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A simple high-performance liquid chromatography assay for simultaneous determination of plasma norepinephrine, epinephrine, dopamine and 3,4-dihydroxyphenyl acetic acid☆

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Abstract

A reversed-phase HPLC assay coupled with electrochemical detection for simultaneously measuring plasma levels of norepinephrine, epinephrine, dopamine, and 3,4-dihydroxyphenylacetic acid (DOPAC) was developed. Separation of the catecholamines and the internal standard isoproterenol was obtained by a mobile phase consisting of 7% methanol in 0.1 M citrate buffer containing 0.3 mM sodium ethylenediaminetetraacetic acid (EDTA), and 0.5 mM 1-octanesulfonic acid, operated under isocratic condition at a flow rate of 1.2 ml/min. The potential of the guard cell was set at $+650$ mV, the first electrode of the analytical cell at $+100$ mV and the second at $+350$ mV. Using a signal-to-noise ratio of >3, the minimum detection limit assessed by direct on column injection was <10 pg for analyte. The assays were linear from basal concentrations to 400 ng/ml. The intra- and inter-assay variations were <10 and 15%, respectively. The assay has been applied successfully to measure plasma concentrations of these catecholamines in humans, rabbits and rats.

Keywords: Analysis; Catecholamines; HPLC; Neurohormones; Pharmacodynamics

1. Introduction

Plasma concentrations of catecholamines and their metabolites are often useful for diagnosis and evaluation of therapeutic and pharmacodynamic effects for psychiatric, neurological and cardiovascular disorders [1–3]. Patients who received anti-depressants such as monoamine oxidase inhibitors, cardiovascular agents such as the calcium antagonists or angiotensin receptor antagonists may have altered plasma catecholamine concentrations [4–7].

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Plasma catecholamine concentrations most commonly have been determined by HPLC with electrochemical detection [8–10]. Extraction of catecholamines from plasma can be effectively achieved using alumina adsorption followed by desorption with a suitable solvent [9,11]. Other extraction methods such as liquid–liquid extraction [12], solid-phase extraction [13], and ultrafiltration [14] have also been used. The current paper describes a simple HPLC assay coupled with electrochemical detection for simultaneous determination of plasma concentrations of norepinephrine, epinephrine, dopamine and 3,4-dihydroxyphenyl acetic acid (DOPAC).

2. Experimental

2.1. Chemicals and solvents

Norepinephrine, epinephrine, dopamine, DOPAC, the internal standard isoproterenol (Fig. 1), and other catecholamines were purchased from Sigma (St. Louis, MO, USA). Various medications which have been used to test for interference of the assay were obtained from their respective pharmaceutical manufacturers. Acid-washed alumina (type WA-4, acid), and sodium 1-octanesulfonic acid were purchased from Sigma, and used as such without purification. All solvents were HPLC grade (BDH Chem., NS, Canada), and other chemicals reagent grade (VWR or Fisher Scientific Canada).

2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-10AD pump (purchased from Man-Tech, Guelph, Ont., Canada), a Rheodyne syringe loading injector (model 9125), with a 100-ml peek injection loop (purchased from Scientific Products & Equipment, Concord, Ont., Canada), a 5-mm 250×4.6-mm i.d. reversed-phase C_{18} column (Ultrasphere-ODS, Beckman Canada, Mississauga, Ont., Canada) preceded by a 5-mm 4×4-mm i.d. C_{18} reversed-phase guard column (Licrocart®, Merck, Germany), an ESA-Coulochem II electrochemical detector with triple glassy carbon electrodes (purchased from Scientific Products & Equipment), and a Spectra Physics 4600 Data Jet Integrator (Toronto, Ont., Canada). The system was operated isocratically at room temperature, with a mobile phase made up of 0.1 M citrate buffer, 0.3 mM disodium ethylenediaminetetraacetic acid (EDTA), 0.5 mM sodium 1-octanesulfonic acid in methanol:water (7:93) with final pH adjusted to 2.5 using 20% sodium hydroxide solution. The mobile phase was filtered through a 0.22-mm filter (Type GVWP, Millipore, Bedford, MA, USA) and degassed prior to use. The flow rate was set at 1.2 ml/min and the operating pressure 1.5 kpsi. The electric potentials of the detector were set as follows: guard cell (model 5020) which was positioned between the pump and the injector at $+650$ mV, the first electrode of the analytical cell (model 5011) at $+100$ mV, and the second at $+350$ mV. The detector range was set at 500 nA, and the output of the second electrode was channelled to the integrator for recording. The integrator was set at attenuation 4 to 8, and chart speed at 0.25 cm/min.

2.3. Preparation of working standards and samples

Stock solutions (1 mg/ml) of norepinephrine, epinephrine, dopamine and DOPAC were prepared in methanol. An aliquot of each of the stock solutions was combined, and diluted with distilled water to produce a spiking stock solution (6 mg/ml). This solution was prepared immediately before it was used to prepare plasma standards at 20, 40, 100, 200 and 400 ng/ml using rat plasma by serial dilution. A working internal standard solution (100 ng/ml) was prepared in distilled water. All solutions were stored at -80°C in the dark.

Blood samples were obtained from healthy volunteers ($n = 3$), New Zealand White rabbits ($n = 6$) and normotensive Sprague–Dawley rats ($n = 5$) for validation. They were immediately centrifuged to separate plasma which was then

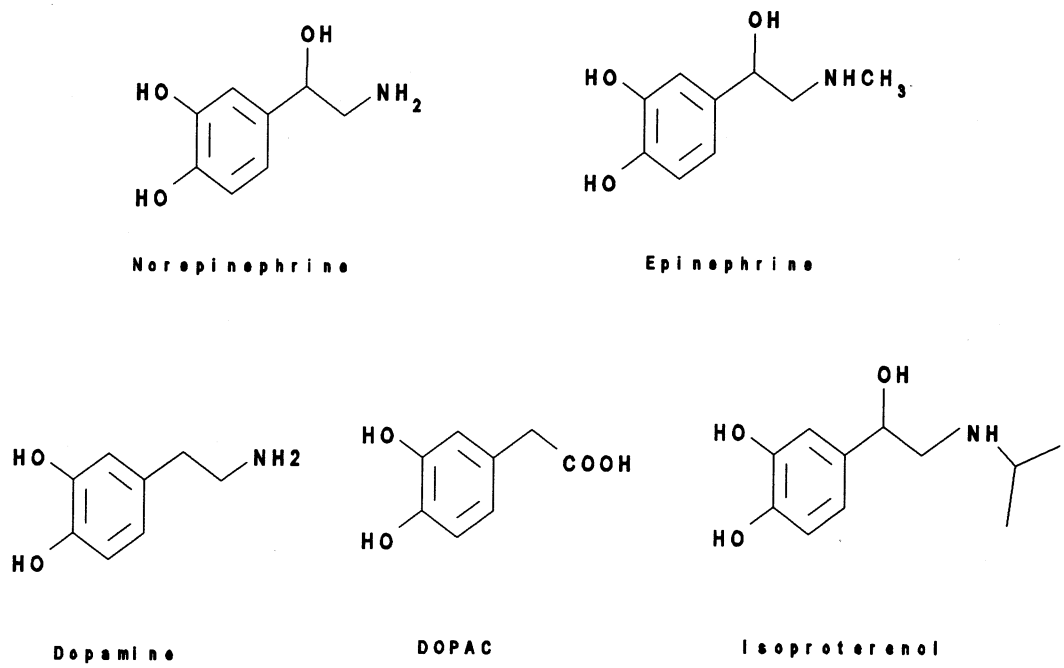

Fig. 1. Chemical structures of norepinephrine, epinephrine, DOPAC, dopamine and the internal standard isoproterenol.

stored at −80°C in the dark. These samples were analyzed within 2 weeks after the collection.

2.4. Extraction of catecholamines from plasma

To a 1.5-ml polyethylene microcentrifuge tube was added 0.1–0.5 ml of plasma sample or standard, 0.5 ml of tris buffer (1.5 M, pH 8.6) containing 0.07 M EDTA, 5 mg of acid-washed alumina, and 50 ml of working internal standard solution (5 ng). The mixture was shaken vigorously for 30 min (Vibrax, Terochem Canada, Markham, Ont.), then centrifuged at 2000 rpm for 1 min at room temperature (Eppendorf centrifuge model 5415, Brinkmann, Westbury, NY, USA). The supernatant fraction was aspirated and discarded. The remaining alumina was washed twice with 1 ml of glass-distilled water, and subsequently with a mixture of 0.04 M phosphoric acid–0.2 M acetic acid (20:80, v/v, pH 1.5–2.0) to desorb the catecholamines. The acidic eluate was transferred to a microcentrifuge tube, and stored at −80°C until analysis.

2.5. Data analysis

Peak height ratios of norepinephrine, epinephrine, dopamine or DOPAC to the internal standard versus plasma concentrations of the standards after background subtraction were analyzed by linear regression analysis. Each concentration was assayed in quadruplicate. Plasma concentrations of each of the catecholamines in unknown samples were calculated from the respective standard curves. Robustness of the assay was evaluated using spiked plasma standards which were repeatedly analysed over a 1-month period, and using samples obtained from healthy volunteers rabbits and rats.

3. Results

3.1. Specificity, recovery, sensitivity and linearity

Norepinephrine, epinephrine, dopamine, DOPAC and the internal standard isoproterenol were well separated from each other and other plasma endogenous materials with retention

Table 1
Mean retention times of catecholamines and related compounds[a]

Compound	Retention time (min)	Compound	Retention time (min)
Norepinephrine	3.5[b]	HVA	15
Epinephrine	4.1	Clomipramine	>30
DOPAC	5.2	Amitriptyline	>30
Dopamine	7.0	Desipramine	>30
5-HIAA	8.5	Moclobemide	>30
Isoproterenol	9.6	Diltiazem	>30
VMA	<2.0	Verapamil	>30

[a] DOPAC, dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; VMA, vanillylmandelic acid; HVA, homovanillic acid.
[b] Each value is determined by direct injection of standard solution of each compound, and represents the mean of three injections.

times of 3.5, 4.1, 7.0 and 9.6 min, respectively (Table 1 and Fig. 2). A series of catecholamines and related drugs acting on the cardiovascular and central nervous systems tested did not interfere with the assay (Table 1). Using plasma standards of 40 and 100 ng/ml for validation, mean recoveries were 90 ± 8.6 and $85 \pm 11\%$ for norepinephrine, 72 ± 4.9 and $71 \pm 11\%$ for epinephrine, 68 ± 12 and $52 \pm 6.5\%$ for dopamine, 68 ± 12 and $52 \pm 6.5\%$ for DOPAC, and the recovery for isoproterenol at the concentration used (ca 5 ng/ml) was $89 \pm 10\%$. Using a signal-to-noise ratio of >3, the minimum detection limit assessed by direct on column injection was less than 10 pg per injection for all the analytes measured. The limit of quantitation varied depending on the baseline plasma concentrations which tended to fluctuate between samples. Based on our experience and depending on many factors such as method of blood sample collection, plasma concentrations of catecholamines can vary by as much as 20-fold. Therefore, it is necessary to use plasma standards of higher concentrations such that the lowest concentration (20 ng/ml) could still produce a peak height ratio greater than three times that of the baseline concentrations. The standard curves for all the analytes were linear from baseline concentrations to 400 ng/ml, with coefficient of determination (r^2) > 0.99. The mean relative standard deviations (% CV) for the concentration range were less than 15% in all cases (Table 2).

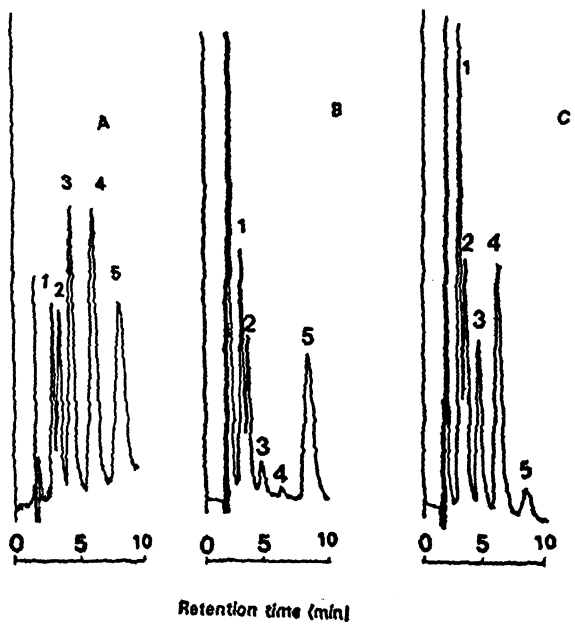

Fig. 2. HPLC-UV chromatograms of the catecholamines in a typical rat plasma sample: (A) 5 ml injection of a standard solution containing 50 pg each of the catecholamines; (B) 20 ml injection of a typical plasma sample; and (C) 5 ml injection of a plasma standard containing 20 ng/ml of each analytes). Notation: (1) norepinephrine; (2) epinephrine; (3) DOPAC; (4) dopamine; (5) isoproterenol.

3.2. Precision and accuracy

Intra- and inter-assay variations determined over a 1-month period were less than 15%. Accuracy of the assay over the concentration range of

Table 2
Standard curves data for catecholamines

Concentration (ng/ml)	Norepinephrine		Epinephrine		DOPAC		Dopamine	
	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
Baseline	4.2[a]	12	1.0	8.5	0.67	15	0.24	12
20	7.4	13	2.6	6.6	3.2	13.6	5.3	4.9
40	14	9.6	4.9	9.9	5.3	4.8	10	7.7
100	35	7.0	13	12	13	12	25	6.5
200	70	1.6	24	2.0	34	9.2	51	2.9
400	150	11	55	13	65	8.2	110	12
Mean % CV		9.0		8.6		11		7.7
r^2	>0.99		>0.99		>0.99		>0.99	
Slope	0.37		0.14		0.17		0.27	
Intercept	−1.3		−0.62		−0.93		−0.54	

[a] Each value represents mean peak height ratio of four replicates.

the standard curves was from 96 to 120% for norepinephrine, 92 to 120% for epinephrine, 92 to 130% for DOPAC, and 96 to 110% for dopamine.

3.3. Plasma samples

Plasma concentrations of the catecholamines determined from three healthy volunteers were 0.94 ± 0.21 ng/ml for norepinephrine, 1.4 ± 0.51 for DOPAC, and 0.20 ± 0.02 ng/ml for dopamine. Epinephrine was measurable only in one volunteer (0.56 ng/ml). Plasma concentrations of the catecholamines were comparable in the three species studied. The results are summarized in Table 3.

Table 3
Plasma concentrations of catecholamine in humans, rats and rabbits

Catecholamines	Humans (ng/ml)	Rabbits (ng/ml)	Rats (ng/ml)
Norepinephrine	0.94 ± 0.21[a]	0.38 ± 0.20	0.73 ± 0.40
Epinephrine	0.56[b]	0.32 ± 0.18	0.69 ± 0.078
DOPAC	1.4 ± 0.51	3.9 ± 1.3	1.4 ± 0.22
Dopamine	0.20 ± 0.02	0.27 ± 0.14	0.48 ± 0.030

[a] Each value is mean ± S.D. of three to four subjects.
[b] Measurable only in one of the three healthy volunteers.

4. Discussion

A main objective of the project is to develop an assay for simultaneous determination of plasma catecholamines and some of the metabolites for pharmacokinetics studies, which are becoming increasingly important for discovery and clinical evaluation of cardiovascular and CNS drugs [4–7]. In order to maximize sensitivity and minimize interference, the assay condition was optimized to obtain maximum recovery from the alumina extraction, and adequate separation of the analytes from drugs and other plasma endogenous materials. The method requires only 0.1–0.5 ml of plasma samples, and thus can be used for both clinical and preclinical studies.

It was determined during the method development that increasing the pH from 2 to 3 decreased the retention times of the basic catecholamines. On the other hand, the acidic catecholamine metabolites such as DOPAC, HMA, and VMA were relatively unaffected. These results are consistent with earlier report by others [15]. At pH 2.5, there was close to baseline separation and a single run can be completed in less than 10 min (Fig. 2). However, at pH higher than 2.7, DOPAC and dopamine had the same retention time. On the other hand, if the pH was 2.3 or below, dopamine and 5-hydroxy indoleacetic acid (5-HIAA) became co-eluted. Thus controlling the pH of the mobile phase is extremely important during day-to-day analysis. This can be accom-

plished readily by examining the mobile phase pH at the beginning of each experiment day. Another factor which needs to be optimized is the concentration of the ion-pairing reagent. As reported in earlier studies [15], we have found that a suitable ion-pairing agent is necessary to retain the basic catecholamines in reversed-phase HPLC, although it has relatively minor effect on the retention of the acidic catecholamine metabolites. For the ion-pairing agent 1-octanesulfonic acid, the optimal concentration was about 0.5 mM which provided adequate separation of the catecholamines for most samples. Higher concentrations of the ion-pairing agent would further improve separation, but at the same time increase cost of analysis and require more regular cleaning of the system due to the higher salt content.

Many HPLC assays for catecholamines have used dihydroxybenzylamine (DHBA) as the internal standard [16,17]. When it was injected into the HPLC system under the current conditions, however, it co-eluted with DOPAC and therefore was not a suitable internal standard under the current conditions. Isoproterenol is a commercially available catecholamine which has a similar detector response as the analytes, and has a retention time of less than 10 min. The recovery of isoproterenol was also comparable to the analytes using the described alumina extraction (90 vs 70–90%). Thus it would be a suitable internal standard for measuring plasma catecholamines.

Among the many different methods of isolating catecholamines from plasma, alumina extraction is one of the most widely used methods [9–11]. It is highly selective for the catecholamines and its metabolites. Bioactive amines which carry similar chemical structures such as serotonin or 5-HIAA were not extracted by the alumina (unpublished results). The method produces a relatively clean sample which allows simultaneous determination of the catecholamines. It has been reported that extraction of DOPAC by alumina is difficult and highly variable depending on the amount of alumina used and the nature of the desorption solvent [9,18]. We have found that the recovery of DOPAC was inversely proportional to the amount of alumina, but the recovery of the other analytes were relatively unaffected. The pH of the desorption solvent was also crucial for the extraction such that at $pH > 2.2$, the recovery of DOPAC was greatly reduced, although the other catecholamines were less affected. Thus it is important to examine the pH of the desorption buffer regularly for routine analysis to ensure that it is between 1.5 and 2. It is probable that the poor recovery is attributed to both adsorption and desorption, and these factors must be controlled during routine analysis.

During the course of method development which requires relatively large volume of plasma for assay validation, we have observed greater than ten times higher plasma concentrations of the catecholamines in animals when the blood samples were collected by cardiac puncture. To overcome the high background of catecholamine concentrations in these plasma samples, the standard curves were constructed covering the range from 20 to 400 ng/ml. Using the described HPLC assay, we were able to simultaneously determine plasma concentrations of norepinephrine, epinephrine, dopamine and DOPAC in humans, rabbits and rats (Table 3). The results are comparable with those reported by others [11,17,19–21]. It is interesting to note that plasma concentrations of the catecholamines are quite similar among the three species tested. These results suggest that species differences may not be a significant factor for plasma neurohormone concentrations, and that neurohormone effects observed in animal studies may have clinical value although further research studies are needed.

In summary, the described HPLC assay is simple, reproducible, rapid, and has adequate sensitivity and specificity to measure plasma norepinephrine, epinephrine, dopamine and DOPAC in both humans and animal species. It is suitable for preclinical and clinical pharmacokinetic and pharmacodynamic studies.

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References

[1] R. Webster, C. Jordan, Neurotransmitters, Drugs and Disease, Blackwell, Oxford, 1989, p. 336.

[2] K. Imai, S. Higashidate, P. Prados, T. Santa, S. Adachi-Akahane, T. Nagao, Relation between blood pressure and plasma catecholamine concentration after administration of calcium antagonists to rats, Biol. Pharm. Bull. 17 (1994) 907–910.

[3] A. Manolis, C. Olympios, M. Sifaki, S. Handanis, M. Bresnahan, I. Gavras, H. Gavras, Suppressing sympathetic activation in congestive heart failure. A new therapeutic strategy, Hypertension 26 (1995) 719–724.

[4] J. Dingemanse, A. Korn, J.-P. Pfefen, T. Guntert, Biochemical effects of high single doses of moclobemide in man: correlation with plasma concentrations, Psychopharmacology 106 (1992) S46–S48.

[5] P. Celada, F. Artigas, Plasma 5-hydroxyindoleacetic acid as an indicator of monoamine oxidase-A inhibition in rat brain and peripheral tissues, J. Neurochem. 61 (1993) 2191–2198.

[6] K. Laflamme, L. Oster, R. Cardinal, J. de-Champlain, Effects of renin-angiotensin blockade on sympathetic reactivity and beta-adrenergic pathway in the spontaneously hypertensive rat, Hypert. 30 (2 Pt 1) (1997) 278–287.

[7] S. Goldsmith, Effect of amlodipine and felodipine on sympathetic activity and baroreflex function in normal humans, Am. J. Hypertens. 8 (1995) 902–908.

[8] Z. Yi, P. Brown, Chromatographic methods for the analysis of basic neurotransmitters and their acidic metabolites, Biomed. Chromatogr. 5 (1991) 101–107.

[9] C. Holmes, G. Eisenhofer, D. Goldstein, Improved assay for plasma dihydroxyphenylacetic acid and other catechols using high-performance liquid chromatography with electrochemical detection, J. Chromatogr. (Biomed. Appl.) 653 (1994) 131–138.

[10] H. He, B. Stein, B. Christman, A. Wood, Determination of catecholamines in sheep plasma by high performance liquid chromatography with electrochemical detection: comparison of deoxyepinephrine and 3,4-dihydroxybenzylamine as internal standard, J. Chromatogr. (Biomed. Appl.) 701 (1997) 115–119.

[11] M.F. Ganhao, J. Hattingh, M.L. Hurwitz, N.I. Pitts, Evaluation of a simple plasma catecholamine extraction procedure prior to high-performance liquid chromatography and electrochemical detection, J. Chromatogr. (Biomed. Appl.) 564 (1991) 55–66.

[12] J. Hartleb, S. Eue, A. Kemper, Simultaneous analysis of homovanillic acid, 5-hydroxyindoleacetic acid, 3-methoxy-4-hydroxy-phenylethylene glycol and vanilmandelic acid in plasma from alcoholics by high performance liquid chromatography with electrochemical detection: critical comparison of solid-phase and liquid-liquid extraction methods, J. Chromatogr. (Biomed. Appl.) 622 (1993) 161–171.

[13] P. Yeung, S. Buckley, S. Pedder, J. Dingemanse, Determination of 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) in plasma by a simple and rapid high performance liquid chromatography assay, J. Pharm. Sci. 85 (1996) 451–454.

[14] F. Cheng, J. Kuo, W. Chang, D. Juang, Y. Shih, J. Lai, Rapid and reliable high-performance liquid chromatographic method for analysing human plasma serotonin, 5-hydroxyindoleacetic acid, homovanillic acid and 3,4-dihydroxyphenylacetic acid, J. Chromatogr. (Biomed. Appl.) 617 (1993) 227–232.

[15] P. Wester, J. Gottfries, K. Johansson, F. Klinteback, B. Winblad, Simultaneous liquid chromatographic determination of seventeen of the major monoamine neurotransmitters, precursors and metabolites. I. Optimization of the mobile phase using factorial designs and a computer program to predict chromatograms, J. Chromatogr. (Biomed. Appl.) 415 (1987) 261–274.

[16] D. Goldstein, G. Feuerstein, J. Izzo Jr, I. Kopin, H. Keiser, Validity and reliability of liquid chromatography with electrochemical detection for measuring plasma levels of norepinephrine and epinephrine in man, Life Sci. 28 (1981) 467–475.

[17] T. Soga, Y. Inoue, Determination of catecholamines in the urine and plasma by on-line sample pretreatment using an internal surface boronic acid gel, J. Chromatogr. (Biomed. Appl.) 620 (1993) 175–181.

[18] G. Eisenhofer, D.S. Goldstein, R. Stull, H.R. Keiser, T. Sunderland, K.L. Murphy, I.J. Kopin, Simultaneous liquid-chromatographic determination of 3,4-dihydroxyphenylglycol, catecholamines, and 3,4-dihydroxyphenylalanine in plasma, and their responses to inhibition of monoamine oxidase, Clin. Chem. 32 (1986) 2030–2033.

[19] B. Friedgen, R. Wolfel, H. Russ, E. Schomig, K.-H. Graefe, The role of extraneuronal amine transport systems for the removal of extracellular catecholamines in the rabbit, Naunyn-Schmiedeberg's Arch. Pharmacol. 354 (1996) 275–286.

[20] R. Conlee, D. Barnett, K. Kelly, D. Han, Effects of cocaine on plasma catecholamine and muscle glycogen concentrations during exercise in the rat, J. Appl. Physiol. 70 (1991) 1323–1327.

[21] S. Goldsmith, Effect of amlodipine on norepinephrine kinetics and baroreflex function in patients with congestive heart failure, Am. Heart J. 134 (1997) 13–19.