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Establishment of an HPLC identification system for detection of counterfeit steroidal drugs

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Abstract

A set of simple HPLC methods employing UV detection were developed for detection of counterfeit drugs by the qualitative and quantitative analysis of nine steroidal drugs, ethinylestradiol, diethylstilbestrol, norethisterone, norgestrel, methyltestosterone, medroxyprogesterone acetate, progesterone, testosterone propionate and nilestriol. The methods were based on studies of the relationships between the retention factors (k) of the nine compounds and the percentages of water to methanol in the mobile phases on a reverse phase Alltima C_{18} column giving reliable separation of the compounds under three sets of chromatographic conditions. The methods were validated using statistical tests and were used on nine commercial samples for detection of possible counterfeit drugs.

Keywords: Steroid hormones; HPLC; Counterfeit drugs

1. Introduction

The prevalence of counterfeit drugs is a vast global problem, especially in developing countries [1,2]. Counterfeit drugs may contain no active ingredient, the wrong active ingredient or the correct ingredient but with lower or higher amount of active. Quick identification of counterfeit drugs in markets would be useful to combat this emerging threat. The Chinese Government is supporting the establishment of a "Fast HPLC Identification System" programme to routinely test more than 400 frequently used categories of chemical medicines in Chinese markets. This report focuses on steroid hormones as a part of the program.

A number of analytical methods for the identification and quantification of steroid hormonal drugs has been reported [3–5]. Official HPLC methods for quality control of the drugs are also found in the pharmacopoeia of many countries [6–8]. However, those methods were established for the quality control of target products by testing the levels of the target compound and its impurities from preparation procedures. Most of the methods are not suitable for detection of counterfeit drugs, for which it is required to qualitatively and quantitatively analyze the target products and to identify the possible replacements. Few analytical methods have been reported for the verification of steroid hormone drugs, especially for those with similar chemical properties. In this paper, a set of simple HPLC methods are presented to distinguish and quantitatively analyze nine steroid hormone drugs, ethinylestradiol (ETH), diethylstilbestrol (DIE), norethisterone (NORE), norgestrel (NORG), methyltestosterone (MET), medroxyprogesterone acetate (MED), progesterone (PRO), testosterone propionate (TES) and nilestriol (NIL) in commercial products. The methods were developed based on the relationships between the retention factors (k) of the hormones and the percentages (C) of water or methanol in mobile phases ($\log k$–C curves) and appear to be useful in the identification of counterfeit drugs.

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Table 1
Commercial pharmaceutical dosage form of nine-hormone drug used as unknown samples in the research

Sample number	Pharmaceutical industries[a]	Drug	Pharmaceutical dosage form
1	A	Ethinylestradiol tablets	50 μg/tablet
2	B	Diethylstilbestrol tablets	1 mg/tablet
3	C	Norethisterone tablets	0.625 mg/tablet
4	D	Compound norgestrel tablets	0.3 mg/tablet (norgestrel)
5	E	Methyltestosterone tablets	5 mg/tablet
6	F	Medroxyprogesterone acetate tablets	2 mg/tablet
7	G	Progesterone injection	1 ml:20 mg
8	H	Testosterone propionate injection	1 ml:25 mg
9	I	Nilestriol tablets	1 mg/tablet

[a] Pharmaceutical industries are identified by letters and samples by numbers.

2. Experimental

2.1. Materials

Reference standards of ETH, DIE, NORE, NORG, MET, MED, PRO, TES and NIL were obtained from the National Institute for Control of Pharmaceutical and Biological Products (NICPBP), Beijing, China. Organic solvents for chromatography, HPLC grade, were purchased from Dima Technology Inc. (Richmond, Canada) and double-distilled water was used. The nine commercial products tested were purchased commercially and the details summarized in Table 1.

2.2. Apparatus

The liquid chromatograph consisted of a Waters 2695 separation module with a Waters 2996 photodiode array detector and an autosampler, or a Shimadzu LC-2010A separation module with SPD-M10A vp detector and an autosampler. Detectors were set to a wavelength range of 190–400 nm. The routine chromatographic separations were performed on an Alltima C18 (5 μm, 150 mm × 4.6 mm, i.d.) column, Alltech Associates, Inc., USA, under isocratic condition at 25 °C. Sonication of samples to aid dissolution was carried out in an ultrasonic cleaner TCQ-250, Beijing Medical Treatment Instrument Factory Second, China.

2.3. Chromatographic conditions

2.3.1. HPLC conditions for identification

The HPLC identification of each of nine individual hormones was performed on an Alltima C18 column at a flow rate of 1.0 ml/min, using two runs with different mobile phases. ETH and DIE were tested using methanol–water (62:38, v/v) as mobile phase 1 and (60:40, v/v) as mobile phase 2 (Table 2), at 221 nm for ETH or 245 nm for DIE. NORE, NORG, MET, MED and PRO were determined at 245 nm with methanol–water (70:30) and (65:35), respectively. TES and NIL were measured using methanol–water (85:15) and (80:20), at 245 nm for TES and at 221 nm for NIL.

2.3.2. HPLC conditions for quantitation

The quantitative analysis of each component was performed with mobile phase 1 as described above (Table 2).

2.4. Procedures

2.4.1. Standard solutions preparation

Standard solutions of DIE, NORE, NORG, MET, MED and NIL were prepared by dissolving each material in methanol to afford solutions of 100 μg/ml. The standard solution of ETH was prepared at a concentration of 20 μg/ml in methanol. Standard solutions of PRO and TES were prepared by dissolving 10.0 mg

Table 2
Chromatographic conditions for the nine analytes

Group	Sample	Mobile phase 1	Mobile phase 2	UV detection (nm)	Flow rate (ml/min)	Injection (μl)
1	ETH DIE	Methanol–water (62:38, v/v)*	Methanol–water (60:40, v/v)	221 245	1	10
2	NORE NORG MET MED PRO	(70:30, v/v)*	(65:35, v/v)	245		
3	TES NIL	(85:15, v/v)*	(80:20, v/v)	245 221		

* Conditions used for quantitation.

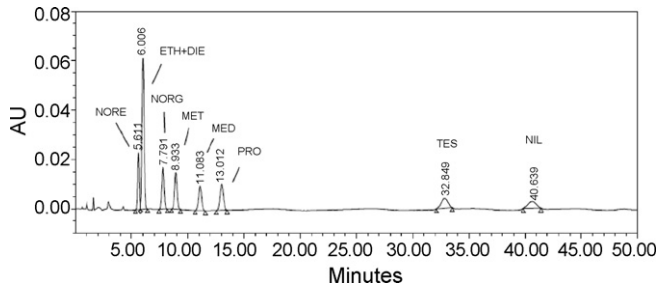

Fig. 1. Chromatogram of the nine hormones in a preliminary HPLC experiment eluted with methanol–water (70:30) at 221 nm.

of each material in THF (20 ml) and were diluted with methanol to give solutions of 100 μg/ml.

2.4.2. Linearity and sensitivity

Working standard solutions were prepared in the range of 40–160% of the concentrations as the sample solutions in Section 2.4.3. Using six different dilutions. Each solution was injected into the HPLC system in triplicate and mean values of peak areas were plotted against concentrations of standards. The curves were adjusted by linear regression using the least mean squares method. Linearity was expressed as a correlation coefficient; the acceptable value must be $R > 0.9990$.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by injecting progressive dilutions of the standard solutions. The LOD and LOQ were defined as a signal/noise ratio of 3:1 and 10:1, respectively.

2.4.3. Sample preparations

2.4.3.1. Commercial tablets. Ten tablets of DIE, NORE, NORG, MET, MED and NIL, respectively were accurately weighed and finely powdered with pestles manually. Portions of the powders theoretically equivalent to 1 mg of DIE, NORE, NORG and NIL were transferred into 10 ml volumetric flasks and dissolved in methanol (8 ml). Powders equivalent to 5 mg of MET and MED were added to 50 ml volumetric flasks and dissolved in methanol (40 ml). After sonication for 5 min, the samples were diluted with methanol to give solutions of 100 μg/ml. The solutions were filtered on a 0.45 μm filter before injection.

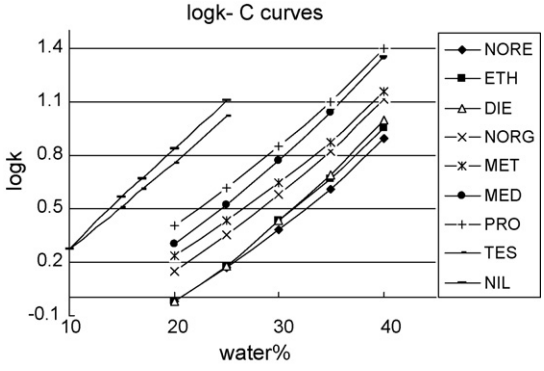

Fig. 2. Effect of water concentration on retention factors ($\log k$) of the nine hormones.

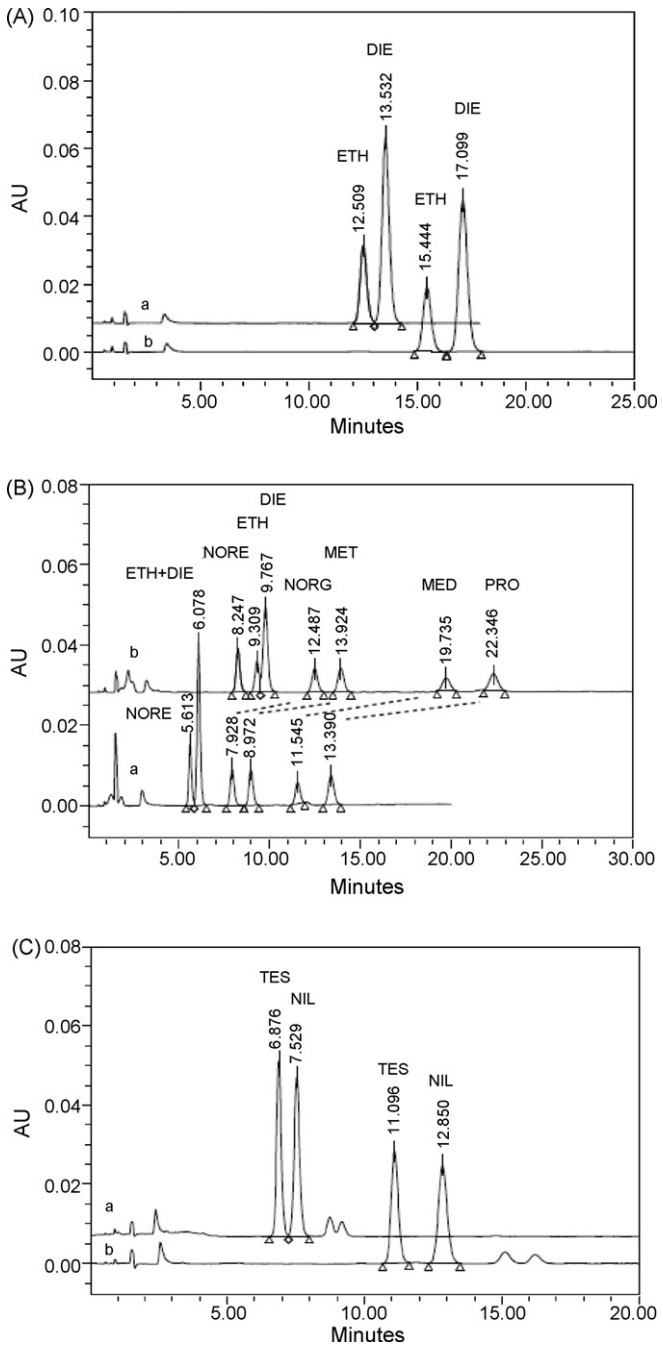

Fig. 3. HPLC chromatograms on a Alltima C18 (5 μm, 150 mm × 4.6 mm, i.d.) column of (A) ETH and DIE using two mobile phases of a mixture of methanol and water (a) 62:38 (v/v) and (b) 60:40 (v/v) at 221 nm; (B) NORE, NORG, MET, MED, PRO, along with ETH and DIE using two mobile phases of a mixture of methanol and water (a) 70:30 (v/v) and (b) 65:35 (v/v) at 221 nm; (C) TES and NIL using two mobile phases of a mixture of methanol and water (a) 85:15 (v/v) and (b) 80:20 (v/v) at 221 nm.

Twenty tablets of ETH were accurately weighed and finely powdered. A portion of the powder equivalent to 500 μg of ETH was transferred into a 25 ml volumetric flask and dissolved in methanol. After sonication for 5 min, the sample was diluted with methanol to give a solution of 20 μg/ml. The solution was filtered prior to HPLC.

Table 3
The % R.S.D.s of retention factors (k) of nine compounds under optimized conditions at three laboratories

Group	Mobile phase methanol–water	Compound	Retention factor (k)			% R.S.D.
			Lab. 1	Lab. 2	Lab. 3	
1	(62:38,	ETH	6.97	4.82[a]	5.26[a]	
	v/v)	DIE	7.62	5.05[a]	5.55[a]	
	(60:40,	ETH	8.84	9.21	9.32	2.8
	v/v)	DIE	10.40	10.14	10.38	1.4
2	(70:30,	NORE	2.58	2.52	2.77	5.1
	v/v)	NORG	4.05	3.91	4.33	5.2
		MET	4.71	4.57	5.05	5.1
		MED	6.35	6.09	6.76	5.3
		PRO	7.53	7.26	7.97	4.7
	(65:35,	NORE	4.25	4.17	4.49	3.9
	v/v)	NORG	6.95	6.79	7.30	3.7
		MET	7.87	7.69	8.28	3.8
		MED	11.57	11.18	12.05	3.8
		PRO	13.23	12.88	13.73	3.2
3	(85:15,	TES	3.38	3.33	3.72	6.1
	v/v)	NIL	3.79	3.77	4.14	5.3
	(80:20,	TES	6.07	5.85	6.55	5.8
	v/v)	NIL	7.18	6.99	7.67	4.9

[a] The data were obtained in methanol–water (65:35).

2.4.3.2. Injections. A measured volume of PRO injection equivalent to 20 mg of active ingredient was added to a 100 ml volumetric flask, dissolved in THF (20 ml) and diluted to volume with methanol. Five milliliters of the solution was transferred to a 10 ml volumetric flask and diluted to volume with methanol to give a solution of 100 μg/ml.

A volume of TES solution equivalent to 25 mg active ingredient was transferred to a 50 ml volumetric flask, dissolved in THF (20 ml) and diluted to volume with methanol. Two milliliters of the solution was transferred to a 10 ml volumetric flask and diluted to volume with methanol to give a solution of 100 μg/ml.

2.4.4. Precision and accuracy

The precision of each proposed method (Table 2) was evaluated through intra-day and inter-day repeatability of responses of sample solutions described in Section 2.4.3. Each solution was determined by six replicates in 1 day ($n = 6$) for intra-day repeatability, and by one injection per day in 6 days for inter-day repeatability ($n = 6$). The precision is expressed as percentage relative standard deviation (% R.S.D.).

To evaluate the accuracy of the proposed methods, recovery tests for all samples were performed by adding known amounts of reference standard solutions to samples followed by analysis using proposed method. The amount of the spiked reference standard was close to the ingredients in the samples. Recoveries were tested in three concentrations that were at 80, 100 and 120% of the concentrations as the sample solutions in Section 2.4.3, and on three replicated samples for each concentration. The percentage of recovery was calculated according to official methods of analysis [9].

3. Results and discussion

3.1. Selection and optimization of mobile phases

This study focused on the development of HPLC methods to easily identity and quantify correct ingredients in the products and to detect possible replacements, especially those using cheaper materials. The methods needed to be suitable for use in local labs, avoiding complex operations (e.g. mobile phase gra-

Table 4
Statistical results of linear regression analysis in the determination of nine compounds by the proposed methods

Compound	Slope of curve	Intercept of curve	Correlation coefficient (r)
ETH	1E + 07	−4181	0.9999
DIE	4E + 07	−23649	1.0000
NORE	3E + 07	−11085	1.0000
NORG	3E + 07	−9464	1.0000
MET	3E + 07	−12348	1.0000
MED	2E + 07	−15797	1.0000
PRO	3E + 07	−15797	1.0000
TES	3E + 07	2495	0.9999
NIL	1E + 07	2793	0.9999

Table 5
Detection limit (LOD) and quantification limit (LOQ)of nine compounds

Compound	Limit of detection (μg/ml)	Limit of quantitation (μg/ml)
ETH	0.43	1.29
DIE	0.10	0.35
NORE	0.04	0.14
NORG	0.06	0.20
MET	0.06	0.20
MED	0.08	0.26
PRO	0.06	0.21
TES	0.06	0.20
NIL	0.41	1.34

Table 6
Results of precision tests of intra-day and inter-day

Compound	% R.S.D.	
	Intra-day ($n=6$)	Inter-day ($n=6$)
ETH	0.32	0.47
DIE	0.42	1.92
NORE	0.33	1.07
NORG	0.07	0.75
MET	0.06	0.51
MED	0.17	0.41
PRO	0.08	0.40
TES	0.08	0.19
NIL	0.28	1.80

dients, complex mobile phases) and special columns because some local labs are short of advanced apparatus and funds. A mixture of methanol and water was selected as the mobile phase, referencing some HPLC conditions of hormone drugs in the Pharmacopoeia of the P.R. of China.

In a preliminary HPLC experiment on the nine hormones eluted with methanol–water (70:30) at 221 nm, TES and NIL had excessively long retention times and broad peaks. The others were not sufficiently resolved (Fig. 1). This suggested that it is impractical to distinguish all nine compounds using one HPLC protocol.

To establish suitable HPLC conditions to distinguish the nine drugs, the relationships between the retention factors (k) and the percentages (C) of water in mobile phases for the nine compounds were studied. The retention factor, k, of a sample component reflects how long a compound remains on the column ($k=(t_r-t_m)/t_m$), where t_r is the retention time of the retained compound, and tm is the retention time of an un-retained component (uracil).

The k of the nine hormones were determined for each of methanol–water mixtures and the results are presented as $\log k$ in Fig. 2. It can be seen from the figure that the k of TES and NIL are very similar and increase similarly with the percentage of water in the mobile phase . They also behave very differently from the others. A proportion of water of at least 15% should give good resolution of these two compounds with reasonable retention times.

The $\log k$–C curves of ETH and DIE overlap when eluting with less than 35% of water in the mobile phase. When the proportion of water exceeded 35%, k of ETH and DIE diverged, which indicates that ETH and DIE could be separated adequately with more than 35% of water in the mobile phase.

NORE has the same retention time as ETH and DIE when eluting with 20–28% water. However, it was well separated with more than 28% water. The $\log k$–C curves of NORG, MET, MED and PRO were separated well with more than 20% water.

The relationships between $\log k$ and water percentages in mobile phases ($\log k$–C curves) were used to select mobile phase which gave good resolution between compounds and rea-

Table 7
Recovery data of standard solutions added to the samples analyzed using the proposed HPLC methods

Sample number	Added (μg/ml)[a]	Measured (μg/ml)[a]	Recovery ± S.D. (%) ($n=3$)	Mean of recovery (%)
1	8.46	8.42	99.5 ± 0.86	99.0
	10.37	10.22	98.6 ± 0.89	
	12.44	12.30	98.8 ± 0.21	
2	41.24	41.14	99.7 ± 0.70	100.8
	50.52	51.22	101.4 ± 0.33	
	60.62	61.47	101.4 ± 0.40	
3	41.24	40.79	98.9 ± 0.82	100.5
	50.52	51.21	101.4 ± 0.23	
	60.62	61.29	101.1 ± 0.78	
4	40.44	40.49	100.1 ± 0.51	101.2
	49.54	50.36	101.7 ± 0.42	
	59.45	60.54	101.8 ± 0.28	
5	40.76	41.44	101.7 ± 0.71	101.4
	49.93	50.47	101.1 ± 0.82	
	59.92	60.74	101.4 ± 0.95	
6	41.76	41.89	100.3 ± 0.30	100.5
	51.16	51.14	100.1 ± 0.98	
	61.39	62.05	101.1 ± 1.01	
7	41.88	42.43	101.3 ± 0.81	101.2
	51.30	51.84	101.1 ± 0.30	
	61.56	62.27	101.2 ± 0.24	
8	40.44	40.14	99.2 ± 0.24	99.4
	49.54	49.17	99.3 ± 0.51	
	59.45	59.2	99.6 ± 0.50	
9	42.32	42.83	101.2 ± 0.15	100.6
	51.84	52.08	100.5 ± 2.2	
	62.21	62.34	100.2 ± 1.1	

[a] Average of three determinations.

Table 8
Results of contents of nine drug determined using the proposed HPLC methods

Sample no.	Drugs	Labeled dosages	Tested mean content (%) (relative deviation, $n=2$)	Specified range of content in Ch.P. (%)
1	Ethinylestradiol tablets	50 μg/tablet	77.10 (0.40%)	80.0–120.0
2	Diethylstilbestrol tablets	1 mg/tablet	86.14 (0. 07%)	90.0–110.0
3	Norethisterone tablets	0.625 mg/tablet	104.31 (0.08%)	90.0–110.0
4	Compound norgestrel tablets	0.3 mg/tablet	99.07 (0.41%)	90.0–115.0
5	Methyltestosterone tablets	5 mg/tablet	97.65 (0.41%)	90.0–110.0
6	Medroxyprogesterone acetate tablets	2 mg/tablet	97.45 (0.45%)	90.0–110.0
7	Progesterone injection	1 ml:20 mg	98.95 (0.26%)	93.0–107.0
8	Testosterone propionate injection	1 ml:25 mg	97.49 (0.29%)	90.0–110.0
9	Nilestriol tablets	1 mg/tablet	105.22 (0.56%)	90.0–110.0

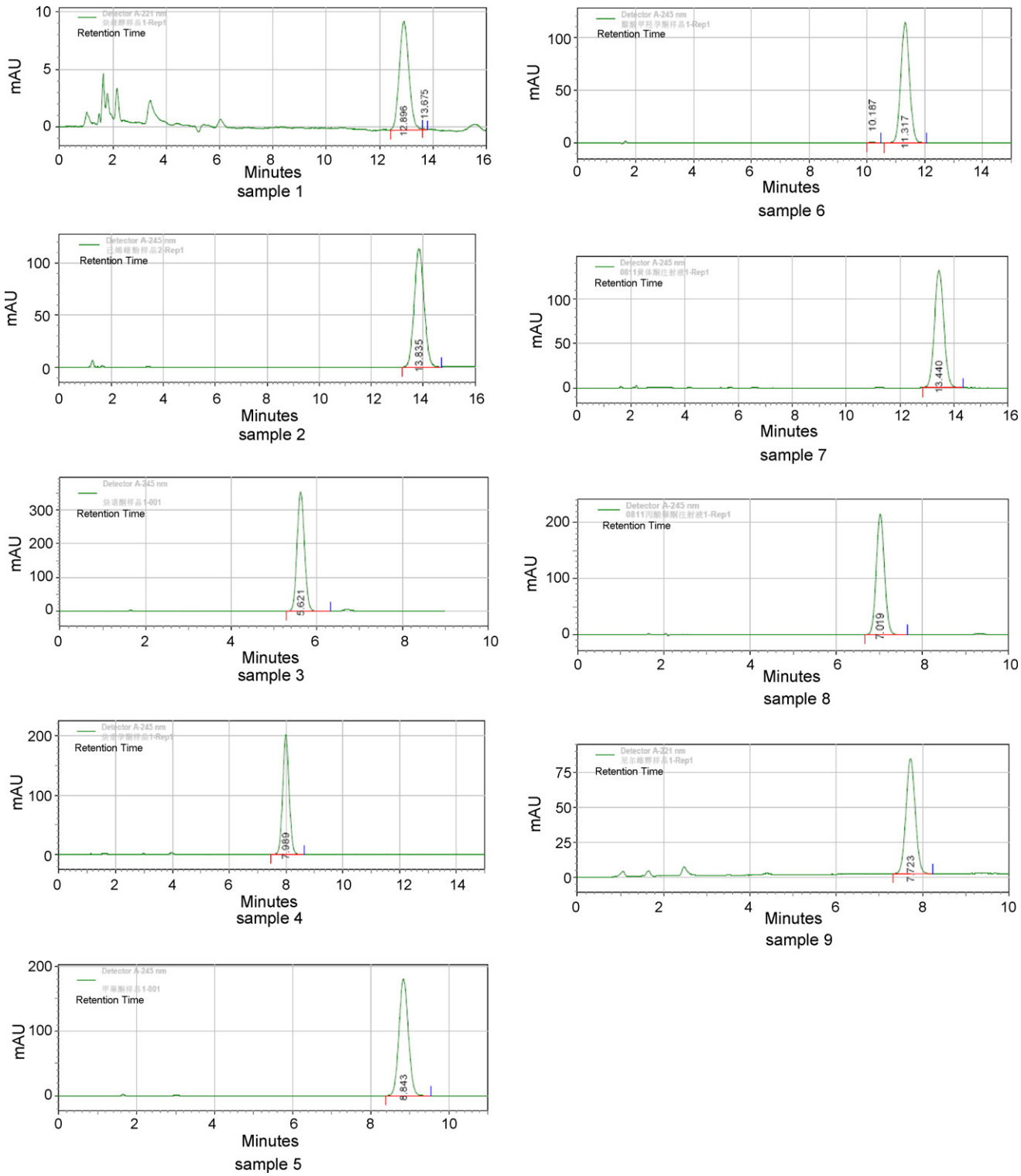

Fig. 4. The chromatograms of nine steroid hormone formulations (samples 1–9) obtained by the proposed quantitation methods.

sonable retention times. The nine analytes were divided into three groups, each with a corresponding mobile phase that gave good separation, i.e., group 1, ETH and DIE, group 2, NORE, NORG, MET, MED and PRO and group 3, TES and NIL (Table 2).

To enhance the specificity of the identification to the compounds, two HPLC mobile phases for each hormone were selected, based on the relationships between k and water percentage in the mobile phases. Methanol–water (62:38) and (60:40) were selected as the mobile phases for the identification of ETH and DIE, and methanol–water (70:30) and (65:35) for identification of NORE, NORG, MET, MED and PRO, and methanol–water (85:15) and (80:20) for the identification of TES and NIL (see Table 2 and Fig. 3).

The retention factors (k) of the compounds were validated using the same type of column at three laboratories in three different regions of China. The k of each compound tested under optimized conditions (see Table 2) at the three laboratories is shown in Table 3 and the % R.S.D.s of the retention factors were less or equal to 6.1%. The results revealed that the retention factors of the nine hormones tested from the three laboratories are consistent and that conditions are stable.

3.2. Linearity and sensitivity

All calibration curves (Table 4) showed linearity over concentration ranges from 8.0 to 32.0 μg/ml for ETH and 40.0–160.0 μg/ml for the other eight compounds. The correlation coefficients obtained with linear regression curves were above 0.9999, which suggests that the selected HPLC methods had excellent linearity over these concentration ranges.

LOD and LOQ values for each compound are shown in Table 5. The values show that the proposed methods are satisfactory for quantitation.

3.3. Precision and accuracy

Precision tests performed on nine sample solutions at the concentrations described in Section 2.4.3 showed that the % R.S.D.s of intra-day and inter-day were less or equal to 0.42 and 1.92, respectively (Table 6). Therefore, the system precision is considered to be satisfactory.

The accuracy was expressed by the recovery of each compound shown in Table 7. The results indicated satisfactory accuracy of the methods for each drug.

3.4. Analysis of commercial formulations

Nine formulations of the steroid hormones of interest were obtained commercially and tested using the HPLC methods as described above (see Table 2).

The HPLC chromatograms of the nine formulations showed that there were no interferences from excipients or solvents in the tablets or injectable solutions (Fig. 4). The percentage of label claimed amount for each product was determined (Table 8). Samples 1 and 2 would be classified as counterfeit drugs according to regulated specifications in the Pharmacopoeia of the P.R. of China. The others would be classified as genuine products.

4. Conclusion

This study developed simple and reliable HPLC methods to determine nine hormone drugs under three sets of HPLC conditions. HPLC methods were found to be more specific than those HPLC methods reported in pharmacopoeias. The methods were shown to be suitable for routine application in screening of commercial pharmaceutical products for counterfeit drugs.

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