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Quantification of caffeine in horse urine and saliva by gas chromatography and ELISA

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Resumo

Quantificação de cafeína em urina e saliva eqüina por cromatografia a gás e teste ELISA.

Foram pesquisados resíduos organoclorados em sangue humano, colhido de 122 indivíduos. Foram compostos dois grupos: um urbano e outro residente em áreas de intensa atividade agrícola. Os resíduos organoclorados pesquisados foram: α - HCH; γ - HCH; Aldrin; Dieldrin; Endrin; Heptacloro; Heptacloro epóxido; HCB; MIrex; o,*p*´DDD; o,*p*´DDT; Oxiclordane; p,*p*´Metoxicloro; p,*p*´DDD; p,*p*´DDE; p,*p*´DDT e Transnonacloro (limite de detecção = 0,010μg/L). Todos os indivíduos responderam a questionário com quesitos sobre idade, sexo, peso, atividade, local de residência, contato ou não praguicidas. Todas as amostras urbanas foram negativas para organoclorados analisados. 10,5% das amostras obtidas nas áreas de atividade agrícola foram positivas. Os resultados das análises do presente trabalho foram comparados com resultados pré-existentes no Rio Grande do Sul. Entre a pesquisa atual e a pesquisas anteriores usadas para comparação, verificou-se diminuição da incidência de organoclorados.

Unitermos: Resíduos organoclorados, sangue.

Abbreviations: ELISA: enzime-linked immunosorbent assay; GC: gas chromatography; NPD: nitrogen-phosphorus detector; IV: intravenous; N2: nitrogen.

Introduction

Caffeine became a powerful choice used with the purpose of increasing cardiopulmonar parameters, not only in humans but in animals, due its central stimulation activity (KUROSAWA *et al*., 1999). As it was already in the list of forbidden substances for the World Anti Doping Agency, studies were done to evaluate the caffeine concentration in vitamin and energy supplements in the attempt of discerning accidental from intentional doping (SALVADORI *et al*., 1994; KAMBER *et al*., 2001). In veterinary sports medicine, caffeine is forbidden by Federation Equestrian International, although studies with this substance continue being accomplished in equine (CARREGARO *et al*., 2004; SAVAGE *et al*., 2005).

To evaluate caffeine and other doping agents, detection methods have become increasingly more sophisticate and accurate. Methods usually employed to quantify caffeine in biological specimens are gas chromatography (CG) and high-performance liquid chromatography (HPLC), both time consuming and expensive (SALVADORI *et al*., 1988). Therefore, it was of interest to apply methods capable of minimizing the assay time and cost without losing quality.

Thin-layer chromatography (TLC) was the first technique employed as screening test for quantification of substances in biological matrices in horses (TOBIN, 1981). However, the only advantage was the cost, since the delay in the procedure and the high incidence of false negatives questioned its use. This was particularly the case when potent pharmacons came into use such as anesthetics, stimulants and narcotics, which produce effects even at very low concentrations.

Later, immunofluoresence tests were utilized due to their relatively high sensitivity and rapid results (TOBIN,

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1988). However, the difficulty in obtaining kits, which required their development, and the need for sample extraction and the use of a fluorimeter led to this technique being replaced by immunoenzymatic assays, which are now widely used in various lines of research and testing.

Enzime-linked immunosorbent assay (ELISA) has been utilized routinely as a pre-selection step in drug assays (THURMAN *et al*., 1990; YEUNG & NEWSOME, 1993; SAWAYA *et al*., 1998 CARREGARO *et al*., 2001). Moreover, it is starting to be used as the only test in drug testing because of its practicality and low cost (EDGAR *et al*., 1996; IL'YASOVA *et al*., 2004). In view of this, ABAD and colleagues (1993) compared the results obtained with ELISA and GC in the determination of nicotine and found a correlation coefficient (r) of 0.88.

In preliminary studies, it was possible to compare GC and ELISA for the determination of caffeine in human plasma, whereby an r of 0.82 was obtained (CARREGARO *et al*., 2001). It was also shown that samples diluted close to the midpoint of the ELISA standard curve (I_{50}), that is, where there is an inhibition of 50% of the maximum absorbance of the colorimetric reaction, give the best correlation. The caffeine levels determined by ELISA were higher than those by GC, probably as a result of caffeine metabolites and of other methylxanthines from human nutrition feed which was not controlled in these experiments. The immunoenzymatic assay does not discriminate the above compounds and is thus susceptible to cross-reactions.

The aim of the present study was to compare GC and ELISA methods in the determination of caffeine in urine, a classical matrix, and saliva, due the facility and noninvasive technique to obtain it, from horses fed with caffeine or any other methylxanthines free meals.

Material and Methods

Seven thoroughbred mares were utilized in the study. They weighed between 380 and 550 kg, and were maintained on pasture grass (*Cynodon dactylon*) supplemented with commercial ration, mineral salt and coast-cross hay (*Cynodon sp*), all known to be devoid of methylxanthines. A dose of 2 mg/kg of caffeine (Sigma Chem. Co., USA) IV was administered diluted in 10 ml of saline solution. Urine and saliva samples were collected, in duplicate, before the administration of caffeine and at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h and 72 h post-administration. A pharmacokinetic study of this drug was carried out in parallel.

For the determination of caffeine by GC, samples were extracted according to the method recommended by GREENE and colleagues (1983). Standard solutions of caffeine were diluted in methanol to establish a standard curve using 5 known concentrations (0.2, 1, 5, 10 and 20 µg/ml) which served as a reference for the assay of samples.

Samples (500 µl) were placed in assay tubes to which 500 µl of deionized water, 1ml of saturated sodium chloride solution and 2ml of dichloromethane were added. The tubes were agitated for 4 min and centrifuged for 1 h at 4 °C, at 626 g. After centrifugation, the organic phase was separated and dried with the help of a stream of N_2, in a warm water bath at 35 °C. Subsequently, the residue was reconstituted with 50 µl of methanol and injected into a chromatograph with a split less injector at 230 °C. The initial temperature of the capillary column was 150 °C and the final 280 °C (20 °C/min). The GC (CP-8400. Varian Ind. Com. Ltda., Brazil) used was equipped with a nitrogen-phosphorus detector (NPD). Hydrogen was used at a rate of 4.7 ml/min and air at a rate of 167 ml/min. The polymer capillary column (CP SIL 5CB) 30 m X 530 µm X 1 m received a constant flow of helium at a rate of 7 ml/min. The injection volume was 1 µl.

The ELISA method was performed using kits (Neogen Corporation, Lexington, USA) specific for caffeine, according to EDGAR and colleagues (1996). At duplicate, 20 µL of standard solution were added to each well to establish standard solutions which were prepared in buffer at concentrations of 1, 5, 10, 50 and 100 ng/ml. In the same way, 20 µL of the samples were added to each well. Afterward, 180 µl of drug-enzyme complex were added, diluted 1:180. The solution formed was homogenized lightly for 1min and incubated at room temperature for 45 min. After this period, the liquid was discarded and the plate completely dried. All the wells were washed three times with 300 µl of washing buffer. Subsequently, 150 µl of substrate were added and the plate was agitated for 30 min at room temperature. The reactions were determined with the use of a plate reader (Multiscan Ascent. Labsystem Rec. Tech., Finland) with a light filter for a wavelength of 650 nm.

The results of the two methods were compared by Pearson's correlation coefficient (r), according to CALLEGARI-JACQUES (2003), utilizing the GraphPad Instat (GraphPad Software Inc.) program.

Results

The standard curves obtained with GC were accepted when at least four out of the five dilutions used fit a linear regression curve with r^2 equal to or higher than 0.999. To determine the efficacy of the extraction method, a recovery test was performed with 10 samples with a known concentration of caffeine (1 µg/ml). Recovery of the drug from urine was 94 % and from saliva 91 %. Concentrations obtained in urine and saliva were therefore multiplied by the correction factor 1.06 and 1.09, respectively. To ELISA, the validate method was similar to anterior, with r^2 specified to minimum 0.99.

Most of the ELISA results were higher than those determined by CG. To determine the correlation between the two methods, all the samples were assayed by ELISA and the results compared to those obtained with GC. After assaying 300 samples, including 160 of urine and 140 of saliva, an r was obtained for the two methods of 0.53, whereby r^2 was 0.28 ($y = 1.576 + 0.846x$) (Figure 1). The results were considered valid when the I_{50} of the standard

curve was up to 5 times the value of the sample I (I_S); otherwise, they were diluted until they were within the maximum limit.

In an attempt to obtain a higher correlation, the total samples (n) were divided into blocks, based on the ratio of the I_{50} of the standard curve and the corresponding I_S. The number of samples in each block was also kept about the same. Therefore, the samples were grouped into 5 blocks with about 60 samples each.

The first block (n = 60), with a ratio between I_{50} and I_S (the inhibition caused by the sample) of up to 0.4, yielded an r of 0.70 with an r^2 of 0.49 (y = 2.135 + 1.334x). The samples of 0.41 to 0.60 (n = 58) showed an r of 0.75 with an r^2 of 0.56 (y = 1.749 + 1.3x). However, the block containing values with ratios of 0.61 to 0.90 (n = 64) showed an r of 0.79 with an r^2 of 0.62 (y = 0.955 + 1.889x). For samples with ratios of 0.91 to 1.50 (n = 57), r was 0.70 with an r^2 of 0.49 (y = 1.156 + 0.855x). The other results within the interval of 1.51 to 5.0 (n = 61), showed an r of 0.69 with an r^2 of 0.47 (y = 0.645 + 0.557x).

Despite that all the blocks showed a significant correlation coefficient, the samples with ratios between 0.6 and 0.9 had a better correlation. Therefore, it was decided to divide them, again, into smaller intervals to see if the r between the two methods would increase. The results that yielded a ratio of 0.5 to 1.0 were therefore divided into 5 blocks.

The block with a ratio between 0.5 and 0.59 (n = 26) had samples with an r of 0.75 and r^2 of 0.56 (y = 1.668 + 1.055x). The samples ratios of 0.60 to 0.69 (n = 23) showed an r of 0.85 with an r^2 of 0.72 (y = 0.844 + 2.019x). However, the block containing values with ratios of 0.70 to 0.79 (n = 25) yielded an r of 0.83 with an r^2 of 0.70 (y = 0.781 + 2.12x). For the interval of 0.80 to 0.89 (n = 15), r was 0.71 with an r^2 of 0.51 (y = 1.264 + 1.631x). The results for the block with ratios of 0.90 to 0.99 (n = 20) showed an r of 0.59 with an r^2 of 0.35 (y = 1.092 + 1.582x) (Table 1).

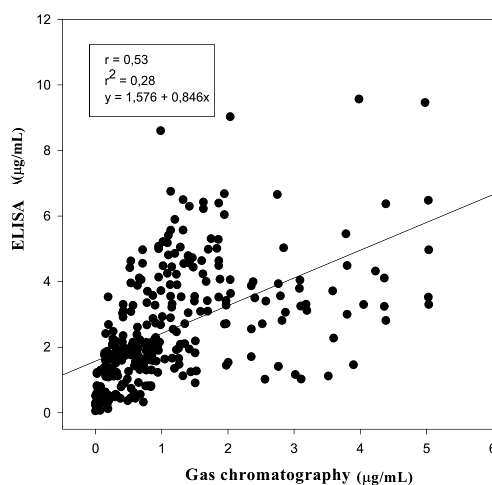

Figure 1. Regression curve between the gas chromatography and ELISA used to quantify caffeine in horses urine and salive samples, in g/mL.

Table 1- Correlation coefficient (r), linear regression coefficient (r^2) and linear regression equation y= a + bx) of the samples of biological matrices of Thoroughbred horses for the determination of caffeine by gas chromatography and ELISA. The blocks were arranged based on the ratio between I_{50} obtained in the standard curves of ELISA and I of the samples, corresponding to each curve. ** p < 0.05

INTERVAL	n	r	r^2	Y = a + bx
0.5 ├── 0.6	26	0.75**	0.56	Y = 1.668 + 1.055x
0.6 ├── 0.7	23	0.85**	0.72	Y = 0.844 + 2.019x
0.7 ├── 0.8	25	0.83**	0.70	Y = 0.781 + 2.120x
0.8 ├── 0.9	15	0.71**	0.51	Y = 1.264 + 1.631x
0.9 ├──┤ 1.0	20	0.59**	0.35	Y = 1.092 + 1.582x

Discussion

The correlation between the two methods studied showed to be positive, albeit discrete. According to CALLEGARI-JACQUES (2003), r varies between -1 and +1. Values between 0 and +1 indicate a positive correlation. However, to consider the two treatments as showing a good positive correlation, an r value above 0.70 is required.

Preliminary studies have been conducted comparing GC and ELISA in the quantification of caffeine concentrations in human plasma samples (CARREGARO *et al.*, 2001). The r value obtained (0.82) demonstrated a good correlation between the two methods. The samples diluted close to the I50 of the standard curve showed higher correlation. However, it was shown that measurements obtained with ELISA were consistently higher than those determined by GC, which was the same as the finding reported by LI and colleagues (1989). The plausible explanation for this finding could be susceptibility to cross-reaction.

THURMAN and colleagues (1990) compared ELISA to GC-MS in the determination of thiazine herbicides in water samples and obtained an r value of 0.91. Nonetheless, the authors pointed out the fact that some thiazines are more detectable when samples are diluted to below the 50% level of absorbance for the ELISA standard curve. Conversely, others have shown a greater correlation when dilution of the samples is above the I_{50} of the standard curve. This could be explained by the reaction of the immunoenzymatic assay, which contains haptens utilized for the production of antibodies with a molecular conformation resembling some thiazines. Therefore, depending on the concentration of each substance, determined by the dilution, antibodies may or may not be activated more, resulting in cross-reaction. Meanwhile, it is noted that ELISA should be considered as good quantification method when combined with GC, because not only is it economical, but it can also be performed on a small volume of sample which is easy to transport.

In the present study, after determining the r between the two methods of quantification, the samples were grouped into blocks according to ratio between I_{50} of the standard curve obtained with ELISA and the IS corresponding to the samples. An increase in correlation coefficient was thereby shown in samples where the ratio fluctuated between 0.6 and 0.9, where an r value of 0.79 was considered good according to CALLEGARI-JACQUES (2003). Based on this rationale, the samples with ratios of 0.5 to 1.0 were divided into sections. In this situation, the r values obtained between the groups differed more than in the previous case. In this manner, the correlation between samples with ratios of 0.9 to 1.0 (r = 0.59) can be rejected. However, the results with ratios between 0.6 and 0.7 and 0.7 and 0.8 showed higher indices of correlation of 0.85 and 0.83, respectively. In comparison with studies previously cited, the results were shown to be somewhat similar.

It should be noted that in this study the biological samples were not submitted to any treatment that could alter the substances present, unlike in the study by YEUNG & NEWSOME (1993). EDGAR and colleagues (1996) cited the possibility of cross reactions in the kits formulated to determine caffeine, since some of the metabolites of this drug are isomers or differ only by the number of methyl groups.

Due to simplicity and economy, ELISA can be used for caffeine quantification in the discussed matrices since it presented good correlation with GC. It is plausible that the higher correlation coefficients obtained here compared to that reported to date, could result from improving the dilution of the samples, attempting to approximate the ratio with the best correlation.

In addition, all the methylxanthines could be converted to caffeine by the addition of new methyl groups, eliminating thus the possibility of cross reactions, which would also alter quantification by GC permitting a more reliable comparison.

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Resumo

O teste ELISA tem sido empregado como teste de pré-seleção na detecção de substâncias utilizadas como antidoping. Todavia, ultimamente tem sido utilizado como única técnica de quantificação em vários países, uma vez que proporciona resultados rápidos e com custo reduzido. O estudo objetivou avaliar a precisão do teste ELISA na quantificação de cafeína tanto em urina quanto em saliva eqüina, comparando-se os valores desse teste aos obtidos em cromatografia a gás (CG). Para o teste ELISA, as amostras foram diluídas para a obtenção de uma concentração próxima ao ponto médio da curva-padrão (I_{50}), correspondente a 50% de absorbância, e posteriormente comparadas aos valores da CG. Para determinar se a proximidade entre o I_{50} da curva-padrão e o I da amostra (I_a) fornece valores mais precisos, as mesmas foram divididas em cinco grupos, dispostos de acordo com a razão entre I_{50}/I_a. As amostras com razão entre 0,6 a 0,7 demonstraram forte correlação (r = 0,85). A possibilidade de reação cruzada no teste ELISA com outras metilxantinas não pode ser descartada, uma vez que as concentrações foram superiores às obtidas por CG.

Unitermos: cafeína, ELISA, cromatografia a gás, eqüino.

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inglês, submetidos ou não à acidificação urinária. Comparação entre as técnicas de quantificação por cromatografia a gás e teste Elisa, in 2002, FCAV - UNESP - Campus de Jaboticabal.

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