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The Extracellular Concentration of the Anti-epileptic Drug Valproate in the Rat Brain as Determined with Microdialysis and an Automated HPLC Procedure

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Abstract—The cellular and extracellular brain concentration of valproic acid in freely moving rats has been estimated after intravenous injection of sodium valproate. Some rats were provided with a stereotaxically implanted dialysis probe in the striatum and a cannula in the heart through which the drug was injected and which allowed regular removal of blood. In other rats tissue levels of valproic acid were determined 5 and 90 min after drug injection. Valproic acid was determined by an automated precolumn derivatization procedure followed by HPLC separation and fluorimetric detection. Extracellular concentration was proportional to the blood concentration at every time interval, indicating rapid exchange of the drug between the two compartments. About 50% of the striatal content of valproate was in the extracellular space. The experiments demonstrated the usefulness of microdialysis to estimate both the extracellular concentration and the average cellular drug levels, provided a sensitive analysis procedure is available.

Microdialysis has most often been applied for the investigation of neurotransmitter release and metabolic pathways in the central nervous system (Ungerstedt 1984; Westerink et al 1987), but has been used in only a few studies for pharmacokinetic purposes. For example, relative and absolute concentrations of cocaine in the striatal extracellular space (ECS) have been estimated (Hurd et al 1988; Nicolaysen et al 1988; Hurd & Ungerstedt 1989); with microdialysis, absolute levels in the ECS can be calculated provided the dialysis efficiency is known. At least three methods have been described to calculate the extracellular concentration from the dialysate levels. Firstly, the in-vitro determined dialysis efficiency is used for the in-vivo measurements; secondly, from concentration measurements at different flow rates, the extracellular concentration can be extrapolated to zero flow rates (Jacobson et al 1985), and, thirdly, the dialysate is continuously reintroduced until a steady state is obtained (Lerma et al 1986).

In the present study microdialysis has been used to estimate the extracellular concentration of the anti-epileptic drug sodium valproate. It is not known with certainty whether the clinical effects of valproate are determined by its accumulation in the intra- or in the extracellular space (Chapman et al 1982). In clinical routine, plasma or serum concentrations of valproic acid are measured because a relation with anti-epileptic action at the cerebral level and with side effects is assumed (Gram et al 1979; Chapman et al 1982; Turnbull et al 1983). Recently we published a method for the automated HPLC analysis of fatty acids and other carboxylic acids including valproic acid, utilizing precolumn derivatization and fluorimetric detection (Wolf & Korf 1988, 1990; Wolf et al 1989). This procedure was used for the analysis of valproate in rat brain dialysate, rat whole blood and rat tissue.

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Materials and Methods

Determination of valproate

Valproic acid was determined in blood and brain dialysates by HPLC as described previously (Wolf et al 1989; Wolf & Korf 1990). The sensitivity of the assay is about 25 pg per injection, corresponding to 20 ng valproic acid per mL dialysate or to 0·25 μg mL^{-1} blood.

Dialysis and blood sampling

U-Shaped cellulose dialysis fibres (200 μm o.d., 20000 mol. wt cut-off) were implanted bilaterally in the rat striatum one or two days before the experiments, as previously described (Korf & Venema 1985).

Conscious male Wistar rats, 200–250 g, obtained from the Centraal Proefdieren Lab, Groningen, The Netherlands, were perfused with artificial cerebrospinal fluid (CSF), containing (mM) NaCl 120, $NaHCO_3$ 15, KCl 5, $CaCl_2$ 1·5, and $MgSO_4$ 1; pH 7·4 was obtained by a constant flushing with 95% O_2/5% CO_2. Sodium valproate (25 mg), was injected through a cannula implanted into the heart. Through the same cannula 20 μL blood samples were taken at regular intervals for the assay of valproic acid. Fig. 1 shows the design of the experiment, including the time of injection and the approximate times of blood withdrawal. We did not succeed in collecting blood samples at every indicated time and in the calculations the exact times were always used. Before and until 90 min after the injection of sodium valproate, a dialysis perfusion rate of 1·4 μL min^{-1} was maintained and 15 min samples were collected. Thereafter the perfusion rate was increased to 20 μL min^{-1} and shorter collection intervals were used, as indicated in Fig. 1. This design was used for a group of four rats. The haematocrit was 44·5 ± 2·4% (mean ± s.e.m. of 8 rats). Therefore a factor of 1·82 was used for the estimation of the plasma concentration of the drug from concentrations of

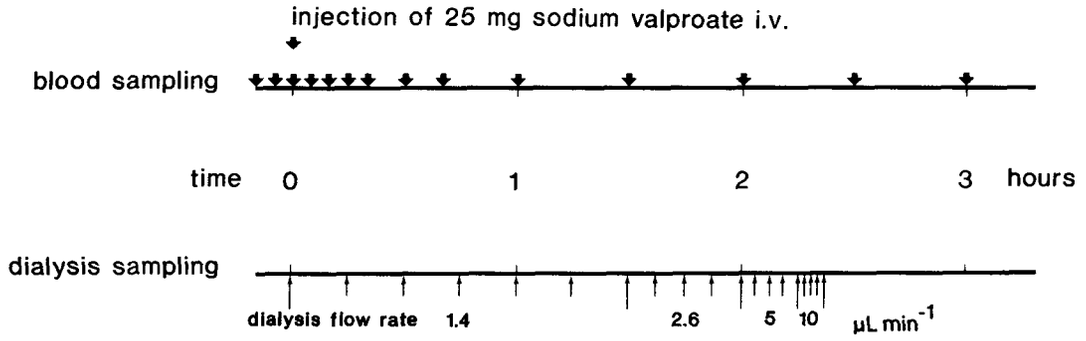

FIG. 1. Design of the experiment. The times of blood withdrawal are indicated with large arrows; the beginning or the end of the collection periods of the dialysate fractions are indicated by thin arrows.

valproate measured in whole blood. In another group of rats, levels of valproic acid in various brain regions were determined 5 or 90 min after intravenous injection of sodium valproate.

Extracellular concentration of valproate

Attempts were made to estimate the extracellular concentration of valproic acid from dialysate levels. We used the following three approaches to data analysis:

1. In the first approach the dialysis efficiency in-vitro was assumed to be identical to the in-vivo efficiency. Accordingly, the efficiency of the dialysis of sodium valproate determined in-vitro at a particular rate of perfusion was used for the calculations with data obtained in-vivo.

2. The second method has been described by Jacobson et al (1985). In this it is assumed that the concentration of valproate in the dialysate is dependent upon the flow rate. Thus at low flow rates there is back diffusion of the valproate from the dialysate to the extracellular space but this is negligible at higher perfusion rates. At any given flow rate the relationship between the dialysate concentration of valproate and the extracellular concentration is given by the equation:

$$C_i = C_o(1 - \exp[-K_oA/F])$$

Where C_i is the concentration of the dialysate and C_o the concentration outside the dialysis tube, i.e. the extracellular concentration. K_o is the average mass transfer coefficient, A is the area of the semipermeable membrane and F is the flow rate. A linear relationship emerges when $-\ln[1-(C_i/C_o)]$ is plotted against the inverse flow rate. It is assumed that the K_o value is constant at flow rates used in the investigation. In the present study this method was used both in-vitro and in-vivo. We have tried to determine whether data reduction for the $-\ln[1-(C_i/C_o)]$ is allowed by series expansion (Bartsch 1974) by using the in-vitro measurements. Data reduction is attractive because a simpler function is obtained.

3. Our third approach has been described by Lerma et al (1986) and is as follows. In a closed circuit the perfusion is continued until a steady state concentration is reached, which is then similar to that in the ECS. However, we could not reach a steady state in-vitro within the time of the experiment (3–4 h). Therefore that approach was abandoned.

Data analysis

Concentrations of the drugs were expressed as valproic acid ($\mu g\ mL^{-1}$) and as mean ± s.e.m. unless otherwise stated. In blood and serum the free + bound (total) fractions were measured, whereas the dialysate (and presumably also the ECS) contained only free valproic acid, because protein was lacking in these fluids. A problem encountered with the in-vivo analysis was the apparent lack of steady state conditions. To correct for this a non-linear curve fitting procedure was applied to the dialysate and to the blood values of individual rats when appropriate. A commercially available computer program (GraphPAD (tm), supplied by ISI) was used for fitting the data. All the dialysate results were recalculated for a flow rate of 1·4 $\mu L\ min^{-1}$, a valid assumption, because of the direct relationship between the flow rate and concentration of the drug found in-vitro. In addition, missing blood concentrations were calculated from the fitted curves obtained from the real data of each rat. Significance of differences was computed with Student's *t*-test.

Equipment

Two Kratos SF400 pumps and a Kratos SF450 gradient mixer were used for solvent delivery. For detection, a Kratos SF980 fluorescence detector with a 5 μL cell was used (excitation wavelength 325 nm, cut-off filter 398 nm). Data were collected with an LDC CI-10 integrator. The separations were performed on a Chromspher ODS column. The acids were automatically derivatized using a Spark PROMIS autosampler. For further details see Wolf et al (1989). The dialysis probes were made locally as described previously (Korf & Venema 1985). For the perfusion an LKB 12000 Varioperpex peristaltic pump was used.

Results

In-vitro dialysis

All three approaches to estimate the extracellular concentrations were evaluated in-vitro. The flow rate most often used in-vivo was 1·4 $\mu L\ min^{-1}$. At this flow rate the in-vitro recovery was 8·87 ± 0·17%, the recovery percentages at rates 2·6, 5 and 10 $\mu L\ min^{-1}$ were 4·89 ± 0·06, 2·09 ± 0·04, and 1·09 ± 0·03%, respectively. In the first approach these recoveries were used for in-vivo calculations.

The Jacobson method (our second approach) resulted in a linear relation when the $-\ln(1-\text{recovery})$ was plotted against the inverse of the flow rate. Fig. 2 (left panel) shows that in-vitro this is so. The term $-\ln(1-C_i/C_o)$ can be

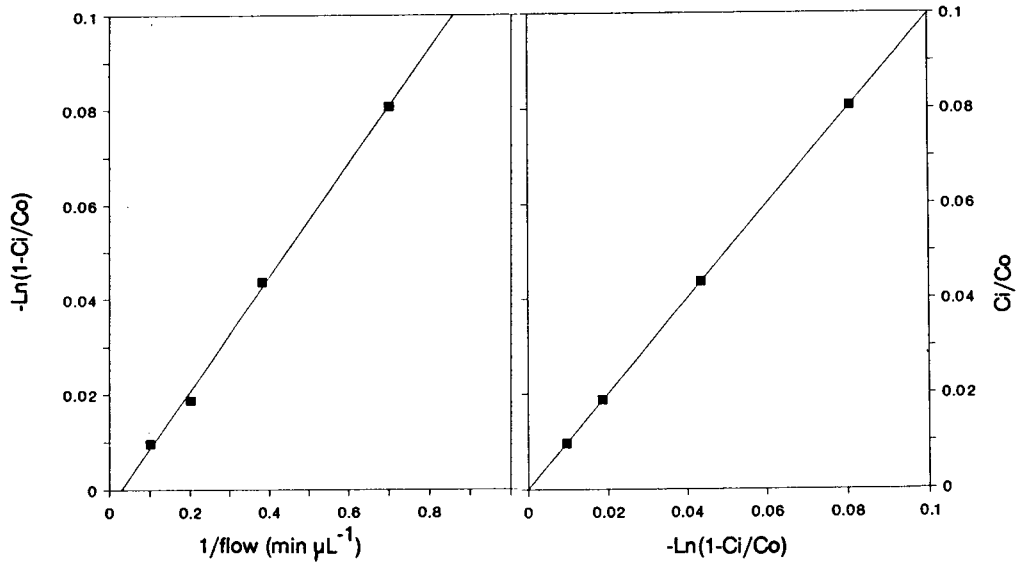

FIG. 2. Left panel: the in-vitro relationship between the concentration of valproic acid in the dialysate and the inverse flow rate as a Jacobson plot: $-\ln(1-\text{recovery})$ vs 1/flow. From this plot the value of K_oA (0·130 µL min^{-1}) and a correlation coefficient of r = 0·997 was calculated. Right panel: the nearly identical values of the first term of the series expansion of $-\ln(1-\text{recovery})$ and $-\ln(1-\text{recovery})$ at a flow rate in-vitro.

approximated by C_i/C_o. In our case the next term contributes about 4% at a flow rate of 1·4 µL min^{-1} and less at higher flow rates, which can be neglected considering the experimental errors. Fig. 2 (right panel) shows the virtual linear relationship between C_i/C_o and $-\ln(1-C_i/C_o)$, thus confirming that data reduction by series expansion is indeed valid. With the Jacobson plot we determined $K_oA = 0{\cdot}130 \pm 0{\cdot}007$ µL min^{-1}. The K_oA value can also be calculated directly from the formula at each flow rate. This resulted in the following mean values: $0{\cdot}133 \pm 0{\cdot}003$, $0{\cdot}130 \pm 0{\cdot}002$, $0{\cdot}105 \pm 0{\cdot}002$ and $0{\cdot}110 \pm 0{\cdot}003$ µL min^{-1} for 1·4, 2·6, 5 and 10 µL min^{-1}, respectively.

Brain, blood and dialysate levels

Average contents (µg g^{-1} wet weight) of valproic acid in the striatum were $60{\cdot}7 \pm 2{\cdot}9$ (n = 16) and $2{\cdot}1 \pm 0{\cdot}3$ (n = 6); for the cortex $55{\cdot}3 \pm 3{\cdot}1$ (n = 14) and $1{\cdot}5 \pm 0{\cdot}7$ (n = 4); and for the cerebellum $60{\cdot}3 \pm 3{\cdot}2$ (n = 16) and $1{\cdot}1 \pm 0{\cdot}3$ (n = 9), 5 and 90 min after i.v. administration, respectively.

Using the in-vitro determined K_oA value (0·130 µL min^{-1})

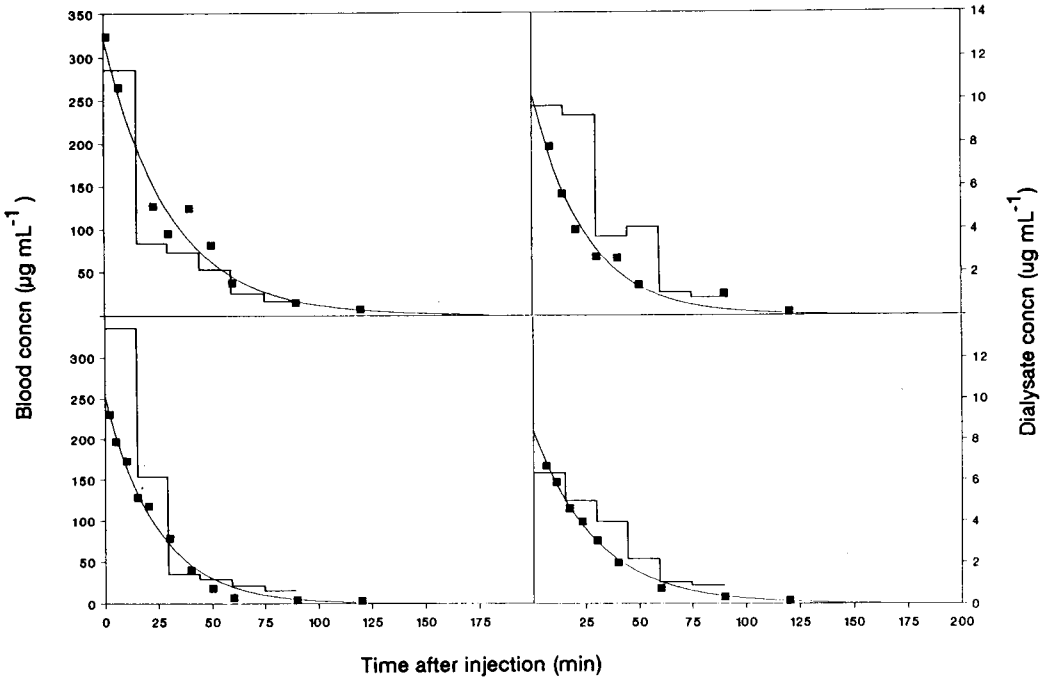

FIG. 3. Time course of the measured valproic acid in whole blood (■), the fitted line through the experimental data (solid line) and the dialysate fractions (histogram) after an i.v. injection of 25 mg of sodium valproate (n = 4).

we calculated the ratio of C_o and the blood concentration of valproic acid. The ratio of C_o to the blood values at a perfusion rate of 1·4 μL min^{-1} during the first 90 min was 0·696 ± 0·066 (n = 24). During this period the concentration in the extracellular space was thus about 70% of the blood concentration of valproic acid. At 90 min the blood levels were 9·33 ± 2·53 μg mL^{-1}. Assuming that all the valproic acid of the blood was in the plasma, then by using the haematocrit as 44·5%, plasma concentrations were 16·80 ± 4·43 μg mL^{-1}.

The data obtained from blood and dialysate measurements and the fitted curves through the data of each of the four rats are given in Fig. 3. According to the known plasma pharmacokinetics of sodium valproate (Dickinson et al 1979; Ogiso et al 1986) the data were fitted for both mono- and biexponential decay. Both fitting procedures resulted in virtually the same residual sum of squares and the same correlation coefficient. Therefore for further calculations only the monoexponential decay curve was used. All the blood levels of valproic acid, obtained either by measurements or by calculation were plotted against the dialysate data, measured at, or extrapolated to, a perfusion rate of 1·4 μL min^{-1} (Fig. 4). The correlation factor found (r = 0·92) suggested that the brain extracellular and the blood valproate achieved rapid equilibrium. A similar high correlation coefficient was obtained by taking all the measured values of valproic acid; these data could be derived directly from Fig. 3, and are therefore not illustrated here. This conclusion allows the correction of dialysate levels to a certain time (here 90 min). This is necessary for the calculation of the extracellular levels of valproate with the Jacobson approach.

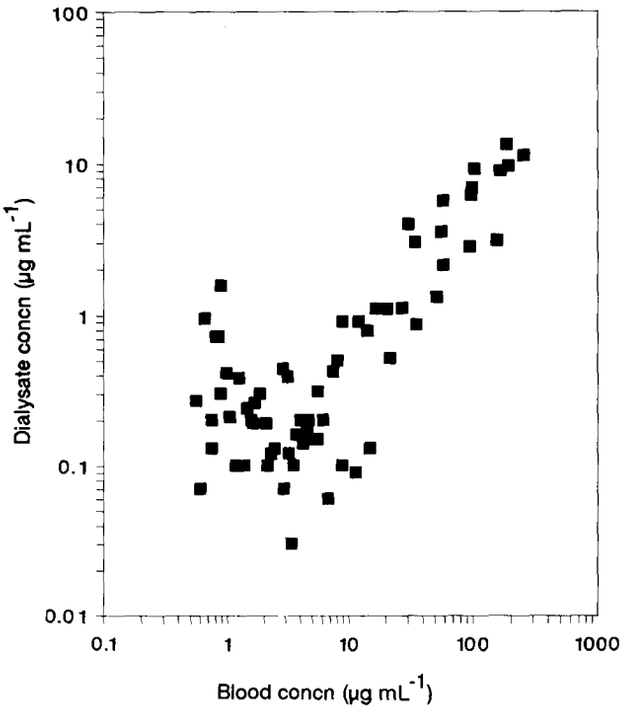

FIG. 4. The blood concentration vs the measured or calculated dialysate concentration of valproic acid. Samples were obtained from four rats for up to 2·5 h after injection of valproate. The perfusion rate was 1·4 μL min^{-1}. r = 0·92, n = 64.

We applied both the Jacobson method and our simplified formula to the dialysis data. Firstly we normalized all the dialysis values using the decay value obtained for the individual rats to t = 90 min. Then we used the original Jacobson formula to calculate C_o using the value for K_oA determined in-vitro. Using the first term of the series expansion of the Jacobson formula we plotted the dialysis against the inverse flow rate. A correlation coefficient of 0·98 was found; the slope is now $C_o K_oA$. Using the in-vitro value of K_oA (0·130 min μL^{-1}) we calculated a C_o for all data. The value at t = 90 min was 5·90 ± 0·90 μg mL^{-1}. Doing the same for the dialysate concentration divided by the value of blood at 90 min for each rat, resulted in a ratio of 0·75 ± 0·17 μL min^{-1}. The K_oA used so far was obtained in-vitro. The theoretical maximum value that C_o can reach is its plasma concentration, that is maximally about 1·82 times the level in blood. It is then assumed that there is a near equilibrium state between the extracellular concentration and the plasma concentration. The minimal K_oA value thus calculated for the flow rates 1·4 and 2·6 μL min^{-1} is 0·030 ± 0·003 (n = 26).

A final attempt to estimate the brain extracellular concentration of valproic acid was made by assuming a pseudo steady state concentration. Thus we assumed that at any time after the injection of sodium valproate the pharmacokinetic rate constants (predominantly metabolism) are small compared with the physicochemical rate constants of diffusion of the drug. The various compartments considered were blood, extracellular space and the cellular space of the striatum, which comprises 2, 18 and 80%, respectively, per unit volume or unit weight of the dissected brain samples (Korf & Postema 1988). The occurrence of a pseudo steady state concentration was demonstrated by the near constant ratio of drug concentration in brain tissue and blood (including the ECS) to blood at both 5 and 90 min (13·1 ± 3·6 and 10·1 ± 1·2 at 5 and 90 min, respectively).

It is unlikely that the extracellular concentration of valproate is the same as the striatal cellular concentration, as can be deduced as follows. Assuming that the mean concentrations of valproic acid in the extra and intracellular spaces are similar, then at t = 90 min there would be an extracellular concentration of 1·91 μg mL^{-1} and thus a K_oA value of 0·264 μg mL^{-1}. In general, however, dialysis from aqueous solutions in-vitro is more efficient than in-vivo, (see Benveniste et al 1989) and therefore the K_oA value determined in-vivo should be lower than that found in-vitro. The actual value of K_oA calculated in-vivo was twice that found in-vitro, and therefore the hypothesis can be rejected and the extracellular valproate concentrations are thus higher than the average intracellular concentrations.

In Table 1 the valproic acid concentrations of the ECS at 90 min, as calculated using the above approaches, are summarized. The values obtained with dialysis are similar and more than 50% of the striatal content of valproate seem to be confined to the extracellular space. A small fraction of valproic acid of the plasma can diffuse directly into the ECS. This could well be the free fraction (i.e. the non-protein bound fraction, amounting to approximately 37%, Chapman et al 1982). The ECS concentrations calculated with the dialysis approach are close to the calculated free concentrations of valproate in plasma (about 5·5–6·0 μg mL^{-1} compared with 6·2 μg mL^{-1}).

Table 1. Calculated extracellular levels of valproate in the rat striatum 90 min. after i.v. injection of the drug.

Method	Assumption(s)	C_o ($\mu g\ mL^{-1}$) ±s.e.m.	% of valproate	
		±s.e.m.	ECS	Cell compartment
Constant dialysis flow rate (1·4 $\mu L\ min^{-1}$)	K_oA in-vivo is the same as in-vitro	6·53±1·87	64	36
Jacobson method	K_oA in-vivo is the same as in vitro	5·44±0·40	53	47
Reduced relationship of Jacobson formula $C_i = C_oK_oA/F$	K_oA in-vivo is the same as in-vitro	5·90±0·90	57	43
All valproate in blood is confined to plasma	Complete transfer of the drug from blood plasma to the ECS	16·96±4·74	no acceptable values	
All valproate in blood is confined to plasma	Only the free fraction (37%) diffuses into the ECS	6·21±1·63	58·5	41·5

Discussion

Application of automated precolumn derivatization techniques allows a highly sensitive assay of valproic acid and avoids time-consuming sample preparation as required in most other assays (e.g. Kupferberg 1982; Wolf et al 1989). The coupling of the drug to the fluorescent label bromomethylmethoxycoumarin is achieved at room temperature (20°C) by catalysis in the injection loop of a specially designed HPLC autosampler. As an example of its usefulness, the valproate assay was applied to monitor dialysate levels collected frequently and at a relatively high perfusion rate. Virtually none of the current assays for valproic acid are sufficiently sensitive to allow such measurements.

As in most other studies (e.g. Nau & Löscher 1982; Löscher & Nau 1983; Hariton et al 1984) we observed no major difference in levels of valproic acid in the brain regions studied. Our study also indicated that, at least at 5 and 90 min after injection, brain levels followed those in the circulation, it can be assumed that there is a rapid exchange of the drug between the various brain compartments and blood. This conclusion implies that at any time after injection of sodium valproate the data may be treated as being in a pseudo steady state; a real steady state is not reached because of the continuously decreasing drug concentrations as the result of metabolism and elimination. The elimination could be described in a mono exponential function as has been shown by others (e.g. Dickinson et al 1979; Löscher & Nau 1983; Ogiso et al 1986). Such a mono-exponential decay is particularly evident in the first 90 min after injection. Thereafter, the blood levels remained low but constant or may even have increased (unpublished data: Ogiso et al 1986).

The current attempt to estimate the extracellular level of valproate not only depends upon dialysis concentrations, but also on the assumed dialysis efficacy in-vivo. In all our calculations we used the in-vitro determined diffusion rate constants. Using constant perfusion rates there are few alternatives (see also below), but using the variable perfusion rates as described by Jacobson et al (1985) the dialysis rate constants may be estimated in-vivo. A major problem, however, is that the extracellular concentration of the compound to be analysed must be constant, which in pharmacokinetic animal studies is rarely the case. Moreover, at the required very low perfusion rates (e.g. below 0·5 $\mu L\ min^{-1}$), the sampling time is too long to be useful in a study like ours. Therefore we applied higher perfusion rates, with the additional advantage that the complex relationship between perfusion flow rates could be avoided and a simple inverse relationship between concentrations and flow rate could be used in calculating absolute levels outside the fluid measured. Both approaches gave virtually identical values at 90 min. Thus the in-vivo diffusion characteristics are maintained at any flow used as occurs in-vitro.

In related studies on cocaine, in-vitro determined dialysis characteristics have also been used for calculations. In those studies the calculated extracellular levels of cocaine correlated highly with the inhibition of dopamine uptake, seen as an enhancement of the amine in the dialysate (Hurd et al 1988; Nicolaysen et al 1988; Hurd & Ungerstedt 1989). An indication of the extracellular concentration of valproic acid has been obtained by the measurements of drug levels in the cerebrospinal fluid (CSF) of both dog and man (Chapman et al 1982; Löscher & Nau 1983). In both species the CSF levels are less than 15% of the total (free + bound) plasma levels and are close to the free plasma fraction of the drug. In the present study we estimated that in the rat the extracellular brain concentration is also close to the free fraction of the drug in the circulation. Approaches to the estimation of drug levels in body compartments where the drugs exert their pharmacological action have recently been summarized (Dingemanse et al 1988). A major conclusion is that circulating drug levels do not reflect reliably the pharmacodynamic compartment and that for most centrally acting drugs CSF levels are a better reflection. A similar conclusion has been reached for cocaine and dopamine uptake inhibition (Nicolaysen et al 1988). Several centrally active drugs interact with molecular entities localized in the outer cell membrane. Membranous molecular entities that are targets include neuronal receptors, transmitter uptake carriers and ion channels. Psychotropic drugs such as uptake inhibitors (e.g. tricyclic antidepressants) or receptor blockers (e.g. antipsychotics) may therefore approach their major targets from the ECS. If so then the extracellular levels are of far more importance than the tissue levels and drugs may thus be evaluated not by their accumulation in the brain, but rather whether ECS levels are sufficient to obtain pharmacological responses.

The results of the present study may have consequences in the determination of the pharmacodynamic profile of valproate. Considering the even distribution of valproate over the various brain regions, with entirely different cell types, we may assume that the intracellular concentration of valproic acid in each brain cell is of the same magnitude and is only

about 1/4 of that in the ECS. These values have to be taken into accout when searching for the molecular mechanism to which the clinical efficacy of valproate is attributed. For instance intracellular concentrations of maximally 1 mM can be reached only at abnormally high valproate doses. Most of the enzymes involved in the metabolism or synthesis of γ-aminobutyric acid have K_i values for valproate of more than 5 mM and will therefore not be affected by the drug in-vivo. Alternatively, inhibition of glia cell uptake of γ-aminobutyric acid (Nilsson et al 1988) or of picrotoxin binding sites (Chapman et al 1982) may be more likely targets for valproate.

Centrally active drugs (including valproate) may reach relatively high extracellular concentrations, despite poor penetration into brain tissue. Most of the current psychotropic drugs are more lipophilic and relatively high doses have to be administrated to reach high extracellular concentrations. Such high doses may also result in the concomitant accumulation in compartments of the body that are not directly involved in the desired, clinical, effects, and may enhance the risk for unwanted side effects. Knowledge of the brain/ECS concentrations ratio may generate search for drugs that accumulate selectively in the extracellular compartment at low dosages.

Acknowledgement

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