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ULTRAVIOLET AND FLUORESCENCE CHARACTERIZATION OF PURINES AND PYRIMIDINES BY POST-COLUMN pH MANIPULATION

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SUMMARY

A method to enhance the fluorescence of purines and pyrimidines using a post-column reactor was developed. Since the ionized forms of these compounds have distinctive optical properties, fluorescence as well as ultraviolet data at various pH could be obtained for the characterization of these biologically important compounds.

This method of identification has several advantages. The on-line technique to protonate or deprotonate the molecules is rapid. The method is non-destructive and permits the collection of chromatographic fractions for further analysis. The eluents normally used for the separations of purines and pyrimidines can be used without modification. In addition, using fluorescence, some methylated purines can be determined at levels as low as 1 picomole.

INTRODUCTION

Alterations in the concentrations of free purines and pyrimidines have been observed in the serum and/or urine of patients with leukemia[1], immunodeficiency[2,3] and other diseases[4,5]. Thus, it is possible that these compounds, alone or in combination, could serve as biochemical markers in the early detection of disease states or monitoring the progress of the disease and/or the response to therapy.

To determine the purine and pyrimidine constituents in physiological fluids, reversed-phase high-performance liquid chromatographic (RPLC) methods have been developed[6–9]. While RPLC has facilitated routine separations of the UV-absorbing low-molecular-weight compounds in complex matrices, the unequivocal identification of the individual chromatographic peaks is difficult; especially when only small amounts of sample are available and/or when the sample contains low levels of the potential marker. Due to the occurrence of ultratrace amounts of these compounds in limited quantities of physiological specimens, unambiguous identifications using powerful off-line techniques (*e.g.*, mass spectrometry) can not usually be obtained. Therefore, identifications must be based on the combined use of alternative characterization methods[10,11]. Furthermore, since most biomedical studies require

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the analysis of a large number of samples, it is imperative to use characterization techniques which are on-line with the analytical separation[10–16].

Although UV and fluorescence detectors are widely used in RPLC systems, they have not been employed to their fullest potential in the identification of unknown chromatographic peaks. Typically, the on-line detectors are employed for the characterization of solutes under conditions fixed by the eluent. However, the purines and pyrimidines are known to undergo characteristic changes in their absorption spectra as a function of pH. Thus, further evidence of peak identity can be obtained from UV absorption data taken at different pH. Moreover, Borresen[17–19], Udenfriend and co-workers[20,21] and others[22–27] have shown that under certain conditions both major and minor purines and pyrimidines emit appreciable fluorescence after UV-excitation. Since the fluorescence of many of these compounds is attributed to the ionized molecule formed in very acidic or basic environments, few of the nucleic acid components fluoresce under the conditions optimized for RPLC separations[11,28].

To utilize the optical properties of the ionized purines and pyrimidines in the identification and/or characterization of these compounds, we used a simple post-column technique[29–31] and obtained UV and fluorescence data at various pH values. In addition, a variety of chromatographic conditions were examined to assess the compatibility with the existing RPLC methods and to optimize the fluorescence since both mobile-phase components and temperature can cause quenching[16–21,32–34].

EXPERIMENTAL

Instrumentation

The liquid chromatographic system consisted of two M 6000A pumps, M 660 programmer, M 440 dual-wavelength detector (Waters Assoc., Milford, MA, U.S.A.) and a controlled-temperature column compartment (DuPont, Wilmington, DE, U.S.A.). The self-correcting ancillary scanning UV and fluorescence detectors were from Kratos, Schoeffel Instrument Division (Westwood, NJ, U.S.A.). The acid–base manipulation of the effluent was performed on-line with a Waters M 6000 pump connected between the dual-wavelength detector and the ancillary detectors. The tracings of detector response were obtained on strip-chart recorders (Houston Instrument, Austin, TX, U.S.A.); peak areas and retention times were obtained from a HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Reagents

All reagents were of the highest purity available. Buffer solutions and aqueous reagents were prepared with HPLC-grade water. Eluents and post-column reagents solutions were degassed by sonication and helium purge. Methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Buffer salts were from Mallinckrodt (St. Louis, MO, U.S.A.), the purine and pyrimidine standards were from Sigma (St. Louis, MO, U.S.A.) and Vega Biochemicals (Tucson, AZ, U.S.A.).

Chromatographic conditions

A variety of chromatographic conditions were studied. The eluents for isocratic elution were prepared with phosphate salts at concentrations of 0.001–0.5 *M* and pH 3.5–6.5; where noted the inclusion of methanol was by volume. For gradient elution

experiments, the initial eluent was 0.02 *M* KH_2PO_4 (pH 5.7) and 100% methanol was the second eluent; a linear program of 0–24% methanol in 35 min at a flow-rate of 1.5 ml/min was used. The reversed-phase columns were from DuPont and Waters Assoc.; both were octadecylsilica type packings.

The post-column reagent solutions used to change the effluent pH were prepared with 18 *M* sulfuric and 15 *M* phosphoric acid; potassium and sodium hydroxides were used for the basic solutions. The pH reported is the apparent value measured at the outlet of the system unless otherwise indicated.

The on-line UV absorption spectra (UAS) at different pH were recorded over the range of 210–350 nm. For the fluorescence excitation spectra (FES), the excitation wavelength was scanned through the range of 220–300 nm while the emission was monitored with a 370 nm cut-off filter.

RESULTS

Determination of conditions for fluorescence detection

Flow-rates

The rates at which the two streams are mixed were found to be important parameters for the fluorescence detection of the nucleic acid components. Using 6-methylguanosine as a model fluorescent compound at pH 2.0, the flow-rates of the eluent (0.001 *M* KH_2PO_4, pH 3.5) and the post-column reagent (0.1 *N* sulfuric acid) were varied systematically. Since a change in the rate at which the post-column reagent is mixed with the column effluent could also change the pH of the solution in the flow-cell, adjustments to the post-column reagent solution pH were made when necessary.

Typically, introduction of the post-column reagent at flow-rates greater than the eluent flow rate caused severe band broadening and poor sensitivity. Using a flow-rate of 2.0 ml/min for the eluent, the sharpest peaks were obtained with a reagent flow of 0.2 ml/min. With an analytical flow-rate of 1.0 ml/min, the best reagent flow was 0.1 ml/min; a 10% trend was observed throughout up to the limiting eluent flow-rate of 4.0 ml/min.

Modified-effluent composition

For simplicity, the term modified-effluent is used to denote the solution which results from mixing the column effluent with the post-column reagent. The purines and pyrimidines used in the evaluation of the modified-effluent effects are listed in Table I.

pH of post-column reagent. Solutions of two acids, sulfuric acid and phosphoric acid and two bases, potassium hydroxide and sodium hydroxide, were evaluated for their potential as post-column reagents in the fluorescence detection of nucleic acid components. At identical pH values, both acids produced equivalent fluorescence peaks for the compounds investigated. In the basic environments, the fluorescence of the purines and pyrimidines was independent of the type of base used.

For some compounds, the fluorescence response as a function of pH takes the form of acidic or alkaline dissociation curves as shown in the plots for m_7-Ade, m_7-GMP and m_7-Ino (Fig. 1). In general, compounds which were devoid of fluores-

TABLE I

CHARACTERISTICS OF SOME BIOLOGICALLY IMPORTANT PURINES AND PYRIMIDINES

Compound	*Ultraviolet absorption maximum (nm)*	*Fluorescence excitation maximum (nm)*	*pH range*	*MDA*,** (nmol)*
Ade	263	280***	1 2	1.0
Ado	257	285	1-2	1.0
ATP	257	285	1-2	1.0§
1-M-Ado	260	285	1-2	1.0
7-M-Ado	272	280	1-2	1.0
Gua	275	295	10-12	0.05§
Guo	257 (274)§§	285	1-2	0.1§
GTP	257 (274)	285	1-2	0.1§
1-M-Gua	250 (274)	290	1 2	0.05§
1-M-Guo	258	290	1 2	0.05§
2-M-Gua	278	290	10-12	0.01§
2-M-Guo	278	290	10 12	0.01§
6-MO-Gua	286	295	1-2	0.003§
6-M-Guo	285	295	1-2	0.001§
7-M-Gua	281	295	10-12	0.02§
7-M-Guo	258	285	1-2	0.003§
M^2-Gua	280	295	10 12	0.01§
7-M-Ino	261	285	7-8	0.002§
Thy	290	295	10-13	1.0
5-M-Cyt	285	295	10-13	0.05

* Emission monitored with 370 nm cut-off filter.
** Minimum amount detected per injection.
*** Observed values presented without correction.
§ In methanolic effluent, sensitivity is increased.
§§ Values in parenthesis are for shoulders in the spectra.

cence in near-neutral environments, fluoresced in the pH range of 1.3 to 10.13. Many of these compounds exhibited a maximum in their fluorescence response over a relatively wide pH range. However, the adenine analogs fluoresced strongly only in a narrow pH zone; for example adenosine, which had maximum fluorescence at pH 2, had drastically reduced fluorescent peaks at pH 3 and pH 1. Analogous behavior was displayed by m_7-Ade (Fig. 1).

Ionic strength of post-column reagent. Since the fluorescence of nucleic acid components in solution can be quenched by phosphate and sulfate ions[17], the concentration limits for these species in the modified effluent were established.

The maximum allowable concentration of total sulfate or phosphate in the modified effluent was determined with the following expression:

$$I_t = \frac{C_1F_1 + C_2F_2}{F_1F_2} \quad (1)$$

where I_t represents the total concentration of the various forms of the acidic species in the modified-effluent, C_1 is the molar concentration of the acidic post-column reagent, F_1 is the post-column reagent flow-rate, C_2 is the molar concentration of the buffer in the eluent and F_2 is the eluent flow-rate. When C_2 is zero (no buffer

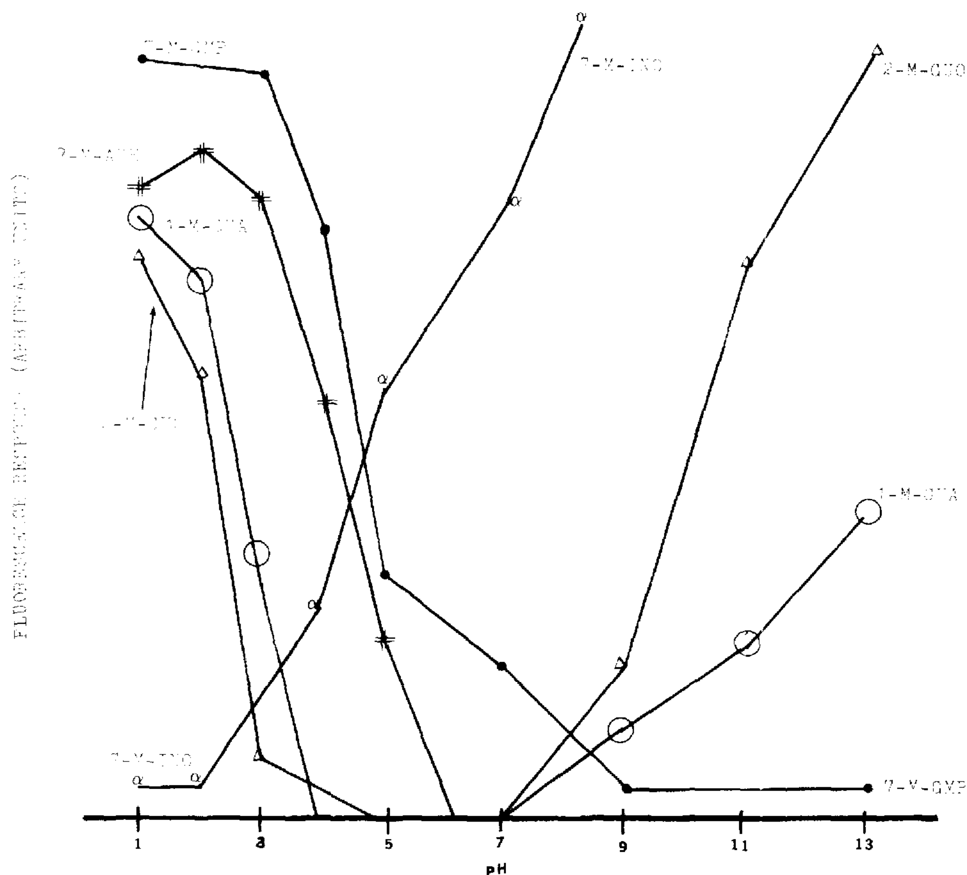

Fig. 1. Relative fluorescence intensities of methylated purines as a function of modified-effluent pH. Excitation: 285 295 nm. Emission: 370 nm.

used in the eluent) and a post-column reagent solution of sulfuric acid–sodium hydroxide is used, quenching was observed when the calculated I_t exceeded 0.5 M. For a phosphoric acid–potassium hydroxide post-column reagent, the limit in total phosphate as expressed by I_t was 0.4 M.

For basic post-column reagent solutions containing phosphate and sulfate salts, identical values for I_t were found at the onset of quenching. In either acidic or basic modified effluents, the addition of 0.2 M potassium chloride, sodium chloride, or ammonium chloride to the post-column reagent had no effect on the fluorescence of the guanine analogues studied. On the other hand, for the adenine analogues (most notably with adenosine), fluorescence quenching was observed when 2.5% or more of 0.1 M potassium chloride or 0.1 M sodium chloride was added to the acidic post-column reagent.

Phosphate buffer in the mobile phase. The contribution of the eluent to the total phosphate concentration in the modified-effluent has been incorporated in eqn. 1. The calculated limit of total phosphate in the modified-effluent (I_t = 0.4 M) was independent of the source, post-column reagent or buffered eluent. Thus, eqn. 1 could

be used as a guide in the preparation of a post-column reagent solution, or to indicate the compatibility of a desired mobile phase. For example, when a 0.5 *M* phosphate buffer (pH 6.0) was mixed with phosphoric acid, it was not possible to achieve a modified-effluent of pH 2 without exceeding the I_t for total phosphate. On the other hand, a mobile phase containing 0.01 *M* phosphate (pH 4.0) could be sufficiently acidified with 10 *M* phosphoric acid while keeping the total phosphate below 0.4 *M*. In some cases, sulfuric acid can be substituted for phosphoric acid as the post-column reagent to achieve the desired acidity. While the expression for the total mixed sulfate and phosphate allowed in the modified-effluent becomes much more complicated, eqn. 1 can be applied as a rough estimate by treating the sulfate present as phosphate.

For basic modified-effluents, only the contribution of total phosphate in the eluent is important since neither K^+ or Na^+ caused quenching.

Organic modifier. The use of methanol in the eluent or the post-column reagent either augmented or did not influence the fluorescence of the compounds listed in Table I. Where the fluorescence emission was enhanced, only 2 5% methanol in the modified-effluent was needed to double (approximately) the fluorescence peak. In addition, as long as the modified-effluent was sufficiently acidic or basic, there appeared to be no limit to the amount of methanol that could be present.

Gradient elution

The post-column technique was not as compatible with gradient elution as with isocratic elution. Although the gradient did not hamper the fluorescence of the compounds studied, there was appreciable noise and drift at maximum detector sensitivity. Thus, for gradient applications, the lowest usable setting was 0.1 μA.

Temperature

Increasing the column temperature from ambient to 35°C decreased the fluorescence of all components studied. By increasing the temperature from 35°C by 5°C intervals, complete fluorescence quenching was observed at 45°C. However, column temperatures as high at 65°C could be used if the post-column reagent is refrigerated and the inlet and outlet tubing is placed in an ice-bath.

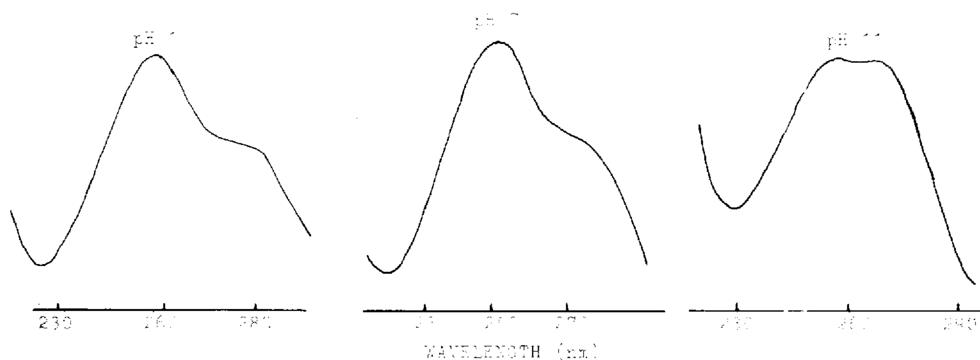

Fig. 2. Ultraviolet absorption spectra (UAS) of guanosine at different pH.

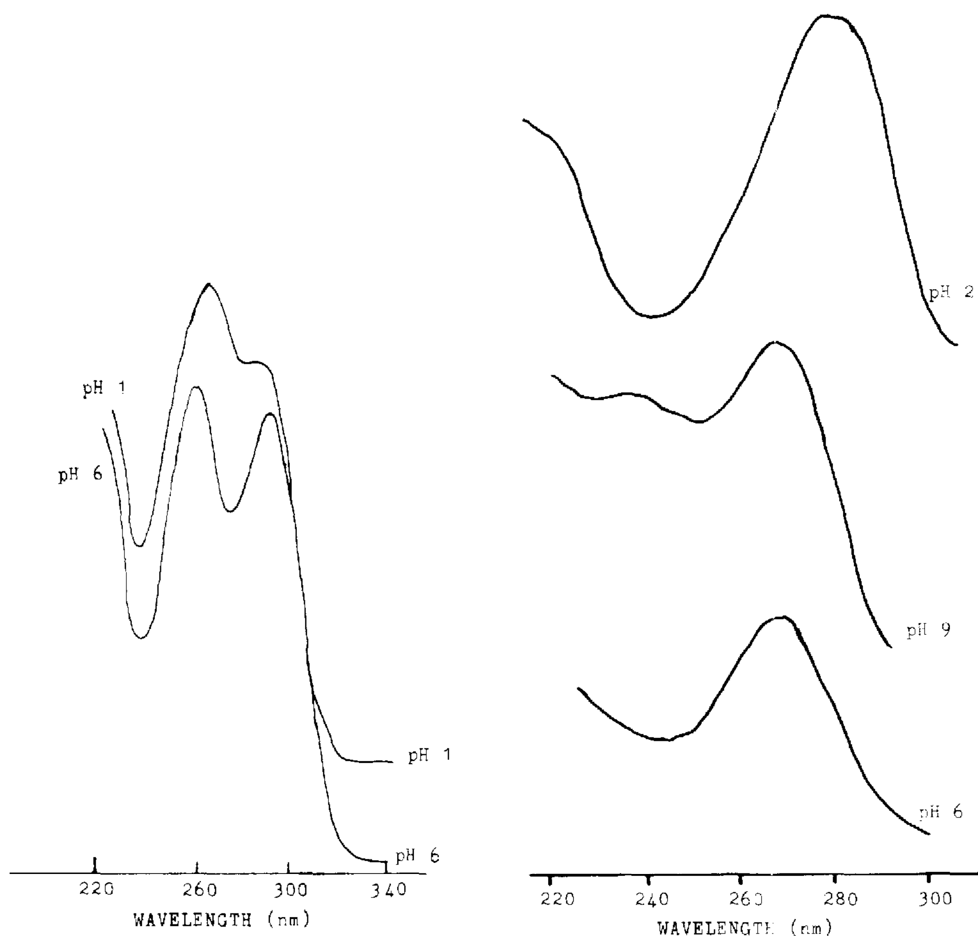

Fig. 3. UAS of 7-methylxanthosine in acidic modified-effluent (upper) and near-neutral effluent (lower).

Fig. 4. Characteristic UAS for cytidine recorded in acidic (upper), in basic (middle) modified-effluents and in the original effluent (lower).

Spectral characterizations

Ultraviolet absorption characteristics

For several groups of purines and pyrimidines, characteristic UAS at different pH were found as shown in Figs. 2, 3 and 4. For guanosine and 7-methylxanthosine, there is a change in the height of the shoulders at 280–300 nm, which result from n–π^* transitions. In general, changes in the relative intensities of the n–π^* transitions as a function of pH are useful for the characterization of the guanines, xanthines and methyl-substituted adenines. In addition, the spectra of cytidine are unique (Fig. 4) in that in basic solution a shoulder occurs at a lower wavelength (*ca.* 240 nm) rather than at a higher wavelength as is seen in the purines.

When scanning detectors are not available, the post-column technique can also be used for the identification and/or characterization of chromatographic peaks with

TABLE II

ULTRAVIOLET ABSORBANCE RATIOS FOR PURINES AND PYRIMIDINES AT 280–254 nm

Compound	*pH*		
	Acidic (1–2)	*Neutral (6 7)*	*Basic (11–13)*
Ade	0.38	0.13	0.61
1-M-Ade	0.21	0.82	0.87
2-M-Ade	0.56	0.15	0.84
6-M-Ade	0.70	0.48	1.20
6-Dimethyl-Ade	1.36	1.29	2.63
7-M-Ade	1.06	0.67	0.80
Cyt	1.54	0.58	3.15
5-M-Cyt	2.62	1.21	3.29
Gua	0.84	1.04	1.20
1-M-Gua	0.81	0.93	1.20
7-M-Gua	0.79	1.86	1.87
Guo	0.70	0.67	0.97
1-M-Guo	0.71	0.53	0.63
2-M-Guo	0.56	0.56	0.82
2-Dimethyl-Guo	0.56	0.63	0.79
7-M-Guo	0.68	1.05	1.52
Ado	0.22	0.10	0.16
2-M-Ado	0.40	0.13	0.19
6-M-Ado	0.41	0.64	0.68
6-Methyl-Ado	0.94	1.13	1.66
Hyp	0.04	0.09	0.17
Ino	0.17	0.12	0.17
Xan	0.21	1.10	1.14
Ura	0.25	0.16	1.40
Cyd	2.10	0.93	2.54
5-M-Cyd	3.57	1.42	1.59

280–254 nm absorbance ratios taken at different pH (Table II). For example, two closely eluting compounds, inosine and adenine, have identical absorbance ratios in near-neutral eluents. However, the adenine peak can be distinguished from the inosine peak since adenine has a much higher absorbance ratio in a basic environment than inosine. Moreover, absorbance ratios at a single wavelength and two different pH, one of which is set by the eluent and the other the modified-effluent, may also be used.

Fluorescence characteristics

The conditions necessary to detect some important nucleic acid components fluorometrically are listed in Table I. Some general trends were observed regarding the fluorescence of purine and pyrimidine compounds. They are summarized as follows:

(1) Nucleotides have lower fluorescence intensities than their corresponding nucleosides.

(2) For nucleotides, fluorescence decreases in the order: monophosphates > diphosphates > triphosphates.

(3) Deoxyribonucleosides are more fluorescent than the corresponding ribonucleoside.

(4) No general trend was observed for the relative fluorescence of substituted ribonucleosides and their bases. The relative fluorescence depends on the base, the position of the substituent and the pH of the modified effluent.

(5) In acidic environments, adenine, guanine and their nucleosides have comparable fluorescence intensities.

(6) In basic environments, guanosine-5'-monophosphate and guanine fluoresce strongly; guanosine, guanosine-5'-diphosphate, guanosine-5'-triphosphate and the adenines do not fluoresce.

(7) As a group, the guanines, especially the methylated analogues of guanine, were the most strongly fluorescent compounds examined.

(8) In near-neutral effluents (no post-column reaction), O_6- and N_7-methylated purine nucleosides are strongly fluorescent. The corresponding nucleotides were less fluorescent while the bases were devoid of fluorescence.

(9) Among the purines investigated, the adenine compounds have the weakest fluorescence under all conditions.

(10) In modified-effluents containing methanol and phosphoric acid, non-fluorescent pyrimidines gave negative peaks when excitated at 220 nm whereas fluorescent pyrimidines gave positive peaks.

Thus, it is possible to use the post-column ionization technique in the identification of two closely eluting compounds directly from their fluorescence characteristics. For example, 7-methyladenine and 2-methylguanosine have retention times which differ by less than 0.5 min in the RPLC separation using our standard conditions. Since in basic solutions the guanosine analogue fluoresces but the adenine analogue does not, the peak can be identified as 2-methylguanosine if it fluoresces and 7-methyladenine if it does not fluoresce.

Comparison of fluorescence and ultraviolet spectra

In general, the UV and fluorescence spectra at identical pH were similar (Fig. 5A). However, dramatic differences were found when the UV and fluorescence spectra for guanine at pH 11 were compared (Fig. 5B).

DISCUSSION

The post-column ionization technique greatly facilitates the accumulation of on-line data which can be used in the identification of purines and pyrimidines in biological matrices. The effect of pH on UV spectra and fluorescence spectra can be used for identification; these data are especially valuable when used in combination with retention behavior and the enzymic peak-shift technique for positive identification of unknown peaks.

The post-column technique has several advantages. The on-line protonation or deprotonation of the molecules is rapid and does not hamper detection due to

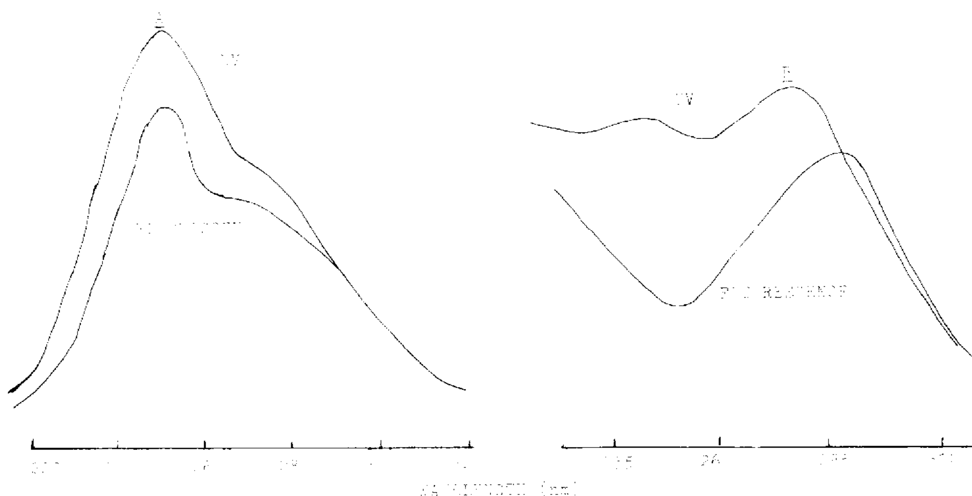

Fig. 5. (A) Comparison of guanine ultraviolet absorption (upper) and fluorescence excitation (lower) spectra recorded at pH 1.5. (B) Guanine spectra at pH 11 illustrating the difference between the fluorescence excitation and ultraviolet absorption spectra.

band broadening. The method is non-destructive and permits collection of chromatographic peaks for further analysis. In addition, the eluents used routinely for the separation of purines and pyrimidines can be used without modification.

The major drawback in the application of the technique for fluorescence characterization of major nucleic acid components is that relatively large or concentrated samples are needed. However, for the characterization of methylated guanines and other methylated purines, the post-column ionization technique used with fluorescence detection is sensitive and specific. Moreover, the high sensitivity (<1 pmol) exhibited with the fluorescence detector is ideally suited for studies of potential biological markers, as well as studies of modifications in nucleic acids.

During the course of this study, it was noted that pyrimidines exhibit unusual fluorescence behavior when excitated at 220 nm. All of the non-fluorescent pyrimidines gave rise to negative fluorescence peaks. However, the fluorescent pyrimidines responded with positive peaks. Su and co-workers[35] have noted similar behavior with other non-fluorescent compounds. The fact that the negative fluorescence peaks occurred only with the pyrimidines is intriguing and warrants further investigation.

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