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DETERMINATION OF CATECHOLAMINES AND O-METHYLATED METABOLITES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION AND ITS APPLICATION TO ENZYME KINETICS

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SUMMARY

In a study of the enzymatic methylation of individual catecholamines, high-performance liquid chromatography was used for the separation from their O-methylated metabolites. A RP-8 column and an aqueous mobile phase containing dodecylsodium sulphonate were used, and the eluate was analysed fluorimetrically (the detection limit was 20 pg). Each catecholamine used and its O-methylated compounds could be detected individually without interference from the catechol-O-methyltransferase and S-adenosylmethionine present. The application of the method to the study of enzyme kinetics is described.

INTRODUCTION

Differences in physiological activity between the catecholamines, as illustrated for instance by adrenaline and noradrenaline, are assumed to be caused by differences in the physico-chemical properties of the side-chain, *e.g.*, stereochemical structure and charge distribution. The character of the side-chain may also have a marked influence on the rate of decomposition of catecholamines by enzymes; however, quantitative data on this subject are scarce.

For this reason, it was decided to study in detail the enzymatic methylation of catecholamines by S-adenosylmethionine (SAM) in the presence of catechol-O-methyltransferase (COMT), as this is an important degradation route of these compounds in the human body. For this study, a method for the determination of these compounds and of their O-methylated products was needed. In the literature, many methods for the determination of these compounds have been described, *e.g.*, gas chromatography, high-performance liquid chromatography (HPLC) and fluorimetry.

Gas chromatography is not attractive because laborious derivatization reactions are necessary prior to the determination[1,2]. Fluorimetry has been widely investigated and used[3,4]. For the oxidative decomposition of catecholamines[5], a fluorimetric method based on the native fluorescence of these compounds appeared

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to be rapid, simple and sensitive[6]. However, for the determination of the methylation products in the presence of the parent catecholamine, a separation is necessary because the O-methylated metabolites have almost the same excitation and emission characteristics as the catecholamine itself. For this reason we considered HPLC as an alternative separation method.

Many workers have considered to the separation of catecholamines[7–20] and their 3-O-methylated metabolites. However, 3-O-methylated metabolite is not the only metabolite formed by enzymatic methylation in the presence of COMT. In the case of, *e.g.*, disturbed metabolism *in vivo*, the 4-O-methylated and 3-O,4-O-dimethylated products may also be formed. Further, in *in vitro* work 3-O- and 4-O-methylated metabolites are formed, and the formation of the 3-O,4-O-dimethylated product cannot be excluded beforehand. On the other hand, it is known that catecholamines are easily oxidised. Thus for a proper study of the kinetics of enzymatic methylation it is necessary to take into account the loss of starting material, by making a mass balance of the catecholamines and the enzymatically formed products.

For the above reasons, the study of the kinetics of the reaction of catecholamines with SAM in the presence of COMT demands a separation of all of these compounds, while SAM and COMT may not interfere in the determination. For *in vivo* measurements a sensitive detector is another requirement. Because electrochemical detection is not suitable for the determination of the 3-O,4-O-dimethylated compounds, the UV method is not sensitive enough and the so-called trihydroxyindole method (fluorimetry) is too laborious, we used the native fluorescence of the catecholamines and their metabolites for detection.

In addition to the method for the determination of these compounds, attention was also paid to its applicability to the study of the kinetics of enzymatic methylation.

EXPERIMENTAL

Chemicals

The following compounds were used as purchased: L-adrenaline D-hydrogentartrate (Fluka, Buchs, Switzerland; purum); dopamine·HCl (Fluka; purum); DL-noradrenaline (C. Roth, Karlsruhe, G.F.R.; purum); DL-metanephrine·HCl (Sigma, St. Louis, MO, U.S.A.); 3-O,4-O-dimethyldopamine (Aldrich Europe, Beerse, Belgium); S-adenosyl-L-methionine chloride, grade II (Sigma); dodecylsodium sulphonate (DNS) (Merck, Darmstadt, G.F.R.); and magnesium chloride (J.T. Baker, Phillipsburg, NJ, U.S.A.; Analyzed Reagent). Research-grade methanol was obtained from Merck.

Demineralised, distilled water was used. The buffer of pH = 3 contained 1.72 g of potassium dihydrogen phosphate (Baker, Analyzed Reagent) and 3 ml of 25% phosphoric acid. The buffer of pH 7.9 consisted of 0.476 g of potassium dihydrogen phosphate and 5.7405 g of disodium hydrogen phosphate (Merck; pro analysi) in 1 l of water. The column was filled with LiChrosorb 5 RP-8 (Chrompack). All of these materials were used without further purification.

The following materials were synthesized according to the method referred to: 3-O-methyldopamine and 4-O-methyldopamine[21]; N-methyladrenaline[22,23]; 3-O-

methylnoradrenaline, 3-O-methyl-N-methyladrenaline[24,25]; paranephrine (4-O-methyladrenaline), norparanephrine (4-O-methylnoradrenaline), 4-O-methyl-N-methyladrenaline[26]; 3-O,4-O-dimethyladrenaline, 3-O,4-O-dimethylnoradrenaline and 3-O,4-O-dimethyl-N-methyladrenaline[27,28].

Preparation of COMT. Adult Wistar rats (200–250 g each) were used. COMT was isolated from the livers and partially purified according to the method of Axelrod and Tomchick[29]. All enzyme activities were measured at 37°C and pH 7.9. Protein determinations were carried out by the method of Lowry *et al.*[30] with bovine serum albumin as a standard.

Apparatus

A Spectra Physics SP 3500 B liquid chromatograph with a column (15 × 0.46 cm I.D.) filled with LiChrosorb 5 RP-8 was used. The column outlet was connected to a Perkin-Elmer Model 204 fluorimetric detector with a thermostated cell holder. The detector signal was recorded with a Kipp BD 40 recorder (Kipp & Zonen, Delft, The Netherlands) and evaluated with an integrator (Spectra Physics Minigrator).

Procedure

The sample solution consisted of a catecholamine and its corresponding 3-O-methylated, 4-O-methylated and dimethylated derivatives in combination with an adequate amount of internal standard, each at a concentration of about 10^{-5} *M* in 0.01 N hydrochloric acid. For the enzymatic methylation the samples also contained SAM (6 μM), COMT (200 μl), magnesium chloride (60 μM) and phosphate buffer. A 10-μl of this solution was injected into the chromatograph and eluted with methanol–pH 3 buffer (1:2) containing dodecylsodium sulphonate (40 mg/l) at a flow-rate of 0.8 ml/min.

Standard reaction. To 200 μl of the partially purified COMT (200–400 μg of protein) were added successively 0.5 ml of a solution of S-Adenosyl-L-methionine (5 mg in 1 ml of water), 2 ml of a solution of magnesium chloride (60 mg in 100 ml of 1:1 water–pH 7.9 phosphate buffer) and at last 1 ml of a solution of dopamine·HCl (about 10^{-4} *M*). All of the reactions were carried out at 37°C. The reaction was started by adding the catecholamine. After specified time intervals, samples of 100 μl were taken. The reaction was stopped by adding the samples (100 μl) to 100 μl of a solution of internal standard (10^{-5} *M* adrenaline in 0.01 N hydrochloric acid). These solutions were ready for immediate injection into the chromatograph Each sample was injected in duplicate.

RESULTS AND DISCUSSION

The determination of the catecholamines adrenaline, noradrenaline, N-methyladrenaline and dopamine in combination with their 3-O-methylated, 4-O-methylated and 3-O,4-O-dimethylated compounds was investigated. In the system used the components are eluted in the following order: the catecholamine, the 3-O-methylated, the 4-O-methylated and last the 3-O,4-O-dimethylated compound. As an example, the separation of dopamine and its methylation products was chosen (see Fig. 1). Adrenaline was used as an internal standard. The retention times were adrenaline

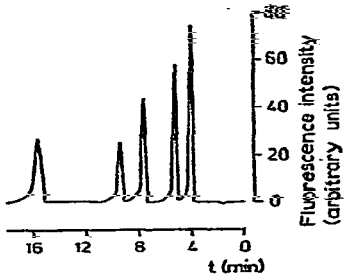

Fig. 1. Chromatogram of a solution of adrenaline, dopamine, 3-O-methyldopamine, 4-O-methyldopamine and 3-O,4-O-dimethyldopamine. Chromatographic conditions: column, 15 × 0.46 cm I.D. filled with LiChrosorb 5 RP-8; mobile phase, methanol–pH 3 buffer (1:2) containing DNS; flow-rate, 0.8 ml/min; pressure drop, 20 MPa; injection volume, 10 μl of 10^{-5} *M* solution. Fluorimetric detection: $\lambda_{exc.} = 284$ nm; $\lambda_{em.} = 316$ nm; native fluorescence.

Compound	*Retention time (min)*	*kappa*
Adrenaline (internal standard)	4.1	1.6
Dopamine	5.3	2.3
3-O-Methyldopamine	7.7	3.8
4-O-Methydopamine	9.5	4.9
3-O,4-O-Dimethyldopamine	15.8	8.9

4.1 min, dopamine 5.3 min, 3-O-methyldopamine 7.7 min, 4-O-methyldopamine 9.5 min and 3-O,4-O-dimethyldopamine 15.8 min.

Injections of various amounts of dopamine, 3-O-methyldopamine, 4-O-methyldopamine and 3-O,4-O-dimethyldopamine in relation to the internal standard, adrenaline, showed that the specific response at the chosen wavelengths was constant up to 10^{-5} *M* (see Fig. 2). The elution pattern was reproducible and the peak areas of dopamine ($3.0 \cdot 10^{-6}$ *M*), 3-O-methyldopamine ($2.2 \cdot 10^{-6}$ *M*), 4-O-methyldopamine ($1.9 \cdot 10^{-6}$ *M*) and 3-O,4-O-dimethyldopamine ($4.1 \cdot 10^{-6}$ *M*) in relation to that of adrenaline ($2.9 \cdot 10^{-6}$ *M*), which was taken as 100, were 25.1 ± 0.43 (s.d.), 21.6 ± 0.48, 18.4 ± 0.19 and 35.3 ± 0.52, respectively (six determinations). The detection limit was 10^{-8} *M* (= 20 pg).

To investigate whether or not COMT and SAM interfere in the determination, the following experiment was performed. Two vials were filled with a solution of dopamine, 3-O-methyl-, 4-O-methyl- and 3-O,4-O-dimethyldopamine (10^{-5} *M* each) and magnesium chloride (10^{-3} *M*) in buffer of pH 7.9. One vial was taken as a blank, and to the other vial also SAM (6 μ*M*) and COMT (200–400 μg of protein) were added (total volume 3.7 ml). Both vials were kept for 2 h at 37°C, then samples were taken and 0.01 *N* hydrochloric acid containing the internal standard was added. By comparison the contents of the two vials, taking into account the conversion of 30% dopamine into 23% 3-O-methyl- and 7% 4-O-methyldopamine in one vial, it appeared that SAM and COMT do not interfere in the determination. For the other catecholamines a similar separation was obtained. The content of methanol in the mobile phase and the flow-rate may be different.

It appeared that this method of determination is very useful for studies of the kinetics of the metabolic degradation *in vitro*, because of its reproducibility, rapidity and simplicity. The study of the kinetics of the enzymatic methylation reaction in

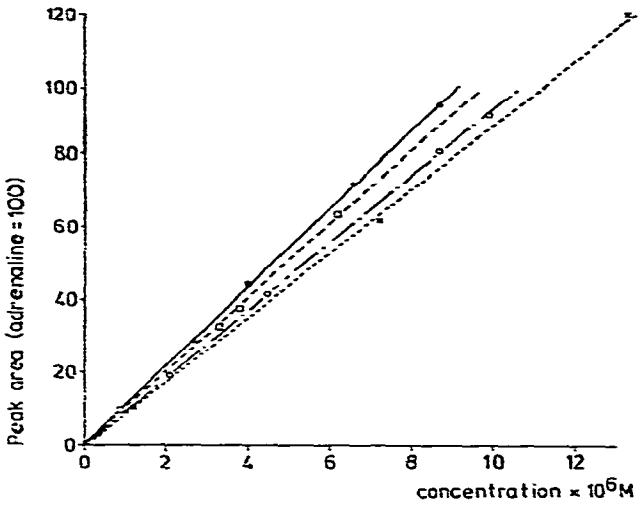

Fig. 2. Analytical evaluation function (also see text).

Line	*Compound*	*L.R.**	*Intercept*	*Slope (M^{-1})*
●——●	3-O-Methyldopamine	0.9998	0.10	10.8×10^6
□----□	4-O-Methyldopamine	0.9993	0.07	10.1×10^6
○-.-.-○	Dopamine	0.9998	−0.04	9.2×10^6
×----×	3-O,4-O-Dimethyldopamine	0.9998	−0.28	8.9×10^6

* L.R. = linear regression (correlation coefficient), r^2.

the presence of COMT, the most important biotransformation route of catecholamines, may produce a relationship between structure and reactivity that may contribute to a rational drug design.

Much has been published about the enzymatic methylation of catecholamines by COMT. However, only a few workers, *e.g.*, refs. 29 and 31–42, have investigated the kinetics of the enzymatic methylation, especially if one takes into account the importance of the subject. Adrenaline was often the subject of these studies[29,34–37,39]; only few workers compared the kinetic data for a series of catecholamines[39]. The kinetic data of the enzyme reaction reported in the literature vary widely. For example, the K_m values of adrenaline vary between $63 \cdot 10^{-5}$ and $0.9 \cdot 10^{-5}$ M[33–35,39]. Possible causes of these variations are SAM and magnesium chloride concentration, pH and ionic strength[37–39]. In the following we discuss other problems mentioned by several workers which may be the cause of the variation in kinetic data, but which were not investigated.

Most workers did not take into account the instability of the catecholamines, especially at higher pH and temperature, in which transition metal ions have an accelerating effect. When COMT is present in the reaction mixture the oxidation is less, in respect of enzyme binding, but cannot be excluded totally.

Senoh *et al.*[32], Flohe and Schwabe[35] and Gulliver and Tipton[42], for instance, studied the enzymatic methylation even above pH 9.5; at this pH one must take into account oxidation or other degradation reactions. A number of workers, *e.g.*, ref. 39, started the reaction by adding the enzyme to the reaction mixture, which means that oxidation can occur before the enzyme is present in the reaction mixture.

Frère and Verly[38] mentioned the instability of the methylated compounds chemically as well as enzymatically.

Chemical reactions, which can occur between oxidation products of catecholamines, such as aminochromes, can lead to the inactivation of COMT[40,41].

From the above, it will be clear that a primary condition for controlling the consequences of the problems mentioned is to make a mass balance of the catecholamines and their enzymatically formed methylated products. It is therefore remarkable that up to now conclusions concerning the kinetics of the enzymatic reaction have been based merely on product formation. Taking into account the complex system, it is likely that an incorrect mass balance led to erroneous conclusions. This serious drawback of such kinetic studies is often caused by the method of determination, which is not suitable for assaying simultaneously the catecholamine and its methylated products.

We have tested our method, which makes possible the simultaneous determination of catecholamine and the 3-O-methyl-, 4-O-methyl- and 3-O,4-O-dimethyl derivatives, for its usefulness in the study of the kinetics.

Fig. 3 shows the decrease in dopamine; under the conditions applied the reaction appeared to be first order in the concentration of the starting material. The deviation in the k value [$(28.3 \pm 0.88) \cdot 10^{-3}$ min^{-1}; $n = 5$] is small, indicating that the procedure followed with the standard reaction, in combination with the method of determination, gives accurate kinetic parameters.

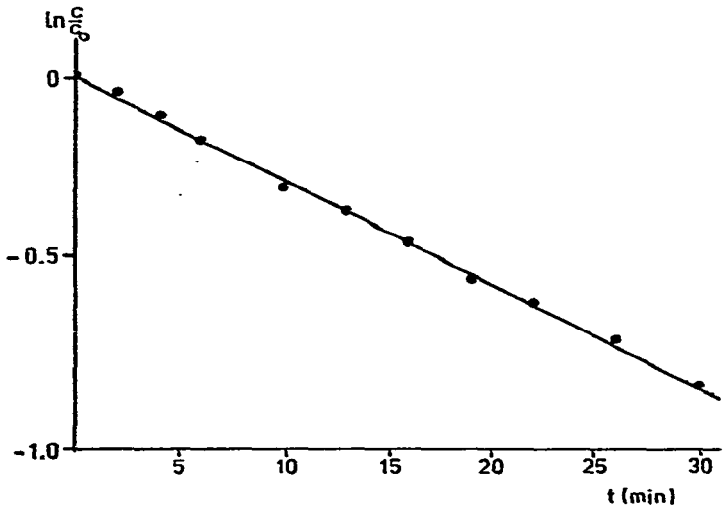

Fig. 3. Decrease in dopamine by enzymatic methylation. First-order reaction: $k = (28.3 \pm 0.88) \times 10^{-3}$ min^{-1} ($n = 5$).

Fig. 4 shows the decrease in the starting material and the formation of 3-O and 4-O-methyldopamine (no 3-O,4-O-dimethyldopamine formation was observed under these circumstances; the ratio of 3-O to 4-O-methyldopamine was 3.3). The mass balance of starting material and products as a function of time is also presented. Fig. 4 shows that the mass balance remains 100% throughout the reaction. However,

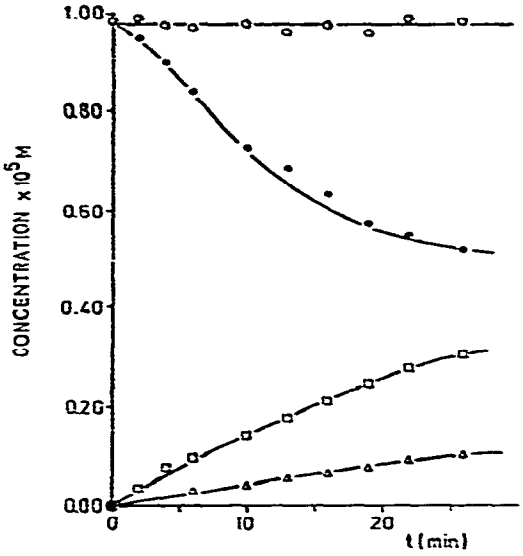

Fig. 4. Enzymatic methylation of dopamine. ●, Decrease of dopamine; □, formation of 3-O-methyldopamine; △, formation of 4-O-methyldopamine; ○, mass balance.

it is more important that if there is a deviation of this 100% line, which may appear under other circumstances, it can be observed with this method. It is a valuable means for testing whether a comparison of the kinetic parameters obtained is meaningful or not.

Research is in progress on structure–reactivity relationships of catecholamines using the method presented here.

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