Journal of Chromatography, 494 (1989) 347–353
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4843

Note

Determination of velnacrine in plasma and urine by high-performance liquid chromatography

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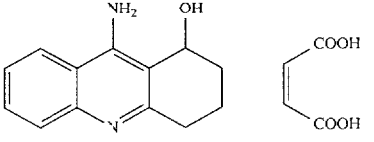
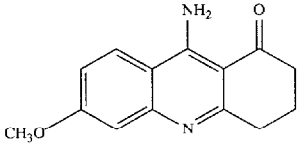
(First received February 21st, 1989, revised manuscript received May 3rd, 1989)

Alzheimer's disease (AD) is recognized as a major cause of impaired cognitive ability among the elderly [1–5] Velnacrine (HP 029), 1,2,3,4-tetrahydro-9-aminoacridin-1-ol maleate (Fig. 1), is a new drug currently being studied for



(A) velnacrine (HP 029)



(B) internal standard

Fig 1 Chemical structures of (A) velnacrine (HP 029), 1,2,3,4-tetrahydro-9-aminoacridin-1-ol maleate, and (B) internal standard, 9-amino-3,4-dihydro-7-methoxyacridin-1(2H)-one

0378-4347/89/$03 50

the treatment of AD The compound inhibits cholinesterase and is active in various animal models predictive of efficacy in AD [6]. On the basis of these results, velnacrine was selected for clinical evaluation in AD patients For pharmacokinetic studies in animals [7] and man [8,9], a sensitive and selective high-performance chromatographic (HPLC) assay for velnacrine in plasma and urine was developed

EXPERIMENTAL

Materials

Velnacrine and internal standard, 9-amino-3,4-dihydro-7-methoxyacridin-1(2H)-one (Fig 1B), were synthesized in the Chemical Research Department of Hoechst-Roussel Pharmaceuticals Acetonitrile, ethyl acetate, methanol, sodium hydroxide, potassium hydroxide and formic acid were analytical grade and purchased from Fisher Scientific (Springfield, NJ, U S A) All solvents were HPLC grade The potassium formate buffer was prepared by mixing formic acid and potassium hydroxide solution until desired pH and concentration were obtained Deionized water (Milli-Q® water purification system, Millipore, Bedford, MA, U S A) was used throughout the study Human plasma was obtained from Biological Specialty (Lansdale, PA, U S A) and processed from heparin-treated blood Human urine was obtained from healthy male volunteers

Sample preparation

Plasma and urine samples (1 ml each) were added to test tubes containing the internal standard (250 ng/ml of plasma and 1000 ng/ml of urine), and 1 ml of 0 1 *M* sodium hydroxide for plasma or 1 *M* sodium hydroxide for urine were added The tubes were vortex-mixed briefly and then 5 ml of cyclohexane–ethyl acetate (1 1, v/v) were added The tubes were then shaken for 10 min on an Eberbach shaker and centrifuged at 2000 *g* for 10 min in a Model J-6B centrifuge (Beckman Instruments, Fullerton, CA, U S A) The organic phase was transferred to a tapered centrifuge tube and evaporated to dryness in a water bath under a nitrogen stream at 40 °C The residue was then reconstituted in 0 2–0 5 ml of mobile phase and a 0 2-ml aliquot of the sample analyzed by HPLC

High-performance liquid chromatography

Plasma and urine samples prepared according to the above procedures were analyzed by an HPLC system employing a Shandon SAS Hypersil 5 μm column (250 mm × 4 6 mm I.D.) The samples were injected by a Waters WISP 710B autosample injector (Milford, MA, U S A.) and eluted with a methanol–600 m*M* potassium formate buffer, pH 2 75 (99 1, v/v) at a flow-rate of 1 5–2 0 ml/min The elution was carried out at ambient temperature and column

effluent was monitored using a Kratos Model 773 UV spectrophotometer (Ramsey, NJ, U S A) with detection at 325 nm The Shandon SAS Hypersil 5 μm column required preconditioning for correct assay performance Prior to first use, a new column is initially flushed with 50 ml of methanol and then with 800 ml of methanol–water–formic acid (80 20 2) at a flow-rate of 0 7 ml/min The column is subsequently equilibrated with mobile phase at a flow-rate of 1 5–2 0 ml/min for at least 2 h before sample injection.

Preparation of calibration curves

Calibration curves were constructed with six different plasma and urine standards covering the expected concentration range (1–500 ng/ml for plasma and 10–5000 ng/ml for urine) Standard curves were established by analyzing five replicates at each concentration Linearity and quantitation limits were determined from weighted least-squares regression analysis [10] The regression equation was used to calculate the plasma and urine concentrations

Validation procedures

The recovery of velnacrine and internal standard from plasma and urine was assessed by adding known amounts to control plasma and urine to give final concentrations of 5, 50 and 500 ng/ml for plasma, and 50, 500 and 5000 ng/ml for urine. The samples (six replicates at each concentration) were extracted and analyzed by HPLC. Extraction efficiency was determined by comparing the peak areas of velnacrine and internal standard, obtained by analyzing spiked plasma and urine, with the peak areas obtained after injection of standards in mobile phase

Within-day and between-day assay variations were assessed by examining the plasma and urine quality control samples used in phase I clinical trials Reproducibility was determined at concentrations of 50 and 500 ng/ml for plasma, and 500 and 5000 ng/ml for urine. Within-day variation was examined by analyzing six replicates at each concentration, and between-day precision was determined from the quality control data acquired over a period of six months

RESULTS AND DISCUSSION

Selectivity

The concentrations of velnacrine in plasma and urine were determined with an isocratic reversed-phase HPLC system employing a Shandon SAS analytical column and UV detection Typical chromatograms for control and spiked human plasma and urine are shown in Figs. 2 and 3 Using this chromatographic system, no endogenous components, extracted from human plasma and urine, interfere with velnacrine or internal standard in the sample assay

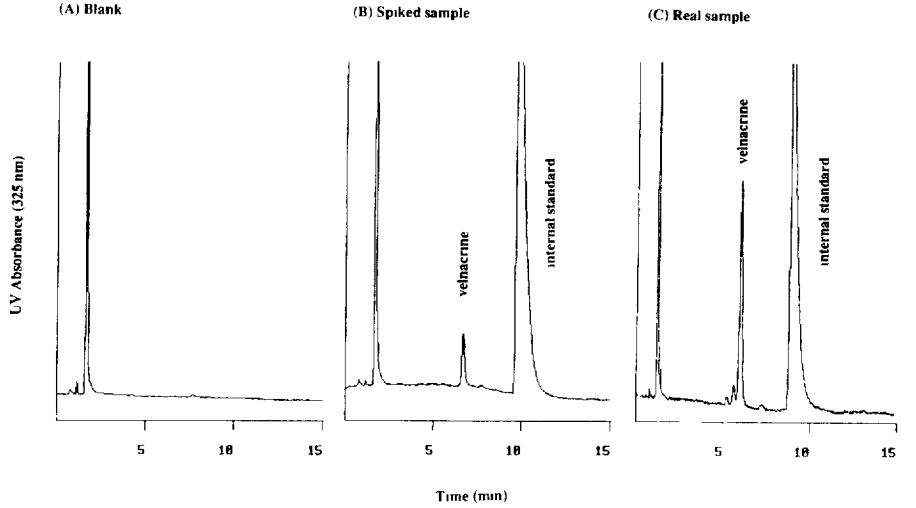


Fig 2 Representative HPLC profiles of (A) blank human plasma, (B) blank human plasma containing 50 ng/ml velnacrine and 200 ng/ml internal standard and (C) human plasma 1 h after oral administration of 100 mg of velnacrine Plasma samples were chromatographed on a Shandon SAS Hypersil 5 μm column at laboratory temperature using methanol–600 m*M* potassium formate, pH 2 75 (99 1, v/v) as mobile phase Column eluate was monitored by UV detection at 325 nm

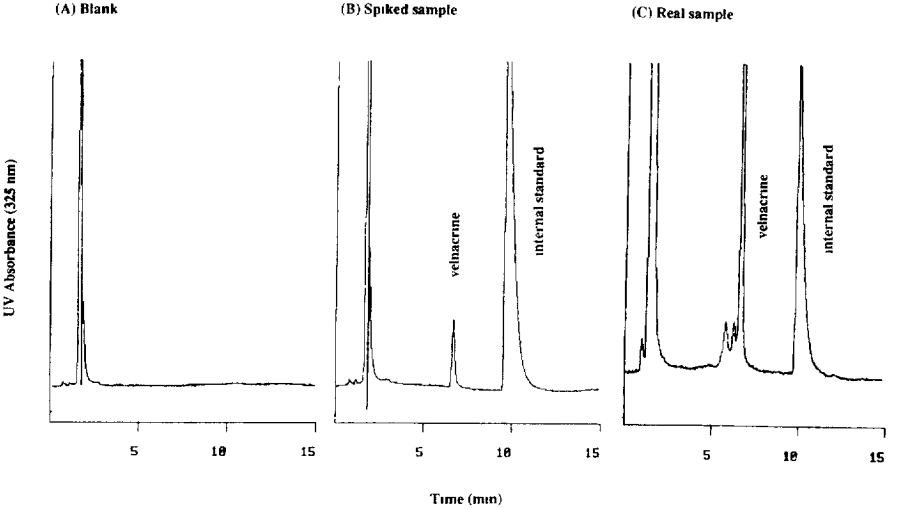


Fig 3 Representative HPLC profiles of (A) blank human urine, (B) blank human urine containing 100 ng/ml velnacrine and 1000 ng/ml internal standard and (C) human urine 0–24 h after oral administration of 100 mg of velnacrine Urine samples were chromatographed on a Shandon SAS Hypersil 5 μm column at laboratory temperature using methanol–600 m*M* potassium formate, pH 2 75 (99 1, v/v) as mobile phase Column eluate was monitored by UV detection at 325 nm

Interfering substances were either eluted at the solvent front under the HPLC conditions or did not absorb UV at 325 nm under these conditions

Recovery

Extraction efficiency was determined by comparing the peak areas from spiked samples with those obtained from injection in mobile phase (Table I) Recovery of velnacrine from plasma was approximately 63% over a concentration range of 5–500 ng/ml, and in urine the recovery was about the same over a concentration range of 50–5000 ng/ml Recovery of internal standard for the plasma (250 ng/ml) and urine (1000 ng/ml) assays was over 95% This could be due to the less polar nature of the molecule The pH of the matrix is important for the extraction of velnacrine, which has a pK_a value of 9 5 [11]. Plasma and urine were basified with 0 1 *M* sodium hydroxide for plasma or 1 *M* sodium hydroxide for urine to facilitate extraction On adding 1 *M* sodium hydroxide to plasma, an emulsion formed and the recovery was reduced to less than 60%

Quantitation limit and linearity

Plasma and urine standards were prepared from human plasma and urine spiked with velnacrine in the ranges 1–500 and 10–5000 ng/ml, respectively Quantitation limit and linear relationship between the peak-area ratios of velnacrine and internal standard versus concentration of velnacrine added to plasma and urine were assessed by a two-stage weighted least-squares regression [10] Plasma and urine calibration curves were described by the regression equations $y = -0.66 + 210\ 5C$ and $y = -0.55 + 339\ 8C$, respectively, where the y is the peak-area ratio and C the velnacrine concentration The observed coefficients of variation (C V.), calculated from the regression equations, were less than 10% for the plasma and urine assays The predicated C V , from the

TABLE I

ASSAY RECOVERY OF VELNACRINE FROM PLASMA AND URINE

Sample	Concentration (ng/ml)	Recovery (mean ± S D , $n=6$) (%)
Plasma	5	65 4 ± 6 8
	50	62 7 ± 2 0
	500	62 6 ± 1 4
Urine	50	61 4 ± 4 8
	500	60 6 ± 0 3
	5000	62 9 ± 1 0

TABLE II

VELNACRINE PLASMA HPLC CALIBRATION DATA

Concentration (ng/ml)	Peak-area ratio (mean ± S D, $n=5$)	Estimated concentration[a] (mean ± S D, $n=5$) (ng/ml)	Weight	Coefficient of variation[b] (%)	
				Observed	Predicted
1	0 00279 ± 0 00027	1 1 ± 0 1	1 532	9 74	83 96
5	0 0131 ± 0 0010	4 8 ± 0 3	1 475	7 38	17 05
10	0 0277 ± 0 0025	10 2 ± 0 7	1 408	7 20	8 70
50	0 141 ± 0 003	50 5 ± 1 1	0 967	2 21	2 09
100	0 280 ± 0 006	99 9 ± 1 9	0 605	2 11	1 36
500	1 42 ± 0 03	506 ± 9	0 014	2 10	1 75

[a]Estimated concentration from the weighted least-squares regression equation $y = -0\ 66 + 210\ 5C$, where y is the peak-area ratio and C the velnacrine concentration
[b]Observed coefficient of variation in peak-area ratio ($n=5$ for each concentration), predicted coefficients of variation were calculated by the weighted least-squares regression method [10]

TABLE III

VELNACRINE URINE HPLC CALIBRATION DATA

Concentration (ng/ml)	Peak-area ratio (mean ± S D, $n=5$)	Estimated concentration[a] (mean ± S D, $n=5$) (ng/ml)	Weight	Coefficient of variation[b] (%)	
				Observed	Predicted
10	0 00528 ± 0 00043	9 8 ± 0 6	1 693	8 08	35 77
50	0 0271 ± 0 0007	50 2 ± 1 5	1 597	2 74	7 32
100	0 0539 ± 0 0013	99 7 ± 2 4	1 484	2 50	3 78
500	0 269 ± 0 004	497 ± 7	0 827	1 30	1 02
1000	0 543 ± 0 007	1004 ± 13	0 398	1 32	0 76
5000	2 73 ± 0 05	5025 ± 67	0 001	1 81	2 66

[a]Estimated concentration from the weighted least-squares regression equation $y = -0\ 55 + 339\ 8C$, where y is the peak-area ratio and C the velnacrine concentration
[b]Observed coefficient of variation in peak-area ratio ($n=5$ for each concentration), predicted coefficients of variation were calculated by the weighted least-squares regression method [10]

TABLE IV

WITHIN-DAY AND BETWEEN-DAY ASSAY PRECISION FOR VELNACRINE

Sample	Precision	n	Concentration (mean ± S D) (ng/ml)	Coefficient of variation (%)
Plasma	Within-day	6	49 ± 3	6 0
			496 ± 14	2 0
	Between-day	30[a]	47 ± 5	10 6
			476 ± 40	8 4
Urine	Within-day	6	540 ± 11	2 0
			5349 ± 113	2 1
	Between-day	20[a]	552 ± 54	9 8
			5443 ± 423	7 8

[a]Individual values are the mean of two measurements

weighted least-squares regression analysis, corresponding to plasma concentrations of 1, 5, 10, 50, 100 and 500 ng/ml, were 84, 17, 8 7, 2 1, 1 4 and 1 8%, respectively. C V for the urine concentrations of 10, 50, 100, 500, 1000 and 5000 ng/ml were estimated as 36, 7.3, 3 7, 1 0, 0 8 and 2 7%, respectively

Quantitation limit for the method is defined as the minimum concentration that can be detected with a pre-defined level of confidence [12] The estimated quantitation limit with a 20% C V of prediction is 4 and 18 ng/ml for plasma and urine, respectively (Tables II and III) The F ratios for the linearity test for plasma and urine are 0.739 and 0 648, respectively, which are much less than the critical value $F_{0\,95,\ 4\,24}=2\,73$, indicating that linearity is fully acceptable

Method precision

The reproducibility of the plasma and urine assays was evaluated by analyzing the quality control samples used in the phase I clinical trials. The results of within-day and between-day precision are expressed as the C V The within-day and between-day variation for plasma at levels of 50 and 500 ng/ml is 2 8–6 1 and 8 4–10 6%, respectively, while the assay variation for urine at levels of 500 and 5000 ng/ml is 2 0–2 1 and 7 8–9 8%, respectively (Table IV)

ACKNOWLEDGEMENTS

The authors thank Dr Bee-Lian Chen, Medical Systems, H-RPI, for his valuable assistance in statistical analysis and Dr. G M Shutske for supplying velnacrine and the internal standard

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