IDENTIFICATION OF CHLORAMPHENICOL OXAMIC ACID AS A NEW MAJOR METABOLITE OF CHLORAMPHENICOL IN RATS

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1. Introduction

Since chloramphenicol (CP) was discovered about 30 years ago and its broad antibiotic spectrum tested in therapeutics, its main metabolic pathways have been described. CP is rapidly absorbed in the rat and excreted in bile as a glucuronide conjugate [1–4]. This compound is readily hydrolysed and converted into CP, which is then reduced to aromatic amines, by bacterial action of the gut flora. In man, CP alcohol appears as a metabolite [5].

Studies on the mechanism of the toxic side effects of CP on bone marrow derived cell have implicated other metabolites. An in vitro covalent binding of CP to rat liver microsomal proteins was shown after activation of CP into an oxamyl chloride which acylates proteins [6]. Although the CP oxamic acid has only been isolated and characterized in vitro from liver microsomes of phenobarbital-treated rats (never with normal microsomes), this suggested an hypothetical metabolic pathway. It was not confirmed by in vivo studies but in vitro [7], a dechlorination of CP by a cytosol of rat liver lead to CP aldehyde as a major metabolite and metabolic pathways for the formation of these metabolites were suggested [7].

As CP oxamic acid has never been isolated and identified in vivo, an hypothetical pathway of CP leading, from CP aldehyde, either to the alcohol or to the oxamic acid could not be confirmed. Here, from metabolic studies in the rat, we describe the in vivo formation of CP oxamic acid. This metabolite has been detected, isolated and identified from urine of CP-treated rats. Determinations have been performed to measure its elimination for 3 days after treatment.

2. Materials and methods

Male OFA rats (~200 g body wt) were held in metabolic cages. After 15 days acclimation they received a single therapeutic dose of CP by stomacchal intubation. All of them received 8 mg CP dissolved in propylene glycol. Besides 3 of them received in the same times 20 μCi [^{14}C]CP (Radio Chemical Center, Amersham, 15 mCi/mmol) and 3 others received 8 mg unlabelled CP plus 80 μCi [^{3}H]CP. [^{3}H]CP (185 mCi/mmol) was synthesized by reduction of the ketone derivative of CP by $NaB^{3}H_4$ (method derived from [6]), the D-*threo* isomer being then purified by high performance liquid chromatography (HPLC). Urine samples were collected each day, before treatment (control) and for 3 days after CP intubation.

2.1. *Extraction*

Urines were extracted twice with 50 ml ethyl acetate (all solvents were analytical grade). The aqueous phase was brought to pH 1.5 with 6 N HCl and re-extracted twice with 50 ml ethyl acetate. After washing with 2 × 4 ml H_2O, the ethyl acetate was evaporated to dryness and the residue dissolved in 2 ml methanol. This extract was chromatographied in silica gel thin-layer chromatography (TLC):$CHCl_3$–MeOH–H_2O–CH_3COOH (50:20:2:2) and compared with a standard [^{3}H]CP oxamate. Standards CP oxamate and [^{3}H]CP oxamate have been synthesized by a method adapted from [6] and the purity checked by mass spectrometry after gas chromatographic analysis (GC–MS) as described below.

The silica band corresponding to CP oxamate was scraped, eluted with water, then brought to pH 1.5 and extracted with twice 30 ml ethyl acetate. After evaporation the dry residue is dissolved in 1 ml methanol.

2.2. *Identification*

^{3}H- and ^{14}C-labelled extracts were transmethylated for 2 h at 80°C in the mixture MeOH–C_6H_6–H_2SO_4. The ^{3}H and ^{14}C hydrolysis products were analysed, respectively, by radio-HPLC or radio-GC and compared to standards of CP base (2-amino-*p*-nitrophenyl-1,3-propanediol) and methylic ester of oxalic acid. Unlabelled urine extracts as well as synthetized CP oxamic acid were methylated with diazomethane, then silylated with BSTFA. The TMS derivatives were analysed by GC on a 1 m glass column of Dexil 300 (3%), (temperature program : 3°C/min from 220–270°C, detection by flame ionization) and the mass spectra were compared.

2.3. *Assays*

The urine extracts from [^{3}H]CP treated animals were analysed by radio-HPLC on a 25 cm × 2.1 mm i.d. column packed with 5 μm C_{18} spherisorb ODS (Spectra-Physics). Mobile phase used was water–methanol (70 + 30), NaH_2PO_4 0.05 M, at 0.8 ml/min flow rate. Fractions of 250 μl were collected, fluors mixture added, and the radioactivity measured in a liquid scintillation spectrometer. Identification and quantitative determinations were performed by comparison with a standard [^{3}H]CP oxamate analysed in the same conditions.

3. Results

Indirect informations on the structure of the urinary metabolite presumed to be CP oxamic acid were obtained from the different steps of the analysis of the radioactive material. It was extracted at acidic pH and has the same R_F in the described TLC as standard CP oxamate. Moreover, after the extraction and and the first steps of purification, hydrolysis of the molecule gave rise to well-defined degradation products: oxalic acid and CP-base. The radio GC of ^{14}C-labelled urine extract, after hydrolysis and transmethylation, shows a peak of same retention time as methylic ester of standard oxalic acid chromatographied in the same conditions (fig.1). In the same way, after HPLC and measure of the radioactivity of the fractions of the eluate, the hydrolysed ^{3}H-labelled urine extract gives raise to a peak of the same retention time as standard unlabelled or tritiated CP-base chromatographed in the same conditions and detected either by radioactivity or by direct UV absorption at 280 nm (fig.2).

To confirm these presumptions, direct identification was performed by GC–MS. Fig.3 and 4 show, respectively, the analysis of TMS derivatives of standard CP oxamic acid and of the urinary extract. Mass spectra correspond to the di TMS, methyl derivative; in the case of the urinary extract (fig.4), silylation was incomplete and another minor peak appears.

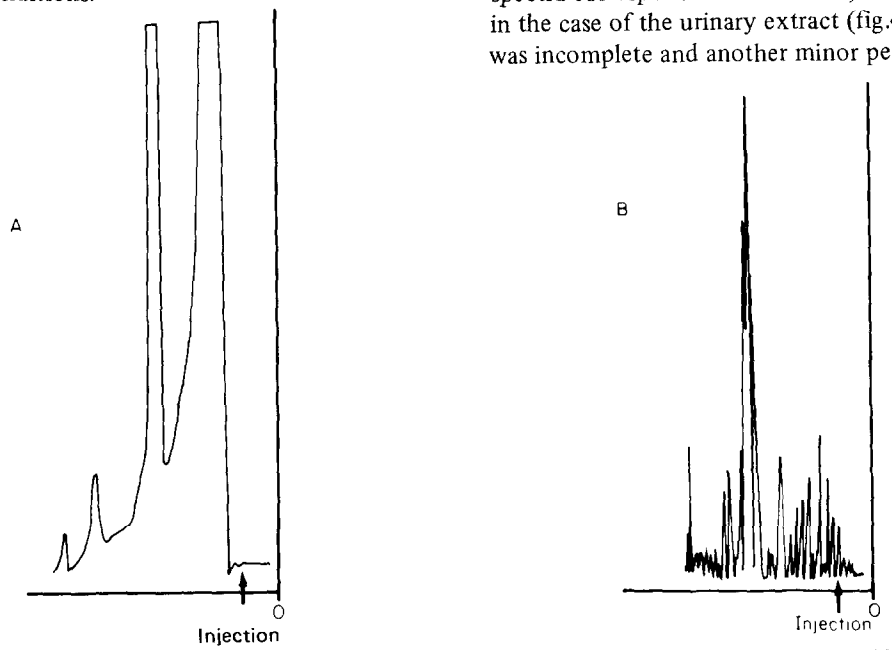
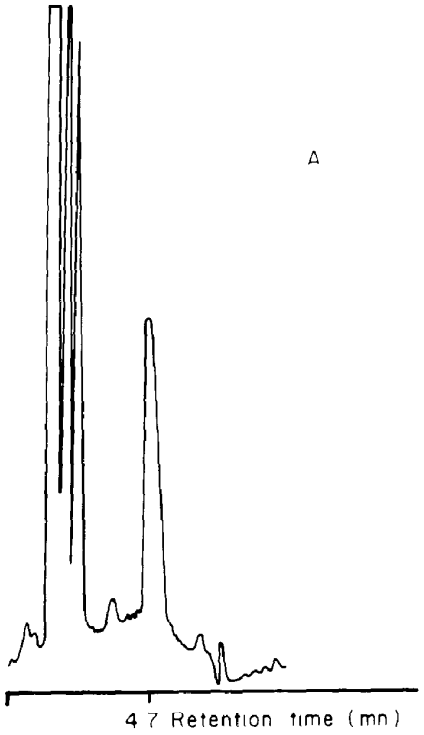


Fig.1. Analysis of the urinary extract from [^{14}C]CP-treated rats. Radio-GC (3 m glass column of DEGS 15%, 110°C) of the hydrolysed and transmethylated extract, spiked with standard oxalic acid (as methylic ester). (A) Flame ionization detection; (B) radioactivity measurement.



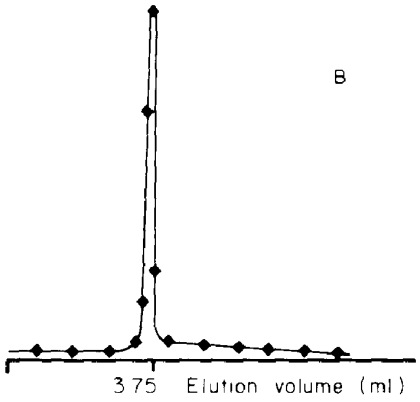


Fig.2. Analysis of the urinary extract from [^{3}H]CP treated rats. Radio-HPLC [25 cm × 2.1 mm column packed with 10 μm lichrosorb RP 18-Merck. Mobile phase: H_2O–MeOH (65:35), NaH_2PO_4, 0.1 M pumped at 0.8 ml/min], of the hydrolyzed extract spiked with standard CP base. (A) UV detection at 280 nm; (B) radioactive counting of the eluate.

Both spectra are identical. The mass fragmentation of CP oxamic acid is detailed in fig.5, with ions at *m/e* 427, 383, 337, 290, 225, 218. The ion 427 represents the loss of 15 amu from the molecular ion, which is classically reported for the loss of CH_3. In the same way, the ions *m/e* 383 and 337 represent the losses of CO_2-CH_3 and NO_2 plus CO_2-CH_3. The ions 290 and 218 correspond to the losses of CH_2O and CH_2O–TMS from the CHOTMS–CH–(NH–CO–COOMe)–CH_2OTMS fragment, plus a rearranged hydrogen atom. The ion at *m/e* 225 represents the $NO_2C_6H_4$ CHOTMS fragment plus a rearranged

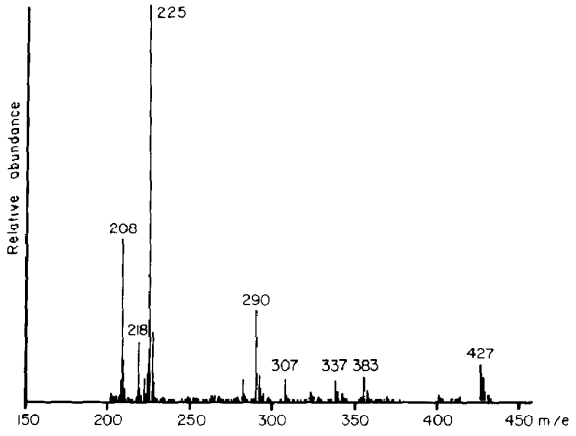
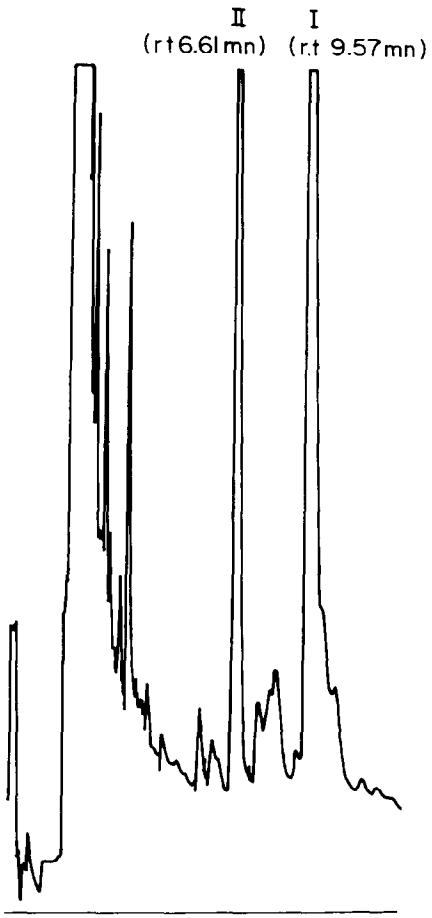


Fig.3. GC–MS analysis of the Me-TMS derivative of the synthesized standard CP oxamic acid. The di TMS derivative represents the major peak (91%).



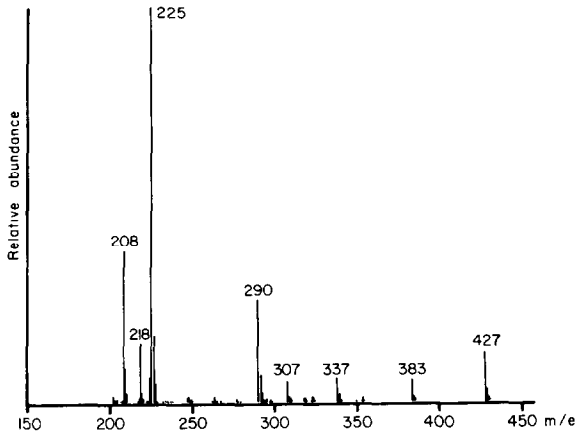


Fig.4. GC–MS analysis of the isolated urinary metabolite after methylation and sylylation. Mass spectrum corresponds to the main peak (I) which is the di TMS derivative and represents ~60% of the total as peak II (~30%) corresponds to an incompletely silylated derivative.

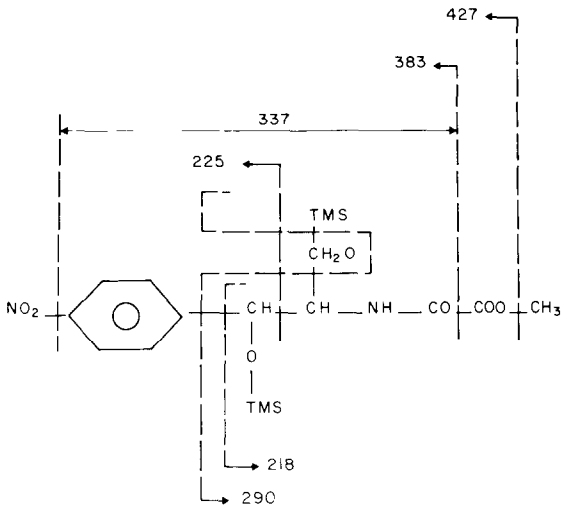


Fig.5. Mass fragmentation of the CP oxamic acid derivative.

hydrogen atom, whereas that at *m/e* 208, described [7] is assumed by this author to derive from the *m/e* 225 ion by the loss of 17 amu.

The identity of the urinary metabolite such isolated to CP oxamic acid is thus clearly established.

It must be noticed that no interference has been observed with control blank urinary sample either in TLC, GC or HPLC analysis.

In vivo quantitative determinations of CP oxamic acid in the urines of [^{3}H]CP treated rats during 3 days after treatment were performed by the radio-HPLC described. In these conditions the retention volume of the labelled urinary extract is 3.25 ml; it is identical to that of standard [^{3}H]CP oxamate and of unlabelled standard CP oxamate, detected by UV absorption at 280 nm. Results are presented in table 1. CP oxamic acid appears to represent a major metabolite corresponding to up to 25% of the radioactivity eliminated in urine.

Table 1
Urinary elimination of CP oxamic acid after a single oral administration of 8 mg CP to rats

Days after treatment	CP oxamic acid (% total radioact. eliminated in urine)
1	25.2 ± 0.2
2	25.7 ± 2.5
3	14.6 ± 6.1
Total 1–3	24.8 ± 0.6

4. Discussion

The identification of CP oxamic acid and its determination as a major metabolite appearing in vivo in CP-treated rats could confirm the first hypothesis on in vitro observations [6], of the formation of a potentially toxic CP–protein conjugate. The covalent binding of CP to protein would occur after activation of the CP to CP oxamyl chloride by liver microsomal enzymes. However the presence of such CP oxamate in urines of treated animals seems more likely to support the metabolic pathway described [7] in rat liver cytosol which implicates the formation of CP aldehyde by a hydrolytic dechlorination. Moreover it appears in this work that the CP aldehyde formed that can give rise to the CP alcohol metabolite described, can also be a precursor to CP oxamyl derivative. In the rat this oxidation appears to be a major route of the metabolic pathway of the CP. The potential pharmacological and toxicological importance of this CP oxamyl derivative remains to be determined.

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