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Biosynthesis in an on-line immobilized-enzyme reactor containing phenylethanolamine *N*-methyltransferase in single-enzyme and coupled-enzyme formats

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Abstract

An immobilized-enzyme reactor (IMER) based upon phenylethanolamnie *N*-methyltransferase (PNMT) has been developed. The activity of the PNMT-IMER and its applicability for on-line *N*-methylation of normetanephrine was investigated. The reactor was connected through a switching valve to a cyano (CN) and ODS stationary phase connected in series. The substrate was injected onto the PNMT-IMER and the unreacted substrate and product were eluted and transported via a switching valve onto the analytical columns. The results from the PNMT-IMER/CN–ODS chromatographic system demonstrate that the enzyme retained its catalytic activity. Known substrates and inhibitors for PNMT were examined and the chromatographic system was utilized to carry out both quantitative and qualitative determinations. The PNMT-IMER/CN–ODS system proves to be useful in basic biochemical studies, an ideal for the high throughput screening of substances for PNMT substrate–inhibitor properties. The PNMT-IMER was then coupled in series using switching-valve technology with a previously developed dopamine β-hydroxylase immobilized-enzyme reactor and used to carry out the on-line two-step synthesis of epinephrine from dopamine.

Keywords: Enzymes; Immobilized enzymes; Phenylethanolamine *N*-methyltransferase; Dopamine β-hydroxylase; Epinephrine; Dopamine

1. Introduction

Enzymes are primary targets of exploitation by the industrial and analytical fields due to their involvement in a wide variety of chemical transformations. However, the use of enzymes in these fields is limited by several factors such as the high cost of isolation and purification stability and difficulty to recover the enzymes for reuse. Enzyme immobilization is one approach that can be used to overcome these problems. In this technology the enzyme is associated with a matrix in order that the enzyme can be stabilized recovered from a reaction mixture and reused. Immobilized enzymes can be used in batchwise experiments or packed into columns and used in flow systems as immobilized-enzyme reactors (IMERs).

IMERs have been developed by numerous groups and have proven to be useful and economic alternatives to conventional enzymatic synthesis or

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screening for inhibitors and substrates. For example, IMERs have been developed based upon α-chymotrypsin [1–3], trypsin [4], lipase [5–7], alcohol dehydrogenase [8,9] and dopamine β-hydroxylase [10]. The IMERs were employed in HPLC systems coupled to analytical columns via switching-valve technology. The systems proved ideal in obtaining both quantitative and qualitative information concerning biosynthetic and metabolic processes.

The enzymes, tyrosine hydroxylase, dopa-decarboxylase, dopamine β-hydroxylase (DBH) and phenylethanolamine *N*-methyltransferase (PNMT) are involved in the synthesis of catecholamines, dopamine, norepinephrine and epinephrine. These enzymes are located in the adrenal medulla and in dopaminergic, noradrenergic and adrenergic nerves [11]. Numerous studies have revealed their involvement in a broad range of physiological processes as well as being implicated in diseases such as Schizophrenia, Parkinson's disease and hypertension [12,13]. As such, the development of drugs that can alter the function of these catecholamine-synthesizing enzymes are key targets for new drug development.

PNMT catalyzes the methylation of norepinephrine and its conversion to epinephrine (Fig. 1). The conversion occurs in the adrenal medulla and in certain nuclei in the central nervous system. The addition of a methyl group significantly alters the pharmacology of the catecholamines. PNMT action has proven important in neuroendocrine and blood pressure regulation, and the importance of developing selective and potent inhibitors has become increasingly evident [14].

We have previously reported the use of the glutaraldehyde-P liquid chromatographic stationary phase for the covalent immobilization of PNMT [15]. The resulting PNMT–Glut-P stationary phase (PNMT-SP) was stable and capable of the transmethylation of normetanephrine. Standard Michaelis–Menten kinetic studies were carried out for both free and immobilized PNMT. Known substrates and inhibitors for PNMT were examined, and the results demonstrated that the PNMT-SP can be utilized for both qualitative and quantitative determinations of enzymatic activity in batch-wise (i.e. non-flow) format.

The aim of the present study was to develop an

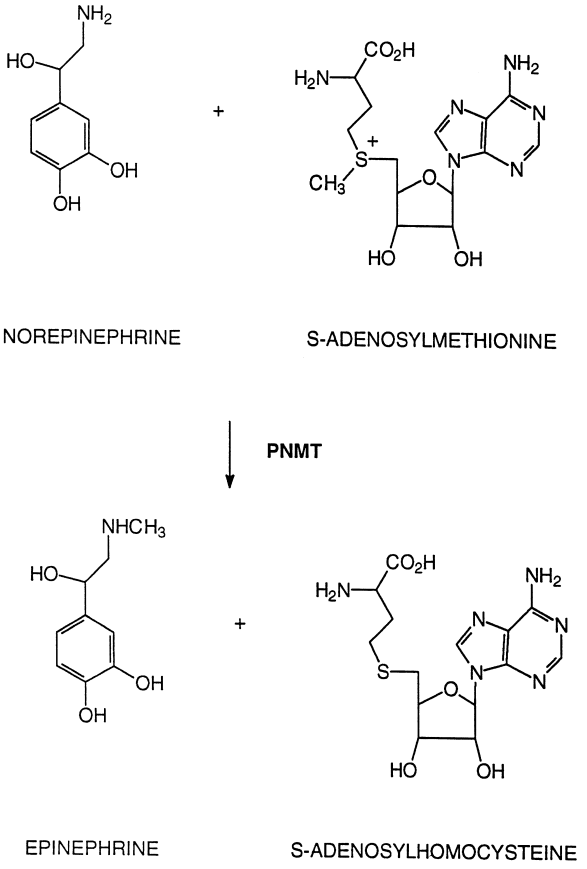

Fig. 1. Reaction of phenylethanolamine *N*-methyltrasferase (PNMT).

IMER containing the PNMT-based liquid chromatographic phase. The experimental goals were the demonstration that the PNMT-IMER could be used as a probe of biochemical and pharmacological properties and as an on-line high-throughput screening for PNMT inhibitors. The PNMT-SP was prepared and packed into a column. The resulting PNMT-IMER was linked to coupled analytical HPLC columns through a switching valve and used for on-line *N*-methylation of known substrates of PNMT. The PNMT-IMER retained its catalytic activity and displayed sensitivity to pH, temperature and inhibitors. The results demonstrate that the PNMT-IMER can be utilized as a chromatographic probe of enzyme–substrate and enzyme–inhibitor interactions. The HPLC system allows for the gene-

ration, separation and identification of substances as well as the identification of inhibitors.

The experimental utilization of the PNMT-IMER was expanded by coupling it to another previously reported IMER based upon DBH (DBH–IMER) [10]. The coupled system was shown to be capable of carrying on-line synthesis of epinephrine from dopamine in a continuous flow system. The immobilized enzyme reactors used independently or as a combination will provide a unique opportunity to explore the interrelationships between these enzymes.

2. Experimental

2.1. Chemicals

PNMT (from bovine adrenal medulla), *s*-adenosyl-L-methionine *p*-toluenesulfonate salt (SAM), DL-normetanephrine hydrochloride, D,L-metanephrine hydrochloride, *S*-adenosyl-L-homocysteine (SAH), methyldopa, dopamine, norepinephrine, epinephrine and other chemicals unless otherwise stated were obtained from Sigma (St. Louis, MO, USA). Glutaraldehyde-P 40 μ*M* affinity packing 300 Å was obtained from J.T. Baker (Phillipsburg, NJ, USA). A 1 cm phenylboronic acid cartridge from Varian (Palo Alto, California, USA) was used for on-line extraction for the coupled IMER system.

2.2. Instrumentation and operating conditions

2.2.1. PNMT-IMER system

Two modular HPLC systems were set-up in order to carry out the chromatographic experiments (Fig. 2A). System 1 consisted of a Thermo Spearation Products P1000 pump (ThermoQuest, San Jose, CA, USA), a Rheodyne 7125 injector with a 100 μl sample loop (Rheodyne, Cotati, CA, USA), and the PNMT-IMER. System 2 consisted of a Thermo Separation Products P1000 pump, a 5 μm cyano (CN) stationary phase packed in a 150×4.6 I.D. mm column (Regis Technologies, Morton Grove, IL, USA), a 5 μm octadecyl (ODS) stationary phase packed in a 250×4.6 I.D. mm column (Regis Technologies) connected in series, a SpectraSystem FL2000 fluorescence detector, and a Thermo Separation Products Chromjet integrator interfaced with a computer equipped with WOW software for data collection. The eluent from system 1 was directed onto system 2 through a Rheodyne 7000 switching valve (SV).

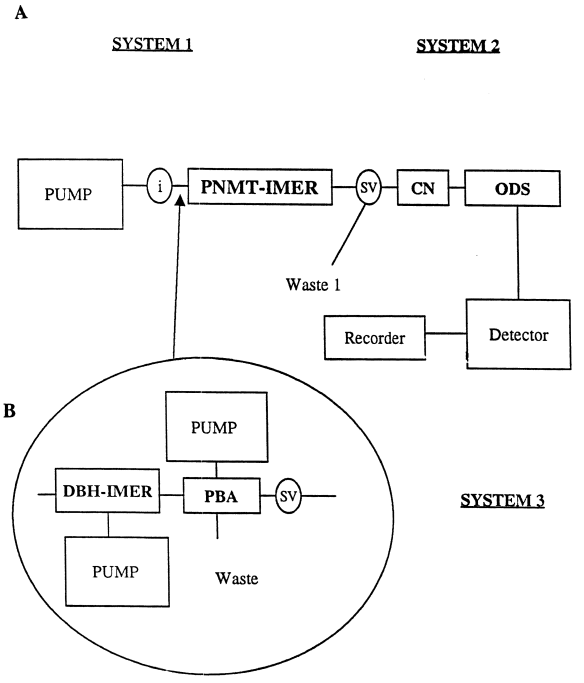

Fig. 2. (A) Schematic representation of on-line phenylethanolamine *N*-methyltransferase immobilized enzyme reactor (PNMT-IMER) HPLC system. (B) Schematic representation of a dopamine beta-hydroxylase immobilized enzyme reactor (DBH–IMER) and phenylboronic acid (PBA) system that can be incorporated to the existing PNMT-IMER system for the on-line synthesis of epinephrine from dopamine.

System 2 was used independently of system 1 by replacing the latter system with a Rheodyne 7125 injector (i) in order to analyze the results obtained from incubations involving non-immobilized PNMT and PNMT immobilized onto the loose Glut-P stationary phase. For the temperature studies, the PNMT-IMER temperature was controlled with a Fiatron System CH-50 column heater (Fiatron, Oconomowoc, WI, USA).

2.2.2. Coupled IMER system

To the existing PNMT-IMER system was added the DBH–IMER coupled to a phenylboronic acid

column (Fig. 2B). Previously reported instrumentation and operating conditions were used for the DBH–IMER [10].

2.3. Chromatographic conditions for PNMT-IMER system

The mobile phase on system 1 consisted of potassium phosphate buffer (0.1 *M*, pH 8.30) with a flow-rate of 0.2 ml/min. A mobile phase consisting of potassium phosphate buffer (25 m*M*) adjusted to pH 2.0 with trifluoroacetic acid was utilized for system 2 to achieve the desired chromatographic separation of the products from the substrates. The solutes were quantitated using fluorescence detection with excitation at $\lambda = 266$ nm and emission at $\lambda = 380$ nm. A flow-rate of 0.7 ml/min and ambient temperature were used for system 2 throughout the study.

2.4. Immobilization of PNMT on loose packing material

PNMT was immobilized onto Glut-P stationary phase utilizing a previously reported method [15]. Briefly, the following procedure was used: (1) The Glut-P stationary phase (300–350 mg) was washed five times with sodium phosphate buffer (0.1 *M*, pH 8.3). In this step, 2 ml of buffer was added to the stationary phase, the suspension was vortex-mixed for 15 min, centrifuged and the supernatant decanted. (2) The enzyme solution [1.96 mg in 2 ml sodium phosphate buffer (0.1 *M*, pH 8.3)] was added to the packing material, the mixture was mixed gently for 15 min and then placed in a rotator/stirrer for 24 h at ambient temperature. (3) At the end of 24 h, the suspension was centrifuged, the supernatant decanted and the packing material washed three additional times with buffer. (4) The amount of enzyme immobilized on the stationary phase was determined by measuring the amount of residual enzyme present in the supernatant using the Bio-Rad protein assay (Bio-Rad Labs, Mississauga, Canada). The difference in the absorbance reading before immobilization and the combined absorbances of the washings after immobilization determined the amount of enzyme bound on the Glut-P stationary phase.

2.5. Preparation of PNMT immobilized enzyme reactor

PNMT immobilized on the Glut-P stationary phase was packed into a 1 cm × 10 mm I.D. guard column (Regis Technologies). The guard column was put into a holder and the column was placed onto the chromatographic system. The PNMT-IMER was washed with phosphate buffer (0.1 *M*, pH 8.3). The eluent was collected in order to determine if any of the enzyme was being washed off the column. The Bio-Rad assay was utilized to measure the amount of non-immobilized enzyme. When the column was not in use it was washed with phosphate buffer (0.1 *M*, pH 8.3) and stored at 4 °C.

2.6. Procedure for on-line injection

2.6.1. PNMT-IMER

A schematic diagram of the coupled HPLC system is presented in Fig. 2. The pump on system 2 is stopped. One hundred μl of a substrate–cofactor mixture is loaded into the injector (i) and the value is switched to the inject position at the same time the switching valve (SV) is switched such that the substrate/product are eluted from the PNMT-IMER and concentrated onto the analytical columns of System 2 for the specified contact time. When the specific contact time elapses the SV is switched back to the original position and the pump on system 2 is started. The unreacted and product formed are separated on the coupled analytical columns.

2.6.2. Coupled IMERs

A representation of the coupled IMER system is illustrated by the incorporation of DBH–IMER (Fig. 2B) into the existing PNMT-IMER system (Fig. 2A). In order to carry out the on-line synthesis of epinephrine from dopamine the following procedure was used: The first pump connected to the DBH–IMER had a mobile phase of sodium acetate buffer (10 m*M*, pH 5.5) with a flow-rate of 0.3 ml/min. All the other pumps in the system were stopped. One hundred μl of a mixture of dopamine and ascorbic acid was loaded into the injector (i) and the valve position switched such that the substrate/product were eluted from the DBH–IMER onto the PBA column where they were trapped.

Following a contact time of 10 min, the second pump was started and mobile phase A (sodium phosphate buffer 25 m*M*, pH 8.4) was pumped through the PBA column at a flow-rate of 0.1 ml/min for 30 s in order to elute the cofactor, ascorbic acid, and any other by-products from the DBH catalyzed reaction. The pump was then switched to mobile phase B (sodium phosphate buffer 25 m*M*, pH 4) with a flow-rate of 0.1 ml/min for 2 min and simultaneously SV1 was switched such that any unreacted substrate and product were eluted onto the PNMT-IMER. The PNMT-IMER system is treated as described above. The only consideration is the addition of SAM into the mobile phase of the pump connected to the PNMT-IMER. Unreacted dopamine and norepinephrine and epinephrine formed are concentrated and separated on the coupled analytical columns.

2.7. Effect of flow-rate and contact time on the PNMT-IMER activity

The effect of the flow-rate through the PNMT-IMER was investigated at flow-rates ranging from 0.1 to 0.4 ml/min at 0.1 ml/min increments. The contact time was 20 min yielding elution volumes of 2, 4, 6 and 8 ml at the respective flow-rates.

The effect of contact time through the PNMT-IMER was also investigated at a fixed flow-rate of 0.2 ml/min. Contact times from 5 to 30 min were investigated at 5 min increments. The recoveries of the substrate and product were determined.

2.8. Effect of pH and temperature on the PNMT-IMER activity

The activity of the PNMT-IMER was measured at a series of pH values (with 0.1 *M* buffers) to determine the optimum pH. The temperature of the PNMT-IMER was kept at 37 °C.

The effect of temperature was also examined for the PNMT-IMER using temperatures ranging from 25 °C (room temperature) to 60 °C. The effect of temperature on PNMT-SP and the PNMT-IMER was compared using Student's *t*-test for unpaired data. All P values <0.05 were considered statistically significant.

2.9. Enzyme activity and inhibition studies on PNMT-IMER

The enzymatic activity on the PNMT-IMER was determined by quantification of the amount of product formed with a given substrate. The temperature of the PNMT-IMER unless otherwise stated was kept at 37 °C with a column heater. Stock solutions of normetanephrine were prepared in water. The substrate concentrations examined ranged from 0.15 to 10 m*M* and that of the cofactor, *S*-adenosyl-L-methionine, ranged from 5 to 100 μ*M*.

Enzymatic activity was examined carrying out injections of a series of substrate/cofactor mixtures. The mixtures were injected onto the IMER at a flow-rate of 0.2 ml/min for a contact time of 20 min. The kinetic parameters were calculated for all forms of the enzyme using standard Michaelis–Menten approach. Lineweaver–Burke plots were used to calculate the Michaelis constant (K_m). The rates of reaction (V_{max}) were calculated using μmol mg^{-1} min^{-1} for non-immobilized PNMT (specific activity) and immobilized PNMT (apparent specific activity). The data was calculated using mg of immobilized or non-immobilized PNMT in order to accurately compare the enzymatic activities in the different formats. Results are expressed as mean±standard error of the mean (SEM).

The effect of known inhibitors, S-adenosylhomocysteine and methyldopa on the enzymatic activity of the PNMT-IMER was also examined. The inhibition of the PNMT-IMER was carried out using injections of a series of substrate–cofactor–inhibitor mixtures.

3. Results and discussion

In the previously reported studies on the development of the PNMT-SP, 135±0.16 μg of PNMT was immobilized onto 50 mg of Glut-P [15]. In this study, the same enzyme:support ratio was utilized such that 0.86±0.13 mg of PNMT was immobilized onto 350±5.4 mg ($n=3$) of Glut-P and packed into a column to form the PNMT-IMER.

Chromatographic studies with the PNMT-IMER and the coupled-system depicted in Fig. 3, demonstrated that the immobilized PNMT was active in the

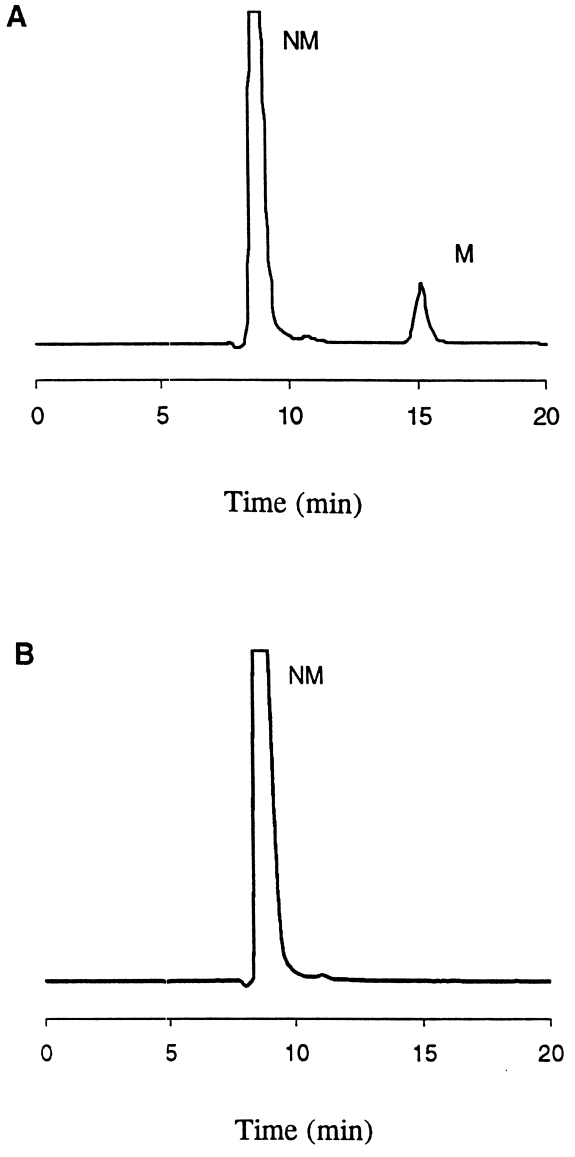

Fig. 3. Representative chromatograms of the on-line *N*-methylation of normetanephrine (NM): (A) reaction mixture (injection of a 1 m*M* NM–20 μ*M* *S*-adenosyl-L-methionine (SAM) mixture); (B) control (injection of 1 m*M* NM and no SAM present). M = Metanephrine.

flow system. When a mixture containing normetanephrine (NM) and *S*-adenosyl-L-methionine (SAM) was injected onto the PNMT-IMER and the eluent from the PNMT-IMER analyzed on system 2, metanephrine (M) appeared in the chromatogram, Fig. 3A. As a positive control, NM was injected onto the coupled system without the cofactor, SAM. Under these conditions, no product formation was observed in the resulting chromatogram, Fig. 3B. The negative control consisted of a column packed with immobilized PNMT that had been heat inactivated before immobilization. Injections of NM–SAM mixtures onto the system containing the inactive PNMT-IMER did not result in the production of M. Thus, the production of M in this system was due to the activity of the PNMT-IMER.

The productivity of the PNMT-IMER depends upon the time that the substrate–cofactor mixture is in contact with the immobilized enzyme. Therefore, the flow-rate through the PNMT-IMER is a key experimental variable. In order to optimize this factor, flow-rates ranging from 0.1 to 0.4 ml min^{-1} at 0.05 ml min^{-1} increments were investigated using a fixed contact time of 20 min. A flow-rate of 0.2 ml min^{-1} allowed for maximal recovery of product formed as well as any unreacted substrate, Table 1.

The affinity (expressed as the Michaelis–Menten constant, K_m) and the rate of reaction (expressed as apparent specific activity, V_{max}) of the immobilized PNMT in the IMER format was determined. For the substrate, NM, the observed K_m value was increased and the V_{max} reduced, both by a factor of approximately 4, relative to the non-immobilized enzyme, Table 2. An increase in K_m indicates a reduced affinity while a decrease in V_{max} indicates a reduced activity. Thus, the immobilization of PNMT negatively affected the enzyme's activity. However, the magnitudes of the observed effects were not solely due to the immobilization of the enzyme. Comparisons of the K_m and V_{max} values obtained with the PNMT-IMER and the PNMT-SP also show a reduction of the affinity and activity of the PNMT-IMER

Table 1
Effect of contact time on phenylethanolamine *N*-methyltransferase immobilized enzyme reactor (PNMT-IMER) activity (at a fixed flow-rate of 0.2 ml/min)

Contact time (min)	Recovery of NM (%)	[M] (μ*M*)
5	45	4.11±0.12
10	87	4.24±0.32
15	95	4.58±0.19
20	94	4.93±0.22
30	96	4.95±0.11

NM: Normetanephrine; M: Metanephrine

Table 2
Kinetic parameters for non-immobilized (PNMT), immobilized (PNMT-SP) and the immobilized phenylethanolamine *N*-methyltransferase reactor (PNMT-IMER)

	PNMT[a]	PNMT-SP[a]	PNMT-IMER
Normetanephrine			
K_m (m*M*)	0.109	0.152	0.384
V_{max} (μmol/mg/min)	1.136	0.823	0.292
SAM			
K_m (μ*M*)	14.17	10.56	7.31
V_{max} (μmol/mg/min)	1.249	0.254	0.424

[a] Data obtained from Ref. [15].

relative to the PNMT-SP. In this case, the values differed by a factor of approximately three, Table 2.

The immobilization of an enzyme places the protein in a new microenvironment that can impede the rate at which the substrate reaches the active site of the enzyme. This is demonstrated by changes in the K_m and V_{max} values between the non-immobilized PNMT and the PNMT-SP, Table 2. However, these values differ by less than 50%. Therefore, the magnitude of changes seen with the PNMT-IMER must be due to the experimental format i.e. the change from a non-flowing system (non-immobilized PNMT and PNMT-SP) to a flowing system (PNMT-IMER). In this case, the key factors may be the kinetics of the distribution of the substrate from the mobile phase to the stationary phase and the shearing forces produced by the moving phase.

The effect of temperature on the PNMT-IMER was also examined. The PNMT-IMER was shown to display maximum product formation at 37 °C with limited changes in production at higher temperatures. Both the PNMT-IMER and PNMT-SP displayed no significant difference in the amount of product formed at temperatures exceeding 37 °C, Table 3. The non-immobilized enzyme however shows a considerable decrease in production of M at temperatures exceeding 60 °C [15]. The increase in stability of the immobilized enzymes is due to the environment that the enzymes are subjected to upon immobilization. Upon immobilization the enzyme is restricted in movement, which account for the lack of thermal denaturation at the higher temperatures.

PNMT is known to be inhibited by its own substrates and products at certain concentrations. The inhibitory effect of two PNMT inhibitors, *S*-adenosyl-L-homocysteine (SAH) and methyldopa was investigated for both PNMT-IMER and non-immobilized enzyme. Fifty percent inhibition was achieved at similar concentrations for both enzyme forms, see Table 4. The PNMT-IMER was shown to be inhibited by SAH at concentrations as low as 5 μ*M* and methyldopa concentrations of 1 μ*M*. The PNMT-IMER can therefore be used to designate the relative affinities of potential PNMT inhibitors.

In this study, two individual IMERs based upon DBH and PNMT were coupled using switching-valve technology and shown to carry out the on-line

Table 3
The effect of temperature on immobilized phenylethanolamine *N*-methyltransferase (PNMT-SP) in non-flow format (i.e. batch-wise) and immobilized PNMT in a column on the on-line system (PNMT-IMER) ($n=3$)

Temperature (°C)	[Metanephrine] (μ*M*)	
	PNMT-SP	PNMT-IMER
25	5.12±0.17[a]	7.44±0.02[a]
30	5.48±0.09[a]	8.33±0.14[a]
37	8.82±0.06	9.57±0.29
45	6.36±0.18	7.45±0.28
60	7.10±0.04	7.06±0.35

[a] *P* values <0.05.

Table 4
The effect of known inhibitors on the activity of non-immobilized phenylethanolamine *N*-methyltransferase (PNMT) and on the phenylethanolamine *N*-methyltransferase immobilized-enzyme reactor (PNMT-IMER) ($n=3$)

Inhibitor	IC_{50}	
	PNMT	PNMT-IMER
Methyldopa	10.4 μ*M*	7.6±0.2 μ*M*
SAH	40.1 μ*M*	50.5±1.5 μ*M*

synthesis of epinephrine from dopamine (Fig. 2). Dopamine was injected onto the DBH–IMER and the reactants and products were eluted onto a phenylboronic acid column for on-line extraction. The substrates and products were transported via a switching valve to the PNMT-IMER. Norepinephrine was then converted into epinephrine by the PNMT-IMER and directed onto the analytical columns for analysis (Fig. 4). The system allows for the analysis of the IMERs individually or as a combination. The construction of a coupled system of this nature provides a number of approaches to basic research into synthetic and metabolic pathways as well as a rapid method for the discovery of new pharmaceutical substances.

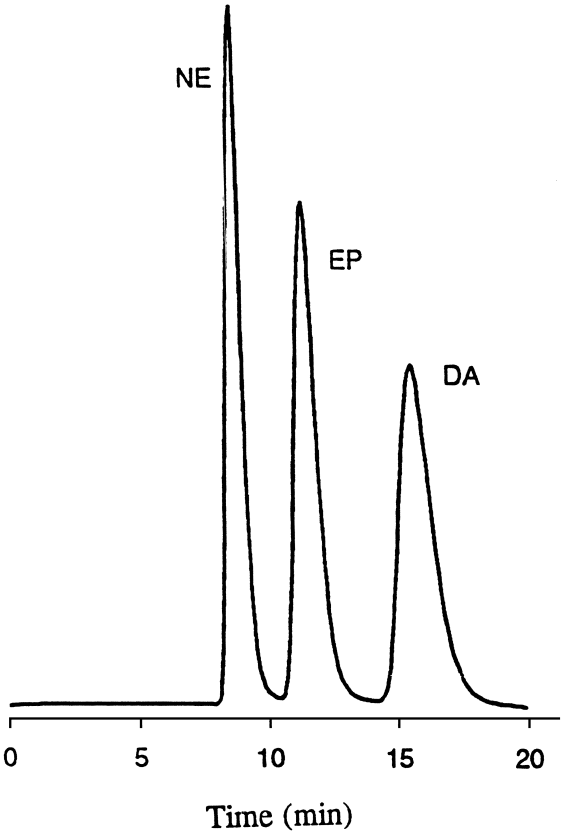

Fig. 4. Representative chromatogram of the coupled IMER system. On-line synthesis of epinephrine (EP) from dopamine (DA). NE = Norepinephrine.

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