*Journal of Pharmaceutical & Biomedical Analysis*
Vol. 7, No. 10, pp. 1207–1212, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00


*Short Communication*

# Fluorimetric assay of stobadine in serum of dogs

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**Keywords**: *Selective liquid–liquid extraction; antiarrhythmic and cardioprotective agent; fluorimetry, assay of stobadine.*

## Introduction

Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole (Fig. 1), is a potential antiarrhythmic and cardioprotective agent with an antihypoxic effect on the myocardium [1, 2]. It is derived from the γ-carboline antidepressant and neuroleptic, carbidine [3, 4], as its active (−)-enantiomer. Biotransformation of stobadine in rat liver microsomes was already described [5].

A preliminary distribution study in animals using the radiolabelled compound $^{3}$H-stobadine showed this drug to have a large distribution volume, and, as a result, low concentrations in blood. Therefore, sensitive and specific radiochemical and TLC methods have been developed [6, 7] which are currently used in pharmacokinetic studies on rats [8].

In the present paper a specific fluorimetric assay is now described for the determination of stobadine in serum of dogs. The assay involves the first step of the radiochemical method.

## Experimental

*Apparatus*

A Tri-Carb 300 CD liquid scintillation counter (Packard, USA) was used for counting

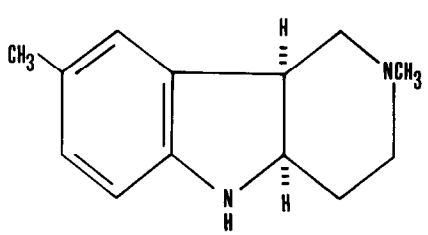


**Figure 1**
Structure of stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole.

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the radioactivity, A Perkin–Elmer 203 fluorescence spectrophotometer was used for detection of the fluorescence of stobadine. A Radelkis OP-211/1 laboratory digital pH meter with a combined glass–calomel electrode was used to measure pH values.

*Chemicals*

An SLD 31 dioxane liquid scintillator (Spolana, CSSR) was used for measuring radioactivity. Britton–Robinson buffers of various pH values were prepared and used in extraction experiments. For re-extraction of stobadine, 0.2 M phosphate buffer of pH 6.5 was used. Solutions were made in double distilled water. n-Hexane and sodium carbonate were of analytical grade. Non-labelled stobadine dihydrochloride and dipalmitate were synthesized at the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences (Prague, CSSR).

*Radiochemicals*

$^3$H-Stobadine dihydrochloride, sp. act. 495 GBq mM$^{-1}$, was synthesized at the Institute for Research, Production and Uses of Radioisotopes (Prague, CSSR), as described in ref. 9. The radiochemical purity (95%) was checked by high-performance liquid chromatography (HPLC).

*Extractions*

The extraction and re-extraction experiments were carried out at ambient temperature in stoppered glass test-tubes silanized with a 5% solution of Surfacil (Pierce, The Netherlands) in benzene by shaking for 5 min. Both phases were separated by centrifuging, and aliquots were mixed with SLD 31 dioxane liquid scintillator and counted for radioactivity. The distribution coefficient $D$ was calculated as the ratio of radioactivity in the organic and aqueous phases.

For recovery studies, drug-free serum was treated with various amounts of non-labelled stobadine and 0.1 μCi of radioactive stobadine. Three millilitres of 2 M sodium carbonate were added to 1 ml of the spiked serum, and the mixture was extracted with 4 ml of n-hexane. The recovery of stobadine was calculated according to the equation:

$$\% \text{ Recovery} = \frac{D}{D + \frac{V_{aq}}{V_{org}}} \times 100, \quad (1)$$

where $D$ is the distribution coefficient, $V_{aq}$ and $V_{org}$ are volumes of the aqueous and organic phase, respectively.

The re-extraction of stobadine into phosphate buffer, performed with a phase ratio of 1:1, was followed by radiometric measurement of both phases and fluorimetric measurement of the aqueous phase. The recovery of stobadine was calculated by using equation (1) above. The excitation and emission wavelengths were 302 and 365 nm, respectively.

*Calibration curve*

Varying quantities of stobadine (50–6000 ng ml$^{-1}$) were added to 1 ml of dog serum. The samples were treated by the proposed analytical procedure and the intensity of fluorescence in relative units was plotted against the concentration of the drug.

*Animal experiments*

Two dogs were used in the experiment. Stobadine dihydrochloride in a dose of 5 mg $kg^{-1}$ (as the free base) was administered orally to one dog which had fasted for 1 day prior to administration. Five days later stobadine dipalmitate (5 mg $kg^{-1}$ as the free base) was administered orally to the same dog. For parenteral administration, $^{3}H$-stobadine dihydrochloride (500 μCi) was added to unlabelled stobadine dihydrochloride (5 mg $kg^{-1}$ as the free base) dissolved in 10 ml of sterile physiological saline, and administered intravenously to the other dog. Blood (10 ml) was drawn at 0.08, 0.25, 0.5, 1, 2, 3, 4, 6, 24 and 48 h following both routes of administration. After leaving the samples overnight at 4°C they were centrifuged to obtain serum.

*Drug analysis*

For determination of total $^{3}H$-radioactivity, 0.2 ml of serum was digested in 0.6 ml 25% potassium hydroxide in 20% ethanol, and 0.2 ml aliquots of the digests were mixed with 15 ml of the liquid scintillator. For the specific assay of stobadine in serum, 1 ml of the sample was analysed radiochemically as described in ref. 6, and 1 ml of the same serum was analysed by the proposed fluorimetric assay. In the radiochemical method, the calculation of the drug concentration in ng $ml^{-1}$ was carried out by comparing the radioactivity extracted into the organic phase with the specific radioactivity of the solution administered. In the fluorimetric method, the calibration curve was used to determine the amount of the drug in each sample.

## Results and Discussion

Because of the relatively high sensitivity of stobadine to oxidation, a simple and rapid analytical method should be used in order to avoid the possible degradation of the drug during analysis.

Considering this fact and keeping in mind the specificity of the assay, the ideal solution of the problem would be to combine the pre-analytical treatment of the biological sample and the subsequent analysis in one single step. This approach has already been applied to the radiochemical analysis of the local anaesthetics $^{3}H$-pentacaine [10], $^{14}C$-carbisocaine [11] and $^{14}C$-heptacaine [12]. All three methods are based on the selective extraction of the parent drugs from the mixture of metabolites, and have been applied in pharmacokinetic studies of these drugs *in vitro* and *in vivo* [13–15]. The results obtained were in good agreement with the results obtained by thin-layer chromatography. Similarly, a specific radiochemical assay has been developed for the determination of $^{3}H$-stobadine in biological samples [6].

Although the classical liquid–liquid extraction methods are gradually being replaced by the modern technique of solid-phase extraction, they still play an important role in isolating drugs and their metabolites from biological fluids. Their main advantage over solid-phase extraction methods lies in the fact that by choosing the proper organic solvent, pH value of the aqueous phase and ionic strength, optimum conditions for selective extraction of the parent drug can be achieved.

A good extraction system must have high selectivity, i.e. give large differences in distribution coefficients for structurally related compounds. To select the solvent and to define the pH for the extraction, the fraction of the drug extracted from aqueous solutions is measured at various pH values in the range 1–14 with each solvent. A simple way of doing this is to extract relatively large amounts of radiolabelled drug (about 10 μg

$ml^{-1}$) with equal volumes of solvent and buffer and to measure the amount of the compound in both phases by counting the radioactivity.

The following observations in Fig. 2 were made: (1) the point of inflexion corresponded to the pH value 8.7, (2) no extraction of stobadine from acidic solution was observed, (3) the extraction yield from alkaline solution increased dramatically in the pH range 7–11. This pH dependence is in good agreement with the fact that stobadine is a weak base with $pK_A$ values 3.2 and 8.7. Protonation of two nitrogen atoms in acidic solution renders the whole molecule electrically charged and, therefore, unextractable into non-polar n-hexane. Only uncharged non-polar molecules can readily extract into n-hexane providing the distribution coefficient exceeds 4. The weak lipophilic properties of stobadine have already been shown to result in a distribution coefficient of 3.4 for the system n-octanol–buffer, pH 7.4.

In general, extraction yields of drugs from biological matrices are lower than those from aqueous solutions. With the aim of increasing the extraction yield, the influence of the ionic strength was investigated. The distribution coefficient was found to increase with increasing concentration of sodium carbonate, and in the presence of 2 M sodium carbonate it reached the value of 11.

As reported in ref. 6, n-hexane is the solvent of choice for quantitative and selective extraction of stobadine from biological matrix after the addition of 2 M sodium carbonate. In choosing the solvent, advantage is taken of the fact that stobadine is converted by the body to derivatives that are less lipid soluble and therefore have lower distribution coefficients between the n-hexane and aqueous phase. n-Hexane extracts only the most lipid soluble non-polar substances. Therefore, n-hexane will extract the parent drug, but leave a large fraction of the relatively polar metabolites and degradation products in the aqueous phase. This decreases the chance of extracting not only interfering metabolites and degradation products, but also normally occurring substances that may interfere with the fluorimetric assay.

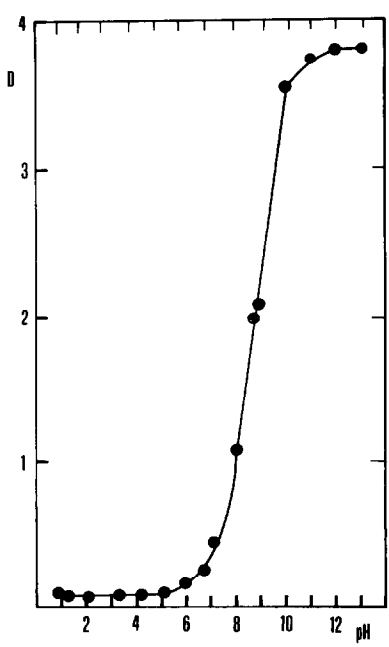


**Figure 2**
Effect of pH on the distribution coefficient ($D$) of $^3$H-stobadine (water–n-hexane).

For fluorimetric measurement, stobadine has to be re-extracted into phosphate buffer of pH 6.5, at which pH value the intensity of fluorescence is at a maximum. The recovery of this step was checked at various concentrations of stobadine. In accordance with the generally accepted theory, at pH 6.5 stobadine is in the completely ionized state and its passage from non-polar n-hexane into phosphate buffer is almost quantitative. It was found that a wide concentration range of stobadine (50–6000 ng $ml^{-1}$) did not affect the extraction and re-extraction yield, for which mean values of 91.55 and 90.26%, respectively, were obtained. The response of the fluorimetric detector was linear in the concentration range of the pure substance from 40 to 6000 ng $ml^{-1}$ in buffer at pH 6.5. The limit of detection was 50 ng $ml^{-1}$ in serum. The relative standard deviation for various concentrations varied between 3.43 and 9.8%.

Due to its simplicity, sensitivity and selectivity, the whole extraction procedure, including the fluorimetric measurement, is suitable for full-range pharmacokinetic studies in dogs. The scheme of the extraction procedure is shown in Fig. 3. The proposed

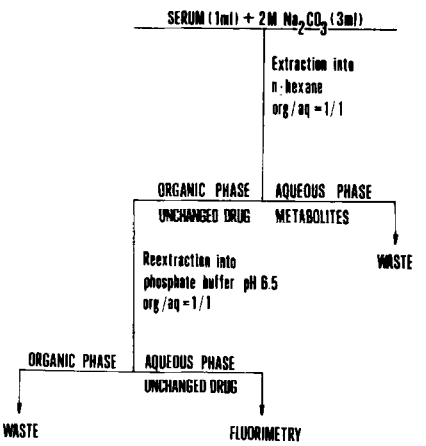


**Figure 3**
Scheme of the extraction procedure for fluorimetric determination of stobadine in serum of dogs.

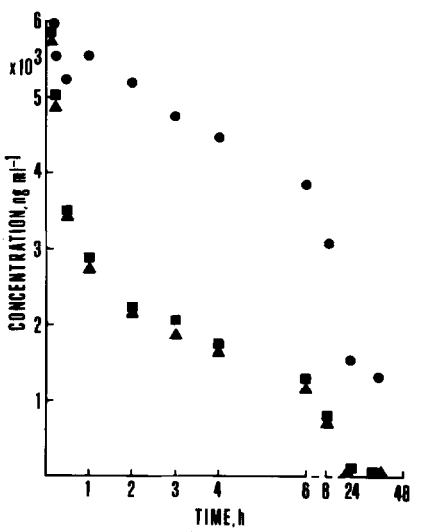


**Figure 4**
Serum levels of total radioactivity (●), and of $^3$H-stobadine determined fluorimetrically (■), and radiochemically (▲) after intravenous administration of $^3$H-stobadine dihydrochloride, in a dose of 5 mg $kg^{-1}$ free base.

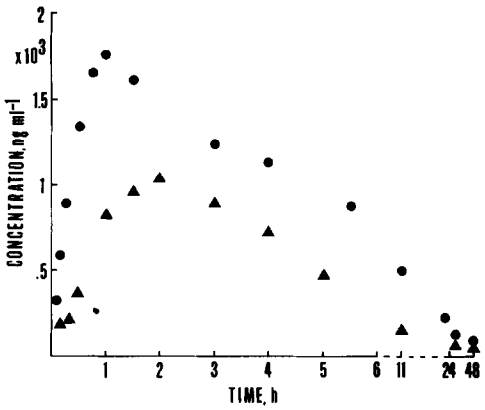


**Figure 5**
Serum levels of stobadine in a dog following single oral administration of stobadine dihydrochloride (●) and stobadine dipalmitate (▲), in a dose of 5 mg $kg^{-1}$ free base.

method was used for the determination of bioavailability of stobadine in dogs after intravenous and oral administration of 5 mg of the drug as base per kg body wt, both as dihydrochloride and dipalmitate salts [16]. Figures 4 and 5 show the stobadine levels in the serum of dogs following both routes of administration of both salts of stobadine. The results show different pharmacokinetics of the two salts of stobadine following oral administration. The total radioactivity–parent drug ratio greatly increased with time indicating rapid metabolism of stobadine.

In conclusion, comparison of the proposed fluorimetric assay and of the previously developed radiochemical method demonstrates that both methods yielded similar serum–time curves after both routes of administration of stobadine. Thus the method proposed is of high specificity and sensitivity and therefore suitable for monitoring stobadine in serum of dogs even 48 h after administration.

*Acknowledgement* — The authors wish to thank Monika Lukacsova for her excellent technical assistance.

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[*Received for review 12 August 1988; revised manuscript received 3 January 1989*]