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Development of a chromatographic method for the isolation and detection of hygromycin B in biological fluids

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Abstract

An affinity chromatography method was developed for the purification of hygromycin B from biological fluids. Lysozyme and α-lactalbumin were immobilized on an *N*-hydroxysuccinimide activated agarose support. Hygromycin B solubilized in water was bound by the proteins and subsequently eluted using 10 m*M* sodium citrate buffer, pH 4.0. Hygromycin B was purified from swine plasma, bovine serum and bovine milk samples using a combination of ion-exchange chromatography for initial clean-up of spiked biological samples followed by affinity chromatography. Thin layer chromatographic analysis of the isolated hygromycin B revealed one band with the same R_F value as the hygromycin B standard.

Keywords: Hygromycin B

1. Introduction

The aminoglycoside antibiotics consist of a group of related antibiotics containing aminosugar residues. The primary effect of the aminoglycoside antibiotics is the inhibition of protein synthesis [1,2]. In humans, toxic levels of the aminoglycoside antibiotics damage the vestibular and auditory systems and the levels of antibiotics administered to patients must be carefully controlled and monitored. In the last few decades, antibiotics have been used in increasing amounts as therapeutic agents in food-producing animals for bacterial infections and to promote growth. Hygromycin B has been approved as a feed additive to control parasitic, respiratory and enteric infections in poultry and swine. Gentamicin and neomycin have also been approved for veterinary use in food-producing animals. Residues from aminoglycoside antibiotics can accumulate over time in the fluids and tissues of farm animals designated for human consumption. Since these residues can be potentially toxic if consumed by humans, rapid and sensitive detection methods are needed for the aminoglycoside residues which may occur in tissues and fluids of farm animals.

There are several reviews that discuss the advan-

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tages and disadvantages of the various methods for detection of aminoglycoside antibiotics in biological samples [3–7]. Microbiological assays are simple to perform and inexpensive to use, but the assays are time-consuming typically requiring 12–48 h for completion. Radioenzyme and radioimmuno assays are less time- consuming (2–3 h) and the assays have the added advantage of good sensitivity and specificity. However, the radiolabeling assays are expensive and require trained personnel for handling and disposal of radioactive material.

Chromatographic methods are more applicable to antibiotic residues purification and detection. Extraction of drug residues from biological samples is usually necessary prior to chromatographic analysis. Solid-phase extraction is frequently used for drug extraction because of the reduction in solvent use and the ease of automation, as compared to solvent extraction methods. Among the chromatographic methods, thin-layer chromatography (TLC) is the most simple to perform and least expensive. TLC was used for detection of aminoglycoside antibiotics in biological fluids after solid-phase extraction using ion-exchange cartridges [8,9]. The TLC method was partially automated and semi-quantitative results were obtained within 2 h. High pressure liquid chromatography (HPLC) analysis can be performed in minutes and also requires sample extraction prior to analysis [10,11]. HPLC methods are easily automated and do not require specially trained personnel. Gas chromatography has not been used extensively for separation and detection of aminoglycoside antibiotics in biological samples [7]. Nakaya reported a GC analysis of kanamycin in bovine muscle [12]. For optimum GC analysis, the samples should be volatile or easily volatilized. GC is more likely combined with mass spectrometry (MS) for confirmatory methods. GC–MS analysis is not suitable for routine monitoring because of the expense and the need for trained personnel. The use of affinity chromatography for purification of aminoglycoside antibiotics from biological samples were not reported until recently [13]. An immunoaffinity chromatography was described for the isolation of hygromycin B from bovine kidney extract.

Affinity chromatography requires specific recognition of a molecule by an immobilized ligand. Immunoaffinity chromatography uses antibodies that bind to a specific drug or class of drugs. Antibodies are typically produced by injecting an appropriate antigen into a rabbit and purifying the resultant antibodies. This technique has the advantage of specific recognition of a drug, but the technique requires the production and purification of the antibodies which can be an expensive process. Any ligand that specifically recognizes and reversibly binds another molecule may be used for affinity chromatography. Lysozyme and α-lactalbumin were chosen for initial study because both proteins bind saccharide substrates. Lysozyme catalyzes the hydrolysis and α-lactalbumin catalyzes the synthesis of (β1→4) glycosidic linkages [14–16]. Covalent enzyme- substrate adducts of lysozyme show distorted covalently linked sugar ring. The distortion of substrate toward a transition state is probably important for lysozyme catalysis [17]. There is a close structural similarity between lysozyme and α-lactalbumin. The amino acid residues which interact with the substrates are conserved in the α-lactalbumin [15]. The two proteins can bind the aminoglycloside antibiotics but no catalytic reaction would occur because of the absence of a (β1→4) glycosidic bond in the antibiotic molecule. Fernandez-Sousa et al. [18] showed that lysozyme activity can be inhibited by aminoglycosidic antibiotics, presumably by binding to the active site of the enzyme.

The objective of this study was to develop a purification method for hygromycin B in biological fluids. Extraction and purification methods for hygromycin B in biological fluids that reduce the use of organic solvents are needed by the regulatory laboratories. Solid-phase extraction of antibiotics from biological samples followed by affinity chromatography for their purification is one approach for organic solvent reduction in the laboratory. In this paper, we report our findings on the development of a chromatographic method for the isolation and detection of hygromycin B in biological fluids. This includes preparation of the immobilized proteins and the application of the affinity chromatography method to purify hygromycin B in bovine serum and milk.

2. Experimental[1]

2.1. Materials and reagents

Lysozyme (hen egg white), α-lactalbumin (Type III from bovine milk), neomycin B, gentamicin and fluorescamine were purchased from Sigma (St. Louis, MO, USA). Hygromycin B was purchased from Calbiochem (La Jolla, CA, USA). Bicinchoninic acid (BCA) protein assay reagent was procured from Pierce (Rockford, IL, USA). Affi-Gel 10, Polyprep column and 2-way stopcock were purchased from Bio-Rad (Rockville Centre, NY, USA) and Clean Screen columns were procured from United Chemical Technologies (Bristol, PA, USA). All other chemicals were of reagent grade. A rotary mixer was obtained from Labindustries (Berkeley, CA, USA).

2.2. Preparation of affinity columns

Lysozyme and α-lactalbumin were immobilized using Affi-Gel 10 (*N*-hydroxysuccinimide ester agarose gel) using a modified procedure as suggested by the manufacturer. Lysozyme (25 mg/ml) was diluted in 0.1 *M* MOPS ([*N*-morpholino] propane sulfonic acid) buffer at pH 7 and filtered through 0.2 μm nylon membrane. Affi-Gel 10 (1 ml) was transferred to a Polyprep column. The gel was washed with 3 column volumes of deionized water. The bottom was capped and 2 ml of lysozyme solution was added to the gel. The top of the column was capped and the mixture was incubated for 4 h at 4°C on a rotator. The excess proteins were drained off the column followed by four, 2 ml washes with 0.1 *M* MOPS (pH 7). The protein concentration was determined for the flow through sample and the MOPS washes using bicinchoninic acid. The unreacted activated carbonyl groups on the agarose gel were blocked by adding 2 ml of 0.1 *M* ethylene diamine solubilized in MOPS by incubating for 1 h at 4°C on a rotator. The excess ethylene diamine was removed from the gel using 0.1 *M* MOPS washes and the gel was stored in distilled water at 4°C. The amount of immobilized lysozyme was determined by measuring the protein concentration of the lysozyme solution before and after immobilization using bicinchoninic acid. α-Lactalbumin was immobilized on Affi-Gel 10 using the same procedure as described above except for the addition of 80 m*M* calcium chloride to the 0.1 *M* MOPS buffer.

2.3. Affinity chromatography of antibiotics in aqueous solutions

Affinity columns were prepared by transferring 1 ml of agarose gel containing immobilized protein to a 5 ml syringe-type disposable column fitted with a frit and 2-way stopcock. The immobilized lysozyme was activated by washing the gel twice with 1 ml aliquots of 0.1 *M* NaOH at a flow-rate of 1 ml/min followed by four 1 ml portions of water. Immobilized α-lactalbumin did not require activation. Aqueous solutions of the antibiotics (1 ml of 25 ppm of the antibiotics solubilized in water) were applied to the affinity columns at a flow-rate of 1 ml/min. The columns were washed with distilled water (4×1.0 ml) and the antibiotics were eluted with 10 m*M* sodium citrate buffer, pH 4.0 (4×1.0 ml). Fluorescamine (1 mg/ml in ethanol) was added to the eluate in equal volume to derivatize the primary amino groups on the antibiotics. The fluorescence was determined using a Perkin Elmer fluorometer at an excitation of 395 nm and an emission of 485 nm after a 15 min reaction.

2.4. Detection of hygromycin B in biological samples

Swine plasma, bovine serum and bovine milk samples were spiked by adding 12.5 ppm, 25 ppm and 250 ppm of hygromycin B, respectively, to 1 ml of the biological fluid. The positive control sample consisted of hygromycin B (same concentration as test sample) in water and the negative control sample consisted of biological fluids without added hygromycin B. Clean Screen columns (copolymeric bonded silica with hydrophobic and cationic functions) were used for initial solid-phase extraction of

[1]Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

antibiotics from the biological fluids [8,9]. The columns were conditioned using 1.0-ml aliquots of 5% diethylamine in methanol, water and 2% phosphoric acid prior to sample application. The adulterated biological samples were acidified using 4 volumes of 2% phosphoric acid and applied to the columns. The columns were washed using 1-ml aliquots of water followed by 0.5-ml aliquots of 5% diethylamine in methanol. The diethylamine samples were collected, solvent evaporated using a vortex evaporator and reconstituted using 1 ml of water. The reconstituted samples were applied to the affinity columns followed by washing sequentially with deionized water (5×1.0 ml) and 10 m*M* sodium citrate buffer, pH 4.0 (5×1.0 ml) to elute the drug. Hygromycin B was detected using either fluorescamine derivatization or thin layer chromatography (TLC).

2.5. TLC analysis

TLC analysis was performed using the method of Medina and coworkers with slight modification [8,9]. Briefly, the eluates were evaporated to dryness and the samples reconstituted with 100–250 μl aqueous ethanol (50%). Aliquots of 25–150 μl were applied to a Whatman LHK-PD silica gel plate with preabsorbent zones. The TLC plate was developed for 20 min in acetone–ethanol–ammonium hydroxide (1:1:1), dried in a vacuum oven, cooled to room temperature and dipped for 4 s in 0.02% fluorescamine (acetone–hexane, 1:15). The plate was dried, sprayed lightly with 0.2 *M* sodium citrate buffer, pH 3.0 and the fluorescent bands were visualized using ultraviolet light at 365 nm wavelength.

3. Results and discussion

3.1. Affinity chromatography of antibiotics in aqueous solutions

An average of 29 mg of lysozyme and 18 mg of α-lactalbumin were immobilized per 1 ml of agarose gel using a 4 h incubation time. The immobilization efficiencies were 53% for lysozyme ($n=12$) and 36% ($n=7$) for α-lactalbumin. Hygromycin B, neomycin B and gentamicin at a concentration of 25 ppm in water were bound by the immobilized lysozyme and eluted with 10 m*M* sodium citrate buffer, pH 4.0 (Fig. 1a). The fluorescence intensity differed among the antibiotics after derivatization using fluorescamine due to the different number of free primary amino groups [19]. Immobilized α-lactalbumin also

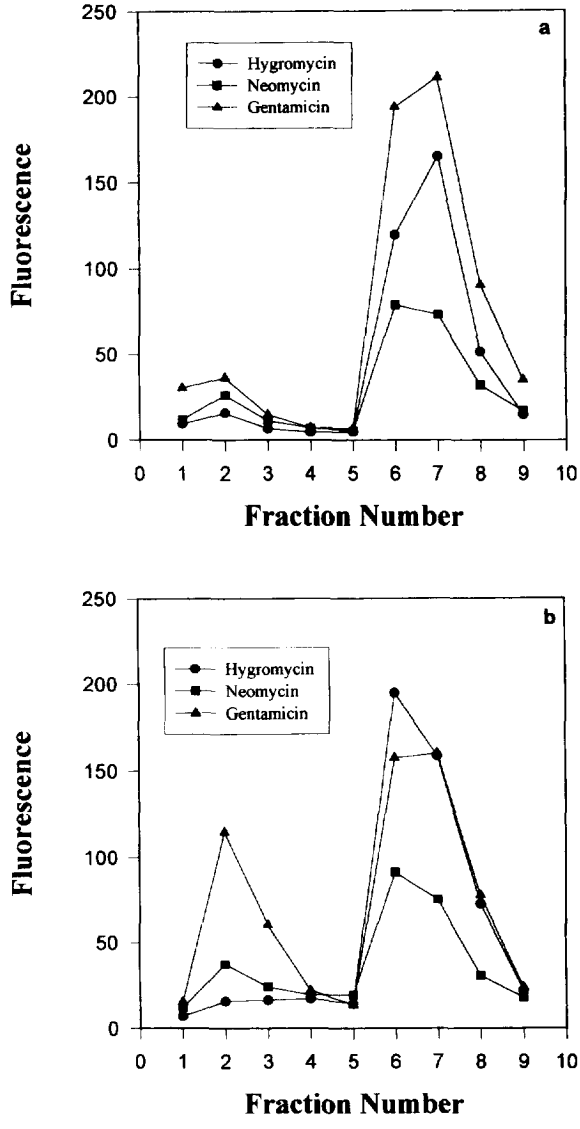

Fig. 1. (a) Affinity chromatography of aminoglycoside antibiotics using immobilized lysozyme ($n=12$). Fraction 1, flow through; fractions 2–5, water washes; fractions 6–9, citrate elution. (b) Affinity chromatography of aminoglycoside antibiotics using immobilized α-lactalbumin ($n=12$). Fraction 1, flow through; fractions 2–5, water washes; fractions 6–9, citrate elution.

bound the three antibiotics used in this study with a lower binding efficiency for gentamicin (Fig. 1b).

The lysozyme and the α-lactalbumin columns showed little or no decrease in binding efficiency over a two-week period (Fig. 2a and Fig. 2b). The lysozyme columns had decreased binding efficiency for hygromycin on day 12, but the binding efficiency was increased upon reactivation of the immobilized lysozyme using 0.1 *M* NaOH. Sodium hydroxide was required for the initial activation and periodic reactivation of the immobilized lysozyme whenever the binding efficiency decreased. Sharon and Eshdat showed a similar reactivation of lysozyme activity by affinity labeling lysozyme using a 2′,3′-epoxypropyl-glycosides of *N*-acetyl-D-glucosamine [20]. The affinity label was an irreversible inhibitor that inactivated the lysozyme catalytic activity between pH 2 and pH 10. However, slow reactivation of the lysozyme catalytic activity was observed in aqueous solutions of triethylamine at pH 11. Lysozyme is a basic protein with a p*I* of 11.0 and its catalytic activity decreases in the acidic pH range [21]. All lysozymes from different sources are basic proteins and any chemical modification which increases the acidity of lysozyme decrease its catalytic activity [15]. Conditions which favored lysozyme catalytic activity (high pH and low ionic strength) also favored binding of hygromycin to the immobilized lysozyme. Hygromycin did not bind to immobilized lysozyme at low pH (0.1 *M* NaOH required for activation of immobilized lysozyme) or high ionic strength. The affinity columns were stored in water at 4°C and the binding efficiencies showed little change after a six-month storage.

3.2. Detection of hygromycin B in biological samples

The lysozyme column was used for further study to examine the application of the affinity column for purification of hygromycin B in biological samples. A combination of solid-phase extraction and affinity chromatography was used for the purification of hygromycin B spiked bovine serum and milk samples (Fig. 3a and Fig. 3b). The bovine serum sample was spiked with 25 ppm of hygromycin B per ml of sample and the milk was spiked with 250 ppm of hygromycin B per ml of sample. The control samples

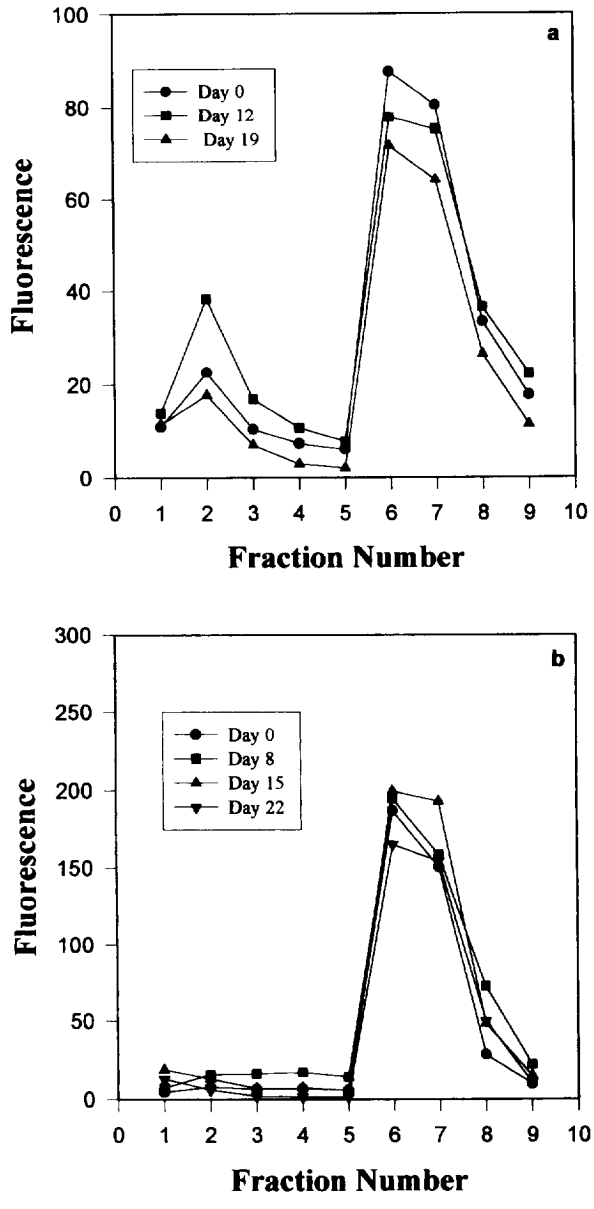

Fig. 2. (a) Affinity chromatography of hygromycin using immobilized lysozyme ($n=4$) at 0, 12 and 19 days of storage. Immobilized lysozyme was activated prior to affinity chromatography using 0.1 *M* NaOH and was reactivated after a decreased binding efficiency after 12 days. (b) Affinity chromatography of hygromycin using immobilized α-lactalbumin ($n=4$) at 0, 8, 15 and 22 days of storage.

(serum and milk blanks) showed no interfering fluorescent compounds. Fig. 3b shows incomplete recovery of hygromycin in bovine milk. This is

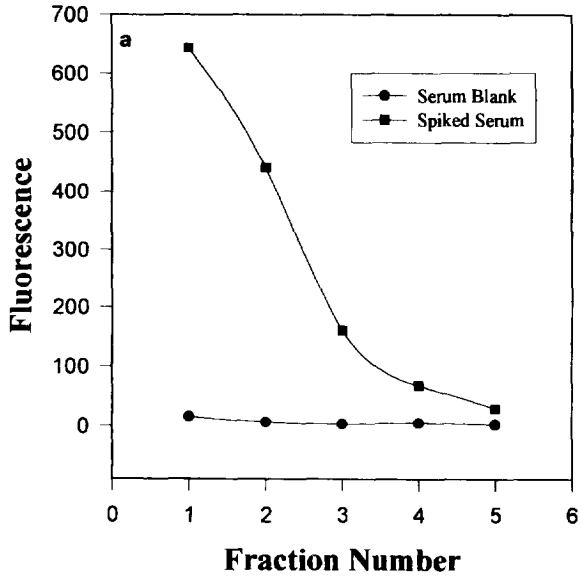

Fig. 3. (a) Clean-up of hygromycin B in bovine serum using solid-phase extraction followed by lysozyme affinity chromatography. Fractions 1–5 show elution of hygromycin using citrate washes. (b) Clean-up of hygromycin B in bovine milk using solid-phase extraction followed by lysozyme affinity chromatography. Fractions 1–5 show elution of hygromycin using citrate washes.

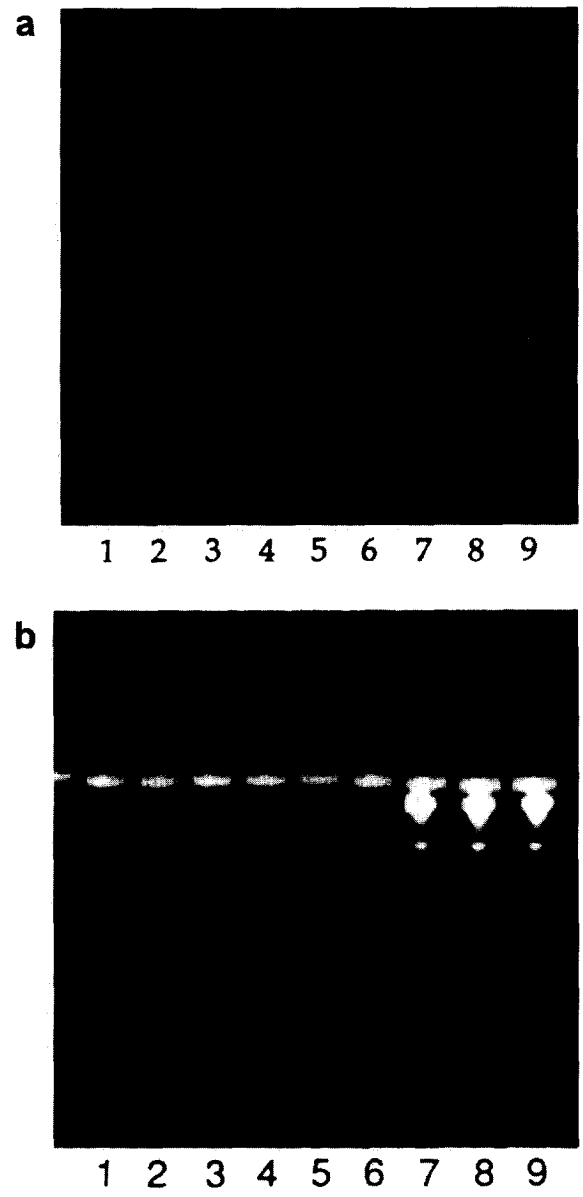

Fig. 4. (a) TLC analysis of hygromycin B (25 ppm) added to bovine serum. Lanes 1, 5 and 9, hygromycin standard; lanes 2 and 6, chromatographed hygromycin standard; lanes 3 and 7, chromatographed serum blank (no hygromycin); lanes 4 and 8, chromatographed hygromycin spiked serum. (b) TLC analysis of hygromycin B (12.5 ppm) added to swine plasma. Lanes 1–4 show bands of samples treated with a combination of solid-phase and affinity chromatography (lane 1, hygromycin standard, lane 2, plasma blank, lanes 3 and 4, hygromycin spiked plasma); lanes 6–9 contains solid-phase extracted samples (lane 6, hygromycin standard, lane 7, plasma blank, lane 8 and 9, hygromycin spiked plasma); lane 5 contains untreated hygromycin standard (25 ppm).

probably due to hygromycin binding to α-lactalbumin present in milk. Other preliminary experiments performed in our laboratory showed that

hygromycin B also binds to immobilized rat albumin but weaker than the two proteins in this study. Aminoglycoside antibiotics are probably bound and transported in vivo by albumins present in blood and milk.

Hygromycin B (25 ppm/ml of sample) added to bovine serum and swine plasma was detected by TLC analysis (Fig. 4a and Fig. 4b) using the procedure described in the TLC section. Fig. 4a shows the TLC analysis for the recovery of hygromycin in serum. The spiked serum sample (25 ppm hygromycin B) shows one band with the same R_F value as the hygromycin B standard (lanes 1 and 2) and there were no bands for the serum blank (lane 3). In Fig. 4b, the TLC analysis shows the contrast for the solid-phase extraction alone and solid-phase extraction followed by affinity chromatography of spiked swine plasma (12.5 ppm hygromycin B). The TLC analysis shows less interfering fluorescent derivatives using a combination of solid-phase extraction and affinity chromatography for the purification of hygromycin B in swine plasma. Hygromycin B concentrations as low as 1 ppm were detected in spiked bovine serum after a combination of solid-phase extraction and affinity chromatography followed by TLC analysis. The detection limit was determined by the sensitivity of the fluorescence assay used in this study. Greater sensitivity can be achieved using other derivatization reagents for aminoglycoside antibiotics that have detection limits in the parts per billion range [11,22].

4. Conclusions

An affinity chromatography method was developed for the purification of hygromycin B in biological fluids. Although solid-phase extraction was necessary prior to affinity chromatography, this method is an improvement over previous methods using solid-phase extraction without further purification. Immobilized lysozyme required activation and periodic reactivation using 0.1 *M* NaOH for optimum binding of the antibiotics. The immobilized α-lactalbumin did not bind the antibiotics as well, probably due to the different substrate specificities for the two proteins. This technique can be combined with a more sensitive detection system for an effective method for antibiotics extraction and detection in biological fluids. This affinity chromatography method is easy to perform, rapid and inexpensive.

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