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Solid-phase Extraction and High Performance Liquid Chromatographic Determination of Steroids Adulterated in Traditional Chinese Medicines

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ABSTRACT

A systematic evaluation and comparison of the recoveries of eight steroids from spiked traditional Chinese medicine (TCM) extract using silica gel solid-phase extraction (SPE) are reported. The eight steroids, betamethasone, cortisone acetate, dexamethasone, hydrocortisone acetate, methylprednisolone, prednisolone, prednisone and triamcinolone, adulterated in TCM were assayed simultaneously by high performance liquid chromatography on a reverse phase column. This chromatographic method was carried out using isocratic elution with acetonitrile and water (3:7). Fludrocortisone acetate was used as an internal standard and detection was at 240 nm. Calibration curves of the eight adulterants were constructed in the range 6-96 μg/mL. Their correlation coefficients ranged from 0.9983 to 0.9996. The relative standard deviations of the eight steroids for intraday and interday analyses were 0.7-4.1% and 1.0-4.4%, respectively. Optimized experimental conditions including solvent composition, TCM extract concentration and different brands of SPE cartridge were also investigated.

Key words: **HPLC, steroids, solid-phase extraction, traditional Chinese medicine, adulterant.**

INTRODUCTION

The adulteration of traditional Chinese medicines (TCM) with synthetic therapeutic substances has been banned by the health authorities for the reason of public safety in Taiwan. Over the years, some of TCM referred to our laboratories from various sources have contained adulterants. The results have been reported year by year[1-2]. The first island-wide monitoring of the prohibited adulteration of TCM, through hospital pharmacies, was carried out in 1992. A higher percentage (26.1%) of adulteration was reported for those TCM without commercial packages[3]. Steroids including prednisolone, betamethasone and dexamethasone were among the top 25 frequent adul-

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terants. Over the past two decades, the adulteration of steroids in TCM has been also reported in several industrialized countries[4-6]. The danger of potential overdose resulting in severe side effects of steroids was well documented[7]. Therefore, the adulteration of these chemical drugs in traditional Chinese medicine can lead to significant clinical consequences.

Solid-phase extraction (SPE) has recently become popular for sample preparation because of its advantages of fast speed, minimal use of solvents, and a wide selection of adsorbents available. The recovery of analytes in the SPE method depends on the stationary adsorbent of the cartridge and the major procedures in extraction, such as cartridge conditioning, sample packing, washing and analyte elution. Most TCM are composed of many crude drugs with complex constituents. Therefore, an economical and efficient method for the detection and assay of adulterants in TCM is needed. To our knowledge, there has not been any report dealing with the separation of steroids of interest as adulterants in TCM.

Our previous studies have established high performance liquid chromatography (HPLC) methods for identification and determination of adulterants in TCM including seventeen analgesics[8], seven sulfonamides[9], four antitrichomonal drugs[10], three androgens[11] and five xanthine bronchodilators[12]. Furthermore, micellar electrokinetic capillary chromatography has been developed for measuring clobenzorex and diazepam[13], as well as fourteen analgesic adulterants[14] in TCM. Furthermore, SPE methods used for the pretreatment of adulterated TCM were also established[12,15-16]. In this study, we employed a combination of SPE and HPLC for the extraction and determination of eight steroids [17]: betamethasone (BE), cortisone acetate (CA), dexamethasone (DE), hydrocortisone acetate (HA), methylprednisolone (ME), prednisolone (PR), prednisone (PN) and triamcinolone (TR) in TCM.

MATERIALS AND METHODS

I. *Reagents and Materials*

BE, CA, DE, HA, ME, PN, TR and fludrocortisone acetate (FA) were purchased from Sigma (St. Louis, MO, USA). PR was purchased from Nakalai (Kyoto, Japan). Acetonitrile, chloroform, dichloromethane and methanol from Labscan (Dublin, Ireland) were LC grade. Ultrapure distilled water with a resistance greater than 18 MΩ was used. Ethanol (Taiwan Tobacco & Wine Monopoly Bureau, ROC) was ChP. grade. The eight brands of silica gel SPE cartridge were obtained from Baker (Phillipsburg, NJ, USA; Brand8), Chrom (Apple Valley, MN, USA; Brand7), Macherey-Nagel (Duren, Germany; Brand5), Merck (Darmstadt, Germany; Brand6), Restek (Bellefonte, PA, USA; Brand3), Supelco (Bellefonte, PA, USA; Brand1), Varian (Harbor City, CA, USA; Brand4) and Waters (Milford, MA, USA; Brand2).

Three commercial concentrated herbal preparations were used as model drugs. The three preparations and their compositions were as follows: Sheau-Ching-Long-Tang (F1): Ephedrae Herba, Paeoniae Radix, Glycyrrhizae Radix, Cinnamoni Ramulus, Asiasari Rhizoma, Zingiberis Rhizoma, Schizandrae Fructus (3.0 g each) and Pinelliae Tuber (6.0 g). Dwu-Hwo-Jin-Sheng-Tang (F2): Citri Leiocarpae Exocarpium (0.3 g); Ginseng Radix, Paeoniae Radix, Angelicae Radix, Glycyrrhizae Radix, Hoelen, Achyranthis Radix, Asiasari Radix, Loranthi Ramus, Gentianae Macrophyllae Radix, Sileris Radix, Ligustici Rhizoma, Eucommiae Cortex, Rehmanniae Radix et Rhizoma (0.4 g each) and Angelicae Tuhou Radix (0.6 g). Ba-Jen-Tang (F3): Glycyrrhizae Radix (1.5 g); Ligustici Rhizoma (2.3 g); Ginseng Radix, Atractylodis Rhizoma, Paeoniae Radix, Hoelen (3.0 g each); Angelicae Radix and Rehmanniae Radix et Rhizoma (4.5 g each). These preparations were purchased from retail outlets in Taipei.

II. *HPLC Apparatus and Conditions*

HPLC was conducted with a HITACHI L-6200 intelligent pump with a HITACHI L-3000

photodiode array detector and Shimadzu SIL-9A auto injector. An Inertsil ODS-80A (250 × 4.6 mm I.D.) column was used. The mobile phase consisted of acetonitrile and water (3:7, v/v). The flow rate was 1.2 mL/min and detection was carried out at 240 nm.

III. *Preparation of Standard Solutions*

An appropriate amount of internal standard solution was added to an ethanol solution containing accurately weighed amounts of the eight steroids to give various concentrations within the range 6.18-98.88, 6.24-99.84, 6.00-96.00, 6.18-98.88, 6.00-96.00, 6.18-98.88, 6.12-97.92 and 6.24-99.84 μg/mL for BE, CA, DE, HA, ME, PN, PR and TR, respectively. Calibration graphs were plotted subsequent to linear regression analysis of the peak area ratios versus concentration.

IV. *Preparation of TCM Extracts*

Three commercial concentrated herbal preparations, Sheau-Ching-Long-Tang, Dwu-Hwo-Jin-Sheng-Tang and Ba-Jen-Tang, traditionally prescribed for cold, analgesic and androgen, respectively[18], were used as model samples for assessing interference and recovery. One gram of each model preparation was accurately weighed and extracted with chloroform (25 mL) at 30°C for 30 min in an ultrasonic bath, filtered, and made to 25 mL with chloroform.

The TCM extracts were diluted with chloroform to afford 1:4 (v/v) and 1:24 (v/v) in order to investigate the matrix effect on the extraction of eight steroids by SPE method. The TCM samples in different dilutions were spiked with standard solutions of each steroids to afford concentration of 120 μg/mL, and extraction of these steroids with the optimum conditions were employed.

V. *Sample Clean-up by SPE*

Each sample solution (5 mL) was loaded onto a SPE column (SiOH, 500 mg, 3 mL column volume), which had been preconditioned before use by sequentially passing a series of solvents consisting of 5 mL of different solvents (methanol or ethanol or isopropanol) and 5 mL of chloroform. The SPE column was eluted with various combination of dichloromethane-isopropanol (10 mL). The eluate was collected and concentrated under reduced pressure to dryness. Finally, the residue was dissolved in 8 mL of ethanol and transferred to a 10 mL volumetric flask to which 0.5 mL of FA solution (1.0 mg/mL) was added and made up to 10 mL with ethanol. This solution was filtered through a 0.45 μm membrane before HPLC analysis.

VI. *Precision*

The intraday and interday variability at three assay concentrations were evaluated for six replicates over six successive days.

VII. *Recovery*

Known concentrations of the eight steroids were spiked into individual model preparation extracts (5 mL). The recoveries of eight steroids were determined by assaying samples with known drug concentrations. These mixtures were extracted and analyzed using the procedure mentioned above. Recoveries were calculated using the following formula:

Recovery (%) = (Amount measured / Amount added) × 100 %

RESULTS AND DISCUSSION

In this study, we developed a procedure with HPLC method for the determination of eight steroids in TCM extract. We also examined the influence of the parameters on extracting these steroids from TCM extract by SPE method. The extraction recoveries of these steroids from TCM samples depended on a number of factors, including the adsorbent treatment and the matrix effect of SPE to TCM extract.

I. *Calibration Graphs and Detection Limits of Chemical Drugs*

Calibration graphs: peak-area ratio, y, vs. concentration, x, μg/mL were obtained over the range of 6.18-98.88, 6.24-99.84, 6.00-96.00, 6.18-98.88, 6.00-96.00, 6.18-98.88, 6.12-97.92 and 6.24-

Table 1. Calibration curves and detection limits of eight steroids

Chemical drugs	Concentration (μg/mL)	Calibration curves	R	Limit of detection (μg/mL)
BE	6.18-98.88	Y=48.11X-0.52	0.9986	0.50
CA	6.24-99.84	Y=45.05X+0.20	0.9986	1.00
DE	6.00-96.00	Y=46.84X-0.26	0.9992	0.50
HA	6.18-98.88	Y=56.81X+0.01	0.9988	1.00
ME	6.00-96.00	Y=55.50X-1.01	0.9996	0.25
PN	6.18-98.88	Y=44.51X-0.71	0.9986	0.25
PR	6.12-97.92	Y=52.93X-0.85	0.9983	0.25
TR	6.24-99.84	Y=50.82X-1.74	0.9990	0.25

Table 2. Intraday and interday analytical precisions of eight steroids

Chemical drug	Concentration (μg/mL)	Intraday (R.S.D., %)[a]	Interday (R.S.D., %)[a]
BE	6.18	0.7	1.7
	24.72	4.1	3.7
	98.88	2.3	3.1
CA	6.24	1.0	4.2
	24.96	2.8	3.5
	99.84	3.4	3.2
TP	6.0	1.6	2.1
	24.0	3.9	4.4
	96.0	1.9	3.5
HA	6.18	1.3	3.4
	24.72	0.4	1.0
	98.88	3.6	2.8
ME	6.0	1.8	3.6
	24.0	3.1	4.1
	96.0	1.5	1.7
PN	6.18	2.9	2.6
	24.72	1.5	3.5
	98.88	2.4	3.9
PR	6.12	2.1	3.7
	24.48	1.3	4.3
	97.92	3.1	3.5
TR	6.24	2.0	3.6
	24.96	1.5	2.3
	99.84	0.9	2.0

[a] n =6.

99.84 μg/mL for BE, CA, DE, HA, ME, PN, PR and TR, respectively. The regression equations of the eight curves and their correlation coefficients were calculated as shown in Table 1. A signal

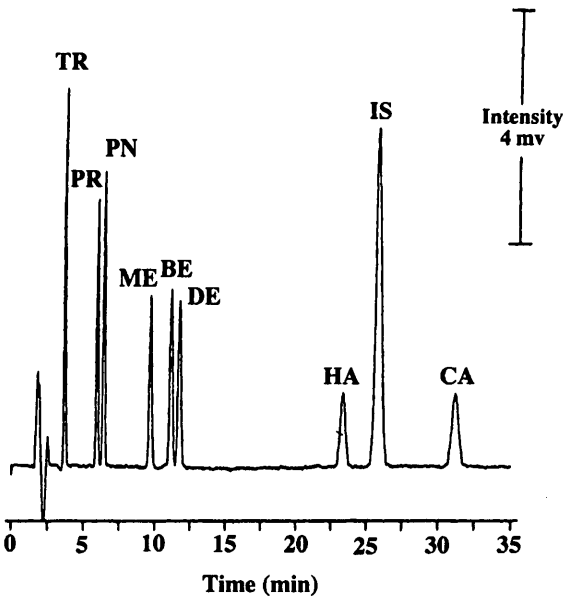


Figure 1. Chromatogram of eight standards with internal standard in TCM after SPE treatment. BE: betamethasone, CA: cortisone acetate, DE: dexamethasone, HA: hydrocortisone acetate, ME: methylprednisolone, PR: prednisolone, PN: prednisone, TR: triamcinolone, IS=fludrocortisone acetate.

three times higher than the noise was regarded as the detection limit. The detection limits of the eight steroids were also shown in Table 1.

Fig. 1 showed a chromatogram in which the retention times for TR, PR, PN, ME, BE, DE, HA, CA and internal standard FA, were 3.6, 5.9, 6.4, 9.7, 11.1, 11.7, 23.3, 31.2 and 25.8 min, respectively. The analysis of the adulterants can be completed within 32 min.

Table 3. Recoveries (%)[a] of eight steroids after SPE with optimum conditions

Concentration (μg/mL)	BE	CA	DE	HA	ME	PN	PR	TR
120	100.5	66.4	103.1	96.7	88.3	94.9	106.4	89.5
240	101.6	67.2	101.9	99.3	98.8	97.0	97.2	92.9
360	110.0	65.7	101.4	103.5	97.1	99.8	100.2	92.5
Mean (%)	104.0	66.4	102.1	99.8	94.7	97.2	101.3	91.6
R.S.D.	4.0	0.9	0.7	2.8	4.6	2.1	3.7	1.7

[a] n =3.

Table 4. Recoveries (%)[a] of eight steroids (120 μg/mL) from cartridges conditioned with different solvents

Conditioning solvent	BE	CA	DE	HA	ME	PN	PR	TR
Methanol	100.5	66.4	103.1	96.7	88.3	94.9	106.4	89.5
Ethanol	94.6	46.8	98.0	98.6	99.4	92.0	92.4	80.9
Isopropanol	51.0	44.7	49.5	68.1	55.3	47.3	48.4	42.9

[a] n =3.

Table 5. Recoveries (%)[a] of eight steroids (120 μg/mL) eluted with different solvent combinations

Elution solvent (CH_2Cl_2:isopropanol)	BE	CA	DE	HA	ME	PN	PR	TR
6:4	100.5	66.4	103.1	96.7	88.3	94.9	106.4	89.5
7:3	99.3	63.3	99.3	86.5	101.3	96.2	96.0	87.7
8:2	106.9	63.4	106.9	84.6	101.4	95.8	94.8	85.0
9:1	100.9	61.9	100.9	75.0	104.2	94.1	91.7	74.8

[a] n =3.

II. *Method Suitability Tests*

The precision of the chromatographic assay method was evaluated by measuring the reproducibility [relative standard deviation (R.S.D.)] and the accuracy was determined by recovery tests. The R.S.D. precision of this method on the basis of peak-area ratios of six replicates were 0.7-4.1 % for intraday and 1.0-4.4 % for interday assays, respectively (Table 2).

Three standard solutions of the eight steroids at concentrations of 120, 240 and 360 μg/mL were prepared in chloroform, respectively. The recoveries were shown in Table 3. The mean values were 104.0, 66.4, 102.1, 99.8, 94.7, 97.2, 101.3 and 91.6 % for BE, CA, DE, HA, ME, PN, PR and TR, respectively. The R.S.D.s of the eight steroids were all lower than 5.0 %.

III. *Conditioning of Cartridges*

The cartridges were conditioned first with various solvents: methanol, ethanol and isopropanol, respectively, and then with chloroform subsequently. The results were listed in Table 4. The best recoveries were obtained with methanol to show 66.4 % for CA and satisfactory for others.

IV. *Sample Elution*

Various solvent mixtures consisting of CH_2Cl_2 and isopropanol in different proportions (6:4, 7:3, 8:2 and 9:1) were used to compare the extraction recoveries. The results were shown in Table 5. Recoveries were 66.4 % for CA and satisfactory for other steroids with CH_2Cl_2-isopropanol (6:4). Except for ME, the recoveries of the other steroids were higher with CH_2Cl_2-isopropanol (6:4) than those with 7:3, 8:2 and 9:1. Although even higher composition (50%) of isopropanol could elute more TCM components, they appeared to interfere with the eight steroids. Therefore, CH_2Cl_2-isopropanol (6:4) was the best elution solvent.

Table 6. The effect of TCM concentration on the recoveries (%)[a] of eight steroids

Formula	TCM Extract:$CHCl_3$	BE	CA	DE	HA	ME	PN	PR	TR
F1	1	98.4	65.8	99.5	94.6	90.3	93.9	100.3	87.5
	1:4	99.8	67.3	100.8	95.6	87.6	95.8	102.1	88.9
	1:24	97.6	65.0	104.3	97.5	85.3	94.7	102.8	87.6
F2	1	100.1	66.9	100.5	98.6	91.3	94.9	100.7	90.2
	1:4	101.2	67.2	98.6	98.1	89.0	95.6	99.8	87.2
	1:24	98.3	66.5	99.7	98.2	87.6	96.5	97.3	85.0
F3	1	99.7	68.3	104.2	95.6	87.7	95.7	98.5	89.1
	1:4	102.1	66.5	102.5	94.3	85.3	95.0	98.0	87.6
	1:24	98.4	64.9	100.7	93.2	86.1	96.1	100.5	86.5

[a] n =3.

Table 7. Recoveries (%)[a] of eight steroids (120 μg/mL) using different brands of silica gel SPE

Brand	BE	CA	DE	HA	ME	PN	PR	TR
Brand1	100.5	66.4	103.1	96.7	88.3	94.9	106.4	89.5
Brand2	102.8	41.3	103.1	107.7	97.7	98.6	97.5	93.3
Brand3	100.9	43.5	98.4	96.5	97.0	97.1	101.2	75.7
Brand4	100.2	44.5	94.4	100.5	106.6	99.1	102.0	99.7
Brand5	98.6	79.3	99.7	99.9	105.7	85.8	90.5	85.7
Brand6	100.3	27.8	99.8	101.1	103.1	90.6	91.0	96.5
Brand7	95.8	27.5	95.2	83.1	91.7	91.2	80.1	82.2
Brand8	93.1	39.7	93.0	86.8	92.9	89.0	89.5	79.7

[a] n =3.

V. *Matrix Effect*

To extract eight steroids from TCM extract with optimum conditions, the TCM samples were spiked with standard solutions. The results (Table 6) revealed that the presence of interferants in TCM does not interfere with the extraction of eight steroids with SPE method. The recoveries of eight steroids showed no significant difference among three concentrations of each TCM. Except for CA (64.9%), the recoveries were quite satisfactory for the others.

VI. *SPE Cartridge Brands*

Eight brands of silica gel SPE cartridges were compared concerning the recoveries of eight steroids. All cartridges were processed by the optimum conditions we developed. The results were shown in Table 7. The recoveries of CA showed greatest differences among different brands of SPE cartridges. The recoveries of CA ranged from 27.5 to 79.3%. To choose the best cartridge (Brand1) was crucial to the recovery of CA. The recoveries for BE, DE, HA, ME, PN, PR and TR were at least 93.1, 93.0, 83.1, 88.3, 85.8, 80.1 and 79.7%, respectively, for eight brands.

CONCLUSION

It is important to develop a fast and efficient method to detect these adulterants in TCM. In this study we have developed a method combining SPE and HPLC. The highest recovery rates were achieved with silica gel cartridge conditioned with methanol and chloroform in sequence, and then eluted with CH_2Cl_2-isopropanol (6:4). The recoveries of BE, CA, DE, HA, ME, PN, PR and TR were 100.5, 66.4, 103.1, 96.7, 88.3, 94.9, 106.4 and 89.5%, respectively. The TCM extracts did not interfere with the eight steroids. This study also compared the efficiency of different brands of silica gel SPE cartridge. More research work on

the applications of SPE method for the extraction of components and adulterants in TCM is still underway in our laboratory.

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中藥製劑中摻加類固醇之固相萃取與高效液相層析研究

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本研究以矽膠固相萃取法萃取摻加於中藥製劑中之八種類固醇betamethasone、cortisone acetate、dexamethasone、hydrocortisone acetate、methylprednisolone、prednisolone, prednisone及triamcinolone，再以高效液相層析法作定量分析比較，評估個別之回收率。本實驗採用逆相層析管柱，沖提液爲乙腈與水(3:7)，檢測波長爲240 nm，內部標準品爲fludrocortisone acetate作分析。八種類固醇檢量線之相關係數(r)爲0.9983至0.9996，均呈現良好線性關係。八種類固醇之同日間及異日間試驗之相對標準差，分別爲0.7-4.1%及1.0-4.4%，顯示再現性佳。固相萃取條件之探討包括萃取溶媒之組成、中藥製劑萃取液之濃度及不同廠牌之固相萃取管等，均加以比較以建立最佳之萃取條件。

關鍵詞：類固醇，固相萃取，高效液相層析，中藥製劑，中藥摻加西藥之檢驗。