J. Enzyme Inhibition, 1998, Vol. 13, pp. 473–483
Reprints available directly from the publisher
Photocopying permitted by license only

RAT LIVER CATECHOL-*O*-METHYLTRANSFERASE KINETICS AND ASSAY METHODOLOGY

NUNO BORGES*, MARIA AUGUSTA VIEIRA-COELHO, ANTÓNIO PARADA and PATRÍCIO SOARES-DA-SILVA

Department of Research and Development, BIAL, 4785 S. Mamede do Coronado, Portugal

(Received 25 November 1997; In final form 9 April 1998)

In mammals, catechol-*O*-methyltransferase (COMT) is distributed throughout various organs, the highest activities being found in the liver and kidney. However, comparisons of the kinetic parameters are difficult to perform, since the experimental procedures in the enzyme assay vary quite considerably. The present work was aimed at studying the optimal liver COMT assay conditions for determining the kinetics of the enzyme. The COMT assay was performed with liver homogenates from 60 days old male Wistar rats with adrenaline (AD) as the substrate. Time course experiments using 100 μM S-adenosyl-L-methionine (SAMe) and 300 μM AD showed linearity of *O*-methylation reaction upto 10 min. Using 100 μM SAMe, V_{max} (nmol mg protein $^{-1}$ h $^{-1}$) and K_m (μM) values progressively decreased respectively from 22.1 and 104.8 at 5 min down to 5.8 and 24.62 at 60 min incubation periods. This decrease was not due to end-product inhibition. Using 2500 μM AD, K_m values (μM) for the methyl donor SAMe increased progressively from 174 at 5 min upto 1192.5 at 60 min; upto 30 min of incubation V_{max} values did not change. When a 5 min incubation period and 500 μM SAMe were used, V_{max} and K_m values for liver COMT were 63.4 nmol mg protein^{-1} h^{-1} and 261.1 μM, respectively. It is concluded that an incubation period of 5 min and a SAMe concentration of 500 μM provide optimal conditions for the liver homogenate COMT assay.

Keywords: Catechol-*O*-methyltransferase; Liver; Adrenaline; Metanephrine

INTRODUCTION

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) is an ubiquitous enzyme in mammals, which catalyses the transfer of a methyl group to a catechol

* Corresponding author. Tel.: 351-2-9866100. Fax: 351-2-9866192.
E-mail: DID.BIAL@mail.telepac.pt.

moiety. This process is important both in the inactivation of exogenous and endogenous compounds, namely catecholamine neurotransmitters.[1]

The organs that usually exhibit higher COMT activities are the liver and the kidney (for a review see Ref. [2]). The liver is considered as playing an important role in the removal of circulating catecholamines in many species.[3]

The interest in COMT inhibition has increased in the last decade, mainly due to the potential use of COMT inhibitors in the treatment of Parkinson's Disease.[4,5] The rationale for the use of COMT inhibitors is based on their capacity to inhibit the methylation of L-DOPA to 3-*O*-methyldopa, which competes with L-DOPA in gaining access to the brain.[5] Among recently developed COMT inhibitors, nitrocatechol derivatives have been shown to be the most potent and selective of all, namely tolcapone and entacapone.[6–8] Both drugs are currently undergoing clinical trials for Parkinson's disease,[9,10] and their use has also been hypothesised in other pathological conditions.[11–14]

Despite the growing interest in this enzyme, to our knowledge no systematic study has been conducted concerning the methodology of COMT evaluation so that each author chooses his own conditions so making the results obtained hardly comparable between workers. In this paper we attempted to establish the optimal conditions for the study of rat liver COMT. A preliminary account of some of these results was presented at the 8th International Catecholamine Symposium, in Asilomar (October 1996, CA, USA).

MATERIALS AND METHODS

Preparation of Liver Homogenates

Livers from 60 days old male Wistar rats weighing 250–280 g (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal), kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C), were used in all experiments. After decapitation under light anaesthesia, the livers were immediately removed and homogenised in 5 mM phosphate buffer, pH 7.8.

Assay of COMT

COMT activity was evaluated by the ability to methylate adrenaline (AD) to metanephrine (MN), as previously described.[15] Aliquots of 0.5 ml of liver

homogenates were preincubated for 20 min with 0.4 ml of phosphate buffer (5 mM). The incubation period was started by the addition of substrate (AD) and the incubation medium also contained pargyline (100 μM), $MgCl_2$ (100 μM) and EGTA (1 mM). The preincubation and incubation were carried out at 37°C protected from light, with continuous shaking and without oxygenation.

At the end of the incubation period the tubes were transferred to ice and the reaction was stopped by the addition of 200 μl of 2 M perchloric acid. The samples were then centrifuged (200 ×*g*, 4 min, 4°C), and 500 μl aliquots of the supernatant filtered on 0.22 μm pore size Spin-X filter tubes (Costar) were used for the assay of MN.

Experiments were carried out varying three parameters: time of incubation, substrate concentration and methyl donor concentration. In the first set of experiments liver homogenates were incubated from 1 to 60 min in the presence of 100 μM of S-adenosyl-L-methionine (SAMe) and 300 μM AD. In the second set of experiments saturation curves for the substrate were obtained using AD concentrations from 5 to 2500 μM. Incubation times varied from 5 to 60 min. We also tested the possibility of end-product inhibition of COMT by another *O*-methylated catecholamine metabolite, normetanephrine, or by the SAMe metabolite, S-adenosyl-homocysteine. For this purpose, liver homogenates were incubated in the presence of 5 to 2500 μM AD, 500 μM SAMe and 100 μM normetanephrine or 100 μM S-adenosyl-homocysteine. The incubation period was 5 min.

Saturation curves for the methyl donor were obtained using SAMe concentrations from 100 to 2500 μM. Incubations times varied from 5 to 60 min.

In some experiments, isolated membrane-bound (MB-COMT) or soluble (S-COMT) forms of liver COMT were used. These fractions were obtained from livers of 60 days old male Wistar rats, as described by Nissinen *et al.*[16] with minor changes. Briefly, livers were homogenised 1:4 (w/v) in 5 mM sodium phosphate buffer, pH 7.8. The homogenates were centrifuged at 15,000 ×*g* for 20 min at 4°C, and the supernatants at 100,000 ×*g* for 60 min at 4°C. The high-speed supernatants were used for determination of the soluble COMT activity. The microsomal fraction was washed twice (100,000 ×*g* for 60 min at 4°C) in sodium phosphate buffer and used to determine the activity of membrane-bound COMT. The assay mixture, the preincubation and incubation times and the MN assay protocol were identical to those used for whole liver homogenates.

Assay of Adrenaline and Metanephrine

The assay of AD and MN was carried out using high pressure liquid chromatography with electrochemical detection, as previously described.[15] The chromatography system consisted of a pump (Gilson model 302; Gilson Medical Electronics, Villiers le Bel, France) connected to a manometric module (Gilson model 802 C) and a stainless steel 5 µm ODS column (25 cm length, Biophase; Bioanalytical Systems, West Lafayette, IN). Aliquots (50 µl) of the filtered supernatant were injected by means of an automatic sample injector (Gilson model 231) connected to a Gilson dilutor (model 401). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (8% v/v), adjusted to pH 3.5 with perchloric acid (2 M) and pumped at a rate of 1.0 ml min^{-1}. The detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141). The detector cell was operated at 0.75 V. The current produced was monitored using the Gilson 712 HPLC software. The lower limits for detection of metanephrine ranged from 350 to 500 fmol (0.5–1.0 pmol mg protein h).

Protein Assay

The protein content of the homogenates was determined by Bradford's method with human serum albumin as standard.[17] The protein content was similar in all samples (approximately 10 mg/500 µl homogenate).

Data Analysis

K_m and V_{max} values for COMT activity were calculated from non-linear regression analysis using the GraphPad Prism statistics software package.[18] Geometric means are given with 95% confidence limits and arithmetic means are given with S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Newman–Keuls multiple comparison test to compare values.

RESULTS

The time course experiments for *O*-methylation of AD revealed a linearity ($r^2 = 0.996$) of COMT activity up to 10 min of incubation; from 15 min onwards the rate of *O*-methylation of AD was found to be non-linear (Figure 1).

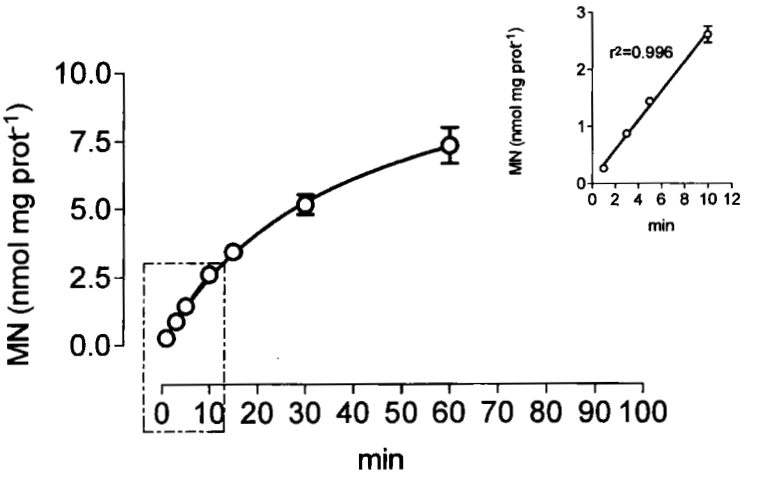

FIGURE 1 Time course for MN formation in liver homogenates. The inset graph shows the linearity of the reaction up to 10 min ($r^2 = 0.996$). Symbols are the means of 4 determinations. Vertical lines indicate S.E.M.

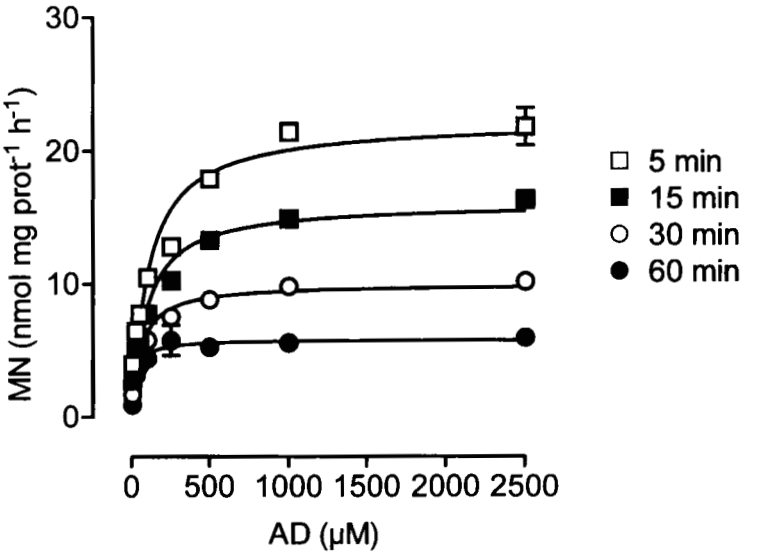

FIGURE 2 Saturation curves obtained with four different incubation times. Kinetic parameters are given in Table I. Symbols are the means of 4 determinations. Vertical lines indicate S.E.M.

Saturation curves showed that the kinetic parameters varied with the time of incubation; the longer the incubation time the lower the rate of *O*-methylation of AD (Figure 2). Both V_{max} and K_m values decreased with an increased incubation time (see Table I). Saturation curves for the methyl

TABLE I Kinetic constants for rat liver homogenate COMT activity using AD or S-adenosyl-L-methyonine as the substrate and four different times of incubation

Incubation time (min)	*Adrenaline*		*S-adenosyl-L-methionine*	
	V_{max} (nmol mg prot^{-1} h^{-1})	K_m (μM)	V_{max} (nmol mg prot^{-1} h^{-1})	K_m (μM)
5	22.12 ± 1.59	104.8 (31.3; 178.4)	107.6 ± 6.0	174.3 (79.7; 269.0)
15	16.00 ± 0.97*	92.6 (36.6; 148.5)	96.7 ± 12.6	525.8 (25.4; 1026.2)
30	9.94 ± 0.41*	55.4 (30.1; 80.7)	116.9 ± 18.6	1108.2 (147.4; 2068.9)
60	5.80 ± 0.16*	24.6 (15.4; 33.8)	49.2 ± 4.9*	1192.4 (530.6; 1854.3)

Values represent the mean of four experiments and the S.E.M. for the V_{max} or the 95% confidence limits for K_m. * Significantly different from respective values at 5 min ($p < 0.05$) as determined by Newman–Keuls test.

donor, SAMe, revealed that V_{max} values did not change and K_m progressively increased up to 30 min (Figure 3 and Table I). At 60 min of incubation, V_{max} was significantly lower and K_m was not different from the value obtained at 30 min (Figure 3 and Table I). From the data presented above, an incubation time of 5 min and a SAMe concentration of 500 μM were chosen. Under these particular conditions, the V_{max} and K_m values obtained were of 63.4 ± 3.3 nmol mg protein^{-1} h^{-1} and 261.1 (127.0, 395.3) μM, respectively (Figure 4).

The *O*-methylated metabolite of noradrenaline, normetanephrine (100 μM), did not affect the saturation curve for *O*-methylation of AD when using an incubation time of 5 min and a SAMe concentration of 500 μM (Figure 4). Both K_m (381.9 (274.3, 489.4) μM) and V_{max} (62.1 ± 1.9 nmol mg protein^{-1} h^{-1}) values were not significantly changed. Under the same experimental conditions, the metabolite of SAMe, S-adenosyl-homocysteine, produced a significant reduction in V_{max} (41.1 ± 4.1 nmol mg protein^{-1} h^{-1}) without changing the K_m (366.6 (36.4, 696.7) μM) (Figure 4).

In the final series of experiments, homogenates of the isolated forms of COMT (MB- and S-COMT) were used instead of whole liver homogenates; the time of incubation was 5 min and the SAMe concentration was 500 μM. Incubation of an enzyme assay mixture containing MB- or S-COMT resulted in a concentration dependent increase in the *O*-methylation of AD (Figures 5 and 6, respectively) with the following kinetic parameters: MB-COMT; $K_m = 3.0$ (1.7, 4.7) μM, $V_{max} = 23.8 \pm 1.1$ nmol mg protein^{-1} h^{-1}: S-COMT; $K_m = 345.2$ (144.5, 546.0) μM, $V_{max} = 58.1 \pm 4.3$ nmol mg/protein h^{-1}.

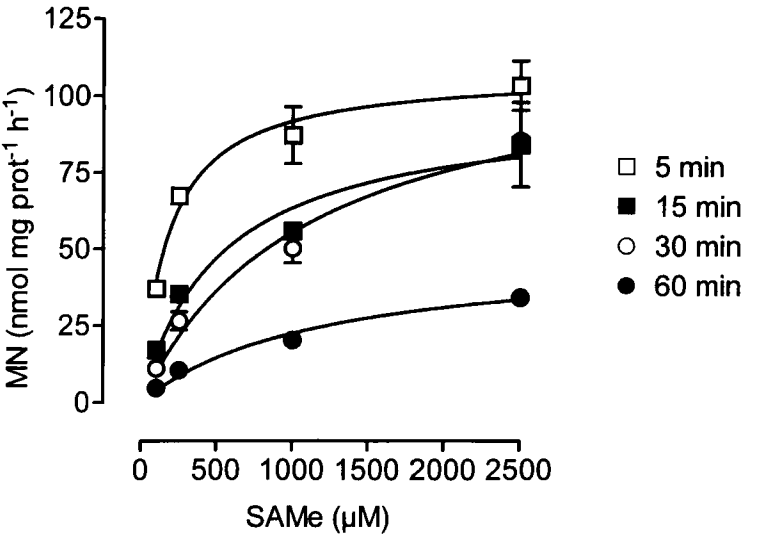

FIGURE 3 Saturation curves for the methyl donor S-adenosyl-L-methionine (SAMe) obtained with four different incubation times. Kinetic parameters are given in Table I. Symbols are the means of 4 determinations. Vertical lines indicate S.E.M.

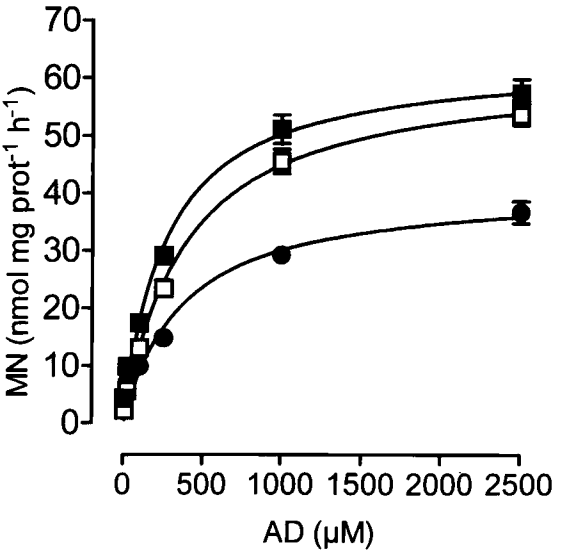

FIGURE 4 Saturation curves for AD using 5 min incubation time and 500 μM SAMe. Control curve (■); normetanephrine (NMN) 100 μM (□); S-adenosyl-L-homocysteine (SAHCys) 100 μM (●). Symbols are the means of 4 determinations. Vertical lines indicate S.E.M.

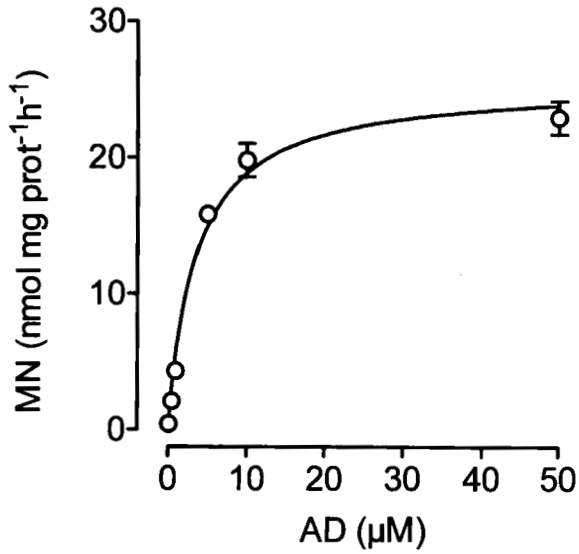

FIGURE 5 Saturation curve and corresponding kinetic parameters for liver membrane-bound COMT using an incubation time of 5 min and a SAMe concentration of 500 μM. Symbols are the means of 4 determinations. Vertical lines indicate S.E.M.

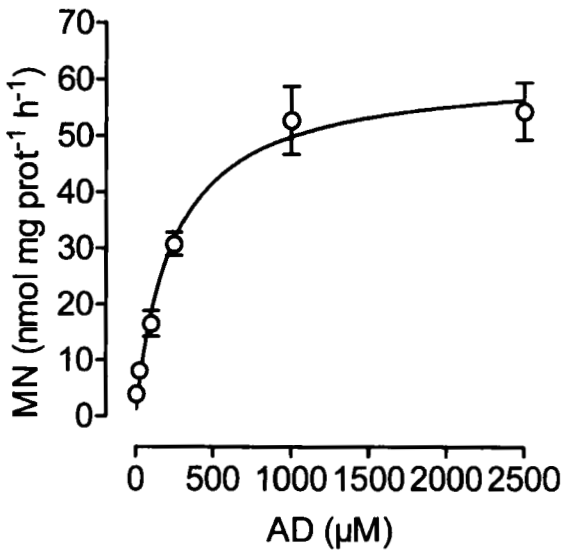

FIGURE 6 Saturation curve and corresponding kinetic parameters for liver soluble COMT using an incubation time of 5 min and a SAMe concentration of 500 μM. Symbols are the means of 4 determinations. Vertical lines indicate S.E.M.

DISCUSSION

The aim of the present work was to define the optimal conditions for the study of rat liver COMT. Although this enzyme has been known for almost 40 years,[1] kinetic parameters in the literature reflect some heterogeneity, probably due to different assay methods.[2,15,19–21]

The time course of *O*-methylation of AD by liver homogenates was initially studied. The results showed that linearity in the assay was achieved up to 10 min of incubation. The analysis of saturation curves using 100 μM SAMe, 300 μM AD and different incubation times, revealed that the values for both kinetic parameters (K_m and V_{max}) decreased when incubation time was increased. These results clearly show the influence of this parameter (contact time with the substrate) on COMT kinetics. The hypothesis that end-product inhibition could account for the changes in the kinetic parameters was ruled out by the results of experiments in which normetanephrine, the *O*-methylated metabolite of noradrenaline, was added to the incubation medium. In this case, no reduction in V_{max} was observed in saturation curves made in the presence of 100 μM of normetanephrine. In the same experiments, it was also shown that the affinity constant was not significantly changed by normetanephrine. The concentration of normetanephrine used exceeds by far the amount of the *O*-methylated derivative formed (54 nmol mg protein^{-1} h^{-1} corresponds to 15 μM) in the presence of the highest concentration of AD tested (2500 μM).

The hypothesis that the V_{max} reduction was due to an insufficient concentration of the methyl donor was tested in experiments in which saturation curves for SAMe, using a high (2500 μM) AD concentration, were made for different incubation times (5, 15, 30 and 60 min). The results show that V_{max} values did not change and K_m progressively increased up to 30 min. At 60 min of incubation, V_{max} was significantly lower and K_m was not different from the value obtained at 30 min. This is in agreement with the view that the affinity for the co-factor SAMe decreases with increase in contact time, as shown in Table I. From this set of data, it was concluded that an incubation time of 5 min and a saturating concentration of SAMe (500 μM) provide optimal conditions for the *O*-methylation of AD by liver homogenates. Longer incubation periods seem to be inadequate, probably due to depletion of SAMe or to accumulation of S-adenosyl-homocysteine, the product of COMT action on SAMe.[22] The latter possibility fits well with the observation of a decrease in enzyme affinity for the co-factor SAMe, as evidenced by an increase in K_m values for SAMe, with an increase in contact time. In order to explore this possibility, a saturation curve for AD was

obtained using those parameters (5 min contact time and 500 μM SAMe) and gave a K_m of 261.1 μM and a V_{max} of 63.4 nmol mg protein^{-1} h^{-1}. These values are greater than those obtained in the experiments shown in Figure 2, when incubation with AD was performed in the presence of 100 μM SAMe for 5, 15, 30 and 60 min. This increase in V_{max} values denotes an increased efficiency in the methylation of AD probably related to a greater availability of SAMe (see V_{max} values at 5 min with 100 and 500 μM SAMe).

In order to determine the type of interaction between the substrate (AD) and the metabolite of SAMe (S-adenosyl-homocysteine), the effect was examined of a single concentration (100 μM) of S-adenosyl-homocysteine on a saturation curve for AD using 500 μM SAMe and a 5 min incubation time. A significant reduction was obtained in V_{max} without changes in K_m, which demonstrates the non-competitive nature of S-adenosyl-homocysteine inhibition on AD methylation. However, these results cannot explain the decrease in K_m values observed with the increase in incubation time. This type of change in COMT affinity with incubation time was also observed recently for rat kidney.[15,23]

The knowledge about COMT has been greatly improved with the discovery and isolation of two distinct forms of this enzyme, S- and MB-COMT.[16,24–27] The liver of most, if not all, of the species studied possessed both forms of the enzyme, although the soluble form accounted for almost all the *O*-methylating activity in this organ.[19,20,28,29] In this study, both forms of the enzyme were isolated from rat liver and their kinetic constants were determined using the optimal conditions found for crude liver homogenates (5 min incubation time and 500 μM SAMe). The results obtained confirm that the two forms display different affinities for the substrate, MB-COMT showing a K_m about 100 times lower, as has been described for other organs.[23,25,26] The V_{max}values cannot be directly compared, as they are expressed per mg of protein and each preparation has a different enzyme/protein ratio.

It is concluded that rat liver COMT kinetic analysis is greatly dependent on parameters such as time of incubation and concentration of the methyl donor, SAMe. It is also suggested that the decrease in *O*-methylation with the increase in substrate and co-factor contact time is most probably related to both a decrease in co-factor availability and the generation of a competitive inhibitor of SAMe and a non-competitive inhibitor of AD, namely S-adenosyl-homocysteine.

References

[1] Axelrod, J. and Tomchick, R. (1958) *J. Biol. Chem.*, **233**, 702.
[2] Guldberg, H. and Marsden, C. (1975) *Pharmacol. Rev.*, **27**(2), 135.

[3] Kopin, I.J. (1985) *Pharmacol. Rev.*, **37**(4), 333.
[4] Kaakkola, S., Gordin, A. and Mannisto, P.T. (1994) *Gen. Pharmac.*, **25**(5), 813.
[5] Mannisto, P.T. and Kaakkola, S. (1990) *Pharmacol. Toxicol.*, **66**(5), 317.
[6] Zurcher, G., Dingemanse, J. and Da Prada, M. (1993) *Adv. Neurol.*, **60**, 641.
[7] Mannisto, P., Kaakkola, S., Nissinen, E., Linden, I.B. and Pohto, P. (1988) *Life Sci.*, **43**(18), 1465.
[8] Nissinen, E., Lindén, I., Schultz, E. and Pohto, P. (1992) *NaunynSchmiedeberg's Arch. Pharmacol.*, **346**, 262.
[9] Davis, T.I., Roznoski, M. and Burns, R.S. (1995) *Mov. Disord.*, **10**(3), 349.
[10] Roberts, J.W., Cora-Locatelli, G., Bravi, D., Amantea, M.A., Mouradian, M.M. and Chase, T.N. (1993) *Neurology*, **43**(12), 2685.
[11] Mannisto, P.T., Lang, A., Rauhala, P. and Vasar, E. (1995) *Eur. J. Pharmacol.*, **274**, 229.
[12] Suzuki, Y.J., Tsuchiya, M., Safadi, A., Kagan, V.E. and Packer, L. (1992) *Free Rad. Biol. Med.*, **13**(5), 517.
[13] Rautelin, H., Renkonen, O.V. and Kosunen, T.V. (1992) *Eur. J. Clin. Microbiol. Infect. Dis.*, **11**(3), 274.
[14] Haramaki, N., Stewart, D.B., Aggarwal, S., Kawabata, T. and Packer, L. (1995) *Biochem. Pharmacol.*, **50**(6), 839.
[15] Vieira-Coelho, M.A. and Soares-da-Silva, P. (1996) *Br. J. Pharmacol.*, **117**(3), 516.
[16] Nissinen, E., Tuominen, R., Perhoniemi, V. and Kaakkola, S. (1988) *Life Sci.*, **42**, 2609.
[17] Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248.
[18] Motulsky, H.G., Spannard, P. and Neubig, R. GraphPad Prisms, GraphPad Prism Software Inc., San Diego, 1994.
[19] Tong, J.H. and D'Iorio, A. (1977) *Can. J. Biochem.*, **55**, 1108.
[20] Borchardt, R.T., Cheng, C.F. and Cooke, P.H. (1974) *Life Sci.*, **14**, 1089.
[21] White, H.L. and Wu, J.C. (1975) *Biochem. J.*, **145**, 135.
[22] Deguchi, T. and Barchas, J. (1971) *J. Biol. Chem.*, **246**, 3175.
[23] Vieira-Coelho, M.A., Borges, N. and Soares-da-Silva, P. (1997) *Br. J. Pharmacol.*, **122**, 34.
[24] Tenhunen, J., Salminen, M., Jalanko, A., Ukkonen, S. and Ulmanen, I. (1993) *DNA Cell Biol.*, **12**(3), 253.
[25] Jeffery, D.R. and Roth, J.A. (1984) *J. Neurochem.*, **42**, 826.
[26] Roth, J. (1992) *Rev. Physiol. Biochem. Pharmacol.*, **120**, 1.
[27] Lotta, T., Vidgren, J., Tilgmann, C., Ulmanen, I., Melen, K., Julkunen, I. and Taskinen, J., (1995) *Biochemistry*, **34**(13), 4202.
[28] Tilgmann, C. and Kalkkinen, N. (1990) *FEBS*, **264**(1), 95.
[29] Salminen, M., Lundstrom, K., Tilgmann, C., Savolainen, R., Kalkkinen, N. and Ulmanen, I. (1990) *Gene*, **93**, 241.