total of 256 experiments in the t_1-domain were collected. In all spectra the HDO-peak was suppressed by presaturation.

Results and discussion

The ^1H spectra of the insulins show complex patterns of many overlapping broad lines and do not allow a detailed interpretation. The methyl protons of alanine and threonine, which are crucial for the discrimination between the insulins (see Table 1), are expected at ca 1·4 and 1·6 ppm, respectively (Bundi & Wüthrich 1979). Fig. 1 shows resolution-enhanced expansions of this region for the three insulins. The methyl signals can be easily assigned on the basis of the primary structural differences: 1·14 ppm Thr B-27, 1·20 ppm Thr B-30, 1·26 ppm Thr A-8, 1·42 ppm Ala B-30, 1·44 ppm Ala B-14 and 1·52 ppm Ala A-8.

A more profound characterization of the insulins can be obtained by two-dimensional spectroscopy (COSY). Fig. 1 shows the 1·1–1·6/3·9–4·5 ppm areas of these spectra. All correlations between the chemical shifts of the methyl protons, and the vicinal methine protons of the alanine and threonine residues are easily observed.

The chemical shifts of the methyl and methine protons of the alanine and threonine residues are virtually identical for all three insulins. Since in general proton chemical shifts are extremely sensitive to the protein conformation (Wüthrich 1976; Dwek 1973), this suggests similar tertiary structures for human, porcine and bovine insulin.

The methyl signal of Thr B-27 is broader than the other threonines; this suggests less mobility i.e. more molecular interaction for Thr B-27. At first sight this is unexpected for a residue so close to the end of the B-chain. However a closer look at the tertiary structure, as revealed by X-ray analysis (Blundell et al 1971), shows that the contact between the monomers in the insulin dimer involves Thr B-27 but not Thr A-8 and B-30. Since the dimeric form is the predominant species present at pH ≈ 3 (Cheshnovsky et al 1983), the larger line-width for Thr B-27 can be understood.

Conclusion. Both the one- and two-dimensional ^1H spectra allow the discrimination between human, porcine and bovine insulin. The tertiary structures of the three insulins in D_2O-solutions are essentially the same.

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Antidiarrhoeal activity of bisnordihydrotoxiferine isolated from the root bark of *Strychnos trinervis* (Vell.) Mart. (Loganiaceae)

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Abstract—Bisnordihydrotoxiferine, a dimeric tertiary indole alkaloid obtained from the root bark of *Strychnos trinervis* (Vell.) Mart. (Loganiaceae), inhibited normal defaecation and castor oil, arachidonic acid, and magnesium sulphate-induced diarrhoea on intraperitoneal administration in mice. The effect may be related to the ability of the compound to decrease normal and castor oil-stimulated gastric emptying, small intestinal transit and water and electrolyte accumulation, and inhibition of normal colonic transit. As prostaglandins are involved in gastrointestinal functions, inhibition of their synthesis is likely to contribute to the anticathartic activity, which has never been reported before for an indole alkaloid.

The dimeric tertiary indole alkaloid bisnordihydrotoxiferine (bisnor) was isolated from the ethanolic extract of the root bark of a Brazilian plant, *Strychnos trinervis* (Vell.) Mart. Bisnor produced no obvious actions in the central nervous system or at the neuromuscular junction when tested in frog isolated rectus abdominis muscle and rat phrenic nerve-diaphragm preparation in concentrations up to 200 μg mL^{-1}. The antidiarrhoeal activity of bisnor and its effect on some of the pathological processes involved in the production of diarrhoea are presented in this communication. Bisnor had been isolated earlier by others (Verpoorte et al 1978; Massiot et al 1983). Some of the results were presented to the 4th Annual Congress of Brazilian Society of Pharmacology and Experimental Therapeutics (Melo et al 1986).

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Materials and methods

Male Wistar rats (140–180 g) and male albino mice (21–25 g) starved for 18–24 h were used. The animals had free access to water. Drugs and bisnor were prepared in 0·1% Tween 80 and administered i.p. in doses below the acute LD50 value of 237·0 mg kg^{-1} (95% confidence limits: 200·9–279·5). Bisnor was inactive by the oral route in doses up to 100·0 mg kg^{-1}.

Inhibition of normal defaecation in mice. Groups of 5 mice were placed individually in polythene cages with filter paper at the bottom. Doses of bisnor were given i.p. to different groups while one group served as vehicle treated control. The number of faeces in each group was counted every hour for the next 4 h. Percent reduction in the number of faeces in the treated groups when compared with the control animals was calculated for each hour.

Evaluation of antidiarrhoeal activity. Doses of bisnor (3·12–25·0 mg kg^{-1}) were injected i.p. to groups of 5 mice 60 min before the administration of the cathartic agents unless stated otherwise. Diphenoxylate or indomethacin was also used when appropriate. The cathartic agents were castor oil (0·1 mL p.o./mouse), magnesium sulphate (2·0 g kg^{-1} p.o.), arachidonic acid sodium salt (15.0 mg kg^{-1} i.p.), and prostaglandin E_2 (PGE_2) at 1·0 mg kg^{-1} i.p. dose. Following their administration the animals were placed separately in polythene cages with filter paper, which was changed every hour. The severity of diarrhoea was assessed for each hour on an arbitrary scale as follows, depending on the consistency and the number of faeces present on each paper (0 = no faeces, 1 = few normal faeces, 2 = few soft faeces, 3 = watery faeces but in numbers less than 30% of the control group with diarrhoea, 4 = same as in 3 but in numbers up to 60% of the control value, and 5 = faeces with similar numbers and consistency as the control group with diarrhoea.) The total score obtained with each treated group was compared with the value in the control group.

Actions of bisnor on patho-physiological alterations in diarrhoea. (i) *Studies on gastric emptying.* Rats in groups of 4 were administered orally a barium meal, 1·0 g/rat in 1·0 mL distilled water, 1 h after injecting bisnor 12·5 mg kg^{-1} i.p. The passage of the barium bolus was continuously monitored with a radioscope to record the time required for its appearance in the duodenum and for its complete removal from the stomach. X-ray photographs were also taken at intervals. Results of control and test groups were compared. The effect of bisnor 12·5 mg kg^{-1} on accelerated gastric emptying caused by castor oil was also investigated in other groups of rats. Castor oil was given orally 15 min before the barium meal to vehicle- and bisnor-treated groups. The transport of the barium meal was then monitored as described earlier.

(ii) *Effect of bisnor on small intestinal transit and water, Na^+, and K^+ secretion.* The methods of Vischer & Casals-Stenzel (1982) were generally followed. Rats were given orally (1·5 mL/animal) a 0·4% suspension of graphit (Indian ink) in 1·5% agar-agar, 60 min after they had received bisnor, 12·5 mg kg^{-1} i.p., or the vehicle. Thirty, 60 and 90 min after the marker, rats in groups of 5 were killed and the gastrointestinal tract was removed and opened. The distance travelled by the marker was measured and expressed as a percent of the total length of the intestine from pylorus to caecum of each animal. The mean value for each group was calculated and the results obtained in the control and tests groups were compared. The inhibitory action of bisnor on stimulated intestinal transit was also performed by giving castor oil, 1·0 mL/rat, along with the graphit-agar suspension. Two doses of bisnor, 12·5 and 25·0 mg kg^{-1} i.p. were used and the effect on accelerated transit was assessed as described for normal transit. Castor oil was employed to stimulate water and ion secretion in the small intestine. One hour after the i.p. administration of vehicle or various doses of bisnor, groups of 5 rats were given castor oil in dose volumes of 1·0 mL/animal. Thirty min later, the small intestine was removed and the fluid present was measured volumetrically. Samples of the fluid were analysed for Na^+ and K^+ concentrations using flame photometry (B260 Micronal). K^+ levels were measured only for the highest dose of 25·0 mg kg^{-1} of bisnor.

(iii) Effect of bisnor on colonic transit, and water and electrolyte secretion. Change in colonic transit was evaluated at a dose of 12·5 mg kg^{-1} i.p. of bisnor injected 60 min before anaesthetizing a group of 5 rats with urethane (1·25 g kg^{-1} i.p.) and introducing into the temporarily exposed caecal end of the colon 0·5 mL of a 0·4% graphit suspension in 1·5% agar kept at 37 °C. The rats were killed after an hour and the distance travelled by the marker in relation to the total length of the colon in control and treated groups, were compared as described earlier. Bisnor in a similar dose was also examined on accelerated colonic transit produced by 1·0 mL/rat of castor oil which was given p.o. at the time of bisnor administration. The activity of bisnor on colonic water and electrolyte transport was attempted by using basically the method of Beubler (1985). 60 min after dosing the rats with 12·5 mg kg^{-1} i.p. of the substance, they were anaesthetized with urethane and the exposed colon in-situ was slowly rinsed with 20·0 mL of 0·9% saline at 37 °C. After 30 min, 2·0 mL of Tyrode solution at 37 °C was injected into the colon at the caecal colonic junction and the rectal colonic end tied off. After 30 or 60 min, rats were killed, the colons were removed and weighed initially and after draining the fluid. The difference in weight gave the amount of fluid present in the colon. Estimates of Na^+ and K^+ concentrations in the fluid were also attempted as described earlier.

Results

Inhibition of normal defaecation. While 6·25 mg kg^{-1} of bisnor had no effect, 12·5 and 25·0 mg kg^{-1} doses inhibited defaecation by 100·0% in the initial 2 h. The activity was reduced to 50·0 and 78·0%, respectively, for the above doses in the third hour. As no faeces were present in the control group at 4 h, the effect of bisnor could not be evaluated for this period.

Antidiarrhoeal property of bisnor. The results are summarized in Table 1. Castor oil produced maximal effect at the second hour. Bisnor or diphenoxylate given 60 min before the oil reduced its cathartic effect in a dose-dependent manner. The ED50 for the second hour was 9·9 mg kg^{-1}. On a weight basis bisnor was 17·36 times less potent than diphenoxylate when estimated graphically. Bisnor also inhibited (ED50 8·7 mg kg^{-1}) the diarrhoea when given 30 min after the oil. The peak cathartic action of magnesium sulphate occurred at the fourth hour. Bisnor reduced the severity of the diarrhoea dose-dependently (ED50 10·2 mg kg^{-1}). Pre-dosing with bisnor or indomethacin significantly diminished the intensity of diarrhoea induced by arachidonic acid as well. The ED50 values of 3·8 mg kg^{-1} for bisnor in this model was lower than the values obtained with other cathartic agents. Neither bisnor (25·0 mg kg^{-1}) nor indomethacin (2·0 mg kg^{-1}) had any significant effect during 4 h measurement of PGE_2-induced diarrhoea which was maximal at 1 h after its injection.

Actions of bisnor on patho-physiological alterations in diarrhoea

(i) Studies on gastric emptying. In normal rats, barium began to appear in the duodenum within a minute of its oral administration. The mean (± s.e.m.) value of 1·0 ± 0·1 min in control rats was delayed significantly ($P < 0·05$) to 8·2 ± 0·6 min in bisnor-treated animals. The period required for complete gastric emptying of the barium bolus was 40·1 ± 40 min in

Table 1. Percent inhibition of castor oil (2)-, magnesium sulphate (4)- and arachidonic acid (1)-induced diarrhoea by bisnor, and in some tests with diphenoxylate (Diphen) or indomethacin (Indo). The numbers above in parentheses indicate the time of evaluation in hours which produced maximum diarrhoea.

Experiment number	Diarrhoea inducer	Test compound (mg kg^{-1} i.p.)		% inhibition	ED50 mg kg^{-1}
1+	Castor oil	Bisnor	6·25	21·7$^{N.S.}$	
		Bisnor	12·50	70·0*	9·90
		Bisnor	25·00	98·1*	
		Diphen	0·31	20·0$^{N.S.}$	
		Diphen	0·62	58·0*	0·57
		Diphen	1·25	87·5*	
2++	Castor oil	Bisnor	6·25	25·0$^{N.S.}$	
		Bisnor	12·50	83·1*	8·7
		Bisnor	25·00	100·0*	
3	Magnesium sulphate				
		Bisnor	6·25	29·0$^{N.S.}$	
		Bisnor	12·50	58·0*	10·2
		Bisnor	25·00	92·5*	
4	Arachidonic acid				
		Bisnor	3·12	40·3*	
		Bisnor	6·25	75·0*	3·8
		Bisnor	12·50	94·6*	
		Indo	2·00	100·0*	

Analysis of variance * $P < 0·05$. N.S. = Not significant. Bisnor administered 60 min (+) or 30 min (++) after castor oil.

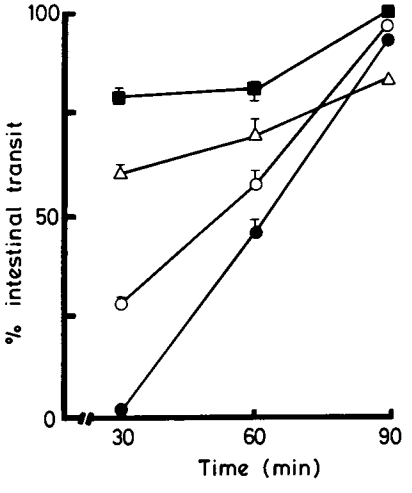

FIG. 1. Inhibitory action of bisnor on castor oil-induced increase in intestinal transit in rats, measured as the distance travelled by the marker in relation to the total length of the intestine. Results are mean ± s.e.m. Key: (△) normal, (■) oil, (○) bisnor 12·5 mg kg^{-1} + oil, (●) bisnor 25·0 mg kg^{-1} + oil.

control and 50·5 ± 4·8 min in treated groups; the difference was not statistically significant at 95·0% confidence level. There was only a difference of 2·0 min between the time of appearance of the marker in the duodenum of control (approx. 1 min) and bisnor-treated (approx. 3 min) animals after castor oil administration. However, while the mean time (± s.e.m.) for complete gastric emptying was 30·7 ± 5·0 min in castor oil-treated control rats, the corresponding value was 46·8 ± 3·7 min in rats dosed with bisnor, which was significantly ($P <$ 0·05) different.

(ii) Effect on small intestinal transit and water and Na^+ and K^+ secretion. Bisnor 12·5 mg kg^{-1} significantly ($P < 0·05$) delayed by 30·4% normal intestinal transit in rats 30 min after administration. There were no significant differences in transit between control and test values for later periods.

The pattern of intestinal transit in normal rats, in castor oil-treated rats and the effects of bisnor on castor oil-stimulated intestinal transit is presented in Fig. 1. The major effect of the oil was found to be in the initial 30 min. During this period the marker travelled about 60·0% of the length of the intestine in normal rats, compared with 79·0% in castor oil-treated groups. During the first hour bisnor significantly ($P < 0·05$) delayed the transit to a rate well below that of even normal value and the largest dose of bisnor abolished the transport of the graphit particles in the initial 30 min. The effects were less at 60 min and by 90 min there were no differences between control and bisnor-treated rats which received castor oil.

The inhibitions produced by various concentrations of bisnor on water and Na^+ secretions are presented in Table 2. The stimulated fluid and Na^+ secretions induced by castor oil were inhibited by bisnor in a dose-related manner. In the presence of 25·0 mg kg^{-1} of bisnor, castor oil-treated animals had similar values as normal control rats. It was also observed that bisnor had a greater inhibitory effect on Na^+ levels than on fluid volume indicating the role of the ions in fluid accumulation. The normal mean (± s.e.m.) K^+ concentration in the intestinal fluid of 19·0 + 1·4 mequiv L^{-1} was not significantly different from the value of 16·0 ± 1·7 mequiv L^{-1} obtained after castor oil administration. Bisnor 25·0 mg kg^{-1} significantly ($P < 0·05$) reduced the K^+ concentration to 7·0 ± 1·1 mequiv L^{-1}, a value well below that of the control rats.

(iii) Effect on colonic transit and water and electrolyte secretion. In normal rats the marker travelled in 1 h approximately half the total length of the colon; the mean ± s.e.m. was 49·3 ± 4·6%. Bisnor significantly ($P < 0·05$) reduced the value to 30·4 ± 4·5, inhibiting the transit by 38·4%. Colonic transit was not increased with castor oil in a significant manner. The distance travelled by the marker was 59·2 ± 3·1% of the total length and it was reduced to 47·8 ± 4·2% with bisnor, an inhibition of 19·2% which was not significant ($P < 0·1$). On average, less

Table 2. Inhibition of castor oil-stimulated fluid and Na^+ secretion in rat intestine by bisnor. The increase in secretions produced by castor oil (30 min) when compared with values in control group were taken as 100% for calculating inhibitions. Results are mean ± s.e.m. of groups of 5 rats.

Treatment	Volume (mL)	Na^+ concn (mequiv L^{-1})	% inhibition	
			Volume	Na^+ concn
Tween 80	0·98 ± 0·20	148 ± 16·5	—	—
Castor oil	2·86 ± 0·13	190 ± 12·1	—	—
Bisnor (6·25 mg) + Castor oil	2·30 ± 0·22	174 ± 11·0	26·6$^{N.S.}$	38·1*
Bisnor (12·5 mg) + Castor oil	1·82 ± 0·43	152 ± 9·0	55·4*	90·5*
Bisnor (25·0 mg) + Castor oil	1·12 ± 0·1	143 ± 8·2	92·5*	100·0*

Analysis of variance * $P < 0·05$. N.S. = Not significant.

than 0·3 mL of the 2·0 mL of Tyrode solution remained in the colon, 30 or 60 min after its injection in control rats, which did not permit the evaluation of the inhibitory activity of bisnor on water and electrolyte secretion. A larger initial volume was not tried as even the presence of 2·0 mL caused local distension and increased segmentation activity.

Discussion

Bisnor showed activity in three models; castor oil, arachidonic acid, and magnesium sulphate-induced diarrhoea in mice in doses at least 20 times less than the acute LD50 dose. The activity extends to normal and pathological conditions as it inhibited the diarrhoea when administered before or after the castor oil. The compound appears to act on all parts of the gastrointestinal tract. Thus it reduced normal and castor oil-induced accelerated gastric emptying and inhibited normal and castor oil-stimulated small intestinal transit. Water, Na^+ and K^+ accumulation were also reduced by the compound. Bisnor inhibited significantly the normal colonic transit as well. Its effect on colonic water and electrolyte secretion could not be analysed with the method used, which also involved the use of anaesthesia, surgery and physical manipulation of the colon, all of which made the interpretation of the results difficult.

Prostaglandins contribute to the patho-physiological functions in the gastrointestinal tract (Sanders 1984). Castor oil and other contact cathartics increase peristaltic activity and produce permeability changes in the intestinal mucosal membranes to electrolytes and water, effects associated with prostaglandin release (Awouters et al 1978; Brunton 1985). Release of prostaglandins is also a major cause of arachidonic acid-induced diarrhoea (Luderer et al 1980; Doherty 1981). Thus, a component of the antidiarrhoeal action of bisnor may be due to the inhibition of prostaglandin synthesis and release but not to its actions, as PGE_2-induced diarrhoea remained unaltered. It is relevant that arachidonic acid-induced diarrhoea was more sensitive to the action of bisnor. However, the activity of bisnor was measured 1 h after arachidonic acid and 2 and 4 h, respectively, after castor oil and magnesium sulphate administration and the ED50 doses of antidiarrhoeal drugs increase with time of evaluation (Awouters et al 1983).

Bisnor also inhibited the effect of magnesium sulphate which is presumed to act by its osmotic properties and by cholecystokinin production (Harvey & Read 1975). Therefore mechanisms other than inhibition of prostaglandin synthesis must also be considered as a number of factors contribute to the functions of the gastrointestinal tract. Examples of these include cholinergic, adrenergic, tryptaminergic and opiate systems and the recently discovered roles of α_2-adrenoceptor stimulants (Doherty & Hancock 1983) and vasoactive intestinal polypeptides (Holzer et al 1986).

The tertiary indole alkaloids have a wide spectrum of pharmacological actions (Neuss 1980). However, this is the first time an antidiarrhoeal activity has been reported. Bisnor also has antimicrobial property (Verpoorte et al 1978) and it may serve as a prototype of a new class of antidiarrhoeal agents with antimicrobial activity but without central actions.

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