Determination of Streptomycin in Eggs Yolk by Capillary Electrophoresis

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Key Words

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Summary

A capillary electrophoresis method for the determination of streptomycin in eggs is described. Analyses were performed on an uncoated silica capillary using a buffer solution of 30 mM sodium dihydrophosphate, 5 mM boric acid and 5 mM sodium tetraborate. Analytes were detected at 200 nm an the calibration curve was linear over the range of 0.16 to 2.0 $\mu g\, g^{-1}$ (r = 0.999). The total analysis time was 7 min. The method has been successfully applied to the quantitative determination of streptomycin in hen eggs after drug ingestion and could used for evaluation of maximum residue limits.

Introduction

Streptomycin belongs to the group of aminoglycoside antibiotics that consist of a disaccharide molecule with a streptidine aminocyclitol moiety. Streptomycin was the earliest aminoglycoside introduced for clinical use in human and veterinary medicine. It is used in parental treatment of systemic bacterial disease of animals or in oral treatment of non-specific enteritis, especially in chickens [1]. Therefore, the use of is compound may result in residues in livestock products, particularly if proper withdrawal times for treated animals have not been practiced. These residues may pose a health threat to consumers, depending on the type of food and the amount of residue present. Antibiotic residues can be found in food products made from meat and their levels, must by quantified by law [2–6]. The Codex Committee on Residues of Veterinary Drugs determines priorities for the consideration of residues of veterinary drugs in foods and recommends Maximum Residue Limits (MRLs) for veterinary drugs. Streptomycin is poorly absorbed from the gastrointestinal tract and the majority of the oral dose is recovered in the faeces. Therefore the typical withdrawal period after intramuscular injection is 30 days and only 5 days after oral administration. Up till now no MRL values for streptomycin in eggs has been recommended [7–10].

Determination of streptomycin residues in animal tissues is a difficult task. In general, microbiological and chemical methods for the determination of antibiotics in animal tissues have been proposed. Microbiological techniques lack sensitivity and specificity, and have a more qualitative than quantitative character. So that chemical methods based on liquid chromatography are widely used. In the case of streptomycin analysis, these methods, as a rule, involved sample derivatization, in order to achieve UV or fluorescence detection, and reversed phase ion-paired LC steps. Thus they are complicated, time-consuming and also interference from other materials is frequent. Moreover, in chromatographic analysis biological samples requires extensive manual sample preparation [11–14]. Therefore, a capillary electrophoresis method seemed worth considering.

Particularly, difficult is analysis of antibiotics in eggs, because egg is not only nutritionally balanced but also a chemical storehouse. Egg yolk has been reported to contain very-low-density lipoprotein, phospholipids and proteins [15–18], which exclude most derivatization techniques.

The aim of this study was to develop a rapid and sensitive capillary electrophoresis assay for the determination of streptomycin in eggs.

Experimental

Reagents

Streptomycin was kindly provided by Pharmaceutical Enterprise "Polfa S. A. Tarchomin" (Warszawa, Poland). Ethyl acetate and acetonitrile was supplied by

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Merck (Darmstadt, Germany), ephedrine hydrochloride was purchased from Sigma (St. Louis, MO, USA). Sodium tetraborate decahydrate, sodium hydroxide and hydrochloric acid were products of POCh (Gliwice, Poland). Acetonitrile was purified by double distillation over anhydrous $CaCl_2$. The calibration of the pHmeter was done with standard buffer solutions purchased from POCh (Gliwice, Poland).

All aqueous solutions were prepared in triple distilled water and were filtered through a 0.45 μm membrane prior to use. Stock solutions of streptomycin and ephedrine hydrochloride, 100 μg mL^{-1}, were prepared in water.

Instrumentation

Analyses were performed on a Beckman P/ACE 2100 electrophoresis apparatus. The capillary cartridge contained a 75 μm ID unmodified silica capillary, which was 67 cm in total length and 61 cm (effective length) to the detector. A deuterium light source with a 200 nm bandpass filter was used and absorbance was monitored at a range of 0.04 AUFS. Data collection was accomplished by using Beckman System Gold software. The voltage was maintained at 25 kV, which gave a current of 140 μA. All experiments were performed at 25μC. The buffer solution was composed of 30 mM sodium dihydrophosphate, 5 mM boric acid and 5 mM sodium tetraborate. The samples were injected using pressure injection mode, typically 7 s at 3.45 k*P*a, at the anode end of the capillary, from a 30 μL sample volume located in the outer rotating tray.

Every new capillary was cleaned with 0.1 M sodium hydroxide for 20 min in order to rehydrate the fused silica material on the internal capillary wall, then with distilled water for 10 min and finally with buffer solution for 10 min. Between each injection, the capillary was regenerated by treatment with 0.1 M hydrochloric acid (0.5 min), to remove adsorbed eggs components, then with 0.1 M sodium hydroxide (2 min) and finally with distilled water (2 min). Prior to each analysis, the capillary was flushed with electrophoretic buffer for 2 min.

Procedure

The egg yolk samples (1 g) were transferred to a 10 mL centrifuge tube and spiked with 30 μL of internal standard (ephedrine hydrochloride, 100 μg mL^{-1}). Each tube was vortexed to mix the yolk with internal standard solution. Then 3 mL of acetonitrile was added and the tubes were shaken mechanically for 10 min to complete the process of deproteinization. After centrifugation for 10 min at 6000 g, the supernatant (acetonitrile phase) was transferred to a clean test tube and evaporated to dryness in a water bath at 60μC. The residue was suspended in 0.1 M sodium hydroxide and extracted with 3 mL of ethyl acetate. The ethyl acetate phase was evaporated to dryness. The residue was suspended in 0.5 mL 0.1 M hydrochloric acid and evaporated to dryness. Finally, the residue was suspended in 0.5 mL of buffer solution, centrifuged at 6000 g for 5 min and stored at –20μC until analysis.

Calibration procedure

The standard curve was established by plotting peak-height ratio (streptomycin/I.S.) versus streptomycin concentrations (μg g^{-1}) and was performed daily.

Using the standard solutions of streptomycin and ephedrine hydrochloride (I.S.), samples of blank control egg yolk were spiked with concentrations of streptomycin ranged from 0.16 to 2.0 μg g^{-1} and with a fixed concentration of internal standard (3 μg g^{-1}). All the samples were prepared and analysed using the procedures described above. A Calibration curve, based on the peak height ratios of streptomycin to I.S., was constructed using six different concentrations of streptomycin analysed six times for each concentration. The data were subjected to linear-regression analysis in order to achieve the appropriate calibration factors. The results are listed in Table I.

Results and Discussion

Typical electropherograms of blank egg yolk extract and extract spiked with streptomycin are shown in Figure 1. As can be seen, base line separation between streptomycin and internal standard (ephedrine hydrochloride) was achieved in a relatively short migration time. The migration times were 3.8 min and 4.6 min for ephedrine hydrochloride and streptomycin respectively. Buffer solutions of various compositions have been investigated in an attempt to separate analytes from interfering peaks. Phosphate-borate buffer solution was chosen as it gave rise to the best separation between streptomycin and internal standard as well as cutting off matrix peaks. The pH was then optimised, and the best separation was achieved at pH 6.35. The best results were obtained using a buffer solution prepared with 30 mM sodium dihydrophosphate, 5 mM boric acid and 5 mM sodium tetraborate. It has been previously found that borates added to aqueous solutions of mono- and oligosaccharides result in an increase of absorbance at 195 nm [19]. This phenomenon is due to complex formation between tetrahydroxyborate ion and polyols. The complex concentration increases with rising borate concentration according to the law of mass action as well as with rising pH due to the higher concentration of alkaline borate ions. Results achieved in this work generally confirm these observations also for streptomycin.

The results were linear over the range 0.16 to 2.0 μg g^{-1} of streptomycin. A straight line passing through the origin was obtained. The regression line calculated using the least square method was $y = 0.3\ (\pm\ 0.001) \times + 0.0012\ (\pm 0.001)$ with the correlation coefficient r = 0.9992 (Table I). Typical limits of detection (three times

Table I. Numerical data for calibration graph for streptomycin using the CE method with ephedrine hydrochloride as internal standard.

Substance	Amount added [$\mu g\ g^{-1}$]						
Streptomycin	0	0.16	0.4	0.8	1.2	1.6	2.0
Ephedrine hydrochloride	3	3	3	3	3	3	3
Series	H/Hw						
A	0	0.041	0.132	0.246	0.374	0.478	0.594
B	0	0.047	0.106	0.232	0.359	0.486	0.616
C	0	0.052	0.118	0.263	0.346	0.46	0.58
D	0	0.058	0.131	0.222	0.358	0.494	0.62
E	0	0.042	0.137	0.236	0.343	0.488	0.592
F	0	0.054	0.115	0.258	0.37	0.468	0.615
AVG	0	0.049	0.123	0.243	0.358	0.479	0.603

Regression equation H/Hw = 0.3 (± 0.001)x + 0.0012 (± 0.001) r = 0.9992

H – peak-high of streptomycin
Hw – peak-high of ephedrine hydrochloride (internal standard)
x – concentration of streptomycin [$\mu g\ g^{-1}$]
r – correlation coefficient

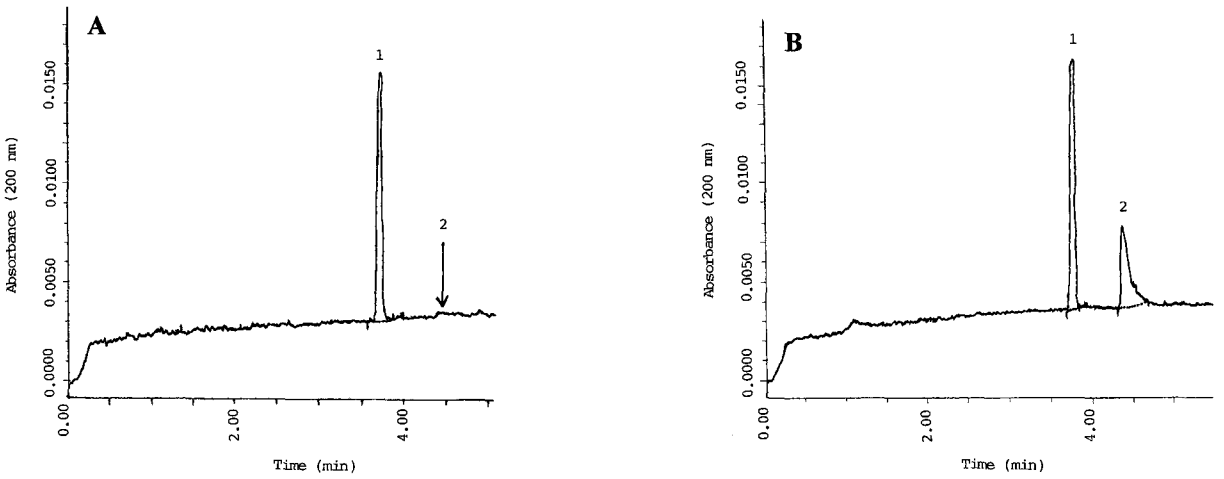

Figure 1
(A) Electropherogram of blank egg yolk extract spiked with ephedrine hydrochloride (1), internal standard, at 10 μgg^{-1}. (B) An egg yolk sample containing streptomycin (2) at 1.6 μgg^{-1} and ephedrine hydrochloride (1), internal standard, at 10 μgg^{-1}.
Separation conditions: applied voltage, 25 kV; 7-s pneumatic injection; unmodified silica capillary (67 cm × 75 μm I.D.) at 25 °C; running buffer solution composed of 30 mM sodium dihydrogenphosphate, 5 mM boric acid and 5 mM sodium tetraborate decahydrate; UV detection at 200 nm. After the elution of the peaks the egg proteins were removed by an acid wash.

Table II. Inter-assay precision for the CE method measured in six replicates. SD, R.S.D and BIAS denote standard deviation, relative standard deviation and bias % respectively.

Amount added [$\mu g\ g^{-1}$]	Amount found	SD [$\mu g\ g^{-1}$]	R.S.D. [%]	BIAS [%]
0.16	0.159	0.007	13.9	0.4
0.4	0.406	0.011	9.7	1.6
0.8	0.805	0.016	6.5	0.7
1.2	1.190	0.012	3.5	0.8
1.6	1.159	0.013	2.7	0.5
2.0	2.005	0.016	2.7	0.3

signal-to-noise) for the assay are 0.12 $\mu g\ g^{-1}$. The limits of quantification, defined as the lowest levels at which the assay was validated, are 0.16 $\mu g\ g^{-1}$.

The absolute recovery of streptomycin was determined by comparing the peak high of the extracted samples with the average peak high of unextracted streptomycin samples and was found to be 71.8 % ± 6.6 %. Precision of the assay, calculated as a relative standard deviation (RSD) for inter-assay variability ranged from 2.7 % for 2.0 $\mu g\ g^{-1}$ to 13.9 % for 0.16 $\mu g\ g^{-1}$. The numerical data are given in Table II.

This method was tested for a limited number of assays on streptomycin residual in hen eggs. The concentrations of streptomycin in eggs, given during four days, at dose of 75 mg kg^{-1} body weight were studied. Eight independent samples were evaluated. Residues in eggs were not found at a measurable level 5 days after feeding, with the exception of one sample, in which streptomycin was found at a level of 0.166 $\mu g\ g^{-1}$.

Because streptomycin lacks chromophores its analysis using HPLC is difficult. Most of the methods previously proposed require either pre or post column derivatization or application of a very specific mobile phase composition. These methods concern assays in a variety of biological matrices and therefore they are difficult to compare. The lowest quantification limit, 20 ppb (ppb = $1 : 10^{-9}$) was achieved for animal tissues [3] then, 25 ppb, for milk [2]. Both methods required laborious derivatization. Two other HPLC methods [4,5] with direct UV detection at 195 nm utilise acetonitrile-water (8:92, v/v) as a mobile phase. Under these circumstances the quantification limits were 500 ppb for human serum[4] and 250 ppb for assays in drugs [5].

One of the main advantages of capillary electrophoresis is the use of aqueous buffers. Hence it is possible to use short-wavelength UV detection. However, in general, the quantification limit is higher tahn for HPLC methods utilising derivatization. As was stated previously, egg yolk is a particularly difficult biological matrix.

Nevertheless, the quantification limit achieved in this work, 160 ppb, placed the result between indirect-detection and direct-detection HPLC methods. Under recent legislation on MRLs the value for streptomycin in eggs has not been set [20]. Current values, in ppb, for other materials are as follow; 200 for milk 1000 for kidney and 500 for muscle liver and fat [7,8]. Therefore, the method proposed in this work might be useful for temporary evaluation of streptomycin concentration in eggs. Moreover, capillary electrophoresis offers number of advantages such as simplicity in sample preparation, short analysis time (10 minutes) and low running cost.

Conclusion

This paper describes a relatively simple, rapid and accurate method for the determination of streptomycin in egg yolk. Time for total electrophoretic determination is less than 10 minutes. Moreover, since the method requires minimal quantities of reagents, the running costs are low.

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