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Determination of PNU 153429, a new polysulphonated derivative of distamycin A, in rat plasma by reversed-phase ion-pair high-performance liquid chromatography with ultraviolet detection[1]

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Abstract

A rapid and selective ion-pair high-performance liquid chromatographic (HPLC) method for the determination of 2,2′ - (carbonylbis(imino - *N* - methyl - 4,2 - pyrrolecarbonylimino(*N* - methyl - 4,2 - pyrrole)carbonylimino)) - bis(1,5 - naphtalenedisulphonic acid), tetrasodium salt (PNU 153429, I) in rat plasma has been developed. I is a new drug currently under investigation for the treatment of rheumatoid arthritis. Aliquots of 100 μl of plasma spiked with 10 μl of internal standard solution (PNU 145156E, I.S.) were added to 100 μl of acetonitrile and vortex mixed. After centrifugation, diluted aliquots of the supernatant were transferred to autosampler vials and analyzed by reversed-phase ion-pair liquid chromatography under isocratic conditions. The retention times of I.S. and I were ≈ 8 and 12 min, respectively. Quantitation was achieved by ultraviolet detection at 323 nm. The assay had a limit of quantitation of 0.1 μg ml^{-1} when 100 μl of plasma were analyzed. The linearity, precision and accuracy of the method were evaluated. No interference from blank rat, mouse, dog, monkey and human plasma was observed. The suitability of the method for in vivo samples was checked by analysis of plasma samples drawn from three cannulated male rats that had received a single 100 mg kg^{-1} i.v. dose of the test compound.

Keywords: 2,2′-(Carbonylbis(imino-*N*-methyl-4,2-pyrrolecarbonylimino(*N*-methyl-4,2-pyrrole)carbony-limino))bis-(1,5-naphtalenedisulphonic acid), tetrasodium salt; Rat plasma; HPLC determination; UV detection

1. Introduction

Rheumatoid arthritis, a disease of unknown aetiology, is characterized by joint inflammation and, in its later stages, cartilage destruction. Inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin-1 α or -1 β have been

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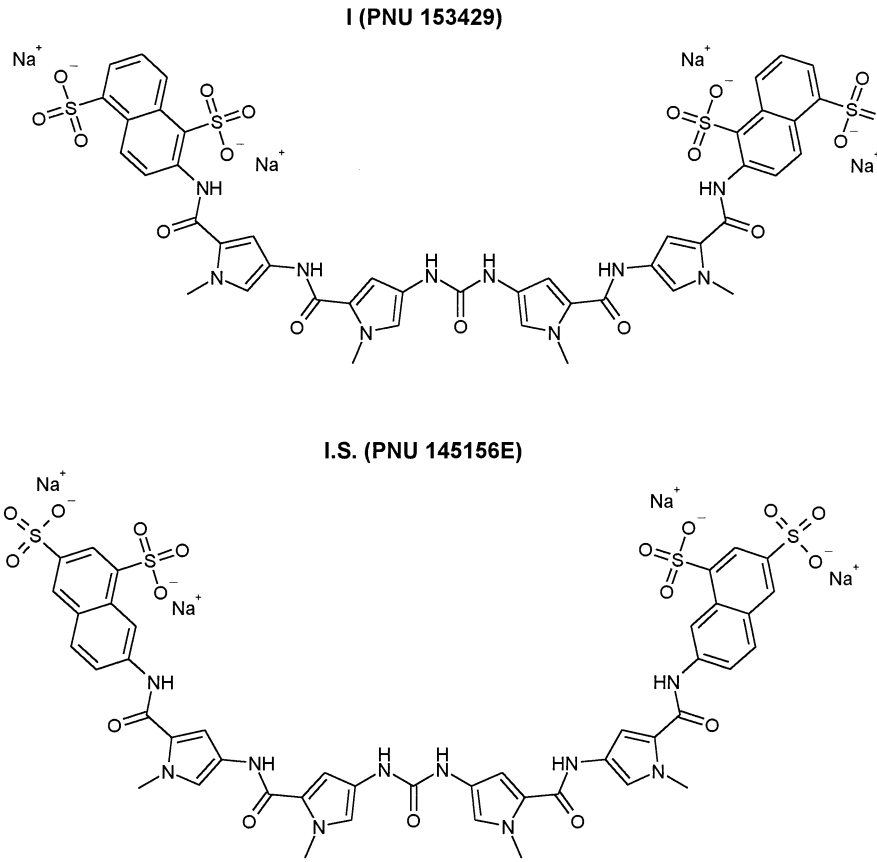

Fig. 1. Structural formulae of I and I.S.

implicated both as regulatory and as effector mediatores of this process [1].

In a research program aimed at developing pharmacological agents able to block the interaction of several cytokines with their cell surface receptors, a number of novel polysulphonated derivatives of distamycin A were synthesized [2]. Among these is 2,2′-(carbonylbis(imino-*N*-methyl-4,2-pyrrolecarbonylimino(*N*-methyl-4,2-pyrrole) carbonylimino))-bis(1,5-naphtalene disulphonic acid), tetrasodium salt (Laboratory code PNU 153429, (I), Fig. 1), a new drug currently under pre-clinical investigation for the treatment of rheumatoid arthritis. I has been shown to inhibit interaction of several cytokines with their cell surface receptors and to induce deoligomerization of the TNF trimers to their inactive monomers. Due to structural analogies (Fig. 1), I might have chemico-physical and pharmacokinetic characteristics similar to those observed for PNU 145156E, namely 7,7′-(carbonylbis(imino-*N*-methyl-4,2-pyrrolecarbonyl imino(*N*-methyl-4,2-pyrrole)carbonylimino))-bis(1,3-naphtalene disulphonic acid), tetrasodium salt. This is currently under Phase I clinical evaluation as an angiogenesis inhibitor. This compound was selected as internal standard (I.S.) for this assay. Determination of I.S. in monkey plasma by high-performance liquid chromatography (HPLC) has already been described [3]. Following a single 20 mg kg^{-1} i.v. dose of I.S. in the monkey, the mean ($\pm$ SD, $n = 3$) maximal concentration observed 5 min post dosing was 382 ± 90 μg ml^{-1} and thereafter the drug was shown to be eliminated very slowly from

plasma with a mean ($\pm$ SD, $n = 3$) terminal half-life of 1135 ± 338 h.

This paper describes the determination of I by ion-pair HPLC with isocratic elution after a rapid procedure for extraction of the analytes from plasma. The HPLC method developed was validated down to a concentration of 0.1 μg ml^{-1} in rat plasma and used for the quantitation of the drug in plasma samples obtained from animals that had received a single 100 mg kg^{-1} i.v. dose of the test compound.

2. Experimental

2.1. Chemicals and solutions

I and I.S. (as tetrasodium salts) were supplied by the Chemistry Department of Pharmacia and Upjohn (Milan, Italy). All other chemicals and solvents were of analytical reagent grade from Carlo Erba Reagents (Milan, Italy) with the exception of 40% (1.5 M) tetrabutylammonium hydroxide (TBAOH) which was purchased from Fluka (Buchs, Switzerland).

Stock solutions of I and I.S. were prepared by dissolving accurately weighed amounts of I and I.S. in bi-distilled water. Suitable working solutions were prepared by dilution with bi-distilled water. Stock and working solutions were found to be stable for at least 1 month if stored at 4°C.

All glassware was silanized before use by treatment with 7% dimethyldichlorosilane in toluene solution followed by rinsing with ethanol, to minimize adsorption.

2.2. Liquid chromatography

The HPLC system used in this study consisted of an isocratic pump (Model Isochrom), an autosampler (Model AS 1000) with a 200 μl loop, a variable wavelength UV detector (Model Spectro Monitor 3200) and a data acquisition system (Model WOW) with Labnet network. Real time chromatograms were obtained on an integrator (Model Chromjet). All these instruments were supplied by Thermo Quest (San Jose, CA).

2.3. Chromatographic conditions

The chromatographic separation was performed with a 150×3.9 mm i.d. Symmetry™ C18, 5 μm, reversed-phase column connected to a 20×3.9 mm i.d. Sentry™ Nova-Pak C18, 4 μm, guard column (both from Waters, Milford, MA, USA). The mobile phase consisted of 0.05 M monobasic potassium phosphate containing the ion-pairing reagent TBAOH (0.060 M) buffered to pH 7.0 with 85% orthophosphoric acid/acetonitrile mixture (48:52, v/v). The flow-rate was 0.5 ml min^{-1}. The detector was set at 323 nm and wired to send a 1 V/AUFS signal to the data system.

2.4. Sample preparation

Aliquots of plasma (100 μl) were transferred to 1.5 ml polypropylene microcentrifuge tubes, then 10 μl of I.S. solution and 100 μl of acetonitrile were added to each tube and vortex mixed. The samples were centrifuged at 12 000 rpm for 2 min and aliquots of 100 μl of the supernatant were diluted with 150 μl of 0.05 M monobasic potassium phosphate solution containing the ion-pairing reagent TBAOH (0.060 M) buffered to pH 7.0 with 85% orthophosphoric acid. The resulting solutions were transferred to autosampler polypropylene vials and an aliquot (200 μl) was injected onto the column.

2.5. Determination of I concentration in quality control and calibration samples

Blank rat plasma samples spiked with known amounts of I and I.S. were analyzed and carried out applying the above procedure. The linearity was evaluated from three calibration curves with six calibration points prepared. These were run on 3 different days in the concentration range 0.1–35 μg ml^{-1} plasma (concentrations were expressed as free acid). The precision and accuracy were evaluated by repeated analyzes of I at three concentrations (≈ 0.5, 1 and 20 μg ml^{-1}) in three replicate samples analyzed on 3 different days. All chromatograms obtained were evaluated by peak-area measurement. The concentration in quality control samples was calculated using the calibra-

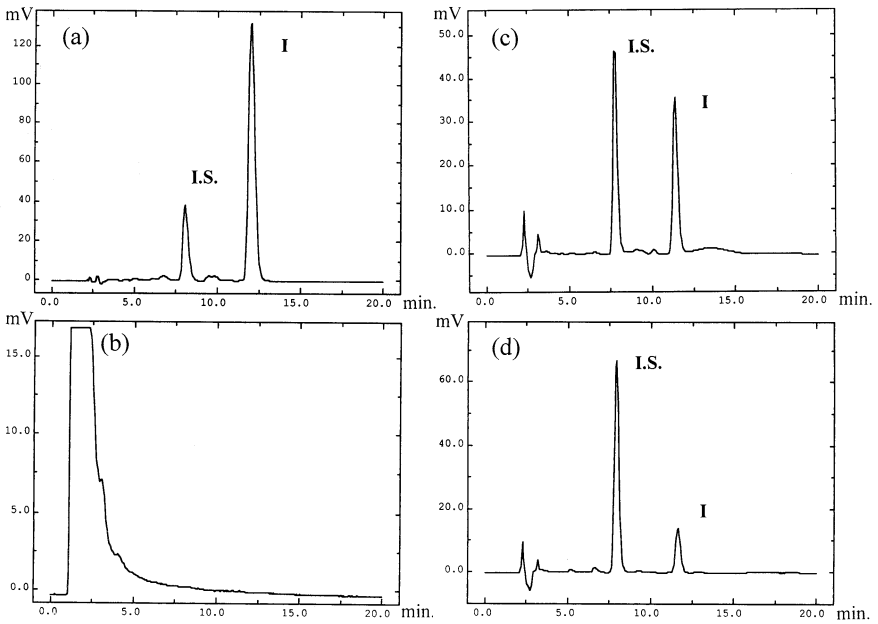

Fig. 2. Chromatogram of (a) standard solution containing 24.4 μg ml^{-1} of I and 5.6 μg ml^{-1} of I.S.; (b) blank rat plasma (0.1 ml); (c) rat plasma (0.1 ml) spiked with 1.5 μg of I and 1.4 μg of I.S.; and (d) plasma (0.1 ml) from a rat given a 100 mg kg^{-1} i.v. dose of I (168 h after administration). Peaks, I = PNU 153429; I.S. = PNU 145156E.

tion curve generated on each day by linear regression (weighting factor $1/y^2$) of the analyte/I.S. peak area ratio against their concentration ratio in plasma.

To evaluate the extraction recovery, the peak area of extracted plasma samples was compared to the peak area obtained with standards dissolved in the mobile phase and injected directly onto the chromatograph.

2.6. Chromatographic system suitability test

The suitability of the chromatographic system for the analysis of I was checked during the validation assay by calculating the column efficiency, the peak symmetry and the resolution factor of the peaks of the two analytes. This evaluation was carried out according to USP [4] using the System Suitability Test software supplied by Thermo Quest.

The column efficiency was expressed as the number of theoretical plates (N). This value must be higher than 4000 for both analytes. The tailing factor (T) must be less than 1.5 for both analytes. The resolution factor (R) between the peaks of the two analytes must be >2.

3. Results and discussion

The chromatograms obtained (a) from a standard solution of I and I.S.; (b) from blank rat plasma; (c) and from spiked rat plasma, are shown in Fig. 2. The retention times of I.S. and I were ≈ 8 and 12 min, respectively.

In comparison with the method previously developed for the determination of I.S. in monkey plasma, the chromatographic conditions adopted have been slightly changed while the extraction procedure has been considerably simplified. The use of a new column (Symmetry instead of Nova-Pak, Waters) allowed us to improve the sharpness, symmetry and reproducibility of the chromatographic peaks. A single deproteination step has been used successfully for the sample preparation instead of a more complex and time-

Table 1
Accuracy and precision of the method for the determination of I in rat plasma

Control sample (μg ml^{-1})	Day	n	Accuracy			Precision		
			Mean found (μg ml^{-1})	Mean recovery (intra-day) (%)	Pooled recovery (inter-day) (%)	SD	RSD (intra-day) (%)	Pooled RSD (inter-day) (%)
0.50	1	3	0.52	104.67		0.003	6.14	
	2	3	0.58	115.00		0.001	1.00	
	3	3	0.46	92.00	103.33	0.002	4.35	11.29
1.10	1	3	1.20	109.39		0.011	9.15	
	2	3	1.21	110.30		0.001	0.48	
	3	3	1.11	100.61	106.77	0.002	1.38	6.43
21.00	1	3	21.12	100.59		0.037	1.73	
	2	3	20.39	97.10		0.028	1.40	
	3	3	24.14	114.95	104.21	0.015	0.64	7.94

consuming liquid–liquid extraction, improving the overall throughput of the method. In any case, the new procedure involves minimal extraction of endogenous compounds from the complex plasma matrix and none of these were found to interfere with the compounds of interest. Mouse, dog, monkey and human plasma can also be assayed by this procedure since the chromatograms obtained were free from interfering peaks at the retention times of the compounds of interest.

The linearity of this HPLC assay was evaluated from three calibration curves run on different days over the concentration range 0.1–35 μg ml^{-1} of rat plasma. A linear regression analysis of the peak area ratio (analyte/I.S.) against the concentration ratio (analyte/I.S.) showed good linearity over the whole range of concentrations tested. The mean calibration curve obtained was described by the equation $y = 0.8189x + 0.0002$ (slope RSD = 6.2%, $n = 3$). Back-calculated concentrations exhibited a RSD $< 12.1\%$. Correlation coefficients (r) ranged from 0.9948 to 0.9985. If the concentration of an unknown sample is suspected to fall outside the range of linearity of the calibration curve, the sample may be assayed using a smaller volume of plasma made up to 0.1 ml with blank rat plasma.

The inter-day precision for concentrations between 0.5 and 21 μg ml^{-1} of rat plasma expressed as RSD ranged from 6.43 to 11.29%, Table 1. At the same concentrations, the intra-day precision was $> 9.15\%$. The intra-day accuracy, evaluated on the same plasma samples and expressed as percentage ratio of the mean amount found to the amount added to plasma, ranged from 92.00 to 115.00%. The pooled accuracy (inter-day) over the 3-day validation period ranged from 103.33 to 106.77%.

The extraction recovery of I and I.S. from rat plasma over the concentration range of 0.1–35 μg ml^{-1} was quantitative. The limit of quantitation (LOQ), chosen as the lowest point on the calibration graph having a back-calculated concentration within 20% of the nominal value, was 0.1 μg ml^{-1} of plasma.

The compounds were found to be stable for at least 48 h when dissolved in the mobile phase and stored at room temperature in plastic vials, thus allowing the use of a non-refrigerated autosampler for automatic injection onto the column of the extracted samples. The present method was used for the determination of plasma levels of I in three cannulated male rats that had received a single 100 mg kg^{-1} i.v. dose of I as a bolus injection. Blood samples (0.2 ml) were drawn into heparinized tubes at 0, 5, 15, 30 min, 1, 2, 4, 24, 48, 96, 168 and 336 h after dosing. The samples were immediately put on ice and centrifuged at $1200 \times g$ for 15 min at $+4$°C. Plasma was separated and stored at -20°C until assayed. A typical chromatogram obtained from one animal at 168 h after dosing is shown in Fig. 2(d). Mean plasma concentrations ($\pm$SD) decreased from 1432.8 ± 77.8 at 5 min to 9.0 ± 1.3 μg ml^{-1} at 336 h after dosing.

4. Conclusion

The method described here is sensitive and selective for the determination of I in rat plasma. It proved to be linear, precise and capable of accurately quantitating the analyte in the concentration range of 0.1–35 μg ml^{-1}. Due to the very rapid extraction procedure and the relatively short HPLC analysis time, more than 80 samples per run/day can easily be analyzed. The suitability of the method was demonstrated in a pharmacokinetic study of I in male rats. This method can, therefore, be very useful in further investigations on the pharmacokinetics of I in pre-clinical studies with the test compound.

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