Journal of Chromatography, 425 (1988) 197–202
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4008

Note

Determination of PI_A and PII_A, the two main components of pristinamycin, by high-performance liquid chromatography in plasma

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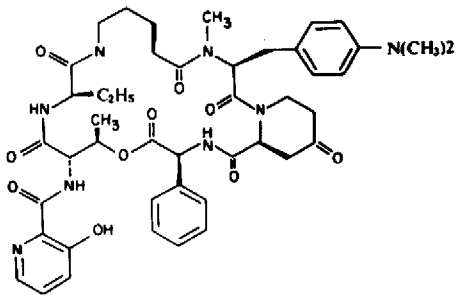
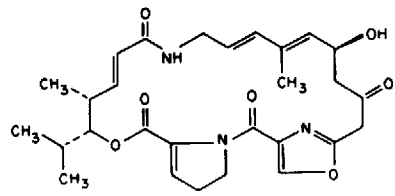
(First received August 20th, 1987; revised manuscript received October 13th, 1987)

Pristinamycin (P) is an antibiotic belonging to the synergistins family [1,2]. This antimicrobial agent is a mixture of two major components, PI_A (a cyclic hexadepsipeptide) and PII_A (a macrocyclic lactone) (Fig. 1). Both have a bacteriostatic activity, and together they show a strong bactericidal synergism [3–7]. The optimal synergism of the mixture of PI_A and PII_A is obtained at well established proportions of each component [8]. For example, against *Staphylococcus aureus* this synergism is optimal in vitro at 30% PI_A and 70% PII_A. These percentages are present in the P administered to patients but, up to now, it has not been possible to verify if these percentages remain unchanged in vivo after oral administration of P.

Indeed, until now, P has been assayed by microbiological procedures [8–10].



PI_A



PII_A

Fig. 1. Structures of PI_A and PII_A.

0378-4347/88/$03.50

However, these are difficult to carry out and their results are unreliable, particularly in respect of the selectivity of the assay for PI_A and PII_A. The lack of a reliable and selective assay for the two major components of P also explains the shortage of precise informations about its pharmacokinetics [1,8,11–13].

In this paper, we propose a procedure for the selective determination of PI_A and PII_A in plasma by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Reagents and chemicals

The PI_A and PII_A components (No. 12535 R.P. Lot SOU 2890 II and No. 12536 R.P. Lot Sou 2891, respectively) were obtained from Rhône-Poulenc (France). Stock solutions of PI_A (1 mg/ml) and PII_A (1 mg/ml) were prepared in methanol and stored at 4 °C. Acetonitrile, hexane, dichloromethane, methanol (all analytical grade), sodium dihydrogenphosphate monohydrate and sodium hydroxide were from E. Merck (Darmstadt, F.R.G.). Water was obtained daily from a Milli-Ro–Milli-Q system (Millipore, Velizy, France).

Sample treatment

An aliquot (1 ml) of plasma was added to 1.5 ml of hexane in a 6-ml screw-capped glass tube. The tube was gently shaken for 10 min by rotation (20 rpm) and centrifuged for 10 min at 1000 *g*. The upper organic layer was aspirated off, and 2 ml of dichloromethane were added to the lower aqueous layer. After shaking for 10 min (20 rpm) and centrifugation at 1000 *g* for 10 min, the upper aqueous layer was aspirated off.

The tubes were put in ice for ca. 5 min and the dichloromethane was transferred to other glass tubes. The organic phase was then evaporated to dryness under nitrogen at 37 °C (10 min). The dry residue was diluted in 60 μl of methanol, and 20 μl were injected into the analytical column.

The extraction recovery was defined by the ratio of the peak height resulting from a supplemented plasma to the peak height resulting from a methanolic solution at the same component concentration. The extraction recovery for PI_A and PII_A was established at 0.1 and 1 μg/ml, with ten replicates for each component and concentration.

Chromatographic conditions

The isocratic liquid chromatograph was constituted from the following units: a Model 302 solvent-delivery module (Gilson, Villiers-le-Bel, France), a sample injection valve equipped with a 20-μl loop (Gilson), a Model HL holochrome variable-wavelength UV–VIS detector (Gilson) and a Model 740 recording data processor (Millipore, Waters Division, Milford, MA, U.S.A.). Separations were performed on a 250 mm × 4 mm I.D. C_{18} reversed-phase analytical column, particle size 5 μm (S.G.E., Villeneuve St. Georges, France) at 25 °C.

The mobile phase was acetonitrile–43 m*M* sodium dihydrogenphosphate monohydrate (53.5 : 46.5, v/v). Sodium hydroxide (0.5 *M*) was then added to adjust the pH to 6. The mobile phase was filtered through a 0.45-μm membrane.

The flow-rate was set at 1 ml/min, and the eluent was monitored at 254 nm. The range setting of the spectrophotometer depended on the concentration of drug measured.

Detection limits and establishment of the calibration curve

The limit of detection was defined as the lowest PI_A or PII_A concentration resulting in a signal-to-noise ratio of 4.

A calibration curve was prepared for each component by diluting both stock solutions in normal plasma, leading to increasing concentrations of PI_A and PII_A: 0.03, 0.1, 0.25, 0.5, 0.75 and 1 μg/ml for PI_A and 0.01, 0.05, 0.1, 0.25, 0.5, 0.75 and 1 μg/ml for PII_A. These plasma samples were then submitted to HPLC analysis, and the peak heights were measured by the integrator. Each standard assay was repeated three times.

Reproducibilities

Both within- and between-day reproducibilities were tested for each component. Two concentrations of a mixture of PI_A and PII_A were included in this study, the first low (0.03 and 0.07 μg/ml, respectively) and the second high (0.3 and 0.7 μg/ml, respectively). Five aliquots of each sample were tested in the same day, and the resulting coefficient of variation (C.V.) indicated the within-day reproducibilities. Aliquots of same sample were tested once a day for five days, and the resulting C.V. indicated the between-day reproducibilities.

Interference

Interferences studies were carried out with many substances that could be coadministered with the P (cloxacillin, vancomycin, guabenxan, bemetizide, verapamil, amiloride, hydrochlorothiazide, indapamide, trinitrin, heptaminol adenosin phosphate, sulindac, diclofenac, analgesics, barbiturics, lorazepam, nitrazepam, sodium alginate, nifuroxazide).

RESULTS

Recovery study

The extraction recovery for PI_A and PII_A is $57.0 \pm 1.7\%$ ($n=10$) and $82.0 \pm 2.3\%$ ($n=10$), respectively. Furthermore, PI_A and PII_A are concentrated 16.7 times. Such concentration of the two compounds results from the small volume of methanol (60 μl) in which PI_A and PII_A are dissolved after evaporation.

Linearity and detection limits

The linearity of plasmatic assays (Fig. 2) was checked from 0.03 to 1 μg/ml for PI_A ($r=0.978$) and from 0.01 to 1 μg/ml for PII_A ($r=0.996$).

The detection limits were established at 30 ng/ml for PI_A and at 10 ng/ml for PII_A.

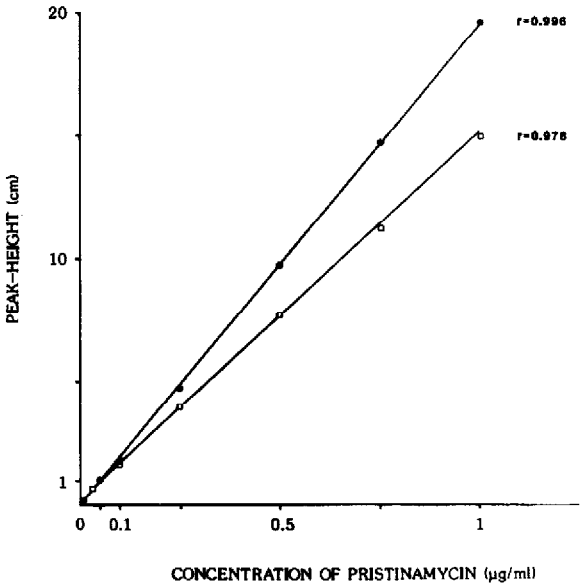


Fig. 2. Linearity of the PI_A (□) and PII_A (●) assays by HPLC in plasma. PI_A assay: $r=0.978$; orthogonal regression equation, $y=14.69x+0.262$ at 0.002 a.u.f.s. PII_A assay: $r=0.996$; orthogonal regression equation, $y=19.67x-0.092$ at 0.01 a.u.f.s. Each point is the mean of three replicates.

TABLE I

PRECISION OF THE HPLC ASSAY

Component	Plasma concentration (µg/ml)	Coefficient of variation (%)	
		Within-day reproducibility ($n=5$)	Between-day reproducibility ($n=5$)
PI_A	0.03	2.3	5
PII_A	0.07	4.2	4.9
PI_A	0.3	4.3	4.8
PII_A	0.7	0.8	5

Precision

The results are reported in Table I. The within-day reproducibility of the plasmatic assays of PI_A and PII_A was characterized by coefficients of variation (C.V.) of 2.3 and 4.2%, respectively, for the detection limits and of 4.3 and 0.8%, respectively, for the highest concentration.

The between-day reproducibility of the plasmatic assays of PI_A and PII_A was defined by C.V. of 5 and 4.9%, respectively, for the detection limits and of 4.8 and 5%, respectively, for the highest concentration.

Selectivity

The calculated selectivity factor relative to PI_A and PII_A was 2.52, which ensured good resolution of both peaks.

Interference study

At each assay, the shape of the peaks of PI_A and PII_A were always carefully checked for skewed peaks, shouldering peaks or tailing peaks resulting from a possible interference with other drugs. Also each blank plasma sample was examined with care to detect any interference with endogenous peaks. During this study no interferences could be observed, either from drugs usually coadministered with the P or from endogenous substances.

Chromatograms and retention times

A typical chromatogram resulting from the analysis of plasma spiked with 0.3 μg/ml PI_A and 0.7 μg/ml PII_A (i.e. ca. 1 μg/ml of P) is shown in Fig. 3.

The chromatogram depicted in Fig. 4 is representative of the analysis of plasma samples from patients treated with a single oral dose of 2 g of Pyostacine® (sample drawn 5.5 h after the dose). PI_A elutes after 6.5 min as a well resolved peak. PII_A is eluted after a 3.4 min retention. It is well resolved from the small endogenous peaks flanking it.

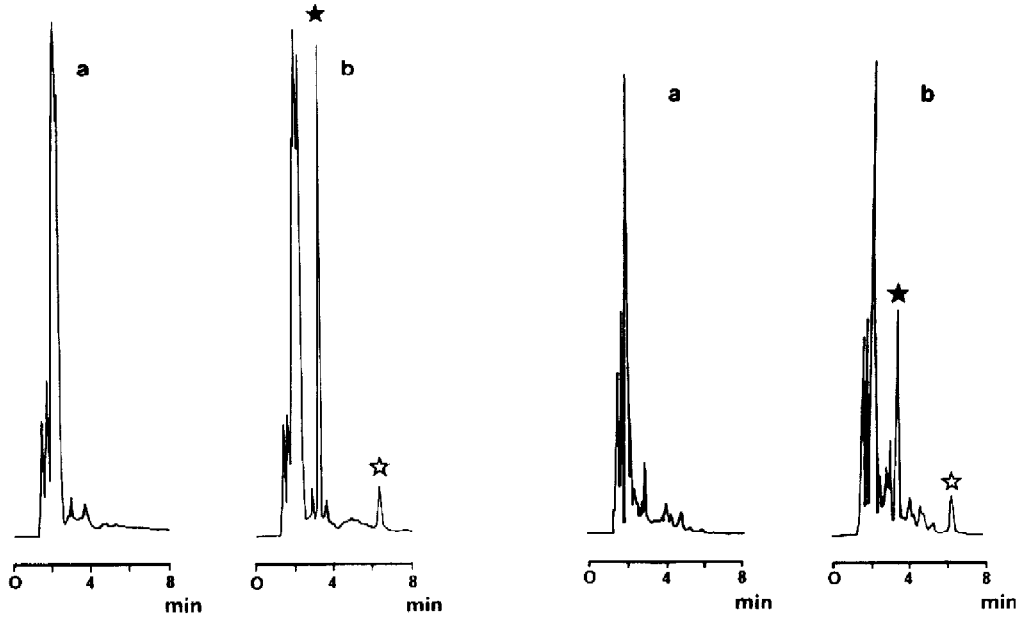


Fig. 3. Chromatograms of (a) a human plasma extract free of PI_A and PII_A; (b) a human plasma extract containing 0.3 μg/ml PI_A (☆) and 0.7 μg/ml of PII_A (★). Detector wavelength, 254 nm; chart-speed, 0.5 cm/min; range, 0.01 a.u.f.s.

Fig. 4. Chromatograms of (a) a human plasma extract before oral administration of Pyostacine ($t=0$ h); (b) a human plasma extract after administration of a single oral dose of 2 g of Pyostacine containing PI_A (☆) and PII_A (★) ($t=5.5$ h). Detector wavelength, 254 nm; chart-speed, 0.5 cm/min; range, 0.01 a.u.f.s.

DISCUSSION

The HPLC assay developed here is selective for PI_A and PII_A: this is essential if knowledge of the proportions of the main components of P present in vivo is to allow an evaluation of its activity [8]. Furthermore, no interference due to usually coadministered drugs could be detected.

The detection limits expected are widely reached for PI_A and PII_A since they represent ca. 1.4 and 5%, respectively, of the maximum plasma concentrations found. These values were achieved by the concentration during the last extraction step.

The reproducibility of the assay is satisfactory when the C.V. is never more than 5%, for the detection limits as well as for the maximum concentrations. The linearity is also good since it is characterized by coefficients of correlation close to 1 when tested for concentrations ranging from the detection limit to the maximum. These two last characteristics of the assay show that the use of an internal standard is not necessary. It is sufficient to construct calibration curves simultaneously for each series of assays.

It is well known that in whole blood PII_A is subject to extensive enzymatic degradation, as more than 75% of the amount of PII_A is degraded in 2 h at 4°C [8–10]. The speed of the assay procedure described (extraction of P from total blood in less than 30 min) was not sufficient to solve this problem. However, immediate centrifugation either after addition of P to the blood (during the assay development) or after sampling (during pharmacokinetic studies) means that the PII_A component is then no longer in contact with erythrocytes and is stable in the plasma [8]. Thus, the resulting plasma concentration is the same as the concentration at the moment of sampling.

The assay proposed here is simple and rapid. The isocratic conditions allow the chromatographic separation of the two main components of P in ca. 7 min, in spite of the dissimilarity of their chemical structures.

With the aid of this selective and accurate assay technique, a pharmacokinetic study of PI_A and PII_A components could be undertaken that would yield precise data concerning the pharmacokinetics of P.

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