Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 7, pp. 557–563, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

A new fluorogenic assay for tyrosine-containing peptides

M. TELLIER, R.J. PRANKERD and G. HOCHHAUS*

College of Pharmacy (Box J-494), University of Florida, Gainesville, FL 32610, USA

Abstract: A new tyrosine-specific LC assay with pre-column fluorogenic derivatization is described for Tyr–Gly as model peptide. *o*-Hydroxylation of the tyrosine residue with tyrosinase in the presence of ascorbic acid, followed by oxidation to the corresponding quinone by potassium ferricyanide at room temperature and condensation with 1,2-diamino-1,2-diphenylethane in the presence of acetonitrile gave a highly fluorescent species. The resulting fluorescence signal was stable over the investigated period of 5 h and exhibited a linear response curve on a reversed-phase LC system. Under optimized reaction conditions, the lower limit of detection for Tyr–Gly was 200 fmol per injection. Examination of a series of dipeptides (L-Tyr–L-X; X = Gly, Ala, Val, Leu, Phe) showed no significant influence of neighbouring amino acids on the enzymatic hydroxylation by tyrosinase. This and the formation of a highly fluorescent signal for Leu-enkephalin suggests the general feasibility of the approach for the determination of tyrosine-containing peptides.

Keywords: *Tyrosinyl dipeptides; fluorogenic derivatization; tyrosinase; 1,2-diamino-1,2-diphenylethane; LC.*

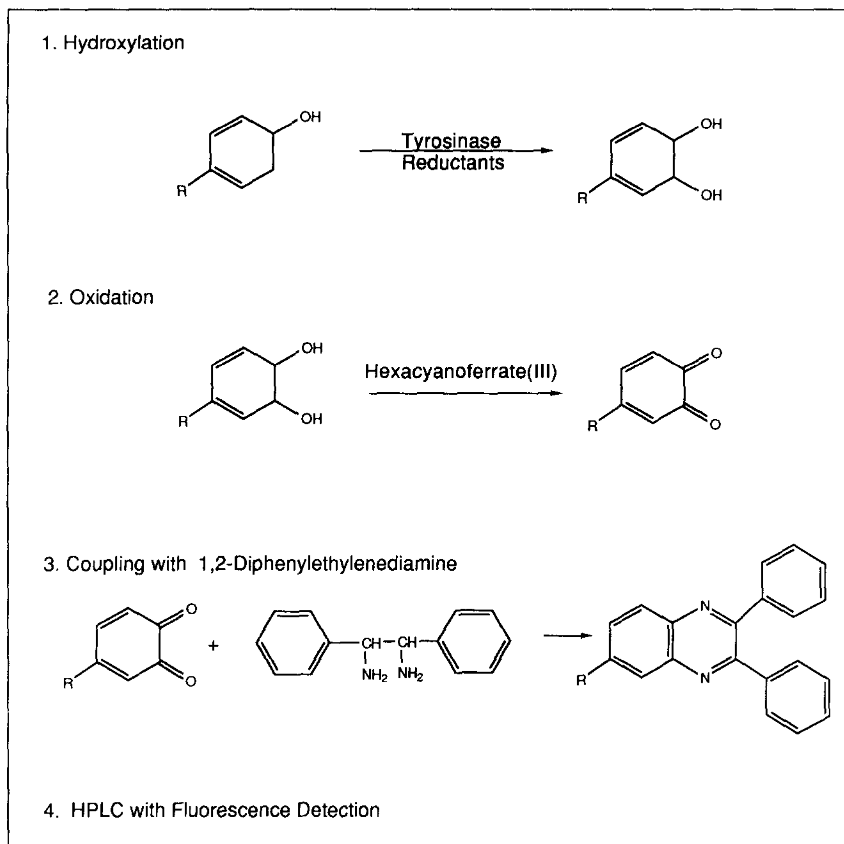
Introduction

Because of the low physiological concentrations of opioid peptides, and those to be expected for therapeutically administered derivatives, ultra-sensitive (pmol ml^{-1}) and specific analytical methods are necessary for their determination in biological fluids. At present, low peptide levels are mainly analysed by radio-immunoassay (RIA) [1]. Although RIAs provide a highly sensitive and reproducible tool for analysis in biological materials, they are hampered by low selectivity because of the potential cross-reactivity of the antiserum to compounds of similar structure and the dependence on radioactive material (tritium or I^{125} tracers) with the inherent potential of health and environmental hazards.

The need for alternative analytical approaches for the measurement of opioid peptides has been stressed by the National Institute on Drug Abuse [2]. Some recent publications have evaluated the potential of a variety of techniques such as LC with electrochemical (EC) detection [3–5], mass spectrometry [6–9] and fluorescence-detection [10–13] without or with the use of laser-induced excitation [14]. LC–EC is generally less sensitive, because of the high oxidation voltages necessary. In contrast, good fluorophoric (e.g. dansylation) or fluorogenic derivatization methods, which are generally based on the reaction with the ubiquitous *N*-terminal amino-group of peptides, are very sensitive, but hampered by low selectivity and the need for complex extraction procedures [13].

Tyrosine is highly conserved in position 1 of all opioid peptides. The aim of the present study was, therefore, to develop a tyrosine-specific fluorogenic derivatization reaction as a means to improve assay specificity. Such attempts have been recently described [11, 12], but the feasibility of these assays for peptides seems to be rather limited because of the harsh reaction conditions. The assay presented here (Scheme 1) utilizes mild, enzymatic reaction of the peptidyl tyrosine-group by mushroom tyrosinase, an enzyme which is limited to the 3-hydroxylation of phenols such as tyrosine or tyrosine-containing peptides and proteins, when suitable reducing agents are present to prevent further oxidation [15]. The transformation of the mono-phenolic tyrosine into a catechol structure enables one to employ the well-characterized arsenal of ultra-sensitive catechol assays in the second step of the assay. As one possible design, the highly fluorogenic condensation reaction with 1,2-diamino-1,2-diphenylethane (after oxidation of the catechol to the *o*-quinone) was selected, as this reaction has been proven to be very valuable for the sensitive analysis of catechols in aqueous

* Author to whom correspondence should be addressed.



Scheme 1

Tyrosinase hydroxylates the aromatic ring of tyrosine in the presence of ascorbic acid (to prevent the formation of the corresponding quinone and consequent polymerization of the reaction products). In the second step, potassium ferricyanide in aqueous buffered acetonitrile (pH 6–7) generates the *o*-quinone, which then reacts with 1,2-diamino-1,2-diphenylethane (DPE), presumably to form a highly fluorescent 2,3-diphenyl-1,4-quinoxaline. The reaction product is finally analysed by LC.

systems [16–18]. Data are presented here that were generated with tyrosine-containing dipeptides as model compounds for opioid peptides. The results demonstrate the potential of this approach for the selective and sensitive determination of these substances.

Experimental

Chemicals and solutions

The dipeptides Tyr–Gly, Tyr–Ala, Tyr–Val, Tyr–Leu, Tyr–Phe and mushroom tyrosinase (T7755) were obtained from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade. The dipeptides were generally dissolved in 0.1 N HCl and further diluted with phosphate buffered saline (PBS, 10 × dilution of stock solution: 160 g NaCl, 4 g KH_2PO_4, 72.7 g $Na_2HPO_4.7H_2O$ and 4 g KCl in 2 l of water) or (1:1, v/v). Tyrosinase was dissolved in PBS (1 mg/100 μl) and stored in aliquots at -20°C. 1,2-Diamino-1,2-diphenylethane (DPE) was synthesized by the method of Irving and Parkins [19]. Briefly, benzaldehyde (1.9 equivalents) was refluxed with ammonium acetate (1 equivalent) for 3 h, the resulting precipitate was collected, and then washed with ethanol. It was hydrolysed with 33% v/v H_2SO_4, benzoic acid and benzaldehyde were removed by steam distillation, and then the DPE precipitated by neutralization with ammonium hydroxide. After recrystallization from petroleum ether, the product had m.p. 118–119°C (literature, 120°C) [19] and a ^1H nuclear magnetic resonance spectrum which supported the assigned structure. A 20 mg ml^{-1} stock solution was prepared in 0.1 N HCl. The oxidant solution contained 63 mg potassium ferricyanide (Fisher, Scientific Co., Fairlawn, NJ, USA) and 119 mg KCl in 5.5 ml PBS. Water was deionized and distilled before use. Other chemicals were of analytical grade.

Apparatus and HPLC conditions

The HPLC system consisted of a Milton Roy CM4000 pump, a Rheodyne 7125 sample injector (20 or 100 μl), a Milton Roy SM3100 variable UV wavelength detector (λ_{max} 280 nm) or a Spectroflow 980 fluorescence detector (ABI Analytical Kratos Division, 5 μl flow cell, λ_{excit} 345 nm, emission cut-off filter, 417 nm). The column was Nucleosil C18, (5 μm, 150 × 4.6 mm) (Keystone Scientific, Bellefonte, PA, USA). For monitoring the tyrosinase reaction products, the mobile phase was 50 mM TRIS (pH 6.8, 1.0 ml min^{-1}) (System I) with UV detection at 280 nm (λ_{max} for catechols). For the fluorescence detection of the more lipophilic DPE-derivatives, the mobile phase was 50 mM TRIS (pH 6.8) and acetonitrile (200:80, v/v, 1.0 ml min^{-1}) (System II).

Purification of tyrosinase

A crude purification of the commercially available tyrosinase was performed by ultrafiltration using Centricon membrane filters (30,000 molecular weight cut-off, Amicon, Danvers, MA, USA). One millilitre of tyrosinase solution was applied to the filter unit and centrifuged at 5000**g** until maximal concentration of the sample was achieved. This centrifugation step was repeated three times after addition of 2 ml of PBS. Finally the concentrate was reconstituted in 1 ml of PBS and aliquots were stored at −20°C.

Fluorogenic reaction

Optimized method. The optimized method was used for the final calibration curve. To 46 μl of dipeptide solution (Tyr–Gly: 0.54–3.25 pmol) in a test tube, 5 μl of purified tyrosinase solution (50 μg) and 5 μl of ascorbic acid solution (88 μmol) were added. After 20 min at room temperature, 135 μl of ferricyanide solution and 110 μl acetonitrile, and 25 μl of the DPE solution were added. After 50 min at room temperature, an aliquot of 100 μl was injected onto the HPLC system with fluorescence detection (System II).

Initial experiments. These experiments, which focused solely on the characterization of the tyrosinase or DPE reactions, were performed with larger volumes than described above, but differed only in the concentrations of the reactants. Exact experimental conditions for the tyrosinase and DPE reactions are described for clarity in the figure legends. The tyrosinase reaction was generally monitored by injecting the reaction mixture directly into LC System I with UV detection at 280 nm, while HPLC System II was used for analysing the DPE-reaction products.

Influence of neighbouring amino acids. To test the influence of neighbouring amino acids on the tyrosinase reaction yield, high concentrations of dipeptides Tyr–Gly, Tyr–Val, Tyr–Phe, Tyr–Leu and Tyr–Ala (2 mM in PBS, final volume 300 μl) were incubated with 400 μg of tyrosinase in the presence of ascorbic acid (25 mM). Control experiments were performed with no tyrosinase added. After 75 min at room temperature, aliquots (20 μl) were injected onto the HPLC system (System I). The fraction of peptide reacted was calculated from the peak height ratio of underivatized peptide present in the incubation and control mixtures.

Results

For clarity, the developed fluorogenic reaction is described in Scheme 1. The validation steps resulting in the described design are described below.

Tyrosinase reaction

The ability of tyrosinase to react with the model peptide Tyr–Gly was first tested. Reaction of Tyr–Gly with tyrosinase at high concentrations resulted in a shift of the UV absorption to higher wavelength, as expected for the formation of catechols (data not shown). Further, the analysis of the reaction mixture by LC revealed the formation of a new peak with shorter retention time (assumed to be *o*-hydroxylated Tyr–Gly) (Fig. 1B), which was not present when each of the reactants was injected separately (Fig. 1A). In the absence of ascorbic acid, the new peak disappeared with time (data not shown), with the formation of a highly coloured insoluble precipitate.

To inhibit the polymerization of the reaction products, several concentrations of ascorbic acid were tested to prevent or reverse the tyrosinase-induced formation of *o*-quinones [15]. Under the employed assay conditions, 25 mM, but not 7 mM of antioxidant, were sufficient to protect the generated catechols with a plateau reached after about 60 min (Fig. 2). Additional experiments suggested that the

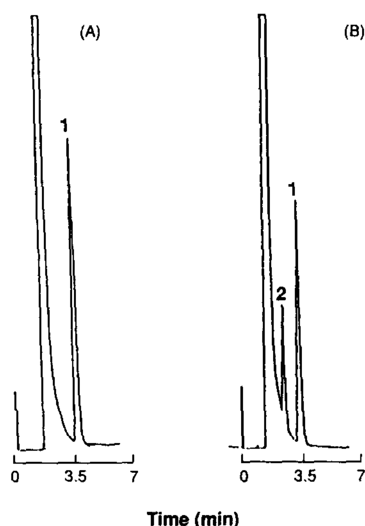


Figure 1
The tyrosinase reaction. (B) Tyrosinase reaction was performed as described in Fig. 2 (7 mM ascorbic acid) and was analysed by HPLC System I. (A) Identical reaction mixture without tyrosinase added. Peak 1: Tyr–Gly; Peak 2: tyrosinase reaction product.

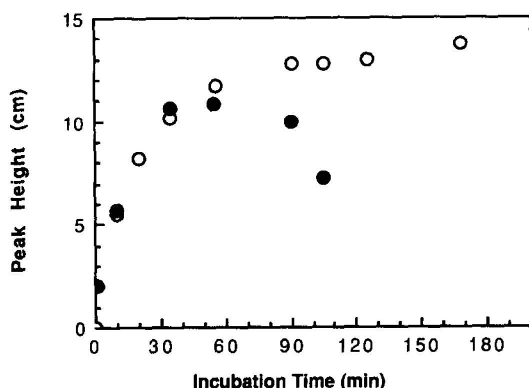


Figure 2
Tyr–Gly (14 mM, 20 μl, concentration in the final incubation mixture: 0.6 mM) and PBS (266 μl) were incubated at room temperature with tyrosinase (400 μg in 40 μl PBS) using two different concentrations of ascorbic acid [100 μl, 25 mM (○) or 7 mM (●) in the final incubation mixture]. At various times throughout the reaction period a 20 μl sample was injected onto HPLC System I.

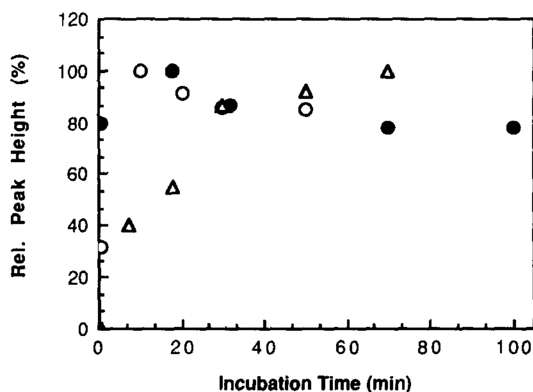


Figure 3
Time curves of tyrosinase incubation for different concentrations of Tyr–Gly. Tyr–Gly [3000 (△), 30 (○), 3 μM (●) in the final incubation mixture] was incubated in total volume of 1600 μl (Vit C: 25 mM, 1.6 mg tyroxinase). After various time intervals, 40 μl were removed and fluorescence intensity following DPE reaction (275 μl of ferricyanide solution, 275 μl water, 450 μl acetonitrile and 100 μl DPE reagent, 30 min incubation) was measured by injecting 20 μl into HPLC System II.

Table 1
Influence of second amino acid on tyrosinase reaction

Dipeptide (1 μmol ml^{-1})	% Reacted (over 75 min)
Tyr–Gly	54
Try–Val	57
Tyr–Leu	73
Tyr–Phe	65
Tyr–Ala	65

optimal protective amount of ascorbic acid depends on the concentration of dipeptide present and a further reduction in ascorbic acid (1.6 mM in the final incubation mixture) was used later in the study when lower concentration of dipeptide were used.

The time profile of product formation (Fig. 3) was dependent on the concentration of enzyme substrate present. Complete reaction was achieved within 10–20 min for micromolar Tyr–Gly concentrations, and a tyrosinase reaction time of 20 min was selected for further experiments with low concentrations of Tyr–Gly.

Initial tests also investigated the influence of the neighbouring amino acid on the tyrosinase reaction. Under the employed assay conditions, no dramatic influence of the neighbouring group could be detected (Table 1), as all of the peptides showed a reaction yield between 50 and 70%.

Reaction with 1,2-diamino-1,2-diphenylethane (DPE)

Experimental conditions previously shown to be optimal for the reaction of DPE with catechols were incorporated into the assay procedure whenever possible. Hence, the selection of the DPE concentration and the addition of KCl were not optimized but taken directly from corresponding catechol assays [16–18] (see Experimental section). Further, acetonitrile which facilitates the condensation

reaction by reducing the thermodynamic activity of water [16] was added. In contrast, the concentration of hexacyanoferrate was increased to 14.3 mM in order to ensure the formation of the peptidyl quinone structure despite the presence of high concentrations of ascorbic acid.

Under the selected conditions, the tyrosinase reaction product (Peak 2 in Fig. 1B), but not underivatized Tyr–Gly (Peak 1 in Fig. 1), reacted with DPE to yield a highly fluorescent product that was excited at 345 nm, and showed an emission maximum of longer than 425 nm. LC analysis (Fig. 4A) revealed one fluorescent peak that eluted after 3.8 min, and whose peak height was directly related to the analyte concentration. In addition, a later eluting peak was observed, which could be related to the presence of ascorbic acid. No further attempts to reduce this signal were attempted, since it did not interfere with the Tyr–Gly peak. Preliminary experiments with Leu-enkephalin, performed under identical conditions, also resulted in a highly fluorescent signal (Fig. 4B) which, however, was not separated from the DPE reaction product of ascorbic acid. The time course of the DPE reaction with Tyr–Gly, as shown in Fig. 5 suggested that an incubation time of 50 min at room temperature was required for the complete development of the signal.

To increase the sensitivity, the volume of the reaction mixtures was reduced to the conditions described in the Experimental section. This, in combination with a five-fold increase in injection volume (100 instead of 20 μl) allowed the injection of 30% of the total sample volume, but resulted also in the generation of several new peaks, due to the higher concentration of tyrosinase present in the DPE reaction mixture. These signals could be greatly diminished by the purification of tyrosinase (see Experimental). Calibration curves performed under these conditions were linear ($r > 0.99$, $n = 3$) over the concentration range of 100–1200 fmol per injection, and the estimated intercepts did not differ significantly from the origin (0.03–0.5 cm peak height). The slope of the calibration curves ranged, however, from 7.09 to 13.9 $pmol^{-1}$ ($n = 3$, conc. expressed in pmol/injection), as small differences in the mobile phase composition had pronounced effects on peak widths. Under these conditions, the limit of detection (3 × background noise) was 200 fmol per injection.

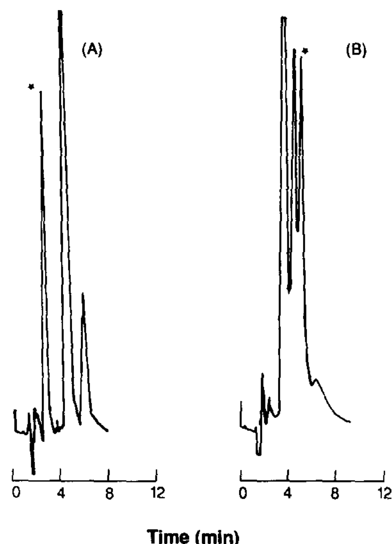


Figure 4
HPLC with fluorescence detection: Tyr–Gly (A) and Leu-enkephalin (B) were assayed as described in the Experimental section, using HPLC System II with fluorescence detection. *, Peptide related peaks.

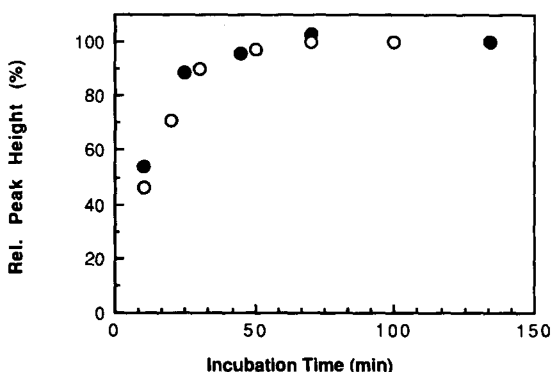


Figure 5
Time course of DPE reaction. A 100 μl volume of a mixture of Tyr–Gly [1.4 μM (●) or 0.7 μM (○) in the final incubation mixture], 50 μg of tyrosinase and ascorbic acid (25 mM) were incubated at room temperature. After 20 min, 550 μl of ferricyanide solution, 450 μl acetonitrile and 100 μl of DPE solution was added, and aliquots of 20 μl were added after defined time intervals.

For comparison, methyldopa ethyl ester (a catechol) was derivatized directly with DPE under the conditions described in the Experimental section. From the resulting calibration curves, a limit of detection of 300 fmol was determined, similar to the sensitivity for Tyr–Gly determined by the tyrosinase–DPE system. LC conditions for Tyr–Gly were not further optimized, as this study was mainly concerned with demonstrating the general

suitability of this approach for the analysis of tyrosine containing peptides.

Discussion

A unique enzyme-based fluorogenic assay for tyrosine containing peptides has been developed. Key to the approach is the enzymatic transformation of peptidyl-tyrosine into the corresponding catechol by tyrosinase. This allows the use of an arsenal of sensitive and specific catechol detection systems for the analysis of tyrosine-containing peptides, and the highly sensitive reaction of DPE with catechols [16–18] was selected as the basis for the fluorogenic reaction.

Tyrosinase was able to react efficiently with Tyr–Gly and the reaction reached completion in less than 20 min at low substrate concentrations. As shown in the results section, neighbouring amino acids did not affect the ability of tyrosinase to hydroxylate the *N*-terminal tyrosinase residue of a number of dipeptides. This parallels findings of Marumo [15] for a number of tripeptides and agrees well with the ability of tyrosinase to hydroxylate Leu-enkephalin (see Results) or higher molecular weight proteins such as insulin [20]. Hence, the outlined hydroxylation and resulting catechol detection is likely to be generally suitable for the detection of tyrosine-containing peptides and proteins. However, further studies have to confirm this hypothesis; in addition, the suitability of the assay principle for non-peptide phenols is currently under investigation.

The second step of the fluorogenic reaction was modified from published procedures for catechols [16–18] and the reaction-time profiles for hydroxylated Tyr–Gly were very similar to those of catechols [16], indicating that the peptide structure does not significantly interfere with the fluorogenic reaction. The sensitivity of the combined tyrosinase–DPE method was 200 fmol per injection; similar to control experiments in which methyldopa ethyl ester was used as a direct substrate for the DPE reaction. These results demonstrated that the tyrosinase reaction proceeded to completion. Taking into consideration, that a 100 times higher sensitivity (2 fmol per assay) was reported for dopa using a superior flow cell design [17], a further increase in sensitivity for the tyrosinase–DPE assay should be possible. However, the limit of detection for this method is already comparable or superior to other fluorogenic assays for opioid peptides [10–13].

As shown in Fig. 4, reaction of DPE with high concentrations of ascorbic acid or related impurities resulted in fluorescence signals which were more lipophilic than the Tyr–Gly derivative. It was not attempted to reduce or eliminate this signal as it did not interfere with the Tyr–Gly peak. However, the interference with the Leu-enkephalin derivatives suggested that the use of alternative reductants such as thiols or bisulphite [15] might be necessary for certain analytes.

The tyrosinase–DPE reaction has some advantages over existing methods. It can be performed under mild enzymatic conditions, in contrast to other tyrosine-specific fluorogenic assays which have to employ harsh reaction conditions at elevated temperatures [11, 12]. The DPE reaction products of hydroxylated Tyr–Gly but also those of dopamine and other catechols [16–18] seem to be stable over a period of hours and consequently not sensitive to variable reaction times during automatic injections. More importantly, the derivatization can be performed in an aqueous system, while other fluorogenic derivatization reactions [13, 14] depend on the careful preparation of water-free samples with its potential pitfalls.

References

[1] L. Terenius and F. Nyberg, *Life Sciences* **41**, 805–808 (1987).
[2] R.S. Rapaka, *Life Sciences* **39**, 1825–1843 (1986).
[3] C. Kim and R. Cheng, *J. Chromatogr.* **494**, 67–76 (1989).
[4] A. Sauter and W. Frick, *J. Chromatogr.* **297**, 215–223 (1984).
[5] L.H. Fleming and N.C. Reynolds, *J. Chromatogr.* **431**, 65–76 (1988).
[6] G.H. Fridland and D.M. Desiderio, *Life Sciences* **41**, 809–812 (1987).
[7] D. Liu, C. Dass, G. Wood and D. Desiderio, *J. Chromatogr.* **500**, 395–412 (1990).
[8] W. Mück and J.D. Henion, *J. Chromatogr.* **495**, 41–59 (1989).
[9] E.D. Lee, W. Mück and J.D. Henion, *J. Chromatogr.* **458**, 313–321 (1988).
[10] M. Ohno, M. Kai and Y. Ohkura, *J. Chromatogr.* **430**, 291–298 (1988).
[11] M. Kai, J. Ishida and Y. Ohkura, *J. Chromatogr.* **430**, 271–278 (1988).
[12] M. Nakano, M. Kai, M. Ohno and Y. Ohkura, *J. Chromatogr.* **411**, 305–311 (1987).
[13] M. Mifune, D.K. Krehbiel, J.F. Stobaugh and C.M. Riley, *J. Chromatogr.* **496**, 55–70 (1989).
[14] K.J. Dave, T.M. Rossi and C.M. Riley, *Pharm. Res.* **7**(Suppl.), S1 (1990).
[15] K. Marumo and J.H. Waite, *Biochim. Biophys. Acta* **872**, 98–103 (1986).

[16] A. Mitsui, H. Nohta and Y. Ohkura, *J. Chromatogr.* **344**, 61–70 (1985).
[17] A. Mitsui, H. Nohta and Y. Ohkura, *J. Chromatogr.* **380**, 229–231 (1986).
[18] H. Nohta, A. Mitsui and Y. Ohkura, *Anal. Chim. Acta* **165**, 171–176 (1984).
[19] M.N.H. Irving and R.M. Parkins, *J. Inorg. Nucl. Chem.* **27**, 270–271 (1965).
[20] J.G. Cory, C.C. Bigelow and E. Frieden, *Biochemistry* **1**, 419–422 (1962).

[*Received for review 24 May 1990; revised version received 10 July 1991*]