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ISOCRATIC SEPARATION OF PHENYLTHIOHYDANTOIN-AMINO ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

It is possible to separate 19 commonly occurring phenylthiohydantoin (PTH)-amino acids in a single run by isocratic elution within 20 min using 25 × 0.46 cm I.D. Ultrasphere-ODS columns. The different factors that influence the resolution of PTH-amino acids, *e.g.*, temperature, pH, ionic strength of the buffer, flow-rate and batch-dependent variations of the eluent composition were investigated and are discussed.

INTRODUCTION

For the identification and quantification of phenylthiohydantoin (PTH)-amino acids resulting from Edman degradation, high-performance liquid chromatography (HPLC) appeared to be a promising method. For the separation of PTH-amino acids reversed-phase material and gradient elution have most frequently been used in the bast[1–4]. Only a few papers on the isocratic separation of PTH-amino acids have been published[5–8]. Zimmerman *et al.* first reported on the isocratic separation method[6], but this method was not favoured later as their subsequent studies utilized gradient elution[4].

Gradient elution permits variations of the separation to be made; however, reproducibility is a serious problem and limits its application to highly specialized laboratories. Isocratic systems are more reliable and suggested the possibility of introducing the separation of PTH-amino acids even in routine practice.

However, our studies on the isocratic separation of PTH-amino acids revealed that there are still a number of factors that influence the separation more seriously than expected, namely temperature, batch-dependent variations in the eluent composition, flow-rate, pH, ionic strength of the buffer and interfering solvents introduced by the sample. Following extensive studies on the effects of these factors, isocratic separation conditions are proposed that may allow acceptable separations even by inexperienced workers. The isocratic separation time is 20 min, and 19 out of 20 PTH-amino acids were separated (Fig. 1).

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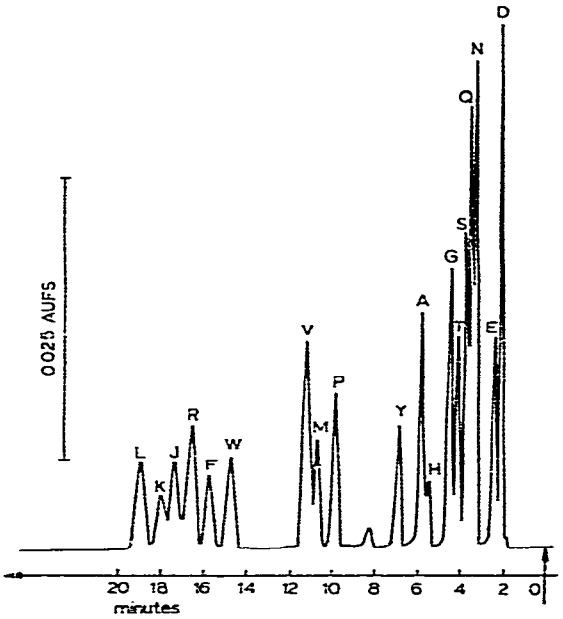

Fig. 1. Isocratic separation of PTH-amino acids on Ultrasphere-ODS. Eluent, 31.5% acetonitrile–6.75% tetrahydrofuran in 5.24 m*M* acetic acid adjusted to pH 5.80 (35% solvent B and 65% solvent A). Solvent A: tetrahydrofuran–5.24 m*M* sodium acetate (pH 5.15) (5:95); solvent B: tetrahydrofuran–acetonitrile (10:90). Sample size, 1 nmol of each PTH-amino acid; flow-rate, 1.0 ml/min at 55°C; detection wavelength, 254 nm. For single letter notation of amino acid residues, see IUPAC–IUB Commission on Biochemical Nomenclature, 1968.

EXPERIMENTAL

Standard solutions of PTH-amino acids (Serva, Heidelberg, G.F.R.) were prepared, about 100 nmol of each being dissolved in 1 ml of HPLC-grade acetonitrile–tetrahydrofuran (9:1). Solutions were stored at −25°C. Acetonitrile (HPLC grade), tetrahydrofuran (Uvasol grade) and analytical-reagent-grade sodium hydroxide and acetic acid were obtained from E. Merck (Darmstadt, G.F.R.). The water used for the experiments was distilled twice.

Analyses were performed with a Beckman device consisting of two solvent delivery systems (Models 100A and 110A), a variable-wavelength detector (Model 165) and a gradient former (Model 420). Separations of PTH-amino acids were carried out at different temperatures using an Altex Ultrasphere-ODS (5 μm) column (25 × 4.6 mm I.D.). The injection volume was 10 μl (*ca.* 1 nmol of each PTH-amino acid). Detection was carried out at 254 nm.

RESULTS

Temperature effects (Figs. 2 and 3)

Separation was investigated at 20, 30, 40, 50, 55, 57 and 60°C. The resolution of PTH-Asp, PTH-Glu, PTH-Asn, PTN-Gln, PTH-Ser, PTH-Thr and PTH-Gly was

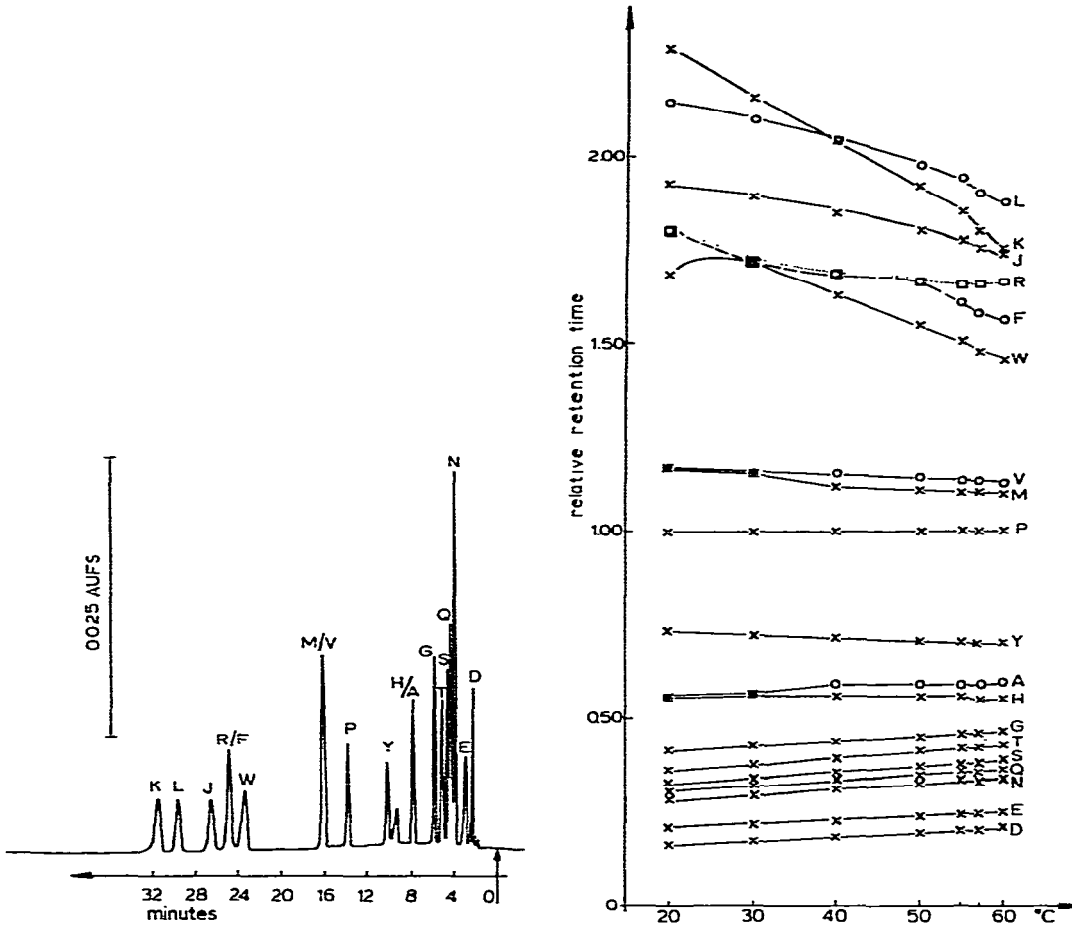

Fig. 2. Separation of PTH-amino acids at room temperature. Conditions as in Fig. 1, except temperature.

Fig. 3. Effect of temperature. Conditions as in Fig. 1, except temperature.

most effective at room temperature. PTH-Ala and PTH-His, PTH-Met and PTH-Val, PTH-Arg and PTH-Phe were not separated. The last peak (PTH-Lys) was eluted after 31.5 min (Fig. 2).

At 30°C PTH-His began to separate from PTH-Ala. PTH-Met appeared as a shoulder on the PTH-Val peak. PTH-Trp, PTH-Arg and PTH-Phe were not separated. PTH-Lys and PTH-Leu eluted as one broad peak forming a shoulder. The retention time of PTH-Lys decreased from 31.5 to 27.2 min. At 40°C PTH-His was clearly separated from PTH-Ala and PTH-Met was partly separated from PTH-Val. PTH-Trp began to separate from PTH-Arg and PTH-Phe. The retention time of PTH-Leu was 23.4 min. At 50°C the PTH-amino acids showed separation patterns as at 55°C, except PTH-Arg and PTH-Phe. PTH-Arg appeared as shoulder on the PTH-Phe peak. The elution time decreased from 23.4 to 20.8 min. At 55°C the optimal separation of PTH-amino acids took place. The change in retention time was minimal.

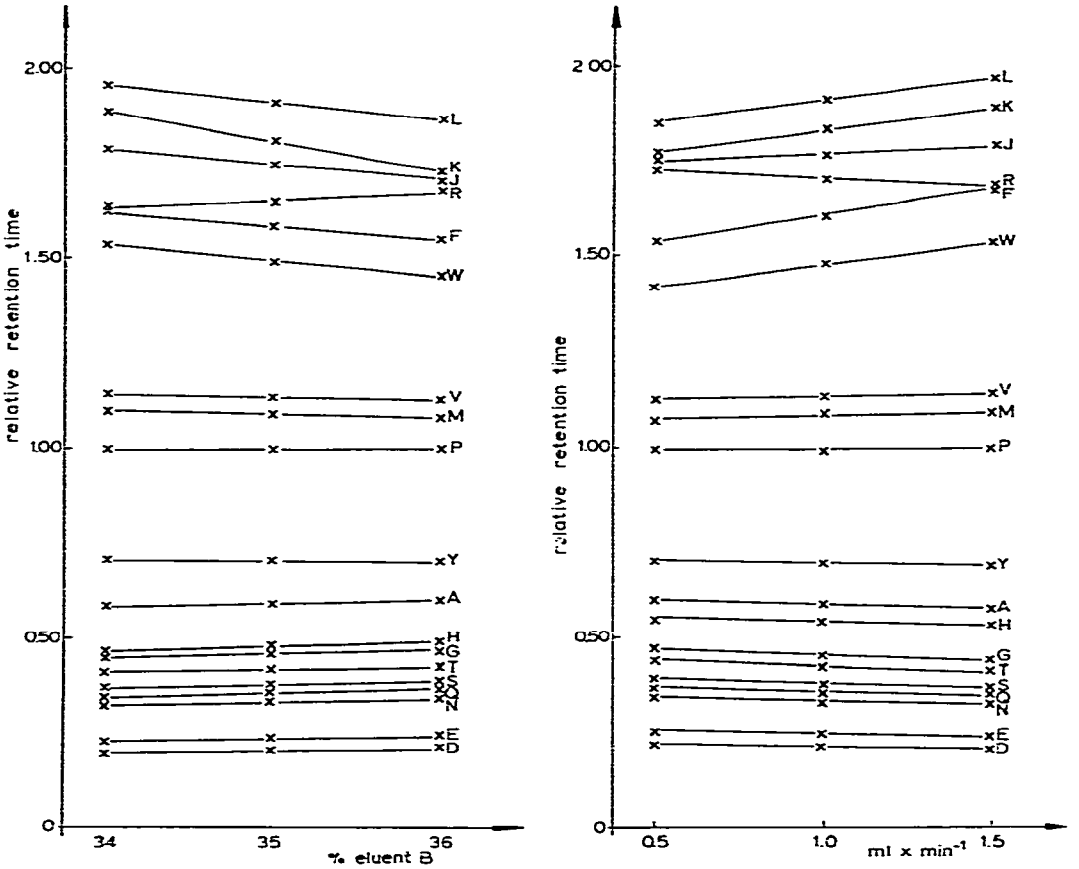

Fig. 4. Eluent effects. Eluent A, tetrahydrofuran–in 5.24 m*M* sodium acetate buffer (pH 5.15) (5:95); eluent B, acetonitrile–tetrahydrofuran (90:10). Other conditions as in Fig. 1.

Fig. 5. Effect of flow-rate. Conditions as in Fig. 1, except flow-rate.

PTH-Leu eluted at 55°C in 19.5 min (Fig. 1). At 57°C PTH-Lys and PTH-Ile began to become closer. PTH-Lys and PTH-Leu eluted together at 60°C.

Effects caused by solvents (Fig. 4)

The alteration of relative retention times of PTH-Leu, PTH-Ile, PTH-Phe, and PTH-Trp were linear and parallel, although overlaps occurred owing to significant changes in the relative retention times of PTH-Arg and PTH-Lys.

A decrease or increase of 1% in eluent B (acetonitrile–tetrahydrofuran, 90:10) affected the separation of PTH-Arg, PTH-Ile, PTH-Phe and PTH-Lys. Following a 1% decrease in eluent B from 35% to 34%, PTH-Arg and PTH-Phe eluted together, and following a 1% increase in eluent B to 36%, PTH-Lys, PTH-Ile and PTH-Arg eluted together. The other PTH-amino acids were hardly affected.

Flow-rate (Fig. 5)

The relative retention times of all PTH-amino acids changed linearly with

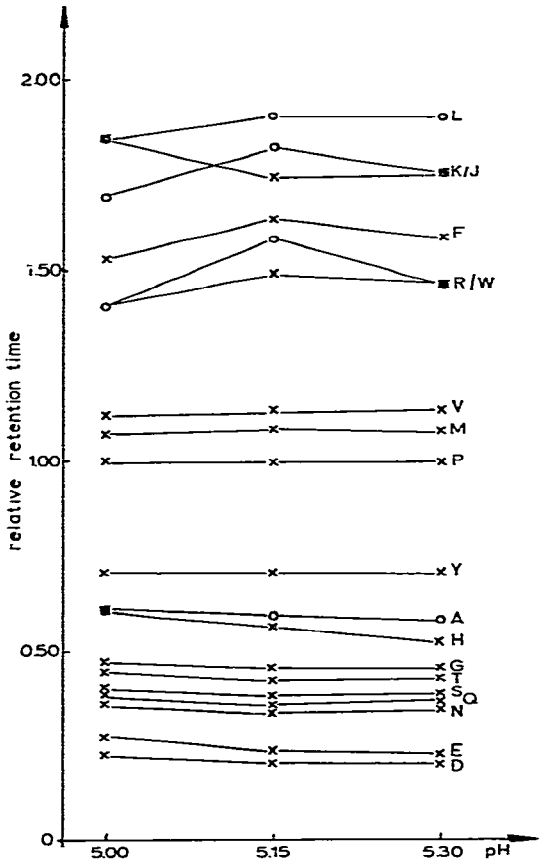

Fig. 6. Effect of pH. Conditions as in Fig. 1, except pH of eluent. Eluent A, tetrahydrofuran–5.24 m*M* acetic acid (5:95) with pH adjusted with sodium hydroxide, final pH 5.00; eluent B, tetrahydrofuran–acetonitrile (10:90).

change in flow-rate. The relative retention times of PTH-Leu, PTH-Ile, PTH-Phe and PTH-Trp increased on increasing the flow-rate. The relative retention time of PTH-Phe increased faster than those of the others, whereas that of PTH-Arg decreased on increasing the flow-rate. Owing to these changes, PTH-Arg and PTH-Phe overlapped.

Lower flow-rates (0.5 ml/min) caused a poor separation of PTH-Ile, PTH-Arg and PTH-Lys. At a flow-rate of 1.50 ml/min PTH-Arg and PTH-Phe eluted together.

Depedence on pH (Fig. 6)

The influence of pH on the separation of PTH-Trp, PTH-Phe, PTH-Arg, PTH-Ile, PTH-Lys, PTH-Leu and PTH-His was greater than that of other factors and neither linearity nor parallelism occurred.

At pH 5.00 of eluent A (final pH of eluents = 5.80) PTH-His eluted PTH-Ala and PTH-Trp and PTH-Arg eluted as one peak; PTH-Leu and PTH-Ile also eluted together. The most favourable separation occurred at pH 5.15 of eluent A (final pH =

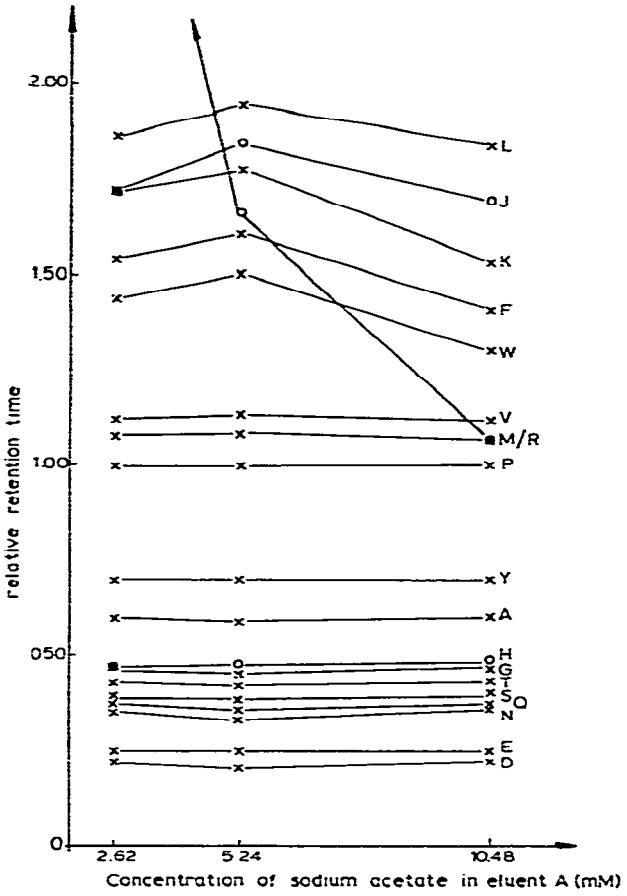

Fig. 7. Effect of ionic strength. Conditions as in Fig. 1.

5.90). At pH 5.30 of eluent A (final pH = 6.03) PTH-Arg eluted as a front shoulder on the PTH-Trp peak and PTH-Lys as a shoulder on the PTH-Ile peak (Fig. 6c).

Effect of ionic strength (Fig. 7)

Optimal separation was achieved at an ionic concentration of 5.24 m*M* of sodium acetate buffer (pH 5.15). On halving the ionic concentration (2.62 m*M* and pH 5.15) the retention time of PTH-Arg particularly increased and eluted as the final peak. Further, PTH-Lys and PTH-Ile were not separated. The separation of PTH-Asn, PTH-Glu and PTH-Ser was poor. On doubling the ionic concentration (10.48 m*M* and pH 5.15) PTH-Arg moved forward considerably and eluted together with PTH-Met; the separation of PTH-Asn, PTH-Gly, PTH-Ser and PTH-Thr deteriorated greatly.

DISCUSSION

Nineteen out of twenty commonly occurring PTH-amino acids could be separated in a single chromatographic run within 20 min under the conditions described

(Fig. 1). Only PTH-Asp and PTH-Cm-Cys eluted together. The separation of PTH-His, PTH-Met, PTH-Val, PTH-Trp, PTH-Arg, PTH-Phe, PTH-Ile, PTH-Lys and PTH-Leu were affected to various extents by temperature, pH, ionic strength, eluent composition and flow-rate. The remaining PTH-amino acids were also affected by these factors but the effect on their resolution was minor.

As the relative retention times of PTH-Pro and PTH-Val remained fairly constant under all of the conditions studied, the retention time of PTH-Pro was taken as unity and the retention times of the other PTH-amino acids were calculated relative to it. PTH-Pro represents a turning point: all PTH-amino acids (group A) with shorter relative retention times behave differently to the other PTH-amino acids (group B), which have longer relative retention times. An increase in temperature (Fig. 3) or the proportion of the organic solvent (Fig. 4) led to an increase in the relative retention times of group A (PTH-Asp, PTH-Glu, PTH-Asn, PTH-Gln, PTH-Ser, PTH-Thr, PTH-Gly, PTH-His, PTH-Ala and PTH-Tyr) and to a decrease in those of group B (PTH-Met, PTH-Val, PTH-Trp, PTH-Phe, PTH-Ile, PTH-Lys and PTH-Leu). The effect of changing the flow-rate was the opposite (Fig. 5), an increase in flow-rate decreasing the relative retention times of group A and increasing those of group B.

The rates of change of the relative retention times of group A were linear and parallel with increasing temperatures, flow-rate and proportion of organic solvent. Group B behaved a slightly differently: changes in their relative retention times were linear with increasing flow-rate and organic solvent, but not parallel, and temperature changes resulted in neither linearity nor parallelism.

With varying pH (Fig. 6) and ionic strength (Fig. 7), changes in group A's relative retention times were not linear but were more or less parallel. Neither linearity nor parallelism occurred for group B.

It is difficult to predict the chromatographic behaviour of PTH-Arg or to classify it in one of the two groups. The greatest effect on the relative retention time was observed on changing the ionic strength, although variations in all the other the conditions used influenced its relative retention time to some extent.

The strongest influence of interfering solvents introduced by the sample was observed with PTH-Thr and PTH-Glu.

The best separation was achieved at 55°C with a flow-rate of 1.0 ml/min and an eluent composition of A:B = 65:35 (Fig. 1).

CONCLUSIONS

The dependence of the separation of PTH-amino acids on the chromatographic conditions is so variable that good reproducibility depends on careful control of the separation conditions. Minor variations in pH, temperature, ionic concentration or composition of the eluent can considerably affect the reproducibility of separation.

The most favourable conditions for the isocratic separation of PTH-amino acids by Ultrasphere-ODS are oven temperature 55°C, flow-rate 1.0 ml/min and eluent 31.5% acetonitrile–6.75% tetrahydrofuran in 5.24 m*M* acetic acid adjusted to pH 5.80. If separations of PTH-Ala and PTH-His, PTH-Met and PTH-Val and PTH-Arg and PTH-Phe are not required, separation at room temperature is strongly recommended.

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