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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF JOSAMYCIN IN SERUM AND URINE

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SUMMARY

A high-performance liquid chromatographic assay for the analysis of josamycin in human serum and urine is presented. The assay involves a simple solid-phase extraction procedure coupled with a phase separation step, separation on a reversed-phase C_{18} column with UV detection by a multi-wavelength programmable detector. The mobile phase was acetonitrile–0.015 *M* phosphate buffer, pH 6.0 (5:2) at a flow-rate of 1.2 ml/min. The column temperature was maintained at 35°C. Linear calibration curves over the concentration ranges 0.1–2.0 mg/l (serum) and 0.5–5 mg/l (urine) were obtained with correlation coefficients of 0.9983 and 1.0000, respectively. The relative standard deviations of five replicate samples at the upper and lower limits of each calibration curve were below 7%. The recoveries at the upper and lower ends of the calibration range for serum were 77% and 70%, respectively, and those for urine were 76% and 80%, respectively.

INTRODUCTION

Josamycin (Fig. 1), a relatively new macrolide antibiotic, is used mainly in the treatment of gram-positive infections[1–5]. Josamycin is reported to be more stable than erythromycin over the normal gastric pH range[6], and is well tolerated with a low incidence of side effects[7,8]. These properties, together with the decreased ability of certain bacteria to become resistant to josamycin compared with other macrolide antibiotics[9,10], confers certain advantages on this drug in the treatment of susceptible infections. Most of the pharmacokinetic data published to-date have been derived

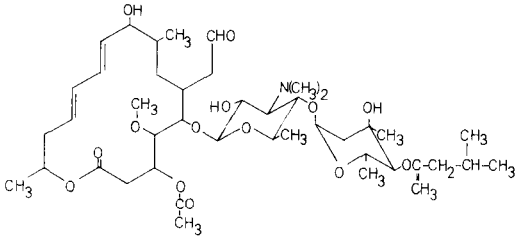

Fig. 1. Molecular structure of josamycin.

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from microbiological assay techniques[11–14], which are tedious to perform. Fourtillan *et al.*[15] adapted the high-performance liquid chromatographic (HPLC) assay of erythromycin developed by Tsuji[16] to assay josamycin in plasma. However, this method involves on-stream post-column derivatisation, extraction and fluorescence detection, requiring complex and expensive equipment. Ducci and Scalori[17] described an HPLC assay of josamycin with a detection limit of 0.3 mg/l, which is insufficiently sensitive for pharmacokinetic studies. Räder *et al.*[18] recently described a sensitive HPLC assay but a dual-pump system and expensive column-switching apparatus are required. The method described here is sufficiently sensitive for pharmacokinetic studies and requires only standard HPLC apparatus.

EXPERIMENTAL

Materials

The modular HPLC system consisted of a constant-flow pump (M45; Waters Assoc., Milford, MA, U.S.A.), an automated sample injector (Waters WISP 710B), a multi-wavelength programmable UV detector (Waters M490), and a dual-pen flatbed recorder (Model 561, Hitachi, Tokyo, Japan). The analysis was performed on a 25 cm × 3.9 mm I.D. stainless-steel column, packed in our laboratory with microparticulate bonded (10 μm) octadecylsilane (C_{18}) material (Techsil, HPLC Technology, Wilmslow, U.K.). The column temperature was maintained at 35°C by a Model LC-22 temperature controller (Bioanalytical Systems, W. Lafayette, IN, U.S.A.) to improve peak shape and resolution of josamycin from pre-eluting peaks observed in serum and urine samples. The solid-phase extractions were performed on 1-ml disposable C_{18} extraction columns (Bond-Elut; Analytichem, Harbour City, CA, U.S.A.).

All reagents were of at least analytical grade. The acetonitrile was of distilled-in-glass UV grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Phosphoric acid and sodium hydroxide were both obtained from Merck (Johannesburg, South Africa). Josamycin was obtained from Yamanouchi (Tokyo, Japan) and oleandomycin phosphate from Pfizer (Pietermaritzburg, South Africa). Water used for extraction and chromatography was purified by filtration through a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

The internal standard solution, containing 0.5 g/l oleandomycin phosphate (Fig. 2) was prepared by dissolving 10 mg oleandomycin phosphate in 2 ml of acetonitrile in a 20-ml volumetric flask and making up to volume with water.

An aqueous josamycin stock solution was prepared by dissolving 10 mg josamycin in 2 ml acetonitrile and making up to volume with water in a 10-ml volumetric flask (solution A). A 1-ml volume of solution A was then diluted to 10 ml with water

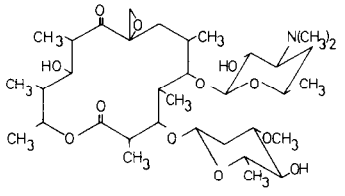

Fig. 2. Molecular structure of oleandomycin.

(solution B). Serum standards containing 0.1, 0.3, 0.6, 1.0, 1.5 and 2.0 mg/l josamycin were prepared by the serial dilution of a serum stock solution containing 1 ml solution B in 50 ml. Urine standards containing 0.25, 0.5, 1.0, 2.5 and 5.0 mg/l josamycin were prepared by the serial dilution of a urine stock solution containing 1 ml of solution B in 20 ml.

The mobile phase was acetonitrile–0.015 *M* phosphate buffer (5:2) degassed and filtered through a 0.4-μm HVLP Millipore filter prior to use. The buffer was prepared by making 0.96 ml phosphoric acid up to 1 l with water and adjusting the pH to 6.0 with sodium hydroxide pellets. The buffer used in the elution mixture (0.05 *M*) was prepared by making 3.2 ml phosphoric acid up to 1 l with water and adjusting the pH to 5.8 with sodium hydroxide pellets.

Methods

A 1-ml volume of sample (serum or urine) was mixed in a vortex mixer with 1 ml of acetonitrile for 5 s. Internal standard (200 μl) was added and mixed for an additional 5 s. Precipitated proteins were separated by centrifugation for 5 min at 1600 *g* and the supernatant was transferred to a 10-ml test-tube, containing 5 ml water. The diluted supernatant was then loaded onto a 1-ml disposable C_{18} extraction column which had been pretreated by wetting with 5 ml acetonitrile followed by 5 ml water, with the aid of a 20-ml custom-made glass reservoir. On completion of the loading process, the column was washed with 20 ml water followed by 4 ml acetonitrile–water (1:1). The column was then dried under vacuum (5–15 mmHg), using a Baker No. 10 extraction system (J. T. Baker, Phillipsburg, NJ, U.S.A.). Drug and internal standard were then eluted into a 2 ml tapered collection tube (Kimble, Owens-Illinois, IL, U.S.A.) with 3 × 0.5 ml aliquots of acetonitrile–0.05 *M* phosphate buffer, pH 5.8 (3:2), the column being dried under vacuum between each aliquot. The sample was evaporated to dryness under vacuum at 40°C in a rotary vacuum centrifuge (Savant, Hicksville, NY, U.S.A.). Reconstitution was effected by the addition of 20 μl of water, mixing for 1 min, addition of 50 μl of acetonitrile, mixing for an additional 1 min, and centrifugation for 30 s at 1600 *g*. An aliquot (30–40 μl) of the clean supernatant was transferred to a WISP limited-volume insert (Waters) with a microsyringe. Aliquots (2–10 μl) of this sample were injected into the chromatographic system. The flow-rate of the mobile phase was maintained at 1.2 ml/min for the analysis of both serum and urine samples, resulting in a pressure of 55 bar. Retention times for josamycin and internal standard (oleandomycin) were *ca.* 6.0 and 8.0 min, respectively. The eluate was monitored at 231 nm for the detection of josamycin (0.02 a.u.f.s. for serum and 0.06 a.u.f.s. for urine) and at 204 nm for the internal standard (0.06 a.u.f.s.). The time constants and threshold values for both wavelengths were 1.0 s and 0%, respectively. Chromatograms of extracts of serum and urine samples collected before and after the administration of 1 g josamycin to a human volunteer are shown in Figs. 3–6.

RESULTS

Calibration curves obtained by plotting the ratio of josamycin peak height to that of internal standard *vs.* josamycin concentration for serum and urine standards were found to be linear over the concentration ranges studied. The linear regression

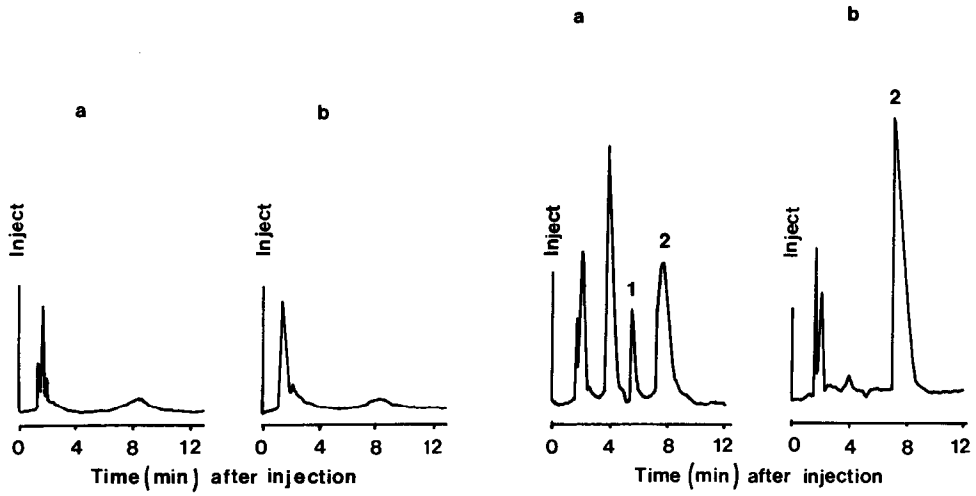

Fig. 3. Chromatograms of a blank serum extract, monitored at 231 nm (a) and 204 nm (b).

Fig. 4. Chromatograms of a serum extract, containing 0.29 mg/l josamycin (1) and internal standard (2), monitored at 231 nm (a) and 204 nm (b).

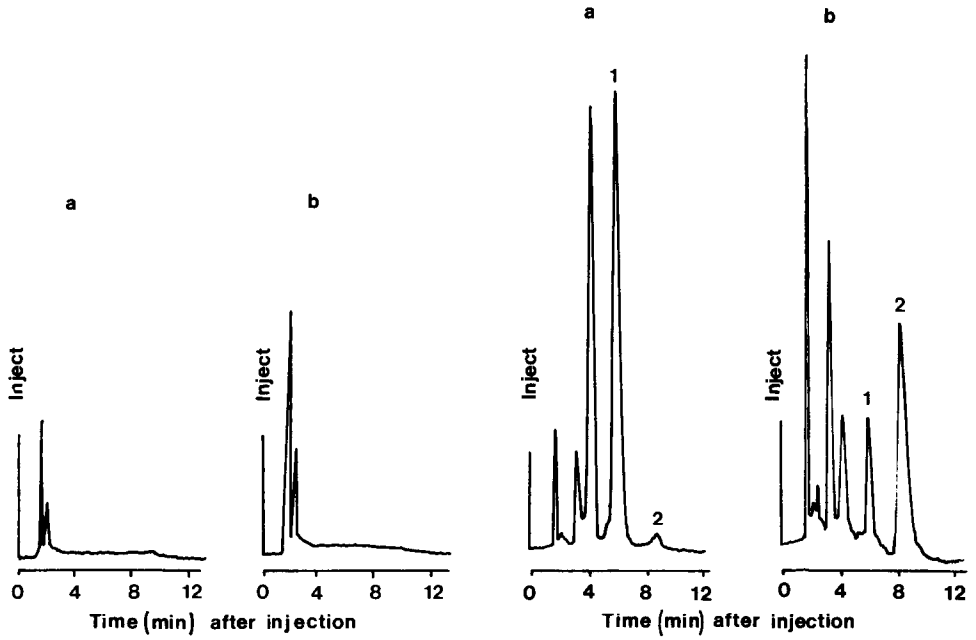

Fig. 5. Chromatograms of a blank urine extract, monitored at 231 nm (a) and 204 nm (b).

Fig. 6. Chromatograms of a urine extract, containing 5.28 mg/l josamycin (1) and internal standard (2), monitored at 231 nm (a) and 204 nm (b).

equations were $y = 1.0855x - 0.0237$ with a correlation coefficient of 0.9983 for serum and $y = 0.3142x - 0.0073$ with a correlation coefficient of 1.0000 for urine.

Accuracy and precision, determined at 0.30 and 1.82 mg/l in serum and 1.49 and 4.54 mg/l in urine, were found to be (mean ± S.D.; $n = 5$) 0.27 ± 0.01 and 1.78 ± 0.10 mg/l, and 1.43 ± 0.05 and 4.96 ± 0.17 mg/l, respectively. Although a slight baseline shift occurred in the area where the internal standard eluted (Fig. 3b), this relatively small aberation did not affect the analysis of serum samples.

The percentage recoveries from spiked serum and urine samples were assessed by extraction of five replicate samples at the upper and lower ends of the calibration ranges without the addition of internal standard. A phase separation was effected by the addition of 20 μl water and 50 μl acetonitrile followed by mixing for 1 min. Due to the partial miscibility of the two solvents after salting out, the volume of the supernatant is unknown. Therefore, as much of this layer as possible was transfered to a 2-ml collection tube, containing internal standard. Samples were then evaporated to dryness under vacuum at 40°C and reconstituted with 20 μl water and 50 μl acetonitrile. After mixing for 1 min, aliquots of this sample were injected into the chromatograph. Reference samples were prepared by the addition of that amount of josamycin equivalent to 100% recovery to collection tubes containing internal standard. Evaporation to dryness and reconstitution were effected as with test samples. The ratios of the peak height for josamycin to that of internal standard for the test samples were then compared with those of the reference samples. The percentage recoveries at the upper and lower ends of the calibration range for serum were 77% and 70%, respectively, and those for urine 76% and 80%, respectively.

The detection limit of josamycin in serum and urine was 0.025 mg/l, based on a signal-to-noise ratio of 3 and determined with a 10-μl injection volume and a channel 1 sensitivity setting of 0.01 a.u.f.s.

Analysis of serum samples stored at −15°C for 7, 14 and 28 days showed the samples to be stable and the method of analysis highly reproducible. Urine samples, on the other hand, showed evidence of degradation.

DISCUSSION

A rapid, sensitive assay with the accuracy and precision required for pharmacokinetic studies has been developed for the analysis of josamycin in human serum and urine. The major metabolite of josamycin is an hydroxylated compound which has been detected in serum and urine by the use of HPLC. Two other metabolites, a second hydroxylated compound and the deisovaleryl metabolite have only been detected in urine[15]. Chromatograms of serum samples collected after the administration of 1 g josamycin to healthy male volunteers show the appearance of an unknown peak at *ca.* 4 min (Fig. 4a). Chromatograms of urine samples collected from the same volunteers show the appearance of two extra peaks at *ca.* 3 min and 4 min (Fig. 6a). As pure samples of these metabolites were not available for chromatographic use, an HP 1040A diode array detector was utilised in an attempt to identify these unknown compounds. Data obtained show the eluting compounds to exhibit UV spectra almost superimposable on that of josamycin. These unknown peaks are therefore likely to be metabolites of josamycin indicating the separation of josamycin from its major and one minor metabolite.

With the aid of custom-built racks and reservoirs, and allowing the sample and washes to percolate through the extraction columns under gravity, over 80 samples can be extracted with ease during a normal working day and assayed overnight.

The utilisation of dual-wavelength monitoring enables the use of an internal standard which shows little or no UV absorbance at 231 nm, the wavelength of maximum absorption of the compound of interest. Chromatographically, oleandomycin is highly suitable as an internal standard in this assay and its use as such has been made possible by the application of dual-wavelength monitoring.

An important characteristic displayed by macrolides[19] when adsorbed on reversed-phase C_{18} material is they are easily eluted by an acetonitrile–buffer mixture but not by an acetonitrile–water mixture. A large water wash after loading is required for the removal of serum salts from the column and it is thus essential that this wash not become contaminated with ions from the diluted sample. Contamination can occur if pasteur pipettes used during loading to remove bubbles from the extraction column are also used during the 20-ml water-wash procedure, or from diluted sample remaining on the side of the reservoir after loading. It is therefore advisable to use clean pasteur pipettes and rinse the reservoirs with HPLC-grade water prior to the commencement of the 20-ml water wash. Contamination of this wash can lead to excessive loss of josamycin during the acetonitrile–water wash prior to elution and serious problems with accuracy and precision.

The composition of the mobile phase was critical for good separation of josamycin and internal standard. An increase in buffer concentration resulted in the more rapid elution of oleandomycin from C_{18} material, with little effect on the retention time of josamycin. Conversely, the retention of josamycin was more sensitive to the proportion of acetonitrile in the mobile phase. A high proportion of acetonitrile promoted rapid elution of josamycin. Good selectivity and resolution on a C_{18} column could easily be effected by manipulation of the molarity of the buffer used and the ratio of acetonitrile to buffer.

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