Journal of Chromatography, 277 (1983) 427—432
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1815

Note

High-performance liquid chromatography of lonidamine in human plasma and urine

ROBERT LECLAIRE, JEAN-GUY BESNER*, PIERRE BAND, SYLVIE MAILHOT, PIERRE GERVAIS, ATTILIO DE SANCTIS and MICHELLE DESCHAMPS

Faculty of Pharmacy, University of Montreal, P.O. Box 6128, Montréal, Québec H3C 3J7 (Canada)

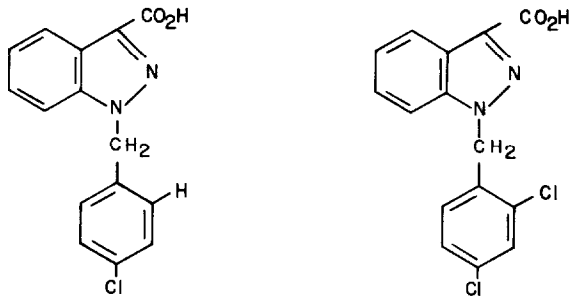
and

LINO LIVERANI

Istituto Di Ricerca F. Angelini, Rome (Italy)

(First received October 8th, 1982; revised manuscript received June 6th, 1983)

Lonidamine is a substituted indazole carboxylic with anticarcinogenic, antispermatogenic and embryotoxic properties [1—4]. In vitro studies using testicular germinal cells [3] and Ehrlich's ascitic tumor cells indicate that lonidamine affects energy metabolism causing a decrease in cellular oxygen consumption mediated by a specific effect on mitochondrial function [5, 6].



AF 1312/TS (internal standard)

LONIDAMINE

Fig. 1. Chemical structures of lonidamine and AF 1312/TS used as internal standard.

In view of the antitumor effects of lonidamine in the Lewis lung tumor and sarcoma 180 [7], of its unique mechanism of action and of the suggestive lack of overlapping toxicity with other chemotherapeutic drugs we have undertaken a clinical phase I pharmacokinetic and toxicologic study of lonidamine which will be reported separately. Lonidamine (L) and AF 1312/TS (IS) have been previously assayed by a fluorometric method in serum and testes after extraction with *n*-heptane [7]; recoveries reported were 54% and 63% respectively, and sensitivity limits were 1 μg/ml of serum and 2 μg/g of tissue. This report describes a sensitive and selective high-performance liquid chromatography (HPLC) assay for the quantitative determination of lonidamine from urine and plasma samples using its analogue AF 1312/TS as the internal standard (Fig. 1). The technique uses an ultraviolet detector set at 300 nm which is the maximum absorbance wavelength of lonidamine and permits the detection of concentrations of 200 ng/ml of biological sample.

MATERIALS AND METHODS

Reagents

All solid chemicals were of reagent grade; anhydrous diethyl ether used for extraction was of analytical reagent grade; acetonitrile for HPLC was of chromatoquality (99%) grade (BDH Chemicals, Montreal, Canada).

Columns

The column used was 250 mm × 4.6 mm I.D. stainless-steel containing a 5-μm Spherisorb ODS packing purchased from Chromatographic Sciences (Montreal, Canada).

Instruments

A high-performance liquid chromatograph equipped with two Constametric III pumps (LDC) and a Rheodyne injector permitting injection of volumes between 1 and 20 μl, was used. The detector was a Perkin-Elmer Model LC-55 set at 300 nm and the recorder a Perkin-Elmer Model 56 set at either 1, 2 or 5 mV depending on the concentration of the sample assayed. Peak areas were calculated with a Varian integrator Model 485 equipped with filtering and baseline tracking devices.

Mobile phase

Throughout the procedures, the isocratic mobile phase contained a mixture acetonitrile—0.1 *M* acetate buffer, pH 3.50 (50:50). The flow-rate was 2.0 ml/min keeping the pressure between 80 and 120 bars, well below the critical pressure of 205 bars.

Analytical standards

Lonidamine and AF 1312/TS were generously supplied by the Angelini Research Institute of Rome, Italy.

Preparation of standards

Stock solutions of lonidamine and AF 1312/TS were prepared by diluting

10 mg of pure substance in 100 ml of methanol; these solutions were stable for more than six months when kept refrigerated at 3—4°C.

Buffer

An acetate buffer of 0.1 *M* was made by adding 6 ml of glacial acetic acid to 500 ml of bidistilled water, the pH was adjusted to 3.50 with 1.0 *M* sodium hydroxide, and the volume completed to 1 l with bidistilled water.

Extraction procedure

To 15-ml PTFE screw-cap tubes containing 1.0 ml of plasma or urine, 5.0 μg of AF 1312/TS were added as internal standard followed by 0.4 ml of 1.0 *M* hydrochloric acid. The samples were then vortexed for 15 sec in order to ensure homogeneity and the extraction was performed with 5.0 ml of anhydrous diethyl ether by agitation for 10 min on an Eberbach reciprocal shaker. The samples were then centrifuged for 10 min at 800 *g* and the organic layer was removed. The extraction procedure was repeated on the residual plasma or urine fraction with 3.0 ml of anhydrous diethyl ether. The two organic fractions were combined and evaporated to dryness under dry nitrogen at 45°C. The residue was dissolved in 200 μl of acetonitrile and 20 μl were used for the assay.

Calibration curves

Plasma. To aliquots of 1 ml of plasma were added amounts ranging from 0 to 25 μg of lonidamine followed by 5.0 μg of AF 1312/TS as internal standard. Each of these concentrations was assayed in triplicate.

Urine. To aliquots of 1 ml of urine were added amounts ranging from 0 to 40 μg of lonidamine followed by 5.0 μg of AF 1312/TS as internal standard. Each concentration was assayed in triplicate.

RESULTS

The mean values obtained for the calibration curves appear in Table I for plasma and urine. In both cases, the standard deviations estimated for each concentration studied were less than 10% of the mean. The regression coefficients were 0.99 for both plasma and urine calibration curves. Routine analysis, performed on different days, on plasma and urine samples spiked with known amounts of lonidamine demonstrated that the reproducibility of this analysis procedure is within 5%. To allow for a more quantitative determination of lonidamine concentration and to circumvent biases induced by possible variations of peak shape over time, surface areas instead of peak heights were used for the quantitative determination of lonidamine. Comparison of peak areas obtained from the extraction of plasma and urine samples spiked with known amounts of lonidamine and AF 1312/TS with those obtained by the injection of standards under the same conditions indicated a recovery of 90 ± 4% for both substances in plasma and urine. Furthermore, the recovery is constant for lonidamine over the range of concentrations studied. This range covers the maximum concentrations obtained during the acute pharmacokinetic studies in man using doses of 300 and 600 mg of lonidamine.

TABLE I

SURFACE RATIOS OF PLASMA AND URINE LONIDAMINE OVER INTERNAL STANDARD OBTAINED FOR CALIBRATION CURVES

Each concentration has been studied in triplicate.

Amount of lonidamine added to plasma (μg/ml)	Mean surface ratios (± S.D.)	Amount of lonidamine added to urine (μg/ml)	Mean surface ratios (± S.D.)
0	0	0	0
0.5	0.06 ± 0.006	0.5	0.09 ± 0.006
1.0	0.12 ± 0.01	1.0	0.18 ± 0.002
1.5	0.18 ± 0.01	2.0	0.38 ± 0.03
2.0	0.29 ± 0.03	4.0	0.77 ± 0.08
4.0	0.68 ± 0.04	6.0	0.96 ± 0.10
6.0	0.98 ± 0.06	8.0	1.53 ± 0.03
8.0	1.56 ± 0.06	10.0	2.03 ± 0.05
10.0	1.78 ± 0.07	12.5	2.70 ± 0.16
12.5	2.14 ± 0.07	15.0	3.04 ± 0.12
15.0	2.56 ± 0.11	20.0	3.45 ± 0.06
17.5	3.07 ± 0.06	25.0	4.60 ± 0.01
20.0	3.29 ± 0.10	30.0	5.37 ± 0.20
22.5	3.84 ± 0.06	35.0	6.43 ± 0.41
25.0	4.41 ± 0.23	40.0	7.28 ± 0.62

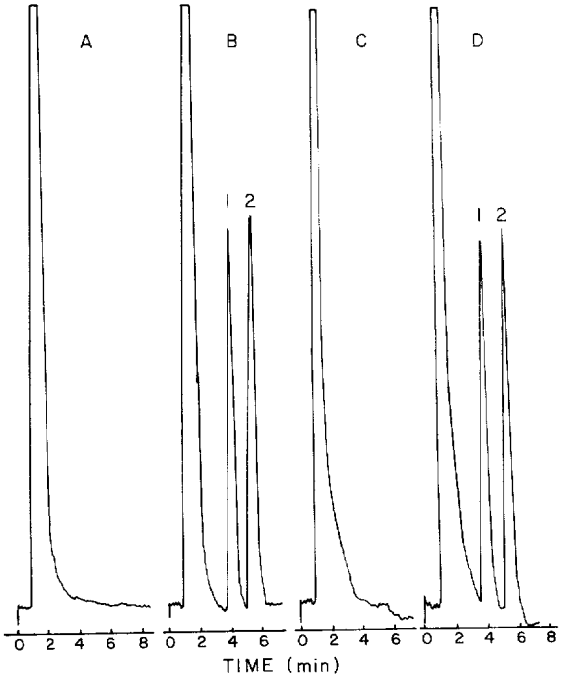


Fig. 2. Typical chromatograms of control human plasma (A), control plasma (B) spiked with AF 1312/TS (1) and lonidamine (2), control human urine (C) and control urine (D) spiked with AF 1312/TS (1) and lonidamine (2).

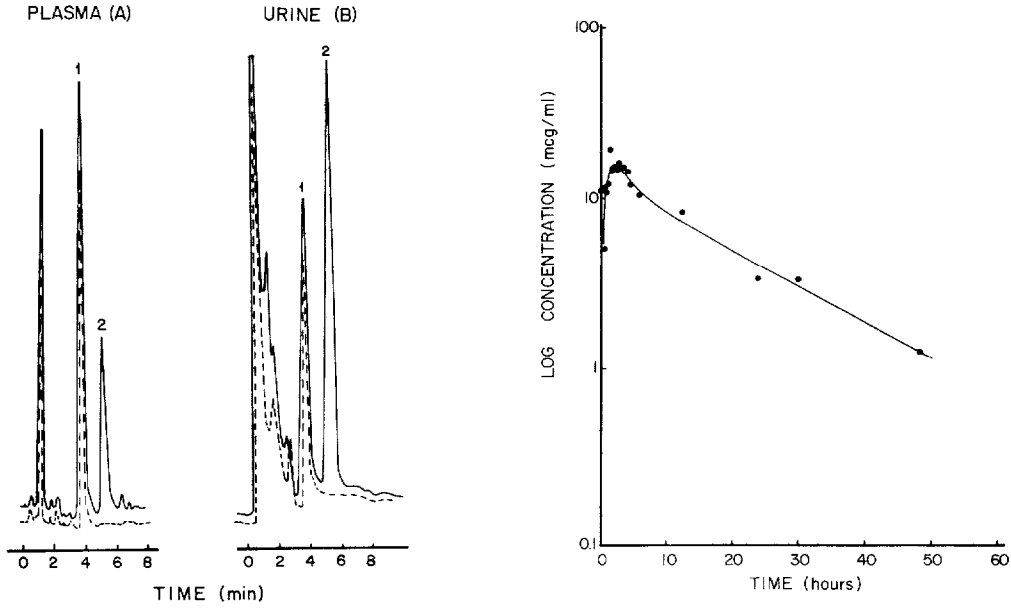


Fig. 3. (A) Chromatogram of plasma samples obtained before (· · · ·) and 1.5 h after (——) oral administration of lonidamine (600 mg) to a cancer patient. (B) Chromatogram of urine samples obtained before (· · · ·) and from 3 to 6 h after (——) drug administration to the same patient.

Fig. 4. Lonidamine plasma concentrations after a single oral dose of 600 mg; the black cicles (•) represent the experimental values and the solid line (—) represents the least square regression curve. Dose, 600 mg; lag time, 0.24 h; K_a, 1.03 h^{-1}; β, 0.048 h^{-1}; K_{12}, 0.26 h^{-1}; K_{21}, 0.21 h^{-1}; $T_{1/2}$, 14.4 h; *VD*, 43.4 l; *TBC*, 2.09 l/h.

Fig. 2 illustrates typical chromatograms obtained by the extraction of blank and spiked plasma and urine samples. The peaks are well defined with no extraneous substances interfering with the assay. The respective retention times for IS and L are 4 and 5.5 min in the conditions already described. Fig. 3 shows chromatograms obtained from plasma and urine samples collected 1.5 h after administration to a cancer patient of a single oral dose of 300 mg. Lonidamine concentrations were 2.70 μg/ml and 7.5 μg/ml for plasma and urine, respectively. Fig. 4 illustrates plasma concentrations measured in one patient who received two capsules of 300 mg of lonidamine. Those preliminary results suggest that lonidamine's single-dose pharmacokinetics should be explained by a bicompartmental open model with first order absorption and a lag time.

DISCUSSION

AF 1312/TS was chosen as the internal standard in view of its very similar chemical structure and of its retention time which permits a clear separation from lonidamine and interfering substances. Our analytical conditions allow the elution of both substances in a region of the chromatogram free from interferences (Fig. 2). The method outlined is rapid and accurate with a sen-

sitivity limit of approximately 0.2 μg/ml of plasma or urine. The analytical procedure reported in this paper differs greatly from the method published by Catanese et al. [8]. Firstly, the extraction procedure, using diethyl ether in acidic medium, permits a better recovery (90%) compared to 54 ± 3% with *n*-heptane. Secondly, this HPLC assay permits the separation of lonidamine from its metabolites (Fig. 3). It also allows the use of an internal standard for the assay which in turn insures the control of possible biases induced by variations in the recovery during extraction and in the volume injected for the assay. The fluorometric assay used by Cantanese et al. [8] does not have these features. Lobl et al. [9] have developed an assay using uniformally tritiated compound. However, this assay is more suitable for the study of the cellular distribution of the indazole carboxylic acid derivatives.

According to our results, the use of reversed-phase HPLC seems to be a method of choice for the evaluation of indazole carboxylic acid derivatives in biological samples since clear separations can be achieved with very little differences in chemical structures. Our analytical method provides the means to study the kinetic parameters of lonidamine. The procedure, rapid and practical, is particularly suitable for multiple sample analysis and is currently used in our laboratory to determine single and chronic pharmacokinetics of lonidamine in cancer patients.

ACKNOWLEDGEMENTS

This work has been supported by Inter-Canada Pharmaceuticals and the F. Angelini Research Institute of Rome, Italy.

REFERENCES

1 F. Coulston, W.J. Dougherty, et al, Exp. Mol. Pathol., 23 (1975) 357—366.
2 B. Silvestrini, C. De Martino, et al, Proc. Serono Symp. Recent Progress in Andrology — L'Aquila 1977, A. Fabbrini, E. Steinberger, Academic Press, New York, Vol. 14, 1978, pp. 453—457.
3 R.W. James, R. Heywood, et al, XII Congr. Soc. Ital. Microscopia Electronica, Ancona, Italy, 1979, Abstract 129.
4 T.J. Lobl, Arch. Andrology, 2 (1979) 353—363.
5 M.G. Paggi, A. Floridi, et al, XII Congr. Soc. Ital. Microscopia Electronica, Ancona, Italy, 1979, Abstract 109.
6 B. Silvestrini, Chemotherapy, 27 (Suppl. 2) (1981) 9—17.
7 A. Caputo, Chemotherapy, 27 (Suppl. 2) (1981) 107—120.
8 B. Catanese, V. Cioli, C. De Martino, B. Silvestrini, Pharmacol. Res. Commun., 10 (1978) 261—269.
9 T.J. Lobl et al., unpublished results, 1977.