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DETERMINATION OF GLAUCINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive method is described for the measurement of *d*-glaucine in pharmacokinetic studies using only 100-μl plasma samples or 200-μl urine samples. It requires a simple extraction clean-up on kieselguhr micro-columns and straight-phase high-performance liquid chromatography with fluorescence detection. Data on selectivity, sensitivity and precision demonstrate the reliability of this method. Its applicability is revealed by single and repeated oral administration pharmacokinetic studies in human subjects.

INTRODUCTION

d-Glaucine (Fig. 1) is an aporphine alkaloid first isolated from *Glaucium flavum* Crantz (Papaveraceae) [1]. The drug is used widely in East Europe as a cough suppressant. The optical isomer, *l*-glaucine, and the racemic *dl*-glaucine seem to have a similar therapeutic effect [2].

Spectrophotometric and high-performance liquid chromatographic (HPLC) methods have been described for quantitative determination of *d*-glaucine [3—5]. None of these methods is sufficiently sensitive and specific for the determination of the drug after administration in the therapeutic range.

The fluorescent property of boldine, another aporphine, was previously described [6]. Glaucine is structurally related and shows a fluorescence spectrum close to that of boldine.

The aim of this paper was to develop a selective and highly sensitive method, based on the fluorescence behaviour of glaucine, in order to obtain pharmacokinetic data.

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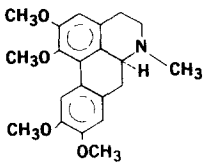
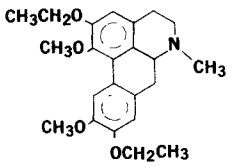


[I]



[II]

Fig. 1. Chemical structures of *d*-glaucine [I] and diethyl boldine (internal standard) [II].

MATERIALS AND METHODS

Chemicals and reagents

d-Glaucine hydrochloride and the internal standard diethyl boldine hydrochloride dihydrate were supplied by Clin-Midy (Montpellier, France). *d*-[6a-^{14}C] Glaucine base (21 mCi/mmol) was obtained from CEA (Saclay, France). Analytical reagent grade *n*-hexane, tetrahydrofuran and diethylamine were purchased from Merck (Darmstadt, F.R.G.); methanol was from Farmitalia Carlo Erba (Milan, Italy), sodium hydroxide, ammonium chloride and ammonia 20% were from Prolabo (Paris, France).

Preparation of micro-columns

Kieselguhr extraction micro-columns were prepared in disposable polypropylene blue tips for Eppendorf pipettes (Hamburg, F.R.G.) They were filled with 0.1 g of kieselguhr (Extrelut Art. 11738, Merck). Glass-wool plugs were inserted to serve as bed support and avoid dispersion of the porous matrix.

Apparatus

The HPLC system consisted of a Model 5000 (Varian, Palo Alto, CA, U.S.A.) solvent delivery pump, a Model 710 B WISP automatic sample injector (Waters Assoc., Milford, MA, U.S.A.) and a Model JY3D spectrofluorimetric detector (Jobin Yvon Instruments, Paris, France) equipped with a 20-μl flow cell. The output signal was fed to a Model SP 4100 (Spectra-Physics, Santa Clara, CA, U.S.A.) integrator for recording and calculation of peak area.

The separation units consisted of a prepacked column of LiChrosorb Si 60 Merck (5-μm irregularly shaped particles), 125 mm $\times$ 4 mm I.D. Chromatographic conditions are given in Fig. 2. Other equipment included a vortex type mixer and an ultrasonic bath for degassing solvents.

Preparation of standards

Standards were prepared by dissolving compounds as salt forms in methanol. Two stock standard solutions were made each month, one containing 1 mg/ml

of *d*-glaucine base and the other containing 1 mg/ml diethyl boldine base. Working standards of 100 μg/ml to 10 ng/ml were prepared weekly by appropriate dilution of the stock solutions.

Procedure for plasma and urine extraction

Before addition of biological fluid each sample tube received an adjusted amount of internal standard as a methanolic solution evaporated to dryness under nitrogen at 50°C.

Plasma. A 100-μl aliquot of plasma was introduced into a sample tube and 200 μl of 0.05 *M* sodium hydroxide aqueous solution were added. The stoppered tube was vortexed for 30 sec, then the sample was applied on a kieselguhr micro-column. After equilibration for 15 min, 5 ml of *n*-hexane were added to the micro-column. The eluate was evaporated at 50°C in a conical tube under a gentle nitrogen steam; the sample residue was reconstituted with 100 μl of mobile phase and a 20-μl aliquot was injected into the HPLC column.

Urine. The procedure for urine was slightly different. To a kieselguhr micro-column were added 250 μl of a mixture of urine—0.1 *M* NH_4Cl—NH_3 buffer pH 8.1 (2:1). Other operations remained unchanged.

Quantitation

The concentration of *d*-glaucine in plasma and urine was determined from eight-point calibration curves of peak area ratios (*d*-glaucine and diethyl boldine) versus *d*-glaucine concentration expressed as free base in plasma and urine carried through the described procedures. A new calibration curve was made with each sample set.

RESULTS AND DISCUSSION

Method

Fig. 2 illustrates the chromatographic separation of *d*-glaucine in biological samples. Diethyl boldine was selected as an internal standard on the basis of the resolution achieved and the similarity of its fluorescence spectrum to that of *d*-glaucine.

The final composition of the mobile phase was the result of systematic work on silica thin-layer chromatography then extrapolation to HPLC plain silica column until adequate resolution was obtained. Diethylamine was added as a mobile phase modifier to avoid peak tailing.

A simple way of extracting glaucine is to operate in a slightly basic aqueous medium (pH 8) with *n*-hexane as extracting solvent. Unfortunately an emulsion often formed in biological samples when this procedure was used. To obtain cleaner extracts of small sample volumes extraction using kieselguhr micro-columns appeared to be suitable. The dilution and the volume of biological samples were adjusted as a function of porous matrix weight. The analytical recovery of extraction was determined by radiotracer techniques. A trace amount of *d*-[^{14}C]glaucine was added to the biological sample before the extraction clean-up step. The recovery measured by liquid scintillation counting prior to HPLC was 79.5% (n = 10, S.D. = 4) in plasma and 84.4% (n = 12, S.D. = 2) in urine.

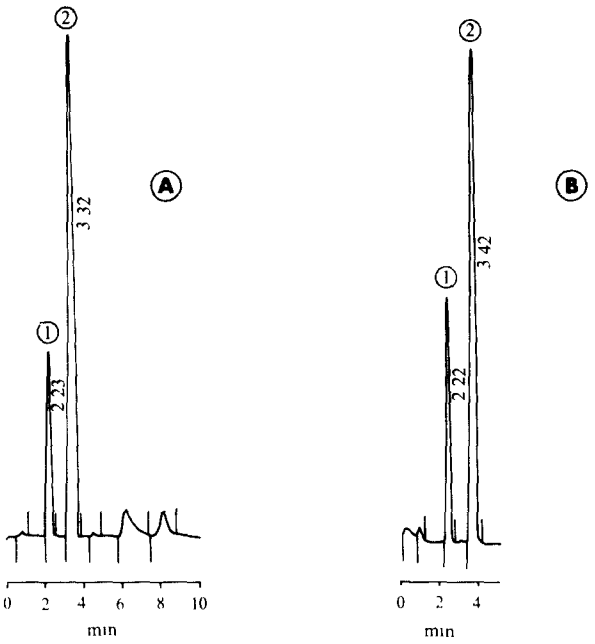


Fig. 2. (A) Typical HPLC chromatogram obtained from urine sample of a treated subject. (B) Typical HPLC chromatogram obtained from plasma sample of a treated subject. Operating conditions: column 125 mm × 4 mm I.D. LiChrosorb Si 60 (5 μm). Mobile phase: *n*-hexane—methanol—tetrahydrofuran—diethylamine (88.5:7.5:4:0.15). Flow-rate: 1.5 ml/min. Injection volume: 20 μl. Fluorescence detection: excitation wavelength 310 nm, emission 340 nm. Peaks: 1 = diethyl boldine (internal standard); 2 = *d*-glaucine.

Calibration curves of peak area ratio versus concentration were obtained by analysing plasma and urine standards containing *d*-glaucine in concentrations usually ranging from 10 to 500 ng/ml, but the curves were rectilinear for at least 0—5 μg/ml. The equations of the curves (10—500 ng/ml) were calculated by least-squares linear regression. A good linear relationship was obtained in the concentration range studied: correlation coefficients for 25 standard curves averaged 0.999 ± 0.0010 (± S.D.), with intercepts not significantly different from zero.

The peaks representing *d*-glaucine and the internal standard were symmetrical and well removed from the injection front and interfering peaks encountered in biological material. Attempts to quantitate *d*-glaucine by either gas chromato-

TABLE I

COMPARISON BETWEEN *d*-GLAUCINE CONCENTRATIONS OBTAINED BY MASS SPECTROMETRY WITH FIELD IONIZATION AND BY HPLC

Concentrations are expressed as ng ml^{-1}.

MS—field ionization	HPLC
97	90
190	175
17	20
205	200

graphy (GC) or gas chromatography—mass spectrometry (GC—MS) were unsuccessful. Adsorption on GC packing materials and thermal decomposition were the basis of the difficulties encountered. Hence, MS with field ionization — a mild ionization technique — was used with favourable results as reference method. The agreement between HPLC and MS methods was acceptable with the sample set checked (Table I).

The HPLC assay proved to be quite sensitive with a lower limit of 5 ng/ml for plasma and 2 ng/ml for urine, and a blank value equal to zero.

Duplicate measurements were performed on plasma and urine from treated subjects. The values were classified into three groups according to the concentration of *d*-glaucine. The differences d_i between the number (N) of duplicate measurements enables the estimation of the standard deviation (S.D.) which characterizes the reproducibility:

$$\text{S.D.} = \sqrt{\frac{\Sigma d_i^2}{2N}}$$

This estimation has been made for concentration ranges where the standard deviation is supposed to remain constant. The results for plasma in Table II indicate, as would be expected, a decrease of precision with decreasing *d*-glaucine concentration. The estimated precision in urine remains equal to 5% (Table III). This result is consistent with a better sensitivity in urine samples than in plasma samples.

TABLE II

PRECISION OF *d*-GLAUCINE DETERMINATION IN PLASMA

Plasma concentration (C_p) range (ng/ml)	Number of duplicate measurements	$\bar{x}_p$*	S.D.	C.V. (%)**
$5 < C_p \leq 20$	10	13.24	2.35	17.8
$20 < C_p \leq 50$	10	34.52	3.00	8.7
$C_p > 50$	10	147.34	4.74	3.2

*Mean value of C_p.

**C.V. (%) = $\frac{100 \text{ S.D.}}{\bar{x}_p}$.

TABLE III

PRECISION OF *d*-GLAUCINE DETERMINATION IN URINE

Urine concentration (C_u) range (ng/ml)	Number of duplicate measurements	$\bar{x}_u$*	S.D.	C.V. (%)**
$15 < C_u \leq 50$	5	23.44	1.30	5.6
$50 < C_u \leq 150$	6	91.5	5.41	5.9
$C_u > 150$	5	329.1	17.40	5.3

*Mean value of C_u.

**C.V. (%) = $\frac{100 \text{ S.D.}}{\bar{x}_u}$.

Application to biological samples

The assay was applied to the determination of *d*-glaucine in plasma and urine after single oral administration and repeated oral administration of the drug to

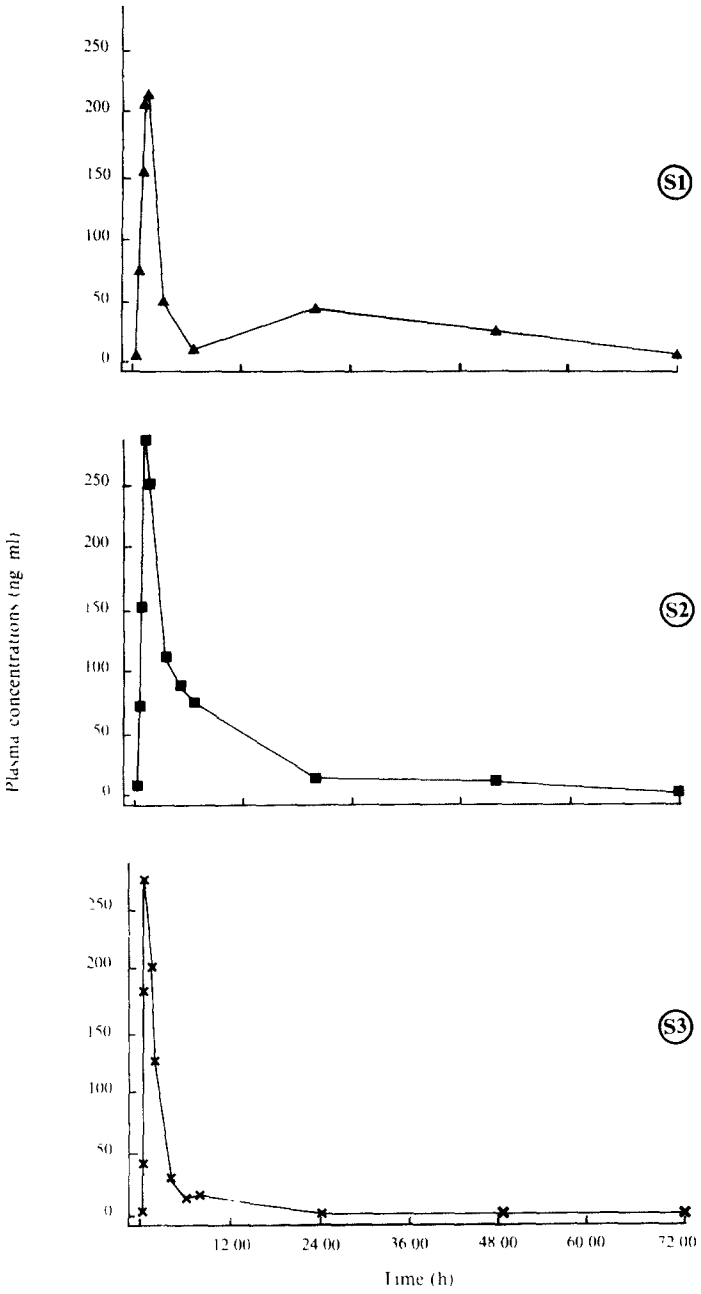


Fig. 3. Plasma concentration—time curves of *d*-glaucine from three healthy volunteers after an oral administration at a dose of 60 mg. Blood samples were collected at the following times. 0.00, 0.17, 0.33, 0.50, 0.75, 1.00, 1 50, 2.00, 4.00, 6 00, 8.00, 24.00, 48.00, 72.00 h after administration. Each concentration value is the mean of duplicate determinations.

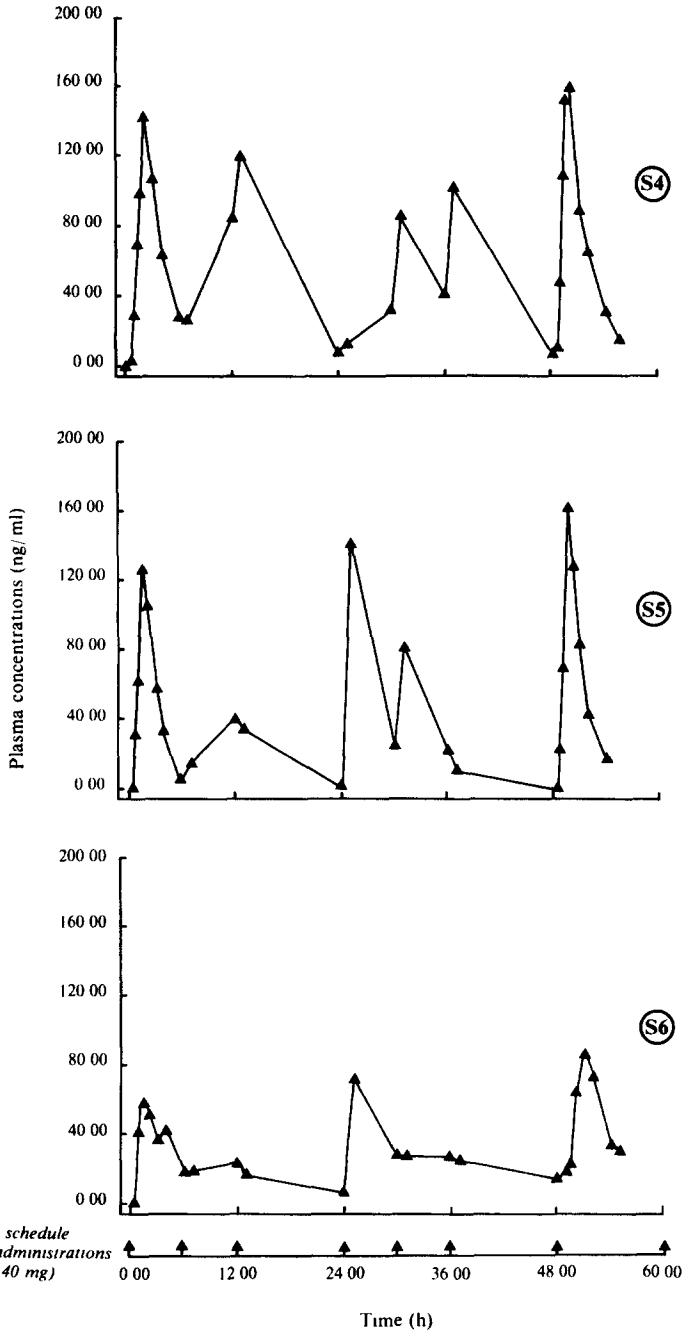


Fig. 4. Plasma concentration—time curves of *d*-glaucine from three healthy volunteers after repeated oral administration. Doses of 40 mg were given at following times: 0, 6, 12, 24, 30, 36, 48 h. Blood samples were collected at: 0.00, 0.17, 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, 4.00, 6.00, 7.00, 12.00, 13.00, 24.00, 25.00, 31.00, 32.00, 36.00, 37.00, 48.00, 48.17, 48.50, 48.75, 49.00, 49.50, 50.00, 51.00, 52.00, 54.00, 55.00, 72.00, 96.00 h after the first administration. Each concentration value is the mean of duplicate determinations.

TABLE IV

URINARY EXCRETION OF *d*-GLAUCINE IN THREE SUBJECTS AFTER A SINGLE ORAL ADMINISTRATION OF 60 mg

Period of time (h)	*d*-Glaucine (μg)		
	S1	S2	S3
0— 8	53.2	140.8	68.5
8—24	6.7	16.9	10.5
24—48	2.0	2.2	0
48—72	0	1 0	0

TABLE V

SOME PHARMACOKINETIC PARAMETERS OF *d*-GLAUCINE OBTAINED AFTER A SINGLE ORAL ADMINISTRATION OF 60 mg

Parameter	S1	S2	S3
Weight (kg)	88	68	89
AUC (0—72 h)* (ng ml^{-1} h)	2076	2088	685
T_{max}** (h)	1.5	2	0.75
C_{max}*** (ng ml^{-1})	200	285	255
Urinary excretion (0—72 h) (percentage of dose)	0.10	0.27	0.13

*Area under the plasma drug concentration—time curve from 0 to 72 h.
**Time to reach maximum plasma drug concentration.
***Maximum plasma drug concentration.

healthy volunteers. Three subjects received a single oral dose of 60 mg and three others received seven doses of 40 mg each, given over a period of three days. The plasma concentration curves are shown in Figs. 3 and 4. Urinary excretion and some pharmacokinetic parameters were calculated after a single administration of the drug (Tables IV and V).

CONCLUSION

An HPLC assay has been developed for the analysis of *d*-glaucine in plasma and urine. This method, which involves rapid sample clean-up on micro-columns, is simple, selective and sensitive and requires only 100 μl of plasma or 200 μl of urine. Our procedure has been used for over one year with human and animal samples and has shown to be reliable and very reproducible in the long run.

The method could be a valuable tool for the further elucidation of human pharmacokinetics and animal toxicokinetics which are of great importance for establishing a relation between, on the one hand, glaucine plasma levels and, on the other hand, clinical and toxic effects [7]. In addition, the method may be suitable for *l*-glaucine and *dl*-glaucine assays.

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REFERENCES

1 F.M. Probst, Justus Liebigs Ann. Chem., 31 (1839) 241.
2 P. Dierckx, G. Leblanc, A. Decoster and D. Criscuolo, Int. J. Clin. Pharmacol. Ther. Toxicol., 19 (1981) 396.
3 P.J. Davis, J. Chromatogr., 193 (1980) 170.
4 L.V. Benzel and L.Y. Ladnaya, Farm. Zh., 38 (1983) 48.
5 L.V. Benzel, L.Y. Ladnaya and D.Y. Rogovsky, Farmatsiya, 32 (1983) 49.
6 T. Gurkan, Mikrochim. Acta, 1 (1976) 173.
7 W. Cautreels, H. Davi and J.P. Jeanniot, in Proceedings of Eighth European Workshop on Drug Metabolism, Liège, September 5—9, 1982, University of Liège, Sart Tilman, 1983, p. 67.