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Solid-phase clean-up and thin-layer chromatographic detection of veterinary aminoglycosides

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Abstract

Chemical methods are needed to confirm the presence of antibiotics detected by microbial inhibition assays in fluids and tissues of farm animals. We have optimized the conditions for the isolation of hygromycin B with a copolymeric bonded solid-phase silica column followed by thin-layer chromatography (TLC) separation and detection of its fluorescence derivative after reaction with fluorescamine. The detection limit of the drug was 50 ng. Serum and plasma samples fortified with hygromycin B were acidified and passed through the copolymerized solid-phase columns previously conditioned with phosphate buffer. Hygromycin B was trapped in the columns and eluted with diethylamine–methanol and analyzed by TLC using acetone–ethanol–ammonium hydroxide as the developing solvent. Hygromycin B bands were derivatized at acidic pH with fluorescamine and visualized under ultraviolet light. Hygromycin B added to bovine plasma was detectable at 25, 50, 100, 250 and 500 ng/ml (ppb). Hygromycin B added to swine serum was detected at 50 ng/ml. However, the serum had to be deproteinized with trichloroacetic acid or acetonitrile prior to solid-phase extraction to gain accurate values. Neomycin and gentamicin (100 ng/ml aqueous solutions) could also be isolated with copolymeric solid-phase columns at a level of 50 ng. Gentamicin, neomycin, gentamicin, spectinomycin, hygromycin B and streptomycin could be separated by TLC, allowing multiresidue detection of these aminoglycosides. The respective R_F values of 0.64, 0.56, 0.52, 0.33 and 0.20 indicate the separation of these five compounds. This procedure provides a rapid and sensitive method for the semi-quantitative estimation of aminoglycosides.

1. Introduction

Hygromycin B is allowed as a feed additive to control parasitic, respiratory and enteric infections of poultry and swine [1]. Microbial assays such as LAST (Live Animal Swab Test) [2] and STOP (Swab Test On Premises) [3] have been used by U.S. regulatory agencies to detect antibiotic residues in urine of live farm animals or kidney and muscle tissues after slaughter. The microbial inhibition tests combined with bioautographic, electrophoretic [4–6] or thin-layer chromatographic methods [7] were reported for the identification of various aminoglycosides. Microbiological and radioimmunoassay methods for the detection of hygromycin B in feeds were described by Hilty [8,9] and Fogelsong and LeFeber [10], respectively. Nakaya et al. [11] reported a high-performance liquid chromatographic (HPLC) method for the

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detection of hygromycin B in pork tissue at 0.3 units (ca. 0.3 μg) per gram of sample. Maitra et al. [12], Shaikh and Allen [13], and Trishchler et al. [14] reviewed microbiological, biochemical, chromatographic and other physico-chemical methods for the detection of other aminoglycosides in biological fluids and tissues of food producing animals. The microbiological assays were non-specific, requiring 2.5–48 h of analysis. The aminoglycosides were detectable at microgram or part-per-million levels. The combination of TLC or electrophoresis and bioautography allowed multi-residue separation of the aminoglycosides but required high concentration of the drugs. The HPLC method utilized a pre- and post-column fluorescent derivatization of the aminoglycosides with reported sensitivities of 150 to 500 ng/ml. Non-isotopic immunoassays were reported for the detection of other aminoglycosides but hygromycin B. Rapid and sensitive chemical or chromatographic methods for the detection of hygromycin B in fluids and tissues of farm animals are needed to confirm the presence of unidentified microbial inhibitors (UMIs) detected by microbiological screening tests.

The objectives of this study were to develop an extraction and solid-phase clean-up method to isolate aminoglycosides from animal plasma or serum and detection by a semi-quantitative TLC procedure. This study presents the optimization of the following conditions: pH of sample pretreatment; pH of the cationic resin necessary to trap hygromycin B from aqueous solutions and fortified serum; solvents to elute hygromycin B; TLC developing solvent; and conditions for the fluorescent derivatization of hygromycin B.

2. Experimental

2.1. Materials and reagents

Glass-distilled solvents and high purity chemicals, such as ammonium hydroxide, tetrahydrofuran, and diethylamine (DEA) were used. Hygromycin B (liquid solution) was obtained from Calbiochem (La Jolla, CA, USA); neomycin sulfate, gentamicin sulfate and fluorescamine were from Sigma (St. Louis, MO, USA). Clean Screen DAU resin, a copolymeric bonded silica with hydrophobic and ionic functions (C_8/sulfonic), was obtained from United Chemical Technology (Bristol, PA, USA). A solid-phase vacuum extractor was purchased from J.T. Baker (Phillipsburg, NJ, USA); LHP-KD silica gel thin-layer plates were from Whatman (Clifton, NJ, USA); twin-trough TLC tank for 10 × 10 plates; chromatographic Immersion Device II; TLC scanner II and automated TLC Sampler III were from Camag (Wrightsville, NC, USA); and the Fisher Biotech ultraviolet viewing box was from Fisher Scientific (King of Prussia, PA, USA).

2.2. Preparation of hygromycin B standards

Stock solutions of 50 mg/ml of hygromycin B and other aminoglycosides in deionized-distilled (DI) water were stored as 100-μl aliquots at −20 or −85°C. Working solutions were made fresh daily from the 50 mg/ml stock solution and allowed to reach room temperature prior to preparation of the working dilutions. Standards used for direct application to TLC plates were diluted in methanol–isopropanol–tetrahydrofuran (5:15:80, v/v) at 0.5, 0.75 and 1.0 μg/ml concentrations resulting in the formation of compact bands. Aliquots of 0.075 or 0.1 ml were applied to TLC plates. Standard solutions of 0.05, 0.1 or 0.25 ng/ml were also prepared daily and diluted with 2% phosphoric acid or 20% TCA solutions for application onto the copolymeric bonded solid-phase column.

2.3. Enhancement of fluorescent signals

Hygromycin B was dissolved in 0.1 *M* citrate buffer (pH 3) or in 0.1 *M* borate buffer (pH 8.6). Fluorescamine was dissolved in buffer–acetone (1:10, v/v). Fluorescent signals were measured after 10–15 min using a 1:10 molar ratio of hygromycin B (1 mg/ml) and fluorescamine (5 mg/ml) at acidic and basic pH using excitation and emission wavelengths of 395 and 480 nm, respectively. The effect of reaction action time

was measured at 0, 5, 10, 15 and 30 min using citrate buffer (pH 3). The stability of aqueous solutions of hygromycin B during storage at 4°C was determined at 0, 24 and 48 h using optimum conditions for derivatization. Thereafter aqueous solutions of hygromycin B (10, 20 and 40 μg/ml) were stored refrigerated for 48 h. Aliquots were taken at intervals of 0, 24 and 48 h and analyzed with TLC. The derivatives were quantitatively measured with the densitometer.

2.4. Optimization of conditions for solid-phase isolation of hygromycin B

The combined effects of amount of resin, sample volume and elution volume were determined to study the recovery of hygromycin B. Hygromycin B (25 μg/ml) dissolved in 1, 2, 3 and 4 ml citrate buffer (pH 3) was passed through 0.5, 1.0, and 1.5% suspensions of copolymeric resin and eluted with 0.2, 0.3, and 0.4 ml of 5% diethylamine in methanol. The amounts of hygromycin B eluted from these columns were measured with a Camag densitometer after TLC development. Utilizing the selected "optimum" conditions, recovery of hygromycin B was determined at 5–50 ng and 12.5–125 ng.

2.5. Preparation of the solid-phase column

CleanScreen copolymeric resin was prepared for chromatographic use by suspending 10 g of resin in 110 ml of methanol–ammonium hydroxide (100:10, v/v) and gently shaking for 5 min; the solvent was decanted, and the resin was rinsed with another 100 ml of methanol, shaken for 5 min and then filtered. The resin was converted to the acid form by shaking for 5 min in 110 ml of methanol–acetic acid; the solution was filtered and the acidification step was repeated once more. Finally, the resin was rinsed with 100 ml of methanol, shaken mildly for 10 min, filtered, and dried in an oven at 80°C. A 5% resin suspension in ethanol was prepared weekly.

The column (12 × 7 mm I.D.) was packed with 130 mg equivalent (2.5 ml suspension) of resin. A frit was placed above the resin bed to hold the resin down. The solid-phase vacuum extractor was adjusted at 2–3 mmHg (7.5–10 kPa) allowing a flow-rate of 1–2 ml/min. The CleanScreen columns were conditioned with 3 × 1 ml of 5% diethylamine (DEA)–methanol, rinsed with 3 × 1 ml of methanol and 3 × 1 ml of deionized (DI) water. The resin was converted to its phosphate form by the addition of 2 × 1 ml of potassium phosphate buffer (0.1 *M*, pH 6), and the excess phosphate washed off with 3 × 1 ml of DI water.

2.6. Plasma and serum sample preparation

Bovine plasma (1, 2, 2.5 ml) fortified with 250, 100 or 50 ng/ml hygromycin B was transferred to 10-ml polypropylene tubes and diluted with 4, 8 and 10 ml of 2% phosphoric acid, respectively. The samples were mixed vigorously with a vortex-mixer and applied directly to the CleanScreen column. Swine serum (2.5 ml) was fortified and diluted with 10 ml of distilled water, acidified with 0.25 ml of 20% trichloroacetic acid (TCA), mixed vigorously, centrifuged at 4°C and 6500 *g* for 30 min, and the TCA supernatant was applied to the column. Likewise, proteins in 2.5 ml swine serum were precipitated with 5 ml of acetonitrile. After centrifugation at 6500 *g* for 15 min, the acetonitrile in the supernatant fluid was evaporated to a volume of 2.5 ml. The aqueous phase was then acidified with 10 ml of 2% phosphoric acid prior to ion-exchange purification. Samples were fortified before and after acidification.

2.7. Solid-phase clean-up

Fortified and blank plasma (unfortified) were acidified and a hygromycin B standard in acid solution were loaded onto the columns and allowed to percolate at a rate of 1–2 ml/min. Hygromycin B was trapped on the column and the columns were rinsed with five 1-ml portions of DI water, followed by 5 × 1 ml of 2-propanol. The columns were aspirated to dryness. Hygromycin B was eluted with 5 × 0.5 ml diethylamine–methanol (5:95, v/v). Eluates were applied directly onto TLC plates, applying 50–100

μl. However, the sensitivity of detection improved when the eluates were dried completely with a vacuum dryer or under a stream of nitrogen and reconstituted with 0.25 ml methanol–isopropanol–tetrahydrofuran (5:15:80, v/v).

2.8. Semi-quantitative thin-layer chromatography

Sample eluates (50–100 μl) were applied with Drummond disposable micropipettes to Whatman LHK-PD silica-gel TLC plates with preabsorbent zones (10 × 10 cm, 9 lanes). A minimum of 50 ng hygromycin B was applied to each lane. The TLC plates were developed vertically in a Camag twin-trough tank containing 10 ml of acetone–ethanol–99.9% NH_4OH (1:1:1, v/v) per chamber reservoir. The TLC plate was developed for 20 min and was removed from the tank and allowed to dry for 35 min at 80°C in a vacuum oven to evaporate ammonia from the TLC plate. A regular oven may also be used. The plate was cooled to room temperature with a stream of nitrogen. Hygromycin B bands were visualized by fluorescent derivatization by dipping the plate into a 0.02% fluorescamine solution for 4 s (0.025 g dissolved in 150 ml acetone–hexane, 10:140, v/v). The TLC plate was dried under nitrogen for 10 min and then sprayed with 0.2 *M* citrate buffer (pH 3.0). Fluorescent bands were detected at 366 nm using the ultraviolet viewing system. The migration distance and fluorescent intensity of hygromycin B standards directly applied onto the TLC plate were compared to those of the samples. Hygromycin B standard passed through the column and unfortified plasma subjected to the same treatment as the unknown samples were used as additional controls.

2.9. Quantitative thin-layer chromatography

Standard hygromycin B solutions in methanol were applied to TLC plates at 25–100 μl volume with the Camag automated Sampler III and the plate was developed as described in the preceding paragraph. Fluorescamine derivatives of hygromycin B were sprayed with borate or citrate buffers to determine pH effects. The fluorescent bands were scanned with the Camag TLC Scanner II using 395 nm excitation and 485 nm emission wavelengths.

A schematic outline of the solid-phase clean-up and TLC analysis of hygromycin B is shown in Fig. 1.

3. Results and discussion

Hygromycin B was used as a model compound for optimizing conditions for extraction, isolation and TLC analysis of aminoglycosides. The structure of hygromycin B is shown in Fig. 2. In our initial studies, hygromycin B and other aminoglycosides were derivatized in a basic pH solution (pH 8.6) supplemented with fluorescamine as was traditionally used for the detection of proteins, peptides, amino acids, amines and amino sugars. However, stability studies showed erratic fluorescent signal intensities when determined in solution or with an ultraviolet scanner for TLC plates. De Silva and Strojny [15], reported that aliphatic primary amines favored alkaline pH conditions for an optimal fluorophore-producing reaction fluorophore, but the primary aromatic amino compounds favored acidic pH 3–4 solutions for optimal reactivity. This report led us to compare the reaction of hygromycin B (1 mg/ml) at basic and acidic pHs. An increase in fluorophore formation in citrate buffer (pH 3) vs. borate buffer (pH 8.6) solutions was observed when measured directly with a fluorometer at 395 nm excitation and 485 nm emission wavelengths. Results of 3 duplicate trials showed that the mean peak heights were 74.4 ± 4.6 mm (S.D.) at acid pH vs. 45.0 ± 3.6 mm (S.D.) at basic pH. The coefficients of variation were 6.2 and 8%, respectively. The mean peak areas were 1546 ± 101 meV (millielectron volt) (S.D.) at acid pH vs. 923 ± 43 meV (S.D.) at basic pH, with coefficients of variation of 6.5 and 4.6%, respectively. Optimum fluorophore formation was found in 10–25 min in acidic solutions (Fig. 3).

In this study, we observed that hygromycin B was unstable in both aqueous and organic solutions. The hygromycin B amounts found after

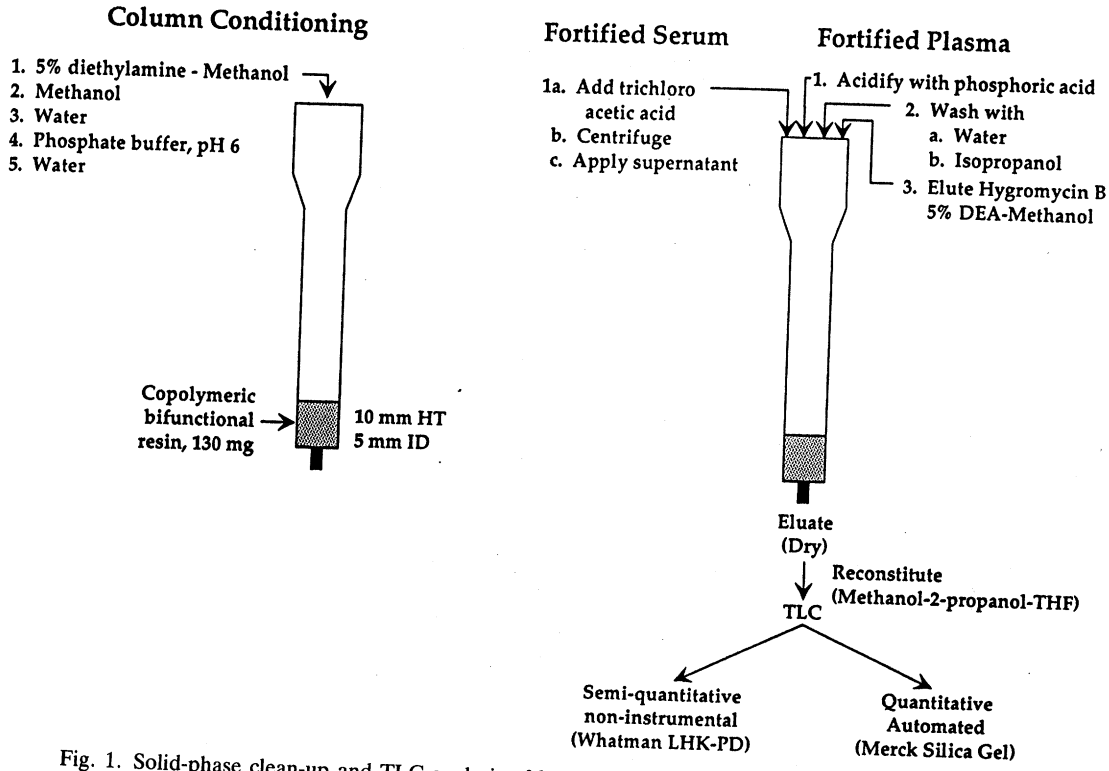
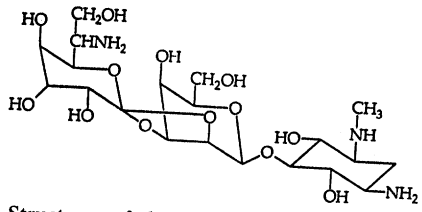


Fig. 1. Solid-phase clean-up and TLC analysis of hygromycin B in bovine plasma and swine serum.

action with fluorescamine to form fluorescent rivatives decreased with storage time. A loss 55 and 35% after 48 h was found for hy-omycin B concentrations of 10 and 40 μg/ml, spectively. Losses after 24-h storage ranged om 10 to 22% with the 10 μg/ml concentration owing the highest decrease in signal, demon-ating the instability of the compound. Losses re much higher in more dilute solutions sug-sting a reduction in the reactivity of the amino oups. In the studies presented here, fresh rking standard solutions were prepared daily from a 50 mg/ml stock solution which was stable for 2–4 weeks.



2. Structure of hygromycin B. Empirical formula: $H_{37}N_3O_{13}$. Molecular mass: 527.5.

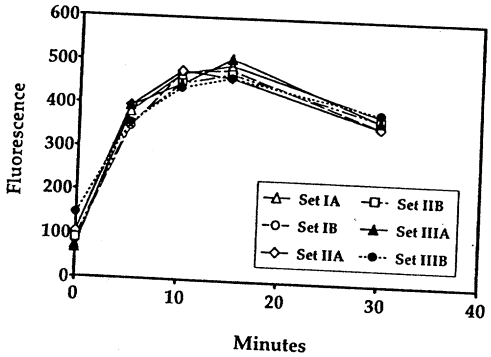


Fig. 3. Comparison of fluorescent signals of hygromycin B derivatized with fluorescamine in citrate buffer (pH 3) vs. time. Experimental conditions in 3 trials of duplicate analysis were: 1 mg/ml hygromycin B was derivatized with fluorescamine and the fluorophores were measured in solution at 395 and 480 nm excitation and emission respectively. Optimum fluorescence signals are found 15–25 min after derivatization.

Results showed that the highest recovery of hygromycin B was obtained by packing columns with 1.5 ml of a 5% resin suspension, applying 3 or 4 ml of hygromycin B (in citrate buffer) on the column and eluting hygromycin B with 0.4 ml of 5% diethylamine in methanol (Fig. 4). Hygromycin B (6.25 μg/ml) in 4 ml citrate buffer was quantitatively recovered when passed through columns containing 1.5 ml of 5% resin suspension (equivalent to 90 mg dry resin) and eluted with 0.6, 0.650 and 0.7 ml DEA-methanol (5:95, v/v). Densitometric scanning of the TLC plate showed a recovery of hygromycin B higher than 80%. Recoveries were slightly lower at 5–50 ng compared to the 1–10 μg range. Hygromycin B concentrations higher than 100 ng showed recoveries of >80% with 0.4-ml elution volumes. In later experiments, higher elution volumes were utilized to improve the recovery of trace amounts of hygromycin B. The minimum detectable amount of hygromycin B and the linearity of the calibration line were determined for solid-phase clean-up and TLC analysis of hygromycin B (Fig. 5). Samples containing added hygromycin B (0.1 μg/ml) were applied to the columns as 1, 2.5 or 7.5 ml volumes. The eluted hygromycin B was applied to the TLC plate in a range of 5–50 ng; the minimum detectability was 5 ng. The calibration lines showed regression coefficients (r^2) of 0.94 and 0.92 for peak heights (mm) and peak areas (meV), respectively. Analysis of 2.5, 5.0 and 25 ml of 0.1 μg/ml hygromycin B applied to the clean-up columns and TLC analysis of an equivalent of 12.5–125 ng of the eluted hygromycin B showed an improvement in linearity of the calibration line ($r^2 = 0.98$) when either peak area or peak height was measured. Again, the loss of hygromycin B at more dilute concentrations was apparent. In later experiments, with lower concentrations of hygromycin B, the elution volume was increased to a total of 2.5 ml of elution solvent applied in 0.5-ml increments to increase the recovery of hygromycin B.

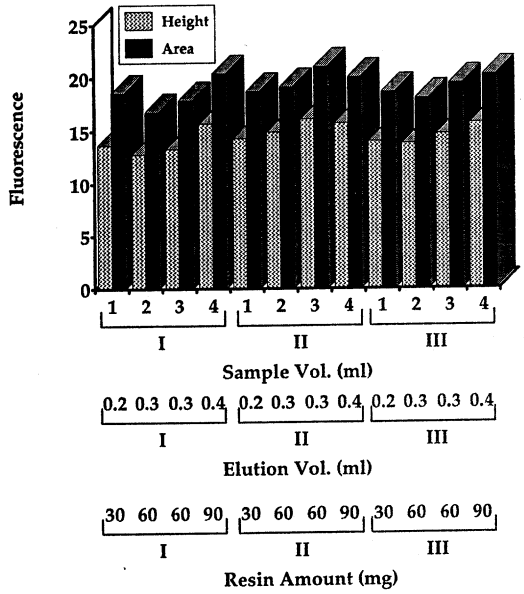


Fig. 4. Optimization of 3 conditions was simultaneously determined for isolation of hygromycin B: sample volume vs. amount of resin vs. volume of elution solvent. Experimental conditions were 25 μg/ml of hygromycin B in 1, 2, 3, and 4 ml of citrate buffer at pH 3 passed through 0.5, 1.0, 1.0 and 1.5 ml of 5% suspension of copolymeric resin and eluted with 0.2, 0.3, 0.3 and 0.4 ml of diethylamine–methanol, respectively. The combined effects of amounts of resin, sample volume and elution volume showed that the best recovery was attained with 3 or 4 ml sample application on 1 or 1.5 ml of resin suspension and elution with 0.4 ml of diethylamine–methanol.

In our early studies, citrate buffer was used to acidify hygromycin B standard solutions. Citrate buffer was not effective when used to acidify the animal serum due to the co-elution of sample contaminants that produce interfering bands in TLC. Removal of proteins from bovine plasma samples by precipitation with TCA or acetonitrile showed that hygromycin B could be detected when these compounds were removed to minimize background interference. Phosphoric acid was tested for acidification of bovine plasma; this had the additional advantage of not requiring a centrifugation step. Phosphoric acid treated bovine plasma was directly applied to the columns and the results showed detection of the added 25 ng/ml of hygromycin B. Fig. 6 shows the TLC results for hygromycin B in bovine

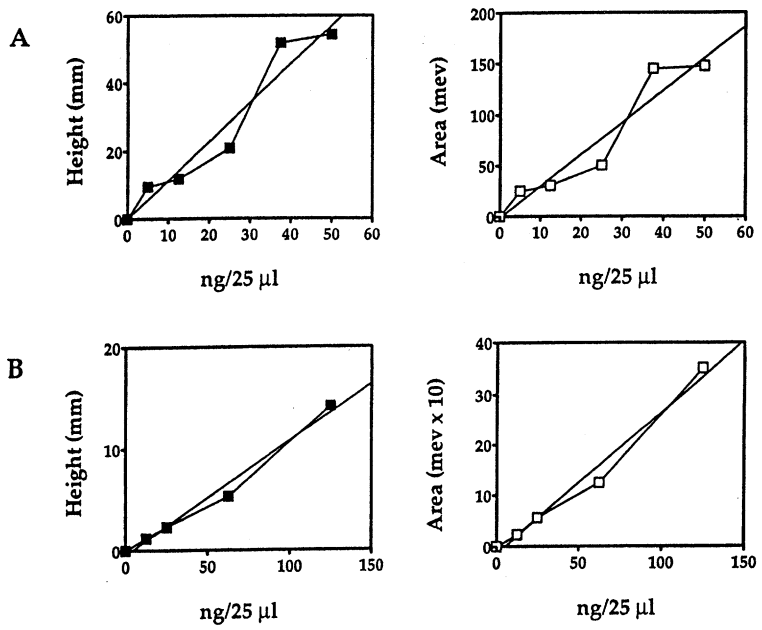


Fig. 5. Calibration curve for hygromycin B passed through the copolymeric resin and analyzed with TLC. Hygromycin B (0.1 μg/ml) in pH 3 buffer was applied to clean-up columns. (A) Sample volumes of 1, 2.5, 5 and 7.5 ml were used and 5–50 ng was quantitatively applied to TLC plates. (B) Sample volumes of 2.5, 5, 12.5 and 25 ml were applied to clean-up columns and an equivalent of 12.5–125 ng hygromycin B was analyzed by TLC.

plasma fortified at 100 and 250 ng/ml. Trace levels (< 100 ng/ml) of hygromycin B were not detected in fortified swine serum treated with phosphoric acid due to the interfering fluorescent background in TLC. Hygromycin B could be detected at concentrations of 100 and 250 ng/ml in swine serum after TCA precipitation and removal of the precipitates by centrifugation. Organic solvent precipitation of proteins using a 2:1 ratio of acetonitrile to sample removed most of the interfering background allowing detection of 50 ng/ml hygromycin B fortified in swine serum. Other approaches to the aqueous clean-up are being investigated on order to remove the interfering background, thereby improving the detection of hygromycin B in swine serum with TLC. We have investigated the use of a non-immuno affinity clean-up for the detection of hygromycin B and other aminoglycosides [16]. Results from this study showed single bands of hygromycin B in the TLC and no other interfering bands.

Aqueous solutions of gentamicin and neomycin at concentrations of 100 ng/ml were semi-quantitatively recovered from the solid-phase columns. The eluted compounds were detected when an equivalent of 50 ng was applied on the TLC plate. The TLC developing solvent used in this study separated gentamicin ($R_F = 0.64$), neomycin ($R_F = 0.56$), spectinomycin ($R_F = 0.52$) and streptomycin ($R_F = 0.20$) from hygromycin B ($R_F = 0.33$), thus allowing multiresidue separation of these aminoglycosides. The minimum detectable amount of hygromycin B and neomycin applied onto Whatman TLC plates is 50 ng, as shown in Table 1. Likewise, aqueous solutions of gentamicin and spectinomycin (100 ng/ml) were also detectable at 50 ng after TLC development and fluorescent derivatization. The solid-phase and TLC pro-

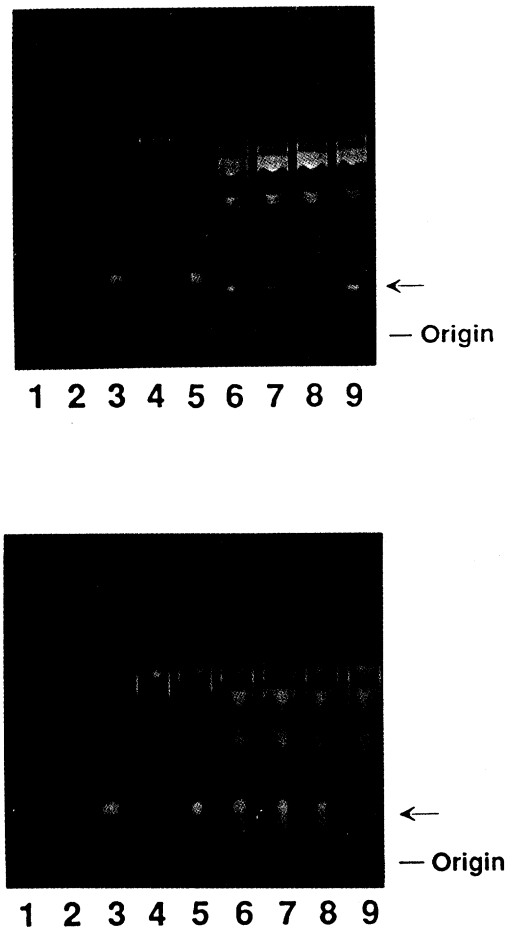


Fig. 6. (Top) Bovine plasma (2 ml) was fortified with 100 ng/ml hygromycin B; the mixture was acidified, purified through solid-phase columns and analyzed with TLC. Lanes 1–3: standard hygromycin B (75, 100, 200 ng) applied directly to TLC; lane 4: reagents passed through column; lane 5: 100 ng/ml hygromycin B in acid solution (2 ml) passed through column, 80 ng applied onto TLC plates; lanes 6–8: fortified plasma samples, 80 ng of estimated purified hygromycin B applied to TLC plate; lane 9: blank (unfortified) plasma passed through column. (Bottom) Bovine plasma (1 ml) was fortified with 250 ng/ml hygromycin B; the mixture was acidified, purified through solid-phase columns and analyzed with TLC. Lanes 1–3: standard hygromycin B (75, 100, 200 ng) applied directly to TLC; lane 4: reagents passed through column; lane 5: 100 ng of 250 ng/ml hygromycin B acid solution (2 ml) passed through column; lanes 6–8: 100 ng of purified hygromycin B fortified in plasma samples; lane 9: blank (unfortified) plasma passed through column.

cedures allowed detection of aminoglycosides in trace amounts after a concentration step of 5–10 × their original levels. Amine-containing interfering compounds were difficult to separate from the aminoglycosides when a higher concentration effect was required for trace analysis.

4. Conclusions

The extraction, isolation and TLC detection procedures described provide a rapid, simple and inexpensive semi-quantitative method for screening hygromycin B at low ng/ml levels in serum or plasma of bovine and swine. Hygromycin B fortified in bovine plasma and swine serum was detectable at the 50 ng level. Swine serum required deproteinization prior to isolation of hygromycin B on the copolymeric resin column. Acetonitrile deproteinization removed more interfering background than TCA in swine serum. Bovine plasma needed only minimal pretreatment, i.e. acidification with phosphoric acid prior to solid-phase clean-up of the fortified sample.

In this study, we have shown that contrary to literature reports on optimum derivatization of primary amino groups with fluorescamine, hygromycin B derivatives had a higher fluorescence in acidic solutions than in basic solutions. Use of acidic solutions increased the sensitivity of the TLC method by 75%, allowing detection at lower levels. The TLC developing solvent also separated other aminoglycosides (neomycin, gentamicin, streptomycin, spectinomycin) and will allow multiresidue detection of these compounds. This chromatographic method is rapid allowing analysis of 12 samples in an 8-h period. The method does not require sophisticated instrumentation and high technical skill. This approach can be used to identify or confirm results indicated positive by microbiological assay[1].

[1] Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 1
Detectability of neomycin and hygromycin B applied to TLC plates with preabsorbent zones, developed in acetone–ethanol–NH_4OH (1:1:1, v/v) and derivatized with fluorescamine

Neomycin/hygromycin B[a] (μg/ml)	Volume applied (μl)	Concentration (ng/lane)	Results
Trial 1			
1	25	25	–
2.5	25	62.5	+
5.0	25	125	++++
Trial 2			
0.5	50	25	–
1.0	50	50[b]	+
2.5	50	62.5	++
5.0	50	125	++++

[a] Samples contain equal amounts of neomycin and hygromycin B with R_F values of 0.56 and 0.33, respectively.
[b] Minimum detectablity for neomycin and hygromycin B.

Acknowledgement

The authors thank Love E. Jones for her technical assistance.

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