International Journal of Pharmaceutics, 46 (1988) 203–209
Elsevier

IJP 01565

Pharmacokinetic studies on the mechanism of renal selectivity of the vasodilator prodrug CGP 22979

J.C. Drieman, H.T.M. Vervoort-Peters and H.H.W. Thijssen

Department of Pharmacology, University of Limburg, Maastricht (The Netherlands)

(Received 12 January 1988)
(Accepted 20 February 1988)

Key words: Prodrug CGP 22979; Compound CGP 18137; Renal vasodilator

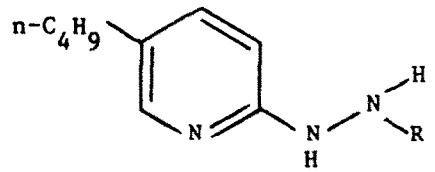
Summary

In this study, the mechanism of action of the renal vasodilator prodrug, CGP 22979 (*N*-acetyl-L-glutamic acid-*N*,[N^2-(5-*n*-butyl-2-pyridyl)hydrazide]) (PD), was studied. First, the pharmacokinetic parameters of the active drug, CGP 18137 (2-hydrazino-5-*n*-butyl pyridine) (D) were established in male Wistar rats; blood half-life ($t_{1/2}$) was 3.1 ± 0.4 min and clearance 156 ± 28 ml · min^{-1}. Half-life of D in vitro in blood, plasma and dialyzed plasma were found to be 2.8, 6 and 60 min, respectively. Addition of pyruvic acid to the dialyzed plasma shortened $t_{1/2}$ to about 9 min. In vitro studies with acylase and γ-glutamyl transpeptidase (GGT) indicate that GGT alone cannot activate PD; both the enzymes acylase and GGT are necessary to convert PD to D. Kidney homogenate was able to convert PD about 7 times faster than liver homogenate. Tissue concentrations of D, following PD administration (in vivo) were found to be significantly higher in kidney tissue compared to liver tissue. No D was found in the blood following either acute (10 or 30 mg · kg^{-1}) or chronic (10 mg · kg^{-1} · day^{-1}) administration of PD. It is argued that, beside the preferential cleavage in the kidney of PD, an important factor in restricting the activity of PD to the kidney is the rapid clearance in the blood of the liberated active compound (D).

Introduction

The prodrug CGP 22979 (PD) is the *N*-acetyl-γ-L-glutamyl derivative of the hydralazine-like vasodilator CGP 18137 (D) (5-*n*-butyl,2-hydrazinopyridin) (see Fig. 1). The compound PD was developed for selective renal vasodilation (Hofbauer et al., 1985). On physiological grounds renal vasodilation is a rational method to treat essential hypertension (Struyker-Boudier, 1980). The renal selectivity was reached by means of prodrug formation of D with the *N*-acetyl-L-γ-glutamyl group. Since renal tissue is rich in γ-glutamyl transpeptidase (GGT) (Albert et al., 1961; Glenner et al., 1962), it was assumed that the vasodilator D will be predominantly released in the kidney and exert its action only there. The principle of renal drug targeting via γ-L-glutamyl prodrugs is based on the work of Orlowski et al. (Wilk et al., 1978; Orlowski et al., 1979). These authors showed enhanced renal concentrations of sulphamethoxazole following its administration as the γ-L-glutamyl or *N*-acyl-γ-L-glutamyl prodrug. The principle of renal selectivity via γ-L-glutamyl prodrugs has since been demonstrated on a variety of compounds (Nagasawa et al., 1980; Magnan et al., 1982; Hofbauer et al., 1985).

Correspondence: H.H.W. Thijssen, Department of Pharmacology, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands.



CGP 18137 (D) : R = H

CGP 22979 (PD): R = $COCH_2CH_2CH(COOH)-NHCOCH_3$

Fig. 1. Structural formulas of D and PD.

Hemodynamic studies with PD in rats showed selective renal vasodilation indeed to occur. Upon chronic administration the blood pressure lowering effect paralleled the renal vasodilation (Smits and Struyker-Boudier, 1985; Nievelstein et al., 1987). Administration of the active drug D, either acutely or chronically, resulted in a generalized vasodilation (Smits and Struyker-Boudier, 1985; Nievelstein et al., 1987). These results are in support of the renal selectivity of PD.

However, target selectivity via the prodrug principle, although attractive, is not as simple as it seems. Computer simulations of selective delivery of active drug via a prodrug indicate that the goal can be obtained under extreme conditions only (Stella and Himmelstein, 1980; Smits and Thijssen, 1986): (a) the activating enzyme should be almost exclusively in the target system; and (2) the active drug, once formed, has to be cleared very efficiently by the target organ.

In the case of the prodrug under study, a complicating factor in explaining its renal selectivity is that the site of action of the active drug is the blood vessel wall (Hofbauer et al., 1985). This means that D, once released from PD in the kidney, is very probably also systemically available.

We investigated the disposition of D in rats after D and PD administration to get more insight into the mechanism of the renal selectivity of PD.

Materials and Methods

Animals

Male Wistar rats (270–300 g, supplied by Winkelman, Borchen, F.R.G.) were used. The animals had free access to lab food and drinking water.

Materials

The compounds CGP 18137 (D), CGP 22979 (PD) and the 3-chloro-analogue of D (CGP 33625) were a gift of Ciba-Geigy (Basel, Switzerland), γ-Glutamyl transpeptidase (GGT), type II, and acylase were obtained from Sigma Chemicals. All other chemicals were of analytical grade and were supplied by Merck Chemicals (Merck, Darmstadt, F.R.G.).

Experiments

Acute experiments with D were performed with pentobarbital-anesthetized rats. Pentobarbital sodium (60 mg/kg) was injected i.p. The left femoral artery and the right femoral vein were provided with a PE-10 catheter. Compound D was administered i.v. via the femoral vein catheter. Blood (150 μl) was sampled via the arterial catheter. Blood (100 μl) was transferred immediately into vessels containing 10 μl of 5% *p*-methoxybenzaldehyde in methanol (v/v), 100 ng of the chloro-analogue of D and 100 μl water. The samples were set aside in the dark.

Tissue distribution of D was studied in conscious animals. D or PD was administered i.v. via a tail vein. At fixed times the animals were sacrificed under light ether anesthesia by blood withdrawal from the descending aorta. Tissues (liver, kidney and others) were quickly removed, weighed and homogenized in 2 vols. of ice-cold 0.02 M Tris-HCl buffer (pH 7.4) in glass vessels containing 20 mg *p*-methoxybenzaldehyde.

Chronic administration of PD was performed by constant rate i.v. infusion using the Alzet osmotic minipump as delivery device. The pump containing PD was implanted subcutaneously and connected to a catheter introduced into the jugular vein. The rats received 10 $mg \cdot kg^{-1} \cdot day^{-1}$ of PD.

The formation of D out of PD in tissue homogenates was examined as follows. A mixture of 1 mM PD, 4 mM *p*-methoxybenzaldehyde and 100 ng of the chloro-analogue of D was prepared in 200 μl 0.1 M Tris-HCl buffer (pH 8.0). The mixture was heated to 37 °C and the reaction was started by the addition of 50 μl of tissue (kidney, liver) homogenate. Denaturated homogenate (5 min at 100 °C) served as control. The procedure to follow the formation of D out of PD by GGT and/or acylase was similar. The amount of enzymes in a reaction mixture were 1 U and 200 U for GGT and acylase, respectively.

Analysis of D

The HPLC assay of hydralazin as described by Ludden et al. (1983) was adapted to assay D. The chloro-analogue of D served as internal standard. The *p*-methoxybenzaldehyde treated samples were allowed to stand in the dark for at least 15 min. The mixtures were extracted with 0.5 ml *n*-hexane. 0.1 ml of the hexane phase was analyzed by HPLC; stationary phase, Lichrosorb 5RP18 (150 × 4.6 mm, Chrompack, The Netherlands); mobile phase, 10% acetic acid (pH 4.0 with ammonia)/acetonitrile; 35/65, v/v; flow, 2 ml/min; UV detection at 365 nm. The retention times were 3.2 min and 4.1 min for internal standard and D, respectively.

Statistics

Data are presented as mean ± S.D. Groups were compared using Student's *t*-test. Differences were considered to be statistically significant for $P < 0.05$.

Results

Experiments with D

To assay D in biological samples it appeared to be necessary to add *p*-methoxybenzaldehyde instantaneously to the samples because of a rapid decline of D upon standing; pilot experiments suggested the half-life of disappearance of D to be less than 5 min in liver homogenates and blood. To get more insight in the mechanism of this elimination, in vitro experiments with blood and plasma were performed (Fig. 2). The data clearly show the rapid in vitro elimination of D from blood and plasma ($t_{1/2}$ 2.8 and 6 min for blood and plasma, respectively). The disappearance from blood was not due to uptake by erythrocytes. Dialyzed plasma was less active ($t_{1/2}$ about 60 min) in eliminating D. After the addition of ketobodies, i.e. pyruvic acid, to the dialyzed plasma, the "eliminating" activity was almost restored ($t_{1/2}$ about 9 min). In phosphate buffer (pH 7.4) D appeared to be stable for at least one hour. These results suggest D, in analogy with hydralazin (Ludden et al., 1982), to react easily with ketones present in biological samples.

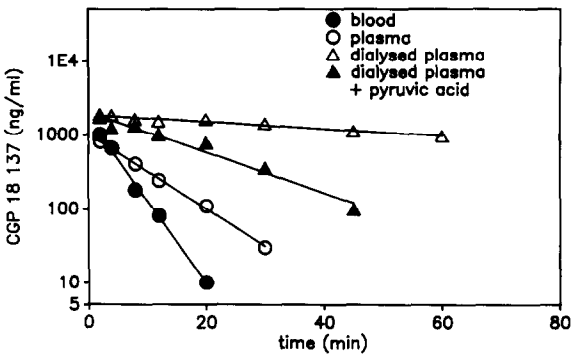


Fig. 2. In vitro disappearance of D in blood, plasma, dialyzed plasma and dialyzed plasma plus pyruvic acid.

The concentration time course of D following its i.v. administration in rats is shown in Fig. 3A. As is seen, the elimination of D from the circulation was very rapid, $t_{1/2}$ 3.1 ± 0.4 min ($n = 4$). A similar rate of decline of D in the blood was observed following the cessation of a 60 min constant rate i.v. infusion of D (Fig. 3B). This precludes distribution phenomena to be accountable for the high disappearance rate of D. Pharmacokinetic constants derived from the i.v. data are summarized in Table 1. Total blood clearance is high (156 ± 28 ml/min) and exceeds cardiac output (about 100 ml/min).

Liver and kidney distribution of D at 15, 30 and 60 min after its i.v. administration is presented in Table 2. Whereas these tissues contained D, no D was detected in blood. The data suggest a preference of D for accumulation in the kidney

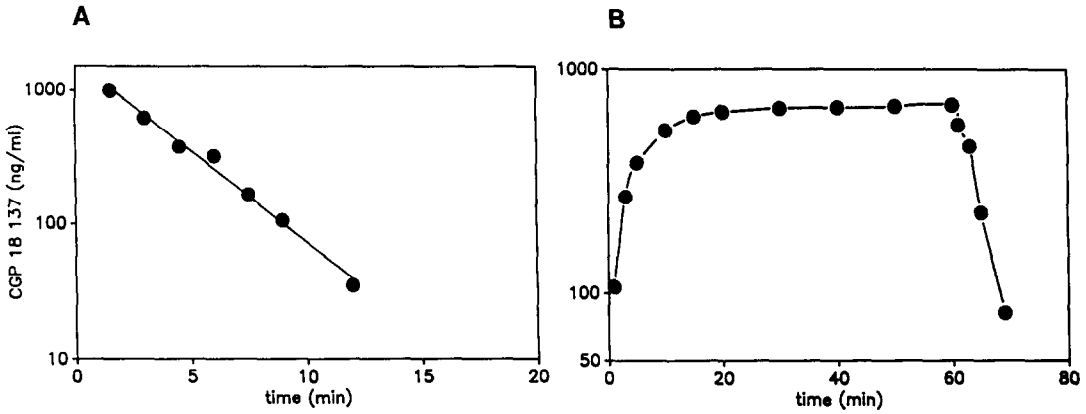


Fig. 3. Blood concentration vs time curves. A: following an i.v. bolus dose of 3 mg · kg^{-1} of D. B: following an i.v. infusion of 0.1 mg · kg^{-1} · min^{-1} of D during 60 min.

over liver. The differences, however, were beyond the level of significance.

Experiments with PD

The in vitro release of D from PD was looked for in liver and kidney homogenates (Fig. 4). As is seen the conversion rate was much higher in kidney homogenates; 5.3 ± 0.5 vs 0.8 ± 0.04 ng · mg protein^{-1} · min^{-1}. The capability of GGT and/or acylase to release D was also investigated. GGT alone failed to release any detectable D within 30 min of incubation. Acylase alone gave some D release (11 ng/30 min). However, the combination of both enzymes highly stimulated D release: 90 ng/30 min.

The in vivo conversion of PD to D was investigated by estimating tissue D distribution after PD i.v. bolus administration and after 7 days administration by constant rate infusion. Bolus injection of PD (10 and 30 mg/kg) resulted in significantly higher kidney D levels as compared

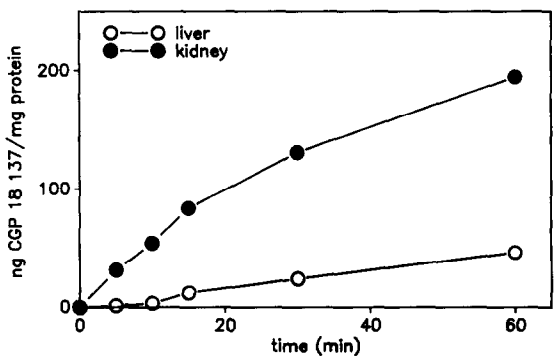


Fig. 4. In vitro conversion of PD by liver and kidney homogenates.

to liver tissue (Table 3). No D could be detected in either blood, lung, spleen, intestine, muscle and testes. In pancreatic tissue, concentrations of D were found to be about half of the liver concentra-

TABLE 1

Pharmacokinetic parameters of CGP 18137 following an i.v. bolus dose of 3 mg · kg^{-1}

Values are expressed as mean ± S.D. of 4 experiments.

$t_{1/2}$	3.1 ± 0.4 min
Cl	156 ± 28 ml · min^{-1} ·
V_d	710 ± 170 ml
AUC	6.6 ± 1.8 μg · ml^{-1} · min

TABLE 2

Tissue concentrations of CGP 18137 (D) following an i.v. bolus dose of 3 mg · kg^{-1} D

Data are expressed as mean ± S.D. of 3–4 experiments.

Tissue	μg · g^{-1}		
	15 min	30 min	60 min
Kidney	1.1 ± 0.4	0.7 ± 0.5	0.16 ± 0.08
Liver	0.5 ± 0.3	0.24 ± 0.15	0.13 ± 0.07
Ratio kidney/liver	2.7 ± 1.5 *	3.0 ± 1.9 *	1.3 ± 0.2 *

* Not significantly different from 1.

TABLE 3

Tissue concentrations of CGP 18137 (D) following i.v. administration of CGP 22979 (PD)

Tissue	$\mu g \cdot g^{-1}$			
	15 min	30 min	60 min	120 min
(A) following 30 mg · kg^{-1} of PD				
Kidney	9.6 ±1.2	8.9±4.3	2.7 ±1.2	0.6±0.1
Liver	2.1 ±0.1	1.2±0.7	0.8 ±0.4	0.2±0.1
Ratio kidney/liver	4.6 ±0.4 *	7.8±1.9 *	3.6 ±0.6 *	3.0±0.7 *
(B) following 10 mg · kg^{-1} of PD				
Kidney	2.3 ±0.3	1.5±0.5	0.19±0.09	–
Liver	0.39±0.06	0.5±0.2	0.02±0.02	–
Ratio kidney/liver	6.2 ±1.8 *	3.0±0.3 *	– **	–

* Significantly different from 1; $P < 0.05$.

** Not relevant due to very low liver concentrations.

tions per gram tissue. Following the chronic administration of PD (10 mg/kg · day during 7 days) D was detected in kidney tissue (130 ± 45 ng · g tissue^{-1}; $n = 5$). In 2 out of 5 animals traces of D were observed in liver homogenates. No D could be detected in pancreas, intestine or lung tissue.

Discussion

The present study demonstrates the renal selectivity of the prodrug PD on the basis of tissue distribution of the active drug D following PD administration.

The concept of target-directed drug delivery via prodrugs is based on target tissue-specific biochemical systems (transmembrane transport systems, transforming enzymes, etc.) that can be called upon for local release of the active drug. As non-target tissues lack these biochemical systems or have only minor activities, the non-target sites in the body are secured from drug exposure. This prodrug concept has been used in the past with varying success (see for a review Stella et al., 1985). Theoretical considerations supported by computer simulations show that target selectivity via prodrugs can be obtained under extreme conditions only (Stella and Himmelstein, 1980; Smits and Thijssen, 1986). One of the prerequisites is to prevent the redistribution of active drug from the target site where it is released. To achieve this goal, the active drug should be cleared by the target site or after its release it should be efficiently cleared by systemic routes.

From our results it is clear that the latter condition holds for the vasodilator D. Drug D is eliminated very rapidly from the circulation. This feature, without doubt, is responsible for the fact that D was not observed in the circulation following PD administration. The elimination probably is due to a chemical condensation reaction between the hydrazine moiety of D and ketobodies in the plasma. Similar reactions have been suggested for hydralazine (Ludden et al., 1982).

The renal selectivity of PD as established before by hemodynamic experiments (Smits and Struyker-Boudier, 1985) is supported by the finding of enhanced renal concentrations of D following PD administration. The renal selectivity of PD is associated with the high renal GGT activity (Hofbauer et al., 1985). Indeed, the in vitro conversion of PD to D was the highest for kidney homogenates. However, as is evident from the relatively high liver levels of D following PD bolus injections (Table 3), renal selectivity of PD is only relative. Because D is unstable in blood, the accumulation of D in the liver must be due to local release. In other words, enough active drug can be released in the liver from γ-glutamyl type prodrugs to provide non-target tissues with drug.

The fact that D accumulates in kidney and liver tissue following both D and PD administration indicates D either to bind tightly to tissue components or to be stored intracellularly. Following PD administration this accumulation could also be a result of intracellular D formation. The latter supposes facilitated or carrier-mediated transport of PD. The renal bound D or the intracellularly accumulated D apparently is available for the smooth muscles of the renal resistance vessels.

Thus far it is believed that the release of active drug from γ-L-glutamyl prodrugs in the kidney is due to cleavage by GGT. This enzyme which is mainly present in the brush border membranes of (proximal) tubular cells transfers γ-glutamyl residues from donor peptides (e.g. GSH) to acceptor

amino acids, dipeptides or water (Meister and Anderson, 1983). The products of this cleavage and transfer reaction are subsequently transported across the brush-border membrane by transport processes. This implies release and reuptake of active drug mainly to occur on the luminal site of the nephron. Before being handled in this way, however, PD has to be deacylated (Orlowski et al., 1979; Cook et al., 1987). The introduction of the *N*-acetyl group on the L-glutamic acid residue is prompted by the studies of Orlowski et al. (1979). It was shown by these authors that *N*-substitution of the glutamic acid residue enhanced the renal selectivity of the model prodrug i.e. *N*-γ-L-glutamyl sulphamethoxazole. It was reasoned that the sequential combination of renal acylases and GGT apparently favors selectivity. This mechanism can only work if both acylase and GGT are operating at the brush-border membrane.

Other mechanisms, however, can also be thought of. Recently a GSH transport system was characterized in the basolateral surfaces of the nephron (Lash and Jones, 1984; Inoue and Morino, 1985). This transport system probably can also handle GSH conjugates of xenobiotics (Lash and Jones, 1985; Kramer et al., 1987). Thus, one could speculate on transmembrane transport of intact PD via this Na^+-dependent GSH transporter. Intracellularly, D is released. A possible candidate for mediating the latter might be the cytosolic enzyme γ-glutamyl cyclotransferase (Orlowski and Meister, 1973). This enzyme is also highly active in the liver and could be responsible for the relatively high amounts of D in this tissue. We are currently performing experiments to get a clue on this.

In summary, the prodrug PD functions as a delivery device for the vasodilator D with preference for renal tissue. However, the prolonged pharmacodynamic selectivity is mainly due to the instability of D in blood, thus preventing D, released for instance from liver tissue, to accumulate in the circulation.

Acknowledgements

This work was generously supported by Ciba-Geigy, Basel, Switzerland.

References

Albert, Z., Orlowski, M. and Szewczuk, A., Histochemical demonstration of gamma-glutamyl transpeptidase. *Nature (Lond.)*, 191 (1961) 767–768,

Cook, N.D., Upperton, K.P., Challis, B.C. and Peters, T.J., The donor specificity and kinetics of the hydrolysis reaction of γ-glutamyl transferase. *Biochim. Biophys. Acta*, 914 (1987) 240–245.

Glenner, G.G., Folk, J.E. and McMillan, P.J., Histochemical demonstration of a gamma-glutamyl transpeptidase-like activity. *J. Histochem. Cytochem.*, 10 (1962) 481–489.

Hofbauer, K.G., Sonnenburg, C., Stalder, R., Criscioni, L., Kraetz, J., Fuhrer, W. and Habicht, E., CGP 22979A, a renal vasodilator with natriuretic properties. *J. Pharmacol. Exp. Ther.*, 232 (1985) 838–844.

Inoue M. and Morino, Y., Direct evidence for the role of the membrane potential in glutathione transport by renal brush border membranes. *J. Biol. Chem.*, 260 (1985) 326–331.

Kramer, R.A., Foureman, G., Greene, K.E. and Reed, D.J., Nephrotoxicity of *S*-(2-chloroethyl)glutathione in the Fischer rat. Evidence for γ-glutamyl transpeptidase-independent uptake by the kidney. *J. Pharmacol. Exp. Ther.*, 242 (1987) 741–748.

Lash, L.H. and Jones, D.P., Renal glutathione transport. *J. Biol. Chem.*, 259 (1984) 14508–14514.

Lash, L.H. and Jones, D.P., Uptake of the glutathione conjugate *S*-(1,2-dichlorovinyl) glutathione by renal basal lateral membrane vesicles and isolated kidney cells. *Mol. Pharmacol.*, 28 (1985) 278–282.

Ludden, T.M., McNay, Jr., J.L., Shepherd, A.M.M. and Lin, M.S., Clinical pharmacokinetics of hydralazine. *Clin. Pharmacokin.*, 7 (1982) 185–205.

Ludden, T.M., Ludden, L.K., Wade, K.E. and Allerheiligen, S.R.B., Determination of hydralazine in human whole blood. *J. Pharm. Sci.*, 72 (1983) 693–695.

Magnan, S.D.J., Shirota, F.N. and Nagasawa, H.T., Drug latentiation by γ-glutamyl transpeptidase. *J. Med. Chem.*, 25 (1982) 1018–1021.

Meister, A. and Anderson, M.E., Glutathione. *Annu. Rev. Biochem.*, 52 (1983) 711–760.

Nagasawa, H.T., Elberling, J.A. and Shirota, F.N., Latentiated forms of the transport-inhibitory α-amino acid adamantine. *J. Pharm. Sci.*, 69 (1980) 1022–1024.

Nievelstein, H.N. M.W., Smits, J.F.M. and Struyker-Boudier, H.A.J. Long-term hemodynamic consequences of renal vasodilation in conscious spontaneously hypertensive rats. *J. Hypertension*, 5, Suppl. 5 (1987) S439–S441.

Orlowski, M. and Meister, A. γ-glutamyl cyclotransferase; distribution, isozymic forms and specificity. *J. Biol. Chem.* 248 (1973) 2836–2844.

Orlowski, M., Mizoguchi, H. and Wilk, S., *N*-acyl-γ-glutamyl derivatives of sulfamethoxazole as models of kidney selective prodrugs. *J. Pharmacol. Exp. Ther.*, 212 (1979) 167–172.

Smits, J.F.M. and Thijssen, H.H.W., Spatial control of drug action: theoretical considerations on the pharmacokinetics of target-aimed drug delivery. In Struyker-Boudier H.A.J.

(Ed.), *Rate-Controlled Drug Administration*, CRC, Boca Raton, FL, 1986, pp. 83–113.

Smits, J.F.M. and Struyker-Boudier, H.A.J., Preferential renal vasodilator effects of CGP 22979A in conscious spontaneously hypertensive rats. *J. Pharmacol. Exp. Ther.*, 232 (1985) 845–848.

Stella, V.J. and Himmelstein, K.J., Prodrugs and site specific drug delivery. *J. Med. Chem.*, 23 (1980) 1275–1282.

Stella, V.J., Charman, W.N.A. and Naringrekar, V.H., Prodrugs, do they have advantages in clinical practice? *Drugs*, 29 (1985) 455–473.

Struyker-Boudier, H.A.J., Dynamic systems analysis as a basis for drug design: application to antihypertensive drug action. In Ariëns, E.J. (Ed.), *Drug Design*. Academic, New York, 1980 pp. 146–191.

Wilk, S., Mizoguchi, H. and Orlowski, M., γ-Glutamyl dopa, a kidney specific dopamine precursor. *J. Pharmacol. Exp. Ther.*, 206 (1978) 227–232.