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4-(6,7-Dihydro-5,8-dioxothiazolo[4,5-*g*]phthalazin-2-yl)benzoic acid *N*-hydroxysuccinimide ester as a highly sensitive chemiluminescence derivatization reagent for amines in liquid chromatography

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Abstract

4-(6,7-Dihydro-5,8-dioxothiazolo[4,5-*g*]phthalazin-2-yl)benzoic acid *N*-hydroxysuccinimide ester was synthesized as a highly sensitive and selective chemiluminescence derivatization reagent for primary and secondary amines in liquid chromatography. Methyl-*n*-octylamine, *n*-nonylamine and *n*-decylamine were used as model compounds to optimize the derivatization, separation and chemiluminescence reaction conditions. This reagent reacts selectively with amines in the presence of triethylamine to give the highly chemiluminescent derivatives, which produce chemiluminescence by reaction with hydrogen peroxide in the presence of potassium hexacyanoferrate(III) in an alkaline medium. The chemiluminescent derivatives of the three amines can be separated within 20 min by reversed-phase liquid chromatography with isocratic elution, followed by chemiluminescence detection. The detection limits (signal-to-noise ratio=3) for primary and secondary amines are at sub-fmol levels for a 20-μl injection. Furthermore, this method was applicable to the determination of amantadine in human plasma.

Keywords: Chemiluminescence detection; Detection, LC; Derivatization, LC; Dihydrodioxothiazolo[4,5-*g*]phthalazinylbenzoic acid hydroxysuccinimide ester; Amines

1. Introduction

Chemiluminescence (CL) detection systems have been successfully introduced into liquid chromatographic (LC) analysis owing to their high sensitivity and wide linear range. Of these, the peroxyoxalate CL system is one of the most popular methods, using fluorescence derivatization reagents, such as dansyl chloride [1,2], 4-halo-7-nitrobenz-2-oxa-1,3-diazole [2,3], *o*-phthalaldehyde [2] and 2,3-naphthalenedialdehyde [4], for the detection of primary (and/or secondary) amines. Although the peroxyoxalate CL system is usually highly sensitive, there are some restrictions in the composition of mobile phase and the combination of fluorophores and peroxyoxalate esters [5,6]. Furthermore, fluorescent impurities contaminating reagents and samples also afford CL peaks in the chromatograms. Recently, a tris(bipyridyl)ruthenium(III) [$Ru(bipy)_3$] CL system has been also reported for the LC determination of

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amines [7,8]. The $Ru(bipy)_3$ CL method is highly selective to tertiary and secondary amines but was not relatively so sensitive compared with other CL determination techniques.

Although luminol is the most widely used chemiluminogenic compound, only a few derivatization reagents, *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) with *N*,*N*′-disuccinimidyl carbonate [9,10], 4-isothiocyanatophthalhydrazide (ILITC) [11] and 6-isothiocyanatobenzo[*g*]phthalazine-1,4(2*H*,3*H*)-dione (IPO) [12], have been reported as luminol-type CL reagents for the determination of amino compounds. These reagents have an *N*-substituted 4-aminophthalic acid or an *N*-substituted 5-aminonaphthalene-2,3-dicarboxylic acid as a light emitter. The CL intensities generated from the luminol-type reagents, however, are known to partially depend on the fluorescence quantum yields of the light emitters [13–16]. Therefore, in order to improve the sensitivities of luminol-type CL reagents, fluorescent compounds having the higher quantum yield should be used as light emitters.

We have already developed some luminol-type CL reagents with higher efficiency based on the above-mentioned strategies; quinoxalinone [17] and benzothiazole derivatives [18] were found to be highly fluorescent compounds, and were therefore utilized as bright emitter luminol-type CL reagents [15,19,20]. More recently, we have developed 5-amino-4-sulfanylphthalhydrazide (ASPH) as a highly sensitive chemiluminogenic derivatization reagent for aromatic aldehydes [21]. The high sensitivity to a variety of aromatic aldehydes has been indicated as the formed derivatives containing the 2-arylbenzothiazole skeleton generate highly intense CL [22]. Therefore, it must be extremely useful to develop a luminol-type CL derivatization reagent containing the 2-arylbenzothiazole skeleton.

In this article, we report a novel luminol-type CL derivatization reagent, 4-(6,7-dihydro-5,8-dioxothiazolo[4,5-*g*]phthalazin-2-yl)benzoic acid *N*-hydroxysuccinimide ester (TPB-Suc, Fig. 1). TPB-Suc has a highly fluorescent 2-arylbenzothiazole skeleton and an *N*-hydroxysuccinimidyl group as a reactive functional site. TPB-Suc reacts with primary and secondary amines in the presence of triethylamine. The resulting TPB derivatives produce CL by reaction with hydrogen peroxide in the presence of potassium hexacyanoferrate(III) in an alkaline medium. The optimum derivatization, LC separation and CL reaction conditions were examined, and a selective and sensitive method for the determination of primary and secondary amines using TPB-Suc, based on LC with CL detection, was developed. Furthermore, this pre-column derivatization LC–CL method could be applied to the detection of amantadine (Fig. 1) in human plasma.

2. Experimental

2.1. Apparatus

Fast atom bombardment (FAB) mass spectra were taken with a Jeol (Tokyo, Japan) DX-300 spectrometer. Uncorrected melting points were obtained with a Gallenkamp (Loughborough, UK) melting point apparatus.

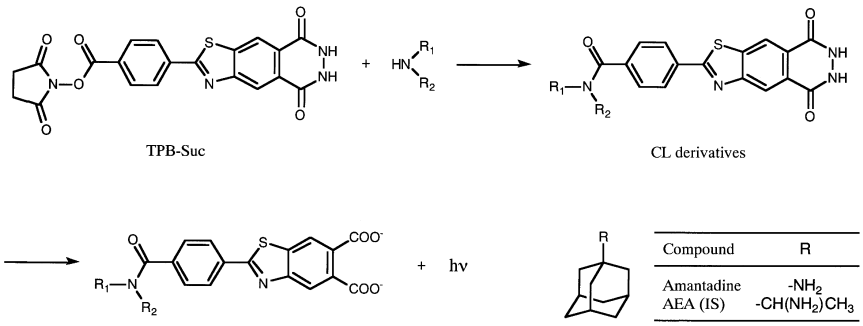

Fig. 1. Derivatization of amines with TPB-Suc and CL reaction of the derivatives.

2.2. Chemicals and solutions

All chemicals and solvents were of the highest purity available and were used as received. Distilled water, purified with a Milli-QII system (Millipore, Milford, MA, USA) was used for all aqueous solutions. Hydrogen peroxide (31%, w/v) was purchased from Mitsubishi Gas Kagaku (Tokyo, Japan). ASPH was prepared as described previously [21].

Stock solutions of amines (10 m*M*) were prepared in *N*,*N*-dimethylformamide (DMF), stored at −20°C, and then further diluted with water to the desired concentration before use. TPB-Suc (2.5 m*M*) and triethylamine (50 m*M*) solutions were prepared in acetonitrile–DMF (3:1, v/v) and acetonitrile, respectively, and were stable for at least a week when stored at −20°C. Hydrogen peroxide (20 m*M*) and potassium hexacyanoferrate(III) (10 m*M*) solutions were prepared in water and 1.5 *M* sodium hydroxide, respectively, and were used within 24 h.

2.3. Synthesis of TPB-Suc

TPB-Suc was synthesized via compound **I** from ASPH [21] by the following methods (Fig. 2).

2.3.1. Compound *I*

ASPH (0.21 g) and 4-formylbenzoic acid (0.15 g) were dissolved in DMF (20 ml), and 1.5 *M* sulfuric acid (20 ml) containing sodium sulfite (0.06 g) and disodium hydrogenphoshite (0.63 g) was added. The mixture was heated at 80°C for 1 h with stirring and further kept stirring for 3 h at room temperature. The resulting precipitates formed by cooling in ice–water, were collected and recrystallized from methanol to give compound **I** as pale yellow needles [(118 mg, 35%), m.p.>300°C]. FAB-MS, m/z=340 ([M+H]$^+$, base peak).

2.3.2. TPB-Suc

Compound **I** (102 mg), *N*-hydroxysuccinimide (69 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (115 mg) were dissolved in DMF (10 ml). The solution was stirred at room temperature for 8 h and was poured into cold water. The resulting precipitates were collected and washed several times with chilled water (ca. 20 ml). Pale yellow crystalline material [(98 mg, 75%), m.p.>300°C] was obtained. FAB-MS, m/z=437 ([M+H]$^+$, base peak).

The total reaction yield of TPB-Suc synthesis was about 25%. TPB-Suc was stable in the crystalline state at room temperature in the dark for at least 3 months in a desiccator containing silica gel.

2.4. Derivatization procedure

To a 100-μl aliquot of a test solution of amines placed in a 3.5-ml Reacti-vial (Pierce, Rockford, IL, USA) were added 100 μl of 2.5 m*M* TPB-Suc solution and 50 μl of 50 m*M* triethylamine. The vial was tightly closed and heated at 80°C for 20 min. A 20-μl portion of the final reaction mixture was injected into the chromatograph. To prepare the reagent blank, 100 μl of water in place of the test solution was subjected to the same procedure.

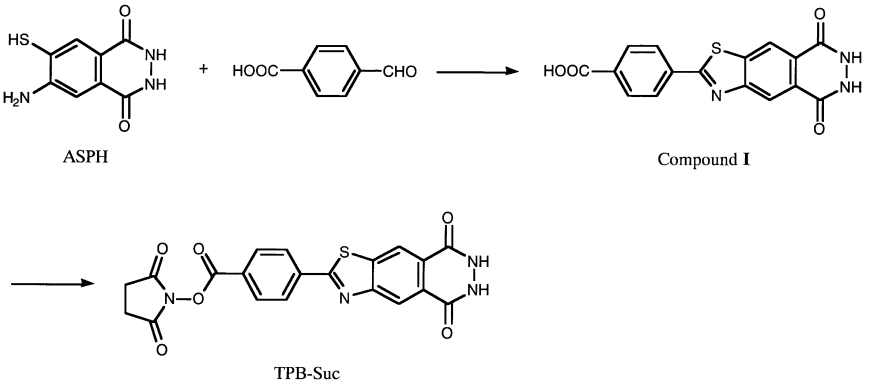

Fig. 2. Synthesis of TPB-Suc.

2.5. LC conditions and chemiluminescence detection system

Chromatography was performed with a Hitachi (Tokyo, Japan) L-7100 liquid chromatograph equipped with a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading sample injector valve (20-μl loop). The TPB derivatives of amines were separated on a reversed-phase column, Cosmosil $5C_{18}$-MS (250×4.6 mm I.D., particle size 5 μm) (Nacalai Tesque, Kyoto, Japan) by isocratic elution with 50 m*M* sodium phosphate buffer (pH 7.0)–acetonitrile (55:45, v/v) as the mobile phase. The flow-rate of mobile phase was set at 1.0 ml/min and the column temperature was ambient (22±4°C).

The effluent from the LC column was first mixed with 20 m*M* hydrogen peroxide solution by the first T-type mixing device, and then with 10 m*M* potassium hexacyanoferrate(III) solution in 1.5 *M* sodium hydroxide by the second T-type mixing device, delivered by two Jasco (Tokyo, Japan) 880-PU LC pumps at flow-rates of 1.0 and 2.0 ml/min, respectively. The generated CL was monitored by a Jasco CL-925 chemiluminescence detector and the signals were recorded on a Hitachi D-2500 Chromato-Integrator. Stainless steel tubing (0.5 mm I.D.) was used for all the LC–CL lines. The length of the tubing between the second mixing device and the detector cell was set at 5.0 cm.

2.6. Analysis of amantadine in human plasma

A healthy volunteer (male, 22 years old, 55 kg) was given a 50-mg single oral dose of amantadine hydrochloride at 09:00 after an overnight fasting. Two hours after administration of the drug, blood sample was collected to the heparinized tube, and the heparinized plasma was obtained by centrifugation at 1000 *g* for 10 min at 4°C. Drug-free plasma samples were obtained from the volunteer as the same manner before the administration of drugs. The plasma samples were immediately frozen and stored at −20°C until assay.

The plasma sample was pre-treated according to the previous fluorometric LC method [23] by using 1-(1-adamantyl)ethylamine (AEA) as an internal standard (I.S.); a 50-μl aliquot of plasma was pipetted into a screw-capped test tube (100×15 mm I.D.) together with 10 μl of the I.S. (1.0 nmol/ml) solution, 50 μl of 1.0 *M* NaOH and 2.0 ml of toluene. The mixture was vortex-mixed for ca. 2 min and centrifuged at 1000 *g* for 5 min. The organic layer (1.5 ml) was transferred to a 3.5-ml Reacti-vial and was evaporated to dryness under a nitrogen stream. The residue, dissolved in 100 μl of 1.0% DMF, was subjected to derivatization (Section 2.4).

The LC separation and CL detection conditions were stated previously (Section 2.5), except that aqueous 55% (v/v) acetonitrile was used as a mobile phase.

3. Results and discussion

Methyl-*n*-octylamine, and *n*-nonylamine and *n*-decylamine were used as representative compounds of secondary and primary amines, respectively, to establish derivatization reaction, LC separation and CL determination conditions.

3.1. LC separation conditions

Fig. 3 shows a typical chromatogram obtained with a mixture of the three amines. A good separation of the TPB derivatives of the amines was achieved under the LC conditions described in Section 2.5. The individual compounds gave corresponding single peaks and could be separated from each other and the reagent blank within 20 min.

3.2. Derivatization of amines with TPB-Suc

It was found that TPB-Suc was easily soluble in DMF and its mixtures, and practically insoluble or hardly soluble in water and other hydrophilic solvents. On the other hand, the derivatization reaction of amines proceeded efficiently by the addition of acetonitrile, dimethylsulfoxide or DMF, and acetonitrile gave the best results. Therefore, a mixture of DMF and acetonitrile was used for the preparation of the reagent solution. Although the peak heights for all amines became higher with increasing concentration of acetonitrile, at least 25% DMF was necessary to dissolve TPB-Suc in the reagent solution. Therefore, TPB-Suc was dissolved in acetonitrile–

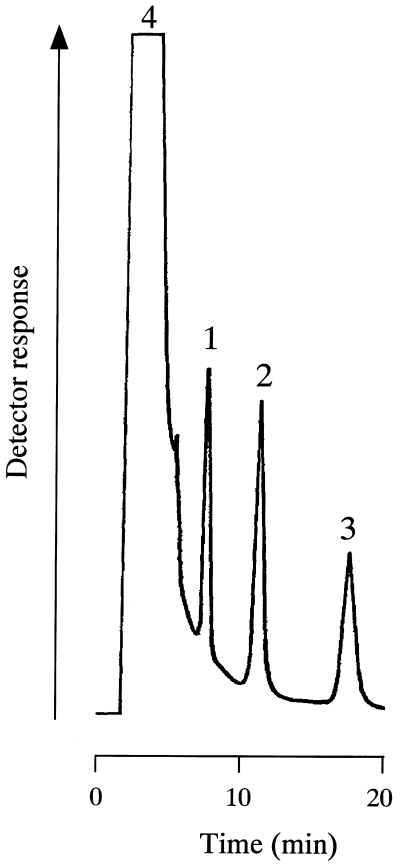

Fig. 3. Chromatogram of the TPB derivatives of amines. Peaks and amounts (fmol per injection volume): 1=methyl-*n*-octylamine (1000); 2=*n*-nonylamine (400); 3=*n*-decylamine (400); 4= reagent blank.

DMF (3:1, v/v) and triethylamine was prepared in acetonitrile.

Maximum and constant peak heights were obtained at concentrations of TPB-Suc solution higher than 2.0 m*M* for both primary and secondary amines; 2.5 m*M* was adopted.

It is well known that the derivatization reaction between amines and *N*-hydroxysuccinimidyl group is accelerated in the presence of a base catalyst. Triethylamine, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, pyridine, quinuclidine, potassium carbonate and sodium hydroxide were examined as catalysts, and triethylamine was the most effective for all the amines examined. The maximum reaction rate was attained at 40–100 m*M* triethylamine; 50 m*M* was recommended in the procedure.

The derivatization reaction occurred at a temperature above 40°C; higher temperatures allowed the reaction to proceed more rapidly. However, temperatures higher than 120°C caused reduction of the CL intensity, probably due to the decomposition of the produced TPB derivatives. The reaction of TPB-Suc with primary amines proceeded rapidly under relatively mild conditions, but with secondary amines it required higher temperatures and a prolonged reaction time. For the simultaneous derivatization of primary and secondary amines, heating at 80°C for 20 min was used as an optimum.

The TPB derivatives of amines in the final mixture were stable, and still gave constant CL intensities after standing for at least 12 h in daylight at room temperature and for 5 days in the dark at 4°C.

3.3. Chemiluminescence reaction of TPB derivatives

The optimum conditions for the CL reaction of the TPB derivatives of amines were examined by setting the flow-rates of the solutions of hydrogen peroxide and potassium hexacyanoferrate(III) at 1.0 and 2.0 ml/min, respectively.

The CL intensities of the TPB derivatives were greatly influenced by the concentrations of hydrogen peroxide, potassium hexacyanoferrate(III) and sodium hydroxide. Their concentrations were varied one at a time to establish the maximum obtainable intensity. Based on these experiments (Fig. 4), 20 m*M* hydrogen peroxide, 10 m*M* potassium hexacyanoferrate(III) and 1.5 *M* sodium hydroxide were selected for optimum and reproducible results.

The CL of TPB derivatives is generated immediately after the addition of the second oxidizing reagent solution to the effluent from the column after passing by the first mixing device. Therefore, the length of tubing between the second mixing device and the detector had a great influence on the CL responses. The peak heights for all amines and the reagent blank were increased by shortening the length of the tubing. On the other hand, the length did not affect the background levels. Therefore, a 5-cm tubing (0.5 mm I.D.) was utilized as the shortest practicably useful length. This indicates that

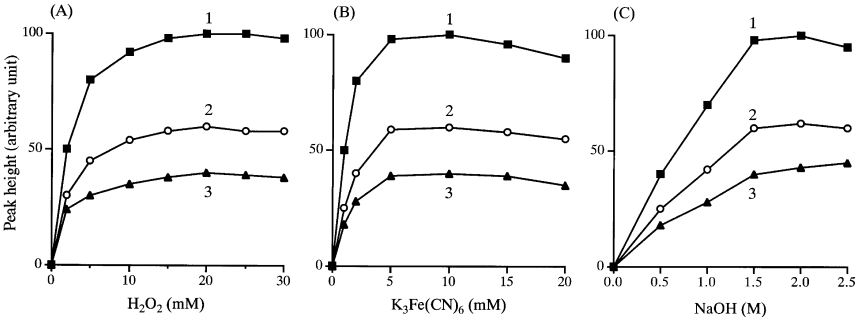

Fig. 4. Effects of the concentrations of (A) hydrogen peroxide, (B) potassium hexacyanoferrate(III) and (C) sodium hydroxide on the CL peak heights. Curves: 1=*n*-nonylamine; 2=*n*-decylamine; 3=methyl-*n*-octylamine.

the CL reaction of the TPB derivatives proceeds very rapidly (passing between the second mixing device and the detector cell was approximately 0.15 s), and is complete within a short period.

3.4. Calibration graph, precision and detection limits

The relationships between the amounts of individual amines and the peak heights were linear over a concentration range of 0.8–8000 fmol per 20 μl injection volume, which corresponded to 0.1–1000 pmol/ml in a sample solution. The linear correlation coefficients were more than 0.999 ($n=3$) for three amines. The between-day precision was established by repeated determinations ($n=8$) using the mixtures of standard amines (4.0 fmol and 4.0 pmol each per 20 μl injection volume); the relative standard deviations were within 4.8% and 2.9%, respectively, for all the amines examined.

The detection limits (amol per 20 μl injection volume, signal-to-noise ratio=3) were 560 (methyl-*n*-octylamine), 210 (*n*-nonylamine) and 410 (*n*-decylamine). The sensitivities of this method to primary amines are at least 10-times higher than those of luminol-type CL methods using ILITC [11] and IPO [12], and to secondary amines are 2–5-times greater than those with ABEI [9,10] and IPO. The novel CL derivatization reagent, TPB-Suc, has almost the same sensitivity as those used in peroxyoxalate CL methods [1–4].

3.5. Reaction of other substances with TPB-Suc

Aliphatic primary and secondary amines, such as amino acids (L-phenylalanine and L-isoleucine) and important short chain amines (β-phenethylamine, histamine and epinephrine), were found to react with TPB-Suc to give the corresponding CL derivatives and their retention times were 2–5 min under the present LC conditions. The TPB derivatives could not be separated completely from the reagent blank component(s) (peak 4 in Fig. 3). Therefore, further refined optimizations of LC conditions or some clean-up procedures including a column-switching technique and/or solid-phase extraction to remove the excess reagent blanks should be required for the determinations of above-mentioned short chain and hydrophilic amines. These studies are now in progress.

Other biologically important substances or environmental chemicals did not produce CL under the recommended procedure at a concentration of 10 nmol/ml. The compounds tested were triethylamine, pyridine, acetylcholine, acetic acid, palmitic acid, ascorbic acid, phosphoric acid, α-ketoglutaric acid, *N*-acetylneuraminic acid, urea, acetone, ethanol, phenol, *n*-decylaldehyde, benzaldehyde, cholesterol, D-glucose, D-fructose, D-ribose and sucrose. These observations suggest that the present CL derivatization method is selective for primary and secondary amines, especially hydrophobic amines.

3.6. Analysis of amantadine in human plasma

Amantadine hydrochloride, 1-tricyclo[3.3.1.$1^{3,7}$]-decylamine monohydrochloride, has been widely used as the first choice drug for Parkinson's disease. For the safety and effective administration of the drug to patients, information concerning the phar-

macokinetic analysis from patients having various types of clinical backgrounds are helpful. Thus, in order to investigate the practicality of TPB-Suc in biological analysis, the present method was applied to the determination of amantadine in human plasma. Amantadine and AEA (I.S.) are so hydrophobic that their TPB derivatives did not elute from the column under the separation conditions as described in Section 2.5. Therefore, more a hydrophobic mobile phase, aqueous 55% (v/v) acetonitrile, was used. Fig. 5 shows typical chromatograms obtained with drug-free plasma, the same plasma spiked with amantadine and AEA, and the plasma after administration of amantadine hydrochloride. The peaks for amantadine and AEA were identified on the basis of their retention times in comparison with standard compounds and co-chromatography with the standards. TPB-Suc gave also a simple chromatogram concerning biological analysis.

The absolute recoveries of amantadine and AEA added to the pooled normal human plasma (200 pmol/ml) were 74.3±3.7 and 75.3±3.8% (mean±standard deviation; $n=5$), respectively, and the drug concentration in the sample was 540 pmol/ml plasma. These values are in good agreements with those obtained by a previously reported fluorometric LC method [23]. These results indicate that this pre-column derivatization LC–CL method using TPB-Suc can be useful for biological substances having primary and/or secondary amino group(s).

4. Conclusion

TPB-Suc was developed as a novel luminol-type CL derivatization reagent for the highly sensitive and selective determination of primary and secondary amines in LC. Because this LC–CL method could be applied to the measurement of amantadine in human plasma, it would be useful to the analysis of biological substances and drugs having primary and/or secondary amino group(s). Furthermore, other CL derivatization reagents containing other reaction sites for carboxylic acids, alcohols, thiols and so forth, would be expected based on the highly chemiluminogenic 2-arylbenzothiazole skeleton obtained from ASPH and corresponding aromatic aldehydes.

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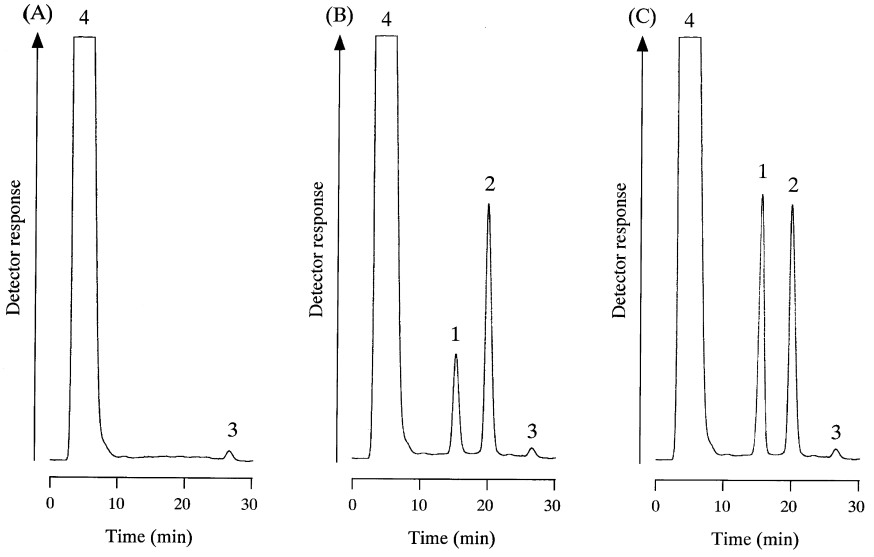

Fig. 5. Chromatograms obtained with plasma samples. Samples: (A) drug-free plasma without I.S.; (B) plasma spiked with amantadine (200 pmol/ml plasma); (C) plasma sample at 2 h after a single oral administration (50 mg) of amantadine hydrochloride. Peaks: 1=amantadine (C, 540 pmol/ml plasma); 2=AEA; 3=endogenous amino compound; 4=reagent blank and/or biogenic amino compounds.

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