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Creation of reversed phase high-performance liquid chromatographic technique to assay platelet-activating factor

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Abstract: Objective: To establish a new assay for platelet-activating factor (PAF), to compare it with bio-assay; and to discuss its significance in some elderly people diseases such as cerebral infarction and coronary heart disease. Methods: To measure PAF levels in 100 controls, 23 elderly patients with cerebral infarction and 65 cases with coronary heart disease by reversed phase high-performance liquid chromatographic technique (rHPLC). Results: rHPLC is more convenient, sensitive, specific, and less confusing, compared with bio-assay. The level of plasma PAF in patients with cerebral infarction was higher than that in the controls ($P<0.01$), and in patients with coronary heart disease. Conclusion: Detection of PAF with rHPLC is more reliable and more accurate. The new assay has important significance in PAF research.

Key words: Platelet activating factor, Reversed phase high-performance liquid chromatographic technique, Coronary heart disease, Cerebral infarction

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INTRODUCTION

Platelet activating factor (PAF), a kind of phospholipids compound, synthesized and secreted mainly by neutrophilic granulocyte, platelet, is a highly potent chemical mediator in inflammation and allergic reactions and induces microvascular leakage in several tissues (Chung, 1997). PAFs are few in vivo, have short half life (only about 30 seconds) and rapidly convert to lyso-PAF that has no bio-activity and is both the metabolite and precursor of PAF (Rao, 1998). Lyso-PAF reformats the membrane phospholipid via acetylation of phospholipase A_2 and converts to PAF; which can also convert to lyso-PAF via deacetylation of phospholipid-2-acetylhydrogenase. Lyso-PAF is more stable than PAF in vivo. PAF has low immunogenicity is difficult to combine with protein carrier; and so is difficult to produce antibody from it (Ammit and O'Neill, 1997). All these make present bio-assay and immune-assay methods do not satisfy clinical needs. Here we try to establish reversed phase high-performance liquid chromatographic technique (rHPLC), a more reliable, convenient and sensitive method, on the basis of bio-assay method established in our lab, to determine PAF level and its significance in some elderly people diseases such as coronary heart disease and cerebral infarction.

MATERIALS AND METHODS

Materials and facilities

They included high-performance liquid chro-

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matograph (Agilent 1100 Series), chromatographic column (Waters Nova-Pak® C1860Å4 μm, 3.9× 150 mm), solvent filtration apparatus, organic filter membrane (purchased from Caledon, Canada), ultrasound cleaning apparatus (Lab-line® Aquawave™) and Millipore™ solute free water apparatus.

Chromatographically pure Lyso-PAF-C_{16} and PAF-C_{18} standard were purchased from Sigma. Chromatographically pure acetonitrile, chloroform and methanol were purchased from Caledon (Canada). Bovine serum albumin (BSA) was purchased from AMRESCO.

Biological materials and apparatus see reference (Xu *et al.*, 1997).

Patients

The control group was consisted of 100 persons who were blood-donors and health-examination people in our hospital, and included 30 men and 30 women (20−50 years old), and 20 men and 20 women (50−80 years old). They were all not afflicted by coroncry thrombosis, cerebral infarction, or other heart diseases.

The afflicted group consisted of twenty-three persons (61−81 years old, average 72.1) who had cerebral infarction, and included 16 males and 7 females, 65 persons (45 males and 20 females, 62−86 years old, average 72) who had coronary heart disease. Excluding drugs for diabetes mellitus, antibiotics and drugs that have effect on platelet were not used within two weeks before the test.

Method

Sample processing: Two ml whole blood decoagulated with 109 mmol/L citric acid salt was centrifugated for 5 min at 3000 rev/min, after which 2 ml chloroform and 4 ml methanol were added into 0.5 ml plasma, it was shaken thoroughly. After addition of 2 ml distilled water and 2 ml chloroform again, it was shaken for the second time and centrifugated for 20 min at 3000 rev/min; after which the chloroform layer was taken out, dried with nitrogen gas flow and stored at –20 °C (Silvestro *et al.*, 1993).

DETECTION WITH RHPLC

Chromatographic conditions: Mobile phase was acetonitrile-water (80:20) including 1 mmol/L methyl-sulfonic acid; flow-rate was 1.0 ml/min, column temperature was 20 °C, detection volume was 20 μl and detection wave-length was 208 nm (Servillo *et al*., 1997).

Construction of working-curve: Lyso-PAF and PAF-C_{18} standard substance were mixed together, multiple-diluted with acetonitrile at 9.75 μg/L–20000 μg/L. After chromatographic analysis, the standard curve was made by comparing peak area with concentration (Fig.1a).

Sample detection: The samples were dissolved in 200 μl acetonitrile with the help of ultrasound for 5 min; the solutions of samples were filtrated with organic membrane. When the reservation time was the same as that of the standard PAF peak, it was the sample peak. According to the curve made before, we can get the different PAF levels in samples and total PAF levels (μg/L) in blood (Sun and Wang, 2000) (Fig.1b).

In Fig.1, Abscissa is reservation time and ordi-

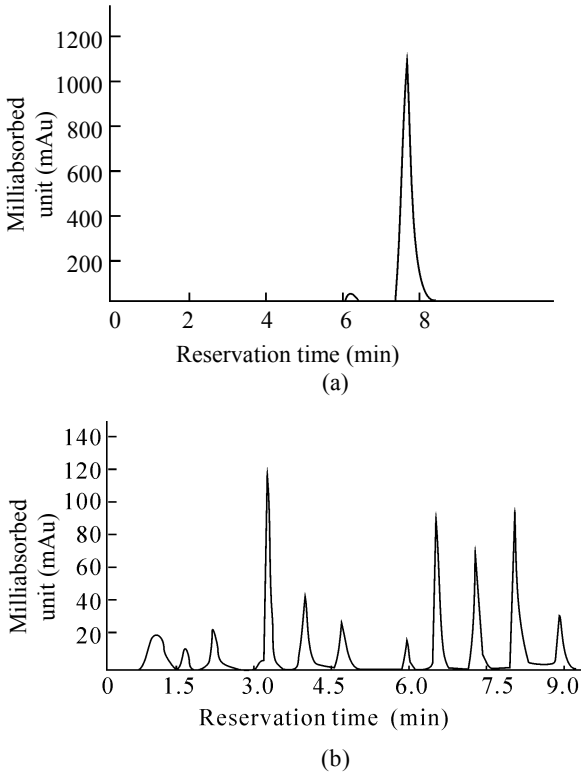

Fig.1 Peaks spectra of (a) standard C_{18}PAF and (b) substance drawn out from plasma

nate is absorption value. The peak that had the same reservation time as that of the standard sample's was the plasma PAF peak.

For comparison with the bio-assay established in our lab, 30 samples were drawn out randomly and detected by rHPLC.

T test and linear correlation analysis were adopted for comparison between groups.

The rHPLC evaluating tests: Adding two kinds of standard 100 ng PAF respectively into the plasma and then treating it the methods above, successive concentration gradient methanol-water, ethanol-water, acetonitrile-water and chloroform-water were taken as mobile phase to find the best combination and proportion of the mobile phase.

Granulocytes from the whole blood were resuspended with D-hanks solution and adjusted to 10^7 ml^{-1} (vital rate≥95%), at 37 °C for 20 min together with 0.2 mg/ml zymosan activated by complement. Processed by the methods described above, the culture medium was assayed by rHPLC to evaluate the feasibility of the drawing method according to the peak's reservation time.

Repetition: The same concentration sample was detected for ten times within one day for ten days successive to calculate the day variation coefficient and group variation coefficient.

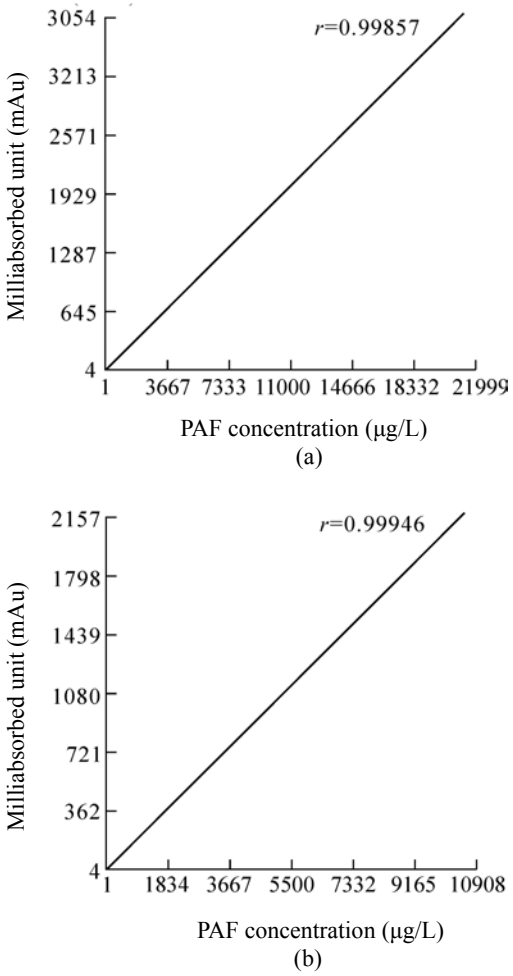

Fig. 2 Working curves of (a) Lyso-PAF and (b) PAF-C_{18}

RESULTS

Acetonitrile had the highest recovery ratio as mobile phase compared with other solvents. The best proportion of acetonitrile:water was 80:20. Chloroform had strong absorption peak at 208 nm. The reservation time of Lyso-PAF and C_{18}PAF was 6.2 min and 7.8 min, respectively.

Lyso PAF and PAF-C_{18} had good linear relation from 9.75 μg/L to 20000 μg/L. The linear equations were Y=68.811552+0.17375996X and Y= 48.21951+0.18889382X. Correlation coefficients were 0.9986 and 0.99946, respectively (Fig.2).

In Fig.2, the absorbed values and PAF concentration are all in good linear from 9.75 μg/L to 20000 μg/L.

After adding 100 ng PAF into 1 ml plasma and 1 ml 0.3% BSA solution respectively, the results showed that the remainders in the plasma and solution after processing posed no obvious interference to the peak shape.

The culture medium of granulocyte peaked in the reservation time of the PAF standard substance after the above processing.

Repetitive test revealed that the value of group CV was 2.1% and the day CV was 3.2%.

Application of rHPLC in diseases such as coronary heart disease and cerebral infarction: PAF levels in cerebral infarction and coronary heart disease were obviously higher than those of control

group (P<0.01) (Table 1).

Comparison between rHPLC and bio-assay: Thirty samples were drawn out randomly and detected by rHPLC and bio-assay established in our lab. Results are listed in Table 2. Their correlation coefficient was r=0.93.

Table 1 PAF levels within control groups and case groups

Groups	Cases	PAF ($\bar{x} \pm s$) (μg/L)	P
Controls I (20−50 years old)	60	136.7 ± 33.4	
Controls II (>50 years old)	40	178.9 ± 41.6	<0.01 (Compared with control I)
Cerebral infarction	23	387.2 ± 78.3	<0.01 (Compared with control II)
Coronary heart disease	28	277.1 ± 56.2	<0.01 (Compared with control II)

Table 2 Results of PAF levels detected by chromatography and bio-assay

Groups	Cases	PAF ($\bar{x} \pm s$) (μg/L)	r Value
Bio-assay group	30	69.7 ± 23.1	r=0.93
rHPLC group	30	181.9 ± 41.6	

DISCUSSION

Bio-assay of PAF is frequently used in China now while radioimmunotechnique is used abroad. Bio-assay lacks specificity and sensitivity because of the rapid transformation of PAF to Lyso-PAF which have no bio-activity in vivo; and many compounds in plasma which have similar functions to PAF can interfere with the detection. Besides, the individual differences of animal platelet, operator dexterity in making PAF, etc. all have influence on the experiment. Aside from the radioactivity in the radioimmunotechnique, the extreme difficulty of making PAF antibody limits the application of the immune method. rHPLC's effective separation and detection of the different PAF ingredients (C_{16}, C_{18} etc.) in the sample makes the test more precise, simple and have good reproduction (Cao and Lin, 2001).

Chloroform cannot be used as mobile phase because of its strong absorption near the PAF peak, and the surplus chloroform in the pre-processing should be dried. The best mobile phase was acetonitrile:water = 80:20. We also found that adding 1 mmol/L methyl-sulfonic acid can separate C_{16}-PAF, C_{18}PAF better and improve peak acuity. Because of PAF rearrangement in water, the sample should be detected within one hour after its dissolution. Mobile phase and sample should be filtered before detection in order to prevent the decrease of column efficiency.

In summary, our findings showed that PAF levels in cerebral infarction and coronary heart disease were obviously higher than those of the control group. The reason may be that, as a kind of phospholipid signal molecule, PAF has strong bio-activity with many kinds of cells and tissues in the organ. Besides causing platelet reaction, it can transmit signal from outside to inside in granulocyte, platelet, etc. and interact with other transmitter, cause the release of arachidonic acid and prostaglandin and the production of oxygen free radical, trigger and amplify the inflammation reaction and thrombosis (Cao and Xu, 2001; Zimmerman *et al*., 2002). When comparing rHPLC and bio-assay, the two methods showed good relation (r=0.93). rHPLC has characteristics of stability and accuracy. Therefore, with the establishment of chromatography, we can study further the significance of PAF in many kinds of diseases and pathological processes.

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