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Short communication

Indirect fluorescence detection of amino sugars with the use of copper complexes of tryptophan and its analogues following high-performance liquid chromatographic separation

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Abstract

A simple, indirect fluorescence detection method has been developed for detecting specific mono-amino sugars (D-glucosamine, D-galactosamine, D-mannosamine) following chromatographic separation. The eluting amino sugars release L-tryptophan (L-Trp) from a copper–tryptophan complex which is introduced postcolumn. Analyte detection is based on measuring the increase in L-Trp fluorescence, which is quenched when complexed with copper. Two tryptophan analogues, 5-hydroxy-L-tryptophan (5-HTP) and DL-5-methoxytryptophan (5-MTP), were also evaluated as postcolumn reagents. 5-MTP was found to be a suitable alternative to L-Trp for the detection of these mono-amino sugars. Detection limits for D-glucosamine, D-galactosamine, and D-mannosamine are in the range of 0.15–0.30 nmol injected.

Keywords: Indirect fluorescence detection; Amino sugars; Copper–tryptophan complex; Fluorescence quenching; Tryptophan analogue compounds; D-Glucosamine; D-Galactosamine; D-Mannosamine

1. Introduction

Amino sugars are biologically important natural products. They are found in various animal species, plants, seeds, fungi, and bacteria [1]. D-Glucosamine (GlcN) and D-galactosamine (GalN) are the most prevalent amino sugars in nature and are considered to play important roles in various biological phenomena [2]. D-Mannosamine (ManN) is a component of the widely distributed sialic acids [3].

Separation and analysis of amino sugars, especially these three amino sugars, have been of interest for many years [2]. These amino sugars have been analyzed by GC with nitrogen-specific flame thermionic detection (FTD) or flame ionization detection (FID) [4–7]. Several methods have been employed for the detection of amino sugars after their separation by HPLC. These methods include monitoring the refractive index (RI) of the eluent [8,9], direct UV photometric detection at 190 nm [10], measuring the UV absorption of amino sugar–copper complexes at 254 nm after eluting the amino sugars from copper-loaded ion exchange resins with aqueous ammonia [11], indirect detection accomplished by adding phenol to the mobile phase [12], photometric or fluorometric detection after pre-column or post-column derivatization [13–20], pulsed amperometric detection (PAD) [8,21–23], and electrospray ionization-ion mobility spectrometry following microbore HPLC separation [24]. In addition to chromatographic methods, the differentiation and quantification of amino sugars have been reported using MS–MS with derivatization [25] or without derivatization [26] of the analytes.

Recently, a simple, indirect HPLC detection method, based on the recovery of L-tryptophan (L-Trp) fluorescence, has been applied to the detection of amino-containing compounds such as the aliphatic biogenic polyamines, amino

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acids, and aminoglycoside antibiotics [27–29]. For this method, a solution containing a copper tryptophan complex is added to the eluent postcolumn. Fluorescence of L-Trp is quenched when bound in the Cu(L-Trp)$_2$ complex. In the presence of the eluting analytes, which are capable of complexing with Cu^{2+}, some fraction of the L-Trp is released from the Cu(L-Trp)$_2$ complex. The fluorescence of L-Trp is thus recovered, as shown by:

$$\text{Cu(l-Trp)}_2 + n\text{A} \leftrightarrow \text{Cu(A)}_n + 2\text{l-Trp}^* \quad (1)$$

where A is the analyte molecule, L-Trp* is the fluorescent form of L-Trp and n is the number of the analyte molecules coordinated to Cu^{2+}.

Here, we describe the application of this indirect fluorescence detection scheme for three amino sugars, GlcN, GluN, and ManN, following their separation by HPLC. Studies were also undertaken to evaluate two tryptophan analogues, 5-hydroxy-L-tryptophan (5-HTP) and DL-5-methoxytryptophan (5-MTP), as alternative fluorescent reagents.

2. Experimental

2.1. Reagents

D-Glucosamine hydrochloride (GlcN) (minimum purity, 99%), D-mannosamine hydrochloride (ManN) (minimum purity 99.4%), L-tryptophan (L-Trp) (minimum purity, 98%), and 5-hydroxy-L-tryptophan (5-HTP) (minimum purity, 99%) were purchased from Sigma (St. Louis, MO, USA). D-Galactosamine hydrochloride (GalN) (minimum purity, 98%) and DL-5-methoxytryptophan (5-MTP) (minimum purity, 95%) were purchased from Aldrich (Milwaukee, WI, USA). Reagent-grade copper sulfate was purchased from Fisher Scientific (Pittsburgh, PA, USA). Reagent-grade sodium borate ($Na_2B_4O_7{\cdot}10H_2O$) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Certified ethylenediaminetetraacetic acid (EDTA) (99.8%) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The copper complexes of tryptophan and its analogues were prepared by adding copper sulfate and the corresponding ligand at a 1:2 (mole/mole) ratio. The deionized water used in the preparation of the standard solutions and mobile phases was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile phases and reagent solutions were vacuum filtered through a 0.45 μm nylon filter (Whatman, Hillboro, OR, USA) prior to use. The pHs of the mobile phases and other solutions were adjusted using dilute solutions prepared from certified sodium hydroxide solution (50%, w/w) and reagent-grade hydrochloric acid (36.5% by weight) purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Apparatus

A Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA) was used for the static fluorescence studies. The chromatographic separations were performed on a Nicolet LC 9560 HPLC system (Madison, WI, USA) using a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 10 μl injection loop. Separation of the amino sugars was achieved on a Hamilton PRP-X100 column (250 mm × 4.1 mm, 10 μm, Reno, NV, USA) containing a polymer-based strong anion-exchanger. A Hitachi 655A-11 LC pump (Tokyo, Japan) delivered the postcolumn reagent via a mixing tee. A Varian flow control dampener (Palo Alto, CA, USA) was placed between the postcolumn reagent pump and the mixing tee to improve flow stability. Chromatographic detection was provided by a Kratos Spectroflow 980 fluorescence detector (Ramsey, NJ, USA) fitted with a 10 μl flow cell. The excitation wavelength of the detector was set at 280 nm. The emission wavelengths detected were selected using longpass filters (320 nm or 350 nm).

3. Results and discussions

3.1. Emission spectra of tryptophan and its analogues

Emission spectra were obtained for L-Trp, 5-HTP, and 5-MTP solutions having pHs of 9.0, 8.6 and 8.4, respectively, with the excitation wavelength set at 280 nm. The wavelengths of maximum emission for L-Trp, 5-HTP, and 5-MTP occur at 360 nm, 338 nm, and 337 nm, respectively. The wavelength of maximum emission varies for these compounds by approximately ±3 nm for solutions ranging in pH from 2.0 to 12.0, which is small compared to the overall breadth of their emission bands. Based on these results, static fluorescence measurements were made using an excitation wavelength set at 280 nm with the emission wavelength set at 360 nm for L-Trp and 340 nm for 5-HTP and 5-MTP.

3.2. Fluorescence quenching of tryptophan and its analogues by copper ion as a function of solution pH

This approach to indirect detection is based on the recovery of the fluorescence of tryptophan or one of its analogues. The recovered fluorescence intensity is the difference between the fluorescence intensity of the species while complexed to copper ion and the fluorescence intensity of the species which is unbound in solution. Experimentally, the pH of the solution is chosen such that the difference in the fluorescence intensity between the bound and unbound species will be as large as possible while minimizing any residual background fluorescence of the solution containing the copper complex.

The fluorescence intensities at 360 nm of a solution containing L-Trp and a solution containing Cu(L-Trp)$_2$ as a function of pH, ranging from 2.0 to 12.0, are presented in Fig. 1a. It

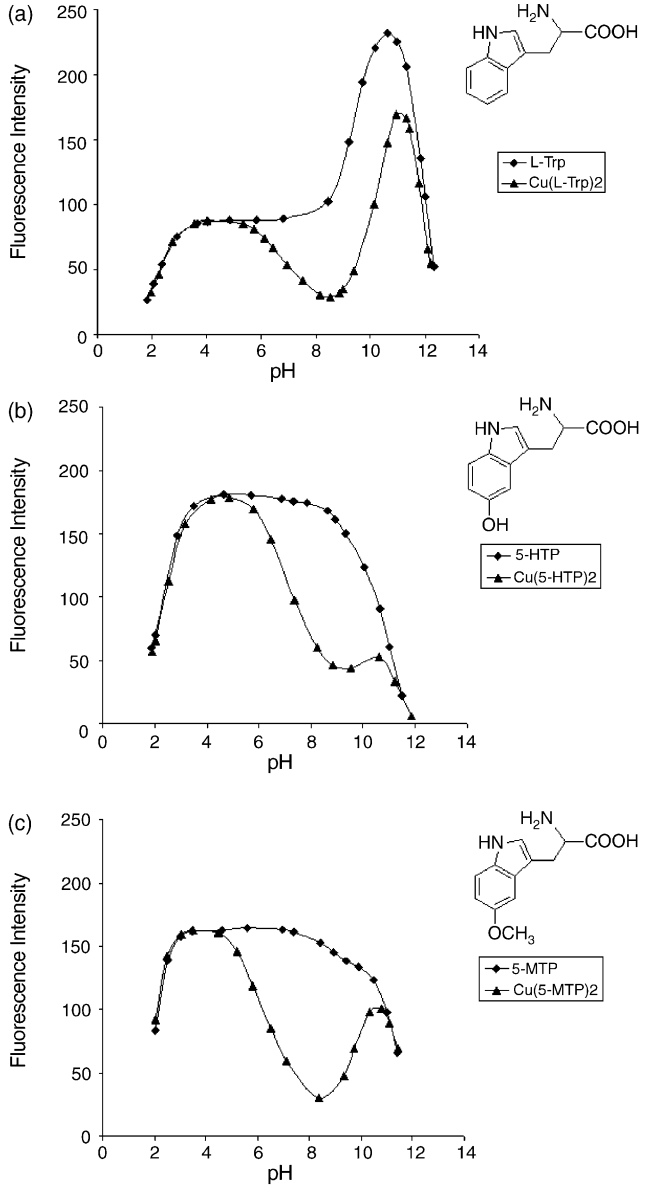

Fig. 1. Fluorescence quenching of (a) L-tryptophan (4 μM), (b) 5-hydroxy-L-tryptophan (4 μM), and (c) DL-5-methoxytryptophan (4 μM) by copper ion (8 μM) as a function of solution pH.

was determined that the minimum fluorescence background occurs at a pH of approximately 8.5, while the difference between the fluorescence intensities of the L-Trp and Cu(L-Trp)$_2$ increases from pH 8.5 to 11.0. Although the fluorescence signal increases over this pH range, using a higher pH solution may not result in improved analyte detection. The fluorescence background may be so large that it is out of the range of the detector and an increase in the level of the background may also affect the noise level. Taking these factors into account, the working pH for the detection of amino sugars with the use of Cu(L-Trp)$_2$ as the postcolumn reagent was chosen to be approximately 9.0.

Plots of the fluorescence intensity at 340 nm of solutions containing 5-HTP and Cu(5-HTP)$_2$ as a function of pH ranging from 2.0 to 12.0 are presented in Fig. 1b. The plots show the fluorescence of 5-HTP is quenched efficiently by copper ion at a pH of approximately 9.0. At this pH, a minimum in the fluorescence intensity of the solution containing the Cu(5-HTP)$_2$ complex is observed. The optimal pH for analyte detection is approximately 8.5 which is where the difference between the fluorescence intensity of 5-HTP and that of Cu(5-HTP)$_2$ is greatest. Fig. 1c shows the fluorescence intensities at 340 nm of 5-MTP and Cu(5-MTP)$_2$ as a function of solution pH. The greatest difference between the fluorescence intensity of 5-MTP and Cu(5-MTP)$_2$ is obtained at a pH of approximately 8.4. These studies indicate that a solution containing Cu(5-HTP)$_2$ at a pH of 8.5 or Cu(5-MTP)$_2$ at a pH of 8.4 might also be suitable for this approach to indirect detection of the amino sugars.

3.3. Stability of potential postcolumn reagents in a basic sodium borate buffer solution

The autoxidation of 5-HTP in strongly basic solution has been reported [30]. The presence of Cu^{2+} ion in the Cu(5-HTP)$_2$ postcolumn reagent solution may act either as an oxidizing agent or a catalytic agent for the oxidation of 5-HTP. It is reasonable to be concerned with the stability of this compound under the solution conditions utilized here.

The stability of Cu(5-HTP)$_2$ in sodium borate buffer solution at pH 8.6 was evaluated by taking aliquots of a Cu(5-HTP)$_2$ stock solution over time. EDTA was added to each aliquot to release 5-HTP from the Cu(5-HTP)$_2$ complex. The concentration of the free 5-HTP in solution was monitored by measuring the fluorescence intensity of the solution. A sample of 5-HTP, initially mixed with Cu^{2+} and EDTA, was also prepared for comparison.

The fluorescence intensity due to 5-HTP decreased over 72 h for the aliquots taken from the Cu(5-HTP)$_2$ stock solution and treated with EDTA. Experiments showed that the decreased fluorescence intensity was not due to a shift in the maximum emission wavelength. Since the fluorescence intensity for 5-HTP in a comparative sample, in which the Cu^{2+} was chelated by EDTA, proved to be stable over the same time frame, it is reasonable to suggest that free Cu^{2+} induced the decomposition of 5-HTP in the basic sodium borate solution. Due to the observed instability of Cu(5-HTP)$_2$ under these solution conditions, it was eliminated from consideration as a new postcolumn reagent.

The presence of a methoxy group at the C-5 position of 5-MTP, instead of a hydroxyl group as in 5-HTP, is expected to improve the stability of this compound in basic solution. A study similar to the one described previously was performed to evaluate the stability of Cu(5-MTP)$_2$ in a pH of 8.4 sodium borate solution. The fluorescence intensity due to the 5-MTP released from the Cu(5-MTP)$_2$ complex by adding EDTA at different times was measured. No significant trend was observed in the fluorescence intensity over

80 hours, indicating that Cu(5-MTP)$_2$ is stable at room temperature under these solution conditions. Based on the results of these studies, Cu(5-MTP)$_2$ was evaluated as a potential postcolumn reagent for the detection of the amino sugars.

3.4. Chromatographic detection of the amino sugars using two different postcolumn reagents

3.4.1. Chromatographic detection of the amino sugars via postcolumn addition of Cu(L-Trp)$_2$ in sodium borate solution, pH 9.0

The three amino sugars, GlcN, GalN and ManN, were separated isocratically using a mobile phase containing 1.6 mM sodium borate at a pH of 9.0 on a PRP-X100 strong anion exchange column. An aqueous solution containing 2×10^{-5} M Cu(L-Trp)$_2$ at a pH of 9.0 was added postcolumn to evaluate the level of detection provided. As in previous studies [27–29], a 350 nm longpass filter was utilized for selecting the L-Trp fluorescence signal. The linear dynamic range for GlcN, GalN and ManN was determined by injecting six standard solutions in triplicate. The injected amounts ranged from 0.5 nmol to 10 nmol of each of the three sugars. The results are presented in Table 1. The detection limits for these three amino sugars were determined to be at the nmol injected level. Lower detection limits in the pmol range for diamines, polyamines and amino acids have been achieved using this indirect fluorescence detection method [27,29]. Since indirect detection of these analytes is based on measuring the fluorescence of L-Trp released from the Cu(L-Trp)$_2$ complex by the analytes, the affinity of the analytes for Cu^{2+} is one of the important factors affecting the amount of L-Trp released. Previous studies [31] have shown that GlcN, GalN and ManN act as bidentate ligands with the amino group being the main donor towards Cu^{2+} and one of the hydroxyl groups being the second donor center. The overall stability constants ($\log \beta_2$) of these amino sugars with Cu^{2+} range from 8.76 to 9.68 [31], which are several orders of magnitude lower than the overall stability constants for copper complexes of polyamines ($\log \beta_2$ = 16.0–18.9) [32] and amino acids ($\log \beta_2$ = 13.7–15.2) [33]. Additionally, polyol compounds, including carbohydrates, have been known to complex with borate ion, via the hydroxyl groups, to form negatively charged borate complexes allowing the separation of these compounds with anion exchange chromatography [34]. The possible complexation of the hydroxyl groups of the amino sugars with borate ion may decrease the affinity of one of the hydroxyl groups for Cu^{2+} and block the complexation of the amino groups with Cu^{2+}. This would result in a smaller stability constant for the binding of these amino sugars to Cu^{2+} under the conditions studied and a higher detection limit. The detection limits based on this indirect detection method are within the range of detection limits which have been reported for these compounds using some precolumn or postcolumn derivatization methods [14–16].

3.4.2. Chromatographic detection using Cu(5-MTP)$_2$ in sodium borate solution at pH 8.4

As indicated previously, the maximum emission wavelengths of L-Trp and 5-MTP are 360 nm and 340 nm, respectively. While earlier studies [27–29] have employed a 350 nm longpass filter for selecting the fluorescence signal of L-Trp, using the same filter for measuring the fluorescence signal for 5-MTP would result in filtering out the maximum emission wavelength for this compound. For this reason, a 320 nm longpass filter was chosen to select the emission intensity for 5-MTP. As expected, since a lower wavelength cutoff filter allows more light to reach the detector, the chromatographic signal measured with the 320 nm longpass filter was greater than observed when using the 350 nm filter. This is true with either L-Trp or 5-MTP as the fluorescent reagent.

A chromatogram showing the separation of the three amino sugars obtained using Cu(5-MTP)$_2$ in 40 mM sodium borate at pH 8.4 as the postcolumn reagent is presented in Fig. 2. The intensity of the chromatographic signal was found to be nearly equivalent to that obtained when using Cu(L-Trp)$_2$ in a pH of 9.0 sodium borate solution with the 320 nm longpass filter. As indicated by Eq. (1), a higher fluorescence efficiency of the fluorescent agent may result in a larger chromatographic signal for the analytes. Based on the results obtained for the quenching of 5-MTP by Cu^{2+} ion (Fig. 1c), which showed the difference in the fluorescence intensities at

Table 1
Results for the detection of three amino sugars with Cu(L-Trp)$_2$ as the postcolumn reagent[a]

Amino sugar	Linear range (nmol)	Regression equation[b]	r[c]	DL[d] (nmol)	RSD[e] (%)
GlcN	0.5–10	$H = 2.6817C + 0.2583$	0.9993	0.15	1.4
GalN	0.5–10	$H = 2.7439C + 0.2685$	0.9992	0.15	2.1
ManN	0.5–10	$H = 1.3704C - 0.2074$	0.9999	0.30	3.1

[a] Mobile phase: 1.6 mM sodium borate, pH 9.0, 1.0 ml/min. Postcolumn reagent: 2×10^{-5} M Cu(L-Trp)$_2$ in 40 mM sodium borate at pH 9.0, flow rate: 1.0 ml/min. Column: Hamilton PRP-X100, anion exchange column. Detector sensitivity: 0.005μAFS. Excitation wavelength = 280 nm. Longpass filter λ_{cutoff} = 350 nm.
[b] H, peak height (mV), C, analyte injected (nmol).
[c] Correlation coefficient.
[d] Detection limit (S/N = 3).
[e] Relative standard deviation (n = 3, for 2.5 nmol GlcN, 2.5 nmol GalN, and 2.5 nmol ManN injected, respectively).

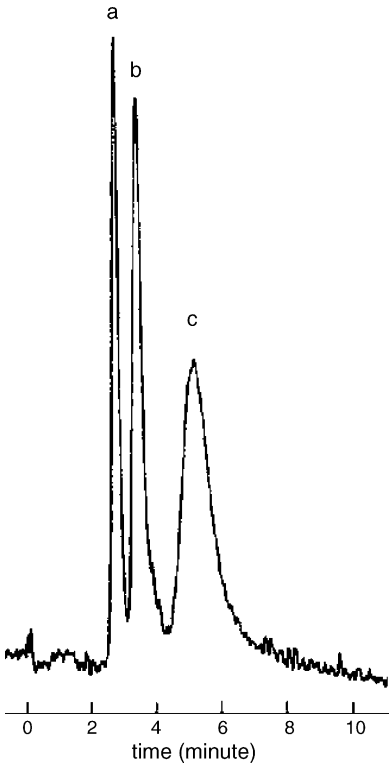

Fig. 2. Chromatogram of the amino sugars with $Cu(5\text{-MTP})_2$ as the postcolumn reagent. Mobile phase: 1.6 mM sodium borate, pH = 9.0, 1.0 ml/min. Postcolumn reagent: 2×10^{-5} M $Cu(5\text{-MTP})_2$ in 40 mM sodium borate at pH = 8.4, flow rate: 1.0 ml/min. Column: Hamilton PRP-X100, anion exchange column. Detector sensitivity: 0.005μAFS. Excitation wavelength = 280 nm. Longpass filter λ_{cutoff} = 320 nm. Peak (a) D-glucosamine (2.5 nmol); (b) D-galactosamine (2.5 nmol); (c) D-mannosamine (2.5 nmol).

pH 8.4 between 5-MTP and $Cu(5\text{-MTP})_2$ was larger than the difference in the fluorescence intensities at pH 9.0 between L-Trp and $Cu(L\text{-Trp})_2$ (Fig. 1a), it is reasonable to expect that using $Cu(5\text{-MTP})_2$ as the postcolumn reagent should result in a more intense signal. The reason why the chromatographic detection results are not consistent with what was predicted based on the static fluorescence studies could be due in part to the difference in the solution conditions or the way the fluorescence intensity was measured. In the static fluorescence studies, the fluorescence intensity of different tryptophan analogues was measured at their maximum emission wavelengths, while the HPLC detector employed in this study responded to the total intensity of light passing through the longpass filter.

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