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Short communication

# Measurement of 4-hydroxycyclophosphamide in serum by reversed-phase high-performance liquid chromatography

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**Abstract**

A reversed-phase high-performance liquid chromatographic method was developed for the measurement of 4-hydroxycyclophosphamide (4-OH-CP) in human serum, since no such assay has been described to date. In the present procedure, the serum sample was treated with semicarbazide at pH 7.4 to derivatize the 4-OH-CP to its aldophosphamide semicarbazone form. Derivatization was performed at 60°C for 60 min and the product was extracted with ethyl acetate–chloroform (75:25, v/v). The derivatives formed were chromatographed on a $C_8$ reversed-phase column with a mobile phase of 0.025 *M* phosphate buffer–acetonitrile (18:82, v/v) and a detection wavelength of 230 nm. The limit of detection of the assay was 0.025 mg/l for 1 ml of serum with a signal-to-noise ratio of 2. The between-assay coefficients of variation at concentrations of 0.2 and 0.4 mg/l were 7.7 and 7.0% respectively. The simplicity and specificity of this method make it directly applicable to clinical studies on 4-OH-CP pharmacokinetics.

## 1, Introduction

Cyclophosphamide, 2-[bis(2-chloroethyl) amino] tetrahydro-2*H*- 1,3,2-oxazaphosphorine 2-oxide (CP), is widely used to treat malignant diseases [1] and, more recently, systematic necrotizing angiitis [2–4]. CP is a prodrug that requires initial metabolic activation by liver microsomal enzymes to form 4-hydroxycyclophosphamide (4-OH-CP) and phosphoramide mustard (PM). 4-OH-CP exists in equilibrium with its tautomer aldophosphamide. 4-OH-CP and PM are responsible for the cytotoxic properties of CP [5]. Knowing the 4-OH-CP concentrations in plasma has helped to improve our knowledge of CP efficacy and toxicity. The few techniques described for quantifying 4-OH-CP in plasma of cancer patients following CP administration were based on fluorometry [6,7], thin-layer chromatography combined with fluorometric detection [8], or GC–MS stable-isotope dilution [9]. No information has been reported on 4-OH-CP levels in patients with systemic vasculitis. In addition, no higher-performance liquid chromatographic (HPLC) method has yet been developed to analyze 4-OH-CP. The major obstacle to such an approach is the instability of 4-OH-CP in biological fluids; this,

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however, could be solved by using semicarbazide [8].

The purpose of our study was to develop a selective and sensitive HPLC method to investigate 4-OH-CP pharmacokinetics in patients with systemic necrotizing angiitis. Our method is based on the formation of the semicarbazone derivative of 4-OH-CP which is then subjected to direct ultraviolet (UV) detection of 4-OH-CP in serum. The 4-OH-CP-semicarbazone derivative was used to evaluate the 4-OH-CP pharmacokinetics.

## 2. Experimental

### 2.1. Chemicals

4-Hydroperoxycyclophosphamide (4-OOH-CP) was kindly supplied by Asta-Pharma (Frankfurt, Germany). In aqueous solution, 4-OOH-CP rapidly forms 4-OH-CP aldophosphamide and hydrogen peroxide. Phenobarbital (Gardenal), used as internal standard, was obtained from Specia (Paris, France) and semicarbazide hydrochloride from Aldrich (Vitry, France). All chemicals (ethyl acetate, chloroform, sodium hydroxide) were provided by Merck (Nogent-sur-Marne, France) except for acetonitrile which was purchased from Touzart et Matignon (Vitry, France). All organic solvents were HPLC grade.

### 2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC 6A pump connected to a Shimadzu SPD 6A UV spectrophotometric detector (Touzart et Matignon) coupled to a Merck D2500 integrator. This system was equipped with a Rheodyne 7125 injection valve and a 50-$\mu$l loop. A 5-$\mu$m Merck Hibar Lichrosorb $C_8$ RP-Select B (25 cm × 4.0 mm I.D.) column was used.

### 2.3. Chromatographic conditions

Acetonitrile–0.025 *M* potassium phosphate buffer (18:82, v/v) adjusted to pH 6 with 1.1 *M* sodium hydroxide, was used as the mobile phase. The UV wavelength for detection was 230 nm. The flow-rate was 0.8 ml/min. The analytical column was operated at ambient temperature.

### 2.4. Extraction procedure

Venous blood samples from patients receiving intravenous CP were treated with 0.67 *M* semicarbazide (pH 7.4) (3/10, v/v) immediately after sample collection. Semicarbazide hydrochloride was used to stabilize the tautomeric equilibrium (4-OH-CP/aldophosphamide) by forming the semicarbazone derivative, thereby preventing spontaneous decomposition to PM [8,10]. Patient sera were divided into 1-ml aliquots, incubated in a water bath at 60°C for 1 h (optimal incubation time for the formation of the 4-OH-CP-semicarbazone derivative) and stored at −20°C until analyzed. Internal standard (75 $\mu$l of a 100 mg/l solution of phenobarbital), and 5 ml of ethyl acetate–chloroform (75/25, v/v) were added to 1 ml of serum, mixed for 15 min and centrifuged at 2000 *g* for 20 min. The organic phase was collected and dried under nitrogen gas at room temperature. The residue was reconstituted with 200 $\mu$l of mobile phase and 200 $\mu$l of chloroform, vortex-mixed for 1 min and centrifuged at 2000 *g* for 15 min. To break up the emulsion, the sample was placed at −20°C for 1 h. This mixture was centrifuged at 1500 *g* for 30 min and 100 $\mu$l was injected onto the chromatographic system.

### 2.5. Calibration curve

4-OOH-CP was used as a substitute for 4-OH-CP. A solution of 4-OH-CP was prepared immediately prior to use by dissolving 4-OOH-CP in distilled water. Standard concentration curves were obtained by adding known amounts of 4-OH-CP in the range 0–0.5 mg/l, stabilized as semicarbazone derivative, to normal freshly frozen serum obtained from the local blood bank. These samples were analyzed as described above. Calculations were based on the peak-area ratio of 4-OH-CP-semicarbazone to that of the internal standard.

### 2.6. Limit of detection

The limit of detection of the assay was 0.025 mg/l for 1 ml of serum with a signal-to-noise ratio of 2. This level should allow 4-OH-CP to be measured in serum over a period of 24 h in patients given an intravenous infusion of CP at a dose ranging from 600 to 1200 mg.

### 2.7. Precision and accuracy

The precision and accuracy of this method were demonstrated by repetitive analysis of plasma spiked with the 4-OH-CP-semicarbazone derivative at different concentrations. The within-day ($n$-10) and between-day ($n = 6$) reproducibility were assessed by the evaluation of the mean and standard deviation (S.D.) values of 2 spiked concentrations (0.2 and 0.4 mg/l). The results are expressed by the coefficient of variation (C.V.) (%) and the mean error (%) in Table 1.

### 2.8. Recovery

The highest extraction recovery for 4-OH-CP was obtained with ethyl acetate (81.3%) as compared to hexane (12.5%) and chloroform (5.4%). Because extraction with ethyl acetate produced interfering peaks, chloroform was added and the ethyl acetate–chloroform ratio was optimized to obtain a high recovery (75%) and clean samples.

Table 1
Linearity of the assay ($n = 6$)

| $x_i$ | $\bar{y}_i$ | $s_i^2$ |
|---|---|---|
| 0.005 | 0.101 | $0.55 \cdot 10^{-4}$ |
| 0.1 | 0.214 | $0.31 \cdot 10^{-4}$ |
| 0.2 | 0.390 | $3.40 \cdot 10^{-3}$ |
| 0.4 | 0.722 | $5.25 \cdot 10^{-3}$ |
| 0.5 | 0.920 | $4.68 \cdot 10^{-3}$ |
| Mean equation | $y = 1.77bx + 0.025$ | |
| Coefficient of variation of slope (%) | 8.0 | |

$x_i$ = concentration (mg/l)
$\bar{y}_i$ and $s_i^2$ mean and variance of the $y$ values at $x_i$ with $y_i$ = (4-OH-CP-semicarbazone)/(internal standard).

## 3. Results and Discussion

### 3.1. Chromatographic separation

Fig. 1 shows chromatograms of the 4-OH-CP-semicarbazone derivative in a serum blank (A) and sample containing 0.2 mg/l (B), patient serum blank (C) and patient serum 15 min after receiving 1000 mg of CP as 1-h intravenous infusion (D). The capacity factors ($k'$) for 4-OH-CP and phenobarbital were 4 and 13, respectively. The analysis time for each serum sample was less than 35 min. Fig. 2 shows the derivatisation reaction of 4-OH-CP with semicarbazide hydrochloride.

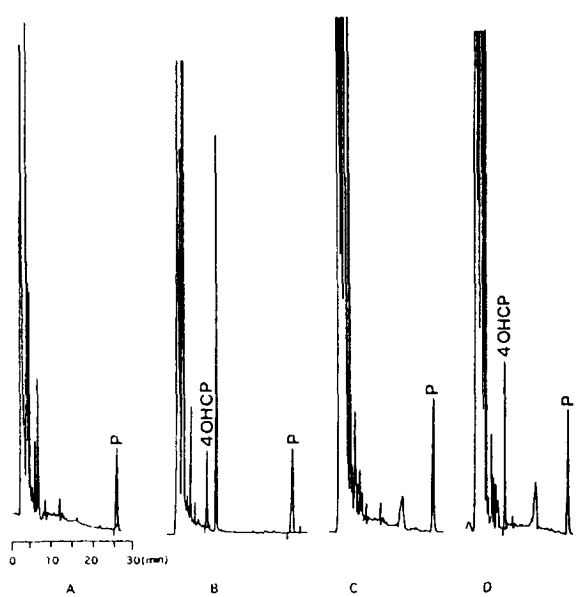


Fig. 1. Chromatograms of 4-OH-CP obtained with 1 ml of serum. (A) Serum blank, (B) sample containing 0.2 mg/l, (C) patient serum blank, (D) patient serum sample drawn 15 min after I.V. infusion of 1000 mg of CP. Peaks: 4OHCP = 4-OH-CP-semicarbazone derivative, P = phenobarbital.

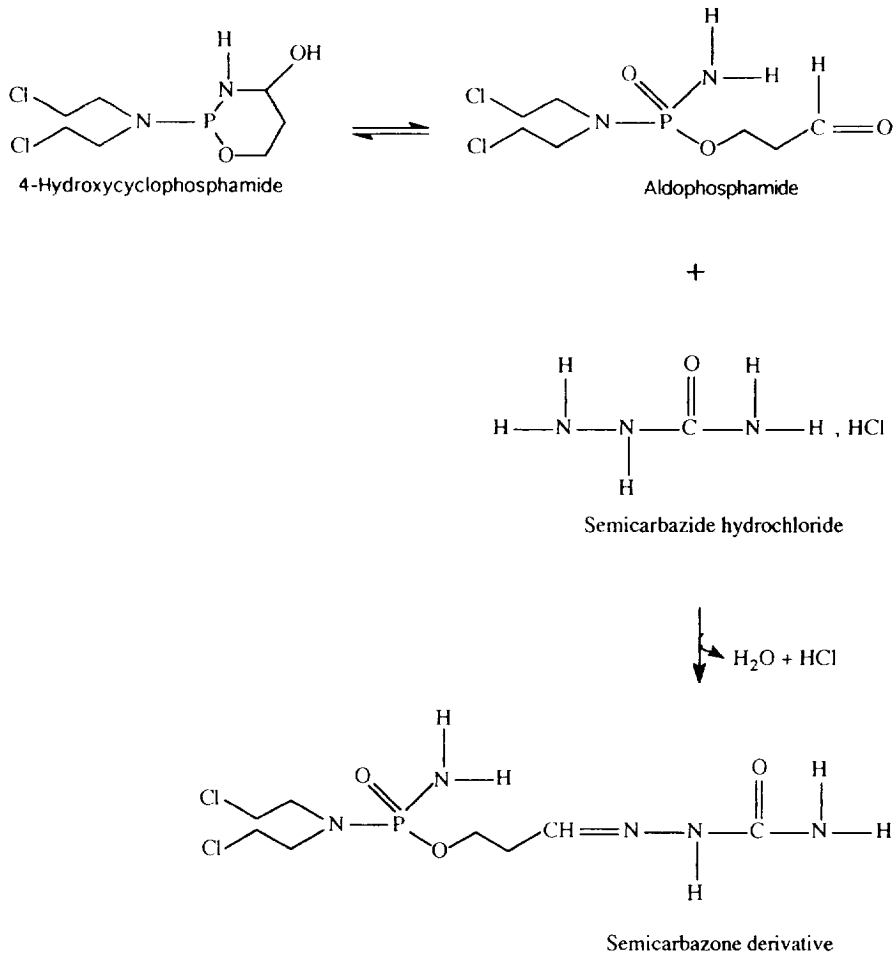


Fig. 2. Derivatisation reaction of 4-OH-CP with semicarbazide hydrochloride.

### 3.2. Linearity and reproducibility

The linearity of the assay for 4-OH-CP-semicarbazone derivative in serum was determined from calibration curves obtained by repeated analysis ($n = 6$) of concentrations of 0.05, 0.1, 0.2, 0.4 and 0.5 mg/l. The mean and variance of each concentration are given in Table 1. The weighted residual sum of squares [11] was 4.38, which is comparable to a $\chi^2$ value with 3 degrees of freedom (7.81 with a risk of 0.05). The assay was linear over the range 0.05–0.5 mg/l. The precision and accuracy of the assay or shown in Table 2.

Table 2
Reproducibility

| Theoretical (mg/l) | Calculated (mean ± S.D.) (mg/l) | C.V. (%) | Mean error (%) |
|---|---|---|---|
| *Within-day* ($n = 10$) | | | |
| 0.2 | 0.211 ± 0.022 | 10.4 | 5.5 |
| 0.4 | 0.421 ± 0.034 | 8.1 | 5.3 |
| *Between-day* ($n = 6$) | | | |
| 0.2 | 0.207 ± 0.016 | 7.7 | 3.5 |
| 0.4 | 0.418 ± 0.029 | 7.0 | 4.6 |

Mean error: (calculated-theoretical)/(theoretical) · 100.

### 3.3. Specificity

No interfering peaks were observed in the HPLC analysis of serum samples from 10 patients receiving a combination drug regimens (methylprednisolone, prednisone, cotrimoxazole, allopurinol, oxazepam, bromazepam, diosmin, acetaminophen).

### 3.4. Stability

The 4-OH-CP-semicarbazone derivative was stable in water and serum at ambient temperature and at 4°C for 24 h. This finding agrees with an in-vitro study which showed that aldophosphamide decayed rapidly during incubation at 37°C for 44 h, whereas the semicarbazone derivative was stable under the same conditions [12]. Aliquots ($n = 6$) of standards were stored at −20°C and analyzed over a 2-month period. A variation of 15% was found which indicated good stability of the 4-OH-CP-semicarbazone derivative in serum.

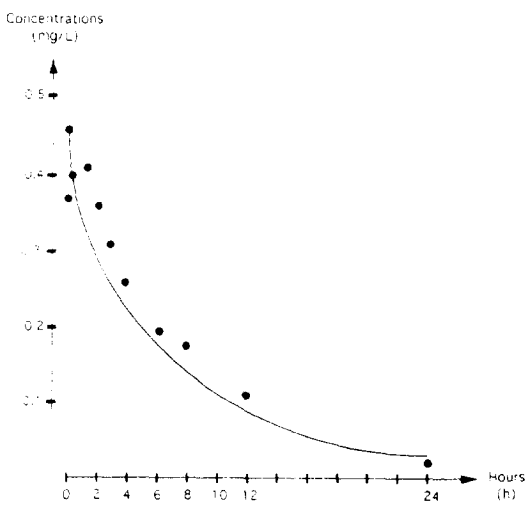


Fig. 3. 4-OH-CP concentrations in the serum of a patient that had received a 1-H intravenous infusion of 1000 mg of CP.

### 3.5. Clinical application

This 4-OH-CP HPLC assay is the first one that can be used to determine the levels of 4-OH-CP in patient sera. Fig. 3 shows the concentration–time profile of the 4-OH-CP serum levels in a patient with systemic necrotizing angiitis who had received 1000 mg of CP as a 1-h intravenous infusion. No information has been reported in other studies on the 4-OH-CP pharmacokinetics in systemic angiitis diseases.

Because of its sensitivity, specificity and reproducibility, this method can be directly applied to pharmacokinetic studies in a clinical setting.

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