and sensitive method for the determination of the fluorescent drugs dipyridamole (coronary vasodilator) and benzydamine hydrochloride (anti-inflammatory) in rat plasma is presented. The method consists of the addition of an internal standard (DNS-L-phenylalanine for dipyridamole and dipyridamole for benzydamine hydrochloride), deproteinization of plasma (1 μl for dipyridamole and 10 μl for benzydamine hydrochloride) with 13 volumes of acetonitrile, direct injection of the supernatant into an ODS column, separation by liquid chromatography and peroxyoxalate chemiluminescence detection using bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate and hydrogen peroxide. Calibration graphs were linear over the ranges 2.5–200 nM and 2.5–100 μM for dipyridamole and benzydamine hydrochloride in plasma, respectively. The detection limits (signal-to-noise ratio = 2) were 345 pM for dipyridamole and 147 nM for benzydamine hydrochloride in plasma. The relative standard deviation for four determinations of dipyridamole at 2.5 nM and benzydamine hydrochloride at 2.5 μM in plasma were 3.7% and 2.6%, respectively. The method was applied to real samples and the time courses of the plasma concentration after oral administration of the two drugs [0.50 mg kg^{-1} (0.99 μmol kg^{-1}) and 10 mg kg^{-1} (29 μmol kg^{-1}), respectively] were obtained.

Keywords: Chemiluminescence; Liquid chromatography; Benzydamine; Dipyridamole; Pharmaceuticals; Plasma

In order to study the pharmacokinetics of drugs, the consecutive measurement of plasma drug concentrations is necessary. For this purpose, many analytical methods have been applied such as radioimmunoassay, gaschromatography–mass spectrometry and liquid chromatography (LC) with electrochemical or spectrophotometric detection. With fluorescent drugs, e.g., dipyridamole {2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine, coronary vasodilator}, perphenazine (antipsychotic) and benzydamine hydrochloride [1-benzyl-3-(3-dimethylaminopropoxy)-1*H*-indazole hydrochloride, anti-inflammatory), LC with fluorimetric detection would seem the most promising approach for their sensitive determination in plasma as it does not require a derivatization step for their detection. However, tedious sample clean-up procedure, including extraction and purification, before the sample injection onto the HPLC column is always required because fluorescent interfering peaks derived from plasma appear on chromatograms.

Recently, the peroxyoxalate chemiluminescence (PO-CL) reaction (Scheme 1) has been successfully applied to the detection of fluorescent compounds separated by LC [1]. As this system generates CL without a light source, the fluorescent compounds can be sensitively detected because of the absence of interferences from the light source. Thus, this detection system has permitted the detection of pmol to amol levels of fluorescent compounds and drugs [2,3]. Moreover, as pointed out



Scheme 1. Proposed mechanism for peroxyoxalate chemiluminescence reaction.

in previous papers [4–6], compounds having fluorescence characteristics in the UV region below ca. 300 nm, i.e., with a high excitation energy, are less excited by this PO-CL reaction. Also, fluorescent compounds that have high oxidation potentials, such as 9,10-dibromoanthracene and 9,10-diphenylanthracene, are less excited [4,7]. This selectivity of CL generation with fluorescent compounds should make it possible to decrease the effect of fluorescent interfering peaks in chromatograms derived from plasma and to avoid the tedious clean-up procedure for plasma samples. In this work, the use of the LC with PO-CL detection for the determination of fluorescent drugs in rat plasma, with dipyridamole and benzydamine as representatives, was investigated in an attempt to establish a facile and sensitive method for the determination of fluorescent drugs in body fluids.

EXPERIMENTAL

Chemicals

Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl) phenyl] oxalate (TDPO) was purchased from Wako (Osaka). Dipyridamole, 5-(*N*,*N*-dimethylaminonaphthalene)-1-sulphonyl(DNS)-L-alanine, cyclohexylammonium DNS-glutamate, cyclohexylammonium DNS-L-methionine, *O*-DNS-L-tyrosine (free acid) and *N*,*O*-di-DNS-L-tyrosine were obtained from Sigma (St. Louis, MO) and DNS-L-phenylalanine piperidinium salt from Pierce (Rockford, IL). Hydrogen peroxide (30%) was purchased from Mitsubishi Gas Kagaku (Tokyo). Imidazole (zone refined) was donated by Tokyo Kasei Kogyo (Tokyo). Distilled water, acetonitrile and ethyl acetate of LC grade were obtained from Wako. Benzydamine hydrochloride was donated by Daiichi Seiyaku (Tokyo). All other chemicals were of analytical-reagent grade.

LC–chemiluminescence detection system

The flow diagram for this system is shown in Fig. 1. The pumps used were a KHP-011 (Kyowa Seimitsu, Tokyo) for the eluent and an LC-6A (Shimadzu, Tokyo, Japan) for the chemilumigenic reagent solution. The injection valve (Rheodyne, Cotati, CA) with a 20-μl loop was from Shimadzu and the analytical column (TSK ODS 80Tm, 150 $\times$ 4.6 mm i.d., 5 μm, maintained at 40 °C) and dummy columns for the eluent and the reagent solutions (TSK ODS-120A, 100 $\times$ 4.6 mm i.d., 5 μm) from Tosoh (Tokyo). A Model 825-CL chemiluminescence monitor (JASCO, Tokyo) with a 93-μl spiral-type flow cell and a recorder (Technicorder Type 3047; Yokogawa Denki, Tokyo) were used. A 25-μl rotating flow mixing device (Kyowa Seimitsu) [8] was heated at 40 °C in a column oven (Model 655A-52; Hitachi, Tokyo). The flow lines (PTFE tubing, 50 $\times$ 0.5 mm and 50 $\times$ 0.8 mm i.d.) were connected to the detector next to the mixing device.

The eluent for dipyridamole and benzydamine was 50 mM imidazole buffer (NO_3^-, pH 6.0)–acetonitrile (1 + 1, v/v). The chemilumigenic reagent solution was a mixture of 0.5 mM TDPO in ethyl acetate and 25 mM hydrogen peroxide in acetonitrile (1 + 1, v/v) at a flow-rate of 1.6 ml min^{-1}. Both solutions were mixed just prior to use. The stock solutions of the fluorescent compounds

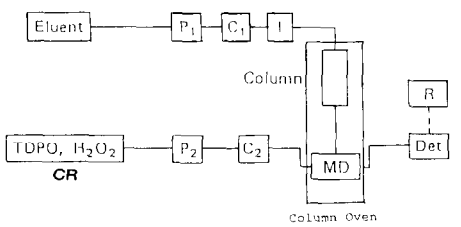


Fig. 1. Flow diagram of LC–peroxyoxalate chemiluminescence detection system. Eluent, 50 mM imidazole buffer (NO_3^-, pH 6.0)–acetonitrile (1+1, v/v); P_1, pump for the eluent; P_2, pump for the chemilumigenic reagent; C_1 and C_2, dummy columns; I, Injector; C, analytical column; CR, chemilumigenic reagent solution [0.25 mM TDPO and 12.5 mM H_2O_2 in acetonitrile–ethyl acetate (1+1, v/v)]; MD, rotating flow mixing device; R, recorder; Det, chemiluminescence monitor.

in acetonitrile or distilled water were diluted with the same solvents to appropriate concentrations. A 20-μl aliquot was subjected to LC analysis.

Standard solutions

Stock standard solutions of dipyridamole, benzydamine hydrochloride and DNS-L-phenylalanine piperidinium salt (internal standard) were prepared by dissolving a few mg of each compound in acetonitrile or distilled water and then diluting to volume with the imidazole buffer.

Sample preparation

A 10-μl volume of the intact rat plasma or the plasma diluted 10-fold with 150 mM imidazole buffer (NO_3^-, pH 6.0) in the case of dipyridamole administration was transferred into a 2-ml Eppendorf sample tube, followed by the addition of 40 μl of the imidazole buffer, 20 μl of the internal standard (5.0 nM DNS-L-phenylalanine for dipyridamole and 0.5 nM dipyridamole for benzydamine hydrochloride) and 130 μl of acetonitorile. The mixture was stirred with a vortex mixer for 10 s and then centrifuged at 2500 *g* for 5 min. To obtain the calibration graphs for both drugs, 40 μl of the imidazole buffer was replaced with the same buffer containing various amounts of dipyridamole (2.5–200 nM) and benzydamine hydrochloride (2.5–100 μM) and treated as described above. A 20-μl aliquot of the supernatant was subjected to LC analysis. For the determination of dipyridamole, peak-height ratios of dipyridamole to DNS-L-phenylalanine were plotted against the concentration of dipyridamole in plasma, and for benzydamine hydrochloride peak-height ratios to dipyridamole were plotted in the same way.

Plasma concentration in rats after oral administration of the drugs

An aqueous solution of each drug was administered to female Wister rats (10 weeks old, 250–260 g) in a single dose of 0.50 mg kg^{-1} (0.99 μmol kg^{-1}) for dipyridamole or 10 mg kg^{-1} (29 μmol kg^{-1}) for benzydamine hydrochloride into the stomach with a tube. After administration, 100 μl of blood were drawn from the jugular vein at intervals of 30 min, 1, 2, 3, 4, 5, 6, 7, 8 and 9 h for dipyridamole and 30 min, 1, 1.5, 2, 3 and 4 h for benzydamine hydrochloride. Plasma samples of 10 μl were transferred into a heparinised 2-ml Eppendorf tube and treated as described above.

RESULTS AND DISCUSSION

In a previous paper [3], a simple method for finding the detection limits of fluorescent drugs using LC with PO-CL detection was reported. Among the fluorescent drugs tested, dipyridamole chemiluminesced most strongly, and the detection limit [signal-to-noise ratio (S/N) = 2] was 160 amol for injection. Benzydamine chemiluminesced less, with a detection limit of 880 fmol. Alimemazine (antihistamine drug) and oxypertine (psycopharmacological drug) chemiluminesced between the first two. In this experiment, dipyridamole and benzydamine were investigated as representatives of stronger and lesser chemiluminescent drugs, respectively.

As suggested in the Introduction, the interferences from the components of plasma in the PO-Cl reaction would be lower than those in fluorescence analysis. Therefore, a simple sample clean-up procedure involving only deproteinization with acetonitrile was tested for the determination of fluorescent drugs in plasma in this experiment.

In a preliminary experiment, several DNS-amino acids (DNS-L-alanine, DNS-glutamic acid, DNS-L-methionine, DNS-L-phenylalanine, *O*-DNS-L-tyrosine and *N*,*O*-di-DNS-L-tyrosine) were tested as internal standards for the drugs. Finally, DNS-phenylalanine and dipyridamole were selected as internal standards for the determination of dipyridamole and benzydamine hydrochloride, respectively, from the points of appropriate retention times.

Blank plasma spiked with each internal standard (corresponding to 1 μl of rat plasma for an injection) gave no interfering peaks for dipyridamole (Fig. 2a) and benzydamine (Fig. 3a) on the chromatograms obtained following treatment of the plasma with only a single deproteinization with acetonitrile. Judging from the chromatograms, two more volumes (20 μl of reaction mix-

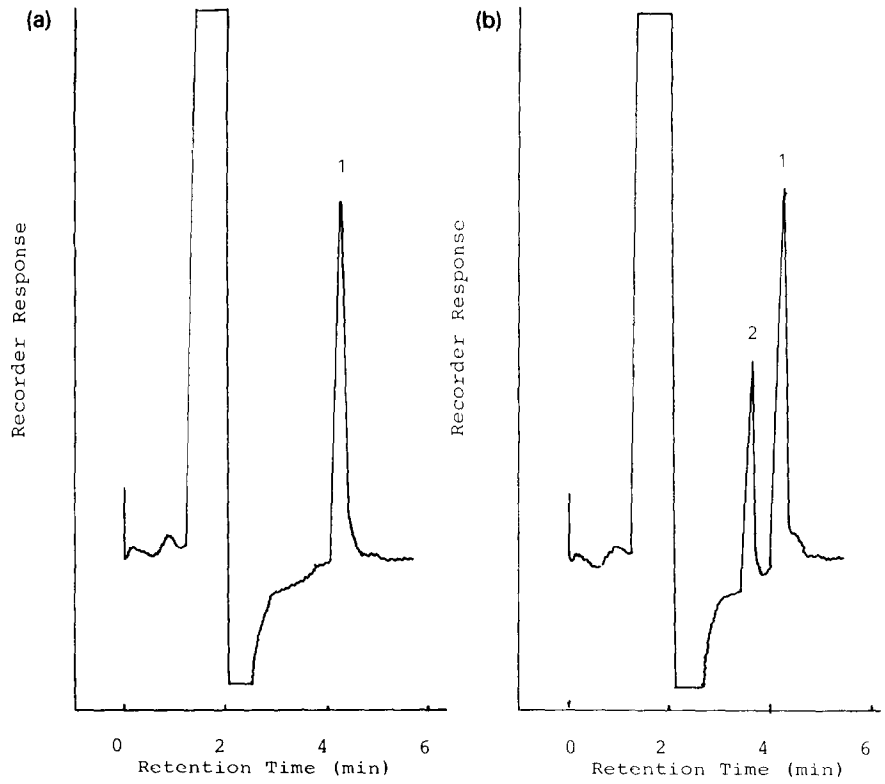


Fig. 2. Chromatograms obtained from rat plasma with or without dipyridamole. (a) Blank rat plasma (corresponding to 0.1 μl) containing 100 nM DNS-L-phenylalanine; (b) rat plasma containing 10 nM dipyridamole and 100 nM DNS-phenylalanine. Peaks: 1 = DNS-L-phenylalanine; 2 = dipyridamole.

ture corresponding to 2 μl of rat plasma) could be injected onto the column. When 25 fmol of dipyridamole or 25 pmol of benzydamine were added to 10 μl of rat plasma (final concentrations 2.5 nM and 2.5 μM, respectively) and treated as described above, each drug was detected together with the internal standards as depicted in Figs. 2b and 3b. The recovery of plasma sample was ca. 90%.

Calibration graphs for dipyridamole and benzydamine hydrochloride obtained by the addition of each drug to rat plasma were linear over the ranges 2.5–200 nM and 2.5–100 μM, respectively. The regression equations were as follows: for dipyridamole, $y = 0.98x + 0.06$ ($r = 0.99$), and for benzydamine hydrochloride, $y = 0.12x - 0.02$ ($r = 0.99$), where y is the peak-height ratio of the drug to the internal standard, x is the concentration of the drug in plasma and r is the correlation coefficient. The detection limits (S/N = 2) were 345 pM for dipyridamole and 147 nM for benzydamine hydrochloride in plasma (34.5 amol and 147 fmol injected amounts, respectively). The relative standard deviations for four determinations of dipyridamole at 2.5 nM and benzydamine hydrochloride at 2.5 μM in plasma were 3.7% and 2.6%, respectively.

Determination of dipyridamole and benzydamine hydrochloride in rat plasma

The above results suggested the possibility of the application of the method to real biological samples. Owing to the simple sample clean-up procedure, time course studies of both drugs were easily performed. Examples of the chromatograms in Fig 4a and b, which were obtained from plasma samples 30 min after the administration of dipyridamole and 2.0 h after the administration of

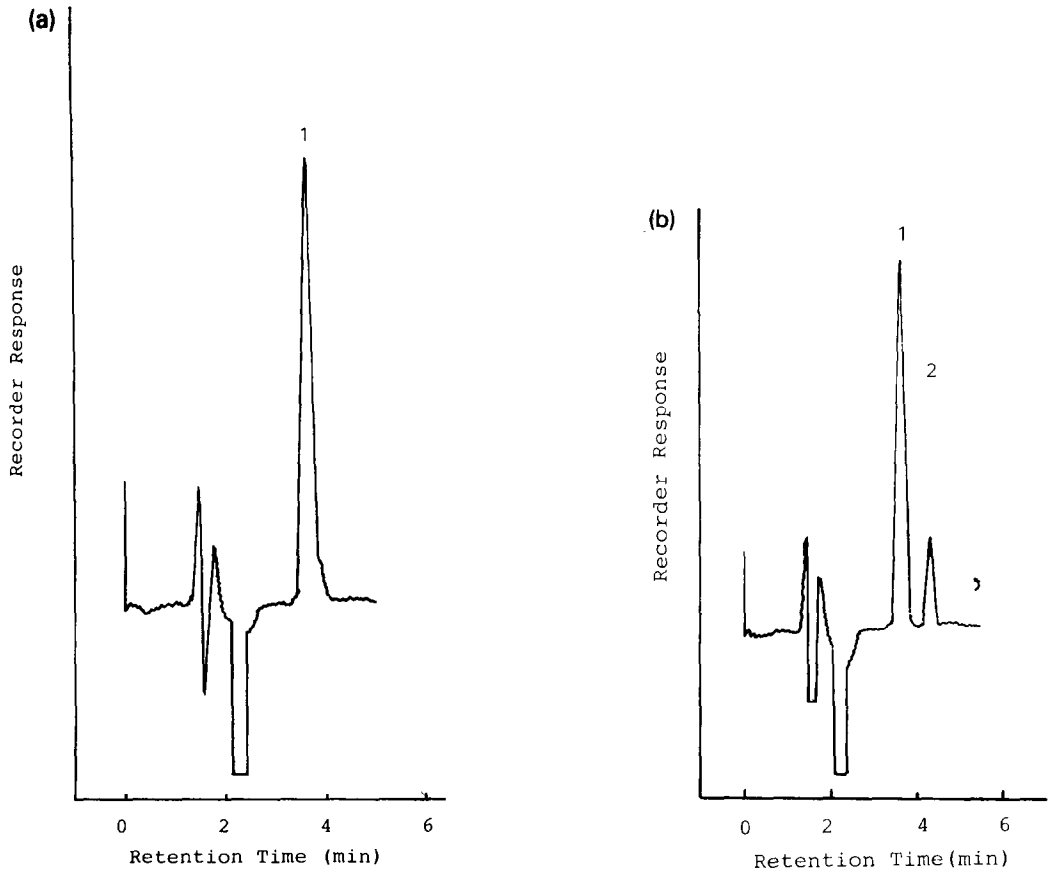


Fig. 3. Chromatograms obtained from rat plasma with or without benzydamine hydrochloride. (a) Blank plasma (corresponding to 1 μl) containing 10 nM dipyridamole; (b) rat plasma containing 2.5 μM benzydamine hydrochloride. Peaks: 1 = dipyridamole; 2 = benzydamine.

benzydamine hydrochloride, respectively. As shown in Fig. 5, the dipyridamole concentration reached its maximum ca. 2 h after oral administration of 0.50 mg kg^{-1} (0.99 μmol kg^{-1}) and its elimination half-life was 2. 0 h. These results are consistent with the data obtained with radioisotope-labelled dipyridamole administered to rats [10 mg kg^{-1} (19.8 μmol kg^{-1})] [9], when dipyridamole was measured in rat plasma after 10-fold dilution. The elimination half-life of dipyridamole administered orally to rats was reported to be 10.2 h [9], whereas in this study it was 2.0 h. This discrepancy seems to be derived from the difference in the measurement of the plasma concentration of dipyridamole in the rat. Kobayashi et al. [9] used ^{14}C-labelled dipyridamole and measured the total radioactivity in the rat plasma, which presumably contained all the metabolites of dipyridamole, and gave a longer half-life, whereas in this work the concentration of intact dipyridamole was measured after separation by LC followed by PO-CL detection.

For benzydamine (oral administration of 10 mg kg^{-1}), the plasma concentration curve was obtained as depicted in Fig. 6. These data are consistent with previous results obtained by fluorimetry when benzydamine hydrochloride was administered to rats at 10 mg kg^{-1} [10].

The present results indicate that a single plasma deproteinization step with acetonitrile is sufficient for the determination of dipyridamole and benzydamine hydrochloride in 1 μl and 10 μl of rat

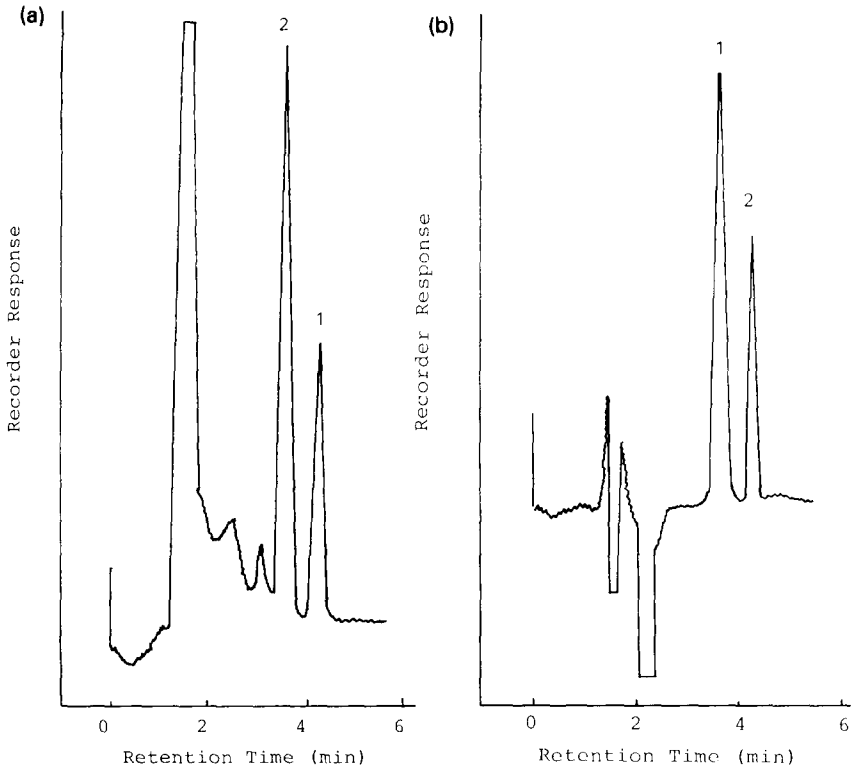


Fig. 4. Chromatograms of dipyridamole and benzydamine hydrochloride in rat plasma after administration of the drugs obtained by the LC with peroxyoxalate chemiluminescence detection. Rat plasma obtained (a) 30 min after the administration of dipyridamole [0.50 mg kg^{-1} (0.99 μmol kg^{-1})] and (b) 2.0 h after the administration of benzydamine hydrochloride [10 mg kg^{-1} (29 μmol kg^{-1})] was treated described as in the text. Peaks: (a) 1 = DNS-L-phenylalanine and 2 = dipyridamole; (b) 1 = dipyridamole and 2 = benzydamine.

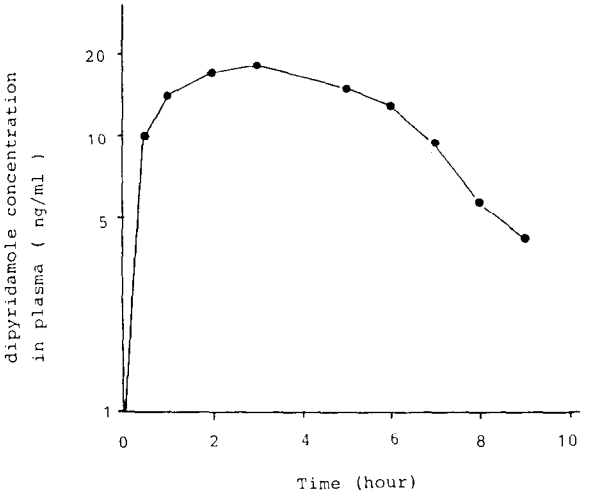


Fig. 5. Time course of plasma concentration of dipyridamole after oral administration to rats. Doses of 0.5 mg kg^{-1} (0.99 μmol kg^{-1}) were administered orally to rats ($n = 3$). Experimental conditions as in the text.

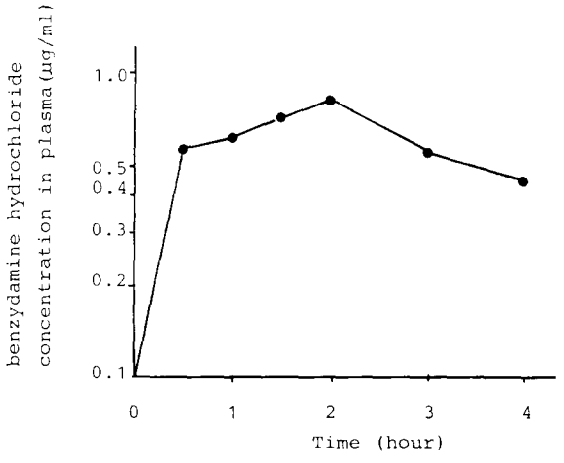


Fig. 6. Time course of plasma concentration of benzydamine hydrochloride after oral administration. Doses of 10 mg kg^{-1} (29 μmol kg^{-1}) were administered orally to rats ($n = 3$). Experimental conditions as in the text.

plasma respectively, and suggest that other fluorescent drugs having the ability to chemiluminesce more than benzydamine by the PO-CL reaction could be determined by this simple and sensitive method. If interferences from the components of plasma were to be encountered on the chromatogram, gradient elution in LC to separate the interfering peaks from the drug peak could be adopted, as in the separation and PO-CL detection of DNS-amino acids [11,12].

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