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SEPARATION OF ALKYLAMINONAPHTHYLENESULFONYL PEPTIDES AND AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

METHODS FOR MEASURING MELANOTROPIN INHIBITING FACTOR BREAKDOWN

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SUMMARY

N,N-Dimethyl, diethyl, dipropyl, dibutyl, and N-monoisopropylaminonaphthylenesulfonyl derivatives of melanotropin inhibiting factor (MIF) and its metabolites were prepared, and their chromatographic behavior was investigated with thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), using five solvent systems on polyamide layers and ten solvent systems on μBondapak C_{18} and μBondapak phenyl columns. A mixture of MIF and its metabolites derivatized with Dns chloride was adequately resolved by two-dimensional chromatography on polyamide layer with solvent systems, formic acid–water (3:97) and benzene–acetic acid (9:1). Bns-MIF and its metabolites were separated with μBondapak C_{18} column with the solvent system acetonitrile–0.01 *M* sodium sulphate buffer, pH 7 (50:50). They were separated into five groups: Gly and Bns acid; Pro–Leu, Leu–Gly and Leu; Pro; Gly-NH_2; and MIF. The alkylaminonaphthylenesulfonyl derivatives had strong fluorescence, which permitted their detection at the level of 10^{-11} to 10^{-9} mol. Dns-MIF and its derivatives had the lowest detectable amounts. HPLC with the aid of the Dns derivatization is reliable and fast, and is the preferable method for study of neuropeptide breakdown.

INTRODUCTION

The melanotropin inhibiting factor (MIF, Pro–Leu–Gly-NH_2) (Fig. 1), which inhibits melanotropin (the melanocyte releasing hormone), has been shown to have

Dns: R=R'=-CH_3

Ethansyl: R=R'=-CH_2CH_3

Propansyl: R=R'=-$CH_2CH_2CH_3$

Bns: R=R'=-$CH_2CH_2CH_2CH_3$

Monoisopropansyl: R=-$CHCH_3$, R=H
CH_3

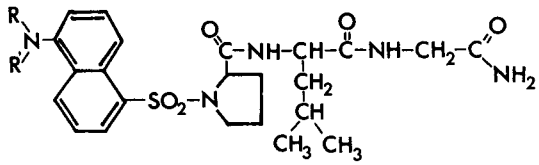

Fig. 1. The structure of N-alkylaminonaphthylenesulfonyl melanocyte-releasing hormone inhibiting factor.

multiple effects, including promotion of the development of morphine tolerance and physical dependence[1], antagonism of morphine-induced catalepsy[2] and potentiation of the therapeutic efficacy of L-Dopa in the treatment of Parkinsonism[3,4]. Since peptidases are very active in brain —500 to 1000 times more than needed for protein turnover— most cerebral peptides are broken down very rapidly. It is therefore possible that some of the effects observed following MIF administration are due not to the intact tripeptide but to some of its breakdown products. In previous studies the breakdown of MIF was estimated by measuring the released amino acids, namely Pro, Leu and Gly, or Gly-NH_2 (ref. 5). Since some of the active products may be peptide metabolites of MIF, we attempted to measure MIF breakdown in detail by measuring the formation of peptides and of the amino acids derived from them. The measuring of MIF breakdown presents some problems. The constituent amino acids of MIF have no strong absorption in the ultraviolet region and have little or no fluorescence. The amino-terminal Pro does not react with the conventional fluorescence tagging reagents, fluorescamine and *o*-phthaldialdehyde. It reacts poorly with ninhydrin, with an absorption at 400 nm; furthermore, prolyl peptides are not easily separated by the amino acid analyzer. Since other proline peptides may present similar problems we attempted chromatographic estimation of derivatized MIF and its metabolites with N,N-dimethyl aminonaphthylenesulfonyl chloride (Dns-Cl). Dns peptides can be resolved on polyamide thin-layer plates[6]. Use of radiolabeled Dns reagent accompanied by autoradiography has been reported to achieve sensitivities in the femtomole range[7]. Seiler and Knögden[8] utilized the dibutyl rather than the dimethyl form of this reagent because of its higher fluorescence and better resolution on thin-layer chromatography (TLC). The disadvantage of the Dns reagent is its lack of specificity. Not only are amino-terminal and the lysine ε-amino groups tagged, but fluorescent derivatives are also produced on the histidine imidazole ring and the tyrosine phenol ring. Such reactions do not present difficulties in the case of peptides such as MIF; this tripeptide and its metabolites when reacting with Dns radical form only a single fluorophor each. We report here the study of the separation of two other novel N,N-dialkylaminonaphthylenesulfonyl-tagged and an N-monoalkylaminonaphthylenesulfonyl-tagged MIF (Fig. 1) and MIF metabolites on TLC and high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and reagents

In this report the names adopted are Dns, ethansyl, propansyl, Bns and monoisopropansyl, respectively, for the N,N-dimethyl, diethyl, dipropyl, dibutyl and N-monoisopropylaminonaphthylenesulfonyl compounds. Dns-Cl was obtained from Pierce (Rockford, Ill., U.S.A.). The other alkylaminonaphthylenesulfonyl chlorides were synthesized by heating 5-amino-1-naphthylenesulfonic acid, the alkyl iodide, and anhydrous potassium fluoride in dimethylformamide at 130–140° for 18 h with vigorous agitation, followed by chlorination of the isolated acid using phosphorus pentachloride[9]. Because of steric factors, only one isopropansyl group becomes attached to the nitrogen instead of two as in the case of the straight chain groups.

The Dns, ethansyl, propansyl, Bns and monoisopropansyl derivatives of MIF and its metabolites were synthesized by a method similar to that developed by Seiler and Knödgen for the synthesis of Dns-amines[8]. Unless otherwise stated, 5 μl of an acetone solution of the reagent (1 mg/ml) was added to 5 μl of 3.6 m*M* amino acid or peptide solution mixed with 5 μl of 0.5 *M* sodium bicarbonate; the mixture was shaken vigorously and kept in the dark at 37° for 1 h. A 0.1-ml volume of ethyl acetate was added to separate the product from water and sodium bicarbonate. The organic layer was used for the chromatography studies.

Reagent-grade solvents obtained commercially were used in TLC without further purification. In HPLC, the HPLC-grade solvents were obtained from Waters Assoc. (Milford, Mass., U.S.A.). The solvent systems for TLC were the following: (1) formic acid–water (3:97); (2) benzene–acetic acid (9:1); (3) ethyl acetate–methanol–acetic acid (20:1:1); (4) isopropanol–1% acetic acid (1:9); (5) acetonitrile–0.01 *M* sodium sulphate buffer pH 7, 41.5:58.5. For HPLC we used acetonitrile–0.01 *M* sodium sulphate buffer pH 7; (6) (45:55); (7) (40:60); (8) (43.5:56.5); (9) (41.5:58.5); (10) (50:50); (11) (20:80); (12) (80:20). Additional solvent systems were (13) methanol–sodium sulphate buffer (50:50); (14) acetone–sodium sulphate buffer (50:50) and (15) isopropanol–1% acetic acid (5:95). All the solvent systems were prepared immediately before use. The solvent systems for HPLC were filtered (0.45 μm) and degassed before use.

TLC polyamide precoated microplates (5 × 5 cm, 25 μm thick) were obtained from Schleicher and Schuell (Keene, N.H., U.S.A.). Prepacked HPLC columns, μBondapak C_{18} and μBondapak phenyl, were from Waters Assoc. They are 30 × 3.9 mm I.D. with a particle size of 10 μm.

TLC

A 1-μl volume of the alkylaminonaphthylenesulfonyl derivative solution was spotted on a polyamide TLC plate with the aid of a Hamilton micro-syringe. The spot, not larger than 3 mm in diameter, was dried in a stream of warm air and developed with solvent systems at room temperature (20°). After the development, the plate was air-dried and observed under ultraviolet light (365 nm). For identification the separated zones were isolated and eluted with ethyl acetate and checked by mass spectrometry.

HPLC

Reversed-phase systems were chosen for HPLC. The HPLC system consisted of a Waters Model 6000 pump, a Waters U6K septumless injector, a Waters Model 440 UV absorbance detector set at 254 nm, an Aminco Fluorometer with excitation at 360 nm and emission at 487 nm and a Houston chart recorder. All runs were performed at room temperature. Samples (1–20 μl) were injected without solvent interruption. Solvents were pumped isocratically at a flow-rate of 3 ml/min, resulting in a pressure of 2000–4000 p.s.i. The separated peak was collected and dried in a stream of filtered nitrogen. The residue was dissolved in ethyl acetate, and its identity was checked by mass spectrometry.

Fluorescence measurements

Fluorescence measurements were carried out with an Aminco-Bowman Spectrophotofluorometer (Amer. Instrument, Silver Springs, Md., U.S.A.) equipped with a ratio photometer. The slits were 3 mm in width. The samples of alkylaminonaphthylenesulfonyl MIF were prepared by reacting 50 μl of 3.6 m*M* MIF with 50 μl of 0.5 *M* sodium bicarbonate and 50 μl of an acetone solution of the reagent at 37° for 1 h. The mixture was dried in a stream of nitrogen, dissolved in 100 μl of solvent system 5, and purified by HPLC with a μBondapak phenyl column and solvent system 5. The corresponding peaks were collected and dried. The residue was dissolved in ethyl acetate and submitted to fluorometric study.

RESULTS AND DISCUSSION

TLC

Elongation of the alkyl group of the N,N-dialkylaminonaphthylenesulfonyl residue decreases the polarity of the compound. The chromatographic behavior of a compound is governed by the dipole moment and by specific interactions of the compound with components of the solvent system and/or the supporting medium. We studied the effect of five solvent systems on the separation of alkylaminonaphthylenesulfonyl MIF and its metabolites on polyamide layer. Three solvent systems (systems 1,4, and 5) were more polar than the stationary phase, and the other two were less so (Table I). Ethansyl, propansyl and Bns derivatives exhibit lower mobilities than the analogous Dns derivatives when the stationary phase is less polar than the mobile phase. In contrast, when the mobile phase is less polar, ethansyl, propansyl and Bns derivatives exhibit greater mobilities than the analogous Dns derivatives. The longer aliphatic chains show the greater effects on the mobilities, in the order of Bns > propansyl > ethansyl > Dns derivatives.

Monoisopropansyl derivatives are more polar than their analogous Dns derivatives because the nitrogen attached to the naphthylene ring is more hydrophilic. With the five solvent systems studied, the mobilities of the monoisopropansyl derivatives are smaller than their analogous Dns derivatives.

All the alkylaminonaphthylenesulfonyl MIF and its metabolites show a confined and strongly fluorescent spot after TLC separation. Solvent systems 1 and 2 were the best combination for separation of alkylaminonaphthylenesulfonyl derivatives. After separation in the first dimension with solvent system 1 (3% formic acid) the plate was dried, turned 90°, and developed with solvent system 2 (benzene–acetic

TABLE I

R_F OF ALKYLAMINONAPHTHYLENESULFONYL MIF AND ITS METABOLITES SEPARATED ON POLYAMIDE

Plate: F 1700 micropolyamide, pre-coated.

Solvent system	*Derivative*	*MIF*	*Pro–Leu*	*Leu–Gly*	*Pro*	*Leu*	*Gly*	*Gly-NH_2*	*OH*
	Dns								
1		0.49	0.17	0.20	0.38	0.17	0.51	0.56	0.40
2		0.71	0.69	0.08	0.60	0.41	0.18	0.47	0
3		0.89	0.95	0.89	0.65	0.82	0.53	0.89	0
4		0.25	0.11	0.11	0.19	0.11	0.15	0.47	0
5		0.40	0.57	0.52	0.74	0.48	0.57	0.51	0.55
	Ethansyl								
1		0.21	0.12	0.16	0.19	0.10	0.29	0.28	0.40
2		0.80	0.70	0.23	0.71	0.50	0.28	0.58	0
3		0.87	0.85	0.83	0.84	0.86	0.77	0.91	0
4		0.14	0.07	0.05	0.07	0.06	0.06	0.17	0.12
5		0.23	0.15	0.23	0.23	0.14	0.17	0.26	0.06
	Propansyl								
1		0.12	0.07	0.05	0.09	0.07	0.10	0.18	0.23
2		0.77	0.75	0.25	0.80	0.61	0.36	0.68	0
3		0.91	0.87	0.86	0.86	0.84	0.74	0.89	0
4		0.05	0.04	0.05	0.05	0.02	0.07	0.09	0.02
5		0.10	0.08	0.10	0.11	0.07	0.07	0.11	0
	Bns								
1		0.08	0.06	0.04	0.07	0.05	0.05	0.09	0.07
2		0.83	0.81	0.38	0.76	0.69	0.43	0.76	0
3		0.95	0.93	0.83	0.93	0.93	0.84	0.97	0
4		0.04	0.05	0.02	0.07	0.04	0.04	0.04	0
5		0.07	0.07	0.11	0.09	0.05	0.07	0.09	0
	Monoisopropansyl								
1		0.10	0.07	0.09	0.07	0.05	0.10	0.13	0.28
2		0.58	0.49	0.14	0.43	0.36	0.11	0.32	0.03
3		0.87	0.79	0.79	0.79	0.86	0.56	0.86	0
4		0.05	0.05	0.03	0.07	0.03	0.06	0.06	0
5		0.29	0.23	0.18	0.28	0.18	0.20	0.24	0.09

acid, 9:1); the Dns-MIF and its metabolites were adequately separated (Fig. 2). The separation power of the other three solvent systems was weaker than solvent system 1 and 2. Dns derivatization and two-dimensional separation on polyamide layers followed by fluorometry in our hands gave a rapid and detailed measure of MIF breakdown.

HPLC

Reversed-phase systems were chosen for their mild, non-destructive interactions. We used two different kinds of reversed-phased column, μBondapak C_{18} and μBondapak phenyl. We studied the HPLC of the alkylaminonaphthylenesulfonyl derivatives with ten different solvent systems containing acetonitrile, methanol, acetone or

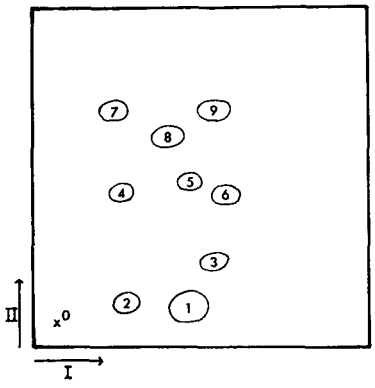

Fig. 2. TLC of Dns-MIF and its metabolites after two-dimensional separation on polyamide. A 50-μl volume of a 3.6 mM solution of MIF and its metabolites was mixed with 50 μl of 0.5 M sodium hydrogen carbonate and 50 μl of Dns-Cl in acetone (1 mg/ml) and placed in the dark at 37° for 1 h. A 1-μl volume of the resultant mixture was spotted on a polyamide TLC micro plate and developed with two-solvent systems in two dimensions, the first dimension with water–formic acid (97:3), the second with benzene–acetic acid (9:1). Zones: 1 = Dns-acid; 2 = Leu–Gly; 3 = Gly; 4 = Leu; 5 = NH_3; 6 = Gly-NH_2; 7 = Pro–Leu; 8 = Pro; 9 = MIF.

isopropanol. With these solvent systems, mold growth was not observed; thus we avoided a problem sometimes encountered in ion-exchange methods.

In general, the capacity factors, k' of derivatives, separated with a μBondapak phenyl column are smaller than with μBondapak C_{18} column. The k' value of a peak eluted by HPLC is defined as $k' = (V_e - V_0)/V_0$, where V_e is the elution volume of the eluted peak, and V_0 is the void volume of the column. Ethansyl, propansyl, Bns, monoisopropansyl, Dns-MIF and the metabolites except glycine can be separated from their fluorescence reagent on μBondapak C_{18} and μBondapak phenyl columns (Tables II, III and IV). The MIF, Gly-NH_2, and Dns acid can be separated from the other amino acids and dipeptides with a less hydrophilic mobile phase. Since Pro–Leu,

TABLE II

CAPACITY FACTORS (k') OF Dns-MIF AND ITS METABOLITES IN DIFFERENT SOLVENT SYSTEMS ON TWO REVERSED-PHASE COLUMNS

C_{18} = μBondapak C_{18} column; Phenyl = μBondapak phenyl column.

Compound	*Solvent system*												
	6		*7*	*8*		*9*		*10*		*11*	*12*	*13*	*15*
	C_{18}	*Phenyl*	C_{18}	C_{18}	*Phenyl*	C_{18}	*Phenyl*	C_{18}	*Phenyl*	C_{18}	C_{18}	*Phenyl*	C_{18}
MIF	24	3.2	>30	5.0	5.3	7.0	8.0	4.5	3.2	>30	0.4	>30	0.6
Pro–Leu	3.4	1.0	4.7	1.2	0.6	1.4	0.6	0.6	0.4	>30	0.4	5.8	0.6
Leu–Gly	1.7	1.0	3.7	0.5	0.6	0.4	0.6	0.6	0.4	—	0.4	3.5	0.6
Pro	1.0	1.0	3.7	0.5	0.6	0.4	0.6	0.6	0.4	>30	0.4	3	0.6
Leu	2.0	3.6	3.7	0.5	0.6	1.0	5.3	0.6	2.5	—	0.4	3.5	0.6
Gly	1.0	1.0	10	0.5	0.6	1.0	0.6	0.6	0.4	>30	0.4	1.0	0.6
Gly-NH_2	5.3	—	>30	2.6	4.0	4.2	5.3	1.8	2.8	—	0.4	>30	0.6
OH	1.0	1.0	3.7	0.5	0.6	0.4	0.6	0.6	0.4	1.4	0.4	1.0	0.6

TABLE III

CAPACITY FACTORS (k') OF ETHANSYL AND PROPANSYL MIF AND ITS METABOLITES IN DIFFERENT SOLVENT SYSTEMS ON TWO REVERSED-PHASE COLUMNS
C_{18} = μBondapak C_{18} column; Phenyl = μBondapak phenyl column.

Compound	*Derivative*	*Solvent system*									
		6		*7*	*8*		*9*		*10*		*13*
		C_{18}	*Phenyl*	C_{18}	C_{18}	*Phenyl*	C_{18}	*Phenyl*	C_{18}	*Phenyl*	*Phenyl*
MIF	Ethansyl	>30	7.6	21	12	11	16	13	6.6	6.8	>30
Pro–Leu		1.8	0.6	3.0	1.2	0.4	2.2	1.5	2.2	0.4	6.8
Leu–Gly		0.6	0.6	2.0	11	0.4	0.4	0.6	0.6	0.4	15
Pro		1.4	0.6	1.0	1	0.4	0.8	0.6	0.6	0.4	5.0
Leu		1.4	3.6	2.2	1.4	7.5	1.6	9.0	0.6	4.0	7.0
Gly		0.6	3.8	0.8	0.6	7.5	0.4	9.0	0.6	4.0	3.0
Gly-NH_2		>30	3.8	8	5.6	2.5	6.6	6.0	3.4	2.6	8.6
OH		0.6	0.6	0.8	0.6	0.4	0.4	0.6	0.6	0.4	1.8
MIF	Propansyl	>30	>30	>30	>30	>30	>30	>30	14	7.8	>30
Pro–Leu		4.0	1.4	7.8	4.6	1.0	5.6	3.0	2.2	0.4	>30
Leu–Gly		2.6	1.0	5.8	2.6	1.0	3.8	1.0	0.6	0.4	>30
Pro		2.2	2.0	3.6	2.2	2.0	2.8	1.8	0.6	1.8	13
Leu		3.0	0.8	5.8	3.0	1.2	4.2	1.8	1.4	6.0	18
Gly		1.0	0.8	1.8	1.4	1.0	1.8	1.0	0.6	6.0	7
Gly-NH_2		>30	>30	>30	>30	>30	>30	>30	7.4	4.4	>30
OH		1.0	0.8	2.0	1.4	1.0	1.4	1.0	0.6	0.4	6.2

TABLE IV

CAPACITY FACTORS (k') OF Bns AND MONOISOPROPANSYL MIF AND ITS METABOLITES IN DIFFERENT SOLVENT SYSTEMS ON TWO REVERSED-PHASE COLUMNS
C_{18} = μBondapak C_{18} column; Phenyl = μBondapak phenyl column.

Compound	*Derivative*	*Solvent system*									
		6		*7*	*8*		*9*		*10*		*13*
		C_{18}	*Phenyl*	C_{18}	C_{18}	*Phenyl*	C_{18}	*Phenyl*	C_{18}	*Phenyl*	*Phenyl*
MIF	Bns	>30	>30	>30	>30	>30	>30	>30	29	12	30
Pro–Leu		13	3.0	11	9.4	7.0	13	9.8	6.2	1.0	11
Leu–Gly		7.0	2.2	2.2	6.2	5.0	9.0	7.5	1.6	1.0	13
Pro		6.0	1.8	10	5.0	3.8	6.2	5.0	3.6	1.0	11
Leu		8.2	2.2	16	6.6	5.0	9.4	7.3	4.0	1.0	9.4
Gly		3.8	1.4	7.0	3.4	3.3	4.6	4.3	2.0	1.0	15
Gly-NH_2		>30	12	>30	>30	>30	>30	>30	15	7.2	>30
OH		3.0	1.4	5.8	2.6	3.0	3.2	3.5	1.6	1.0	11
MIF	Monoisopropansyl	6.2	3.2	11	7.0	5.3	8.2	8.0	4.0	3.2	>30
Pro–Leu		1.4	1.0	0.6	0.4	0.6	1.0	0.6	0.6	0.4	5.8
Leu–Gly		0.6	1.0	0.6	0.8	0.6	0.4	0.6	0.6	0.4	3.5
Pro		2.8	1.0	0.9	0.8	0.6	0.4	0.6	0.6	0.4	3.0
Leu		2.2	3.6	1.0	0.6	0.6	0.4	5.3	0.6	2.5	3.5
Gly		0.6	1.0	0.8	0.4	0.6	0.4	0.6	0.6	0.4	1.0
Gly-NH_2		2.8	—	4.2	3.0	4.0	3.2	5.3	2.2	2.8	>30
OH		0.6	1.0	0.8	0.4	0.6	0.4	0.6	0.6	0.4	1.0

Leu–Gly, Pro, Leu and Gly are more polar than Gly-NH_2 and MIF, their k' values are smaller. When MIF and its metabolites were reacted with Bns chloride and separated with μBondapak C_{18} column with solvent system acetonitrile–0.01 *M* sodium sulphate buffer, pH 7.0 (50:50), they were separated into five groups, Gly and the Bns acid in one group (Fig. 3, peak b); Pro–Leu, Leu–Gly, and Leu in another group (Fig. 3, peak d); Pro (Fig. 3, peak c); Gly-NH_2 (Fig. 3, peak e); and MIF (Fig. 3, peak f). The retention time is 0.7, 1.5, 1.3, and 8.3 min respectively. We used an isocratic system for the separation. MIF and its metabolites could be separated better by gradient solvent systems, which take a longer time because of the regeneration of the column.

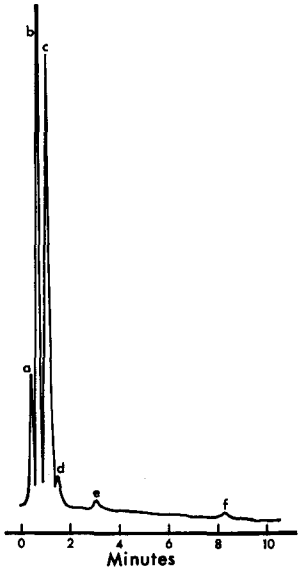

Fig. 3. HPLC of Bns-MIF and its metabolites with μBondapak C_{18} column and 0.02 a.u.f.s. A 50-μl volume of 3.6 m*M* of MIF and its metabolites was mixed with 50 μl of 0.5 *M* sodium hydrogen carbonate and 50 μl of Bns chloride in acetone (1 mg/ml). After the mixture reacted for 1 h at 37° in the dark, 15 μl was submitted to HPLC study with the solvent system acetonitrile–0.01 *M* sodium sulphate buffer, pH 7.0 (45:55). Peaks: a = solvent front; b = Gly and Bns acid; c = Pro; d = Pro–Leu, Leu and Leu–Gly; e = Gly-NH_2; f = MIF.

HPLC with reversed-phase systems has advantages, particularly in the conservation of the naphthylenesulphonamides, which are relatively unstable on active surfaces in the presence of oxygen. Our findings agree with those of Seiler and Knödgen[8] that Bns derivatives are preferable to Dns derivatives in the analysis of amino acids and peptides because of their longer retention time on the reversed-phase column.

Fluorescence

In crystalline form, Dns-Cl is yellow; ethansyl, propansyl and Bns are orange-brown and monoisopropansyl is dark green. In ethyl acetate, all of them are yellow. All the reagents have a $\lambda_{max.}$ of fluorescence at 450 nm. Activation $\lambda_{max.}$ (nm) of the

reagent was the following: Dns and monoisopropansyl, 350; ethansyl, Bns and propansyl, 300; fluorescence $\lambda_{max.}$ (nm) in each case was 450. For the MIF derivatives activation $\lambda_{max.}$ (nm) was for Dns, Bns, propansyl and ethansyl, 350; for monoisopropansyl, 400; fluorescence $\lambda_{max.}$ (nm) for Dns, 470; for Bns, propansyl, ethansyl and monoisopropansyl, 500 nm. The peptide (MIF) derivatized with alkylaminonaphthylenesulfonyl chloride results in a shift of the $\lambda_{max.}$ of activation and fluorescence to longer wavelengths; that is, it becomes yellow in color. This was also noticed in the polyamide layers under ultraviolet light (365 nm). The alkylaminonaphthylenesulfonyl acids are blue under ultraviolet light.

Sensitivity

Picomoles of amino acids derivatized with Dns-Cl can be detected by TLC on polyamide layers. With the aid of radioactively labeled Dns-Cl, the minimum detectable amount can be brought to the femtomole range[7]. In HPLC, Dns derivatives give the highest sensitivity, followed in decreasing order by monoisopropansyl, ethansyl, propansyl and Bns compounds. Dns derivatives are detectable at about 10 times lower level than the Bns ones (Table V). The detectable amount is in the picomole range. It is claimed that the fluorescence quantum yields of Bns derivatives exceed those of the Dns derivatives by about 15%[8]. Their determinations were done in ethyl acetate, with the exciting wavelength 333 nm and fluorescence wavelength 530 nm. The discrepancy between our results and those of Seiler and Knödgen is due to the difference in the solvent systems and the wavelength of excitation and fluorescence. Our fluoromonitor has a preset excitation at 360 nm and fluorescence at 487 nm, which are close to, but not at, the excitation and fluorescence $\lambda_{max.}$ of Dns derivatives. The solvent systems we used for the separation may have quenching effects on the fluorescence of Bns derivatives. With Dns, Pro has the lowest detectable amount and Gly-NH_2 has the highest. Our method, while sensitive in comparison with conventional chromatographic methods, is still high with respect to peptide level *in vivo*. This can be improved by coupling to mass spectrometry with integrated ion-current technique[12].

In our experiments MIF was broken down by brain tissues with a rate of

TABLE V

MINIMUM DETECTABLE AMOUNT OF ALKYLAMINONAPHTHYLENESULFONYL-MIF AND ITS METABOLITES

Compound	*Minimal amount detected (10^{-12} mol)*				
	Dns	*Ethansyl*	*Propansyl*	*Bns*	*Monoisopropansyl*
MIF	219	1014	1217	3388	320
Pro–Leu	30	343	365	597	99
Leu–Gly	58	762	1352	1792	446
Pro	10	49	91	80	12
Leu	19	124	182	4480	78
Gly	n.d.*	66	182	149	27
Gly-NH_2	562	686	4057	7261	448

* n.d. = Not determined since glycine either can not be separated from Dns-hydroxide or it sticks to the column with the different solvent systems we tried.

170 nmol/mg brain protein per hour. Briefly, the brain tissue was homogenized with 19 parts of 0.1 *M* Tris buffer, pH 7.5. An amount of 180 nmol MIF in 100 μl of Tris buffer was equilibrated at 37° and incubated with 50 μl of the enzyme solution for 1 h. The reaction was stopped by placing the mixture in a boiling water bath for 10 min. The precipitate was centrifuged at 4000 *g* for 20 min, and 50 μl of the supernatant was reacted with 8 μl of 1 *M* sodium hydrogen carbonate and 20 μl of Dns-Cl in acetone (5 mg/ml) in a conical tube. The solution was mixed well and incubated at 37° in the dark for 1 h, the tubes were centrifuged, and 10 μl of the supernatant was submitted for HPLC studies. Further details with respect to the peptidase activity on MIF in brain tissues will be published elsewhere[13].

Peptidase activities on neuropeptides have usually been studied by the measurement of the released amino acid by amino acid analyzer[5], which is an indirect method or by radioimmuno assay, which suffers from the problems of specificity. We find the method employing HPLC for MIF a reliable, fast, and preferable one for studies of peptide breakdown; it measures the peptides formed, not only the amino acids.

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