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Determination of the amino acid neurotransmitters in the dorsal root ganglion of the rat by capillary electrophoresis with a laser-induced fluorescence-charge coupled device

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Abstract

A sensitive, rapid and accurate analysis of the amino acid neurotransmitters in dorsal root ganglion (DRG) of rat was accomplished by capillary electrophoresis with a laser-induced fluorescence-charged coupled device (LIF-CCD) and fluorescein isothiocyanate (FITC) derivatization. Since the CCD is an image detector with a matrix array of photoelements, the photogenerated charge generated from single photoelements was summed up by on-chip charge binning, and used to improve the detection sensitivity. A self-compiled data processing program was used to calculate digitally the summation of the multiwavelength data and transform three-dimensional electropherograms into two-dimensional electropherograms. Use of this program improved the detection sensitivity, the extraction of useful statistical data and the performance of the quantitative analysis. Owing to its excellent detectability and the unique characteristic of its two-dimensional format, which allows to perform analog and digital summation, high detection sensitivity with detection limits ranging from 1.2 to 17.2 n*M* was obtained for the amino acid neurotransmitters by LIF-CCD. This method was demonstrated to be a powerful tool for analyzing the complex biological samples through quantitative determination of the six amino acid neurotransmitters in the DRG of rat. © 1998 Elsevier Science B.V.

Keywords: Amino acids

1. Introduction

Capillary electrophoresis (CE) is a newly emerging technique for rapid and high-resolution separation of biomolecules [1,2]. Recently, it has become a popular method for the separation and determination of amino acids [3,4]. In CE separation of amino acids, the detection methods include UV–Vis [5], LIF [6], amperometry [7,8] and laser thermo-optical absorbance [9]. However, LIF is of particular interest since it offers the highest sensitivity. Application of CE–LIF to the analysis of amino acids is dramatically increasing due to its high efficiency and sensitivity.

Though many publications deal with CE separation of amino acids, only a few studies have been reported on the analysis of amino acid neurotransmitters. With the developments in neuroscience, some amino acids have been shown to play an important role in the nervous system. They are

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usually classified into excitatory and inhibitory amino acids according to their different functions in the nervous system [10]. Excitatory amino acids include glutamate and aspartate, while inhibitory amino acids include glycine and γ-amino-*n*-butyric acid. Recently, analysis of these amino acids in neurobiological samples using CE methods has been of great interest. For example, CE–UV–Vis has been used to analyze the amino acid neurotransmitters in rat brain [11]. CE–LIF has been used to analyze amino acid neurotransmitters in microdialysates [12] and perform in vivo monitoring [13,14]. In addition, CE–amperometry has also been used to determine the amino acid neurotransmitters in brain and monitor excitatory amino acid release coupled with microdialysis [15,16]. However, to date, analysis of these amino acid neurotransmitters using CE–LIF-CCD has not been reported. CCD is a spectroscopic array detector with high sensitivity, wide response range and multidimensional format [17]. Use of CCD as the detector for CE–LIF can provide a three-dimensional electropherogram with a complete fluorescence spectrum for the analyte, and this can be used to perform the wavelength resolution of the fluorescence emission and improve the detector ability [18]. Moreover, since CCD is an image detector with a matrix array of photoelements, the CCD readouts from single photoelements can be summed up by on-chip analog summation called charge binning, or by digital summation in the computer [17,19,20]. Hence, the detection sensitivity can be improved dramatically.

The dorsal root ganglion (DRG), where the primary sensory neurons are located, is the site for the input and adjustment of the primary sensory afferent information. In our previous reports, the DRG neuron was used as a model to study the properties of receptors and investigate the modulation of afferent information in primary sensory neurons using a whole-cell patch-clamp technique [21–23]. Analysis of the amino acid neurotransmitters in the DRG contributes to the explanation of the probable mechanisms underlying the primary afferent information. However, analysis of these amino acids is difficult, because the components in the DRG are very complex, and DRG tissue is very small, producing very small amounts of neurotransmitters. Classical methods such as histochemistry or immunohistochemistry were often used to perform the qualitative analysis of neurotransmitters in nerve tissues [24]. However, these methods seem inconvenient and cannot provide quantitative results very accurately. On the contrary, CE is a simple and useful method to perform the quantitative determination of amino acid neurotransmitters owing to its high resolution and speed.

In this paper, a rapid, sensitive and quantitative analysis of the amino acid neurotransmitters in DRG, using CE with LIF-CCD and FITC derivatization, is presented for the first time. Six amino acid neurotransmitters were determined accurately through the use of an internal standard. A self-compiled program compiled with MACRO language was applied to calculate the summation of the multiwavelength data and transfer the three-dimensional electropherogram into a two-dimensional electropherogram, which permits it to extract useful statistical data and perform the quantitative analysis. By the use of analog and digital summation, the detection sensitivity was improved greatly. Average detection limits in the n*M* range were obtained for the amino acid neurotransmitters.

2. Experimental

2.1. CE apparatus

The CE system was built in-house. A high-voltage power supply (0–30 kV, Huazhong University of Sci. and Tech., China) was used to provide the separation voltage for electrophoresis. The electrical connections were made at both ends of the capillary with platinum wires. Experiments were carried out using a 75 μm I.D.×375 μm O.D. fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, China). Before use the capillary was washed with 0.1 *M* NaOH, deionized, double-distilled water and the separation buffer, using a self-constructed gas-pressure apparatus. The capillary length was 51 cm, and a short section (ca. 3 mm) of the polyimide coating was removed to form a detection window at a point 5 cm from the cathodic end of the capillary by burning the coating with a flame and wiping it with a wet sponge to minimize scratching of the capillary. The typical electrophoretic buffer was 15 m*M* borate at

pH 9.2, and the electrophoretic current was typically 21.6 μA. Sample introduction was accomplished by siphoning at 10 cm height for 10 s. All CE experiments was conducted at room temperature (25°C).

2.2. Detection apparatus

A 488-nm air-cooled argon ion laser (40 mW, Ion Laser Technology, Shanghai, China) was used as the fluorescence excitation source for the LIF detection system. The laser beam was focused with a 10× lens (Shanghai Optical Corp., Shanghai, China), which was mounted on a three-axis stage, on the detection window of the capillary. The fluorescence emission was collected at right angles to the excitation source, and focused with two lenses on the spectrograph entrance slit of a polychromator (HR320, Instruments, SA, Inc., USA) which has a high dispersion with 1200 grooves/mm grating.

A thermoelectrically cooled CCD (Model 1530-P, EG&G, Princeton Applied Research, USA) was then used to detect the grating-dispersed fluorescence signal. The CCD photosensitive region consists of 512×512 photoelements (pixels). Each element measures 19×19 μm, while the full image zone is 9.7×9.7 mm. Since the grating diffracted the fluorescence signal along the 512 pixels, an interval of ~200 nm could be imaged with a spectral distribution of ~0.4 nm/pixel. No binning was used in the wavelength dimension, i.e., a serial binning of 1 was selected. Hence, each CCD readout produced 512 point spectra and no spectral information was lost. However, charge binning [19,20] in the spatial dimension was employed to improve the detection sensitivity. Since the fluorescence signal was mainly distributed from row 50 to 450 in the spatial dimension, parallel binning of 400 was used.

The CCD chip was cooled with a Peltier device to −40°C to reduce the dark current and background noise. A 80486 Gateway 2000 computer was used to read out CCD and store data. The pixel information from CCD was transferred via the fiber optic link to the Model 1564 OMA 4 Controller board in the computer, where it was processed for display, manipulation and storage. Data acquisition and CCD operation parameters were controlled with the application software OMA SPEC 4000. The MACRO language provided by this application software was used to compile a subprogram to calculate the summation of the multiwavelength data and transfer three-dimensional electropherograms into two-dimensional electropherograms.

2.3. Reagents

Unless stated otherwise, all chemicals were of analytical-reagent grade (Shanghai Reagent Corp., Shanghai). γ-Amino-*n*-butyric acid (GABA) and γ-Glu–Gly were purchased from Sigma (St. Louis, MO, USA). Alanine (Ala), aspartate (Asp), glutamate (Glu), glycine (Gly), taurine (Tau) were obtained from Shanghai Biochemical Reagent Corp. (Shanghai, China), and FITC isomer I from Beijing Reagent Corp. (Beijing, China). The 10 m*M* stock solutions of the amino acids and γ-Glu–Gly were prepared in borate buffer and diluted to the desired concentration prior to use. The 50 m*M* stock solution of FITC was prepared in acetone, with traces of pyridine (0.002%, v/v) used to catalyze the derivatization reaction, and stored at 4°C in the dark. Borate solutions were prepared by dissolution of $Na_2B_4O_7 \cdot 10H_2O$ in double-distilled water, and the pH values were adjusted with 1.0 *M* H_3BO_3 or NaOH. All solutions were prepared by double-distilled, deionized water and filtered through a 0.22-μm cellulose acetate filter (Shanghai Institute of Medicine and Industry, China).

2.4. Precolumn derivatization of standard amino acids

Precolumn derivatization of standard amino acid solutions was conducted in microvials. A final volume of 1.0 ml was used for each experiment. After appropriate amounts of the amino acid solutions were diluted and mixed in borate buffer (15 m*M*, pH 9.2), FITC was added to give a final concentration 30 times higher than the total concentration of amino acids. Typically, the analytes were derivatized with FITC for 6 h at 40°C. The reactants were then stored in the dark at 4°C before use.

2.5. Sample treatment and derivatization

After a rat was decapitated, the vertebrate column in thoracic and lumbar segments was dissected out

and longitudinally divided into two halves along the median lines on both dorsal and ventral sides. The DRG was then taken out using dissecting forceps. After the attached nerves and surrounding connective tissues were removed, this tissue (~2 mg) was minced with iridectomy scissors, and homogenated in borate buffer (15 m*M*, pH 9.2) for 20 min.

The homogenate was then acidified with concentrated perchloric acid, and finalized to 500 μl. After a centrifugation at 15 000 *g* for 10 min, 100 μl of the supernatant was transferred to a 500-μl microvial. This was followed by the addition of 25 μl of 1.0 m*M* γ-Glu–Gly and 350 μl of borate buffer (15 m*M*, pH 9.2). Finally, 25 μl of 50 m*M* FITC solution was added to give a final concentration of 2.5 m*M*. The mixture was then allowed to react at 40°C for 6 h. After derivatization, the sample was stored in the dark at 4°C.

3. Results and discussion

3.1. Precolumn derivatization conditions

Precolumn derivatization of amino acids was conducted in borate buffer, because borate buffer can easily provide the optimum pH value for the derivatization reaction, and the concentration of the borate buffer has little influence on the derivatization efficiency [25]. Furthermore, it can maintain a compatible supporting electrolyte with that in the separation capillary.

The reaction temperature influences the derivatization efficiency greatly. Elevation of the reaction temperature contributes to improving the fluorescence intensities of FITC-derivatized amino acids in CE–LIF [25]. The effect of reaction temperature on the derivatization efficiency was tested with equimolar (5.0 μ*M*) standard amino acids at room temperature (25°C) and an elevated temperature of 40°C. No CE signal was observed when derivatization reaction was controlled at room temperature, indicating that the reaction was incomplete. While, at 40°C, well-defined electrophoretic peaks were obtained for the analytes. Comparison with the two electropherograms obtained at different temperatures, inferred that an elevated temperature helped to get higher derivatization efficiency. Better detection sensitivity with an average 100-fold enhancement was obtained.

The derivatization efficiency was also largely influenced by the concentration of FITC. Though low concentrations of FITC resulted in few interfering electrophoretic peaks, the reaction was incomplete and the detection sensitivity was very low. As the FITC concentration increased, the derivatization efficiency became better, and when the concentration of FITC exceeded, by nearly 30 times, that of the total concentration of analytes, the maximum efficiency was reached and it became stable. In addition, trace pyridine was added and used to accelerate the derivatization reaction. With trace pyridine, the derivatization reaction reached equilibrium in about 6 h, while without pyridine 14 h was required. Furthermore, trace pyridine helped to stabilize the reaction products. In our experiments, the FITC-derivatized amino acids still remained stable 24 h after equilibrium was reached, and nearly identical CE results were obtained.

3.2. Separation conditions

Borate solution is usually used as the supporting electrolyte for the separation of amino acids by CE. This buffer gives a high and reproducible electroosmotic flow, and provides high efficiency and speed for the separation of amino acids. In our experiments, the initial stage to separate these amino acid neurotransmitters was to optimize the pH of the borate buffer. Though low pH values resulted in a short separation time, the resolution reduced and overlapped peaks of Tau and Gly were observed. While at high pH values, the currents in the separation capillary increased too much, resulting in an unsteady electroosmotic flow. Considering both the resolution and the separation time, the optimum pH value was chosen to be 9.2.

The influence of separation voltage on the separation of the six amino acid neurotransmitters was also investigated. At a voltage of 15 kV, Tau and Gly were poorly resolved since the separation time was too short. As the separation voltage decreased, the difference in retention time of the analytes was extended, indicating that low voltage was beneficial to improving the resolution. However, lower separation voltage led to longer separation time and

worse separation efficiency. The optimum separation voltage was chosen to be 12.5 kV.

Under the above separation conditions, the effect of borate concentrations on the separation was also investigated. With 7.5 m*M* borate buffer, the velocity of electroosmosis was low, resulting in long separation time and low efficiency. When 30 m*M* borate buffer was used, the current in the separation capillary was quite high and unfavorable for the dissipation of Joule heat from the high potential fields. Consequently, zone broadening from convection was enhanced, resulting in low separation efficiency. Hence, 15 m*M* borate at pH 9.2 was used as the typical electrophoretic buffer.

3.3. Separation and detection of amino acid standards by CE–LIF-CCD

The heart of the CCD detector is the CCD image sensor device. This solid-state sensor is composed of a matrix array of pixels [17]. For the Model 1530-P 512 CCD, the photosensitive array consists of 512 columns×512 horizontal rows of charge-coupled pixels. When it is exposed to a spectral source, i.e., the shutter is open, photons will strike the face of the array and generate a charge in the pixels. The photogenerated charge is collected during this exposure time, and then the shutter is closed to allow for transferring the charge. If no binning is taking place, the accumulated charge per pixel is then shifted one row at a time towards a serial transfer register (shift register), and read out by using the on-chip output amplifier. After a row has been completely shifted into the shift register, then amplified and converted, 512 readouts are acquired; each readout corresponds to a different spectral element. This process continues until all 512 rows have been completely read out. Thus, an integrated sample point (a CCD count) is completed. Therefore, as the CCD counts increase, i.e., the shutter opens and then closes continuously, a three-dimensional electropherogram of time–intensity–wavelength can be obtained by a CE system with a CCD multiwavelength detector [18,26,27]. Fig. 1 illustrates the three-dimensional electropherograms obtained for the standard analytes using CE-LIF-CCD under the optimum separation conditions described above. Charge binning in the spatial dimension (parallel binning) with 400 rows of charge

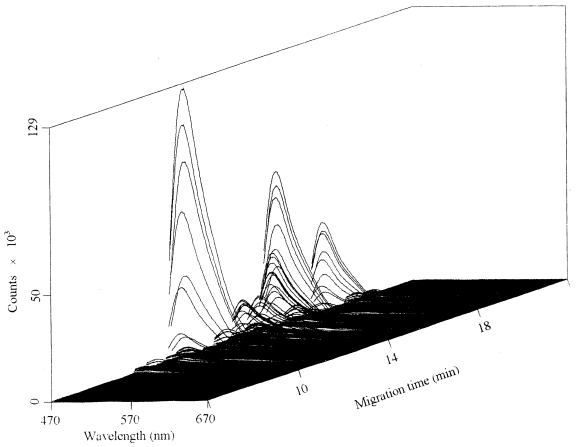

Fig. 1. Three-dimensional electropherogram of the equimolar standard analytes obtained by CE–LIF-CCD. Background electrolyte, borate buffer, 15 m*M*, pH 9.2; voltage, 12.5 kV; injection, 10 s at 10 cm. Amino acid concentrations, 5.0 μ*M*; γ-Glu–Gly concentration, 50 μ*M*.

packets combined was used in this study. Since binning is an analog summation accomplished by combining some photogenerated charge packets into a single charge packet on-chip, it will reduce the total readout noise and increase the S/N ratio, resulting in an enhancement of the detection sensitivity [19]. Compared to no parallel binning, about 20-fold increase of the S/N ratio was obtained through the parallel binning of 400.

Though the three-dimensional electropherogram can provide multiple useful information, it is inconvenient to perform the data processing under the three-dimensional mode. Hence, it is important to translate the three-dimensional electropherograms and extract the useful analytical information from the multidimensional data. Three-dimensional electropherograms can be translated into two-dimensional electropherograms of fluorescence intensity vs. retention time by extracting the data of a single wavelength. However, this method leads to a loss of the detection sensitivity, and cannot maintain the maximum response for each analyte, since the fluorescence spectra of each analyte are not completely identical. Three-dimensional electropherograms could also be transformed into two-dimensional electropherograms based on the principle of projection at the plane of fluorescence intensity vs. retention time. Fig. 2 is the corresponding two-dimen-

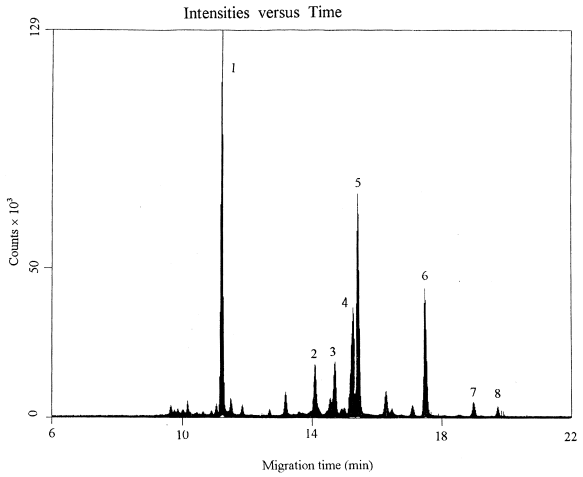

Fig. 2. Corresponding two-dimensional skiagraph of Fig. 1 based on the principle of projection at the plane of fluorescence intensity vs. retention time. All conditions as in Fig. 1. Peaks: (1) FITC, (2) GABA, (3) Ala, (4) Tau, (5) Gly, (6) γ-Glu–Gly, (7) Glu, (8) Asp.

sional skiagraph of Fig. 1. However, this projection method obviously affects the reproducibility and sensitivity of the detection. A low S/N ratio was observed from the two-dimensional skiagraph. Here, a calculation subprogram compiled with the MACRO language was presented, and used to sum all the multiwavelength data of each analyte, and obtain the two-dimensional electropherogram. In general, the noise for the photogenerated charge data acquired by CCD is random, and summation of the photogenerated charge data can make the random noise cancel each other out, while the fluorescence intensity is enhanced. Therefore, the S/N ratio is improved greatly. Fig. 3 illustrates the corresponding two-dimensional electropherogram of Fig. 1 after the multiwavelength data were processed. Audio-visual peaks and enhanced detection sensitivity were observed.

The migration times, the R.S.D.s of the migration times and the number of theoretical plates for the analytes are shown in Table 1. The number of theoretical plates was calculated from retention time and peak half-widths. It can be seen that high efficiencies were obtained for the separation of the amino acid neurotransmitters by CE.

Many factors affect the accuracy and reproducibility of CE quantitation. Using internal standards helps to overcome this problem. In this paper, γ-Gly–Glu was used as the internal standard to perform the quantitative analysis since its electrophoretic velocity is similar to that of the amino acid neurotransmitters, and its electrophoretic peak was well defined. The calibration curve was constructed by derivatizing amino acid standards at each concentration and normalizing the corresponding peak heights to that of the internal standard. Linear regression analysis gave linear correlation coefficients greater than 0.988 for the analytes. Detection limits for the analytes were obtained, from the electropherogram (not shown), of 0.1 μM equimolar amino acids at an S/N of 3. Table 2 lists linear correlation coefficients, detection limits for the analytes, and a comparison with the results in previous reports. It shows that high sensitivity was obtained through the use of LIF-CCD with FITC derivatization.

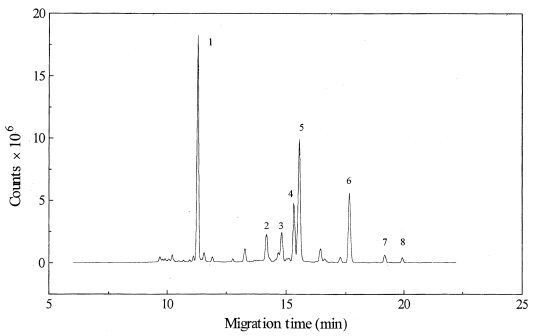

Fig. 3. Corresponding two-dimensional electropherogram of Fig. 1 with the data processed using the calculation program. All conditions as in Fig. 1. Peaks: (1) FITC, (2) GABA, (3) Ala, (4) Tau, (5) Gly, (6) γ-Glu–Gly, (7) Glu, (8) Asp.

Table 1
Migration times (t_m), R.S.D.s of t_m ($n=7$) and separation efficiencies (N, number of theoretical plates) for the six amino acids neurotransmitters

Analytes	t_m (min)	R.S.D. (%)	N
GABA	14.12	0.25	156 000
Ala	14.83	0.29	178 000
Tau	15.30	0.28	203 000
Gly	15.58	0.34	191 000
Glu	19.17	0.38	282 000
Asp	19.95	0.30	328 000

Table 2
Linear correlation coefficients (*r*) and a comparison of detection limits for the amino acid neurotransmitters in LIF-CCD with those in previous reports

Analytes	r (n=7)	Detection limits			
		LIF-CCD[a] (n*M*)	LIF[b] (n*M*)	ECD[c] (n*M*)	UV[d] (μM)
GABA	0.997	3.6	0.29	—	3.8
Ala	0.991	4.1	16	49	3.7
Tau	0.993	3.0	—	—	4.4
Gly	0.998	1.2	5.2	—	4.3
Glu	0.988	9.8	58	110	8.7
Asp	0.992	17.2	1.1	79	10.2

[a]FITC-labeled (this work).
[b]CBQCA-labeled [28].
[c]NDA-labeled [16].
[d]NDA-labeled [11].

3.4. Determination of amino acid neurotransmitters in DRG

Fig. 4 illustrates a typical three-dimensional electropherogram obtained for the homogenate of a DRG. The corresponding two-dimensional electropherogram is shown in Fig. 5 after the multiwavelength data were summed digitally. As marked in Fig. 5, six amino acid neurotransmitters were identified. Other amino acids in the biological sample also led to some electrophoretic peaks. However, these ordinary amino acids did not affect the identification of the amino acid neurotransmitters since they were observed to elute before the neurotransmitters (except serine which eluted between GABA and Ala) under the separation conditions described above.

Use of the internal standard and the calibration curves enabled quantitative analysis of the amino acid neurotransmitters in DRG. Six homogenates from six individual DRG were analyzed in this study and, for each homogenate, three parallel injections were performed to give the average results. The measured concentrations of the amino acids in the six homogenates are listed in Table 3 and the corresponding measured amounts in DRG are listed in Table 4.

In summary, we have demonstrated that CE-LIF-CCD is an efficient and sensitive method to perform the analysis of multiple components in complex

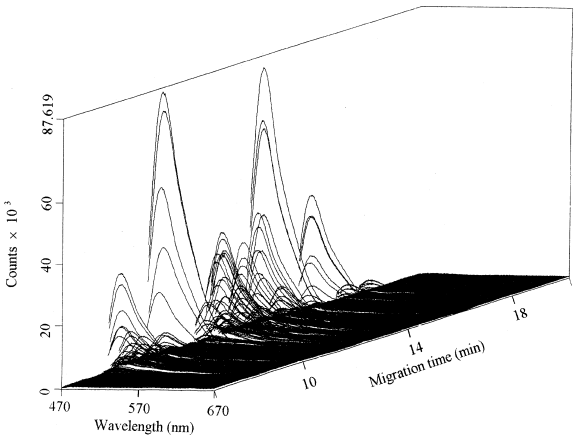

Fig. 4. Typical three-dimensional electropherogram obtained for the homogenate of a rat DRG. All conditions as in Fig. 1.

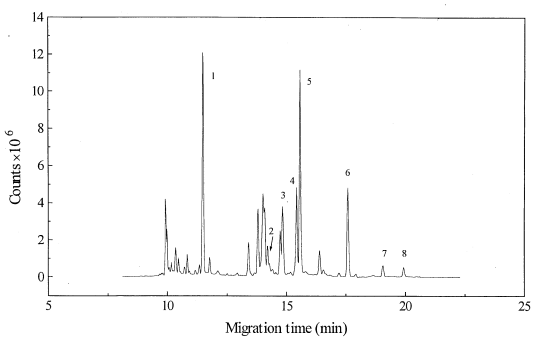

Fig. 5. Corresponding two-dimensional electropherogram of Fig. 4 with the data processed using the calculation program. All conditions as in Fig. 1. Peaks: (1) FITC, (2) GABA, (3) Ala, (4) Tau, (5) Gly, (6) γ-Glu–Gly, (7) Glu, (8) Asp.

Table 3
Determination of the amino acid neurotransmitters in the homogenates of six DRG samples

Analytes	In homogenate (μM)						
	1	2	3	4	5	6	Mean±S.D.
GABA	3.25(4.6)	2.87(2.3)	3.85(1.9)	3.72(2.8)	4.10(4.0)	3.46(5.1)	3.54±0.44
Ala	6.63(2.7)	5.25(2.0)	7.13(4.2)	9.63(3.1)	8.50(6.1)	6.75(3.5)	7.32±1.54
Tau	4.82(1.6)	4.22(3.9)	5.96(5.8)	5.90(5.1)	6.75(4.4)	3.92(3.1)	5.26±1.12
Gly	7.24(7.0)	6.82(2.6)	6.25(2.6)	6.53(2.4)	6.56(4.8)	5.91(4.2)	6.55±0.46
Glu	3.18(3.7)	4.09(4.5)	3.41(3.0)	5.45(6.3)	4.55(3.1)	3.46(2.8)	4.02±0.87
Asp	4.22(5.1)	4.22(3.3)	5.00(3.8)	6.39(4.7)	5.42(2.2)	4.82(2.0)	5.02±0.82

All measured values are the average of three runs for each sample. Shown in parentheses are the relative standard deviations (R.S.D. (%)) of three parallel determinations for the same sample. S.D., standard deviation for the six samples.

Table 4
Measured amounts of the amino acid neurotransmitters in six DRG samples

Analytes	In DRG (mg/g)						
	1	2	3	4	5	6	Mean±S.D.
GABA	0.361	0.345	0.463	0.362	0.395	0.454	0.397±0.050
Ala	0.636	0.546	0.740	0.810	0.707	0.764	0.701±0.095
Tau	0.649	0.617	0.869	0.697	0.789	0.623	0.707±0.102
Gly	0.586	0.598	0.547	0.462	0.460	0.565	0.536±0.063
Glu	0.503	0.702	0.585	0.756	0.625	0.647	0.636±0.088
Asp	0.604	0.656	0.776	0.803	0.673	0.816	0.721±0.088

S.D., standard deviation for the six samples.

biological samples. By using this method, satisfactory results for the separation and determination of amino acid neurotransmitters in rat DRG were obtained.

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