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Separation and Determination of Testosterone and Testosterone Esters in Selected Pharmaceutical Formulations

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Abstract □ A rapid quantitative procedure is presented for the separation of testosterone esters from their hydrolysis products through the use of the acetonitrile–infusorial earth column. The method was applied to testosterone cypionate, testosterone enanthate, and testosterone propionate. Recovery and replication of reference standard testosterone and its three esters through the proposed method ranged from 99.1 to 100.3%, and the percent relative standard deviation ranged from 0.6 to 1.0%. Two samples can be separated into testosterone and testosterone ester fractions in about 1.5 hr. The analyses of 20 injectable and one buccal tablet formulations made by 12 different manufacturers are reported.

Keyphrases □ Chromatography, column—analysis, testosterone and testosterone esters, various pharmaceuticals, acetonitrile–infusorial earth column □ Testosterone—analysis, acetonitrile–infusorial earth column chromatography, separation from testosterone esters, various pharmaceuticals □ Testosterone esters—analysis, acetonitrile–infusorial earth column chromatography, separation from testosterone esters, various pharmaceuticals

The "American Drug Index" (1) lists 57 different drug products that contain testosterone esters; they are mostly injectables and are made by 27 different manufacturers. The active ingredients are testosterone cypionate in 11 formulations, testosterone enanthate in 34, testosterone ketolaurate in one, and testosterone propionate in 11.

The official assay procedure in the USP (2) for testosterone drug substance and injection requires GLC analysis. Cleanup of testosterone propionate oil injectables by reversed-phase chromatography has been described (3), and this method is the basis for the modified cleanup procedure in USP XIX (4) for testosterone cypionate and testosterone propionate injectables. These USP procedures measure total testosterone plus esters. The final determinative step for these two compounds is spectrophotometric measurement of their isoniazid hydrazones, a procedure that is slightly altered from that originally described (5).

The USP–NF (6) presents a high-pressure liquid chromatographic (HPLC) method for testosterone propionate tablets. Other steroid separation methods include column adsorption chromatography (7), GLC (8, 9), TLC (10), paper chromatography (11), HPLC (4), column partition chromatography (12), and use of an acetonitrile–infusorial earth column (13).

This paper reports a relatively rapid quantitative analytical procedure for the separation of testosterone from its cypionate, enanthate, and propionate esters using an acetonitrile–infusorial earth column. The final determinative step compares the standard and sample isoniazid hydrazones. The proposed procedure was applied to the analysis of 21 pharmaceutical formulations made by 12 different manufacturers.

EXPERIMENTAL

Apparatus—The following were used: UV–visible recording spectrophotometers[1] with 1-cm stoppered quartz cells, a high-pressure liquid chromatograph[2] (sensitivity of 0.005 absorbance unit full scale with a 254-nm UV detector[3] and a reversed phase column[4]), glass chromatographic columns for partition chromatography (2.2 × 25 cm, constricted at one end to 0.4 × 5 cm), an aluminum tamping rod, an electrobalance[5], and TLC[6] equipment. Volumetric flasks and pipets were either NBS calibrated or Class A glassware.

Materials—Alcohol USP, distilled-in-glass grade[7] acetonitrile, chloroform, *n*-heptane, absolute methanol, and reagent grade acetic acid were used along with acid-washed infusorial earth[8]. Also used were USP reference standard testosterone cypionate, testosterone enanthate, and testosterone propionate and NF reference standard testosterone.

Reagents—Mutually saturated acetonitrile–*n*-heptane was prepared as follows. Acetonitrile, 30 ml, was mixed with 500 ml of *n*-heptane (sufficient for two determinations) in a separator, agitated vigorously for 2 min, and allowed to stand until both layers were clear. These mutually saturated solutions were used whenever acetonitrile or *n*-heptane was called for in these directions.

Sample Preparation—*Tablets*—Twenty tablets were weighed,

[1] Cary models 15 and 17.
[2] Waters model 6000 pump.
[3] Waters 440 detector.
[4] Separations Group, Vidac TP.
[5] Cahn models G-2 and 25.
[6] TLC plates 6060, Eastman Organic Chemicals.
[7] Burdick & Jackson.
[8] Celite 545, Johns-Manville Product Corp.

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Table I—Recovery Replication of Steroid Esters (Percent Recovered) through the Proposed Procedure

Run	Testosterone Cypionate			Testosterone Enanthate			Testosterone Propionate			Testosterone,
	Fraction 1[a]	Fraction 2[b]	Total	Fraction 1[a]	Fraction 2[b]	Total	Fraction 1[a]	Fraction 2[b]	Total	Fraction 2[b]
1	98.8	1.2	100.0	100.3	0.4	100.7	100.0	0.6	100.6	98.5
2	98.0	1.1	99.1	99.3	0.3	99.6	97.9	0.6	98.5	99.3
3	98.3	1.1	99.4	99.4	0.3	99.7	99.1	0.6	99.7	99.2
4	99.5	1.1	100.6	101.2	0.5	101.7	98.7	0.9	99.6	99.4
5	98.3	1.3	99.6	100.8	0.4	101.2	98.3	0.4	98.7	100.4
6	98.7	1.5	100.2	101.2	0.4	101.6	97.8	1.0	98.8	101.5
7	98.5	1.4	99.9	99.8	0.6	100.4	98.1	0.7	98.8	99.3
8	97.5	1.5	99.0	97.9	0.8	98.7	98.1	0.4	98.5	99.9
9	98.1	1.3	99.4	98.3	0.8	99.1	98.5	0.4	98.9	100.2
Average		1.3	99.7		0.5	100.3		0.6	99.1	99.7
RSD[c]			0.6			1.0			0.7	1.0

[a] Elution fraction 1. [b] Elution fraction 2. [c] Relative standard deviation calculated from the range by the method of Dean and Dixon (14).

ground to pass through a 60-mesh sieve, and mixed thoroughly. Then a sample containing ~12.5 mg of steroid was accurately weighed and transferred to a 50-ml volumetric flask, diluted to volume with methanol, and mixed. Two 5-ml aliquots, each containing ~1.25 mg of steroid, were evaporated just to dryness. The sample residues were dissolved in 1.5 ml each of acetonitrile and *n*-heptane for cleanup as directed under *Column Preparation*.

Injectables—The contents of several containers were well mixed, and duplicate 2.0-ml sample portions were diluted accurately with chloroform so that aliquots of the final sample solution containing ~1.25 mg of testosterone ester could be evaporated just to dryness on the steam bath. The sample residues were dissolved in 1.5 ml each of acetonitrile and *n*-heptane for cleanup as directed under *Column Preparation*.

Column Preparation—A glass wool plug was inserted in the bottom of a chromatographic column. Then a 4-g portion of infusorial earth, thoroughly mixed with 4 ml of acetonitrile, was transferred to the column and packed firmly with a tamping rod.

The sample solution, prepared as directed, was mixed thoroughly with 3 g of infusorial earth to yield a light fluffy mixture. This mixture was transferred to the column above the acetonitrile layer and packed firmly. The sample beaker, tamping rod, spatula, and funnel were dry washed with about 1 g of infusorial earth, which was added to the column. The same equipment was dry washed with glass wool, which was placed on top of the sample layer plus washings and packed firmly.

Column Elution of Steroids—The sample beaker was washed with 250 ml of *n*-heptane in small portions, which were transferred to the column to maintain a liquid head approximately 12 cm above the column bed. About 60 ml of *n*-heptane eluate was collected in the first fraction, the beakers were changed, and about 190 ml was collected in the second fraction. The last wash was allowed to drain completely from the column, the tip was rinsed with alcohol, and both effluents were saved for further analysis.

The first 60-ml *n*-heptane fraction was diluted accurately to volume with chloroform to give a final concentration of ~0.012 mg of steroid ester/ml. This solution was allowed to come to room temperature and was readjusted to volume before further dilution or taking of aliquots. The second 190-ml *n*-heptane fraction was carefully evaporated just to dryness on the steam bath. The residues were dissolved and diluted accurately to a volume with absolute methanol to yield a concentration of ~0.012 mg of steroid/ml. In every case, aliquots were taken carefully just to dryness for the isoniazid determination.

Isoniazid Method—The procedure of Umberger (5) was used, except that the hydrochloric acid concentration was doubled to increase the sensitivity.

UV Method—Aliquots of testosterone fractions 1 and 2 were taken carefully to dryness on the steam bath. The residues were dissolved and diluted with methanol to ~0.010 mg of testosterone/ml or 0.012 mg of testosterone esters/ml and scanned spectrophotometrically from 360 to 220 nm *versus* the same solvent. They were compared with a reference standard solution of steroid of about the same concentration.

Completeness of Testosterone Elution—After column elution with 250 ml of *n*-heptane in accordance with the proposed method, an additional step was employed to check the completeness of testosterone elution in all method development studies. The sample beaker was washed with 125 ml of chloroform in several portions, which were added to the column to maintain the liquid level close to the column top. The last portion was allowed to drain completely, the tip was rinsed with alcohol, and the eluate was taken carefully just to dryness. When dissolved and reacted with 4 ml of isoniazid, the residues gave no visible color.

Elution Studies—Approximately 2 mg, accurately weighed, of reference standard testosterone and each of the three testosterone esters were dissolved in 1.5 ml each of acetonitrile and *n*-heptane and placed on the column as described. These compounds were eluted at a rate of ~7 ml/min with 300 ml of *n*-heptane and collected in 15-ml portions for analysis. A second determination was made of testosterone with the rate of elution set at about 1 ml/min. Each 15-ml portion of the eluate was taken carefully just to dryness. Each residue was dissolved in and diluted with methanol and spectrophotometrically scanned in the UV region. Elution curves were plotted for each experiment.

Replication Studies of Recoveries of Testosterone and Its Esters—Reference standard testosterone and its esters, equivalent to ~10 mg (accurately weighed) of testosterone, were dissolved and diluted to 50 ml with methanol. Nine 5-ml aliquots of each steroid were taken carefully just to dryness. The residues were dissolved in 1.5 ml each of *n*-heptane and acetonitrile, placed on the column, and eluted according to the described procedure.

For testosterone esters, the 60-ml fraction of *n*-heptane was diluted to 100 ml with chloroform, and 5-ml aliquots were evaporated carefully

Table II—Replication and Separation of Testosterone (Percent Recovered) from Its Esters by the Proposed Procedure

Run	Testosterone Enanthate, Isoniazid	Testosterone, Isoniazid	Testosterone Cypionate		Testosterone		Testosterone Propionate		Testosterone	
			Isoniazid	UV	Isoniazid	UV	Isoniazid	UV	Isoniazid	UV
1	99.8	103.7	99.4	100.4	101.5	99.7	97.1	98.0	103.3	100.2
2	99.0	102.3	99.6	100.6	99.1	96.8	98.6	99.2	99.8	96.8
3	101.1	101.1	99.4	100.6	100.2	97.7	99.8	100.7	99.5	94.5
4	100.0	103.5	99.2	100.4	100.1	100.9	97.9	98.5	99.4	99.7
5	100.6	100.4	99.2	100.7	101.1	100.6	99.3	99.8	98.9	98.8
6	100.2	99.6[a]	98.3	99.8	100.3	100.6	98.9	99.5	99.7	101.1
7	100.5	98.4[a]	98.9	98.0	99.3	99.3	97.3	99.5	100.7	100.7
8	98.8	98.1[a]	100.8	98.3	99.8	100.2	100.2	98.8	97.4	102.0
9	99.3	101.2[a]	ND[b]	ND[b]	ND[b]	ND[b]	98.2	99.0	100.3	100.4
10	97.9	103.4[a]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
Average	99.7	101.2	99.4	99.8	100.2	99.5	98.6	99.2	99.9	99.4
RSD[c]	1.1	1.8	0.9	0.9	0.8	1.4	1.0	0.9	1.9	2.5

[a] Testosterone eluted from column with 125 ml of chloroform following elution of the ester. [b] Not determined. [c] Relative standard deviation calculated from the range by the method of Dean and Dixon (14).

Table III—Recoveries of Testosterone Esters from the Acetonitrile Column

Testosterone Cypionate			Testosterone Enanthate			Testosterone Propionate		
Amount, mg	Percent Recovered		Amount, mg	Percent Recovered		Amount, mg	Percent Recovered	
	UV	Isoniazid[a]		UV	Isoniazid[a]		UV	Isoniazid[a]
2.757[b]	99.6	97.1 (1.1)	2.769	102.0	98.9 (0.3)	2.585[b]	100.1	100.1 (0.8)
4.165[b]	98.8	97.9 (2.1)	4.216	102.8	100.7 (0.3)	3.634[b]	100.5	99.7 (0.5)
4.216	99.3	97.8 (1.4)	5.627	100.4	99.8 (0.4)	3.655	100.3	101.4 (0.2)
5.541	100.6	98.0 (1.3)	—	—	—	4.834[b]	100.1	99.3 (0.7)
5.603[b]	98.5	96.8 (2.1)	—	—	—	5.015	101.3	101.2 (0.2)
Average	99.4	97.5 (1.6)		101.7	99.8 (0.3)		100.5	100.3 (0.5)

[a] The values in parentheses represent the percent of testosterone found in fraction 2. [b] Fraction 1 was evaporated to dryness and diluted with absolute methanol as required for determination by UV and isoniazid.

Table IV—Volatility Study (Percent Recovered by Isoniazid Method)

Minutes	Testosterone, Live Steam	Testosterone Cypionate		Testosterone Enanthate		Testosterone Propionate	
		Steam Bath	Live Steam	Steam Bath	Live Steam	Steam Bath	Live Steam
10	—	99.4	—	97.9	—	89.5	—
20	—	98.9	—	96.2	—	87.6	—
40	80.0	97.4	100.0	96.5	97.2	83.3	81.0
60	78.2	99.8	98.0	96.5	98.8	63.5	80.1

Table V—Base Degradation of Steroids (Percent Recovered by Isoniazid Method)

Hours[a]	Testosterone Cypionate		Testosterone Enanthate		Testosterone Propionate	
	Fraction 1	Fraction 2	Fraction 1	Fraction 2	Fraction 1	Fraction 2
1	94.5	2.0	97.5	0.7	91.1	2.9
2	ND[b]	ND	96.8	1.5	93.8	4.8
4	92.4	6.1	95.4	3.9	90.1	8.6
8	85.9	10.4	93.3	6.7	82.1	16.7
24	72.2	25.6	80.9	15.3	ND	ND
48	52.7	43.5	ND	ND	ND	ND
78	ND	ND	ND	ND	18.6	76.9

[a] Time of exposure of testosterone ester to base. [b] Not determined.

to dryness and used for the isoniazid determination. The 190-ml *n*-heptane fraction containing testosterone was evaporated to a small volume, made up to 100 ml with chloroform, and mixed; then 5-ml aliquots were taken carefully just to dryness for the isoniazid reaction. Results of this study (Table I) ranged from 99.1 to 100.3% recovery, and the relative standard deviations calculated from the range (14) were 0.6–1.0%.

Recovery and Replication Studies of Testosterone and Testosterone Ester Separation—About 10 mg of testosterone standard (accurately weighed) and the equivalent weight of its cypionate, enanthate, and propionate ester standards were dissolved and diluted to 50 ml with chloroform. Several 10-ml aliquots were taken carefully just to dryness on the steam bath. The residues were dissolved in 1.5 ml each of acetonitrile and *n*-heptane, placed on the column, and separated using the procedure described.

The first and second *n*-heptane fractions were taken carefully just to dryness on the steam bath. In some cases, the column was eluted with 60 ml of *n*-heptane and 125 ml of chloroform (instead of 190 ml of *n*-heptane), and the chloroform fraction was taken carefully just to dryness. All residues were dissolved in absolute methanol and diluted to 100 ml. Selected samples were determined by UV spectrophotometry, and 5-ml aliquots of all samples were evaporated just to dryness for colorimetric determination by isoniazid.

Results of this study (Table II) showed that recoveries ranged from 94.5 to 103.7% for testosterone and from 97.1 to 101.1% for its esters and that the values obtained by the UV and isoniazid methods compared favorably. Relative standard deviations of separated steroids calculated from the range were 0.8–2.5%.

TLC and HPLC determinations of Samples 5–8 (Table III) for the separation of testosterone and testosterone cypionate showed no cross-contamination. An 80:20 acetonitrile–water mixture was used to elute testosterone cypionate in 10.5 min, and a 40:60 acetonitrile–water system was used to elute testosterone by HPLC. TLC of testosterone and its cypionate on silica gel G with chloroform gave R_f values of 0.35 and 0.7, respectively.

Recovery Study of Large Quantities of Testosterone Esters—Three accurately weighed portions of each testosterone ester standard, ranging from 2 to 6 mg, were dissolved in 1.5-ml aliquots of acetonitrile and *n*-heptane and placed on columns using the procedure described. The first fractions from several columns were taken carefully to dryness on the steam bath. The residues were dissolved and diluted with absolute methanol to obtain a concentration of ~0.012 mg/ml for UV and isoniazid determinations.

For other selected samples, the first fraction was diluted to 100 ml with chloroform, and appropriate aliquots were taken just to dryness by evaporation for UV and isoniazid determinations. Each residue was treated with 4 ml of isoniazid reagent to develop the color. The results (Table III) showed that as much as 5.6 mg of testosterone esters could be recovered satisfactorily in fraction 1.

Solvent Volatility Study—About 1.25 mg, accurately weighed, of each testosterone ester was dissolved and diluted to 100 ml with methanol. Five-milliliter aliquots were placed in 100-ml beakers and evaporated just to dryness on the steam bath. Replicate residues were left on the steam bath for selected periods after dryness. The residues were dissolved and diluted to volume, and aliquots were taken for the isoniazid reaction.

The experiment was repeated with replicate aliquots of testosterone and its esters but with the beakers placed over the openings on the steam bath to allow heating by live steam for 40 and 60 min. The residues were dissolved and diluted to volume with methanol for determination by isoniazid. Table IV shows that significant testosterone and testosterone propionate losses occurred when they were exposed to steam temperatures for extended periods after solvent volatilization.

Base Degradation Study—About 12.5 mg, accurately weighed, of each testosterone ester was dissolved in ~25 ml of methanol. Then 0.5 ml of 10% aqueous tetramethylammonium hydroxide was added. The mixture was diluted to 50 ml with methanol and mixed. At selected times, 5-ml aliquots were removed and 0.5 ml of acetic acid was added to each aliquot. The acidified methanol aliquots were evaporated carefully just to dryness on the steam bath.

Each residue was dissolved in 1.5 ml each of acetonitrile and *n*-heptane, and the mixture was placed on the column and treated as already described. The results (Table V) showed that testosterone ester hydrolysis by tetramethylammonium hydroxide in methanol proceeded at a fairly

Table VI—Analysis of Typical Pharmaceutical Formulations

Sample[a]	Active Ingredient	Other Ingredients[b]	Amount Declared, mg/ml	Percent of Declared (by Isoniazid)	
				Steroid Ester	Testosterone
1	Testosterone enanthate	(2) (7)	100	100.4, 100.4	1.3, 1.3
2	Testosterone enanthate	(2) (7)	200	100.0, 100.5	1.3, 1.1
3	Testosterone enanthate	(2) (7)	200	82.4, 81.3	13.5, 12.9
4	Testosterone enanthate	(2) (7)	100	103.2, 103.4	1.7, 1.8
5	Testosterone enanthate	(2) (6) (7)	180	100.1, 100.4	1.9, 1.9
6	Testosterone enanthate	(2) (7)	100	104.6, 105.3	0.3, 0.2
7	Testosterone enanthate	(2) (6) (7)	90	99.3, 100.6	1.2, 1.4
8	Testosterone enanthate	(2) (7)	100	99.4, 100.0	1.1, 0.9
9	Testosterone enanthate	(2) (7)	200	100.7, 100.9	1.3, 1.2
10	Testosterone enanthate	(2) (3) (7)	200	97.3, 98.0	1.6, 1.7
11	Testosterone enanthate	(2) (3) (7)	200	98.4, 97.4	1.6, 1.6
12	Testosterone enanthate	(2) (7)	100	101.0, 100.6	1.0, 1.1
13	Testosterone enanthate	(2) (4) (7)	100	98.1, 97.9	1.5, 1.4
14	Testosterone cypionate	(2) (3) (5)	200	100.3, 100.3	0.3, 0.2
15	Testosterone cypionate	(2) (3) (5)	200	98.7, 97.3	0.4, 0.5
16	Testosterone cypionate	(2) (3) (5)	200	97.2, 97.5	0.1, 0.1
17	Testosterone cypionate	(2) (5)	100	98.4, 99.4	0.5, 0.4
18	Testosterone cypionate	(2) (5) (9)	100	103.0, 102.3	0.4, 0.4
19	Testosterone propionate	(8) (10) (11)	50	100.6, 100.3	3.6, 3.9
20	Testosterone propionate	(1) (2) (4) (11)	100	87.3, 86.3	2.1, 2.8
21[c]	Testosterone propionate		10[d]	97.6, 96.7	0.1, 0.1
22	Testosterone		100	0.1, 0.1	101.1, 101.6
23	Testosterone		25	0.2, 0.2	99.0, 98.6

[a] Samples 1–20 were injectables, Sample 21 was a tablet, and Samples 22 and 23 were suspensions. [b] (1) = absolute alcohol, (2) = benzyl alcohol, (3) = benzyl benzoate, (4) = chlorobutanol, (5) = cottonseed oil, (6) = estradiol valerate, (7) = ethyl oleate, (8) = methylparaben, (9) = oxystearin, (10) = propylparaben, and (11) = sesame oil. [c] Analysis of duplicate samples by UV: 96.0 and 96.6% of declared. [d] Amount is in milligrams per tablet.

rapid rate at room temperature and that the undecomposed steroid ester and testosterone could be separated and recovered by the proposed method.

RESULTS AND DISCUSSION

The effects of various hydrocarbon solvents and flow rates on testosterone elution from acetonitrile–infusorial earth columns have been studied (13). *n*-Heptane was selected as a solvent for use in the column chromatography because it gives optimum separation of testosterone from its esters. Symmetrical elution curves for testosterone and the three esters studied showed that testosterone enanthate required 20 ml, testosterone cypionate required 23 ml, and testosterone propionate required 45 ml of *n*-heptane for complete elution.

A 7-ml/min normal flow rate of *n*-heptane eluted 2 mg of testosterone from the acetonitrile column in a band between 88 and 195 ml. A 1-ml/min flow rate of *n*-heptane eluted the same amount of testosterone in a band between 105 and 184 ml. While the faster elution rate resulted in band broadening, adequate separations between testosterone and its esters were obtained. Testosterone propionate elution was completed at 45 ml of *n*-heptane, and elution of testosterone from the column did not occur before 88 ml. The recommended cut-off volume at 60 ml of *n*-heptane provided an adequate safety factor and resulted in pure fractions.

The values reported for all determinations represent an average of two isoniazid color developments.

Table I shows 0.3–1.3% hydrolysis of the testosterone ester reference standards. TLC confirmed the presence of testosterone in the second *n*-heptane fraction of all three esters. Both HPLC and TLC of portions of the final diluted solutions after separation on the acetonitrile column showed no testosterone cross-contamination with its esters in either *n*-heptane fraction. The agreement between the results obtained by the UV and isoniazid methods (Table II) was well within the expected range and recoveries obtained for testosterone by eluting the column with an additional 190 ml of *n*-heptane or with 125 ml of chloroform. Fewer manipulations were required if chloroform was used to elute testosterone from the acetonitrile column, but less sample interference resulted from elution with 190 ml of *n*-heptane.

Table III shows that up to 5.6 mg of the three testosterone esters could be eluted completely in the first 60 ml of *n*-heptane collected from the acetonitrile column. It also shows that the results of chloroform dilution of the first *n*-heptane fraction and evaporation of small aliquots to dryness were in agreement with the results obtained by evaporating the *n*-heptane (fraction 1) carefully just to dryness, dissolving the residue in absolute methanol for UV measurement, and carrying subsequent aliquots to dryness for isoniazid determination.

The solvent volatility study (Table IV) showed that when the samples were evaporated carefully just to dryness under air on the steam bath, no steroid loss occurred. However, significant losses of 10–30% of testosterone and testosterone propionate resulted when the sample residues were left on the steam bath for extended periods after the solvent had been volatilized.

The rate of testosterone ester hydrolysis by tetramethylammonium hydroxide (Table V) was faster for testosterone cypionate than for testosterone enanthate and fastest for testosterone propionate. At 8 hr, the hydrolysis rate for testosterone enanthate was ~0.8%/hr; for testosterone cypionate, it was ~1.3%/hr; and for testosterone propionate, it was ~2.1%/hr. TLC and IR spectrophotometry confirmed the presence of enanthic acid in the chloroform fraction from the acetonitrile column.

Analyses of typical pharmaceutical formulations, mostly injectables, by the proposed method are reported in Table VI. Good replication of sample analysis was obtained. The amount of testosterone found varied from 0.1 to 13.5% of the declared amount of testosterone ester, with only three of the 21 products analyzed showing more than 2.0%. Sample 14, analyzed in duplicate by direct dilution and the isoniazid method, gave 123.8 and 125.1%, while Sample 15 gave 121.6 and 120.0% of the declared amount. These results, when compared with those shown in Table VI, showed that a 20–25% interference was removed by the proposed cleanup procedure. Apparently the cleanup procedure is applicable to injectables containing a wide variety of excipients (Table VI) including cottonseed and sesame oils.

The samples that contained benzyl alcohol could not be cleaned up by the proposed procedure for determination by UV spectrophotometry since it eluted in the first *n*-heptane fraction along with the testosterone esters. While benzyl alcohol interfered with the UV spectrum of the testosterone esters, there was no interference when isoniazid was used as the final determinative step.

Samples 22 and 23 (Table VI) for testosterone suspensions are included to show the versatility of the acetonitrile column.

The proposed procedure is relatively rapid and is specific for testosterone ester determination. Two samples can be prepared for analysis, placed on separate columns, and separated into two fractions containing testosterone ester and testosterone in 1.5 hr. The results of the duplicate analyses of 21 different pharmaceutical formulations showed that the proposed procedure provided adequate sample cleanup with satisfactory accuracy and replication of the analytical data. This method can be used to assess hydrolytic degradation of testosterone esters in pharmaceutical formulations by measuring intact testosterone ester and/or testosterone present in the sample.

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Application of Thermochromism in Spectrophotometric Analysis: Selective Determination of Berberine in Pharmaceuticals by Solvent Extraction

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Abstract □ A solvent extraction and spectrophotometric method for selective determination of berberine in pharmaceuticals is proposed. Berberine forms an ion associate with tetrabromophenolphthalein ethyl ester, which is extracted into ethylene dichloride. Secondary and tertiary amines and alkaloids are coextracted with the berberine and complicate the berberine determination. The absorbance of the secondary and tertiary amines and alkaloids into ethylene dichloride, however, decreases nearly to zero when the temperature is elevated from 25 to 60°. Thus, berberine can be determined successfully in the presence of the secondary and tertiary amines and alkaloids by using thermochromism.

Keyphrases □ Berberine—analysis, solvent extraction, spectrophotometric method using thermochromism, various pharmaceuticals □ Thermochromism—analysis, berberine in various pharmaceuticals, solvent extraction, spectrophotometry □ Spectrophotometry, solvent extraction—analysis, berberine in various pharmaceuticals, using thermochromism □ Antibacterial agents—berberine, analysis in various pharmaceuticals, solvent extraction, spectrometry, using thermochromism

Bromthymol blue, bromcresol green, and bromphenol blue usually are used for the solvent extraction and spectrophotometric determination of thiamine (1), quaternary ammonium compounds (2), and quinine ethylcarbonate (3), respectively. These dyes are diprotic acids with a narrow optimum pH range and poor extractability of the ion associates into organic solvents.

Tetrabromophenolphthalein ethyl ester anion (I) is a monoprotic acid and can form ion associates (II) with quaternary ammonium cations (III) and charge transfer complexes (IV) with amines (V), which are effectively extracted into organic solvents through a wide pH range (4). Tetrabromophenolphthalein ethyl ester shows a high sensitivity for quaternary ammonium cations and amines, but the absorption spectra overlap each other. Thus, it is difficult to determine spectrophotometrically the quaternary ammonium salts and amines separately when they coexist. An attempt was made to separate the amine procaine from the quaternary ammonium salt benzethonium by solvent extraction, but it was difficult to remove the amine completely (5).

The author found, however, that the color of the charge transfer complex in ethylene dichloride reversibly disappeared with the elevation of temperature. Even at 60°, the red complex was rarely found while the absorbance of the ion associate at 610 nm was unchanged at 60°. Thus, a quaternary ammonium salt could be determined spectrophotometrically at 60° without the disturbance of amines.

This paper describes the extraction and spectrophotometric determination of berberine in pharmaceuticals after the temperature is increased to 60° to eliminate the effect of the coexisting amines.

EXPERIMENTAL

Apparatus—A double-beam spectrophotometer[1] attached to a temperature-controlled cell holder was used to measure absorbances at a constant temperature using quartz 1-cm cells with stoppers. An x–y recorder[2] was used for the spectra. Constant cell temperature was maintained by circulating constant-temperature water through the temperature-controlled cell holder by means of a temperature-controlled circulator[3]. The solvent temperature in the cell was checked by dipping a thermoelement of a thermometer[4] in the solvent. A pH meter, a shaker, and a centrifuge were also used.

Reagents—*Standard Berberine Solution*—A stock solution of 1×10^{-3} M berberine was prepared by dissolving 0.4075 g of berberine hydrochloride[5] (dried at 105°) in distilled water and diluting to 1 liter with distilled water. The stock solution was standardized by the official method (6). The solution was used after accurate dilution.

Tetrabromophenolphthalein Ethyl Ester Solution—A 2.0×10^{-3} M solution of tetrabromophenolphthalein ethyl ester was prepared by dissolving 0.1400 g of tetrabromophenolphthalein ethyl ester potassium salt[6] in ethanol to give a 100-ml solution.

Buffer Solution (pH 8.5)—The borate–phosphate buffer was prepared by adding 1 N H_2SO_4 or 1 N NaOH to the 0.3 M potassium dihydrogen phosphate containing 0.1 M sodium borate.

Procaine[7], ephedrine[8], quinine[9], papaverine[9], eserine[10], and emetine[5] were used as the hydrochloride and the sulfate. Ethylene dichloride was used as the extractant. All chemicals were reagent grade.

[1] Hitachi model 556.
[2] Hitachi model 057.
[3] Komatsu-Yamato model CTE-240.
[4] Anritsu model HP-4F.
[5] Nakarai Kagaku Yakuhin Co., Kyoto, Japan.
[6] Tokyo Kasei Kogyo Co., Tokyo, Japan.
[7] Daiichi Seiyaku Co., Tokyo, Japan.
[8] Sanwa Kagaku Co., Nagoya, Japan.
[9] Katayama Kagaku Kogyo Co., Osaka, Japan.
[10] E. Merck, Darmstadt, West Germany.

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