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Probenecid-induced accumulation of 5-hydroxyindoleacetic acid and homovanillic acid in rat brain

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The accumulation of 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) in rat brain has been examined after probenecid infusion over 8 h. At plasma probenecid concentrations of 200–400 μg mL^{-1} a steady state level in the accumulation of 5-HIAA and HVA was achieved, the increase above the endogenous levels being 135% and 65%, respectively. When the plasma concentration of probenecid rose above 400 μg mL^{-1} there was further accumulation of both 5-HIAA and HVA probably induced by increased neuronal activity or toxicity due to probenecid. The explanation for the plateau of 5-HIAA and HVA obtained over the plasma probenecid concentration interval of 200–400 μg mL^{-1} could be that the levels were reached when there was complete inhibition of active transport, and when the rate of formation of the metabolites equalled the rate of elimination by alternative routes i.e. bulk flow and diffusion. Therefore when probenecid is used to inhibit the active transport of acid monoamine metabolites across the blood-brain barrier, its plasma concentration should be within the range of 200–400 μg mL^{-1}.

Probenecid is known to inhibit competitively the active transport of weak organic acids across different membranes, for example in the kidney and across the blood-brain barrier (Beyer et al 1951; Neff et al 1967). The subsequent accumulation of 5-hydroxyindole-3-acetic acid (5-HIAA) and homovanillic acid (HVA) in brain tissue and cerebrospinal fluid (CSF) after treatment with probenecid has been used to establish brain monoamine turnover in animals and man (Neff et al 1967). The site of action of probenecid is unclear, but most investigations point to the choroid plexus in the fourth ventricle (Sampath & Neff 1974; Wolfson et al 1974; Jakupcevic et al 1977; Bulat & Zivkovic 1978; Yuwiler et al 1982). Earlier studies have shown that different doses of probenecid give different degrees of blockade of the transport across the blood-brain barrier (van Praag & Korf 1974; Elghozi et al 1983).

In animals a supramaximum dose is usually given to obtain total blockade of the transport, with varying results. Variability in probenecid blockade has also been a problem in human studies (Roos & Sjöström 1969; Tamarkin et al 1970; Sjöström 1972, 1973). One reason for the divergent results could be that the inhibition of 5-HIAA and HVA transport has been related to the dose of probenecid instead of to the plasma concentration.

Earlier studies have shown increased accumulation of 5-HIAA and HVA with time after a single intraperitoneal (i.p.) dose of probenecid (Neff et al 1967; Goodwin & Post 1973; Elghozi et al 1983; Hutson et al 1984). Over that time the blood concentration of probenecid increased owing to the absorption phase. This means the accumulation of the monoamine metabolites in the brain may depend on the degree of inhibition of the active transport by probenecid. Such a problem could possibly be avoided by close monitoring of probenecid plasma concentrations.

In the present study we have examined the accumulation of 5-HIAA and HVA in rat brain after different doses of probenecid, with simultaneous monitoring of the plasma concentration. The accumulation of 5-HIAA and HVA was measured in whole brains after treatment with a single intravenous bolus dose of probenecid, or after i.v. infusion over 8 h. Different infusion rates were used to obtain different plasma levels of the drug. The rats were decapitated, and the accumulation of metabolites measured. Probenecid was administered as a constant i.v. infusion to eliminate differences in accumulation with time, which may be dependent on the absorption of probenecid from the gastrointestinal tract and on the disposition of the substance in the body.

MATERIALS AND METHODS

Animals and drugs

Male Sprague-Dawley rats, 235–316 g, were used and had free access to food and water until the start

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of the experiments. The day before, two silicon rubber cannulae (Silastic, 0·02 in. i.d.; 0·037 in. o.d.) were placed in the jugular veins under light ether anaesthesia, allowing an exact amount of probenecid to be introduced via one catheter and exact sampling of blood via the other. During the study the rats were conscious and unrestrained.

Probenecid was dissolved in 1 M sodium hydroxide diluted with physiological saline and adjusted with 0·1 M hydrochloric acid to pH 7·4. A single i.v. bolus dose (100 mg kg^{-1}) or an 8 h constant i.v. infusion was administered. Different infusion rates (65–245 μg min^{-1}) were used.

A blood sample was taken for analysis of probenecid 15, 30 or 60 min after the i.v. bolus dose, and the animal was then decapitated. During the infusion blood samples were collected at 360, 420 and 480 min, each being replaced by an equal volume of saline. The maximum cumulative blood volume withdrawn from any animal did not exceed 5% of the total blood volume. After completion of the infusion the rats were decapitated.

The blood samples were immediately centrifuged, and the plasma kept frozen at −50 °C until analysed for probenecid. The entire brain was removed, freed from blood and membranes, weighed, and immediately frozen at −70 °C. The brain was homogenized in 5 mL 0·1 M hydrochloric acid together with internal standards 5-hydroxyindole-3-propionic acid (5-HIPA) and vanillic acid (VA). The homogenate was centrifuged at 20 000 rev min^{-1} at 4 °C, and the supernatant was frozen at −50 °C until analysed for 5-HIAA, HVA, and probenecid.

Chemical assays

Probenecid was determined in plasma and in brain supernatants by a gas liquid chromatographic method with electron capture detection (Hartvig et al 1982). A glass column packed with 3% OV-17 on Gas CHROM was used under the following conditions: oven temperature 230 °C, injection temperature 270 °C, detector temperature 300 °C, nitrogen flow 40 mL min^{-1}. The *N,N*-diethyl analogue of probenecid was used as internal standard. The detection limit was 1·0 μg mL^{-1} with a relative standard deviation of 8%.

5-HIAA and HVA were determined in the supernatant by a high-performance liquid chromatographic method with electrochemical detection. 5-HIPA and VA were used as internal standards for 5-HIAA and HVA, respectively. A stainless steel column (4·6 × 195 mm) packed with ODS-hypersil C_{18} μm (Shandon) was used. The electrochemical detector consisted of a carbon paste electrode (OD-O) with a detector potential maintained at +0·72 V versus an Ag/AgCl reference electrode. The mobile phase was a mixture of tetrabutylammonium phosphate buffer (pH 6·7, μ = 0·2) and methanol–acetonitrile–water (0·05 : 0·05 : 1). The flow-rate was adjusted to 1·5 mL min^{-1} at a pressure of 2300 psi. The standard curves were made on pooled brain homogenates from 14 rats to eliminate intra-individual differences in 5-HIAA and HVA. The detection limits of 5-HIAA and HVA were 10 ng mL^{-1}, with a relative standard deviation of 11%.

Calculations

The accumulation (E) of 5-HIAA and HVA obtained in the brain was fitted to the plasma concentration of probenecid (C) using Hill's equation (Hill 1910):

$$E = (E_{max} \times C^s)/(C^s + C50^s) \quad (1)$$

where E_{max} = the maximum accumulation when probenecid concentration approaches infinity, C50 = the probenecid concentration producing the half-maximum accumulation, and s = a constant influencing the slope of this relationship. The fit of equation 1 to the experimental data was obtained by the non-linear digital regression programme NONLIN (Metzler et al 1974). Different initial estimates were used to avoid local minima in the sum of square of surfaces. The goodness of fit of computed data to observed data was based on coefficient of correlation (r), coefficient of determination (r^2), and standard deviation of parameters.

RESULTS

Establishment of steady state plasma levels by infusion

During constant intravenous infusion for 8 h, plasma concentrations of probenecid reached different levels depending on the rate of infusion (Fig. 1). Steady state levels were apparently reached after 8 h at rates of 65–140 μg min^{-1}. Higher rates did not produce a plateau during the 8 h, probably owing to the Michaelis-Menten elimination kinetics of probenecid.

At the time of decapitation the unbound concentration of probenecid in plasma was calculated from the experimentally determined total drug levels, utilizing the non-linear relationship between free and bound plasma concentrations of probenecid in rat (Emanuelsson & Paalzow 1986). As shown in Fig. 2, the concentration in brain increased in a non-linear

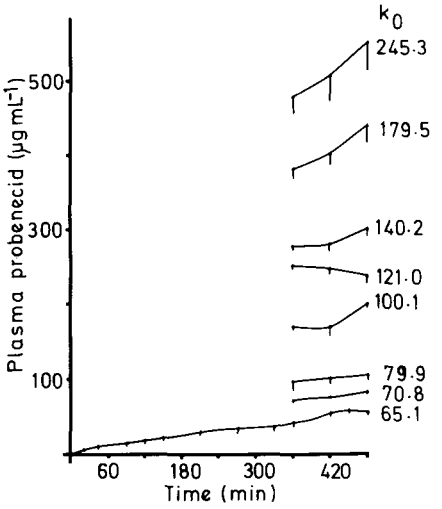

FIG. 1. Plasma concentration ($\mu g\ mL^{-1}$) of probenecid vs time (min). Probenecid was administered as constant i.v. infusion over 8 h, at different rates (k_0, $\mu g\ min^{-1}$). Each point represents mean ± s.d. for 4 rats.

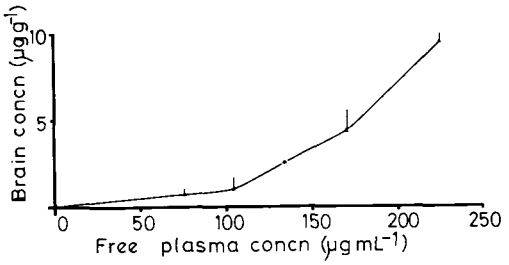

FIG. 2. Concentration of probenecid in the brain vs free concentration of probenecid in plasma at the time of decapitation. Each point represents the mean ± s.d. for 4 rats.

manner when plotted against the free concentration of probenecid in plasma. This was especially clear when the free drug level in plasma exceeded 100 $\mu g\ mL^{-1}$. To obtain brain levels of probenecid > 2 $\mu g\ g^{-1}$, a total plasma concentration of probenecid greater than 400 $\mu g\ mL^{-1}$ was needed.

Relationship between plasma probenecid and brain monoamine metabolites levels

The plasma probenecid concentration in rats given an i.v. bolus dose of 100 mg kg^{-1} declined as shown in Fig. 3 remaining within 200–400 $\mu g\ mL^{-1}$ during the first hour.

Rats receiving this bolus dose were decapitated 15, 30 or 60 min after the injection. Brain levels of 5-HIAA, HVA, and probenecid were estimated. Fig. 4 shows the accumulation of 5-HIAA and HVA

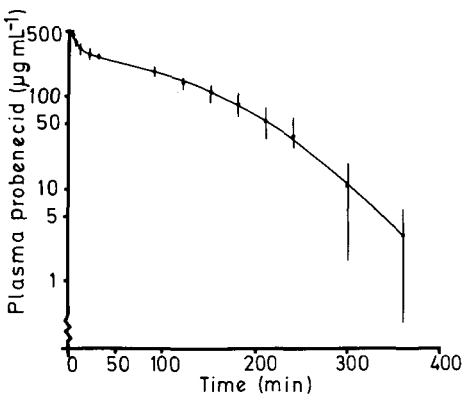

FIG. 3. Plasma concentration ($\mu g\ mL^{-1}$) of probenecid vs time. Probenecid was administered as a single i.v. bolus dose of 100 mg kg^{-1}. Each point represents the mean ± s.d. for 6 rats.

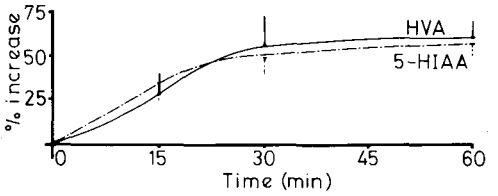

FIG. 4. Increase (%) in accumulation of 5-HIAA and HVA above the endogenous level vs time (min). Probenecid was given as a single i.v. bolus dose 100 mg kg^{-1}. Each point represents the mean ± s.d. for 4 rats.

with time. A plateau level was reached after approximately 30 min, with maximum values of 55% and 60% above the endogenous levels for 5-HIAA and HVA, respectively. The mean endogenous level of 5-HIAA in brain was determined to be 242 ng g^{-1} and that of HVA to be 56 ng g^{-1}.

After i.v. infusion the 5-HIAA brain levels increased with increasing plasma concentration of probenecid (Fig. 5), reaching a maximum of 135% above the endogenous level between plasma probenecid concentrations of 200 and 400 $\mu g\ mL^{-1}$. With higher plasma levels a further pronounced increase in 5-HIAA concentration was noted.

The same type of accumulation curve was obtained for HVA as for 5-HIAA, with a plateau level of HVA 65% above the endogenous level between plasma probenecid concentrations 200–400 $\mu g\ mL^{-1}$. Plasma probenecid concentrations exceeding 400 $\mu g\ mL^{-1}$ produced a dramatic increase in cerebral HVA levels (Fig. 5).

The Hill equation (eqn 1) was fitted to the obtained effect (E) of probenecid on 5-HIAA and HVA accumulation at different concentrations of

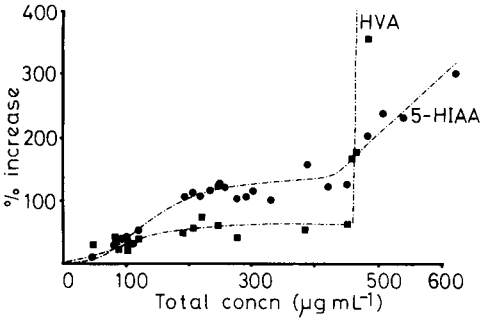

FIG. 5. Increase (%) in the accumulation of 5-HIAA and HVA above the endogenous level vs plasma concentration of probenecid at the time of decapitation. Probenecid was administered as a constant i.v. infusion over 8 h. Each point represents data from 1 rat. The continuous lines show the fit of Hill's equation (eqn 1) to the experimental data.

Table 1. Estimation ± s.d. of Hill's equation (eqn 1) to the accumulation obtained (increase %) of 5-HIAA and HVA vs plasma concentration of probenecid at the time of decapitation (0–400 μg mL^{-1}). Probenecid was administered as a constant i.v. infusion over 8 h.

	5-HIAA	HVA
E_{max} (increase %)	134·0 ± 13·9	64·8 ± 21·9
s	3·4 ± 1·2	2·4 ± 1·6
C50 (μg mL^{-1})	134·6 ± 16·8	109·0 ± 52·9

probenecid (0–400 μg mL^{-1}). The fit of Hill's equation to the experimental data is also shown in Fig. 5, and the parameter estimates (E_{max}, C50 and s) are shown in Table 1. The maximum increases in the accumulation (E_{max} ± s.d.) were 135 ± 14% and 65 ± 22% for 5-HIAA and HVA, respectively.

DISCUSSION

Neff et al (1967) found linear accumulation of 5-HIAA in rat brain with time after i.p. administration of 200 mg kg^{-1} probenecid. This inhibiting effect of probenecid on the transport of 5-HIAA from rat brain to the blood has since been used to study the activity of central dopaminergic and 5-HT neural transmission: the higher the activity the greater the accumulation of acidic metabolites (Roos & Sjöström 1969; Tamarkin et al 1970; Sjöström 1972, 1973).

Probenecid is a valuable tool in inhibiting the transport of acidic monoamine metabolites across the blood-brain barrier, but its use has been hampered by the lack of pharmacokinetic data, which have only recently become available. Different doses of probenecid have been administered in animals (Guldberg et al 1966; van Wijk et al 1979; Elghozi et al 1983) and man (Goodwin & Post 1973; Sjöström 1972, 1973; van Praag & Korf 1974; Post & Goodwin 1974), but the plasma concentration of probenecid needed to achieve complete inhibition of the transport of metabolites across the blood-brain barrier remains obscure.

Transport of organic acids within the CNS seems to be achieved by a system similar to that acting in the kidney with regard to both substrate specificity and vulnerability (Pappenheimer et al 1961; Barany 1972). The choroid plexus, particularly in the fourth ventricle, has been suggested as the anatomical site of transport from the cerebrospinal fluid into the blood (Ashcroft et al 1968; Cserr & van Dyke 1971; Wolfson et al 1974; Yuwiler et al 1982). The efflux of 5-HIAA and HVA from the choroid plexus probably takes place not only by active transport but also by diffusion and by bulk flow (Ashcroft et al 1968). Sampath & Neff (1974) showed that 5-HIAA was taken up in the isolated choroid plexus by both passive diffusion and by active transport. At higher concentrations of 5-HIAA the diffusion dominated the transport, but at lower concentrations active transport was the main mechanism.

In the present investigations we used our knowledge of the pharmacokinetics of probenecid in the rat (Emanuelsson & Paalzow 1986) to study the inhibition of the transport of 5-HIAA and HVA more closely. Only the free levels of HVA were estimated, although it is known that HVA also exists as the sulphonyloxy conjugate (HVA-SO_4) (Dedek et al 1979; Hutson et al 1984; Sarna et al 1984; Curzon et al 1985). Both HVA and HVA-SO_4 have to be considered to make an appropriate estimation of the total brain dopamine metabolism, as using only the HVA concentration may result in underestimation. Probenecid influences the accumulation of both conjugated and free HVA, but to different degrees (Sarna et al 1984; Curzon et al 1985). The way that probenecid affects the accumulation of HVA-SO_4 together with its effects on the transport mechanisms of HVA, must be known if correct conclusions about the total dopamine metabolism are to be drawn from results obtained. The aim of this study was to evaluate the influence of probenecid on the active transport of 5-HIAA and HVA across the blood-brain barrier, and not to make a quantitative estimation of dopamine and 5-HT brain metabolism. Intravenous infusion was used to establish different plasma concentrations of probenecid, and the accumulation of the acidic metabolites in the whole rat brain was followed.

With rising probenecid concentration the accumulation of 5-HIAA and HVA increased up to a

maximum of 135% and 65% above the endogenous levels, respectively, and these figures were noted at probenecid plasma concentrations of 200–400 μg mL^{-1}. The lower accumulation of HVA compared with 5-HIAA might be explained by the further metabolism of HVA to HVA-SO_4 (Dedek et al 1979) in parallel with alternative transport routes across the blood-brain barrier. When the plasma concentration of probenecid rose above 400 μg mL^{-1} a secondary, pronounced accumulation of both metabolites was observed (Fig. 5).

After a single i.v. bolus dose of probenecid the accumulation of both metabolites reached a plateau within 30 min (Fig. 4).

When using and standardizing probenecid as an inhibitor for the investigation of activity of 5-HT and dopaminergic central neurons, it is therefore important to monitor the plasma level of probenecid and keep it within the range 200–400 μg mL^{-1}.

The steady state level of metabolites obtained in the present investigation could be explained by complete inhibition of the active transport system by probenecid. A level is reached when the rate of formation of the metabolites equals the rate of elimination by alternative mechanisms, i.e. further metabolism, bulk flow and diffusion from the CNS. The ability to reach steady state levels of accumulated acidic metabolites indicates that the first order elimination (diffusion and bulk flow) is the major pathway of elimination of these substances from CNS with increasing probenecid plasma concentration (Tsuchiya & Levy 1972). For a drug that is eliminated by parallel first-order transport and Michaelis-Menten active transport, the first-order process takes over more and more of the elimination as the dose increases (van Rossum et al 1983). These reports support our conclusion that the accumulation of 5-HIAA and HVA increases as the concentration of probenecid rises up to about 200 μg mL^{-1}. With further increase in plasma probenecid concentration up to about 400 μg mL^{-1} active transport is completely inhibited and the transport of 5-HIAA and HVA across the blood-brain barrier is dominated by passive diffusion and bulk flow.

The second, dramatic increase in accumulation of brain 5-HIAA and HVA is unexplained but it is probably due to the marked increase in probenecid concentration in the brain (Fig. 2), where it may produce increased neuronal activity or toxic effects.

It is the free fraction of probenecid that can diffuse across the blood-brain barrier and enter the cerebral tissue. Fig. 2 shows the relationship between the concentration of probenecid in the brain and the free concentration of probenecid in plasma. When the unbound plasma probenecid concentration increased above 100 μg mL^{-1}, a marked increase in probenecid levels in the brain was noted. A free plasma probenecid concentration of 100 μg mL^{-1} is almost equivalent to a total concentration of 400 μg mL^{-1}. The reason for the marked increase in probenecid in the brain at free plasma concentrations exceeding 100 μg mL^{-1} is unknown, but there could be a toxic or an unspecific effect influencing the transportation processes across the membranes.

Studies on possible pharmacological effects of probenecid include that of Lyness & Mycek (1978), who showed reduced spontaneous motor activity in the rat after a dose of 200 mg kg^{-1}. In another study, probenecid raised the threshold for nociceptive responses and increased the brain concentration of noradrenaline (Paalzow & Paalzow 1974). Cramer et al (1972) and Bowers & Study (1979) reported that probenecid increased cyclic AMP levels in human CSF.

Another explanation for the secondary increase in accumulation of 5-HIAA could be that at higher plasma concentrations probenecid displaces tryptophan from its binding site on the albumin molecule (Curzon et al 1976; van Wijk et al 1979). When the free concentration of tryptophan in blood is raised, its transportation into the brain also increases. Higher brain concentrations of tryptophan might increase the synthesis of 5-HT and this could explain the increased accumulation of 5-HIAA since it is the main metabolite. However, the theory is inconsistent with findings by Curzon et al (1985), Korf et al (1972) and Sarna et al (1983). Curzon et al (1985) found that the mean rat brain level of tryptophan was only slightly altered after doses of 200 mg kg^{-1} probenecid, and Korf et al (1972) reported that even if the brain level of tryptophan was increased the 5-HT metabolites remained unaffected.

The finding of a plateau level in the accumulation of monoamine metabolites after probenecid infusion has not previously been reported, probably owing to the unmonitored probenecid plasma concentration (Neff et al 1967; Elghozi et al 1983). Our findings support the use of monitored plasma levels of probenecid when using it for estimation of neuronal activity.

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