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ANALYSIS OF DANSYL DERIVATIVES OF DI- AND POLYAMINES IN MOUSE BRAIN, HUMAN SERUM AND DUODENAL BIOPSY SPECIMENS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A STANDARD REVERSED-PHASE COLUMN

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SUMMARY

The concentrations of putrescine, spermine and spermidine were measured in human serum, children's duodenal biopsy specimens and mouse brain homogenates by high-performance liquid chromatography. The chromatographic analysis was performed on dansyl derivatives of the polyamines using a reversed-phase system with an ion-pairing retention mechanism (heptane sulphonate). Capacity factors were determined at different concentrations of acetonitrile. Simple linear gradients were set up for fast (15 min) or routine (25 min) analysis. Three fluorescence detectors were compared for these determinations and their detection limits determined. The minimum detectable amount of polyamines was 25 fmol compared to 500 fmol with standard detectors. While samples prepared from tissues did not require a high sensitivity, a detector of better performance was needed to assay the polyamines in human serum.

INTRODUCTION

Putrescine (Put), spermidine (Spd) and spermine (spm) are polyamines implicated in many processes affecting cell growth and maturation. In animals, experiments have been concentrated on the study of polyamine metabolism in rapidly growing tissues: embryonic growth of different organs, compensatory growth and experimental tumours [1]. The involvement of polyamines in the

postnatal maturation of the rat small intestinal mucosa has also been demonstrated [2]. Most human studies on polyamines have attempted to relate disturbances of polyamine levels in serum, urine or cerebrospinal fluid with malignant disease, while data on tissue polyamines are scanty [1].

As our laboratory is interested in the study of polyamine metabolism in mice and in humans, we aimed, in the present paper, at developing a method for the measurements of these compounds in biological fluids (serum), and animal (mouse brain) and human (intestine) tissues. Seiler's [3] review of the assay procedures already presented high-performance liquid chromatography (HPLC) as a powerful method [3]. With this technique, these compounds were rapidly separated (20—50 min), and measured in amounts of 10—100 pmol. In addition, the method was shown to be suitable for full automation. The usefulness of HPLC was later apparent for the analysis of unusual polyamines [4], acetylated derivatives [5,6] and in numerous studies on polyamine biosynthesis [7].

As in the case of the amino acid analysis, HPLC approaches are multiple. Separation can be achieved with an ion-exchange column or a reversed-phase column. Post- and pre-column derivatization methods are used to produce UV-absorbing or fluorescent derivatives. Various reagents are available: ninhydrin, o-phthalaldehyde, fluorescamine, and dansyl, dabsyl, tosyl or benzoyl chlorides [3—10].

Among these numerous combinations, reversed-phase separation and a pre-column dansylation procedure were selected for the following reasons. The analysis can be performed with a standard liquid chromatograph without additional devices. Using dansyl chloride (Dns-Cl) the derivatization step allows concentration of the sample prior to injection. This well documented derivatization [3,7,11] seems to give the greatest sensitivity in HPLC analysis [10] by formation of di-, tri- and tetradansyl derivatives for Put, Spd and Spm, respectively. The chosen separation mode was a reversed-phase system. The performance of these stationary phases has been recently improved. They are now available in 3-μm particles for high-speed separations or packed in narrow-bore columns to operate at low flow-rates. These methods of low dispersion enable the modern analyst to increase detectability and to reduce sample volume, solvent consumption and separation time down to several minutes, i.e. minimizes the analysis cost.

The present work was derived from the recent paper of Brown et al. [10]. The regulation of retention was investigated to set up linear gradients depending upon specific requirements. The method will be illustrated by the analysis of polyamines in various biological samples: brain homogenate of young mice (10—30 days), children's duodenal biopsy tissue and human serum. As a high detector sensitivity was required for serum analysis, the sensitivity and selectivity of three fluorescence detectors were compared. In a subsequent paper the performances of three types of columns (standard, high-speed and microbore) will be determined as a second alternative for improving the detectability.

MATERIALS AND METHODS

Apparatus

The HPLC equipment was mainly purchased from Altex Scientific (Berkeley, CA, U.S.A.). It was composed of two solvent metering pumps (A and B, Model 110 A) controlled by a microprocessor (Model 421), a high-pressure mixing chamber and a manual injection valve (Model 210) fitted with a 20-μl sample loop. A 5-μm Ultrasphere-ODS column (150 $\times$ 4.6 mm) from the same manufacturer was protected by a pre-column (42 $\times$ 3.2 mm) dry-packed with Vydac-201 RP (30—44 μm) from Macherey, Nagel & Co. (Düren, F.R.G.) for routine analysis. The performance of this column system was found to be unchanged after one year of use at a rate of two days per week.

Different fluorescence detectors were used. Two filter fluorimeters from Gilson (Middelton, WI, U.S.A.) were compared. The Spectra-Glo model (detector A) was equipped with a 15-μl cell and standard filters for fluorescamine. Model 121 (detector B) had a 9-μl flow cell and similar filters: 310—410 nm and 475—650 nm for excitation and emission, respectively. Detector C was a Model LS-4 fluorescence spectrophotometer from Perkin-Elmer (Norwalk, CT, U.S.A.) with a 3-μl flow cell. With this apparatus optimal wavelengths of 333 and 522 nm were determined by stopping the flow and scanning the excitation and emission spectra.

Electrical signals were processed by a C-R1A Chromatopac recording data calculator from Shimadzu (Kyoto, Japan) or sent to a double-pen recorder (10 mV).

Reagents and solvents

Putrescine, spermidine and spermine were from Sigma (St. Louis, MO, U.S.A.). Dansyl chloride and sodium 1-heptanesulphonate were from Aldrich-Europe (Beerse, Belgium). Carbonate salts, sulphosalicylic acid, acetic and perchloric acids, heptane, methanol and acetone were pro analysi chemicals from E. Merck (Darmstadt, F.R.G.). Acetonitrile-UV was an HPLC-grade solvent from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Procedures

Solvent A for the HPLC analysis was prepared as follows: 20 mM sodium 1-heptanesulphonate and 20 mM acetic acid solution in water delivered by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.). This advantageously replaces the commercial ion-pairing reagent PIC-B7 from Waters Assoc. (Milford, MA, U.S.A.). Solvent B was HPLC-grade pure acetonitrile.

Standard solutions of the polyamines were prepared from 1 mM stock solutions in water and stored at -20°C. They were found to be stable for a period of several months.

Mouse brain homogenates were prepared from C57 black deermice. The heads of the decapitated animals were immediately frozen by immersion in liquid nitrogen and stored at -70°C. One hemisphere (100—140 mg) was rapidly treated by the addition of 10 vols. of 0.2 M cold perchloric acid and homogenized in a glass Potter. The homogenate was left for 15 min at 0°C and proteins were pelleted by centrifugation at 5000 g for 20 min. The pellet

was resuspended in the same volume of acid, homogenized and centrifuged in the same conditions. The pooled supernatants were stored overnight in an ice and water bath. A last centrifugation was performed before derivatization. Aliquots of 200 μl of the clear supernatant were used for derivatization, i.e. 6.8—9.5% of the starting material [10].

Children's duodenal biopsy tissue (2—7 mg) was homogenized in water (1 mg wet tissue per 200 μl) at 0°C using a glass homogenizer. Solid sulphosalicylic acid was added up to a 40 mg/ml final concentration and precipitation was allowed to proceed for 30 min at 0°C. After centrifugation at 2000 g for 15 min, aliquots of 200 μl of the supernatant i.e. 14—50% of the original sample, were submitted to the dansylation procedure.

Human serum was deproteinized by sulphosalicylic acid and treated as described for duodenal biopsy material.

RESULTS

Control of retention and selectivity

In the ion-pairing separation of Brown et al. [10], a concave gradient was used to effect the retention using PIC-B7 in 50% acetonitrile as solvent A and pure acetonitrile as solvent B.

Before setting up a simple linear gradient, which is easily reproducible from laboratory to laboratory, we started by reconstituting the pre-mixed PIC-B7 reagent (as described in the experimental part) to study the influence of acetonitrile on retention. The results of these isocratic measurements are presented in a double-logarithmic plot in Fig. 1. This type of presentation was found to be linear, i.e. more useful than the classical plot of log k' as a function of % B. This allows linear regression parameters to be determined which facilitate inter- or extrapolations. High capacity factors are easily obtained by lowering the acetonitrile concentration, which also increases the selectivity. Such high values are required as many peaks were observed before the elution of Put (Figs. 2—4) or in a blank chromatogram (see Fig. 3 of ref. 10). At high detection sensitivity, and depending upon the sample, a large number of non-polyamine fluorescent peaks were visible throughout the whole chromatogram (Fig. 4). Some of these could coelute with polyamines, i.e. the straight lines of Fig. 1 were crossed by those from impurity peaks, coming mainly from all the chemicals involved in the derivatization procedure. Some of these interfering peaks were eliminated by purification of the dansyl reagent as described by Seiler and Demisch [11].

Gradient set-up and peak identification

Two examples of gradient analysis are presented in Fig. 2, differing by a factor of two in time between injections. The amounts of polyamines are similar to those measured in samples from duodenal biopsies of children. Part A of this figure shows an incomplete separation of Put (peak 1) from the first eluting peaks when starting with an initial solvent composition of 70% (v/v). A partial contamination of Spd (peak 2) due to the steepness of the gradient was also observed. This run took only 12 min. A complete resolution of the peaks of polyamines from impurities was obtained by lowering both
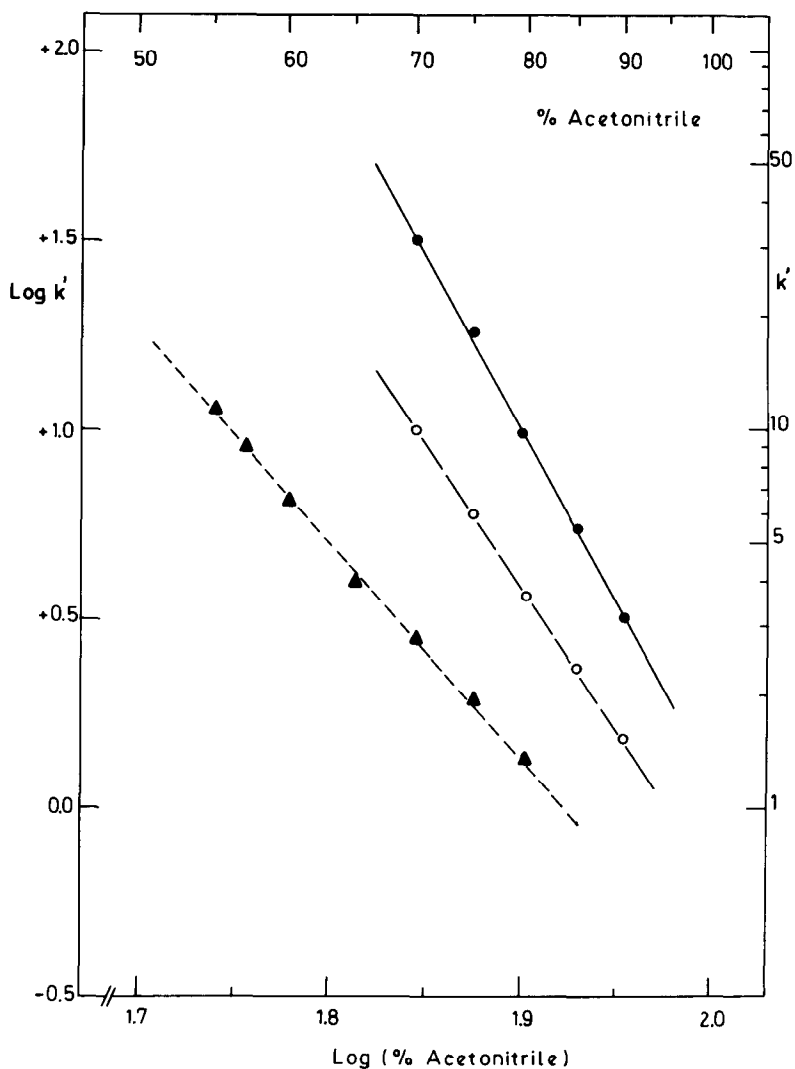
Fig. 1. Dependence of capacity factor mobile phase composition. Sample: 20 μl of dansylated Spm (●), Spd (○) or Put (▲) at 10 nmol/ml. Stationary phase: 5-μm Ultrasphere-ODS, 150 × 4.6 mm. Mobile phase: solvent A = 20 mM heptanesulphonate and 20 mM acetic acid, in water; solvent B = acetonitrile. Flow-rate: 2 ml/min. Detector B: Model 121 from Gilson with standard filters for fluorescamine. Parameters of the linear regressions: intercepts of 18.55, 14.86 and 10.90 for Spm, Spd and Put, respectively; slopes of −9.24, −7.51 and −5.66 for Spm, Spd and Put, respectively.

initial composition and gradient slope (part B). A complete analytical run was achieved in 25 min with a limited loss of sensitivity. This type of gradient was adopted for routine analysis. Retention times were found to be perfectly constant, i.e. a variation coefficient below 1%, so that peak identification by the data processor was possible.

Quantitative analysis of various biological samples
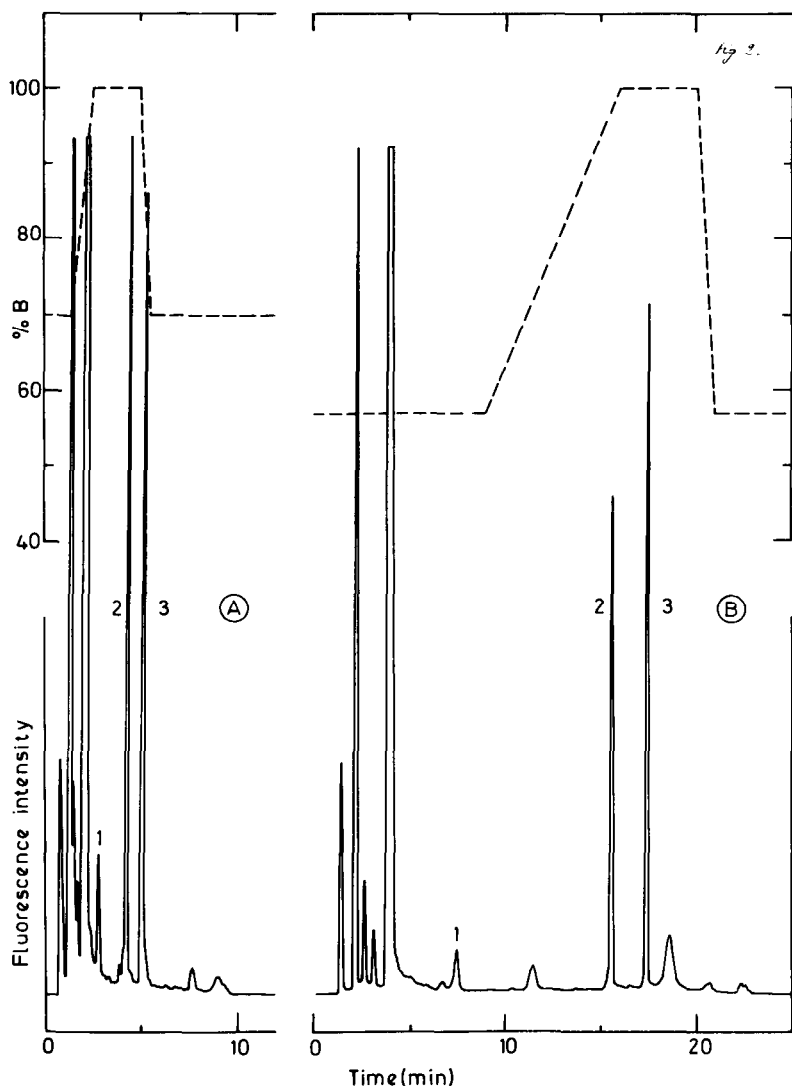   When the concentrations of the dansylated polyamines were assayed in

Fig. 2. Linear gradient separation of a diamine (putrescine) and polyamines (spermidine and spermine) on a 5-μm Ultrasphere-ODS column using a filter fluorometer (detector B). Sample: 20 μl of dansylated Put (peak 1, 1 nmol/ml), Spd (peak 2, 4 nmol/ml) and Spm (peak 3, 4 nmol/ml). Full and dotted lines refer to the recorded fluorescence intensities and to the programmed gradient, respectively. Part A (12-min analytical run): the solvent composition is changed after 1.5 min (70% to 100% B in 1 min) and 5 min (100% to 70% B in 0.5 min). Part B (25-min analytical run): the solvent composition is changed after 9 min (57% to 100% B in 7 min) and 20 min (100% to 37% B in 1 min). Maximum operating pressure at 2 ml/min: 18 MPa (2500 p.s.i.).

three types of biological extracts, the response was found to vary over a wide range of sensitivities depending on the amount of starting material available and the content of polyamines in the investigated sample. Samples prepared from mouse brain or human serum were found to contain amounts of poly-amines differing by two orders of magnitude, as can be seen from the concen-trations of the standard solutions in Table I.

TABLE I

LEVELS OF POLYAMINES IN VARIOUS BIOLOGICAL SAMPLES

The results are expressed as mean values of n measurements ± S.D., nmol/g weight tissue or nmol/ml of serum.

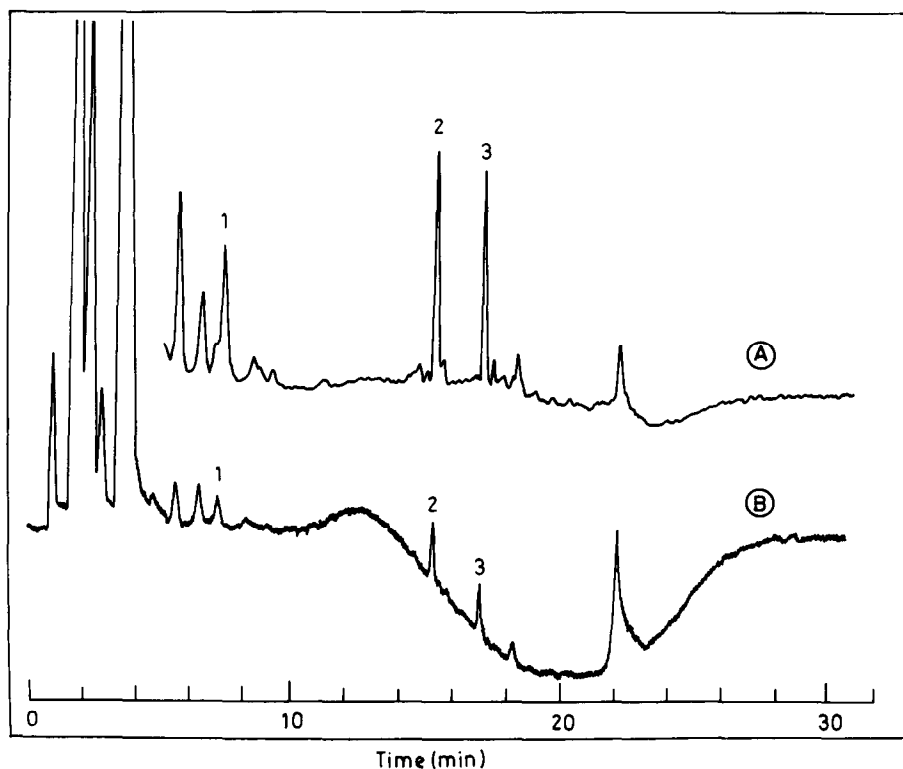| Standards used for calibration (nmol/ml)	Detector type	Sample origin	Type	Putrescine (Put)	Spermidine (Spd)	Spermine (Spm)
5—10	A	Brain from mouse aged 15 days ($n = 5$)	Normal (C_{57} black) Epileptic (DBA/2)	53.6±5.8 70.0±13.8	402±21 470±24	566±38 566±38
1—5	B	Children's duodenal biopsy tissue ($n = 7$)	Normal	52±33	402±61	1362±333
0.05—0.1	C	Human serum ($n = 3$)	Normal	1.1±0.3	0.06±0.01	0.05±0.02

Fig. 3. Comparative response of detectors C (part A) and B (part B) for the injection of 1 pmol of putrescine (peak 1), spermidine (peak 2) and spermine (peak 3) using the 25-min gradient shown in Fig. 2B. Part A (detector C): LS-4 spectrofluorimeter from Perkin-Elmer set at 333 nm (excitation) and 522 nm (emission) using an intermediate scaling factor. Part B: (detector B): Model 121 instrument of Gilson operating at maximum sensitivity.

Mouse brain homogenates and children's intestinal biopsy tissues from normal individuals could be analysed with a low-cost filter fluorimeter. Detector B showed a detection limit of 0.6 pmol for Put and of 0.3 pmol for Spd and Spm, assuming a limiting value of 3 for the signal-to-noise ratio. Detector A was found to be four to five times less sensitive than detector B.
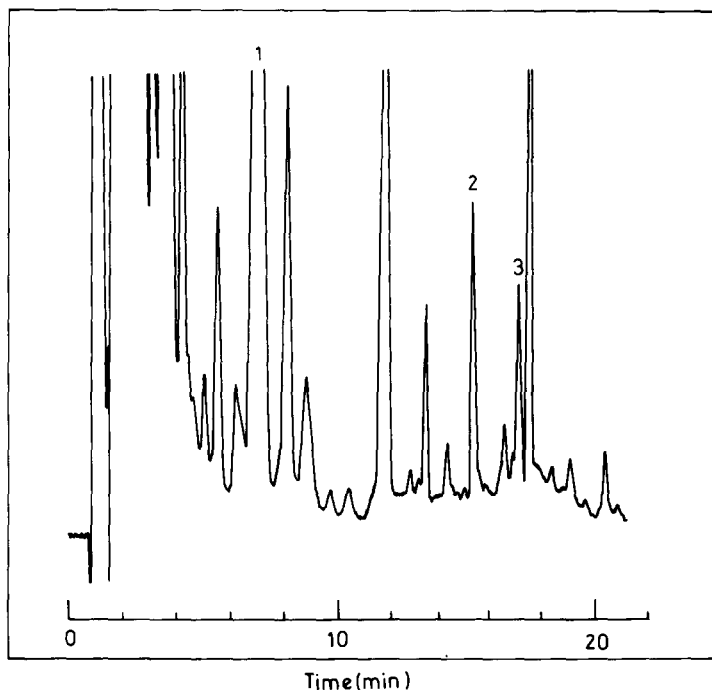
Fig. 4. Analysis of a serum sample using detector C. Peaks as in Fig. 2.

A major drawback of these filter detectors was the significant drift of their baseline when used at high sensitivity in the gradient elution mode. This disadvantage is shown in Fig. 3 where detectors B and C were connected in series for the analysis of a mixture of the three polyamines in equimolar amounts (1 pmol of each dansyl derivative was injected). This effect was proportional to the slope of the gradient and varied from solvent to solvent. It was probably related to changes of refractive index.

The results reported in Table I are in good agreement with those reported in the literature [12,13].

The complete analysis of human serum could not be realized using detector A or B. For this analysis a more sensitive detector was necessary, namely, detector C, which was able to detect less than 0.025 pmol. A typical chromatogram obtained with this detector is shown in Fig. 4. For this type of trace analysis, concentration by peak compression technique would be helpful [14], particularly if a microbore column is used.

CONCLUSIONS

The concentration of polyamines as their fluorescent dansyl derivatives was easily measured by high-performance liquid chromatography in various biological samples. The regulation of retention by heptane sulphonate and acetonitrile as mobile phase components allows high capacity and selectivity factors to be attained. The much higher hydrophobicity of polyamines (spermine and spermidine) as compared to a diamine (putrescine) justified the use of a gradient elution system to shorten the analysis time. Simple linear

gradient led to satisfactory results and a relatively short analysis time. The minimal detectable amount was 25 fmol compared to 500 fmol with standard detectors. This method is well suited for the analysis of polyamines in organ fragments such as those from brain or duodenal biopsies. Preliminary results showed its usefulness for studying the role of polyamines in the mechanism of epilepsy [15] and in the maturation of human intestinal mucosa. The method is presently being extended to the analysis of amines resulting from amino acid decarboxylation, by modification of the elution programme. The application of the method described in the present paper to serum analysis, however, requires a higher degree of sensitivity, which could be reached by the use of a spectrofluorimeter equipped with a small flow cell, instead of using a low-cost filter fluorimeter. Other alternatives for sensitivity enhancement will be considered in a subsequent publication.

ACKNOWLEDGEMENT

REFERENCES

1 J. Janne, H. Poso and A. Raina, Biochim. Biophys. Acta, 473 (1978) 241.
2 G.O. Luk, L.J. Marton and J.B. Baylin, Science, 210 (1980) 195.
3 N. Seiler, Clin. Chem., 25 (1977) 1519.
4 L. Servillo, C. Esposito and R. Porta, Ital. J. Biochem., 27 (1978) 334.
5 N. Seiler and B. Knödgen, J. Chromatogr., 221 (1980) 227.
6 P.K. Bondy and N.Z. Canellakis, J. Chromatogr., 224 (1981) 371.
7 M. Tabor and C.W. Tabor, Methods Enzymol., 94 (1983).
8 G.G. Shaw, I.M.S. Al-Deen and P.M. Elworthy, J. Chromatogr. Sci., 18 (1980) 166.
9 J.-K. Lin and C.-C. Lai, J. Chromatogr., 227 (1982) 369.
10 N.D. Brown, M.P. Strickler and J.M. Whaum, J. Chromatogr., 245 (1982) 101.
11 N. Seiler and L. Demisch, in K. Blau and G. King (Editors), Handbook of Derivatives for Chromatography, Heyden, London, 1978, p. 350.
12 T.W. Jasper, W.G. Luttge, T.B. Benton and A.D. Garnica, Dev. Neurosci., 5 (1982) 233.
13 M.S. Moruzzi, M.G. Monti, G. Piccinini and G. Mezzetti, Life Sci., 31 (1982) 1639.
14 K. Šlais, D. Kouřilová and M. Krejčí, J. Chromatogr., 282 (1983) 363.
15 J. Laschet, J. Bontemps, G. Dandrifosse and T. Grisar, Arch. Int. Physiol. Biochem., 92 (1984) 41.