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Development and validation of a HPLC electrochemical detection method to measure COMT activity as a tool in drug development

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The determination of catechol-O-methyltransferase (COMT) activity is considered valuable for various pharmaceutical and biomedical research projects. A specific high performance liquid chromatography-coulometric electrochemical detection method, for the assay of COMT activity was developed by measuring the formation of normetanephrine from norepinephrine. The chromatographic separation was achieved on a C_{18} reversed phase column with a mobile phase consisting of 10 mM sodium dihydrogen phosphate buffer, 4 mM sodium 1-octanesulfonate, 0.17 mM ethylenediaminetetra-acetic acid disodium salt, 6 % methanol and 4 % acetonitrile (pH ± 4.0). The detection of normetanephrine was achieved through electrochemical detection, with a coulometric cell potential setting of +450 mV. The flow rate was at 1 ml/min and the total run time was 45 min. The method was validated according to validation guidelines (Shabir 2006; European Medicines Agency 2011; US FDA 2018). The method was found to be linear ($R^2 > 0.99$) over the analytical range (100 to 2500 ng/ml) for all the analytes. All the other validation parameters (sensitivity, precision, accuracy, recovery and stability) were acceptable and within range. The method was applied for the determination of COMT activity in rat liver homogenate test samples. The known selective COMT inhibitor entacapone was used as test inhibitor. The results confirmed the ability of entacapone to inhibit COMT activity by decreasing the production of all the metabolites of norepinephrine.

1. Introduction

The development of new drugs rely on good analytical methods to facilitate the development process. Throughout the drug discovery and development processes, reliable analytical assay methods play a crucial role, whether used to determine *in vitro* inhibition potencies during library screening and optimization of potency or to determine if drug candidates possess *in vivo* potency at a specific protein target. For the development of novel inhibitors of the enzyme catechol-O-methyltransferase (COMT), specific and sensitive analytical methods are needed. For the assay of COMT activity, a variety of analytical methods has been developed over the years comprising enzyme-linked immunosorbent assays (ELISA), spectrophotometric and spectrofluorometric assays, and high performance liquid chromatographic (HPLC) methods. There is a variety of ELISA methods which are commercially available as test kits, but these are expensive and only a certain number of samples can be analysed per ELISA kit. The spectrophotometric methods generally have low sensitivity, are expensive and need large sample sizes. Although an HPLC method might be relatively expensive, it is highly sensitive, specific for a particular analyte and yields reliable and accurate measurements. For HPLC, the three detection methods most often used are ultraviolet (UV), fluorescent (FLD) and electrochemical detection (ECD) (Kaplan et al. 1992; Reenilä et al. 1995; Tsunoda et al. 2001). The COMT enzyme is a key enzyme in the metabolism of the neurotransmitters norepinephrine and epinephrine (Fig. 1) which are important chemical messengers (Sembulingam and Sembulingam 2012; Wilkinson and Brown 2015). A better understanding of these metabolic pathways and how drugs influence them can assist in the discovery and development of improved or more potent drugs for a variety of illnesses.

The aim of this study was to develop and validate a method to measure COMT activity in our laboratory which will be selective, specific, reliable and affordable under our conditions.

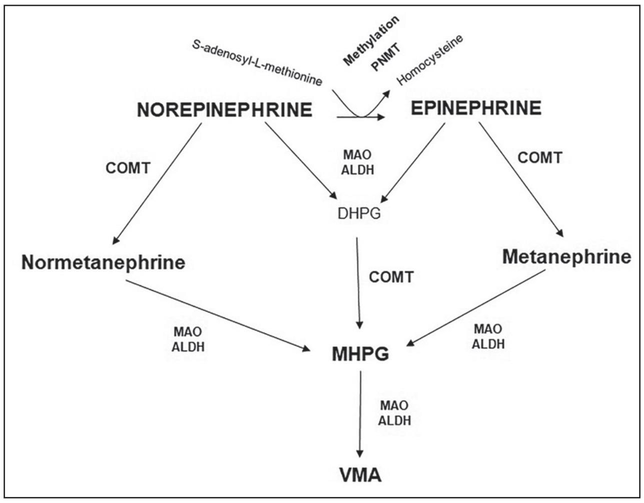

Fig. 1: Simplified diagram illustrating the metabolic pathways of norepinephrine and epinephrine. PNMT: Phenylethanolamine-N-methyltransferase; COMT: Catechol-O-methyltransferase; MAO: Monoamine oxidase; ALDH: Aldehyde dehydrogenase; DHPG: 3,4-Dihydroxyphenylglycol; MHPG: 3-Methoxy-4-hydroxyphenylglycol; VMA: Vanillylmandelic acid (Zianni et al. 2004).

2. Investigations and results

2.1. Methods (standards, mobile phase and sample preparation)

2.1.1. Preparation of standard solutions

A standard stock solution with a concentration of 100 µg/ml for each of the analytes, norepinephrine (NE), normetanephrine (NMN), 3-methoxy-4-hydroxyphenylglycol (MHPG), vanil-

lylmandelic acid (VMA), epinephrine (EPI) and metanephrine (MN) was prepared using a sodium phosphate buffer (di-sodium hydrogen orthophosphate; 25 mM; pH 7.8) as the solvent.
From this stock solution, eight solutions ranging from 100 ng/ml to 2500 ng/ml were prepared (Table 1). These solutions were used to establish the linear regression of the method.

Table 1: Concentrations of the different analytes (ng/ml well as in μM)

Conc. (ng/ml)	Conc. (μM)					
	VMA	MHPG	NE	NMN	EPI	MN
100	0,50	0,27	0,59	0,55	0,55	0,51
150	0,76	0,41	0,89	0,82	0,82	0,76
200	1,01	0,54	1,18	1,09	1,09	1,01
300	1,51	0,81	1,77	1,64	1,64	1,52
500	2,52	1,35	2,96	2,73	2,73	2,54
750	3,78	2,03	4,43	4,09	4,09	3,80
1000	5,05	2,70	5,91	5,46	5,46	5,07
2500	12,62	6,76	14,78	13,65	13,65	12,68

2.1.2. *Preparation of the internal standard solution*

A stock solution of the internal standard, 3,4-dihydroxy-benzylamine (DHBA) was prepared at a concentration of 100 μg/ml using sodium phosphate buffer as the solvent as described for the standards above. The working internal standard solution with a final concentration of 2000 ng/ml was prepared in 1 M $HClO_4$ with an appropriate dilution from the internal standard stock solution. This solution was used to terminate the enzyme reactions as the final steps in the sample preparation.

2.1.3. *Mobile phase preparation*

A mobile phase consisting of 10 mM sodium dihydrogen phosphate buffer, 4 mM sodium 1-octanesulfonate, 0.17 mM ethylenediaminetetra-acetic acid disodium salt, 6 % (v/v) methanol and 4 % (v/v) acetonitrile was prepared. The pH of the mobile phase was set at ±4.00 with ortho-phosphoric acid (85 %). The mobile phase was filtered through a 0.22 μm nylon filter before use (Agela Technologies).

2.1.4. *HPLC Instrument and electrochemical detector settings*

Instrumentation settings are given in Table 2.

Table 2: Instrumentation settings

HPLC instrument settings:	
Flow rate	1.0 ml/min
Injection volume	25 μl
Run time	45 min
Column temperature	23 °C (column compartment)
Electrochemical detector settings:	
Cell potential settings	Test electrode 1 (E1): -150 mV (to eliminate background noise)
	Test electrode 2 (E2): +450 mV (to analyse the analytes)
	Guard Cell (E^{GC}): +350 mV
	Detection range: 500 nA
	Filter: 0.5 s
	Offset: 0 %
	Signal output: 0.1 V
Data collection rate	20 Hz

2.1.5. *Sample preparations for standards and test samples modified from Aoyama et al. (2005)*

The standard samples were prepared in 1.5 ml microcentrifuge tubes. For the standards, DMSO replaced the test inhibitor drug while distilled water replaced the COMT enzyme solution.

Table 3: Sample preparation steps for standards

Steps	Reagents	Volume (Total = 137.5 μl)
1.	2 mM $MgCl_2$	10 μl
2.	Standard (100 – 2500 ng/ml)	35 μl
3.	200 μM *S*-(5′-sdenosyl)-*L*-methionine (SAMe)	25 μl
4.	DMSO	5 μl
5.	Sample was vortexed for 5 s and incubated at 37 °C for 10 min	
6.	Distilled water instead of COMT	50 μl
7.	Samples were vortexed for 5 s and allow to incubate at 37 °C for 60 min	
8.	1 M $HClO_4$ (to terminate reaction, containing the internal standard)	12.5 μl

Table 4: Sample preparation steps for test liver tissue samples

Steps	Reagents	Volume (Total = 137.5 μl)
1.	2 mM $MgCl_2$	10 μl
2.	250 μM substrate (norepinephrine)	35 μl
3.	200 μM SAMe	25 μl
4.	Test inhibitor (entacapone) dissolved in DMSO	5 μl
5.	Sample was vortexed for 5 s and incubated at 37 °C for 10 min	
6.	COMT solution	50 μl
7.	Sample was vortexed for 5 s and incubated at 37 °C for 60 min	
8.	1 M $HClO_4$ (To terminate reaction, containing the internal standard)	12.5 μl

After the stop solution (1 M $HClO_4$) was added, the samples were centrifuged at 4 °C for 10 min at 20 817 rcf. The supernatants were subsequently transferred to HPLC vial inserts. The Chromeleon® Chromatography Management System software was then programmed to inject 25 μl of the sample into the HPLC system.

2.2. *Method validation parameters*

Electrochemical detection techniques coupled to an HPLC instrument offers high sensitivity and selective detection for catecholamines and their metabolites (Peaston and Weinkove 2004; Dong 2006; Swartz 2010).
The method was validated according to the guidelines of the FDA for linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy (repeatability), recovery and stability (US FDA, 2013).

2.2.1. *Linearity (calibration curve)*

Linearity was evaluated by preparing eight standard concentrations as described in section 2.1.1. Six replicates of each standard were injected to establish linear regression for each analyte. The linear regression value (coefficient of determination, R^2) for the calibration curve should not be less than 0.95 for endogenous biomolecules (Shabir 2006). For calibration, the internal standard method was used with DHBA as the selected internal standard.

2.2.2. *Quantification and detection limits (sensitivity)*

The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) can be defined as the minimum concentration

Table 5: Linear regression line equation and coefficient of determination

Analytes	$y = mx + c$	Coefficient of determination (R^2)
Norepinephrine (NE)	$y = 0,0012x + 0,0251$	$R^2 = 0,9997$
Normetanephrine (NMN)	$y = 0,0013x + 0,0100$	$R^2 = 0,9999$
3-Methoxy-4-hydroxyphenyl-glycol (MHPG)	$y = 0,0015x + 0,1119$	$R^2 = 0.9926$
Vanillylmandelic acid (VMA)	$y = 0,0009x - 0,0266$	$R^2 = 0.9987$
Epinephrine (EPI)	$y = 0,0014x + 0,0547$	$R^2 = 0,9988$
Metanephrine (MN)	$y = 0,0012x + 0,0100$	$R^2 = 0,9999$

where the signal-to-noise ratio is at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava and Gupta 2011).

2.2.3. *Precision and accuracy*

Four concentrations ranging from low to high (100, 200, 500 and 2500 ng/ml) were selected and six samples of each concentration was prepared and injected to determine precision and accuracy. Precision was expressed in %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15 % (US FDA 2013). The accuracy for each concentration level was determined by comparing the mean test concentration to that of the true concentration value. The accuracy was expressed as percentage recovery. The acceptability criterion for accuracy for each concentration level was 90 to 110 % (Shabir 2006; US FDA 2013).

2.2.4. *Recovery*

The percentage recovery of the extraction procedure was determined by preparing four spiked concentrations (100, 200, 500 and 2500 ng/ml) in solution A. The peak areas were compared to those of the prepared standards. This was done in triplicate for each concentration and the mean percentage recovery was calculated. The percentage recovery for each concentration must be above 90 % and consistently reproducible to adhere to the acceptance criterion.

2.2.5. *Stability*

Stability was determined by injecting the eight standard solutions and two test samples directly after sample preparation. The initial injection set was assayed immediately and served as reference

Table 6: Accuracy and precision results

Analyte conc. (ng/ml)	Intra-day (n=6)			Inter-day (n=6)		
	Measured conc. (ng/ml) (mean ± SD)	Precision (%RSD)	Accuracy (%)	Measured conc. (ng/ml) (mean ± SD)	Precision (%RSD)	Accuracy (%)
Norepinephrine (NE)						
100 (LLOQ)	101.36 ± 1.60	1.58	101.36	90.47 ± 5.95	6.57	90.47
200	209.95 ± 1.49	0.71	104.97	182.91 ± 11.50	6.29	91.46
500	508.62 ± 2.11	0.42	101.72	487.60 ± 12.12	2.43	97.46
2500	2557.27 ± 19.77	0.77	102.29	2471.01 ± 56.82	2.30	98.84
Normetanephrine (NMN)						
100 (LLOQ)	105.70 ± 2.94	2.78	105.70	94.61 ± 4.29	4.53	94.61
200	208.81 ± 2.70	1.29	104.40	191.57 ± 7.12	3.71	95.78
500	501.37 ± 2.39	0.48	100.27	498.64 ± 6.29	1.26	99.73
2500	2519.36 ± 16.37	0.83	100.76	2481.08 ± 67.05	2.70	99.24
Vanillylmandelic acid (VMA)						
100 (LLOQ)	108.03 ± 4.06	3.76	108.03	91.74 ± 7.38	8.04	91.74
200	204.29 ± 8.74	4.28	102.15	188.9 ± 15.68	8.30	94.49
500	503.63 ± 14.81	2.94	100.73	484.08 ± 33.99	7.02	96.82
2500	2711.35 ± 13.76	0.51	108.45	2260.41 ± 41.12	1.82	90.42
3-Methoxy-4-hydroxyphenylglycol (MHPG)						
100 (LLOQ)	103.54 ± 3.60	3.48	103.54	96.58 ± 2.29	2.37	96.58
200	207.25 ± 7.25	3.50	103.62	193.01 ± 5.66	2.93	96.50
500	511.40 ± 4.96	0.97	102.28	498.60 ± 12.12	2.43	99.72
2500	2501.59 ± 45,71	1.83	100.06	2498.41 ± 63.59	2.55	99.94
Epinephrine (EPI)						
100 (LLOQ)	106.88± 1.85	1.73	106.88	90.72 ± 6.77	7.46	90.72
200	200..41 ± 3.72	1.86	100.20	181.32 ± 15.85	15.85	90.66
500	500.95 ± 2.86	0.57	100.19	467.45 ± 17.79	3.81	93.49
2500	2502.44 ± 16.16	0.65	100.10	2469.27 ± 49.21	1.99	98.77
Metanephrine (MN)						
100 (LLOQ)	106.05 ± 3.40	3.20	106.05	93.12 ± 1.67	1.79	93.12
200	208.75 ± 9.06	4.34	104.37	191.62 ± 5.04	2.63	95.81
500	503.31 ± 8.72	1.73	100.66	496.71 ± 10.14	2.04	99.34
2500	2527.62 ± 23.64	0.94	101.10	2472.69 ± 56.08	2.27	98.91

values. The samples were reinjected after 24 h and 48 h, while keeping the samples at room temperature in the HPLC auto-sampler tray. Percentage stability for each analyte is given as the mean percentage stability (±SD) of the samples analysed at the given time point.

2.3. Method validation results

2.3.1. Linearity/calibration curve

The calibration curves constructed for all analytes were evaluated by means of linear regression analysis. Six replicates of each standard were injected and the mean used to establish linear regression for each analyte. Linearity was excellent over the calibration ranges (100 to 2500 ng/ml), with corresponding coefficient of determination (R^2) consistently greater than 0.99 for all analytes (Table 5).

2.3.2. Quantification and detection limits (sensitivity)

The limit of detection (LOD) and the limit of quantification (LOQ) were determined to be 10 ng/ml and 100 ng/ml, respectively, for all the analytes that were analysed and evaluated in this study.

2.3.3. Precision and accuracy

The results for the determination of precision and accuracy of all four selected concentrations are provided in Table 6. Both the precision and accuracy were within the acceptable criteria ranges set by the method validation parameters in section 2.2.3. For all analytes, at these four concentrations the %RSD for both the intra-day and inter-day precision was smaller than 9 %, which is well below the 15 % criteria. The accuracy of all concentration levels for all of the analytes tested, ranged from 90.47 to 108.45 %, which is within the set criteria of 90 to 110 %.

Table 7: Percentage recovery results

Analytes	*Conc. (ng/ml)*	*% Recovery (mean ± SD)*
Norepinephrine (NE)	100 (LLOQ)	98.60 ± 4.40
	200	98.80 ± 2.54
	500	97.90 ± 2.84
	2500	98.29 ± 2.10
Normetanephrine (NMN)	100 (LLOQ)	95.02 ± 9.14
	200	97.20 ± 1.01
	500	100.91 ± 1.53
	2500	99.61 ± 2.38
Vanillylmandelic acid (VMA)	100 (LLOQ)	101.88 ± 3.34
	200	96.43 ± 5.97
	500	100.81 ± 3.55
	2500	99.34 ± 3.84
3-Methoxy-4-hydroxyphenylglycol (MHPG)	100 (LLOQ)	99.22 ± 1.84
	200	97.24 ± 1.50
	500	106.68 ± 4.37
	2500	97.82 ± 3.90
Epinephrine (EPI)	100 (LLOQ)	98.32 ± 6.21
	200	98.81 ± 5.50
	500	97.44 ± 1.74
	2500	99.73 ± 2.41
Metanephrine (MN)	100 (LLOQ)	94.15 ± 1.87
	200	95.69 ± 1.17
	500	102.48 ± 1.97
	2500	99.65 ± 2.34

2.3.4. Recovery

The mean absolute recovery for each analyte, measured in triplicate for all four concentrations tested, were constantly above 90%, with the mean recovery indicated in Table 7.

2.3.5. Stability

In Table 8, the results of the stability study are provided as the mean percentage values. For all the analytes, the stability of eight standard solutions as well as the stability of the analytes in three test rat liver homogenate samples are demonstrated. All the analytes in the standard solutions as well as in the test tissue samples were stable when tested at 24 h and 48 h after being stored at room temperature in the auto-sampler tray. In contrast poor stability was recorded for the analytes after 48 h in the presence of rat liver homogenate. The 48 h poor stability of the analytes in the rat liver homogenate samples can be due to the storage at room temperature. Room temperature of ±23 °C is high enough to cause natural degradation of the analytes in the samples (Shabihkhani et al. 2014). This can be resolved by using a temperature controlled auto-sampler which can help to keep the samples stable for longer, but good laboratory practice will be to analyse the samples rather within 24 h.

Table 8: Stability results for the eight standards

% Stability (mean ± SD) of monoamines analytes at room temperature after 24 h and 48 h.		
Time:	***24 hours****	***48 hours****
Norepinephrine (NE)	98.54 ± 2.52	101.85 ± 4.07
Normetanephrine (NMN)	101.51 ± 0.95	104.37 ± 3.02
Vanillylmandelic acid (VMA)	95.40 ± 5.96	103.74 ± 9.82
3-Methoxy-4-hydroxyphenylglycol (MHPG)	102.52 ± 1.70	108.61 ± 3.09
Epinephrine (EPI)	98.45 ± 2.73	101.38 ± 4.15
Metanephrine (MN)	99.81 ± 1.86	104.50 ± 4.60
Stability results for the three test rat liver homogenate samples		
Time:	***24 hours****	***48 hours****
Norepinephrine (NE)	99.27 ± 3.71	83.84 ± 4.89
Normetanephrine (NMN)	99.37 ± 2.47	85.26 ± 13.14
Vanillylmandelic acid (VMA)	100.43 ± 4.40	81.88 ± 4.21
3-Methoxy-4-hydroxyphenylglycol (MHPG)	94.73 ± 6.06	70.74 ± 9.92
Epinephrine (EPI)	90.06 ± 5.95	58.88 ± 13.32
Metanephrine (MN)	Stability could not be calculated because MN was below LOD in most of the rat liver samples tested.	

*Compared to 100% at t = 0, mean ±SD.

Table 9: Relative retention times of the monoamines and their metabolites and the internal standard

Analytes	**Relative retention times (minutes)**
1. Vanillylmandelic acid (VMA)	± 3.488
2. 3-Methoxy-4-hydroxyphenylglycol (MHPG)	± 5.0424
3. Norepinephrine (NE)	± 13.015
4. Epinephrine (EPI)	± 17.044
5. Internal Standard (DHBA)	± 22.009
6. Normetanephrine (NMN)	± 24.347
7. Metanephrine (MN)	± 34.152

2.4. Chromatographic results

Table 9 presents the relative retention times of norepinephrine, epinephrine, their metabolites and the internal standard for this method.

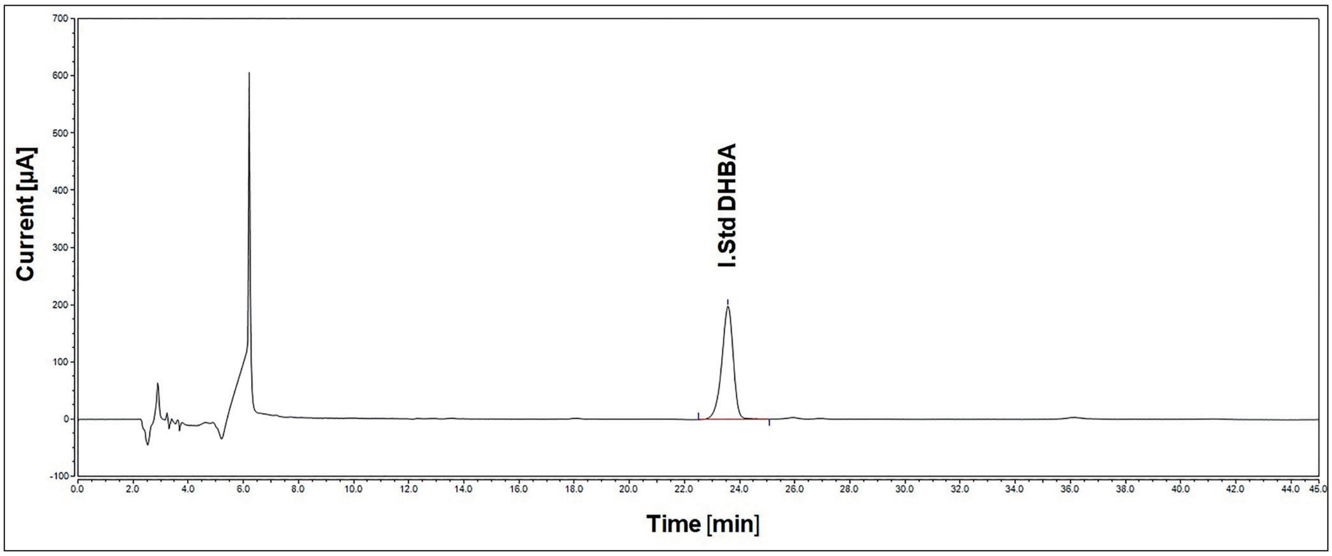

Fig. 2: Blank sample with internal standard (DHBA).

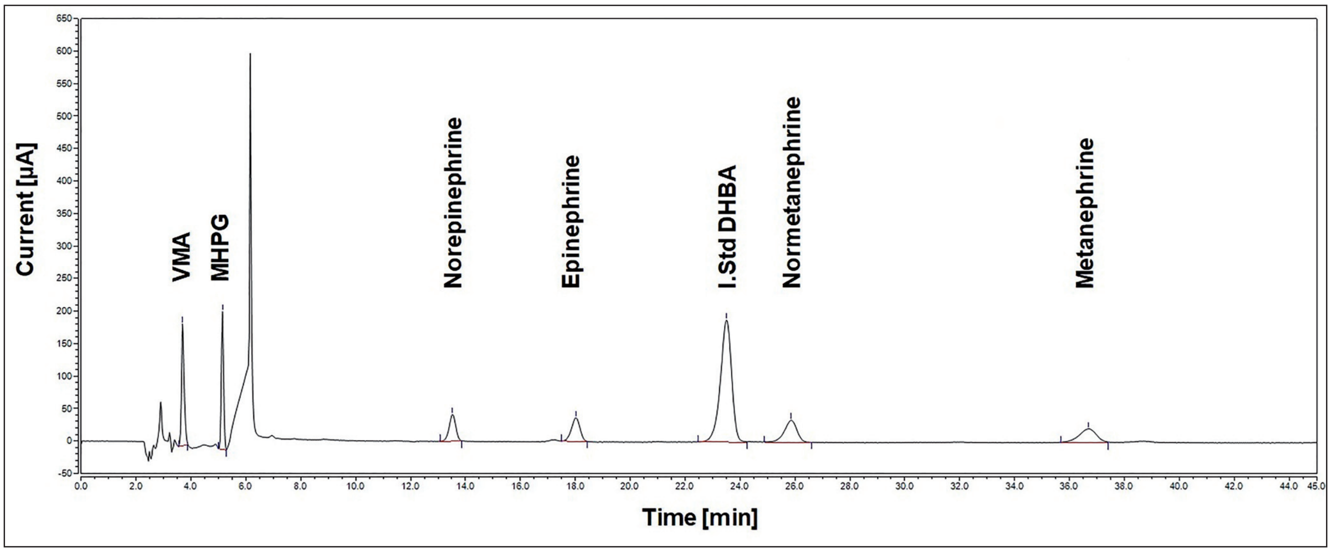

Fig. 3: Standard solution containing 100 ng/ml of each analyte.

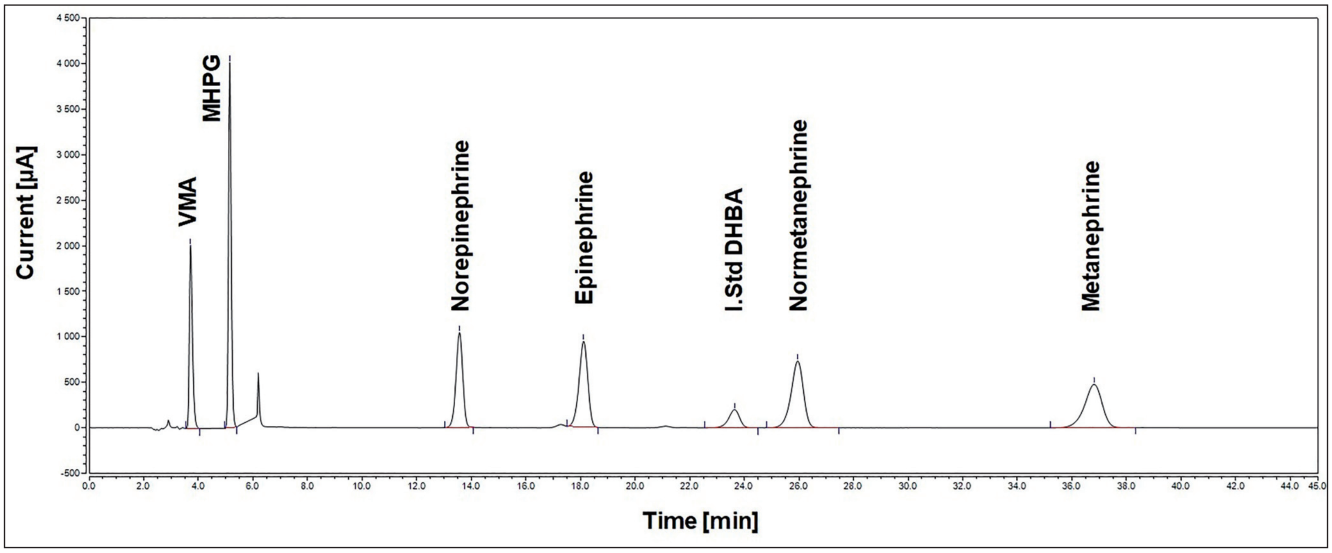

Fig. 4: Standard solution containing 2500 ng/ml of each analyte.

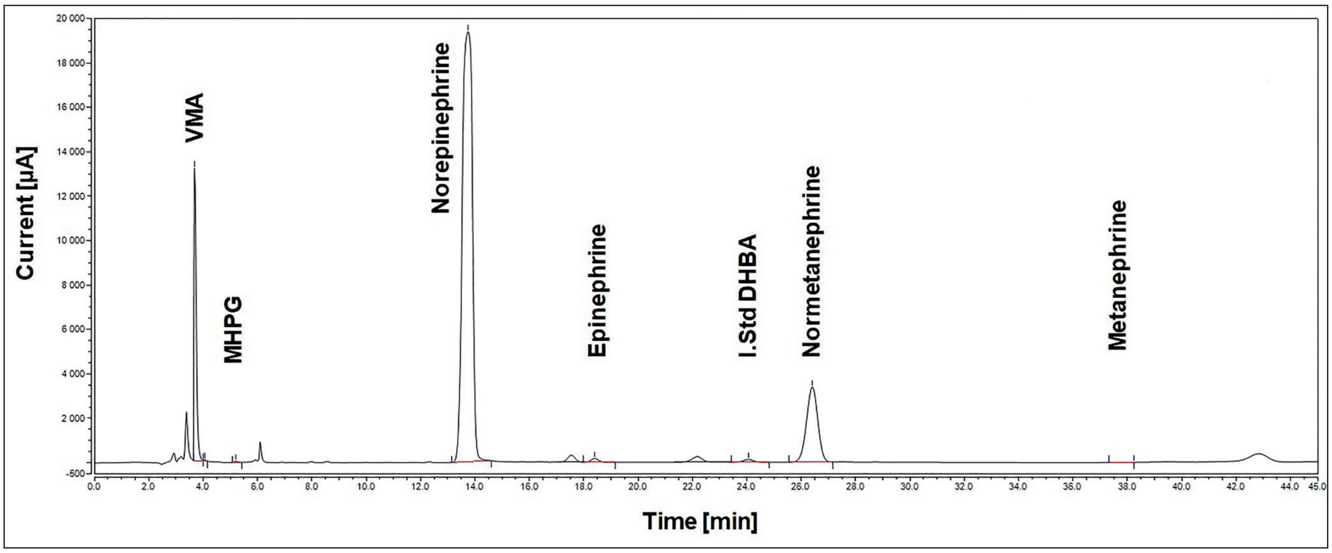

Fig. 5: Rat liver homogenate sample treated with 0 µM entacapone.

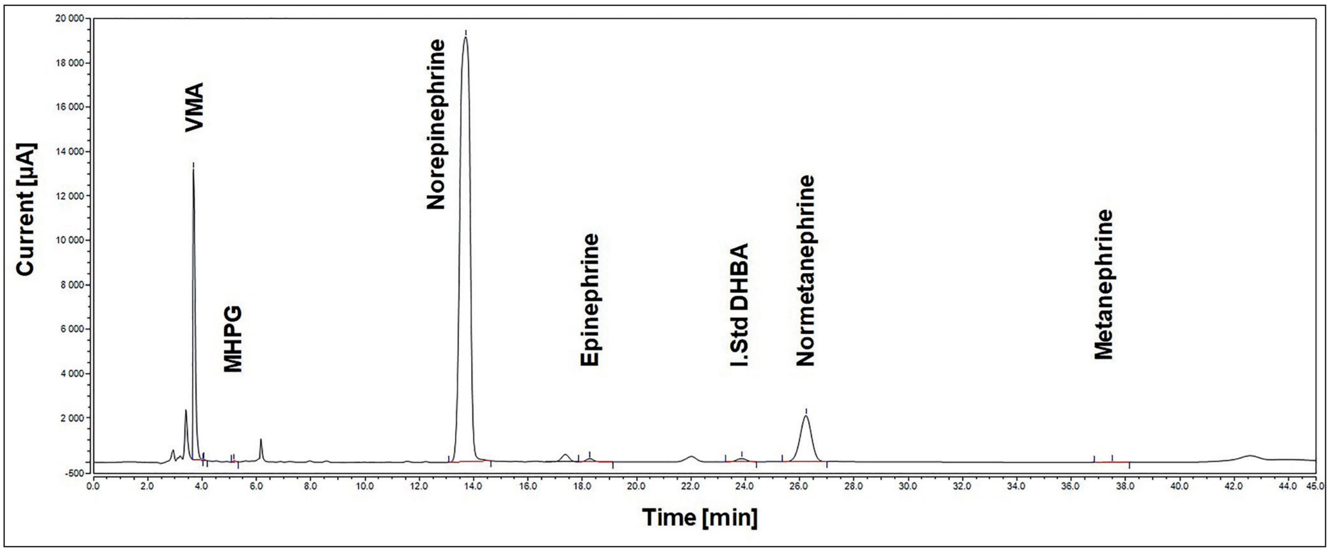

Fig. 6: Rat liver homogenate sample treated with 0.3 µM entacapone.

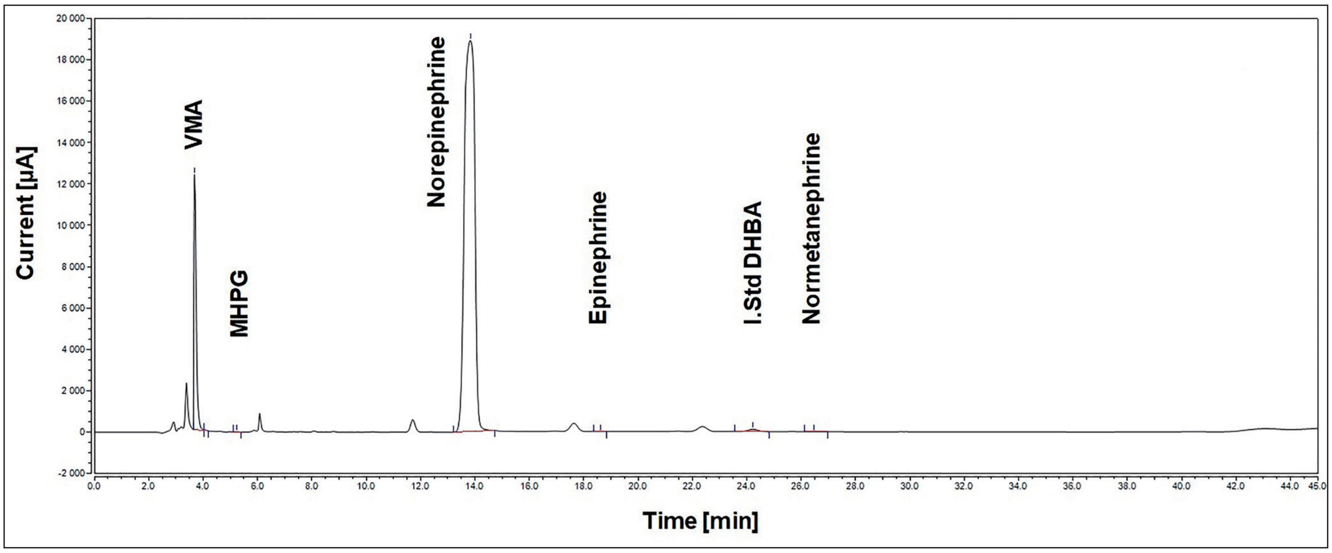

Fig. 7: Rat liver homogenate sample treated with 100 µM entacapone (metanephrine in this sample was below LOD).

The following three chromatograms are representative of the following samples: a blank sample containing only the internal standard, DHBA (Fig. 2); a standard solution containing 100 ng/ml of each analyte (Fig. 3); a standard solution containing 2500 ng/ml of each analyte Fig. 4).

2.5. *Application of the method to prepared rat liver tissue test samples*

The following three chromatograms are representative of the prepared rat liver homogenate test samples that were treated with norepinephrine and incubated for 20 min (see section 2.1.5). The samples were also treated with a range of concentrations (from 0 µM to 100 µM) of the selective COMT inhibitor, entacapone (Katzung 2018).

In chromatographic separation the ratio (fluctuations) of the retention times of the analytes and internal standard must correspond to that of standard solution within a tolerance of ±2.5 % for LC (European Commission 2002).

Table 10 shows the results of the COMT assay that was performed to illustrate the inhibition of COMT by entacapone. Entacapone successfully inhibited the COMT activity by decreasing not only the production of normetanephrine, but also indirectly decreasing the production of MHPG and VMA. The reduction of metabolite concentration correlated with the increased concentration of entacapone.

Table 10: COMT assay results done on rat liver homogenate with entacapone used as the inhibition drug

Entacapone conc. added (µM)	Norepinephrine	NMN	MHPG	VMA	Epinephrine	MN
	Concentration measured (µM)					
0	758,60	126,70	0,28	125,44	4,26	0,05
0.01	595,65	109,29	0,22	103,33	3,57	0,16
0.1	582,46	84,15	0,15	100,80	3,33	0,38
0.3	715,66	46,52	0,11	122,82	3,45	0,12
1.0	563,87	1,66	0,02	88,93	0,52	BLOD
3.0	644,78	0,32	0,03	104,53	0,20	BLOD
10.0	653,03	0,06	0,02	102,91	0,06	BLOD
100.0	657,90	0,05	0,02	99,79	0,03	BLOD

BLOD (Below limit of detection)

3. Discussion

In this study, a specific HPLC method, coupled to a coulometric electrochemical detection method, was developed for the measurement of COMT activity in rat liver homogenate. This method was validated for linearity, sensitivity, precision, accuracy, recovery and stability. The linearity over the relevant calibration ranges for all analytes was excellent and the coefficient of determination (R^2) was consistently above 0.95. The LLOQ and LLOD were respectively 100 ng/ml and 10 ng/ml for all analytes. The percentage recovery was acceptable for all the analytes at all the concentrations measured. The percentage stability was also acceptable for both the standards and test samples at 24 h, but not for the test samples at 48 hours. The method was found to be very sensitive and specific for all the analytes tested, although metanephrine was below LOD in some of the rat liver homogenate test samples.

The newly developed and validated method was applied to measure COMT activity in rat liver homogenate and to illustrate with the inhibition of COMT by the known selective COMT inhibitor entacapone, at a concentration range of 0 µM to 100 µM. Entacapone inhibited the activity of COMT in a concentration-dependent manner and decreased the production of normetanephrine, MHPG and VMA. A decrease in epinephrine and metanephrine were also observed in the study. In future studies other known COMT inhibitor may also be used to further validate and test this new method. We propose that this new method can be applied to future studies where COMT activity can be measured with norepinephrine as a substrate.

4. Experimental

4.1. *Chemicals and reagents*

l-Noradrenaline hydrochloride, d,l-normetanephrine hydrochloride, 3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt, vanillylmandelic acid, epinephrine bitartrate salt, d,l-metanephrine hydrochloride and the 3,4-dihydroxy-benzylamine (internal standard) were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionised water and HPLC grade methanol (MeOH) and acetonitrile (ACN); sodium dihydrogen phosphate buffer; 1-octanesulphonic acid sodium salt; ethylenediaminetetra-acetic acid disodium salt; orthophosphoric acid (H_3PO_4 85 %). Chemicals used for the sample preparation were magnesium chloride, *S*-(5′-sdenosyl)-*L*-methionine (SAMe) chloride dihydrochloride, dimethyl sulfoxide (DMSO); perchloric acid (70%). All the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa) and Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa).

4.2. *Materials*

The analytical HPLC column used was a Venusil ASB C_{18} (purchased from Bonna-Agela Technologies, USA), 4.6 x 250 mm, a particle size of 5 µm, pore size of 300 Å and a surface area of 200 m^2/g. The developed and validated method was applied to prepared Sprague-Dawley rat liver homogenate samples.

4.3. *Instrumentation*

The chromatographic system consisted of an Ultimate 3000 UHPLC system, equipped with an ISO-3100SD isocratic pump and WPS-3000TSL analytical autosampler, coupled to an ECD-3000RS rapid separation electrochemical detector with 2-Channel Coulometric Cell 6011RS and Chromeleon® chromatography management system version 7.2 (all obtained from Thermo Fisher Scientific, Waltham, MA USA).

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