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Detection and Determination of Phenformin in Chinese Medicinal Capsules by GC-MS and HPLC

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ABSTRACT

Phenformin was prohibited by the ROC Department of Health in 1978. In this investigation, a GC-MS was used for the detection of phenformin in herbal preparation produced in Mainland China followed by determination by HPLC. For GC-MS, a HP-5MS capillary column was used. The initial column temperature was at 100˚C for 2 min, ramping 10˚C/min to 280˚C, then holding at 280˚C for 15 min. Ionization source temperature was at 230˚C, and 70 eV electron impact mode was employed. HPLC was performed on a Cosmosil 5C18 AR (5 cm × 4.6 mm), and a μBondpak phenyl (30 cm × 3.9 mm) column using 0.01M potassium dihydrogenphosphate (pH 6.55) - acetonitrile (60:40, v/v) as eluent, with flow rate at 1.0 mL/min and detection wavelength set at 235 nm. Metformin was used as the internal standard. The interday and intraday precisions were less than 1.6%. The limit of detection and the limit of quantitation were 0.065 μg/mL and 0.20 μg/mL, respectively. The spike recovery was 102.7%. The average content of phenformin in the preparation was 3.45 mg per capsule.

Key words: phenformin, traditional Chinese medicine, GC-MS, high performance liquid chromatography

INTRODUCTION

Traditional Chinese medicines (TCM) are sourced from animals, plants and minerals. The complexity and pharmacological activity of constituents in TCM have attracted much research. People often turn to TCM when modern western medicine fails. Nowadays, TCM has become a major alternative treatment. Since about 80% of the world's population take herbal medicines, quality controls of the herbal medicines are very important. In general, TCM is considered to possess mild action with no appreciable harmful effects, and easy to take TCM in excess of the recommended dose for long term use. In order to enhance their efficacy, TCM is often illegally adulterated with western medicine. The quantities of adulterants were even over the normal dosage range and were not the ones required or responsible for the therapy claimed on the label found in many cases. Therefore, it is dangerous to take those TCMs without any recognition about the adulterants. For the sake of safety, adulteration of TCM was banned by the Department of Health (DOH) in Taiwan. In view of the responsibility of NLFD to supervise the qualities of foods and drugs, we have many investigations related to the adulteration of TCM and the results have been documented in the annual reports[1-5] of the NLFD since 1970. Recently, herbal medicines produced in Mainland China have become widespread in Taiwan, through the increasing frequency of cross-strait exchanges. Their quality are incessantly supervised by the NLFD under the directives of the DOH.

In this study, capsules of Zhenge Jiangt Angsan (ZJA, 珍蛤降醣散) produced in Mainland China and provided from a patient were suspected to contain anti-diabetic western drugs. Screen tests using TLC, UV, and then confirmed by GC-MS, indicated that the adulterant was phenformin. Furthermore, an HPLC method was developed to quantify the phenformin in order to understand the amount adulterated in ZJA. Phenformin (Figure 1) was used as a hypoglycemic agent. However, its administration frequently resulted in gastrointestinal upset including vomiting, anorexia, and diarrhea. Moreover, it has been associated with a severe and even a fatal disturbance in lactic metabolism that results in lactic acidosis. Additionally, the use of phenformin may also be associated with an increase in cardiovascular disease[6, 7]. Therefore, the FDA banned its sale in the US in 1975[8], while the DOH banned its importation, manufacture and sales in Taiwan in 1978.

MATERIALS AND METHODS

I. *Materials*

Ten capsules of ZJA were kindly offered by the National

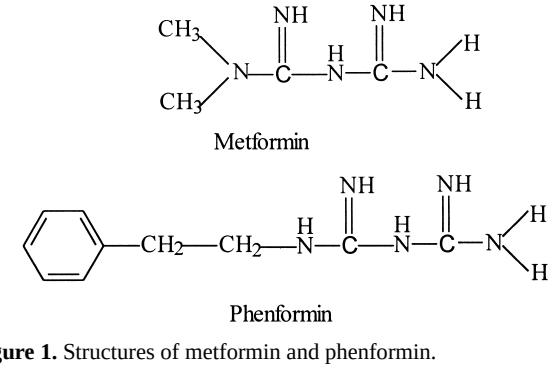

Figure 1. Structures of metformin and phenformin.

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Cheng Kung University Hospital (originally obtained from a patient).

Phenformin HCl was pharmacopoeia grade. Metformin HCl was purchased from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogenphosphate and diethylamine were purchased from Wako (Osaka, Japan). Acetonitrile, methanol and n-butanol of LC grade were purchased from Labscan (Dublin, Ireland). Glacial acetic acid was purchased from E. Merck (Darmstadt, Germany).

II. *Equipments*

The high performance liquid chromatography system was composed of a Waters 600 pump and a controller, a 486 tunable absorbance detector (Millipore, Boston, MA, U.S.A.), a Hitachi L-7200 autosampler (Hitachi, Tokyo, Japan), and a Shiunn Haw computing integrator (Scientific Information Service). The GC/MS system was composed of an HP-6890 GC system, an HP-5973 mass selective detector, an HP 6890 Series Injector, and an HP MSD Chemstation (Hewlett Packard, Palo Alto, CA, U.S.A.). U-3210 Spectrophotometer (Hitachi, Tokyo, Japan) was used also.

III. *Method*

(I) *Thin Layer Chromatography*

TLC plates were silica gel Merck 60 F_{254} 20 × 20 cm (E. Merck). The mobile phase was n-butanol-water-glacial acetic acid (7:2:1, v/v), and detection wavelength was 254 nm. Dragendorff spray was used as a chromogenic reagent.

(II) *Gas Chromatography and Mass Spectrometry*

A 30 m × 0.25 mm HP-5MS (crosslinked 5%-diphenyl-95%-dimethylpolysiloxane) capillary column was used (0.25 μm film thickness). The operation conditions were as follows: splitless injection; 50.0 mL/min purge flow; oven, with initial temperature at 100˚C for 2 min, ramping 10˚C/min to 280˚C, then holding at 280˚C for 15 min; injection temperature 250˚C; ionization source temperature 230˚C; 70 eV electron impact mode; solvent delay 4 min; injection volume 1 μL; using helium as carrier gas at 1 mL/min.

(III) *High Performance Liquid Chromatography*

A Cosmosil 5C18-AR (5 cm × 4.6 mm I.D.) reverse phase column (Nacalai Tesque, Kyoto, Japan) and a μBondpak phenyl (30 cm × 3.9 mm I.D.) column were used. The mobile phase was composed of 0.01 M potassium dihydrogenphosphate buffer (the pH was adjusted to 6.55 by the addition of diethylamine) and acetonitrile (40:60). The flow rate was 1 mL/min and the detection wavelength was 235 nm.

(IV) *Preparation of Standard Solution*

Phenformin standard was accurately weighed and dissolved in methanol to afford the concentration (1 mg/mL). The calibration curve was established using 5, 10, 20, 30, 50 and 100 μg/mL of phenformin standard solution as calibrators with metformin (20 μg/mL) as the internal standard.

(V) *Screening and Confirmation of the Sample Solution*

The sample of ZJA (1 cap) was extracted with 10 mL methanol by ultrasonic shaking for 30 min as the sample solution. A portion of the sample solution extract was analyzed by TLC, UV and confirmed by GC/MS.

(VI) *Determination by HPLC*

Three capsules were accurately weighed and then extracted with 10 mL methanol by ultrasonic shaking for 30 min, respectively. After filtering, the residue was extracted once more and the combined extract was adjusted to 25 mL with methanol. To 10 mL aliquot, an appropriate amount of the internal standard metformin was added, and the volume was adjusted to 25 mL with methanol and then filtered (0.45 μm Millipore) before injection.

(VII) *Recovery*

The powders of capsules were thoroughly mixed, equally divided into four portions and then accurately weighed. They were all spiked with different amounts of standard except one used as a control. The spiked amounts of standard were 10.7 mg, 20.1 mg and 30.1 mg, respectively. The extraction method followed that described above, and the internal standard was added to each solution at a concentration of 20 μg/mL.

(VIII) *Reproducibility Test*

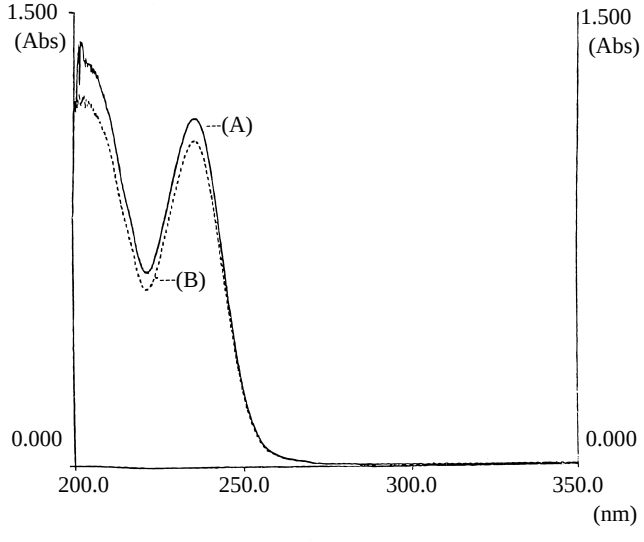

Figure 2. UV spectra of phenformin solution (A) and the methanol extract of TLC spots (Rf = 0.49) isolated from Zhenge Jiangt Angsan (B).

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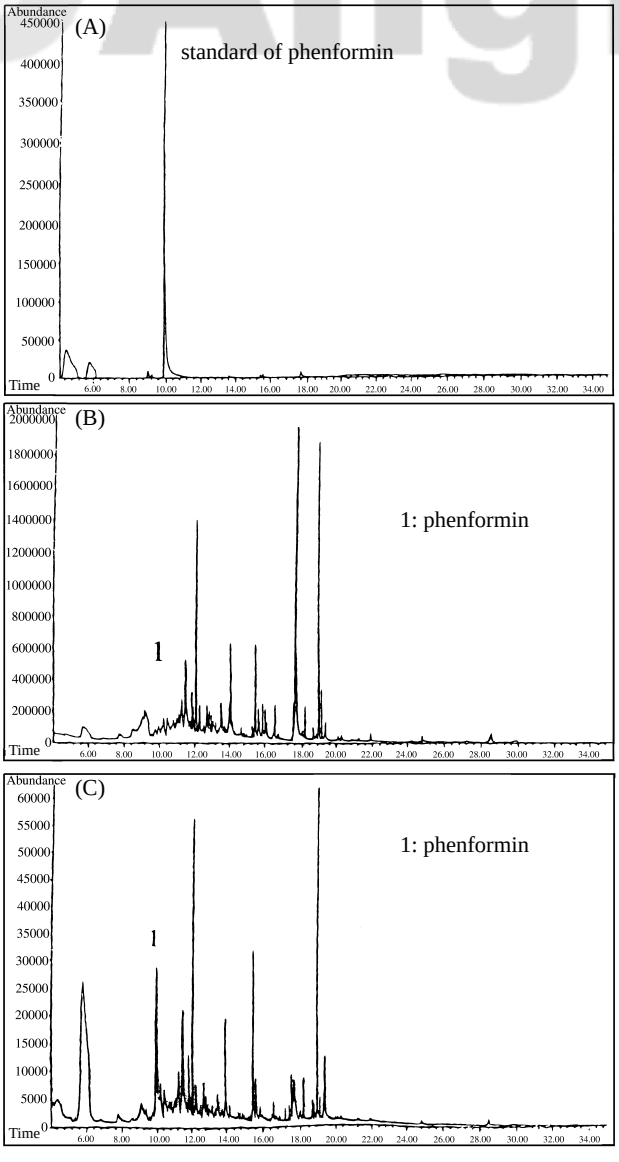

Figure 3. Total ion chromatograms of phenformin standard solution (A), the sample solution of Zhenge Jiangt Angsan (B) and ion chromatogram of extract of Zhenge Jiangt Angsan at the mode of *m/z* 91(C).

Intraday (running five times within 24 hr), and interday tests (running five times within successive 5 days with each at least 24 hr interval) were performed using the above calibrators. The reproducibilities were evaluated by the relative standard deviations.

(IX) *The Detection Limit and Qantitation Limit*

The concentration of the standard solution of which the ratio of peak height to noise 3/1 was defined as the detection limit, and the ratio 10/1 was defined as the quantitation limit.

RESULTS AND DISCUSSION

Through a simple screening test using TLC with n-butanol-water-glacial acetic acid (7:2:1, v/v) as the developing solvent, when detected as orange color after sprayed with

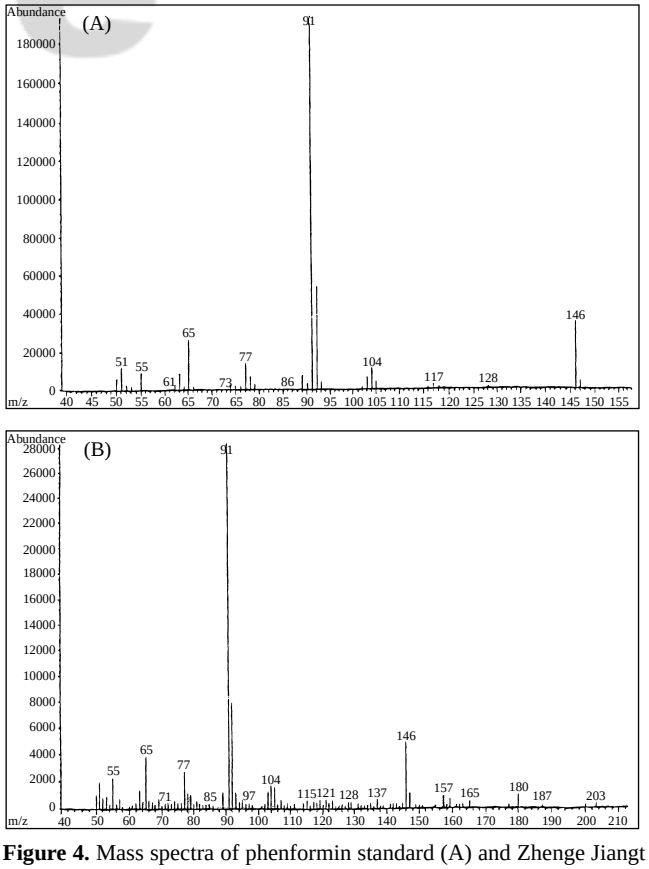

Figure 4. Mass spectra of phenformin standard (A) and Zhenge Jiangt Angsan extract (B).

Dragendorff reagent, phenformin was found as an adulterant in the suspected sample of ZJA containing Pearl(珍珠), Ge Jie(蛤蚧) and various Chinese herbs.

I. *Identification of Phenformin by UV and GC/MS*

The band corresponding to Rf (0.49) of phenformin was scrapped and extracted with methanol. The maximal absorption was at 235 nm. Its UV spectrum and UV spectrum of phenformin standard solution are shown in Figure 2 and both are consistent with the maximal absorption at 235 nm.

Some reports[9-17] indicated that phenformin ($C_{10}H_{15}N_5$) having polar group, it could not vaporized easily without derivatization in GC analysis. In the routine work, derivatization of TCM needs more investigation owing to its complicated constituents. However, the concentration of phenformin as high as 1000 μg/mL could be found in this GC/MS system. The total ion chromatograms and mass spectra of standard phenformin and ZJA are shown in Figures 3-4. Retention time for phenformin was 9.9 min. The mass spectrum could not show its molecular ion peak, but prominent ion peak at *m/z* 91 was a distinct peak. The peak ion *m/z* 91 used as an identified peak in this experiment. Though the chromatogram of ZJA was complicated, the corresponding retention time for ZJA contained the same fragment ion *m/z* 91. Utilization of selected ion chromatogram offered by the MSD Chemstation at *m/z* 91, the total ion chromatogram of ZJA with an obvious peak at 9.9 min could distinguish phen-

formin from the other herbal constituents. By further comparison of mass fragments with library search software, phenformin was identified.

II. *Assay by HPLC*

Analyses of biguanides have been reported in journals. The analytical methods include HPLC[18-24], GC[9-17], CE[18] and HPLC-ESIMA[18]. A method without the previous time-consuming derivatizing process, or post-column derivatization, is the main requirement for the determination of adulterant in TCM with complicated constituents. In this study, the HPLC method using 0.01 M potassium dihydrogenphosphate (the pH was adjusted to 6.55 by the addition of diethylamine) and acetonitrile (40:60) as the mobile phase was used. The HPLC chromatograms of the standard phenformin and ZJA were shown in Figure 5. The total analytical time was 15 min. The retention times for metformin and phenformin were 5.48 and 9.36 min, respectively. Metformin with a structure similar to phenformin was selected as an internal standard, and could separate from phenformin without interference. The calibration curve with Y = 0.0244X + 0.0042 (correlation coefficients = 0.999), showed a good linearity within the range of 5~100 μg/mL. The contents of phenformin in 3 capsules of ZJA calculated from the calibration curve are listed in Table 1.

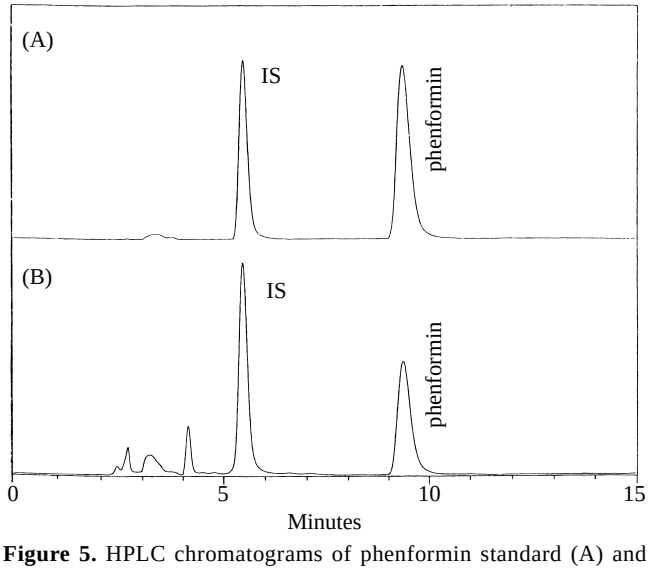

Figure 5. HPLC chromatograms of phenformin standard (A) and Zhenge Jiangt Angsan extract(B).

Table 1. The contents of phenformin in 3 capsules of Zhenge Jiangt Angsan

Sample	1	2	3	Average content
Content (mg/cap)	3.45 ± 0.04	3.47 ± 0.03	3.42 ± 0.04	3.45 ± 0.04
(RSD, %)	(1.16)	(0.86)	(1.17)	(1.16)

*n=5, RSD: relative standard deviation.

Table 2. Reproducibility of interday and intraday analysis (n=5)

Concentration	Relative Standard Deviation(%)	
(μg/mL)	intraday	Interday
5	0.50	1.06
10	0.72	1.19
20	0.28	0.66
30	0.17	1.51
50	0.48	0.71
100	0.28	0.60

Although the contents of phenformin in capsules do not exceed those in the market package[6], the use of ZJA must be careful. If a patient takes 3-10 caps/time for three times a day, the amount of phenformin taken would be 10.5-105 mg/day. According to the manufacture's recommended dose, that might exceed the therapeutic dose[25]. Administration of ZJA without knowing the presence of phenformin over a long term might result in life-threatening complications as described previously.

III. *Reproducibility*

The relative standard deviations (RSD) of intraday and interday calculated from results of five replicates are shown in Table 2. The small values of all RSD values (less than 1.6%), indicated very good reproducibility.

IV. *Recovery*

The recoveries of three spiked amounts ranged from 97.7% to 105.3% were shown in Table 3. The average recovery was 102.7%, indicating the method was accurate.

V. *The Limit of Detection and the Limit of Quantitation*

The limit of detection and quantitation were 0.065 μg/mL and 0.20 μg/mL, respectively. The method showed a satisfactory sensitivity.

CONCLUSION

In this research, a banned western chemical drug adulterated in TCM was identified as phenformin and confirmed by TLC, UV and GC/MS. The high performance chromatographic method developed to quantify the adulterant in TCM was shown to be rapid, reliable and accurate.

Table 3. Recovery of phenformin (n=5) spiked in Zhenge Jiangt Angsan

Amount spiked(mg)	Found(mg) Mean ± SD(RSD%)	Recovery Mean ± SD(RSD%)	Average recovery Mean ± SD(RSD%)
10.7	10.45 ± 0.03(0.29)	97.65 ± 0.25(0.26)	
20.1	21.17 ± 0.04(0.19)	105.30 ± 0.18(0.17)	102.69 ± 3.57(3.48)
30.1	31.64 ± 0.03(0.09)	105.12 ± 0.11(0.10)	

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應用氣相層析質譜儀及高效液相層析儀分析中藥製劑摻加phenformin西藥成分之研究

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摘　要

治療糖尿病之phenformin（芬法敏）由於使用後會造成乳酸中毒，行政院衛生署雖已於民國67年即禁用，然卻於某大陸藥品中檢出，因而引起我們研究的興趣。本研究係以氣相層析質譜儀鑑定該成分，續以高效液相層析儀分析其含量。氣相層析質譜儀設定條件如下：HP-5MS毛細氣相層析管，以70eV離子電能作電子撞擊式（EI mode）分析，起始溫度100˚C維持2分鐘，再以每分鐘10˚C之升溫速率，至280˚C並維持15分鐘。高效液相層析分析條件如下：Cosmosil 5C18 AR（5 cm × 4.6 mm）及μBondpak phenyl（30 cm × 3.9 mm）為分析之管柱，使用移動相為磷酸二氫鉀（pH 6.55）：乙腈（40:60）。流速1 mL/min，偵測波長235 nm。以metformin為內部標準品。分析結果如下：同日間及異日間之測試，其相對標準偏差在1.6%以下。偵測極限及最低檢出量分別為0.065 μg/mL及0.20 μg/mL，添加回收率102.7%，檢體中平均含量為3.45 mg／膠囊。

關鍵詞：phenformin, 中藥摻加西藥，氣相層析質譜儀，高效液相層析法