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Distribution of apomorphine enantiomers in plasma, brain tissue and striatal extracellular fluid

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Abstract

Steady-state concentrations of apomorphine enantiomers were measured in the extracellular fluid collected from rat brain striatum by microdialysis. The free and total concentrations of both enantiomers were also measured in plasma as well as the total concentrations in different brain regions (striatum, cortex and cerebellum). We noticed no regional difference in the total concentrations of the two enantiomers. The extracellular concentrations were much lower, amounting to 8% for *R*(−)-apomorphine and 4% for *S*(+)-apomorphine, of the total brain tissue concentrations. The microdialysis samples contained 12 times more (*R*(−)-apomorphine and 5 times more *S*(+)-apomorphine than the free apomorphine measured in plasma. The extracellular concentrations of *R*(−)-apomorphine (129 ± 20 pmol/ml) were significantly higher ($P = 0.001$, $n = 6$), than those of *S*(+)-apomorphine (70 ± 10 pmol/ml). These results indicate that both enantiomers of apomorphine concentrate equally in brain cells, and that a stereoselective uptake system could operate for *R*(−)-apomorphine at the blood–brain barrier level. © 1997 Elsevier Science B.V.

Keywords: Apomorphine; Enantiomer; Biodistribution; Uptake, stereoselective, active

1. Introduction

In recent years, apomorphine has gained interest as an effective and safe diagnostic tool to assess dopamine responsiveness of parkinsonian syndrome (D'Costa et al., 1991; Gasser et al., 1992; Hughes et al., 1990). The drug has also increasingly been used in a 'rescue therapeutic strategy' for reversing sudden 'off' periods in Parkinson's disease since it displays a potent and rapid action (Goetz and Diederich, 1992), attributed to its dopamine D_1 and D_2 receptor agonist properties.

However, studies indicate that apomorphine displays complex pharmacological, biochemical and behavioural attributes which cannot simply be explained by its dopamine receptor agonist effects (Colpaert et al., 1976). In clinical practice also, it has some aspects which would not be expected from a dopamine agonist. It alleviates conditions normally worsened by levodopa, like Huntington's chorea and when combined with levodopa, it consistently reverses the 'on-off' fluctuations in Parkinson's disease which are normally precipitated by levodopa itself (Dannielle et al., 1995). It has been suggested, among other hypotheses, that these discrepancies could be due to uptake mechanisms which concentrate apomorphine in different regions of the central nervous system, preferably dopaminergic regions, initiating concentration-dependent pharmacological activities which cannot be correlated with in vitro experiments. Though apomorphine accumulation by brain compared to plasma has been reported (Biachi and Landi, 1985), previous results on the regional distribution and the role of the dopaminergic uptake system have been contradictory (Butterworth et al., 1975; Kebabian, 1978; Melzacka et al., 1978; Westerink and Horn, 1979). Although these contradictions could be due to the use of different doses, different routes of administration and different time schedules, it is also conceivable that the equilibrium between different compartments was not achieved. Therefore a need to conduct the studies at steady-state conditions obtained by a continuous infusion was put forward for the present experiment.

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The aim of this work was to study the biodistribution of apomorphine enantiomers in brain extracellular fluid using a microdialysis technique. Brain homogenates of different brain regions, and free as well as total concentrations in plasma were measured after subcutaneous infusion of apomorphine to achieve steady-state conditions. Since the physico-chemical properties of *R*(−)-apomorphine and *S*(+)-apomorphine are identical, any difference in local accumulation of the optical antipodes could only be attributed to a stereoselective uptake, hence a pharmacologically significant process.

Information on apomorphine biodistribution is not only helpful in understanding various pharmacological responses observed after apomorphine administration and thereof their clinical significance, but also for the interpretation of data in studies carried out on the central dopaminergic system using apomorphine.

2. Materials and methods

2.1. Chemicals

R(−)-Apomorphine hydrochloride was obtained from Federa (Brussels, Belgium), *S*(+)-apomorphine from Bioblock Scientific (Illkirch, France) and the internal standard *N*-propyl-norapomorphine from Research Biochemicals International (Natick, MA, USA). All reagents were analytical or high-performance liquid chromatography (HPLC) grade and were used as received. The water used throughout was purified by a Millipore Milli-Q system (Bedford, MA, USA).

2.2. Chromatography

Analysis was performed using a slightly modified HPLC method as described before (Sam et al., 1994). The chromatography system consisted of a pump model 6000A, connected to an injector type U6K from Waters Associates (Milford, MA, USA). The detection system consisted of a coulometric electrochemical detector from ESA Coulochem (Bedford, MA, USA) model 5100A using a dual electrode analytical cell model 5010 and a guard cell model 5020. The cells were protected by carbon pre-filters.

The output of the detector was channelled to a computer terminal equipped with Kontron PC Integrator software (version 2.60) for data recording and processing. The mobile phase was freshly prepared daily, filtered through a 0.45 μm filter and then degassed in an ultrasonic bath for 5 min.

Chromatographic separation was achieved using a cartridge placed under radial compression with an internal diameter of 8 mm, 10 cm long, filled with reversed-phase C_{18} material (Nova-Pack) of particle size 4 μm from Waters Associates. A protective guard column filled with the same C_{18} material was placed before the column.

Separation was carried out at 20–22°C. The mobile phase consisted of acetonitrile 20% (v/v) and phosphate buffer (NaH_2PO_4 0.01 M, EDTA 1 mM, pH 3.25) 80% (v/v). The flow rate was 1.8 ml/min. The detection of apomorphine and the internal standard was carried out in the oxidative mode at an operating potential of 0.25 V. The guard cell placed before the injector was operated at a potential of 0.30 V. The detection limit was about 3.5 pmol/ml.

2.3. Microdialysis and apomorphine administration

Microdialysis samples were collected from the striatum of freely moving rats. Male albino Wistar rats (≈ 275 g) were anaesthetised with an intraperitoneal injection of ketamine/diazepam mixture (50 and 5 mg/kg) and placed on a stereotaxic frame. Intracranial guide cannulas (CMA Microdialysis, Stockholm, Sweden) were stereotaxically implanted in the striatum in the following co-ordinates: A +1.2, L +2.5 V +2.8 towards bregma, and firmly fixed on the skull using dental acrylic paste. The cannula was later replaced by a 3 mm probe (CMA 12, CMA Microdialysis, Stockholm, Sweden). A tube to be used for subcutaneous infusion of apomorphine was passed and fixed through the acrylic material and surgically implanted and sutured subcutaneously on the back of the animal. The rats were allowed to recover and the striatum was perfused with Ringers solution (Na^+ 147 mM, K^+ 4 mM, Ca^+ 2.25 mM and Cl^- 147 mM) at a flow rate of 1 μl/min for 18 h prior to the next day experiments. After three baseline collections (60 min), apomorphine was infused subcutaneously through the tube described above at an infusion rate of 2.5 μl/min to give a total amount of apomorphine base of 5 mg/kg weight in 240 min.

The behaviour of the animals was carefully observed during the administration of the apomorphine enantiomers.

Dialysates were collected at 20-min intervals into 5 μl of 0.1 M perchloric acid with 0.001% mercaptoethanol, 0.1% EDTA and *N*-propyl-norapomorphine as internal standard, and assayed for apomorphine.

2.4. Plasma and tissue sample collection

After 240 min of apomorphine infusion during which 12 microdialysate samples were collected, the animals were immediately anaesthetised and a heart puncture was carried out. The blood was collected in tubes containing EDTA and 5 μl of 1% mercaptoethanol per ml of blood. Plasma was separated from the cells and used for analysis of total and free apomorphine. The brain was immediately removed from the skull, placed on dry ice and samples of cortex, cerebellum and striatum were dissected, weighed and homogenised in 1 ml of ice-cold 0.1 N perchloric acid with 0.005% mercaptoethanol and 0.1% EDTA using a Teflon pestle in a glass Potter immersed in ice-cold water.

The whole dissection procedure of the brain was carried out within 10 min.

2.5. Sample processing and analysis

The microdialysate samples were injected directly into the HPLC system for apomorphine analysis. For the plasma and brain homogenates samples, 250 μl aliquots were taken, adjusted to pH 7.4 and spiked with the internal standard. The samples were then vortex mixed with diethylether (5 ml) for 30 s, and centrifuged for 5 min at $1000 \times g$. The diethylether layer (4.0 ml) was then back extracted in 250 μl of 0.1 M HCl with 0.001% mercaptoethanol, and analysed.

Free apomorphine was determined in ultrafiltered plasma ($2000 \times g$, 25°C for 10 min) using Centrifree membrane filters (molecular cut-off 30 000) from Amicon (Beverly, MA, USA). Aliquots of the filtrate were spiked with the internal standard and extracted as plasma above. Since these samples were much cleaner, the etherial extracts were dried in nitrogen and reconstituted in 0.1 M HCl (affording a concentration step) and analysed.

2.6. Recovery during microdialysis

Recovery studies were performed using in vivo retrodialysis. Apomorphine solution (1 μM) in Ringer's solution (with 0.001% mercaptoethanol) was perfused through the microdialysis probe into the striatum of freely moving rats at a flow rate of 1 μl/min. Dialysates were collected at 20-min intervals into 5 μl of 0.1 M perchloric acid with 0.001% mercaptoethanol, 0.1% EDTA and the internal standard. Recovery was calculated as the percent loss of apomorphine in the dialysates.

2.7. Data analysis

The amounts of apomorphine were expressed as pmol/ml in the dialysate and plasma or as pmol/g in the brain tissue. The microdialysis data were corrected for the in vivo relative recovery of the probe. Unless otherwise stated, all values are expressed as mean ± S.E.M. obtained in six animals. The data were compared to each other using Student's *t*-test with the significance level set at 0.05.

3. Results

3.1. Behavioural response

As expected, administration of *R*(−)-apomorphine induced sniffing, licking, agitation, chewing and over-reactivity. This behaviour which started about 20 min after the start of *R*(−)-apomorphine infusion, was intense, repetitive (stereotype) and was exhibited throughout the infusion period. On the contrary, administration of *S*(+)-apomorphine did not evoke any obvious behavioural changes reflecting their different affinity and activity on dopamine receptors.

3.2. Kinetics of apomorphine in the microdialysate

In the present experiment, we were able to follow the appearance of apomorphine in the extracellular compartment of the rat striatum during a subcutaneous infusion. Table 1 reports the evolution of apomorphine concentration in the microdialysate for the two enantiomers in six animals.

Table 1
Concentration of apomorphine (pmol/ml) in the microdialysate

Time (min)	*R*(−)-Apomorphine (*n* = 6)						*S*(+)-Apomorphine (*n* = 6)					
	1	2	3	4	5	6	1	2	3	4	5	6
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
40	15.0	8.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
60	24.0	15.0	10.0	0.0	7.0	27.0	0.0	5.5	9.0	5.0	4.0	0.0
80	25.1	22.0	18.5	12.8	12.5	33.5	7.0	5.5	10.0	7.5	6.3	5.0
100	31.0	29.7	41.5	15.0	13.4	33.5	12.0	11.5	17.5	6.0	6.5	7.0
120	29.0	29.5	42.0	19.5	18.1	34.5	14.0	15.5	15.5	9.5	9.5	14.0
140	32.2	30.5	43.0	21.5	19.0	33.0	13.8	21.5	24.0	12.0	9.0	12.5
160	33.5	30.7	43.5	27.5	21.2	35.5	15.5	22.0	24.5	12.0	14.0	17.0
180	31.5	31.7	45.5	28.9	21.3	35.5	16.3	21.5	26.0	10.0	18.0	15.5
200	33.2	32.5	43.0	29.4	23.0	35.5	18.0	24.0	25.5	13.0	17.0	19.0
220	32.0	30.5	41.0	26.5	21.4	36.5	17.0	22.0	25.5	10.0	19.5	16.5
240	33.0	32.7	43.5	27.6	23.5	34.0	17.5	21.5	26.5	12.0	19.0	16.5
C_{ss}	32.7	31.9	42.5	27.8	22.6	35.3	17.5	22.5	25.8	11.7	18.5	17.3

Concentration of apomorphine enantiomers (pmol/ml) in the microdialysate collected in 20-min intervals from rat striatum during subcutaneous infusion of apomorphine (5 mg/kg) for 240 min. Steady-state concentrations C_{ss} were calculated as the means of the last three data points.

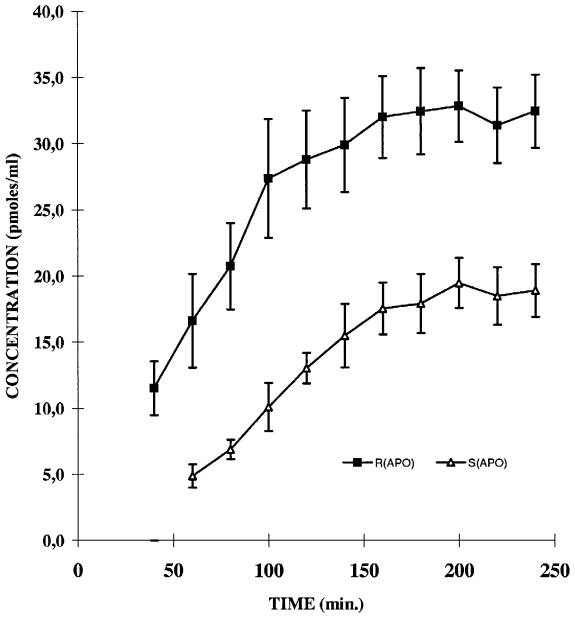

Fig. 1. Mean concentrations ($n = 6$) of $R(-)$-apomorphine (closed squares) and $S(+)$-apomorphine (open triangles) in the microdialysate samples (20 μl) collected at 20 min intervals, plotted against time. Apomorphine (5 mg/kg) was administered as a subcutaneous infusion (2.5 μl/min) for 240 min.

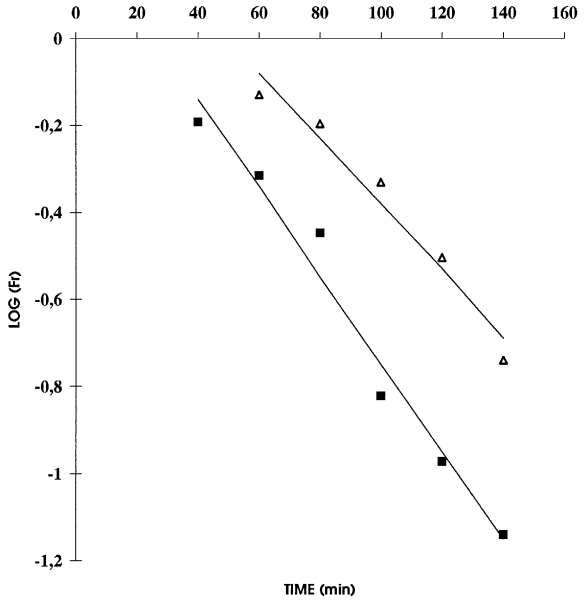

Fig. 2. Relationship between the logarithm of the fractions of concentrations to steady state (Fr) and time. The linear relationship gave correlation coefficients of 0.98 and 0.97 and slopes of −0.01 and −0.0076 for $R(-)$-apomorphine (closed squares) and $S(+)$-apomorphine (open triangles), respectively.

Since equilibrium was achieved in less than 2 h after the start of the infusion in all animals we took the means of the last three data points to determine the mean steady-state concentrations (C_{ss}) in the microdialysate for each animal. The mean microdialysate concentrations of $R(-)$-apomorphine were 32.2 ± 2.8 pmol/ml ($n = 6$), which was significantly higher ($P = 0.001$) compared to those of $S(+)$-apomorphine with a value of 18.9 ± 2.0 pmol/ml ($n = 6$).

Fig. 1 shows the mean concentrations of apomorphine enantiomers in the microdialysate plotted against time. Mean concentrations of $R(-)$-apomorphine were significantly higher than those of $S(+)$-apomorphine at all time points ($P > 0.002$ in all cases). Apomorphine concentration building up in the microdialysate followed a somewhat delayed and apparently exponential curve. The delay was due to lag time (t_o) required for the central and peripheral compartments transfer and the void volume of the microdialysate tubing. Although the pattern is not strictly monoexponential, we were able to analyse the data on the basis of monoexponential kinetics.

In a monoexponential system, the evolution of compartmental concentrations during an infusion is given by:

$$C_t = R_o/VK\ (1 - e^{-Kt}) \quad (1)$$

where C = represents concentration at any time t; R_o = is the infusion rate; V = the volume of distribution of the compartment; K = first order rate constant.

After 4–7 times half-life, C_t approaches C_{ss}, the steady-state concentrations at equilibrium and Eq. (1) becomes:

$$C_t = C_{ss}(1 - e^{-Kt}) \quad (2)$$

Rearrangement gives:

$$1 - (C_t/C_{ss}) = e^{-Kt} \quad (3)$$

The value on the left side of Eq. (3) is the fraction to reach equilibrium (Fr) at any time:

Therefore: $Fr = e^{-Kt}$

A plot of log(Fr) versus time affords estimation of K, and t_o.

The relationship between the logarithm of Fr against time for the mean concentrations of the two enantiomers is given in Fig. 2, and Table 2 reports the kinetic data

Table 2
Pharmacokinetic parameters of apomorphine in the striatum

Parameter	$R(-)$-Apomorphine	$S(+)$-Apomorphine
Kinetic constant K (h^{-1})	1.38	1.0
Steady-state concentration C_{ss} (pmol/ml)	32.2	18.9
Lag time t_o (min)	26.6	50.0

Pharmacokinetic parameters of apomorphine enantiomers in the striatum of rat following a subcutaneous infusion of apomorphine enantiomers (5 mg/kg) for 240 min. The parameters were estimated using monoexponential kinetics.

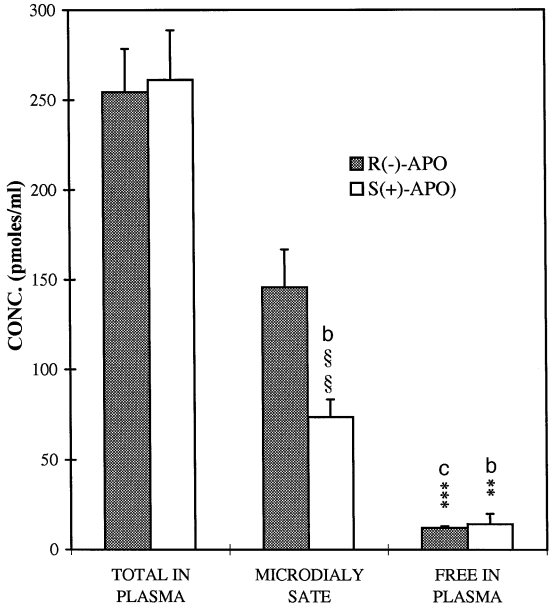

Fig. 3. The absolute extracellular steady-state concentrations of $R(-)$-apomorphine and $S(+)$-apomorphine obtained in rat striatum after continuous subcutaneous infusion of 5 mg/kg apomorphine for 240 min compared to the free and total concentrations in plasma. Each value is the mean ± S.E.M. obtained in six animals. Statistical comparison of free concentrations in plasma compared to microdialysate concentrations for each enantiomer are indicated by [b] ($P < 0.01$) and [c] ($P < 0.001$); and between the two enantiomers in the microdialysate by [b] ($P < 0.01$).

estimated from this approach. These data show that $R(-)$-apomorphine had a shorter lag time compared to $S(+)$-apomorphine indicating that there could be a stereoselective uptake for $R(-)$-apomorphine across the brain–blood barrier. This is also reflected in the first-order kinetic constants for the two enantiomers.

These results show that there is a stereoselective biological phenomenon which could involve a carrier system distinguishing between the two enantiomers across the blood–brain barrier.

3.3. Absolute extracellular concentrations of apomorphine in the striatum

The in vivo relative recovery of apomorphine was $25 \pm 4.6\%$, the absolute concentration (C_{ab}) in the microdialysate was therefore calculated as:

$$C_{ab} = 4C_{ss}$$

These resulted in absolute mean values of 129 ± 20 pmol/ml for $R(-)$-apomorphine and 70 ± 10 pmol/ml for $S(+)$-apomorphine.

3.4. Free and total apomorphine in plasma

Total apomorphine in plasma was similar for both enantiomers. $R(-)$-Apomorphine amounted to 250 ± 20 pmol/ml and $S(+)$-apomorphine to 260 ± 30 pmol/ml.

Table 3
Concentrations of apomorphine (pmol/g) in different brain regions

Brain region	$R(-)$-Apomorphine	$S(+)$-Apomorphine
Striatum	1930 ± 180	1790 ± 300
Cortex	1460 ± 120	1770 ± 210
Cerebellum	1620 ± 170	1400 ± 370

Concentration of apomorphine enantiomers (pmol/ml) in different brain tissues of rat ($n = 6$) dissected immediately after subcutaneous infusion of apomorphine (5 mg/kg) for 240 min.

The free apomorphine in plasma which could cross the blood–brain barrier and therefore be in equilibrium with the extracellular compartment if a simple diffusion process was going on was a small fraction of the total for both enantiomers: $R(-)$-apomorphine 4.8% and $S(+)$-apomorphine 5.4% corresponding to absolute values of 12 ± 1 pmol/ml and 14 ± 6 pmol/ml, respectively. The extracellular concentrations for both enantiomers were much higher compared to those of free apomorphine found in blood, as shown in Fig. 3.

3.5. Total tissue concentrations in various brain regions

Apomorphine was concentrated extensively in different brain regions but concentrations were the same in dopaminergic (striatum) and non-dopaminergic (cortex, cerebellum) regions. The data are summarised in Table 3.

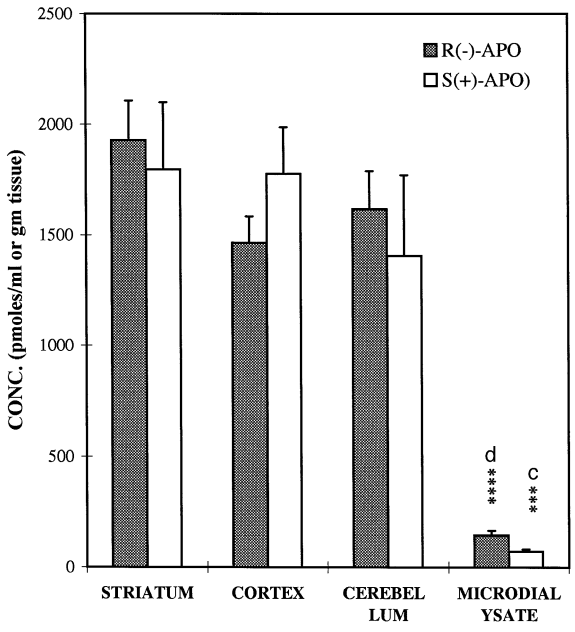

Fig. 4. Concentrations of $R(-)$-apomorphine and $S(+)$-apomorphine in the striatum, cortex and cerebellum obtained after continuous subcutaneous infusion of apomorphine (5 mg/kg) for 240 min compared to steady-state concentrations in the microdialysate from the striatum. Each value is the mean ± S.E.M. obtained in six animals. Microdialysate concentrations differed significantly from striatal tissue concentrations for each enantiomer: [c] ($P < 0.001$) and [d] ($P < 0.0001$).

There was no significant enantiomeric variation in concentration for the three brain regions (Fig. 4) regardless of the significant difference in extracellular concentrations between the two enantiomers.

4. Discussion

The present study reports the distribution of apomorphine in blood and brain of rats after a continuous subcutaneous infusion. Our data show that apomorphine concentrates in brain tissue (more than 12 times), as compared to the extracellular fluid of the striatum where dopamine receptors are located. Though this can be due to simple partitioning related to the high lipophilicity of apomorphine at physiological pH, or due to tissue protein binding (Smith et al., 1985), the eventuality that this is achieved through active transport across the neuronal membrane cannot be ruled out as a mechanism by which these high intracellular concentrations are achieved. However, the possibility that such an uptake system involves a dopaminergic uptake system is ruled out because apomorphine was equally concentrated in dopaminergic and non-dopaminergic regions.

The high intracellular concentrations of *R*(−)-apomorphine could explain the tyrosine hydroxylase inhibitory activity of the drug proposed in in vivo experiments which has not been established in in vitro experiments (Goldstein et al., 1970). Although apomorphine consistently shows higher or equal affinity to dopamine receptors as compared to dopamine itself, it is not as effective when given alone in parkinsonism. However, when apomorphine is combined with levodopa at lower doses it is effective and is useful in reversing the 'off' phenomenon experienced by patients on levodopa alone. This efficacy could therefore point out other mechanisms than just receptor interaction.

Since apomorphine has a free radical scavenging activity in in vitro experiments (Sam and Verbeke, 1995), the high intracellular concentrations may be important during cellular oxidative stress, chemical reactions which are likely mediated intracellularly. This oxidative stress has been suggested as important in the pathogenesis and treatment of parkinsonism.

The free apomorphine in the extracellular fluid collected in the striatum was much higher than the free apomorphine concentrations in plasma. However, since this extracellular compartment is in equilibrium with the cellular compartment which was rich in apomorphine, it is difficult to conclude if these high concentrations are completely due to active transport across blood–brain barrier or a re-distribution from the cellular compartment. Finding that the concentration of *R*(−)-apomorphine was double that of *S*(+)-apomorphine, indicates a possible stereoselective active uptake for *R*(−)-apomorphine across the blood–brain barrier. An alternative explanation could be found in the higher affinity of *R*(−)-apomorphine (Van Tol et al., 1991; Seeman and Van Tol, 1993) for the dopamine receptors located on the cell membranes in the striatum, which could also complicate the equilibrium kinetics of this enantiomer in the extracellular fluid. To clarify this, it will be interesting to study the extracellular concentration in non-dopaminergic regions.

Table 4
Concentrations of apomorphine in brain and plasma in previous studies compared to this study

Dose (mg/kg) and route	Time (min)	Concentration		
		Striatum	Cerebellum	Plasma
10 (i.p.) [a]	20	60.2 (30.1)	27.3 (13.7)	
20 (s.c.) [b]	20	7.1 (1.8)	2.9 (0.7)	
5 (i.p.) [c]	20	2.2	0.4	
10 (s.c.) [d]	15	12.7 (6.4)	9.4 (4.7)	
5 (s.c.) [e]	10	7.2		1.2
5 (s.c.i.) [f]	240	1.9	1.6	0.3

[a] Westerink and Horn (1979); [b] Butterworth et al. (1975); [c] Melzacka et al. (1978); [d] Kebabian (1978); [e] Biachi and Landi (1985); [f] this study; s.c.i. = subcutaneous infusion. Values in parentheses normalised for a dose of 5 mg/kg.

Our data confirm previous results that apomorphine concentrates in the brain tissue as compared to plasma (Biachi and Landi, 1985) and that there is no regional difference in uptake (Kebabian, 1978), but disagree with others which indicated that apomorphine preferably concentrates in the dopaminergic regions, i.e. the striatum (Melzacka et al., 1978), or that distribution is uneven (Westerink and Horn, 1979; Butterworth et al., 1975). However, since we used a steady-state equilibration approach, it is somehow difficult to directly compare all results. Table 4 summarises previous data obtained from rats and compared to this study. When concentrations are normalised to doses, it is suggestive that after subcutaneous injection, concentrations are inversely related to the sampling time indicating that maximum concentrations in the regions sampled were attained much earlier than estimated at 20 min. This relationship may be tissue dependent and indeed, rates of uptake after single injection may be subject to differences in regional blood flow rather than equilibration kinetics. However, after a continuous infusion, the extent of uptake could be more dependent on transport systems, drug partitioning and biological membrane diffusion parameters. The low concentrations in this study reflect the time of administration of 4 h. It is not clear why the intraperitoneal route gave variable results, but factors related to the absorptive area and first-pass metabolism or local oxidation of apomorphine may be involved.

Pharmacokinetics data from humans on apomorphine show that concentrations of apomorphine achieved in the ventricular cerebrospinal fluid (CSF) is much less com-

pared to the total plasma levels (Przedborski et al., 1995; Hofstee et al., 1994). These data do not contradict our data since they compared the free and bound apomorphine in plasma against the free form in the CSF. In this study comparisons of the free apomorphine in the two compartments show that indeed apomorphine concentrates in cerebral extracellular fluid and give more information on apomorphine uptake across the blood–brain barrier.

Whether there exists a clearly defined active and stereoselective uptake of apomorphine needs further investigations. We are now investigating this possibility using cell cultures to narrow down the different interacting mechanisms.

In conclusion, apomorphine enantiomers are equally and uniformly concentrated in different brain tissues as compared to the extracellular fluid. They also concentrate in CSF compared to plasma, but this is more pronounced in the case of *R*(−)-apomorphine indicating an involvement of stereoselective uptake mechanisms.

The neurotropic properties of *R*(−)-apomorphine give answer to some of its pharmacological properties. *S*(+)-Apomorphine shares the same neurotropic property, and therefore should display similar cellular chemical reactions, like free radical scavenging activity, which do not involve stereoselective systems.

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