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REVERSE PHASE HPLC ANALYSIS BY DANSYLATION OF AMINO ACIDS IN MO--ETC(U)
OCT 79 R A MILLER , R BONGIOVANNI , T BOEHM

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Chromatographic analysis of the amino acid content of saliva previously has been done by cation exchange and thin layer chromatography. This report describes the application of HPLC in the analysis of salivary amino acids using precolumn derivatization with dansyl chloride. The derivatization procedure allowed dansylation without prior deproteinization and subsequent extraction into methanol. The use of the fluorescent derivative increases the sensitivity of this technique over the classical cation exchange chromatography technique using ninhydrin reagent. The chromatography of these salivary amino acids

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demonstrated both identifiable as well as unidentifiable peaks. Of the eleven amino acids identified, seven were quantified. All eleven of the identified amino acids have also been reported in human saliva by use of cation exchange chromatography. The amino acid levels found in baseline monkey saliva are quantitatively similar to the levels found in unstimulated human saliva. However, the response of the monkeys to pilocarpine demonstrated quantitative differences in salivary amino acid levels from the levels observed in human salivary specimens after wax stimulation. After administration of atropine sulfate (antidote), only five of the amino acid levels in monkey saliva returned to baseline.

REVERSE PHASE HPLC ANALYSIS BY

DANSYLATION OF AMINO ACIDS IN MONKEY SALIVA

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INTRODUCTION

This investigation arose from a study of the chemical composition of saliva and the changes which occur when normal salivary secretion is altered by pharmacological agents. The determination of amino acids in physiological fluids has previously been done by various methods of chromatography. Interest in our laboratory required an amino acid assay which was simple, rapid, highly sensitive, and utilized a non-dedicated analytical system. Classically, amino acids have been analyzed by cation exchange chromatography in combination with post column derivatization by ninhydrin (1). While this method has both the sensitivity and resolution required for the analysis of physiological samples, the system is totally dedicated to amino acid analysis. Battistone and Burnett (2) used thin-layer chromatography, another frequently used technique, for the analysis of salivary amino acids. However, their results were more qualitative than quantitative. By derivatizing the amino acids with dansyl chloride prior to chromatographic separation on polyamide sheets, Varga and Richards (3) were able to quantitate amino acids in the picomole range.

More recently, dansylation of the amino acids prior to separation has been used in liquid chromatography. Initial liquid chromatographic separations of dansyl amino acids used a polyamide column which was able to separate 18 amino acids in 40 hours (4). Engelhardt, Asshaver, Neue, and Weigand (5) shortened the separation time to approximately one hour by using normal phase high-speed liquid chromatography. However, this

technique required a two-step elution. The common protein amino acids were separated in 30 minutes by Bayer, Grom, Kaltenegger, and Uhmann (6), who employed a biphasic system using gradient elution in addition to a second column with an isocratic solvent system. In addition, the authors reported on reverse phase separation of dansyl amino acids using gradient elution. Other investigators have used dansylation in combination with reverse phase chromatography for the analysis of amino acids in physiological fluids (7-9). However, these studies did not include the analysis of amino acids in saliva.

The technique described in this report makes use of dansyl chloride (1-dimethyl-aminonaphthalene-5-sulfonyl chloride) to convert free salivary amino acids to their fluorescent derivatives which are separated by reverse phase high pressure liquid chromatography (HPLC). Free amino acid determinations were made on baseline, pilocarpine-stimulated, and atropine-blocked saliva samples collected from cynomolgus monkeys.

SAMPLE COLLECTION

For the quantitative determination of free amino acids, whole saliva secretions were obtained from five cynomolgus monkeys. Each monkey was sedated with Sernalin (12 mg phencyclidine/7 kg I.M.). Whole saliva was collected for 30 minutes to establish baseline levels (unstimulated). After the baseline collection, pilocarpine (1 mg) was administered subcutaneously and saliva was collected for 15 minutes (stimulated) after allowing five minutes for the drug to take effect. This was followed by the administration of atropine (0.1 mg) subcutaneously. After allowing another five minutes for the drug to take effect, saliva was collected for an additional 15 minutes. Immediately following collection,

the volume of saliva was measured to determine flow rate. In order to remove particulate matter, the saliva was centrifuged at 1800 rpm for 15 minutes. The saliva was stored at -40C until analysis to decrease the breakdown of salivary protein to the free amino acids.

SAMPLE PREPARATION

The amino acid standards and saliva samples were prepared for HPLC analysis by a modification of Bongiovanni's procedure for dansylation (7). A reaction tube was prepared by adding 300 µl of 0.5 M sodium bicarbonate buffer pH 9 to a test tube and evaporating to dryness at 120C in a heating block. An aliquot of saliva (90 µl) was added to the previously prepared reaction tube in addition to 10 µl of the internal standard (0.2 µmoles/ml norvaline) and 100 µl of 5% TCA. (Note: Originally, samples were deproteinized with 5% TCA prior to the addition to the reaction tube; since very little protein precipitated, this step was omitted in subsequent analysis. The necessity of the TCA in the reaction tube other than to partially neutralize excess buffer, has not been determined.) The reaction tube was vortexed and 200 µl of dansyl chloride (1.5 mg/ml acetone) added. The reaction was carried out for one hour at 55C. The dansylated mixture was evaporated to dryness under a stream of nitrogen and reconstituted with 1 ml of methanol. The sample was filtered on a 0.45µ Fluoropore filter to remove the undissolved salts and protein.

CHROMATOGRAPHY

A 50 µl aliquot of the derivatized sample was injected into the HPLC (Waters Associates, Model 244 with Solvent Programmer) equipped

with a fluorometer (Farrand Optical Co., Model A-4). The amino acids were separated on two μBondapak C_{18} reverse phase columns (30 cm x 4 mm) (Waters Associates) in combination with an ODS guard column (7 cm x 2 mm) (Whatman Inc.). The solvent system consisted of 0.1 M sodium acetate buffer pH 7.5 in pump A and acetonitrile (UV grade, Burdick and Jackson) in pump B. A linear gradient (curve No. 6 on the solvent programmer) was programmed from 15/85 acetonitrile/sodium acetate to 45/55 acetonitrile/sodium acetate over a 90 minute period with a flow rate of 1.3 ml/minute, allowing sufficient time for the elution of tyrosine.

The dansylated amino acids were detected at 365 nm using 0.01 Absorbance Units Full Scale (AUFS). Fluorescence was determined using 360 nm excitation wavelength and a 480 nm cut-off filter (3-72).

RESULTS AND DISCUSSION

The chromatogram of the dansylated amino acid standards is shown in Figure 1. The amino acid peaks are listed in Table 1 along with their elution times. Lysine (#18) was not included in the standard mixture but its elution order has been noted on the chromatogram and its elution time is listed in Table 1. Each amino acid peak was identified by spiking the standard mixture with the individual dansylated amino acids. Dansyl hydroxide, which elutes at 14 minutes and dansyl amide, which elutes at 54 minutes, are not labeled on the chromatogram. In this study, norvaline was used as an internal standard and was included in the sample blank illustrated in Figure 2. The blank contained a peak eluting with a retention time very close or the same as the retention time of phenylalanine (#16). Fortunately, the peak height and/or peak area remains

constant when equal concentrations of dansyl chloride are used during the derivatization procedure. The standard curve for phenylalanine is shown in Figure 3. As expected, the standard curve does not pass through zero but does remain linear according to the linear regression equation $y = 2.9x + 0.9$. However, caution should be emphasized in quantitating phenylalanine at lower concentrations (less than 0.05 μmoles/ml) with this technique. Low concentrations can be quantitated by eliminating the interfering peak. This can be accomplished by extracting the dansylated blank, standards, and samples with n-heptane (10) or by changing the solvent system. Using 1% acetic acid with acetonitrile will shift the phenylalanine away from the interfering peak allowing quantitation at lower concentrations.

The separation of salivary amino acids by HPLC produced chromatograms which had major peaks that could be identified by comparison to known standards. The amino acids tentatively identified were: aspartic acid, glutamic acid, serine, glycine, alanine, proline, tryptophan, phenylalanine, cystine/cysteine, lysine, and tyrosine. A representative chromatogram of a baseline saliva is shown in Figure 4.

The amino acids identified in the cynomolgus monkey saliva have been reported in either stimulated or unstimulated human saliva (11, 12). The expectation prior to this experiment was that some correlation for free amino acids would be shown between humans and the cynomolgus monkey because of the phylogenetic approximation. Two factors should be considered when making a comparison. First, the assumption made is that these values are unstimulated. However, as can be noted in Table 2, monkey #241 showed extremely high flow rates and considerable variation

in baseline samples. This variation from the norm suggests some stimulation present in the baseline specimen in spite of consistent results with the remaining animals (Table 2, top).

A second factor is the pharmacological effect of the anesthesia on the salivary gland secretion. The effect of phencyclidine upon this physiology has not been studied, however, considering the consistency of the values obtained, it was felt that the anesthesia effect was similar in both the stimulated and unstimulated animals.

The amino acid profiles found in the saliva samples collected after the administration of atropine were qualitatively similar to the amino acid profiles of the baseline and stimulated saliva samples.

In this study, only seven amino acids were quantitated in the saliva. Using norvaline as an internal standard, the amino acid concentrations were determined by comparing the peak heights of the samples to known standards. The concentrations of glycine, alanine, and lysine in baseline monkey saliva were similar to the values reported for unstimulated human saliva (Table 3). On the other hand, phenylalanine and tyrosine were considerably higher (10 X) in concentration in monkey saliva than in human saliva. Initially, the high levels of phenylalanine and tyrosine were questioned. However, both amino acids were high in concentration and it was felt that the values obtained were real. This acceptance was due to two facts:

1. The two amino acids are very similar in structure.

2. The two structures are found in the same metabolic pathway.

In addition, the concentration of tyrosine obtained from the atropine

sample was similar to the value reported for unstimulated human saliva. It is interesting to note that the pilocarpine stimulation of monkey saliva shows an amino acid concentration response different from that reported for wax stimulation of human saliva. Lysine was the only amino acid to have the same response to pilocarpine and wax stimulation. Of the seven amino acids quantitated, five returned to baseline when the antidote was administered. After antidote administration, phenylalanine and tyrosine continued to decrease in concentration and to approach the concentrations reported for these two amino acids in unstimulated human saliva.

SUMMARY

This report describes the application of HPLC in the analysis of salivary amino acids using precolumn derivatization with dansyl chloride. The derivatization procedure allowed dansylation without prior deproteinization and subsequent extraction into methanol. The use of the fluorescent derivative increases the sensitivity of this technique over the classical cation exchange chromatography technique using ninhydrin reagent. The chromatography of these salivary amino acids demonstrated both identifiable as well as unidentifiable peaks. Of the eleven amino acids identified, seven were quantified. All eleven of the identified amino acids have also been reported in human saliva by use of cation exchange chromatography. The amino acid levels found in baseline monkey saliva are quantitatively similar to the levels found in unstimulated human saliva. However, the response of the monkeys to pilocarpine demonstrated quantitative differences in salivary amino acid levels from the levels observed

in human salivary specimens after wax stimulation. After administration of atropine sulfate (antidote) only five of the amino acid levels in monkey saliva returned to baseline.

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* * * *

MILITARY DISCLAIMER

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the U. S. Army Medical Department.

* * * *

In conducting research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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LEGENDS

FIG. 1. HPLC chromatogram of amino acid standards with key to standards in Table 1. Columns: two μBondapak C_{18}; eluent: acetonitrile /0.1 M sodium acetate pH 7.5; 90 min. linear gradient: 15/85 to 45/55, acetonitrile/sodium acetate flow rate: 1.3 ml/min.

FIG. 2. HPLC chromatogram of the sample blank which included the internal standard Norvaline. Columns: two μBondapak C_{18}; eluent: acetonitrile /0.1 M sodium acetate pH 7.5; 90 min. linear gradient: 15/85 to 45/55, acetonitrile/sodium acetate; flow rate: 1.3 ml/min.

FIG. 3. Standard curve of phenylalanine. The relative fluorescence is equal to the peak height of the phenylalanine divided by the peak height of the internal standard.

FIG. 4. HPLC chromatogram of the amino acids in baseline monkey saliva. Columns: two μBondapak C_{18}; eluent: acetonitrile /0.1 M sodium acetate pH 7.5; 90 min. linear gradient: 15/85 to 45/55, acetonitrile/sodium acetate; flow rate: 1.3 ml/min.

FIG. 5. See text for details. HPLC chromatogram of the amino acids in pilocarpine stimulated saliva. Columns: two μBondapak C_{18}; eluent: acetonitrile /0.1 M sodium acetate pH 7.5; 90 min. linear gradient: 15/85 to 45/55, acetonitrile/sodium acetate; flow rate: 1.3 ml/min.

TABLE 1

Amino Acid Standards

Standard Number	Amino Acid	Retention Time (min.)
1.	Aspartic Acid	10.50
2.	Glutamic Acid	11.78
3.	Histidine	19.13
4.	Serine	22.90
5.	Threonine	24.85
6.	Glycine	26.16
7.	Alanine	27.61
8.	Arginine	28.27
9.	Proline	31.32
10.	Valine	35.50
11.	Norvaline	37.96
12.	Methionine	38.74
13.	Isoleucine	42.32
14.	Leucine	43.32
15.	Tryptophan	48.17
16.	Phenylalanine	51.40
17.	Cystine and Cysteine	72.73
18.	Lysine	73.98
19.	Tyrosine	87.07

TABLE 2

Salivary Flow Rates (ml/min)

Monkey No.	Baseline	Pilocarpine	Atropine
703	0.13	1.07	0.17
706	0.26	1.67	0.17
702	0.09	1.30	0.16
709	0.02	1.65	0.18
A-98	0.02	1.22	0.15
Mean	0.10	1.38	0.17
241	1.23	1.27	0.19
241	0.42	1.32	0.63
241	0.30	1.69	0.40
Mean	0.65	1.43	0.41

TABLE 3

Concentration of Amino Acids in Saliva

(μmoles/ml)

	MONKEY			HUMAN	
Amino Acids	Baseline	Pilocarpine Stimulated	Atropine	Unstimulated[a]	Wax Stimulated[b]
Glycine	0.183	0.238	0.140	0.144	Decrease
Proline	0.202	0.071	0.140	0.088	Increase
Phenylalanine	1.448	0.404	0.391	0.012	Increase
Alanine	0.057	0.057	0.080	0.032	Decrease
Tyrosine	0.911	0.172	0.014	0.015	Increase
Cystine/Cysteine	0.211	0.016	0.056	-----	Increase
Lysine	0.024	0.014	0.029	0.032	Decrease

[a]See Reference 11.

[b]See Reference 12.

Fig 1

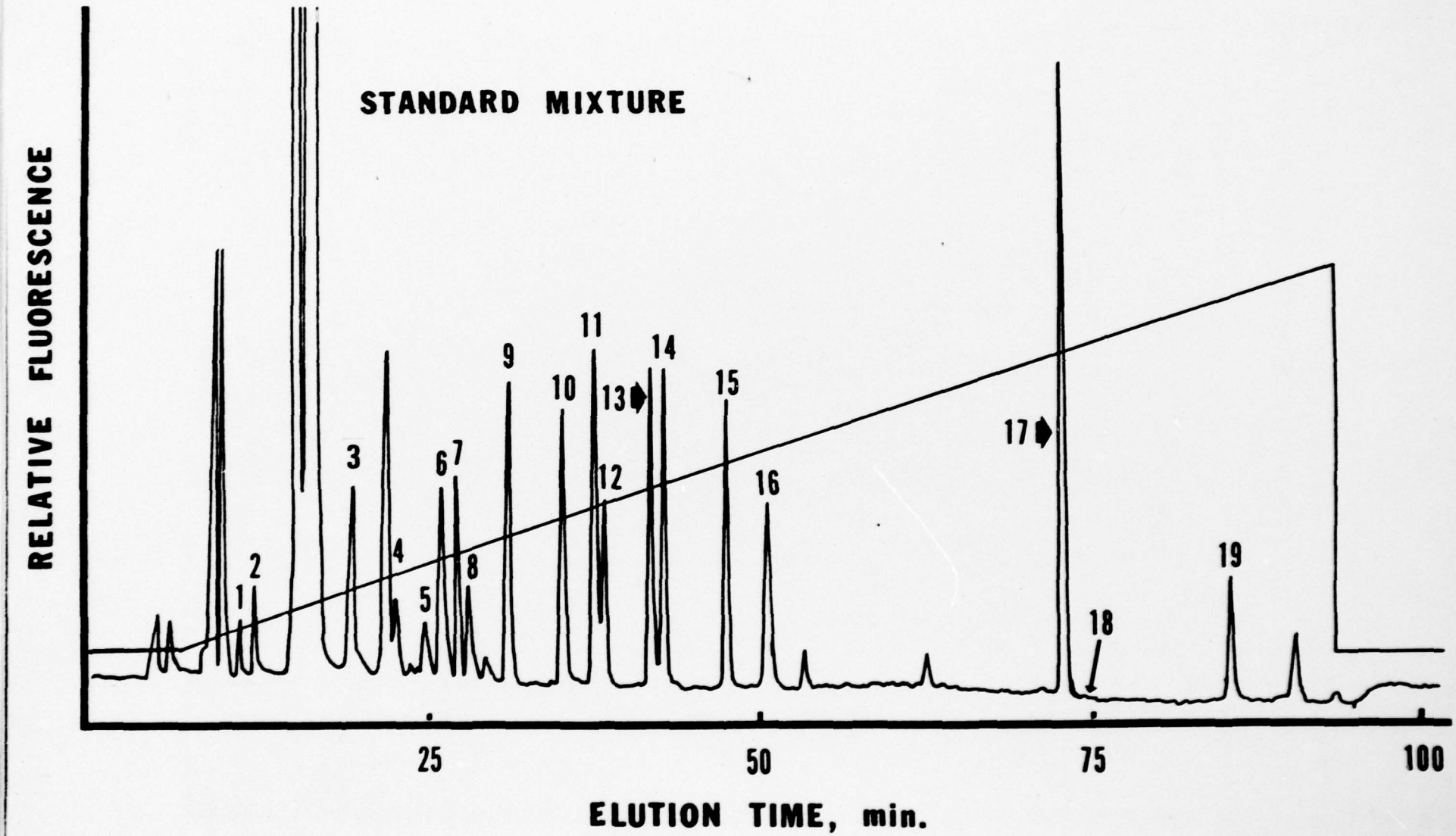
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Fig 2

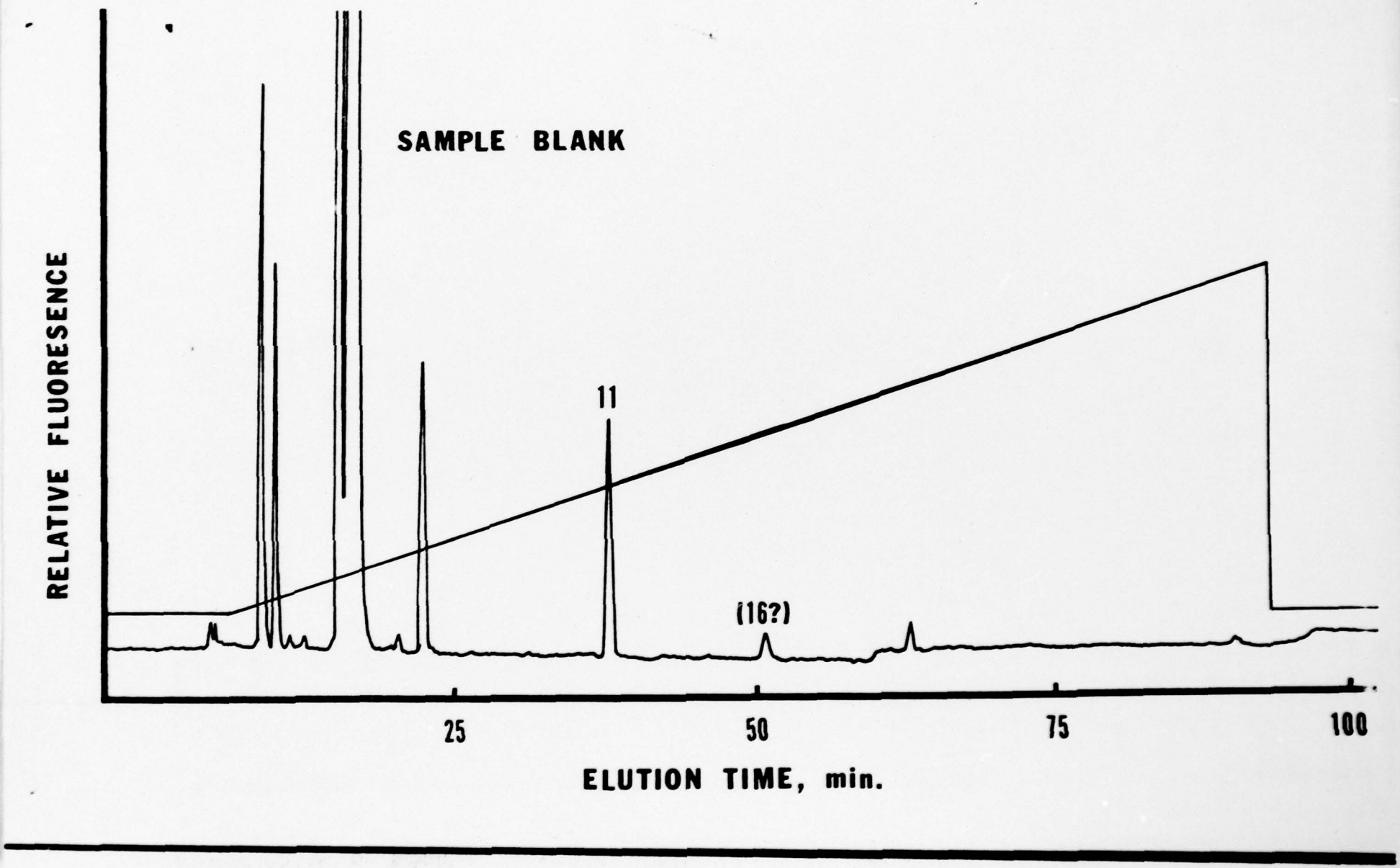
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Fig 3

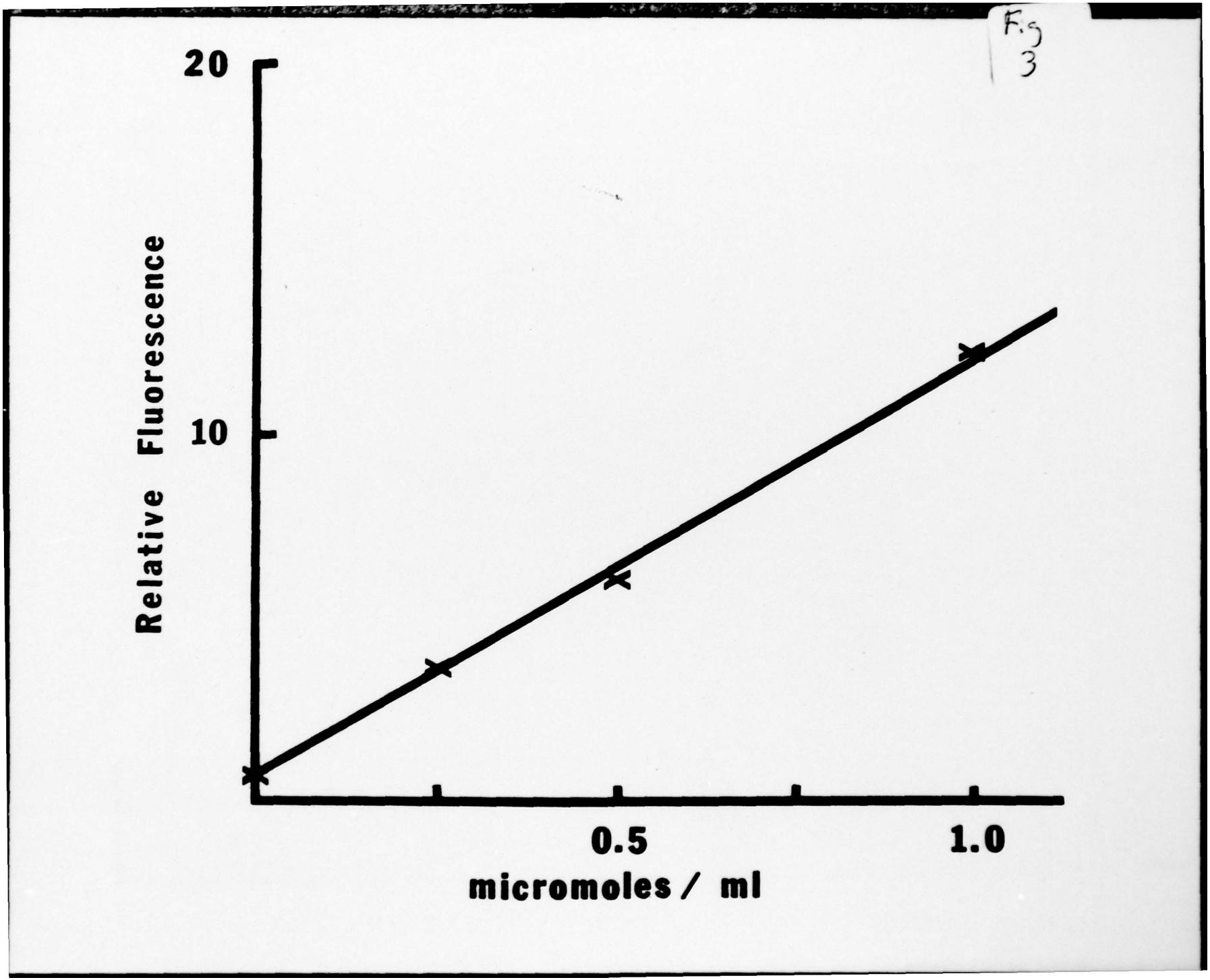

Fig 4

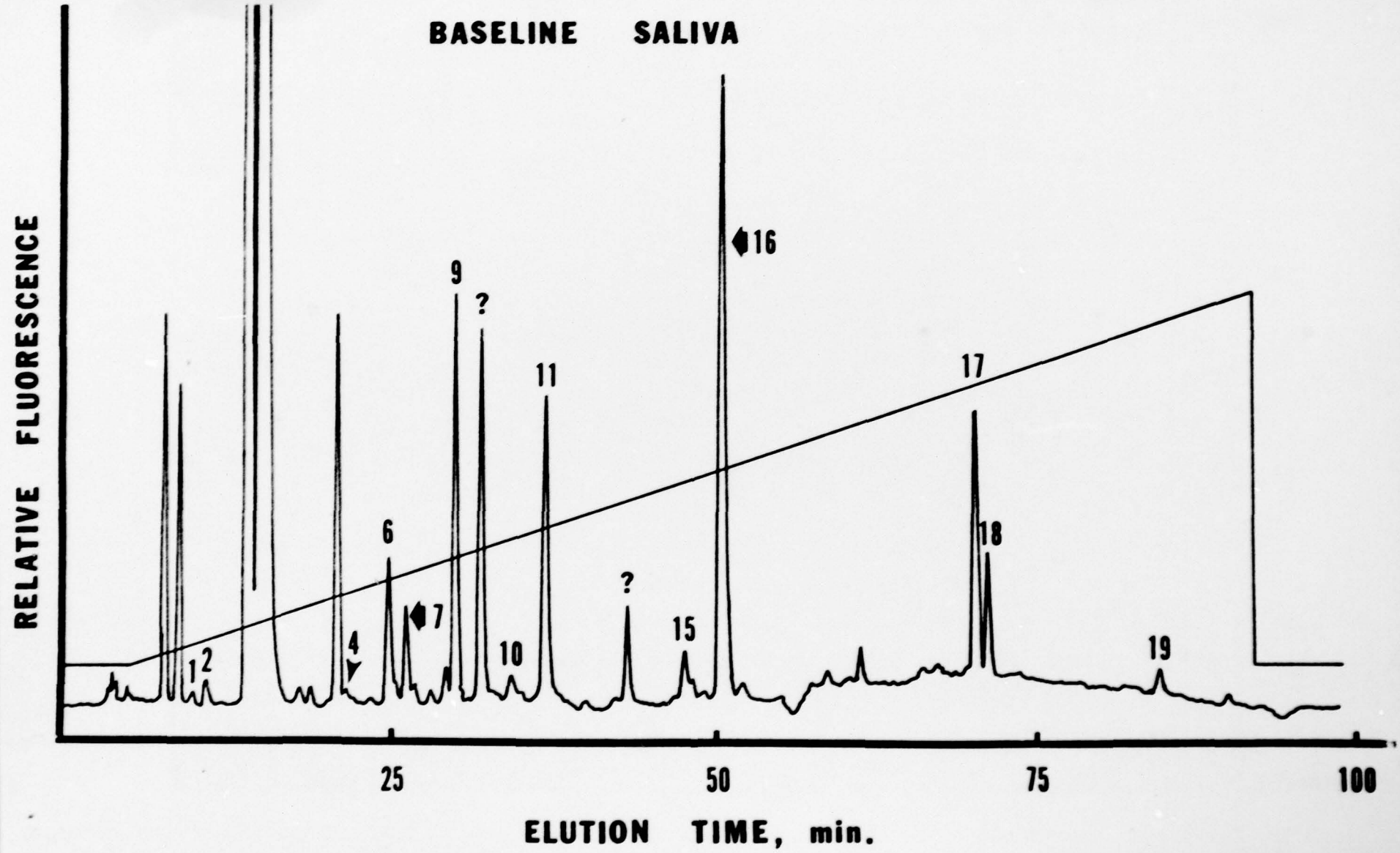
BASELINE SALIVA
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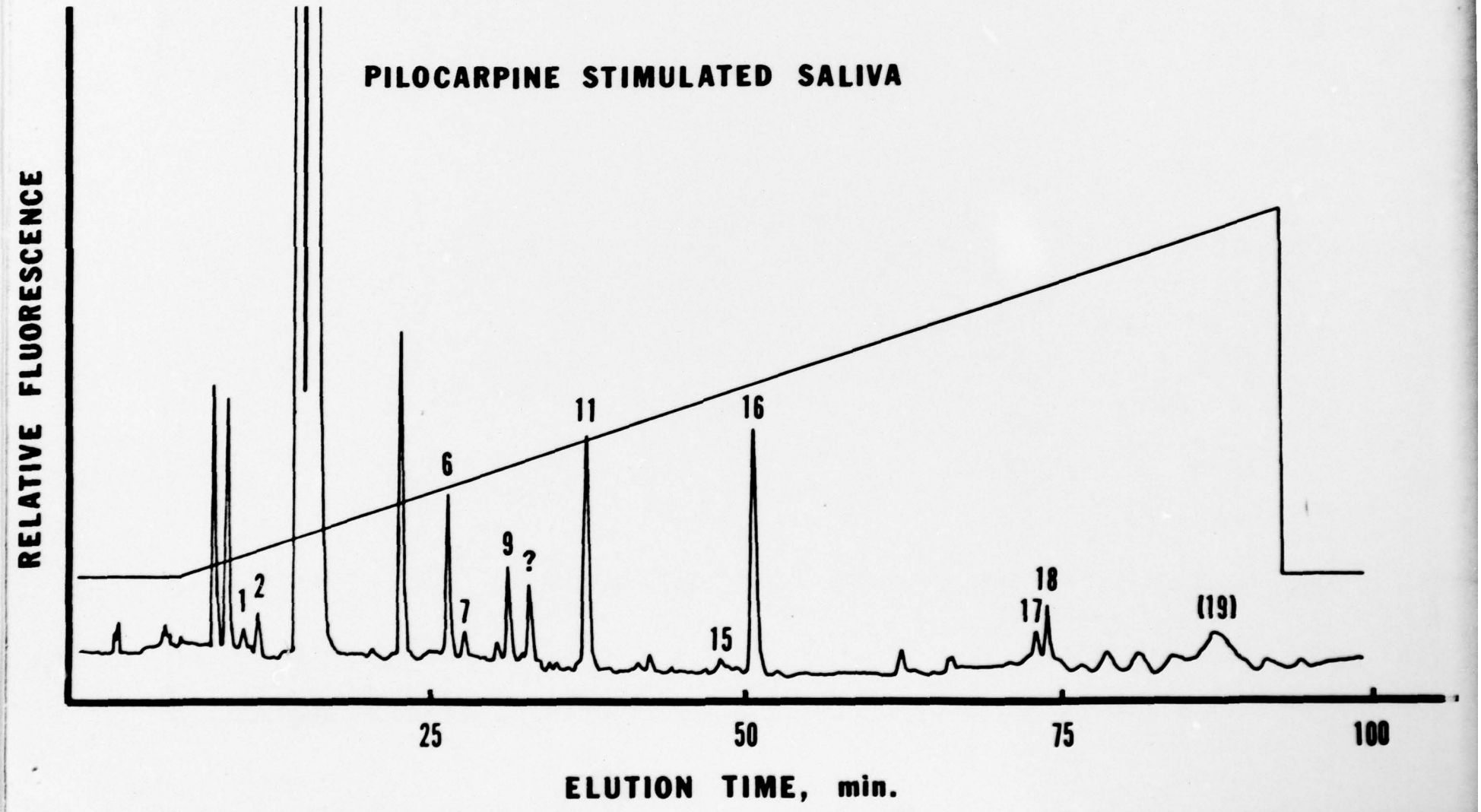
Fig 5


PILOCARPINE STIMULATED SALIVA
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