Journal of Chromatography, 433 (1988) 269–275
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4397

Note

Determination of stobadine, a novel cardioprotective drug, using capillary gas chromatography with nitrogen–phosphorus detection after its selective solid-phase extraction from serum

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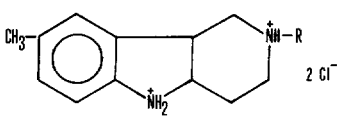
(First received June 1st, 1988; revised manuscript received July 21st, 1988)

Stobadine, the dihydrochloride of (−)-*cis*-2,8-dimethyl-2,3,4,4*a*,5,9*b*-hexahydro-1*H*-pyrido[4,3-*b*]indole (Fig. 1), is a new cardioprotective agent of the γ-carboline structural group [1] that has recently been undergoing clinical studies. For the determination of this drug in biological fluids two methods had previously been described, spectrofluorometry [2] and gas chromatography (GC) with a packed column and flame ionization detection [3]. However, these methods were developed for special purposes (toxicological investigations and in vitro metabolic studies, respectively), and they are not appropriate for low-concentration determinations, which are necessary for clinical pharmacokinetic studies of therapeutic doses of stobadine.

This paper describes a simple GC assay procedure that permits the quantitative determination of stobadine in serum down to 5 ng ml^{-1} using 1 ml of serum. A highly selective solid-phase extraction, previously described by our laboratory for some basic drugs [4–6], was used in combination with capillary column separation and nitrogen-specific detection of the underivatized drug.



R CH_3 STOBADINE
C_2H_5 INTERNAL STANDARD

Fig. 1. Molecular structures of stobadine and the internal standard.

0378-4347/88/$03.50

EXPERIMENTAL

Instrumentation

A Hewlett-Packard Model 5880A gas chromatograph was used, with a 5880A level-four terminal and a Model 7673A Hewlett-Packard autosampler. The chromatograph was equipped with a thermionic nitrogen selective detector. An HP 1 fused-silica capillary column (12.5 m×0.2 mm I.D., film thickness 0.33 μm) (Hewlett-Packard, Vienna, Austria) was used. The temperature of the column oven was held at 100°C for 1 min following injection; it was then increased at 20°C min^{-1} to 190°C and held for 2 min. The temperatures of the splitless injection port and the detector were 300°C. Nitrogen was used as a carrier gas at a flow-rate of 2 ml min^{-1} and as an auxiliary gas at 30 ml min^{-1}. The purge activation time was 30 s.

Materials

Stobadine and the racemic N-ethyl analogue of stobadine, (±)-*cis*-2-ethyl-8-methyl-2,3,4,4*a*,5,9*b*-hexahydro-1*H*-pyrido[4,3-*b*]indole (Fig. 1), used as an internal standard, were synthesized at the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences (Prague, Czechoslovakia).

Separcol SI C_{18} extraction columns (Centre of Chemical Research, Slovak Academy of Sciences, Bratislava, Czechoslovakia), packed with 100 mg of sorbent, were used in the extraction step. Each cartridge was used two to five times.

Analytical-grade acetonitrile and methanol were obtained from Lachema (Brno, Czechoslovakia).

All glassware was cleaned in hydrochloric acid, then silanized with a 5% solution of Surfasil (Pierce, Oud Beijerland, The Netherlands) in benzene.

Human plasma stabilized with citric acid, used for recovery studies, was from the Department of Hematology and Transfusion, School of Medicine, Comenius University (Bratislava, Czechoslovakia). Serum and urine for recovery studies and serum for construction of the calibration graph were obtained from human volunteers in the laboratory.

Evaluation of organic solvents for solid-phase extraction

The procedure described previously for determination of mexiletine [6] was utilized. Separcol SI C_{18} cartridges were prepared by flushing with 2 ml of methanol and 1 ml of distilled water. Then 1 ml of water spiked with 1 μg of stobadine was applied. After spontaneous passage of the water through the cartridge (ca. 1 min), the sorbent was washed with 1 ml of water. The residual water was displaced from the cartridge under mild pressure of nitrogen. For the displacement of retained stobadine, 1, 2, 3 and 5 ml of methanol or acetonitrile were then used. The organic solvents were collected into 3- and 5-ml cone vials (Reacti-Vial, Pierce), and 10 μl of the methanolic solution of the internal standard (100 μg ml^{-1}) were added. The organic solvent was evaporated to dryness at 50°C under a gentle stream of nitrogen. To the dry residue 50 μl of methanol were added, and the vials were stoppered and agitated on a vortex mixer for 10 s. The solution was placed into autosampler vials, and 1 μl of the solution was injected into the gas

chromatograph. The stobadine/internal standard peak-area ratios were compared with those obtained after mixing 1 μg of stobadine with 1 μg of the internal standard. All evaluations were carried out in triplicate.

Recovery studies

Volumes of 10 μl and 100 μl of an aqueous solution of stobadine (10 μg ml^{-1}) were added to 1 ml of human serum, plasma, or urine or to 1 ml of water and agitated on a vortex mixer for 5 s. The Separcol SI C_{18} cartridges were activated by the procedure described, and the sample was applied. The cartridges were then washed with 1 ml of water and 1 ml of acetronitrile. The residual solvent was displaced under mild pressure of nitrogen, and the retained stobadine was eluted with 2.5 ml of methanol. Methanol was collected into 3-ml cone vials, and 25 μl of the methanolic solution of the internal standard (10 μg ml^{-1}) were added to the eluate. The solution was evaporated and the described procedure was carried through. The stobadine/internal standard peak-area ratios were then compared with those obtained after mixing 100 or 1000 ng of stobadine with 250 ng of the internal standard. All evaluations were carried out in triplicate.

Calibration curve

Various amounts of stobadine (5 ng to 5 μg) and 250 ng of the internal standard, all aqueous solutions, were added to 1 ml of human serum. The solutions were agitated on a vortex mixer for 5 s, then the analytes were extracted as described for recovery studies. The stobadine/internal standard peak-area ratio was used to calculate the calibration curve.

Determination of unknown samples

Samples of 2–4 ml of serum were divided to two parts and placed in 4-ml screw-top vials. An aqueous solution of the internal standard (100 μg ml^{-1}) was added to give a final concentration of the internal standard of 250 ng ml^{-1}. The samples were agitated on a vortex mixer for 5 s and carried through the procedure described under *Recovery studies.*

Validation of the method

Intra-assay precision. Four concentrations of stobadine (5, 10, 50 and 100 ng ml^{-1}), six samples each, were assayed according to the described procedure. The data from this experiment were summarized at each concentration to yield the amounts found ($\pm$S.D.) and coefficients of variation (C.V.).

Inter-assay precision. Analyses of four concentrations of stobadine (50, 200, 500 and 1500 ng ml^{-1} of serum) were performed on the first, second, fourth, fifth and eighth day after their preparation. The data from this experiment were treated in the same way as the intra-assay data.

Long-term quality control. A 6-ml volume of the standard serum sample of stobadine (1000 ng ml^{-1}) was prepared and divided into three 2-ml portions. These were frozen and analysed after one, four and sixteen weeks following their preparation as two samples of 1 ml. The data were treated as the amounts of stobadine found in each sample analysed.

RESULTS AND DISCUSSION

After serum samples have been applied and the cartridges washed with water, the methanol and acetonitrile eluents from Separcol SI C_{18} cartridges still contain a lot of contaminating compounds and thus cannot be used to measure stobadine concentrations. It was therefore necessary to study the ability of both liquids to elute stobadine in water. The elution curves for the extraction of stobadine by methanol and acetonitrile are shown in Fig. 2. The different elution abilities of the two liquids are clearly visible. Compared with our previous findings, this difference is greater than that recorded in the case of mexiletine [6], and it approaches the difference observed in the case of local anaesthetics [4]. From the practical point of view, this difference means that a combination of the two organic solvents, i.e. acetonitrile (5% recovery of 1 ml) and methanol (100% recovery of 2.5 ml), can be used in the case of body fluids.

Table I lists the recoveries of stobadine from different fluids in concentrations of 100 and 1000 ng ml^{-1}, using the complete selective solid-phase extraction, i.e. application of sample, washing cartridges with 1 ml of water and 1 ml of aceto-

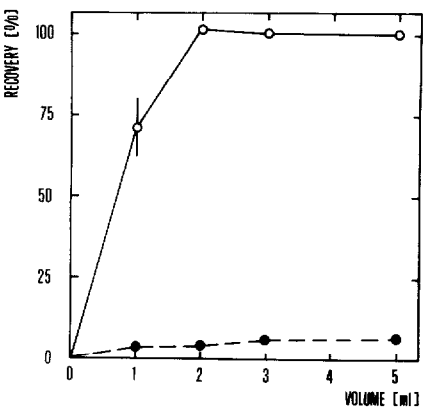


Fig. 2. Dependence of the recovery of the solid-phase extraction method on the amount of eluting methanol (○) and acetonitrile (●) (in the majority of cases, standard deviation bars were smaller than diameters of points representing mean values).

TABLE I

RECOVERY OF THE SELECTIVE SOLID-PHASE EXTRACTION METHOD OF STOBADINE FROM VARIOUS FLUIDS

Fluid	Recovery (mean ± S.D., $n=3$) (%)	
	100 ng ml^{-1}	1000 ng ml^{-1}
Water	84 ± 1	85 ± 4
Serum	70 ± 6	73 ± 5
Plasma	59 ± 2	59 ± 1
Urine	42 ± 8	13 ± 2

nitrile and eluting stobadine with 2.5 ml of methanol. The recoveries are different for the different fluids. The recoveries from water are in the range expected, but those from body fluids are lower, decreasing in the sequence serum, plasma, urine. The decreased recoveries from blood derivatives can be explained by the binding of the drug to serum or plasma proteins and by its elimination together with the proteins. However, this explanation does not account for the differences between plasma and serum and it does not sufficiently substantiate the low and concentration-dependent recoveries of stobadine in urine. In view of these findings, a study of ion-pair formation of basic drugs in body fluids and its influence on recovery in solid-phase extraction has recently been undertaken in our laboratory.

Regardless of the reason for the loss of stobadine during the solid-phase extraction procedure, it is clear that serum is better than plasma and that this type of extraction is entirely unsuitable for stobadine determination in urine.

Fig. 3 shows two chromatograms, one after selective solid-phase extraction of blank human serum, the other of serum spiked with stobadine and the internal standard. Both peaks are baseline-separated with retention times of 5.5 and 6 min, respectively. No interference from endogenous compounds in real samples was observed, and chromatograms of real samples did not differ from chromatograms of spiked samples. The main metabolites of stobadine are N-demethylstobadine and stobadine N-oxide [3]. The physicochemical and chromatographic properties of these compounds indicate that they would not interfere with the unchanged drug.

The calibration curve constructed from eight points situated within four orders (the highest serum concentrations expected were in the microgram range [7]) showed non-linearities at low as well as at high concentrations. Therefore, the

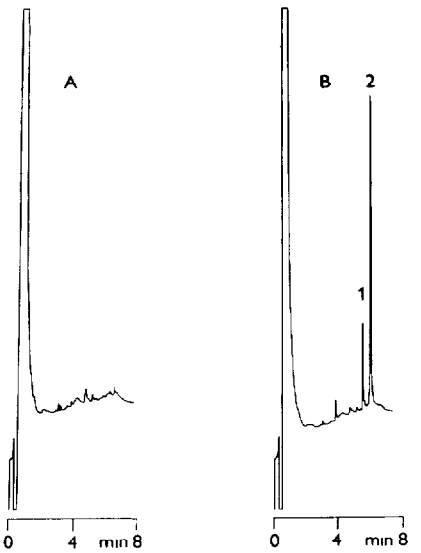


Fig. 3. Chromatograms of serum extracts. (A) Serum free from drugs, (B) serum spiked with stobadine (50 ng ml^{-1}) and the internal standard (250 ng ml^{-1}). Peaks: 1 = stobadine; 2 = internal standard.

TABLE II

INTRA-ASSAY PRECISION FOR THE DETERMINATION OF STOBADINE IN HUMAN SERUM SAMPLES

Amount added (ng ml^{-1})	Amount found (mean ± S.D., $n=6$) (ng ml^{-1})	Coefficient of variation (%)
5.0	4.9 ± 0.4	8.3
10.0	9.8 ± 0.6	5.7
50.0	51.7 ± 4.1	7.9
100.0	103.3 ± 10.5	10.2

TABLE III

INTER-ASSAY PRECISION FOR THE DETERMINATION OF STOBADINE IN HUMAN SERUM SAMPLES

Amount added (ng ml^{-1})	Amount found (mean ± S.D., $n=5$) (ng ml^{-1})	Coefficient of variation (%)
50.0	53.8 ± 5.5	10.2
200.0	190.1 ± 12.5	6.6
500.0	515 2 ± 37.1	7.2
1500.0	1449.2 ± 113.0	7.8

TABLE IV

LONG-TERM QUALITY CONTROL FOR THE DETERMINATION OF STOBADINE IN HUMAN SERUM (AMOUNT ADDED, 1000 ng ml^{-1})

Storage time (weeks)	Amount found* (ng ml^{-1})
1	1003, 1080
4	967, 1063
16	978, 1087

*Each 2-ml portion was divided into two 1-ml samples.

power function was used to fit the experimental data. The equation is as follows: $y=143.65x^{1\,1}$. The closeness of the exponent of the regression equation to 1.0 means that the curvature is only slight. The coefficient of correlation was 0.998.

Extensive accuracy and precision data were obtained, and they are presented in Tables II to IV. In all cases, coefficients of variation were 10% or lower. The data indicate integrity of the sample during storage and reproducibility of the method over a number of samples and different times.

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