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CORRELATION OF ANALGESIA WITH LEVELS OF TILIDINE AND A BIOLOGICALLY ACTIVE METABOLITE IN RAT PLASMA AND BRAIN

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Abstract—The analgesic ED_{50} of tilidine administered orally to rats was 15·4 mg/kg at 15 min and 17·2 mg/kg at 30 min. The drug was metabolized quickly and extensively, and it and its principal metabolite readily crossed the blood–brain barrier. Analgesia after the oral administration of tilidine fumarate to rats was correlated with levels of this metabolite in plasma and brain, and with the tilidine levels in plasma. It appears probable that the conversion of tilidine into its metabolite, which has analgesic properties, is closely related to the analgesic state.

After the synthesis of tilidine, ethyl 2-(dimethylamino)-1-phenyl-3-cyclohexene-1-carboxylate, by Satzinger [1], its pharmacology was described by Herrmann *et al.* [2], and its clinical activity as an analgesic agent was reported by Teicher and Stelzer [3]. Vollmer and Poisson [4] showed that peroral tilidine administration to humans was followed by rapid and extensive conversion to metabolites (I and II). Using gas chromatography to assay serial plasma samples, Vollmer *et al.* [5] learned that tilidine was metabolized primarily by *N*-monodemethylation* to metabolite I, a compound synthesized by Satzinger [1].

In the present study, we administered the calculated analgesic ED_{50} of tilidine to rats to provide groups of animals which did and did not exhibit analgesia at various intervals after treatment. The same animals tested for evidence of analgesia provided plasma and brain samples which were assayed for tilidine and metabolite I. The aim of this study was to learn whether the analgesic activity of tilidine might be correlated with the levels of the drug and this metabolite in rat plasma and/or brain.

MATERIALS AND METHODS

Oral analgesic activity of tilidine. The method employed has been described by D'Amour and Smith [6]. Male, Sprague–Dawley rats (125–145 g) obtained from Charles River Farms (Wilmington, Mass.) were used. The animals were allowed free access to food and water except during actual testing. The tails of the rats were blackened with India ink 4–5 cm from their tips, and they were sequentially gently restrained to facilitate placement of the blackened area of the tail above a heater coil. The apparatus incorporated a photoelectrically controlled timing circuit and a heater coil which was automatically activated when the rat's tail was placed over the coil. The rats reacted characteristically to the heat by flicking their tails away from the hot coil and thereby deactivating the timer and heater. Their reaction times were measured quantitatively by the number of seconds that elapsed between placement of the tail over the heater coil and demonstration of the end-point. Pre-drug control times were calculated by averaging two reaction times for each rat. Animals exhibiting a positive analgesic response at various times after drug administration were those rats whose pre-drug reaction time had doubled. To prevent thermal injury, the heater coil was deactivated if the animal did not react within 30 sec. Doses of tilidine fumarate (calculated as base) were dissolved in distilled water and administered orally (5–40 mg/kg) to groups of ten rats. The dosage volume was 1 ml/kg. An additional group, which received distilled water, served as control. Rats were tested 15, 30, 60 and 120 min after dosing. Changes in mean reaction time in the tilidine-treated group were compared to the control group by Student's *t*-test or to their pretreatment latency by a paired *t*-test. The analgesic ED_{50} (95% confidence limits) of tilidine was calculated by the method of Litchfield and Wilcoxon [7].

Collection of brain and plasma samples in rats tested for analgesia. Groups of ten rats received 20 mg/kg of tilidine fumarate (calculated as base), and analgesia was assayed as previously described in each group at either 15, 30 or 60 min after drug administration. The order of testing times was randomly determined. Based on the criterion for analgesia at each testing interval after drug administration, rats were designated as exhibiting analgesia (analgesic rats) or not exhibiting analgesia (non-analgesic rats). On randomly assigned days, either analgesic rats or non-analgesic rats were selected

* K.-O. Vollmer, personal communication.

for study. After each test, two rats were anesthetized with ether before blood was collected by cardiac puncture, and the brains were removed. Approximately 4 min elapsed between initiation of the analgesic determination and completion of the sample collection. The samples were chilled prior to assay which was always performed on the same day. Seven pairs of analgesic rats and seven pairs of non-analgesic rats were selected for assay at each time interval after drug administration.

Extraction of rat plasma. Equal volumes (2·5 ml) of plasma from each of two rats (5·0 ml total volume) were mixed vigorously (Vortex) with 2·5 ml of cold 5% trichloroacetic acid. The resulting suspension was mixed gently for 3 min with 15 ml of cold chloroform and centrifuged at 750 *g* for 10–15 min at 0°. After the chloroform was separated, the solid material was resuspended in the aqueous phase and was re-extracted as above with a 5-ml portion of chloroform. The combined chloroform extracts were dried by filtration through anhydrous sodium sulfate.

Extraction of rat brain. The brains from two rats were minced and combined (approximately 4 g total), then homogenized in a chilled Potter–Elvehjem homogenizer with 15 ml of 2% trichloroacetic acid. The homogenate was mixed gently for 3 min with 10 ml of cold chloroform and centrifuged at 750 *g* for 10–15 min at 0°. After separating the chloroform, the solid material was resuspended in the aqueous phase and was re-extracted as above with an additional 10-ml portion of chloroform. The combined chloroform extracts were filtered through anhydrous sodium sulfate.

Concentration of extracts. The filtered chloroform extracts from the plasma or brain samples were concentrated to 10 ml by evaporation under a stream of dry air at room temperature. The chloroform was washed successively with 3 ml of 1 N sodium hydroxide, 5 ml of 0·1 N sodium hydroxide, then was extracted first with 3 ml and finally with 1 ml of 0·1 N hydrochloric acid. The combined acidic extracts were made alkaline by the addition of 0·5 ml of 1 N sodium hydroxide and the liberated bases were extracted with three 1-ml portions of ether. The ether was evaporated carefully to dryness in a stream of dry air. The air stream was removed immediately upon evaporation of the ether to minimize losses of the volatile bases. The residue from the evaporation was dissolved in 20 μl chloroform containing 1 mg/ml of hexadecane, the internal standard for gas chromatographic assay.

Gas chromatography. The separation of tilidine and metabolite I was performed by gas chromatography with flame ionization detection as described by Vollmer *et al.* [5]. A 6 ft × 1/8 in. steel column packed with 1% LAC-4-R-886 on 60/80 mesh Chromosorb G (AW) impregnated with 6% KOH was employed. The column was conditioned prior to use by heating overnight at 190° followed by the injection at normal operating temperature of several 4-μl loading samples of a solution of 32 mg/ml each of tilidine and its metabolite in chloroform. A loading sample was injected daily prior to performing the assays. Normal chromatographic conditions were a column temperature of 128°, an injector temperature of 190° and a carrier gas flow rate of 80 cm^3/min. With these parameters, the retention times of tilidine and its metabolite were 7·0 and 9·5 min, respectively; the hexadecane internal standard appeared as a sharp spike at 0·5 min. Quantitation was obtained from the ratios of the peak areas of tilidine or metabolite I to the peak height of the hexadecane internal standard. These ratios were related to those obtained from calibration curves constructed from the corresponding ratios produced by known amounts of the two bases added to plasma or brain homogenates. The calibration curves were linear over the range of the assays, and concentrations as low as 2 ng/ml could be detected.

Statistical evaluation of results. Analysis of variance performed on the results of the chromatographic assays showed the variances to be homogeneous only upon logarithmic transformation of the data. This homogeneity was observed not only in time but also in analgesic vs non-analgesic comparisons. As a consequence of the transformation, the mean values calculated were geometric rather than arithmetic means. The comparison between analgesic and non-analgesic groups was made by applying Student's t to the means of the transformed data. The ratios of the geometric means were tested against unity.

Table 1. Effect of tilidine fumarate on reaction time to heat stimulus applied to rat tail

Dose* (mg/kg, p.o.)	Mean wt† (g ± S. E.)	Reaction time (sec ± S. E.) after administration 15 min	30 min	60 min	120 min
Control	142·3 ± 2·3	4·0 ± 0·7	3·2 ± 0·3	4·0 ± 0·6	4·8 ± 0·9
5	139·3 ± 3·0	5·2 ± 0·8	5·2 ± 1·1	4·8 ± 0·8	4·9 ± 0·6
10	134·5 ± 3·6	8·0 ± 2·5	7·6 ± 2·6	5·1 ± 0·7	4·7 ± 0·7
20	129·9 ± 2·5	14·6 ± 4·4‡	9·2 ± 2·5‡	5·3 ± 0·7	4·3 ± 0·6
40	138·6 ± 3·7	19·3 ± 3·7§	17·6 ± 3·3§	11·7 ± 3·3‡	12·9 ± 3·5‡

* Calculated as tilidine base.
† Ten rats/group.
‡ $P < 0.05$ vs control.
§ $P < 0.001$ vs control.

RESULTS

Oral analgesic activity of tilidine fumarate and determination of its ED_{50}. The onset of analgesia after tilidine administration was within 15 min, and the lowest dose which significantly increased the reaction time of the tilidine-treated group compared with the control group was 20 mg/kg. Group reaction time was significantly elevated for 30 min after the 20 mg/kg dose and for as long as 120 min after the 40 mg/kg dose compared with the control group (Table 1). Since the proportion of animals meeting the criterion of analgesia was not greater than 50 per cent beyond the 30-min testing interval (Table 2), the duration of analgesic activity in rats would appear to be brief. The analgesic ED_{50} calculated at the time of peak activity (15 min) was 15·4 (9·6 to 26·1) mg/kg, and the analgesic activity calculated at 30 min was not significantly less, ED_{50} = 17·2 (8·1 to 59·2) mg/kg.

Table 2. Proportion of rats meeting analgesic criterion after tilidine administration at various dose levels

Tilidine dose (mg/kg, p.o.)	Number of rats* in analgesia at 15 min	30 min	60 min	120 min
Control	0	0	1	0
5	1	3	1	3
10	3	2	2	2
20	7	5	2	2
40	8	8	4	5

* Ten rats/group.

Correlation of plasma and brain levels of tilidine and metabolite I with analgesic condition of rats. Tilidine, administered at a dose (20 mg/kg) which approximated its analgesic ED_{50}, produced analgesia in groups of rats tested at various time intervals after its administration. The criterion of analgesia was satisfied by 39·2 per cent (12 determinations; N = 120) of the animals at 60 min after drug administration, a value significantly lower ($P < 0·01$) than the 15-min group (63·6 per cent; 14 determinations; N = 140) and the 30-min group (57·9 per cent; 14 determinations; N = 140).

Animals were selected from these groups for assay of tilidine and metabolite I in plasma and brain. The mean pretreatment latency (5·6 ± 0·3 sec; N = 42) of rats which exhibited analgesia, and the mean pretreatment latency (4·9 ± 0·2 sec; N = 42) of rats which did not exhibit analgesia after the administration of tilidine did not differ significantly from each other. When the response latencies of tilidine-treated groups were combined, for all three time periods after administration, the mean post-treatment latency (5·4 ± 0·4 sec; N = 42) of non-analgesic rats did not differ significantly from the mean of their pretreatment latencies (*vide supra*). However, the response latencies of rats, selected as exhibiting analgesia, approached the maximum latency possible (30 sec).

Plasma levels of tilidine and metabolite I in rats. The concentrations of tilidine and metabolite I found in the rat plasma samples are given in Table 3. Although the concentrations of tilidine in the plasma of the analgesic animals were higher than in the non-analgesic animals at each time period, the ratio of analgesic to non-analgesic concentrations did not differ significantly. The consistently higher values for the analgesic animals are reflected by the means of all three time periods. However, the mean tilidine plasma concentration of 0·057 μg/ml in the analgesic animals is significantly greater ($P < 0·03$) than the 0·032 μg/ml in the non-analgesic animals. The concentrations of metabolite I in the plasma of the analgesic animals were significantly higher than those of the non-analgesic animals at each time interval. The mean metabolite I plasma concentration of 0·249 μg/ml was also significantly greater ($P < 0·001$) than 0·121 μg/ml in the non-analgesic animals.

Brain levels of tilidine and metabolite I in rats. The concentrations of tilidine and metabolite I found in the rat brains are given in Table 4. The mean concentration of 0·29 μg/g tilidine in the brains of the analgesic rats was not significantly greater than the 0·21 μg/

Table 3. Concentration of tilidine and metabolite I in rat plasma after a 20 mg/kg tilidine dose in animals which either did or did not exhibit analgesia at each time period

Component	Time after dose (min)	Analgesic (μg/ml* in plasma)	Non-analgesic (μg/ml* in plasma)	Ratio† (analgesic/non-analgesic)
Tilidine	15	0·063	0·042	1·50 ($P < 0·32$)
	30	0·094	0·046	2·05 ($P < 0·84$)
	60	0·031	0·017	1·80 ($P < 0·15$)
Mean		0·057	0·032	1·77 ($P < 0·03$)
Metabolite I	15	0·347	0·179	1·94 ($P < 0·03$)
	30	0·322	0·158	2·04 ($P < 0·02$)
	60	0·138	0·064	2·16 ($P < 0·02$)
Mean		0·249	0·121	2·05 ($P < 0·001$)

* Geometric means of seven values (rounded off).

† Ratios of unrounded values; P = probability of significant difference from ratio of 1·00; pooled coefficient of variation (36 degrees of freedom) 112% for tilidine and 73% for metabolite I.

Table 4. Concentration of tilidine and metabolite I in rat brain after a 20 mg/kg tilidine dose in animals which either did or did not exhibit analgesia at each time period

Component	Time after dose (min)	Analgesic (μg/g* in brain)	Non-analgesic (μg/g* in brain)	Ratio† (analgesic/non-analgesic)
Tilidine	15	0·36	0·23	1·56 ($P < 0·24$)
	30	0·46	0·30	1·52 ($P < 0·29$)
	60	0·15	0·12	1·17 ($P < 0·68$)
Mean		0·29	0·21	1·41 ($P < 0·13$)
Metabolite I	15	2·37	1·37	1·73 ($P < 0·14$)
	30	2·32	1·14	2·04 ($P < 0·06$)
	60	0·77	0·24	3·26 ($P < 0·003$)
Mean		1·61	0·72	2·26 ($P < 0·001$)

* Geometric means of seven values (rounded off).

† Ratios of unrounded values; P = probability of significant difference from ratio of 1·00; pooled coefficient of variation (36 degrees of freedom) 101% for tilidine and 99% for metabolite I.

g in the non-analgesic rats, nor was significance shown for the ratios of concentration at the three time periods. The mean concentration of 1·61 μg/g of metabolite I in the brain of the analgesic rats was significantly greater ($P < 0·001$) than the 0·72 μg/g in the non-analgesic rats. Although the ratio of 3·26 at 60 min alone is highly significant ($P < 0·003$), the values at 15 min and 30 min of borderline significance also contribute to the significance of the mean.

DISCUSSION

Very little data have been obtained until recently on the central nervous system levels of analgesic drugs. According to Mulé [8], the principal reason for the dearth of experimentation appears to be the sensitivity of the available chemical techniques. Some investigators [9–12] have reported good correlations between analgesic drug and/or metabolite concentrations in plasma and/or brain and analgesic efficacy or duration, although others [13, 14] did not observe correlations in brain samples of animals studied in their laboratories. In the present investigation, we have used the sensitive gas chromatographic technique, introduced by Vollmer *et al.* [5], for the assay of tilidine and its metabolite, and have shown levels of tilidine in plasma and metabolite I in plasma and brain to be correlated with the analgesic state of the animals.

The rapid induction of analgesia after the oral administration of tilidine fumarate is consistent with: (1) the rapid drug absorption observed by assaying rat plasma, (2) the rapid and extensive conversion of tilidine to a metabolite which also has analgesic properties [4] and (3) the facile penetration of tilidine and this metabolite into the rat brain. In confirmation of the literature, administration of tilidine to unfasted animals in our study did not appear to alter its analgesic activity, since the ED_{50} which we obtained did not differ significantly from the ED_{50} obtained, at the time of peak activity, by Herrman *et al.* [2] after oral administration of tilidine to fasted rats tested in a procedure similar to ours. When individual time periods are disregarded, there appears to be no level of tilidine or metabolite I concentration in plasma or brain which may be regarded as a threshold for the elicitation of analgesia. A similar conclusion can be drawn from the experiments of Paalzow and Arbin [12] regarding levels of the analgesic drug, pentazocine, in plasma and brain of mice which either did or did not exhibit analgesia.

It was possible to correlate tilidine and metabolite I levels with analgesia. Onset of analgesia corresponded with the occurrence of maximal levels of metabolite I in plasma and brain of all rats. However, neither analgesia nor drug levels were assayed earlier than 15 min after dosing. Peak plasma and brain levels of tilidine were not obtained until 30 min, and this delay may reflect the rapid metabolism of tilidine. The lowest levels of tilidine and metabolite I were found in plasma and brain at 60 min, at which time the proportion of animals exhibiting analgesia was lowest.

The analgesic state of these animals was reflected by neither the plasma nor the brain tilidine levels at the individual test periods; but on the average (pooled tilidine levels for these test periods), the plasma level of tilidine was greater in rats exhibiting analgesia. It is possible that rats exhibiting analgesia absorbed tilidine more completely after its oral administration. On the other hand, analgesia was more closely temporally correlated with plasma and brain metabolite I concentrations, and it is most probable that the degree of biotransformation of tilidine into its metabolite is related positively to the analgesic state of the animals. Paalzow and Arbin [12] made the opposite observation regarding plasma levels of pentazocine and hydroxypentazocine. In their study, mice lacking analgesia seemed to metabolize pentazocine more extensively than animals which showed an effect. Whereas the metabolite of tilidine has analgesic activity [4], all known metabolites of pentazocine were reported by Berkowitz and Way [11] to have practically no analge-

sic activity in comparison to pentazocine itself.* It is of interest that the elevation and decline of tilidine and its metabolite in brain tissue are closely correlated with changes in plasma levels, suggesting that the drug may be bound in a freely reversible manner to brain tissue.

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