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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC EVALUATION OF PCF 39, A NEW IMMUNOMODULATOR AGENT

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SUMMARY

An high-performance liquid chromatographic analysis of PCF 39, N^2-[5-(hypoxanthin-9-yl)pentyloxycarbonyl]-L-arginine, with ultraviolet detection, has been devised and validated. The main pharmacokinetic results encountered for rats treated intravenously with PCF 39 at a dose of 100 mg/kg are described.

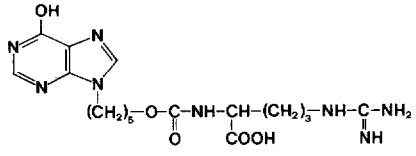
INTRODUCTION

PCF 39 (Fig. 1) is a new drug which in animal immunopharmacological investigations has been demonstrated to normalize the immune defence of immunodepressed mice and to activate the endogenous production of lynphochines and other non-anticorpal immunologic mediators; experimental evidence suggests a macrophage activation as the mechanism of the PCF 39 activity[1–3]. An analytical method meeting all the requirements for quantitative evaluation of the drug in biological samples was standardized in order to investigate its pharmacokinetics in the rat, mouse and human beings. This paper describes the analytical procedures and the main pharmacokinetic results with the rat.

EXPERIMENTAL

Materials

Solvents and chemicals, all of analytical or HPLC grade, were supplied by Merck (Bracco, Milan, Italy). PCF 39 was supplied by Sigma Tau (Rome, Italy).



$C_{17}H_{26}N_8O_5$ M.W. = 422.44

Fig. 1. Chemical structure of PCF 39.

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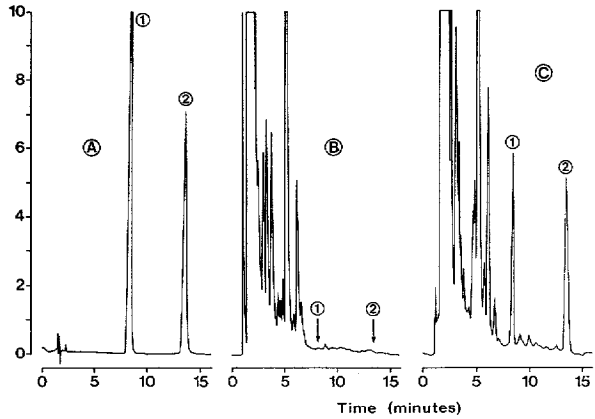

Fig. 2. Chromatograms of PCF 39 (1) and caffeine (2). (A) Authentic standards; (B) blank plasma; (C) plasma of rat treated with the drug. For chromatographic conditions, see Experimental.

A Varian Model 5020 liquid chromatograph and a Varian Model 2050 UV variable detector were used for the analysis. The column was a Supelcosil LC-18 DB, 5 μm, 250 mm × 4.6 mm I.D., supplied by Supelco (Supelchem, Milan, Italy). The statistical computations were performed on a Macintosh Plus personal computer (Apple computer, Reggio Emilia, Italy).

Methods

A 1 ml volume of plasma (or urine or tissue homogenate) was deproteinized with 2 ml methanol. After stirring for 10 min and centrifuging at 2400 *g* for 10 min, an aliquot of the supernatant was separated and evaporated under a nitrogen flow at 40°C. The solution was then reconstituted with 75–150 μl water for high-performance liquid chromatography (HPLC). An appropriate amount of internal standard (caffeine) was added after the extraction to monitor recovery, and before extraction in the routine analysis.

The mobile phase consisted of methanol–0.05 *M* phosphate buffer at pH 7.2 (18:82). The flow-rate was 1.8 ml/min. Absorbance was monitored at 250 nm. Retention times were 8.27 min for PCF 39 and 13.40 min for caffeine. Fig. 2 shows a typical HPLC chromatogram of the two substances.

Sprague-Dawley male rats, weighing 230–270 g, supplied by Charles River (Calco, Italy) were used in all the experiments.

PCF 39 was administered in doses of 25, 50 and 100 mg/kg intravenously. The rats were sacrified by cervical dislocation, and the serial plasma concentrations and tissue distribution in the gastric and intestinal wall, liver, kidneys, lungs, myocardium, lymph nodes, thymus and spleen were evaluated as described above. Similarly, the parent drug was evaluated in urine excreted over 0–3, 3–6, 6–9 and 9–24 h periods following administration. The cumulative biliary excretion of the drug was also investigated, in anesthetized rats through a biliary fistula.

TABLE I

RECOVERY OF PCF 39 FROM RAT PLASMA IN TRIPLICATE TRIALS

Internal standard (I.S.) was added in such a way as to achieve a ratio drug/I.S. of 1 : 1 for each drug level. During routine analysis, I.S. was such as to remain in a ratio drug/I.S. ranging from 1 : 4 to 4 : 1. The linear regression method gave the following correlation: $Y = 0.167 + 0.929\ X$, where $r^2 = 0.9978$.

PCF 39 added (= X) (μg/ml)	*PCF 39 recovered (= Y)*			*Recovery (%)*
	Mean (μg/ml)	*S.D.*	*% C.V.*	
0.5	0.50	0.008	1.54	99.3
1	0.99	0.03	3.07	99.3
2.5	2.42	0.07	2.73	97.0
10	9.85	0.23	2.37	98.5
25	24.91	0.14	0.55	99.6
50	45.46	1.04	2.29	90.9
100	92.27	2.64	2.87	92.3
250	232.87	11.60	4.98	93.1
			Mean	96.2 ± 4.08
			% C.V.	4.24

RESULTS AND DISCUSSION

The hydrophilic nature of PCF 39 did not allow it to be extracted from biological samples by solvent partition. This problem was solved by deproteinization, followed by sample concentration and injection. This procedure enabled high recovery of PCF 39, *i.e.*, 96.2%, and linear in the range of 0.5–250 μg/ml, with a correlation

TABLE II

LINEARITY OF DETECTOR RESPONSE WITH A FIXED DRUG-TO-INTERNAL STANDARD RATIO (1 : 1), ASCERTAINED BY A CONSTANT DETECTOR RESPONSE FACTOR (DRF)

$$\text{DRF} = \frac{\text{I.S. peak area}}{\text{analyte peak area}} \cdot \frac{\text{analyte concentration}}{\text{I.S. concentration}}$$

PCF 39 injected (ng)		*DRF (mean ± S.D.) (n = 4)*
40		0.667 ± 0.0043
100		0.672 ± 0.0017
200		0.677 ± 0.0021
400		0.684 ± 0.0006
1000		0.686 ± 0.0017
2000		0.686 ± 0.0030
4000		0.684 ± 0.0035
	Mean	0.679
	S.D.	0.0076
	% C.V.	1.11

TABLE III

DETECTOR RESPONSE FACTOR AT DIFFERENT PCF 39 TO INTERNAL STANDARD (I.S.) RATIOS WITH AMOUNTS RANGING BETWEEN 250 AND 1000 NG

Linearity was ascertained as in Table II.

Drug / I.S. (ng)		*DRF*
250/1000		0.684
500/1000		0.688
1000/1000		0.689
1000/500		0.688
1000/250		0.681
	Mean	0.686
	S.D.	0.0034
	% C.V.	0.50

coefficient, r^2, of 0.9978 (Table I). The extraction recovery of the caffeine internal standard (I.S.) was similar to that of PCF 39. The linearity was verified in the range 40–4000 ng of PCF 39 and I.S., injected at drug-to-internal standard ratios of 1:1 and 1:4–4:1 respectively (Tables II and III).

The reproducibility tests gave an inter-assay coefficient of variation of 1.11% with a fixed ratio drug/I.S. and 0.50% with a variable ratio drug/I.S. When replication of the same drug concentration was carried out, an intra-assay coefficient of variation (C.V.) of 0.35% was obtained. The C.V. rose to 4.24% when the entire process of extraction was completed (Tables I–III).

The lowest detectable amount of PCF 39 was 1 ng, which is 30 ng/ml in terms of plasma or tissue concentration.

When injected intravenously, PCF 39 was cleared from plasma with a three-phase behaviour from the highest value of 225 μg/ml observed at 3.75 min to the lowest, 52 ng/ml, observed after 6 h(Fig. 3). The three phases showed $t_{1/2}$ values of 2.98, 21.3 and 156.9 min respectively. This may be governed by (a) the physical

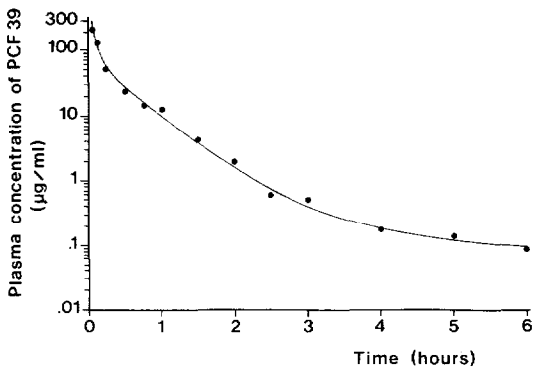

Fig. 3. Plasma concentration *vs.* time curve after i.v. administration of PCF 39 to rats. $C_p t = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$, where $A = 403$ μg/ml, $\alpha = 13.9\ h^{-1}$, $B = 68.4$ μg/ml, $\beta = 1.9\ h^{-1}$, $C = 0.46$ μg/ml and $\gamma = 0.26\ h^{-1}$.

dilution of the drug, (b) both rapid and slow plasma–tissue equilibration processes, (c) urinary excretion, which occurs mainly in the first 3 h and (d) biotransformation processes.

Cumulative urinary excretion was as high as 55%, and biliary excretion in 5 h rose to 12% on average. Among the tissues investigated, the kidneys, liver, lymph nodes and thymus showed the highest concentrations of the drug [60.6, 94.2, 38.4 and 28.3 μg/g of wet organ respectively after intravenous (i.v.) administration of PCF 39 at a dose of 100 mg/kg].

In conclusion, this method achieved a sufficient degree of validation for pharmacokinetic application. With this method a skillful operator can routinely analyse around 30 samples a day by manual injection and use of an automatic integrator. As only routine instrumentation is required, the method is simple, easy and inexpensive.

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