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Simple and sensitive method for the determination of chlorpheniramine maleate in human plasma using liquid chromatography–mass spectrometry

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Abstract

A convenient liquid chromatographic–single quadrupole mass spectrometric (LC–MS) method was developed and validated for the determination of chlorpheniramine maleate (INN name: chlorphenamine) in human plasma. The method had advantages of a single liquid–liquid extraction with diethylether and high sensitivity. The linearity was also excellent over the concentration range of 0.52–20.8 ng/ml of chlorpheniramine maleate. The intra- and inter-day precision and accuracy ranged between 0.0 and 13.9%, showing a good reproducibility. This developed method was successfully applied to analysis of chlorpheniramine maleate in clinical studies.
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Keywords: Chlorpheniramine maleate

1. Introduction

Chlorpheniramine maleate (CPM) (3-(4-chlorophenyl)-*N*,*N*-dimethyl-3-(2-pyridyl)propylamine monomaleate) is a commercially available antihistamine. It has been widely used for symptomatic relief of common colds and allergic diseases. Pharmacokinetic studies have revealed that plasma chlorpheniramine concentrations in humans are low, for example, the maximum levels of 6.2 and 7.0–8.2 ng/ml after a single oral administration of 2 and 4 mg, respectively [1], 5.8–11.3 ng/ml after a 4-mg administration [2] and 3.9 ng/ml after a 2.67-mg administration [3]. Some devised methods have been reported to determine human plasma chlorpheniramine by using gas chromatography (GC) [4], gas chromatography–mass spectrometry (GC–MS) [5] and high-performance liquid chromatography (HPLC) [1–3,6,7]. Some of these methods need time-consuming derivatization or several extraction cycles. However, an HPLC–tandem mass spectrometry (MS–MS) which has been reported recently [8] is considered the most practical because of simple pretreatment, high sensitivity and short run time. In the present study, we developed an LC–MS method which is as convenient and sensitive for the

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determination of human plasma CPM as the LC–MS–MS method [8].

In addition, this method was applied to the determination of CPM in human plasma from a clinical, postmarketing surveillance study.

2. Experimental

2.1. Chemicals and reagents

As a standard substance for quantitation of CPM (Fig. 1), (+)-chlorpheniramine maleate of biochemical grade was purchased from Wako (Osaka, Japan). The internal standard (AD-5423, 2-(4-ethyl-1-piperazinyl)-4-(4-fluorophenyl)-5,6,7,8,9,10-hexahydrocycloocta[b]pyridine, Fig. 1) was synthesized in the authors' Company. Methanol and acetonitrile with HPLC grade were purchased from Nacalai Tesque (Kyoto, Japan). A 4-***M*** NaOH solution of volumetric analysis grade, ammonium acetate of analytical reagent-grade and diethylether of residual pesticide grade were from Wako. Water was purified using Milli-Q Labo (Millipore, Tokyo, Japan). The 4-***M*** NaOH solution was diluted with water to 0.2 ***M*** in a volumetric flask.

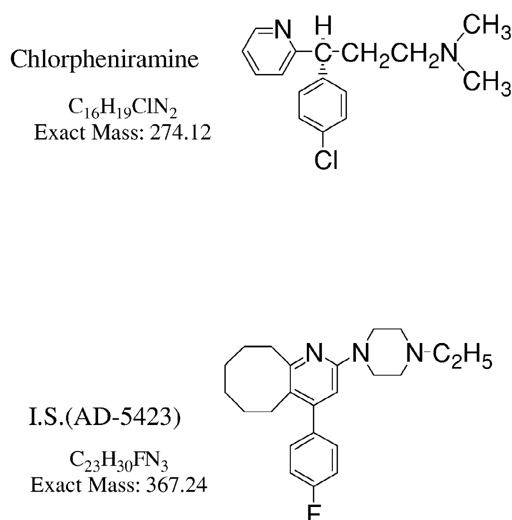


Fig. 1. Chemical structures of chlorpheniramine and I.S.

2.2. Plasma samples

Plasma for development and validation of this analytical method was obtained by centrifuging blood from six healthy volunteers in our laboratory. Sodium heparin was used as anticoagulant. The obtained plasma was pooled and stored frozen at −20 °C until use.

Plasma samples for quantitation were drawn from six healthy volunteers at 2, 2.5, 3 and 3.5 h after a single oral administration of 2-mg chlorpheniramine maleate in a clinical study on postmarketing surveillance. The samples were stored frozen at −20 °C until analysis. This study was granted by the Ethical Committee on Clinical Investigation, Tohoku University School of Medicine and was performed in accordance with the Declaration of Helsinki and its amendments.

2.3. Instrumentation

LC–MS was carried out using a single quadrupole mass spectrometer, an Agilent-1100 LC–MSD system (Agilent Technologies, Waldbronn, Germany). The stainless-steel column (250×2.0 mm I.D.) packed with Develosil PhA (5 μm particle size) was used (Nomura, Seto, Japan). Mobile phase A was 20 m*M* ammonium acetate in water and mobile phase B consisted of acetonitrile–methanol (1:1, v/v). Each mobile phase was filtered through a 0.45-μm membrane and degassed under reduced pressure. Linear gradient elution was employed with a 17 min run time and its sequence was as follows: A–B (45:55) held for 5 min after injection, 20:80 at 6.3 min and held up to 10 min, and thereafter 45:55. Flow-rate was maintained initially at 0.3 ml/min for 5 min, increased linearly to 0.35 ml/min at 6.3 min, and returned to 0.3 ml/min at 10 min. Column oven temperature was 40 °C. Autosampler temperature was 10 °C.

The LC–MS system was operated using electrospray ionization (ESI) probe in the positive ion mode with the capillary voltage set at 3000 V, fragmentor voltage: 80 V, drying N_2 gas temperature: 320 °C, drying N_2 gas flow: 7.0 l/min, neblizer N_2 gas pressure: 35 p.s.i.g. (1 p.s.i.g.=6894.76 Pa), gain: 3.0, and dwell time: 289 ms. Selected-ion monitoring (SIM) data were obtained by following two ions:

m/z 275 (chlorpheniramine) and *m/z* 368 (I.S.). LC–MSD control and data analysis were performed by Chem Station Rev. A 06.0.1 software, running on a Vectra XA computer (Hewlett-Packard).

2.4. Standard solution

A stock solution of CPM was prepared in methanol (50.2 μg/ml) and stored at 4 °C. The stock solution was diluted with methanol–water (30:70, v/v) to prepare working solutions at the final concentrations of 5.20, 20.8, 50.2, 104 and 208 ng/ml. An I.S. stock solution was also prepared in methanol (50 μg/ml) and stored at 4 °C. The I.S. stock solution was diluted with methanol–water (30:70, v/v) to prepare a working solution at the final concentration of 100 ng/ml.

2.5. Sample preparation and pretreatment

2.5.1. Specificity

To 0.5 ml of drug-free human plasma were added 0.1 ml of methanol–water (30:70, v/v) (double blank) or 0.05 ml of the lowest (0.52 ng/ml) CPM or 5.20 ng/ml CPM working solution and 0.05 ml of the working I.S. solution, 0.1 ml of 0.2 *M* NaOH solution and 5 ml of diethylether in a glass-stoppered 15-ml centrifuge tube. The tube was shaken for 10 min and then centrifuged for 10 min at 850 *g*. The organic layer (4 ml) was transferred to another disposable glass tube and evaporated to dryness in vacuo. The residue was dissolved in 0.1 ml of A–B (45:55, v/v) and a 20-μl aliquot of the solution was injected into the LC–MS system.

2.5.2. Linearity and reproducibility

Plasma samples spiked with the CPM and I.S. working solutions were processed according to the procedure described above for the construction of calibration curves and the assessment of accuracy and precision. The calibration curve was obtained by plotting the peak-area ratio (y) of CPM to the I.S. against the concentration (x) of CPM, using least-squares regression with weighting of $1/x^2$.

Intra- and inter-day reproducibility was assessed based on accuracy and precision. The accuracy was estimated to calculate the relative error (RE,%) [(mean observed concentration)/(spiked concentration)×100]. The precision was estimated to calculate the relative standard deviation (RSD, %) [(standard deviation)/(mean observed concentration)×100].

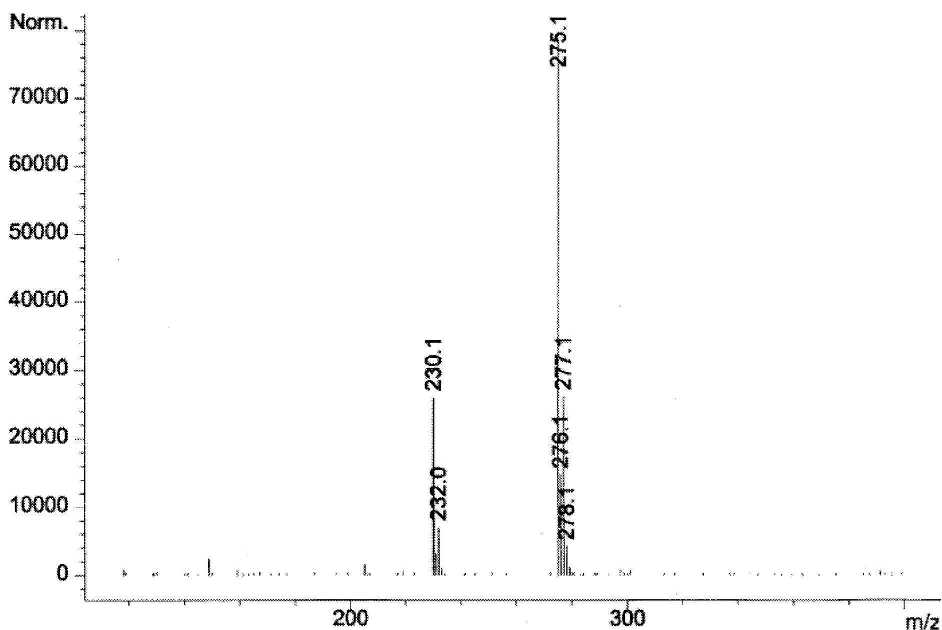
2.5.3. Stability

The processed plasma samples (0.52, 5.20 and 20.8 ng/ml) in the mobile phase solution (A–B, 45:55) were left in the autosampler at 10 °C for 24 h and then the stability was determined. The freeze–thaw stability was determined after two repeated freezing and thawing cycles. The long-term stability was determined after storing human plasma samples (5.20 and 20.8 ng/ml) in the freezer at −20 °C for 75 days. The stability was estimated as residual fraction [(concentration detected at time of measurement)/(concentration detected at time of preparation)×100, %].

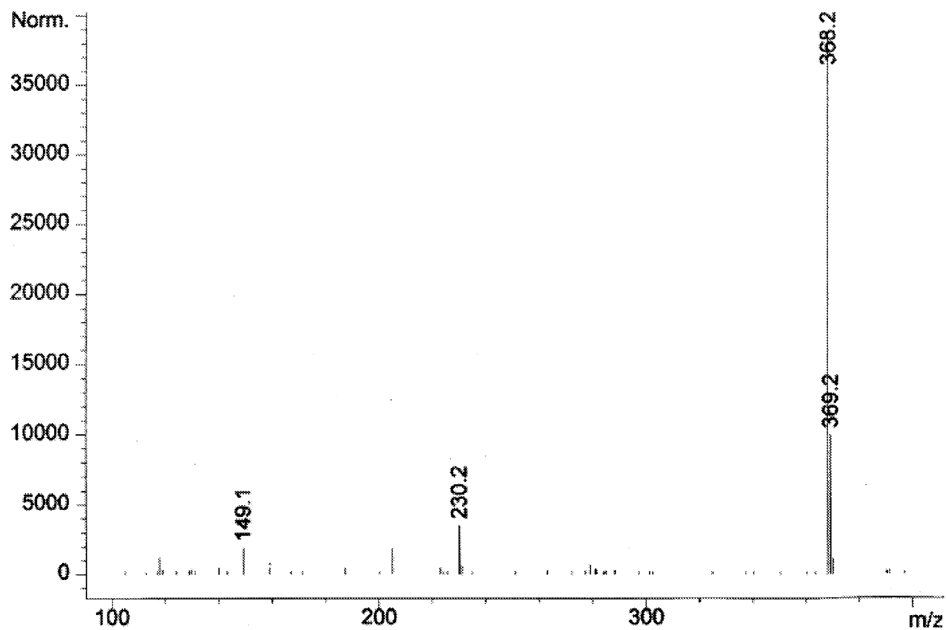
3. Results and discussion

3.1. Method optimization

MS conditions were optimized without an HPLC column using mobile phase of 20 m*M* ammonium acetate in water–acetonitrile of various ratios between 6:4 and 4:6. The polarity was set to the positive ESI mode because CPM and I.S. were both basic compounds. The fragmentor and capillary voltages were semiautomatically scanned using flow injection analysis (FIA) of the LC–MSD system, to achieve the highest sensitivity of CPM. Other MS parameters were also semiautomatically using FIA, such as drying N_2 gas temperature, drying N_2 gas flow, neblizer N_2 gas pressure and signal gain to achieve a stable baseline and optimal signal-to-noise ratio of the FIA chromatogram. A phenethyl-substituted (PhA) semimicro column was optimum to obtain LC–MS symmetrical peaks of CPM and I.S., suggesting mild π–π interactions with their two aromatic rings (Fig. 1). Linear gradient elution together with this column made it possible for the analyte peaks to be separated from interference peaks and for a flow-rate to be set to 0.3–0.35 ml/min. This flow-rate is slower than that (1 ml/min) in the reported LC–MS–MS method [8]. The final LC and



• • • • • • • (1) Chlorpheniramine



• • • • • • • • • • (2) AD-5423 (internal standard)

Fig. 2. Mass spectra of chlorpheniramine and AD-5423 (internal standard).

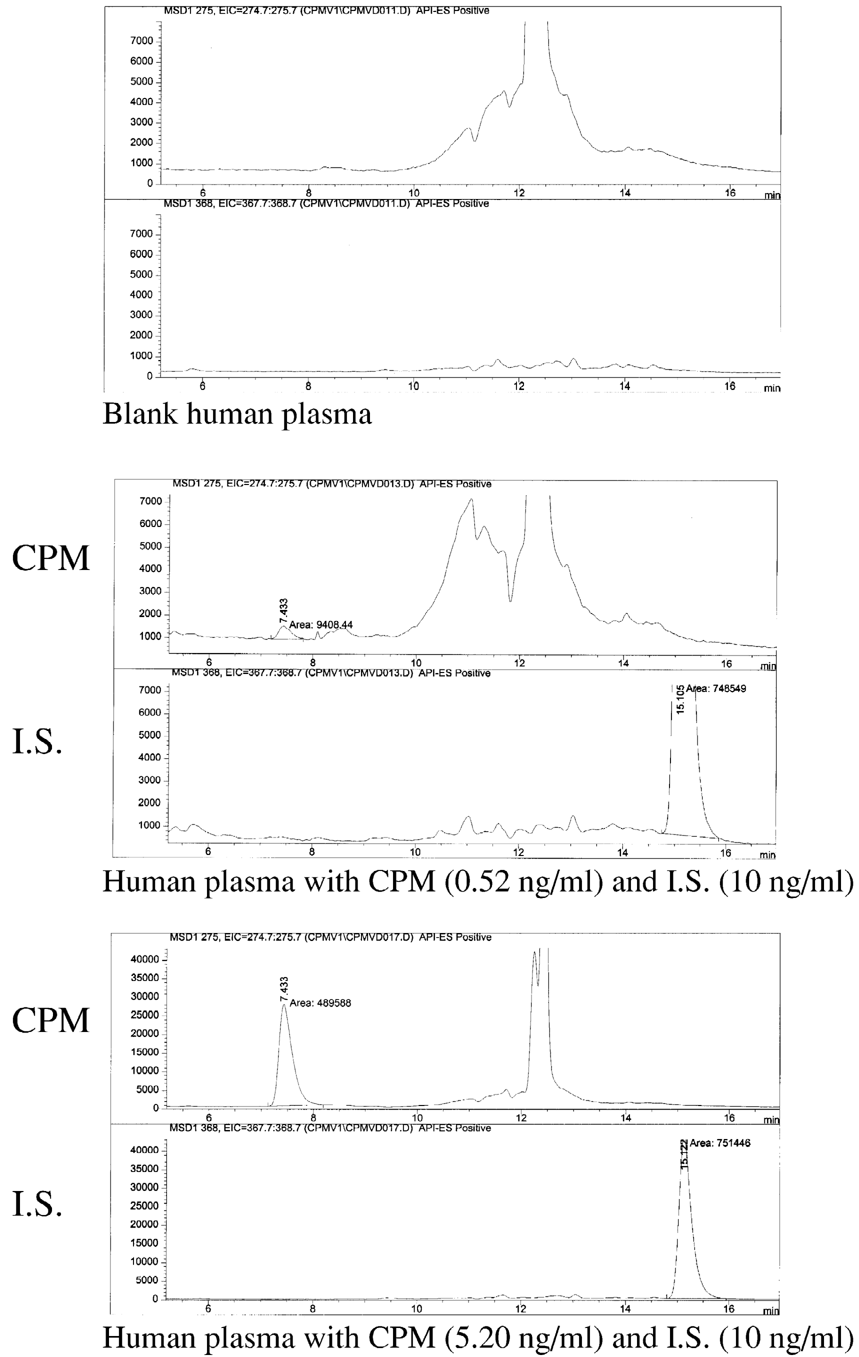


Fig. 3. Typical LC–MS chromatograms of human plasma.

Table 1
Parameters of CPM calibration curves using human plasma

	Slope (a)	Intercept (b)	Correlation coefficient (r)
Day 1	0.0301	−0.00329	0.999
Day 2	0.0324	−0.00293	0.999
Day 3	0.0340	−0.00118	0.998
Mean	0.03220	−0.00247	
SD	0.00196	0.00113	

Concentration = $a\times$(area ratio of CPM to I.S.) + b.

MS parameters were set as shown in Instrumentation.

3.2. Mass spectra

Mass spectra of CPM and I.S. under the positive ESI mode are shown in Fig. 2. Chlorpheniramine and I.S. showed intense $[M+H]^+$ ions at m/z 275 and 368, respectively. A minor fragment ion was observed at m/z 230 in the spectrum of chlorpheniramine, probably corresponding to dedimethylaminochlorpheniramine.

3.3. Specificity

Typical chromatograms of drug-free human plasma and human plasma spiked with CPM (0.52 ng/ml, 5.20 ng/ml) and I.S. (10 ng/ml) are shown in Fig. 3. The peaks of chlorpheniramine and I.S. eluted at 7.4 and 15.1 min, respectively. The run time was 17 min, which is longer than that (4.5 min) in the LC–MS–MS method [8]. The CPM and I.S. were well separated from each other and from endogenous components. On the other hand, the interference with specificity by other drugs was not tested. We think, however, there is not any interference with specificity, because the drugs that may be administered concomitantly, such as cough and cold preparation or antiallergic agent, do not have masses close to 275, for example, acetoaminophen: 151.16, aspirin: 180.18, ibuprofen: 206.29, indomethacin: 357.79, and ketoprofen: 254.29.

3.4. Linearity and reproducibility

As shown in Table 1, calibration curves over the concentration range of 0.52–20.8 ng/ml were expressed as $y=(3.22\times10^{-2}\pm1.95\times10^{-3})x+(-2.48\times10^{-3}\pm1.14\times10^{-3})$ (mean±SD) and the correlation coefficients (r) exceeded 0.995. Thus, good linearity of this method was seen in this concentration range.

Table 2 shows the intra-day precision and accuracy of detected CPM concentrations in human plasma. The intra-day precision ranged between 4.3 and 5.5%

Table 2
Intra-day precision and accuracy for CPM in human plasma

Spiked concentration (ng/ml)	Detected concentration (ng/ml)			Precision RSD (%)	Accuracy RE (%)
		Mean	SD		
0.52	0.54	0.55	0.03	5.5	5.8
	0.52				
	0.55				
	0.59				
	0.57				
5.20	4.99	5.36	0.23	4.3	3.1
	5.40				
	5.34				
	5.52				
	5.56				
20.8	26.0	23.7	1.3	5.5	13.9
	22.9				
	23.2				
	23.4				
	23.2				

Table 3
Inter-day precision and accuracy for CPM in human plasma

Spiked concentration (ng/ml)	Detected concentration (ng/ml)								Inter-day	
	Day 1		Day 2		Day 3		Mean		Precision RSD (%)	Accuracy RE (%)
		Mean		Mean		Mean	Mean	SD		
0.52	0.56 0.56	0.56	0.56 0.57	0.57	0.46 0.42	0.44	0.52	0.07	13.5	0.0
5.20	5.34 5.33	5.34	5.49 5.54	5.52	4.91 5.30	5.11	5.32	0.21	3.9	2.3
20.8	21.9 22.8	22.4	23.1 23.5	23.3	21.6 21.4	21.5	22.4	0.90	4.0	7.7

and intra-day accuracy ranged between 3.1 and 13.9%. The precision and accuracy for the lowest concentration were within 20%, proving the lower limit of quantitation (LLQ) [9] to be 0.52 ng/ml although the injection volume was only 20 μl. This LLQ corresponding to 0.37 ng/ml as chlorphenir-amine is similar to that (0.2 ng/ml) reported by the LC–MS–MS method [8]. In addition, the inter-day precision and accuracy ranged between 3.9 and 13.5% and between 0.0 and 7.7%, respectively (Table 3).

The above results demonstrate that reliable and reproducible plasma concentrations of CPM are determined over the range of 0.52 to 20.8 ng/ml by the present LC–MS method.

3.5. Stability

The residual fraction of CPM (0.52, 5.20 and 20.8 ng/ml) in the mobile phase after standing overnight was 92.6–96.4%, which shows that CPM is stable at least for 24 h in the autosampler (Table 4). After two freeze–thaw cycles the residual fraction of plasma CPM (5.20 and 20.8 ng/ml) was 104.1–105.3% (Table 5), showing CPM stability after the cycles. Moreover, CPM in plasma (5.20 and 20.8 ng/ml) stored in the freezer was stable for 75 days based on the residual fraction of 98.9–118.9% (Table 5).

3.6. Application

This developed method was applied to the determination of CPM in human plasma from a clinical study of post marketing surveillance. The quantitated mean plasma concentrations of CPM after a single oral administration of 2 mg of CPM are shown in Fig. 4. The maximum mean concentration of CPM at 3 h after the administration was 4.32 ng/ml (average of six volunteers). The CPM value can be converted to 3.03 ng/ml of chlorpheniramine, which is almost the same as those reported previously [1,3].

Table 4
Stability of CPM in autosampler at 10 °C

Spiked concentration (ng/ml)	Initial concentration (ng/ml)	After 24 h		
		Concentration (ng/ml)	Residual fraction (%)	
				Mean
0.52	0.56	0.53	94.6	
	0.56	0.53	94.6	94.6
5.2	5.34	4.96	92.9	
	5.33	4.94	92.7	92.8
20.8	21.9	21.0	95.9	
	22.8	22.1	96.9	96.4

Table 5
Long-term and freeze–thaw stability of CPM in human plasma at −20 °C

Day	5 ng/ml spiked			20 ng/ml spiked		
	Detected concentration (ng/ml)	Mean	Residual fraction (%)	Detected concentration (ng/ml)	Mean	Residual fraction (%)
Initial	5.35 5.28	5.32	100.0	22.2 23.2	22.7	100.0
Day 7	5.48 5.04	5.26	98.9	22.6 22.4	22.5	99.1
Day 22	5.36 5.37	5.37	100.9	24.3 22.0	23.2	102.2
Day 33	5.63 5.70	5.67	106.6	23.7 30.2	27.0	118.9
Day 75	5.38 5.42	5.40	101.5	24.2 22.7	23.5	103.5
Freeze–thaw (2 cycles)	5.44 5.63	5.54	104.1	23.8 23.9	23.9	105.3

4. Conclusions

We developed and validated an LC–MS method for the determination of CPM in human plasma. This method is as convenient and sensitive as the reported by LC–MS–MS method [8] with regard to a single liquid–liquid extraction and LLQ, and superior in cost performance of the system instrument. Our method will be useful in clinical pharmacokinetic studies of CPM.

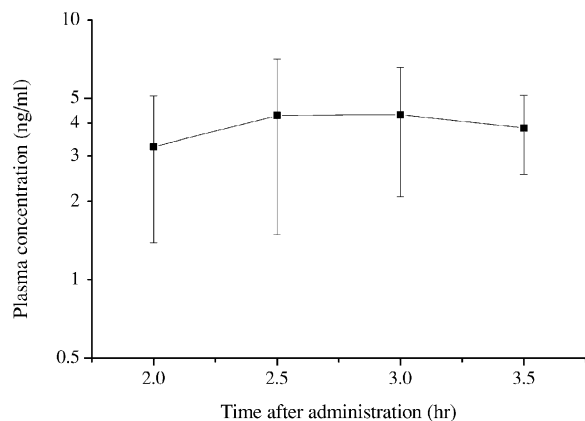


Fig. 4. Plasma levels of CPM in human after an oral administration at the dose of 2 mg (each plot represents mean (SD) of six subjects).

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