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Non-derivatization approach to high-performance liquid chromatography–fluorescence detection for aminoglycoside antibiotics based on a ligand displacement reaction

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Abstract

An indirect fluorescence detection method has been developed for detecting the aminoglycoside antibiotics following chromatographic separation. This approach to detection is based on a displacement reaction between the aminoglycosides and a copper(II)-L-tryptophan (L-Trp) complex, Cu(L-Trp)$_2$. The aminoglycosides, which contain multiple amino groups, have strong affinities for the Cu(II) ion and displace L-Trp from the Cu(L-Trp)$_2$ complex. The resulting increase in L-Trp fluorescence, which is quenched when coordinated to Cu(II), is indicative of the presence of the aminoglycoside. Fluorescence titration data indicate that there is a stoichiometric ratio of 1:1 between the reaction of the aminoglycosides with Cu(L-Trp)$_2$. This HPLC detection scheme is implemented postcolumn by mixing a buffered Cu(L-Trp)$_2$ solution with the column eluent prior to detection. The aminoglycosides were separated with the use of a column packed with a polymeric strong cation-exchanger. Separation and detection variables were optimized and are discussed. The detection limits for the aminoglycosides tested ranged from 4.2 to 14.5 ng injected ($S/N=3$). A linear working curve was achieved for amikacin in the range of 29–586 ng for a six point linearity test. The developed separation and detection scheme was further tested by analyzing commercial pharmaceutical formulations of these antibiotics.

Keywords: Metal complexes; Pharmaceutical analysis; Antibiotics; Aminoglycosides; Kanamycins; Neomycin; Amihacin

1. Introduction

Since the discovery and clinical use of streptomycin in 1944, numerous aminoglycosides have been developed and introduced as pharmaceuticals [1]. These aminoglycosides have a very broad antimicrobial spectrum extending from gram-positive cocci to gram-negative bacilli. The aminoglycosides bind to the surface of the bacteria and are transported through the cell wall. Once within the cell, they inhibit protein synthesis by microorganisms, resulting in a rapid, concentration-dependent bactericidal action [2,3].

Monitoring aminoglycoside concentration is important in drug pharmacokinetic studies, as well as studies concerning the clinical efficacy and side effects of these drugs [4]. Traditionally, HPLC analysis of aminoglycosides involves either precolumn or postcolumn derivatization, since they do not possess functional groups which strongly absorb or fluoresce [5–7], as demonstrated by the representative molecular structures of the four amino-

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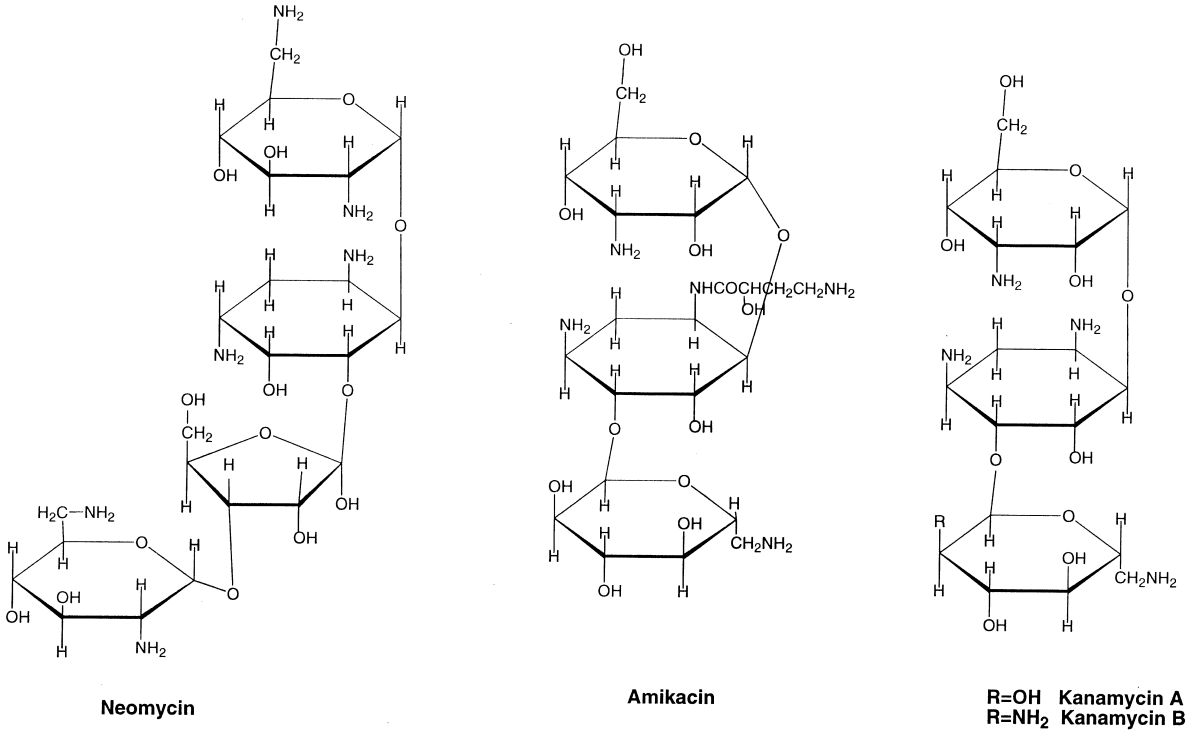

Fig. 1. Molecular structures of four aminoglycosides.

glycosides given in Fig. 1. Common labeling agents used for derivatization of the aminoglycosides for HPLC are 1-fluoro-2,4-dinitrobenzene (FDNB) [8,9], nitrophenylation reagents [10] and *o*-phthaladehyde (OPA) derivatives [11]. Limitations of detection approaches involving derivatization include time-consuming and labor-intensive sample preparation and the instability of the derivatives. Various experimental factors such as the labeling agent concentration, pH, temperature and solvent composition need to be optimized for the derivatization. Derivatives may degrade within a few hours after the derivatization reaction [4], which increases the complexity and lowers the reproducibility of the analysis. In the case of the aminoglycosides, because there are several amino groups in these compounds, multiple derivatives may be produced [6]. Numerous efforts have been spent or are being investigated to seek nonderivatization methods for the analysis of aminoglycoside antibiotics, among which pulsed electrochemical detection [12–14] and mass spectrometric detection [15,16] have been studied extensively. Recently, detection of various aminoglycosides following chromatographic separation based on light scattering has also been reported [17].

A simple and sensitive, indirect HPLC detection method for amino-containing compounds such as aliphatic biogenic polyamines and amino acids was developed based on monitoring the fluorescence of L-tryptophan (L-Trp) [18,19]. Fluorescence of L-Trp is originally quenched in the form of the Cu(L-Trp)$_2$ complex. The analyte molecules react with the Cu(L-Trp)$_2$ complex to displace L-Trp with the resulting increase in fluorescence being indicative of the presence of the analyte. This nonderivatization method offers simplicity and reasonably good sensitivity for the analytes investigated. In this study the detection scheme is applied to the detection of a variety of aminoglycosides following chromatographic separation, with the aim to develop an effective and simple method for detecting aminoglycoside antibiotics.

Commercial pharmaceutical formulations were also analyzed using the developed method.

2. Experimental

2.1. Reagents

The aminoglycosides amikacin sulfate ($C_{22}H_{43}N_5O_{13}\cdot 2H_2SO_4$), kanamycin A sulfate ($C_{18}H_{36}N_4O_{11}\cdot H_2SO_4$), kanamycin B sulfate ($C_{18}H_{37}N_5O_{10}\cdot 2H_2SO_4$), neomycin sulfate ($C_{23}H_{46}N_6O_{13}\cdot 2H_2SO_4$), tobramycin sulfate ($C_{18}H_{37}N_5O_9\cdot 2H_2SO_4$) and geneticin sulfate ($C_{20}H_{40}N_4O_{10}\cdot 2H_2SO_4$) were purchased from Sigma (St. Louis, MO, USA). The aminoglycosides spectinomycin sulfate ($C_{14}H_{24}N_2O_7\cdot H_2SO_4$), ribostamycin sulfate ($C_{17}H_{34}N_4O_{10}\cdot 2H_2SO_4$), hygromycin B ($C_{20}H_{37}N_3O_{13}$) and neamine ($C_{12}H_{26}N_4O_6$) were purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). L-Trp was purchased from Aldrich (Milwaukee, WI, USA). Reagent-grade copper sulfate was purchased from Fisher (Pittsburgh, PA, USA). Reagent-grade sodium acetate and sodium borate ($Na_2B_4O_7\cdot 10H_2O$) were purchased from Baker (Phillipsburg, NJ, USA). The deionized water used in the preparation of standard solutions and eluents was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile phases and postcolumn reagents were filtered through a 0.45 μm nylon filter (Whatman, Hillsboro, OR, USA) prior to use. Dilute aqueous solutions of reagent-grade sodium hydroxide (Fisher, Pittsburgh, PA, USA) and hydrochloric acid (Baker) were used to adjust the pH of the mobile phase and postcolumn reagent. Commercial aminoglycoside formulations were obtained locally.

2.2. Apparatus

A SIM AMINCO luminescence spectrometer (SIM Instruments, Urbana, IL, USA) was used for the static fluorescence studies. The excitation and emission wavelengths were set at 280 nm and 350 nm, respectively. The chromatographic separations were performed using a Nicolet LC 9560 HPLC system (Madison, WI, USA) fitted with a Reodyne 7125 injector (Cotati, CA, USA) having a 10-μl injection loop. Separation of the aminoglycosides was achieved with the use of a Hamilton PRP-X200 column (250×4.6 mm I.D.; Reno, NV, USA) containing a polymer-based strong cation-exchanger with a particle size of 10 μm. A Hitachi 655A-11 LC pump (Tokyo, Japan) was employed to deliver the postcolumn reagent via a 3 μl 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. A Kratos Spectroflow 980 fluorescence detector (Ramsey, NJ, USA) fitted with a 10 μl detection flow cell was used for chromatographic detection. The excitation wavelength of the detector was set at 280 nm and a 350 nm longpass glass filter was utilized for selecting emission wavelength.

3. Results and discussions

3.1. Chromatographic separation

The instrumental set up is given in Fig. 2.

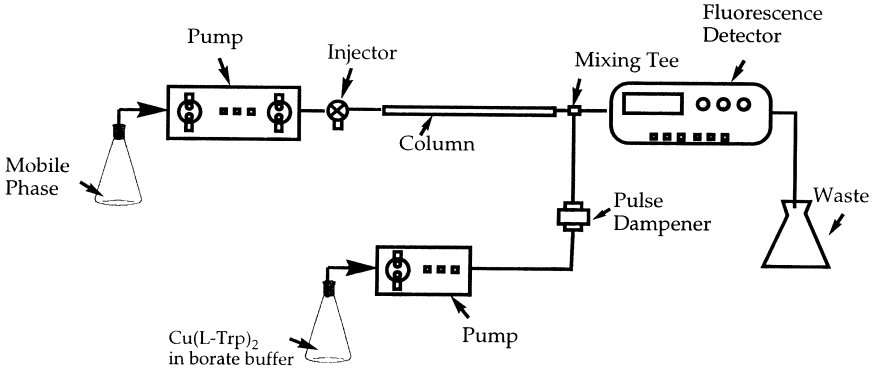

Fig. 2. Experimental setup.

Compared to other postcolumn detection methods, which often require a specially designed reaction chamber, only a mixing tee with a mixing volume of 3.0 μl is necessary in this scheme, which limits extracolumn bandbroadening. The use of a flow dampener for delivery of the postcolumn reagent has been found to be important for minimizing baseline fluctuations.

Due to the strong hydrophilicity of the aminoglycosides, these compounds are difficult to retain in the reversed-phase mode. Current separation methods for these compounds include reversed-phase ion-pair chromatography [15], and either cation-exchange or anion-exchange chromatography [20]. In this study a strong cation-exchange column was utilized. The retention of most of the aminoglycosides on the Hamilton SCX column is based on the cationic nature of these compounds within an appropriate pH range for the protonation of the amino groups. Most aminoglycosides have a first acidic association constant in the range from 8.8 to 10.0 [21–23]. To assure appropriate and adequate ionization of the aminoglycosides, the pH of the mobile phase was adjusted to 5.5 with the use of acetate buffer. As one of the strongest commonly used monovalent ion-exchangers, K^+ was chosen to elute the analyte compounds. Care should be taken in choosing the ion-exchanger, as some divalent ions, such as Cu^{2+}, Co^{2+} and Zn^{2+} may interfere with this approach to analyte detection by binding to L-Trp and the aminoglycosides. Other divalent ions, such as Ca^{2+}, and Ba^{2+}, may form hydroxides which can precipitate at the detection pH used in this study. KBr was used to adjust the strength of the mobile phase. A plot of the capacity factors of four of the aminoglycosides tested versus the KBr concentration of the mobile phase is given in Fig. 3. A mixture of amikacin, kanamycin A, kanamycin B and neomycin can be baseline separated using a mobile phase containing 0.7 *M* KBr. This mobile phase condition is also capable of providing a baseline separation of a mixture containing hygromycin B, ribostamycin, tobramycin and spectinomycin, or a mixture of geneticin and neamine. With the exception of spectinomycin, the elution order is consistent with the number of amino groups of the aminoglycosides, with the aminoglycosides possessing more amino groups being retained longer. Spectinomycin, however, has a constant capacity factor of 2.4, which is independent on the KBr concentration of the mobile phase in the range 0.6–1.0 *M*, suggesting a retention mechanism other than ion-exchange.

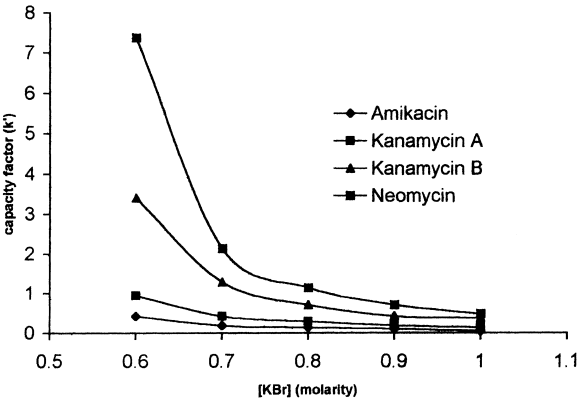

Fig. 3. Capacity factors (k′) for selected aminoglycosides plotted versus the concentration of KBr in the mobile phase.

3.2. Studies on the detection of aminoglycosides

3.2.1. Stoichiometric studies of the interaction of several aminoglycosides with Cu(L-Trp)$_2$

A series of experiments were performed to determine the stoichiometry between Cu(L-Trp)$_2$ and several aminoglycosides in solution. A solution of Cu(L-Trp)$_2$ was prepared by dissolving the appropriate masses of solid L-Trp and $CuSO_4 \cdot 5H_2O$ in an aqueous solution of sodium borate. Solutions containing different concentrations of the aminoglycosides were added to 0.05 m*M* Cu(L-Trp)$_2$ solutions containing 4 m*M* sodium borate adjusted to a pH of 8.1. This resulted in the ''titration'' of the Cu(L-Trp)$_2$ with these compounds in aqueous solution. The fluorescence intensities measured for these solutions versus the concentration of neomycin are plotted in Fig. 4. The aminoglycosides, kanamycin A, kanamycin B and amikacin give similar fluorescence ''titration'' pattern as shown in Fig. 4.

The data presented in Fig. 4 demonstrate that the fluorescence intensities of the solutions increase as the amount of aminoglycosides added increase until the recovered fluorescence signal reaches a plateau when a stoichiometric amount of the aminoglycoside compound is added to the reagent solution.

A stoichiometric ratio of 1:1 for [A]:[Cu(L-Trp)$_2$]

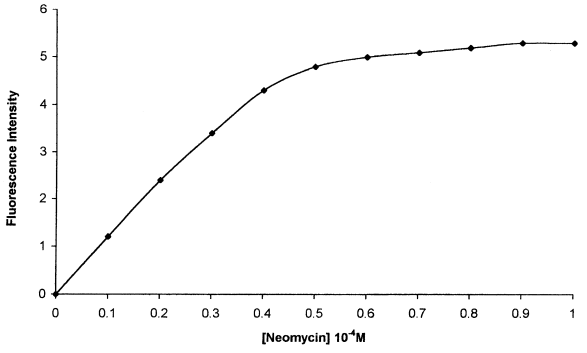

Fig. 4. Stoichiometric study of Cu(L-Trp)$_2$ with neomycin. The concentration of Cu(L-Trp)$_2$ is 0.05 m*M*, adjusted to a pH of 8.1, in 4 m*M* soduim borate.

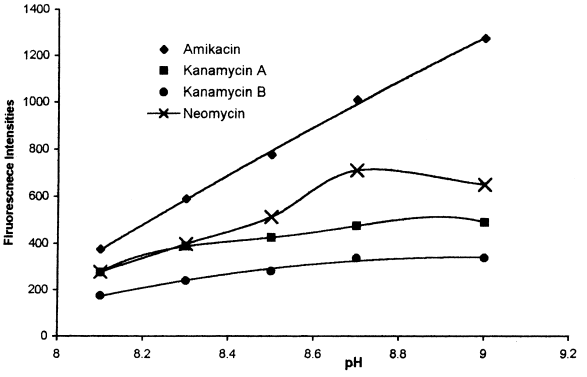

Fig. 5. Chromatographic peak intensities of four aminoglycosides versus pH. The amounts of samples injected were amikacin: 293 ng; kanamycin A: 242 ng; kanamycin B: 242 ng; neomycin: 307.5 ng.

(where A represents aminoglycosides) is determined from the titration data, corresponding to the reaction:

$$Cu(L\text{-}Trp)_2 + A = Cu(A) + 2L\text{-}Trp^*$$

L-Trp* represents fluorescently active L-Trp. This reaction is in good agreement with electrochemical studies and other spectroscopic results, in which the aminoglycosides were found to form 1:1 complexes with Cu(II) [21–23]. Thus, every aminoglycoside molecule displaces two fluorescent L-Trp molecule from the complex Cu(L-Trp)$_2$.

3.2.2. Optimization of the detection conditions

Possessing multiple amino groups, the aminoglycosides are effective chelators of Cu(II) ions [21–23]. As shown in Fig. 2, a buffered Cu(L-Trp)$_2$ solution was pumped postcolumn to the column eluent. Upon mixing with a Cu(L-Trp)$_2$ solution, the eluted aminoglycosides displace the L-Trp from the complex and the fluorescence of L-Trp is thereby recovered. Thus an increase in L-Trp fluorescence is indicative of the presence of the aminoglycosides.

Detection pH, which is one of the most important parameters for this approach to detection, was optimized by adjusting the pH of the postcolumn reagent, which is 0.05 m*M* Cu(L-Trp)$_2$ in a sodium borate solution. Under the conditions of this experiment, 4 m*M* sodium borate was found to provide sufficient buffering capacity to maintain the detection pH. As shown in Fig. 5, the fluorescence signal intensities of the aminoglycosides generally increase in the pH range of 8.1–9.0, except for neomycin, for which the fluorescence signal intensity decreases slightly above pH 8.8. A comprehensive interpretation of how the following three pH dependent factors affect detection, was given in previous studies [18,19]: (1) Fluorescence of free L-Trp steadily increases above pH 8.3, since the deprotonated free L-Trp has a higher fluorescence efficiency than zwitterionic L-Trp; (2) L-Trp binds to Cu(II) ion most efficiently at a pH of 8.1 with the greatest fluorescence quenching observed at this point. Dissociation of Cu(L-Trp)$_2$ increase both above and below pH 8.1; (3) The affinities of the analytes for Cu(II) ion, which in this study are aminoglycosides, are pH dependent, since the binding between the analytes and Cu(II) ion is affected by both the degree of analyte protonation and Cu(II) complexation with hydroxide ions.

For the three factors given here, the first one tends to lead to a chromatographic signal intensity decrease when the detection pH is away from 8.1, while the other two factors tend to lead to an increase in the signal intensity as the pH increases. The change of pH affects the three factors for different aminoglycosides to a different extent, due to differences in their molecular structures and number of amino groups. As shown in Fig. 5, the second and third factors generally dominate the chromatographic peak intensities as the pH increases, except for neomycin, where the first factor dominates when the detection pH is higher than 8.7.

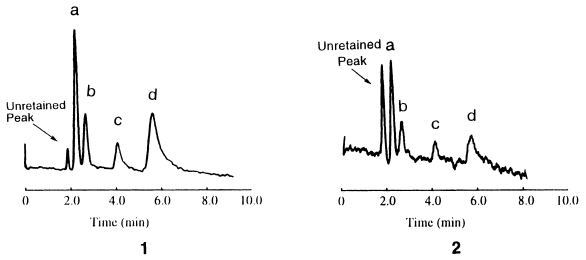

Fig. 6. Representative chromatograms of the aminoglycoside standards. Separation was achieved with the use of a Hamilton PRP-X200 SCX column. Mobile phase: 0.7 *M* KBr, 1.5 m*M* sodium acetate buffer at pH 5.5, 1.2 ml/min; Postcolumn reagent: 0.05 m*M* $Cu(L\text{-}Trp)_2$, 4 m*M* sodium borate buffer at pH 8.8, 1.8 ml/min. Peak (a) amikacin; (b) kanamycin A; (c) kanamycin B; (d) neomycin. The amounts of samples injected were (1) a: 293 ng; b: 242 ng; c: 242 ng; d: 307.5 ng; (2) a: 29.3 ng; b: 24.2 ng; c: 24.2 ng; d: 30.7 ng.

Due to the slow precipitation of $Cu(OH)_2$ in the postcolumn reagent when the pH is above 9.0, the detection pH was set at 8.8 to assure long term stability of the reagent. The flow-rates of the postcolumn reagent were evaluated in the range of 1.0 to 2.2 ml/min, for the separation of mixtures of amikacin, kanamycin A, kanamycin B and neomycin. From a flow-rate of 1.0 to 2.2 ml/min, the signal intensities decrease steadily, due to the effect of dilution of the analyte upon mixing with the postcolumn reagent. A compromise was made between the decreased signal and increased baseline fluctuation observed at lower flow-rates. The flow-rate of the postcolumn reagent was set at 1.8 ml/min.

3.2.3. Evaluation of the optimized detection conditions for the aminoglycosides

Standard aminoglycoside solutions were prepared in distilled water, and the dynamic ranges were obtained for the chosen compounds under the optimized separation and detection conditions. Representative chromatograms showing the separation of the four, aminoglycosides amikacin, kanamycin A, kanamycin B and neomycin at different concentrations are given in Fig. 6. Table 1 lists the statistical performance of the detection. The detection limits for the four aminoglycosides range from 4.2 ng for amikacin to 14.5 ng for kanamycin B injected on column, corresponding to 0.4 ppm for amikacin to 1.4 ppm for kanamycin B with a 10 μl injection volume. Representative chromatograms for the separation of six other aminoglycosides, hygromycin B, ribostamycin, spectinomycin, tobramycin, geneticin and neamine are shown in Fig. 7, along with detailed separation and detection conditions.

Commercial aminoglycoside samples in intravenous injectable and ophthalmic formulations were obtained and analyzed based on the developed separation and detection method. Samples were diluted with distilled water but no further sample treatment was required. The representative chromatograms for these samples are shown in Fig. 8. Actual and experimental values for the concentration were found to be within experimental variability. For an intravenous amikacin sample, the determined concentration is 252 ± 5.5 mg/ml, compared to an expected concentration of 250 mg/ml; for an ophthalmic neomycin sample, the measured concentra-

Table 1
Characteristic parameters of the calibration graphs and analytical figures of merit for the analysis of aminoglycosides

Aminoglycoside	Range (ng)	Regression Eq.[a]	r[b]	DL[c] (ng)	RSD[d](%)
Amikacin	29–586	H = 55.5 + 3.7C	0.9986	4.2	2.2
Kanamycin A	24–483	H = 6.4 + 2.0C	0.9997	9.3	2.9
Kanamycin B	24–483	H = 5.6 + 1.0C	0.9994	14.5	3.5
Neomycin	31–615	H = 4.0 + 1.6C	0.9998	11.8	3.6

[a] H, peak height, C, analyte mass injected (in ng).
[b] Correlation coefficient.
[c] Detection limit.
[d] Relative standard deviation ($N = 5$, for 293 ng amikacin, 242 ng kanamycin A, 242 ng kanamycin B and 307.5 ng neomycin injected respectively).

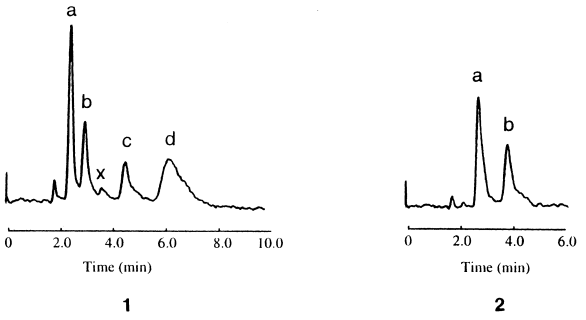

Fig. 7. Representative chromatograms of the aminoglycoside standards. Separation was achieved with the use of a Hamilton PRP-X200 SCX column. Mobile phase: 0.7 *M* KBr, 1.5 m*M* sodium acetate buffer at pH 5.5, 1.2 ml/min; Postcolumn reagent: 0.05 m*M* Cu(L-Trp)$_2$, 4 m*M* sodium borate buffer at pH 8.8, 1.8 ml/min. Chromatogram 1: Peak (a) hygromycin B (527 ng); (b) ribostamycin (454.5 ng); (c) tobramycin (467.5 ng); (d) spectinomycin (332.3 ng). Chromatogram 2: Peak (a) geneticin (496.7 ng); (b) neamine (322.4 ng). Peak x is an unknown present in our spectinomycin standard.

tion is 3.58±0.13 mg/ml, compared to an expected concentration of 3.5 mg/ml.

The detection method developed is free from many of the problems encountered when using derivatization approaches, such as labor-intensive sample preparation and derivative instability. Also, compared to a conventional postcolumn detection method, in which a specially designed postcolumn reaction chamber is used to provide an adequate reaction time between the analytes and the post-column reagent, only a minimum volume mixing tee was utilized in this study, which simplifies the instrumental setup and reduces extracolumn bandbroadening due to the extra mixing volume introduced. The detection limits obtained for the aminoglycosides tested with the developed detection methods in this study are comparable to precolumn derivatization methods using either fluorescence [8] or UV–Vis detection [9,24], which generally have

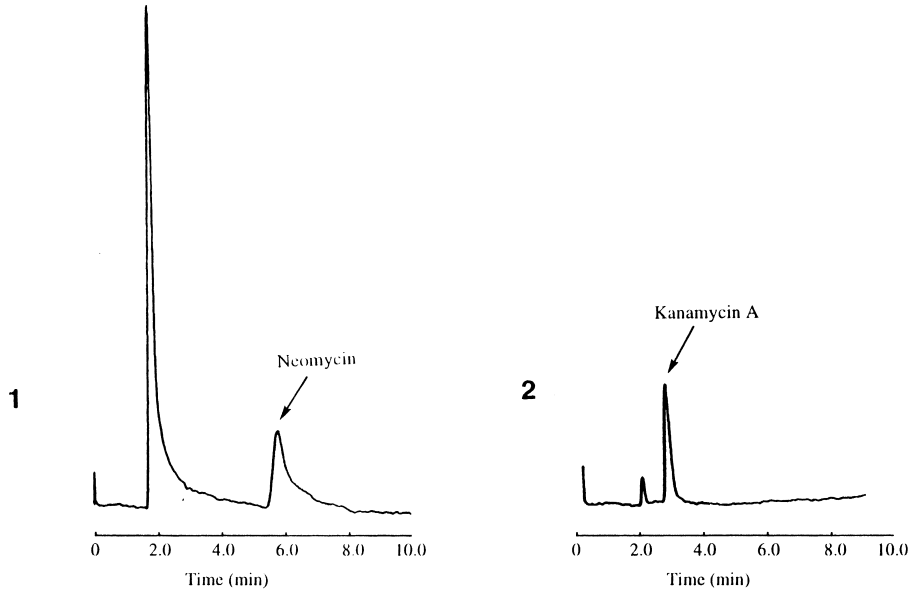

Fig. 8. Representative chromatograms of aminoglycosides in commercial formulations. Analyses were performed with the use of a Hamilton PRP-X200 SCX column. Mobile phase: 0.7 *M* KBr, 1.5 m*M* sodium acetate buffer at pH 5.5, 1.2 ml/min; Postcolumn reagent: 0.05 m*M* Cu(L-Trp)$_2$, 4 m*M* sodium borate buffer at pH 8.8, 1.8 ml/min. Chromatogram 1: a 1:100 volume diluted *o*-phthalmic neomycin sample; chromatogram 2: a 1:10000 volume diluted intravenous kanamycin A sample; chromatogram 3: a 3:100 volume diluted ophthalmic tobromycin sample.

detection limits around 10 ng injected on column or higher. The detection limits in this study are also comparable to detection limits obtained for the well-developed pulsed electrochemical detection for the aminoglycosides [12–15], which range from 4 to 15 ng. Normally detection limits as low as several picograms can be achieved in selected ion monitoring (SIM) mode in HPLC–MS detection in reversed-phase chromatography [25]. However, for optimized detection of aminoglycosides in a SIM mode in HPLC–MS, the detection limits reportedly are in a range of 1 – 50 ng [26], possibly due to the poor compatibility between the nonvolatile ion pairing agents used in the ion pair chromatography.

Still in a preliminary stage, studies are being carried out to further optimize the detection scheme in a number of aspects. Since the sensitivity of detection is essentially limited by the fluorescence of the monitoring agent, several agents with greater fluorescence efficiencies are being evaluated to further improve the detection limits. Another approach to improve detection is to include the reagent in a chromatographic mobile phase, or a capillary zone electrophoresis running buffer. This will not only simplify the instrumentation, but also avoid dilution of the analytes upon mixing with the postcolumn reagent used in previous studies.

However, it should also be noted that the detection conditions may not be compatible with the chromatographic separation conditions, considering the complication brought about by the interaction between the Cu(II)–L-Trp complex and the stationary phase. In this sense, the addition of the detection agent to a capillary zone electrophoresis running buffer may be more feasible.

4. Conclusions

Indirect fluorescence detection based on a fluorescence displacement reaction was applied to the detection of aminoglycoside antibiotics following chromatographic separation. A stoichiometric ratio of 1:1 was determined for the displacement reaction between the aminoglycosides and $Cu(L\text{-}Trp)_2$. This non-derivatization detection scheme does not require labor-intensive and time-consuming sample preparation. Pharmaceutical formulations of the aminoglycoside antibiotics were analyzed using the developed detection method. Detection limits obtained for the aminoglycosides are in the range of 4.5–14.5 ng injected, corresponding to a sub ppm to low ppm concentration level with a 10 μl injection volume. The method has been successfully applied to formulated products.

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