Short communication

Determination of steroids adulterated in liquid herbal medicines using QuEChERS sample preparation and high-performance liquid chromatography

Nantana Klinsunthorn [a,b], Amorn Petsom [a], Thumnoon Nhujak [a,*]

[a] *Chromatography and Separation Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[b] *Regional Medical Sciences Center Samut Songkhram, Department of Medical Sciences, Ministry of Public Health, Samut Songkhram 75000, Thailand*

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ABSTRACT

QuEChERS sample preparation was optimized using solvent extraction with acetonitrile and dispersive-solid phase extraction with primary and secondary amine sorbents, and validated for high-performance liquid chromatographic determination of nine steroids commonly used to adulterate herbal medicines: such as triamcinolone, prednisolone, hydrocortisone, methylprednisolone, betamethasone, dexamethasone, beclomethasone, fludrocortsone acetate and cortisone acetate. Satisfactory extraction recoveries of 91–113% for all nine steroids were obtained, along with an acceptable precision in extraction recoveries shown by R.S.D. of $\leq$4.6 and 3.2% for intraday and interday, respectively. The QuEChERS sample preparation developed here allows the reliable detection of adulterated steroids with the limits of detection in the range of 0.06–0.17 ppm. Adulterated steroids in three out of six real commercial liquid herbal medicines were found, such as 1.6 and 8.8 ppm dexamethasone and 0.43 ppm prednisolone.

1. Introduction

Herbal medicines are increasingly being used in both preventative and treatment based medicines and tonics because consumers perceive herbals as "natural", safe, harmless and free from adverse side effects [1]. In order to accelerate fast and effective treatment, synthetic drugs, such as steroids, may be intentionally mixed with the herbal medicine. However, the adulteration of herbal medicines with synthetic steroids is prohibited [2,3]. An overdose of steroids can result in adverse side effects, such as hyperglycemia, hypocalcemin, hypokalemia, high blood pressure, muscle wasting, etc. [4]. Therefore, an effective analytical method to monitor the levels of synthetic steroids adulterated in herbal medicines, as with all food stuffs, is important.

Previously, the identification and quantification of steroids in traditional medicines [1,2,5], animal feed [6], cosmetics [7] and urine [8,9] have been reported using high-performance liquid chromatography (HPLC) with a UV detector [5–7] or a mass spectrometer [1,2,8,9]. However, prior to HPLC analysis, sample preparation is required to remove some matrices that may interfere with the detection of the steroids of interest, reduce the separation efficiency or shorten the column life. The typical procedures of sample preparation have previously involved extracting the samples with organic solvent(s), followed by cleaning-up the extract with solid phase extraction (SPE) using an SPE cartridge [5–9]. The disadvantages of these procedures include the use of large amounts of organic solvents for each extraction (25–50 ml), the time required for the preparation or shaking (30–60 min) of each sample, the use of expensive SPE cartridges, and several steps of SPE solvent elution. In addition, chloroform, which is traditionally used for solvent extraction of traditional medicines [5], is banned in some laboratories due to it being designated as hazardous to health. Moreover, in some cases of our routine analysis at the Regional Medical Sciences Center, we have noticed emulsion formation of the samples occurred with chloroform and other solvents used for extraction, requiring extra phase separation steps to be included. Therefore, a more practical and user/environmental friendly sample preparation methodology is required for the analysis of herbal medicine samples.

Recently, a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) technique has been accepted for the preparation of samples for evaluation of the level of residual pesticides in a variety of sample matrices, such as vegetables and fruits [11–14], and other foods [15–17]. This approach contains two easy steps [11–17]. First, the sample is extracted with an organic solvent (1–10 ml) in the presence of anhydrous salts. Second, a simple cleaning-up is carried out using dispersive-SPE (d-SPE) sorbents in a centrifuge tube. Each step is easily performed by vortexing and centrifuging for a few minutes (2–6 min) and could be partially automated for high throughput analysis demands.

Therefore, the aim of this work was to optimize and to validate the QuEChERS sample preparation methodology for the HPLC

* Corresponding author. Tel.: +66 2 2187609; fax: +66 2 2187598.
  E-mail address: thumnoon.n@chula.ac.th (T. Nhujak).

determination of steroids adulterlated in liquid herbal medicines. The validated method will be used for analysis of real samples.

2. Experimental

2.1. Chemicals

All nine steroids used were purchased from Sigma–Aldrich (MO, USA): triamcinolone (TAL), prednisolone (PNL), hydrocortisone (HCS), methyprednisolone (MPS), betamethasone (BTS), dexamethasone (DXS), beclomethasone (BCS), fludrocortisone acetate (FCA) and cortisone acetate (CSA). Acetonitrile (ACN), ethyl acetate (EtOAc), acetone, NaCl and anhydrous $MgSO_4$ were obtained from Merck (Darmstadt, Germany). The following sorbents for d-SPE were obtained from Varian (Harbor city, USA): primary and secondary amine (PSA), octadecylsilane (C_{18}), alumina, graphitized carbon black (GCB), and hydrophilic–lipophilic balances copolymer (HLB). The blank sample of liquid herbal medicines was obtained from Green Chat Natural Herbes (Thailand) Co, LTD. The real samples were obtained from the Regional Medical Sciences Center Samut Songkhram and their brand names cannot be disclosed.

2.2. HPLC separation

HPLC analysis was performed on an Agilent 1200 series system (Agilent Technologies, USA), equipped with a G1315D PDA UV–Vis detector scanning from 200 to 400 nm and monitoring at 240 nm, a G1311A quaternary pump, a G1322A vacuum degasser and a G1329A autosampler. The chromatographic separation of steroids was carried out using a Hypersil BDS C_{18} column (300 mm × 4.6 mm I.D., 5 μm) with a 1.0-ml/min flow rate of a (v/v) gradient elution of ACN:water mobile phase starting from 33:67 for 10 min to 50:50 in 10–20 min. All sample solutions were injected using a 10-μl sample loop. It should be noted that the reversed phase-HPLC with a C18 column and ACN:water was used in previous works on steroids separation [5,7]. However, the gradient elution of ACN:water was optimized in this work, in order to achieve baseline resolution ($R_S \geq 1.5$) of all nine steroids within appropriate time of analysis (20 min). The following retention times were obtained: 4.78, 6.81, 7.13, 10.01, 10.73, 11.20, 12.59, 17.32 and 18.86 min, for TAL, PNL, HCS, MPS, BTS, DXS, BCS, FCA and CSA, respectively.

2.3. Standard solution preparation

Stock solutions of 1000 ppm steroids dissolved in ACN were separately prepared by weighing an appropriate amount of each standard and then dissolving these in ACN. Each working standard solution containing nine steroids was prepared by pipetting the appropriate amounts of each stock solution.

2.4. QuEChERS sample preparation

125 mg (±5%) NaCl and 500 mg (±5%) anhydrous $MgSO_4$ were filled in a 15 ml PTFE centrifuge tube. Then 2 ml of ACN and 2 ml of a liquid herbal medicine sample were added into the tube. The mixture was shaken vigorously for 10 s, vortexed for 1 min, and then centrifuged for 5 min at 25.2 × g. A 1 ml aliquot from the upper part of the extract was transferred into a microcentrifuge tube containing 50 mg (±10%) of PSA sorbent and 50 mg (±10%) $MgSO_4$. The mixture was then shaken, vortexed and centrifuged for 5 min at 15.4 × g. The harvested ACN extract was then filtered using a 0.45 μm nylon filter prior to HPLC analysis.
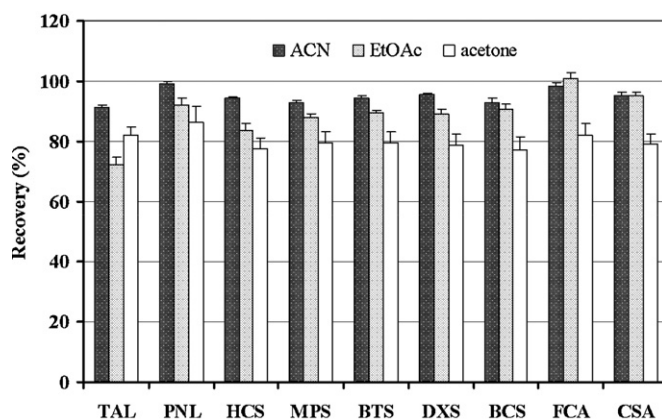

Fig. 1. Average recovery ($n = 3$ batches) of steroids obtained from the QuEChERS sample preparation using ACN, EtOAc and acetone for the solvent extraction of a blank sample spiked with nine steroids at 10 ppm each. Other QuEChERS conditions are given in Section 2.4.

3. Result and discussion

3.1. QuEChERS optimization

ACN, EtOAc and acetone, commonly used in the QuEChERS technique, were compared for solvent extraction of a blank sample (three batches) of a commercial liquid herbal medicine spiked with the nine standard steroids at 10 ppm each. The experiments were performed as mentioned in Section 2.4, except that the type of solvent was varied. In comparison with that obtained using EtOAc and acetone (Fig. 1), ACN provided a better extraction efficiency for all nine steroids with recoveries in the range of 91–99%, whilst acetone was the worst. Therefore, ACN was chosen as the extraction solvent for all further work. In addition, a final extraction solution in ACN is compatible with the water:ACN mobile phase used for the HPLC separation of steroids in this work.

$MgSO_4$ and NaCl salts, particularly with a weight ratio of 4:1 is widely used to induce phase separation in the QuEChERS solvent extraction step [10–12]. In this work, the 4:1 $MgSO_4$:NaCl salts with total amounts of 1000, 750 and 625 mg gave the similar extraction recovery of each steroid (89–97, 89–95 and 91–99%, respectively), but lower recovery (83–91%) was obtained from 500 mg of salts. Therefore, 500 mg $MgSO_4$:125 mg NaCl was chosen in this experiment.

In order to remove matrix components, d-SPE was performed using PSA, C_{18}, alumina, GCB and HLB as sorbents. When GCB or PSA was used as the d-SPE clean-up matrix, they were found to effectively remove the brown color of the ACN extract, whilst a slightly brown extract was still observed using C_{18}, alumina and HLB. This is consistent with the HPLC chromatograms from the three latter sorbents that were found to contain more interference peaks (data not shown). In addition, a poor recovery was found using HLB and GCB sorbents (67–82% and 12–68%, respectively) as shown in Fig. 2, implying that the steroids were retained on these sorbents. Indeed, GCB has previously been reported to not only effectively remove pigments but sterols as well in food samples [10,11]. Since steroids and sterols have somewhat similar structures, steroids, especially those with a high hydrophobicity (higher retention time), may preferentially be retained on GCB. Overall, across the nine evaluated steroids, the PSA, C18 and alumina sorbents provided a comparably high recovery for each steroid (90–100%), except for the low hydrophobic TAL that had a lower recovery (76%) using alumina. Taking account of the high recovery levels and effective removal of pigments, PSA was chosen as the sorbent for d-SPE.

Table 1
Calibration graphs, LOD, and LOQ.

Analyte	Concentration range (ppm)	Calibration plot			LOD (ppm)	LOQ (ppm)	HorRat at LOQ
		Slope	Intercept	r^2			
TAL	0.20–15	19,000 ± 250	500 ± 250	0.9991	0.06	0.20	0.3
PNL	0.30–15	22,000 ± 150	10 ± 200	0.9998	0.09	0.30	0.2
HCS	0.30–15	23,000 ± 100	250 ± 150	0.9999	0.10	0.30	0.2
MPS	0.40–15	21,160 ± 130	−390 ± 170	0.9998	0.13	0.45	0.4
BTS	0.40–15	22,600 ± 350	−700 ± 550	0.9988	0.13	0.45	0.3
DXS	0.40–15	22,900 ± 250	−350 ± 400	0.9997	0.13	0.40	0.4
BCS	0.50–15	20,700 ± 350	−850 ± 560	0.9985	0.17	0.55	0.5
FCA	0.30–15	22,850 ± 300	−600 ± 350	0.9989	0.09	0.30	0.4
CSA	0.30–15	22,500 ± 300	−500 ± 300	0.9992	0.09	0.30	0.4
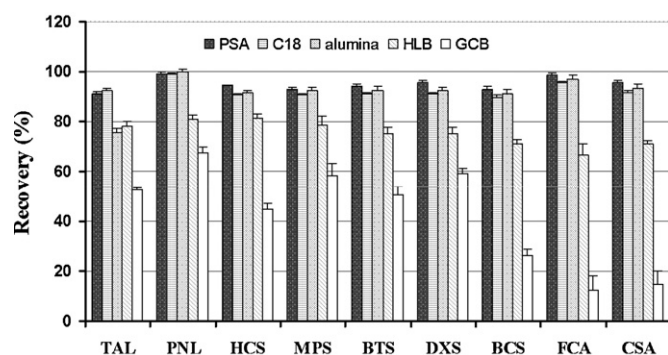
Fig. 2. Average recovery ($n = 3$ batches) of steroids obtained from the QuEChERS sample preparation using five different sorbents for d-SPE of a blank sample spiked with steroids at 10 ppm each. Other QuEChERS conditions are given in Section 2.4.

The amount of PSA was then varied at 25, 50 and 100 mg for the d-SPE. However, PSA at 25 mg gave a poorer extraction of the brown color and a high number of interference peaks on the HPLC chromatogram, whilst a comparable recovery of steroids was obtained with the use of 50 or 100 mg PSA (data not shown). Therefore, 50 mg PSA was chosen for use as the d-SPE.

3.2. QuEChERS method validation

In this work, limit of detection (LOD) and limit of quantitation (LOQ) were obtained from the concentration of the analyte, after QuEChERS sample preparation, giving a signal-to-noise ratio of 3 and 10, respectively. From the results of ten batches of a blank sample spiked with steroids at the concentrations near LOD and LOQ (Table 1), the QuEChERS technique provided LOD and LOQ for steroids in a range of 0.06–0.17 and 0.20–0.55 ppm, respectively, which is sufficient for the HPLC determination of steroids intentionally mixed with herbal medicines.

In terms of the repeatability of the LOQ, a HorRat value (a Horwitz ratio) obtained in a range of 0.2–0.5 meets the acceptance criteria for AOAC, where the HorRat is the ratio of the observed

R.S.D. calculated from the actual performance data, R.S.D.$_r$ (%), to the corresponding predicted relative standard deviation calculated from 0.67 times theoretical values determined by the Horwitz function for inter-laboratory precision, P.R.S.D.$_r$ (%) = $0.67 \times 2\,C^{-0.1505}$, and where C is the concentration added, expressed as a mass fraction [18–20]. By plotting the peak area against the analyte concentration range (listed in Table 1), a highly linear relationship was obtained with $r^2 > 0.998$.

Accuracy and precision in the QuEChERS extraction recovery were evaluated for steroids spiked in the blank samples at the four levels of 10 ppm and 2, 4 and 10 times the LOQ value (from Table 1) for each analyte. From Table 2, satisfactory accuracy of recovery, that is the range of 91–113%, was obtained, with 97% of the recovery data being within in the acceptable recovery of 80–110% for the analyte concentration in the range 0.1–10 ppm [19]. An accepted level of precision was also obtained with R.S.D. of <5% and the HorRat values of 0.1–0.6 for all steroids.

Intraday and interday precisions in the recovery were also determined using the blank sample spiked with the nine steroids at a concentration level of ten times their respective LOQ value (from Table 1). For the intraday precision, the recovery R.S.D. value was obtained from 10 batches of the QuEChERS sample preparation, whilst that for interday precision was evaluated from five days. From Table 3, an acceptable precision in the recovery was obtained with R.S.D. $\leq$ 4.6 and 3.2 for intraday and interday, respectively, and with HorRat values of <0.6 in all cases (data not shown).

3.3. Application to real samples

Steroids adulterated in six samples of liquid herbal medicine from different suppliers were determined by HPLC using QuEChERS sample preparation in triplicate batches. Using a spiking technique and comparing the UV spectra of peaks with those of the steroid standards, samples L1 L2 and L3 were found to be adulterated with 8.8 ± 0.2, 1.6 ± 0.1 ppm dexamethasone (UV λ_{max} of 240 nm) and 0.43 ± 0.01 ppm prednisolone (UV λ_{max} of 245 nm), respectively (Fig. 3), whilst adulterated steroids were not detected, subject to the LOD of this assay system, in the other three samples.

Table 2
Accuracy and precision in the QuEChERS extraction recovery of steroids spiked in blank sample at four levels ($n = 10$ batches).

| Analyte	% Recoveries (% R.S.D.) at				HorRat at			
	$2 \times$ LOQ	$4 \times$ LOQ	$10 \times$ LOQ	10 ppm	$2 \times$ LOQ	$4 \times$ LOQ	$10 \times$ LOQ	10 ppm
TAL	113.0 (1.8)	100.2 (2.1)	99.5 (1.3)	91.1 (2.5)	0.2	0.2	0.1	0.3
PNL	102.5 (3.0)	98.2 (2.2)	98.5 (1.6)	99.3 (1.2)	0.3	0.2	0.2	0.2
HCS	98.1 (2.6)	95.3 (2.2)	96.1 (2.5)	94.5 (1.5)	0.2	0.2	0.3	0.2
MPS	97.5 (3.4)	96.0 (2.2)	97.5 (1.6)	92.8 (4.6)	0.3	0.2	0.2	0.6
BTS	103.1 (4.7)	98.4 (1.7)	97.0 (1.4)	94.4 (3.2)	0.4	0.2	0.2	0.4
DXS	98.9 (3.6)	95.4 (2.1)	96.2 (1.3)	95.6 (2.3)	0.3	0.2	0.2	0.3
BCS	97.4 (3.3)	99.4 (3.3)	94.4 (1.6)	92.8 (1.9)	0.3	0.3	0.2	0.3
FCA	101.3 (6.8)	97.5 (2.6)	98.4 (1.6)	98.6 (1.9)	0.6	0.2	0.2	0.3
CSA	102.1 (2.5)	98.8 (3.2)	98.5 (1.9)	95.4 (2.2)	0.2	0.3	0.2	0.3

% R.S.D. in parentheses.

Table 3
Intraday and interday precisions in the QuEChERS extraction recovery of steroids spiked in the blank sample at $10 \times$ LOQ R.S.D.

Analyte	% R.S.D. for QuEChERS extraction recoveries Intraday (10 batches each day)					Interday
	Day 1	Day 2	Day 3	Day 4	Day 5	Overall
TAL	1.9 (97.8)	3.1 (98.6)	2.9 (98.3)	3.0 (95.8)	3.5 (95.9)	3.2 (98.8)
PNL	1.4 (98.5)	2.2 (96.9)	2.5 (97.5)	1.6 (96.9)	1.9 (99.7)	1.9 (97.8)
HCS	1.6 (97.7)	2.2 (96.5)	3.4 (96.9)	2.5 (96.0)	4.0 (101.1)	3.1(97.3)
MPS	1.4 (92.4)	2.3 (92.7)	2.4 (92.0)	2.4 (91.3)	2.8 (93.6)	2.4 (92.1)
BTS	2.0 (104.3)	2.3 (105.1)	2.4 (104.7)	3.4 (104.6)	3.5 (108.3)	2.9 (104.5)
DXS	1.9 (100.3)	2.9 (99.2)	3.9 (100.4)	2.3 (100.9)	4.1 (103.4)	3.0 (100.2)
BCS	1.9 (95.7)	3.4 (96.3)	1.4 (95.7)	2.1 (94.7)	2.5 (96.4)	2.4 (95.5)
FCA	1.5 (95.9)	4.2 (97.2)	4.6 (97.0)	2.7 (94.1)	2.2 (95.1)	2.5 (95.9)
CSA	1.7 (94.4)	3.3 (93.9)	3.6 (95.2)	2.3 (91.8)	3.0 (94.2)	2.7 (94.4)

The mean recovery in parentheses.
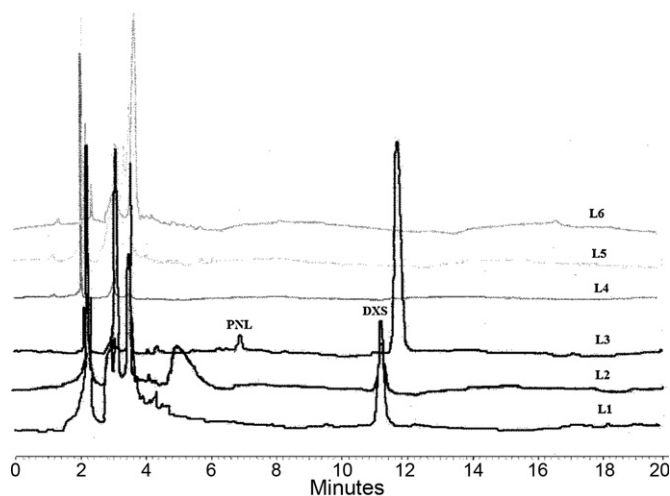
Fig. 3. HPLC chromatograms of real samples of liquid herbal medicines after the QuEChERS extraction.

4. Conclusions

QuEChERS sample preparation was developed for the HPLC determination of steroids adulterated in liquid herbal medicines, using ACN as the solvent extraction and PSA as the d-SPE sorbent. This QuEChERS method gave a high accuracy and precision and allowed the detection of steroids in the sample down to 0.1 ppm. In comparison with the previous method using solvent extraction with chloroform and cartridge SPE [5], the QuEChERS sample preparation provides a faster, easier and cheaper method. In future work, the QuEChERS sample preparation method will be evaluated for the HPLC determination of steroids adulterated in powder samples of herbal medicines.

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