

# Comparison of the quantification of caffeine in human plasma by gas chromatography and ELISA

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## Abstract

In the present study we evaluated the precision of the ELISA method to quantify caffeine in human plasma and compared the results with those obtained by gas chromatography. A total of 58 samples were analyzed by gas chromatography using a nitrogen-phosphorus detector and routine techniques. For the ELISA test, the samples were diluted to obtain a concentration corresponding to 50% of the absorbance of the standard curve. To determine whether the proximity between the  $I_{50}$  of the standard curve and that of the sample would bring about a more precise result, the samples were divided into three blocks according to the criterion of difference, in modulus, of the  $I_{50}$  of the standard curve and of the  $I_{50}$  of the sample. The samples were classified into three groups. The first was composed of 20 samples with  $I_{50}$  up to 1.5 ng/ml, the second consisted of 21 samples with  $I_{50}$  ranging from 1.51 to 3 ng/ml, and the third of 17 samples with  $I_{50}$  ranging from 3.01 to 13 ng/ml. The determination coefficient ( $R^2 = 0.999$ ) showed that the data obtained by gas chromatography represented a reliable basis. The results obtained by ELISA were also reliable, with an estimated Pearson correlation coefficient of 0.82 between the two methods. This coefficient for the different groups (0.88, 0.79 and 0.49 for groups 1, 2 and 3, respectively) showed greater reliability for the test with dilutions closer to  $I_{50}$ .

## Key words

- Gas chromatography
- ELISA
- Caffeine
- Plasma
- Quantification

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Caffeine is one of the most frequently consumed substances in the world; it is present in coffee-, chocolate- and cola-based beverages (1). Together with theophylline and theobromine, it belongs to the xanthine group. The methylxanthines exert a pharmacological function in various systems, such as the central nervous system, causing anxiety and fear (2), stimulation of the cardiovascular system, leading to tachycardia and ar-

rhythmia (3) and relaxation of the smooth musculature (4).

Caffeine is quickly absorbed, reaching its maximum concentration within 1 h, and is metabolized by the liver. Its half-life is 3 to 7 h. The main metabolite is paraxanthine, which represents 95% of the elimination form of the substance (5).

Caffeine is employed therapeutically in cases of asthma and chronic obstruction of

the lungs. However, it also has toxic effects. After ingesting approximately 15 mg/kg, a person may show severe alterations in the nervous and circulatory system, such as sleeplessness, anxiety, delirium, vomiting and convulsions (6). Another important characteristic is physical dependence, commonly observed after daily ingestion of at least 400 mg of caffeine over a period of two weeks. Moreover, daily ingestion of 250 mg during a long period of time can also lead to dependence (7). Migraine, fatigue, lethargy and sleeplessness are the main symptoms which can be observed up to 48 h after intake (8).

Physical dependence is more common than assumed. To investigate this hypothesis, plasma samples from patients of the Psychiatric Hospital of the University of Kentucky were quantified for caffeine. The methods usually employed to quantify caffeine in biological specimens are gas chromatography and high-performance liquid chromatography, which are time consuming and expensive (9). On the other hand, the ELISA method is of easy and rapid execution and inexpensive (10).

The objective of the present study was to compare the results obtained with gas chromatography and ELISA to determine the precision of the latter method for caffeine quantification. Caffeine was quantified in 64 human plasma samples originating from the University of Kentucky Hospital. All samples arrived frozen and were maintained thus throughout the experiment, being thawed only to collect the aliquot to be used. For the gas chromatography method (11), standard caffeine (Sigma Chemical Co., St. Louis, MO, USA) solutions were prepared in methanol (Mallinckrodt Inc., Paris, KY, USA) in order to establish the standard curve on the basis of five known concentrations (50, 100, 250, 500 and 1,000 ng/ml of plasma). Each sample received 1,000 ng/ml of cyclizine (Sigma), this being the internal control standard. The plasma samples and the standard curve samples were placed in test tubes (100

µl/sample) in duplicate. Nine hundred microliters of deionized water, 1 ml of saturated sodium solution and 2 ml of dichloromethane (Sigma) were added to each tube. The samples were shaken for 4 min and centrifuged for 1 h at 626 g and 4°C. The organic phase was dried under a nitrogen stream at 35°C. The samples were then reconstituted with 50 µl of methyl alcohol, shaken for 15 s and submitted to a gas chromatograph (Varian Star 3400CX - Varian Analytical Inst., Palo Alto, CA, USA) equipped with a nitrogen-phosphorus detector. Hydrogen at a flow of 4.7 ml/min and air at a flow of 167 ml/min were used as the mobile phase. The apparatus contained a 15 m x 530 µm x 1 µm polymer capillary (Megabore DB17; J & W Scientific, Folsom, CA, USA), which received a constant flow of helium at the rate of 21 ml/min. The initial temperature of the capillary was 70°C and the final temperature 280°C.

The measurement of caffeine by ELISA is based on a previously described methodology (10). Each ELISA well received 20 µl of the sample or of the internal standard solution, and 180 µl of the drug-enzyme complex diluted 1:180. The solution was lightly mixed for 1 min and incubated at room temperature for 45 min. Each well was washed three times with 300 µl of washing buffer. The next step consisted of the addition of 150 µl of substrate. The plate was shaken for 30 min at room temperature and absorbance at 650 nm was measured with a microwell reader (Bio-Tek Inst., Lake Havasu City, AZ, USA).

The 64 samples of human plasma were first examined using gas chromatography, considered to be the standard method to quantify this drug. The identification of caffeine and cyclizine was confirmed on the basis of the chromatograms of the internal standard which contained only methyl alcohol, caffeine and cyclizine. Only 6 of the 58 samples did not conform to the standard curve, i.e., their concentrations were below

50 ng/ml, which was the minimum concentration of the standard curve. Thus, in these cases the samples were disregarded for the purpose of statistical analysis.

With the objective of developing a rapid and economic method, we quantified the samples again by ELISA. The standard curve was obtained with five dilutions of 1 to 100 ng/ml of plasma. The samples were diluted so as to show a value close to  $I_{50}$  (concentration which determines 50% inhibition of the maximum absorbance obtained with the standard curve of the experiment). The average  $I_{50}$  of the experiment was 7.27 ng/ml, quite close to the  $I_{50}$  determined by the manufacturer (Neogen Corporation, Lexington, KY, USA), of 6 ng/ml.

As can be seen in Figure 1, the methods were compared by the Pearson correlation coefficient ( $r$ ) (12), which showed a value of  $r = 0.82$  ( $P < 0.01$ ) (SAS System). The equation that describes the curve was  $y = 1855.39 + 1.02x$  ( $R^2 = 0.82$ ). To determine whether the proximity between the  $I_{50}$  of the standard curve and that of each sample would lead to a more precise result for ELISA, the samples were divided into three blocks according to the criterion of sample  $I_{50}$  deviation from the  $I_{50}$  of the standard curve (Figure 2). The first block, consisting of 20 samples which deviated from the  $I_{50}$  by 0 to 1.5 ng/ml, obtained an  $r = 0.88$  ( $P < 0.01$ ). The second block, consisting of 21 samples which deviated from the  $I_{50}$  by 1.51 to 3 ng/ml, showed an  $r = 0.79$  ( $P < 0.01$ ). The third block, consisting of 17 samples which deviated from the  $I_{50}$  by more than 3.0 ng/ml, showed an  $r = 0.49$  ( $P < 0.05$ ).

The results obtained by gas chromatography demonstrate that this method is good for the quantification of the substance. This is borne out by the determination coefficients ( $R^2$ ) of the standard curves, always above 0.999, certifying the precision of the method for extraction as well as for analysis.

The use of cyclizine as internal standard was also advantageous, as demonstrated by

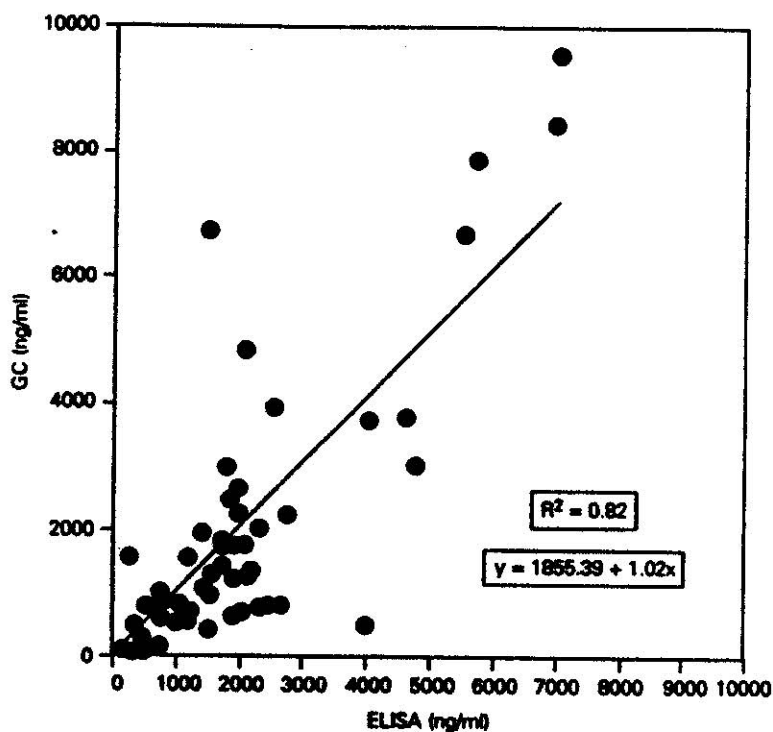


Figure 1. Regression curve between the gas chromatography (GC) and ELISA methods used to quantify caffeine in human plasma, in ng/ml.

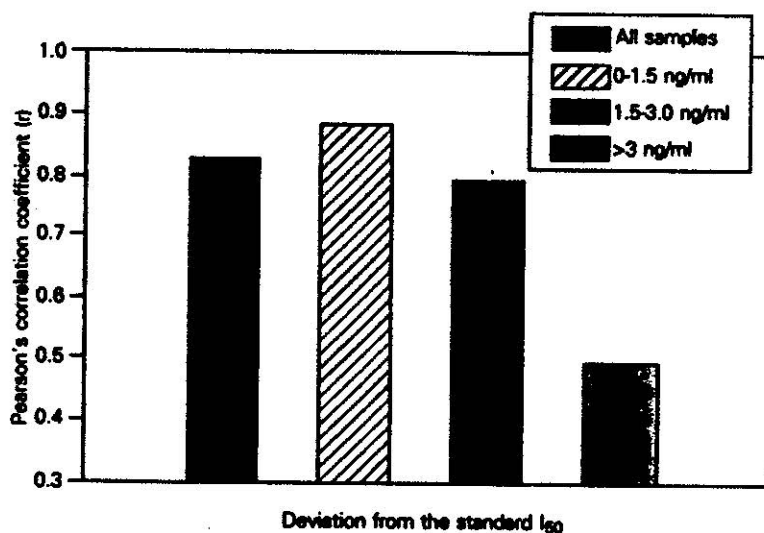


Figure 2. Pearson's correlation coefficient between the results obtained by gas chromatography and by ELISA to determine caffeine concentration in 58 human plasma samples. The blocks express the deviation from the standard  $I_{50}$  (in modulo).

repetitiveness and acuity tests. For the former, only one sample of known concentration was examined 10 times. The coefficient of variation obtained without the internal correction factor (cyclizine) was 20.81%. After adjusting the results obtained, the value fell to 6.3%. For the latter, 10 samples of identical and known concentrations were examined. The coefficient of variation was 13.61% without correction and was reduced to 10% after correction. This proves the necessity of using cyclizine as an internal standard. Another important conclusion is the repetitiveness and acuity of the method, now also established by these results, which were found to be below the 15% level determined elsewhere (13) as the maximum for a reliable analysis.

The ELISA proved to be adequate since the  $R^2$  was always higher than 0.99. However, most of the concentrations obtained by ELISA were higher than those obtained by gas chromatography on the basis of pair comparison. This can be explained by the fact that the antibody of the plate allows a cross-reaction with other methylxanthines such as theobromine or theophylline. The

metabolites of caffeine can also modify the intensity of the reaction.

Statistical analysis of the data shows that the two methods present a good correlation. Separating the samples tested by ELISA into blocks, it was noted that variations between the  $I_{50}$  of the standard curve and the  $I_{50}$  of the sample above 3 ng/ml no longer represented a reliable quantitative result. On the other hand, we conclude that the use of ELISA to quantify caffeine in human plasma, with dilutions close to the  $I_{50}$ , i.e., with a difference, in modulo, of up to 3 ng/ml, can be considered a reliable, simple, economic and fast option for screening analysis or even for quantification assays. These results corroborate observations which recommend ELISA tests for the determination of doping in horses (14).

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