ISSN : 0974-7419 Volume 16 Issue 1

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 16(1) 2016 [020-035]

Simultaneous analysis of doping drugs in human plasma and urine using HPLC- DAD

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ABSTRACT

A highly sensitive RP-HPLC method has been developed for simultaneous separation and quantitation of seven doping drugs, including four diuretics 'Hydrochlorothiazide (HCTZ), Furosemide (FUR), Indapamide (IDP) and spironolactone (SPIRO)', Salbutamol (SAL) as β-agonist, Testosterone (TSE) as anabolic and Betamethasone (BMS) as corticosteroid in spiked human plasma and urine, by using Zorbax eclipse HC-C18 column (250 mm x 4.6mm x 5µm) with mobile phase acetonitrile: phosphoric acid pH 3 (50:50, v/v) under isocratic conditions with flow rate of 1.0 ml min^{-1} and at room temperature. Diode array detector was adjusted at '225, 272, 235, 242 and 244' and 239 nm for quantitative determination of 'HCTZ, SAL, FUR, IDP and TSE' and both 'SPIRO and BMS', respectively. The linearity range for the studied drugs in the plasma was 100-9000, 100-1800, 100-5000, 200-9000 and 1000-9000 $ng.ml^{-1}$ for 'HCTZ and SAL', for 'FUR and TSE', for IDP, for SPIRO and for BMS, respectively. LODs and LOQs values were found to be '31.16, 29.99, 28.14, 29.84, 31.98, 28.55 and 250.99' and '94.42, 90.88, 85.27, 90.42, 96.91, 86.52 and 760.58' ng ml^{-1} for HCTZ, SAL, FUR, IDP, TSE, SPIRO and BMS, respectively. Also; the investigated drugs could be determined in spiked urine samples after direct dilution and solid phase extraction (SPE), where in the last way (SPE) HCTZ and SAL could not be determined, since they give irreproducible results. In direct dilution way; the linearity range was 150 – 5000, 50 – 5000, 150 – 1500 and 100 – 5000 $ng.ml^{-1}$ for 'HCTZ and BMS', 'SAL, IDP and TSE', 'FUR' and for 'SPIRO', respectively and the 'LODs and LOQs-values' were '39.41, 11.98, 35.52, 12.70, 14.11, 29.01 and 40.72' and '119.42, 36.30, 107.64, 38.48, 42.76, 87.91 and 123.39' $ng.ml^{-1}$ for HCTZ, SAL, FUR, IDP, TSE, SPIRO and BMS, respectively. In SPE method; the linearity range was 250-3000, 150- 6000 and 150-7000 $ng.ml^{-1}$ for FUR, IDP and for 'TSE, SPIRO and BMS', respectively and the 'LODs and LOQs-values' were '70.11, 39.15, 42.71, 45.91 and 49.01' and 212.45, 118.64, 129.42, 139.12 and 148.52 $ng.ml^{-1}$ for FUR, IDP, TSE, SPIRO and BMS, respectively. It was shown that SPE is more sensitive, for determination of FUR, IDP, TSE, SPIRO and BMS, than direct dilution (only 1:4 dilution compared to 1:50 fold dilution in direct dilution), however, HCTZ and SAL could not be determined.

KEYWORDS

Doping control;
HPLC-DAD;
Urine;
Plasma;
SPE.

INTRODUCTION

A great number of drugs with minimum required performance limits (MRPL) are currently included in the list of prohibited substances, as shown in Error! Reference source not found.), published by World Anti-Doping Agency (WADA)[1,2]. Numerous technical approaches are needed to analyze the great diversity of doping agents and confirm the findings. Doping analysis has to conform to the requirements of International Standards for Laboratories established by WADA including e.g. chain of custody, validation of screening and confirmation methods and criteria for identification[3,4]. The progressive appearance of new abused drugs challenges the doping laboratories to update their detection methods. Today, doping analysis requires the use of several different chromatographic, mass spectrometric and immunological methods. Consequently, using a large number of separate analytical procedures results in a more complex, time-consuming and laborious screening procedure.

Diuretics are pharmaceutical drugs which are used to increase urine flow by promoting the excretion of water by the kidneys. They are used often for the treatment of heart conditions, liver, kidney or lung disease to alleviate salt or water retention. Their potent ability to excrete water has caused diuretics to be misused in sport; where rapid weight loss is required to meet a weight category. It has also been used to ensure the urine is diluted; so that, the detection of other banned substances is made more difficult. Thus the use of diuretics in sport is banned at competition. The urine dilution effect of diuretics also allows them to be classified as masking agents and precludes their use both in and out of competition[5]. There are several classes of diuretic drugs—Thiazides (e.g., HCTZ), loop diuretics (e.g., FUR), potassium sparing diuretics (e.g., AMI), carbonic anhydrase inhibitors (e.g., acetazolamide), osmotic diuretics (e.g., mannitol) and mercurial diuretics (e.g., mersalyl). The drugs are all relatively polar and hence are amenable to analysis by HPLC using C18 type phases with diode array (DAD) or fluorescence detection[6,7].

β_2-adrenoceptor agonists (SAL) used in the treatment of bronchial asthma, however, athletes must obtain a therapeutic use exemption (TUE) documenting their compromised lung function. Because of SAL's potential anabolic effects when taken orally, it is only permitted after inhaled administration, and it is carefully monitored by WADA through urine analysis, both in and out of competition. Currently, urine concentrations greater than 1000 $ng.mL^{-1}$ are considered adverse findings and are indicative of oral administration, regardless of whether the athlete has a TUE[8].

In addition to its antiasthmatic effects, β_2-agonists have been pharmacologically proven to be able to improve nitrogen retention, reduce body fat and promote muscle growth. As a result; there is much interest in the determination of these substances in body fluids. However, the hydrophilicity of the β_2-agonists coupled with the low concentrations found in urine and plasma makes the analysis relatively difficult[9].

SAL is a prohibited substance with two threshold values. Urine concentrations over 100 and below 1000 $ngmL^{-1}$ are considered to imply stimulant dosage while over 1000 $ngmL^{-1}$, salbutamol is regarded as anabolic agent[10, 11].

Anabolic steroids are chemical, synthetic derivatives of TSE modified to enhance the anabolic rather than the androgenic actions of the hormone[12]. TSE is a steroid hormone, synthesized in the human body from cholesterol. It serves distinct functions at different stages of life. During embryonic life, androgen action is central to the development of the male phenotype. At puberty, the hormone is responsible for the secondary sexual characteristics; that transform boys into men. TSE regulates many physiological processes in the adult male including muscle protein metabolism, sexual and cognitive functions, erythropoiesis, plasma lipid levels, and bone metabolism[13].

Anabolic steroids are used in the treatment of osteoporosis and the anemia associated with chronic renal failure. It increases hemoglobin and the mass of red blood cells. They are used as a doping agent to build muscle mass, increase bone density and stimulate appetite. Furthermore, it might also enhance erythropoietic-stimulating factor to increase the

erythrocyte production. Other effects include increased levels of low-density lipoproteins and decreased levels of high-density lipoproteins[14].

Corticosteroids are very potent anti-inflammatory products used in the treatment of chronic asthmatic symptoms. However, they have severe potential side effects when used without medical control, such as permanent skin atrophy and pustule psoriasis. Moreover, some systematic side effects are hypertension, diabetes mellitus, osteoporosis, allergic contact dermatitis, Cushing's syndrome … etc. They have an effect on the nervous system, and can cause euphoria and improve an athlete's ability to concentrate in performance of endurance events and power events. Corticosteroids can alleviate pain in general. Due to the higher physical exertion, pain and injuries are often consequences of sports, and hence corticosteroids are widely used as pain and inflammatory relieving agents. They are now on the list of substances banned by the International Olympic Committee (IOC)[15]. Systemic use of corticosteroids is forbidden in all circumstances but, when medically necessary, local and intra-articular injections are allowed under medical supervision[16].

WADA MRPL for the accredited laboratories[17] is indeed not a threshold value, nor is it a limit of detection (LOD) or a limit of quantification (LOQ), but rather a parameter to assess laboratory performance, this meaning that; adverse analytical findings may result from concentrations below the MRPL, provided the identification criteria[18] are satisfied. This means that; since a urinary threshold value is presently set up only for a few compounds (e.g. ephedrines, SAL), the analysis of urine samples may not allow to discriminate between remote recreational/therapeutic use of stimulants and a real doping offence.

The aim of this work is to develop analytical separation technique to screen seven prohibited drugs of different pharmacological classes and determine them at very low limits in spiked urine and plasma.

EXPERIMENTAL

Chemicals and reagents

All reagents and solvents were of HPLC grade, including 'Acetonitrile, Methanol and water' (Sigma Gmbh, Germany) Phosphoric acid (Reidel-deHaën, Germany). Frozen human plasma batch No. 071937 was obtained from VACCERA, Giza, Egypt. Human urine samples were obtained from healthy volunteers after getting their informed consent.

All samples were stocked in plastic tight closed tubes at - 20°C prior to analysis (1 week maximum storage time).

Reference samples

'HCTZ and SPIRO', 'FUR, SAL, BMS and IDP' and TSE were kindly provided by Kahira Pharmaceutical Co., Arab Drug Company (ADCO) and CID pharmaceutical Co., (Cairo, Egypt). The purities were certified to be 99.66%, 99.67%, 99.55%, 99.63%, 99.59, 99.55% and 99.65%, respectively.

Instrumentation

Waters Alliance e2695 HPLC binary pump (WatersTechnology, Milford, MA) equipped with an in-line vacuum degassing auto-sampler with capacity for 120 vials with programmable temperature control, heated column compartment and photodiode array detector (model 2998). All components of the HPLC system were controllable through the Empower 3 chromatography data software. Zorbax eclipse HC-C18 column (250 mm x 4.6mm x 5µm). 0.2 µm disposable membrane filters (Millipore corp., Milford, MA, USA). Jenway pH meter 3310 pH /mV/°C. Table- top Centrifuge PLC-05, Germany Industrial Corp., associated with Cannic, USA. Sonicator (Crest ultrasonics, scotch road. Mercer, country airport, Trenton, NJ 08628). Micropipette. Solid Phase Extraction: 12 Port Vacuum Extraction Manifold Assy (Phenomenex) with Vacuum Pump, Beco,Germany. SPE C18 cartridges.balance. vortex

LC parameters

At room temperature, under isocratic condition acetonitrile: phosphoric acid pH 3 (50:50, v/v) used mobile phase with a flow rate of 1.0 ml min^{-1}. Immediately before use; all solvents were filtered through 0.2 µm membrane filter and degassed in an ultrasonic bath. Zorbax eclipse HC-C_{18} column (250 mm x 4.6mm x 5µm) was equilibrated with mobile

phase. Sample solutions, after pretreatment and prior to injection, were filtered through 0.2 µm syringe filter. DAD was adjusted at 225, 272, 235, 242, 244 nm for quantitative determination of HCTZ, SAL, FUR, IDP and TSE, respectively and at 239 nm for both SPIRO and BMS.

Preparation of standard solutions

Stock standard solutions (1mg.ml^{-1}): HCTZ, SAL, FUR, IDP, TEST, SPIRO and BMS stock solutions were prepared by transferring 100 mg of each separately into 100-ml volumetric flask and completed to mark with acetonitrile.

Working standard solutions:(Solution A) (100 µg.ml^{-1})

Standard working mixture solution A was prepared by transferring 10 ml of each of their corresponding previously prepared stock standard solutions into 100-ml volumetric flask and completed to mark with the mobile phase.

(Solution B) (10 µg.ml^{-1})

Standard working mixture solution B was prepared by transferring 10 ml of their previously prepared working standard solutions (A) into 100-ml volumetric flask and completed to mark with the mobile phase.

The studied drugs were chosen according to the list of banned substances in the Olympic Movement Anti-doping Code and needed to be detected emphatically in routine analysis.

Sample preparation

For plasma

Accurately measured volumes (20 - 100 µl) of solution B and (10-200 µl) of solution A were transferred, separately, into 20 ml stoppered shaking tubes and the volume was completed to 2-mls with plasma. 4-mls acetonitrile were added to each tube, then the mixtures were vortexed for 2 minutes and centrifuged for 20 minutes at 4500 rpm. 2 mls taken from the upper layer in each tube, were transferred to a beaker, left to evaporate till dryness at room temperature and reconstituted in 2-mls of the mobile phase. The reconstituted solutions, having concentrations in a range of (100 - 10000 ng.ml^{-1}) for the drug mixture were then filtered through 0.2 µm millipore syringe filters.

For urine

For direct dilution, urine was spiked with (10-100 µl) of solution B and (12-100 µl) of solution A and diluted (1:50) with water to prepare spiked urine samples (2 mls) of concentrations (50-5000 ng.ml^{-1}), then filtered through 0.2 µm millipore syringe filters.

For SPE, 400µl urine was spiked with (30-100 µl) of solution B and (12-140 µl) of solution A and completed to 2 mls with water (1:4 dilution) to prepare spiked urine samples of concentrations (150-7000 ng.ml^{-1}). C18 extraction cartridges were conditioned with 1 ml methanol, then 1 ml water, then urine sample was loaded, then washed with water and the drugs were eluted with 2 mls acetonitrile and then filtered through 0.2 µm millipore syringe filters.

METHOD VALIDATION

HPLC-DAD

Linearity

Aqueous mixture: Different aliquots of the working standard solution A & B were transferred, separately, into 50-ml volumetric flasks, diluted to volume with mobile phase to obtain concentrations of (50-10000) ng.ml^{-1} for the mentioned drugs. The solutions were filtered through 0.2 µm millipore syringe filters.

In plasma: The reconstituted solutions prepared in *2.4.1.*, having concentrations in a range of (100 - 10000 ng.ml^{-1}) for the drugs mixture were filtered through 0.2 µm millipore syringe filters.

In urine: The spiked urine samples, previously treated either with SPE (concentration 150-7000 ng.ml^{-1}) or direct dilution (concentration 50-5000 ng.ml^{-1}) prepared in *2.4.2.*, filtered through 0.2 µm millipore syringe filters.

A 10 µl volume of each solution was injected, in triplicates, and the drugs were separated and quantified, using the above mentioned chromatographic conditions and the average peak areas were calculated. The calibration curves, corresponding the re-

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TABLE 1: List of prohibited substances and methods 2006 established by world anti-doping agency (WADA)[1]

Substances and methods prohibited at all times
S1. Anabolic agents
1a. Exogenous anabolic steroids
1b. Endogenous anabolic steroids
Other anabolic agents
S2. Hormones and related substances
S3. β_2-Agonists
S4. Agents with anti-estrogenic activity
S5. Diuretics and another masking agents
Prohibited methods
M1. Enhancement of oxygen transfer
M2. Chemical and physical manipulation
M3. Gene doping
Substances and methods prohibited in-competition
S6. Stimulants
S7. Narcotics
S8. Cannabinoids
S9. Glucocorticosteroids
Substances prohibited in particular sports
P1. Alcohol
P2. β-Blockers

lationship between average peak areas and their concentrations were plotted and the regression equations for the seven investigated drugs were then computed.

Accuracy

The previously mentioned procedure, under *3.1.1.* was repeated for the analysis of different concentrations of the pure investigated drugs mixture, their mixture in plasma, in urine by SPE and direct dilution. The concentrations were calculated, using the corresponding regression equation and the percentage recoveries were then calculated as shown in TABLES (2-8).

Precision

Repeatability (intraday precision)

The intraday variation was evaluated by applying the previously mentioned procedure under 3.1.1. for analysis 150, 500 and 1000 ng.ml^{-1} for HCTZ, SAL and 200, 500 and 1000 ng.ml^{-1} for the rest of drugs (n = 6).

It is applied for the analysis concentration 200, 1000 and 1500 ng.ml^{-1} for all drugs in plasma except for BMS 1000, 2000 and 7000 ng.ml^{-1}, performed as triplicates in plasma.

It is applied for the analysis concentration 250, 1000 and 3000 ng.ml^{-1} for TEST, SPIRO and 250,500,2000 ng ml-1 for FUR, 250,1500 and 3000 for IDP and 500,2000 and 6000 for BMS performed, performed as triplicates in urine after SPE.

TABLE 2 : Accuracy of the proposed HPLC method for the analysis of pure samples of HCTZ, SAL, FUR, IDP, TEST, SPIRO and BMS

HCTZ			SAL			FUR			IDP		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
50.00	49.55	99.10	50.00	49.66	99.32	50.00	49.72	99.44	50.00	49.55	99.10
60.00	59.87	99.78	60.00	59.66	99.43	60.00	59.81	99.68	60.00	59.91	99.85
70.00	69.42	99.17	70.00	69.39	99.13	70.00	69.83	99.76	70.00	69.91	99.87
80.00	79.47	99.34	100.00	99.69	99.69	100.00	99.52	99.52	100.00	99.72	99.72
90.00	89.26	99.18	150.00	148.99	99.33	200.00	199.16	99.58	200.00	198.24	99.12
150.00	150.12	100.08	200.00	200.56	100.28	300.00	298.29	99.43	300.00	300.57	100.19
500.00	502.95	100.59	500.00	502.05	100.41	500.00	497.45	99.49	500.00	497.60	99.52
1000.00	991.30	99.13	1000.00	998.80	99.88	700.00	697.27	99.61	700.00	697.06	99.58
10000.00	9964.00	99.64	10000.00	9957.00	99.57	1000.00	998.50	99.85	1000.00	999.50	99.95
Mean ± S.D.	99.56 ± 0.52		Mean ± S.D.	99.67 ± 0.44		Mean ± S.D.	99.59 ± 0.14		Mean ± S.D.	99.66 ± 0.37.	

***Average of three determinations**

TABLE 3: Accuracy of the proposed HPLC method for the analysis of pure samples of HCTZ, SAL, FUR, IDP, TSE, SPIRO and BMS (cont'd)

TSE			SPIRO			FUR		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
50.00	50.42	100.84	100.00	99.58	99.58	100.00	100.87	100.87
60.00	60.70	101.17	200.00	198.78	99.39	200.00	198.58	99.29
70.00	69.37	99.10	300.00	299.37	99.79	500.00	498.20	99.64
100.00	100.69	100.69	500.00	498.60	99.72	700.00	698.67	99.81
200.00	201.54	100.77	700.00	698.88	99.84	1000.00	997.32	99.73
500.00	498.00	99.60	1000.00	1002.21	100.22	10000.00	9995.42	99.95
700.00	698.81	99.83						
1000.00	999.60	99.96						
Mean ± S.D.	100.25 ± 0.72		Mean ± S.D.	99.76 ± 0.28		Mean ± S.D.	99.88 ± 0.53	

***Average of three determinations**

TABLE 4 : Determination of human plasma samples spiked with HCTZ, SAL, FUR, IDP, TSE, SPIRO and BMS using the proposed HPLC method

HCTZ			SAL			FUR			IDP		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
100.00	98.68	98.68	100.00	98.32	98.32	100.00	101.56	101.56	100.00	98.51	98.51
200.00	196.46	98.23	200.00	200.86	100.43	200.00	202.72	101.36	200.00	197.22	98.61
300.00	294.72	98.24	600.00	588.96	98.16	300.00	296.25	98.75	300.00	304.20	101.40
500.00	495.10	99.02	800.00	785.68	98.21	1500.00	1513.81	100.92	400.00	405.68	101.42
2000.00	2014.40	100.72	1000.00	1014.82	101.48	1600.00	1598.24	99.89	2000.00	2021.00	101.05
4000.00	3982.40	99.56	1200.00	1215.24	101.27	1800.00	1800.90	100.05	5000.00	5030.50	100.61
5000.00	4979.50	99.59	2000.00	1992.03	99.60						
8000.00	7920.80	99.01	4000.00	3982.41	99.56						
9000.00	8996.40	99.96	8000.00	7938.42	99.23						
			9000.00	8946.90	99.41						
Mean ± S.D.	99.22 ± 0.82		Mean ± S.D.	99.57 ± 1.19		Mean ± S.D.	100.42 ± 1.06		Mean ± S.D.	100.27 ± 1.35	

***Average of three determinations**

TABLE 5 : Determination of human plasma samples spiked with HCTZ, SAL, FUR, IDP, TSE, SPIRO and BMS using the proposed HPLC method (cont'd)

FUR			SPIRO			IDP		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
100.00	98.05	98.05	200.00	203.04	101.52	1000.00	982.34	98.23
200.00	197.86	98.93	500.00	505.20	101.04	1800.00	1771.38	98.41
300.00	296.22	98.74	600.00	609.72	101.62	2000.00	1975.82	98.79
1000.00	992.33	99.23	800.00	785.92	98.24	3000.00	2968.83	98.96
1800.00	1797.84	99.88	1000.00	989.93	98.99	8000.00	7937.61	99.22
			2000.00	2007.41	100.37	9000.00	8954.13	99.49
Mean ± S.D.	98.97 ± 0.67		3000.00	2980.82	99.36			
			8000.00	8016.04	100.20	Mean ± S.D.	98.85 ± 0.48	
			9000.00	8989.22	99.88			
			Mean ± S.D.	100.14 ± 1.15				

***Average of three determinations**

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TABLE 6 : Determination of human urine samples spiked with HCTZ and SAL after direct dilution using the proposed HPLC method

HCTZ			SAL		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
150.00	153.47	102.31	50.00	51.32	102.64
250.00	253.81	101.52	100.00	102.54	102.54
2000.00	2029.20	101.46	150.00	153.71	102.47
3000.00	2982.93	99.43	250.00	244.71	97.88
4000.00	3980.84	99.52	300.00	293.61	97.87
5000.00	5049.51	100.99	400.00	393.56	98.39
			500.00	492.22	98.44
			1500.00	1488.63	99.24
Mean ± S.D.	100.87 ± 1.16		2000.00	1980.20	99.01
			5000.00	5019.00	100.38
			Mean ± S.D.	99.89 ± 1.98	

***Average of three determinations**

TABLE 7 : Determination of human urine samples spiked with FUR using the proposed HPLC method using SPE and direct dilution

FUR					
Through SPE			Through direct dilution		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
250	245.03	98.01	150	153.12	102.08
300	286.71	95.57	250	245.08	98.03
400	387.76	96.94	700	699.93	99.99
500	486.05	97.21	1000	1006.91	100.69
1000	951.54	95.15			
2000	2030.22	101.51	1500	1491.60	99.44
3000	2972.70	99.09			
Mean ± S.D.	97.64 ± 2.17		Mean ± S.D.	100.05 ± 1.49	

***Average of three determinations**

TABLE 8 : Determination of human urine samples spiked with IDP using the proposed HPLC method using SPE and direct dilution

Through SPE			Through direct dilution		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
150	156.95	104.63	50	51.21	102.42
250	243.75	97.50	100	99.51	99.51
400	385.96	96.49	150	146.49	97.66
500	489.45	97.89	250	245.61	98.24
1500	1489.52	99.30	400	391.72	97.93
4000	3986.41	99.66	500	492.80	98.56
5000	5034.00	100.68	700	700.14	100.02
6000	5977.23	99.62	1000	988.53	98.85
Mean ± S.D.	99.47 ± 2.49		1500	1481.25	98.75
			2000	1989.82.	99.49
			4000	3968.81	99.22
			5000	5033.00	100.66
			Mean ± S.D.	99.28 ± 1.31	

***Average of three determinations**

It is applied for the analysis concentration 250, 500 and 1500 ng.ml^{-1} for FUR and 250, 1000 and 3000 ng.ml^{-1} for the rest of drugs performed as triplicates in urine after direct dilution.

The concentrations were calculated using the corresponding regression equation and the percentage recoveries and standard deviations were then calculated as shown in TABLES (9-12).

Intermediate precision (interday precision)

The interday variation was evaluated by applying the previously mentioned procedure under 3.1.1. for analysis 150, 500 and 1000 ng.ml^{-1} for HCTZ, SAL and 200, 500 and 1000 ng. ml^{-1} for the rest of drugs (n = 6).

It is applied for the analysis concentration 200, 1000 and 1500 ng.ml^{-1} for all drugs in plasma except for BMS 1000, 2000 and 7000 ng.ml^{-1}, performed as triplicates in plasma.

It is applied for the analysis concentration 250, 1000 and 3000 ng.ml^{-1} for TEST, SPIRO and 250,500 and 2000 ng.ml^{-1} for FUR, 250,1500 and 3000 ng.ml^{-1} for IDP and 500,2000 and 6000 for BMS performed, performed as triplicates in urine after SPE.

It is applied for the analysis concentration 250, 500 and 1500 ng.ml^{-1} for FUR and 250, 1000 and 3000 ng.ml^{-1} for the rest of drugs performed as triplicates in urine after direct dilution.

TABLE 9 : Determination of human urine samples spiked with TEST using the proposed HPLC method using SPE and direct dilution

Through SPE			Through direct dilution		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
150	163.02	108.68	50	52.29	104.58
250	243.40	97.36	100	103.24	103.24
300	288.33	96.11	150	146.89	97.93
400	398.64	99.66	250	245.88	98.35
500	513.15	102.63	300	293.04	97.68
1500	1510.05	100.67	400	388.08	97.02
4000	4000.81	100.02	2000	2001.81	100.09
6000	6033.63	100.56	4000	3999.20	99.98
7000	6999.32	99.99	5000	5077.00	101.54
Mean ± S.D.	100.63 ± 3.56		Mean ± S.D.	100.05 ± 2.62	

***Average of three determinations**

TABLE 10 : Determination of human urine samples spiked with SPIRO using the proposed HPLC method using SPE and direct dilution

Through SPE			Through direct dilution		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
150	161.99	107.99	100	104.72	104.72
250	244.23	97.69	150	151.37	100.91
300	306.84	102.28	250	243.03	97.21
400	416.00	104.00	300	303.69	101.23
500	486.95	97.39	400	396.48	99.12
1500	1508.41	100.56	700	702.31	100.33
2000	1959.83	97.99	2000	2010.60	100.53
4000	3988.42	99.71	3000	2939.74	97.99
6000	6019.80	100.33	5000	5036.00	100.72
7000	6990.94	99.87			
Mean ± S.D.	100.78 ± 3.26		Mean ± S.D.	100.31 ± 2.16	

***Average of three determinations**

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TABLE 11 : Determination of human urine samples spiked with BMS using the proposed HPLC method using SPE and direct dilution

Through SPE			Through direct dilution		
Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*	Taken (ng ml^{-1})	Found (ng ml^{-1})	% Recovery*
150	140.72	93.81	150	146.59	97.73
250	271.20	108.48	250	246.18	98.47
300	295.32	98.44	300	305.67	101.89
400	419.96	104.99	400	395.00	98.75
500	519.91	103.98	500	498.95	99.79
2000	1997.00	99.85	700	694.96	99.28
3000	2998.84	99.96	1000	987.41	98.74
4000	3986.83	99.67	3000	2994.93	99.83
6000	6000.62	100.01	4000	3995.60	99.89
7000	6998.61	99.98	5000	4944.11	98.88
Mean ± S.D.	100.92 ± 4.02		Mean ± S.D.	99.33 ± 1.13	

***Average of three determinations**

TABLE 12 : Results of assay validation parameters of the proposed HPLC method for the determination of the seven pure drugs

Parameter	HCTZ	SAL	FUR	IDP	TEST	SPIRO	BMS
Retention time	5.20 ± 0.24	6.3 ± 0.26	8.00 ± 0.31	9..50 ± 0.19	13.07 ± 0.40	18.50 ± 0.29	26.00 ± 0.39
Linearity (ng ml^{-1})	50 – 10000	50 – 10000	50 – 1000	50 – 1000	50 – 1000	100–1000	100 – 10000
Slope	115.41	82.17	321.92	154.87	138.68	135.89	31.48
Standard error of slope	0.88	0.58	2.08	0.68	0.93	1.11	0.19
Confidence limit of slope	113.33 ---- 117.49	80.80 ---- 83.55	317.01— 326.82	153.27---- 156.47	136.40 --- 140.96	132.82 --- 138.98	30.97 --- 32.00
Intercept	20544.14	7024.96	-3285.81	-574.77	-4996.83	-6874.57	4291.72
Confidence limit of intercept	13575.34 --- 27512.94	2421.01 ----- 11628.91	-1036.73 ---- -5534.9	-1307.29 --- 157.74	-6077.25 ---- -3916.42	-8597.14 ---- -5152.00	2158.42 --- 6425.02
Correlation coefficient	0.9997	0.9998	0.9998	0.9999	0.9998	0.9998	0.9999
Standard error of estimation	8113.09	5349.14	1973	642.66	884.41	839.24	1623.58
LOD	15.08	14.58	14.05	14.42	15.99	28.55	30.17
LOQ	45.68	44.18	42.58	43.69	48.45	86.52	91.42
Repeatability[a] (SD_r)	99.93 ± 0.74	99.87 ± 0.54	99.64 ± 0.19	99.53 ± 0.42	100.11 ± 0.59	99.78 ± 0.42	99.55 ± 0.23
Intermediate precision[b] (SD_{int})	99.87 ± 0.62	99.72 ± 0.69	99.29 ± 0.32	99.29 ± 0.25	100.41 ± 0.89	99.42 ± 0.39	99.33 ± 0.12
Accuracy± S.D.	99.56 ± 0.52	99.67 ± 0.44	99.59 ± 0.14	99.66 ± 0.37	100.25 ± 0.72	99.76 ± 0.28	99.88 ± 0.53

[a] The intraday and [b] the interday relative standard deviations of samples of concentration 150, 500, 1000 ng ml^{-1} for HCTZ , SAL and 200, 500, 1000 ng ml^{-1} for each of FUR, IDP, TEST, SPIRO and BMS performed as triplicates

The concentrations were calculated using the corresponding regression equation and the percentage recoveries and standard deviations were then calculated as shown in TABLES (9-12).

Selectivity

A Laboratory prepared mixture of the seven drugs were prepared at a concentration of 10 µg.ml^{-1} of each drug, filtered through 0.2 µm filter, then 10-µl volume of the resultant solution was injected in triplicates, and determined, using the chromatographic conditions described under *3.1.1.* and average peak area for each was calculated. Concentrations were calculated, using the corresponding re-

gression equation and the percentage recoveries.

Limit of detection and limit of quantitation

The lowest detectable concentrations of the seven drugs, either in pure form or in spiked plasma and urine were determined by applying the previously mentioned chromatographic conditions under 3.1.1. as shown in TABLES (9-12). The USP guideline for determination of LOD and LOQ was followed. The estimation based on the standard deviation of response. LOD and LOQ are, thus;

$$\mathbf{LOD = 3.3 \times \sigma / S}$$

$$\mathbf{LOQ = 10 \times \sigma / S}$$

Where; σ is the standard deviation of response and S is the slope. Here, the standard deviation of the y-intercepts of the regression lines can be used as the standard deviation of response.

RESULTS AND DISCUSSION

A RP-HPLC method was suggested for simultaneous quantitative determination of HCTZ, SAL, FUR, IDP, TSE, SPIRO and BMS in pure form and in spiked human plasma and urine. The proposed method is based on the difference in retention times between the investigated drugs.

The specified chromatographic conditions were adjusted; a 10-µl volume was injected into HPLC. Several mobile phases were tried to obtain satisfactory results, good separation of the doping drugs, symmetric peak shape and short run time.

The best results were obtained upon using mobile phase acetonitrile : phosphoric acid at pH 3 (50:50, v/v) with a flow rate 1 $ml.min^{-1}$ at wavelengths 225, 272, 235, 242, 244 and 239 nm for the drugs' mixture. It was found that pH 3 was optimum for good separation; also on slight change in the mobile phase pH shows no significant difference.

These wavelengths were chosen according to the maximum wavelengths (λ_{max}) or the wavelengths that show maximum sensitivity for the studied drugs. The advantage of using DAD is that we can obtain a maximum sensitivity with minimum interference through choosing the λ_{max} for each drug as the running wavelength upon its determination in the mixture.

The retention times, shown in TABLE (9), offer the advantage of rapid analysis and reduction of consumed solvents. Calibration curves for each of the investigated drugs, either in pure form or in plasma and urine were constructed, representing the relationship between the calculated average peak areas and the corresponding concentrations and the regression equations were computed as shown in TABLES (9-12).

In plasma; the high selectivity of the proposed method allowed simultaneous quantitative determination of the seven investigated diuretics. Its high selectivity and sensitivity permits its use for quantitative analysis in human plasma for antidoping purpose.

The sample pretreatment step was essential to remove plasma proteins that clog membrane filter and contaminate the column. The aim was to obtain the highest possible recovery, while using the simplest available extraction procedures. A rapid procedure is to mix the biological fluid with at least two volumes of methanol or acetonitrile causing protein precipitation[19]. We tried methanol, acetonitrile or a mixture1:1, but we found that, acetonitrile is more effective, given clean chromatograms for the blank plasma samples at the specified retention times of the drugs. The results of assay validation are presented in TABLE (10).

The roles of measurements and the limits for acceptance are given by pharmacopoeia[20]. From these data, one can conclude that; the proposed method meets all criteria for pharmaceutical analysis.

The results of the system suitability tests shown in TABLE (13) assured the adequacy of the proposed HPLC method for simultaneous analysis of the doping drugs either in pure form, plasma and urine. It should be noted that; we obtain system suitability parameters for each drug at the wavelength showing maximum sensitivity. The run time was 27 mins, which offers an advantage of rapid analysis and reduction of the consumed solvents.

In bioanalysis, many substances (endogenous substances, metabolites, degradation products, co-administrated drugs, etc.) can potentially interfere in the determination of the analytes of interest. The extent of the specificity experiments is mainly determined by the application of the method, it should

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TABLE 13 : Results of assay validation parameters of the proposed HPLC method for the determination of the seven drugs in spiked human plasma

Parameter	HCTZ	SAL	FUR	IDP	TEST	SPIRO	BMS
Retention time	5.31 ± 0.40	6.40 ± 0.39	8.20 ± 0.35	9.65 ± 0.25	13.22 ± 0.45	18.65 ± 0.32	26.11 ± 0.21
Linearity (ng ml^{-1})	100 – 9000	100 – 9000	100 – 1800	100 – 5000	100 – 1800	200–9000	1000 – 9000
Slope	267.67	135.09	462.11	168.85	188.28	100.46	46.41
Standard error of slope	1.69	1.41	5.01	2.69	0.94	1.10	0.67
Confidence limit of slope	263.66 ---- 271.68	131.83 ---- 138.35	448.19— 476.03	161.36---- 176.34	185.29 --- 191.27	97.86 --- 103.07	44.54 --- 48.28
Intercept	20903.02	23602.16	30479.68	26227.59	3756.64	-17535.76	11676.66
Confidence limit of intercept	2452.24--- 39353.79	10218.97 ---- 36985.34	14218.14 ---- 46741.22	9682.80 --- 42772.39	958.68 ---- -6554.59	-28522.16 --- -6549.36	-21392.88 --- -1960.45
Correlation coefficient	0.9998	0.9995	0.9997	0.9995	0.9999	0.9996	0.9996
Standard error of estimation	16648.10	13862.43	8895.01	11640.17	1350.84	10461.13	5201.31
LOD	31.16	29.99	28.14	29.84	31.98	28.55	250.99
LOQ	94.42	90.88	85.27	90.42	96.91	86.52	760.58
Repeatability[a] (SD_r)	99.36 ± 1.47	101.06 ± 0.55	100.30 ± 1.47	98.52 ± 0.14	99.19 ± 0.25	100.13 ± 1.28	98.79 ± 0.57
Intermediate precision[b] (SD_{int})	99.58 ± 1.54	101.15 ± 0.25	100.30 ± 1.57	98.34 ± 0.11	99.06 ± 0.39	100.22 ± 1.51	98.61 ± 0.56
Accuracy± S.D.	99.22 ± 0.82	99.57 ± 1.19	100.42 ± 1.06	100.27 ± 1.35	98.97 ± 0.67	100.14 ± 1.15	98.85 ± 0.48

[a] The intraday and [b] the interday relative standard deviations of samples of concentration 200, 1000, 1500 ng ml^{-1} for HCTZ , SAL, FUR, IDP, TEST, SPIRO and 1000, 2000,7000 ng ml^{-1} for BMS performed as triplicates

TABLE 14 : Results of assay validation parameters of the proposed HPLC method for the determination of the five drugs in spiked human urine through SPE

Parameter	FUR	IDP	TEST	SPIRO	BMS
Retention time	7.99 ± 0.25	9.35 ± 0.35	12.99 ± 0.45	18.11 ± 0.30	25.55 ± 0.39
Linearity (ng ml^{-1})	250 – 3000	150 – 6000	150 – 7000	150–7000	150 – 7000
Slope	218.19	101.52	72.29	62.83	35.63
Standard error of slope	2.43	0.69	0.54	0.54	0.26
Confidence limit of slope	211.95— 224.44	99.84---- 103.21	71.01 --- 73.57	61.58 --- 64.08	35.03 --- 36.24
Intercept	80495.71	24964.03	23148.94	16745.53	8637.39
Confidence limit of intercept	71487.19 ---- 89504.22	19647.75 --- 30280.31	18814.23 ---- 27483.64	12649.79 ---- 20841.27	6586.41 --- 10688.37
Correlation coefficient	0.9997	0.9998	0.9998	0.9997	0.9998
Standard error of estimation	6257.87	4359.85	4143.53	4154.29	2016.29
LOD	70.11	39.15	42.71	45.91	49.01
LOQ	212.45	118.64	129.42	139.12	148.52
Repeatability[a] (SD_r)	98.96 ± 2.23	98.71 ± 1.05	98.31 ± 0.82	98.18 ± 0.47	101.28 ± 2.34
Intermediate precision[b] (SD_{int})	99.11 ± 2.49	99.07 ± 1.82	98.11 ± 0.46	97.77 ± 0.74	101.84 ± 2.87
Accuracy± S.D.	97.64± 2.17	99.47 ± 2.49	100.63 ± 3.56	100.78 ± 3.26	100.92 ± 4.01

[a] The intraday and [b] the interday relative standard deviations of samples of concentration 250, 1000, 3000 ng ml^{-1} for TEST, SPIRO and 250,500,2000 ng ml^{-1} for FUR, 250,1500, 3000 for IDP and 500,2000,6000 for BMS performed as triplicates

TABLE 15 : Results of assay validation parameters of the proposed HPLC method for the determination of the seven drugs in spiked human urine through direct dilution

Parameter	HCTZ	SAL	FUR	IDP	TEST	SPIRO	BMS
Retention time	4.88 ± 0.40	6.00 ± 0.38	7.91 ± 0.34	9.30 ± 0.40	12.85 ± 0.38	18.00 ± 0.28	25.68 ± 0.35
Linearity (ng ml^{-1})	150 – 5000	50 – 5000	150 – 1500	50 – 5000	50 – 5000	100–5000	150 – 5000
Slope	138.89	77.75	168.25	69.69	56.24	50.49	22.05
Standard error of slope	2.10	0.86	2.81	0.56	0.64	0.36	0.17
Confidence limit of slope	133.05 ---- 144.74	75.77 ---- 79.73	159.31— 177.19	68.44---- 70.94	54.73 --- 57.74	49.64 --- 51.35	21.65 --- 22.45
Intercept	57428.54	25255.74	55862.09	15081.16	12406.62	3876.81	1904.36
Confidence limit of intercept	39887.22--- 74969.85	21721.13 ----- 28790.36	48037.09 ---- 63687.08	12551.09 --- 17611.23	9027.96 ---- 15785.28	2104.76 ---- -5648.86	986.64 --- 2822.08
Correlation coefficient	0.9995	0.9995	0.9996	0.9997	0.9995	0.9998	0.9997
Standard error of estimation	9298.35	3967.84	3121.57	3009.99	3408.56	1733.94	933.72
LOD	39.41	11.98	35.52	12.70	14.11	29.01	40.72
LOQ	119.42	36.30	107.64	38.48	42.76	87.91	123.39
Repeatability[a] (SD_r)	100.15 ± 0.99	98.81 ± 0.54	98.77 ± 0.71	98.90 ± 0.69	98.95 ± 0.52	99.19 ± 1.01	99.01 ± 0.72
Intermediate precision[b] (SD_{int})	99.99 ± 1.54	98.44 ± 0.34	98.55 ± 0.86	98.86 ± 0.46	98.73 ± 0.49	99.00 ± 0.94	98.27 ± 0.79
Accuracy± S.D.	100.87 ± 1.16	99.89 ± 1.98	100.05 ± 1.49	99.28 ± 1.31	100.05 ± 2.62	100.31 ± 2.16	99.33 ± 1.13

[a]The intraday and [b] the interday relative standard deviations of samples of concentration 250, 500, 1500 ng ml^{-1} for FUR and 250,1000,3000 ng ml^{-1} for the rest of drugs performed as triplicates

TABLE 16 : System suitability parameters for the analysis of the seven drugs using the proposed HPLC method

Parameter	HCTZ	SAL	FUR	IDP	TEST	SPIRO	BMS
Capacity factor (K′)	0.75	1.12	1.67	2.2	3.36	5.22	7.67
Tailing factor (T)	1	1.02	1	1	1	1	1
Number of theoretical plates (N)	4900	7168.44	11377.78	12037.22	30368.87	34782.25	43264.00
Selectivity* (α)	1.49	1.49	1.32	1.53	1.55	1.47	1.47
Resolution** (R)	4	5.08	5.33	7.71	14.88	16.33	16.33

*** Selectivity was calculated according to the capacity factors of two successive peaks; ** Resolution was calculated according to the retention times of the drugs in two successive peaks**

be noted that ICH makes no difference between the terms 'selectivity' and 'sensitivity'. Several validation documents[22, 23, 24] require different sources of blank matrices to be analyzed. One must demonstrate that there is no interference in the chromatographic region of the analytes.

The proposed method was tested for specificity by comparing chromatograms of 3 different sources of blank human plasma. The chromatograms were free from any interfering peaks at the retention times of the studied drugs. Thus the proposed method can be used for quantitative determination of the seven drugs in plasma, i.e. for antidoping purpose without interference by endogenous plasma components.

The spiked human plasma samples stored at -20 °C, were injected over a period of 1 month did not suffer any appreciable changes in the assay values and were able to meet the criteria mentioned above. Hence, the samples were stable during 1 month. In addition, the drugs mixture was found to be stable in human plasma and the stability is maintained at room temperature for more than 12 hours.

The extraction efficiency was verified by the accuracy of the proposed method showing in TABLE

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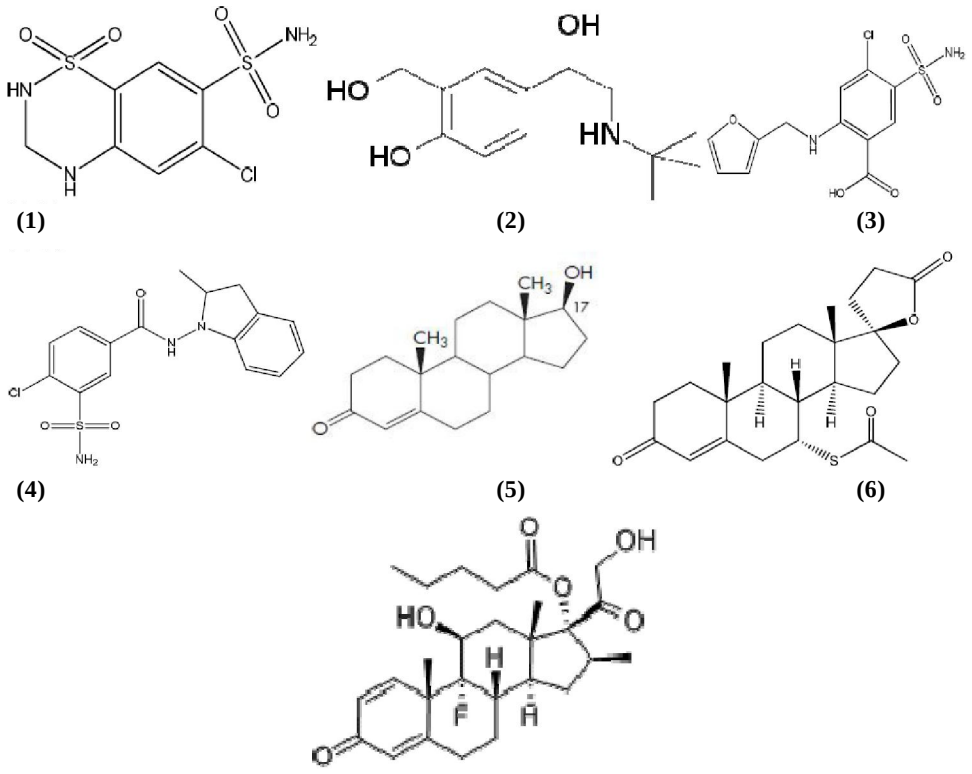

Figure 1 : Chemical structures of the studied doping drugs (1) Hydrochlorothiazide (HCTZ) (2) Salbutamol (SAL) (3) Furosmide (FUR) (4) Indapamide (IDP) (5) Testosterone (TSE) (6) Spironolactone (SPIRO) (7) Betamethasone (BMS)

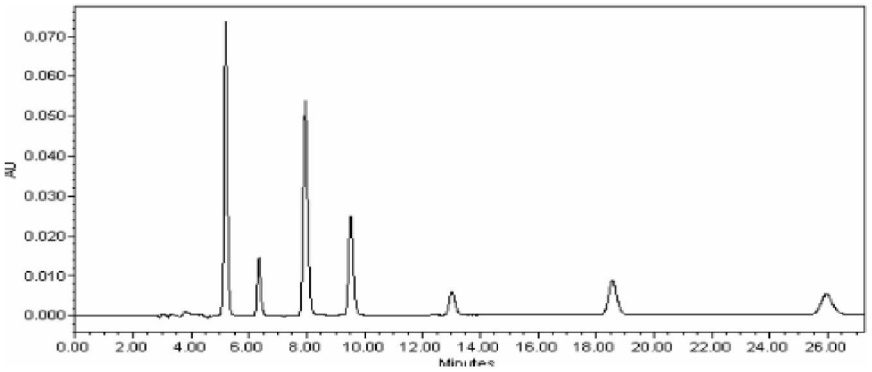

Figure 2 : Chromatogram showing simultaneous separation of the seven drugs (10µg ml^{-1}) at λ_{225} nm

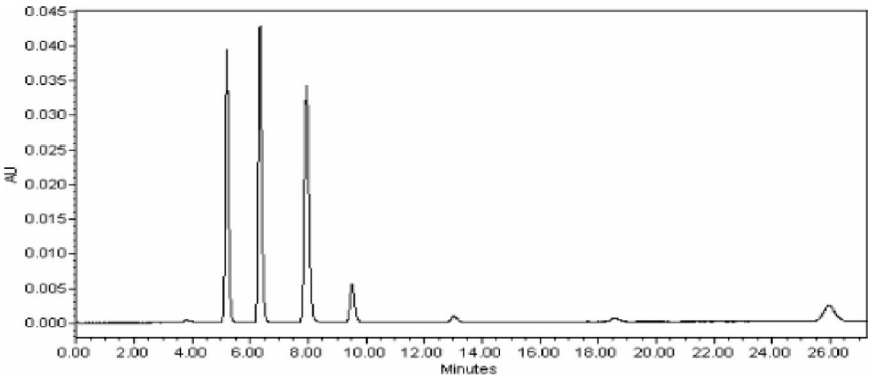

Figure 3 : Chromatogram showing simultaneous separation of the seven drugs (10µg ml^{-1}) at λ_{272} nm

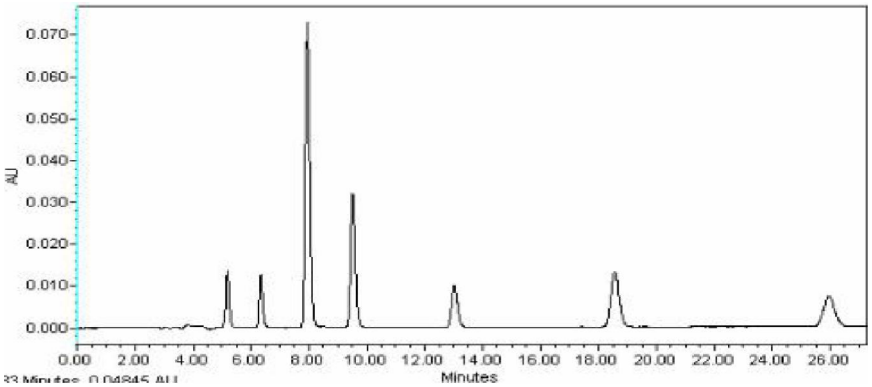

Figure 4 : Chromatogram showing simultaneous separation of the seven drugs (10µg ml^{-1}) at λ_{235} nm

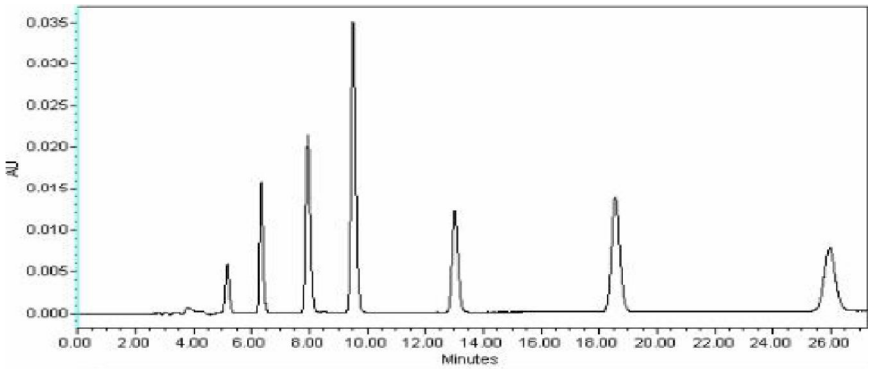

Figure 5 : Chromatogram showing simultaneous separation of the seven drugs (10µg ml^{-1}) at λ_{242} nm

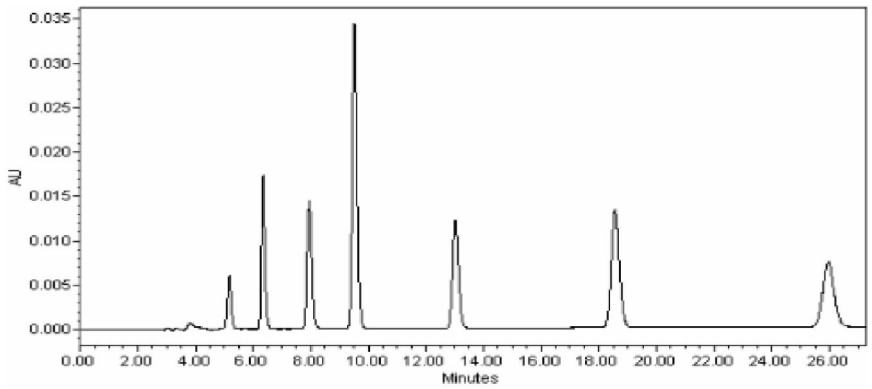

Figure 6 : Chromatogram showing simultaneous separation of the seven drugs (10µg ml^{-1}) at λ_{244} nm

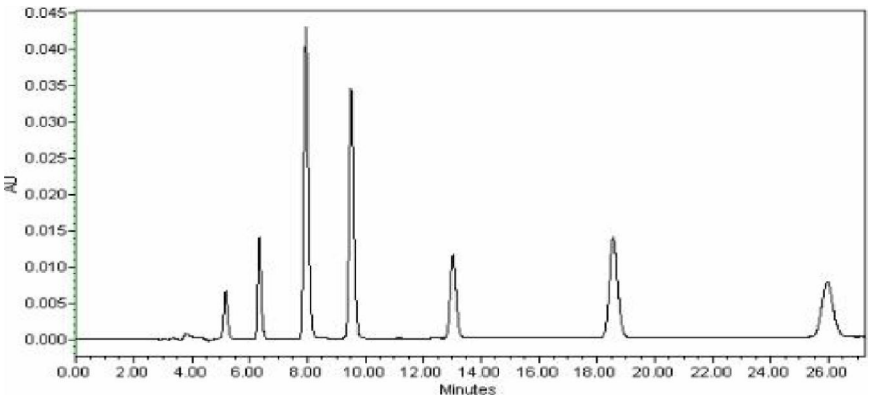

Figure 7 : Chromatogram showing simultaneous separation of the seven drugs (10µg ml^{-1}) at λ_{239} nm

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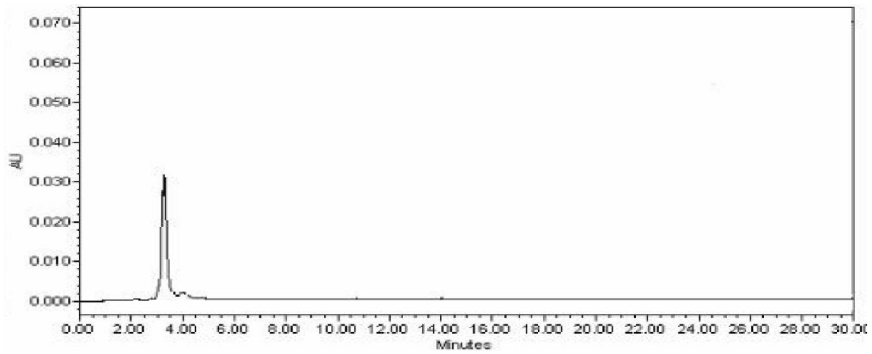

Figure 8 : Chromatogram of blank plasma at λ_{225} nm

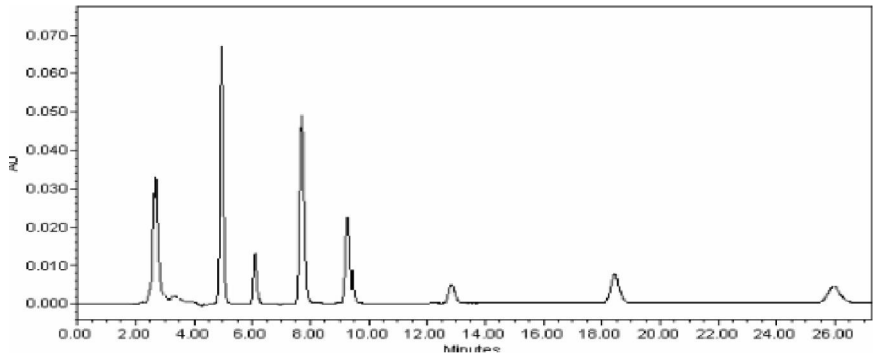

Figure 9 : Chromatogram of plasma spiked with the seven drugs (each of 2000 ng ml^{-1}) at λ_{225} nm

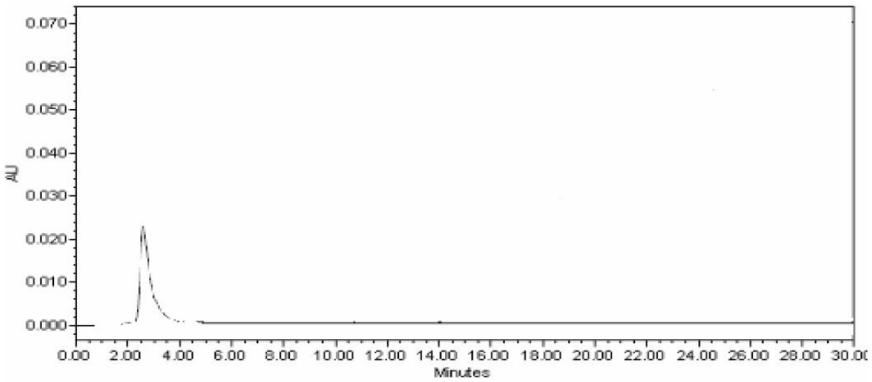

Figure 10 : Chromatogram of blank urine after SPE at λ_{244} nm

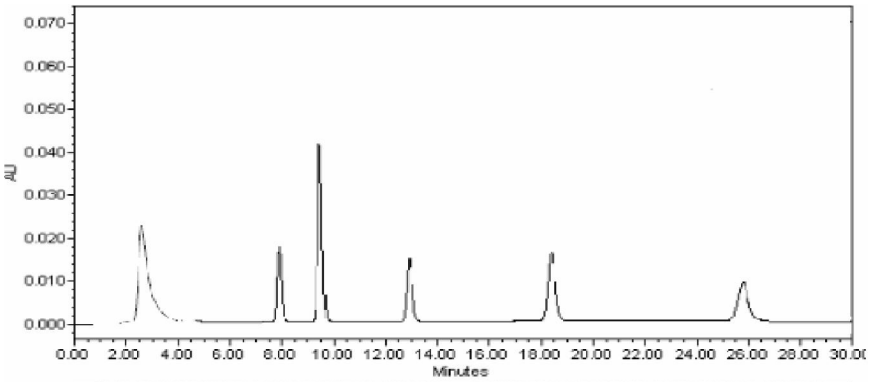

Figure 11 : Chromatogram of urine spiked with the seven drugs at λ_{244} nm after SPE

(3), while results of assay validation of the seven drugs are illustrated in TABLES (10).

In urine, the background signal of urine samples, due to the proteins (wide band at the head of the

chromatograms) and several endogenous compounds (peaks at diverse retention times), can seriously affect the detection of drugs. Also, direct injection without dilution or pretreatment may harm the column, cause bacterial growth, damage the packing material thus shortening the life of the column or can force frequent regeneration of the stationary phase. Both direct dilution and SPE were validated and compared. It was found that SPE (1:4dilution) more sensitive than direct dilution (1:50 dilution), however HCTZ and SAL produced irreproducible results because of their hydrophilicity. The pretreatment efficiency was verified by the accuracy of the proposed method showing in TABLE (4-8), while results of assay validation of the seven drugs are illustrated in TABLES (11,12).

The proposed HPLC method was applied for simultaneous determination of the four diuretics in spiked human plasma.

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