High-Performance Liquid Chromatographic Analysis of Amino Acid- and Peptide-Derived Chloramines

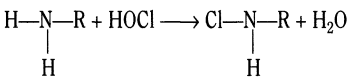
Suzanne M. Furness-Green, Tracy R. Inskeep, Jennifer J. Starke, Lu Ping*, Heather R. Greenleaf-Schumann, and **Thomas E. Goyne†**
Department of Chemistry, Valparaiso University, Valparaiso, IN 46383

Abstract

An isocratic reversed-phase high-performance liquid chromatographic method is reported for the analysis of amino acid- and peptide-derived chloramines (these are important intermediates in two very different processes: destruction of microbes by the neutrophil and disinfection of water with chlorine). Specifically, results are reported for the chloramines derived from the following amino acids and dipeptides: taurine, Ala, Gly, Ser, Thr, Phe, Val, AlaGly, GlyGly, PheGly, SerGly, and ValGly. Analyses are performed on a 250 × 4.6-mm C_{18} column using a buffered water–acetonitrile mixture as the mobile phase. For samples containing hydrophilic chloramines, an ion-pairing agent is added to the mobile phase. Two detection methods are used: direct ultraviolet (UV) detection of the chloramine at 254 nm and indirect UV detection of I_3^- at 350 nm following postcolumn reaction with iodide. Unexpectedly, the decomposition of amino acid-derived chloramines is found to greatly accelerate during chromatographic elution on a reversed-phase column.

Introduction

Organic chloramines are formed by the reaction of amines with hypochlorous acid, as shown in Equation 1.

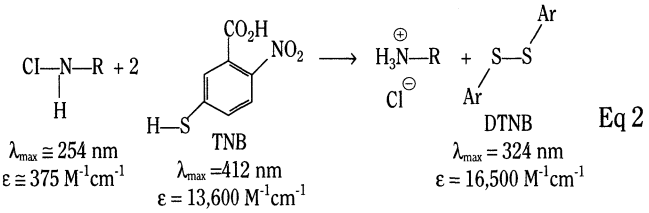


Eq 1

The chloramines derived from amino acids, peptides, and proteins are important intermediates in two very different processes: the disinfection of potable and waste waters with chlorine (1–13) and the destruction of microbes by the neutrophil and related host defense cells (14–27). In the case of potable water and waste water chlorination, there is concern that these chloramines and/or their decomposition products may pose a public health risk. In the case of the host defense system, the chloramines that are generated by the neutrophil and related cells are of interest not only because they are toxic to invading microbes but also because they can injure host tissue, especially during chronic inflammation. Progress toward a thorough understanding of either system has been hampered by the lack of adequate analytical methods to characterize and quantitate organic chloramines.

A number of colorimetric reactions and electrochemical methods have been used to analyze the organic chloramines found in chlorinated waters (28). These methods have the drawback that they cannot distinguish one organic chloramine from another and are subject to interference by inorganic chloramine (NH_2Cl) and other oxidants. Two methods have been reported in which organic chloramines are first derivatized and then analyzed by high-performance liquid chromatography (HPLC) (29–31). The dansylsulfinic acid method reported by Scully et al. (29,30) is severely limited because it has been shown to proceed via formation of dansyl chloride, which subsequently reacts with any amine in the system to yield the corresponding dansyl derivative (30). More recently, the Scully group has used HPLC to study the chlorination products of several amino acids (11,32,33) and peptides (12,13).

In 1990, we began the development of a reversed-phase HPLC method to directly analyze amino acid- and peptide-derived chloramines. In addition to direct ultraviolet (UV) detection of the chloramine at 254 nm, we developed a more sensitive and selective postcolumn detection method using 5-thio-2-nitrobenzoic acid (TNB) as the postcolumn colorimetric agent (20).

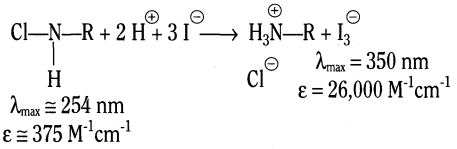


Eq 2

While this work was in progress, Jersey and Johnson reported a reversed-phase HPLC method for directly analyzing chloramines (1,2) using iodide as the postcolumn reagent and employing amperometric detection of the I_3^-. Yoon and Jensen have adapted

*Permanent address: Chemistry Department, Hangzhou University, ZJ310028, P.R. China.
† Author to whom correspondence should be addressed.

their method by using UV detection of I_3^- in place of amperometric detection (7).



Eq 3

As discussed below, iodide is more convenient to handle than TNB. We thus switched Jersey and Johnson's method (with UV detection) for all subsequent work.

In addition to corroborating Jersey and Johnson's results for amino acid-derived chloramines, we have extended their method to a number of dipeptide-derived chloramines. We have also employed an ion-pairing agent to achieve adequate resolution of hydrophilic chloramines, which otherwise elute near the void volume. Finally, we have made the unexpected finding that the decomposition of amino acid-derived chloramines is greatly accelerated during chromatographic elution on a reversed-phase column.

Experimental

Materials

Amino acids and peptides were purchased from Sigma (St. Louis, MO) and dissolved in water to yield 0.100M stock solutions that were stored at –20°C. Reagent-grade sodium hypochlorite and sodium borohydride and semiconductor-grade potassium hydroxide (99.99%) were obtained from Aldrich (Milwaukee, WI). The sodium hypochlorite reagent was purified by vacuum distillation as described by Albrich and coworkers (34). (Briefly, the reagent was acidified to pH 7.5 with 1.0M H_3PO_4 and then distilled under an aspirator vacuum into an ice-cooled receiving flask. The distillate was made basic with NaOH (pH 12) and stored in microcentrifuge tubes at –20°C). Reagent-grade potassium iodide, acetic acid, and HPLC-grade acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Tetrabutylammonium phosphate (0.5M solution), HPLC-grade potassium dihydrogen phosphate, and HPLC-grade phosphoric acid (1.0M) were purchased from Regis (Morton Grove, IL). Water was purified using a Barnstead Easy Pure system (Barnstead, Boston, MA).

Preparation of 5-thio-2-nitrobenzoic acid

5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB) was purchased from Sigma and reduced to 5-thio-2-nitrobenzoic acid (TNB) with sodium borohydride as described by Aune and Thomas (35). Specifically, a 1mM solution of DTNB dissolved in 50mM sodium phosphate buffer (pH 6.6) containing 5mM ethylenediaminetetraacetic acid (EDTA) was purged with nitrogen and then charged with 40 equivalents of sodium borohydride. After 1 h of stirring at 37°C, UV analysis of the reaction mixture revealed an essentially complete reaction. If stored at 4°C in a tightly sealed container (to minimize air oxidation), this solution was stable for a period of several years. Immediately prior to use, the concentration of TNB was determined using UV spectroscopy (see Equation 2), and the solution was diluted to 5.0×10^{-5}M with H_2O.

Preparation of chloramines

Chloramines were prepared by adding sodium hypochlorite to a 10-fold molar excess of the appropriate amine (20). Specifically, sodium hypochlorite (approximately 0.2M stock solution, enough to yield a final concentration of 2.58×10^{-3}M) was added to a solution containing 2.67×10^{-2}M amino acid or peptide in 50mM potassium phosphate buffer (pH 7.40). Sodium hypochlorite concentrations were based on the molar absorptivity ε_{292} = 362$M^{-1}cm^{-1}$ (36). UV–vis spectroscopy was used to measure the chloramine concentration using the published molar absorptivity values listed in Table I. Once the reaction mixture was prepared, it was stored on ice to minimize chloramine decomposition. Samples were used before appreciable chloramine decomposition could take place (based on the reported half-lives listed in Table II).

Kinetics analysis

Chloramine decomposition rates were determined using a Hewlett-Packard 8452A diode-array spectrometer (Hewlett-Packard, Palo Alto, CA) fitted with a water-jacketed cuvet holder and a 1-cm path length cuvet. A Lo-Temptrol 154 circulating water bath (Precision Scientific, Chicago, IL) was used to maintain the temperature at 25°C ± 0.1.

HPLC analysis

The HPLC system consisted of a Waters 600E system controller and pump for the mobile phase (Waters, Milford, MA), a Rheodyne model 7125 injection port (Rheodyne, Cotati, CA) fitted with a PEEK sample loop, and a Regis Rexchrom C_{18} column (250 × 4.6-mm i.d., 5 µm, 100 Angstrom) protected by a matching Regis Rexchrom guard cartridge (1 cm × 3.0-mm i.d.). In some experiments, the column temperature was controlled using an Alltech water jacket (Alltech, Deerfield, IL) fitted to the circulating water bath described above. The column effluent first passed through a

Table I. Spectroscopic Properties of Amino Acid- and Peptide-Derived Chloramines

Chloramine	λ_{max} (nm)	ε (M^{-1} cm^{-1})	Reference
Taurine-Cl	250	398	42
	250	398	43
	252	429	20
Ala-Cl	254	375	44
	255	373 ± 30*	37
Gly-Cl	254	375	44
	256	350	45
Cl-GlyGly	254	357	44
Ile-Cl	255	358 ± 25*	46
	255	368 ± 7*	37
Leu-Cl	255	338 ± 23*	46
	255	340 ± 20*	37
Ser-Cl	255	371 ± 11*	37
Thr-Cl	256	357	44
	255	345 ± 4*	37
Val-Cl	255	357 ± 12*	37

* The values reported in references 37 and 46 are based on ε_{292} = 350M^{-1} cm^{-1} for hypochlorite. The values shown here are corrected from the reported values based on ε_{292} = 362$M^{-1}cm^{-1}$ for hypochlorite (36).

Waters model 440 fixed-wavelength detector set at 254 nm (1-cm path length). The postcolumn reagent was pumped with an LDC minipump (LDC, Riviera Beach, FL), first through 9 m of Teflon tubing (1.59-mm [1/16-in.] o.d., 1.02-mm [0.040-in.] i.d.), which acted as a pulse dampener, and then into a mixing tee where it combined with the column effluent. The postcolumn reactor consisted of another 2.5 m of Teflon tubing (1.59-mm [1/16-in.] o.d., 0.51-mm [0.020-in.] i.d.). The effluent from the postcolumn reactor finally passed through an ISCO V4 variable-wavelength UV–vis detector (ISCO, Lincoln, NE) set at 350 nm (1-cm path length). The output from both detectors was fed into a chromatographic data system consisting of a Zenith 386SX computer running the Waters Baseline 810 hardware/software package. The integration start and stop points selected by the computer were always visually inspected and manually adjusted as needed.

Table II. Decomposition Rates of Amino Acid-Derived Chloramines

Chloramine	Half-life (min)	pH	Temperature (°C)	Reference
Ala-Cl	43.3	7	25	39
	43	6.85	25	3
	71.44	6	25	47
	96	7.50	20	48
Arg-Cl	32.54	6	25	47
Asn-Cl	10.00	6	25	47
Asp-Cl	9.45	6	25	47
Cys-Cl	*	6	25	47
Gln-Cl	28.88	6	25	47
Glu-Cl	26	6.89	25	49
	24.66	6	25	47
Gly-Cl	473	10.30	25	49
	27,480	6.85	25	3
	277	7	25	39
	',800	7.55	20	48
His-Cl	34	7.58	25	48
Ile-Cl	58.6	7	25	46
	73.72	6	25	47
Leu-Cl	35.0	7	25	46
	79	7.85	23	48
	34.48	6	25	47
	36.1	5–12	25	50
Lys-Cl	58.73	6	25	47
	49.0	10.17	25	49
Met-Cl	*	6	25	47
Phe-Cl	38.08	6	25	47
	52	6.87	25	48
Pro-Cl	13	6.85	25	3
	1.46	6	25	47
Ser-Cl	33.64	6	25	47
	33	7.25	25	48
	39	6–9	25	51
Thr-Cl	117.46	6	25	47
	71.1	7.08	25	49
Trp-Cl	*	6	25	47
Tyr-Cl	33.97	6	25	47
Val-Cl	71.44	6	25	47

* Too fast to measure manually.

The mobile phase consisted of 50mM potassium phosphate buffer (pH 7.40) containing 7.5% acetonitrile and, where indicated, 40mM tetrabutylammonium phosphate. Two postcolumn reagents were employed: a 5.0×10^{-5}M TNB solution described above and 90mM potassium iodide in 0.294M acetate buffer (pH 4.0) (2). The mobile phase and postcolumn reagent were delivered at flow rates of 1.0 and 0.5 mL/min, respectively. Sample composition was matched to that of the mobile phase by spiking each sample with acetonitrile (and tetrabutylammonium phosphate, if present in the mobile phase) immediately prior to injection. Complete loop-filling was ensured by injecting a 10-fold excess of sample into the sample loop. Column dead times (t_0) were taken to be the retention time of the first baseline disturbance (at 254 nm), when a 10-µL sample of water was injected using 100% methanol as the mobile phase at a flow rate of 1.0 mL/min.

Results and Discussion

Development of the chromatographic method

Typical chromatograms of the chloramines derived from glycine, alanine, and valine are shown in Figure 1. Notably, glycine chloramine and alanine chloramine eluted near the void volume. This is because these species bear a net negative charge at the neutral pH of the mobile phase (because the pK_a of the chloramine group is less than 1 [37], the nitrogen is not protonated at neutral pH). Valine chloramine also bears a net negative charge under these conditions but was retained on the column to a degree due to its large, hydrophobic side-chain. The chromatographic behavior exhibited in Figure 1 is as one would expect and is what we observed for all of the amino acid- and peptide-derived chloramines that were analyzed; all eluted near the void volume unless they possessed a large, hydrophobic side-chain. Our results, which are summarized in the middle data column of Table III, are in agreement with the findings of Jersey and Johnson (see Appendix G in reference 2).

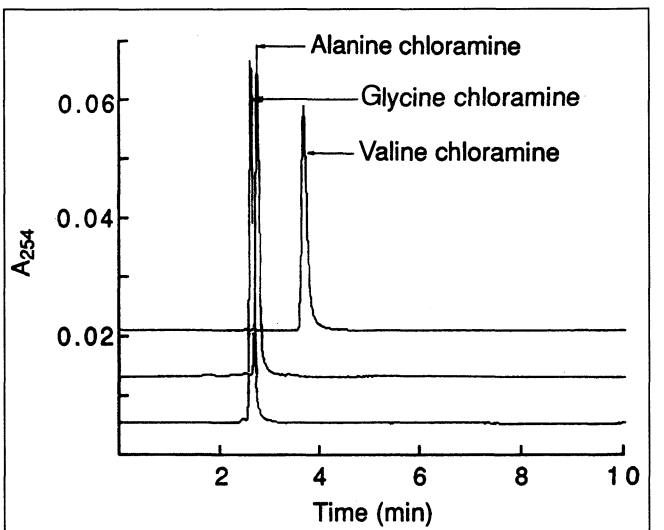


Figure 1. Typical chromatograms of amino acid-derived chloramines (20 µL, 2.3×10^{-3}M) in the absence of ion-pairing agent (direct detection). Column temperature: 25.0°C ± 0.1.

In order to cleanly resolve carboxylates, which elute near the void volume in reversed-phase chromatography, a common strategy is to lower the pH of the mobile phase until the carboxyl group is protonated. This solution was not suitable in this case because chloramines tend to rapidly disproportionate below pH 5, as shown in Equation 4 (20).

$$2\,RNHCl + H^+ \longrightarrow RNCl_2 + RNH_3^+ \qquad \text{Eq 4}$$

Instead of lowering the pH, our approach was to add an ion-pairing agent (tetrabutylammonium phosphate) to the mobile phase. Because of the expense of this reagent, a series of experiments were carried out to establish the minimum concentration that would yield reproducible chromatographic behavior. Retention times drifted badly using either a 20 or 30mM ion-pairing agent but were stable using 40mM (data not shown). It is worth noting that 40mM tetrabutylammonium is the concentration suggested by Snyder, Glajch, and Kirkland (38).

Table III. Capacity Factors for Amino Acid- and Peptide-Derived Chloramines*

	Capacity factor	
Chloramine	$[Bu_4N^+] = 0$	$[Bu_4N^+] = 40mM$
Taurine-Cl	0.81 ± 0.01	6.16 ± 0.04
Taurine-Cl_2	3.89 ± 0.03	–
Ala-Cl	0.69 ± 0.01	3.03 ± 0.02
Gly-Cl	0.62 ± 0.01	2.34 ± 0.02
Ser-Cl	0.58 ± 0.01	1.82 ± 0.02
Thr-Cl	0.60 ± 0.01	2.28 ± 0.02
Phe-Cl	7.79 ± 0.05	–
Val-Cl	1.26 ± 0.01	–
Cl-AlaGly	0.78 ± 0.01	2.67 ± 0.02
Cl-GlyGly	0.68 ± 0.01	2.08 ± 0.02
Cl-PheGly	13.0 ± 0.2	–
Cl-SerGly	0.66 ± 0.01	1.92 ± 0.02
Cl-ValGly	1.94 ± 0.02	10.8 ± 0.1

* HPLC conditions as described in the Experimental section for direct detection. Column temperature: 25.0°C ± 0.1. All samples were 10 µL of 2.58×10^{-3}M chloramine. Capacity factors are reported as the average ± standard deviation of three injections and are calculated based on a t_0 value of 1.60 ± 0.01 min (average ± standard deviation of three injections).

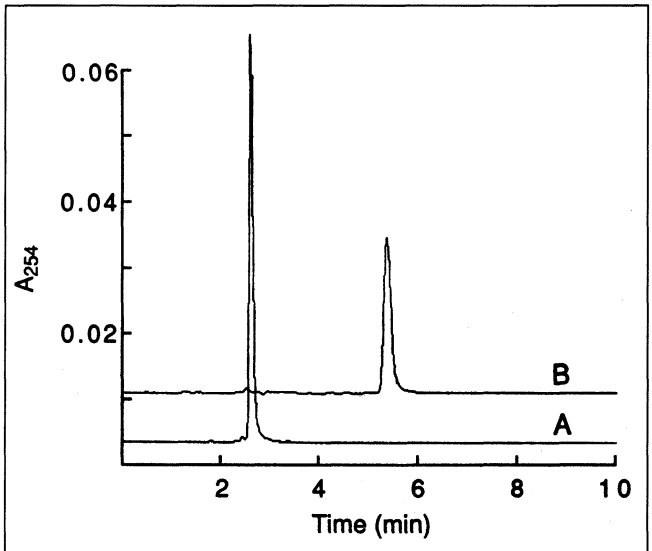
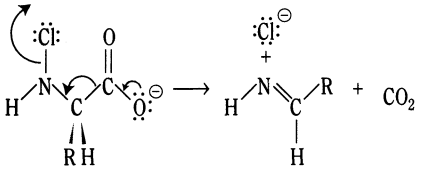


Figure 2. Typical chromatograms of glycine chloramine (10 µL, 2.6×10^{-3}M) in the absence (A) and presence (B) of 40mM tetrabutylammonium ion using direct detection. Column temperature: 25.0°C ± 0.1.

On-column chloramine decomposition

Figure 2 shows chromatograms for glycine chloramine in the presence and absence of tetrabutylammonium ion. Figure 3 shows corresponding chromatograms for alanine chloramine. The behavior of glycine chloramine was entirely expected; the chloramine peak eluted at a longer retention time using the ion-pairing agent but was otherwise unaltered. The alanine chloramine peak, on the other hand, shifted to a longer retention time by the ion-pairing agent, and the peak area was drastically diminished. Based on evidence to be described below, we believe that the striking difference between Figures 3A and 3B is due to rapid decomposition of alanine chloramine as it passed through the reversed-phase column in the presence of the ion-pairing agent. In fact, it is well-documented that amino acid-derived chloramines decompose via the reaction shown in Equation 5 (3,39,40). Note that the imine subsequently hydrolyzes to form ammonia and an aldehyde.



Eq 5

As shown in Table II, the rate of this decomposition reaction varied widely from one amino acid to the next. Although the reported half-life of alanine chloramine (more than 43 min) is far too long to account for the almost complete disappearance of a peak with a retention time of just over 6 min (Figure 3B), it

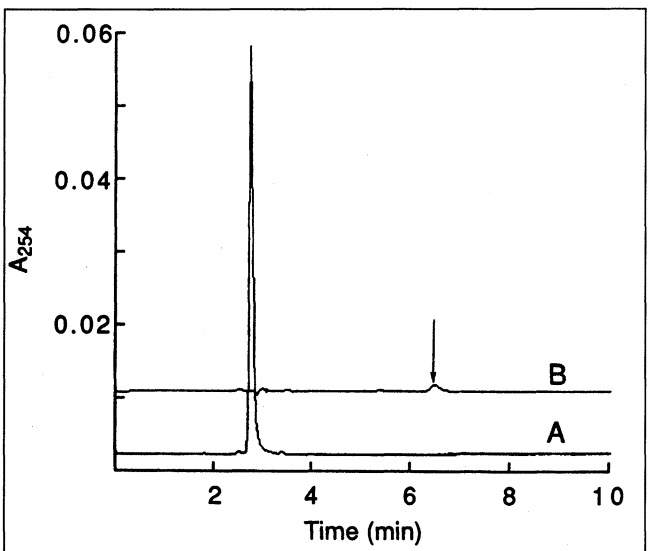


Figure 3. Typical chromatograms of alanine chloramine (10 µL, 2.6×10^{-3}M) in the absence (A) and presence (B) of 40mM tetrabutylammonium ion using direct detection. Column temperature: 25.0°C ± 0.1.

occurred to us that the decomposition might be accelerated by the presence of acetonitrile and/or the tetrabutylammonium ion. This possibility was tested by measuring the decomposition rate in the presence of these two mobile phase components. The results shown in Table IV do indeed reveal that alanine chloramine decomposes more rapidly in the presence of an ion-pairing agent and/or acetonitrile; however, the half-life is still too long to account for the dramatic decrease in peak area seen in Figure 3B.

Another possible explanation for the smaller peak area seen in Figure 3B than in 3A is that the ion-pairing agent merely increased the time that the chloramine was in the column and that decomposition was in fact accelerated by exposure to the stationary phase or the column wall. To test this possibility, alanine chloramine was injected in the absence of an ion-pairing agent, and the retention time was artificially lengthened by stopping the pump for 4 min midway through the elution. The resulting chloramine peak was no smaller, within experimental error, than the peak shown in Figure 3A (data not shown). The dramatic difference between the behavior of alanine chloramine in Figure 3B and when its retention time was artificially lengthened ruled out the possibility that the C_{18} stationary phase or the column wall alone caused the accelerated decomposition.

Table IV. Dependence of Chloramine Decomposition Rates on Reaction Medium

	Concentration		
Chloramine	$[Bu_4N^+]$ (mM)	% CH_3CN	Half-life* (min)
Ala-Cl	0	0	44.5 ± 0.5 (3)
	40	0	43.5 ± 0.5 (3)
	0	5.0	33.3 ± 0.2 (3)
	40	5.0	31.6 ± 1.1 (3)
Ser-Cl	0	0	35.3 (1)
	40	5.0	25.0 (1)

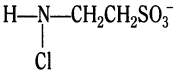
* The number of determinations is shown in parentheses. Errors are one standard devition.

Based on the several lines of evidence described below, we believe that the ion-pairing agent accelerated the decomposition of alanine chloramine by allowing the chloramine to intimately associate with the nonpolar stationary phase. This hypothesis is supported by the reported findings that decomposition rates for alanine chloramine and leucine chloramine are accelerated as the reaction medium becomes progressively less polar (by the addition of a variety of organic solvents) (39–41). We believe that further support is afforded by comparison of peak areas for a series of amino acid- and peptide-derived chloramines (see Table V). Before describing our interpretation of these results, it is necessary to explain how the relative molar concentrations (columns 4 and 6 in Table V) were calculated. First, because all of the chloramines studied have fairly similar molar absorptivities at 254 nm (see Table I), relative peak areas are roughly proportional to relative concentrations. Relative concentrations can be calculated exactly from relative peak areas using Equation 7.

$$\frac{\text{peak area taurine} - \text{Cl}}{\text{peak area chloramine}} = \frac{\varepsilon_{\text{taurine}-\text{Cl}} \cdot [\text{taurine} - \text{Cl}]}{\varepsilon_{\text{chloramine}} \cdot [\text{chloramine}]} \qquad \text{Eq 6}$$

$$\frac{[\text{chloramine}]}{[\text{taurine} - \text{Cl}]} = \frac{\varepsilon_{\text{taurine}-\text{Cl}} \cdot \text{peak area chloramine}}{\varepsilon_{\text{chloramine}} \cdot \text{peak area taurine} - \text{Cl}} \qquad \text{Eq 7}$$

In Table V, taurine chloramine was chosen as the relative standard because it consistently yielded the highest, most reproducible peak areas. It should be added that taurine chloramine (whose structure is shown in Equation 8) cannot undergo the decomposition reaction shown in Equation 5 and, in fact, is reported to be more stable than amino acid-derived chloramine (20).



Eq 8

Table V. HPLC Peak Areas and Relative Concentrations*

		$[Bu_4N^+] = 0$		$[Bu_4N^+] = 40mM$	
Chloramine	ε	HPLC peak area (μV sec)	[Chloramine]/[Taurine-Cl]	HPLC peak area (μV sec)	[Chloramine]/[Taurine-Cl]
Taurine-Cl	408†	399,500 ± 1,600 (3)	1.00	342,000 ± 11,000 (2)	1.00
Ala-Cl	374†	306,000 ± 9,000 (3)	0.84	16,000 ± 8,000 (2)	0.05
Gly-Cl	350††	374,000 ± 27,000 (3)	1.09	249,000 ± 4,000 (3)	0.85
Ser-Cl	371	282,000 ± 4,000 (3)	0.78	83,900 ± 500 (2)	0.27
Thr-Cl	351†	325,000 ± 12,000 (3)	0.95	29,800 ± 8,100 (2)	0.10
Val-Cl	357	290,000 ± 8,000 (3)	0.83	no peaks	–
Cl-AlaGly	374§	315,000 ± 15,000 (3)	0.86	260,000 ± 3,000 (3)	0.83
Cl-GlyGly	357	354,000 ± 2,000 (3)	1.01	258,800 ± 500 (3)	0.87
Cl-SerGly	371§	396,000 ± 13,000 (3)	1.09	224,000 ± 4,000 (3)	0.72
Cl-ValGly	357§	330,000 ± 3,000 (3)	0.95	293,000 ± 5,000 (3)	0.98

* See Table III for experimental conditions.
† Average of all values listed in Table I.
†† Believed to be the more accurate of the two values listed in Table I (this is the more recent of the two values from the same research group).
§ The value for the peptide is not known. The value for the corresponding amino acid-derived chloramine is used in its place.

Because of uncertainties in the molar absorptivities shown in Table I as well as uncertainties in measured peak areas, the concentration ratios reported in Table V are probably not accurate to better than ± 10%.

A number of patterns emerge from the relative concentrations listed in Table V. First, with the exception of glycine chloramine, all of the amino acid-derived chloramines have markedly lower relative peak areas in the presence of the ion-pairing agent. We believe this is due to the fact that glycine chloramine is by far the most stable amino acid-derived chloramine (see Table II). Second, the relative concentrations of the corresponding dipeptides changed very little in the presence of the ion-pairing agent. We believe this was due to the fact that peptide-derived chloramines cannot undergo the decomposition reaction shown in

Equation 5 and are in fact observed to be more stable than their amino acid-derived counterparts (12,13).

The behavior of phenylalanine chloramine lends additional support to the hypothesis that the rate of chloramine decomposition is greatly accelerated by intimate association with the stationary phase. As can be seen in Table III, phenylalanine chloramine is strongly retained on the column, even in the absence of the ion-pairing agent, due to its large, hydrophobic side-chain. Thus, one would predict its peak area to be small, even in the absence of the ion-pairing agent. Note, however, that direct detection at 254 nm cannot be used to quantitate phenylalanine chloramine because both the chloramine moiety and the phenylalanine side-chain absorb at this wavelength. Instead, we used iodide as a postcolumn colorimetric reagent to allow selective detection of chloramine (see below for a complete discussion of the postcolumn method). The results, which are summarized in Table VI, reveal that the phenylalanine chloramine peak area was indeed much smaller than that for either taurine chloramine or Cl-PheGly. Jersey and Johnson's results also appear to support our hypothesis that chloramines derived from hydrophobic amino acids decompose more rapidly during reversed-phase chromatography. Figure 1 in Appendix G of their initial report (2) shows a chromatogram of a number of chloramines derived from various amines and amino acids (no ion-pairing agent). Peak areas for hydrophobic amino acid-derived chloramines were markedly smaller than for chloramines derived from hydrophilic amino acids or simple amines.

The temperature dependence of the chloramine peak area also supported the hypothesis that variations in peak area were due to variations in chloramine decomposition rate. Table VII lists retention times, peaks areas, and relative concentrations of taurine chloramine and alanine chloramine in the presence of the ion-pairing agent as a function of temperature. As one would expect, retention times were only slightly affected by temperature. The peak area for alanine chloramine decreased dramatically as temperature increased, as one would expect if the sample were undergoing a thermally activated decomposition reaction. The taurine chloramine peak area also decreased as temperature increased, although to a much lesser extent. Relative concentrations (the last column in Table VII) were calculated from relative peak areas using Equation 7. Two dipeptides (Cl-Gly-Gly and Cl-Ala-Gly) have been subjected to HPLC analysis in the presence of 40mM tetrabutylammonium ion at 20, 25, and 30°C (data not shown). In neither case did the chloramine peak area appreciably decrease as the temperature increased, in agreement with the observed stability of peptide-derived chloramine compared with their amino acid-derived counterparts.

Although we suggest that the rapid on-column decomposition of amino acid-derived chloramines was due to intimate association between the chloramine and the nonpolar stationary phase, it should be stressed that this explanation is merely conjecture. A reviewer has suggested the following alternative hypothesis, which we have tested. This hypothesis rests on two assumptions: (*a*) that monochloramines undergo rapid disproportionation, as shown in Equation 4, and (*b*) that dichloramines decompose more rapidly than the corresponding monochloramine. In our study, chloramine samples that were injected onto the HPLC column were always prepared by adding sodium hypochlorite to a 10-fold excess of the appropriate amino acid. As a result, chloramine samples contained a ninefold excess of the parent amino acid. This approach was chosen so as to minimize dichloramine formation (i.e., to shift the equilibrium shown in Equation 4 to the left) (20). During chromatographic elution, hydrophilic chloramines, which elute near the void volume (see Table III), presumably do not separate from the excess parent amine. Hydrophobic chloramines, which are retained on the column, presumably are separated from the parent amino acid and would thus be expected to decompose more quickly. We used fraction collection and a manual ninhydrin method to demonstrate that this is true. Specifically, we detected the elution times for some parent amino acids (Phe and Val in the absence of ion-pairing agent in the mobile phase and Ala, Ser, and Thr in the presence of ion-pairing agent) and found that, in each case, the amino acid eluted well before the chloramine (data not shown). Next, we injected samples of five chloramines (Ala-Cl, Phe-Cl, Ser-Cl, Thr-Cl, and Val-Cl) onto the HPLC column and collected the chloramine peak into a micro cuvet and then immediately monitored the decomposition kinetics by UV spectroscopy. We separately measured the decomposition rate of samples containing each of these five chloramines along with a ninefold excess of the parent amino acid dissolved in mobile phase. The results (data not shown) did not reveal a significant increase in the decomposition rate of an HPLC-purified chloramine compared with that chloramine in the presence of excess amino acid. Thus, we do not believe that the striking on-column decomposition of hydrophobic chloramines that we observed

Table VI. Peak Areas of Phe-Cl, Cl-Phe-Gly, and Taurine Chloramine*

Chloramine	Peak area at 350 nm (μV min)	$\frac{[\text{Chloramine}]^{\dagger}}{[\text{Taurine-Cl}]}$
Taurine-Cl	1,043,000 ± 34,000	1.00
Phe-Cl	68,000 ± 20,000	0.06
Cl-PheGly	1,068,000 ± 10,000	1.02

* HPLC conditions are described in the Experimental section for postcolumn detection with iodide. Temperature: 23°C ± 2. Triplicate injections of 10 μL of 2.58×10^{-4}M chloramine samples.
† Ratio of peak areas.

Table VII. Temperature Dependence of Chloramine Chromatographic Behavior*

Chloramine	Temperature (°C)	Retention time (min)	Peak area (μV min)	$\frac{[\text{Chloramine}]}{[\text{Taurine-Cl}]_{20°C}}$
Taurine-Cl	20	11.14 ± 0.01	61,000 ± 600	1.00
	25	10.93 ± 0.01	60,700 ± 600	0.99
	30	10.82 ± 0.01	55,000 ± 1,400	0.91
Alanine-Cl	20	6.15 ± 0.01	14,000 ± 1,400	0.24
	25	6.25 ± 0.01	4,400 ± 90	0.08
	30	no peak	0	0

* HPLC conditions are described in the Experimental section for direct detection. Temperatures maintained ± 0.01°C. Triplicate injections of 20 μL of 2.58×10^{-3}M chloramine samples.

was due to a shift in the chloramine–dichloramine equilibrium. Rather, we continue to suspect that decomposition was somehow accelerated by the hydrophobic environment of the stationary phase (although this remains conjecture).

Transfer of electrophilic chlorine from chloramines to amines

It should be noted that a mixed-mode HPLC column (Alltech Mixed Mode RP 18/anion column) whose stationary phase contained both C_{18} and weak anion exchange groups proved to be completely unsatisfactory for the analysis of chloramines. Using this column, no chloramine peaks could be detected under a wide variety of chromatographic conditions (although a standard mixture of benzyl alcohol and benzoic acid behaved normally). We believe that chloramines did not elute from this column due to chlorine transfer reaction from the chloramine to the amino groups that comprise the weak anion exchange sites on the stationary phase. This type of chlorine exchange reaction, which is shown in Equation 9, is well-documented for a variety of chloramines (20).

$$Cl{-}\underset{H}{N}{-}R_1 + H{-}\underset{H}{N}{-}R_2 \rightleftharpoons H{-}\underset{H}{N}{-}R_1 + Cl{-}\underset{H}{N}{-}R_2 \qquad \text{Eq 9}$$

Using the HPLC method described here, we observed chlorine transfer equilibration between glycine chloramine and taurine. In the first experiment, a solution containing 2.58×10^{-3}M glycine chloramine and 2.67×10^{-2}M glycine was mixed with a solution containing 2.67×10^{-2}M taurine. Aliquots were removed at various time intervals and injected into the HPLC. As shown in Table VIII, the chlorine transfer reaction reached equilibrium within 15 min. Table IX shows the results of the inverse experiment (a solution containing 2.58×10^{-3}M taurine chloramine and 2.67×10^{-2}M taurine was mixed with a solution containing 2.67×10^{-2}M glycine). A comparison of Tables VIII and IX reveals that both reactions reached similar equilibrium mixtures. This potential for rapid chlorine transfer is another complicating factor one must consider when designing an analysis method for chloramines.

Table VIII. Chlorine Transfer Between Glycine Chloramine and Taurine*

Elapsed time (min)	Mole fraction Gly-Cl†	Mole fraction taurine-Cl†
1	0.40	0.60
15	0.06	0.94
60	0.06	0.94
300	0.06	0.94

* HPLC conditions are described in the Experimental section for direct detection. The mobile phase contained 40mM tetrabutylammonium ion and 25% acetonitrile. All injections were 15 µL.
† Mole fractions calculated from Gly-Cl and taurine-Cl peak areas using Equation 7.

Table IX. Chlorine Transfer Between Taurine Chloramine and Glycine*

Elapsed time (min)	Mole fraction Gly-Cl†	Mole fraction taurine-Cl†
1	0.02	0.98
15	0.07	0.93
240	0.11	0.89

* See Table VII.
† Mole fractions calculated from Gly-Cl and taurine-Cl peak areas using Equation 7.

Problems caused by mobile phase impurities

During the early phase of this work, the water used to prepare the mobile phase was of a lower purity than that obtained from the Barnstead Easy Pure system described in the Experimental section. This resulted in a poor chromatographic baseline and poor sample-to-sample reproducibility (data not shown). On several occasions, we encountered situations in which peak areas were smaller and more variable than normal, although retention times were normal. We believe that a major factor contributing to this variability was the accumulation of oxidizible mobile phase contaminants on the column. In this scenario, each time a chloramine sample is injected, some fraction of it is consumed by reaction with the oxidizible contaminant(s). The chloramine peak area will be diminished accordingly, and the degree to which it is diminished will depend on both the concentration and nature of the contaminants. This concentration will steadily increase through the course of a day as the contaminant is continuously pumped onto the column but will also decrease each time a chloramine sample is injected. We developed two strategies to deal with this problem. First and foremost, we switched to high-purity water and other reagents for preparation of the mobile phase. Another less satisfactory measure that resulted in only partial, temporary relief was the injection of a large amount of sodium hypochlorite onto the column (typically 20 µL of approximately 0.1M NaOCl). We attribute this relief to the ability of the sodium hypochlorite to react with most or all of the accumulated oxidizible contaminants. The possibility of on-column bimolecular reaction (as opposed to on-column unimolecular degradation) is yet another complicating factor one must bear in mind when using HPLC to analyze chloramines.

Chromatographic ruggedness of the method

To determine the ruggedness of this method, the effect of several chromatographic variables on retention times and peak areas was investigated. As shown in Table VII, peak areas can be very sensitive to changes in temperature under conditions that favor chloramine decomposition. Otherwise, retention times and peak areas are not appreciably changed by small changes in temperature (± 2°C). When the pH of the mobile phase was adjusted from 7.4 to 7.1 or 7.7 (in the presence of 40mM tetrabutylammonium ion), the retention times for taurine chloramine, glycine chloramine, and Cl-ValGly changed by less than 10%. Peak areas were not appreciably influenced by these pH changes. Likewise, retention times did not change when one Rexchrom C_{18} column was replaced with another.

Postcolumn colorimetric reaction

In addition to the direct UV detection of chloramines, we employed postcolumn colorimetric detection to increase sensitivity and selectivity. As stated in the Introduction, we first

employed 5-thio-2-nitrobenzoic acid (TNB) as the postcolumn reagent (see Equation 2). As can be seen from Equation 2, the detector wavelength could be set to monitor either the appearance of product at 324 nm or the disappearance of reagent at 412 nm. While this work was in progress, Jersey and Johnson reported their postcolumn method using iodide as the postcolumn reagent (see Equation 3). Iodide offers an advantage over TNB insofar as it is commercially available, whereas TNB must be prepared by borohydride reduction from the commercially available disulfide.

Figure 4 shows the chromatograms that resulted from direct and postcolumn detection of phenylalanine chloramine. This figure captures both the advantages of postcolumn detection and the limitations of our apparatus. The first advantage was that detection was selective; other species that absorb at 254 nm (in this case, the large peak due to unreacted phenylalanine and several impurity peaks) did not interfere at 350 nm. The second advantage was sensitivity; the 350 nm peak for phenylalanine chloramine is much larger than the 254-nm peak. (That the peak identified with the arrow in the 254-nm chromatogram is, in fact, phenylalanine chloramine is established by the 0.55-min time lag between that peak and the major peak in the 350-nm chromatogram. This time lag was established using other, unambiguous chromatograms.) The large ripple in the 350-nm baseline was due to pulsations from the single-piston pump that delivered the postcolumn reagent. This instrument artifact was somewhat attenuated by the 9-m length of Teflon tubing that served as a pulse dampener but nonetheless limited the sensitivity of our postcolumn detection to approximately 2 nmol. The amplitude of this ripple is independent of sample size and thus is negligible for larger samples. Using UV detection of I_3^- and a more suitable postcolumn pump, Yoon and Jensen achieved a detection limit of 6 pmol for glycine chloramine (7). Finally, it should be noted that we did not optimize the length of the Teflon tubing that served as the postcolumn reactor (i.e., we did not find the minimum length that would yield complete reaction and yet contribute minimal band broadening).

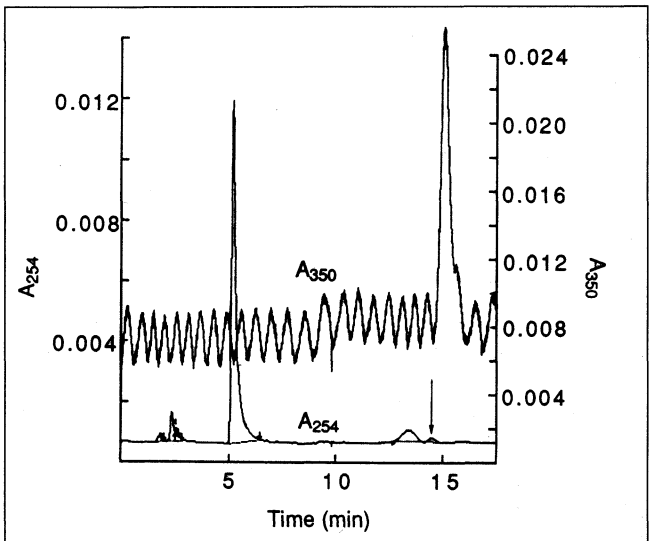


Figure 4. Chromatograms of phenylalanine chloramine (10 µL, 2.6×10^{-3}M) using direct detection at 254 nm and postcolumn detection with iodide at 350 nm. Conditions are as described in the Experimental section for postcolumn detection. Column temperature: 23°C ± 2.

In addition to phenylalanine chloramine and Cl-PheGly (see Table VI), we have used postcolumn detection with the following chloramines (data not shown): Gly-Cl, Cl-GlyGly, Cl-AlaGly, Val-Cl, Cl-ValGly, and taurine-Cl.

Conclusion

In agreement with the reports of Jersey and Johnson (1,2) and Yoon and Jensen (7), our results show that reversed-phase HPLC can be used to directly analyze amino acid-derived chloramines. However, we also discovered a unique and, to us, unexpected problem: the more strongly a chloramine associates with the nonpolar stationary phase, the more quickly it decomposes. Thus, chromatographic conditions that yield satisfactory resolution can be expected to yield poor sensitivity. Because the decomposition appears to be driven by the same factor that determines reversed-phase chromatographic resolution, we cannot suggest a remedy to this problem. Taken together with our results that suggest that weak anion exchange chromatography is completely unsuited for the analysis of chloramines (because of the exchange of electrophilic chlorine from the analyte to the amino groups on the stationary phase), it appears that HPLC is a less than ideal tool for analyzing amino acid-derived chloramines. Fortunately, peptide-derived chloramines are not destabilized by intimate contact with the nonpolar stationary phase. Thus, HPLC is a robust method for analyzing these compounds.

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