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## Note

# Determination of josamycin in plasma, erythrocytes and leucocytes via high-performance liquid chromatography by direct injection of plasma or homogenized blood cells, using alternating sample clean-up columns

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Josamycin is a macrolide antibiotic that is particularly indicated for the treatment of infections of the skin and of the respiratory tract, the ear, nose and throat. Its important pharmacokinetic properties include accumulation in certain cells and an increase in blood plasma levels after repeated ingestion [1—5]. Previously described chromatographic methods all involve time-consuming extraction steps. As josamycin is a relatively lipophilic substance it seemed reasonable to attempt direct injection of plasma, with sample clean-up on a special pre-column [2,3,6,7].

Injection of plasma directly into the high-performance liquid chromatographic (HPLC) system without pretreatment of the sample has been described for several substances. Nevertheless, some problems exist in adapting this technique to fully automatic mode. Three main problems may occur: (1) the adsorption on the clean-up column is poor and some of the drug is washed out as waste; (2) the photometric absorption maximum of the drug is in the low UV range ($\leq$ 235 nm), where most of the components of the plasma also absorb; (3) poor UV absorption or a low concentration of the drug in the sample make it necessary to inject large volumes of plasma, which results in overload of the clean-up column.

In the case of josamycin we observed high adsorption on the clean-up column, a UV maximum at 230 nm and low UV absorption. Some analytical problems were therefore to be expected. However, the results obtained during the validation of the method demonstrated that all the analytical disadvantages of the drug were easily compensated for by the excellent adsorption of the drug on the clean-up columns. It therefore appears that when working with clean-up columns and backflush the adsorptive behaviour of the drug on the

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clean-up column is of decisive importance. If the adsorption is very good all other disadvantages can easily be overcome.

This has allowed us to develop a method for the fully automatic and selective determination of josamycin in plasma. As this method proved to be highly sensitive, with a detection limit of 5 ng per injection, it could also be used for the determination of josamycin in erythrocytes and leucocytes, if the blood cells are isolated and counted, and if the volume of the cell fraction is determined by special methods [8,9].

## EXPERIMENTAL

### *Materials*

Acetonitrile was purchased from Riedel de Haën (Seelze, F.R.G.). Methanol, sodium acetate (anhydrous), disodium hydrogen phosphate dihydrate and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, F.R.G.). RPMI medium 1640 was purchased from Gibco Europe (Nunc, Wiesbaden, F.R.G.). Water was highly purified with the Milli Q system from Millipore (Eschborn, F.R.G.). Standard josamycin was delivered by Yamanouchi (Japan).

Sodium acetate solution (0.05 *M*) was prepared by dissolving 4.10 g of sodium acetate in water to make 1000.0 ml. Purified water was used as mobile phase for sample clean-up. The mobile phase for analysis was prepared by mixing sodium acetate solution (0.01 *M*) and acetonitrile (60:40, w/w = 54:46, v/v). The josamycin stock solution was prepared by dissolving 50 mg of josamycin in methanol to make 50.0 ml.

The phosphate buffer (pH 5.4, 0.05 *M*) was obtained by dissolving 0.596 g of disodium hydrogen phosphate dihydrate and 6.621 g of potassium dihydrogen phosphate in water to make 1000.0 ml.

The following columns were used: for sample clean-up, 5 × 4.6 mm I.D., Nucleosil $C_{18}$, 30 μm, filled by Bischoff Analysentechnik; for pre-columns, 20 × 4.6 mm I.D., Shandon Hypersil CPS, 5 μm, filled by Bischoff Analysentechnik; main column, 250 × 4.6 mm I.D., Shandon Hypersil CPS, 5 μm, filled by Bischoff Analysentechnik.

The lifetime of clean-up columns and pre-columns largely depends on whether plasma samples or homogenized blood cell suspensions are injected. Plasma can be injected ca. 100 times, blood cell suspensions ca. 20 times. The lifetime of the main column is not influenced by the procedure and lies within the usual range.

### *Apparatus*

Our system has been established as a module model and is controlled by the system Controller PLK 1 of Izumi Denki. Other modules of the system are: solvent-delivery module 112 (Beckmann), autosampler ASI 120 (Ismatec) with Rheodyne injection valve and a 200-μl sample loop, UV detector Spectroflow 773 (Kratos) set at 230 nm, Vista CDS 401 (Varian) as computing integrator and column-switching valves (Latek). Connections between modules, columns and capillaries were free of void volume. The flow-rate of the "clean-up pump" was 2 ml/min and that of the "analytical pump" 1.3 ml/min. The flow must be pulse-free.

*Procedure*

The samples are injected (valve 1) on a 0.5-cm column for sample clean-up, and the column is then washed with water (pump A) for 5 min. Josamycin is adsorbed on the Nucleosil $C_{18}$ material (30 $\mu$m), while all hydrophilic substances are eluted. After the columns have been switched (valve 2 and valve 3), josamycin is eluted in the backflush mode with mobile phase for analysis (pump B; run time, 10 min). While josamycin is being eluted from clean-up column 1, clean-up column 2 is reconditioned with water for the next sample clean-up (Fig. 1) [7].

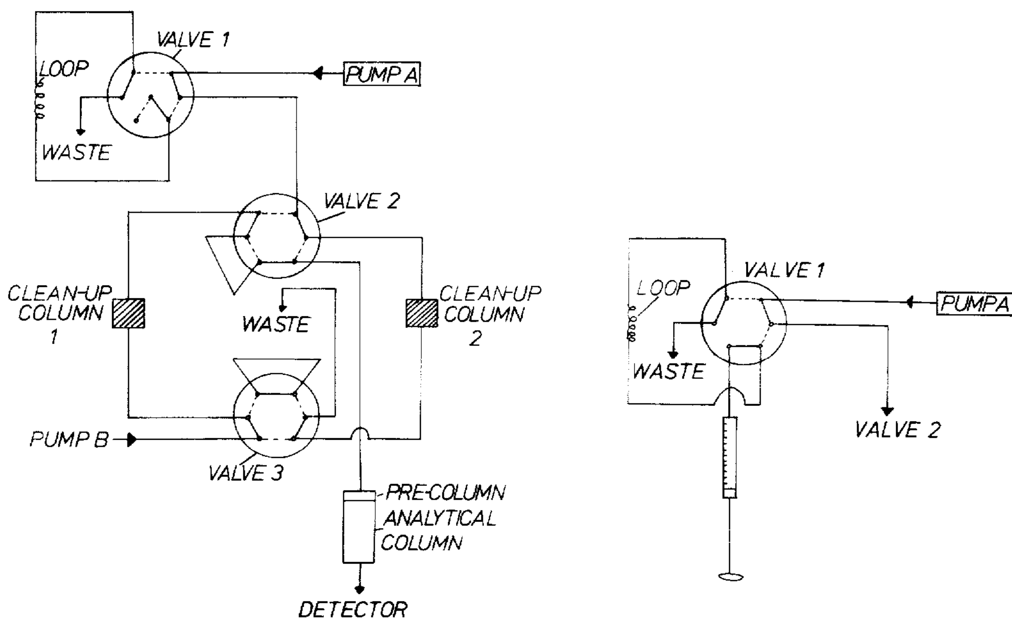


Fig. 1. Column switching system.

Fig. 2. Connection of injection cannula and dosing valve.

No sample preparation is necessary in the experiments with plasma as the plasma is directly injected into the HPLC system. In the experiments with leucocytes and erythrocytes the cells are first isolated and then counted, and their total volume is determined by special methods [8, 9]. They are suspended and lysed with 500 $\mu$l of 0.05 *M* phosphate buffer and then homogenized. A sample, equivalent to $2.5 \cdot 10^7$ blood cells, is then injected. In some cases manual injection of the cell suspension is necessary and then the injection cannula is directly connected to the valve (Fig. 2).

A double determination of each sample is carried out and compared with the peak height of an external standard (josamycin in blank plasma or in RPMI medium) (Fig. 3). The addition of an internal standard such as josamycin propionate to the sample is not essential because of the high precision, accuracy and reproducibility of the determination.

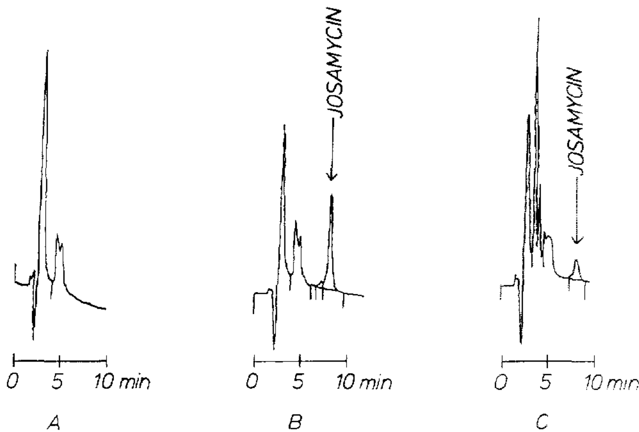


Fig. 3. Typical chromatograms of (A) blank plasma; (B) blank plasma spiked with 1.0 μg/ml josamycin; (C) plasma sample corresponding to 0.25 μg/ml josamycin.

## RESULTS AND DISCUSSION

### *Validation*

The method was validated with calibration curves and by determining the recovery, the accuracy and the detection limit. The relevant data are given in Table I.

TABLE I

CALIBRATION CURVE DATA FOR JOSAMYCIN IN PLASMA, WATER AND RPMI BUFFER

The curves are linear and pass through the origin.

| Sample | Concentration range (μg/ml) | *n* | $r^2$* | RG** (mean ± S.D.) (%) |
|---|---|---|---|---|
| Plasma | 0.5—5.0 | 11 | 0.997 | 3.30 ± 1.69 |
| Plasma | 0.1—3.0 | 9 | 0.9932 | 5.62 ± 3.99 |
| Water | 0.5—5.0 | 12 | 0.9959 | 3.32 ± 2.60 |
| RPMI buffer | 0.1—0.8 | 8 | 0.9996 | 1.82 ± 1.89 |

*$r$ = Correlation coefficient.

**RG is the criterion for the quality of the regression, i.e. the mean value of the percentage differences between the measured value and the value calculated from the linear regression.

### *Recovery*

The recovery from plasma was 100%. The calibration curves from plasma and from water were almost exactly superimposable. For the determination of josamycin in blood cells the method had to be validated individually for each cell type. For this purpose the blood cell fraction was suspended in RPMI 1640 medium (Flow Labs.) and incubated with josamycin at 37°C for 45 min. The number of blood cells was carefully determined and was ca. $2.5 \cdot 10^7$ cells for all experiments. After incubation the cell suspension was lysed, thoroughly homogenized and injected.

*Accuracy*

The accuracy of the method was tested with spiked samples. The amount of added josamycin was unknown to the analyst (Table II).

TABLE II

DETERMINATION OF JOSAMYCIN IN PLASMA

| Amount added (μg/ml) (unknown to the analyst) | Found (μg/ml) | Error (μg/ml) |
|---|---|---|
| 1.32 | 1.14 | 0.18 |
| 1.32 | 1.23 | 0.09 |
| 2.63 | 2.79 | 0.13 |
| 2.63 | 2.72 | 0.09 |
| 3.94 | 4.09 | 0.15 |
| 3.94 | 3.90 | 0.04 |

*Lower limit of detection*

The detection limit is 5 ng per injection (25 ng/ml).

*Biological samples*

The plasma levels and the results for the accumulation of josamycin in plasma after repeated ingestion are similar to those that have been reported earlier for josamycin and for other macrolide antibiotics [2,3,6,12]. What was surprising, however, was the marked accumulation of josamycin in leucocytes [4]. The accumulation factors for josamycin in the leucocyte fractions can be taken from Table III. They agree quite well with the results that were obtained by using radioactive material [11]. No accumulation was observed in erythrocytes.

TABLE III

DETERMINATION OF JOSAMYCIN IN POLYMORPHONUCLEAR LEUCOCYTES

| Concentration of josamycin in the medium | Time of incubation at 37°C (min) | Concentrations of cell-associated josamycin (accumulation factor)* | |
|---|---|---|---|
| | | Before cell wash | After cell wash (twice) |
| 2 μg/ml (= 1 $F$) | 10 | 22 $F \pm 4$ ($n = 5$) | 9 $F \pm 3$ ($n = 4$) |
| 4 μg/ml (= 1 $F$) | 10 | 21 $F \pm 3$ ($n = 8$) | 7 $F \pm 2$ ($n = 6$) |
| 4 μg/ml (= 1 $F$) | 45 | 25 $F \pm 7$ ($n = 9$) | 17 $F \pm 3$ ($n = 5$) |

*1 $F$ = initial concentration; $XF$ = factor of accumulation; $n$ = number of experiments.

## CONCLUSIONS

An analytical method for the determination of josamycin in plasma has been developed, which has the following important advantages in comparison with previously described methods: (1) sample preparation is not necessary, as the plasma is directly injected into the system; (2) the recovery is 100%, a value

which could not be achieved for this analysis with extraction procedures in our tests; (3) accuracy and reproducibility are reliable, so that the addition of an internal standard is not necessary; (4) the detection limit could be lowered to 25 ng/ml or 5 ng per injection.

This method is selective for josamycin and allows it to be separated from its metabolites [10]. Because of the low detection limit and because of the high affinity of josamycin for the clean-up column, this method can also be used for the determination of josamycin in isolated blood cell fractions. These fractions can also be injected without an extraction step, though the recovery is diminished to 81% in the case of polymorphonuclear cells. The reproducibility and accuracy are not as good as in plasma but still acceptable. The injection of the cell suspension directly into an HPLC system may therefore be a viable method for other substances.

In the course of this study it became evident that josamycin is strongly accumulated in isolated leucocyte fractions, whereas in erythrocyte fractions no significant accumulation occurs. The HPLC results were compared under all test conditions with those from another method, which employed radiolabelled josamycin [11]. The agreement between the two methods is very good.

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