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Determination of dansyl amino acids using tris(2,2'-bipyridyl)ruthenium(II) chemiluminescence for post-column reaction detection in high-performance liquid chromatography

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ABSTRACT

Dansyl amino acids are determined using a tris(2,2'-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] chemiluminescence (CL) post-column reaction. The derivatized amino acids are separated on a reversed-phase column (Zorbax ODS) using a mobile phase containing 15% acetonitrile in 25 m*M* trifluoroacetate at pH 7.5. The eluted dansyl amino acids are combined at a mixing tee with 2.0 m*M* $Ru(bpy)_3^{2+}$ CL reagent. The combined solution passes through a flow cell, which has a dual platinum electrode at a potential of 1250 mV. On the surface of the electrode $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)_3^{3+}$ which reacts with dansyl amino acids to emit light. CL intensities vary only slightly with CL reagent or with mobile phase flow-rates. CL intensities increase significantly with an increase in pH over the pH 6–8 region. A slight increase in CL intensity is obtained with increasing acetonitrile in the mobile phase. The working curve for dansyl-derivatized glutamate covers three orders of magnitude with a detection limit of 0.1 μM (2 pmol injected).

INTRODUCTION

1 - Dimethylaminonaphthalene - 5 - sulphonyl chloride (dansyl chloride, Dns-Cl) is a well known derivatizing agent for amino acid analysis and peptide sequencing. The derivatization method is well established [1]. The reaction scheme is shown in Fig. 1. In general, dansyl derivatives of amino acids can be detected either by absorbance at 254 nm or by fluorescence (excitation maximum 385 nm, emission maximum 460–495 nm).

In recent years chemiluminescence (CL) has become an attractive detection method for liquid chromatography due to very low detection limits and wide linear working ranges which can be obtained while using relatively simple instrumentation [2]. 5-Amino-2,3-dihydro-1,4-phthalazinedione (luminol) CL has been applied to the detection of various organic species separated by

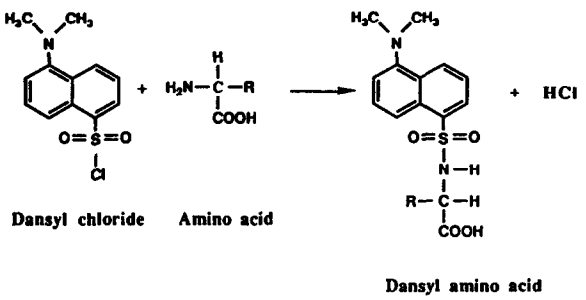

Fig. 1. Reaction scheme for the dansyl derivation of amino acids.

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HPLC [3–7] and for the detection of metal ions separated by ion exchange [8]. N,N'-Dimethyl-9,9'-diacridinium nitrate (lucigenin) has been used to detect corticosteroids separated by reversed-phase chromatography [9]. Peroxyoxalate CL has been widely used for HPLC detection of fluorescent analytes (*e.g.* polycyclic aromatic hydrocarbons) and fluorescent derivatives (*e.g.* dansyl and *o*-phthalaldehyde derivatives of amino acids and amines) [10,11]. The CL reaction with tris(2,2'-bipyridyl)ruthenium(III), $Ru(bpy)_3^{3+}$, has been used for the detection of aliphatic amines [12], amino acids [13–15] and clindamicin antibiotics [16] separated by HPLC.

The CL system of interest in this study is $Ru(bpy)_3^{3+}$. This CL reaction has been applied for the determination of oxalate [17,18], aliphatic and alicyclic amines [15,19,20], amino acids and proteins [13–15,21,22], NADH and species coupled to NADH production [18,23], and tris(2,2'-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] itself [24]. Since CL intensities for amines reacting with $Ru(bpy)_3^{3+}$ increase 10-fold going from primary to secondary amines and another 10–50-fold going from secondary to tertiary amines, dansyl derivatives should give rise to intense emission with $Ru(bpy)_3^{3+}$ due to the secondary and tertiary amine groups. The oxidative–reduction reaction scheme for chemiluminescence from $Ru(bpy)_3^{2+}$ has been postulated by Rubinstein *et al.* [25].

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$$

$$Ru(bpy)_3^{3+} + \text{reductant} \rightarrow \text{product} + [Ru(bpy)_3^{2+}]^*$$

$$[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu$$

The initial oxidation of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ is performed at an electrode surface. The electrogenerated chemiluminescence (ECL) intensity is directly proportional to the amount of reductant, which is the dansyl derivative in this case.

In this paper, we report a method for the determination of dansyl amino acids using $Ru(bpy)_3^{2+}$ CL reaction after HPLC separation on a reversed-phase column. The effects on CL intensity for dansyl derivatives determined in a flow injection system will be described for flow-rates of CL reagent and mobile phase, pH of mobile phase and organic modifier composition of mobile phase.

EXPERIMENTAL

Instrumentation

Flow injection analysis and HPLC experiments were done with the chemiluminescence detection system shown in Fig. 2. For flow injection analysis a Rainin Rabbit peristaltic pump (Woburn, MA, USA) was used to deliver the $Ru(bpy)_3^{2+}$ CL reagent. An Altex Model 110 A HPLC pump (Berkeley, CA, USA) was used to deliver the buffered mobile phase through an Altex Model 210 injector equipped with a 20-μl injector loop. The sample in the buffer stream then combined with the $Ru(bpy)_3^{2+}$ CL reagent at a mixing tee.

The HPLC system was composed of a silica pre-column, a Partisil ODS guard-column (70 mm × 2.0 mm I.D.) and a DuPont Zorbax ODS

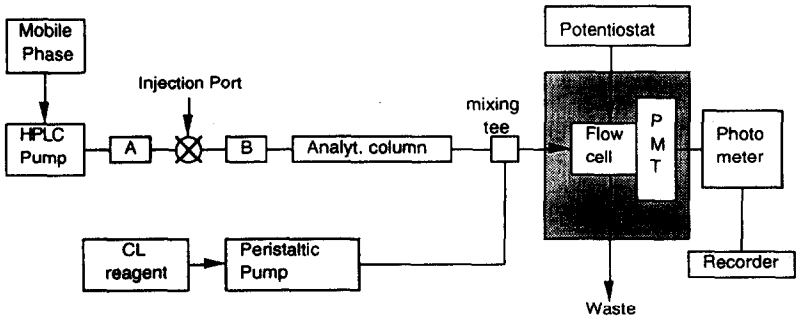

Fig. 2. Schematic diagram for chemiluminescence detection in flow injection and HPLC system (guard and analytical columns are not present in flow injection system). A = Silica pre-column; B = guard column.

column (5 μm, 250 mm × 4.6 mm I.D.). The mobile phase [25 m*M* trifluoroacetic acid (TFA), pH 7.5 with 15% (v/v) acetonitrile] was delivered at a flow-rate of 1.0 ml/min. After passing through the HPLC system the column effluent was combined at a mixing-tee with the $Ru(bpy)_3^{2+}$ solution [2 m*M* $Ru(bpy)_3^{2+}$ in 2.5 m*M* TFA with no organic modifier] which was delivered with a peristaltic pump at a flow-rate of 1.0 ml/min. The combined solution then passed through the flow cell.

In both the flow injection and HPLC systems described above, the flow cell was assembled from a conventional LC–electrochemical detection dual platinum electrode (Bioanalytical Systems, West Lafayette, IN, USA) and a Plexiglas window for detection of CL emission. A PTFE spacer was inserted between the electrode and the Plexiglas to create a 10-μl flow cell volume. In addition to the dual working electrode, a stainless-steel counter electrode at the cell exit and a screw-in Ag/AgCl reference electrode (Bioanalytical Systems) were employed. The cell employed here contained a platinum working electrode, but glassy carbon working electrodes yield equivalent results. Cyclic voltammograms of $Ru(bpy)_3^{2+}$ solutions show the same peak potentials with both electrodes. ECL emission intensities are about 20–30% higher with platinum electrodes.

The flow cell was mounted directly across from the photomultiplier tube (PMT) window. A collimating lens was placed between the flow cell and the PMT window in order to improve the collection efficiency. The entire flow cell was enclosed in a dark box. During all experiments, the working electrode was held at a potential of +1.25 V (*vs.* Ag/AgCl) in order to oxidize $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$. The potential was maintained using a Bioanalytical Systems BAS 100A electrochemical analyzer. The photomultiplier used was a Hamamatsu R928 photomultiplier (Middlesex, NJ, USA) biased at 900 V. The PMT currents were measured using a Pacific Instruments Model 124 photometer (Concord, CA, USA) followed by a Curken Instrument Corp. 250 series strip chart recorder (Hawleyville, CT, USA). Optimization of separation conditions was carried out with the HPLC system using an Altex Model 153 UV–Vis detector to monitor the column effluent.

Reagents

Dansyl chloride, dansyl alanine and free amino acids were purchased from Sigma (St. Louis, MO, USA). Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate and TFA were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile was HPLC grade and distilled over dansyl chloride in order to remove potential interferences. The remaining sample solutions were prepared from reagent-grade or better chemicals and purchased from commercial sources. Water for all solutions was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The HPLC mobile phase, 25 m*M* TFA adjusted to pH 7.5 with 1.0 *M* sodium hydroxide solution and mixed with acetonitrile (85:15, v/v), was prepared and then degassed prior to use. A stock dansyl chloride solution (5.0 m*M*) was prepared with distilled acetonitrile.

Dansylation

Dansylation was carried out according to the modified procedure of Tapuhi *et al.* [1]. Dansyl chloride was dissolved in distilled acetonitrile (5.0 m*M*). The various amino acids were dissolved in 40 m*M* lithium carbonate buffer (pH 9.5 adjusted with HCl). A 1-ml volume of the dansyl chloride solution was rapidly added to 2 ml of the amino acid solution; this resulted in a 10-fold or greater excess of dansyl chloride reagent. The mixture was sonicated for 10 min and then allowed to stand at room temperature. During derivatization the reaction ampoule was wrapped in aluminum foil since dansyl derivatives are known to be photosensitive. A 20-μl aliquot was removed from the ampoule after 50 min and analyzed by the HPLC–CL detection system. Tapuhi *et al.* [1] have reported dansyl chloride derivatization for the amino acids we used here to be greater than 90% complete under these conditions.

pH Studies

Solutions of each analyte were prepared in 100 m*M* potassium phosphate buffered at different pH values covering the range from pH 6 to 8. CL

reagent solutions of 2.0 m*M* $Ru(bpy)_3^{2+}$ were also prepared in 100 m*M* potassium phosphate buffered at different pH values. Concentrations used for each analyte were 5.0 μM for dansyl alanine and 50 μM for dansyl chloride. The carrier stream was changed for each pH tested, and the carrier stream and CL reagent were at the same pH.

Organic modifier studies

A 50 μM concentration of Dns-Ala was prepared in 25 m*M* TFA (pH 7.5). The amount of acetonitrile was varied from 0 to 50% in the 25 m*M* TFA mobile phase. The carrier stream was changed for each percentage acetonitrile tested. The CL reagent solution was always 2 m*M* $Ru(bpy)_3^{2+}$ in 25 m*M* TFA, pH 7.5 with no organic modifier. Diffusion coefficients were measured in each mobile phase having a different percentage of acetonitrile. Electrochemical experiments were performed in a three-electrode configuration consisting of a dual platinum electrode (area 0.16 cm^2), a platinum counter electrode and an Ag/AgCl reference electrode. A Bioanalytical Systems BAS 100A electrochemical analyzer was used to obtain cyclic voltammograms for 1.0 m*M* $Ru(bpy)_3^{2+}$ in buffers containing 0–40% (v/v) acetonitrile at scan rates of 25, 50, 75, 100 and 150 mV/s. Diffusion coefficients were calculated from the slopes of Randles–Sevcik plots for the cyclic voltammetric data in each buffer [26].

RESULTS AND DISCUSSION

Aliphatic amines have been known to be detected with $Ru(bpy)_3^{2+}$. Although aromatic amines such as aniline and N-methylaniline quench CL emission, N,N′-dimethylaniline is known to be quite reactive [19]. Since CL intensities for amines with $Ru(bpy)3^{2+}$ increase 10-fold going from primary to secondary amines and another 10–50-fold going from secondary to tertiary amines, it was the tertiary and secondary amine groups of dansyl amino acids that originally caught our attention. The tertiary amine group has a structure similar to N,N-dimethylaniline. The secondary amine group is adjacent to a SO_2 group. Generally little is known about how the substituent adjacent to the nitrogen atom affects the CL signal although an adjacent carbonyl leads to very low CL intensity. However, it was recently reported that certain sulfonamides (chlorothiazide, trichloromethiazide and hydrochlorothiazide) gave strong emission upon reaction with $Ru(bpy)_3^{3+}$ CL reagent [27].

Using the flow injection system shown in Fig. 2, detection limits (signal-to-noise ratio 2) for dansyl chloride and Dns-Ala were obtained with both 100 m*M* phosphate buffer (pH 7.5) and 25 m*M* trifluoroacetate (pH 7.5). The detection limits for dansyl chloride and Dns-Ala were 1.0 μM (20 pmol) and 0.1 μM (2 pmol), respectively, in both mobile phases. However, a two-fold higher slope in the working curves was obtained with phosphate buffer than with TFA. The lower detection limits for Dns-Ala relative to that of dansyl chloride may result from the fact that the dansyl amino acids contain both secondary and tertiary amine functionalities whereas the dansyl chloride contains only tertiary amine group. Because both tertiary and secondary amine groups react with $Ru(bpy)_3^{2+}$ to emit light, the combined emission intensity from both amine groups of Dns-Ala results in the lower detection limit.

Flow-rate effect

The effect of flow-rate on the CL signal was characterized using the flow injection system shown in Fig. 2. Fig. 3 shows the CL dependence on the flow-rates for 50 μM Dns-Ala in 25 m*M* TFA (pH 7.5). The HPLC carrier stream was maintained at a flow-rate of 1.0 ml/min, while the $Ru(bpy)_3^{2+}$ reagent flow-rate was varied. Increasing the flow-rate from 0.5 to 2.0 ml/min led to an almost constant CL signal. It might be anticipated that decreased residence times of the CL reagent and resulting decreased diffusion distances in the flow cell would lead to the CL intensities showing significant dependence on the flow-rate. However, since the amount of $Ru(bpy)_3^{2+}$ is in large excess with respect to the analyte and the electron transfer is so efficient in solution phase, the flow-rate of CL reagent is not a significant factor on the CL signal. When the CL reagent flow-rate was maintained at 1.0 ml/min, the CL signal was varied with the change of

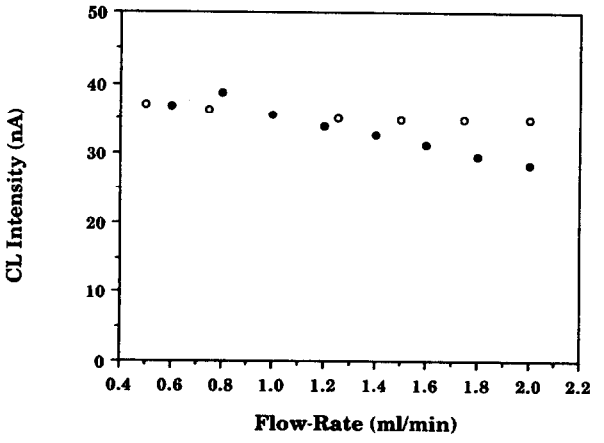

Fig. 3. Effect of flow-rates on CL for 50 μM Dns-Ala. ○ = Varying CL reagent flow-rate; ● = varying mobile phase flow-rate.

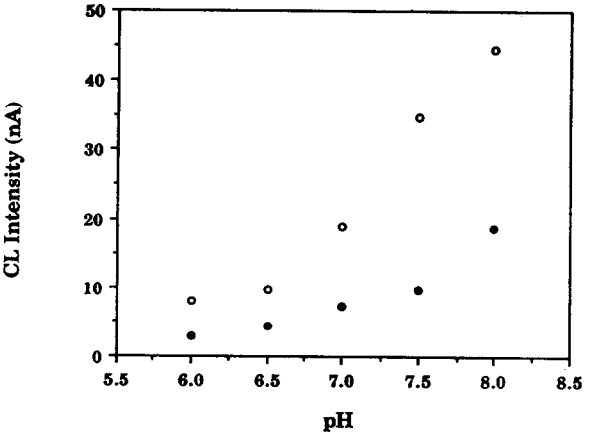

Fig. 4. Effect of pH on CL for 50 μM dansyl chloride (●) and 5.0 μM Dns-Ala (○).

flow-rate of mobile phase carrier stream. An increased CL was observed up to 0.8 ml/min and a continuous CL decrease was obtained after that range. Overall, the signal varied little with change of flow-rate for Dns-Ala.

pH Effect

In order to obtain the optimum condition for the separation of a mixture of dansyl amino acids via reversed-phase HPLC, the choice of optimal parameters (composition, molarity and pH of the mobile phase) should be made. Therefore the effect of mobile phase pH on the CL intensities for 50 μM dansyl chloride and 5.0 μM Dns-Ala was investigated. This work was centered around pH 7.5, since the optimum pH for the separation has been reported to be 7.6 for 25 mM TFA mobile phase [28]. Fig. 4 shows the results obtained. As the pH of mobile phase increases, the CL signal increases significantly for both species. The noise level remained constant, so higher CL intensities correspond to higher signal-to-noise ratios. This variation in CL intensity with pH is similar to the results for amino acids reported by Brune and Bobbitt [15,21]. Although the CL intensities increase for Dns-Ala and dansyl chloride at a higher pH, dissolution of the silica gel matrix of the column support starts at pH > 8. For that reason, we used a pH 7.5 mobile phase. The CL reagent was always at the same pH as the mobile phase in this work. Because the CL reagent was added after the separation, it would have been possible to buffer that reagent at a more alkaline pH to obtain higher CL intensities. Mixing must be very efficient then to avoid degradation in precision. Additionally, if immobilized $Ru(bpy)_3^{2+}$ was used for the detection step [18,23], then no post-separation solution additions are required at all if the separation pH and detection pH are the same.

Effect of organic modifier composition

In a reversed-phase separation, a significant amount of an organic solvent as modifier can be required in the mobile phase. Therefore, it was necessary to investigate the effects such an organic solvent would have on the CL reaction. Acetonitrile and methanol are commonly used as organic modifiers in reversed-phase HPLC. Acetonitrile was used as an organic modifier in this experiment because it was also used as the solvent for the dansyl derivatization reaction of amino acids. Increasing the acetonitrile content from 0 to 50% (v/v) in the 25 mM TFA carrier stream (pH 7.5) led to an slight increase in the observed CL intensities for 50 μM Dns-Ala. This result is represented in Fig. 5. The relative standard deviation was about 3% for all these measurements. Brune and Bobbitt [15] have reported slightly enhanced $Ru(bpy)_3^{2+}$ CL intensities with up to 20% acetonitrile in the mobile phase. Diffusion coefficients were measured for

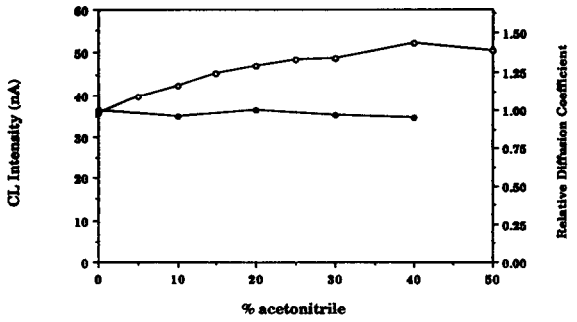

Fig. 5. Effect of acetonitrile composition on CL for 50 μM Dns-Ala. ○ = CL intensity; ● = relative diffusion coefficient.

each buffer having different compositions of acetonitrile in order to know whether or not a change in the mass transfer rate could be occurring and be responsible for the observed increase in the CL signal. The measured diffusion coefficients are almost constant over the 0–40% acetonitrile range (Fig. 5). This result shows that the presence of acetonitrile in the mobile phase buffer may not be affecting the mass transfer rate but instead may affect the quantum efficiencies of the CL process. Bard and co-workers [25,29] have reported that the ECL efficiency (photons emitted/$Ru(bpy)_3^{3+}$ generated) of this reaction is around 5% in acetonitrile and 2% in aqueous solution. Therefore the increased ECL efficiencies are responsible for the observed increase in CL intensity.

HPLC of dansyl-derivatized amino acids

TFA buffer is known to be highly selective for the separation of peptides [30–32] and phenylthiohydantoin derivatives of amino acids [33]. From the studies by Levina and Nazimov [28], optimum separation of dansyl amino acids was obtained with 25 mM TFA at pH 7.6. The HPLC system we used is shown in Fig. 2. In order to minimize resolution degradation all tubing used was 0.01 in. I.D. (1 in. = 2.54 cm) up to flow cell inlet. Separation of the dansyl-derivatized amino acids was performed with a Zorbax ODS (5 μm) column using a mobile phase containing 15% acetonitrile in 25 mM TFA at pH 7.5. Fig. 6 shows the separation of a mixture of six dansyl derivatized amino acids.

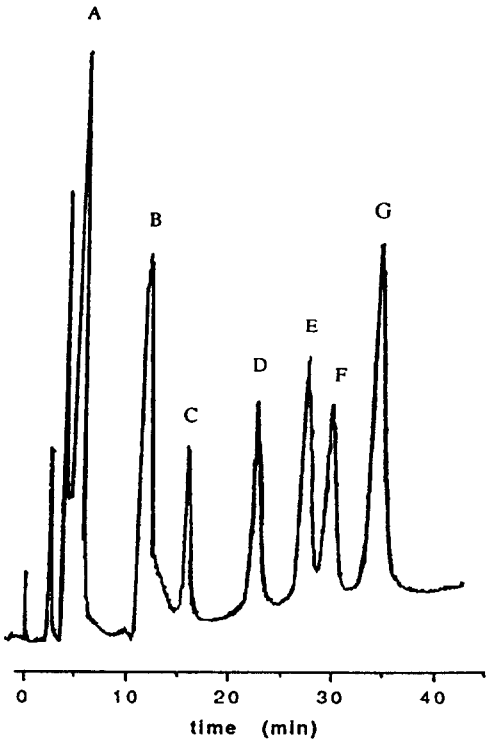

Fig. 6. Isocatic separation of six dansyl amino acids on Zorbax ODS column (5 μm) with a mobile phase containing 15% acetonitrile in 25 mM trifluoroacetate (pH 7.5). Flow-rate 1.0 ml/lmin. Each compound is 25 μM (500 pmol). Peaks: A = Dns-Glu; B = Dns-OH; C = Dns-Asn; D = Dns-Ser; E = Dns-Thr; F = Dns-Gly; G = Dns-Ala.

The six dansyl amino acids were well resolved from each other. The first two peaks are caused by the derivatization solvents. Li_2CO_3 (40 mM) at pH 9.5 was used to dissolve amino acids in the derivatization procedure; therefore, hydroxide ion in the sample resulted in CL response. A working curve for dansyl-derivatized glutamate has been obtained using the same separation conditions. Fig. 7 shows the CL signal for Dns-Glu concentrations from 0.1 to 250 μM. Each point is a mean of 3 or more injections of a sample. The relative standard deviations at 1 μM and above range from 1.5–4.5%. Least squares parameters for the 5 to 250 μM concentration range are given in Table I for both linear and logarithmic plots. The signals represented were based on peak heights instead of peak areas since the result based on peak areas was the same as that obtained based on peak heights.

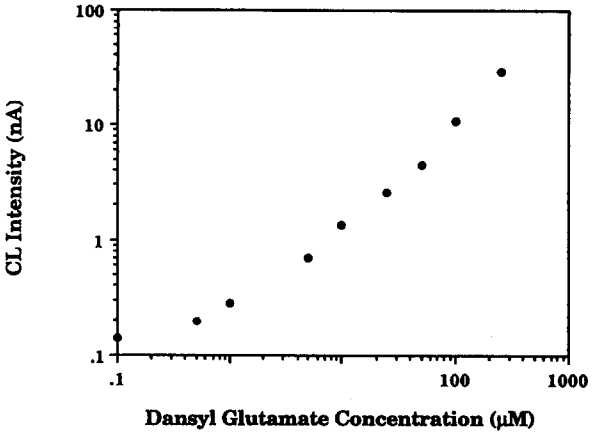

Fig. 7. Dns-Glu working curve using HPLC–CL system.

The chromatographic detection limit (signal-to-noise ratio 2) obtained was 0.1 μM (2 pmol) for Dns-Glu.

CONCLUSIONS

This detection method has a wide dynamic range and detection limits which are comparable to those reported using other methods. The detection limit obtained for Dns-Glu (2 pmol) is 25 times lower than that achieved with UV absorbance detection (50 pmol) and only slightly higher than that reported using fluorescence (sub-pmol) detection. Peroxyoxalate CL has considerably lower detection limits (10 fmol) for dansyl amino acids; however, $Ru(bpy)_3^{2+}$ CL has advantages of reagent stability, greater compatibility with common reversed-phase HPLC solvent systems, and the possibility to use the $Ru(bpy)_3^{2+}$ as an immobilized regenerable CL reagent [18]. Furthermore, it is likely that significantly improved $Ru(bpy)_3^{2+}$ detection limits could be achieved with a dansyl-like derivatization reagent designed specifically for $Ru(bpy)_3^{2+}$ CL. Although underivatized amino acids can be detected with $Ru(bpy)_3^{3+}$ CL, the dansyl derivatives have detection limits improved by three orders of magnitude. In our same flow system we observe detection limits of 2 nmol for underivatized glutamate and 1 nmol for underivatized alanine as compared to 2 pmol for Dns-Glu. The separations reported here were done with isocratic elution; however, gradient elution could be easily applied to our HPLC–CL method for the complete separation of all dansyl derivatives of the 20 common amino acids in a short time period since organic modifier in the mobile phase does not degrade the CL reaction.

TABLE I

LEAST-SQUARES PARAMETERS FOR WORKING CURVES (5–250 μM Dns-Glu)

	Linear	Logarithmic
Slope	0.117 nA/M	0.936
Slope standard error	0.003	0.049
Intercept	−0.397 nA	−4.23
Intercept standard error	0.372	0.223
Correlation coefficient	0.997	0.989
Standard error estimate	0.691	0.061

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