Pergamon

0731-7085(94)00098-0

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 11, pp. 1355–1361, 1994
Elsevier Science Ltd
Printed in Great Britain
0731-7085/94 $7.00 + 0.00

Lysine vasopressin undergoes rapid glycation in the presence of reducing sugars

EDWARD TARELLI,* PATRICK H. CORRAN, BRIAN R. BINGHAM, HEATHER MOLLISON and ROBIN WAIT†

Division of Chemistry, National Institute for Biological Standrds and Control, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK
†CAMR, Porton Down, Wilts SP4 0JG, UK

Abstract: Lysine vasopressin (LVP) readily reacts with reducing saccharides both in lyophilized preparations and in aqueous solution. Incubation of LVP with, for example, lactose over a pH range of 3.0–8.5 in phosphate buffer or simply in water, gives rise to a number of reaction products, some of which form rapidly (in a matter of hours) even in the frozen state. Reaction mixtures were analysed by reversed-phase HPLC and the structures of the products were deduced from the amino-acid composition of isolated components, by comparison with product profiles obtained with analogues under similar conditions and by FAB mass-spectral analysis of derivatives isolated after reduction with cyanoborohydride. The primary products arise from the formation of Schiff's bases at one or both of the two amino functions. The α-amino group of the N-terminal cystine is considerably more reactive than is the ϵ-amino group of lysine and it is the N-terminal adduct which rapidly forms even at −20°C. It is concluded that caution must be shown in using reducing sugars in formulations containing peptides and proteins, particularly the vasopressins and oxytocin.

Keywords: *Lysine vasopressin; rapid glycation.*

Introduction

Lysine vasopressin [1] (LVP) is a nonapeptide secreted by the posterior pituitary. It plays an important role in homeostasis as the principal antidiuretic hormone but also exhibits pressor activity. Like all members of the vasopressin/oxytocin family, it contains an internal disulphide (cys^1–cys^6)loop and the C-terminal is amidated. There are two primary amino groups, the α-amino group of cys^1 and the ϵ-amino group of lys^8 (Fig. 1).

When lyophilizing small amounts of peptides such as LVP for use therapeutically or as bioassay standards it is common practice to dilute the peptide with excipients which may act as stabilizing agents and/or may prevent loss from adhesion to glass surfaces; their use results in a more visible and aesthetically acceptable lyophilized solid. One category of excipient in normal use is that of sugars and sugar alcohols, particularly lactose, α,α′-trehalose and mannitol, and these excipients are usually present in a large excess over the active substance (>100:1 w/w ratios are common). Over many years lactose has found particular favour for these applications because of its ready availability in pure form and good drying characteristics. There are many instances where it has fulfilled this function in a highly satisfactory manner [2].

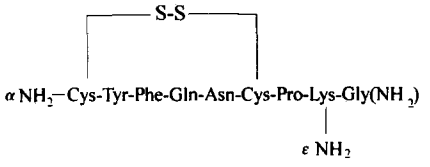

Figure 1
Structure of lysine vasopressin showing free amino groups and C-terminal amide.

The work reported here was stimulated by experience with trial preparations for potential bioassay standards of LVP and other neurohypophyseal nonapeptides in which the peptide had been lyophilized in the presence of a number of different bulking agents. Those preparations containing lactose as the sole excipient showed reduced biological activity and unacceptable stability although when examined by HPLC in acidic conditions, there appeared to be no obvious explanation for the reduced stability. In order to establish the

*Author to whom correspondence should be addressed.

cause of this lability, a study of the behaviour of a specimen nonapeptide, LVP, in the presence of reducing and non-reducing sugars was undertaken.

Experimental

Synthetic lysine vasopressin (batch JLB 4029 679) was a gift from Sandoz Ltd (Basle, Switzerland) and was obtained through Dr S. Poole (NIBSC). Sodium phosphate salts, sodium azide and lactose were AnalaR grade from BDH (Poole, UK). Oxytocin, sodium cyanoborohydride and 4-dimethylamino-azobenzene-4′-sulphonyl chloride (DABSyl chloride) were obtained from The Sigma Chemical Co. (Poole, Dorset, UK). Arginine vasopressin and desmopressin (1-desamino-8-*D*-arginyl vasopressin) were obtained from Ferring Pharmaceuticals (Feltham, UK).

Trial preparations of LVP in ampoules were prepared by lyophilizing aliquots (1 ml) of a solution of LVP (20 μg ml^{-1}) and lactose (5 mg ml^{-1}) in 50 mM citrate or 50 mM acetate buffer (pH 4.5) according to published methods [3]. Solutions containing LVP (1 mM) and lactose (100 mM) were prepared in 0.01% (w/v) solution of sodium azide in water or 100 mM phosphate buffer. The solutions were filtered through a 0.22-μm filter and were then kept at appropriate temperatures for the required times. For isolation of products for structural identification purposes sodium cyanoborohydride (3 mg) was added to a reaction mixture containing 1 mg of LVP in 1 ml of 100 mM phosphate (pH 8). Incubation was continued at 37°C for a further 24 h and acetic acid (50 μl) was then added. The solution was then fractionated as described below.

For preliminary experiments products were separated by reversed-phase HPLC on Hypersil MOS (Shandon, Runcorn, UK) with a mobile phase of methanol–0.1 M triethylammonium phosphate (pH 3) (60:40, v/v). Subsequent experiments were analysed isocratically on 100 or 150 mm × 5 mm i.d. columns of Hypersil ODS (Shandon) or Spherisorb S50DS2 (Phase Separations, Queensferry, Clwyd, UK) packed in-house or 100 × 3 mm i.d. cartridges of Spherisorb S50DS2 (Chrompack, London, UK) with a mobile phase of 12.5–15% acetonitrile (depending on circumstances) in 0.1 M sodium phosphate (pH 7) [4]. For preparative chromatography a 150 × 4.6 mm column of the polymeric reversed-phase packing PLRP-S300 (Polymer Laboratories, Church Stretton, UK) was eluted with a gradient of 12–25% acetonitrile in 0.05 M triethylammonium acetate (pH 7.0). The eluent was monitored at 210 or 280 nm depending upon the sensitivity required. Fast atom bombardment (FAB) mass spectroscopy was performed on a Kratos MS 80 RFA mass spectrometer fitted with an Ion-tech gun using xenon as bombarding gas (8–9 keV, 1 mA current). One microlitre of the sample (about 20 μg of the peptides dissolved in 10 μl of 0.5% heptabutyric acid) was mixed with 1 μl of glycerol on the stainless steel target. Masses were assigned by comparison with caesium iodide clusters.

Amino-acids were determined as the DABSyl derivatives following vapour-phase acid hydrolysis over 6 M HCl (100°C, 14 h) [5].

Results and Discussion

Preliminary experiments (data not shown) in which LVP and analogues were lyophilized with moderate excesses (4–10 fold) of reducing sugars (glucose and lactose) and non-reducing sugars (trehalose and sucrose) and incubated at 37°C and 65% relative humidity, showed that additional peptide components were produced in the presence of reducing sugars, but not with non-reducing sugars, and that the reducing sugars were consumed at a rate commensurate with the appearance of these components. Reaction rates under these conditions proved very irreproducible and, since for lactose an identical range of products was observed from reactions carried out in solution, further experiments were performed using solutions in water or phosphate buffer at pH 3.0, 5.5, 7.0 and 8.5 (containing 0.01% w/v of NaN_3 as bacteriostat). Reaction rates were compared at temperatures of −20 (frozen), 4, 20 and 37°C.

When analysed by RP-HPLC at an acidic pH the products were poorly separated and more strongly retained than LVP. Re-injection showed that they were not stable under these conditions; however, chromatography at pH 7 gave a better separation, with the separated components stable for up to 24 h.

Reaction course

Solutions of LVP and lactose in water or in phosphate buffer at 37°C all produced chromatograms which showed a number of products in addition to LVP. A representative

chromatogram is shown in Fig. 2. In the early stages, only one product was observed (A in Fig. 2) and its formation was subsequently followed by the appearance of B and then C. The time courses for the formation of A, B and C are shown in Fig. 3 and these results can be summarized as follows: the formation of A was rapid, was observed within 1 h of incubation and was most pronounced in the solution at pH 7.0; B was formed subsequent to A and its formation was most pronounced in the solution at pH 8.5; the rate of appearance of A and B was slower at pH 3.0 than under any of the other conditions; C was the next product formed, but little or none was observed at pH 3.0; other minor products were produced in the later stages of the reaction (but were not formed to any extent at pH 3.0); and the early

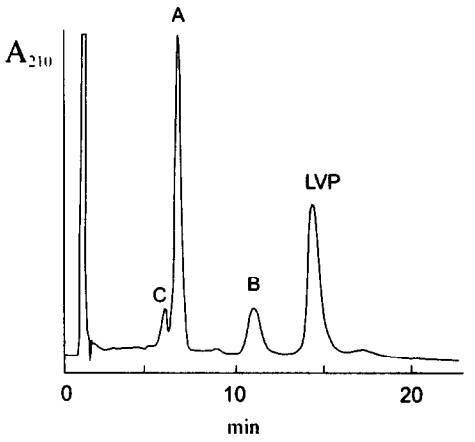

Figure 2
RP-HPLC of LVP/lactose reaction in water after 21 days at 37°C.

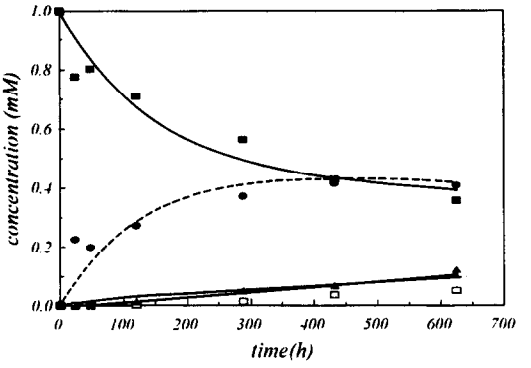

Figure 3
Time course of reaction for LVP/lactose reaction in water at 37°C. Data fitted according to scheme in Fig. 4, with $k_1 = 0.035\ M^{-1}\ h^{-1}$, $k_2 = 0.0032\ h^{-1}$, $k_3 = 0.0035\ M^{-1}\ h^{-1}$, $k_4 = 0.0001\ h^{-1}$. ■: LVP; ●: Product A; ▲: Product B; □: Product C.

reactions (forming A and B) appeared from their time-course profiles (Fig. 3) to be equilibria.

The reaction, as expected, proceeded more slowly at 4 and 20°C but the same products were formed in the same order. This is most simply explained by the scheme of inter-related equilibria set out in Fig. 4. The lines fitted to the observed values in Fig. 3 have been calculated by applying the kinetic scheme of Fig. 4 with the values shown in the legend.

Reversibility of the reaction

Since these reactions were slow at low pH, the mixtures formed after 26 days in pH 8.5 and pH 7.0 buffers were acidified with 1% acetic acid to a pH of approximately 3.0 and then re-analysed. In both cases, levels of products A and C were rapidly reduced (<1 h) and this reduction was accompanied by a concomitant increase in the peak corresponding to LVP. The area of B increased initially by an amount approximately equivalent to the area of C lost, and then declined much more slowly than A or C. C had essentially disappeared after a few hours incubation at 37°C. These results support the reversible nature of these reactions and the acid lability of the products, particularly A and C.

Reaction at lower temperatures

Control samples were routinely analysed. These controls were the original aqueous solutions of LVP plus lactose, stored at −20°C, thawed prior to analysis and immediately returned to storage at −20°C.

These samples were also found to contain product A and, with time, small amounts of B and C. Repeated freezing and thawing of the samples may have contributed to this effect but when solutions were kept at 4°C or were kept frozen at −20°C for 21 days before analysis, considerable amounts of product A were also

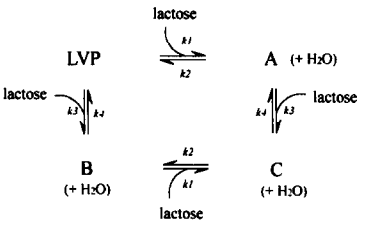

Figure 4
Possible equilibrium scheme of reactions between LVP and lactose.

present (15–50% of total absorbance depending on the pH). The rate of freezing of solutions did not affect these results; similar profiles were obtained from solutions frozen rapidly (in an alcohol–dry ice bath) or frozen more slowly in a freezer at −20°C. These reactions were observed in all the solutions but were most pronounced at pH 7.0 and 8.5 and least pronounced at pH 3.0. Approximately twice as much of product A was formed in the frozen samples as in solution at 4°C. The most likely explanation is that an equilibrium is shifted by removal of water as ice.

It is clear from these results that a very facile reaction is taking place in these solutions resulting in the formation of product A. A second slower reaction also occurs resulting in product B and subsequently product C is formed. All these reactions are reversible and A and C are of higher acid lability than B. The most likely explanation of the observations is that A and B are formed independently and each then reacts further to form C. The simplest scheme is the set of interlinked equilibria illustrated in Fig. 4. From the initial rates of reaction and equilibrium positions the rate constants k_1–k_4 can be estimated and used to derive the lines shown in Fig. 3.

The full set of fitted rate constants for reactions at 37°C and all pH values is given in Table 1. The fit to the reaction scheme is least satisfactory in the case of product C perhaps because of the effects of later irreversible reactions.

Nature of the adducts

Lactose is a reducing sugar and consequently exhibits chemical characteristics associated

Table 1
Estimated rate constants* for the reactions (at 37°C) shown in Fig. 4

Reaction conditions	k_1 (M^{-1} h^{-1})	k_2 (h^{-1})	k_3 (M^{-1} h^{-1})	k_4 (h^{-1})
Water	0.0350	0.0032	0.0035	0.0001
pH 3.0	0.0005	0.0017	0.0005	0.0017
pH 5.5	0.1000	0.0083	0.0050	0.0001
pH 7.0	0.2050	0.0216	0.0100	0.0033
pH 8.5	0.0600	0.0040	0.0075	0.0005

*k_1 and k_3 are second-order rate constants, k_2 and k_4 are pseudo-first-order rate constants (water is the second reactant). Estimates of rate constants were derived from initial rates and estimated equilibrium positions and adjusted by eye to give the most satisfactory fit to the experimental points; the figures shown are, therefore, only approximate. The quality of the fit is particularly insensitive to the value of k_4.

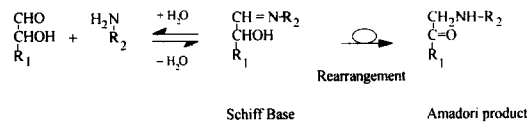

Figure 5
Formation of Schiff base and Amadori rearrangement product.

with an aldehyde. This includes the ability to reversibly react with a primary amine, with the elimination of water, to form a Schiff's base which can subsequently undergo an Amadori rearrangement which is irreversible (Fig. 5) [6–8]. This constitutes the initial stages of the "Maillard Reaction" which is often observed in heat processed foods containing lactose, glucose (or other reducing sugar) and protein. The reaction is known to occur *in vivo* (non-enzymic glycation), especially in relatively long-lived proteins such as haemoglobin and albumin which co-exist with 3–6 mM glucose in normal serum. Glycation levels are elevated in diabetes where serum glucose concentrations are raised, and these modified species are useful markers for identifying and monitoring the disease [9].

The N-terminal amino group of LVP (and of the structurally related oxytocin) are known to exhibit exceptional reactivity towards acetone with which they form a five-membered cyclic isopropylidine derivative involving both the α-amino group of cystine and the neighbouring amide nitrogen [10, 11]. This structure has been confirmed by synthesis [12]. The adduct has been utilized to protect the α-amino group in the construction of an affinity support material in which LVP was specifically coupled through the ϵ-amino group of the lysine residue; this was successfully used to affinity purify neurophysins [13]. The condensation with acetone probably proceeds via the initial formation of an imine (Schiff's base) at the α-amino group.

The pK_a of the α-amino group is anomalously low in LVP and oxytocin (~pH 6); (this low value must follow from the isoelectric point of oxytocin [14] and can be determined by electrophoretic titration [15]). This is probably the major influence on the reaction rate, especially near neutrality, since the active species involved are thought to be the protonated carbonyl and the free amine.

Structural identification of products

As the amount of peptide available for this

study was limited and the reaction mixtures were complex, particularly in the later stages, structural analyses were performed only on the first-formed major products. In addition, since the reactions were reversible, the products A, B and C were isolated as stable derivatives by treatment with sodium cyanoborohydride, which selectively reduces imines [10]. The pattern and retention of the reduced products on HPLC were virtually identical to those of the unreduced reaction mixture. The reduced products A′, B′ and C′ were isolated by preparative HPLC and analysed by amino-acid analysis and FAB mass-spectrometric analysis. The amino-acid analyses (Table 2) showed that A′ and C′ contained much reduced levels of half-cystine and that B′ and C′ contained virtually no lysine. Other amino-acids were present in A′, B′ and C′ in approximately the same proportions as in LVP; recoveries of tyrosine and cystine following hydrolysis are often poor and the authors have found poor recoveries of aspartic and glutamic acids to be an occasional problem of several pre-column derivatization methods for amino-acid analysis. Characteristic products are known to arise from the hydrolysis of glycated lysine [16, 17]; it is not clear what products would be expected following hydrolysis and DAB-Sylation of the reduced derivatives of cystine and lysine, but no peaks clearly corresponding to additional non-protein amino-acids were observed. These results accord with A, B and C being lactose adducts of LVP resulting from the formation of Schiff's bases at the amino group of cystine (A), the amino group of lysine (B) and at both of these amino groups (C). These structures are consistent with FAB mass-spectral data in which LVP gave a molecular ion at an *m/z* value of 1056.5 ($M + H^+$) (calculated 1056.4). A′ and B′ produced molecular ions at *m/z* 1382.6 ($M + H^+$) and at 1404.6 ($M + Na^+$) corresponding to compounds incorporating one reduced Schiff's base adduct with a disaccharide (calculated 1382.6 for $M + H^+$), whereas C produced molecular ions at 1708.4 ($M + H^+$) and at 1730.3 ($M + Na^+$) corresponding to the incorporation of two reduced lactose molecules (calculated 1708.6 for $M + H^+$).

Table 2
Amino-acid analyses of products A′–C′

Mean of two hydrolyses: calculated relative to Phe = 1				
aa	A′	B′	C′	Expected
Asp	0.64	0.82	0.53	1
½Cys	0.33	1.13	0.33	2
Glu	0.74	0.92	0.60	1
Gly	1.03	1.04	0.94	1
Lys	0.80	0.21	0.12	1
Phe	*1.00*	*1.00*	*1.00*	*1*
Pro	0.93	1.10	1.18	1
Tyr	0.55	0.53	0.65	1

The assignment of the structures of these products was further confirmed by comparison of the reaction products with analogues of LVP which differed in the number of amino groups. Oxytocin and arginine vasopressin, both of which possess only one equivalent α-amino group, gave essentially one product on reaction with lactose. These products corresponded in relative retention on HPLC to product A derived from LVP. Desmopressin, which possesses no amino groups (the 'N-terminal' residue is β-mercaptopropionic acid) did not react with lactose, except to incorporate a small amount of radioactivity (data not shown) from ^{14}C lactose into a product with relative retention on HPLC corresponding to product B. Desmopressin is known to contain traces of the *D*-ornithine[8] analogue and this finding is consistent with the assignment of product B and the identification of the equivalent component for desmopressin as the reaction product with traces of 1-desamino-*D*-orn[8]-vasopressin.

Re-examination of original lyophilized material in ampoules

A few samples of the original preparations of LVP lyophilized with lactose remained from the early stages of the work; some of these samples had been subjected to accelerated degradation at elevated temperatures. These were analysed at pH 7.0 (Fig. 6) to see if reaction products with lactose were detectable. Ampoules of lyophilized LVP in acetate buffer (pH 4.5) and stored at −20°C contained a trace of a peak corresponding to product B (the ε-lys[8] adduct). After storage at 37°C for 3 months, 1–2% of peaks A and B were visible together with an additional component X which eluted just after peak A in the chromatogram. X corresponded to one of the peaks which appeared in the later stages of reaction in the model experiments, and was present in about the same amount as A and B. One possibility is that X is an Amadori compound which is likely to be slowly formed over time. When stored at 56°C for 3 months these same

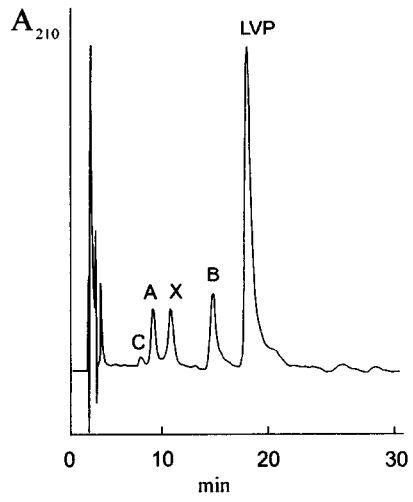

Figure 6
RP-HPLC of LVP preparation in ampoules in acetate buffer (pH 4.5) after 3 months at 56°C.

three components were visible in much greater amounts (6–8% for A and X, and 15% for B) together with a small amount (about 1%) of product C. These products were also present in the samples prepared with citrate in ampoules, but in much smaller quantities (2–7% after 3 months at 56°C). Products A and B were formed in the original material in ampoules at approximately equal rates, whereas in model reactions in solution close to neutrality or in dried samples maintained at 65% relative humidity, product A was formed at a much faster rate than B; this difference may be a result of pH or may perhaps be an effect of water activity.

Summary and Conclusions

To summarise, the results have shown that LVP (and structurally related analogues) reacts with lactose in aqueous solution over a range of pH 3.0–8.5 to give sequential condensation products (Schiff's bases) resulting from reactions at the amino group of cystine followed by that of lysine. These reactions also occur in the lyophilized state. In solution the reaction for cystine is most rapid near neutrality, whereas for lysine the reaction is fastest under more basic conditions. The α-amino group of the cystine residue reacts rapidly at temperatures as low as −20°C. This adduct may be cyclic, a five-membered acetal ring by analogy with the acetone adduct, or may exist as a glycosylamine [18]; however, it is not possible to determine from the results which is the case. The primary reaction, however, must be the reversible formation of an imine at this site. The later-formed products observed on chromatographic analyses probably arise from an Amadori rearrangement and/or further Maillard reactions, and their structures have not been investigated further.

In conclusion, these results have shown that LVP and related peptides are not stable in the presence of lactose and that this reducing disaccharide should be avoided for use as an excipient for these compounds. As noted earlier a similar reaction occurs with glucose but, although in principle modification by glucose at the cystine residue could occur *in vivo*, the short half-life in plasma (2–4 min) and reduced intracellular concentrations of glucose (<1 mM) [19] make it unlikely to be of any significance. The reactivity at the N-terminal appears to be unusual but is not perhaps unique; other structures may exhibit similar reactivity and the possibility of facile glycation (or an analogous reaction) should, therefore, be kept in mind when handling peptide materials, for example in choosing formulation matrices.

Acknowledgements — We would like to thank N. Sutcliffe for the amino-acid analyses, Dr S. Poole and Sandoz Ltd for supplying LVP and Dr C.A. White for assistance in preliminary work.

References

[1] H.N. Antoniades, in *The Plasma Proteins, Structure, Function and Genetic Control*, Vol. 3, 2nd edn (F.W. Putman, Ed.), pp. 381–383. Academic Press, New York (1977).
[2] World Health Organization, *Lists of International Biological Standards, International Biological Reference Preparations and International Reference Reagents*. W.H.O., Geneva (1982).
[3] P.J. Campbell, *J. Biol. Stand.* **2**, 249–258 (1974).
[4] K. Krummen and R.W. Frei, *J. Chromatogr.* **132**, 27–36 (1977).
[5] J.Y. Chang, R. Knecht and D.G. Braun, *Meths. Enzymol.* **91**, 41–48 (1983).
[6] *Maillard Reactions in Food*, (C. Eriksson, Ed.). Pergamon Press, Oxford (1981).
[7] *The Maillard Reaction in Aging, Diabetes and Nutrition*, (J.W. Baynes and V.M. Monnier, Eds). Alan R. Bliss, New York (1989).
[8] F. Ledl and R. Schleicher, *Angew. Chemie Internat. Edn* **29**, 565–706 (1990).
[9] P.C. Cohen, in *Special Topics in Endocrinology and Metabolism*. Alan R. Bliss, New York. **4**, 69–92 (1982).
[10] D. Yamashiro, R.T. Havran, H.L. Aanning and V. Du Vigneaud, *Proc. Nat. Acad. Sci. U.S.A.* **57**, 1058–1061 (1967).
[11] D. Yamashiro, H.L. Aanning and V. Du Vigneaud, *Proc. Nat. Acad. Sci. U.S.A.* **54**, 166–171 (1967).

[12] R.T. Havran and V. Du Vignaud, *J. Amer. Chem. Soc.* **91**, 2696–2698 (1969).
[13] I.C.A.F. Robinson, D.H. Edgar and J.M. Walker, *Neuroscience* **1**, 35–39 (1976).
[14] F. Nachtmann, K. Krummen, F. Maxl and E. Riemer, in *Analytical Profiles of Drug Substances* (K. Florey, Ed.), pp. 563–599. Academic Press, London (1981).
[15] N. Sutcliffe and P.H. Corran, *J. Chromatogr.* **636**, 95–103 (1993).
[16] P.A. Finot, J. Bricout, R. Viani and J. Mauron, *Experientia* **24**, 1097–1099 (1968).
[17] P.A. Finot, R. Viani, J. Bricout and J. Mauron, *Experientia* **25**, 134–135 (1969).
[18] C.I. Neglia, H.J. Cohen, A.R. Garber, P.D. Ellis, S.R. Thorpe and J.W. Baynes, *J. Biol. Chem.* **258**, 14279–14283 (1983).
[19] *Harper's Biochemistry* 23rd edn (R.K. Murray, D.K. Granner, P.A. Mayes and V.W. Rodwell, Eds). Prentice Hall International, London (1993).

[*Received for review 17 May 1994; revised manuscript received 4 July 1994*]