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ORIGINAL RESEARCH

Extraction and chromatographic separation methods in pharmacokinetic studies of Stobadine®—an indole-related antioxidant and free-radical scavenger†

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ABSTRACT: This overview provides comprehensive information on the most relevant results of Stobadine preclinical disposition studies. In order to investigate pharmacokinetic processes of the drug in rats, dogs and in human volunteers, several bioanalytical assays based on radiometric, spectrofluorometric, as well as chromatographic determination methods were developed and implemented. In small laboratory animals, the drug absorption, distribution, metabolism and elimination were investigated by administering ^{3}H-labeled Stobadine. Spectrofluorometry was used alternatively for the determination of cold/unlabeled Stobadine in extracts of biomaterials sampled from larger animal species. The chromatographic separation methods proved, however, to be the most advantageous for determining details of the drug disposition and fate in the body. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION

The primary focus in the research of the scientific workers of the Institute of Experimental Pharmacology of the Slovak Academy of Sciences has been concentrated long-term on preclinical studies of therapeutic substances with membrane-stabilizing effects. Currently, one of these compounds, Stobadine®, [85202-17-1], has reached the third, i.e. clinical, phase of testing.

Stobadine (Fig. 1), a cardio- and neuroprotective agent with antidysrhythmic and antihypoxic effects, protects the brain and myocardium from ischemia, and the lungs and other tissues from damage caused by free, mostly oxygen-derived radicals (Beneš and Štolc, 1989; Bilčíková *et al.*, 1990; Bauer and Štolc, 1993; Horáková and Štolc, 1998; Sotníková *et al.*, 1998). As part of the preclinical pharmacokinetic studies, several bioanalytical determination methods for Stobadine have been elaborated and applied (see Appendix).

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†*Dedicated to professor Dr Dr E. Mutschler*. The authors wish to express their gratitude to Professor Dr Dr E. Mutschler for his valuable contribution to the celebration of the 50th Anniversary of the Institute of Experimental Pharmacology during the joint International Symposia on 'Recent Advances in the Mechanism of Drug Action' and 'Drug Action on Reactive Oxygen Species with Special Attention to Stobadine', both held in the castle of the Slovak Academy of Sciences in Smolenice, Slovak Republic (Sotníková and Šoltés, 1997, 1998).

The aim of this paper is to overview these bioanalytical methods not only from methodological aspects, but also from the point of view of reassessing the obtained pharmacokinetic data themselves, as well as their relationship to Stobadine pharmacodynamics.

METHODOLOGICAL SECTION AND RESULTS

Drug synthesis and labeling

Stobadine, developed at the Institute of Experimental Pharmacology, SAS, Bratislava (Štolc *et al.*, 1983), was prepared in Slovakofarma JSC, Hlohovec, Slovak Republic, in cooperation with the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic. Two salt forms of Stobadine have been used throughout the preclinical studies, namely its water soluble dihydrochloride salt

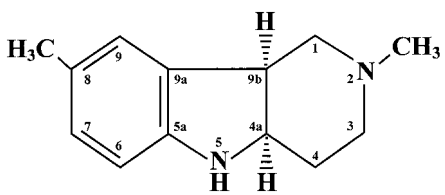


Figure 1. Chemical structure of Stobadine (base; molecular weight = 202.3 Da). CAS reference number: [85202-17-1]. CAS registered name 'Stobadine': *cis*-(−)-2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1-H-pyrido[4,3-b]indole (*Chemical Abstracts*, 1994).

(sometimes referred to in the literature as DH 1011) and the water insoluble dipalmitate (referred to as DP 1031).

Stobadine labeling by tritium (^{3}H) was performed at the Institute for Research, Production and Use of Radioisotopes, Prague, from the drug 6-bromo-derivative by a catalytic reductive dehalogenation reaction in which the bromine atom (in 6-C position) was replaced by that of tritium (Marko *et al.*, 1989). The ^{3}H-Stobadine supply (dihydrochloride salt dissolved in methanol), of radiochemical purity = 96%, activity = 40 MBq/mL, and specific activity = 495 GBq/mmol, is delivered and stored under argon gas.

Control of purity and stability of the drug

The antioxidative property and free-radical scavenging action of Stobadine are attributed to the relatively high readiness of its molecule to release one hydrogen radical from the 5-N position (cf. Fig. 1) (Staško *et al.*, 1990) during the reaction with a free radical such as hydroxyl (Ondriaš *et al.*, 1989; Štefek and Beneš, 1991; Steenken *et al.*, 1992; Orviský *et al.*, 1997), peroxyl (Steenken *et al.*, 1992), or alkoxyl (Steenken *et al.*, 1992). In the presence of oxygen, the formed radical (5-N$^{\cdot}$) is transformed through an intermediate (5-N-O$^{\cdot}$) into the drug oxidized derivative(s) (Staško *et al.*, 1990). Although this very reactivity of Stobadine can be used to advantage in preventing several pathophysiological conditions, a marked decomposition potential of the drug molecules, induced for example by the action of atmospheric oxygen, should be kept in mind.

The solid Stobadine dipalmitate, the salt form designed to be the active species in the final oral drug formulation, demonstrates an excellent stability during storage conditions. However in pharmacokinetic studies, when ^{3}H-Stobadine and/or Stobadine (their dihydrochloride salts dissolved usually in physiological saline) are administered to animals, the limited drug stability in daylight and in oxygen-containing atmosphere (Bezáková *et al.*, 1993) should be taken into consideration.

Thin-layer chromatography (TLC) combined with liquid-scintillation counting (LSC) has been used to verify the radiochemical purity of the supplies of ^{3}H-Stobadine (the batch solutions) as well as their stability during storage (Šoltés and Trnovec, 1987). To develop the TLC plates (Silufol® UV_{254}; Kavalier, Sázava. Czech Republic) three different multicomponent mobile phases of (1) $CH_3OH + CHCl_3 + ClCH_2CH_2Cl + (C_2H_5)_3N$, 60:20:20:3.5; (2) $CHCl_3 + ClCH_2CH_2Cl + (CH_3)_2CHOH + (C_2H_5)_3N$, 60:20:20:5; and (3) benzene + $(CH_3)_2CHOH + CH_3OH$ + 26% aqueous ammonia. 60:20:20:1.5 (v/v/v/v) were established as the most appropriate. Under any of the experimental conditions (1, 2 or 3), the retention factor (R_f) of Stobadine equals the optimal value ($R_f = 0.5$). The content of ^{3}H-radioactivity in the TLC spot(s) was counted by a TriCarb-300 CD or 2500 TR-liquid-scintillation spectrometer (Packard Instrument Co., Meriden, CT). A purity of $\geq$95% was set as the criterion for working with the given batch of the labeled drug.

The stability of ^{3}H-Stobadine dissolved and stored in various aqueous buffered solutions with different pH values ($3.0 \leq pH \leq 12.2$) was checked by monitoring the content of the drug and/or of its (radioactive) decomposition products on operating a high-performance liquid chromatographic (HPLC) apparatus interconnected with a Trace II-type radioactivity detector (Packard Instrument Co.) (Sčasnar *et al.*, 1989a). A compact-glass-cartridge (CGC) column (3.3×150 mm) packed with Separon® SGX CN sorbent (mean particle size = 5 μm; Tessek Ltd, Prague) was run with an eluent of 8.0% (v/v) ethanol in aqueous NaH_2PO_4 (50 mmol/L) with addition of tri-*n*-butylamine (50 mmol/L); the eluent pH was set to 2.6 with concentrated H_3PO_4. Under these experimental conditions the drug molecules were eluted at a retention time (R_t) of 6.0 min. On analyzing the ^{3}H-Stobadine samples kept for up to 4 h in buffered solutions, two decomposition products at $R_t = 3.5$ and 9.0 min were observed. The study clearly showed that in the pH region from 3.9 to 8.4 the drug was relatively stable, while at other pH values, especially those within the alkaline pH region, its stability decreased considerably. Thus on treating the biomaterials investigated for their drug content, it is an inevitable prerequisite to minimize the time of ^{3}H-Stobadine/Stobadine handling and storage in strongly acidic or strongly alkaline conditions.

Determination of ^{3}H-Stobadine in animals without drug isolating/separating procedure

Evidence has been provided that the molecules of Stobadine are metabolized *in vivo*. In numerous biomaterials investigated, the content of the parent ^{3}H-Stobadine found was significantly lower than the total ^{3}H-radioactivity counted in the given sample. An exceptionally significant finding was the observation that after i.v. bolus administration of ^{3}H-Stobadine dihydrochloride to rats, in the dose of 2.0 mg/kg,† the ^{3}H-radioactivity level of the sum of plasma Stobadine metabolites was practically unchanged over a relatively long period of time (days), despite the rapid decline of the parent drug plasma concentration (with a terminal elimination half-life $t_{1/2elim}$ = 85.6 min; cf. also Table 1) (Kállay *et al.*, 1990). Thus in pharmacokinetic studies for the determination of the parent Stobadine, it is mandatory to apply a drug isolating/separating procedure by which the analyte traced is set free of its metabolite(s). However, curiously enough, as seen in the following subsections, there are some direct assay methods yielding data virtually representing the content of the parent drug.

†In the whole paper 'dose' represents the drug base.

Table 1. Pharmacokinetic parameters of Stobadine in rats (applied dose of ^{3}H-Stobadine dihydrochloride: 2.0 mg/kg)

	Assay		
	Extraction method		TLC
Drug administration mode and parameter	Kállay *et al.* (1990)	Kállay *et al.* (1991)	Bittererová *et al.* (1990)
i.v. bolus[a]			
$t_{1/2elim}$ (min)	85.6		78.1
V_c (L/kg)	3.84		3.0
V_{SS} (L/kg)	4.78		8.5
Cl_{app} (mL/min kg)	105.3		117.5
p.o. single			
c_{peak} (ng/mL)			41
t_{peak} (min)	60	60	45
$t_{1/2elim}$ (min)		95 ± 8.2	
p.o. repeated			
$t_{1/2elim}$ (min)		78 ± 7.5	

$t_{1/2elim}$ = drug terminal elimination half-life; V_C and V_{SS} = volume of drug distribution in the central compartment and at steady-state; Cl_{app} = apparent body clearance; c_{peak} = peak drug concentration; t_{peak} = time in which c_{peak} attained.
[a] Relationship of ^{3}H-Stobadine concentration (c) in plasma to time (t) after i.v. drug administration approximated by a bi-exponential four-parameter functional dependence, ie an open two-compartment pharmacokinetic model conferred on the data (Gibaldi and Perrier, 1982).

Brain uptake of ^{3}H-Stobadine

The uptake of Stobadine into the rat brain was determined by injecting a mixture of ^{3}H-labeled drug and a freely diffusible internal standard ^{14}C-butan-l-ol into the carotid artery of the animals (Kállay *et al.*, 1990). Within 5 s the rats were decapitated and in the ipsilateral cerebral hemisphere the ratio of ^{3}H/^{14}C radioactivity was counted by liquid-scintillation spectrometry. The established brain uptake index = 78 ± 2.9% indicates that Stobadine passes relatively readily across the blood-brain barrier (Bezek *et al.*, 1990) and thus during actual treatment of patients a substantial amount of the parent drug could reach the brain tissue.

Scanning of ^{3}H-Stobadine in animals by a radiographic method

On applying the mode of i.v. bolus injection of the labeled drug, the content of the tracer determined at a very early time interval involves dominantly parent drug molecules. Figure 2 represents a radiogram corresponding to the actual situation 5 min after ^{3}H-Stobadine dihydrochloride administration into the rat tail vein (Kállay *et al.*, 1990). [The drug dose applied was 2.9 mg/kg; the amount of (β)-radioactivity = 44 MBq per animal.] As seen, the distribution of the radioactive compound(s) in the animal body, representing mostly ^{3}H-Stobadine molecules, is heterogenic. A relatively high drug content can be evidenced in lung, brain, myocardium and liver tissue. The extensive drug entrapping by the lung(s) relates to a known phenomenon—pneumophilicity of the basic substance(s). The high content of the ^{3}H-label in the liver corresponds well with the natural function of this organ, ie uptake of foreign substances from the blood stream, their biotransformation and subsequent elimination in the form of metabolites. The radiographic scans taken at longer time intervals (following i.v. drug administration route) (Kállay *et al.*, 1990) served well for proper selection of tissues/organs whose investigation is of much relevance on studying the pharmacokinetics of the parent Stobadine and/or of its metabolites. (The rat organs processed were lung, brain, heart, liver, kidney, small and large intestine, spleen, muscle and testis)

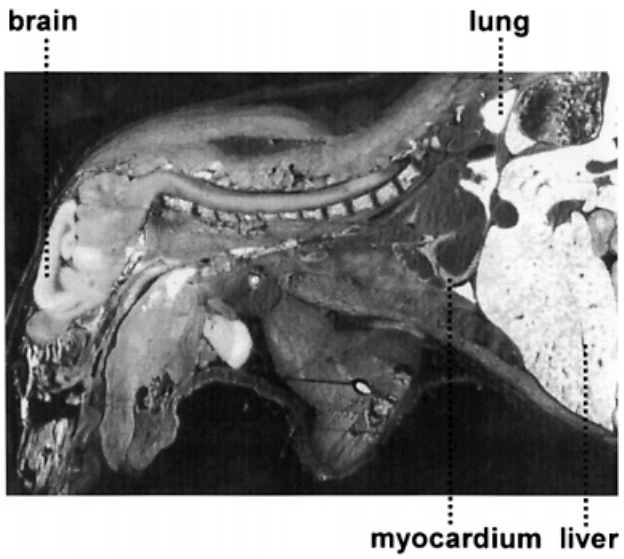
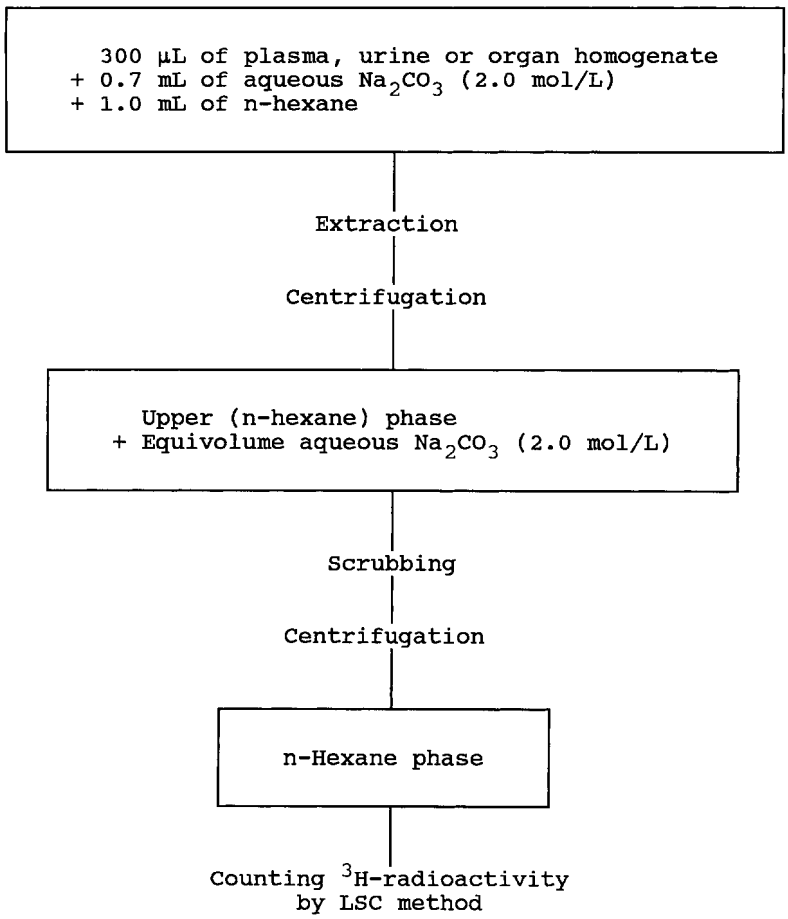


Figure 2. Radiogram representing the labeled drug distribution in the rat 5 min after i.v. administration of ^{3}H-Stobadine dihydrochloride.



Scheme 1. Sketch of the sample treatment procedure applied on determining the parent ^{3}H-Stobadine in various biological materials.

Bioanalytical assay methods involving drug extracting procedure(s)

Experimental pharmacokinetics of a novel (potential) drug are usually studied by applying the pharmaceutical labeled by a (β)-radioisotope. Primarily, the total radioactivity representing the mixture of the labeled parent drug and its metabolites is determined. To separate the molecules of the parent substance from the sum of its metabolites, a single- or multi-step liquid extraction is usually applied.

Bioanalysis of ^{3}H-Stobadine by the extraction method

In drug disposition studies performed on small laboratory animals, the use of ^{3}H-labeled Stobadine allows a number of simplifications and provides several advantages.

Extraction method principle: Scheme 1 is self-explanatory, displaying the method developed (Ščasnár and Štefek, 1987) and extensively used (cf. Appendix) on determining the drug distribution and/or its fate in the body of rats and rabbits. The specificity of the procedure represented in Scheme 1 was verified by combining the TLC separation and LSC quantification methods on analyzing the *n*-hexane extract/phase derived from plasma, urine or from the homogenate of various rat organs (lung, heart, liver, kidney). As demonstrated (Ščasnár and Štefek, 1987), the parent ^{3}H-Stobadine molecules were isolated selectively (>90%) with a high recovery (>90%). This extraction method is characterized by its simplicity, rapidity and efficiency, and it is sufficiently selective and sensitive for determination of low levels of ^{3}H-Stobadine in various biological samples.

Extraction method application(s): an interesting study design was used in investigating the pharmacokinetics of ^{3}H-Stobadine dihydrochloride in rats (Kállay *et al.*, 1991). The drug was administered orally in a dose of 2.0 mg/kg to a group of animals, and the time course of the parent ^{3}H-Stobadine plasma concentration was determined ($t_{1/2elim} = 95 \pm 8.2$ min; cf. also Table 1). During three consecutive days, another group of rats was treated with the labeled drug by applying an oral dose of 6.0 mg/kg in order to quickly saturate the rat tissues. From the 4th up to 15th day, the maintenance daily dose of labeled Stobadine applied p.o. was 1.0 mg/kg. During

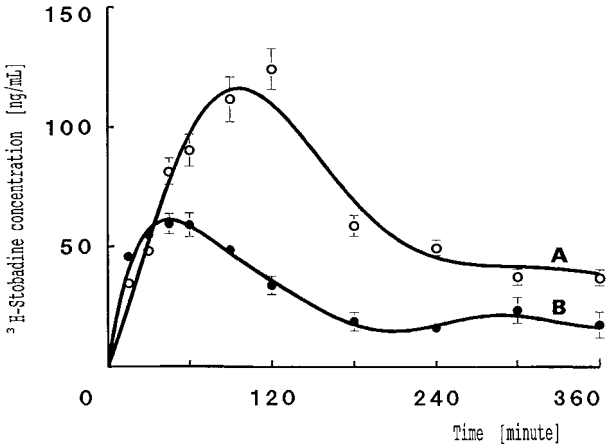


Figure 3. Time courses of the labeled drug plasma concentration in non-pregnant (A) and pregnant (B) rabbits following single p.o. administration of ^{3}H-Stobadine dihydrochloride.

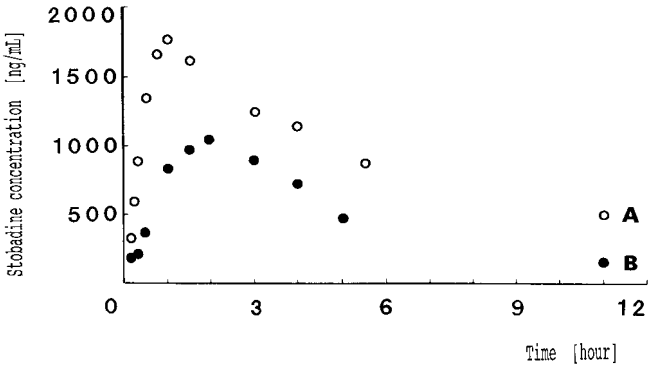


Figure 4. Stobadine levels determined in dog serum following single p.o. administration of dihydrochloride (A) or dipalmitate (B) salt of Stobadine.

this time period the peaks and troughs of the total ^{3}H-radioactivity in plasma indicated that virtually steady-state conditions had been achieved. The treatment of animals from the 16th up to the 25th day continued by applying cold/unlabeled Stobadine dihydrochloride at the dose of 1.0 mg/kg. Finally, on the 26th day, the initial ^{3}H-Stobadine dihydrochloride dose (2.0 mg/kg) was applied and the time course of the parent drug plasma level was determined. In this experimental design, ie repeated p.o. drug administration mode, the calculated $t_{1/2elim}$ value was 78 ± 7.5 min (cf. also Table 1).

In another study (Ujházy *et al.*, 1994, 1995), ^{3}H-Stobadine dihydrochloride was administered orally in a single dose of 5.0 mg/kg to a group of non-pregnant rabbits and to a group of pregnant animals on the 27th day of gestation. The time courses of the parent ^{3}H-Stobadine level determined in plasma are represented in Fig. 3. As evident, the drug concentration profile in the last stage of pregnancy indicated a recirculation of ^{3}H-Stobadine in the maternal body [cf. Fig. 3, curve B, the (second) peak at 300 min]. To describe the drug fate in non-pregnant animals, a third-order linear model was fully satisfactory, while in the case of pregnant rabbits even a fifth-order linear model had to be applied. The apparent body clearance of the drug in non-pregnant rabbits was found to be 139.6 mL/min kg. During pregnancy its value rose significantly and at the 27th day of gestation it equaled 312.6 mL/min kg.

Bioanalysis of Stobadine by a fluorimetric method

The molecules of Stobadine exhibit native fluorescence providing an adequate property for spectrofluorometry (SF) as a (nonradiometric) bioanalytical method.

SF method(s) principle: both SF methods developed (Marko, 1985; Ščasnár *et al.*, 1989b) for the parent drug isolation from serum (as well as from urine) exploit the extraction procedure outlined in Scheme 1. However, since the intensity of Stobadine fluorescence is pH dependent, with the maximum in the region close to neutral pH (Marko, 1985), the drug molecules from the *n*-hexane phase are better back-extracted into a phosphate buffer (0.1 mol/L, pH 6.5) to obtain a liquid sample appropriate for fluorescence measurements. By using a Perkin-Elmer 203 spectrofluorometer (Perkin-Elmer, Norwal, CT) at the excitation and emission wavelengths 302 and 365 nm, respectively, the limit of Stobadine determination in dog serum was found to be 50 ng/mL with RSD $< 10\%$ (Ščasnár *et al.*, 1989b). On analyzing the samples derived from dog urine, this limit was 500 ng/mL, mostly due to non-standard fluorescence of (co-)extracted endogenic compounds (Marko, 1985).

SF method application(s): Figure 4 shows the concentrations of Stobadine determined in dog serum following a single oral administration of Stobadine dihydrochloride or dipalmitate in the dose of 5.0 mg/kg (Ščasnár *et al.*, 1989b). On applying a solution of the dihydrochloride salt, the concentration reached a peak value of 1770 ng/mL (c_{peak}) at 1.0 h (t_{peak}) after drug dosing. However the value of c_{peak} reached at 2 h after Stobadine dipalmitate (powder) administration was significantly smaller, ie 1040 ng/mL (cf. also Table 2).

Drug determinations applying chromatographic separation methods

In bioanalysis of drugs, radiochemical as well as spectro-(photo/fluoro)-metric assays have certainly yielded much useful knowledge. However, since the step of liquid extraction fails to be adequately specific, some ensuing conclusions may be biased. Chromatographic methods, with their inherent characteristic of efficient separation of multicomponent mixtures, provide the required specificity.

Table 2. Pharmacokinetic parameters of Stobadine in dogs after oral administration of different drug salt forms at the dose of 5.0 mg/kg

	Stobadine salt form				
	Dihydrochloride			Dipalmitate	
Parameter	Šoltés *et al.* (1991)[a]	Ščasnár *et al.* (1989b)	Kukan *et al.* (1994)	Šoltés *et al.* (1991)[a]	Ščasnár *et al.* (1989b)
$t_{1/2abs}$ (min)	24 ± 2.5		21 ± 7.2	32 ± 4.6	
$t_{1/2elim}$ (min)	312 ± 28.8			196 ± 20.6	
t_{lag} (min)	2 ± 5.2			15 ± 1.2	
c_{peak} (ng/mL)	1282 ± 44.0	1770		823 ± 27.2	1040
t_{peak} (min)	97 ± 5.3	60		100 ± 7.1	120
Cl_{tot} (mL/min)	7 ± 0.4			15 ± 0.8	
F_{rel} (%)				46.4	

$t_{1/2abs}$ = drug absorption half-life; $t_{1/2elim}$ = terminal elimination half-life; t_{lag} = lag time; c_{peak} = peak drug concentration; t_{peak} = time of c_{peak} attained; Cl_{tot} = total body clearance; F_{rel} = relative bioavailability.

[a] Relationship of Stobadine concentration (c) in serum to time (t) after p.o. drug administration approximated by a function representing a two-compartment open linear pharmacokinetic model with a first-order absorption and a time delay (t_{lag}) between administration and onset of drug absorption (Peck *et al.*, 1984).

Bioanalysis of ^{3}H-Stobadine by the TLC method

The TLC method is the simplest chromatographic assay for ^{3}H-labeled Stobadine bioanalysis. This method, in combination with the LSC quantification of the parent drug and/or its metabolites, has been applied with good results in a number of pharmacokinetic studies (Bittererová, 1989; Bittererová *et al.*, 1990).

TLC method principle: to 1.0 mL of (rat) plasma investigated for the ^{3}H-Stobadine content, 100 μL of aqueous trichloroacetic acid solution (2.0 mol/L) is admixed. By this step plasma proteins precipitate quantitatively (Blanchard, 1981); simultaneously, due to sample acidification, the corresponding trichloroacetic drug salt is formed. The precipitated plasma proteins are separated by centrifugation. (The adsorption of Stobadine trichloroacetate to the protein precipitant is insignificant.) To a 500 μL aliquot of the withdrawn supernatant an aqueous NaOH solution (50 μL; 2.0 mol/L) and chloroform (100 μL) are added. The liquids, in a 1.5 mL Eppendorf capped tube are shaken intensively for about 5 min. (From the alkalized supernatant. Stobadine, the base, is partitioned quantitatively into the chloroform phase). After centrifugation, 50 μL of the chloroform extract (the bottom phase) is spotted onto the aluminum TLC plate of Silufol® UV_{254}. A marker of methanolic solution of Stobadine dihydrochloride (2 μL; 1 mg/mL) is spotted onto the plate simultaneously. The developing solvent system applied is a four-component mixture of $CHCl_3$ + $ClCH_2CH_2Cl$ + $(CH_3)_2CHOH$ + $(C_2H_5)_3N$, 60:20:20:5 [v/v/v/v; cf. above solvent system (2)]. The localization of Stobadine/^{3}H-Stobadine on the developed TLC plate is indicated by the quenched reflectance of the fluorescence indicator (UV_{254}). The position of the parent drug ($R_f = 0.5$) does not interfere either with Stobadine metabolites (2-*N*-desmethyl Stobadine $R_f = 0.2$; Stobadine 2−*N*→O derivative $R_f = 0$) or with UV light-(254 nm)-absorbing endogens ($R_f > 0.9$) co-extracted by chloroform. The cut-out part of the aluminum TLC plate containing the analyte determined is digested (for 5 min) directly in the measuring LSC vessel by applying a water-ethanolic solution of KOH (0.2 mL; $H_2O + C_2H_5OH +$ KOH, 1.2 L:0.4 L:0.32 kg) and the digest is subsequently dispersed by adding methanol (2 mL). After addition of an SLD 31® solution (15 mL; Spolana, Neratovice, Czech Republic), the ^{3}H-radioactivity is evaluated by the LSC method. The calibration of this method is performed by processing rat plasma samples containing a precisely adjusted amount of the labeled drug. The precision of ^{3}H-Stobadine determination found was ± 0.57%. The method sensitivity, which lies in the ppb (sub-ppb) concentration range of ^{3}H-Stobadine, is sufficiently low to be appropriate in most experimental pharmacokinetic studies.

TLC method application(s): by using this relatively laborious but very precise method, the ^{3}H-Stobadine concentration-time courses were determined in plasma of intact rats (Bittererová *et al.*, 1990) and in animals with experimentally induced heart dysfunction, as well as in rats with induced liver or kidney function damage (Bittererová, 1989). The dependence of pharmacokinetic parameters obtained after various drug administration modes, i.v. bolus and p.o. single or repeated, was investigated by applying drug doses ranging from 0.4 μg/kg to 12.0 mg/kg. For illustration, Table 1 presents the pharmacokinetic parameters determined when ^{3}H-Stobadine dihydrochloride was administered to rats in the dose of 2.0 mg/kg.

Bioanalysis of Stobadine by the HPLC method

The HPLC (GC as well) assay of a drug and/or its

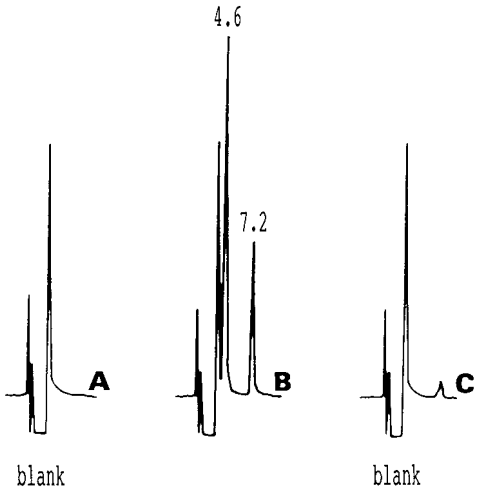


Figure 5. HPLC analytical profiles of samples derived from a dog (A, B), following single p.o. administration of Stobadine dipalmitate, and from a human volunteer (C).

metabolites in a biological sample consists mostly of two stages (Mehta, 1986; Šoltés, 1989):

(i) transformation of the biological sample into the analytical one;
(ii) evaluation of the analytical sample itself.

HPLC method principle: an appropriate amount (2.5 μg) of the 2-*N*-propyl analog of Stobadine, applied as an internal standard, and a carbonate buffer (400 μL; 1.3 mol/L; pH 9.5) are admixed to a given volume of serum (up to 4.0 mL) investigated for Stobadine content. The resulting sample is applied onto a pre-conditioned solid-phase extraction (SPE) cartridge (Separcol® SI C 18; AnaPron Ltd., Bratislava). In this way both analytes, Stobadine and the internal standard, are quantitatively entrapped by the solid microparticulate material packed in the cartridge. The main part of co-trapped endogenic compounds is flushed out from the packing by applying 1 mL of acetonitrile. Finally the analytes still retained by the cartridge sorbent are eluted by 2.5 mL of methanol. After blowing off the liquid by a stream of nitrogen, the solid residue is redissolved in a small volume (25 μL) of dichloromethane and a sample aliquot (10 μL) is injected onto the CGC column (3 × 150 mm) packed with Separon® SGX silica sorbent (Tessek). By applying such a procedure in analyzing dog serum [cf. Fig. 5, panels (A) and (B)], the chromatogram reveals clearly the relative peaks of the internal standard and Stobadine at 4.6 and 7.2 min; both are sharp and symmetrical [cf. Fig. 5, panel (B)]. On analyzing the samples from human volunteers, a minor peak of an endogenic compound eluting immediately after Stobadine was observed [cf. Fig. 5, panel (C)]. The photometric detection set at 302 nm was fully satisfactory for determining relatively high concentrations of Stobadine in dog serum; the wavelength setting at 247 nm did however improve the limit of Stobadine detection. At a drug level of 500 ng/mL, the RSD found for dog serum was 4.4%; at a concentration of 50 ng/mL it was 8.1%. The lowest detectable drug concentration in serum of human volunteers (at 247 nm detector setting) was 10 ng/mL.

Table 3. Time (t_{peak}) of reaching Stobadine peak concentration (c_{peak}) in healthy volunteers after single oral ingestion of the therapeutic substance—Stobadine dipalmitate (dose of drug base normalized to body weight)

Drug dose (mg/kg)	t_{peak} (h)	c_{peak} (ng/mL)	Number of subjects[a]
<0.4		<10	1
0.8	1.5–2.0	12–38	3
1.6	1.0–2.5	82–114	3
2.4	1.5	45–289	2

[a] Stobadine dipalmitate ingested orally was well tolerated by all human volunteers (Dzúrik and Fedelešová, 1987).

HPLC method application(s): the HPLC technique was used to determine the bioavailability of Stobadine in the dog(s) after single oral administration of the two different drug salts—dihydrochloride or dipalmitate (Šoltés *et al.*, 1991). The dose applied was 5.0 mg/kg. The pharmacokinetic parameters calculated, or rather their errors (see Table 2), imply that a very good precision of the analytical determinations was achieved.

In a further part of the preclinical pharmacokinetic study of Stobadine, the levels of the parent drug were examined in serum samples of healthy volunteers who received a drug formulation containing Stobadine dipalmitate. The results showed (see Table 3) that on monitoring the Stobadine level in patients, it is necessary to take a blood sample within the time interval of 1.0–2.5 h after administration of the drug and that the expectable c_{peak} (in dependence on the patient's body weight) will be in the interval of 10–300 ng/mL (Šoltés *et al.*, 1991).

GC methods for Stobadine bioanalysis

Three qualitatively different GC designs applicable for Stobadine bioanalysis have been introduced so far (cf. Appendix). The first one (Štefek and Beneš. 1987), which comprises a glass column (3.0 mm × 2.5 m) packed with 3% OV-17 on Chromosorb® W (80–100 mesh) and a flame-ionization detector, was used to advantage in studying Stobadine metabolism (Štefek *et al.*, 1986, 1987, 1989 Štefek and Beneš, 1987, 1989 Štefek, 1993).

The second GC bioanalytical method (Marko, 1988) is based on using an HP 1 fused-silica capillary column (0.2 mm × 12.5 m; film thickness = 0.33 μm) and a thermionic nitrogen-selective detector. By combining

these design attributes along with SPE sample processing, the limit of Stobadine determination in a spiked serum sample (1.0 mL) was 5 ng/mL. Such sensitivity as well as the attained intra- and inter-assay precision ($CV \leq 10.2\%$) is adequate for Stobadine pharmacokinetic studies in various organisms, including man. Nevertheless, further development and improvement of this method has resulted in the presently used arrangement operating (on-line) with a mass-spectrometric (MS) detector.

The development of the GC-MS method was prompted by the need to determine not only Stobadine but also its pro-drug forms, designed for local application(s) as transdermal (drug) formulation(s) (Koprda *et al.*, 1995, 1996, 1998): for *in vitro* (transdermal) measurements, diffusion chambers according to Franz (1975) were used. The skin from the abdominal region of 5 day-old rats separated the donor and receptor compartments. The donor compartment contained the investigated pro-drug, ie the oxalate salt of 5-*N*-acetylated Stobadine, dissolved (2.5×10^{-2} mol/L) in a solvent system of, for example, ethanol + dipelargonate of propylene glycol, 1:9 (v/v). The receptor compartment was filled with phosphate buffered saline (pH 7.4). The kinetics of the pro-drug permeation through the skin (thermostated at 32°C) was determined on analyzing 0.2 mL sample aliquots withdrawn in 2 h intervals from the chamber receptor site.

GC-MS method principle: the internal standard Darodipine (500 ng), dissolved in 10 μL of methanol, is added to the sample withdrawn. This solution, diluted by phosphate buffered saline (0.8 mL), is alkalized by addition of 26% aqueous ammonia (0.2 mL). The analytes are extracted (5 min) with chloroform (3.0 mL). After centrifugation, the chloroform (bottom) phase is transferred into a heated vial and the liquid is blown off by a stream of nitrogen. The solid residue is redissolved in 200 μL of toluene and this final sample, in a volume of 1.0 μL is (splitless) injected into an HP 5890A gas chromatograph equipped with an HP 5970B mass-selective detector (Hewlett-Packard Company, Palo Alto, CA). The separation is performed in a fused-silica capillary column (0.2 mm × 12 m), lined inside with a 0.33 μm film of 5% crosslinked phenylmethylsilicone stationary phase. The column is heated from 120 to 210°C by a linear gradient of 30 deg/min and then of 10 deg/min up to 300°C. The temperature of the sample injection port is 260°C, the same as that of the MS detector. Helium is used as the carrier gas (pressure = 35 kPa at the column inlet; flow-rate 2.0 mL/min). For quantitative analysis, selective ion monitoring is operative at 58 and 201 *m/z* and the molecular ion of the 5-*N*-acetyl pro-drug derivative = 244 *m/z;* for Stobadine detection *m/z* = 58 and 202. The setting for the internal standard Darodipine is at 252 and 371 *m/z.* Qualitative analysis is done in the 10–500 *m/z* mass-spectrum span. Figure 6 demonstrates a typical result obtained on testing

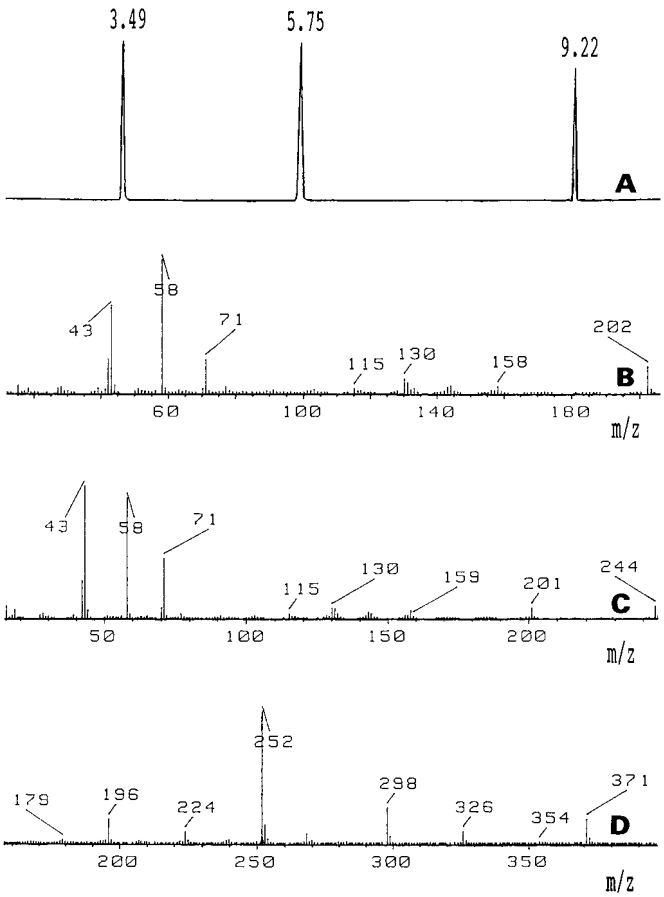


Figure 6. Quantitative (A) and qualitative (B, C and D) GC-MS analysis of the calibrating sample mixture composed of Stobadine (B), 5-*N*-acetyl (Stobadine) pro-drug derivative (C), as well as Darodipine (D), used as the internal standard.

the performance of the GC-MS device by running a sample mixture composed of appropriate amounts of Stobadine, the 5-*N*-acetyl pro-drug derivative, as well as Darodipine. The analytes are eluted at the respective R_t of 3.49, 5.75 and 9.22 min as symmetric peaks. Under the experimental conditions used, the limits of determinations are 1 and 0.5 ng/mL for the 5-*N*-acetyl pro-drug derivative and Stobadine, respectively.

GC-MS method applications: this specific and highly sensitive GC-MS method has been used to study the kinetics of the pro-drug permeation as well as that of Stobadine release in the skin. The pro-drugs investigated so far are the 5-*N* derivatives of Stobadine, substituted by a -CO-R group, with -R representing a saturated linear or branched alkyl substituent or an unsaturated alkyl-aryl chain (Beneš and Pronayová, 1994). Further components in the donor solution of the transdermal (drug) formulation, serving as pro-drug solubilizers and/or skin penetration enhancers, were phosphate buffered saline (pH 7.4), dimethylsulfoxide, ethanol, propylene glycol, dipelargonate of propylene glycol, monoethylether of

diethyleneglycol (Transcutol®), dodecyl-azacycloheptan-2-one (Azone®), etc. (Boháčik, 1996).

DISCUSSION OF OBSERVATIONS

Bioanalytical methods

Even on considering only their endogenous constituents, biological samples are multicomponent mixtures. The determination of the concentration of a given foreign substance in a biological sample is inevitably a rather complicated analytical task. It is not unusual that the sensitivity requirements of an analytical method would lie within the concentration of ppb (or even sub-ppb). Simultaneously, the limited volume of the biological sample examined determines the application of two potential alternatives, ie the use of radiopharmaceutics with high specific activity or chromatographic analysis working with a highly sensitive detection unit.

Bioanalysis of drugs—radiometric approach

Bioanalytical methods of ^{3}H-labeled Stobadine exploit the fact that, by its chemical nature, the drug molecule is a weak base. It dissociates in two steps [pK_{a1} = 2.9, pK_{a2} = 7.2 (Bezákova *et al.*, 1993); pK_{a2} = 8.7 ± 0.1 (Štefek *et al.*, 1989)]. Under strongly alkaline conditions, the drug molecule (Stobadine base) bears no charge. Yet on the other hand, the protonation of Stobadine nitrogen atom(s) in (strong) acid(s) renders the drug molecule electrically charged/ionized. This reversibility of the ionized-uncharged molecular state was used for partitioning Stobadine molecules between the biological (aqueous) and the organic (solvent) phases on determining the drug content in various biomaterials (see above).

Chromatography in drug bioanalysis

Prechromatographic separation/isolation of analytes from biological samples has been performed by employing several physical and/or chemical processes, such as (ultra)-filtration, liquid extraction and chemical derivatization. Recently, however, for preparation of analytical samples from biological materials (chromatographic) sorbents have ever more frequently been used, so that analytes are conveniently (pre-)separated and concentrated by exploiting adsorption, absorption (partitionation), ion-exchange, chelation, and other separation processes (McDowall, 1989). The whole set of these procedures is termed solid-phase extraction (SPE).

Apolar solutes can efficiently be entrapped by hydrophobized (eg C_{18}) silica gel. Prior to subsequent elution/displacement of the trapped (basic) analytes, eg by methanol, inclusion of the sorbent washing with acetonitrile has proved to be highly advantageous (Šoltés, 1992a, 1992b; Šoltés and Sébille, 1997). The chromatographic (HPLC, GC) patterns of samples determined on their Stobadine content, prepared by implementing the CH_3CN washing step, were found to be much smoother than those without the acetonitrile wash (Marko, 1988; Šoltés *et al.*, 1991). Similar observations were reported in the case of bioanalysis of several other basic drugs (Šoltés *et al.*, 1983, 1984; Marko *et al.*, 1985, 1990a, 1990b).

Pharmacokinetics of the drug

It is the major subject of pharmacokinetics to study the disposition and fate of drugs in the body. Further, this speciality is also concerned with the following particular processes — drug absorption, distribution, metabolism and elimination. The pharmacokinetic parameters to be determined are, for example, drug absorption half-life ($t_{1/2abs}$), distribution volume(s), terminal elimination half-life ($t_{1/2elim}$), absolute or relative bioavailability, as well as the effect of dosage, dosage form and administration mode and site upon the values of the parameters determined.

Drug absorption

The absorption half-lives of the two Stobadine salt forms were established on the basis of p.o. administration of the drug(s) to dogs. As given in Table 2, the value of $t_{1/2abs}$ = 24 ± 2.5 min for Stobadine dihydrochloride corresponds well with that calculated for dipalmitate ($t_{1/2abs}$ = 32 ± 4.6 min). Thus it can be anticipated that the orally applied solution of dihydrochloride passes very quickly through the stomach into the intestine. There, in the environment with alkaline pH, Stobadine salt is converted to a base which is subsequently resorbed into the blood stream. [The intestinal wall is a lipoidal membrane barrier through which only the uncharged/non-ionized molecules can penetrate (Taylor, 1986).] Although a certain lag time was found for these processes (t_{lag} = 2 ± 5.2 min), it was statistically nonsignificant. Yet when dipalmitate is administered orally, the passage of this (water insoluble) salt form to its absorption site is retarded. Before crossing the intestinal wall, the drug has to be solubilized. These processes also account for the observed lag time = 15 ± 1.2 min, during which the drug molecules are not available for the body. An interesting finding is the equivalence of the $t_{1/2abs}$ = 24 ± 2.5 min determined for the dihydrochloride drug salt form with the $t_{1/2abs}$ value = 21 ± 7.2 min, established on applying the ^{3}H-Stobadine dihydrochloride into the (chronic) dog ileal loop (Kukan *et al.*, 1994). This good agreement indicates that in the ileal loop, at pH ≈ 7.8, the molecules of dihydrochloride are converted to the drug base, which readily passes through the gut wall.

Salt forms of Stobadine and Stobadine pro-drug forms: the rate (and extent) of drug absorption depends, among other factors, on the hydro- or liposolubility of its molecules. In the case of (parent) Stobadine, modification of the drug chemical properties was achieved by producing and using its salts, i.e. dihydrochloride and dipalmitate. The application of the latter was however accompanied by reduction of the drug bioavailability (cf. Table 2; $F_{rel.} = 46.4\%$). Nevertheless, the excellent stability of the (solid) dipalmitate strongly favors the involvement of this salt form in manufacture the final oral drug formulation (Bauerová *et al.*, 1995, 1999).

A qualitatively different way is to apply a pro-drug form, eg a 5-*N*-acyl (Stobadine) derivative. By using this approach during the phases of pro-drug penetration into the skin as well as permeation through it, active Stobadine molecules should be released by hydrolytic reaction(s) of the enzymatic system present in the human skin (Wiechers and de Zeeuw, 1991). However, despite achieving a constant and adequately intensive flux of 5-*N*-acetylated Stobadine (its oxalate salt) through the 5 day-old rat (hairless) skin [14 ± 1.0 μg/cm^2 h (Boháčik, 1996)], the presence of Stobadine was not confirmed in the permeate. This may however be due to the well-known fact of a still immature metabolic activity of the (rat) newborn tissue(s)/organ(s) (Morgan, 1997). which was used in the *in vitro* model studies.

Mode of Stobadine administration: during the pre-clinical pharmacokinetic studies, several modes of administration of the drug were applied, such as i.v. bolus, i.p., p.o. single and repeated. Of these, oral ingestion of Stobadine (dipalmitate) was established as optimal for treating diseases in which the damaging action of oxygen-derived radicals is primarily involved. However, transdermal pro-drug forms may have a great impact in those cases where the noxa is operative within a small well-localized subdermal region of the patient's body.

Drug distribution

The study described above showed that $78 \pm 2.9\%$ of ^{3}H-Stobadine (dihydrochloride) passes across the rat blood-brain barrier. This finding corresponds very well with the result represented in Fig. 2, demonstrating a significant amount of the tracer in the rat brain. The radiographic scan (cf. Fig. 2) indicates also that Stobadine molecules are quickly distributed not only into the brain but also into the myocardium and lung, ie the three tissues/organs which are directly or indirectly affected by diseases or disorders associated with the action of free, oxygen-derived, radicals.

Stobadine binding to plasma proteins: the (reversible) binding interactions between ^{3}H-Stobadine dihydrochloride and rat plasma as well as human serum were investigated using the method of equilibrium dialysis. The results obtained indicated that, in the drug concentration range applied, the nature of Stobadine binding to rat plasma proteins was nonspecific and unsaturable. The value of the drug total binding ranged between 12 ± 4.7 and $23 \pm 2.3\%$.

Human serum, as well as human α_1-acid glycoprotein (α_1-AGP) or human serum albumin (HSA) interacted with ^{3}H-Stobadine dihydrochloride, also nonspecifically. In the drug concentration range investigated, the extent of the total binding to (full) serum = $60 \pm 2\%$; to α_1-AGP = $50 \pm 1\%$; and to HSA = $45 \pm 1\%$. The calculated capacity of the total binding was in the case of α_1-AGP $(1.02 \pm 0.02) \times 10^5$ L/mol, and in that of HSA $(4.0 \pm 0.1) \times 10^4$ L/mol (Oravcová, 1991). In the light of the parameters determined and of the binding mechanism assumed, it can be concluded that the interactions of Stobadine with plasma proteins would not markedly affect the fate of the drug in the patient's body.

Stobadine affinity to tissues/organs: the extent of Stobadine binding interactions with rat plasma proteins is low ($\leq 23 \pm 2.3\%$). However the levels of the parent drug determined in the rat body indicate that Stobadine has an extensive (binding) affinity to all the tissues/organs processed. Such interactions are clearly also manifested by the large drug distribution volume(s) observed (cf. Table 1). Within a relatively short time interval following either i.v. or p.o. drug administration routes, Stobadine levels in the rat organs studied significantly exceeded the corresponding drug plasma concentrations. With increasing time, the values of the organ to plasma concentration ratios reached values of several tens (Kállay *et al.*, 1990). Nevertheless, after an appropriately extended time interval, neither the labeled Stobadine nor its radioactive metabolites were detectable in the rat body. Thus, during treatment, there appears to be no risk of accumulation or irreversible binding of these compounds within the living organism.

Stobadine placental transfer and distribution in fetuses: the extent of Stobadine placental transfer was determined in rabbits (see above) since their hemoendothelial placenta resembles that of human beings. The results of this study proved that Stobadine crossed the placental barrier of rabbits and, starting with min 60 after drug dosage, the fetal plasma drug levels exceeded those of the dams. Moreover, in all tissues/organs of both the pregnant does and their fetuses, parent Stobadine levels were comparable to or even greater than in corresponding plasma samples. It could thus be advantageous to apply Stobadine to protect the tissues/organs, especially the brain and heart, from damage induced by oxidative stress during late organogenesis and delivery.

Drug metabolism

The liver is the main organ responsible for the biotransformation (and elimination) of drugs. The relevant

mechanisms have been investigated by using different *in vitro* approaches. In decreasing order of tissue organization, the following techniques are used: isolated perfused liver, liver slices, isolated hepatocytes, microsomes, and isolated proteins.

Stobadine in vitro/in vivo metabolic studies: incubation of Stobadine with fortified rat liver microsomal preparation yielded two metabolites (Štefek and Beneš, 1987; Štefek *et al.*, 1987). Their chemical structure indicated that in the Stobadine molecule the position of 2-N (cf. Fig. 1) was enzymatically attacked. Demethylation reaction yielded 2-*N*-desmethyl Stobadine, while by oxygenation reaction(s) the Stobadine 2-N→O derivative was generated. Concerning the latter compound, both stereoisomers bearing the 2-N→O group oriented axially or equatorially were established (Štefek *et al.*, 1987). All three chemical structures were confirmed by comparing the physico-chemical parameters of the compounds synthesized with those of the substances isolated from the biomaterials investigated. To prepare the anticipated Stobadine metabolites, knowledge derived from the synthesis of some other 2,3,4,4a.5.9b-hexahydro-1H-pyrido[4,3-b]indole derivatives was exploited (Nagai *et al.*, 1979). The given metabolites were found also in rat urine after i.p. administration of Stobadine dihydrochloride (Štefek *et al.*, 1986).

First-pass effect and enterohepatal recirculation of Stobadine: a marked first-pass effect has to be considered, since, for example, upon ^{3}H-Stobadine dihydrochloride administration to rats, within the early time interval (0-240 min) less than one-fifth of the orally applied drug dose reached the animal systemic circulation (Kállay *et al.*, 1990). On the other hand, the shorter $t_{1/2elim} = 78 \pm 7.5$ min, determined after 25 days of repeated oral drug administration compared to the value of 95 ± 8.2 min observed at single oral drug dosing (cf. also Table 1), may indicate a trace induction of the drug metabolizing enzymes in the rat liver (Kállay *et al.*, 1991).

The relatively high levels of total ^{3}H-radioactivity in both the liver and small intestine, determined after oral application of ^{3}H-Stobadine (dihydrochloride) to rats (Kállay *et al.*, 1990), might suggest enterohepatal recirculation of the drug (and/or of its metabolites).

Drug excretion

Biotransformation has to be classified as the major elimination route of Stobadine since within the first 3 days following ^{3}H-Stobadine dihydrochloride i.v. or p.o. administration to rats less than the respective 5.4 or 5.5% of the drug dose applied was counted in the excreta. The fraction of the parent Stobadine excreted within the initial 6 h reached only $4.7 \pm 0.38\%$ (i.v.) or $3.6 \pm 0.53\%$ (p.o.) (Kállay *et al.*, 1990).

Stobadine excretion in breast milk: ^{3}H-Stobadine dihydrochloride was administered in a single oral dose of 5.0 mg/kg to lactating rats on postpartum day 10 to follow the excretion of labeled compounds in breast milk. The ^{3}H-radioactivity was determined both in the gastric content of suckling pups and in their body. The results showed that during the first 24 h after administration of the ^{3}H-labeled drug to lactating rats less than 0.4% of the applied ^{3}H-radioactivity was recovered in the pups (Ujházy *et al.*, 1999a). Thus, by taking into account both the physico-chemical properties of the drug and its pharmacokinetics, it can be concluded that Stobadine excretion in breast milk is of practically no significance.

Stobadine excretion in urine/feces: as already described above, within 3 days following either i.v. or p.o. administration of ^{3}H-Stobadine dihydrochloride to rats, the parent drug content in the excreta (urine + feces) was 5.4 and 5.5%, respectively. At both modes of drug application, the ratio of Stobadine excreted in urine and feces was strongly shifted in favor of elimination by urine, namely 93:7 (i.v.) and 77:23 (p.o.).

CONCLUDING REMARKS

The three specific requirements which have to be met to achieve effective introduction of a novel synthetic antioxidant into practical use are as follows:

1. Strong antioxidative/free-radical scavenging activity of the drug preparation.
2. Rapid resorption of the drug molecules and their adequately long availability in the tissues/organs which are most sensitive to oxidative stress, ie brain, heart, lung, liver, kidney.
3. Low toxicity of the synthesized compound.

Remarkably enough, Stobadine® possesses all three characteristics to an optimally balanced extent.

Another synthetic compound, very similar to Stobadine by its chemical structure, is Carbidine® [33162-17-3], namely a dihydrochloride of 2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1H-pyrido[4,3-b]indole. This clinically approved drug (racemate), exerting significant antidepressive and neuroleptic actions (Barkov, 1971, 1973; Barkov and Kutcherova, 1972, 1975), consists basically of Stobadine (dihydrochloride) and of its *cis*-(+)-antipode in a mass ratio 1:1. In the early 1970s, Carbidine was introduced into clinical practice in the former Soviet Union. Since then, the drug has been available either in tablet form (25 mg) or in ampoules [2 mL of 1.25% (w/v) solution]. For oral drug dosing, $\frac{1}{2}$–2 tablets three times daily are ingested. Intramuscularly, up to 4 mL three to four times in 2 h intervals followed by 4 mL three times a day are injected in the course of treatment (Trinus, 1976).

APPENDIX

Table A1. Analytical methods used for the determination of Stobadine in various biomaterials

Biomaterial	Internal standard	Sample processing	Derivatization agent	Analytical technique	Detection / Quantitation	Reference(s)
Rat						
Brain	^{14}C-Butan-l-ol				/LSC	Kállay *et al.* (1990)
Skin permeate					/LSC	Boháčik (1996)
Whole body					Radiography	Kállay *et al.* (1990)
Plasma		LLE			/LSC	Ščasnár and Štefek (1987) Kállay *et al.* (1990) Kállay *et al.* (1991) Krištofová *et al.* (1991) Ujházy *et al.* (1999b)
Organ(s)		LLE			/LSC	Ščasnár and Štefek (1987) Kállay *et al.* (1990) Krištofová *et al.* (1991)
Urine		LLE			/LSC	Ščasnár and Štefek (1987) Kállay *et al.* (1991)
Feces		LLE			/LSC	Kállay *et al.* (1991)
Plasma		PP, LLE		TLC	UV/LSC	Bittererová (1989) Bittererová *et al.* (1990)
Liver microsomes[a]	2-*N*-ethyl analog	PP, LLE	TFAA	GC	FID	Štefek and Beneš, (1987) Štefek *et al.* (1987) Štefek and Beneš (1989) Štefek *et al.* (1989)
Skin permeate[b]	Darodipine	LLE		GC	MS	Koprda *et al.* (1995) Boháčik (1996) Koprda *et al.* (1996)
Guinea pig						
Liver microsomes[a]	2-*N*-ethyl analog	PP, LLE	TFAA	GC	FID	Štefek and Beneš (1989) Štefek *et al.* (1989)
Snake						
Skin (shed) permeate					/LSC	Boháčik (1996)
Rabbit						
Plasma		LLE			/LSC	Ujházy *et al.* (1994) Ujházy *et al.* (1995) Ujházy *et al.* (1999c) Ujházy *et al.* (1999d)
Organ(s)		LLE			/LSC	Ujházy *et al.* (1999c) Ujházy *et al.* (1999d)
Liver microsomes[a]	2-*N*-ethyl analog	PP, LLE	TFAA	GC	FID	Štefek and Beneš (1989) Štefek *et al.* (1989)
Dog						
Serum		LLE, LLE		SF	Ex_{302}, Em_{365}	Marko (1985) Ščasnár *et al.* (1989b)
Urine		LLE, LLE		SF	Ex_{302}, Em_{365}	Marko (1985)
Serum	2-*N*-propyl analog	SPE		HPLC	UV	Šoltés *et al.* (1991)
Plasma	2-*N*-ethyl analog	SPE		GC	NPD	Marko (1988)
Serum	2-*N*-ethyl analog	SPE		GC	NPD	Marko (1988)
Urine	2-*N*-ethyl analog	SPE		GC	NPD	Marko (1988)
Plasma[c]	5-*N*-acetyl analog	LLE		GC	MS	Bauerová *et al.* (1999)
Human						
Serum	2-*N*-propyl analog	SPE		HPLC	UV	Šoltés *et al.* (1991)
Skin (p.m.) permeate					/LSC	Boháčik (1996)

Abbreviations: LLE = liquid–liquid extraction; PP = protein precipitation; SPE = solid-phase extraction; TFAA = trifluoroacetic anhydride; GC = gas chromatography; HPLC = high-performance liquid chromatography; SF = spectrofluorometry; TLC = thin-layer chromatography; LSC = liquid-scintillation counting; UV, FID, NPD, or MS = (spectro)photometric, flame-ionization, nitrogen–phosphorous, or mass-spectrometric detector; Ex/Em = excitation/emission wavelengths; p.m. = post mortem.

[a] The method is focussed on the determination of Stobadine metabolites generated *in vitro*.

[b] Along with Stobadine, the pro-drug was also determined.

[c] The GC-MS detection limit of Stobadine determined in samples derived from dog plasma was 1 ng/mL.

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REFERENCES

Barkov, N. K. 1971. Pharmacological properties of carbidine (in Russian). *Farmakologija i Toksikologija* **34**:647–650.

Barkov, N. K. and Kutcherova, N. F. 1972. On the relation between the chemical structure and pharmacological activity of the carboline derivatives (in Russian). *Khimitcheskij i Farmaceutitcheskij Zhurnal* **6**(8):23–27.

Barkov, N. K. 1973. On the mode of carbidine action (in Russian). *Farmakologija i Toksikologija* **36**:154–157.

Barkov, N. K. and Kutcherova, N. F. 1975. Further study of the links between the chemical structure and pharmacological activity of the carboline derivatives (in Russian). *Khimitcheskij i Farmaceutitcheskij Zhurnal* **9**(4):6–10.

Bauer, V. and Štolc, S. 1993. Isolated cells and tissues as models for studies of drug mechanism of action. *Plzeňský lékařský Sborník*, **67**:17–25.

Bauerová, K., Bezek, Š., Bohov, P., Ďurišová, M. and Kukan, M. 1995. Evaluation of relative bioavailability of two stobadine preparations in dogs. *Therapeutic Drug Monitoring* **17**:423.

Bauerová, K., Bohov, P., Ďurišová, M. and Bezek, Š. 1999. Bioequivalence study: Contribution to the development of an oral application form of stobadine dipalmitate. *Methods and Findings in Experimental and Clinical Pharmacology* **21**:499–503.

Beneš, L. and Štolc, S. 1989. Stobadine. *Drugs of Future* **14**:135–137.

Beneš, L. and Pronayová, N. 1994. Free radical scavenger prodrugs-potentially potent brain penetrating agents. *Pharmazie* **49**:69–70.

Bezáková, Ž., Blešová, M. and Bachratá, M. 1993. Stobadine dichloride-study of physical-chemical properties and stability (in Slovak). *Farmaceutický Obzor* **62**:247–252.

Bezek, Š., Trnovec, T., Ščasnár, V., Ďurišová, M., Kukan, M., Kállay, Z., Laginová, V. and Svoboda, V. 1990. Irradiation of the head by ^{60}Co opens the blood-brain barrier for drugs in rats. *Experientia* **46**:1017–1020.

Bilčíková, L., Mátyás, Š. and Bauer, V. 1990. Effect of stobadine and histamine H_1 and H_2 blockers on histamine-induced contraction of guinea-pig airways *in vitro*. *Respiration* **57**:104–108.

Bittererová, J., Šoltés, L., Kállay, Z. and Trnovec, T. 1990. Thin-layer chromatographic determination of ^{3}H-labelled stobadin in rat plasma for pharmacokinetic purposes. *Pharmazie* **45**:437–438.

Bittererová, J. 1989. Stobadine pharmacokinetics under normal and pathologically changed conditions in rats (in Slovak). PhD Thesis. Pharmaceutical Faculty of the Comenius University. Bratislava.

Blanchard, J. 1981. Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis. *Journal of Chromatography* **226**:455–460.

Boháčik, L'. 1996. Effect of chemical permeation enhancers and of drug forms on transdermal permeation (in Slovak). PhD Thesis, Pharmaceutical Faculty of the Comenius University, Bratislava

Chemical Abstracts. 1994. *Index Guide*, American Chemical Society, Columbus, Ohio, U.S.A.

Dzúrik, R. and Fedelešová, V. 1987. Report on the clinical study of the preparation stobadine in volunteers: a single administration (in Slovak). In: *Stobadine, a New Medicine with Antiarrhythmic, Protective and Antihypoxic Effect on Myocardium*. Institute of Experimental Pharmacology, SAS, Bratislava, Slovak Republic, 2358–2369.

Franz, T. J. 1975. Percutaneous absorption. On the relevance of *in vitro* data. *Journal of Investigative Dermatology* **64**:190–195.

Gibaldi, M. and Perrier, D. 1982. *Pharmacokinetics*. Marcel Dekker. Inc., New York and Basel.

Horáková, L'. and Štolc, S. 1998. Antioxidant and pharmacodynamic effects of pyridoindole stobadine. *General Pharmacology* **30**:627–638.

Kállay, Z., Bittererová, J., Brejcha, A., Faberová, V., Bezek, Š. and Trnovec, T. 1990. Plasma concentration, tissue distribution and excretion of the prospective cardioprotective agent cis-(−)-2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1H-pyrido-[4,3-b]indole dihydrochloride in rats. *Arzneimittel-Forschung/Drug Research* **40**:974–979.

Kállay, Z., Šoltés, L. and Trnovec, T. 1991. Pharmacokinetics of stobadin and of the sum of its metabolites in rats during repeated administration. *Biopharmaceutics and Drug Disposition* **12**:201–205.

Koprda, V., Boháčik, L'., Bohov, P. and Hadgraft, J. 1995. Skin permeation of stobadine acyl derivatives. *Abstracts of the 4th International Conference on Prediction of Percutaneous Penetration.* Vol. 4a, La Grande Motte, France, C26.

Koprda, V., Boháčik, L'., Bohov, P. and Hadgraft, J. 1996. Skin permeation of stobadine acyl-derivatives. In: *Prediction of Percutaneous Penetration*, Vol. 4B (Brain, K. R., James, V. J., Walters, K. A., Eds.) STS Publishing Ltd., Cardiff, United Kingdom, 93–95.

Koprda, V., Mihalová, D. and Boháčik, L'. 1998. The effect of Azone on penetration of pyridoindole acyl-derivatives across 5-day rat skin. In: *Perspectives in Percutaneous Penetration*, Vol. 6A. (Brain, K. R. and Walters, K. A., Eds.) STS Publishing Ltd., Cardiff, United Kingdom, 29.

Krištofová, A., Ujházy, E., Bezek, Š., Balonová, T. and Nosál', R. 1991. Stobadine toxicity and transplacental movement. *Archives of Toxicology*, Suppl. **14**:280–284.

Kukan, M., Bezek, Š., Trnovec, T., Gabauer, I. and Styk, J. 1994. On the absorption of drugs using chronic dog ileal loop method. *Methods and Findings in Experimental and Clinical Pharmacology* **16**:437–442.

Marko, V. 1985. Spectrofluorometric analysis in biological fluids of the dihydrochloride of (−)-cis-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-I-H-pyrido[4,3b]indole (DH 1011), a prospective antiarrhythmic. *Pharmazie* **40**:192.

Marko, V., Štefek, M. and Šoltés, L. 1985. Gas chromatographic determination of pentacaine in rat serum. *Journal of Chromatography* **339**:410–413.

Marko, V. 1988. Determination of stobadine, a novel cardioprotective drug, using capillary gas chromatography with nitrogen-phosphorus detection after its selective solid-phase extraction from serum. *Journal of Chromatography* **433**:269–275.

Marko, V., Filip, J., Uhrín, D., Trnovec, T. and Beneš, L. 1989. Synthesis of tritium-labelled stobadine, a new cardioprotective agent. *Journal of Labelled Compounds and Radiopharmaceuticals* **27**:35–43.

Marko, V., Šoltés, L. and Novák, I. 1990a. Selective solid-phase extraction of basic drugs by C_{18}-silica. Discussion of possible interactions. *Journal of Pharmaceutical and Biomedical Analysis* **8**:297–301.

Marko, V., Šoltés, L. and Radová, K. 1990b. Polar interactions in solid-phase extraction of basic drugs by octadecylsilanized silica. *Journal of Chromatographic Science* **28**:403–406.

McDowall, R. D. 1989. Sample preparation for biomedical analysis. *Journal of Chromatography* **492**:3–58.

Mehta, A. C. 1986. Sample pretreament in the trace determination of drugs in biological fluids. *Talanta* **33**:67–73.

Morgan, D. J. 1997. Drug disposition in mother and foetus. *Clinical and Experimental Pharmacology and Physiology* **24**:869–873.

Nagai, Y., Irie, A., Masuda, Y., Oka, M. and Uno, H. 1979. Synthesis of 2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]-indole derivatives and their central nervous system activities. *Journal of Medicinal Chemistry* **22**:677–683.

Ondriaš, K., Mišík, V., Gergel', D. and Staško, A. 1989. Lipid

peroxidation of phosphatidylcholine liposomes depressed by the calcium channel blockers nifedipine and verapamil and by the antiarrhythmic-antihypoxic drug stobadine. *Biochimica et Biophysica Acta* **1003**:238–245.

Oravcová, J. 1991. Interactions of drugs and of their optical isomers with plasma proteins (in Slovak). Ph.D. Thesis, Institute of Experimental Pharmacology, SAS, Bratislava.

Orviský, E., Šoltés, L. and Stančíková, M. 1997. High-molecular-weight hyaluronan—a valuable tool in testing the antioxidative activity of amphiphilic drugs stobadine and vinpocetine. *Journal of Pharmaceutical and Biomedical Analysis* **16**:419–424.

Peck, C. C., Beal, S. L., Sheiner, L. B. and Nichols, A. I. 1984. Extended least squares nonlinear regression: possible solution to the "choice of weights" problem in analysis of individual pharmacokinetic data. *Journal of Pharmacokinetics and Biopharmaceutics* **12**:545–558.

Ščasnár, V. and Štefek, M. 1987. Solvent extraction and radiometric determination of ^{3}H-stobadine in biological material. *Journal of Radioanalytical and Nuclear Chemistry. Art.* **111**:117–122.

Ščasnár, V., Zemánek, M., Šoltés, L. and Lukácsová, M. 1989a. High-performance liquid radiochromatography: Its application for stability study of (^{3}H)stobadin. *Journal of Radioanalytical and Nuclear Chemistry, Art.* **134**:433–436.

Ščasnár, V., Bezek, Š. and Trnovec, T. 1989b. Fluorimetric assay of stobadine in serum of dogs. *Journal of Pharmaceutical and Biomedical Analysis* **7**:1207–1212.

Šoltés, L., Beneš, L. and Berek, D. 1983. Selective preseparation of pentacaine from biological material by SEP-PAK C18 cartridge. *Methods and Findings in Experimental and Clinical Pharmacology* **5**:461–465.

Šoltés, L., Berek, D. and Štefek, M. 1984. Efficient and selective isolation of local anaesthetics from biological fluids using C18 silica gel. *Journal of Chromatography* **286**:223–227.

Šoltés, L. and Trnovec, T. 1987. Determination of radiochemical purity of ^{3}H-stobadin by means of thin-layer chromatography. *Pharmazie* **42**:863–864.

Šoltés, L. 1989. High-performance liquid chromatographic determination of beta-adrenoceptor blocking agents in body fluids. *Biomedical Chromatography* **3**:139–152.

Šoltés, L., Kállay, Z., Bezek, Š. and Fedelešová, V. 1991. A high-performance liquid chromatographic method for the determination of stobadin pharmacokinetics in serum. *Biopharmaceutics and Drug Disposition* **12**:29–35.

Šoltés, L. 1992a. Enhanced selectivity of the isolation of basic drugs from body fluids by means of extraction minicolumns and sophisticated elution schemes. *Biomedical Chromatography* **6**:43–49.

Šoltés, L. 1992b. Prechromatographic preparation of biological samples by extraction in a liquid-solid sorbent system for determination of basic drugs (in Slovak). *Chemické Listy (Chemical Letters)* **86**:299–301.

Šoltés, L. and Sébille, B. 1997. Effective one/two step purification of various materials by solid-phase extraction. *Biomedical Chromatography* **11**:348–351.

Sotníková, R., Okruhlicová, L'. and Noskovič, P. 1998. Endothelial protective effect of stobadine on ischemia-reperfusion-induced injury. *General Physiology and Biophysics* **17**:253–264.

Sotníková, R. and Šoltés, L. 1997. Meeting Report "Recent Advances in the Mechanism of Drug Action". *ISSX Newsletter* **17**(3):5.

Sotníková, R. and Šoltés, L. 1998. Meeting Report "The International Symposium on Drug Action on Reactive Oxygen Species with Special Attention to Stobadine". *ISSX Newsletter* **18**(1):5 and 8.

Staško, A., Ondriáš, K., Mišík, V., Szöcsová, H. and Gergel', D. 1990. Stobadine—a novel scavenger of free radicals. *Chemické Zvesti (Chemical Papers)* **44**:493–500.

Steenken, S., Sundquist, A. R., Jovanovic, S. V., Crockett, R. and Sies, H. 1992. Antioxidant activity of the pyridoindole stobadine: Pulse radiolytic characterization of one-electro-oxidized stobadine and quenching of singlet molecular oxygen. *Chemical Research in Toxicology* **5**:355–360.

Štefek, M., Beneš, L. and Kočiš, P. 1986. *In vivo* biotransformation of DH 1011 (in Slovak). *Československá Fyziologie*. **35**:367.

Štefek, M. and Beneš, L. 1987. Gas chromatographic determination of stobadine, N-desmethylstobadine and stobadine N-oxide in rat liver microsomal incubations. *Journal of Chromatography* **415**:163–169.

Štefek, M., Beneš, L., Jergelová, M., Ščasnár, V., Turi-Nagy, L. and Kočiš, P. 1987. Biotransformation of stobadine, a gamma-carboline antiarrhythmic and cardioprotective agent, in rat liver microsomes. *Xenobiotica* **17**:1067–1073.

Štefek, M. and Beneš, L. 1989. Hydrogen peroxide-dependent liver microsomal N-demethylation and N-oxygenation of stobadine, a gamma-carboline antiarrhythmic and cardioprotective agent. *Xenobiotica* **19**:627–634.

Štefek, M., Beneš, L. and Zelník, V. 1989. N-oxygenation of stobadine, a gamma-carboline antiarrhythmic and cardioprotective agent: the role of flavin-containing monooxygenase. *Xenobiotica* **19**:143–150.

Štefek, M. and Beneš, L. 1991. Pyridoindole stobadine is a potent scavenger of hydroxyl radicals. *FEBS Letters* **294**:264–266.

Štefek, M. 1993. *In vitro* studies on the interaction of the pyridoindole antioxidant stobadine with rat liver microsomal P450. *Xenobiotica* **23**:983–993.

Štolc, S., Bauer, V., Beneš, L. and Tichý, M. 1983. Medicine with antiarrhythmic and antihypoxic activity and its method of preparation. Czechoslovak Patent: 229067; cf. also Swiss Patent: 651754 (1985); Swedish Patent: 8204693-9; Belgian Patent: 894148 (1985); German Patent: P-3231088; Spanish Patent: 553017; Japanese Patent: 1514040.

Taylor, D. C. 1986. Sites and mechanism of intestinal drug absorption. *Pharmacy International* **7**:179–183.

Trinus, F. P. 1976. *Pharmacotherapeutical Handbook* (in Russian). Zdorovija, Kijev, Second Edn, 27–28.

Ujházy, E., Faberová, V., Dubovicky, M., Zemánek, M., Dedík, L. and Durišová, M. 1994. Placental transfer of stobadine at different gestational stages in rabbits. *Teratology* **50**:33A.

Ujházy, E., Faberová, V., Dubovický, M., Zemánek, M., Janšák, J., Dedík, L. and Ďurišová, M. 1995. Placental transfer of stobadine at different gestational stages in rabbits—approximation by pharmacokinetic model (in Slovak). *Československá Fyziologie* **44**:15–17.

Ujházy, E., Dubovický, M., Bezek, Š., Šoltés, L., Žačiková, S. and Janšák, J. 1999a. Stobadine excretion into milk: Study in rats. *Fundamental and Clinical Pharmacology* **13**:368.

Ujházy, E., Dubovický, M., Šoltés, L., Bezek, Š. and Zemánek, M. 1999b. Stobadine placental transfer and excretion in breast milk: Studies in rats. *Biomarkers and Environment* **3**:8.

Ujházy, E., Dubovický, M., Šoltés, L., Faberová, V., Zemánek, M. and Gajdošík, A. 1999c. Placental transfer of stobadine in rabbits. *Life Sciences* **65**:2011–2014.

Ujházy, E., Dubovický, M., Šoltés, L., Faberová, V., Zemánek, M. and Gajdošík, A. 1999d. Placental transfer of antioxidant stobadine at different gestational stages in rabbits. *Archives of Toxicology*; MS B-123/99.

Wiechers, J. W. and de Zeeuw, R. A. 1991. Prodrugs and penetration enhancement: Implications for dermal metabolism. In: *Prediction of Percutaneous Penetration* (Scott, R. C., Guy, R. H., Hadgraft, J., Boddé, H. E., Eds.). IBC Technical Services Ltd., London, Vol. **2**:259–269.

