Asian Journal of Chemistry Vol. 22, No. 5 (2010), 3608-3614

A New Caffeine Test for Diagnosis of Cirrhosis by GC/MS

C. MESAROS†, M. CULEA*, A.M. IORDACHE*‡, I. VISOVAN, C. COZAR and C. COSMA
Department of Biomedical Physics, Babes-Bolyai University, Cluj-Napoca-400084, Romania
E-mail: mculea@phys.ubbcluj.ro; andres_iro2002@yahoo.com

A method for caffeine levels determination in children suffering from liver diseases by gas chromatography-mass spectrometry (GC/MS) is presented. The selected ion monitoring (SIM) mode was used in caffeine test measurements by adding known amounts of the ^{15}N labelled internal standard. The method was validated in the range 0-20 μg/mL caffeine. A single dose of 4 mg/kg p.o. of caffeine was followed by blood concentrations measurements at two points, 1 and 9 h. Caffeine clearance, 0.74 ± 0.49 mL min^{-1} kg^{-1}, measured in patients with cirrhosis was reduced and half live time, $t_{1/2}$ = 14.73 ± 12.36 h, was increased as compared with controls of 1.28 ± 0.31 mL min^{-1} kg^{-1} and $t_{1/2}$ = 5.73 ± 1.58 h.

Key Words: Caffeine test, GC-MS, Cirrhosis, Clearance.

INTRODUCTION

Gas chromatography-mass spectrometric (GC/MS) applications for measuring drugs are very important for purity control[1,2], for pharmacokinetic studies[3,4], in metabolic studies[5] or clinical applications for treatment and diagnosis[6]. Caffeine is used for measuring the metabolic capacity of the liver but also as a marker of river waters pollution[7]. Fasting plasma or saliva caffeine concentrations have been suggested to be a simple guide to the severity of chronic liver disease[8-10]. Caffeine has the advantage of being well tolerated when administered orally and the saliva level parallels the serum concentration, making a non-invasive test feasible[10,11]. Because caffeine is metabolized by the hepatic P-450 cytochrome oxidase system, clearance of caffeine is an excellent quantitative test of hepatic function in human beings. The caffeine is demethylated under the influence of the hepatic microsomal cytochrome P-450 producing dimethyl xanthines as theophylline and theobromine[9], so these xanthines should be avoided to be internal standards in quantitative work. Caffeine test consists in caffeine oral intake followed by measurements of the caffeine concentration or urine metabolites in blood, saliva, urine or of labelled CO_2 in the exhalation air. Metabolism of caffeine is decreased in patients with primary billiary cirrhosis and alcoholic liver cirrhosis. The caffeine clearance test was used

†Department of Biophysics and Medical Biotechnics, University of Medicine and Pharmacy, Targu Mures, Romania.
‡National R&D Institute of Cryogenics and Isotopic Technologies - ICSI Rm. Valcea, 4 Uzinei St., Rm. Valcea, 240050, Romania

also to assess the improvement in hepatic metabolic capacity after nutritional supplementation of branched-chain amino acids or after drug-induced hepatitis[12,13]. The determination of caffeine clearance can serve as a useful parameter for the assessment of hepatic functional reserve in hepatocellular carcinoma patients post treatment[14]. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) studies of labelled xanthines have demonstrated that theophylline is converted to caffeine in premature human newborns by N-7 methylation. In adults, the inverse process exists wherein caffeine is demethylated to give theophylline[15]. The effects of liver disease on some pharmacokinetic parameters of caffeine as clearance and half-life time was usually studied by HPLC[16] and immunoassay methods[8] and some times by GC/MS[17].

The aim of this work is to develop a method to characterize some pharmacokinetic parameters as plasma clearance and half-life time of caffeine in children to test hepatic dysfunctions.

EXPERIMENTAL

^{15}N-theophylline, 74.2 atom % ^{15}N, labelled at the nitrogen in the position 7, synthesized in the National Institute for Research and Development for Isotopic and Molecular Technology Cluj-Napoca, was used as internal standard[6]. The purity was ascertained by infrared spectroscopy, mass spectrometry and melting point. Caffeine was administered p.o. in children with different hepatic dysfunctions. Caffeine, as a sterile caffeine sodium benzoate solution in water for injection use, contained 125 mg of caffeine and 125 mg of sodium benzoate/mL.

General procedure

Extraction procedure: 1 mL of plasma containing caffeine was placed into a 5 mL screw-cap vial and 10 µL of internal standard ^{15}N-theophylline, 2 mL of the extraction solvent, chloroform: isopropanol 20:1 v/v and 0.5 g NaCl were added. After mechanical mixing for 1 min, the sample was centrifuged for 3 min. The organic layer (lower layer) was transferred to second vial and evaporated to dryness under a stream of argon. The residue was dissolved in 100 µL solvent and then 3 µL were injected into the GC.

Method validation: The method was validated in the range 0-20 µg/mL caffeine. Aliquots of distilled water containing known amounts of caffeine 3, 5, 10, 15, 20 µg mL^{-1} and 10 µg of ^{15}N-theophylline were taken through above procedure. Each sample was prepared in duplicate and measured twice. The regression curve, plotted as peak-area ratio of m/z 194/181 *versus* caffeine concentration, gave the following linearity parameters: slope 0.5207, intercept 0.1058, coefficient of corre-lation r = 0.98.

Precision gave RSD values lower than 5 % for 5 µg/mL (n = 7) and lower than 3 % for 3 µg/mL (n = 5). Accuracy showed values lower than 10 % (Table-1). Each value was obtained as an average between two measurements of the same sample. The limit of detection was 0.1 µg/mL caffeine in blood sample, signal to noise ratio 4:1.

TABLE-1
PRECISION AND ACCURACY OF THE METHOD

Concentration added (μg mL^{-1})	n	Concentration measured (μg mL^{-1})	RSD (%)	Accuracy (%)
3	5	3.1	2.96	3.36
5	7	5.5	5.06	10.00

Population: Caffeine concentration measurements were performed in 33 hospitalized children suffering from hepatic dysfunctions and controls. Three different groups were studied: **A**, formed by 18 children with hepatitis aged 3-19 years old, **B**, consisting from 5 children with cirrhosis, aged between 5-12 years old, and **C**, 10 children as control aged between 5-15 years old. The main dose was 4 mg/kg, p.o., for all groups. Blood samples were taken, at 0, 0.5, 1, 3, 6, 9 and 12 h. Blood samples were drawn into heparinized plastic tubes and immediately centrifuged. Plasma was stored at -20 ºC.

Calculation: Regression curves obtain by the GC/MS method in the selected ion monitoring (SIM) mode was used for pharmacokinetic parameters study. Caffeine elimination constant was calculated as follows:

$$k_{el} = (\ln C_1 - \ln C_2)/\Delta t$$

where C_1 = higher caffeine blood concentration, C_2 = lower caffeine blood concentration, Δt = the time elapsed between venous blood samples.

Two points caffeine clearance was calculated as $Cl = k_{el} \times V_d$ and caffeine half-life as $t_{1/2} = \ln 2/k_{el}$, using a constant volume of distribution (V_d) of 0.6 L/kg body weight[8]. Clearance values calculated as dose/area under curve (AUC) were compared with the two-point values.

Detection method: A Hewlett Packard (Palo Alto, CA, USA) 5989B mass spectrometer coupled to a 5890 gas chromatograph were used in the conditions: EI (electronic impact) mode, selected ion monitoring (SIM) mode, electron energy 70 eV, electron emission 300 µA and ion source temperature 200 ºC. The GC/MS interface line was maintained to 280 °C and quadrupol analyser at 100 ºC. The gas chromatograph-mass spectrometer (GC/MS) assay used a HP-5MS fused silica capillary column, 30 m × 0.25 mm, 0.25 µm film-thickness, programmed from 200-250 °C at a rate of 10 °C/min, the flow rate 1 mL/min, with helium 5.5 as carrier gas. Injector temperature was 200 °C. Retention time for caffeine and ^{15}N-theophylline, the internal standard, were 3.5 and 2.8 min, respectively. 3 µL of sample were injected. The molecular ion m/z 194 for caffeine and the molecular ion m/z 181 for the internal standard were monitored for quantitative analyses in the SIM mode.

RESULTS AND DISCUSSION

Caffeine clearance, measured in patients with cirrhosis and chronic hepatitis, was reduced and half live time was increased in children with liver disease as compared with control. The decreased metabolism observed in patients with various forms of liver disease was correlated to the disease status. The ion chromatogram in SIM

mode for the molecular ions, the basic peaks for the both components, caffeine (m/z 194) and the internal standard (m/z 181), are presented in Fig. 1. The mass spectrum of the caffeine is shown in Fig. 2.

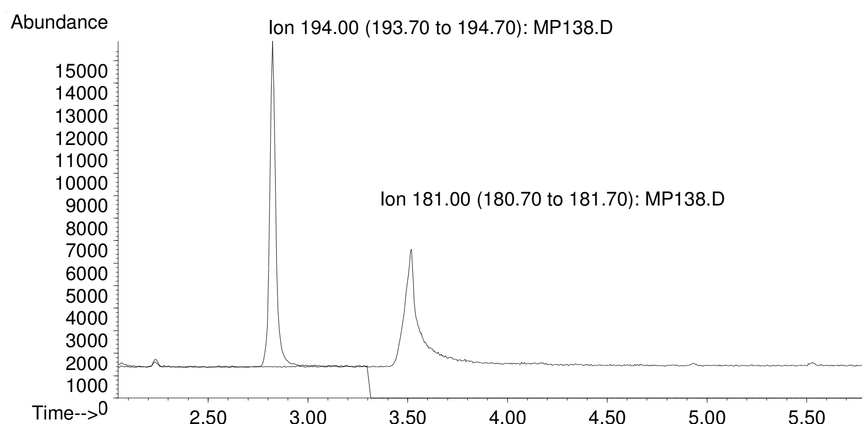

Fig. 1. Caffeine separation chromatogram by GC/MS in the SIM mode; basic ions m/z 194 for caffeine and m/z 181 for the internal standard were used

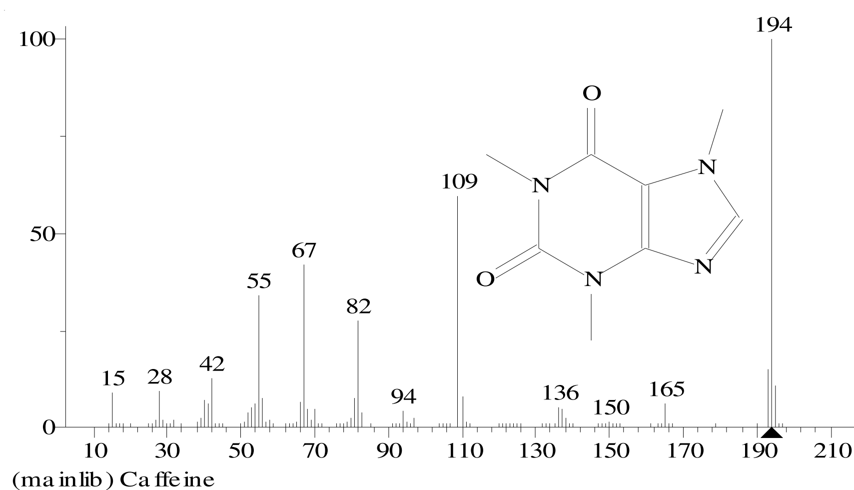

Fig. 2. Mass spectrum of caffeine; M = 194

The mean values of the two pharmacokinetic parameters studied, Cl and $t_{1/2}$, for patients with hepatic diseases and control, show high differences especially between control and cirrhosis cases. Caffeine plasma concentrations at 8 h time interval in hepatitis, control and cirrhotic patients are presented in Fig. 3. Fig. 4 presents the plasma mean values of caffeine concentration in patients in comparison with the mean value of caffeine concentration obtained in control. The elimination curve of plasma caffeine obtained for a control subject is presented in Fig. 5.

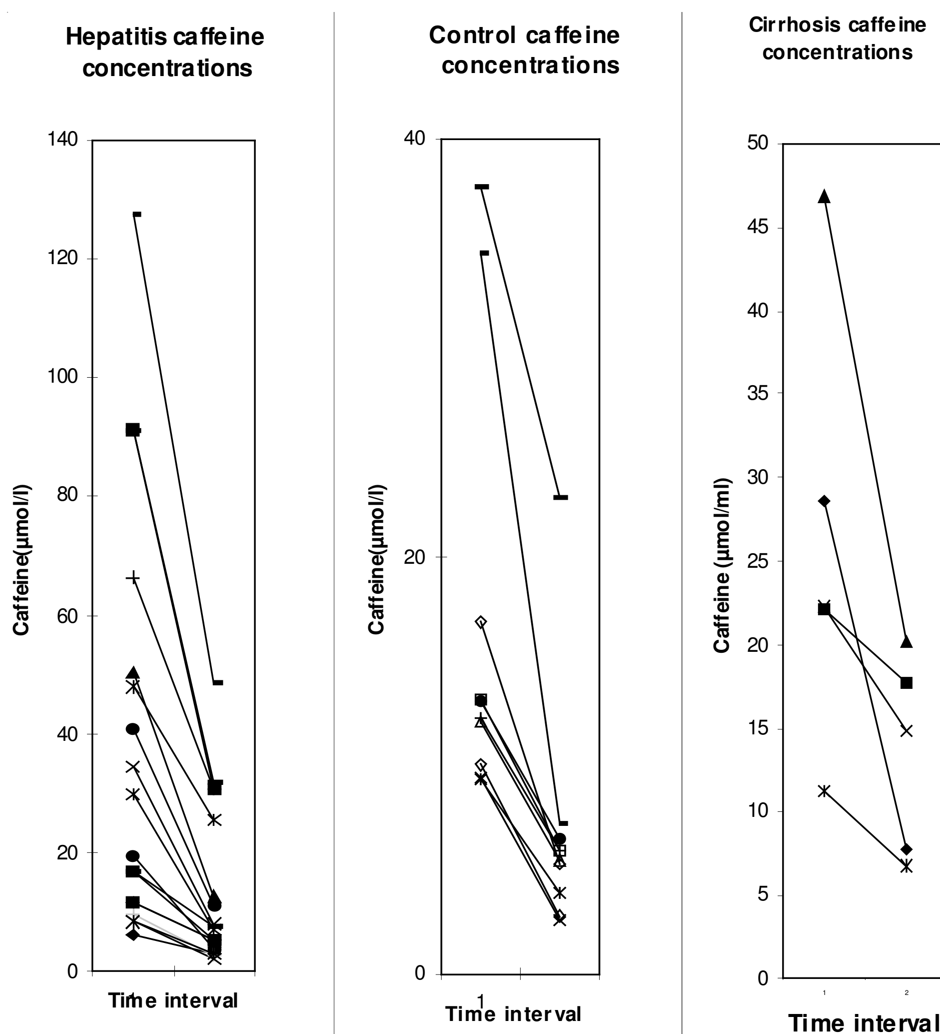

Fig. 3. Caffeine plasma concentrations at time interval of 8 h in hepatitis, control and cirrhotic patients (two point concentration in given subject is connected with a straight line)

The method developed is simple, precise and rapid, useful in the analysis of xanthines. The isotopic labelled internal standard and SIM method used avoid the overlapping with different contaminants or metabolites. Good linearity, precision, accuracy and sensitivity were obtained in the range 0-20 µg/mL of the drug. Significant changes (student's paired t-test $p < 0.01$) were observed in caffeine metabolism in children with decompensate cirrhosis. The clearance values of 0.74 ± 0.49 mL min^{-1} kg^{-1} and half-life times of 14.73 ± 12.36 h are changed because of the reduction in "functioning hepatocyte mass". Patients with noncirrhotic liver disease showed

intermediate values (Cl = 1.23 ± 0.45 mL min^{-1} kg^{-1} and $t_{1/2}$ = 6.33 ± 2.17 h) but higher values of caffeine plasma concentrations especially in the first hour after dose (Fig. 4).

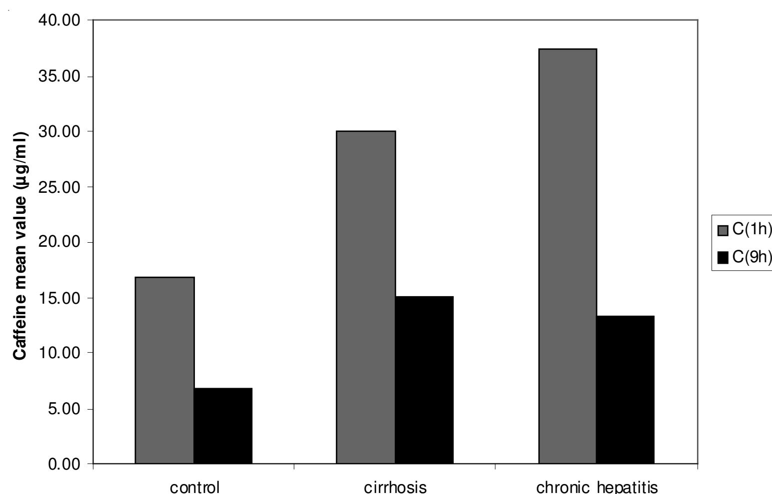

Fig. 4. Mean level of caffeine after 1 and 9 h in the blood of patients in comparison with control

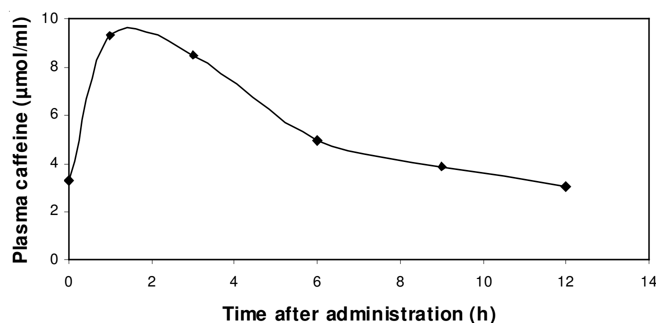

Fig. 5. Elimination curve of caffeine in a control subject

The control levels for clearance and half life time were 1.3 ± 0.4 mL min^{-1} kg^{-1} and $t_{1/2}$ = 4.4 ± 1.9 h in the literature[9] and in present data of 1.28 ± 0.31 mL min^{-1} kg^{-1} and $t_{1/2}$ = 5.73 ± 1.58 h (n = 10).

Plasma concentrations of caffeine were measured in eighteen patients with chronic hepatitis and 5 patients with cirrhosis and in 10 healthy subjects after caffeine (4 mg/kg p.o.) loading. The total body clearance measured by two-point study (sampling times 1 and 9 h) and seven-point study (sampling times 0, 0.5, 1.0, 3.0, 6.0, 9.0, 12 h) were highly significantly correlated (r = 0.94, p less than 0.001). The elimination half-life ($t_{1/2}$) of caffeine was significantly longer in cirrhotic patients than in the other two groups and clearance was substantially reduced in these patients.

These findings suggest that caffeine pharmacokinetic parameters can be estimated using two-point blood sampling procedure and GC/MS determination, following a single load. The higher concentrations of caffeine observed in the first hour after caffeine loading in hepatitis compared with controls could be used for a rapid test for hepatitis when precise and accurate methods are used.

Conclusion

The method developed is simple, precise and rapid, useful in the analysis of xanthines. The use of isotopic labelled internal standard avoids the overlapping with different contaminants. Good validation parameters were obtained in the range of interest. Significant changes ($p < 0.01$) were observed in caffeine metabolism in children with decompensated cirrhosis. The clearance values in cirrhosis are changed because of the reduction in "functioning hepatocyte mass". Patients with noncirrhotic liver disease showed intermediate values but higher values of caffeine plasma concentrations.

ACKNOWLEDGEMENT

This work has been supported by the Romanian Research Foundation (CEEX, project number 166/2006).

REFERENCES

1. M. Culea, N. Palibroda, V. Mercea and A.D. Abraham, *Rev. Roum. Biochim*., **23**, 273 (1986).
2. S. Nicoara, M. Culea, A. Nica, E. Culea and O. Cozar, *Indoor Built Environ*., **14**, 405 (2005).
3. M. Culea, N. Palibroda and A.D. Abraham, *Rev. Roum. Biochim*., **24**, 169 (1987).
4. J. Jodynis-Liebert, J. Flieger, A. Matuszewska and J. Juszczyk , *J. Clin. Pharmacol.*, **44**, 338 (2004).
5. M. Culea and D.L. Hachey, *Rapid Commun. Mass Spectrom*., **9**, 655 (1995).
6. M. Culea, N. Palibroda, P. Panta Chereches and M. Nanulescu, *Chromatographia*, **53**, S387 (2001).
7. A.P. Ferreira, *Cad. Saude Publica Rio de Janeiro*, **21**, 1884 (2005).
8. G. Host, A. Wahllander, U. Von Mandach and R. Preisig, *Hepatology*, **7**, 338 (1987).
9. N.R. Scott, D. Stambuk, J. Chakraborty, V. Marks and M.Y. Morgan, *Clin. Sci*., **74**, 377 (1988).
10. A. Hostege, M. Staiger, K. Haag and W. Gerok, *Klin. Wochenschr*., **67**, 6 (1989).
11. W.F. Balistreri, H.H. A-Kader, K.D, Setchell, D. Gremse, F.C. Ryckman and T.J. Schroeder, *Ann. Clin. Lab. Sci*., **22**, 162 (1992).
12. P. Tangkijvanich, V. Mahachai, S. Wittayalertpanya, V. Ariyawongsopon and S. Isarasena, *Southeast Asian J. Trop Med. Public Health*, **31**, 152 (2000).
13. Y.C. Bechtel, H. Lelouet, M.P. Brientini, M. David-Laroche, J.P. Miguet, G. Paintaud and P.R. Bechtel, *Therapie*, **55**, 619 (2000).
14. M.C. Bastien, F. Leblond, V. Pichette and J.P. Villeneuve, *Can. J. Physiol. Pharmacol*., **78**, 912 (2000).
15. J.L. Brazier, B. Ribon, M. Desage and B. Salle, *Biomed. Mass Spectrom*., **7**, 189 (1980).
16. S. Wittayalertpanya and V. Mahachai, *J. Med. Assoc. Thai*, **84**, S189 (2001).
17. Y. Benchekroun, M. Desage, B. Ribon and J.L. Brazier, *J. Chromatogr.*, **532**, 261 (1990).

(*Received*: 23 June 2009; *Accepted*: 16 January 2010) AJC-8313