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Short Communication

Sensitive determination of josamycin and rokitamycin in plasma by high-performance liquid chromatography with fluorescence detection

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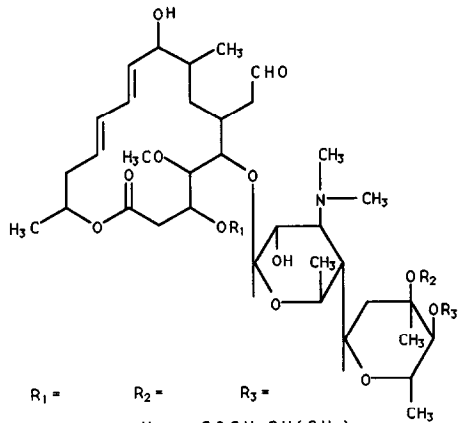
ABSTRACT

Rokitamycin and josamycin were successfully derivatized with dansylhydrazine in 20 min at 60°C. Rokitamycin and josamycin levels were determined in plasma after ion-pair extraction into hexane–isoamyl alcohol with lauryl sulphate and precolumn derivatization. Resolution was obtained by liquid chromatography with fluorescence detection (352/537 nm) in 12 min. The limit of detection was 20 ng/ml macrolide starting from 1 ml of plasma, and linearity was demonstrated between 50 and 400 ng/ml. Inter-run coefficients of variation were 10.2% at 100 ng/ml and 9.1% at 300 ng/ml. The system was reliably used for pharmacokinetic studies in plasma.

INTRODUCTION

Rokitamycin is a new semi-synthetic 16-membered macrolide antibiotic whose structure is similar to that of josamycin (Fig. 1). Rokitamycin is metabolized by humans into leucomycin A_7 (LMA_7) and leucomycin V (LMV), which are active metabolites (Fig. 1). Rokitamycin and its metabolites are currently determined by microbial bioassay [1] or high-performance liquid chromatography (HPLC) with UV detection [2], like josamycin [3–5]. However, in our experience, these methods are sometimes not sufficiently sensitive and are prone to endogenous or exogenous interference, especially when applied to determination of tissue concentrations. Since dansylhydrazine has previously been used to derivatize the aldehydes or ketones of steroids [6–10] or bile



	R_1 =	R_2 =	R_3 =
Josamycin	$COCH_3$	H	$COCH_2CH(CH_3)_2$
Rokitamycin	H	COC_2H_5	COC_3H_7
LMA7	H	H	COC_3H_7
LMV	H	H	H

Fig. 1. Macrolide structures.

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acids [10,11], the presence of an aldehyde group in all of these macrolides prompted us to study their determination by HPLC with fluorescence detection using dansylhydrazine as the reagent for precolumn derivatization. The method is described for the determination of rokitamycin using josamycin as the internal standard, but the reverse is also possible.

EXPERIMENTAL

Reagents

Rokitamycin and its metabolites, LMA_7 and LMV, were obtained from Pierre Fabre Labs. (Boulogne-Billancourt, France) and josamycin was from Pharmuka (Genevilliers, France). Sodium acetate, sodium chloride and ammonium acetate were bought from Merck (Darmstadt, Germany). Sodium lauryl sulphate, neostigmine methyl sulphate and trimethylchlorosilane (TMCS) were purchased from Sigma (L'Isle, d'Abeau, France). UV-grade hexane, isoamyl alcohol and dansylhydrazine were obtained from Fluka (Buchs, Switzerland). Quality controls and calibration standards were prepared using Biotrol plasma (ref. A02254, Chenevières, France). All reagents and chemicals were of analytical grade.

Apparatus

The liquid chromatograph consisted of a Shimadzu LC-6A pump, a Rheodyne Model 7125 injector with a 20-μl sample loop, a Shimadzu RF535 fluorescence detector, a Jones column oven and a Shimadzu CR5A integrator (Touzart et Matignon, Vitry, France). A Nucleosil C_{18} column (150 mm × 4.6 mm I.D., 3 μm particle size) was inserted into the system (SFCC, Neuilly-Plaisance, France). Excitation and emission spectra of the macrolide derivatives were recorded with a Perkin-Elmer LS3 fluorimeter (Montigny-le-Bretonneux, France). Tissues (human tonsil or lung) were ground into a powder using a Spex 6700 grinder (Polylabo, Strasbourg, France) in a bath of liquid nitrogen. Screw-capped glass tubes (110 mm × 16 mm I.D.) were silanized with 1% TMCS in toluene for 1 h at 70°C and then washed twice with methanol.

Preparation of plasma standards

A 1000 mg/l stock solution in methanol was prepared for each macrolide and stored at −80°C. Working solutions of 2 mg/l were prepared in methanol and made fresh daily. Silanized tubes were filled with 0, 25, 50, 100, 150 and 200 μl of the rokitamycin standard solution, evaporated to dryness, and spiked with 1 ml of Biotrol plasma to yield a six-point standard curve, ranging from 50 to 400 ng/ml. Once the plasma has been spiked with rokitamycin, it must be extracted immediately, since rokitamycin may be metabolized by esterases into LMA_7 and LMV [1].

Quality controls at 100 and 300 ng/ml were prepared using a 0.2 m*M* final concentration of neostigmine methyl sulphate as the esterase inhibitor, and kept frozen at −80°C for at most two months. Stability (or efficacy of the esterase inhibitor) was not demonstrated for a greater period of time.

Plasma extraction procedure

Sample (1 ml) were transferred to 110 mm × 16 mm I.D. silanized glass tubes. To each sample (collected in a heparin–neostigmine methyl sulphate-containing tube) or standard, 100 μl of a 2 mg/l josamycin methanolic solution was added, followed by 50 μl of 0.1 *M* sodium acetate–acetic acid buffer (pH 4.65), 100 ml of saturated sodium chloride and 100 μl of 0.01 *M* sodium lauryl sulphate. Then, 5 ml of a hexane–isoamyl alcohol (90:10, v/v) mixture was added to each tube. After capping, the samples were transferred to a reciprocating shaker and agitated for 15 min. The samples were centrifuged for 10 min at 1000 *g*. The organic layer was transferred to silanized glass tubes. The aqueous layer was extracted again with 5 ml of hexane–isoamyl alcohol and treated as above. The organic layers were combined and the samples were evaporated to dryness under nitrogen with mild heating.

Derivatization procedure

To each tube, 200 μl of freshly prepared 0.002% (w/v) dansylhydrazine solution in toluene–methanol–acetic acid (90:10:1.13, v/v) were

added. The tubes were capped, heated at 60°C for 20 min in a water-bath, and then evaporated to dryness under nitrogen and heating at 40°C. After derivatization, samples were stable for at least 6 h, but should be reconstituted only just before injection. The stability of dried samples was not evaluated after 6 h. Samples were reconstituted by adding 20 μl of ethyl acetate to dissolve all the yellow residue, followed by extraction of the derivatives with 60 μl of 0.1 *M* hydrochloric acid. After a few minutes, the two phases separated and 20 μl of the lower aqueous phase were injected.

Chromatographic conditions

The mobile phase was acetonitrile–0.05 *M* ammonium acetate (72:28, v/v), pumped at 0.8 ml/min. The column oven was set at 32.5°C. Fluorimetric detection was performed with the excitation wavelength at 352 nm and the emission wavelength at 537 nm.

RESULTS

HPLC analysis

Fig. 2 shows the chromatograms of drug-free plasma and plasma containing 200 ng/ml rokitamycin. Each macrolide derivative appears as two peaks; the second one is much higher than the first because of the *syn–anti* isomerism of the dansylhydrazones [9]. This was confirmed by collecting each peak and reinjecting it onto the column: the new trace showed the appearance of both peaks. For this reason, the second peak was always used for quantitation. The active metabolites of rokitamycin, LMA_7 and LMV, were well separated: the retention times of their derivatives were, respectively, 5.4 and 3.9 min *versus* 11.5 min for dansylrokitamycin and 9.5 min for dansyljosamycin.

Precision and acuracy

Table I shows the inter-asssay reproducibility and the accuracy of rokitamycin determinations measured as a function of the quality controls. Bias was found to be negligible, and reproducibility, expressed as the coefficient of variation

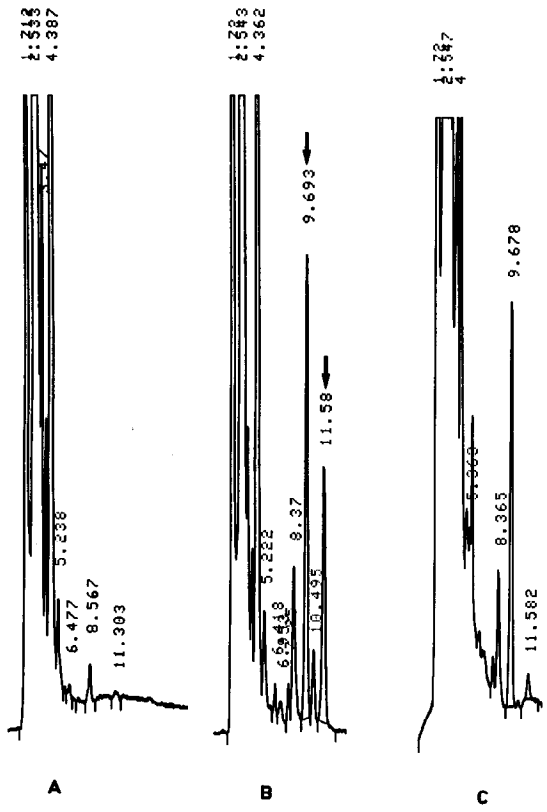

Fig. 2. Chromatograms of (A) a drug-free human plasma sample, (B) 200 ng/ml josamycin (at 9.7 min) and 200 ng/ml rokitamycin (at 11.6 min) in human plasma (8 mV full scale) and (C) 21 ng/ml rokitamycin in a plasma sample from a patient 3 h after a 400-mg oral dose (8 mV full scale).

TABLE I

REPRODUCIBILITY AND ACCURACY OF ROKITAMYCIN DETERMINATION

Parameter	Value	
	100 ng/ml	300 ng/ml
Number of samples	29	29
Mean (ng/ml)	103.8	297.2
Bias (%)	3.8	−1.0
Standard deviation (ng/ml)	10.6	27.1
Coefficient of vatiation (%)	10.2	9.1
Range (ng/ml)	81–119	237–335

(C.V.), was 10.2% at 100 ng/ml and 9.1% at 300 ng/ml.

Plasma extraction efficiency

Comparison of a six-point plasma standard curve with a methanolic standard curve showed an average recovery of 63% over the range 50–400 ng/ml for rokitamycin and 76% for josamycin in the same range.

Derivatization efficiency

The derivatization yield was measured by comparing the peak height of a methanolic standard with that of a dansylmacrolide obtained with an excess of macrolide over dansylhydrazine; it was calculated to be 80% for rokitamycin and josamycin.

Limit of detection

The limit of detection, at a signal-to-noise ratio of 3, of the dansylmacrolides, measured by injection of two-fold dilutions of pure solutions (unextracted) of the derivatives onto the column, was equivalent to 6 ng/ml in plasma. However, such a low level of macrolides could not be derivatized and the limit of detection was 20 ng/ml in 1 ml plasma as shown in Fig. 2C. The limit of quantification (the lowest amount that can be successfully extracted and derivatized with a C.V. less than 15%) was 50 ng/ml.

Reproducibility

The method has been used for two years with few problems, provided that the reagent solutions are freshly prepared frequently, and that the purity of the solvents and the cleanliness of the glassware are rigorously assured. The C.V. of the slope of the calibration line over time was 6.5% (n = 29).

DISCUSSION

Extraction procedure

Previously described procedures for the extraction of josamycin [3] and rokitamycin [2] were tried, but the former gave a low recovery for rokitamycin and the latter was time-consuming. For these reasons, an ion-pair extraction was studied, because all these macrolides have a tertiary amino group in their structure, whose pK_a is *ca.* 7.5 in water. Thus, an ion-pair was formed using lauryl sulphate as the counter-ion, at pH 4.5, where both the macrolide and lauryl sulphate are strongly ionized. Extraction of the ion-pair by ethyl acetate or toluene, chloroform and mixtures of these with hexane afforded low recoveries. Since the octanol–water distribution coefficient is known to be very high for rokitamycin, mixtures of isoamyl alcohol and hexane were tested, which gave high recoveries. The hexane–isoamyl alcohol mixture chosen (90:10) gave a high extraction yield and relatively easy evaporation. A higher content of isoamyl alcohol or a longer-chain alcohol, such as octanol, took too long to evaporate. Shorter alcohols, such as butanol, extracted many more "yellow products", leading to a viscous unmanageable residue. The addition of saturated sodium chloride improved phase separation. The extraction method has been successfully applied to lung and tonsil samples.

Derivatization

It is well known that dansylhydrazine derivatization proceeds under acidic conditions [6–11], and different acids have been used. In this study, three acids were compared: acetic acid, trichloroacetic acid and trifluoroacetic acid, which differ in their pK_a values. Acetic acid gave the highest yield. The effect of the acetic acid concentration in the reaction medium was determined at 0.2, 0.5, 1 and 2 M. The highest derivatization yield was obtained with 0.5 M acetic acid.

Formation of dansylhydrazones produces water (Fig. 3), which should be removed to increase the derivatization yield. The use of toluene–methanol as the reaction solvent enabled solubilization of dansylhydrazine and macrolides, and the formation of an azeotrope with water ensured its elimination, provided that the temperature was higher than 60°C. The reaction kinetics were examined from 2 to 60 min (Fig. 4), revealing the appearance of a plateau at 20 min.

The final evaporation step of the reaction me-

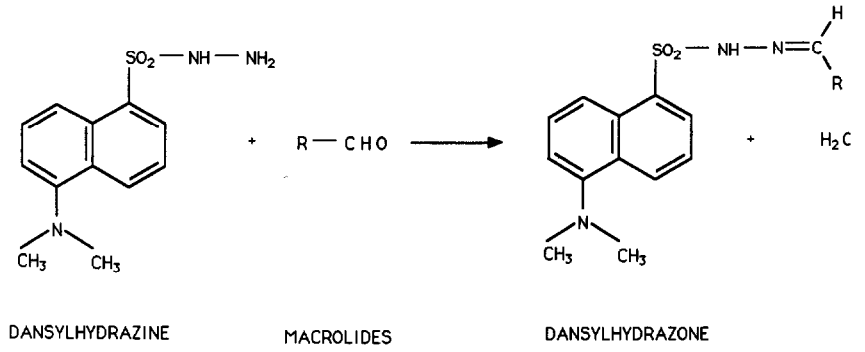

Fig. 3. Derivatization of the macrolides by dansylhydrazine.

dium was important, because it produced an increasing concentration of the reactants, necessary to obtain a quick and complete reaction between low levels of macrolides and dansylhydrazine. This procedure has been described previously [9] and proved successful, as the lowest amount of macrolide that could be derivatized was 25 pmol (20 ng/ml in 1 ml).

Chromatography and detection

Injection of the residue reconstituted in the mobile phase produced some interfering peaks. These were probably due to the derivatization of many endogenous compounds coextracted with the macrolides, such as steroids and related compounds, because the derivatization conditions and detection wavelengths are very similar [6–11]. A considerable improvement was obtained by treating the dry residue with ethyl acetate and hydrochloric acid, as described above. All the "yellow products" remained in the organic phase, while the dansylmacrolides, because of protonation their amino group, were extracted into the acidic aqueous phase, yielding a very clean chromatogram. However, excess dansylhydrazine and the presence of by-products ruled out the possibility of confidently determining the metabolites LMA_7 and LMV, although they were extracted and derivatized like the parent compound. Their quantitation would have required another chromatographic system, but no such attempts were undertaken in this investigation.

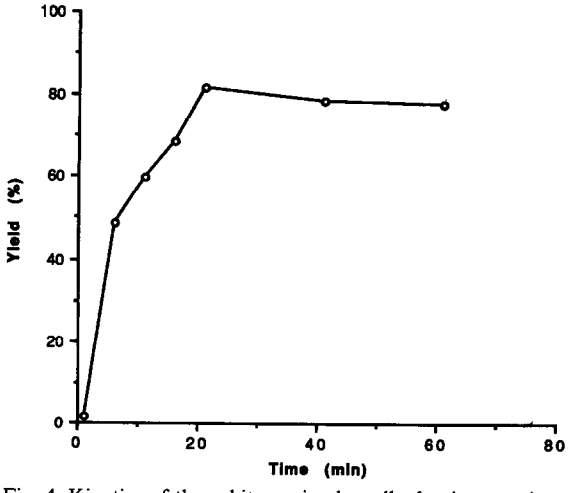

Fig. 4. Kinetics of the rokitamycin–dansylhydrazine reaction at 60°C (mean of two determinations).

CONCLUSIONS

Dansylhydrazine was found to be of great interest in the determination of several macrolides possessing an aldehyde function. The method compared favourably with the routinely used microbiological assay and HPLC with UV detection for sensitivity, reproducibility and selectivity. By paying close attention to some of its subtleties, this procedure was found to be reliable over two years of use in determining josamycin and rokitamycin levels in plasma and tissues, *e.g.* tonsil and lung.

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