variations do effect the frequency of piezoelectric crystal vibration in the detector cell, such effects can be compensated simply by including a reference detector in the system.

Surface Adsorption Effects. To detect surface adsorption, we placed indicator crystals bonded with either the C_{18} or C_{22} hydrocarbons in the detector cell as indicator crystals while plain as well as the trimethyl(dimethylamino)silane bonded crystals were used as reference detectors. Figure 3B indicates the change in the indicator/reference frequency ratio after injection of 0.2 mL of 10% CCl_4 and 0.2 mL of 10% toluene into a mobile phase of 20% aqueous methanol. In the case of CCl_4 both crystals decreased in frequency due to a liquid density change. However, the C_{18} bonded indicator crystal exhibited a larger decrease because surface adsorption also occurred. This disproportionate change in the frequency of the indicator crystal caused the variation in the frequency ratio observed. The injection of toluene caused only the reference crystal to increase its frequency because of a decrease in liquid density. Surface adsorption at the indicator crystal has predominated over density effects and lowered the indicator frequency causing a change in the frequency ratio.

While detector response was observed for injections of CCl_4 and toluene under these experimental conditions, the introduction of large nonpolar molecules such as oleic acid, butylbenzene, and naphthalene gave no adsorption response. The frequency shift of C_{18} crystals was also found to be greater than for C_{22} bonded surfaces. Clearly what modification of the quartz surfaces that was achieved allowed detectable adsorption of only small nonpolar molecules and with poor efficiency. Such a response does prove, however, that the effects of adsorption on crystal resonance frequency can be separated from those attributable to liquid density variations. Such results also indicate that the selective detection of various solute classes is potentially possible.

Further development of this approach toward LC detection must first center on improved methods of surface modification to encourage adsorption. It can be calculated from Sauerbrey's equations and constants (*6*) that the indicator crystal response to CCl_4 shown in Figure 3B was caused by the absorption of only 1.3 μg of the 31.8 mg of solute present. This low efficiency and sensitivity are also complicated by the fact that the treated crystals became saturated with solute after several consecutive injections and ceased giving a response. Soaking in a nonpolar solvent was found to regenerate the crystals, indicating that slow desorption rates are probably the cause of such behavior.

The most likely origin of the above absorption problems is poor coverage of hydrocarbon molecules on the quartz surfaces. Improved surface modification techniques may therefore be expected to yield more sensitive detection, and further research efforts will be pointed in this direction.

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High-Performance Liquid Chromatographic Determination of 5-Halopyrimidinone Interferon Inducers

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High-performance liquid chromatography with microparticulate, bonded, reversed-phase columns separates closely related 5-halopyrimidinones that are interferon inducers. A method was developed to quantitate serum levels of 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone, an important analogue of this new series. The method is, with minor modifications, suitable to measure other 5-halophenylpyrimidinone analogues. Results show that the quantitation of serum levels as low as 2 μg mL^{-1} is possible with ultraviolet detection at 235 nm. Protein precipitation and extraction prior to chromatography improve the daily sample throughput by removing interfering peaks with capacity factors greater than 45. Preliminary results indicate a species-dependent variation in the half-lives of elimination of the free compound after its administration, orally, to experimental animals. Rats clear the drug with a half-life of 4.5 h; cats clear the drug with half-lives ranging from 10 to 18 h depending on the dose administered. The differences in metabolic clearance may be relevant to observed toxicity differences between these species.

Recent clinical trials indicated that interferon could be an effective antiviral and antineoplastic agent. Although this glycoprotein expresses its biological activity at subnanogram levels, it is difficult to produce and purify. Despite its demonstrated potential, shortages of interferon hinder the expansion of clinical trials. Various conventional and unconventional mass production techniques could, conceivably, alleviate this shortage; however, they have not done so yet.

Another approach to evaluate and develop the therapeutic potential of interferon relies on the inherent capacity of cells to secrete the substance, endogenously, after exposure to an inducing agent that can derepress the, ordinarily, silent interferon gene. Whether one induces endogenous interferon production or whether one produces it for exogenous administration, the induction step and some form of interferon inducer are pivotal. Nevertheless, the development of safe and effective interferon inducers is at an embryonic stage.

5-Halopyrimidinones can induce interferon in vivo in several species, and in vitro in mammalian cell cultures, including

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human cells (*1–3*). Compared with other inducing agents (*4–8*) some 5-halopyrimidinones have a favorable activity and toxicity profile in experimental animals. We have evaluated the high-performance liquid chromatography of these compounds and developed a quantitative method to monitor the serum concentrations after their administration, orally, to experimental animals. The quantitative method was validated with 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone, but it is, with minor modifications, applicable to other phenylpyrimidinones in this series. The generic value of the method is discussed in terms of some current problems of interferon induction.

EXPERIMENTAL SECTION

Materials. 5-Halopyrimidinones (Experimental Chemistry Laboratories, The Upjohn Co., Kalamazoo, MI) were synthesized as described earlier (*3*). Theophylline, xanthine, uric acid, caffeine, theobromine, and mono- or dialkyl analogues of these compounds were obtained commercially (Sigma, St. Louis, MO). Ethyl acetate, 2-propanol, methanol, and acetonitrile, distilled-in-glass (Burdick and Jackson, Muskegon, MI), were used as received. Rodent and dog plasma for assay development were obtained commercially (Pel-Freeze Biologicals Inc., Rodgers, AR). ACS grade ammonium sulfate, anhydrous sodium sulfate, acetic acid, and potassium hydroxide (J. T. Baker, Philipsburg, NJ) were used as received.

Apparatus. For high-performance liquid chromatography we used a constant flow reciprocating pump (Laboratory Data Control, Riviera Beach, FL), a sampling valve with a 20-μL loop (Rheodyne Model 7200, Anspec Corp., Ann Arbor, MI), a variable-wavelength detector (Model LC-55, Perkin-Elmer, Norwalk, CT), and a variable-sensitivity recorder (Varian A-25, Walnut Creek, CA). Microparticulate reversed-phase columns, μBondapak C18 (Waters Associates, Milford, MA), RP-8 (E. Merck Laboratories, Elmsford, NY), and Partisil 5 ODS (Whatman Inc., Clifton, NJ) were used as received. A two-speed reciprocating horizontal shaker (Eberbach and Sons, Ann Arbor, MI) was used for extracting serum or plasma samples.

High-Performance Liquid Chromatography. Compounds cited in the text were dissolved in methanol/water, 80/20, to yield concentrations of 100 μg mL^{-1}, approximately. These solutions were chromatographed on different microparticulate reversed-phase columns. After preliminary experiments, a mobile phase composed of methanol/water/acetic acid 50/50/0.1 (v/v/v) was used to evaluate the chromatographic performance of the different columns under normalized conditions of mobile phase composition and flow rate. The detector wavelength was fixed at 235 nm. Modifications to alter resolution between closely related pairs of 5-halopyrimidinones were based on the initial chromatograms obtained under these conditions.

Quantitative Analysis. For the quantitative analysis of 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone, the corresponding 5-iodo analogue was used as an internal standard. For biological experiments using 2-amino-5-iodo-6-phenyl-4(3*H*)-pyrimidinone as a test compound, the 5-bromo analogue is an appropriate internal standard. The internal standard was dissolved in methanol/water, 80/20 (v/v), to give a concentration of 1.0 mg mL^{-1}.

Plasma or serum (1.0 mL) and the internal standard (0.10 mL, 1.0 mg mL^{-1}) were mixed in a centrifuge tube (15 mL). A saturated ammonium sulfate solution (2.0 mL) and granular ammonium sulfate (0.5 g) were added, followed by 2-propanol (5.0 mL). The mixture was shaken for 30 min and then centrifuged (1000*g*, 10 min, 25 °C). The 2-propanol layer was evaporated to dryness after transfer to another centrifuge tube. The residue, after evaporation, was reconstituted with 2-propanol/water (3.0 mL, 1/2 (v/v)) and the pH was adjusted to 10.0 with potassium hydroxide (8 M). The alkaline, aqueous supernatant was extracted with hexane (5.0 mL) to remove neutral and basic lipids. There was no significant (<2%) extraction of 5-halopyrimidinones into the hexane phase. The hexane layer was discarded, and the pH was adjusted to 3.0 with formic acid (50–100 μL). The 5-halopyrimidinones were extracted twice with ethyl acetate (5.0 mL), and the pooled extracts were desiccated over anhydrous sodium sulfate. The ethyl acetate was evaporated with a nitrogen stream. The residue, containing extracted 5-halopyrimidinones, was dissolved in methanol/water, 80/20 (1.0 mL), and a portion (20 μL) was chromatographed on a μBondapak C18 column, 30 cm × 0.4 cm i.d., using the mobile-phase methanol/water/acetic acid 40/60/0.1 (v/v/v), flowing at 1.5 mL min^{-1}. The detector wavelength was 235 nm. Attenuation was adjusted between 0.04 and 0.4 absorbance units full scale. Under these conditions, 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone elutes at $K' = 14.1$ and the internal standard 2-amino-5-iodo-6-phenyl-4(3*H*)-pyrimidinone elutes at $K' = 16.6$. The sensitivity of the method for quantitation is 2 μg mL^{-1} serum assuming a signal/noise ratio of 5/1 at 0.04 absorbance units full scale attenuation. The sensitivity can be improved by reconstituting the extracted 5-halopyrimidinones into a volume smaller than 1.0 mL, prior to injection. Calibration curves were prepared by fortifying control serum or plasma with 5-halo-6-phenylpyrimidinone to give concentrations of 15–120 μg mL^{-1}. The procedure described above was applied to calibration standards and samples.

With minor amendments in the mobile-phase composition, the method is applicable to other members of the analogue series. Preliminary results indicate that the method is applicable for measuring urinary 5-halopyrimidinones without interference from xanthine oxidase metabolites.

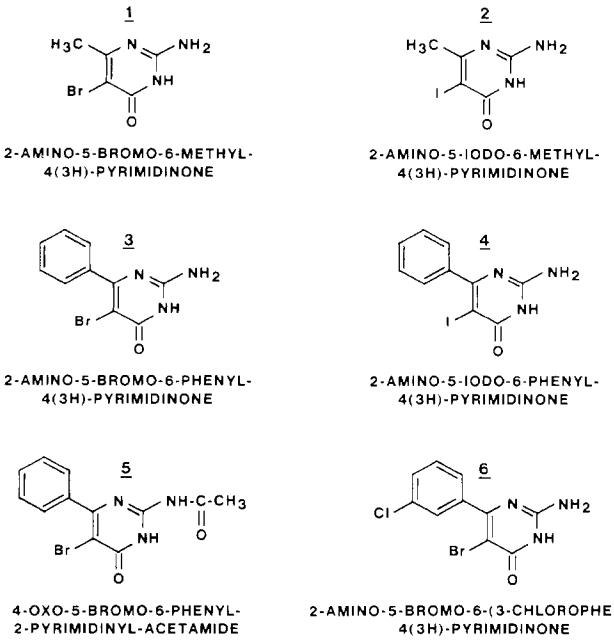

Figure 1. Structures of 5-halopyrimidinones.

RESULTS AND DISCUSSION

Figure 1 shows the structures of some typical 5-halopyrimidinone analogues. Figure 2 shows their separation by high-performance liquid chromatography on three different microparticulate chemically bonded reversed-phase columns with the isocratic mobile-phase methanol/water/acetic acid, 50/50/0.1 (v/v/v). On the basis of these initial chromatograms we could optimize the resolution of any particular pair of 5-halopyrimidinones by altering the amount of methanol moderator. Compared to acetonitrile or 2-propanol modifiers, methanol gave consistently superior Gaussian peak shapes with the lowest capacity factors. For example, a μBondapak C18 column separated all six compounds with base line resolution in 30 min with a mobile phase of methanol/water/acetic acid, 40/60/0.01 (v/v/v). We selected this system for routine quantitative analysis of biological samples on both theoretical and practical grounds. Compared to the μBondapak C18 column, changes in mobile-phase composition to optimize resolution of the early eluting peaks 1–4 on an RP-8 column resulted in unsuitable increases in the K' value for the later eluting peaks 5 and 6. Karger et al. (*9*) have stressed the desirability of minimizing the K' value for quantitative trace analysis. Table I compares the capacity

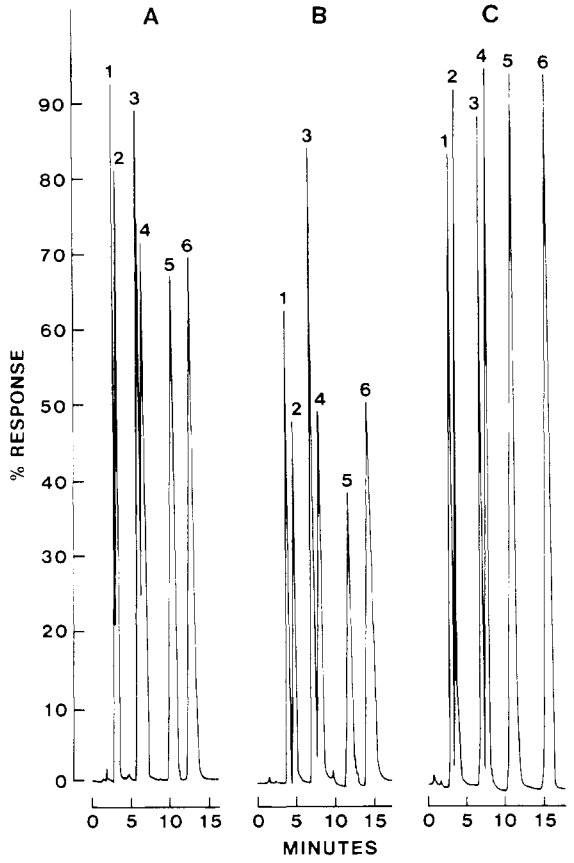

Figure 2. Separation of 5-halopyrimidinones. 5-Halopyrimidiones depicted in Figure 1 were separated on three different reversed-phase columns: A = μBondapak C18, B = Partisil 5 ODS, C = Merck RP-8. Mobile phase = methanol/water/acetic acid, 50/50/0.1 (v/v/v); flow rate = 1.5 mL min^{-1}; UV = 235 nm 0.4 absorbance units full scale; approximately 2 μg of each compound injected.

factors, on both columns, for several 5-halopyrimidinones and related compounds including xanthine oxidase metabolites that could interfere, potentially, in a quantitative method. Although the 5-μm reversed-phase columns resolved the 5-halopyrimidinones, operating pressures were typically 4800–5000 psig compared to 1600 to 2000 psig for an equivalent separation on a 10-μm μBondapak C18 column. These data and the selection of a particular column type are based on a limited comparison of two columns of each type.

Quantitation of serum concentrations of 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone was based on the chromatographic separations described. Protein precipitation and extractions prior to high-performance liquid chromatography prevented column fouling from the cumulative deposition of insoluble materials and improved the daily sample throughput by removing interfering late eluting peaks ($K' > 45$). An internal standard, 2-amino-5-iodo-6-phenyl-4(3*H*)-pyrimidinone, eliminated the need for quantitative transfers during sample processing; however, quantitative experiments indicated that 86.3 ± 4.5% ($v = 11$) 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone was recovered after the protein precipitation and extraction step.

Four separate calibration curves were prepared over 20 days. Within the range 15–110 μg mL^{-1}, the correlation coefficient was $r^2 = 0.995$ or greater for each curve. The slopes were linear and ranged from 0.0104 to 0.0113 with a mean ± relative standard deviation (rsd) of 0.0107 ± 0.0004. The percent relative standard deviations of the interassay ordinate values for the four curves were ±3.9%, ±4.8%, ±8.2%, and ±6.7% for concentrations (abscissa) of 118, 59, 29.5, and 14.8 μg mL^{-1}, respectively.

Table I. Capacity Factors for Xanthine Oxidase Metabolites and 5-Halopyrimidinones

compound	capacity factor (K')[a] column A[b]	column B[c]
uric acid	2.1	1.9
hypoxanthine	2.1	2.3
3-methyluric acid	2.2	2.2
xanthine	2.2	2.2
7-methylxanthine	2.2	2.4
7-methyluric acid	2.2	2.1
3-methylxanthine	2.4	2.5
1,3-dimethyluric acid	2.8	2.5
theobromine	2.9	2.6
1-methylxanthine	2.9	2.6
β-hydroxyethyltheophylline	3.3	3.3
theophylline	3.6	3.8
1,3-dimethylxanthine	3.6	
hydroxypropyltheophylline	3.9	4.5
2-amino-5-bromo-6-methyl-4(3*H*)-pyrimidinone	4.3	8.8
	4.3	
caffeine	4.7	4.7
2-amino-5-iodo-6-methyl-4(3*H*)-pyrimidinone	4.9	11.6
8-chlorotheophylline	6.3	6.3
2-amino-5-chloro-6-(2-methoxyphenyl)-4(3*H*)-pyrimidinone	10.3	
methyl *p*-hydroxybenzoate	12.0	16.4
2-amino-5-iodo-6-(2-methoxyphenyl)-4(3*H*)-pyrimidinone	12.9	
isobutylmethylxanthine	14.1	17.2
2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone	14.1	21.3
2-amino-5-iodo-6-(2-fluorophenyl)-4(3*H*)-pyrimidinone	15.2	26.2
2-amino-5-chloro-6-(3-methoxyphenyl)-4(3*H*)-pyrimidinone	15.8	
2-amino-5-iodo-6-phenyl-4(3*H*)-pyrimidinone	16.6	
2-amino-5-bromo-6-(3-methoxyphenyl)4(3*H*)-pyrimidinone	16.6	
2-amino-5-chloro-6-(3-fluorophenyl)-4(3*H*)-pyrimidinone	17.7	
2-amino-5-bromo-6-(3-fluorophenyl)-4(3*H*)-pyrimidinone	18.9	
ethyl *p*-hydroxybenzoate	19.3	30.6
2-amino-5-iodo-6-(3-methoxyphenyl)-4(3*H*)-pyrimidinone	20.3	
2-amino-5-iodo-6-(3-fluorophenyl)-4(3*H*)-pyrimidinone	21.0	
2-amino-5-bromo-4-oxo-6-phenyl-2-pyrimidinylacetamide	29.5	35.8
2-amino-5-bromo-6-(3-chlorophenyl)-4(3*H*)-pyrimidinone	38.9	54.6

[a] $K' = (V_r - V_0)/V_0$; mobile phase, methanol/water/acetic acid, 40/60/0.1 (v/v); flow rate, 1.5 mL min^{-1}. [b] Column A = μBondapak C18. [c] Column B = RP-8.

2-Amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone is moderately stable in plasma. At both 2 and 6 days after incubation with dog plasma at 37 °C, the compound was recovered quantitatively (118 and 59 μg mL^{-1}). At 13 days of incubation, a concentration-dependent loss was evident, and recoveries were 81.7% and 72.2% for 118 and 59 μg mL^{-1}, respectively.

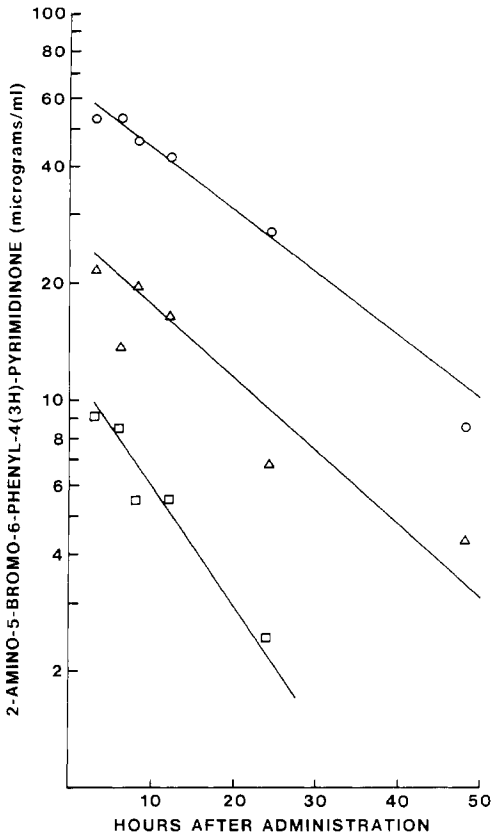

Figure 3. Serum concentrations of 2-amino-5-bromo-6-phenyl-4-(3*H*)-pyrimidinone in cats. Drug was suspended in an aqueous vehicle and administered orally to cats to yield doses of: (○) 50 mg kg^{-1}; (△) 10 mg kg^{-1}; (□) 5 mg kg^{-1}.

The compound was stable in 0.1 N sodium hydroxide at 37 °C for 96 h without measurable decomposition. The compound decomposed by 20% in 0.1 N sulfuric acid at 37 °C after 48 h. A new peak appeared in the chromatogram coincident in retention volume with the dehalogenated pyrimidinone 2-amino-6-phenyl-4(3*H*)-pyrimidinone.

Figure 3 shows results from a typical experimental application. Serum levels of 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone rose with increasing oral doses of the interferon inducer. Serum interferon levels rose with increasing serum drug concentration. For example, at 3 h after administration, interferon levels were 15000, 6000, and 150 interferon units mL^{-1}. Differences in the rate of clearance of the compound from circulatory system were evident between rats and cats. The half-life of elimination in rat serum for an oral dose of 100 mg kg^{-1} was 4.45 ± 1.1 h (mean ± rsd, n = 4). The half-lives of elimination from cat serum were 17.4, 18.5, and 10.6 h for oral doses of 50, 10, and 5 mg kg^{-1}, respectively. A typical chromatogram from these experiments is depicted in Figure 4. The peaks eluted near the solvent front are metabolites of 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone.

The chromatographic method described may contribute to the solution of some current problems in the development of interferon inducers and, by extension, to the development of the therapeutic potential of interferon. For example, the chemical requirements for low molecular weight interferon inducers are uncertain. *N,N*-Dioctadecyl-*N'*,*N'*-bis(2-hydroxyethyl)propanediamine (*5*), bis(diethylamino)-fluorenone (*6*), quinacrine (*7*), and anthraquinone (*8*) can induce interferon, either in vivo or in vitro. Structure–activity studies on a few unrelated compounds are, typically, fruitless. However, structure–activity studies within a class, such as the 5-halopyrimidinones, could clarify some chemical requirements

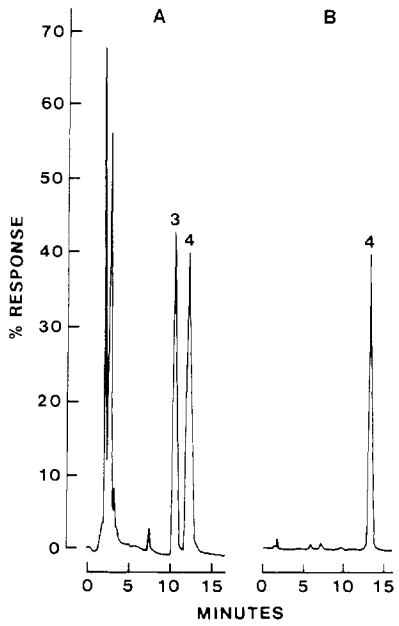

Figure 4. Typical chromatograms of biological extracts: (A) serum of cat dosed with 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone, peak 3; peak 4 = the internal standard, 2-amino-5-iodo-6-phenyl-4(3*H*)-pyrimidinone; early eluting peaks (t_r < 5 min) are metabolites of 3; (B) cat control serum; 4 = internal standard peak.

for interferon induction, as well as the significance that drug absorption, distribution, metabolism, and excretion have on the activity/toxicity profile.

Karger (*10*) has summarized why reversed-phase high-performance liquid chromatography is ideal for many problems. Our results confirm its value for separating and analyzing similar analogues of a new series of interferon inducers. Ion exchange chromatography has been used extensively for separating purines and pyrimidines (*11*); however, the hydrophobic contribution of the halogen substituent excluded its use as a practical method for separating the 5-halopyrimidinones. It is noteworthy that several recent reports suggest that reversed-phase chromatography may supplant ion exchange chromatography for separating nucleotides, nucleosides, and bases (*12–15*).

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