called autoregulation. Autoregulation is due to an automatic adjustment of the renal vascular tone to the change of perfusion pressure. The present experiments showed that nicardipine, a Ca channel blocker, abolished autoregulation of renal blood flow and depressed the change of renal vascular resistance. Previously, we obtained similar results using other Ca channel blockers, such as verapamil (Ogawa & Ono 1986), nifedipine (Ogawa & Ono 1987) and diltiazem (Ogawa et al 1987). Furthermore, we observed that the inhibitory effects of these Ca channel blockers on autoregulation were antagonized by simultaneous infusion of Bay k 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate), a Ca channel activator (Ogawa & Ono 1986, 1987; Ogawa et al 1987). Recently, we showed that TMB-8 (8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate), an inhibitor of intracellular Ca^{2+} release, had no effect on autoregulation of renal blood flow (Ogawa & Ono 1988a). Therefore, the inhibitory effect of nicardipine on autoregulation appears to be due to its Ca channel blocking action. Indeed, nicardipine markedly inhibited renal vasoconstriction caused by YC-170 (Takenaka et al 1988), a Ca channel activator, in this experiment.

Sakamoto et al (1978) reported that nicardipine has an inhibitory activity upon cyclic (c) AMP phosphodiesterase. We have previously reported that both forskolin which activates adenylate cyclase and IBMX which inhibits cAMP phosphodiesterase had no effect on autoregulation of renal blood flow (Ogawa & Ono 1988b). Therefore, the inhibitory effect of cAMP phosphodiesterase by nicardipine seems not to be involved in the abolition of autoregulatory function.

In conclusion, it is considered that nicardipine inhibits autoregulatory pressure-dependent elevation of renal vascular resistance by its Ca channel blocking action. Guyton et al (1970) reported that increased renal vascular resistance is necessary for sustaining elevated systemic blood pressure. The inhibitory effect of this Ca channel blocker on autoregulation of renal blood flow may be a factor in its hypotensive effect.

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Serum protein binding of noscapine: influence of a reversible hydrolysis

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Abstract—The binding of the antitussive drug noscapine to human serum, pure albumin and α_1-acid glycoprotein has been investigated by ultrafiltration and equilibrium dialysis, using radiolabelled noscapine. The binding in serum pooled from volunteers was $93 \pm 0{\cdot}2\%$ (at 100 ng mL^{-1}). After incubation for 24 h the binding decreased to about 85% (ultrafiltration $87{\cdot}0 \pm 1{\cdot}0\%$; equilibrium dialysis $84{\cdot}3 \pm 1{\cdot}2\%$), because of the conversion of noscapine to noscapinic acid. Only unbound drug underwent this hydrolysis, and as noscapine is extensively bound in healthy volunteers, this elimination process is probably unimportant. The major binding protein of noscapine was albumin ($K = 3060\ M^{-1}$, $n = 5{\cdot}62$), but the binding to α_1-acid glycoprotein was also substantial ($K = 31500\ M^{-1}$, $n = 1{\cdot}73$). The interindividual variation in binding was low and binding was linear at the concentrations observed after therapeutic doses (0–500 ng mL^{-1}).

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Noscapine is a phthalideisoquinoline alkaloid with cough suppression as its only pronounced pharmacological effect. Its antitussive effect is of the same order of magnitude as that of codeine in both healthy volunteers (Empey et al 1979) and patients with chronic bronchitis (Matthys et al 1985).

The lactone ring on the noscapine molecule can undergo a pH-dependent reversible hydrolysis to noscapinic acid (Fig. 1). The two forms are present in approximately equal concentrations in buffer solutions of physiological pH (Pawelczyk & Zajac 1975). However, Johansson et al (1983) could not detect noscapinic acid in whole blood or albumin solution spiked with noscapine and incubated to equilibrium. They proposed that this difference between buffer and blood/albumin solution was due to strong binding of noscapine to proteins. In this case, the inability to detect noscapinic acid would imply high protein binding inconsistent with a previously reported figure for binding in serum of 65% (Idänpään-Heikkilä 1968).

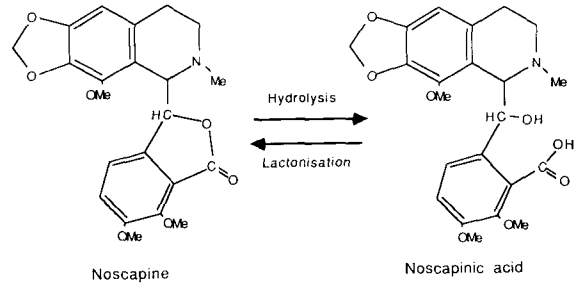

FIG. 1. Interconversion between noscapine (left) and noscapinic acid (right).

Investigating the fate of noscapine in the plasma should improve our understanding of noscapine pharmacokinetics. If plasma protein binding is modest, non-enzymatic hydrolysis may contribute substantially to the elimination of noscapine, the mechanism of which is still relatively unknown (Göber et al 1977; Tsunoda & Yosimura 1979, 1981). If, on the other hand, binding is high, variability in the degree of binding may be a source of the relatively high intersubject differences found in noscapine pharmacokinetics in man (Dahlström et al 1982).

This investigation aims to gain better understanding of also noscapine interactions in serum. Its specific goals were: (1) to estimate the amount of serum binding and identify the major binding proteins, (2) to assess the interindividual variations in the degree of binding, (3) to determine the lactone-acid equilibrium in serum, and (4) to develop a model of noscapine interactions in serum, from which in-vivo and in-vitro predictions can be made.

Materials and methods

[^{3}H]Noscapine was prepared by Amersham International Ltd at two specific activities (0·39 and 14 Ci mmol^{-1}). The purification of crude material was accomplished by TLC (Waldi et al 1961) or HPLC (Karlsson et al 1988) to >98 and >95% purity, respectively. No difference in drug binding was detected between the two preparations. The stability of noscapine after incubation was confirmed by HPLC analysis (Johansson et al 1983). Plasma and serum pooled from ten healthy volunteers were used either fresh, or frozen immediately after separation from the blood cells and thawed just before each experiment. The pH was adjusted to 7·4 by gassing serum and plasma with 5% CO_2 (v/v) in air. Serum concentrations of albumin and α_1-acid glycoprotein were determined at the Kliniska laboratoriet, Stockholm, Sweden. Pure human serum albumin (Kabi AB, Sweden) and human α_1-acid glycoprotein (Behringwerke AG, W. Germany) were reconstituted in sodium phosphate buffer (pH 7·40, 0·039 M). [^{3}H]Noscapine was dissolved and stored in 50 μM HCl or 100 μM NaOH to obtain the lactone and acid forms, respectively. Unless indicated otherwise, the lactone form was used.

Dialysis was carried out in acrylic microcells consisting of two chambers separated by a dialysis membrane. Standard membranes (Technicon Chemicals, Orcq, Belgium) were equilibrated overnight before use, in buffer at room temperature (20°C). The adsorption of [^{3}H]noscapine to the dialysis cells and membranes was 5·8 ± 1·8% (n = 8). In a typical dialysis experiment, 1 mL of saline phosphate (Ehrnebo et al 1974) or Tris-Ringer buffer (Kristensen & Gram 1982) and 1 mL of serum spiked with [^{3}H]noscapine were added to the two chambers. The cells were allowed to equilibrate, at room temperature or 37°C, while slowly rotating for 24 h. The free fraction (f_u) was calculated as the volume-shift corrected (Tozer et al (Appendix IV) 1983) ratio of post-dialysis drug concentration in the buffer to that in the serum chamber. Protein concentration was measured, to check for protein leakage and serum dilution, using the biuret reaction (Gornall et al 1949). The serum dilution during equilibrium dialysis was also determined from the volume of fluid remaining in the cell chambers after dialysis.

An Amicon filter micropartition system (MPS) with anisotropic, hydrophilic YMT membranes (Amicon, Danvers, MA, USA) was used for ultrafiltration. One mL of protein solution (serum, plasma, albumin or α_1-acid glycoprotein solution) spiked with [^{3}H]noscapine was centrifuged at 1500 *g* until the filtrate amounted to about 20% of the initial volume. Aliquots of the filtrate and the initial solution to which the drug had been added were analysed. The free fraction f_u was calculated as the ratio of the drug concentration in the filtrate to that in the protein solution. Measurements were performed at room temperature or 25°C. Protein leakage, estimated from measurements according to the method of Lowry et al (1951), and binding of [^{3}H]noscapine to filter membranes were both negligible.

Protein binding determinations were performed in triplicate and the radioactivities of all the samples (0·1–0·25 mL) were measured by liquid scintillation in 5 mL of aqueous scintillant (Beckman HP/B), at an efficiency of 50%. Values are reported as mean ± s.d.

Binding constants for noscapine binding to albumin and α_1-acid glycoprotein were estimated by the LIGAND computer program (Munson & Rodbard 1980). A continuous simulation computer program (ACSL, Reference manual 1986) was used to calculate the change in f_u over time. In these simulations, the rate constants for hydrolysis of noscapine (k_h) and lactonization of noscapinic acid (k_l) were 0·063 and 0·0915 h^{-1}, respectively. These were obtained from the mean values reported by Johansson et al (1983, $k_h = 0{\cdot}061$ h^{-1} and $k_l = 0{\cdot}078$ h^{-1}, interpolation in their Fig. 5 and Table 2) and Pawelczyk & Zajac (1975, $k_h = 0{\cdot}065$ h^{-1} and $k_l = 0{\cdot}105$ h^{-1}, interpolation in their Fig. 4). Equation 1a gives the fraction of noscapine eliminated through hydrolysis (f_h), assuming that only unbound noscapine is subject to hydrolysis and that the reformation of noscapine from noscapinic acid is negligible. In equation 1a $f_{u(\text{total body})}$ denotes the fraction of noscapine unbound in the body, A_b is the total amount of noscapine in the body and k the overall elimination rate constant. Expressing k in terms of total body water (TBW) and clearance (Rowland & Tozer 1980) gives equation 1b. TBW was set at 42 L:

$$f_h = \frac{\text{rate of hydrolysis}}{\text{rate of elimination}} = \frac{k_h \cdot f_{u(\text{total body})} \cdot A_b}{k \cdot A_b} \quad (1a)$$

$$f_h = \frac{TBW \cdot f_u \cdot k_h}{\text{Clearance}} \quad (1b)$$

Results

The values of f_u for noscapine (100 ng mL^{-1}) and noscapinic acid (100 ng mL^{-1}) in spiked pooled serum were 7·0 ± 0·2% and 87·9 ± 3·2%, respectively, when determined by ultrafiltration immediately after addition of drug. To obtain the same extent of conversion of [^{3}H]noscapine to [^{3}H]noscapinic acid as in the equilibrium dialysis experiments, serum was again incubated at room temperature for 24 h before centrifugation. The f_u in serum at such conditions was, at 10–1,000 ng mL^{-1} (n = 5), 13·0 ± 1·0% (range 12·0–14·8%). The interindividual variation in [^{3}H]noscapine binding to plasma, determined after 24 h incubation, was low; the f_u in spiked plasma (100 ng mL^{-1}) from ten individuals was 21·6 ± 0·9% (range 19·9–22·6%, duplicate determinations).

[^{3}H]Noscapine was stable during the equilibrium dialysis, except for the reversible hydrolysis to noscapinic acid. A

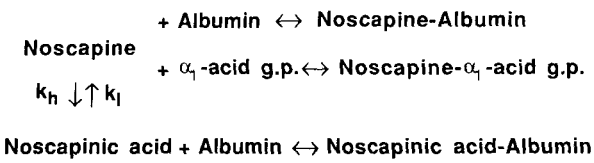

FIG. 2. Schematic model of the hydrolysis of noscapine and the lactonisation of noscapinic acid, and the interactions between these molecules and plasma proteins (g.p. = glycoprotein). The model involves a variable n denoting the number of drug molecules combining with each protein molecule, but for simplicity this is not illustrated in the figure. The symbol ⟷ denotes equilibria which in this context are regarded as instantaneous, and k_h and k_l are the rate constants for hydrolysis and lactonisation, respectively.

Table 1. Binding constants for noscapine and noscapinic acid (mean ± s.d.) in pure protein solutions determined by displacement of 3 ng mL^{-1} labelled drug by nine concentrations of unlabelled drug in logarithmic dilutions (range 30 ng mL^{-1}–100 μg mL^{-1}). The estimates given were obtained from the simultaneous fit of three such experiments for each interaction. Protein concentrations were 45 g L^{-1} (albumin) and 0·67 g L^{-1} (α_1-acid glycoprotein). To prevent hydrolysis or lactonization, all determinations were performed by ultrafiltration immediately after the addition of drug to the protein solutions. K = binding affinity constant, n = number of binding sites, P_t = total protein concentration N.E. = not estimated and f_u is predicted value in the therapeutic range of noscapine (0–500 ng mL^{-1}). K_D = binding dissociation constant obtained from 1/K.

	[^{3}H]Noscapine		[^{3}H]Noscapinic acid
Parameters	Albumin	α-acid glycoprotein	Albumin
K (M^{-1})	3060 ± 746	31500 ± 6670	N.E.
n	5·62 ± 1·34	1·73 ± 0·28	N.E.
n·K·P_t	11·2	0·954	0·20
f_u	0·082	0·51	0·83
K_D (μg mL^{-1})	135	13·1	N.E.

constant value for f_u was reached after 24 h and maintained until 48 h. The volume shift after 24 h was approximately 20%, with good agreement between the two methods of determination. The f_u values of [^{3}H]noscapine at therapeutic concentrations (10–500 ng mL^{-1}, n = 4) in serum, as determined by equilibrium dialysis, were 15·7 ± 1·2% at room temperature (range 13·5–16·5%) and 15·4 ± 0·7% at 37°C (range 13·9–16·3%). There was no dependency on the drug concentration. There was no significant difference in binding between experiments with sodium phosphate and those with Tris-Ringer buffer.

The f_u of total radioactivity is dependent on several factors: the interconversion between [^{3}H]noscapine and [^{3}H]noscapinic acid, and the binding of either one or both of these two to serum proteins. Fig. 2 shows a model for the interactions of the added ligand. This model is based on several assumptions: all binding equilibria are instantaneous, conversion can only take place for the unbound molecules, [^{3}H]noscapinic acid is only bound to a single type of albumin binding site, and [^{3}H]noscapine is bound to one type of binding site on albumin and another type on α_1-acid glycoprotein. The binding of the drugs to pure proteins was evaluated by ultrafiltration and the binding constants were determined (Table 1).

The time courses of the binding of radioactivity after addition of [^{3}H]noscapine and [^{3}H]noscapinic acid to serum were determined by ultrafiltration (Fig. 3). Fig. 3 also shows the time courses predicted by the model. Constants in the simulations were obtained from the measured serum protein concentrations. The binding constants were estimated from pure protein solutions and the conversion rate constants from the literature.

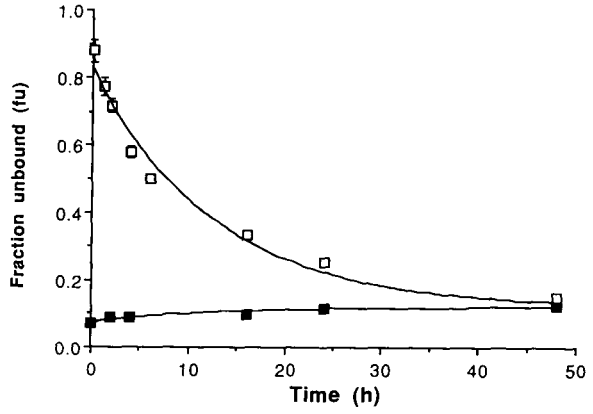

FIG. 3. The time courses of the free fraction of radioactivity, determined by ultrafiltration, after initial addition of [^{3}H]noscapine (■) or [^{3}H]noscapinic acid (□) to pooled human serum. The means ± s.d. of triplicate determinations are shown. The continuous lines show the time courses of f_u predicted by the model (Fig. 2). The simulations used the binding constants shown in Table 1 and drug and protein concentrations were as follows: [Drug] = 100 ng mL^{-1}, [Albumin] = 46 g L^{-1}, [α_1-acid-glycoprotein] = 0·7 g L^{-1}, pH = 7·4, 25°C. Rate constants for hydrolysis (k_h) and lactonization (k_l) were set at 0·063 h^{-1} and 0·0915 h^{-1}, respectively (see Methods).

The predicted time course of binding after addition of the acid form deviated slightly from the observed values, but in general the agreement between predictions and observations were good. An alternative model was considered, where both bound and unbound noscapine and noscapinic acid were subject to hydrolysis and lactonisation. It did not matter whether bound molecules were modelled to remain bound or to dissociate when converted, as the binding equilibria were considered instantaneous. This alternative model gave an f_u of 38% at equilibrium and a concentration ratio of lactone to acid of 1·4.

Discussion

Noscapine exhibits a high degree of binding (93%) in human serum, with albumin and α_1-acid glycoprotein as the only major binding constituents. The degree of binding in serum observed at room temperature was similar to that at 37°C. The binding in serum, pure albumin and α_1-acid glycoprotein solutions was constant at therapeutic concentrations of noscapine (up to 500 ng mL^{-1}). The total serum binding of other basic drugs binding α_1-acid glycoprotein with similar binding constants, e.g. methadone (Romach et al 1981), alprenolol and imipramine (Piafsky & Borgå 1977) is highly dependent on the α_1-acid glycoprotein concentration. In contrast, the total binding of noscapine is only marginally affected by elevated levels of α_1-acid glycoprotein, as predicted from the extensive binding of the drug to albumin. The interindividual variation in the free fraction was low, further implying that plasma protein binding is not an important source of the 2–4 fold variation in the disposition of noscapine, observed by Dahlström et al (1982) in 5 healthy volunteers.

Idänpään-Heikkilä (1968) found the human serum binding of noscapine in-vitro to be about 65%, using the Sephadex bath method. This markedly lower estimate of drug binding could perhaps be explained by a small, uncorrected hydrolysis during incubation, a higher drug concentration and/or lower protein concentrations (levels not reported). However, the discrepancy is probably largely due to differences in methods used. The difference between the free fractions of [^{3}H]noscapine found in plasma and serum has no obvious explanation. However, the absolute difference in the degree of binding would be smaller,

since the measured values also reflect different degrees of conversion to [^{3}H]noscapinic acid.

Ultrafiltration after equilibration and equilibrium dialysis gave similar serum binding values. The values thus obtained do not, however, only reflect the binding of the added [^{3}H]noscapine, as the free fraction of radioactivity in-vitro was found to increase with time. The corresponding decrease in binding, like the increase in binding with time after initial addition of [^{3}H]noscapinic acid, could be explained by an interconversion between the unbound moities of the two forms. The degree of binding and the rate of change in f_u agreed well with the predictions made from estimated binding constants and published rates of hydrolysis and lactonization.

The major pathways of noscapine elimination are unknown, although more than 10 metabolites have been identified in urine from the rat, rabbit and man (Göber et al 1977; Tsunoda & Yosimura 1979, 1981). Consideration of the rate of noscapine hydrolysis (Pawelczyk & Zajac 1975) and the rate of noscapine elimination in healthy volunteers (Dahlström et al 1982), suggests that a non-enzymatic hydrolysis of noscapine in-vivo would, if proceeding as in an aqueous solution of the same pH and temperature, account for about 75% of the total elimination. There are several indications, however, that hydrolysis may not play such a major role in the elimination of noscapine. Firstly, the structures of the metabolites which have been found show no sign of being secondary to noscapinic acid. Secondly, no noscapinic acid was detected in the plasma from volunteers after intake of noscapine (Johansson et al 1983). Thirdly, the considerable interindividual variation in the observed rate of elimination (Dahlström et al 1982) could not reflect differences in the rates of hydrolysis. The extent of non-enzymatic hydrolysis of noscapine in man can be approximately estimated from the proposed binding model together with the value of plasma clearance (92 L h^{-1}) reported by Dahlström et al (1982). If an f_u of 7% can be considered representative of the population, and if neither plasma protein nor tissue-bound noscapine is subject to hyrdolysis, the upper limit of the fraction of noscapine eliminated through this pathway (f_u) would be about 1%, according to equation 1b.

It is concluded that in spite of high serum protein binding, interindividual variations in f_u are probably of minor importance in explaining the varying kinetics of noscapine and that non-enzymatic hydrolysis is unlikely to be a major elimination pathway of noscapine.

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