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Short Communication

High-performance liquid chromatographic assay for N^2-[5-(hypoxanthin-9-yl)pentyloxycarbonyl]-L-arginine (ST 789) in plasma by cyclization with benzoin and fluorimetric detection

G. Bruno*, S. Curti and A. Longo

Research and Development Division, Department of Analytical Methods, Sigma-Tau S.p.A., Via Pontina km. 30,400, 00040 Pomezia, Rome (Italy)

A. Marzo

Research and Development Division, Department of Drug Metabolism and Pharmacokinetics, Sigma-Tau S.p.A., Via Pontina km. 30,400, 00040 Pomezia, Rome (Italy)

E. Arrigoni Martelli

Research and Development Division, Department of Analytical Methods, Sigma-Tau S.p.A., Via Pontina km. 30,400, 00040 Pomezia, Rome (Italy)

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ABSTRACT

This paper describes a new highly sensitive assay for N^2-[5-(hypoxanthin-9-yl)pentyloxycarbonyl]-L-arginine, an immunomodulatory agent, required for clinical pharmacokinetic investigation. A pre-column derivatization by cyclization with benzoin in aqueous medium produces the fluorescent 2-substituted amino-4,5-diphenylimidazole fluorescing at 450 nm (excitation wavelength 310 nm). L-Arginine-acetyl-L-carnitinamide chloride (ST 857, II), another arginine derivative, was used as an internal standard. A C_{18} DB column (5 μm, 250 mm × 4.6 mm I.D.) and a 45:55 (v/v) mixture of 0.05 *M* ammonium phosphate at pH 7.2 and methanol as mobile phase were used. Linearity was ascertained in the range 5–100 ng. Extraction recovery from plasma proved to be higher than 90% in the range 5–50 ng/ml. Intra-day precision, expressed as coefficient of variation, was in the range 4.7–6.0%. The limit of quantification proved to be 5 ng/ml and the limit of detection 2 ng/ml at a signal-to-noise ratio of 5. The method is specific.

INTRODUCTION

N^2-[5-(Hypoxanthin-9-yl)pentyloxycarbonyl]-L-arginine (ST 789, I) (Fig. 1) is a new drug, which in animal immunopharmacological investigations has been demonstrated to normalize the immunodefence of immunodepressed mice and to activate the endogenous production of lymphokines and other immunological mediators: experimental evidence suggests that macrophage activation is the

* Corresponding author.

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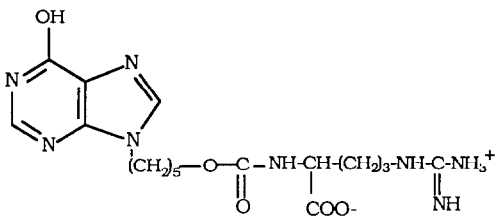
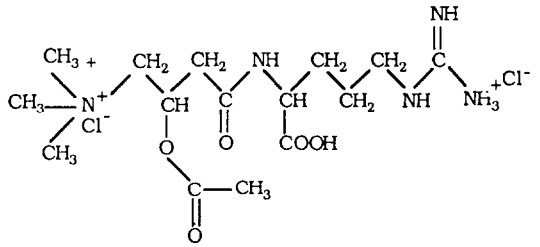


I (ST789) $C_{17}H_{26}N_8O_5$ Mr = 422.44



II (ST857 LS.) $C_{15}H_{31}Cl_2N_5O_5$ Mr = 432.35

Fig. 1. Chemical structures of I and II (internal standard).

mechanism of the activity of compound I [1–3].

In a previous paper [4] we described an HPLC assay for non-derivatized I employing UV detection at 250 nm and caffeine as an internal standard, allowing the detection of 30–50 ng/ml as the lowest detectable concentration. It was employed in a series of preclinical pharmacokinetic investigations [5].

The recent approval of an IND (authorization for Introduction of New Drugs in Human Experimentation) for compound I called for a new highly sensitive assay of this drug for pharmacokinetic and bioavailability investigations in humans. This paper describes a selective, sensitive assay for I based on pre-column derivatization by cyclization with benzoin [6,7], producing a 2-substituted amino-4,5-diphenylimidazole. Endogenous arginine was previously separated by solid-phase extraction. Compound II was used as internal standard. The sensitivity and other analytical specifications allow this method to be used for pharmacokinetic and bioavailability investigations in humans.

EXPERIMENTAL

Chemicals

All reagents used for the derivatization were from Sigma (St. Louis, MO, USA). The analytical- and HPLC-grade solvents were from Merck (Bracco, Milan, Italy).

Apparatus

The chromatographic equipment was a Varian 9000 STAR system consisting of a solvent-delivery system (Model 9010) and a fluorescence detector (Model 9070), controlled by software for data collection (Varian, Humboldt, Sunnyvale, CA, USA).

Chromatographic conditions

The reversed-phase cartridges (C_{18}; 1 ml) and the HPLC column (C_{18} DB) were from Supelco (Bellefonte, PA, USA). Compounds I and II (internal standard) were supplied by Sigma-Tau (Pomezia, Rome, Italy). The HPLC column was a C_{18} DB, 5 μm (250 mm × 4.6 mm I.D.). The mobile phase consisted of 0.05 *M* ammonium phosphate buffer (pH 7.2)–methanol (45:55, v/v). The flow-rate was 1.2 ml/min. The fluorimetric detector was set at an excitation wavelength of 310 nm and an emission wavelength of 450 nm. Under these conditions the retention times of I and internal standard (II) were 16.2 and 12.0 min, respectively.

Extraction

Compound I was extracted from plasma by liquid–solid partition on reversed-phase cartridges according to the following procedure: plasma (1 ml) was loaded on a Supelclean C_{18} cartridge, previously equilibrated with methanol and water (3 ml of each); then the column was washed with water (4 ml) and eluted with methanol (2 ml). The eluate was concentrated, dissolved in 100 μl of water, and 10 μl of a 5 μg/ml solution of internal standard were added; the sample was ready for the derivatization.

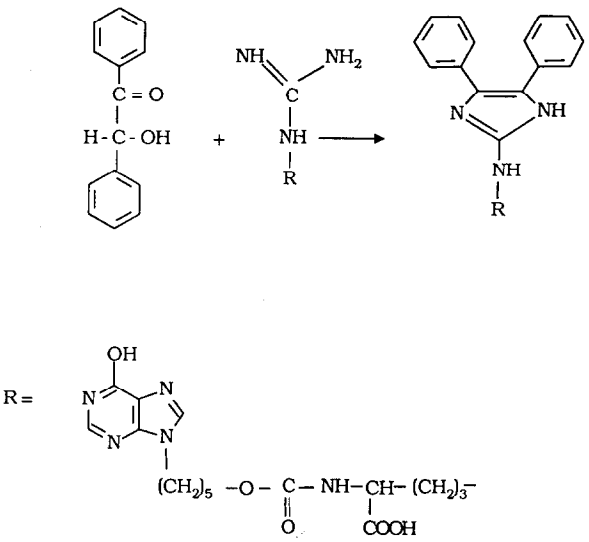


Fig. 2. Derivatization of I with benzoin producing a 2-substituted amino-4,5-diphenylimidazole.

Derivatization

The following solutions were used for the fluorescence derivatization (Fig. 2): (1) 4 m*M* benzoin (50 μl); (2) 0.1 *M* β-mercaptoethanol–0.2 *M* sodium sulphite (50 μl); (3) 2.0 *M* potassium hydroxide (100 μl); (4) 2 *M* hydrochloric acid–0.5 *M* Tris–HCl pH 9.2 (100 μl).

To 100 μl of sample solution, solutions 1, 2 and 3 were added; the mixture was heated at 100°C for 5 min, cooled in ice-water for 2 min and acidified with solution 4.

RESULTS

Linearity and accuracy

The linearity of the assay was ascertained in the range 5–100 ng/ml (in terms of plasma concentration) producing the following results where $y =$ detector response expressed as I/internal standard peak-area ratio and $x =$ I added (ng/ml): $y = 4.0 \cdot 10^{-3}x - 3.2 \cdot 10^{-3}$ with a linear regression coefficient $r = 0.9997$ (Table I).

The accuracy of the assay was determined in the range 5–100 ng/ml: it ranged etween 7 and 4% (Table I).

TABLE I

LINEARITY AND ACCURACY OF THE ASSAY OF I IN HUMAN PLASMA

$y = +4.0 \cdot 10^{-3}x - 3.2 \cdot 10^{-3}$; $r = 0.9997$.

I added (x) (ng/ml)	I/internal standard ratio	I found (y) (ng/ml)	Accuracy (%)
5	0.0177	5.2	4.0
10	0.0351	9.5	5.0
20	0.0716	18.6	7.0
50	0.2030	51.6	2.4
100	0.3982	99.7	0.3

Extraction recovery

Recovery of I from plasma at concentrations of 5 and 50 ng/ml was 96.2 ± 6.4% (mean ± S.D.) (Table II).

Precision, selectivity and sensitivity

Precision was evaluated by spiking human blank plasma with I at the concentrations of 5 and 100 ng/ml (six samples for each concentration) and internal standard and performing all the analytical manipulations above described. The intra-day C.V. was in the range 4.7–6.0% (Table III).

TABLE II

EXTRACTION RECOVERY OF I FROM HUMAN PLASMA

I added (ng/ml)	I/internal standard ratio		Recovery (%)
	Reference	Sample	
5	0.0358	0.0339	94.7
		0.0369	103.1
		0.0326	91.1
		0.0367	102.5
50	0.2723	0.2466	90.6
		0.2548	93.6
		0.2862	105.1
		0.2427	89.1
Mean			96.2
S.D.			6.4

TABLE III

PRECISION OF THE ASSAY OF I IN HUMAN PLASMA

I added (ng/ml)	I/internal standard ratio
5	0.0179
	0.0165
	0.0196
	0.0190
	0.0188
	0.0174
Mean	0.0182
S.D.	0.0011
C.V. (%)	6.0
100	0.5091
	0.4973
	0.4422
	0.4835
	0.4829
	0.4834
Mean	0.4831
S.D.	0.0226
C.V. (%)	4.7

Selectivity was very good as no interfering endogenous peak was encountered and the two peaks corresponding to I and II were well separated (Fig. 3).

The limit of quantification (LOQ) was 5 ng/ml plasma with a C.V. of 6.0%. The limit of detection (LOD) was 2 ng/ml.

DISCUSSION

Data reported in this paper demonstrate that compound I can be determined by HPLC using fluorimetric detection after derivatization with benzoin. The main problem encountered was the clean-up procedure for the elimination of arginine. This was achieved by solid-phase extraction. Compound I was extracted from plasma by means of a liquid–solid partition with a recovery of more than 90%.

The data reported in this paper demonstrate that the new method is ten to twenty times more sensitive than the previous one based on the

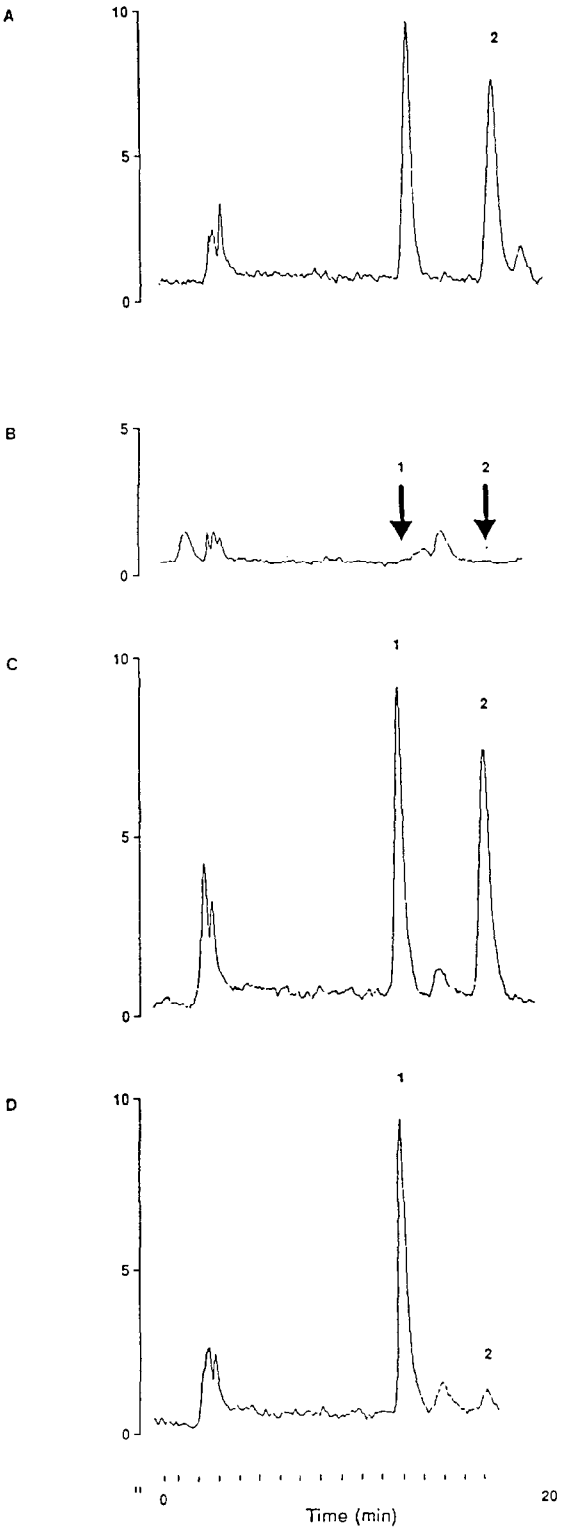


Fig. 3. Typical HPLC recordings of ST I (2) and internal standard (1). (a) Reference solution containing I (100 ng) and II (20 ng); (b) blank human plasma; (c) plasma spiked with I (100 ng) and II (20 ng); (d) plasma spiked with I (5 ng) and II (20 ng).

detection by UV absorption of the underivatized I [4]. The linearity, reproducibility, extraction recovery, selectivity and accuracy allow this method to be used for pharmacokinetic and bioavailability investigations in humans.

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