RESEARCH ARTICLES

Modified Method for Serum Paraxanthine/Caffeine Ratio: An Index of CYP1A2 Activity

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Abstract

The ratio of paraxanthine/caffeine is generally used to be an index of CYP1A2 activity. The assay of serum paraxanthine/caffeine ratio was modified from the method of Koch J.P. et al. The validation of a reverse phase high performance liquid chromatography (HPLC) method with UV detection for both paraxanthine and caffeine in serum was described. The optimum time of blood sampling after caffeine intake was detected in a pilot study. Each subject took a 180 mg single oral dose of caffeine solution. Blood samples were collected before and 1,2,3,4,5,6 and 8 hours after caffeine intake and analyzed further by HPLC. The assay validation was shown as these parameters. The lower limit of detection of the assay was 0.125 μg/ml and 0.25 μg/ml, for paraxanthine and caffeine, respectively. Accuracy expressed as % recovery, those range were 97.73 – 105.49 % and 95.84 – 100.63 %, for paraxanthine and caffeine, respectively. The precision expressed as relative standard deviation, the results were 2.88% and 5.25% for intraday and interday assay of paraxanthine, and 3.07% and 5.78% for intraday and interday assay of caffeine. Linearity of calibration curve of both were covered 0 – 8 μg/ml (R^2= 0.9999). Serum samples were stable when stored at –70°C for 24 weeks. The best sampling time of serum paraxanthine/caffeine ratio was 5 hours after caffeine intake. This method is simplified and reliable for serum paraxanthine/caffeine ratio determination as an index of CYP1A2 activity.

Keywords : paraxanthine/caffeine ratio, CYP1A2, HPLC

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การดัดแปลงวิธีวิเคราะห์หาอัตราส่วนพาราแซนธีนต่อแคฟเฟอีนในซีรัม: ดัชนีวัดการทำงานของเอนไซม์ไซโตโครม พี 450 1เอ2

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บทคัดย่อ

อัตราส่วนพาราแซนธีนต่อแแคฟเฟอีนนิยมใช้เป็นดัชนีวัดการทำงานของเอนไซม์ไซโตโครมพี 450 1เอ2 วิธีการวิเคราะห์หาอัตราส่วนพาราแซนธีนต่อแคฟเฟอีนในซีรัมโดยวิธี HPLC ได้ดัดแปลงจากวิธีการของ Koch J.P. และคณะ และทำการศึกษานำร่องเพื่อหาเวลาที่เหมาะสมในการเจาะเลือดโดยให้อาสาสมัครแต่ละคนรับประทานแคฟเฟอีนขนาด 180 มิลลิกรัม เจาะเลือดก่อนและหลังรับประทานแคฟเฟอีน 1, 2, 3, 4, 5, 6 และ 8 ชั่วโมง ความเข้มข้นต่ำสุดของพาราแซนธีนและแคฟเฟอีนที่วิเคราะห์ได้คือ 0.125 และ 0.25 ไมโครกรัมต่อมิลลิลิตรตามลำดับ ความถูกต้องของการวิเคราะห์แสดงในรูปของเปอร์เซ็นต์การคืนกลับซึ่งมีค่าระหว่าง 97.73 – 105.49 % และ 95.84 – 100.63 % สำหรับพาราแซนธีนและแคฟเฟอีนตามลำดับ ความเที่ยงตรงของการวิเคราะห์แสดงในรูปของสัมประสิทธิ์ความแปรปรวน ความเที่ยงตรงของการวิเคราะห์พาราแซนธีนในซีรัมมีค่าสัมประสิทธิ์ความแปรปรวน 2.88 % และ 5.25 % สำหรับการวิเคราะห์ในวันเดียวกันและการวิเคราะห์ต่างวันกันตามลำดับ ความเที่ยงตรงของการวิเคราะห์แคฟเฟอีนในซีรัมมีค่าสัมประสิทธิ์ความแปรปรวน 3.07% และ 5.78 % สำหรับการวิเคราะห์ในวันเดียวกันและวิเคราะห์ต่างวันตามลำดับ ความเป็นเส้นตรงของการวิเคราะห์สารทั้งสองครอบคลุมความเข้มข้น 0-8 ไมโครกรัมต่อมิลลิลิตร ตัวอย่างซีรัมมีความคงตัวดีเมื่อเก็บตัวอย่างไว้ที่ -70 องศาเซลเซียส นาน 24 สัปดาห์ และพบว่าระยะเวลาที่เหมาะสมในการเจาะเลือดเพื่อหาระดับพาราแซนธีนและแคฟเฟอีนในซีรัมคือ 5 ชั่วโมงหลังรับประทานแคฟเฟอีน วิธีการวิเคราะห์นี้ง่ายและมีความน่าเชื่อถือ เหมาะที่จะใช้วัดอัตราส่วนพาราแซนธีน/แคฟเฟอีนในซีรัมเพื่อตรวจวัดการทำงานของเอนไซม์ไซโตโครมพี 450 1เอ2

คำสำคัญ : อัตราส่วนพาราแซนธีนต่อแคฟเฟอีน, ไซโตโครมพี450 1เอ2, HPLC

Introduction

In vivo study, CYP 1A2 activity can be measured[1]. Phenacetin, a substrate of CYP 1A2 was assayed for CYP1A2 activity in previous report, but it has carcinogenic activity[2]. Presently, caffeine (1,3,7-trimethylxanthine:137X) is the most popular probe for CYP 1A2 activity determination because it has low toxicity[3,4]. Biotransformation of caffeine is virtually confined to the liver and about 16 metabolites are excreted in urine[1,5]. Caffeine undergoes demethylation to paraxanthine (1,7- dimethylxanthine:17X) as the major metabolite (84%) while others are minor metabolites (Fig.1)[6].

CYP1A2 is the major enzyme for caffeine metabolism. A number of parameters based on caffeine metabolism have been selected as marker of CYP 1A2 activity, including caffeine breath test, urinary caffeine metabolites ratio and plasma or serum caffeine metabolites ratio[1,7]. The first is tested by the measurement of $^{13}CO_2$ or $^{14}CO_2$ following a dose of caffeine labeled in the N3-methyl group[6]. The requirement for isotope labeled and specialized equipment is needed to measure the labeled carbon-dioxide in exhaled air[1]. The second is the determination of caffeine and its metabolites in urine after caffeine intake[8].This method based on either secondary or tertiary metabolites, are not ideal indices of CYP1A2 activity because these metabolites are not exclusively formed by CYP1A2. Futhermore, these methods might vary substantially with factors such as urinary flow, inter-ethnic differences in renal function and sampling time of urine collection[1,3]. The last one is the determination of caffeine and its metabolites in plasma or serum. Paraxanthine/caffeine ratio has been recognized as a good index for CYP1A2 activity because paraxanthine is a major metabolite and catalyzed by CYP1A2 only[7,9]. In previous report[(3,4)], blood samples were collected at 4 or 6 hours postdosing of caffeine in which a high level of paraxanthine appeared.

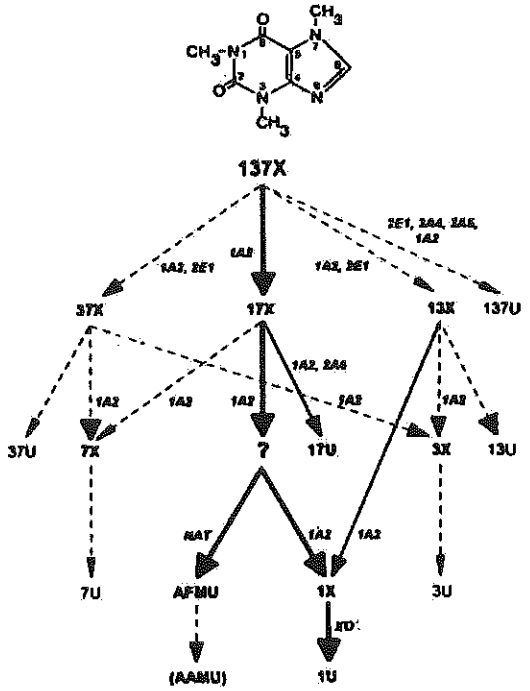

Figure 1 Metabolic pathway of caffeine

The main purpose of this study was to develop a high performance liquid chromatography (HPLC) method for the determination of paraxanthine and caffeine in serum. We detected the best sampling time for paraxanthine/caffeine ratio assay in a pilot study.

Material and method

Samples

Pool human sera were obtained from healthy volunteers who abstained from caffeine containing food and beverage 3 days. In pilot study, serum samples were collected from three female healthy volunteers who abstained from caffeine containing food and beverage 3 days and not exposed to compounds or drugs which interfering caffeine metabolism. Each subject took a 180 mg single oral dose of caffeine solution. Blood samples were subsequently collected at 1,2,3,4,5,6 and 8 hours after caffeine administration. The sera were separated and stored at –70 °C until analysis.

Reagents

Caffeine (anhydrous, BP grade, betch no71015), paraxanthine and 8-chlorotheophylline as the internal standard were obtained from Sigma Chemical Co.Ltd. Trichloroacetic acid and acetic acid were purchased from MERK, methyl alchohol HPLC grade from Lab Guard and acetonitrile HPLC grade from Scharlau Chemie S.A. Tetrahydrofuran was obtained from Farmitalia Carloerba. Double distilled water was used throughtout this investigation.

Apparatus

HPLC apparatus from Spectra System Thermo Separation Products was consisted of a model P1000 for delivering the mobile phase, a model automatic injector AS 3000 for injection samples and UV detector with a model of UV 1000 used to monitor paraxanthine, caffeine and 8-chlorotheophylline at the wavelength of 273 nm. A μ bondapak C18 stainless steel column (30 cm, 3.9 mm, I.D. Water Associates) was suitable in the condition. A computer system with PC1000 software was used to analyse peak and set the standard system

Method

Serum samples

Stock standard solution of paraxanthine and caffeine were prepared and used to spike into the pool serum. The series of serum paraxanthine and caffeine standard concentration were 0 (blank serum), 0.5, 1, 2, 4 and 8 μg/ml. Serum samples obtained from volunteers who took 180 mg of caffeine were prepared to assay.

Sample preparation

The sample preparation was modified from Koch J.P. et al[10]. Serum protein was precipitated before the samples injected into the chromatographic system. Procedure for preparing the sample was achieved by the following, 500 μl of serum sample added with 200 μl of internal standard, 500 μl of methanol and 500 μl of 10% trichloroacetic acid. The sample was mixed on a vortex mixer for 1 minute and centrifuged at 4,000 rpm for 15 minute. 50 μl of the supernatant was injected into the HPLC system.

Chromatographic condition

The mobile phase for paraxanthine and caffeine assay was the mixture of acetic acid, tetrahydrofuran, acetonitrile and water (1:3:40:456). The mixture was adjusted to pH 5.6 and filtrated over a Milllipore filter before used in the assay. The mobile phase was delivered to HPLC system at flow rate 1.2 ml/min and UV wavelength 273 nm. Quantitation was based on peak area integration by software P1000 computer system.

Method validation

Analytical method validation was modified from the method described by Koch J.P et al[10] and Guidance for industry: Bioanalytical method validation (U.S. Department of Health and Human Services FDA, CDER, CVM. May 2001, BP)[11].

The analytical method developed was validated to ensure the acceptability of the performance. The parameters determined are lower limit of detection, accuracy, precision, specificity, linearity and stability.

Statistical analysis

The statistical program was employed by computerization of the mean, standard deviation, percentage of relative standard deviation and linear regression analysis.

Result

Specificity

Chromatogram of paraxanthine, 8-chlorotheophylline (internal standard) and caffeine were shown in figure 2. The retention time of paraxanthine, 8-chloro-theophylline and caffeine were 8, 10.2 and 14.1 minute, respectively. All peaks were not disturbed from endogenous and solvent peak.

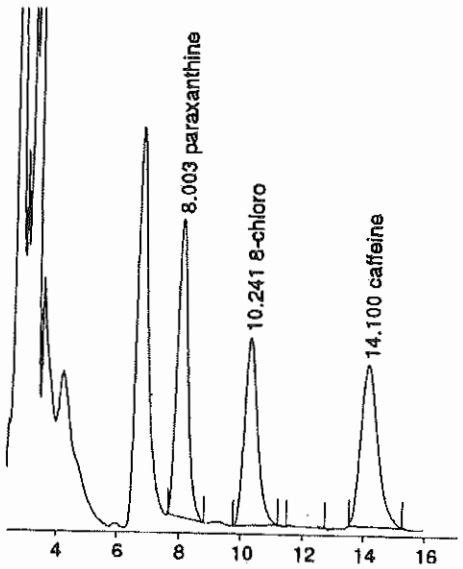

Figure 2 Chromatogram of serum paraxanthine, 8-chlorotheophylline and caffeine spiked in pool serum

Accuracy

The accuracy of the method for analysing paraxanthine and caffeine in serum was determined in terms of the percentage of analytical recovery averaged 100.63 ± 4.24% and 98.34 ± 2.4%, respectively. The efficiency of extraction procedure was expressed in term of the percentage of physical recovery. The average of percent physical recovery of paraxanthine and caffeine were 87.61 ± 1.8% and 91.17 ± 2.01%, respectively. The recovery of both compound as shown in table 1.

Precision

The precision of this assay at different concentrations was represented by percent of relative standard deviation (%RSD). The averages %RSD of intra-day and inter-day of paraxanthine were 2.88% and 5.25%, respectively. The averages %RSD of intra-day and inter-day of caffeine were 3.07% and 5.78%, respectively. The precision of both compounds was shown in table 2.

Linearity

The calibration curve for the spiked paraxanthine and caffeine in pool serum was linear over the concentration range 0-8 μg/ml and correlation coefficient (R^2) was 0.9999 aand 0.9996, respectively, as shown in figure 3 and 4.

Table 1 Percent recovery of serum paraxanthine and caffeine assay at low, medium and high concentrations

Standard conc. μg/ml	%Physical recovery paraxanthine	caffeine	%Analytical recovery paraxanthine	caffeine
1	87.12 ± 4.85	89.14 ± 3.89	105.49 ± 5.1	98.56 ± 5.1
2	86.10 ± 9.01	91.2 ± 3.2	98.67 ± 9.19	95.84 ± 2.47
8	89.60 ± 8.46	93.16 ± 6.95	97.73 ± 7.05	100.63 ± 2.4
Average of % recovery	87.61 ± 1.8	91.17 ± 2.01	100.63 ± 4.24	98.34 ± 2.4

Table 2 Intra-day and inter-day precision of serum paraxanthine and caffeine assay at low, medium and high concentrations

Standard conc. μg/ml	Intra-day precision (%RSD) paraxanthine	caffeine	Inter-day precision (%RSD) paraxanthine	caffeine
1	3.85	4.89	6.08	9.24
2	0.74	1.72	7.91	3.06
8	4.06	2.6	1.77	5.32
Average of %RSD	2.88	3.07	5.25	5.78

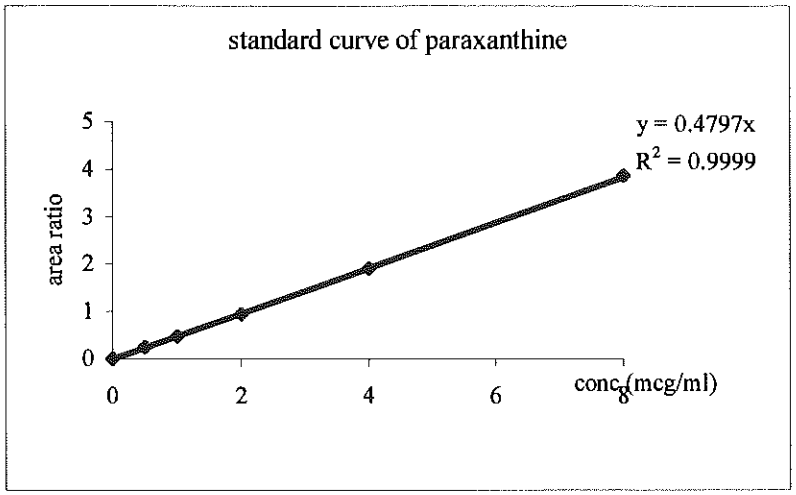

Figure 3 Standard curve of serum paraxanthine

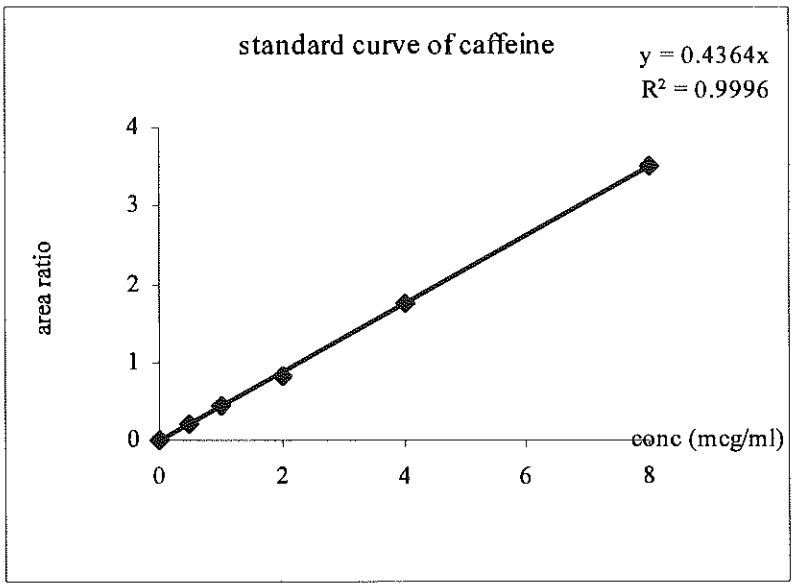

Figure 4 Standard curve of serum caffeine

Lower limit of detection

The lower limit of detection that can be assayed by this method is 0.125 μg/ml for paraxanthine and 0.25 μg/ml for caffeine.

Stability

The stability of paraxanthine and caffeine was determined at the concentration of 2 μg/ml and stored at –70 °C for 24 weeks. The area ratio of both compounds at any storage time closed to the value at time 0, as shown in figure 5.

The appropiate time to determine paraxanthine/caffeine ratio was identified in normal volunteers. The level of paraxanthine and caffeine at before and after caffeine intake was shown in figure 6. At 5 hours after caffeine administration, paraxanthine level was as high as it could be determined. All subjects who took a 180 mg of caffeine did not manifest any adverse effects.

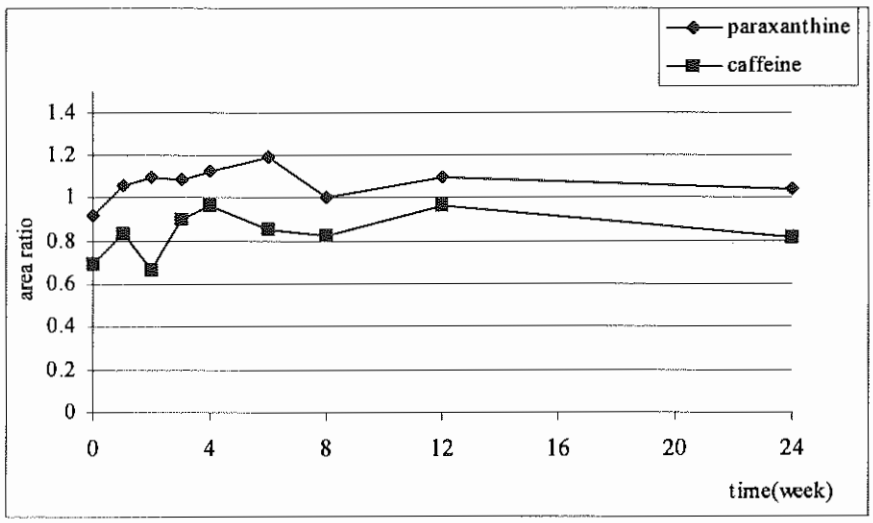

Figure 5 Stability of serum paraxanthine and caffeine at 2 μg/ml

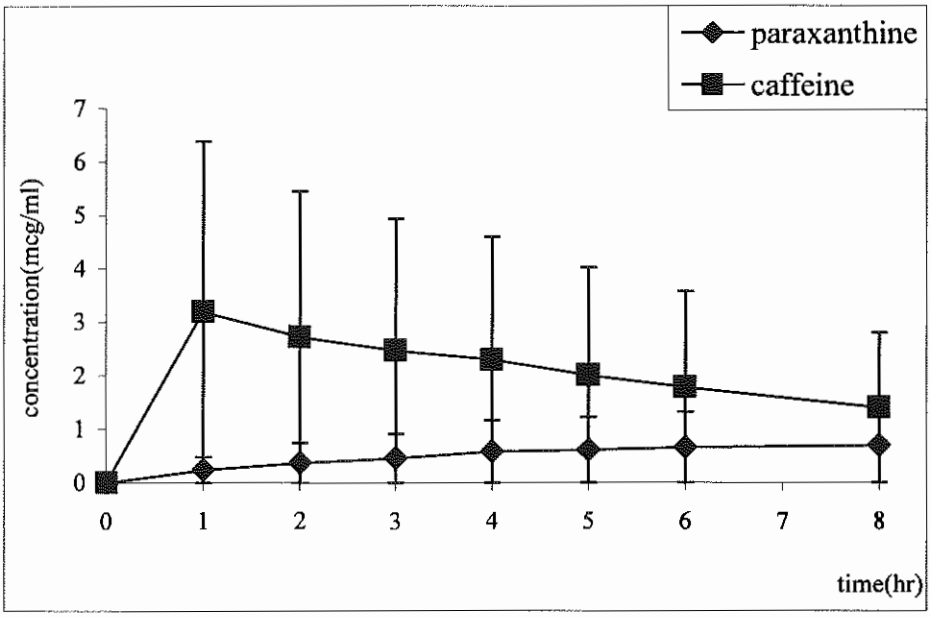

Figure 6 Average serum concentration of paraxanthine and caffeine at each time after caffeine administration in normal volunteers.

Discussion and conclusion

Caffeine is often used as a probe for CYP 1A2 activity.[3] Paraxanthine is a major metabolite (84%) and catalyzed by CYP1A2[1]. Serum paraxanthine/caffeine ratio was chosen to be an indicator of CYP1A2 activity. The determination of serum paraxanthine and caffeine using HPLC was developed[10]. In this report, the method was modified to be simple and practical for routine assay. This modified method was validated following the bioanalytical standard method of validation[10-11].

It has been approved with the acceptance criteria of each standard parameter.

Lower limit of detections of both compounds are acceptable. Percent RSD of the detection was less than 20%[11]. In general, percent RSD of intra-day and inter-day variation that present the precision of the method should not be exceed 10% and 15% respectively[10-11]. The result shows good precision of both paraxanthine and caffeine determinations. With the accuracy of the assay, percent recovery should be within the limit of 80-120%[11]. The result also presents good accuracy of both. Specificity indicated by the characteristic of chromatogram, paraxanthine, 8-chlorotheophylline and caffeine did not disturb by each other and other serum peaks. That shows good performance for determination of each standard in the same sample. The calibration curve of both were linear, R^2 closed to 1.0, and covered the range of paraxanthine and caffeine in serum. The good stability of both compounds was shown when stored at -70 °C. All results indicate that the method is high performance and reliable for a routine assay of CYP1A2 activity.

In a pilot study, serum paraxanthine and caffeine level at each time point was analysed. The profile concentration vs time curve of both was interpreted. The result suggested that the blood samples at 5 hours after caffeine intake should be collected to determine paraxanthine/ caffeine ratio. Paraxanthine level was as high as it can be determined. If the time is too prolong, paraxanthine will be metabolized to other compound by CYP 1A2[1], as shown by the metabolic pathway of caffeine in figure 1[6]. The study expressed that this simplified method can be used to determine CYP1A2 activity in population who exposed the CYP1A2 interference agents.

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