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Rapid Diagnosis of Cirrhosis by Isotopic Dilution Gas Chromatography-Mass Spectrometry

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Abstract — *A GC/MS method for caffeine levels determination in children suffering of liver diseases is described. Caffeine and ^{15}N-theophylline, the internal standard, were separated on a HP-5 capillary column 30mx 0.25mm diameter, 0,25µm film support in the temperature program: 200-250^{o}C, at 10^{o}C/min. The mass spectrometer was used in the selected ion monitoring (SIM) mode. The method was validated in the range 0-20µg/ml caffeine. A dose of 4mg/kg p.o. was followed by blood caffeine concentrations measurements at 1 hour and 12 hours samples. The extraction and the analysis were very rapid. 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.*

Keywords : *caffeine, cirrhosis, isotopic dilution, GC/MS*

1. Introduction

Caffeine, 1,3,7 trimethylxanthine, has been introduced as a compound for measuring the metabolic capacity of the liver, being well tolerated when administrated orally. Clearance of caffeine is an excellent quantitative test of hepatic function in human beings, because caffeine is metabolized by the hepatic P-450 cytochrome oxidase system. Caffeine test consists in caffeine oral intake followed by measurements of blood, saliva, labelled CO_2 in the exhalation air, urine caffeine concentration or urine metabolites. Metabolism of caffeine is decreased in patients with primary billiary cirrhosis, alcoholic liver cirrhosis and the equilibrium between the various metabolic pathways of caffeine is impaired [1, 2]. The caffeine clearance test was used also to assess the improvement in hepatic metabolic capacity after nutritional supplementation of branched-chain amino acids or after drug-induced hepatitis. The determination of caffeine clearance can serve as a useful parameter for the assessment of hepatic functional reserve in hepatocellular carcinoma patients post treatment.

Fasting plasma or saliva caffeine concentrations have been suggested to be a simple guide to the severity of chronic liver disease. Liver function in infants and children has traditionally relied on static indices of hepatic structure, cellular integrity or function and is based on the release of substances from damaged tissues. The development of dynamic tests based on the measurement of substances metabolized or cleared from blood by the liver offers a more precise quantitative estimation of hepatic functional capacity. The majority of the administrated caffeine is demethylated under the influence of the hepatic microsomal cytocrome P-450 producing dimethylxantines as theophylline and theobromine, so these xanthines should be avoided to be internal standards in quantitative work. GC/MS studies of labeled xanthines have demonstrated that teophylline 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 .

The effects of liver disease on some pharmacokinetic parameters of caffeine as clearance and half-life time was usually studied by HPLC and immunoassay methods and some times by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) [3].

The aim of the present investigation was to validate a rapid and precise GC/MS method for plasma caffeine level determination for the characterization of some pharmacokinetic parameters as plasma clearance and half-life time in children by using ^{15}N-theophylline as internal standard.

The application of the method was focused on hepatitis and cirrhosis, being rare to children and much more studied.

1.1. Chemicals and Reagents

^{15}N-theophylline, 74,2 atom % ^{15}N, labeled at the nitrogen in the position 7, synthesized in the National Institute for Reasearch and Development for Isotopic and Molecular Technology Cluj-Napoca, was used as internal standard. The purity was ascertained by infrared spectrscopy, 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 containing 125 mg of caffeine and 125 mg of sodium benzoate per 1 ml ampoule was obtained from pharmacy. All other reagents were from Comchim (Bucuresti, Romania). Chloroform and isopropanol were purified by distillation.

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1.2. Equipment

A Hewlett Packard (Palo Alto,CA, USA) 5989B mass spectrometer coupled to a 5890 gas chromatograph were used in the conditions: EI mode, electron energy 70 eV, electron emission 300µA and ion source temperature 200°C, selected ion monitoring (SIM) mode. The GC/MS interface line was maintained to 280°C, and quadrupol analyser at 100 °C. The gas chromatograph-mass spectrometer (GC/MS) assay used a HP-5MS fused silica capillary column, 30mx0.25mm, 0.25µm film-thickness, programmed from 200°C to 250°C at a rate of 10°C/min, the flow rate 1ml/min, with helium 5.5 as carrier gas. Injector temperature was 200 °C. The molecular ion m/z 194 for caffeine (fig.1) and the molecular ion m/z 181 for the internal standard, were monitored for quantitative analyses in the selected ion monitoring (SIM) mode.Retention time for caffeine and ^{15}N-theophylline, the internal standard, were 3.5 min and 2.8 min (fig. 2). 3 µl of sample were injected.

1.3. Extraction Procedure

1ml of plasma containing caffeine was placed into a 5 ml screw-cap vial and 10µl 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.

1.4. Method Validation

The method was validated in the range 0-20µg/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 to m/z181 versus caffeine concentration, gave the following linearity parameters: slope 0.5207, intercept 0.1058, r = 0.97.

Precision gave R.S.D values lower than 5% for 5µg/ml (n=7) and lower than 3% for 3µg/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 0.1µg ml^{-1}caffeine in blood sample, signal to noise ratio 4:1.

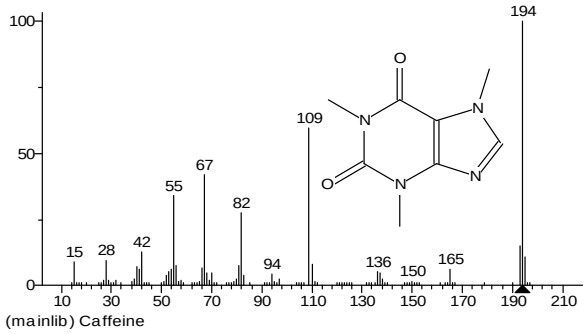

Figure 1. The mass spectrum of caffeine; M=194

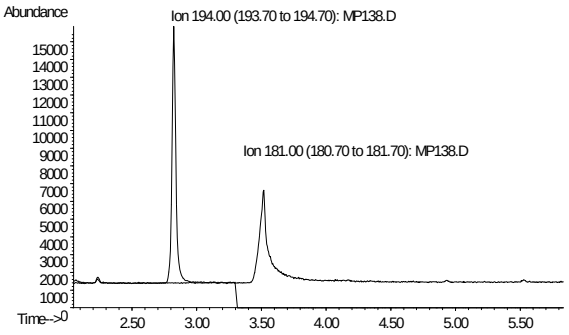

Figure 2. The chromatogram of separation of caffeine and labelled theophylline by GC/MS by using SIM mode; m/z 194 for caffeine and m/z 181 for the internal standard

1.5. Population

Caffeine concentration measurements were performed in 31 hospitalized children suffering of hepatic dysfuctions and controls. Three different groups were studied: A, formed by 19 children with hepatitis aged 3-15 years old, B, consisting from 4 children with cirrhosis, aged between 5-12 years old, and C, 8 children as control aged between 5-15 years old. The main dose was 4mg/kg, p.o., for all groups. Blood samples were taken, at 0, 30 min, 1, 3, 6, 9 and 12 h. Blood samples were drawn into heparinized plastic tubes and immediately centrifuged. Plasma was stored at -20°C. Written informed consents were obtained from each subject parent prior to this study.

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

1.6. Calculation

Regression curves obtain by the GC/MS method in the 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 liters per kg body weight. Clearance values calculated as dose/area under curve (AUC) were compared with the two-points values.

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

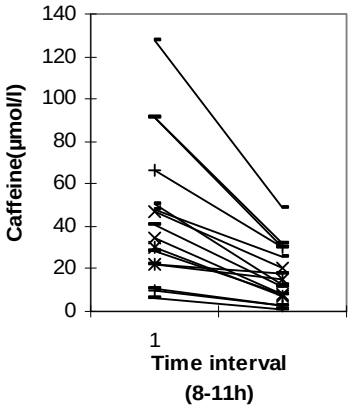

Figure 3. Caffeine plasma concentrations at time interval (1=1h and 2=9h) in hepatitis (n=17)

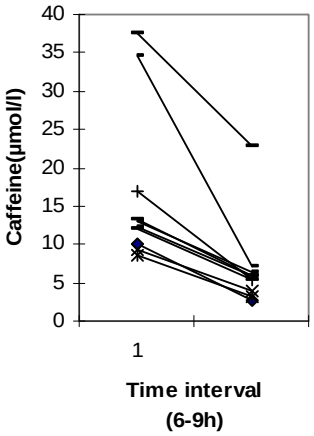

Figure 4. Caffeine plasma concentrations at time interval (1=1h and 2=9h) in control (n=8)

The method is simple, precise and rapid, useful in the analysis of xanthines. Isotopic labeled internal standard used avoids metabolites overlapping. Good linearity, precision, accuracy and sensitivity were obtained in the range 0-20µg/ml of 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 0.55±0.41 ml min^{-1} kg^{-1} and half-life times of 19.11±14.9h are changed because of the reduction in "functioning hepatocyte mass". The caffeine level values in different patients compared with control are shown in Fig. 3 and 4. The control values 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 and our data of 1.36±0.23 ml min^{-1} kg^{-1} and $t_{1/2}$=5.23±0.85 h (n=8). Patients with noncirrhotic liver disease showed intermediate values (Cl=1.19±0.45 ml min^{-1} $kg^{-1}$and $t_{1/2}$=6.62±2.37 h) but higher values of caffeine plasma concentrations especially in the first hour after dose (Fig. 3).

Plasma concentrations of caffeine were measured in nineteen patients with chronic hepatitis, and four patients with cirrhosis and in eight healthy subjects after caffeine (4 mg/kg p. o.) loading. The correlations of total body clearance between two-point study (sampling times 1h and 9 h) and seven-point study (sampling times 0, 0.5, 1, 3, 6, 9, 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. Caffeine clearance test was unable to distinguish the difference of liver function between the control subjects and in patients with hepatitis ($p>0.05$). The higher concentrations of caffeine observed in the first hour after caffeine loading in hepatitis compared with controls (Fig. 3) could be a

possible test for hepatitis when very precise and accurate methods as isotopic dilution GC/MS are used.

Further statistical work will is necessary for caffeine test especially in hepatitis.

3. ACKNOWLEDGMENTS

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