ELSEVIER

Journal of Chromatography B, 691 (1997) 173–178

JOURNAL OF CHROMATOGRAPHY B

Derivatization and high-performance liquid chromatographic analysis of pentaazapentacosane pentahydrochloride

Susan Heimbecher[a], Yung-Chi Lee[a], S. Esmail Tabibi[b], Samuel H. Yalkowsky[a,*]

[a]*Department of Pharmaceutical Sciences, College of Pharmacy, University of Arizona, Tucson, AZ 85721, USA*
[b]*National Institute of Health, National Cancer Institute, Pharmaceutical Resources Branch, Bethesda, MD 20892, USA*

Received 11 June 1996; revised 10 September 1996; accepted 16 September 1996

Abstract

A rapid high-performance liquid chromatographic method for determination of the dansyl derivative of pentaazapentacosane (PAPC) pentahydrochloride has been developed. The chromatographic system uses a reversed-phase C_8 column, a mobile phase of acetic acid buffer and acetonitrile and UV detection. The dansylation conditions were optimized with a pH of 11.0 and a 20-fold dansyl chloride excess. The yield of dansyl PAPC increased 10-fold as the reaction pH was changed from 9.5 to 10.5. Under derivatization conditions of pH 8.5–11.0 and 1–30-fold excess dansyl chloride only perdansyl PAPC was found.

Keywords: Pentaazapentacosane pentahydrochloride; Polyamines; Dansylation

1. Introduction

Pentaazapentacosane pentahydrochloride (PAPC·HCl) is a synthetically produced aliphatic pentaamine that is being investigated for use as an anticancer agent. As shown in Fig. 1, it is analogous in structure to several naturally occurring polyamines, e.g., spermine and spermidine, that have been implicated in the regulation of cell growth [1].

Like spermine and spermidine, PAPC does not absorb in the UV region and therefore, for most assays, it must be derivatized. Because dansyl chloride is frequently used for aliphatic polyamines [2–4] it was chosen as the derivatizing agent. Dansylation procedures for aliphatic polyamines have used buffers with pH values of less than 9 to greater than 11 [2–13]. Over this range significant differences in the yield of dansylated product and assay sensitivity are expected. Preliminary work with PAPC showed that the typical conditions for amine derivatization (excess dansyl chloride and pH 9–10 [5–7]) were inadequate for analytical purposes. Therefore the effect of pH, dansyl chloride concentration and reaction time on the dansylation of PAPC was investigated in this study.

It has been suggested that partially derivatized polyamines can be formed, especially at low pH or low dansyl chloride concentration [6,10]. However the presence or absence of partially dansylated products has not been specifically demonstrated because most procedures require sample extraction following dansylation and partially dansylated products may be lost in this step. In this study, dansyl

*Corresponding author.

PII S0378-4347(96)00452-5

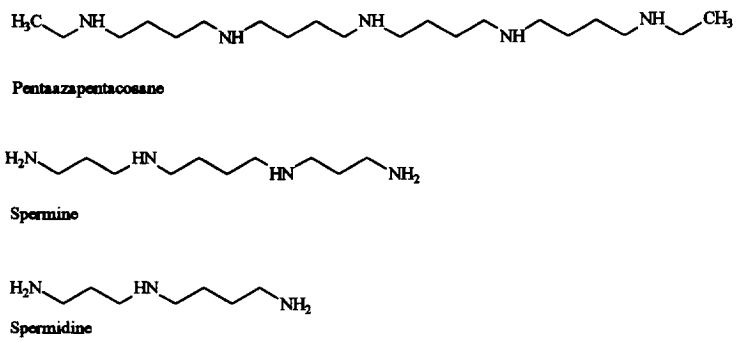

Fig. 1. Structures of PAPC, spermine and spermidine.

PAPC is analyzed without an extraction step to allow detection of partially dansylated products should they exist.

2. Experimental

2.1. Chemicals

PAPC·HCl was supplied by the National Cancer Institute (Bethesda, MD, USA). Piperazine·HCl, 98% and piperidine·HCl, 99% were purchased from Aldrich (Milwaukee, WI, USA). Dansyl chloride (Dns-Cl), 95%, glycine, 99% and horse skeletal muscle myoglobin, 95–100%, were purchased from Sigma (St. Louis, MO, USA). Triethylamine (TEA), HPLC grade, and glacial acetic acid, ACS reagent grade, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium hydroxide GR, was purchased from EM Science (Gibbstown, NJ, USA). Spectrometric grade acetonitrile was purchased from Baxter (Muskegon, MI, USA).

2.2. Derivatization procedure

Duplicate samples of PAPC·HCl (molecular mass 539.9) were derivatized at five different pH values and six different Dns-Cl concentrations. The following solutions were prepared; stock solution of 38.8 μg PAPC·HCl/ml water (equal to 72 nmol/ml); 1.2 mg/ml of dansyl chloride in acetonitrile (equal to 4.4 μmol/ml) and a buffer of 3 ml TEA to 250 ml water (equal to 85.5 μmol TEA/ml). The TEA solution was adjusted to pH 8.5, 9.5, 10.0, 10.5 or 11.0 with glacial acetic acid (TEA buffer was used to minimize precipitation in the mobile phase).

Dansylation was carried out by adding 2.0 ml TEA buffer (at pH 8.5–11.0), 2.0 ml PAPC solution and 0.2, 0.5, 1.0, 2.0, 3.0 or 5.0 ml dansyl chloride solution to a 10-ml volumetric. The samples were then diluted to volume with acetonitrile and water to give a final ratio of 60:40 organic to aqueous. The final concentrations in the derivatization solutions were 14 nmol/ml PAPC, 17 μmol/ml TEA, and 0.09, 0.22, 0.44, 0.88, 1.3 or 2.2 μmol/ml dansyl chloride. The samples were protected from light and allowed to react at room temperature for 4, 8 and 24 h. The reaction was stopped by adding a 2.0-ml aliquot of the reaction solution to 2 ml of glycine (2 mg/ml) in a 10-ml volumetric. After 10 min the neutralized solutions were diluted to volume with 0.04 *M* pH 5 acetic acid–acetonitrile (30:70).

2.3. HPLC analysis

Samples were analyzed with a Beckman System Gold M406 liquid chromatograph. The system includes a 100-μl injection loop, a Beckman 110B solvent delivery pump and a Beckman 168 diode array detector (Beckman Instruments, Fullerton, CA, USA). Data analysis was performed using System Gold Chromatographic Acquisition Software (Beckman Instruments). The column was an adsorbosphere RP-C_8 column, 5 μm, 150×4.6 mm I.D. (Alltech Associates, Deerfield, IL, USA).

The mobile phase was 0.04 *M* pH 5 acetic acid–acetonitrile (10:90). The acetic acid buffer pH had been adjusted with ammonium hydroxide. The HPLC conditions included a flow-rate of 1.5 ml per

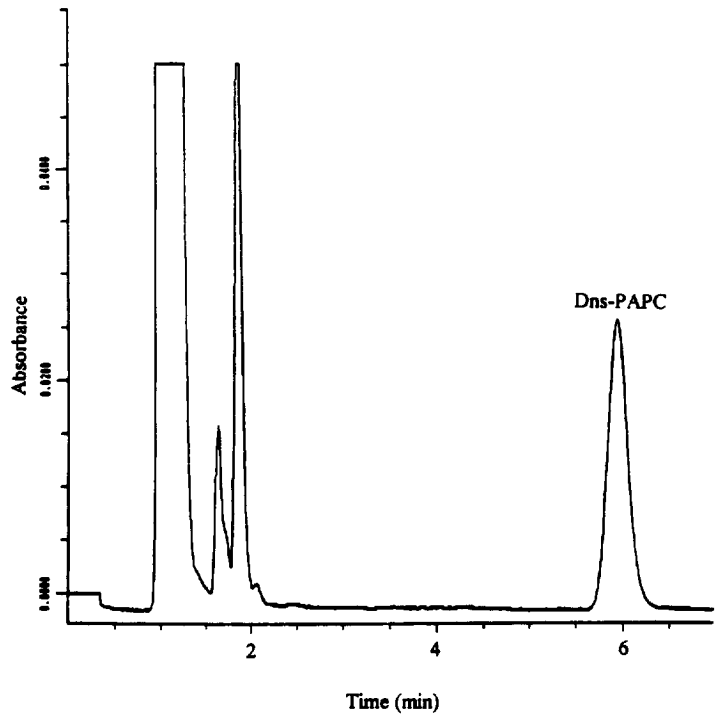

Fig. 2. Chromatogram of dansylated PAPC (Dns-PAPC). The amount injected was 0.3 nmol. The sample had been dansylated in a solution containing TEA buffer (pH 10.5) and 2.2 μmol/ml dansyl chloride for 24 h. For separation conditions see Section 2.3.

minute, UV detection at 254 nm, and an injection volume of 100 μl. As shown in Fig. 2, dansylated PAPC (Dns-PAPC) eluted at 6 min under these conditions.

2.4. Quantitation of dansylated PAPC

The amount of dansylated PAPC detected is reported as percent relative recovery. This is calculated by assigning a value of 100% to the Dns-PAPC peak with the greatest peak area. The amount detected for all other samples is then calculated relative to this value.

2.5. Mass spectrometry

The fraction of HPLC mobile phase corresponding to the 6 min peak was collected. Mass analysis of this sample was done with a Finnigan TSQ 7000 (Finnigan, San Jose, CA, USA) by electrospray mass spectrometry. Calibration was against the multicharge envelop of equine skeletal muscle myoglobin.

3. Results and discussion

3.1. Dansylation time

No significant difference was seen between samples derivatized for 4, 8 or 24 h and therefore the average values for these time points are used. This result indicates that at room temperature dansylation is completed within 4 h.

3.2. Dansyl chloride concentration

An excess of Dns-Cl is typically added in order to compensate for side reactions [3,14]. In aqueous solutions significant amounts of Dns-OH are formed as the pH is increased above pH 9.5 [15]. On the other hand, reagent peaks may interfere with the assay if too large an amount of Dns-Cl is used [8]. In

this study the concentrations of Dns-Cl in the reaction solution (0.09–2.2 μmol/ml) correspond to stoichiometric ratios of approximately 1–30 based upon five amines per PAPC molecule. Fig. 3 shows the effect of Dns-Cl concentration on Dns-PAPC recovery at several different pH values. Each data point in the figure is the average of six measurements. As expected, when Dns-Cl concentrations are low an increase in Dns-Cl results in an increase in Dns-PAPC. However, for every pH tested, the effect of increasing Dns-Cl concentration levels off at about 0.9 μmol/ml (a 13-fold Dns-Cl excess).

3.3. pH

The pH of the solution, as well as the amine pK_a, are critical factors for dansylation. Because Dns-Cl will only react with uncharged amines, dansylation increases as the pH is increased. However, as mentioned above, the formation of Dns-OH also increases with pH. Therefore, the solution pH should ideally be well above the analyte pK_a but not so high that significant Dns-OH is formed [15]. Fig. 4 shows that the amount of Dns-PAPC formed consistently increases with pH. In all cases, very little Dns-PAPC is formed below pH 9.5. However, as the pH is increased from 9.5 to 10.5 the amount of Dns-PAPC increases and, at high Dns-Cl concentrations this is quite dramatic (a 10-fold increase). For these samples a slight change in pH could result in significant analytical variability. Finally, for the high Dns-Cl concentrations no significant change in the amount of Dns-PAPC is seen from pH 10.5 to 11.0. Therefore, using a pH within this latter range, along with sufficient Dns-Cl, should give both a good yield and consistent results.

3.4. Perdansylated PAPC

PAPC·HCl has five secondary amines with estimated pK_a values ranging from 8.5 to >11.0. Because dansyl chloride only reacts with uncharged amines, a partially dansylated product might be expected at a low pH. Also, incomplete derivatization may be expected at low dansyl chloride concentrations [6]. However, for all the conditions tested only one Dns-PAPC chromatographic peak is seen. Since all other peaks on the chromatogram are also found in the blank there is no evidence of partially dansylated PAPC. In order to ensure that these species were not missed due to interference, the early eluting peaks were further separated by increasing the aqueous portion of the mobile phase to 40%.

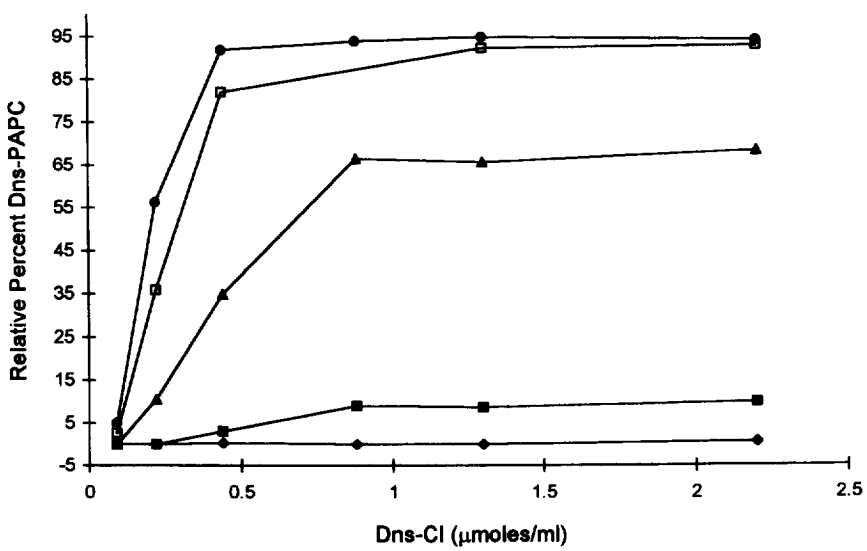

Fig. 3. Dns-PAPC vs. Dns-Cl Concentration for five different pH values. pH 8.5 (♦), pH 9.5 (■), pH 10.0 (▲), pH 10.5 (□), pH 11.0 (●). The data point for pH 10.5 at 0.88 μmol/ml Dns-Cl was an outlier and is not shown.

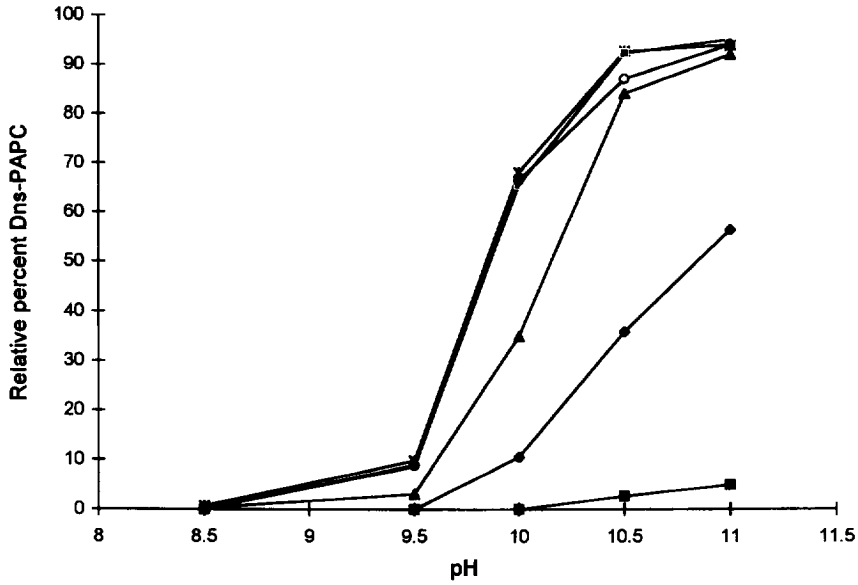

Fig. 4. Dns-PAPC vs. pH for six different Dns-Cl Concentrations. Dns-Cl concentration=0.09 (■), 0.22 (♦), 0.44 (▲), 0.88 (●), 1.3 (□) and 2.2 (★) μmol/ml. The individual data points for the three highest Dns-Cl concentrations are not clearly visible due to overlap. The data point for pH 10.5 at 0.88 μmol/ml Dns-Cl (○) was interpolated from the line in Fig. 3.

Again, under these conditions only one Dns-PAPC peak was obtained.

Mass spectrometry of this peak gave a mass number of 1522, which is equivalent to perdansyl PAPC. In addition, the chromatographic peak area of Dns-PAPC was compared with that of a dansylated monoamine (piperidine) and a diamine (piperazine). Assuming that UV absorbance increases linearly with the number of dansyl groups, the peak area of pentadansylated PAPC should be five times that of a monodansylated compound. As shown in Table 1, the results are reasonably consistent with complete dansylation of PAPC. The slightly lower than expected values may be due to differences in the chromatographic conditions. The absorbance of Dns-PAPC at 254 nm increases slightly in a solution of ACN–buffer (55:45), the mobile phase used for the other dansylated compounds. However, due to the elution time a mobile phase of ACN–buffer (90:10) was used for Dns-PAPC.

Table 1
Relative peak area per picomole on column for a mono, di and penta dansylated compound

Compound	Relative AUC/pmol at 254 nm		Mobile phase
	Calculated	Found	
Monodansyl-piperidine	1.0	1.0	55:45 ACN–buffer
Didansyl-piperazine	2.0	1.8	55:45 ACN–buffer
Pentadansyl-PAPC	5.0	4.4	90:10 ACN–buffer

3.5. Method validation

3.5.1. Detection limit

The method validation was performed under the optimized dansylation conditions of 4 h reaction time, pH 11.0 and 1.3 μmol/ml Dns-Cl concentration. The minimal detection limits for Dns-PAPC were found by injecting 0.7 pmol on to the column. The signal-to-noise ratio at this level was 2:1.

3.5.2. Linearity

The linearity between the polyamine concentration and the measured peak area was determined by analyzing PAPC concentrations of 6.1–122 μg/ml (equal to 11.3–226 nmol/ml). Sample concentrations of 2.4 μg/ml were not within the linear range. At each concentration five replicates were dansylated and assayed as described in experimental Section 2.2 and Section 2.3. This procedure was repeated a

Table 2
Reproducibility of assay

PAPC concentration (μg/ml)	Day 1 Within-run (n=5) C.V. (%)	Day 2 Within-run (n=5) C.V. (%)	Combined results Inter-day (n=10) C.V. (%)
6.10	3.7	2.9	5.6
12.20	1.6	6.5	4.6
24.38	7.6	1.9	6.7
48.76	2.5	1.3	2.5
121.9	1.9	1.4	1.7

second day. The regression equation for the concentration of PAPC standard solutions (y) vs. the peak areas (x) is $y=3.6143x+0.7128$ ($r=0.99995$) for day 1 and $y=3.5847x+0.03599$ ($r=0.99972$) for day 2.

3.5.3. Reproducibility

Samples containing five different concentrations of PAPC in water (five replicates each) were assayed as described in experimental Section 2.2 and Section 2.3. This procedure was repeated a second day. As shown in Table 2 the within-run coefficient of variation ranged from 1.3 to 7.6% and the inter-day coefficient of variation ranged from 1.7 to 6.7%.

4. Conclusion

The optimized dansylation conditions for PAPC·HCl include a pH of 11.0, a Dns-Cl concentration of 1.3 μmol/ml and a dansylation time of 4.0 h. Under these robust conditions samples containing 11–226 nmol of PAPC can be quantitated. Dansylation below pH 10.5 will give smaller and, likely more variable chromatographic peaks. Under a variety of derivatization conditions only the perdansylated product was found.

Acknowledgments

The authors would like to thank Dr. Thomas McClure for the mass spectrometry analysis. This work was supported under Contract No. N01-CM-27757 from the National Cancer Institute.

References

[1] U. Bachrach, C.M. Caldarera and V. Zappia (Editors), Advances in Polyamine Research, Vol. 3, Raven, New York, 1981.
[2] J. Bontemps, J. Laschet, G. Dandrifosse, J. Van Cutsem and P. Forget, J. Chromatogr., 311 (1984) 59.
[3] N. Seiler, J. Chromatogr., 63 (1971) 97.
[4] N. Seiler and B. Knödgen, J. Chromatogr., 164 (1979) 155.
[5] J.M. Wilkinson, in W.S. Hancock (Editor), CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins, Vol. 1, CRC, Boca Raton, FL, 1984, p. 339.
[6] N. Seiler, in N. Marks and R. Rodnight (Editors), Research Methods in Neurochemistry, Vol. 3, Plenum Press, New York, 1975, p. 409.
[7] K. Imai, T. Toyo'oka and H. Miyano, Analyst, 109 (1984) 1365.
[8] P.M. Kabra, H.K. Lee, W.P. Lubich and L.J. Marton, J. Chromatogr., 380 (1986) 19.
[9] Y. Saeki, N. Uehara and S. Shirakawa, J. Chromatogr., 145 (1978) 221.
[10] G.W. Peng, M.A.F. Gadalla, A. Peng, V. Smith and W. L. Chiou, Clin. Chem., 23 (1977) 1838.
[11] M.A. Desiderio, P. Davalli and A. Perin, J. Chromatogr., 419 (1987) 285.
[12] M.C. Gennaro, E. Mentasti, C. Sarzanini and V. Porta, Chromatographia, 25 (1988) 117.
[13] M. Henriks-Echerman and T. Laijoki, J. Chromatogr., 333 (1985) 220.
[14] N. Seiler, in D. Glick (Editor), Methods of Biochemical Analysis, Vol. 18, Wiley, New York, 1970, p. 265.
[15] C. Gros and B. Labouesse, Eur. J. Biochem., 7 (1969) 463.