IDENTIFICATION OF MENAQUINONE-4 METABOLITES IN THE RAT

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Four metabolites of menaquinone-4 [MQ-4] were isolated from rat urine, bile and liver. From rat urine following intravenous or oral administration of [^{14}C]MQ-4, two major metabolites were isolated and their aglycones were identified as 2-methyl-3-(5'-carboxy-3'-methyl-2'-pentyl)-1,4-naphthoquinone [K acid 1] and 3-(3'-carboxybutyl)-2-methyl-1,4-naphthoquinone [K acid 2]. The aglycone of a third minor metabolite isolated from bile was tentatively identified as 2-methyl-3-(15'-carboxy-3',7',11'-trimethyl-2',6',10',14'-hexadecatetranyl)-1,4-naphthoquinone [MQ-4-COOH]. The structures of the three aglycones, which were excreted into the urine or bile mainly as glucuronide conjugates, indicated that oxidative degradation of the alkyl side chain of MQ-4 had occurred by ω - and β -oxidation. In addition, 2,3-epoxy-MQ-4 was identified in the liver of rats which were pretreated with warfarin and then dosed with [^{14}C]MQ-4.

KEYWORDS — menaquinone-4; rat; K acid 1; K acid 2; 2,3-epoxy-menaquinone-4; metabolite

INTRODUCTION

The vitamin K group includes several homologues which differ in the alkyl side chain at the 3-position of the naphthoquinone ring. Of these homologues, MQ-4 is considered to be a physiologically active form in animals.¹⁾ However, in contrast to phylloquinone, only limited information on the biotransformation of MQ-4 has been available.^{2,3)} Furthermore, the radioactive MQ-4 used in the previous studies contained a significant amount of cis-isomer, while natural

MQ-4 occurs entirely in the all-trans form. Therefore, it is desirable to use synthetic MQ-4 of all-trans configuration to study the biotransformation of the vitamin.

The present paper deals with the isolation and identification of the metabolites in normal and warfarin-pretreated rats given a single dose of all-trans [^{14}C]MQ-4. The chemical characterization of the metabolites was based on comparative gas chromatographic/mass spectrometric [GC/MS] studies after acidic extraction and methylation.

MATERIALS AND METHODS

Chemicals and Synthesis — [^{14}C]MQ-4 with a specific activity of 1.50 MBq/mg was synthesized according to the procedure recently reported.⁴⁾ The compound labeled with ^{14}C at the carbon atom 3' of the side chain had a chemical and radiochemical purity >98% tested by TLC. The percentages of the trans-form and cis-form were 96 and 4 by HPLC analysis, respectively. The reference compounds and their derivatives were prepared in our laboratories according to the known synthetic procedures.⁵⁻⁷⁾ β -Glucuronidase (type H 1, Sigma, U.S.A.) and HCl-methanol reagent 10 (Tokyo Kasei, Japan) were purchased.

Animals — Groups of three male Wistar rats (Shizuoka Laboratory Animal Center, Japan) weighing 200-250 g were used without fasting.

Drug Administration and Collection of Samples — [^{14}C]MQ-4 (10 mg) was solubilized with Nikkol HCO-60 in 2.5 or 10 ml of distilled water. The [^{14}C]MQ-4 solution was given to the rats orally or intravenously at a dose of 4 mg/kg. Urine and feces were collected separately every 24 hours. To estimate radioactivity, the bile ducts were cannulated with polyethylene tubing (PE50, Clay Adams, U.S.A.) and serial bile samples were taken every two hours for 10 h and then at 24 h. Warfarin (10 mg/kg) was given intraperitoneally to rats at 1 h before oral administration of [^{14}C]MQ-4. At 2 h after the dosing with [^{14}C]MQ-4, the rats were killed and the livers were removed.

Measurement of Radioactivity — The radioactivity in each sample of urine, bile, feces and solvent extract was measured with a liquid scintillation counter (LSC-903, Aloka).

Extraction and Derivatization of Metabolites — The urine and bile samples were freeze-dried, and 5-10 ml of HCl-methanol reagent was added to the residues for esterification. The mixture was vortex-mixed and stirred at room temperature for 15 h. The solvent was evaporated off and the residue was extracted with ethyl acetate. The liver homogenates (20%) of the warfarin-pretreated rats were adjusted to pH 2-3 with 1 N HCl and extracted with tetrahydrofuran/ethyl ether

(1:1).

Structure Determination of Metabolites — The organic phases were evaporated and the residues were subjected to preparative TLC and GC/MS analysis. All MS analyses were run on a JMS-DX300 GC/MS system (JEOL, Japan) coupled on line with a JMA-2000 data system (JEOL). A glass GC column (200 × 0.2 cm i.d.) packed with 2% OV-1 on Chromosorb WAW-DMCS (100-120 mesh) was temperature-programmed (200-280°C). The mass spectrometer was operated in the electron impact [EI] mode (electron energy 70 eV, emission current 300 μA).

RESULTS AND DISCUSSION

Excretion of Radioactivity — The cumulative urinary excretion of radioactivity during 5 days after oral and intravenous administration of [¹⁴C]MQ-4 was 6.0% and 10.3% of the dose, respectively. Most of the urinary recovery occurred during the first collection interval. Based on the urinary excretion ratio, the absorption of [¹⁴C]MQ-4 after oral administration amounted to about 60% of the dose. The major route of excretion of radioactivity was the feces via the bile. It was found that 63.3% of the given dose was recovered in bile within 24 h after intravenous administration. However, only 1.8% of the dose was excreted into bile in the case of oral administration to bile duct-cannulated rats, suggesting that the bile is essential for absorption of MQ-4.

The 24 h pooled urine and bile were taken for further analysis.

Metabolite Pattern in the Urine — The major fraction of radioactivity excreted into urine consisted of acidic metabolites and their conjugates. These conjugates were considered to be mainly glucuronides since they were mostly hydrolyzed by β-glucuronidase.

To prevent the production of artifacts, lyophilized urine was treated with HCl-methanol reagent. By this procedure, the conjugates were hydrolyzed to their hydroquinone compounds and simultaneously converted into the methyl esters. The solvent extract of the rat urine treated with HCl-methanol reagent showed 2 peaks on the TLC-radiochromatogram using a solvent system of chloroform-methanol-acetic acid (28:4:1). One main peak (*R_f* = 0.80) corresponded to the authentic methyl esters of both K acid 1 and K acid 2, and the other sub-peak (*R_f* = 0.60) to both free acids. These quinones were difficult to separate from each other by the above TLC procedure.

Mass Spectral Identification of Urinary Metabolites — For structural elucidation of urinary metabolites, the main peak (*R_f* = 0.80) on preparative TLC was extracted with ethyl acetate and the extract was subjected to GC/MS. The extract was composed of two compounds, metabolite 1 methyl ester and metabolite 2 methyl

ester, which showed the same retention time on GC as K acid 1 methyl ester and K acid 2 methyl ester, respectively.

The mass spectrum of metabolite 1 methyl ester showed prominent peaks at m/z 312(M^+), 297, 281, 238 and 225(base peak), 197, 115, 105 and 76, the fragmentation pattern of which was identical with that of K acid 1 methyl ester. In addition, an increase by 2 mass units due to ^{14}C was recognized, for example, in the peaks at m/z 314(M^+), 299, 283, 240 and 227. The fragmentation patterns confirmed that metabolite 1 is K acid 1.

On EI of metabolite 2 methyl ester, its mass spectrum showed prominent peaks at m/z 254, 239, 226 and 212 (base peak) together with peaks at m/z 286 (molecular ion), 199, 128, 115, 105 and 76, the fragmentation pattern of which was identical with that of K acid 2 methyl ester. In this spectrum, an increase by 2 mass units was recognized, for example, in the peaks at m/z 288(M^+), 256, 241, 228 and 214. The fragmentation patterns confirmed that metabolite 2 is K acid 2.

Metabolite 3 in the Liver of Warfarin-Pretreated Rats — The liver extract of warfarin-pretreated rats at 2 h after dosing with [^{14}C]MQ-4 showed 2 spots on the TLC-autoradiogram using a solvent system of acetonitrile-water (1:1); one ($R_f = 0.5$) was identified as unchanged MQ-4 but the other spot ($R_f = 0.57$) was a new metabolite not seen in the normal rat liver. The new metabolite [metabolite 3] corresponded to authentic 2,3-epoxy-MQ-4 in terms of the R_f value of TLC. The amount of radioactivity of metabolite 3 was 23% of that of unchanged MQ-4. For structural elucidation, metabolite 3 was isolated by preparative TLC and subjected to GC/MS. Metabolite 3 showed one peak at the same retention time as that of the authentic compound on GC and also its fragmentation pattern strikingly resembled that of 2,3-epoxy-MQ-4 in the EI-MS. These data indicate that metabolite 3 is 2,3-epoxy-MQ-4.

Urinary and Biliary Excretion Rate of Metabolites — Another aglycone [metabolite 4] as the methyl ester was recognized in bile treated with HCl-methanol reagent, and it corresponded to the methyl ester of authentic MQ-4-COOH in GC and TLC.

Table I shows the ratio of MQ-4 and the major aglycones in rat bile and urine after treatment with HCl-methanol reagent. In the 24 h pooled bile after intravenous dosing, 6.9% of the radioactivity in the sample was unchanged MQ-4. The main aglycones were K acid 2 (10.9%), K acid 1 (9.1%) and MQ-4-COOH (6.8%). In the 24 h pooled urine after dosing, MQ-4 and MQ-4-COOH were not detected. The main aglycones were K acid 1 (47.2%) and K acid 2 (9.8%) in the urine after intravenous administration; after oral dosing the amounts were 32.7% and 24.2%,

respectively.

Table I. Ratio of MQ-4 and the Major Aglycones in Rat Bile and Urine after Hydrolysis with Methanolic HCl

Compound	i.v.		p.o.
	Bile	Urine	Urine
	(% of radioactivity in sample)		
MQ-4	6.9	n.d.	n.d.
MQ-4-COOH	6.8	n.d.	n.d.
K acid 1	9.1	47.2	32.7
K acid 2	10.9	9.8	24.2

Each value represents the mean of three animals.
n.d.: not detected

Metabolic Pathways of MQ-4 — Four metabolites of MQ-4 were isolated from rat urine, bile and liver, and identified. On the basis of their structures, the metabolic pathways of MQ-4 are proposed as shown in Fig.1. This pathway begins with ω -methyl oxidation of the side chain followed by β -oxidation of the resultant MQ-4-COOH to K acid 1 and K acid 2. In the presence of warfarin, 2,3-epoxy-MQ-4 accumulated in the liver as in the case of 2,3-epoxy-phyllquinone.⁸⁾ This suggests that 2,3-epoxy-MQ-4 is converted to MQ-4 in the normal rat and that the reaction is inhibited by warfarin.

Fig.1. Possible Metabolic Pathways of MQ-4 in Rats

In this study, we determined the metabolic pathways of MQ-4 in rats and found that the metabolism of MQ-4 is similar to that of phylloquinone.^{8,9)} In conclusion, the unsaturated alkyl side chain at the 3-position of the naphthoquinone ring is also metabolized in common with the saturated chain.

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