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A direct HPLC method to estimate streptomycin and its putative ototoxic derivative, streptidine, in blood serum: Application to streptomycin-treated humans

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Abstract

Streptomycin is an aminoglycoside antibiotic with a well-known antituberculosis activity; it is commonly used in clinical practice because it is effective and cheap. However, streptomycin has severe ototoxic effects. The delayed and gradual onset may suggest that a metabolic derivative of the antibiotic could be a potential contributor to ototoxicity. As in a rat experimental model this compound was found to be streptidine, we investigated whether this ototoxic metabolite was also present in the blood of streptomycin-treated patients. To this end, we implemented and optimized a direct reverse-phase HPLC technique to identify and estimate streptomycin and streptidine in serum of streptomycin-treated patients. All criteria for validation of the method were implemented in standard curves in serum of healthy non-treated volunteers by addition of increasing concentration of both compounds and their determination in a trichloroacetic acid deproteinized extract. We found that recovery of streptomycin or streptidine was ≥91.5%. Linearity was $r^2 \geq 0.99$. The intraday and interday precisions were ≤9.7 and ≤10.6%, respectively. The relative intraday and interday error ranged from −9.0 to 8.3% for both compounds in human serum. Studies in patients included five male individuals treated from 35 to 90 days with 1 g/day of streptomycin, presenting inner ear malfunction from mild to severe, in whose serum streptidine was always present, and could be successfully separated from streptomycin. Therefore, the validated method used can be a valuable tool to measure and follow these compounds in serum of streptomycin-treated patients.

Keywords: Human serum; Streptidine; Streptomycin; Streptomycin metabolism

1. Introduction

The onset of ototoxic side effects of aminoglycosides is delayed and gradual. Thus, some investigators have postulated a metabolic transformation or activation to produce a molecular species derived from the antibiotic which may show greater toxicity than the drug itself, or alternatively these derivatives may act in combination with the antibiotic, producing the ototoxic effect [1]. Studies with kanamycin [2], gentamicin [3,4] and streptomycin (STP) [5] have been performed, but the resulting metabolites have not been identified and characterized.

In pigmented rats using a direct HPLC method, we have demonstrated the presence of streptidine (STD) *in vivo* following chronic treatment with STP [6]. Further, we showed that chronic treatment with STD, as assessed by swimming behaviour observations, produces disruption of utricular organ-related vestibular function earlier described by us in rats treated with STP [7–9]. These effects of STD on swimming are similar as those produced by STP, even when the dose of STD was only 10% that of the STP dose [6].

The putative source *in vivo* of STD is hydrolysis of STP in the rat blood. A similar metabolic transformation has been previously suggested for other aminoglycosides [1,3,4].

These findings, in concert with a similar specific decrease in the number of sensory cells in the utricular macula produced in either STP-treated animals or by a lesser dose of STD [6], sup-

Abbreviations: EDTA, ethylenediaminetetraacetic acid; PAR, peak area ratio; %R.S.D., relative standard deviation percent; STD, streptidine; STP, streptomycin; TCA, trichloroacetic acid

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port our hypothesis that blood-borne STD is the major ototoxic agent producing disruption of vestibular function in STP therapy [10]. Other studies have demonstrated that this antibiotic accumulates in the perilymph and remains there for a short time period (0–4 h) after which it is excreted [11,12].

The *in vivo* presence of STD in the blood of STP-treated animals contradicts the assertion that STP, and possibly other aminoglycoside antibiotics, are excreted in urine essentially unchanged [13]. This finding also corroborates the observation of specific ototoxic effects of STP without any nephrotoxic effects reported for other antibiotics [14,15] as the general health of the animals in our study was preserved.

To establish whether a hydrolytic mechanism for STP may be present in human blood, in preliminary experiments we incubated *in vitro* the antibiotic in human serum, and developed an extraction procedure and a high performance liquid chromatography method to separate, identify and measure directly STP and its metabolite, STD. Indeed, *in vitro* hydrolysis of STP to its derivative STD was demonstrated [16].

The HPLC technique used in the above mentioned study in deproteinized human serum samples was essentially as described by Granados et al. [16] and Granados and Meza [6] for human and rat serum samples. STP is currently measured using microbiological turbidimetric assays or thin-layer chromatography described in the *British Pharmacopoeia* [17] or a sole microbiological assay used by the *USP Pharmacopoeia* [18]. Because these methods do not offer the necessary combination of speed, specificity, simplicity, sensitivity and precision there is a need for a direct method.

In the present study, we investigated whether a similar hydrolytic mechanism is present in serum from patients being treated with STP that could give rise to STD by enzymatic hydrolysis. We used the direct isocratic HPLC method described by us in the separation and estimation of STP and STD in human serum [16] and rat serum [6].

In this report, we implemented and optimized the method reported by Granados and Meza [6] to separate and estimate STP and STD in a serum deproteinized extract which will be ideal for clinical studies that require a rapid, sensitive and reproducible determination of STP and its putative ototoxic metabolite, STD, in low amounts after treatment with the antibiotic in human serum.

2. Materials and methods

2.1. Reagents

Reference standards used were streptomycin sulphate from Sigma (St. Louis, MO, USA) and streptidine sulphate prepared from streptomycin sulphate (above) as previously described [19]. Sodium 1-hexanosulfonate was from Regis (Morton Grove, IL, USA); tribasic sodium phosphate, acetonitrile HPLC grade and phosphoric acid were from Mallinckrodt Baker, S.A. (México D.F., México). Monobasic potassium phosphate, trichloroacetic acid and EDTA were from Sigma. Water used was double distilled in an all-glass still after passage through an ion-exchange column.

2.2. Equipment and HPLC conditions

The chromatographic system consisted of a pump Waters 600E (Waters Assoc., Molford, MA, USA) equipped with an autosampler Waters 717plus and a PDA detector Waters 996. The analytical column was a Prodigy column ODS3 reversed-phase, 5 μm, 250 mm × 4.6 mm (Phenomenex, Torrance, CA, USA). The column flow-rate was maintained at 1.3 mL/min. The column temperature was set to 25 °C (±0.1). The detector wavelength was of 200 ± 0.2 nm. Upon completion of daily analysis, the column was washed with a mixture of acetonitrile:water (65:35).

The mobile phase for measuring STP and STD contained buffer (20 mM sodium 1-hexanesulfonate and 25 mM tribasic sodium phosphate, pH 6.0, solvent A) and acetonitrile (solvent B) (85:15, v/v). The pH solution was adjusted with phosphoric acid (85%) and filtered through a 0.22 μm filter (Millipore Corp., Bedford, MA) prior to use.

2.3. Separation and estimation of streptomycin and streptidine in human serum

2.3.1. Human serum

Human serum samples were obtained from STP-treated patients provided *by* the Department of Tuberculosis of the National Institute of Respiratory Diseases (INER), Mexico, and from healthy non-treated volunteers supplied by a certified clinical analysis laboratory. Patients and volunteers were between 25 and 55 years of age.

2.4. Extraction procedure

The extraction was implemented in serum from healthy volunteers to which increasing concentrations of STP from 10 to 80 μg/mL or STD from 2 to 16 μg/mL were added. They were deproteinized by addition of 20% trichloroacetic acid (TCA, 120 μL/mL of serum). Supernatant was separated from precipitate by centrifugation at 14,000 rpm for 40 min. A volume of 20 μL of each solution was injected and analyzed for 9 min.

2.5. Extraction of STP and STD in patient serum for HPLC determination

Human serum samples from STP-treated patients were treated as in the above paragraph. These deproteinized supernatant samples were used for the HPLC analysis. Concentrations of STP and STD were calculated from standard curves of STP and STD in healthy human serum.

2.6. Stability

For demonstrating the stability of standards in serum extracts of two different concentrations of STP (15 and 65 μg/mL) and for STD (4 and 14 μg/mL) were stored at 4 °C for 48 h or for a week. Samples were compared against freshly prepared 100% controls analyzed in the same analytical run in replicates of six.

2.7. Assay validation

2.7.1. Linearity

Linearity was calculated by determination of six incubations at six different concentrations of STP (between 10 and 80 μg/mL) and six different concentrations of STD (from 2 to 16 μg/mL) in healthy human serum, both compounds either together or separately.

2.7.2. Percent recovery and precision

Extraction efficiency was estimated by comparing replicate peak area ratios (PARs) of extracted human serum *versus* non-extracted standard solutions in phosphate buffer (50 mM; pH 6.8) for 15, 35 and 65 μg/mL concentrations of STP and 4, 8.4 and 14 μg/mL concentrations of STD. The relative standard deviation percent (%R.S.D.) was determined by calculating from standard deviation means of six replicate samples × 100/the mean of six replicate samples.

The precision was determined over concentration ranges of 10–80 μg/mL (STP) and 2–16 μg/mL (STD). For intraday precision the assay was replicated six times. Interday precision was performed for six replicate samples on three different days, following the above procedure.

2.7.3. Accuracy

It was determined over concentration ranges of 10–80 μg/mL (STP) and 2–16 μg/mL (STD) in healthy human serum from six different runs performed in six replicates of three different days. The mean value of the three runs was calculated and compared with the spiked value (amount added) to determine the percentage difference between both, and the percent of bias was calculated: %Bias = [(measured value − actual value)/actual value] × 100.

2.7.4. Specificity

Specificity of the method was investigated by analysis of four independent sources of the blank samples, which consisted of a healthy human serum sample without the addition of STP or STD and checking for interference by endogenous matrix components.

2.7.5. Limits of detection and quantification

Limits were estimated from a signal-to-noise ratio in the region where STP and STD appear in four different blank samples. A limit of detection from a signal-to-noise of 3:1, and a limit of quantification from signal-to-noise of 10:1 were obtained.

3. Results and discussion

3.1. Percent recovery

Peak area ratio values of extracted serum standards and standard buffer solutions were quantitative (>99%) from both compounds for all concentration ranges in either sample matrix (Table 1). Therefore, the rest of parameters analyzed are trustful for STP and STD.

3.2. Stability

Stability of STP after storing at 4 °C for 48 h or a week showed that the concentrations of this compound was ≥95% compared with freshly samples with the same concentrations of STD and STP analyzed in the same analytical run. The concentrations of STD after storing at 4 °C for 48 h measured ≥98%, however, after storing at 4 °C for a week a decrease of 20% was demonstrated. Consequently determination of STD in patient serum should be done within a week.

4. Method validation

4.1. Successful separation of STP from STD to discern between both compounds

The HPLC technique satisfactorily separated STD from STP in a mixture of both compounds. Retention times were around 4.01 ± 0.17 min for STD and 6.10 ± 0.45 min for STP (Fig. 1B).

4.1.1. Linearity

The calibration curves for STP and STD showed linearity within six different concentrations between 10 and 80 μg/mL (STP) or between 2 and 16 μg/mL (STD) in human serum. The linear regression equations of calibration curves by adding

Table 1
Streptidine and streptomycin recovery ($n = 3$)

Solution	STD (μg/mL)		Recovery (%)	STP (μg/mL)		Recovery (%)
	Expected	Found		Expected	Found	
Serum	4	4.58	114.5	15	14.13	100.9
	8.4	8.34	99.2	35	34.44	98.4
	14	14.17	101.1	65	65.17	100.2
Mean ± S.D.			104.93 ± 6.80			99.83 ± 1.05
Buffer	4	4.49	112.2	15	14.79	98.6
	8.4	8.38	99.7	35	34.56	98.7
	14	13.78	98.4	65	65.85	101.3
Mean ± S.D.			103.43 ± 6.22			99.53 ± 1.24

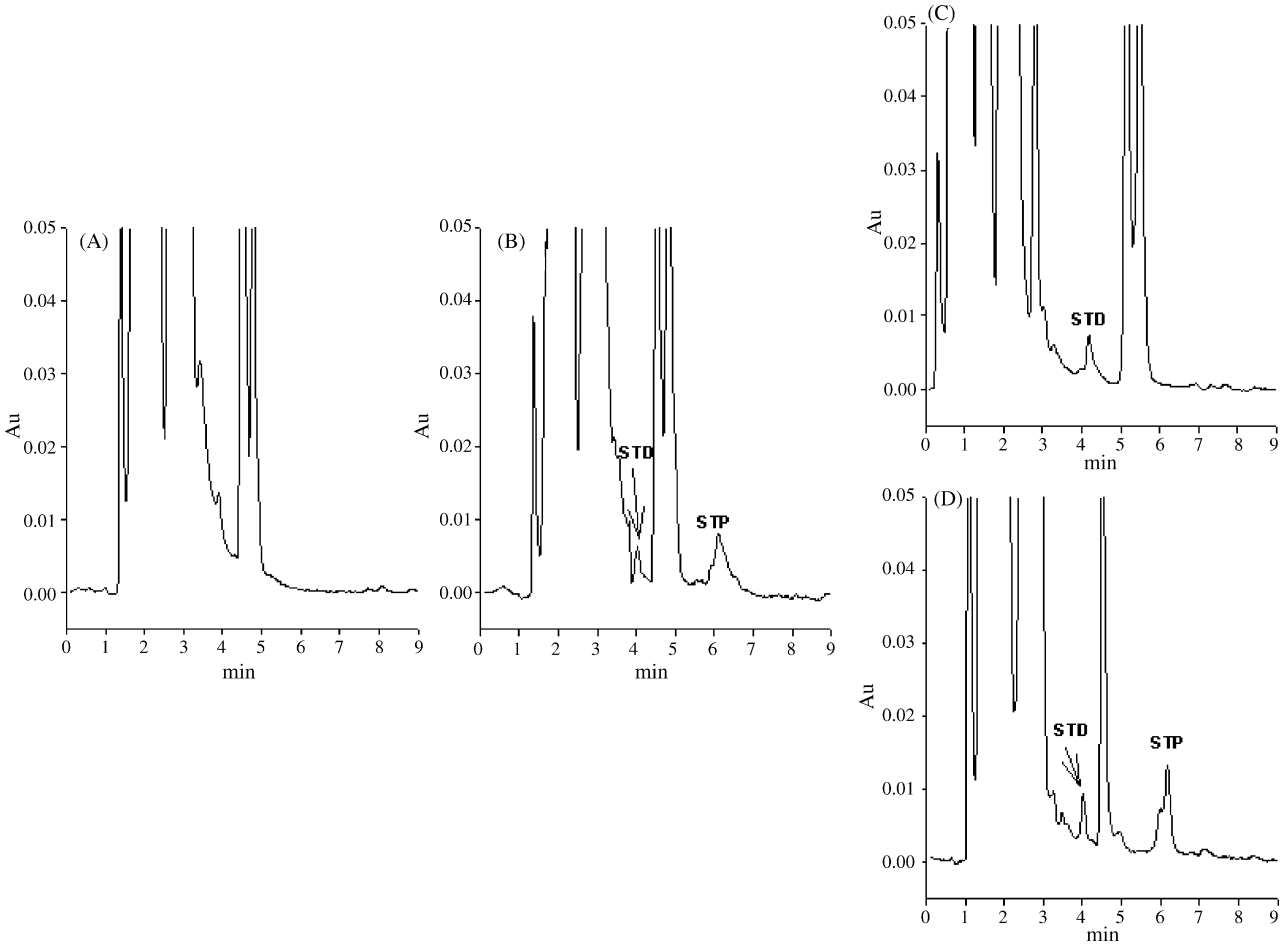

Fig. 1. Typical chromatograms from STP-treated patients: (A) blank human serum extract; (B) human serum standard containing 2 μg/mL of STD and 10 μg/mL of STP; (C) STP-treated patient after 88 days of treatment, who only presented the signal of STD; (D) STP-treated patient that, presented both STD and STP after 35 days of treatment. Separation was done using a Prodigy ODS3 column and a mobile phase of buffer:acetonitrile, pH 6.0 (85:15, v/v), at a flow rate of 1.3 mL/min. Retention times for STD and STP were approximately 4.01 and 6.10 min, respectively. Au: arbitrary units.

standards to human serum after precipitation of proteins were $y = 1039.73x + 2377.73$, $r^2 = 0.992$, and $y = 1409.88x - 358.93$, $r^2 = 0.995$ for STP and STD, respectively. This finding is important because it has earlier been reported that after intramuscular administration of STP (1 g), the concentrations of the antibiotic in the human blood is between 20 and 50 μg/mL [20].

4.1.2. Precision

The intraday and interday precision data for STP and STD, respectively, are listed in Table 2. The intraday %R.S.D. values were ≤6.4 and 9.7% for STP and STD, respectively. The interday precision was ≤10.6% for STP and ≤5.0% for STD. These results imply that the variability coefficient between samples is excellent since they do not go beyond 10%.

4.1.3. Accuracy

The accuracy analyses for the above data for STP and STD, respectively, are shown in the same Table 2. The relative intraday and interday errors ranged from −9.0 to 8.3% for STP and STD in human serum. Therefore, our estimates are within the expected values of the concentration range of both compounds, as we can observe in %recovery in all concentration ranges in both compounds (see last column of Table 2).

All parameters of our method validation agreed with specifications for a validation of chromatographic methods in biomedical analysis [21], where each parameter of validation of the STP or STD in a biological fluid (human serum) was ≤15%.

4.1.4. Specificity

Fig. 1A shows a typical chromatogram of a blank sample (healthy human serum), where interference for detecting STD and STP by endogenous matrix components was not observed. This observation was seen in four independent blank samples, which indicates that the specificity of the method for estimating STD and STP in human serum is satisfactory.

4.1.5. Limits of detection and quantification

Limits of detection (signal-to-noise ratio of 3) were 0.5 and 1.2 μg/mL for STP and STD, respectively, and the limit of quantification (signal-to-noise ratio of 10) for STP was 3 and 1.9 μg/mL for STD using an injection volume of 20 μL. The limits of quantification reported by Whall were 2.0 and

Table 2
Intraday and interday assay precision and accuracy for STP sulfate and STD sulfate in human serum ($n = 3$)

	Concentration (μg/mL)	Intraday precision (%R.S.D.)	Intraday accuracy (%Bias)	Interday precision (%R.S.D.)	Interday accuracy (%Bias)	Recovery (%)
STP	10.0	6.4	4.1	10.6	8.3	108.3
	20.0	5.1	−3.6	2.5	−3.6	96.3
	30.0	6.4	5.4	8.3	−5.5	94.5
	40.0	4.6	−1.5	2.7	−4.1	95.8
	50.0	4.2	4.8	4.3	2.7	102.8
	80.0	2.7	0.4	3.2	0.2	100.2
Mean ± S.D.						99.6 ± 4.78
STD	2.0	8.7	−9.0	3.1	−8.5	91.5
	5.0	7.9	1.0	5.0	3.6	103.8
	7.0	7.5	5.4	2.0	4.5	104.5
	10.0	7.0	−3.9	4.8	1.2	101.2
	13.0	9.7	−4.8	4.5	0.8	100.8
	16.0	4.5	1.4	1.7	−1.0	99.0
Mean ± S.D.						100.1 ± 4.28

0.5 μg/mL of STP and STD, respectively, in a non-biological sample (aqueous solution) using an injection volume of 25 μL [22]. Kurosawa et al. reported a detection limit of 2.0 μg/mL for STP in human serum using a 100 μL aliquot injection volume of supernatant [23]. Furthermore, our HPLC method is significantly more sensitive, requires less sample and permits the identification and measurement of STD, a metabolic derivative by enzymatic hydrolysis of STP in human serum of STP-treated patients.

In addition, this method includes a very simple deproteinization of serum using 20% TCA, and a non-derivatized HPLC technique that takes only 10 min, reducing the time of analysis significantly. It is also sensitive and accurate and can be used for pharmacokinetic studies as well as for routine monitoring of STP and STD in serum during treatment of patients. Other HPLC methods for determination of STP in human serum are time consuming, require either pre-treatment of the sample [24] or the use of post-column derivatization with ninhydrin [24]. A fluorescence polarisation immunoassay has been also developed [25], but none of them discern STP from STD.

4.2. Determination and estimation of STD and STP in STP-treated patients

To confirm the clinical applicability of the method, serum samples were obtained from patients undergoing STP therapy and they were analyzed by the proposed method.

In Fig. 1C and D, typical chromatograms of the serum extract of STP-treated patients are shown. In (C) a patient with 88 days of treatment with STP that only presented the vestibulotoxic metabolite, STD, while STP was absent. In (D) a STP-treated patient (35 days of treatment), who presented both compounds, the STP peak ($T_R = 6.10$ min) and a small peak, with the same retention time as standard STD peak ($T_R = 4.01$ min).

The STP and STD concentrations derived from five male STP-treated patients are presented in Table 3. We observed in all these patients that STD is released from STP starting on the 35th day of the treatment with the antibiotic and that its concentration increased with time. The presence of STP was only observed in two patients. Although a deeper analysis of these results, as a function of the state of ototoxicity, falls beyond the intention of this study, it is clear that: (1) the deleterious action of both compounds depends on the day of treatment with STP, and (2) some patients are prone to show only vestibular damage symptoms while others only present damage of audition processes and that STP and STD seem to act in concert.

As to the application of the technique, our results show that STD can be released from STP by hydrolysis in human serum after prolonged STP administration and its concentration can be

Table 3
STD and/or STP found in serum of STP-treated patients[a]

Patient	Age	Days of STP treatment	Symptoms	STD (μg/mL)	STP (μg/mL)
1	44	35	Absent	5.34 ± 0.17	8.04 ± 1.10
2	45	45	Vertigo	1.30 ± 0.18	↓LD
		88	Vertigo	4.78 ± 0.30	↓LD
3	51	60	Loss of high frequencies	1.61 ± 0.13	↓LD
4	51	90	Bilateral mild hearing loss	4.17 ± 0.14	↓LD
5	37	90	Vertigo	3.26 ± 0.24	4.57 ± 0.41

[a]Data are mean values of six-time repeat of the same sample ± S.D. ↓LD: below limit detection.

followed with time. We hereby demonstrate, for the first time, a possible metabolite (STD) of the antibiotic, possibly contributing to its ototoxicity.

5. Conclusion

We have shown that STP can undergo some metabolic transformations in the blood of patients under treatment with the antibiotic by a simple HPLC technique. The method involves a very simple protein extraction of serum to estimate STP and STD in human serum. Even though no internal standard was used in the method, precise results were obtained in one step. The present work also demonstrates that this analysis, requiring only 20 μL of patient serum, is sensitive and accurate.

Therefore, this method can be used for routine therapeutic monitoring after STP administration and the appearance of its ototoxic metabolite (STD) along time of treatment in serum of STP-treated patients. These observations stress and summarize the importance of our method to detect the metabolic product (STD) of STP in order to prevent (further) ototoxicity in patients treated with STP and suggesting the interruption or continuation of the treatment, or even the replacement by other therapeutic scheme.

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