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# HIGHLY SENSITIVE DETERMINATION OF CHLORPHENIRAMINE AS FLUORESCENCE DERIVATIVE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A new method has been developed for the quantitative analysis of chlorpheniramine in human blood using high-performance liquid chromatography with a fluorescence detector. Benzyl chloroformate was found to be suitable as a fluorometric derivatizing reagent. A linear calibration curve ranging from 0 to 40 ng was obtained for chlorpheniramine, and the minimum detectable concentration was 0.1 ng/ml (whole blood) at a signal-to-noise ratio of 2. It was confirmed that chlorpheniramine levels in the blood of healthy adult volunteers could be precisely determined up to 96 h after a single oral administration of a tablet containing 4 mg of chlorpheniramine.

## INTRODUCTION

Chlorpheniramine maleate (CPM), which contains a pyridine substituent, is a potent tertiary amine antihistamine. It has been widely used for symptomatic relief of the common cold and allergic diseases.

Recent progress in analytical techniques such as high-performance liquid chromatography (HPLC) [1], gas chromatography–mass spectrometry (GC–MS) [2] and gas chromatography (GC) [3] has led to the minimum detectable amount being lowered to the nanogram range. Midha et al. [4] reported a new radioimmunoassay (RIA) technique with a detection sensitivity in the subnanogram range. This technique, however, requires antisera, and thus may not be useful in pharmacokinetic examinations because of cross-reactivity. Fluorometric techniques have been used by several investigators for the detection of tertiary amines [5,6]. The minimum detectable concentrations for CPM by these techniques, using an ion-pair reagent, were 3 ng [5] and 50 ng/ml [6], respectively. This is not sensitive enough to detect CPM in the blood after normal dosage.

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It is generally known that tertiary amines undergo dealkylation when heated with chloroformate, and form corresponding carbamates [7–9]. Gübitz et al. [9] reported that 2-naphthyl chloroformate is a suitable fluorescence reagent for the derivatization of drugs containing a tertiary amino group, and they applied the reaction to the pre-column derivatization of some antihistamines. Sternson and Cooper [10] suggested that the reaction with pentafluorobenzyl chloroformate is not suitable for the detection of tertiary amines containing a pyridine nucleus, because the reagent appears to attack pyridine resulting in ring-opening and generation of a variety of degradation products.

The purpose of this paper is to describe an HPLC fluorometric method for determining CPM in human blood, using benzyl chloroformate (BCF) as a derivatizing agent. The minimum detectable concentration was 0.1 ng/ml, at a signal-to-noise ratio of 2.

## EXPERIMENTAL

### *Reagents and chemicals*

CPM and triprolidine hydrochloride (TLH) were kindly donated by Sankyo (Tokyo, Japan) and diphenhydramine hydrochloride (DHH) by Kowa (Nagoya, Japan). BCF (95% liquid) was purchased from Aldrich (Milwaukee, WI, U.S.A.). All solvents were UV grade, and other reagents were of the highest analytical quality (Wako, Tokyo, Japan). These reagents were not purified before use.

### *Derivatization procedure and calibration*

Each of nine samples containing 0.4, 1.0, 3.0, 5.0, 10, 15, 20, 30 and 40 ng of CPM in chloroform (200 ng/ml) were taken in a sealed acceptor tube (10 ml) specific for the Kuderna-Danish evaporative concentrator. Then 50 mg of potassium carbonate (anhydrous) and 0.4 $\mu$l of BCF ($3.3 \cdot 10^{-9}$ mol) were mixed with each sample, and chloroform was added to bring the total sample volume to 200 $\mu$l. The volume of BCF was measured using a microsyringe (10A-RN; Scientific Glass Engineering, Austin, TX, U.S.A.). Each sample was heated at 80°C for 1.5 h in a dry bath. After cooling, the solvent was evaporated at 50°C using an aspirator. The residue was dissolved in 500 $\mu$l of 84% methanol solution, and then 100 $\mu$l of this solution were injected into the chromatograph as the standard to establish a calibration curve. TLH, DHH, 2-(*p*-chlorobenzyl)pyridine (CBP) and CPM extracted from blood samples were also derivatized by the same procedure.

### *Chromatographic conditions*

The HPLC system consisted of a Model LC-3A (Shimadzu, Japan), a guard column (50 mm × 2.1 mm I.D.), a commercially packed ODS column (Du Pont, Wilmington, DE, U.S.A.), a Zorbax® ODS column (25 cm × 4.6 mm I.D.) and a fluorescence detector (RF-500; Shimadzu) with an aluminium-mirrored rectangular solid 120-$\mu$l flow-cell (Shimadzu). The wavelength was set at 350 nm for excitation and 500 nm for emission. Methanol–water (84:16, v/v) was used as

TABLE I

NAMES AND CONTENTS OF THE DRUGS USED

| Compound Cold Tablets Marupi | | Contac® Spansule Capsule | |
|---|---|---|---|
| Acetaminophen | 200.0 mg | Dextromethorphan hydrobromide | 30.0 mg |
| N-methylephedrine hydrochloride | 16.0 mg | CPM | 4.0 mg |
| | | Phenylpropanolamine hydrochloride | 50.0 mg |
| CPM | 2.0 mg | | |
| Caffeine | 24.0 mg | | |
| Thiamine mononitrate | 6.0 mg | | |
| Riboflavine | 2.8 mg | | |
| Ascorbic acid | 120.0 mg | | |

the mobile phase. The solvent flow-rate was 1.7 ml/min. The column temperature was maintained at 50°C.

*Extraction*

An extraction column was made of a 10-ml glass syringe with a stainless-steel hypodermic needle (23 mm × 0.5 mm) and filled with 3.0 g of Extrelut® (E. Merck, Darmstadt, F.R.G.). A sample of 1 ml of human venous blood was taken. By adding 0.1 *M* potassium hydroxide solution and distilled water gradually and alternately, the total volume was brought to exactly 4.0 ml and pH to ca. 9.5. This solution was poured into the extraction column, and then eluted with 15 ml of carbon tetrachloride. The column eluate was evaporated under a stream of nitrogen at room temperature.

*Recovery*

Six samples of blank blood were spiked with 0.4, 2.5, 5.0, 10.0, 25.0 and 30.0 ng/ml CPM. The recovery of CPM was determined from the ratio of the peak height of the extracted derivative of CPM to that of the non-extracted CPM. The standard was prepared by dissolving the CPM in chloroform.

*Blood level*

A commercially available tablet containing 4 mg of CPM (Polaramine®; Essex Nippon, Osaka, Japan) was orally given with 250 ml of tap water to each of three male volunteers (27, 31 and 34 years old; 61, 57 and 65 kg body weight, respectively). In addition, one of two kinds of a commonly administered drug containing CPM (Compound Cold Tablets Marupi; Dainippon Pharmaceutical, Osaka, Japan and Contac® Spansule Capsule, Sumitomo Pharmaceutical, Osaka, Japan) were orally given to each of other two male volunteers (30 and 34 years old; 68 and 64 kg body weight, respectively. The contents of these drugs are indicated in Table I.

Blood samples (3 ml) were collected from the median-cephalic or the median-basilic vein before (for the control) and at 0.5, 1, 2, 3, 5, 24, 48, 72 and 96 h after the administration of the tablet. In two subjects the experiment could not be performed more than 24 or 48 h after administration. Each blood sample was

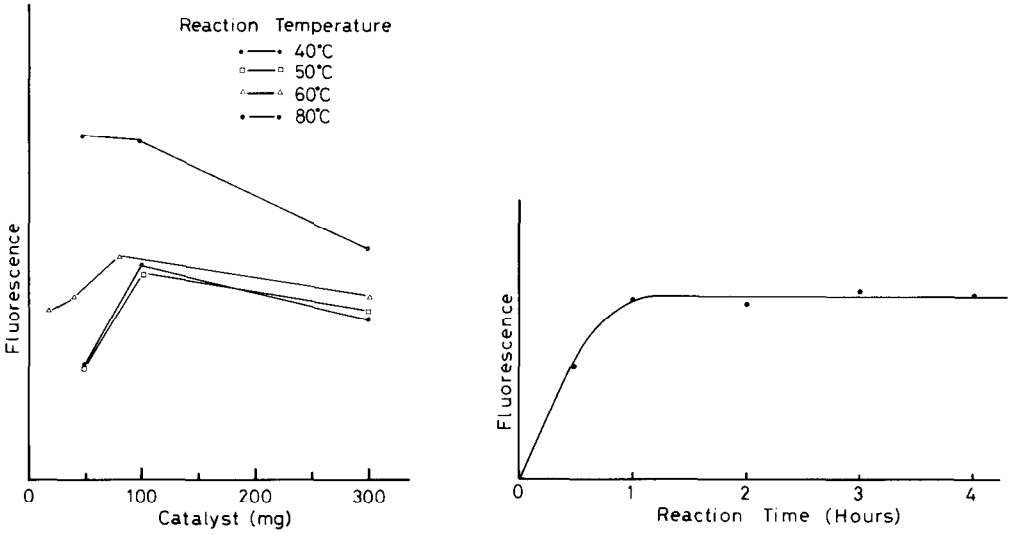



Fig. 1. Relation between the fluorescence intensity and the amount of catalyst.

Fig. 2. Kinetics of the reaction of BCF with CPM.

stored in a 10-ml heparinized glass tube covered with a glass stopper at −20°C until analysis. From each tube, two 1.0-ml aliquots were taken for analysis, and the data obtained were averaged.

## RESULTS

### *Preliminary examination*

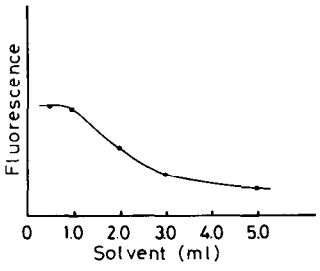
In this study, BCF was used as a fluorescence reagent for the derivatization of CPM. First, the effect of temperature on the reaction between CPM, BCF and catalyst (potassium carbonate) was examined. In Fig. 1, the intensity of the fluorescence is plotted against the amount of catalyst (10, 50, 100 and 300 mg) at four different temperatures: 40, 50, 60 and 80°C; the highest intensity was obtained using 50 mg of catalyst at 80°C. The reaction at 100°C was also examined but the results were inconsistent, probably owing to evaporation. Thus, the conditions stated above were used throughout the following analysis.

Other derivatizing conditions such as the reaction time (Fig. 2), the solvent volume (Fig. 3) and the amount of BCF (Fig. 4) were also examined. From the results, 1.5 h, 200 μl and 0.4 μl ($=3.3\cdot10^{-9}$ mol), respectively, were chosen as the derivatizing conditions.

### *Linearity, sensitivity and recovery*

A linear relationship was found between the peak height of the CPM derivative ($y$) and the CPM concentration ($x$), as given by the equation $y=3.0223x+0.228$ ($r=0.9997$, $n=10$) for the blood CPM concentration range 0–40 ng/ml.

Fig. 5 shows four chromatograms from various blood samples. There was no



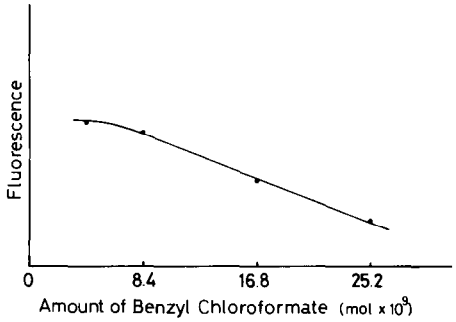




Fig. 3. Relation between fluorescence and the volume of the reaction solvent.

Fig. 4. Relation between fluorescence and the amount of BCF.

peak in the chromatogram of the blank blood (Fig. 5B) to interfere with the determination of CPM. The minimum detectable concentration of CPM was 0.1 ng/ml (whole blood) at a signal-to-noise ratio of 2. This CPM derivative showed stable fluorescence for more than 24 h at 4°C.

Table II summarizes the relation between the spiked concentration of CPM, its recovered concentration and the ratio of recovery determined from the peak heights. These results reveal that the recovery ratio of this method is high enough for CPM in human blood to be analysed quantitatively.

*Statistical validation*

Table III lists the intra-assay variation of CPM recovered from blood: the mean ranged from 2.6 to 14.4% over the concentration range 0.4–30.0 ng/ml. Similarly, Table IV shows that mean of the inter-assay variation of CPM recovered from blood ranged from 0.7 to 13.0% over the concentration range 0.4–10.0 ng/ml.

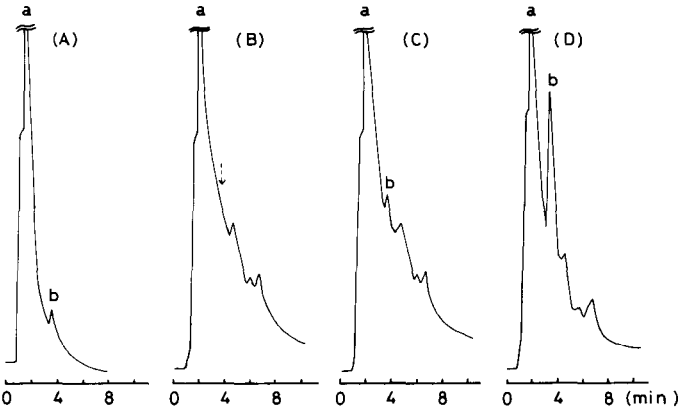



Fig. 5. Chromatograms of derivatives from (A) a standard of 0.4 ng/ml CPM, (B) blank blood, (C) blood spiked with 0.4 ng/ml CPM and (D) a blood sample (7.8 ng/ml) from a volunteer (57 kg body weight), taken 3 h after a 4-mg dose. Peaks: a = reagent decomposition products; b = CPM.

TABLE II

RECOVERY STUDIES FOR CPM

| Spiked concentration (ng/ml) | Chlorpheniramine recovered (mean ± S.D., $n=3$) | |
|---|---|---|
| | ng/ml | % |
| 0.4 | 0.38 ± 0.06 | 95.8 ± 14.4 |
| 2.5 | 2.30 ± 0.17 | 92.0 ± 6.9 |
| 5.0 | 4.33 ± 0.55 | 86.7 ± 11.0 |
| 10.0 | 9.30 ± 0.44 | 93.0 ± 4.4 |
| 25.0 | 23.10 ± 1.38 | 92.4 ± 5.3 |
| 30.0 | 27.80 ± 0.81 | 92.8 ± 2.6 |

*Time profiles of blood CPM concentration*

The blood concentration of CPM in the five volunteers and its time profiles after oral dosage are shown in Table V and Fig. 6. Those obtained from volunteers A, B and C were very similar. The half-lives of CPM in the blood in these volunteers were estimated as being 20.0 h for A, 18.0 h for B and 15.5 h for C. These values were within the same range as those reported previously [1, 4, 11–13].

The time profile in volunteer D was slightly different from those in volunteers A, B and C. This may be due to the lower dose of CPM (2 mg) and the difference

TABLE III

INTRA-ASSAY VARIATION OF CPM IN BLOOD

In all cases $n=3$.

| Amount added (ng/ml) | Amount recovered (ng/ml) | Standard deviation (%) |
|---|---|---|
| 0.4 | 0.4 | 14.4 |
| 2.5 | 2.3 | 6.9 |
| 5.0 | 4.3 | 11.0 |
| 10.0 | 9.3 | 4.4 |
| 25.0 | 23.1 | 5.3 |
| 30.0 | 27.8 | 2.6 |

TABLE IV

INTER-ASSAY VARIATION OF CPM IN BLOOD

| Amount added (ng/ml) | Amount recovered (ng/ml) | $n$ | Standard deviation (%) |
|---|---|---|---|
| 0.4 | 0.4 | 5 | 13.0 |
| 1.0 | 0.8 | 5 | 11.2 |
| 2.0 | 1.9 | 5 | 4.2 |
| 5.0 | 4.2 | 3 | 3.5 |
| 10.0 | 9.6 | 5 | 0.7 |

TABLE V

SUMMARY OF BLOOD CONCENTRATION DATA OF CPM IN FIVE VOLUNTEERS

N.A. = sample not analysed.

| Volunteer | Age (years) | Weight (kg) | Dose of CPM (mg) | Blood levels (ng/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0.5 h | 1.0 h | 2.0 h | 3.0 h | 5.0 h | 24.0 h | 48.0 h | 72.0 h | 96.0 h |
| A* | 27 | 61 | 4 | 0.6 | 1.2 | 5.7 | 7.0 | 5.9 | 2.8 | 0.9 | 0.6 | 0.3 |
| B* | 31 | 57 | 4 | 3.7 | 4.6 | 6.2 | 7.8 | 6.8 | 2.7 | 2.2 | 1.4 | 0.9 |
| C* | 34 | 65 | 4 | 2.1 | 3.8 | 6.3 | 8.6 | 5.7 | 2.9 | 1.5 | 1.0 | 0.6 |
| D** | 30 | 68 | 2 | 3.3 | 6.2 | N.A. | 3.9 | 3.2 | 1.5 | 0.2 | N.A. | N.A. |
| E*** | 34 | 64 | 4 | 5.2 | 5.4 | 8.2 | 5.5 | 6.0 | 5.3 | N.A. | N.A. | N.A. |

*Administration of Polaramine®.
**Administration of Compound Cold Tablets Marupi.
***Administration of Contac®Spansule Capsule.

of the bioavailability of CPM in the Compound Cold Tablets Marupi. As indicated by the time profile in volunteer E, the blood concentration of CPM 24 h after the administration was higher than that recorded from any other volunteer. This may be caused by the Spansule capsule.

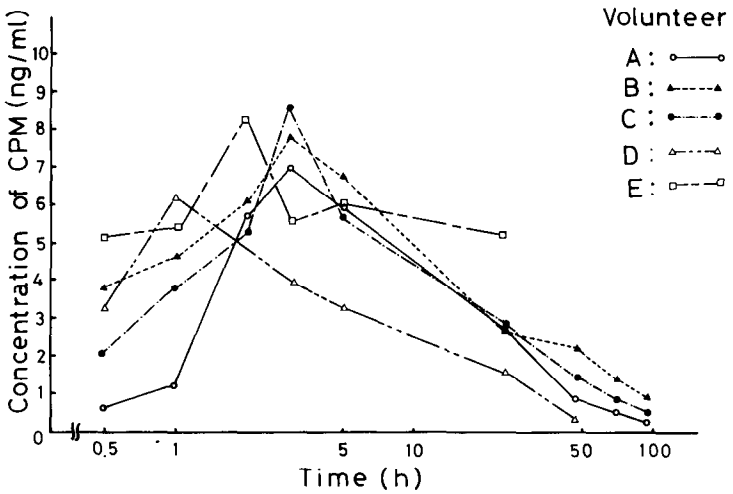



Fig. 6. Change in the blood concentration of CPM in five volunteers.

## DISCUSSION

### *Sensitivity of the method*

This study confirmed that CPM levels in the blood of healthy adults could be accurately determined up to 96 h after a single oral administration of a tablet containing 4 mg. This means that this is a sensitive method with a high recovery rate and selectivity for detecting CPM. The minimum detectable concentration of CPM by this technique was 0.1 ng/ml (whole blood), which is almost in the

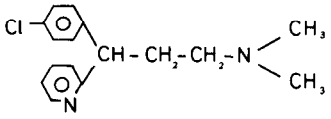
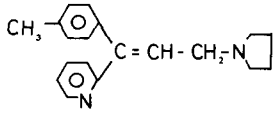
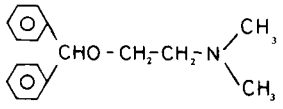
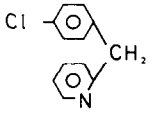
| Compound (Abbreviation) | Structure |
|---|---|
| Chlorpheniramine (CPM) |  |
| Triprolidine (TLH) |  |
| Diphenhydramine (DHH) |  |
| 2-(p-Chlorobenzyl) pyridine (CBP) |  |

Fig. 7. Structures of the compounds studied.

same range as that obtained by the RIA method [4] and lower than that determined by conventional chromatography methods [1–3].

*Reaction of CPM with tertiary amines*

For analysis of a tertiary amine such as CPM, sodium coumarin 6-sulphonate [6] and dimethoxyanthracene sulphonate [5] were used as fluorescence reagents by previous investigators. Sternson and Cooper [10] reported that all compounds with both an $\alpha$-pyridyl substituent and a dimethylaminoalkyl group fail to yield analytically useful products after reaction with the chloroformate reagent. Gübitz et al. [9] succeeded in analysing some tertiary amines without an $\alpha$-pyridyl substituent by using 2-naphthyl chloroformate as a fluorescence reagent, and they have stated that the reaction of these pyridyl-substituted compounds with chloroformate does not take place homogeneously owing to the formation of by-products following ring-opening. This makes the derivatization procedure not applicable.

Fig. 7 shows the chemical structures of three kinds of tertiary amine (CPM, TLH and DHH) and CBP. It was found through this study that CPM showed fluorescence after treatment with BCF, and that its intensity was proportional to the CPM concentration. It was also revealed that TLH and CBP, which have an $\alpha$-pyridyl substituent, showed fluorescence following reaction with BCF (Fig.

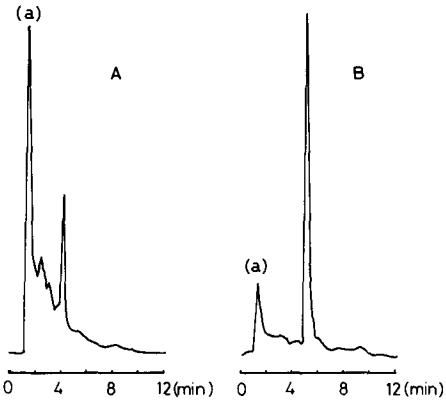



Fig. 8. Chromatograms of the derivatives from (A) TLH and (B) CBP. Peak a = reagent decomposition products.

8A and B), but DHH did not. This indicates that these $\alpha$-pyridyl-substituted compounds react with BCF to produce a fluorescent substance by opening the pyridine ring.

It is well known that there are two metabolites of CPM, monodesmethylchlorpheniramine and didesmethylchlorpheniramine, which have not been detected in plasma samples [1–4]. This suggests that the interference due to these compounds was not taken into consideration in this method. In addition, any other coadministered compounds in drugs (Table I) did not show the same fluorescence as CPM. This also suggests that these compounds did not interfere with CPM analysis. In conclusion, this method utilizing BCF as a fluorometric derivatizing reagent is highly sensitive for the quantitative analysis of CPM in human blood.

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