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SEPARATION OF Dns-AMINO ACID AMIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid high-performance liquid chromatographic method is described for the separation of Dns-amino acid amides. The separation is accomplished by reversed-phase chromatography on an Ultrasphere ODS column with a gradient program consisting of 5 m*M* sodium phosphate buffer, pH 7.0, and acetonitrile, and UV detection at 206, 254, or 275 nm. Seventeen Dns-amino acid amides were separated both from each other and from Dns-OH and Dns-NH_2. In this system, most of the mono-Dns-amino acids, which are normally present in large excess in samples derived from biological sources, are clearly separated from the Dns-amino acid amides. In addition, the efficiency of two liquid distribution methods for the bulk separation of Dns-amino acid amides and Dns-amino acids was determined. The system described here is recommended for the identification of amino acid amides released enzymatically from amidated peptides.

INTRODUCTION

A large number of biologically active peptides are amidated at their C-terminus. These include, among others, gastrointestinal hormones (*e.g.* gastrin, cholecystokinin), anuran skin peptides (bombesin, ranatensin), and a variety of neuropeptides such as luteinizing hormone-releasing hormone, vasopressin, oxytocin, thyrotropin releasing hormone, and substance P. The C-terminal amino acid amide can be removed from most of these peptides by enzymatic cleavage[1–5]. Therefore, the availability of methods for the analysis of amidated amino acids is important both for the structural elucidation of amidated peptides and for the identification of new, potentially biologically active, amidated peptides. Since biologically active peptides usually occur in only minute amounts, a high sensitivity of detection is required. Recently, the separation of Dns-amino acid amides on polyamide thin-layer plates analogous to the separation of Dns-amino acids[6] was described[5]. This method was used as a chemical assay in the purification and isolation of amidated biologically active peptides from small intestine and brain[7–9]. However, the limited capacity of polyamide plates makes the analysis of samples from crude extracts difficult. Usually such extracts yield large amounts of other compounds besides Dns-amino acid amides. Therefore, a high-

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performance liquid chromatographic (HPLC) method was developed for separation of Dns-amino acid amides.

MATERIALS AND METHODS

L-Phenylalanine amide–HCl, L-proline amide–HCl, L-serine amide-HCl, L-tryptophan amide–HCl, L-valine amide–HCl, L-leucine amide–HCl, L-tyrosine amide, glycine amide–HCl, and L-methionine amide–HCl were obtained from Sigma. L-Alanine amide–HCl, L-glutamic acid amide (L-isoglutamine), aspartic acid amide (isoasparagine), and L-asparagine amide–HCl (L-aspartic diamide–HCl) were from Chemalog. L-Histidine amide–2HCl was from Bachem. L-Threonine amide was obtained from Vega while L-isoleucine amide–HCl was from United States Biochemical Corp. L-Lysine amide–2HCl was synthesized in the laboratory of the late Dr. Roderich Walter.

The Dns-amino acid amides were synthesized from the amino acid amides and Dns-chloride (Dns-Cl; Pierce) by an established procedure in 50 m*M* sodium carbonate buffer, pH 10.0[5]. The Dns-amino acid amides were used directly without prior isolation. Dns-amino acids were obtained from Sigma.

Separations were performed on an Altex Ultrasphere ODS column, 250 × 4.6 mm I.D., with a Spectra-Physics Model SP8700 solvent delivery system. The flow-rate was 1 ml/min. The effluent was monitored at 206, 254, or 275 nm with an LKB Uvicord S detector.

Distribution coefficients were determined by shaking standard mixtures of Dns-amino acid amides and Dns-amino acids with 0.5 ml each of 50 m*M* sodium carbonate buffer, pH 10.0, and 0.5 ml ethyl acetate or benzene for 2 min. Aliquots from the aqueous and organic phases were removed, evaporated to dryness, resuspended in 5 m*M* sodium phosphate (pH 7.0)–acetonitrile (4:1) and separated by HPLC as described below. The relative amounts of each Dns derivatized compound in the aqueous and organic phases was determined by comparison of peak areas.

In the experiments on enzymatically released amino acid amides, 5 nmoles of the amidated peptide were incubated with a freshly prepared solution of toad skin carboxamidopeptidase[1–4] at pH 7.5 and 25°C for 20 h. Thereafter, the pH was adjusted to 10.0 and the solution was allowed to react with a 10-fold excess of Dns-Cl in acetone. The volume was then adjusted to 0.2 ml and the Dns-amino acid amide was extracted with 0.5 ml of benzene. After evaporation to dryness, the Dns-amino acid amide was resuspended in starting mobile phase and identified by HPLC.

RESULTS

In preliminary experiments, a linear gradient system of 5 m*M* sodium phosphate buffer, pH 7.0, and acetonitrile was found to give better separations than an 0.1 % phosphoric acid–acetonitrile system. The former system was further modified to give optimal separation of the Dns-amino acid amides as follows: Solvent A, 5 m*M* sodium phosphate, pH 7.0; Solvent B, acetonitrile; gradient program: 0 min, 20 % B; 8 min, 20 % B; 20 min, 35 % B; 30 min, 40 % B; 40 min, 80 % B; 45 min, 80 % B. Fig. 1 shows a typical chromatogram of a standard mixture containing 10 nmoles each of the Dns derivatized amino acid amides. Table I shows the retention times of the Dns-

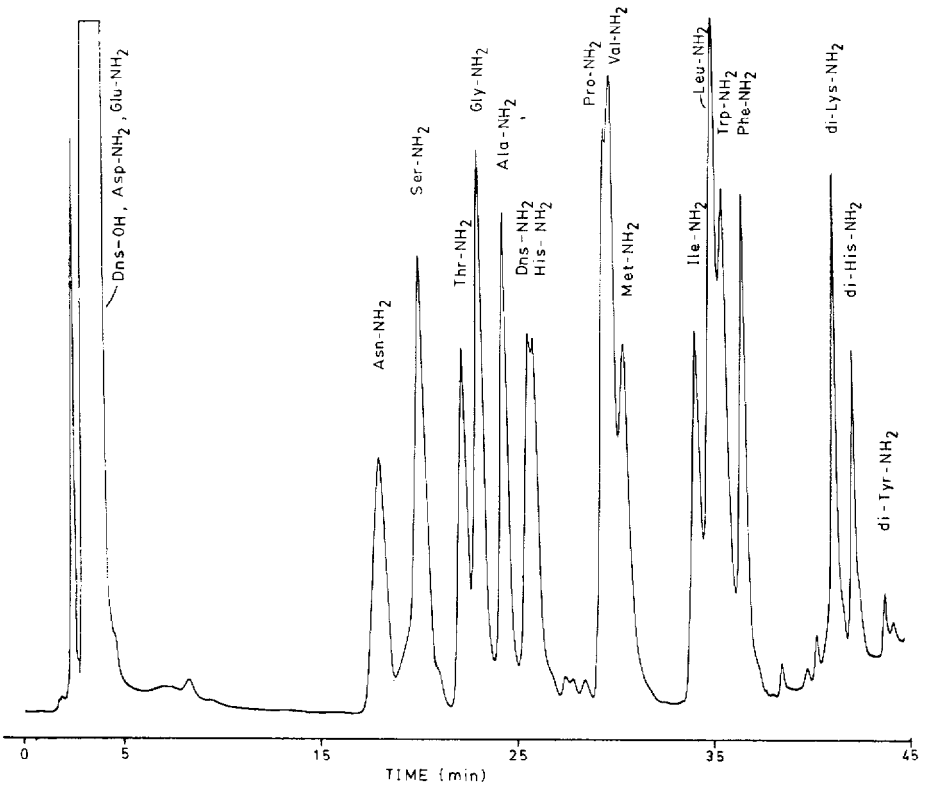

Fig. 1. HPLC separation of Dns-amino acid amides. Operating conditions: column, Ultrasphere ODS, 250 × 4.6 mm I.D., 5 μm; flow-rate, 1 ml/min: Solvent A, 5 m*M* sodium phosphate, pH 7.0; Solvent B, acetonitrile; gradient program, 0 min, 20% B, 8 min. 20% B, 20 min. 35% B, 30 min. 40% B, 40 min. 80% B, 45 min. 80% B; sample, 10 nmoles of each Dns-amino acid amide in 25 μl; detection, UV at 254 nm.

amino acid amides and, for comparison, those of some Dns-amino acids. When a large excess of Dns-acid (Dns-OH) was present, detection of Dns-glutamic acid amide and Dns-asparatic acid amide was difficult. Di-Dns-lysine amide, di-Dns-histidine amide, and di-Dns-tyrosine amide were difficult to quantify, not only because of interference with impurities eluted at high acetonitrile concentrations, but also because of their limited solubility in the starting buffer. This solubility problem was particularly evident with di-Dns-tyrosine amide. The sensitivity of detection was below 200 pmoles at 254 and 275 nm and below 50 pmoles at 206 nm. A significant baseline shift was evident at 206 nm.

The separation of the Dns-amino acid amides from Dns-amino acids is of particular importance since in biological samples free amino acids are usually present in very large excess over amino acid amides released enzymatically from amidated peptides. For this reason, bulk separation of Dns-amino acid amides and Dns-amino acids is often necessary before chromatography. Distribution between 50 m*M* sodium carbonate buffer and ethyl acetate was described before[5], but we found that the Dns-amino acids were incompletely removed by this procedure. Table II shows the relative amounts of Dns-amino acids and Dns-amino acid amides extracted into the organic

TABLE I

RETENTION TIMES FOR Dns-AMINO ACID AMIDES AND Dns-AMINO ACIDS

Chromatographic conditions as in Fig. 1.

Amino acid amides	*Amino acids*	*Retention time (min)*
	Glu	1.9
	Asp	1.9
		3.0 (Dns-OH)
	Asn	3.3
$Glu\text{-}NH_2$		3.5
$Asp\text{-}NH_2$		3.7
	Ser	3.9
	Gly	4.4
	Thr	4.5
	Ala	5.1
	Pro	6.6
	Val	8.9
	Met	11.3
	Ile	14.5
	Leu	15.2
	Trp	17.1
	Phe	17.4
$Asn\text{-}NH_2$		18.0
$Ser\text{-}NH_2$		19.9
$Thr\text{-}NH_2$		22.0
$Gly\text{-}NH_2$		22.8
$Ala\text{-}NH_2$		24.0
		25.0 ($Dns\text{-}NH_2$)
	Di-Lys	25.2
$His\text{-}NH_2$		25.5
$Pro\text{-}NH_2$		28.9
$Val\text{-}NH_2$		29.5
$Met\text{-}NH_1$		30.2
	Di-Tyr	30.7
$Ile\text{-}NH_2$		33.9
$Leu\text{-}NH_2$		34.7
$Trp\text{-}NH_2$		35.3
$Phe\text{-}NH_2$		36.2
$Di\text{-}Lys\text{-}NH_2$		40.7
$Di\text{-}His\text{-}NH_2$		41.8
$Di\text{-}Tyr\text{-}NH_2$		43.3

phase by distribution between equal volumes of 50 m*M* sodium carbonate buffer, pH 10.0, and either ethyl acetate or benzene. In both systems, Dns-amide is largely extracted into the organic phase whereas Dns-acid is quantitatively retained in the aqueous phase. Dns-Glutamic acid amide and Dns-aspartic acid amide remain in the aqueous phase.

Fig. 2 shows chromatograms of 5 nmoles of oxytocin and substance P after incubation with toad skin carboxamidopeptidase[1,3,4], subsequent Dns derivatization, and distribution between 200 μl of 50 m*M* sodium carbonate, pH 10.0, and 500 μl of

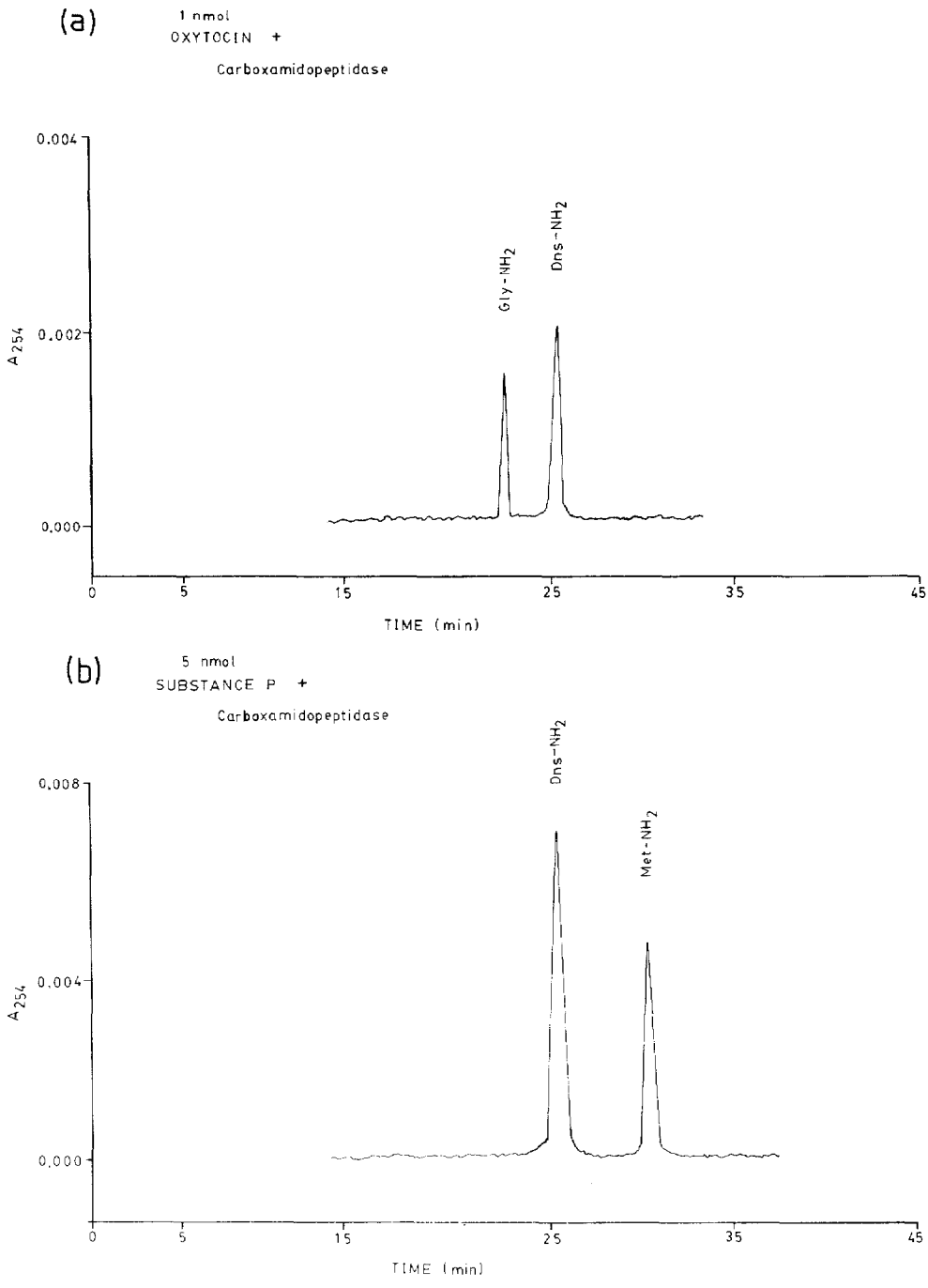

Fig. 2. Release of amino acid amides from peptides by carboxamidopeptidase. Oxytocin (1 nmole) (a) or substance P (5 nmoles) (b) was incubated with toad skin carboxamidopeptidase at pH 7.5 for 20 h at 25°C. The solution was then adjusted to pH 10.0 and treated with a 10-fold excess of Dns-Cl in a total volume of 0.2 ml. The Dns-amino acid amide was then extracted with 0.5 ml of benzene, evaporated to dryness, resuspended in the starting mobile phase, and subjected to HPLC as described in Fig. 1.

TABLE II

DISTRIBUTION COEFFICIENTS OF Dns-AMINO ACID AMIDES AND Dns-AMINO ACIDS

Dns-amino acid amide			*Dns-amino acids*		
Amide	*Percentage in upper phase**		*Amino acid*	*Percentage in upper phase**	
	Benzene	*Ethyl acetate*		*Benzene*	*Ethyl acetate*
Asn-NH_2	$\leqslant 2$	67	Asn	<0.5	<0.5
Ser-NH_2	11	88	Gln	<0.5	<0.5
Thr-HN_2	37	90	Ser	<0.5	<0.5
Gly-NH_2	56	95	Glu	<0.5	<0.5
Ala-NH_2	63	97	Asp	<0.5	<0.5
His-NH_2	88	95	Thr	<0.5	<0.5
Pro-NH_2	$\geqslant 98$	>99	Arg	<0.5	0.8
Val-NH_2	95	>99	Ala	<0.5	0.7
Met-NH_2	89	>99	Pro	<0.5	0.5
Ile-NH_2	96	>99	Val	<0.5	4
Leu-NH_2	96	>99	Met	<0.5	4
Trp-NH_2	96	>99	Ile	<0.5	8
Phe-NH_2	96	>99	Leu	<0.5	8
			Trp	<0.5	10
			Phe	<0.5	11

* A mixture of amino acid amides (or amino acids) was Dns derivatized. The products were diluted with 50 m*M* sodium carbonate, pH 10, to a volume of 0.5 ml. Benzene (or ethyl acetate) (0.5 ml) was added and the mixture was swirled for 1 min. An aliquot (0.4 ml) was removed from both the upper and lower phases and evaporated to dryness. The residue was dissolved in 0.2 ml of 20% acetonitrile in water and subjected to HPLC separation. Peak heights of each Dns derivatized constituent were compared in each phase.

benzene. The C-terminal glycine amide and methionine amide, respectively, could be demonstrated in addition to the Dns-amide (Dns-NH_2) side-product.

DISCUSSION

The method described here can be used for the analysis of free amino acid amides in biological material. However, it is mainly designed as a tool for the analysis of terminal amino acid amides cleaved enzymatically from amidated peptides. Thus, the method can be used in the structural analysis of purified biologically active peptides containing an amidated C-terminus and in the detection of amidated peptides in tissue extracts. Unlike peptides that are generated during intracellular protein degradation, biologically active peptides frequently contain an amidated C-terminus. The usefulness of a chemical assay method for C-terminal amino acid amides in the search for novel, biologically active peptides was shown previously[7–9]. This approach is facilitated by the utilization of enzymes that specifically cleave amidated C-terminal amino acids from peptides[1–4].

Because of the generally low concentrations of the amidated peptides in biological material, a high sensitivity of detection is essential. With a UV detector, the

lower limit of detection in the HPLC method is *ca.* 50 pmoles, which is, in our hands, about 5-fold higher than in the thin-layer procedure. In addition, the HPLC method can accommodate much larger sample sizes, which enhances the ability to detect trace concentrations of Dns-amino acid amides. In the present experiments, no radiolabelled Dns-Cl was used. However, the use of [^{3}H]Dns-Cl, combined with radioactive flow-detection, could yield a highly sensitive method that is much more rapid and convenient than any comparable thin-layer procedure.

Another advantage of the HPLC method is related to the frequent presence of large amounts of other compounds that cannot easily be separated from the Dns-amino acid amides by bulk methods. For example, large amounts of unidentified products were found in crude brain extracts after carboxamidopeptidase treatment and Dns derivatization that were not separated from Dns-amino acid amides by either ethyl acetate or benzene extraction (data not shown). Such preparations are very difficult to analyze by the standard thin-layer procedure[5] because of the very low capacity of the polyamide plates, but are much more easily accommodated by the HPLC method.

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