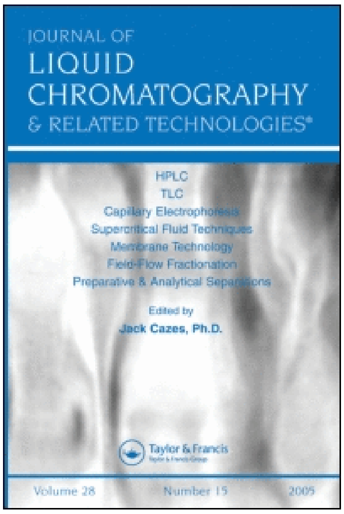

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SIMULTANEOUS ANALYSIS OF STREPTOMYCIN, DIHYDROSTREPTOMYCIN AND THEIR RELATED SUBSTANCES BY CAPILLARY ZONE ELECTROPHORESIS

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SIMULTANEOUS ANALYSIS OF STREPTOMYCIN, DIHYDROSTREPTOMYCIN AND THEIR RELATED SUBSTANCES BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

A capillary zone electrophoresis method was developed and validated for simultaneous separation of streptomycin (STM) and dihydrostreptomycin (DHS) with direct UV detection at 205 nm. Good selectivity was obtained within a short analysis time.

Electrophoresis conditions were as follows: 50 mM sodium tetraborate including 5% (v/v) methanol at pH 10.25; fused-silica capillary, 44 cm (effective length 36 cm) × 50 μm I.D.; voltage, 15 kV; temperature, 25°C. The method allows STM and DHS to be completely separated from their main related substances and each other.

The robustness of the method was examined by means of a full-fraction factorial design to test the influences of buffer pH, concentration of buffer and of methanol. The parameters for validation such as linearity, precision, limit of detection, and limit of quantitation are also reported.

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INTRODUCTION

Streptomycin (STM) and dihydrostreptomycin (DHS) are two members of the aminoglycosides antibiotics (AGs), which are widely used against both gram-positive and gram-negative bacteria. STM is often produced by direct fermentation processes. Most of the DHS is derived from STM. Commercial samples of both STM and DHS contain several related substances. Figure 1 shows chemical structures.

Recently, the perspectives of LC analysis of AGs were reviewed by R. Tawa et al.[1] Streptomycin and dihydrostreptomycin have been analyzed by ion-pair reversed phase chromatography with UV detection.[2-4]

Capillary electrophoresis (CE) is a powerful separation and quantitation technique that often provides higher resolving power, shorter analysis time, and lower operational cost than LC. In recent years, two papers described the use of CE with indirect or direct UV detection for the separation of different AGs, one from another.[5,6]

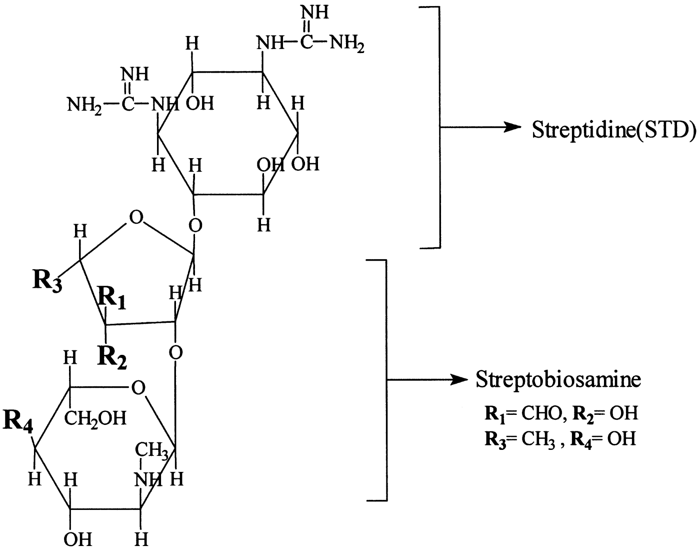
Another paper described the determination of the residual quantity of streptomycin in bulk dihydrostreptomycin with a micellar system consisting of myristyltrimethylammonium bromide in a borate buffer. The use of this cationic surfactant necessitated reversal of polarity. Other, unidentified compounds, were present in a dihydrostreptomycin standard.[7] Streptomycin was also determined in egg yolks by capillary zone electrophoresis (CZE) with a mixed phosphate-borate buffer.[8]

The present paper reports a CZE method using direct UV detection for the analysis of STM and DHS. It enables simultaneous separation of STM, DHS, and their main related substances within 10 min. The robustness of the method was examined by applying a full-fraction factorial design. The most important parameters governing the separation (such as buffer pH, the concentration of buffer and of organic modifier) were investigated. Quantitative data are reported.

EXPERIMENTAL

Materials and Reference Samples

All reagents were of analytical grade (Acros Organics, Geel, Belgium or Merck, Darmstadt, Germany). The fused silica capillary was from Polymicro Technologies (Phoenix, AZ, U.S.A.): 44 cm (effective length 36 cm) × 50 μm I. D. Throughout the study, Milli-Q water was used (Millipore, Milford, MA, U.S.A.). All the solutions were filtered through 0.2 μm nylon filters (Euroscientific, Lint, Belgium).



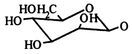
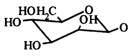
	R_1	R_2	R_3	R_4
Streptomycin (STM)	CHO	OH	CH_3	OH
Dihydrostreptomycin (DHS)	CH_2OH	OH	CH_3	OH
Hydroxystreptomycin (HOSTM)	CHO	OH	CH_2OH	OH
Dihydrodeoxystreptomycin (DHDS)	CH_2OH	H	CH_3	OH
Mannosidostreptomycin (STM B)	CHO	OH	CH_3	
Mannosidodihydrostreptomycin (DHS B)	CH_2OH	OH	CH_3	

Figure 1. Chemical structures of DHS, STM and their related substances.

The house standards for STM and DHS were available in the laboratory. STM sulfate and DHS sulfate were obtained from VMD (Arendonk, Belgium) and Dopharma (Raamsdonksveer, The Netherlands), respectively. Dihydrodeoxystreptomycin (DHDS) was prepared according to Ikeda et al.,[9] mannosidodihydrostreptomycin (DHS-B) by sodium borohydride reduction of streptomycin B (STM-B),[2,10] and streptidine (STD) by acid hydrolysis of DHS.[11] Hydroxystreptomycin (HOSTM) was from Abbott Laboratories (Chicago, IL, U.S.A.).

Instrumental and Operating Conditions

CE experiments were carried out on Spectraphoresis 1000 equipment (Thermo Separation Products, Fremont, CA, U.S.A.), which was driven by CE software (version 3.0.1) operating under IBM OS/2 ™ (version 1.2).

The vacuum system of the instrument applies a constant negative pressure of 0.75 psi for sample injection. Hydrodynamic injection was performed for 4 seconds during the method development and robustness study.

UV detection was at 205 nm. pH measurements were performed on a Consort C831 pH-meter (Turnhout, Belgium). The pH of buffers was adjusted using 1 M NaOH before making up to volume. All samples were dissolved in water. The capillary was washed at the beginning of the day with 0.1 M NaOH for 5 min at 60°C followed by a water wash for 5 min at 60°C. Before every analysis, the capillary was washed for 2 min with running buffer.

Software

The set-up of the applied full fraction factorial design, together with the analysis of the measured response variables and the multivariate regression calculation, was supported by the statistical graphics software system 'STATGRAPHICS' version 6.0 (Manugistics, Rockville, MD, USA).

RESULTS AND DISCUSSION

Method Development

Method development was performed with an artificial mixture containing STD, DHDS, DHS, STM, and HOSTM (see Figure 1). DHS B and STM B were not available in sufficient amounts to be included in the mixture and, therefore, were only used in the final stage. Since selectivity depends to a great extent on the type of buffer, three different buffers, namely sodium carbonate, sodium phosphate, and sodium tetraborate, were first compared. Each was prepared at a concentration of 50 mM, pH 10.5.

The first two buffers did not separate several components of the mixture and the baseline was unstable when carbonate was used. Subsequent experiments were performed with sodium tetraborate, not only because it performed best of all three buffers, but also because detection sensitivity was higher due to formation of complexes between borate and the compounds.[12]

The next step was the optimization of the pH of the background electrolyte. In preliminary experiments, pH was varied between 4 and 11. It was found that only at a pH exceeding 10, separation of all compounds could be obtained.

The most important separation DHS/STM showed to be best at a pH of 10.75. Then, the influence of pH was examined from 9.75 to 10.75 (steps 0.25). The pH 10.25 was retained because it gave a better separation for all compounds and during the run samples were stable in this alkaline buffer.

The effects of concentration of buffer (30-60 mM, steps 10 mM), of organic modifiers such as acetonitrile (0-15%, steps 5%) and methanol (0-15%, steps 5%) were also investigated. Instrumental parameters such as capillary

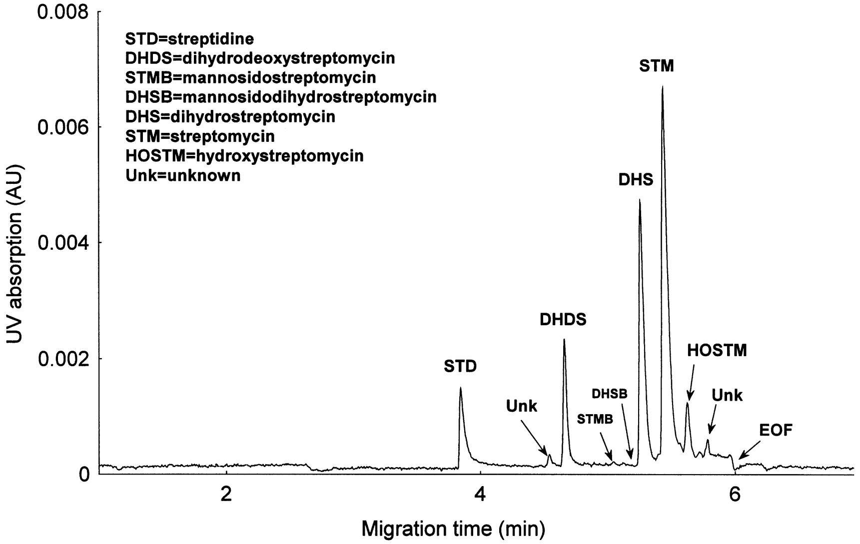


Figure 2. Electropherogram of a spiked STM sample. Running buffer, 50 mM sodium tetraborate and 5% methanol adjusted to pH 10.25; voltage, 15 kV; temperature, 25°C; detection wavelength, 205 nm.

temperature and applied voltage were optimised. Finally, chosen electrophoresis conditions were as follows: running buffer: 50 mM sodium tetraborate and 5% methanol adjusted to pH 10.25; voltage, 15 kV; temperature, 25°C; detection wavelength, 205 nm.

Figure 2 shows a typical electropherogram of a spiked STM sample. All the compounds are positively charged since they migrate faster than EOF. This means that the extent of negative charge imparted to the molecule by borate complexation does not exceed the two positive charges of the guanidine groups. A pKa value of 13.6 has been reported for guanidine groups.[13]

A comparison with the performance of LC[4] shows that the CZE method can obtain a similar separation and takes much less time than LC. The latter analysis lasted about 1 hour.

Method Robustness

Robustness is an important aspect of method validation. One evaluates the influence of small changes in the operating or environmental conditions of the analytical procedure on measured or calculated responses. The changes introduced when performing a robustness test reflect the changes that can occur when a method is used in different laboratories, by different experimenters, or using different equipments.[14]

Experimental designs involve simultaneous alteration of all parameters according to a predefined matrix of experiments. Among them, full-factorial designs are well adapted to the determination of the relative importance of each variable in comparison to the estimated responses. As a matter of fact, in a full-factorial design, l^n experiments are needed to evaluate the influence of n variables at l levels.

In this study, the influence of each of three relevant electrophoretic parameters governing the separation process, was examined by applying a full-fraction factorial design at two levels. This involves $2^3 = 8$ different experimental measurements. One central point combination was included in the design and so 9 measurements had to be performed as well as duplicate experiments 10-18.

Randomization of runs was performed by the software system 'STATGRAPHICS'. One injection was done for each experiment and experiments were duplicated using the same buffer solution. In this work, response variables were analyzed ignoring interactions greater than two. The system would, thus, only estimate interactions between two parameters. The standard error was calculated using all degrees of freedom that remain after the system has determined the desired effects.

Table 1

Values Corresponding to -1, 0 and +1 Levels

Electrophoretic Parameter	Lower Value (-1)	Central Value (0)	High Value (+1)
Buffer pH	10.0	10.25	10.5
Concentration of methanol (%)	2.5	5	7.5
Concentration of buffer (mM)	40	50	60

The three relevant electrophoretic parameters examined as variables were: (1) buffer pH; (2) concentration of methanol; (3) concentration of buffer. The values for the design are given in Table 1. As response variables in the factorial design, migration time and selectivities among important compounds were measured, namely STD, DHDS, DHS, STM, and HOSTM (only part of these data are shown). STM B was not checked because it was not available in sufficient quantities. Analysis of the measured response variables permitted the obtaining of standardized pareto charts.

A standardized pareto chart consists of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. The codes A, B, and C correspond to the buffer pH, the concentrations of methanol and of buffer, respectively. The combination of two codes indicates the interaction effect between the two parameters. The bars are displayed in order of the size of the effects, with the largest effects on top. The chart includes a vertical line at the critical t-value for an alpha of 0.05.

Effects for which the bars are smaller than the critical t-value are considered as not significant and thus not affecting the response variables. Effects may be positive or negative.

Figure 3 shows influences of the parameters on the selectivities (a) between DHS and STM ($\alpha_{D\text{-}S}$); and (b) between STM and HOSTM ($\alpha_{S\text{-}H}$). The related substance HOSTM was chosen because it migrates most closely to the main component. From Figure 3a, it can be seen that the buffer pH has a positive influence on $\alpha_{D\text{-}S}$. This means that an increase in pH improves the selectivity $\alpha_{D\text{-}S}$. The buffer concentration and its interaction with pH (AC) have significant negative influences. The former means that an increase in buffer

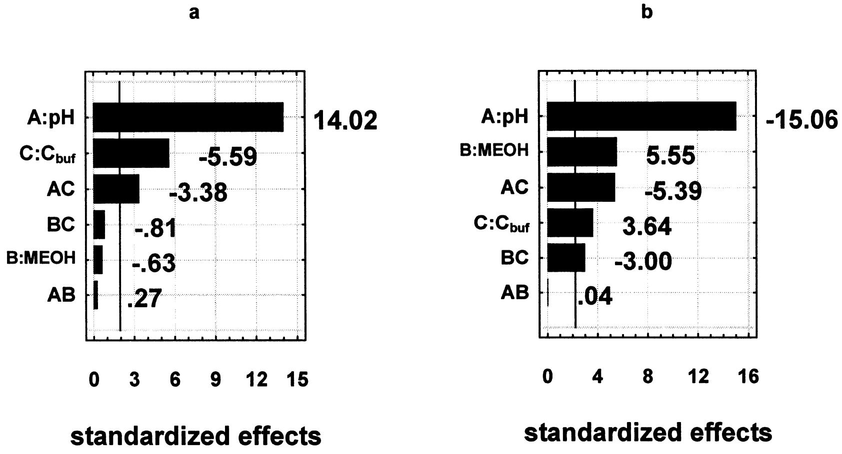


Figure 3. Standardized pareto charts, representing the estimated effects of parameters and parameter interactions on the selectivities (a) between DHS and STM ($\alpha_{D\text{-}S}$) and (b) between STM and HOSTM($\alpha_{S\text{-}H}$).

concentration decreases $\alpha_{D\text{-}S}$. The latter means that a change in pH affects $\alpha_{D\text{-}S}$ more at a lower buffer concentration than at a higher buffer concentration.

The effect of pH on $\alpha_{S\text{-}H}$ is opposite. Figure 3b shows that the pH has a significant negative influence on $\alpha_{S\text{-}H}$: decreasing the pH improves the selectivity $\alpha_{S\text{-}H}$. Both the concentrations of buffer and of methanol have positive influences. The interaction effect between buffer pH and concentration (AC) is also negative for $\alpha_{S\text{-}H}$. The interaction between concentrations of methanol and of buffer is also significantly negative, however, it is less important.

In order to estimate better the influence of the most important parameters, response surface plots were constructed. This type of graph plots response variables *versus* two variable parameters in a three-dimensional space.

Figure 4 shows how the migration times of compounds DHS and STM vary as a function of the concentrations of methanol and buffer (these two parameters are the most important for the migration time of DHS and STM, data not shown). The buffer pH was kept constant at 10.25. Since the planes never overlap, it can be concluded that selectivity between the two most important compounds is consistent within these boundaries.

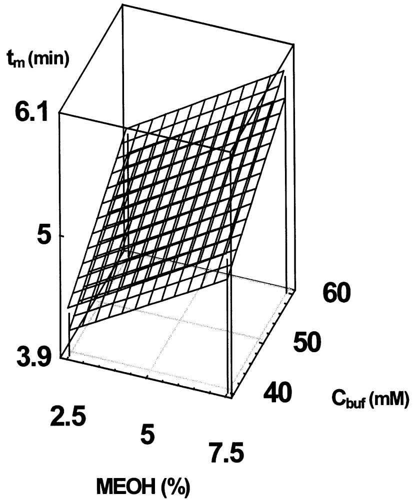


Figure 4. Estimated response surface plot for migration time (t_m) of DHS (lower plane) and STM (upper plane). The buffer pH was kept constant at 10.25.

Quantitative Analysis

The quantitative features of this method were examined and the results are shown in Table 2. Repeatability studies were performed with a 1 mg/mL solution being injected for 4 s, which is suitable for assay of STM or DHS. In limit of detection (LOD) and limit of quantitation (LOQ) tests, a solution of STM (2 mg/mL) and DHS (2 mg/mL) were diluted gradually. The solutions with a dilution of 0.18% for DHS and STM relative to the originally injected solution, and 0.5% for DHS and STM relative to the originally injected solution, correspond to the LOD and LOQ, respectively.

When injecting a 2 mg/mL solution of STM, it was noticed that the small impurity between STM and HOSTM, starts to co-migrate with STM. The injection volume was about 17 nL for LOD and LOQ. Because DHS and STM lack chromophores, their LOD and LOQ are rather high.

Table 2

Quantitative Features for Dihydrostreptomycin and Streptomycin

Parameter	
Within-day repeatability (n=6)[a]	
Migration time	RSD = 0.91% (DHS) 0.92% (STM)
Corrected area	RSD = 0.61% (DHS) 0.49% (STM)
Day-to-day repeatability (n=18, three days)[a]	
Migration time	RSD = 0.98% (DHS) 1.01% (STM)
Corrected area	RSD = 2.8% (DHS) 4.3% (STM)
Linearity y = corrected area, x = sample concentration in mg/mL range = 0.40 - 1.60 mg/mL	DHS: $y = 2409.5x - 142.1$, $r = 0.9996$, $S_{y,x} = 49.2$ STM: $y = 2236.0x - 114.9$, $r = 0.9996$, $S_{y,x} = 45.4$
Number of concentrations = 5, total number of analyses = 15	
LOD (S/N = 3)[b]	for both DHS and STM: 61.2 pg (0.18%, relative)
LOQ, RSD = 11.3% (n=8)[b] (DHS) 14.2% (n-8)[b] (STM)	for both DHS and STM: 170 pg (0.5%, relative)

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[a] 1 mg/mL, hydrodynamic injection, 4 s. [b] 2 mg/mL, hydrodynamic injection, 10 s.

CONCLUSION

A CZE method was developed for separation of STM and DHS from their related substances. The separation is much faster than that of LC and selectivity is similar to LC.[3,4] A full-fraction factorial design pointed out that the pH of the background electrolyte has the largest effect on the selectivity of the most critical separations. The response surface plot showed that the separation of the main compounds STM and DHS was robust.

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