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Dynamic monitoring of total plasma homocysteine in spontaneously hypertensive rats by LC-MS

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Hypertension has been recognized to be closely related to plasma homocysteine levels (tHcy). Spontaneously hypertensive rats (SHR) are used widely for hypertension research, but it is unclear whether hypertension is related to high levels of tHcy in rat plasma. To test whether hyperhomocysteinemia occurs in SHR we dynamically measured plasma total homocysteine (tHcy) in SHR by liquid chromatography-tandem mass spectrometry (LC-MS). This analytical method has good linearity within the range of 1–100 μmol/L for tHcy in rat plasma with a correlation coefficient of $R = 0.9975$. After dynamic monitoring (12 weeks) on the plasma tHcy in SHR and Wistar-Kyoto rats, we found that there was no significant difference in tHcy level between SHR and Wistar-Kyoto rats, which was 6.98 ± 1.82 μmol/L and 8.04 ± 1.64 μmol/L, respectively. And there was no significantly high level of plasma tHcy in SHR.

1. Introduction

Homocysteine is an intermediate in the metabolism of the indispensable amino acid methionine (House et al. 1999). Plasma total homocysteine (tHcy), which includes all forms of homocysteine (homocysteine, homocysteine-protein disulfides and homocystine), is independently associated with cardiovascular diseases as well as atherosclerotic lesions in hyperlipidemic patients (Refsum et al. 2004). High levels of tHcy could lead to abortion, birth defects even apoplexy, senile dementia and other geriatric illnesses like osteoporosis, etc (Boushey 1995). And as the tHcy increases to 5 mmol L^{-1}, systolic and diastolic pressure would increase to 0.5 and 0.7 mmHg accordingly (Lim and Cassano 2002).

The spontaneously hypertensive rat (SHR) is an animal model of primary hypertension. Hypertensive development begins around 5–6 weeks of age, reaching systolic pressures between 180 and 200 mmHg in the adult phase. It is the most studied model of hypertension used in genetic hypertensive drug research safety and efficacy testing (Pinto et al. 1998). But do those animals have a high level of tHcy in its plasma? To test the hypothesis that hyperhomocysteinemia occurs in SHR we dynamically measured plasma levels of homocysteine in SHR.

There are several methods for plasma tHcy detection such as: high-performance liquid chromatography (Minniti et al. 1998, Nolin et al. 2007, Ichinose et al. 2009); capillary electrophoresis (Kang et al.1997); enzyme-linked immunosorbent assay (ELISA) (Fermo et al. 2000); Gas chromatographic–mass spectrometric (GC-MS) (Ducros et al. 1999; Hušek et al. 2000, Shinohara, et al. 2001) and liquid chromatography–electrospray tandem mass spectrometry (HPLC-MS) (Kuhn et al. 2006; Rafii et al. 2007; Tuschl et al. 2005; Guan et al. 2003; Li et al. 2008). Because there is an oxidized form of tHcy in plasma, most methods add a reducing agent such as DL-Dithiothreitol (DDT) before the analysis, and precolumn derivatization is usually needed for the fluorescence detector in HPLC analysis. Those were time-consuming derivatization protocols and reducing reagents. The LC-MS method is very sensitive and reliable. In this paper, we used it as the analytical method to find out whether the SHR has high plasma tHcy.

2. Investigations and results

2.1. Validation of the plasma tHcy assay

Under the conditions described in the experimental section, the assay was highly specific, and no endogenous plasma materials interfered with peak tHcy. Fig. 1 shows representative LC-MS chromatograms of tHcy in standard solutions with a retention time of 0.9 min. The mass spectrometer was set to monitor ions with m/z of 136, which correspond to the protonated molecular ions of Hcy. The calibration cure was linear from 1–100 μmol/L ($Y = 242.219452C + 545.654229$, $R = 0.9975$). The standard solutions of Hcy (2.5, 10 and 50 μmol/L) were prepared and there are five copies of each concentration. The intra-day precision was calculated when doing the analysis within a day, and the inter-day precision was calculated when doing analysis within 5 days. Results are shown in Table 1.

2.2. Dynamic monitoring of tHcy

Ten SHR and ten Wistar-Kyoto rats were involved, aged 12 weeks, when the plasma was collected from the caudal vein and analyzed by LC-MS. The concentration of tHcy in SHR and Wistar-Kyoto rats at 178 days were: 8.04 ± 1.64 μmol/L (SHR), 6.98 ± 1.82 μmol/L (WKY); and at 238 days, concentrations were 8.45 ± 1.90 μmol/L (SHR), 7.49 ± 1.95 μmol/L (WKY) (Fig. 2).

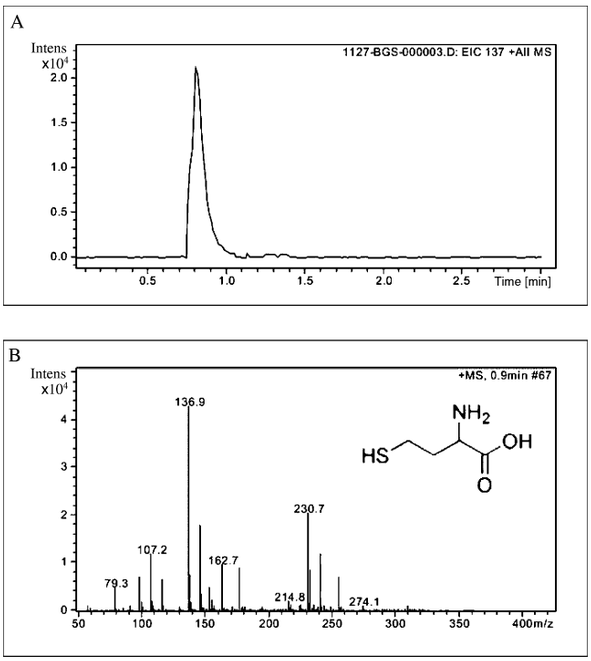

Fig. 1: Representative LC-MS chromatograms of Hcy: A LC-MS chromatogram of Hcy; B mass-spectrogram of Hcy

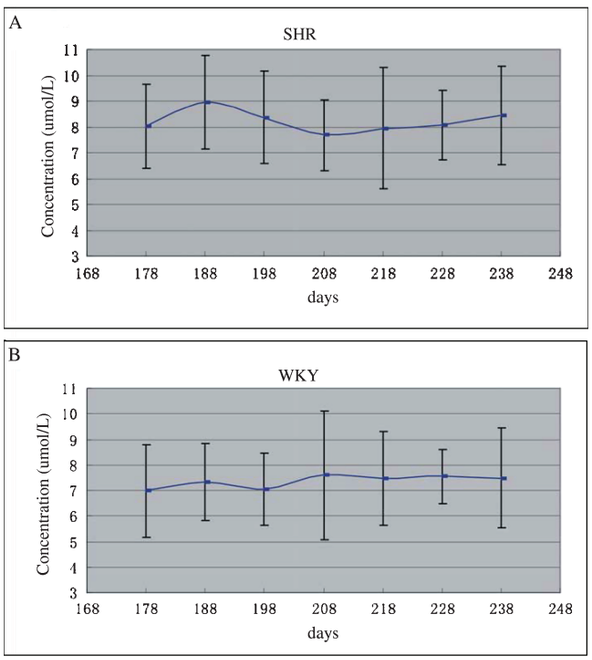

Fig. 2: Dynamic monitoring tHcy from 178 days to 238 days, A the plasma tHcy of SHR, B the plasma tHcy of Wistar-Kyoto rats

Table 1: Intra-day and inter-day precision of tHcy determination ($n = 5$)

Conc. (μmol/L)	Intra-day precision		Inter-day precision	
	Conc. ($\bar{x} \pm s$)	*RSD*	Conc. ($\bar{x} \pm s$)	*RSD*
2.5	2.54 ± 0.24	9.39	1.97 ± 0.17	8.85
10	10.76 ± 0.94	8.72	10.64 ± 0.89	8.34
50	50.17 ± 1.21	2.42	50.95 ± 1.50	2.95

2.3. Statistical results

All the data were collected for Spherically Symmetric Test ($P = 0.19$), and showed that they could meet the condition. The results of repeatedly measured analysis of variance are as below: the value of P in multivariate analysis of variance exceeded 0.05, which indicated that there was no significant difference in the concentration of tHcy among all the time intervals; the value of P in the interaction effects of time and administration method exceeded 0.05, which indicated that there was no significant difference in the concentration of tHcy in SHR among different administration methods as time went by (Table 2).

Table 2: Repeated measures analysis of variance of dynamic monitoring tHcy in SHR and Wistar-Kyoto rats

Effect		Sig.
Time	Pillai's Trace	.674
	Wilks' Lambda	.674
	Hotelling's Trace	.674
	Roy's Largest Root	.674
Time * group	Pillai's Trace	.968
	Wilks' Lambda	.975
	Hotelling's Trace	.982
	Roy's Largest Root	.385

3. Discussion

In the literature, plasma levels of homocysteine were reported to be 4.1 ± 0.1 μmol/L in 26-week-old male SHR (Kondziella et al. 2008). And, by dynamically monitoring the level of tHcy in the SHR, we found that there was no significant difference in the level of tHcy between the SHR and normal rats. So hypertension in SHR has no relation with homocysteine.

4. Experimental

4.1. Chemicals and reagents

DL-Hcy (purity > 95.0%) was purchased from Sigma Aldrich. LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). All other chemicals were of analytical grade and used without further purification. Ultra-pure water (resistance > 18 mΩ) prepared by a Millipore Milli-Q purification system (Bedford, USA) was used to make mobile phase and all other solutions.

4.2. Animals

Ten male spontaneously hypertensive rats (SHR), weighing between 250–350 g, and aged 12 weeks, as well as ten normotensive Wistar-Kyoto rats of the same age as the SHRs, weighing between 270–350 g were all obtained from the Institute of High Blood Pressure, Shanghai. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College, and were in accordance with the Guide for the Care and Use of Laboratory Animals.

4.3. Instrumentation and conditions

All analysis was performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany), equipped with a quaternary pump, a degasser, an autosampler, a thermostat column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany); equipped with an electrospray ion source and controlled by ChemStation software.

Chromatographic separation was achieved on a 150 mm × 2.1 mm, 3.5 μm particle, Agilent Zorbax SB-C_{18} column at 30 °C, with aqueous 40% methanol vs. 60% water (40:60, V/V) at 300 μL/min.

The samples were measured using the positive electrospray ionization technique operated in the selected reaction mode. With full scan MS, protonated $[M + H]^+$ molecule in a positive ion mode was obtained: m/z 136 (Hcy). Drying gas flow and nebulizer pressure was set to 7 L/min and 30 psi. The

dry gas temperature and capillary voltage of the system was adjusted to 350 °C and 3,000 V, respectively.

4.4. Calibration curves and quantitation

For calibration of tandem MS analysis, standard solutions of known Hcy, concentrations were prepared at 1.0, 2.5, 5.0, 10, 25, 50, 100 μmol/L. Each sample was prepared in triplicate. The samples were analyzed as described above. The peak areas (m/z 136 for Hcy) were determined in triplicate. The calibration graphs were obtained by least-squares linear fitting of the peak-area versus the analytical concentration added to the calibration sample, and used on each solution sample.

4.5. Sample preparation

(40 μL) Plasma was added to 1.5 mL EP tubes, and 10 μL of 90% trichloro-acetic acid was added for protein precipitation. The mixture was vortex-mixed for 1 min and the precipitated proteins were removed by centrifugation (12000 rpm/10 min). The supernatant was separated before LC-MS analysis. Supernatant (30 μL) was transferred quantitatively into a vase, and then diluted with the mobile phase (120 μL). A 5 μL aliquot of this was injected into LC-MS.

4.6. Accuracy and precision

Accuracy and precision were determined by assaying five preparations of 0.1-ml portions of pooled rat plasma spiked with several concentrations of Hcy (2.5, 10, 50 μmol/L). After preparation of the sample for LC-MS as described above, the peak areas were measured.

4.7. Dynamic monitoring of plasma tHcy in spontaneously hypertensive rat

Ten 12-week-old male spontaneously hypertensive rats (SHR), were assigned to the Group-SHR and ten age-matched Wistar-Kyoto rats of the same gender and bodyweight were assigned to the Group-WKY. Three or four rats were housed per cage. All animals had free access to standard rat chow and water ad libitum under controlled temperature (22 °C) and constant light-dark cycles of 12 h: 12 h.

Blood (0.5 mL) was drawn from the vena caudalis of each rat of both groups, every 10 days, and then put into ice cold tubes with heparin for anticoagulation. After intensive mixing, the samples were centrifuged for 10 min at 3000 rpm, then they were stored at 80 °C for further analysis with the developed LC-MS method.

4.8. Statistical analysis

All the data of the tHcy detection were analyzed with repeated measures analysis of variance, which was used to distinguish the variation tendency of tHcy among the groups.

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