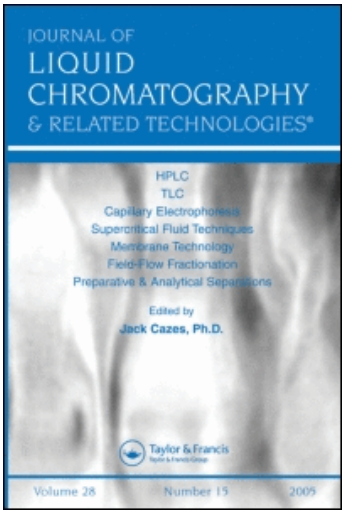


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# The Use of Large Volume Injections for the Isocratic Separation of Phenylthiohydantoin Amino Acids by Microbore Liquid Chromatography

M. R. Silver[a]; T. D. Trosper[a]; M. R. Gould[a]; J. E. Dickinson[a]; G. A. Desotelle[a]
[a] EM SCIENCE, A Division of EM Industries, Inc., Gibbstown, New Jersey

**To cite this Article** Silver, M. R. , Trosper, T. D. , Gould, M. R. , Dickinson, J. E. and Desotelle, G. A.(1984) 'The Use of Large Volume Injections for the Isocratic Separation of Phenylthiohydantoin Amino Acids by Microbore Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 7: 3, 559 — 568
**To link to this Article: DOI:** 10.1080/01483918408073985
**URL:** http://dx.doi.org/10.1080/01483918408073985

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 559-568 (1984)

# THE USE OF LARGE VOLUME INJECTIONS FOR THE ISOCRATIC SEPARATION OF PHENYLTHIOHYDANTOIN AMINO ACIDS BY MICROBORE LIQUID CHROMATOGRAPHY

M.R. Silver, T.D. Trosper, M.R. Gould, J.E. Dickinson and G.A. Desotelle

EM SCIENCE
A Division of EM Industries, Inc.
480 Democrat Road
Gibbstown, New Jersey 08027



## ABSTRACT

The use of microbore columns in high performance liquid chromatography (HPLC) has remained limited primarily due to difficulties in adapting conventional HPLC analysis to the smaller volumes and lower flow rates of microbore chromatography while maintaining high quality chromatographic results.

Although conventional HPLC techniques are the method of choice for PTH-amino acid analysis, these techniques are rapidly approaching their useful limits in the field of microsequencing. A suitable method for the isocratic room temperature separation of PTH-amino acids employing microbore liquid chromatography is discussed herein. As described, the system employed for this type of analysis makes it readily adaptable to the field of protein sequencing. A comparison of a large volume mobile phase injection and a large volume "non-eluting" solvent injection with microbore columns is also presented.

## INTRODUCTION

The general applicability of microbore columns in liquid chromatography is by no means unique and has, in fact, been discussed quite extensively be several investigators (1-5). More specifically, the use of stainless steel microbore columns for the separation and identification of phenylthiohydantoin (PTH) amino



 0148-3919/84/0703-0559$3.50/0

acids has been discussed to some extent as early as 1980 (5). However, in the last several years, numerous technological advances related to microbore HPLC allow for refinements of this specific application.

The advantages of microbore columns, relative to their classical analytical counterparts, include extremely high efficiencies, low solvent consumption under normal operating conditions, and the capability of obtaining high linear velocities at very low flow rates. Moreover, the most distinct advantage of microbore columns is the increase in relative mass sensitivity which can be achieved when sample concentrations are limited. This increase in relative mass sensitivity has been examined and discussed in detail by Scott and Kucera (3).

In the past, the use of microbore columns for routine analysis was somewhat limited. This was due to the small sample injection volumes required (0.5 µl to 5.0 µl) and a lack of readily available instrumentation which did not require expensive modification. This latter point has been gradually rectified with the introduction of commercially available microbore chromatographic systems.



As with any attempt to develop an analytical methodology which has a direct application for routine use in the laboratory, careful consideration must be given to both the advantages and disadvantages of the system employed. This becomes quite apparent when attempting to adapt an analysis system from conventional high performance liquid chromatography to microbore liquid chromatography.

Although modern HPLC techniques have met with considerable success for the analysis of PTH-amino acids, These conventional methods are rapidly approaching their useful limits as research trends advance towards sequencing "microquantities" of protein, i.e. less than 0.5 nanomoles. However, the use of microbore columns with their inherent increase in relative mass sensitivity will extend the usefulness of liquid chromatography into the field of microsequencing; provided that relatively simple analysis systems can be devised.

The basic procedure for the identification of PTH-amino acids is predicated on the comparison of the retention times of the sample PTH-amino acid and the same PTH-amino acid in a standard mixture. Therefore, any microbore chromatographic procedure employed for this type of analysis must provide sufficient resolution of all standard components to make sample identification reliable. Furthermore, analysis time must

be of sufficient length (i.e. thirty minutes or less) in order for the identification process to handle the volume of samples generated by automated sequencing equipment.

Finally, sample working volumes must be large enough (i.e. 50-100 microliters) to obtain complete and reproducible dissolution of the sequencing fractions. This final condition dictates the use of larger injection volumes than are typically used with microbore columns in order to obtain an accurate representation of the sample PTH-amino acid at a reasonable detector attenuation. Thus, the size of the injection volume can be a difficult problem to overcome.

The use of large volume injections with microbore columns for the isocratic separation of twenty PTH-amino acids at room temperature is reported here. A comparison is presented between the use of a 20 µl mobile phase injection and a 20 µl sample volume injection employing a "non-eluting" solvent technique similar to that described by Broquaire and Guinebault (6).



## EXPERIMENTAL

### Equipment, Solvents and Standards

The microbore chromatographic system used for this analysis was composed of a MACS$^{tm}$ 100 single piston pump, a MACS 700 variable wavelength U.V. detector equipped with 0.5 µl flow cells (1.0 mm pathlength), obtained from EM SCIENCE a Division of EM Industries, Inc., A Kipp and Zonen model BD 40 chart recorder, A Hewlitt Packard 3390A integrator, a Rheodyne model 7125 manual injection valve (volume of sample loop 20 µl).

The microbore column used in this study was 500 mm in length with a 1.0 mm internal diameter. This particular column was packed in the laboratory with LiChrosorb RP-18, 7 micron sorbent (E. Merck, Darmstadt, West Germany). Column efficiency, under optimal conditions determined as plates per meter (P/M) was calculated from the following formula;

$$N = 5.54 \left( \frac{t_r}{t_{w0.5h}} \right)^2$$

where N plates per column, $t_r$ is the retention time in seconds of ethylbenzene ($k' = 1.52$), and $t_{w0.5h}$ is the width at half height of

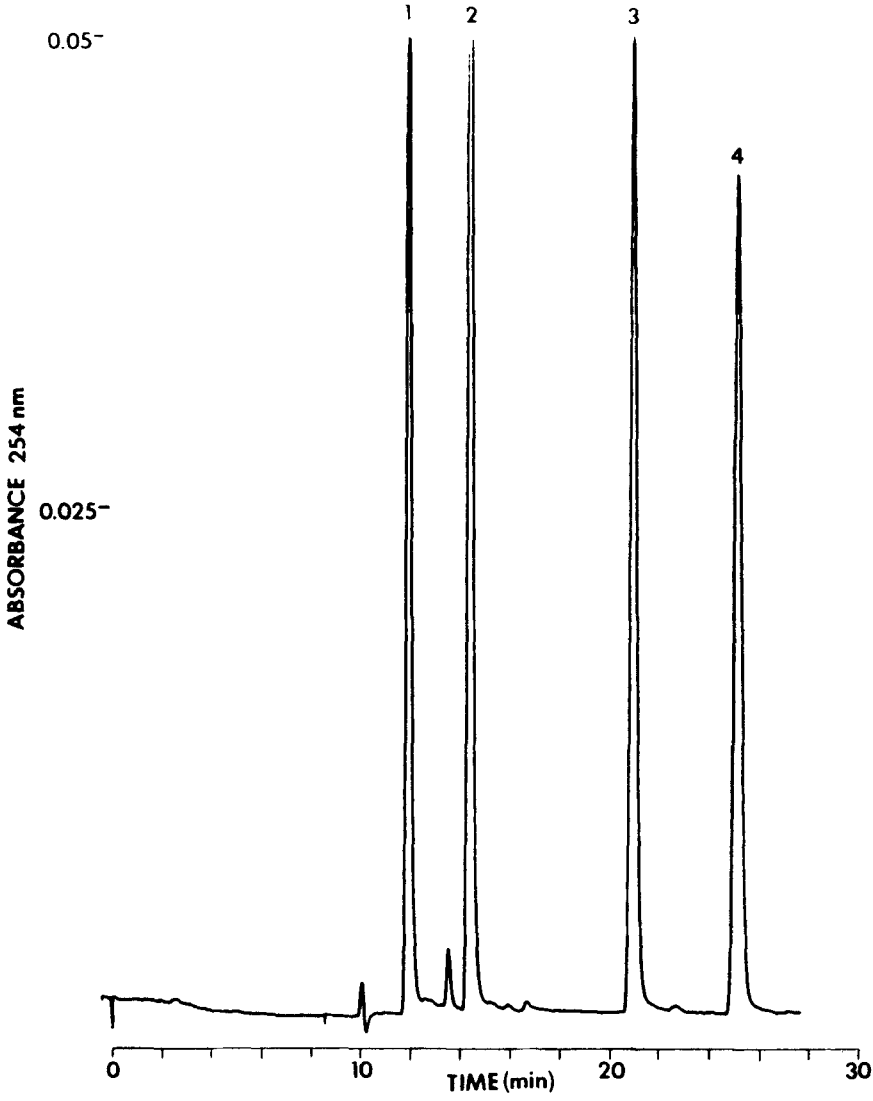


FIGURE 1 Separation of a standard mixture of phenol, ethylphenol, toluene and ethylbenzene.

ethylbenzene expressed in seconds. All values are based on the separation of a standard mixture (Figure 1) of phenol (1.0 g/l, peak #1) ehtylphenol (2.0 g/l, peak #2) toluene (4.0 g/l, peak #3) and ethylbenzene (4.0 g/l, peak #4) using a methanol/water (80:20) solvent system, flow rate 30 µl/minute, 0.5 µl sample injection volume, U.V. detection at 254 nm, at a sensitivity of 0.5 Absorbance Units Full Scale (AUFS), and a chart speed of 5 mm/minute.

Low-absorbance grade sodium acetate (AcONa), OmniSolv® HPLC grade acetonitrile and OmniSolv water used for the mobile phase were obtained from EM SCIENCE, a Division of EM Industries, Inc. (Gibbstown, New Jersey). All PTH-amino acid standards were purchased from Pierce Chemical Co. (Rockford, Illinois) with the exception of PTH-glutamic acid methyl ester (EoMe) and PTH-aspartic acid methyl ester (DoMe) which were synthesized in the laboratory.



## Chromatographic Conditions

The chromatographic conditions employed for the analysis of the PTH-amino acid standards were as follows:

Sample: 1.0 nanomole of each of 20 PTH-amino acid standards
Injection Volume: 20 µl
Mobile phase: Acetonitrile/AcONa 0/01M pH 4.5 (42/58 v/v)
Flow rate: 75 µl/min. (2200 psi)
U.V. detection: 269 nm
Detector sensitivity: 0.2 AUFS
Chart speed: 1.0cm/min.
Temperature: Ambient

The "non-eluting" solvent used for sample injections was composed of 25% mobile phase and 75% water.

## RESULTS AND DISCUSSION

The separation of the standard mixture used for the calculation of column efficiency is shown in Figure 1. The value of N as calculated from the ethylbenzene peak (#4) was 37,250 plates (74,500 P/M).

The chromatogram shown in Figure 2 demonstrates the effects of a 20 µl mobile phase injection containing 1.0 nanomole of each standard PTH-amino acid. The standard single letter abbreviations are used for peak identification. The lack of resolution and peak tailing observed in this chromatogram is ultimately attributable to elution of the sample before the injection is complete.

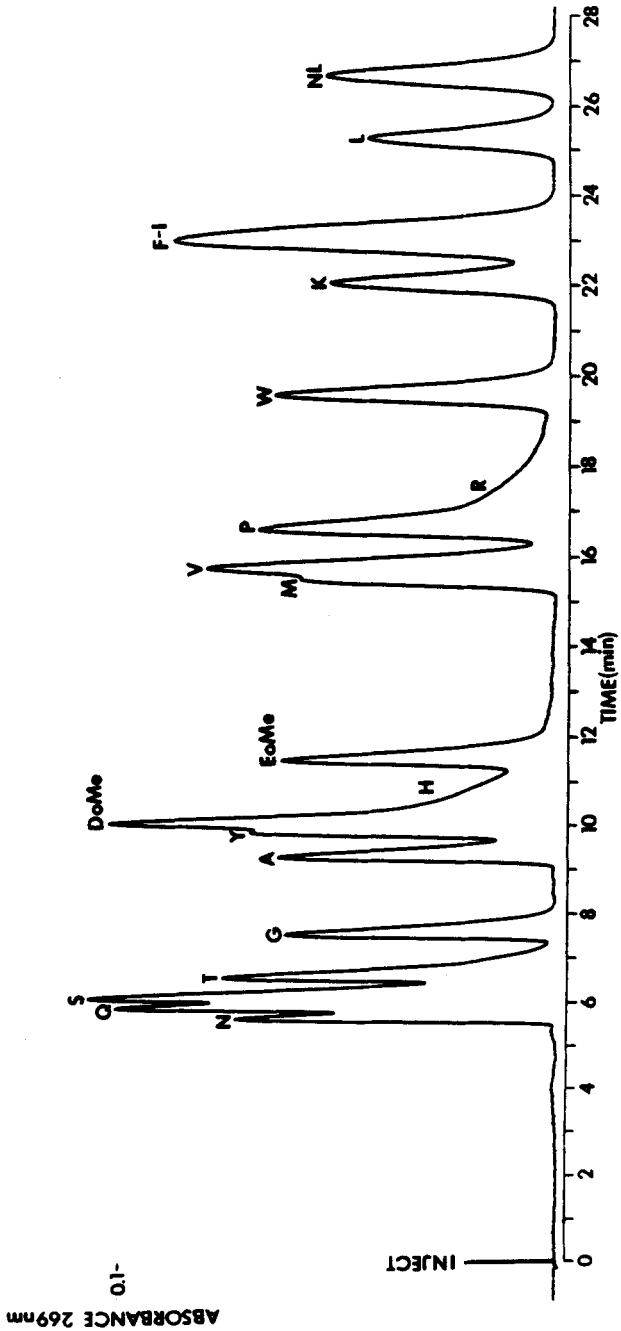


FIGURE 2 Separation of twently PTH-amino acids using a 20 µl mobile phase injection.

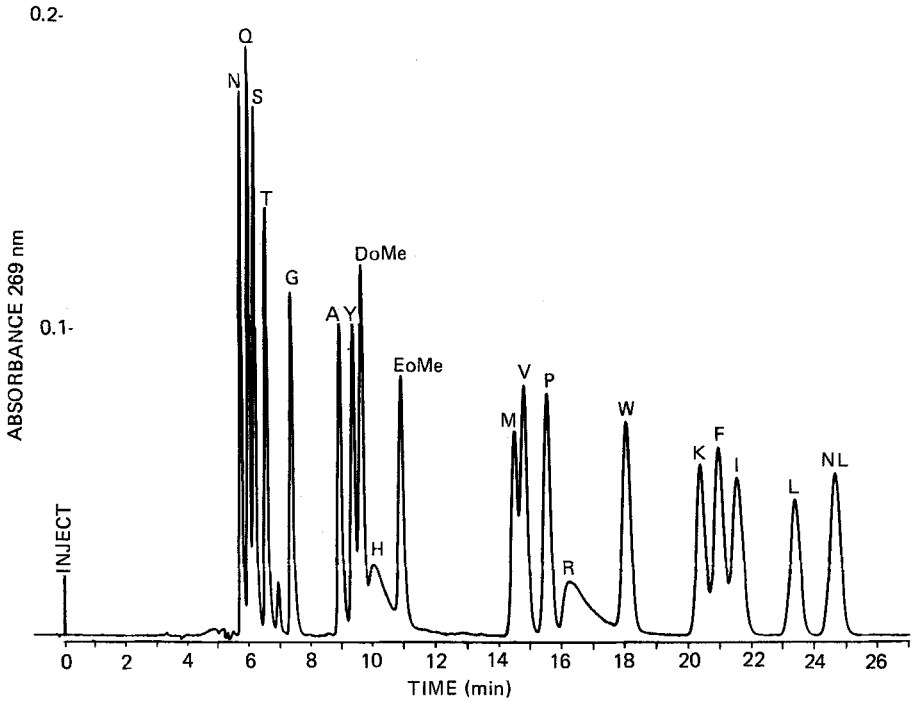


FIGURE 3 Separation of twenty PTH-amino acids using a 20 µl "non-eluting" solvent injection.

In contrast, the chromatogram shown in Figure 3 demonstrates the effects of a 20 µl "non-eluting" solvent injection containing an identical quantity of each standard. In this chromatogram, resolution of all components is greatly increased over the mobile phase injection making identification of the individual standards reliable. This dramatic increase in resolution is due to a concentrating effect whereby the sample remains at the head of the column until the injection is complete and thus maintaining column efficiency.

A comparison of the two injection techniques reveals not only a loss of efficiency with the mobile phase injection but also an increase in overall analysis time resulting from a shift in retention times of the individual components. Furthermore, There is a measurable decrease in sensitivity of the early eluting peaks due primarily to peak broadening. These results are expected in accordance with the data reported for large bore columns by Broquaire and Guinebault (6).

TABLE I

Reproducibility Of The "Non-eluting" Injection Technique

| PTH-Amino Acid | $t_r$ (minutes) | Mean $t_r$ | Standard Deviation | PTH-Amino Acid | $t_r$ (minutes) | Mean $t_r$ | Standard Deviation |
|---|---|---|---|---|---|---|---|
| N | 5.88 | 5.87 | 0.017 | M | 14.67 | 14.67 | 0.025 |
| Q | 6.12 | 6.12 | 0.021 | V | 14.93 | 14.94 | 0.029 |
| S | 6.36 | 6.36 | 0.021 | P | 15.85 | 15.86 | 0.013 |
| T | 6.75 | 6.74 | 0.022 | R | 16.24 | 16.22 | 0.026 |
| G | 7.69 | 7.67 | 0.024 | W | 18.25 | 18.23 | 0.026 |
| A | 9.32 | 9.30 | 0.027 | K | 20.68 | 20.68 | 0.057 |
| Y | 9.70 | 9.73 | 0.025 | F | 21.11 | 21.10 | 0.069 |
| DoMe | 9.90 | 9.88 | 0.026 | I | 21.87 | 21.86 | 0.074 |
| H | 10.17 | 10.15 | 0.027 | L | 23.71 | 23.70 | 0.082 |
| EoMe | 11.06 | 11.04 | 0.026 | NL | 24.88 | 24.87 | 0.086 |



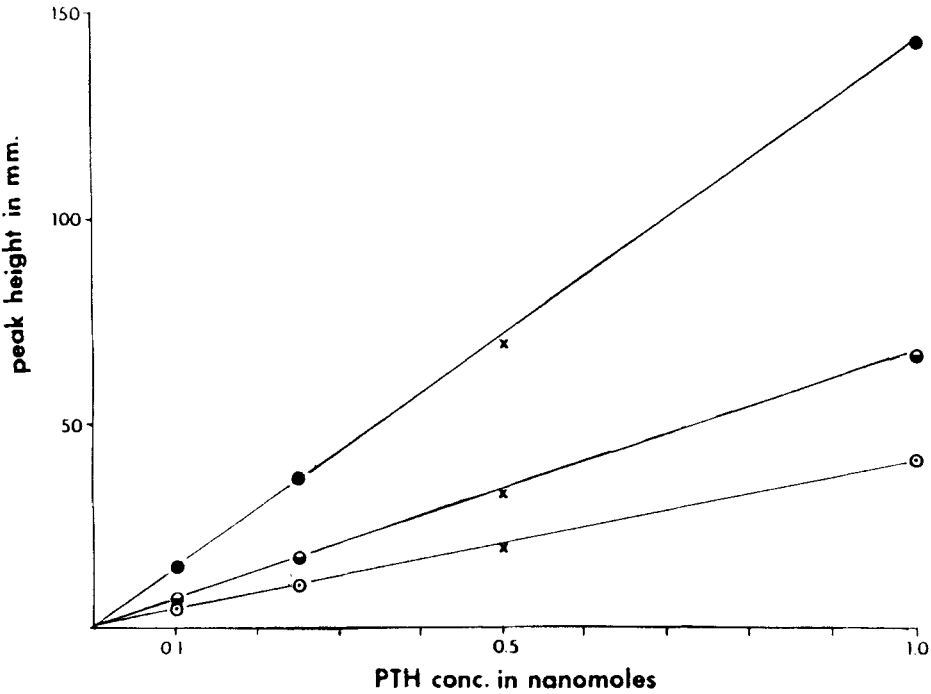


FIGURE 4 Linearity of the isocratic microbore system represented by Asparagine (●), Proline (◒), and Leucine (⊙). Peak heights obtained for a 0.5 nanomole sample are represented by x.

Table I demonstrates the reproducibility of the "non-eluting" injection technique in conjunction with the isocratic system. The mean retention times were calculated from four replicates of 1.0 nanomole injections. The maximum time between replicates was 5.0 minutes. Figure 4 demonstrates the linearity of the system based on peak height in millimeters. Three representative PTH-amino acids were chosen from the beginning (N), middle (P), and end (L) of chromatograms generated from 0.1, 0.25 and 1.0 nanomole samples. The peak heights obtained for the same three PTH-amino acids from a 0.5 nanomole sample were found to be in good agreement with the values determined graphically.

The techniques described herein demonstrate that the use of large volume injections in conjunction with microbore liquid chromatography is a viable alternative to conventional HPLC. The system employed for the analysis shown in Figure 3 provides a rapid and relatively simple technique for the separation of PTH-amino acids which meets the criteria necessary for direct application to protein sequence analysis. Furthermore, serious consideration should be given to the advantage of increased mass sensitivity which will be addressed in a future study.



## ACKNOWLEDGEMENTS

MACS and OMNISOLV are trademarks of EM Industries, Inc.

LiChrosorb is a registered trademark of E. Merck, Darmstadt, West Germany.

## REFERENCES

1. Scott, R.P.W. and Kucera, P., Mode of Operation and performance characteristics of microbore columns for use in liquid chromatography. J. Chromatogr. 169, 51, 1979.

2. Scott, R.P.W., Kucera, P. and Munroe, M., Use of microbore columns for rapid liquid chromatographic separations. J. Chromatogr. 186, 475, 1979.

3. Scott, R.P.W. and Kucera, P., Use of microbore columns for the separation of substances of biological origin. J. Chromatogr. 185, 27, 1979.

4. Ishii, D., Asai, K., Hibi, K., Jonokuchi, T. and Nagaya, M., A study of micro high performance liquid chromatography. 1 Development of techniques for the miniaturization of high performance liquid chromatography. J. Chromatogr. 144, 157, 1977.

5. Godtfredsen, S.E. and Oliver, R.W.A., On the analysis of phenylthiohydantoin amino acids by high performance liquid chromatography. Carlsberg Res. Commun. 45, 35, 1980.

6. Broquaire, M. and Guinebault, P.R., Large volume injection of biological samples dissolved in a non-eluting solvent: a way to increase sensitivity and a means of automatic drug determination using HPLC. J. Liq. Chromatogr. 4, 2039, 1981.