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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SPIRAMYCIN BY DIRECT INJECTION OF PLASMA, USING COLUMN-SWITCHING FOR SAMPLE CLEAN-UP

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SUMMARY

A fully automated high-performance liquid chromatographic method is described for the determination of spiramycin 1 in plasma. First, 1 ml of plasma is diluted with 1.5 ml of 4% acetonitrile containing spiramycin 2 as internal standard, and 1 ml is then injected via an automatic sampling unit. A pneumatic valve, which is remote-controlled by the programmable timer of an integrator, switches the sample, initially injected onto a precolumn for sample clean-up, to an analytical column for sample separation. This method was compared with a microbiological assay and has been successfully applied to pharmacokinetic studies on spiramycin in humans.

INTRODUCTION

Spiramycin, a member of the macrolide group of antibiotics, is derived from the culture filtrate of *Streptomyces ambofaciens* [1, 2], and consists of a mixture of three closely related compounds, namely spiramycin 1, 2 and 3 (Fig. 1) [3]. Antibiotics obtained from macrolide-producing organisms commonly consist of several homologous components. A high-performance liquid chromatographic (HPLC) method, which separates the three components of spiramycin, has been published [4], but it was developed in order to separate and determine the components of spiramycin in bulk powders, and in pharmaceutical preparations.

The measurement of drugs and their metabolites by HPLC in biological fluids, such as plasma or urine, involves sample extraction steps that are long and tedious to carry out, and are not applicable to compounds that are incompletely extracted.

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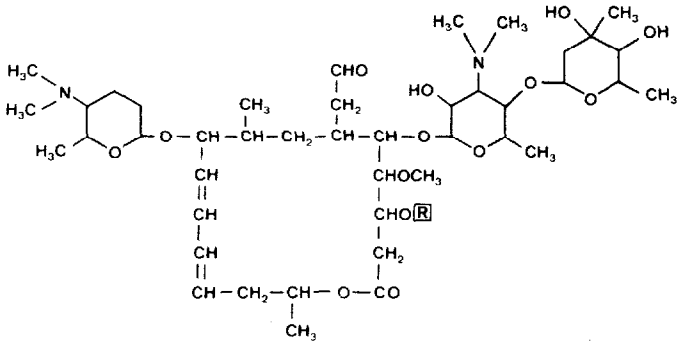

Fig. 1. Structures of spiramycin 1 (R = H), spiramycin 2 ($R = COCH_3$) and spiramycin 3 ($R = COC_2H_5$).

Several methods have been published on column-switching techniques that use precolumns for simultaneous sample clean-up and enrichment [5—7]. These methods have in common the direct injection of biological fluids onto a reversed-phase precolumn, which is then washed with water to eliminate proteins, salts and other highly polar compounds. The system is then switched to the back-flush mode, which initiates elution of the compounds retained at the top of the precolumn, and separation is performed on an analytical column. A method for the determination of a cephalosporin antibiotic in plasma by column-switching has been published [8], but it uses an anion-exchange column and two reversed-phase columns.

As far as we are aware, no HPLC method for the determination of spiramycin in plasma has been published. This paper describes a method for the direct measurement of spiramycin in plasma using the column-switching technique. Recovery, reproducibility, linearity and sensitivity were studied. The method was applied in a pharmacokinetic study in humans, and the results were compared with those obtained from a microbiological assay.

MATERIALS AND METHODS

Apparatus

The chromatographic system consists of a Chromatem 380 pump (Touzart and Matignon, Vitry sur Seine, France), which delivers solvent to the analytical column, and a Gilson 302 pump (Gilson, Villiers-Le-Bel, France), which delivers solvent to the precolumn. The analytical column (15 × 0.46 cm I.D.) is filled with Nucleosil 5 C_8, with a particle size of 5 μm (Macherey-Nagel, Duren, F.R.G.) and the precolumn (5 × 0.7 cm I.D.) is filled with Perisorb RP-18, with a particle size of 30—40 μm (E. Merck, Darmstadt, F.R.G.). A Rheodyne 7010 six-port valve fitted with a Model 7001 pneumatic actuator and a solenoid valve for remote actuation (Rheodyne, Cotati, CA, U.S.A.) is used for column switching. Samples are injected with a WISP 710B automatic injector (Waters, Paris, France) and are detected at 230 nm with an LDC Spectromonitor III UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Peak heights are measured with an ICAP 10 integrator (LTT, Paris, France), which also has a

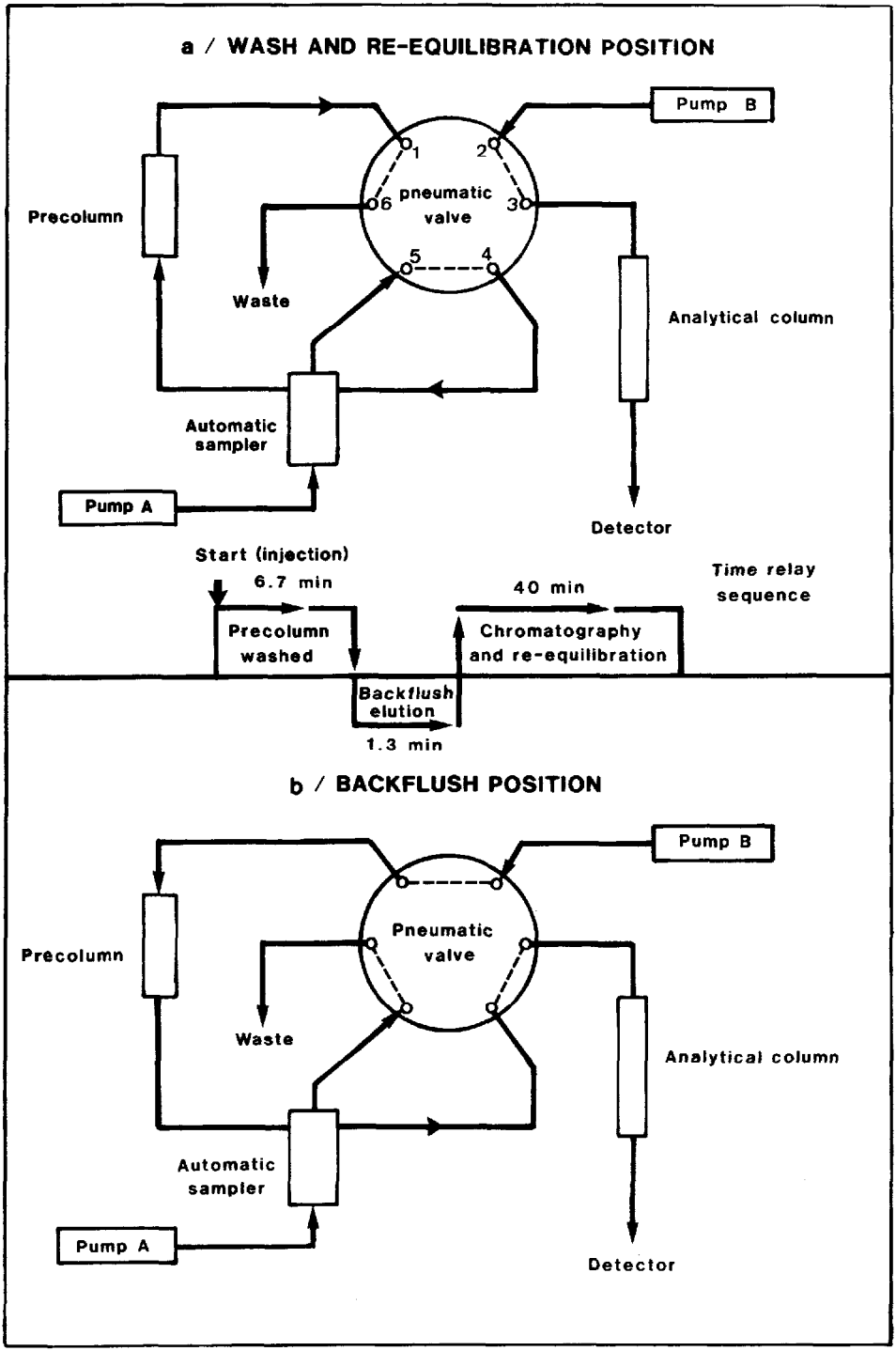

Fig. 2. Schematic diagram of the column-switching assembly. Precolumn, Perisorb RP-18, 30–40 μm (5 × 0.7 cm I.D.); analytical column, Nucleosil 5 C_8, 5 μm (15 × 0.46 cm I.D.). Mobile phases: pump A, 4% acetonitrile at 2.5 ml/min (clean-up mobile phase); pump B, 26% acetonitrile in 2‰ perchloric acid at 1.8 ml/min (elution and chromatography mobile phase). The sequence of the time-relay is shown by arrows. (a) Precolumn washing, re-equilibration and chromatography; (b) backflush elution.

programmable external events time-relay used for the remote control of column-switching.

The complete apparatus is illustrated in Fig. 2, and consists of an autosampler which, on sample injection, starts the programmable time-relay of the integrator. The integrator remotely controls the pneumatically driven valve, which connects the precolumn with the injection system and pump A. The precolumn can also be connected in the back-flush mode with the analytical column and pump B, when the pneumatic valve is switched.

Sample preparation and column-switching

Plasma (1 ml) is pipetted into the glass sample vial of the WISP, followed by 1.5 ml of 4% acetonitrile containing the internal standard (spiramycin 2). Then 1 ml of this mixture is injected automatically onto the precolumn, which is equilibrated with 4% acetonitrile (pump A, 2.5 ml/min). After injection the precolumn is washed for 400 sec, then the time-relay switches the pneumatic valve so that the precolumn is in the solvent stream of pump B (Fig. 2). Pump B delivers 26% acetonitrile in $2^0/_{00}$ perchloric acid at a flow-rate of 1.8 ml/min, in the back-flush mode, for elution and chromatography on the analytical column. The pneumatic valve is switched 80 sec later, so that pump B delivers directly to the analytical column, and pump A delivers the solvent for washing the precolumn. The precolumn is washed and re-equilibrated in parallel with the elution of the products on the analytical column. The pneumatic valve stays in this position until the end of the analysis (40 min), and is then ready for the injection of the next sample.

Preparation of standard curves and recovery of spiramycin 1 from plasma

Human plasma (1 ml) was spiked with 0.25 ml (750 ng) of spiramycin 2 and 0.25 ml (50, 100, 200, 300 and 500 ng) of spiramycin 1, both in 4% acetonitrile. A further 1 ml of 4% acetonitrile was added, and 1 ml of the mixture was injected. Three samples for each concentration of spiramycin 1 were prepared and injected on two separate days. A plot of the ratios of the peak heights of spiramycin 1 and spiramycin 2 against the concentration of spiramycin 1 added (six standards for each concentration) was used to calculate the linear regression equation. Plasma (1 ml) from subjects treated with spiramycin was diluted with 1.25 ml of 4% acetonitrile followed by 0.25 ml (750 ng) of spiramycin 2 (internal standard). The amount of spiramycin in plasma was calculated from the ratio of the peak heights of spiramycin 1 and spiramycin 2 with reference to the regression equation.

For recovery experiments, aqueous solutions of spiramycin 1 and spiramycin 2 were prepared as for spiked plasma, except that water (1 ml) was used. Recovery of spiramycin 1 from plasma was measured by comparing peak heights of spiramycin 1 obtained from aqueous solutions injected directly onto the analytical column, and peak heights obtained from spiked plasma (100—1000 ng/ml) injected using column-switching.

RESULTS AND DISCUSSION

The absorbance maximum for spiramycin in the eluent was found to be 230 nm, and at this wavelength a very large interfering peak was observed when

the precolumn was washed with water. Roth et al. [5] used water to wash their precolumn, but no interfering peak was noted. In their method, smaller amounts of plasma (150 μl) were injected, and detection was by fluorometry at 330/370 nm. Similarly, Voelter et al. [6], who injected up to 1 ml of plasma, did not observe interfering peaks, but detection was at 257 nm. It would thus appear that with detection at longer wavelengths, water can be used to wash the precolumn, without the appearance of interfering peaks. In the present study, with detection at 230 nm, 4% acetonitrile was used to wash the precolumn, and no interfering peak was observed.

The dilution of plasma with 4% acetonitrile had two functions: firstly, diluted plasma was less likely to block the syringe needle of the automatic injector, and secondly, dilution with 4% acetonitrile appeared to stabilise spiramycin in human plasma. Previous studies [9] have shown that the lactone moiety of spiramycin is rapidly hydrolysed in rat plasma incubated at 37°C. No degradation of spiramycin in human plasma diluted with 4% acetonitrile and incubated at ambient temperature was observed in the present work.

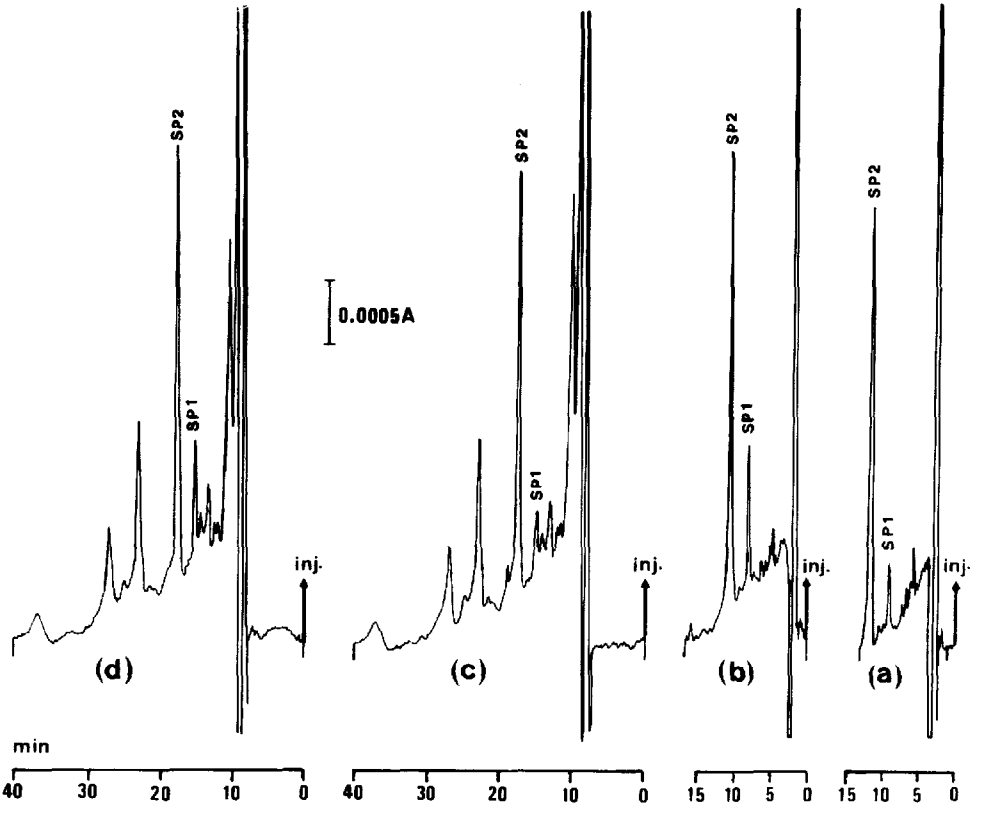

Fig. 3. Recovery of spiramycin 1 from plasma. The figure shows chromatograms of: (a) an aqueous solution of spiramycin 1 (SP 1) (100 ng/ml) diluted with 4% acetonitrile and injected directly onto the analytical column; (b) an aqueous solution of SP 1 (200 ng/ml) diluted and injected directly; (c) a plasma sample spiked with SP 1 (100 ng/ml) diluted with 4% acetonitrile and injected using column-switching; (d) a plasma sample spiked with SP 1 (200 ng/ml) diluted and injected using column-switching. Samples were spiked with spiramycin 2 (SP 2) internal standard (750 ng/ml).

TABLE I

RECOVERY OF SPIRAMYCIN 1 FROM SPIKED PLASMA INJECTED USING COLUMN-SWITCHING, COMPARED WITH AQUEOUS SOLUTIONS INJECTED DIRECTLY ONTO THE ANALYTICAL COLUMN

Concentration in spiked plasma (ng/ml)	Recovery (%)
100	71
200	75
500	80
1000	96

Recovery of spiramycin from plasma

The recovery of spiramycin 1 from plasma spiked with 100—1000 ng/ml spiramycin 1 and injected using column-switching was compared with aqueous solutions injected directly onto the analytical column. The injection by way of the precolumn did not lead to peak broadening, and the chromatograms were comparable (Fig. 3). The recovery of spiramycin 1 from plasma injected using column-switching was 71—96% over the concentration range studied (Table I). The slight loss is either due to the use of 4% acetonitrile for the washing of the precolumn, which may lead to some pre-elution of the sample or, alternatively, sample recovery may be slightly concentration-dependent when the precolumn is eluted with 26% acetonitrile. Previous studies using column-switching for drug dosage did not use internal standards [5—7], but recovery was almost 100% when water was used to wash the precolumn.

In the present study an internal standard (spiramycin 2) was used to compensate for any variability in the recovery from the precolumn. Spiramycin 2 was used as internal standard because of its similar chemical structure. The commercial preparation of spiramycin (Rovamycine®, Lab Spécia, France) contains 4% spiramycin 2, but no increase in the internal standard peak was noted when plasma of subjects treated with Rovamycine was analysed. It would therefore appear that either this amount is too small to detect or spiramycin 2 is rapidly deacetylated in vivo to give spiramycin 1.

TABLE II

REPRODUCIBILITY OF SPIRAMYCIN 1 DETERMINATION IN SPIKED PLASMA, AND THE REGRESSION EQUATION CALCULATED FROM A PLOT OF THE RATIO OF THE PEAK HEIGHTS OF SPIRAMYCIN 1 AND SPIRAMYCIN 2 AGAINST THE CONCENTRATION OF SPIRAMYCIN 1 ADDED

Regression equation: $y = mx+b$, where m (slope) = 1.4622, b (intercept) = −0.0409 and r (correlation coefficient) = 0.9965. Number of samples (n) = 6.

Concentration in spiked plasma (ng/ml)	Mean concentration found (ng/ml)	Coefficient of variation (%)
50	43	8.4
100	99	11.4
200	206	6.2
300	303	5.4
500	495	3.9

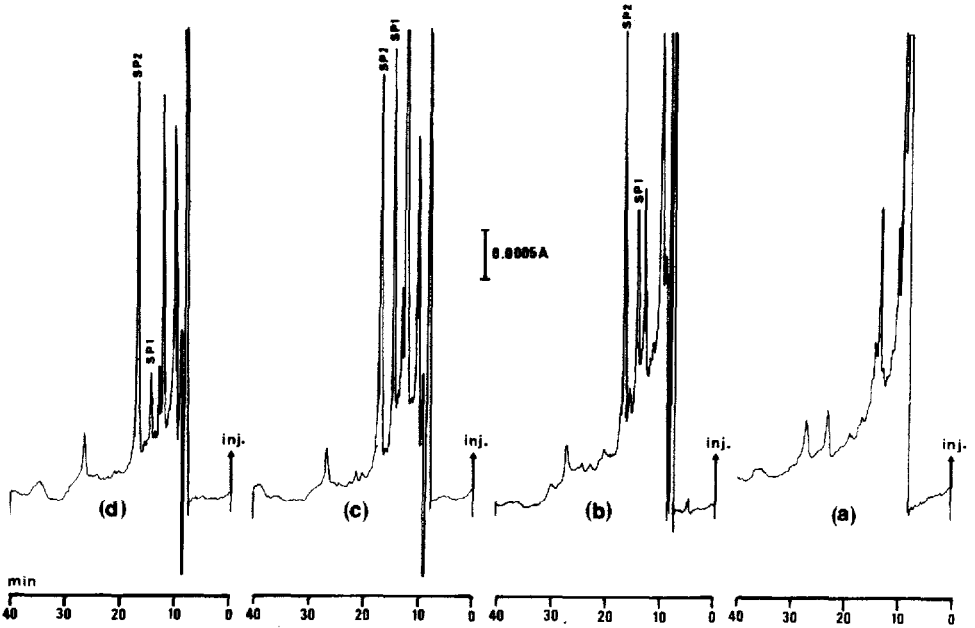

Fig. 4. Chromatograms of spiramycin 1 (SP 1) and spiramycin 2 (SP 2) internal standard. The figure shows chromatograms from plasma of: (a) a blank sample; (b) a sample spiked with 300 ng/ml SP 1; (c) a 2-h sample from subject 1 treated with spiramycin (Rovamycine) (the SP 1 concentration found was 637 ng/ml); (d) a 12-h sample from the same subject (the SP 1 concentration found was 156 ng/ml. Samples b, c and d were spiked with SP 2 internal standard (750 ng/ml).

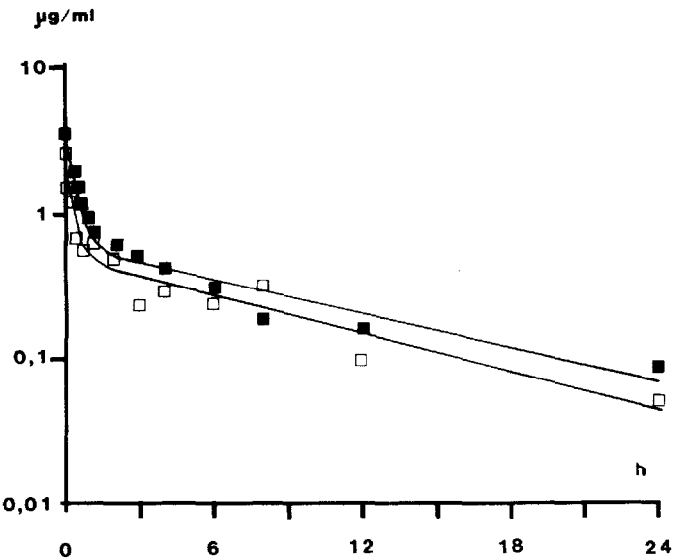

Fig. 5. Comparison of plasma concentration–time curves of spiramycin 1 determined by HPLC (■) and microbiological assay (□) in subject 1 after intravenous infusion of Rovamycine (500 mg). Time-zero is end of infusion. The elimination half-life was calculated as 7.5 h from concentrations determined by HPLC and as 6.7 h from concentrations determined by microbiological assay. The curves are best-fit least-squares estimations.

Precision, linearity and sensitivity

Standard curves were generated for spiramycin 1 as described above. The estimated mean concentration and the coefficient of variation (C.V.) for the range of drug concentrations were calculated, and are given in Table II. The C.V. did not exceed 12% for any of the concentrations studied. The linear regression equation and the coefficient of correlation for the standard curve are also given in Table II. It was possible to measure 50 ng/ml with a C.V. of 8.4%, using the present method.

Clinical application

The HPLC method was compared with an existing microbiological method for the measurement of spiramycin, which measures bacterial growth inhibition [10]. Two subjects were given intravenous infusions of Rovamycine (500 mg), and their blood was sampled at regular intervals for drug determination. Chromatograms of blank plasma, spiked plasma and plasma from a subject treated with Rovamycine are shown in Fig. 4, and the plasma concentration—time curve for this subject is shown in Fig. 5. Concentrations of spiramycin 1 in plasma, measured by HPLC, and total antibiotic activity in plasma, measured by microbiological assay, are also compared in Fig. 5. Good agreement was found between the two methods; thus the antibiotic activity of Rovamycine is probably due to spiramycin 1, and no active metabolite would appear to be formed during metabolism in vivo.

CONCLUSION

In previous studies using column-switching and direct injection of plasma for drug determination [6, 7], the precolumn remained in back-flush elution with the analytical column during the entire analytical phase of the analysis. Two alternative working precolumns were used in the method of Roth et al. [5], where one precolumn was eluted in the back-flush mode at the same time as the other was re-equilibrated.

In the present method, the precolumn is washed and re-equilibrated in parallel with the analytical phase, as the back-flush mode is of short duration (80 sec) after the initial phase of sample clean-up on the precolumn (400 sec). Thus two precolumns are unnecessary, and re-equilibration of the precolumn is achieved during the analytical phase. The precolumn and analytical column were washed overnight with 50% methanol, after the injection of a pharmacokinetic study (ca. thirty samples); this increased the lifetime of columns.

The present method should be applicable to the measurement of other drugs, as it has been developed for a drug with poor absorbance characteristics and required the injection of relatively large amounts of plasma.

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