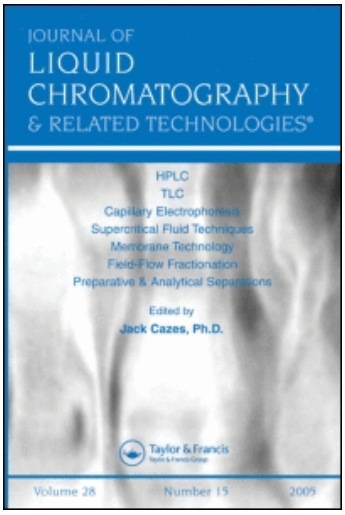

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Determination of Amino Acids by High-Performance Liquid Chromatography with Electrochemical Detection Using Ferrocene Derivatization Reagents

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DETERMINATION OF AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION USING FERROCENE DERIVATIZATION REAGENTS

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ABSTRACT

Two new reagents possessing ferrocene as an electrophore and isothiocyanate reactive toward the amino group were prepared and evaluated for pre-column derivatization of amino acids in high-performance liquid chromatography with electrochemical detection. The utilities of these reagents were investigated employing glycine as a model compound. Ferrocenylisothiocyanate was more favorable with respect to reactivity and electrochemical properties. The newly developed method was applied to the determination of 4-aminobutyric acid in biological specimens.

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) is a useful method for the determination

of trace components in complex matrices because of its excellent selectivity and sensitivity [1]. In recent years, various pre- and post-column labeling methods have been developed to extend its applicability [2-5]. In previous studies of this series, we proposed a novel ferrocene reagent having an active ester for pre-column labeling of amines [5]. The method was applied to the determination of ornithine decarboxylase activity by measuring putrescine enzymatically formed. As the ferrocene derivative undergoes facile oxidation and the product is in turn readily reduced, it can be detected selectively in the presence of other electroactive compounds, such as phenols, catechols and aromatic amines. The reaction with amines proceeded quantitatively to reach a plateau in 1 h, but the reactivity with amino acids was not satisfactory. These data prompted us to prepare derivatization reagents for amino acids in HPLC/ED having an isothiocyanate group reactive toward the amino group in amino acids [6] and a ferrocene moiety highly responsive to an electrochemical detector. This paper also describes the application of the newly developed method to the determination of 4-aminobutyric acid (GABA) in biological specimens.

MATERIALS AND METHODS

Materials

(Dimethylaminomethyl)ferrocene was purchased from Aldrich Co. (Milwaukee, WI, U.S.A.). Ferrocenecarboxylic acid was supplied by Tokyo Kasei Kogyo Co. (Tokyo, Japan). Amino acids were kindly supplied by Ajinomoto Co. (Tokyo). α-Ketoglutaric acid and GABAse were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) and Boehringer Mannheim Yamanouchi Co. (Tokyo),

respectively. All other reagents and chemicals were purified by recrystallization or distillation prior to use. Silica gel 60 and silica gel HF_{254} (E. Merck AG, Darmstadt, F.R.G.) was used for column chromatography and thin layer chromatography (TLC), respectively.

Instruments

HPLC was carried out on a TOSOH CCPD chromatograph (TOSOH Co., Tokyo) equipped with an EICOM ECD-100 amperometric detector (EICOM Co., Kyoto). Glassy carbon and Ag/AgCl electrodes were used as a working electrode and a reference electrode, respectively. A TSKgel ODS-80TM (5 μm) column (15 x 0.4 cm i.d.)(TOSOH Co.) was used at a flow rate of 1 ml/min at ambient temperature. The void volume was determined by the use of $NaNO_2$. Fluorescence was measured on Shimadzu RF-5000 (Shimadzu Co., Kyoto).

Preparation of derivatization reagents

Melting point was measured on a micro hot-stage apparatus (Yanagimoto Mfg Co., Kyoto) and was uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on JEOL FX-100 (JEOL Co., Tokyo) at 100 MHz using tetramethylsilane as an internal standard (IS). Abbreviations used are s=singlet and t=triplet. Low- and high-resolution mass (MS) spectral measurements were run on Hitachi M-80 (Hitachi Co., Tokyo). Infrared (IR) spectra were measured on JASCO A-202 (JASCO Co., Tokyo).

Ferrocenylisothiocyanate (I)

Ferrocenylamine (300 mg), obtained from ferrocenecarboxylic acid [4], was suspended in carbon disulfide/H_2O (2:3; 1.5 ml) and

stirred for 5 min under ice-cooling. After addition of 83% KOH (0.45 ml), the whole was kept at 100° C for 25 min in a sealed tube. Chloroethyl formate (0.48 ml) was added to the reaction mixture under ice-cooling and then kept at room temperature for 1 h. The mixture was further stirred with 67% KOH (0.45 ml) for 1 h and then extracted with ether. The organic layer was washed with H_2O, dried over Na_2SO_4 and then evaporated down. The residue was subjected to column chromatography (10 x 1.1 cm i.d.) and the eluate with benzene was dried to give ferrocenylisothiocyanate (I) as a dark red oily substance (10 mg). High-resolution MS m/z: 242.8716 (M^+)(Calcd. for $C_{11}H_9FeNS$, 242.8737). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2120 (NCS). ^{1}H-NMR ($CDCl_3$) δ: 4.08 (2H, t, J=2 Hz, 3, 4-H), 4.30 (5H, s, 1'-5'-H), 4.43 (2H, t, J=2 Hz, 2, 5-H). The purity of I was confirmed by TLC (Rf 0.44; n-hexane/benzene (10:1)) and HPLC (t_R 10.0 min; acetonitrile/1.5% NaOAc (pH 3.0)(2:1)).

2-Ferrocenylethylisothiocyanate (II)

2-Ferrocenylethylamine (120 mg), obtained from (dimethylaminomethyl)ferrocene [4], was treated with carbon disulfide as described above. The residue was subjected to column chromatography (10 x 1.1 cm i.d.) and the eluate with benzene was dried to give 2-ferrocenylethylisothiocyanate (II) as dark red prisms (5 mg). mp 43-45° C. High-resolution MS m/z: 270.9197 (M^+)(Calcd. for $C_{13}H_{13}FeNS$, 270.9237) IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1960 (NCS). ^{1}H-NMR ($CDCl_3$) δ: 2.74 (2H, t, J=7 Hz, ferrocenyl-CH_2), 3.60 (2H, t, J=7 Hz, -CH_2NCS), 4.13 (9H, s, ferrocenyl H). The purity of II was confirmed by TLC (Rf 0.83: benzene) and HPLC (t_R 15.5 min: acetonitrile/1.5% NaOAc (pH 3.0)(3:2)).

Derivatization of amino acids

Each derivatization reagent (ca. 37 eq.) in acetonitrile (0.1 ml) was added to a solution of amino acid (1 μg) in acetonitrile/H_2O (1:1; 0.1 ml) containing triethylamine (0.2 μl). The whole was kept at 70° C in a capped tube and an aliquot of the solution was subjected to HPLC/ED. The derivatization rate was determined by comparison of the peak area of the ferrocene equivalent to the amino acid used.

Procedure for the determination of GABA in brain

Male Wistar strain rats and Hartley strain guinea pigs weighing respectively, 150-200 g and 400-600 g, were used. Fresh cerebrum or cerebellum (0.33 g) was homogenized with ice-cooled 10% $HClO_4$ (4.8 ml). 5-Aminovaleric acid (IS; 5 μg) in 10% $HClO_4$ (0.5 ml) was added to 0.5 ml of the homogenate. After centrifugation at 1500 g for 15 min, 0.1 ml of the supernatant was taken into an assay tube, alkalized with triethylamine (ca. pH 8.0) and then derivatized with I at 70° C for 30 min. After the derivatization reaction, the excess reagent was extracted with ether and an aliquot of the aqueous layer was subjected to HPLC/ED. Protein was determined by the method of Lowry et al. [7] using bovine serum albumin as a reference.

Enzymic method using α-ketoglutaric acid and GABAse followed by fluorometric detection was done according to the procedure described by Graham et al. [8].

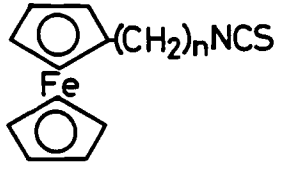
Recovery test for GABA in brain

The guinea pig brain (cerebrum) was homogenized with 10% $HClO_4$ and centrifuged as described above. The precipitate was

extracted several times with 10% $HClO_4$ to remove endogeneous amino acids. The remaining precipitate was homogenized with 10% $HClO_4$ and used as a control assay homogenate (250 μg protein/0.1 ml). The spiked samples were prepared by addition of 1 μg or 5 μg each of GABA to a control assay homogenate (0.1 ml). Pretreatment and derivatization with I followed by HPLC/ED were carried out in the manner described above.

RESULTS AND DISCUSSION

The design of promising derivatization reagents for amino acids in HPLC/ED requires two structural features, viz., a functional group reactive toward amino acids and an electrophore highly responsive to an electrochemical detector. In this study, two isothiocyanates (I, II) possessing ferrocene as an electrophore were prepared. Ferrocenylisothiocyanate (I) and 2-ferrocenylethylisothiocyanate (II) were synthesized from ferrocenylamine and 2-ferrocenylethylamine, respectively (Fig. 1). The reactivities of I and II besides N-succinimidyl 3-ferrocenylpropionate [5] toward amino acids were examined employing glycine as a model compound. Derivatization of glycine



I : n=0
II : n=2

FIGURE 1. Structures of derivatization reagents for amino acids.

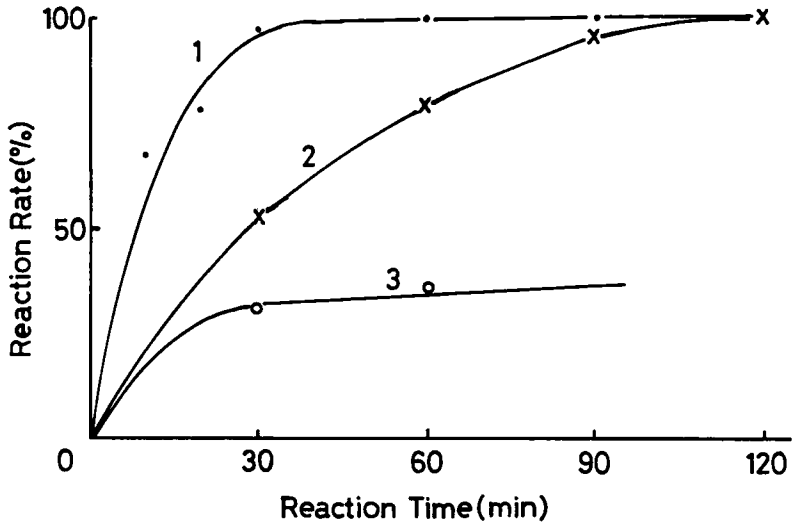


FIGURE 2. Time course for derivatization of glycine.
1. I; 2. II; 3. N-succinimidyl 3-ferrocenylpropionate
The peak area of the ferrocene equivalent to glycine was taken as 100%.

with the former two was performed in acetonitrile/water/triethylamine at 70° C. The reactions with I and II proceeded almost quantitatively to reach a plateau in 30 min and 90 min respectively, while the reaction rate with the active ester was not over 40% under the condition previously reported [5](Fig. 2). The derivatives with the former two reagents were stable at room temperature for at least one day. The electrochemical properties of the derivatives formed with I and II were investigated with an amperometric detector.The hydrodynamic voltammograms of these derivatives are illustrated in Fig. 3. The derivative with I exhibited two-step oxidation, due to ferrocenyl (+0.45 V) and thioureido ($\geq$ +0.60 V) residues,

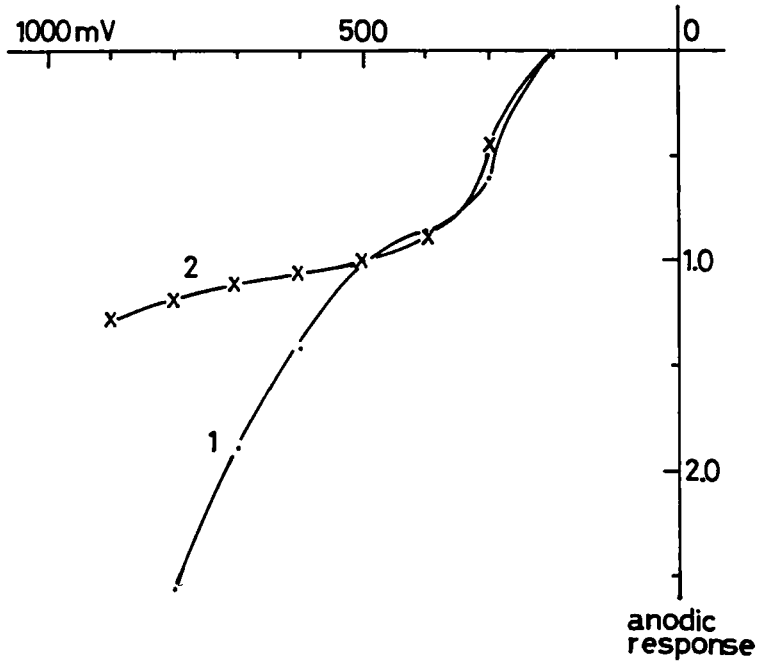


FIGURE 3. Hydrodynamic voltammograms of glycine derivatives.
1. I; 2. II
The response of each compound at +0.50 V was taken as 1.0.

while that with II exhibited only a slightly increasing value above +0.50 V. The thioureido residue directly linked to the ferrocene in derivative with I would be more easily oxidized than that of derivative with II. The characteristic hydrodynamic voltammogram of the former is helpful to identify the peaks. It is evident from these data that I is more favorable with respect to the reactivity and electrochemical properties.

The utility of I was then tested for the determination of GABA in guinea pig or rat brain. On treatment with I at 70° C, GABA and 5-aminovaleric acid (IS) were derivatized in 30 min.

TABLE 1

Capacity Factors (k') of Main Amino Acids Known to Be in the Brain[a]

Amino acid	k'	Amino acid	k'
Glutamine	0.25	Tyrosine	1.78
Lysine	0.25	GABA	2.67
Aspartic acid	0.44	IS[b]	3.50
Glutamic acid	0.47	Isoleucine	6.94
Glycine	0.89	Leucine	7.22
Alanine	1.28		

[a] Conditions; mobile phase, acetonitrile/0.5% NaOAc (pH 4.0)(2:3); applied potential +0.50 V. t_0 was 1.8 min. The k' value of taurine was 0.28 under these conditions.
[b] 5-Aminovaleric acid.

Derivatives of these two and other main amino acids known to be in the brain [9-14] were well separated by HPLC on TSKgel ODS-80TM using acetonitrile/0.5% sodium acetate (pH 4.0) as a mobile phase (Table 1). The brain was homogenized with 10% perchloric acid and after centrifugation, the supernatant was subjected to derivatization with I followed by HPLC/ED. A typical chromatogram is illustrated in Fig. 4. The peak with retention time 6.2 min was unequivocally identified as GABA derivative by comparison with the authentic sample in chromatographic behaviors using methanol/1.5% sodium acetate (pH 3.0)(1:1)(t_R 9.5 min) and hydrodynamic voltammogram. When the amount ratio of GABA to IS (0.5 μg/tube) was plotted against the peak height ratio, a linear relationship was observed in the range of 0.1-2.5 μg/tube, the regression equation being y=2.61x. In addition, GABA added to a

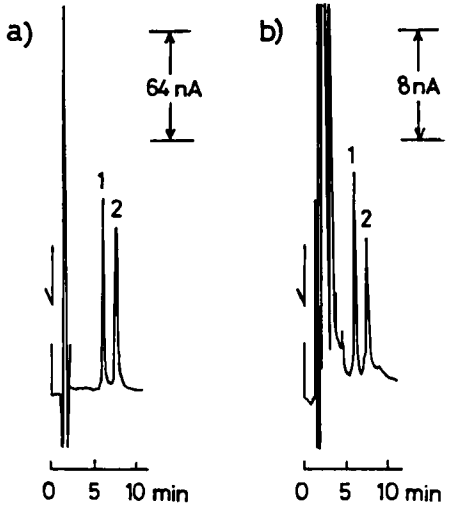


FIGURE 4. High-performance liquid chromatograms of GABA derivatives.
a) Authentic sample; b) sample from rat cerebellum
1. GABA; 2. IS
Conditions: mobile phase, acetonitrile/0.5% NaOAc (pH 4.0)(2:3), applied potential +0.50 V.

control assay homogenate at two levels (1.0 and 5.0 μg) showed recoveries of $\geq$ 85.0% (relative standard deviation$\leq$ 3.4%, n$\geq$8). The recovery rate of IS (1 μg/control assay homogenate) was 95.5% (relative standard deviation=2.8%, n=10). As illustrated in Fig. 5, the values obtained by HPLC/ED were in good accord with those by enzymic method with fluorometric detection [8]. These results demonstrate that the proposed method is satisfactory with respect to accuracy and precision. The concentrations of GABA in cerebrum (guinea pig) and cerebellum (rat) were found to be 2.67 μmol/g wet tissue (106 μmol/g protein; n=2) and 1.67 μmol/g wet tissue (56 μmol/g protein; n=2), respectively.

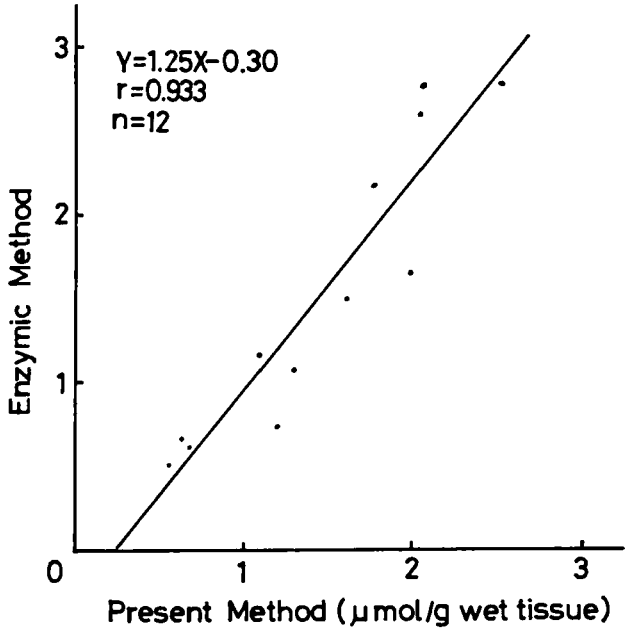


FIGURE 5. Correlation of GABA levels in guinea pig or rat brain determined by the present method and enzymic method.

HPLC using derivatization with O-phthalaldehyde (OPA) followed by fluorometric or electrochemical detection is popular for the determination of GABA in biological specimens [11-14]. The derivative with OPA is unstable [9] and the detection limits are 0.6 and 2 pmol for fluorometric [14] and electrochemical detection [11], respectively. On the contrary the detection limit of the proposed method was 0.05 pmol at the applied potential of +0.50 V (signal to noise ratio of 5) and derivative is stable as described above. Pre-column derivatization with I appears to be of great use for the quantitation of picomol level of amino acids by HPLC/ED. Further applications of the proposed method to the

determination of amino acids in biological fluids are being conducted and the details will be reported elsewhere.

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