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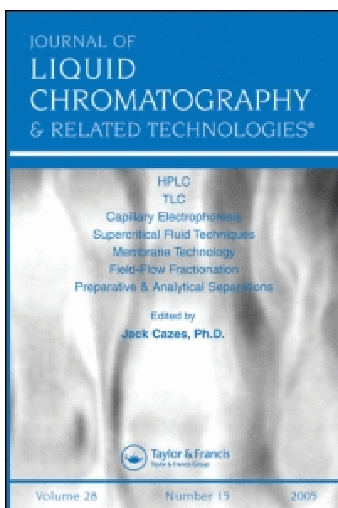

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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A Sensitive High Performance Liquid Chromatography Assay for Trospectomycin. An Aminocyclitol Antibiótic, in Human Plasma and Serum

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To cite this Article Simmonds, R. J. , Wood, S. A. and Ackland, M. J.(1990) 'A Sensitive High Performance Liquid Chromatography Assay for Trospectomycin. An Aminocyclitol Antibiótic, in Human Plasma and Serum', Journal of Liquid Chromatography & Related Technologies, 13: 6, 1125 — 1142
To link to this Article: DOI: 10.1080/01483919008049238
URL: http://dx.doi.org/10.1080/01483919008049238

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JOURNAL OF LIQUID CHROMATOGRAPHY, 13(6), 1125-1142 (1990)

Original Article

A SENSITIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR TROSPECTOMYCIN, AN AMINOCYCLITOL ANTIBIOTIC, IN HUMAN PLASMA AND SERUM

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ABSTRACT

This report describes a sensitive, selective and robust assay for the quantification of trospectomycin, an aminocyclitol antibiotic, in human plasma and serum.

This is the first published High Performance Liquid Chromatography (HPLC) bioanalytical method for a member of this class of compound.

The method involves the selective solid phase extraction of 6'-n-propyl spectinomycin and the internal standard 6'-n-butyl spectinomycin from 0.5 ml of biofluid, efficient reversed phase high pressure liquid chromatography with post column oxidation, reaction with o-phthaldialdehyde and fluorescence detection. The limit of quantification from 0.5 ml of biofluid is 10 ng/ml.

INTRODUCTION

Trospectomycin (6'-n-propyl spectinomycin sulphate), a new analogue of spectinomycin, is a broad spectrum antibiotic for

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use against both Gram negative and Gram positive bacteria. A specific assay for trospectomycin was required to support human volunteer and patient trials. A sensitivity of 20 ng/ml was necessary, to enable pharmacokinetic analysis and routine application, as more than five thousand samples had to be processed.

Trospectomycin is an aminocyclitol antibiotic, and has the structure shown in fig. 1.

HPLC methods exist to assay bulk drug formulations of an aminocyclitol antibiotic, namely spectinomycin itself (1,2), but these could not be adapted easily for bioanalytical purposes, being limited by the sensitivity of electrochemical detection (1) or by the use of complex precolumn derivatisation procedures unsuitable for complex biofluids (2). Spectinomycin has also been measured in fermentation broths by reversed phase HPLC with a two stage post column reaction and fluorometric detection (3). The potential of this approach has not been exploited for bioanalysis, possibly because it was not possible to obtain more efficient chromatography reproducibly without using amine modifiers in the mobile phase (which would interfere with detection) and the need to optimise the post column reaction system so that low nanogram amounts of analyte can be quantified.

Aminocyclitol drugs do present a considerable challenge in HPLC method development. Their polar nature and instability at high pH have repercussions upon both extraction from the biological matrix and the methods possible for achieving efficient chromatography. The lack of a strong chromophore or electroactive group makes sensitive detection problematical. These unhelpful characteristics have so far prevented the development of a sensitive HPLC method for this class of compound. We here report the first example of a viable

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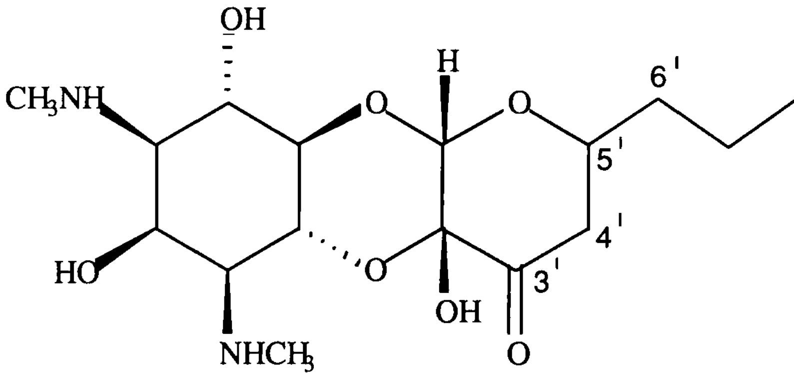

FIGURE 1

Structure of Trospectomycin, 6-n-propyl Spectinomycin.

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bioanalytical assay, capable of routine operation with ng/ml sensitivity.

Selective solid phase extraction, in contrast to liquid/liquid extraction, was effective at physiological pH, and efficient reversed phase chromatography without the addition of a competing amine was carried out by using a highly acidic mobile phase. This allowed post column oxidation of the secondary amine group and subsequent derivatisation with o-phthaldialdehyde to a highly fluorescent product. The reliability of this procedure was optimised by using a commercially available post column reaction system (PCRS). Any small variations remaining in the method were compensated for by including a purpose made analogue of trospectomycin as an internal standard.

MATERIALS

Chemicals and Reagents:

Trospectomycin, 6'-n-propyl spectinomycin sulphate, was obtained from the Upjohn Company, Kalamazoo, USA.. The internal standard, 6'-n-butyl spectinomycin hydrochloride, was synthesised in the Pharmaceutical Research Laboratories, Upjohn Ltd., Crawley, UK.. O-phthaldialdehyde and mercaptoethanol were obtained from Sigma Chemical Company Ltd., Poole, UK.. Boric acid and potassium hydroxide (Analar grade), trifluoroacetic acid (TFA) and sodium hypochlorite, 12 % w/v (Spectrosol grade), were obtained from BDH Ltd., Poole, UK.. Methanol and acetonitrile (HPLC grade) were obtained from Fisons, Loughborough, U.K.. Double glass distilled water or equivalent was used for all reagents, solutions, and the mobile phase.

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Equipment:

Analytichem Bond Elut C18 (ODS) cartridges, and Vac-Elut ten-place vacuum manifolds were obtained from Jones Chromatography (Hengoed, UK.). A Denley (Billingshurst, UK.) bench top centrifuge, model BM 402, was used for preparation of plasma and any subsequent centrifugation of samples.

METHODS

Preparation of Standard Solutions for Calibration Curve and Internal Standard:

Trospectomycin and internal standard were dissolved in water to give solutions of c.a. 1 mg/ml. These stock solutions

were stored at -20°C, and replaced monthly. Aliquots of trospectomycin stock solution were diluted with water to give solutions in the range 1-100 μg/ml, which were used for spiking aliquots of control plasma or serum. Aliquots of internal standard stock solution were diluted to 25 μg/ml. Dilute solutions were stored at 4 C and prepared weekly.

Preparation of Reagents for Post-Column Reaction:

Boric acid (93 g) was dissolved in 5 litres of water. Approximately 75g of potassium hydroxide was added with stirring to bring the pH to 10.2. This solution was then used to prepare the oxidising solution (reagent A) by diluting 10 ml of sodium hypochlorite solution to one litre, and derivatising solution (reagent B), by dissolving 800 mg of o-phthaldialdehyde in 40 ml of methanol, diluting to one litre, and adding 1 ml of mercaptoethanol. Reagents were degassed by sparging with helium, and used within 24 hours.

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Extraction of Plasma and Serum:

Plasma, with heparin as anticoagulant, or serum, whether control samples or from a clinical study, were stored at -20 C before analysis. After thawing, samples were inspected: those with particulate or agglomerated material were centrifuged for one minute. Only clear biofluid was extracted. Aliquots (0.25-0.5 ml) were transferred to small test tubes and spiked with 25 μl of internal standard solution, and diluted with water to 1 ml.

Bond Elut cartridges were primed successively with 2 ml acetonitrile, 2 ml of aqueous 60% acetonitrile, 0.2 % TFA and 2 ml water. Prepared samples were passed slowly through the

cartridges, which were then rinsed with 2 ml water and 2 ml 50 % methanol/water. Cartridges were partially dried by drawing through air for 20-30 seconds. Trospectomycin and the internal standard were eluted with 0.6 ml of an aqueous solution of 60 % acetonitrile, 0.2 % TFA. After elution, the pressure in the Vac-Elut manifold was further reduced in order to evaporate eluates to 0.2 ml *in situ*.

HPLC

HPLC was carried out using a Waters (Watford, UK.) 510 twin piston pump, Waters WISP injector, Shimadzu (Houghton-Le-Spring, UK.) CTA 60 column oven, and a Perkin Elmer (Beaconsfield, UK.) LS1 fluorescence detector. Data were collected and processed using a Spectra Physics (Hemel Hempstead, UK.) SP 4270 integrator. The analytical column was a Dupont Zorbax (Hichrom, Reading, UK.) C8, 250 * 4.6 mm I.D., maintained at 45 C. The mobile phase was 0.5 % TFA in 16 % acetonitrile/water, flow rate 1.5 ml/ min.. Between 25 to 150 µl of cartridge eluate was injected.

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Post Column Derivatisation and Detection:

Two Kratos (Spectrochrom Ltd., Litchborough, UK.) Spectraflow 400 twin piston pumps (reagent pumps) and a Kratos PCRS 520 temperature control system were used and set up as shown in Fig. 2. Reaction coil 1 was 2.0 ml in volume, maintained at 75 C. Reaction coil 2 had a volume of 0.5 ml, and was maintained at room temperature. Both reagent A and reagent B were pumped at 1.0 ml/min.

Fluorescent species in the eluate were detected with the fluorimeter set at 340 nm (excitation) and 462 nm (emission).

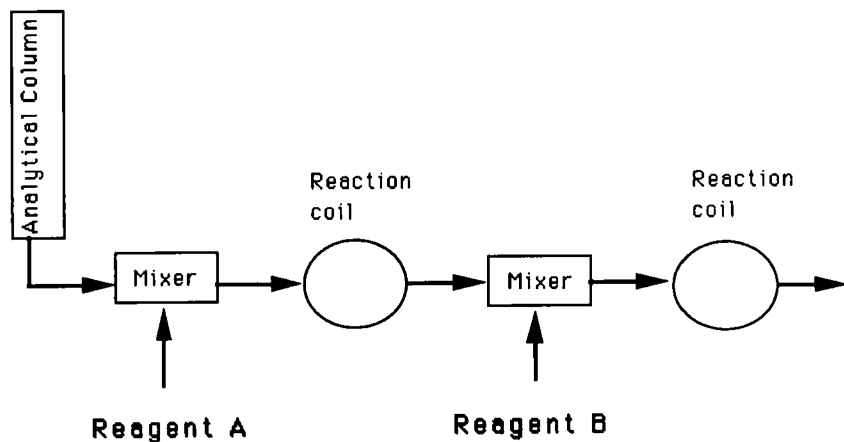

FIGURE 2

Post Column Reaction System

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Analyte peaks were automatically integrated. Trospectomycin concentrations were calculated from standard curves produced by programmed SP 4270 software.

RESULTS AND DISCUSSION

HPLC and Post Column Reaction:

Trospectomycin and 6'-n-butyl spectinomycin were detected as sharp peaks at 7-8 and 13-16 minutes respectively with an apparent retention values of three (trospectomycin) and six (internal standard) (fig. 3). Accurate assessment of retention

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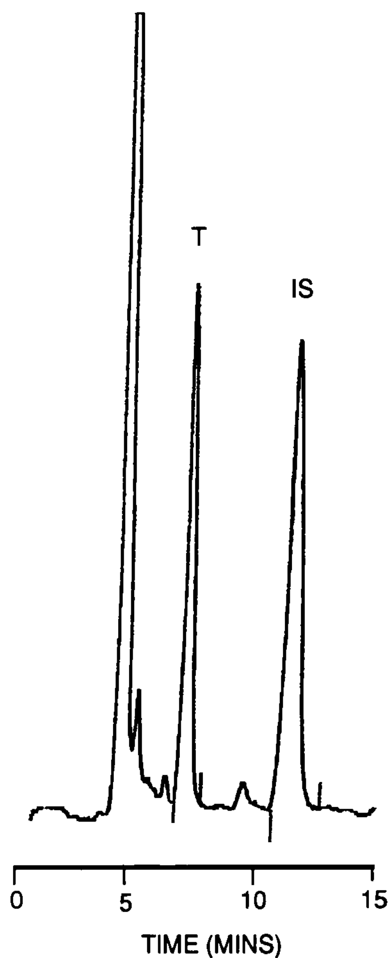

FIGURE 3

HPLC of Trospectomycin (T) and 6-n-butyl spectinomycin (IS). Conditions as text. Chromatogram of extract of plasma spiked with 5μg/ml of both analytes. 50μl injected.

is not possible because of the large "dead volume" associated with the PCRS. Efficiency was of the order of 3 - 5,000 theoretical plates on column, as measured with trospectomycin, and it is evident that the PCRS, which features knitted Teflon reaction coils, causes little peak spreading.

Other stationary phases were tried, including Spherisorb phenyl and Microsorb C18, and similar efficiency and retention was seen. However, Zorbax C8 was found to be the most robust in routine use. Lower concentrations of TFA in the mobile phase were associated with a loss of efficiency and peak shape; 0.5 % was optimum in practice. Despite the highly acidic mobile phase, no degradation of the packing material was seen. A guard column was not found necessary, and, with occasional flushing with 80 % acetonitrile/water, no loss of performance was observed over a period of two months continuous use.

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Despite the complexity of the system, baseline stability was good in terms of short term and long term noise. Long term drift, once the system had been equilibrated, was not a problem and represented less than 5 % of full scale deflection over a period of 24 hours.

Post column reaction and detection were repeatable, within a run and between runs. The PCRS reagents were sufficiently stable to enable analytical runs of over 24 hours (70-100 samples), and their preparation was not critical. The concentrations of the reagent solution, the flow rates chosen, the volume of the reagent coils and the temperature of oxidation were all chosen to be non critical, and were not necessarily optimum for the greatest sensitivity. Nevertheless, 2 ng of trospectomycin could be detected on column, and this represents a practicable limit of detection of the complete system.

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Extraction from plasma and serum

A range of reversed phase and polar bonded phase was tested, with a variety of rinsing steps and elution solvents. TFA was necessary in the final step to ensure elution from the sorbent. Greater than 50 % methanol could be used in the cartridge rinse step with selective elution of biofluid components and no loss of analytes. Extracts of control plasma and serum gave very clean chromatograms and peaks of endogenous origin interfering with either trospectomycin or the internal standard were not seen, even at high sensitivity (fig. 4). No long running peaks were seen in extracts, which were stable in an autosampler for at least 24 hours. The whole extract (c.a. 0.2 ml) could be injected without loss of chromatographic performance.

The extraction procedure proved rapid and reliable. Recovery of trospectomycin and the internal standard, estimated by comparing peak heights generated by unextracted standards and standards extracted from plasma or serum, was reproducible, and at least 90 % even at the lowest concentrations. The non critical nature of extraction procedures enabled up to 100 plasma or serum samples to be processed in a batch. The partial evaporation of cartridge eluates caused no problems such as the loss of analytes, but complete evaporation and redissolution in HPLC mobile phase did result in apparent variations in the recovery of trospectomycin at concentrations above 5 $\mu g/ml$.

There was no readily available compound with the correct properties to act as an internal standard, and mimic trospectomycin's idiosyncratic behaviour in extraction and reversed phase HPLC. Other analogues of trospectomycin were tried: 6'-n-pentyl spectinomycin eluted just after trospectomycin on HPLC, but analogues with ethyl, propyl, butyl

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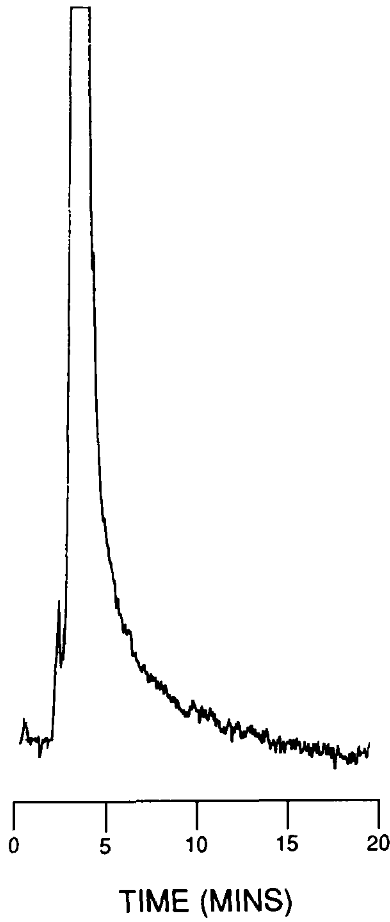

FIGURE 4

HPLC of extract of control plasma. Conditions as text, 100 μl injected.

and pentyl moieties substituted in the 3' position showed unexpected low retentions with HPLC. The internal standard finally chosen, 6'-n-butyl spectinomycin, behaves well during extraction from plasma or serum, but retention is rather longer than optimum, though the peak runs well clear of any interferences.

Assay characteristics were very good, despite the possible variability introduced by the complex post column reaction system. Graphs of peak height ratio (trospectomycin/internal standard) were linear over the range 0.025 μg/ml to 15 μg/ml trospectomycin (r greater than 0.999 for twenty points). Replicate calibration graphs of seven points in duplicate, in the range 0.1 to 10 μg/ml, gave an average value for r of 0.9996 (fig. 5), with a coefficient of variation for slope of 3%. Satisfactory precision (C.V. less than 10%) was shown throughout the concentration range. Precision at low levels was measured using quality control (QC) samples prepared independently by spiking control plasma to give a nominal trospectomycin concentration of 22 ng/ml (fig. 6). Within run and between run variation was 5 % and 3.7 % C.V. respectively, measured over 13 routine analytical runs. A limit of quantification of about 0.01 μg/ml (C.V. of better than 15 %) was seen in practice (fig. 7). Sensitivity can be improved by increasing the volume of biofluid extracted (fig. 8). The assay was used routinely at a contract facility over a period of months and several thousand samples. No problems were found, either in extraction or HPLC.

The method can be modified to measure trospectomycin in plasma from other species, such as the rat or dog, and with minimal changes to measure other biofluids, such as urine, or even for tissue residues. Little or no interference to the assay has been seen, either from compounds endogenous to biological samples or from possible degradation products of

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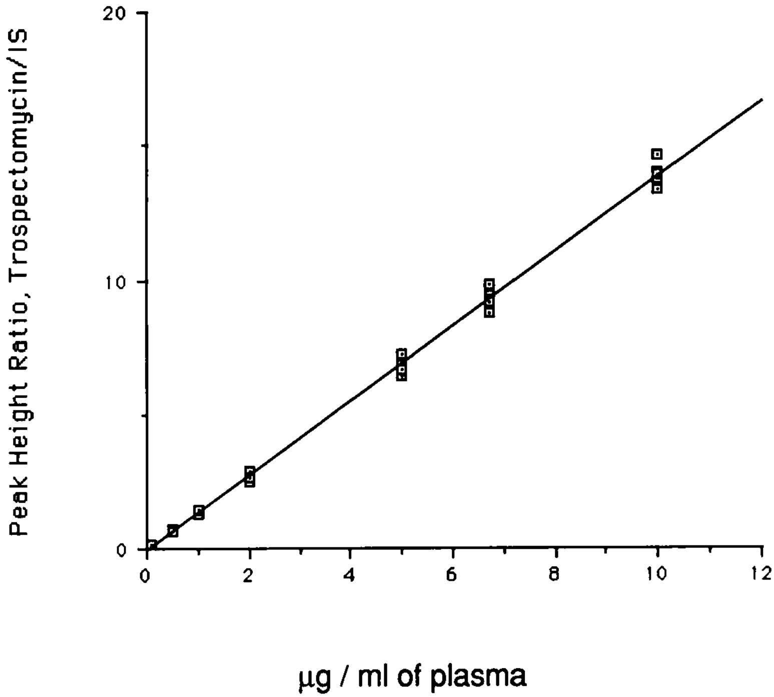

FIGURE 5

Calibration curve, spiked concentration vs peak height ratio. All results from three consecutive curves.

r = 0.999 slope = 1.390 int. = 0.0045

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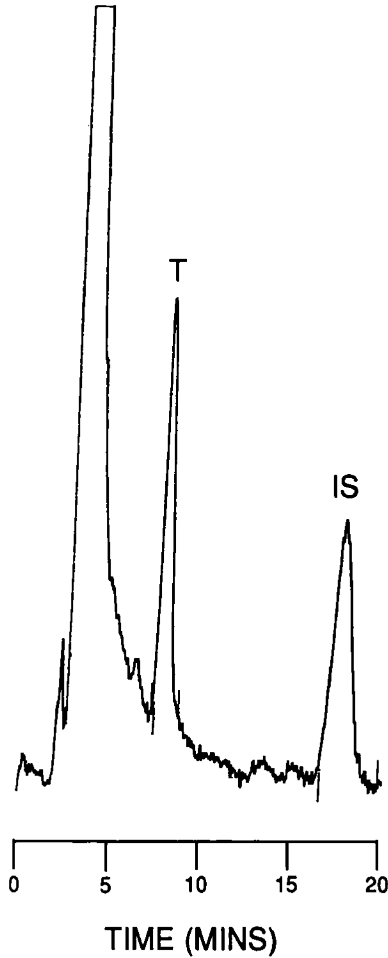

FIGURE 6

HPLC of extract of QC sample, Trospectomycin peak (T) represents 22 ng/ml. 100 µl injected.

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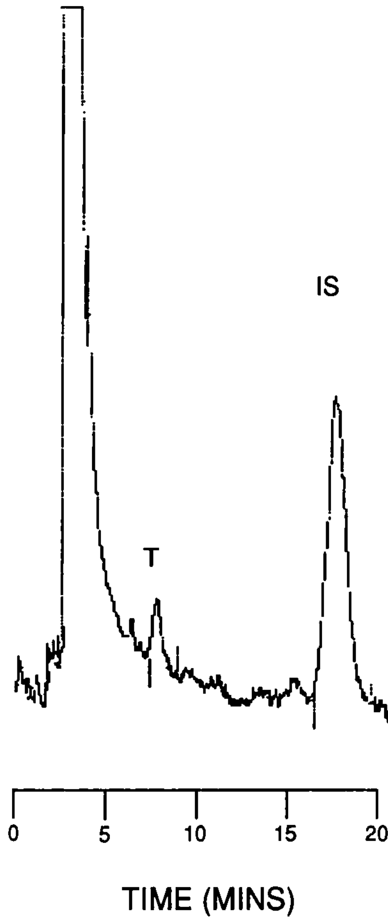

FIGURE 7

HPLC of human volunteer study sample. Extract of 0.5 ml of plasma sampled 72 hr after 75 mg IM dose. Trospectomycin represents 13 ng/ml.

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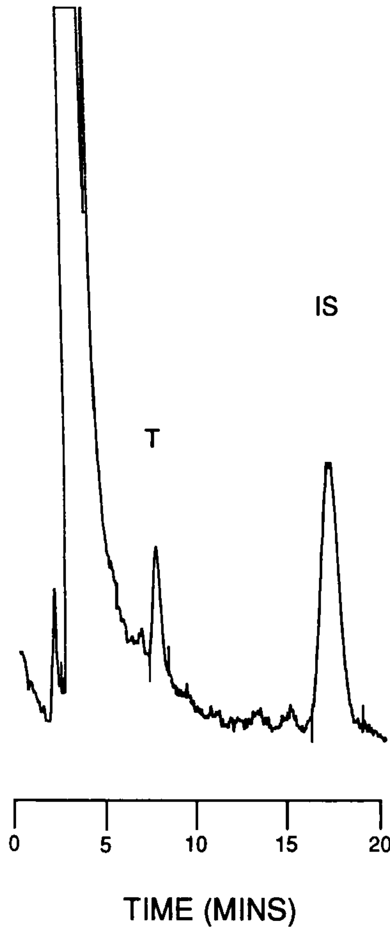

FIGURE 8

HPLC of human volunteer study sample. Extract of 1.0 ml of plasma sampled 72 hr after 75 mg IM dose. Trospectomycin represents 14 ng/ml.

trospectomycin, such as 6'-n-propyl actinospecinoic acid, which is not extracted, and actinamine which is eluted in the void volume by HPLC. It is thought that any drugs co-administered with trospectomycin, or adjuvants such as Cetomacrogol are unlikely to interfere.

In conclusion, this method, the first to our knowledge for this class of compound, has shown itself to be robust, rapid, and reliable, and of sufficient sensitivity to assay small biofluid samples from human or animal samples with great specificity.

The method depends upon:
1. Efficient extraction of analytes.
2. Novel HPLC for this class of compound that is both efficient and reliable.
3. Use of a close analogue as an internal standard to compensate for any variation in any aspect of the method.
4. Use of highly developed and commercially available post column reaction system.

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In addition, the method is flexible, and can be readily modified to measure trospectomycin in different biofluids, for example, urine, or for tissue residue analysis, or for other aminocyclotol antibiotics.

REFERENCES

1. Elrod, E., Bauer, J.F., Messner, S.L. "Determination of Spectinomycin Dihydrochloride by Liquid Chromatography with Electrochemical Detection". Pharm. Res. 5, 10, 664 1988.

2. Tsuji, K., Jenkins, K. "Derivatisation of Secondary Amines with 2-Naphalene-Sulfonyl Chloride for High-Performance Liquid Chromatographic Analysis of Spectinomycin". J. Chromatog. 333, 365, 1985.

3. Myers, H.N., Rindler, J.V. "Determination of Spectinomycin by High-Performance Liquid Chromatography with Fluorometric Detection". J. Chromatog, 176, 103, 1979.

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Received: November 7, 1989
Accepted: January 22, 1990