

Determination and stability of some international screening limited drugs in equine blood by LC-MS/MS

Eylem Funda GÖKTAŞ^{1,a,✉}, Erol KABİL^{1,b}, Ertuğrul GÜNEŞ^{1,c}, Serol KORKMAZ^{1,d},
Tuba Asena KADIOĞLU^{1,e}, Ayşen KURT CÜCÜ^{2,f}

¹İstanbul Pendik Veterinary Control Institute, Doping Control Laboratory; ²Marmara University, Faculty of Pharmacy, Department of Analytical Chemistry, İstanbul, Turkey.

^aORCID: 0000-0002-1269-8114; ^bORCID: 0000-0002-0339-0353; ^cORCID: 0000-0001-9872-1882;

^dORCID: 0000-0001-8970-6883; ^eORCID: 0000-0003-2048-9854; ^fORCID: 0000-0001-9527-4730.

✉Corresponding author: eylemfundagoktas@gmail.com

Received date: 13.03.2019- Accepted date: 10.07.2019

Abstract: Stability of some international screening limited therapeutic substances for doping control in equine blood has been investigated with a validated method by using LC-MS/MS after chemical hydrolysis with orthophosphoric acid. Whole blood samples were extracted with a WAX cartridge on auto-SPE. Stability test of the drug substances performed at the same time in working solution and matrix at +4°C, -20°C, +20°C in dark and +20°C in light conditions for 1, 2, 3 and 4 weeks. For short-time stability, the effects of 6 h storage at +55°C was also investigated. Repeated data were statistically analyzed with ANOVA. All substances in working solution were stable at -20°C and +4°C. In the blood matrix, higher temperatures (+20°C) in light and dark caused degradation of substances at the end of four weeks and short-term study (P < 0.05). In conclusion, these international screening limited substances in blood matrix could be unstable related to temperature and storage time, although in working solution they could be mostly stable in various temperatures for four weeks.

Keywords: Doping control, equine blood, LC-MS/MS, stability.

Uluslararası tarama limiti olan bazı ilaçların at kanında LC-MS/MS ile tayini ve stabilitesi

Özet: Doping kontrolü amacıyla uluslararası tarama limiti kapsamında olan bazı terapötik maddelerin at kanında valide bir metotla LC-MS/MS cihazı kullanılarak stabilitesi araştırılmıştır. Tam kan örneklerinde ortofosforik asitle kimyasal hidroliz sonrasında bir WAX kartuş kullanılarak otomatik katı faz ekstaksiyonuyla ekstrakte edilmiştir. Etkin maddelerinin stabilite testi, çalışma çözeltisinde ve matriks içerisinde +4°C, -20°C, +20°C karanlıkta ve +20°C gün ışığında 1, 2, 3 ve 4 hafta için eşzamanlı olarak çalışılmıştır. Kısa zamanlı stabilite çalışması +55°C'de 6 saat saklamanın etkisini kapsayacak şekilde araştırılmıştır. Yapılan tekrarlı analizler ANOVA ile istatistiksel olarak değerlendirilmiştir. Çalışma çözeltisindeki tüm maddeler -20°C ve 4°C'de stabildi. Işıktaki ve karanlıkta yüksek sıcaklık, dört hafta sonunda ve kısa süreli çalışmada kan matriksindeki maddelerin bozunmasına neden olmuştur (P < 0.05). Sonuç olarak, bu uluslararası tarama limitli maddeler sıcaklık ve zamana bağlı olarak kan matriksinde stabil olmayabilirken, çalışma çözeltisinde farklı sıcaklıklarda dört hafta boyunca çoğunlukla stabildir.

Anahtar sözcükler: At kanı, doping kontrol, LC-MS/MS, stabilite.

Introduction

Most of the doping laboratories that control the samples collected from racing horses are conducting the analysis of prohibited substances according to the International Agreement on Breeding, Racing and Wagering (IABRW) guidance document, which is determined and updated by International Federation of Horseracing Authorities (IFHA) (7). There is no limit level for prohibited substances, however, some endogenous substances in horse metabolism, feed or environmental contaminants have a threshold and therapeutic

substances with international screening limits should be analyzed at their specified value as a requirement. The limits for substances are regulated based on therapeutic effects on equines health and prevent their misuse (26). Most of the samples taken from equines are urine and/or blood. When determining the limit values and substances, the type of the sample and drug metabolism are considered. The studied therapeutic substances and the international screening limits in blood are shown in Table 1.

Post-race samples are separated into two parts as called A and B (7). Sample A is transported as quickly as possible for doping control. Sample B stored under suitable conditions is analyzed if sample A is detected as positive. According to the current legislation, blood samples are not frozen and stored in the refrigerator (+4°C) unless separated to serum or plasma (7, 26). The time elapsed between the analysis of sample A and B; the storage conditions of sample B, the description of the effect of temperature changes during transport and consequently knowing the stability of the positive detected substance are critical for the proper interpretation results of the analysis (20). If sample A is identified as positive, sample B should be identifiable in the same manner. In biological fluids sample during storage and transportation, drug concentration could be decreased or increased related to temperature, pH, thermal or chemical degradation, hydrolysis, enzymatic metabolism, compound interference, microbiological or matrix effects (9, 11). Due to these effects, confirmatory analysis with sample B, reported as positive before, could be determined as negative. Especially for limited substances, the storage conditions and time are crucial for the quantitative drug presence in the sample to avoid false positive/negative results (9, 11, 24, 25). So, the stability knowledge of the substances in the sample gives vital information about reporting time.

In previous studies, short or long-term stability were conducted in urine (6, 22, 23, 24, 25), whole blood (1, 2, 5, 8, 12), plasma or serum (1, 2, 19) for doping control (10, 18, 19, 24, 25), trace element analysis (21) and toxicological researches (4, 5, 6). Nevertheless, there are few studies were focused on the threshold or limited doping agents except some anabolic steroids (10, 18), diuretics (24) and caffeine (25) in urine matrix. Beside of previous studies, we aimed to analyze the international screening limited Flunixin, Meloxicam, Dembrexine, N-Butylscopolammonium, Carprofen, Butorphanol, Lidocaine, Mepivacaine, Acepromazine, 7 deuterated internal standards (IS) with the validated method by LC-MS/MS and evaluate firstly their stability in diluted working standard and equine blood matrix at different temperatures and conditions in four week period. In addition to that, short-term stability of 6 h storage at +55°C was investigated as a simulation of hot-day storage effects.

Material and Methods

Chemicals and standard solutions: Flunixin, Flunixin d3 and Carprofen obtained from Sigma Aldrich (Schnelldorf, Germany); Meloxicam, Dembrexine, Butorphanol tartrate, Scopolamine d3 Hydrobromide trihydrate and Cortisol-9,11,12,12 d4 purchased from Chiron (Chiron AS, Trondheim Norway); Meloxicam d3,

Carprofen-d3, 3-OH Lidocaine-d5, 3-OH Mepivacaine-d3, Acepromazine, N-Butylscopolammonium provided from TRC (Toronto Research Chemicals, North York ON, Canada); Mepivacaine obtained from EDQM (Strasbourg, France); Lidocaine was purchased from LGC (Molsheim France). All standards purity was $\geq 98\%$. Water was purified with an Elga-purelab flex water purification system (Elga-Veolia Water Solutions&Technologies, UK). n-Hexane was obtained from VWR Chemicals (VWR International Fontenay Sous Bois, France), Ethyl acetate and Methanol were obtained from J.T. Baker (Gliwice, Poland). Potassium dihydrogen phosphate and orthophosphoric acid, acetonitrile (ACN) and formic acid (FA) were purchased from Merck (Darmstadt, Germany). Acetic acid was obtained from Sigma Aldrich (Schnelldorf, Germany). All chemicals were of HPLC grade purity. The solid-phase extraction (SPE) cartridge Oasis Wax (3 cc Vac Cartridge, 60 mg Sorbent per Cartridge, 60 μm Particle Size, 100/pk) were obtained from Waters, USA. For sample preparation auto-pipette (Eppendorf Multipette Xstream), vortex mixer (Allsheng MTV-100), laboratory centrifuge (Thermo Scientific Heraeus Cryofuge 5500i), nitrogen evaporator (Biotage Turbo Vab LV), SPE system auto-SPE (Gilson Aspec 274) were used. For storage, Sanyo Medicoool (MPR-414F) refrigerator and Sanyo Biomedical (MDF-U537D) freezer were used. Blood samples taken from race horses which were determined as negative in the doping control laboratory were used. Permission of the ethics committee was received for this study (Local Ethical Committee for Experimental Animals of Pendik Veterinary Control Institute 03/2019).

Preparation of solutions and instrument conditions: Primary stock standard solutions were prepared in methanol at a concentration of 1 mg mL⁻¹ (Carprofen, Dembrexine, Flunixin, Acepromazine, Lidocaine, Mepivacaine, N-Butylscopolammonium, Butorphanol, Meloxicam d3 (IS), 3 Hydroxy Lidocaine d5 (IS), 3 Hydroxy Mepivacaine d3 (IS), Carprofen d3 (IS) and Scopolamine d3 Hydrobromide trihydrate (IS)), 100 $\mu\text{g mL}^{-1}$ (Flunixin d3 and Cortisol d4) except 1 mg mL⁻¹ Meloxicam in DMSO. The working standard mix solution was prepared by dilution of suitable aliquots of primary stock standard solutions and used to spike the blood samples. Cortisol d4 was used as an internal standard for Dembrexine, Acepromazine and Butorphanol. The working solution mix (10 mL) was contained 0.1 $\mu\text{g mL}^{-1}$ Flunixin, 0.1 $\mu\text{g mL}^{-1}$ Meloxicam, 0.1 $\mu\text{g mL}^{-1}$ Carprofen, 0.002 $\mu\text{g mL}^{-1}$ Acepromazine, 0.002 $\mu\text{g mL}^{-1}$ Lidocaine, 0.5 $\mu\text{g mL}^{-1}$ Dembrexine, 0.005 $\mu\text{g mL}^{-1}$ Mepivacaine, 0.01 $\mu\text{g mL}^{-1}$ N-Butylscopolammonium, 0.002 $\mu\text{g mL}^{-1}$ Butorphanol. Internal standard mix solution (10 mL) was contained 5 $\mu\text{g mL}^{-1}$ concentration of deuterated IS.

Table 1. International screening limits (ISL) of nine drugs recommended by IFHA's Advisory Council on Equine Prohibited Substances and Practices, March 2016 (7).

Substance	ISL (ng/mL in plasma)
Flunixin (FLU)	1
Meloxicam (MEL)	1
Dembrexine (DEM)	5
N-Butylscopolammonium (SCO)	0.05
Carprofen (CAR)	100
Butorphanol (BUT)	0.01
Lidocaine (LID)	0.05
Mepivacaine (MEP)	0.05
Acepromazine (ACE)	0.02

Table 2. Optimisation data of drug substances and internal standards in MRM mode.

Compound Name	Precursor ion	Fragmentor	Product ion	Collision energy	Polarity
Meloxicam	352.2	100	141	15	Positive
			115	16	
Flunixin	297.2	130	279	21	Positive
			264	33	
Carprofen	274.1	90	228	9	Positive
			193	31	
Acepromazine	327.3	150	254.1	20	Positive
			222.1	40	
			86.1	20	
Lidocaine	235.3	100	86.1	11	Positive
			58.1	33	
Dembrexin	378	100	262.7	25	Positive
			81	25	
Mepivacaine	247.3	110	98.1	16	Positive
			70.1	48	
N-Butylscopolammonium Bromide	360.2	150	194	16	Positive
			103	50	
Butorphanol	328.4	140	310.2	20	Positive
			157.1	46	
Cortisol D4	367.2	120	121	21	Positive
			97	41	
Flunixin D3	300.1	140	282.1	21	Positive
			264.1	35	
Scopolamine D3 Hyrobromid trihydrate	307.2	90	159.1	11	Positive
			141	27	
3-OH Mepivacaine-D3	266.2	110	101.1	17	Positive
			73.1	37	
3-OH Lidocaine-D5	256.2	100	91.2	17	Positive
			63.2	41	
Carprofen-d3	275	80	231	13	Negative
			228	41	
Meloxicam-d3	355.1	120	141	39	Positive
			115	9	

LC-MS/MS analyses were performed on an Agilent series 1200 liquid chromatography (Santa Clara, CA, USA) coupled to 6460 triple-quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source. The study was modified by Moulard et al. (14) LC MS/MS method. For separation, Sunfire C18 column (Waters, USA) with dimension 2.1x150 mm and particle size 3.5 μm was used. The gradient was applied with H₂O-0.1 % FA (phase A) and ACN-0.1 % FA (phase B). The initial conditions of the gradient kept until 5 min was 80 % A and 20 % B. The phase A was decreased to 50 % at 20 min, and at 25 min to 0 %. Such conditions were maintained until 27 min and the system was re-equilibrated with initial compositions of the mobile phase. The total run-time of the method was 31 min. The flow rate was 0.3 mL min⁻¹, the injection volume was 20 μL , and the column temperature was 35°C. The ESI interface conditions with multiple reaction monitoring (MRM) modes were; the gas temperature 325°C, gas flow 10 L min⁻¹ (N₂), sheath gas temperature 350°C, sheath gas flow 12 L min⁻¹, capillary voltage 3.5 kV in negative mode and 4.5 kV in positive mode, nebulizer gas 50 psi and max. pressure limit was 600 bar. Diagnostic ions of drug substances were detected in MRM mode as listed in Table 2.

Sample preparation: The study was modified by Popot et al. (16) extraction method. 3 mL whole blood samples plasma into 15 mL polypropylene tube after centrifuge at 4400 rpm for 15 min 30 μL of IS mix, 100 μL orthophosphoric acid and 1.5 mL deionized water was added, mixed in the vortex for 10 min for hydrolysis. Then, 2.5 mL phosphate buffer (0.5 M, pH 6.5) was added and pH adjusted to 6 with 5 % HCl. The sample was mixed again and centrifuged (3500 rpm, 15 min). The tubes were placed into auto-SPE and preconditioned (2 mL deionized water, 2 mL methanol). The sample was loaded on the cartridge, washed with 3 mL deionized water/methanol (95/5) and dried for 2 min under vacuum then eluted from the cartridge with 3 mL ethyl acetate/methanol-2 % acetic acid (50/50) and evaporated to dryness (N₂, 45°C). The dry residue dissolved with 2x100 μL methanol, transferred into a glass vial and dried again. 50 μL mobile phase solution (80/20 H₂O-0.1 % FA/ACN-0.1 % FA) added and mixed for 3 min before injected to LC MS-MS.

Stability studies: Stability design have been demonstrated according to 2002/657/EC Commission Decision (3, 15). For this purpose; 60 mL drug-free blood sample was collected and spiked with mix standard solution and IS mix solution on ISL levels and shaken for 10 min. After that 3 mL of blood sample was taken and analyzed as fresh solution data for matrix stability. For each storage period, 15 mL of blood sample divided into glass bottles and stored at +4 \pm 2°C (in the refrigerator), -20 \pm 2°C (deep freeze), +20 \pm 2°C in light and +20 \pm 2°C in

dark at climate room and temperature controlled with a data logger. The same application was designed for the working solution mix stability with the separation of 750 μL of standard mix solutions to different storage conditions and fresh solution analyzed as initial data for stability test. The aliquots were tested with three replicates after 1, 2, 3 and 4 weeks' storage (estimated reporting time). Short-term stability was evaluated as the influence of 6 h storage at +55 \pm 2°C for simulating the transfer effects on hot days. 6 mL of drug-free whole blood spiked with 60 μL mix standard solution. 3 mL was kept in an incubator at +55 \pm 2°C for 6 h and the other 3 mL kept at +4°C. Then both of them were analyzed together (n=3).

The stability test was designed according to 2002/657/EC, but there is no criterion for the assessment of stability in the guideline. Therefore, for the estimation of the instability of drugs while for the evaluation of degradation over a time period the \pm 15% precision acceptance criterion was used (20). In many other stability studies, the results were interpreted according to this criterion (9, 12, 13, 17, 23). The results outside the specified criteria are shown on the lines of the graphics. Analysis of Variances (ANOVA) was used for statistical evaluation of the effect of temperature, light-darkness and time of the storage by comparing the initial concentration and measured concentration at varying temperature using SPSS software package (SPSS version 21 for Windows).

Results

Results of the method for quantitation of some ISL drugs were shown in Figure 1 at the limit level spiked for each drug. The method was used in our laboratories routine analyses and validated according to 2002/657/EC (3). The validation summary for studied substances were shown in Table 3. In the results of validation data, CV % values were calculated as 1.5-7.2 % for precision and 1.6-19.7 % for with-in laboratory reproducibility (Table 2).

For estimation of the results, responses of substances were shown depend on temperature and time in Figure 2 and 3. Initial and end of four weeks concentrations were statistically compared in Table 4. Over four weeks, all substances in working solution were stable in conditions of +4°C and -20°C (P> 0.05). But N-Butylscopolammonium in the light condition of +20°C after 3rd week and Dembrexin in both of light and dark conditions of +20°C after 2nd week deviated out of the criterion of \pm 15% (Figure 2, P< 0.05).

In blood matrix, while all substances were stable at +4°C for four weeks (Figure 3, P> 0.05), N-Butylscopolammonium was significantly degraded at 4th week (Table 4, P< 0.05). There were significant differences in all substances at +20°C in both light and dark conditions when comparing initial data with 4th week data (Table 4, P< 0.05). In addition, N-

Butylscopolammonium, Acepromazine and Carprofen were significantly unstable at -20°C during four weeks (Figure 3, P< 0.05).

When blood samples were stored at +55°C during six hours for short-term stability, substances (except Flunixin

and Carprofen) were degraded at an amount of up to 50 %. Though, Flunixin and Carprofen were increased at high temperature (Figure 4).

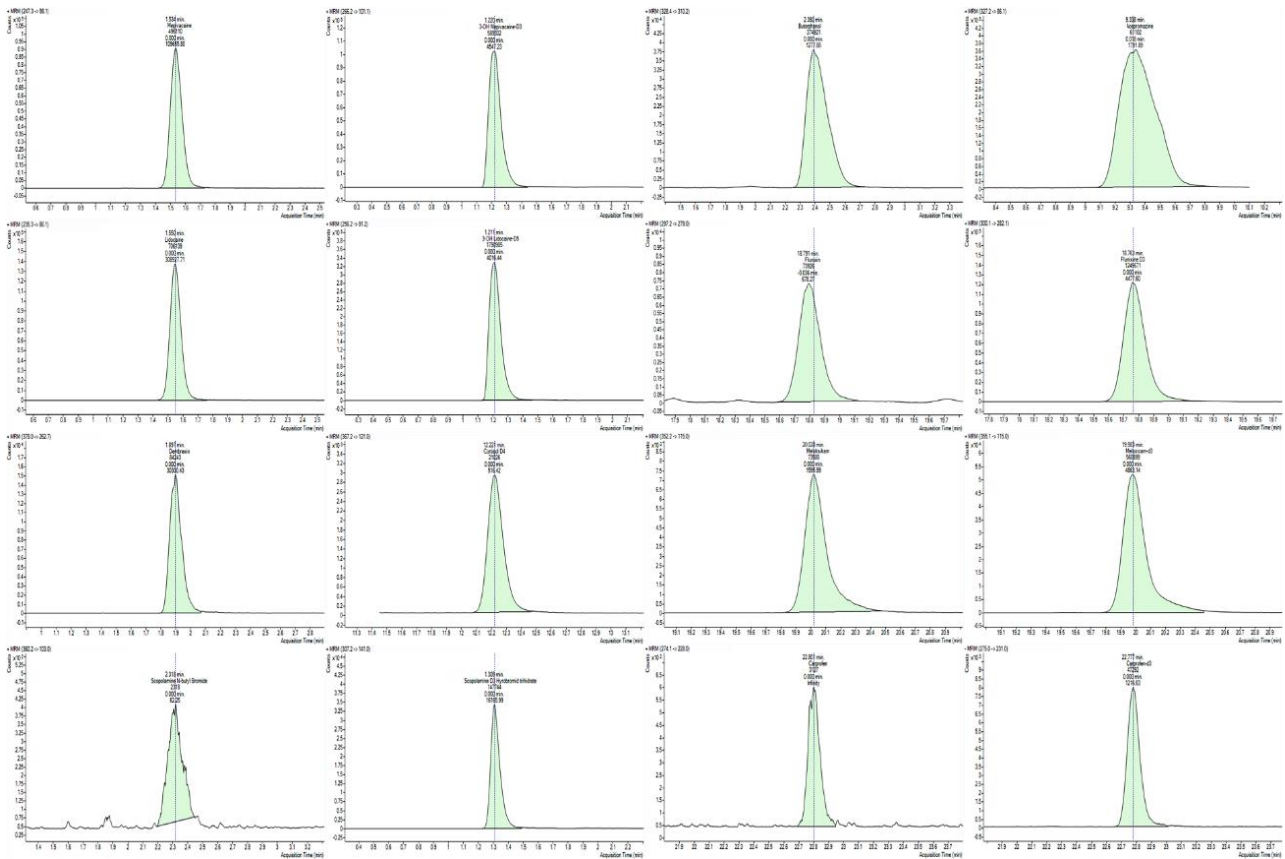


Figure 1. Chromatograms of spiked equine blood samples at ISL levels and internal standards.

Table 3. Results of method validation.

Substances	Conc. spiked (ng/mL)	Interday (n=6)		Intraday (n=18)		Within lab. reproducibility CV (%) ^b	CC α (ng/mL) ($\alpha=1\%$)	CC β (ng/mL) ($\beta=5\%$)
		Conc. measured (mean \pm SD)(ng/mL)	Precision CV (%) ^a	Conc. measured (mean \pm SD)(ng/mL)	Precision CV (%) ^a			
MEP	0.05	0.0494 \pm 0.0015	3.1235	0.0496 \pm 0.0013	2.5585	3.0827	0.0522	0.0544
LID	0.05	0.0487 \pm 0.0010	1.9965	0.0494 \pm 0.0012	2.4186	2.4747	0.0520	0.0540
DEM	5.00	5.0397 \pm 0.0799	1.5863	5.0243 \pm 0.0841	1.6733	1.6530	5.1385	5.2769
SCO	0.05	0.0492 \pm 0.0008	1.6536	0.0492 \pm 0.0019	3.9028	4.7741	0.0537	0.0575
BUT	0.01	0.0108 \pm 0.0006	5.4450	0.0103 \pm 0.0007	7.1511	7.8057	0.0113	0.0126
ACE	0.02	0.0195 \pm 0.0014	7.2780	0.0205 \pm 0.0033	16.2990	19.7906	0.0250	0.0300
FLU	1.00	1.0272 \pm 0.0375	3.6550	0.9970 \pm 0.0404	4.0536	3.6661	1.0596	1.1192
MEL	1.00	0.9429 \pm 0.0550	5.8288	0.9992 \pm 0.0778	7.7836	5.5974	1.1140	1.2279
CAR	100.00	101.6593 \pm 6.0112	5.9131	100.2595 \pm 7.3812	7.3621	7.8057	113.9520	127.9039

^a For ≥ 10 -100 ng/mL CV(%) shall be not exceed 20. For mass fractions lower than 100 ng/mL the application of the Horwitz Equation gives unacceptable high values. Therefore. the CVs for concentrations lower than 100 ng/mL shall be as low as possible.

^b For 100 ng/mL CV(%) shall be not exceed 23. For mass fractions lower than 100 ng/mL the application of the Horwitz Equation gives unacceptable high values. Therefore. the CVs for concentrations lower than 100 ng/mL shall be as low as possible.

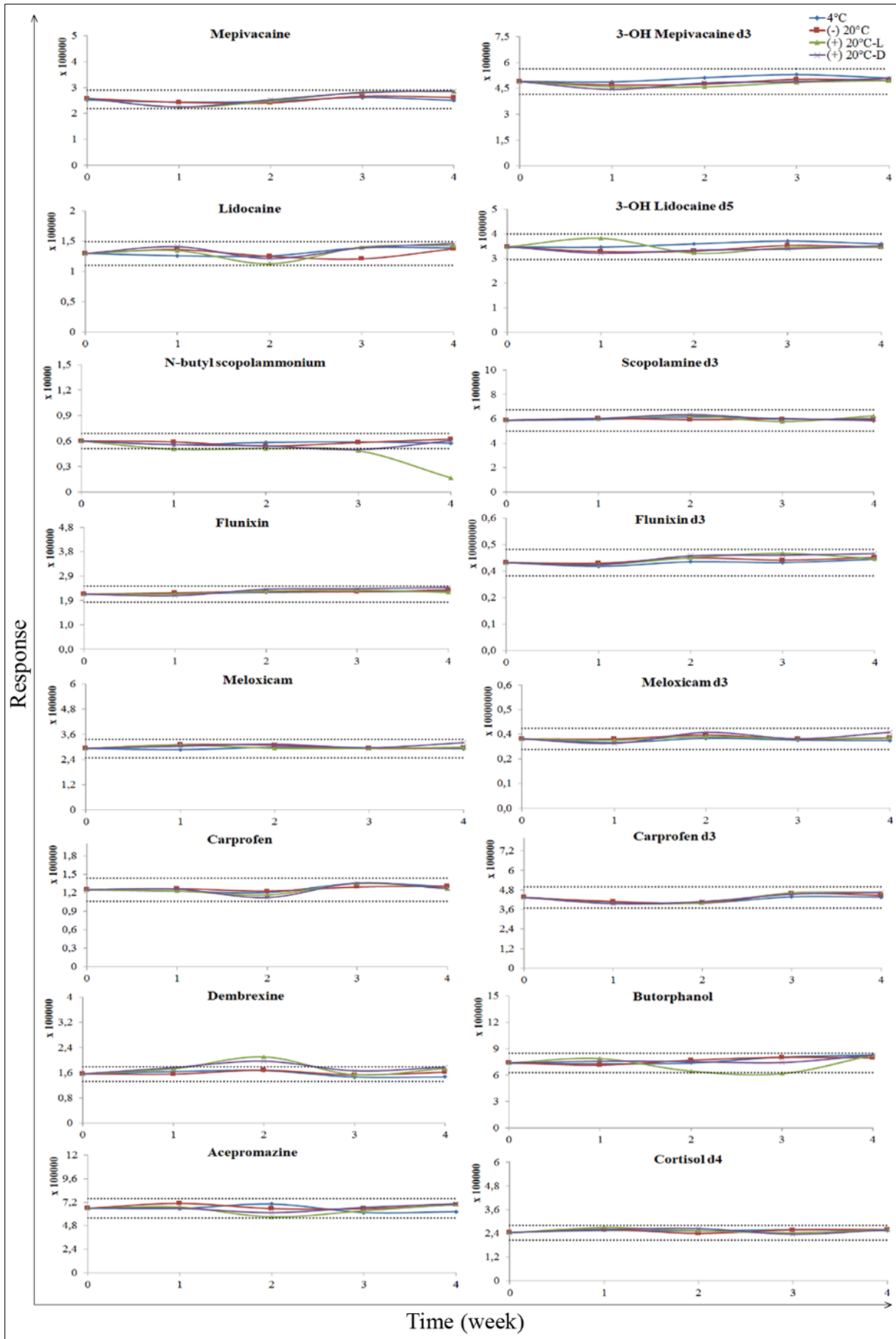


Figure 2. Stability results of working solution mix of nine ISL substances and their internal standards.

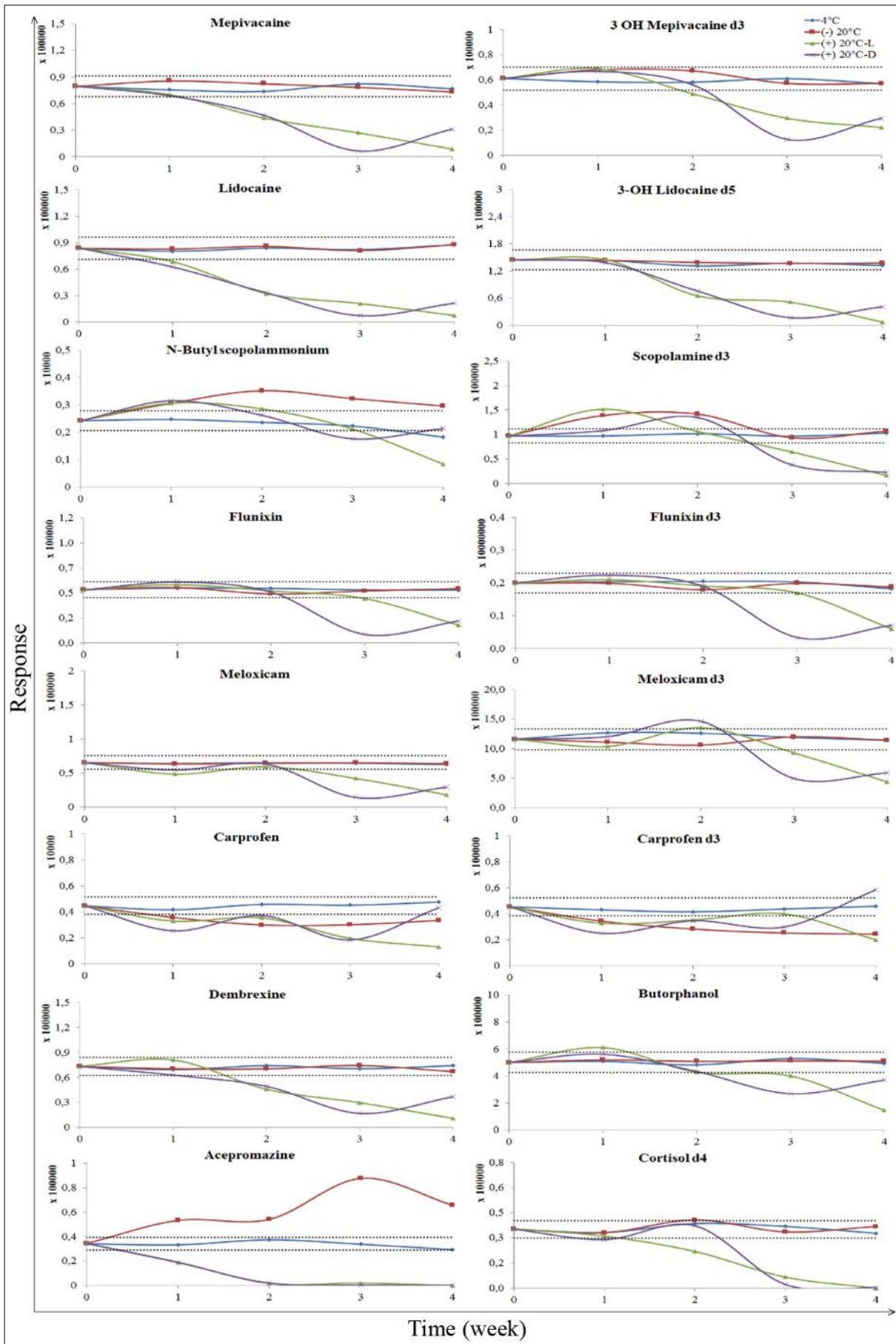


Figure 3. Nine ISL substances and their IS's stability results in blood matrix.

Table 4. The effects of different storage conditions on stability after four weeks.

Substances	Matrix (mean±SD) (ng/mL)				Working Solution (mean±SD) (ng/mL)					
	Initial	+4°C	-20°C	+20°C-Light	+20°C-Dark	Initial	+4°C	-20°C	+20°C-Light	+20°C-Dark
MEP	0.057±0.008	0.062±0.001	0.063±0.002	0.029±0.001 ^a	0.034±0.001 ^a	0.050±0.001	0.051±0.001	0.051±0.001	0.052±0.001	0.052±0.001
LID	0.043±0.005	0.04±0.001	0.043±0.001	0.017±0.002 ^a	0.024±0.001 ^a	0.051±0.002	0.051±0.001	0.051±0.001	0.051±0.001	0.051±0.001
DEM	3.067±0.142	3.016±0.077	2.992±0.025	0.02±0.001 ^a	0.05±0.001 ^a	5.198±0.169	5.108±0.038	5.104±0.050	5.104±0.129 ^a	5.222±0.069
SCO	0.066±0.006	0.047±0.003 ^a	0.076±0.001 ^a	0.035±0.004 ^a	0.059±0.001 ^a	0.049±0.001	0.051±0.001	0.050±0.001	0.035±0.001 ^a	0.050±0.001
BUT	0.008±0.001	0.008±0.001	0.008±0.001	0.001±0.000 ^a	0.005±0.000 ^a	0.010±0.001	0.010±0.001	0.011±0.001	0.011±0.001	0.011±0.001
ACE	0.015±0.001	0.016±0.001	0.025±0.003 ^a	0.008±0.005 ^a	0.005±0.001 ^a	0.019±0.001	0.020±0.001	0.021±0.001	0.020±0.001	0.020±0.001
FLU	0.991±0.010	0.99±0.011	0.995±0.005	0.738±0.055 ^a	0.005±0.001 ^a	1.018±0.055	1.022±0.021	1.031±0.016	1.032±0.009	1.050±0.023
MEL	1.008±0.069	1.015±0.009	1.015±0.013	0.615±0.046 ^a	0.707±0.037 ^a	0.978±0.012	1.001±0.017	1.001±0.012	1.007±0.021	1.036±0.021
CAR	107.430±12.180	98.592±3.215	72.473±1.874 ^a	33.500±5.168 ^a	85.746±1.686 ^a	103.188±8.020	108.325±4.961	109.825±3.483	108.173±2.917	107.583±2.884

^a Significant difference (P < 0.05) when compared to the initial data mean.

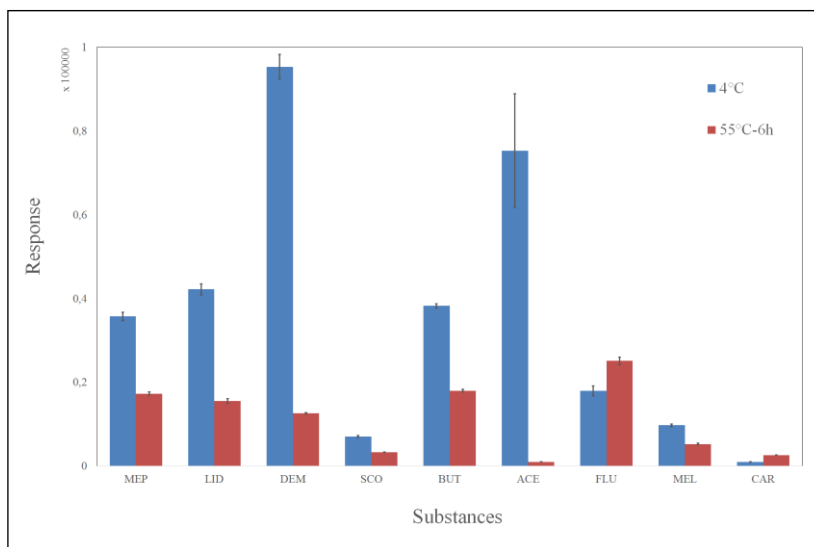


Figure 4. Short-term stability of ISL substances.

Discussion and Conclusion

The stability knowledge of standards used as references and working solutions at different storage conditions is requisite to check the analysis method and to compare the spiked sample with real sample. For this reason, the stability of both working solution and internal standards used in this study was examined and compared unlike previous studies. Results of this study found out that substances in working solution were stable and matrix effects were clearly occurred related to storage temperature and time. But, at 4th week N-Butylscopolammonium in working solution was degraded at +20°C in light. Van der Merwe et al. (22) similarly reported that substances can be affected by light.

In blood matrix stability study, reproduction of microorganisms has occurred in the samples kept in +20°C light and dark after 2nd week. The stability of the substances significantly degraded in +20°C conditions in a short time like other studies on urine and blood (19, 22, 24). It could be a result of thermal or microbiological degradation (11). Considering the short-term stability study, the samples have a negative effect if the samples are above +20°C, hence the results may be misleading.

There were deviations from the criterion value ($\pm 15\%$) at -20°C for Acepromazine, Carprofen and N-Butylscopolammonium. Acepromazine showed a negligible increase in concentration overtime during the storage at -20°C (Figure 3). This effect could be re-investigated. The materials used as internal standard are showed similar effects. These results demonstrated that blood samples should not be frozen as indicated on legislation (7). The previous study for 10-week stability in the blood, a decrease of Carprofen (30% at -20°C) and Meloxicam (70% at +20°C) occurred similar to our study, and it was stated that the time between the analyses of A

and B samples was 4 weeks as a proposal (19). However, the instability of N-Butylscopolammonium after the 3rd week, some antipsychotic drugs after 1st week (17) and diuretics after 4th week (24) showed that the analysis time between sample A and B should be kept as short as possible because the substances were differently affected at storage conditions.

Short-term stability demonstrated the high temperature decreased all ISL substances except Flunixin and Carprofen (24). Schenk et al. (19) suggested that the temperature may increase some substances with protein participation. So, the increase of Flunixin and Carprofen might be related to thermo-hydrolysis of proteins in blood matrix.

The best of our knowledge, this is the first report about the stability of ISL substances based on limit value in whole equine blood related to different temperatures, time and light conditions. It was presented the effects of temperature and time on substances in matrix by the comparison of working solution and matrix stabilities. In accordance with results, samples might be stored and transported at +4°C in dark condition and immediately analyzed to avoid false negative/positive results. Future studies about the stability of doping agents may be increased for correct interpretation.

Acknowledgements

This study was supported by funds from the Republic of Turkey Ministry of Agriculture and Forestry, İstanbul Pendik Veterinary Control Institute.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Atanasov VN, Stoykova S, Runiov A, et al** (2012): *Stability of diazepam in blood samples at different storage conditions and in the presence of alcohol*. *Forensic Sci Int*, **215**, 159-163.
2. **Clauwaert KM, Van Bocxlaer JF, De Leenheer AP** (2001): *Stability study of the designer drugs "MDA, MDMA and MDEA" in water, serum, whole blood, and urine under various storage temperatures*. *Forensic Sci Int*, **124**, 36-42.
3. **Commission Decision** (2002): Concerning the performance of analytical methods and the interpretation of results. European Commission, 2002/657/EC.
4. **Dugan S, Bogema S, Schwartz RW, et al** (1994): *Stability of drugs of abuse in urine samples stored at -20°C*. *J Anal Toxicol*, **18**, 391-396.
5. **Giorgi SN, Meeker JE** (1995): *A 5-year stability study of common illicit drugs in blood*. *J Anal Toxicol*, **19**, 392-8.
6. **Hippenstiel MJ, Gerson B** (1994): *Optimization of storage conditions for cocaine and benzoylecgonine in urine: a review*. *J Anal Toxicol*, **18**, 104-109.
7. **International Federation of Horseracing Authorities** (2019): International agreement on breeding, racing and wagering and appendixes (February 2019). <https://www.ifhaonline.org/resources/ifAgreement.pdf> (14 February 2019).
8. **Jane Ellis M, Livesey JH, Evans MJ** (2003): *Hormone stability in human whole blood*. *Clinical Biochem*, **36**, 109-112.
9. **Jiménez C, Ventura R, Segura J, et al** (2004): *Protocols for stability and homogeneity studies of drugs for its application to doping control*. *Anal Chim Acta*, **515**, 323-331.
10. **Jiménez C, de la Torre R, Segura J, et al** (2006): *Stability studies of testosterone and epitestosterone glucuronides in urine*. *Rapid Commun Mass Spectrom*, **20**, 858-864.
11. **Jiménez C, de la Torre R, Ventura M, et al** (2006): *Stability studies of amphetamine and ephedrine derivatives in urine*. *J Chromatogr B Analyt Technol Biomed Life Sci*, **843**, 84-93.
12. **Kita K, Ishii T, Hotta K, et al** (2018): *A dried blood spot assay with UPLC-MS/MS for the simultaneous determination of E6005, a phosphodiesterase 4 inhibitor, and its metabolite in human blood*. *J Pharm Biomed Anal*, **157**, 208-216.
13. **Moody DE, Monti KM, Spanbauer AC** (1999): *Long-term stability of abused drugs and antiabuse chemotherapeutical agents stored at -20°C*. *J Anal Toxicol*, **6**, 535-40.
14. **Moulard Y, Bailly-Chouriberry L, Boyer S, et al** (2011): *Use of benchtop exactive high resolution and high mass accuracy orbitrap mass spectrometer for screening in horse doping control*. *Anal Chim Acta*, **700**, 126-136.
15. **Peris-Vicente J, Esteve-Romero J, Carda-Broch S** (2015): *Validation of analytical methods based on chromatographic techniques: An overview*. 1757-1808. In: LA Jared, A Berthod, VP Estévez, AM Stalcup (Ed), *Analytical Separation Science*. Wiley-VCH Verlag GmbH & Co. KGaA, Germany.
16. **Popot MA, Garcia P, Hubert C, et al** (2014): *HPLC/ESI-MS(n) method for non-amino bisphosphonates: application to the detection of tiludronate in equine plasma*. *J Chromatogr B Analyt Technol Biomed Life Sci*, **958**, 108-116.
17. **Saar E, Gerostamoulos D, Drummer OH, et al** (2012): *Assessment of the stability of 30 antipsychotic drugs in stored blood specimens*. *Forensic Sci Int*, **215**, 152-158.
18. **Saudan C, Entenza JM, Baume N, et al** (2006): *Short-term stability of testosterone and epitestosterone conjugates in urine samples: quantification by liquid chromatography-linear ion trap mass spectrometry*. *J Chromatogr B Analyt Technol Biomed Life Sci*, **844**, 168-174.
19. **Schenk I, Machnik M, Guddat S, et al** (2008): *Stability of a representative selection of drugs in horse blood*. 225-235. In: E Houghton, FI Keskin, JF Wade, N Yazıcıoğlu (Ed), *Proceeding of the 17th International Conference of Racing Analysts and Veterinarians*, Antalya, Turkey.
20. **Shah VP, Midha KK, Dighe S, et al** (1992): *Analytical methods validation: Bioavailability, bioequivalence, and pharmacokinetic studies*. *J Pharm Sci*, **81**, 309-312.
21. **Tevis DS, Jarrett JM, Jones DR, et al** (2018): *Assessing the stability of Cd, Mn, Pb, Se, and total Hg in whole human blood by ICP-DRC-MS as a function of temperature and time*. *Clin Chim Acta*, **485**, 1-6.
22. **Van der Merwe PJ, Pieterse JW** (1994): *Stability of zeranone, nandrolone and trenbolone in bovine urine*. *Analyst*, **119**, 2651-2653.
23. **Van der Merwe PJ, Grobbelaar E** (2003): *Stability of 19-norandrosterone and 19-noretiocholanolone glucuronide in urine under various storage conditions*. *SA Sports Medicine*, **15**, 14-17.
24. **Van Eenoo P, Lootens L, Spaerkeer A, et al** (2007): *Results of stability studies with doping agents in urine*. *J Anal Toxicol*, **31**, 543-548.
25. **Ventura R, Jiménez C, Closas N, et al** (2003): *Stability studies of selected doping agents in urine: caffeine*. *J Chromatogr B Analyt Technol Biomed Life Sci*, **795**, 167-177.
26. **Wong JK, Wan TS** (2014): *Doping control analyses in horseracing: a clinician's guide*. *Vet J*, **200**, 8-16.