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High-performance liquid chromatographic determination of taurine in human plasma using pre-column extraction and derivatization[1]

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Abstract

Plasma samples (100 μl) were treated with 150 μl of acetonitrile and centrifuged at 5800*g* for 10 min and 50 μl of 10 mM borate buffer (pH 9.2) were added to the supernatant solution. This was followed by the addition of a 50 μl aliquot of 5 mM fluorescamine in acetonitrile and immediate vortex mixing. A 20 μl sample was injected on to a reversed-phase HPLC system using a Bondclone C-18 10 μm analytical column (300 mm × 3.9 mm). The mobile phase was tetrahydrofuran–acetonitrile–phosphate buffer (15 mM, pH 3.5) (4:24:72, v/v/v). The taurine derivative was detected by measuring the UV absorbance of 385 nm. Platelet-poor plasma samples were spiked with known amounts of taurine and inter- and intra-assay calibration curves were obtained. The method was applied to the determination of taurine in platelet-rich plasma.

Keywords: Derivatization; Fluorescamine; Human plasma; Reversed-phase chromatography; Taurine

1. Introduction

Taurine is a naturally occuring β-sulphonated amino acid that is not incorporated into proteins, but found free or in some simple peptides [1]. It has been implicated in many physiological functions, pharmacological actions [2] and pathological conditions. Among the physiological roles attributed to taurine are membrane stabilization [3], antioxidation [4,5], neuromodulation [6] and regulation of calcium homeostasis [7]. Although intracellular taurine concentration is stringently controlled [8], plasma levels are altered during trauma [9], sepsis [10] and cancer [11]. Recent evidence suggests that the level of taurine in plasma may be a useful indicator of myocardial infarction [12]. As the role of taurine in various disease states becomes more widely recognized, the need for a simple, rapid assay for routine plasma taurine estimation becomes more important.

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Recent HPLC methods involving derivatization for the determination of taurine have employed *ortho*-phthalaldehyde (OPA), which requires the presence of a thiol such as mercaptoethanol to ensure that derivatization occurs instantaneously. Although taurine has been determined in this way with both ultraviolet (UV) absorbance [13] and fluorescence detection [14], there are stability problems associated with this reagent. Dansyl chloride has also been used to determine taurine [15], but this derivatization forms many side products, requires quenching and has a long reaction time. Taurine has been determined by derivatization with dansyl chloride, forming a derivative that absorbs in the visible region [16], but the reaction requires high temperatures and the presence of salts can have detrimental effects on the reaction yield.

Other available derivatizing agents for amino acids include phenyl isothiocyanate, but the yield can be adversely affected by the presence of salts, divalent cations and buffers [17]. 9-Fluorenylmethyl chloroformate forms stable derivatives, but hydrolysis products of the reagent interfere unless removed prior to analysis [17].

Fluorescamine was first introduced for the determination of primary amines and amino acids in 1972 [18] and has been used for the quantitation of taurine (Fig. 1) using fluorescence detection [19]. In this work, UV absorption at 385 nm was chosen because whereas the fluorescence intensity may decrease over a few hours, the absorbance remains unchanged for up to 1 week. Fluorescamine was selected because its reaction with primary amines proceeds instantaneously at ambient temperature in alkaline medium, the reagent and its major hydrolysis products do not interfere with UV detection and the derivatives are stable.

2. Experimental

2.1. Chemicals

Taurine (99%) and fluorescamine (98%) were obtained from the Aldrich Chemical (Gillingham, Dorset, UK). All other amino acids were obtained from BDH Chemicals (Poole, Dorset, UK), as were perchloric acid (70%), disodium tetraborate and potassium dihydrogenphosphate. Boric acid was purchased from Merck (Darmstadt, Germany). Acetonitrile, methanol and tetrahydrofuran were of HPLC grade and were purchased from Labscan (Dublin, Ireland). Super-purity acetonitrile from Romil Chemicals (Loughborough, UK) was used for the deproteinization step. Water was deionized using an Elgastat purification system.

2.2. Materials

Hypersep C-18 cartridges (200 mg, 3 ml) were received as a gift from Shandon Scientific (Runcorn, UK). Anion- (SCX) and cation- (PRS) exhange columns (200 mg, 3 ml) were kindly donated by IST (Mid-Glamorgan, UK). Microcon-3 concentrators and Micropure separator inserts were purchased from Amicon (Stonehouse, UK).

2.3. Preparation of reagents and standard solutions

Taurine standard solutions were prepared daily from a stock aqueous solution of 1 mg ml^{-1} that was prepared on a weekly basis. Fluorescamine solution (5 mM) was prepared in acetonitrile and kept at room temperature. Such a solution is stable for 12 weeks [20]. Borate buffer was prepared by adjusting 100 mM disodium tetraborate solution to pH 9.2 with 10 mM boric acid. The 15 mM phosphate buffer was made up each week by dissolving potassium dihydrogenphosphate in water and adjusting the pH to 3.5 with phosphoric acid. The mobile phase was tetrahydrofuran–acetonitrile–phosphate buffer (pH 3.5) (4:24:72, v/v/v). After mixing, the pH of the mobile phase was

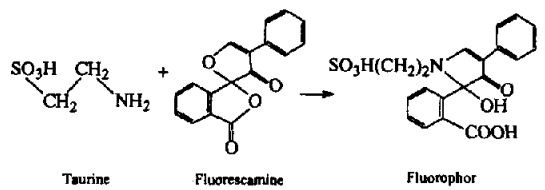

Fig. 1. Derivatization of taurine with fluorescamine.

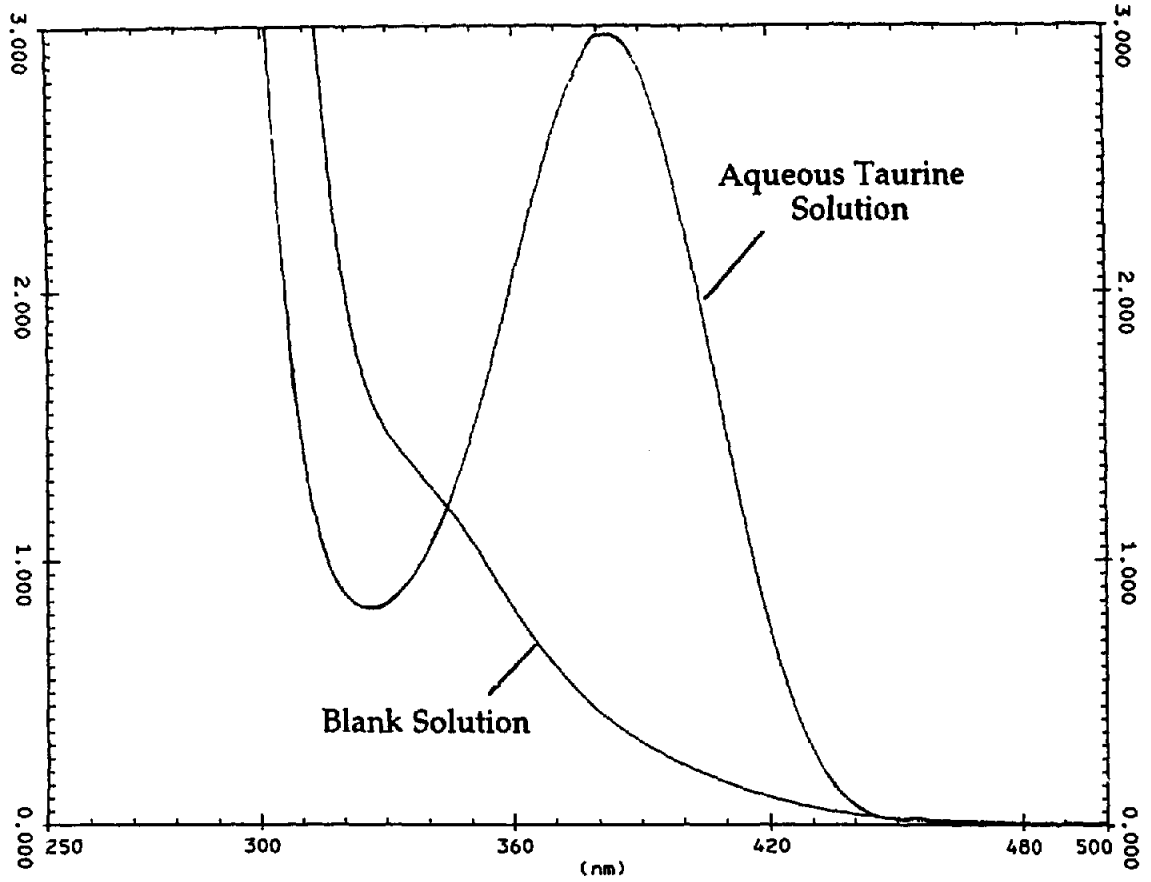

Fig. 2. UV absorbance spectrum of taurine derivative.

assessed, then it was filtered under vacuum through a 0.45 μm Millipore filter and sonicated for 20 min.

2.4. HPLC system

The high-performance liquid chromatograph was equipped with a Waters (Miliford, MA, USA) model 510 dual-piston pump, a Waters Model 486 tuneable absorbance detector and a Waters model 746 data module. The Rheodyne injection port (Cotati, CA, USA) was fitted with a 20 μl loop. A C-8 guard column was fitted prior to the Bondclone C-18 10 μm stainless-steel analytical column (300 mm × 3.9 mm i.d.). The flow rate of the eluent was 1 ml min^{-1}, the system pressure was approximately 1100 psi and all measurements were made at ambient temperature. UV detection was carried out at 385 nm, the maximum absorbance wavelength for the taurine derivative (Fig. 2).

2.5. Sample preparation

Blood samples were taken from fasting volunteers in glass tubes containing sodium citrate as anti-coagulant. Large-bore butterfly syringes were used so as to minimize cell damage, since this can result in platelet ruption and hence give rise to falsely elevated levels of basal taurine present in the plasma. Platelet-rich plasma (PRP) was obtained by centrifugation for 5 min at 170g at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation for 15 min at 1500g at room temperature. Care was taken during pipetting so as not to disturb the buffy coat layer.

For the preparation of spiked platelet-poor samples, 5 μl of the taurine standard solution were added to 95 μl of plasma to give standards with 0, 5, 10, 20 and 30 μg ml^{-1} of taurine added. Each sample (100 μl) was treated with 150 μl of super-purity acetonitrile, vortex mixed and centrifuged for 10 min at 5800*g*. Borate buffer (50 μl, 100 mM, pH 9.2) was added to adjust the supernatant to approximately pH 9. Then 50 μl of fluorescamine in acetonitrile (5 mM) were added and the solution was immediately vortex mixed. Samples were analysed on the HPLC system within 6 h.

3. Results and discussion

3.1. Optimization of protocol

3.1.1. Optimization of sample preparation

Initial experiments centred on ultrafiltration as a method of sample preparation. Microcon-3 concentrators with a molecular mass cut-off of 3000 were used in conjunction with Micropure separator inserts for removal of large molecules prior to derivatization and analysis. The centrifugation time was found to be very long (appproximately 140 min) and, although there was a concentrating effect, few of the lower molecular mass interferents were removed. Clean-up with reversed-phase solid-phase extraction cartridges resulted in a large dilution factor and poor clean-up and recovery. With the anion- and cation-exchange columns, the aim was selectively to retain or selectively to elute the taurine. It was not possible to retain the taurine on the columns under any circumstances and, in fact, extra impurities were introduced. Perchloric acid proved to be an efficient deproteinization agent, but it was subsequently difficult to raise the pH reproducibly prior to derivatization. Boiling was found to give poor reproducibility and methanol was required in a 3:1 ratio to the sample in order to effect complete precipitation of proteins. Deproteinization by acetonitrile proved to be the most facile and reproducible method of sample preparation and was the easiest to execute.

3.1.2. Optimization of reaction conditions

The pH at which the derivatization of taurine takes place is crucial to the reaction. The pH must be $\geqslant 8.5$, and the optimum is 9. Fluorescamine was dissolved in acetonitrile because of its compatibility with the deproteinized supernatant and its suitability as a non-hydroxylic but water-miscible solvent. Immediate mixing is essential for maximum response.

3.1.3. Optimization of mobile phase

The original mobile phase consisted of acetonitrile and phosphate buffer. Originally, this was optimized with the composition acetonitrile–phosphate buffer (pH 2.5, 15 mM) (35:65, v/v). Although this gave good chromatography for aqueous standards, in plasma samples taurine co-eluted with other components. Methanol was used as an organic modifier at various ratios with little success, but tetrahydrofuran changed the selectivity, which improved the resolution between taurine and other endogenous compounds. Hence the final composition was tetrahydrofuran–acetonitrile–phosphate buffer (15 mM) (4:24:72, v/v/v). A variety of pH values were examined, 1.6, 2.5, 2.8, 3.5 and 4.0, and at pH 3.5 taurine was adequately separated from other closely eluting amino acid derivatives such as alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, serine, threonine and valine.

3.2. Quantitative analyses of standards

3.2.1. Calibration and calculation

The spiked samples of platelet-poor plasma were quantified by external standardization. The slope and intercept of the calibration graphs were determined by unweighted linear regression of the taurine peak height versus the concentration of taurine added.

3.2.2. Precision

Precision was defined in terms of the variability between batches (inter-assay) and within batches (intra-assay). Inter-assay variation was assessed singly in four replicate runs covering the concentration range 5–30 μm ml^{-1}. Intra-assay variability was determined in quadruplicate in the same

Table 1
Precision data

Amount added (μg/ml)	Mean amount found ± SD (μg/ml)	RSD (%)	Difference between added and found (%)
Intra-assay (repeatability)			
5 ($n = 3$)	4.56 ± 0.41	9.01	−8.87
10	10.30 ± 0.66	6.38	−3.03
20	20.50 ± 0.59	2.90	−2.50
30	29.63 ± 1.17	3.95	+1.21
		Mean: 5.56	Mean: 4.38
$y = 350.28x - 192.77$			
$r = 0.9998$			
Inter-assay (reproducibility)			
5	4.82 ± 0.26	5.33	+3.56
10	10.22 ± 0.58	5.69	−2.21
20	20.00 ± 0.69	3.43	−0.02
30	29.95 ± 0.31	1.05	+0.15
		Mean: 3.87	Mean: 2.75
$y = 347.74\ (\pm 19.85)x - 179.11\ (\pm 125.78)$			
$r = 0.9995 \pm 0.0002$			

concentration range. The precision of the method was described by the mean relative standard derivation (RSD), and this was found for taurine when the peak heights were interpolated as unknowns on the regression lines. For inter-assay variation (reproducibility), the interpolations were based on the four regression lines generated from the four replicate runs, and for intra-assay variation (repeatability), the interpolations were based on a single regression line generated from the quadruplicate run. Because human plasma contains a basal level of taurine, platelet-poor unspiked plasma shows a small peak in the chromatogram that was assumed to correspond to taurine. An unspiked platelet-poor sample was run with each calibration and the height of the taurine peak was subtracted from the taurine peak in each of the spiked samples. Precision data, calculated on the basis of the subtracted results, are presented in Table 1, and they demonstrate that the reproducibility (mean RSD = 3.87%) and repeatability (mean RSD = 5.56%) of the methods are within accepted values for clinical analyses. These results were compared with data from unsubtracted values (i.e. where the peak in the blank sample was not subtracted from the spiked standards), and it was found that the overall precision values were higher (4.08% and 6.75% for reproducibility and repeatability, respectively) and there was greater variability in precision amongst individual values.

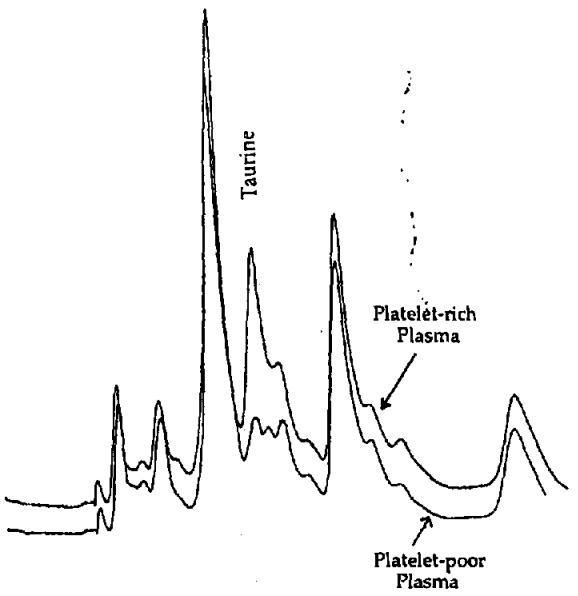

Fig. 3. Platelet-poor and platelet-rich plasma from one volunteer. Column, Bondclone C-18, 10 μm, 300 × 3.9 mm i.d.). Mobile phase, tetrahydrofuran–acetonitrile–phosphate buffer (15 mM, pH 3.5) (4:24:72). Sample preparation as described in text.

Table 2
Comparison of previous methods and results

Ref.	Derivatizating agent/ detection method	Method of collecting and treating blood	Taurine levels found ($\mu g/ml^{-1}$)
[21]	OPA/fluorescence	*Human:* Antecubital vein, vacutainer 7 ml glass tubes with EDTA. PRP: 1000*g*, 15 min, 4°C PPP: 12 100*g*, 30 min. 4°C	PPP: 5.6 ± 0.5 PRP: 16–19 (PC: 200–450 000 per ml plasma)
[22]	OPA/fluorescence	*Human:* Method not quoted	Quoted: 7.4 ± 1.5
[23]	OPA/fluorescence	*Human:* Heparinized tubes, 2000*g*, 10 min	5.0 ± 0.9
[24]	OPA/UV	*Rat:* Whole blood	41.9 ± 4.9
[13]	OPA/UV	*Rat:* Inferior vena cava using heparinized syringes *Chick:* Cardiac puncture, 1400*g*, 10 min	12.6 ± 1.6 13.5 ± 0.04
[25]	Dansyl chloride/ fluorescence	*Feline:* Chilled heparinized syringes, 2677*g*	3.0 ± 0.3

3.2.3. Linearity and accuracy

The correlation coefficient of the regression line for the mean intra-assay values was 0.9998 (subtracted data) and 0.9985 (unsubtracted data). Accuracy (presented in Table 1), as defined as the percentage difference between the amount added and the amount found by back-calculation, was usually less than 5% with mean values of 4.38% and 2.75% for within-batch and between-batch analyses, respectively.

3.2.4. Recovery

Recovery may be calculated in absolute or relative terms. In the calculation of absolute recovery, the peak response of an extracted standard is compared with that of unextracted standards that are prepared to the same theoretical concentration as the extracts. Relative recovery is calculated by comparing peak responses of extracted matrix standards against those of extracted aqueous standards. This procedure was used to account for the presence of acetonitrile in the sample to be injected. Using this method, the relative recovery of taurine from plasma was found to be 89.7%.

3.2.5. Limit of quantitation

The limit of quantitation was found to be 5 μg ml^{-1} taurine in plasma samples.

3.2.6. Selectivity

Taurine is adequately separated from endogenous plasma components, as can be seen in Fig. 3. A number of amino acids are inherently present in both platelet-poor and platelet-rich plasma and the ten thought most probable to interfere (as mentioned in Section 3.1.3) were subjected to the same extraction and separation conditions and, by adjustment of the mobile phase pH and aqueous-to-organic ratio, it was possible to resolve these compounds from the taurine peak. In fact, it is expected that the method described could be applied to the simultaneous determination of taurine and other amino acids.

3.3. Quantitative analyses of samples

The main difference between platelet-poor and platelet-rich plasma from the same volunteer is the height of the taurine peak (Fig. 3). The level of taurine in PRP is usually of the order of four times greater than in PPP [21], but this depends not only on the number of platelets present but also on how the sample was taken and treated. The role of platelets should be considered, since the platelet count can vary from 100 000 to 500 000 per ml of plasma with factors such as age, sex, health and sample treatment playing a part.

In the literature, it is often unclear whether or not platelets are present in plasma samples being analysed for taurine because protocols for blood collection may not be described or may often omit certain procedural steps (Table 2). Different syringes and collecting vials for the blood samples are used, different g-forces and temperatures are common during the centrifugation step and pipetting of the supernatant can cause cell rupture if sufficient care is not taken.

It was found that the platelets could be removed from plasma with minimum rupture if the samples are collected with the large-bore butterfly syringes. This procedure ensures that taurine in platelet-poor plasma is minimized. However, it should be noted that there is always a basal level of taurine present in human plasma. Other workers have circumvented this problem and obtained taurine-free, platelet-free plasma by using plasma from kittens raised on a taurine-free diet. Feline plasma naturally contains low concentrations of taurine (see Table 2).

Samples of PRP from three different sources were determined by interpolation of the peak heights as on a calibration curve (5–30 $\mu g\ ml^{-1}$). Results from the individual in Fig. 3 gave a value of 15.0 $\mu g\ ml^{-1}$, while values from two pooled PRP samples were calculated to be 16.4 and 18.5 $\mu g\ ml^{-1}$. All three results are within the expected range according to literature values.

4. Conclusions

Fluorescamine can be used for the pre-column derivatization of taurine and allows the estimation of taurine levels in platelet-rich plasma. Other analyses have difficult and labour-intensive sample preparation and some involve derivatization procedures that require quenching and/or the presence of co-solvents in order for the reaction to take place. Many are prone to interference from the derivatizing agent itself and there are discrepancies in the stability of derivatives. The method presented here offers a simple, efficient and rapid method for the determination of taurine in platelet-rich plasma, with comparable sensitivity to fluorescence methods, where a result is feasible within 1 h of taking blood from a patient. With escalating work into the quantitation of taurine in human plasma for medical purposes, the simplicity of this assay will be of great benefit to clinical research.

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References

[1] R.J. Huxtable, Fed. Proc., Fed. Am. Soc. Exp. Biol., 39 (1979) 2678.
[2] R.J. Huxtable and L.A. Sebring, Trends Pharm. Sci., (1986) 481.
[3] H. Pasantes-Morales and C. Cruz, J. Neurosci. Res., 11 (1984) 303.
[4] P.P. Stapleton and F.J. Bloomfield, J. Biomed. Sci., 3 (1993) 79.
[5] J. Milei, R. Ferreira, S. Llesuy, P. Forcada, J. Covarrubias and A. Boveris, Am. Heart J., 123 (1992) 339.
[6] K. Kuriyama, Fed. Proc., Fed. Am. Soc. Exp. Biol., 39 (1979) 2680.
[7] J. Azari and R.J. Huxtable, Eur. J. Pharm., 61 (1980) 217.
[8] N.E. Vinton, S.A. Laidlaw, M.E. Ament and J.D. Kopple, Am. J. Clin. Nutr., 44 (1986) 398.
[9] M. Jeevanandam, D.H. Young, L. Ramias and W.R. Schiller, Am. J. Clin. Nutr., 51 (1990) 1040.
[10] L.I. Woolf, A.C. Grovers, J.P. Moore, J.H. Duff, R.J. Finley and R.L. Loomer, Surgery, 79 (1979) 283.
[11] G.E. Grey, A.M. Landel and M.M. Meguid, Nutrition, 10 (1994) 11–15.
[12] S.K. Bhatnagar, J.D. Welty and A.R. Al Yusef, Int. J. Cardiol., 27 (1990) 361–366.
[13] D.W. Porter, M.A. Banks, V. Castranova and W.G. Martin, J. Chromatogr., 454 (1988) 311–316.
[14] G.M. Anderson, T.M. Durkin, M. Chokraborty and D.J. Cohen, J. Chromatogr., 431 (1988) 400–405.
[15] T.J. Amiss, K.L. Tyczkowska and D.P. Aucoin, J. Chromatogr., 526 (1990) 375–382.
[16] V. Stocchi, F. Palma, G. Piccoli, B. Biagiarelli, L. Cucchiarini and M. Magnani, J. Liq. Chromatogr., 17 (1994) 347–357.
[17] C.T. Mant, N.E. Zhou and R.S. Hodges, J. Chromatogr.

Libr., 51B (1992) 75–85.
[18] S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele, Science, 178 (1972) 871.
[19] T. Sakai and T. Nagasawa, J. Chromatogr., 576 (1992) 155–157.
[20] S. De Barnardo, M. Weigele, V. Toome, K. Manhart, W. Leimgruber, P. Bohlen, S. Stein and S. Udenfriend, Arch. Biochem. Biophys., 163 (1974) 390.
[21] K.H. Tachiki, H.C. Hendrie, J. Kellams and M.H. Aprison, Clin. Chim. Acta, 75 (1977) 455.
[22] M. Eslami and J.D. Stuart, J. Liq. Chromatogr., 7 (1984) 1117–1131.
[23] L.L. Hirschberger, J. de La Rosa and M.H. Stipanuk, J. Chromatogr., 343 (1985) 303–313.
[24] T. Hirai, H. Oyhama and R. Kido, Anal. Biochem., 163 (1987) 339–342.
[25] T.J. Amiss, K.L. Tyczkowska and D.P. Aucoin, J. Chromatogr., 526 (1990) 375–382.