行政院國家科學委員會專題研究計畫　期中進度報告

液相層析/串聯質譜儀連線偵測極長鏈脂肪酸於新生兒之先天性過氧化酶體失調之篩選應用(1/2)

計畫類別：個別型計畫
計畫編號：NSC91-2113-M-039-001-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：中國醫藥學院醫學系

計畫主持人：賴建成
共同主持人：鄔哲源，蔡輔仁，蔡長海

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中　華　民　國 92 年 5 月 30 日

行政院國家科學委員會補助專題研究計畫 □成果報告 ☑期中進度報告

液相層析/串聯質譜儀連線偵測極長鏈脂肪酸於新生兒之先天性過氧化體失調症(II)(2/2)

計畫類別：☑ 個別型計畫 □ 整合型計畫
計畫編號：NSC 91－2113－M－039－001－
執行期間：2002 年 08 月 01 日至 2003 年 07 月 31 日

計畫主持人：賴建成
共同主持人：蔡輔仁、蔡長海、鄔哲源
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：☑精簡報告 □完整報告

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執行單位：中國醫藥學院醫學系

中　華　民　國　　年　　月　　日

1. 前言

中國醫藥學院附設醫院 (中國附醫) 於 2000 年初安裝液相層析/串聯式質譜儀工作平台 (liquid chromatography/tandem mass spectrometry;LC/MS/MS)，期望利用先進的質譜技術提升國內的遺傳醫學水平，初期針對胺基酸、脂肪酸及有機酸代謝疾病進行篩檢，同年 10 月完成本院 2100 例新生兒篩檢分析，建立國內第一套濃度標準範圍。其中於六月篩檢出一例罕見疾病：異戊酸血症，並及早進行治療，在優生保健部、醫學研究部、兒科部、護理部及婦產部共同努力下，中國附醫於 2000 年 12 月 20 日正式對外宣佈提供台灣第一個第二代新生兒篩檢服務，同時與罕見疾病基金會簽約合作共同推動第二代新生兒篩檢。中國附醫為國內第一個利用液相層析/串聯式質譜儀技術完成正式報告及臨床應用並首度提供此項技術於正式之新生兒篩檢服務，於國內居於領先地位。

目前中國附醫之新生兒篩檢一次 2 分鐘的分析可篩檢 30 種以上之遺傳疾病，平均一天一位技術員可篩檢上百位之新生兒，若能應用至其他各種遺傳疾病篩檢分析，則相對於傳統方法，新的檢驗方法將會更準確、快速、經濟、大量自動化及簡便。

2. 研究目的

Currently, the amino acids, acylcarnitine and 17-hydroxyprogesterone profiling of dry blood specimens using LC/MS/MS has been developed and recognized as a useful tool for screening inherited metabolic defects of newborns in our laboratory. In this study, our most important goal was to provide a high throughput and specific method with potential to screen for many of the peroxisomal disorders (ex. adrenleukodystrophy (ALD), adrenomyelinoneuropathy (AMN), peroxisomal biogenesis defects (PBD), Refsum disease and etc.) with a 3-mm blood spot (~3.6 μl blood impregnated on filter paper) from newborns using high performance liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS).

3. 文獻探討

There is considerable pressure to expand universal newborn screening programmes to cover a broader range of inborn errors of metabolism. Peroxisomes catalyze the β-oxidation of fatty acids and related substrates. The specificity of peroximal β-oxidation overlaps that of mitochondrial β-oxidation. Substrates that are preferentially or exclusively oxidized in peroxisomes include very long chain fatty acids (VLCFA), polyunsatured fatty acids, dicarboxylic fatty acid,

prostaglandins and the side chain of cholesterol. Peroxisomal disorders are a heterogeneous collection of inherited disorders characterized by impaired, reduced or total absence of peroxisomes in cells[1]. Patients with these disorders can be detected by an accumulation of VLCFA such as eicosanoic (C20:0), docosanoic (C22:0), tetracosanoic (C24:0) and hexacosanoic (C26:0) acids in their red blood cells, plasma or cultured skin fibroblasts. Zellweger syndrome[2] is the most severe of this group of disorders. It is apparent at birth and results in death within the first year. Neonatal adrenoleukodystrophy (ALD)[3] and infantile Refsum disease[4] and hyperpipecolic acidemia[5] are less severe, and some patients are in stable condition in the third or forth decade, albeit with deficits in vision, hearing, and cognitive function. They demonstrate similar, although generally milder, symptoms and biochemical abnormalities. Death often occurs in childhood. Other inborn errors appear to be caused by single peroximal enzyme defects. Some them mimic the disorders of peroxisome biogenesis to a considerable extent but the underlying cause is entirely different. Rhizomelic chondrodysplasia punctata appears to be an intermediate case with deficiency of a subset of peroximal enzymes.

Currently, neonatal screening procedures for the peroximal disorders are based on its determination of VLCFA by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). Quantification of VLCFA by GC[6,7] or GC/MS[8,9] takes 1-2 days. Elevated ratios of C26:0/C22:0 and C24:0/C22:0 indicate a peroximal disorder. The GC/MS method has been applied to the analysis of large blood spots containing 50-100 μl of blood[10-12]. An elevated C26:0/C22:0 ratio has been observed in blood spots obtained from Guthrie cards collected at birth from Zellweger syndrome patients[11] and in cord blood of X-linked ALD[12,13]. This gives justtification to developing a rapid, reliable method for screening for peroximal disorders on a sample size as small as that contained in a 3-mm neonatal blood spot. Analyses of VLCFA by GC and GC/MS methods, while highly sensitive and specific, is still slow and difficult to automate.

Recently, electrospray ionization (ESI)[14] has rapidly emerged as a very promising technique for the analysis of compounds with medium or high polarity. Similar to atmospheric pressure chemical ionization (APCI), ESI produces ions at atmospheric pressure, but without the need of high temperature that could decompose labile compounds. Because of its low detection limit, high specificity,

soft ionization and more importantly, abundant structural information, electrospray mass spectrometry (ESI-MS) and its related techniques have been considered one of the ideal devices for drug and screening analysis.

Today, tandem mass spectrometry (MS/MS) has already emerged as a powerful analytical tool in clinical biochemical genetics[15]. MS/MS was developed as a technique for expanding the scope and efficiency of newborn screening for inherited metabolic disorders[16-18]. Liquid chromatography coupled with electrospray/multidimensional mass spectrometry (LC-ESI/MSn)[19-21] represents a powerful alternative combining rapidly, easy process, high throughput, accuracy, specificity and sensitivity. One advantage of using MS instead of UV as the detector is that baseline separation may not be needed for a clear identification, and the relative concentration of each compound can be calculated based on their peak areas. A few publications have proposed the detection of very long chain fatty acids using liquid chromatography/tandem mass spectrometry (LC/MS/MS)[22,23]. An faster LC/MS/MS can be used to replace the slow GC and GC/MS assays. The strategy developed was to liberate all of the VLCFA from the dried blood sample and to derivatize them if possible. An LC/MS/MS assay was suitable for measuring VLCFA in a 3-mm blood spot (~3.6 μl blood). The amino acid, acylcarnitine and 17-hydroxyprogesterone profiling of dry blood specimens using LC/MS/MS has been recognized as a useful tool for screening inherited metabolic defects of newborns in our laboratory[24-26]. A LC/MS/MS coupled with the microtiter plate technology to perform lower-sample volume screening for peroximal disorders in the newborn filter-paper blood specimens was designed in this study. We have sought the first development of an alternative method for the determination of VLCFA in newborn that takes advantage of the analytical versatility, specificity, and high throughput unique to the combination of HPLC and MS/MS.

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4. 研究方法

Chemicals

Glacial acetic acid, very long chain fatty acids (VLCFA) and related compounds were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC grade methanol and acetonitrile were obtained from LAB-SCAN Analytical Science (Labscan Ltd. Dublin, Ireland). Deionized (18MΩ) water (Milli-Q water system, Millipore Inc., Bedford, MA, U.S.A.) was used in the preparation of the samples and buffer solution. Prior to use, the mobile phase was filtered through a 0.45 μm membrane filter (Gelman Sciences, Michigan, U.S.A.). Blank human whole blood samples were obtained from China Medical College Hospital, Taichung, Taiwan.

Standard solutions

Standard stock solutions were prepared at the concentration of 0.1 mg/ml in ethanol and kept in the dark at -20℃ when not in use. For the calibration, standard samples containing VLCFA were prepared at 500, 300, 250, 200, 150, 100, 50 and 30 ng/ml. 30 μl of a blank sample (mobile phase), and of each standard, were loaded on to newborn screening cards. All newborn screening cards for calibration were dried at room temperature for at least three hours, and then stored in polypropylene bags at room temperature until analyzed.

Collection of filter-paper blood specimens

Standardized filter-paper forms (Standardized Schleicher & Schull filter-paper S&S 903; Dassel, Germany), impregnated with whole capillary blood from peroximal disorder patients or 2-5-day-old infants, were collected from the Department of Genetics, China Medical College Hospital, Taichung, Taiwan. We collected blood spot samples from these patients in order to establish corresponding abnormal VLCFA profiles. All filter papers containing blood samples were dried at room temperature for at least three hours, and then stored in polypropylene bags at room temperature until analyzed.

Sample extraction

One 1/8-inch circles from each blood spot (equivalent to 3.6 μl of whole blood) were excised from a 0.5-inch (12.7mm)-diameter dried blood spot and placed into a flat bottom 96 well block (individual 250μl wells, Corning Incorporated, USA) using an automated Wallac DELFIA DBS puncher (Turku, Finland). A stock solution containing a known concentration of internal standard (100 μmol/L, margaric acid) in ethanol was prepared and added to each well (3.6 μl). Internal standard margaric acid was only used in this extraction step. Acetonitrile (180 μl) and 5N hydrochloric acid (20 μl) were added to each well. The wells were sealed, shaked and heated at 85 ℃ for 50 minutes. Subsequently, using a multi-channel pipette, the extracts were transferred into a clean V-bottomed 96 well microplate (individual 220μl wells, Corning Incorporated, USA). Each 96-well microplate was placed in an evaporator (SPEDry-96, JONES CHROMATOGRAPHY Ltd, U.K.) and the solutions evaporated to dryness under a gentle stream of dry nitrogen.

Derivatization of VLCFA

Oxalyl chloride (50 μl) was added to the residue, then it was sealed and heated at 50℃ for 5 min. The volatile material was evaporated to dryness under a gentle stream of dry nitrogen and several derivatizing agents (dimethylaminoethanol, 2-amino-2-methyl-1-propanol and choline, 50 μl) were added for testing. After 5 min at 20℃, the volatile material was evaporated under a gentle stream of dry nitrogen. The derivatized VLCFA were reconstituted in 50μl of acetonitrile/ water/ acetic acid (70:30:0.2) solution. The plate was covered with aluminum foil and placed on an autosampler tray for HPLC/ESI-MS/MS or other analysis.

HPLC system

The HPLC separation was performed on a Surveyor LC system (Thermo-Finnigan, San Jose, CA). HPLC analysis was performed on a 5 μm C-18 microbore column (50╳2.0 mm I.D.), operated at ambient temperature. A guard column (C-8 cartridge) was used to prolong the life of the HPLC column. The mobile phase was acetonitrile/ water/ acetic acid=70:30:0.2 (v/v) and the flow rate was 200 μl/min. At the end of each series, the column was thoroughly rinsed with a mixture of acetonitrile-deionized water (30:70, v/v) at a flow rate of 200 μl/min for 2 hours, and stored. The autosampler was fitted with a 10 μl loop and equipped with a

96 well sample plate stack. The HPLC and autosampler systems were all synchronized via the PC P4 workstation (Xcalibrate 1.3 software).

Electrospray and Mass Spectrometry (ESI-MS)

A Finnigan LCQ Deca XP quadrupole ion trap mass spectrometer (Finnigan Corp., San Jose, CA, U.S.A.), equipped with a pneumatically assisted electrospray ionization source, was used. The mass spectrometer was operated in the positive ion mode by applying a voltage of 4.5 kV to the ESI needle. The temperature of the heated capillary in the ESI source was set at 280 ℃. To avoid space charge effects, the number of ions stored in the trap was regulated by the automatic gain control, which was set at 1×10^8 ions for full scan mode, 5×10^7 for MS/MS mode, and 2×10^7 for ZoomScan mode. The flow rate of the sheath gas of nitrogen was set at 45 (arbitrary units). Helium was used as the damping gas at a pressure of 10^{-3} torr. Voltages across the capillary and the octapole lenses were tuned by an automated procedure to maximize signal for the ion of interest.

In MS/MS analysis, typical values for the relative collision energy (peak-to-peak amplitude of the resonance excitation) ranged from 0.4 to 0.8 eV. Mass spectra collected in full-mass scan mode were obtained by scanning over the range m/z 95 to 500. The maximum ion collection time was 0.3 s for each step and 3 scans were added for each spectrum.

Matrix assisted laser desorption ionization-quadrupole-time of flight (MALDI-Q-TOF) mass spectrometry

MALDI-Q-TOF was performed with a hybrid quadrupole-time of flight (Q-TOF) mass spectrometer (Qstar XL hybrid Quadrupole TOF system, Applied Biosystems-MDS Sciex).

5. 結果與討論

目前過氧化體失調的的檢測方式大都技術偵測極長鏈脂肪酸，雖然具有靈敏度及專一性高之優點，但常會有步驟緩慢及自動化困難之情形。大量分析、高專一性、低偵測極限與富含分析物結構物訊息是液相層析-電灑游離/質譜/質譜連線技術的優點，因此選擇此技術開發應用於新生兒極長鏈脂肪酸的偵測，而不同型式的先天性過氧化次分析中同時篩檢出來。因此相對於傳統方法，新的先天性檢方法將會更準確,快速,經濟及簡便。目前本實驗室發展液相層析/質譜/質譜連線

偵測氨基酸、醯基肉毒鹼及 17 羥孕酮已成功應用於新生兒遺傳代謝疾病之偵測。

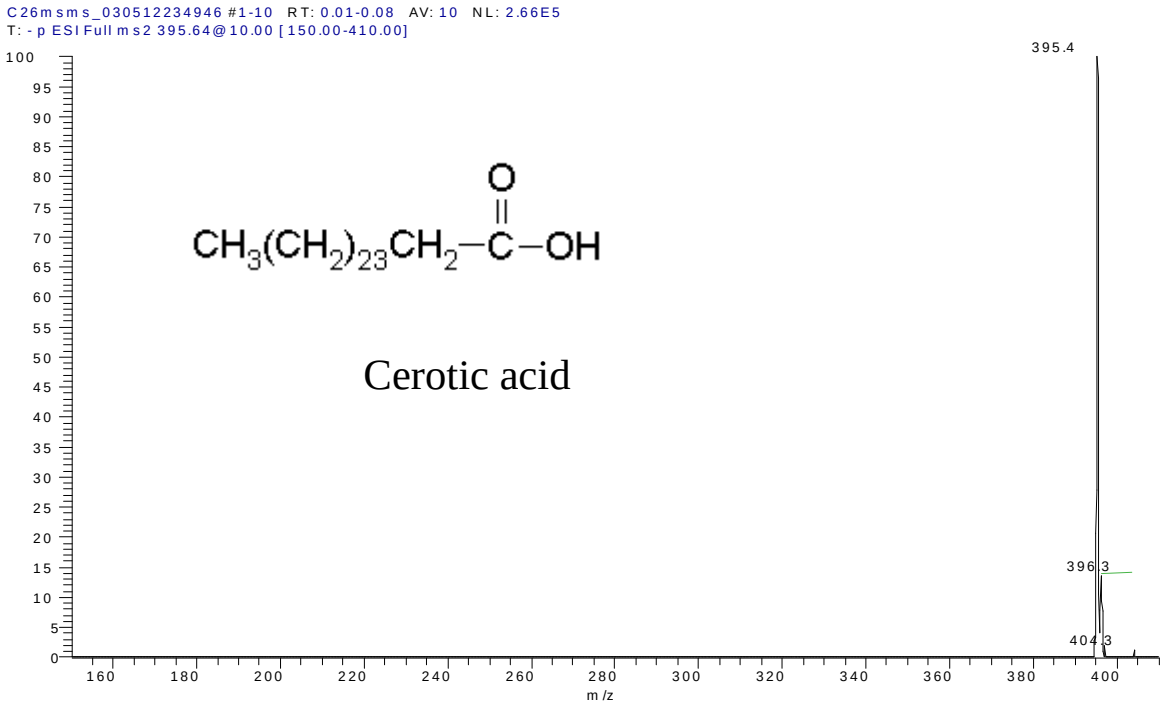
為萃取並釋放出血卡中血球及血液中等等所含之所有極長鏈脂肪酸，如: 十四、十六、十八、二十、二十二、二十四及二十六烷酸等，需實驗條件較劇烈之前處理方式，於此利用打孔機將含有樣品之濾紙打下一點 (直徑 1/8-inch、約 3.6 µl 之血液) 置於 96 孔樣品盤中，加入含內標準品之試劑後，我們採用了酸化後加熱將樣品中含極長鏈脂肪酸之脂質全轉換成極長鏈脂肪酸，避免傳統複雜之鹼化與多次萃取方式。

針對長鏈脂肪酸之質譜偵測方式，我們採取電灑法與介質輔助雷射脫附法兩種方式去嘗試，希望能得到較適當且快速之分析方法。

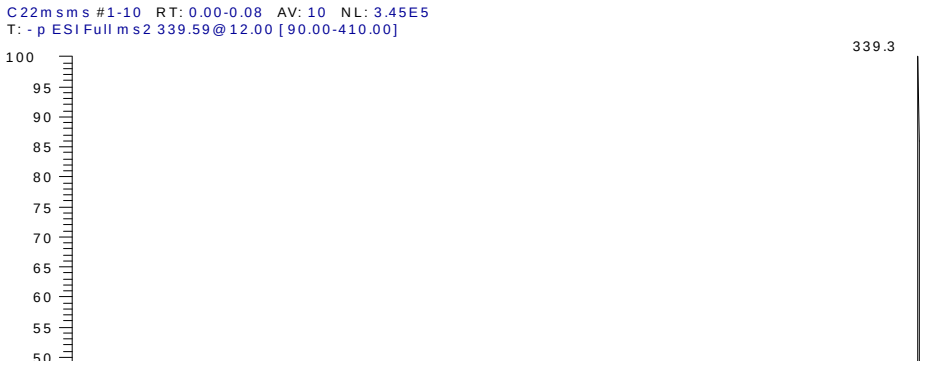
電灑法-串聯式質譜儀：

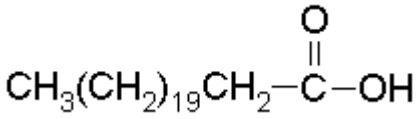
因長鏈脂肪酸本身帶有一羧基，初步以直接進樣電灑分析來測試,如圖 1 所示，於質譜/質譜圖譜中可見到清楚的$[M-H]^-$母離子，嘗試逐步提高碰撞能量，仍未可得到相關之特徵裂解離子，圖 1a 的 26 烷酸與圖 1b 的 22 烷酸質譜/質譜圖譜顯示於離子裂解模式下直接進樣無圖譜

(a)



(b)





Behenic acid

圖 1.利 用 直 接 進 樣 電 灑 質譜/質譜模式偵測 (a)26 烷酸 cerotic acid (b)22 烷酸 behenic acid (濃度 5 ppm、流速 5 μl/min)

在缺乏特徵裂解離子下並無法利用靈敏度及專一性高之選擇反應偵測(SRM)模式，因此嘗試以衍生化的方式來獲得具有特徵裂解離子之母離子，利用不同之醇類與極長鏈脂肪酸進行酯化反應，如 dimethylaminoethanol、2-amino-2-methyl-1-propanol、choline 等等，如圖 2 所示為經二甲基乙醇胺酯化之極長鏈脂肪酸 arachidic acid 之質譜/質譜圖，但為了增加靈敏度，目前仍嘗試其他之衍生化方式。

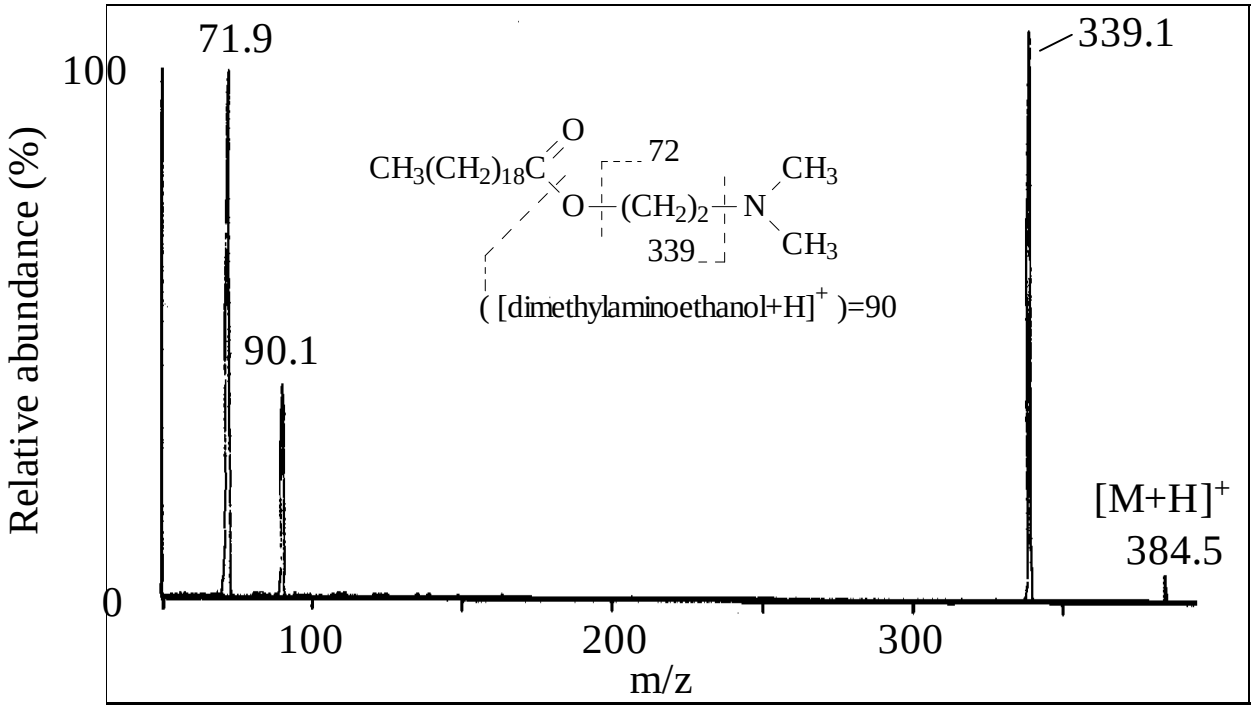


圖 2. 經二甲基乙醇胺酯化之極長鏈脂肪酸 arachidic acid 之質譜/質譜圖

介質輔助雷射脫附游離-四極式-飛行時間式串聯質譜儀：

為了比較不同游離化方式得到的好處，目前正持續進行下列幾種不同方式之分析方式：

- 選擇適當介質以直接分析極長鏈脂肪酸，如 9-aminoacridine、porphyrin。
- 以醋酸鈉對極長鏈脂肪酸進行皂化後，選擇適當介質分析。