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Oxysterols As Indices of Oxidative Stress in Man After Paraquat Ingestion

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The aim of this study is to evaluate oxidative stress in man after paraquat ingestion by analyzing 7α- and 7β-hydroperoxycholest-5-en-3β-ol (7α- and 7β-OOH) as well as oxysterols, cholesterol oxidation products, as indices of lipid peroxidation. Lung, kidney, and liver were collected at autopsy from seven patients with paraquat poisoning and seven controls matched for age and sex. We identified for the first time 7-ketocholesterol (7-keto) and 7-hydroxycholesterol (7α-OH and 7β-OH) in human kidney by LC–MS. Next, we quantified 7α-OOH and 7β-OOH by HPLC with postcolumn chemiluminescence as well as oxysterols by HPLC–UV. Both 7α-OOH and 7β-OOH detected in lung and kidney from the controls were as low as the paraquat group. In contrast, we found both 7-keto and 7β-OH in lung and 7-keto in kidney from the paraquat group were significantly higher than from the controls. This is the first report on accumulated oxysterols in lung and kidney from human paraquat poisoning. It seems to reflect greater oxidative stress in the pathology of paraquat intoxication.

Keywords: Paraquat; 7-Ketocholesterol; 7β-Hydroxycholesterol; Lipid peroxidation; Human

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridinium) is widely used as a herbicide and well known to generate free radicals such as superoxide anion and hydroxyl radical via a redox-cycling reaction.[1–3] It has been suggested that reactive oxygen species (ROS) and subsequent lipid peroxidation are major contributors to lung injuries such as pulmonary edema, interstitial pneumonia, and pulmonary fibrosis in human paraquat intoxication.[4] However, there are few markers of lipid peroxidation in fatal cases of human paraquat intoxication. In previous studies, diene-conjugated linoleic acid of plasma phospholipid of four patients,[5] serum malondialdehyde in one patient,[6] and pulmonary and hepatic malondialdehyde in seven cases[7] of fatal paraquat intoxication were used as markers.

Previously, we found an evident accumulation of cholesterol hydroperoxides, 7α- and 7β-hydroperoxycholest-5-en-3β-ol (7α- and 7β-OOH) in the kidney of rats administered paraquat even at a dose of 10 mg/kg, and showed that 7α-OOH and 7β-OOH were the sensitive pathogenic markers of oxidative stress.[8] Paradoxically, we failed to observe any accumulation of either 7α-OOH or 7β-OOH in the lung of rats treated with paraquat at a low dose.[8]

It is said that cholesterol is peroxidized or auto-oxidized to produce 7α-OOH, which is then converted to 7β-OOH, and that 7α-OOH is reduced to 7α-hydroxycholesterol (7α-OH) and 7β-OOH to 7β-hydroxycholesterol (7β-OH). Both 7α-OOH and 7β-OOH may be oxidized to 3β-hydroxycholest-5-en-7-one (also termed 7-ketocholesterol; 7-keto). The putative metabolic pathway of these sterols from cholesterol is shown in Fig. 1. We hypothesize that oxysterols, the production of cholesterol oxidation, increase in the lung from fatal paraquat intoxication as a consequence of ROS-mediated reactions. To

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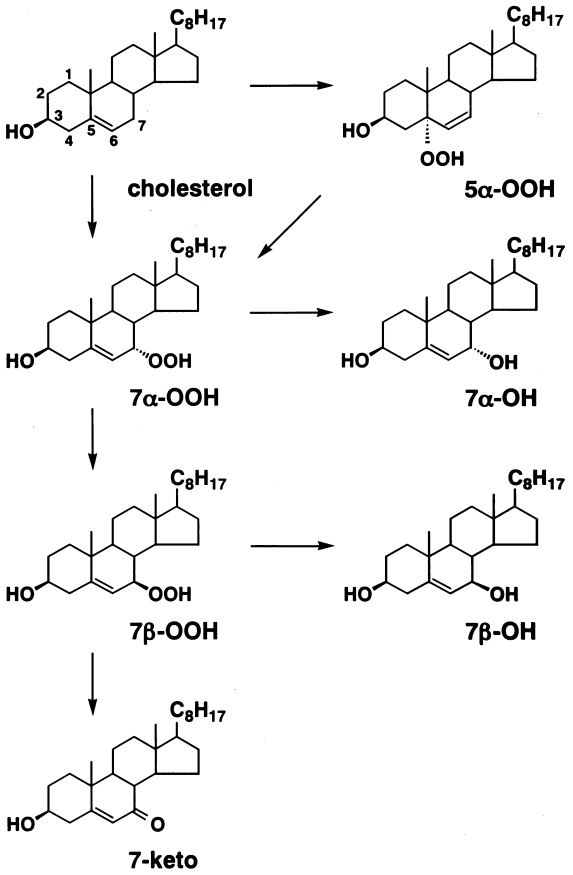

FIGURE 1 A putative pathway to form cholesterol hydroperoxides and oxysterols. 5α-OOH, 5α-hydroperoxycholest-6-en-3β-ol; 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-keto, 7-ketocholesterol.

prove this hypothesis, we measured 7α-OOH and 7β-OOH as well as 7α-OH, 7β-OH, and 7-keto in seven cases of fatal paraquat intoxication compared to age and sex matched controls. The present study was undertaken to determine if paraquat affects cholesterol hydroperoxides or oxysterols in human tissue after paraquat ingestion.

MATERIALS AND METHODS

Materials

3,5-Di-*tert*-butyl-4-hydroxytoluene, luminol (3-aminophthaloylhydrazine) and cytochrome c (from horse heart, type VI) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Cholesterol hydroperoxides, 5α-hydroperoxycholest-6-en-3β-ol (5α-OOH), 7α-OOH and 7β-OOH, and β-sitosterol 5α-hydroperoxide (as internal standard, IS) for HPLC with postcolumn chemiluminescence (HPLC-CL) were prepared as described previously.[9] 7-Keto, 7α-OH, 7β-OH, and β-sitosterol as IS for HPLC–UV were purchased from Steraloids (Wilton, NH).

TABLE I Demographic data for subjects

No.	Age	Sex	Survival time after ingestion (days)	Paraquat intake (g) or blood concentration (ppm)	Pathological findings
1	52	M	2	Unknown	Pulmonary edema
2	70	M	2	70 ppm	Pulmonary edema, acute tubular necrosis. Centrolobular necrosis of the liver
3	36	F	5	1.5–2.0 g	Pulmonary edema with neutrophilic infiltration, partial hyaline degeneration, tubular necrosis
4	59	M	6	1.5–2.0 g	Pulmonary hemorrhage, ARDS(−)
5	60	F	16	1.5–2.0 g	Filling in alveolar space with fibroblast, prominent pulmonary fibrosis
6	42	M	17	3.75 ppm	Prominent pulmonary fibrosis, gastrointestinal bleeding, renal swelling and clouding
7	47	M	33	2.13 ppm	Pulmonary fibrosis, partial honeycomb lung with emphysematous change

Subjects

Five male and two female patients (mean age 52.3, range 42–72-years-old) were studied after their deliberate ingestion of paraquat. On hospital admission, plasma paraquat concentration was measured by HPLC. They died 2–33 days after hospital admission. Lung, liver, and kidney from each patient were taken at autopsy performed 1–3 h after death. Microscopic findings are shown in Table I. Seven controls matched for age and sex were used. They died from exsanguination in an accident. The present study meets the ethical guidelines of the Japanese society of Legal Medicine.

Extraction

Total lipid was extracted by adding to approximately 0.2 g of tissue 5 ml of ice-cold chloroform/methanol (2:1, v/v) containing 0.005% (v/v) butylated hydroxytoluene (as antioxidant), 500 pmol β-sitosterol 5α-hydroperoxide as an IS for HPLC-CL, and 60 nmol β-sitosterol as an IS for HPLC–UV. The mixture was homogenized under ice-cold conditions. The next procedure of extraction and purification with Sep-Pak ($-NH_2$) was performed as described previously.[10]

LC–MS

A Hitachi L-7000 series liquid chromatography system fitted with TSKgel ODS-80Ts column (Tosoh, Tokyo, Japan) (250 × 4.6 mm internal diameter) and a model M-1200AP LC–MS system which incorporated an atmospheric chemical ionization system (Hitachi, Tokyo, Japan) were used to identify 7-OH and 7-keto. The mobile phase, methanol containing 10 mM ammonium acetate, was delivered at a flow rate of 0.7 ml/min.

HPLC-CL Analysis

Cholesterol hydroperoxides were quantified by HPLC-CL as described previously.[10] A TSK gel Octyl-80Ts column (Tosoh, Japan) was used with methanol/water/acetonitrile (89:9:2) as a mobile phase.

HPLC–UV Analysis of Oxysterols

Oxysterols were determined by HPLC comprised of an L-7100 pump (Hitachi), SPD-10Avp UV detector (Shimadzu, Kyoto, Japan) set at 210 and 245 nm, and a Chromatopac C-R8A integrator (Shimadzu). An Inertsil ODS-2 column (GL Sciences) was used (5 μm, 150 × 4.0 mm internal diameter). Acetonitrile/methanol/water (46:45:9) at the flow rate of 0.7 ml/min was supplied to make the mobile phase. All oxysterols were detected at 210 nm, while 7-keto was detected at both 245 and 210 nm. The area of absorbance at 245 nm was 2.6 times as large as at 210 nm (hence the determination of 7-keto at 245 nm).

Standard curves were prepared by analyses of 25–200 ng of 7α-OH, 50–200 ng of 7β-OH, and 7-keto using 250 ng of IS (β-sitosterol).

Statistical Analysis

All data are expressed as mean ± SD. Differences between groups were assessed with Student's *t*-test.

RESULTS

Table I shows demographic data on the poisoning cases. There were two cases of acute toxic phase, two cases of subacute toxic phase, and three cases of remaining phase. Pulmonary edema, renal tubular necrosis, and lobular liver cell necrosis were observed. Pulmonary fibrosis was observed in three cases (death over 16 days).

Cholesterol Hydroperoxides

The separation of mixtures of standard cholesterol hydroperoxides (5α-OOH, 7α-OOH, and 7β-OOH) and β-sitosterol 5α-hydroperoxide as the IS was successfully achieved using a TSK gel Octyl-80Ts column (Fig. 2). Figure 2 also shows HPLC-CL chromatograms of a human lung sample. Peaks 1 and 2 at their respective retention times (Rts) of 6.9 and 7.4 min corresponded with standards of 7β-OOH and 7α-OOH, respectively.

Lipid extracts in lung from the controls contained 7α-OOH (mean, 0.13 nmol/g) and 7β-OOH (mean, 0.33 nmol/g), in kidney 7α-OOH (mean, 0.14 nmol/g) and 7β-OOH (mean,

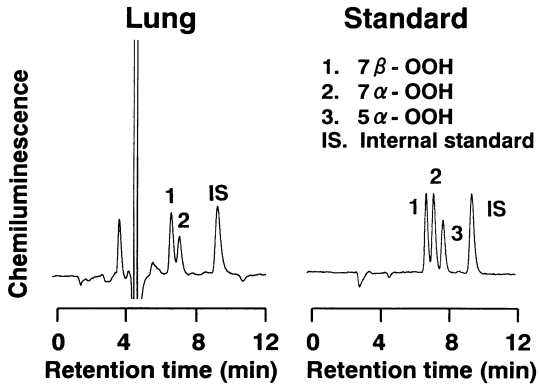

FIGURE 2 HPLC analysis of standard cholesterol hydroperoxides and lung sample with chemiluminescence detection. 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol. 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 5α-OOH, 5α-hydroperoxycholest-6-en-3β-ol.

TABLE II 7-Hydroperoxycholesterol concentrations in human lung and kidney from paraquat and control groups

Tissue		7α-OOH (nmol/g)	7β-OOH (nmol/g)
Lung	Paraquat	0.11 ± 0.11	0.23 ± 0.22
	Control	0.13 ± 0.15	0.33 ± 0.29
	p	0.83	0.54
Kidney	Paraquat	0.06 ± 0.04	0.12 ± 0.09
	Control	0.14 ± 0.10	0.36 ± 0.32
	p	0.08	0.10

Values are mean ± SD ($n = 7$). 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol.

0.36 nmol/g) (Table II) but not 5α-OOH as previously described.[8] Neither 7α-OOH nor 7β-OOH in both kidney and lung from the paraquat group was higher than those from the controls.

LC–MS

Figure 3 shows a total ion chromatogram (TIC) of lipid extracts from the human kidney of paraquat poisoning by LC–MS (Column 1). Peaks A and B appeared at an Rts of 8.4 and 9.6 min, respectively. Figure 3 also shows the mass spectra of standard 7-hydroxycholesterol (7-OH) (Column 2) and 7-keto (Column 4), together with the peaks A (Column 3) and B (Column 5) from kidney. Standard 7-OH had an ion $[M+H-H_2O]^+$ at m/z 385 and a fragment ion at m/z 367, while standard 7-keto had an ion $[M+H]^+$ at m/z 401 and an ion $[M+H-H_2O]^+$ at m/z 383. The Rt and mass spectrum of peak A were almost identical to 7-OH, while those of peak B were almost identical to 7-keto. Accordingly, peaks A and B were identified as 7-OH and 7-keto.

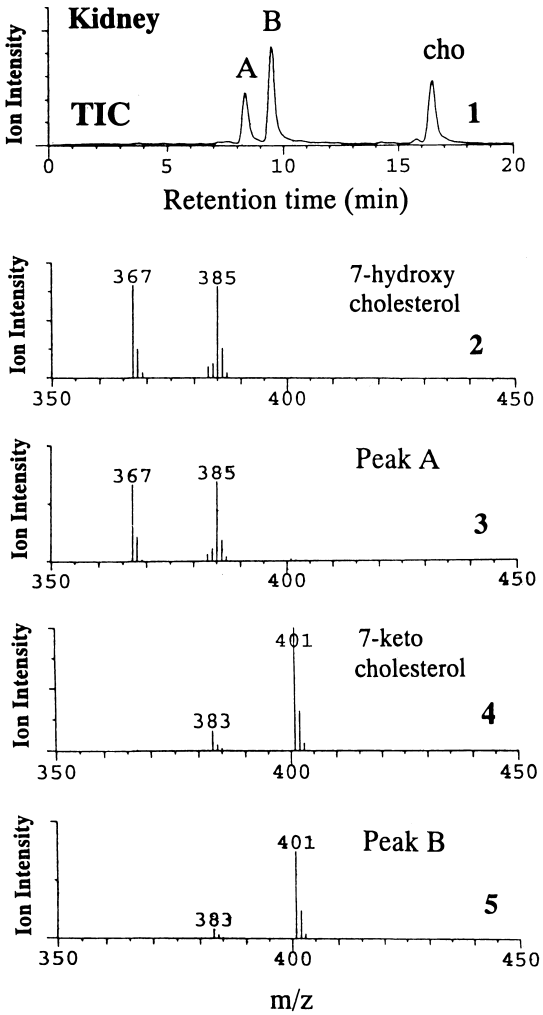

FIGURE 3 TIC of lipid extracts from human kidney by LC–MS. Mass spectra of standard 7-hydroxycholesterol, 7-ketocholesterol, and peaks A and B.

Oxysterol

Chromatographic profiles of oxysterols in a human lung sample are shown in Fig. 4. These peaks 1–4 appearing at Rts of 9.7, 10.1, 10.8, and 34.3 min, respectively, corresponded with 7α-OH, 7β-OH, 7-keto, and cholesterol at 210 nm (lower column), and peak 3 was identified as 7-keto (upper column).

Oxysterol concentrations in lung, kidney, and liver are shown in Table III. Lipid extracts in lung from the control subjects contained 7-keto (mean, 162 nmol/g), 7β-OH (mean, 79 nmol/g) and 7α-OH (mean, 85 nmol/g). Accordingly, 7-keto in lung was two times higher than both 7β-OH and 7α-OH. Furthermore, in both kidney and liver from the controls, 7-keto was 1.5–2 times higher than both 7β-OH and 7α-OH. 7-Keto concentration in both lung and kidney was more than 300 times higher than the sum of 7α-OOH and 7β-OOH, while 7β-OH was about 200 times as high as the sum of 7α-OOH and 7β-OOH. 7-Keto and 7β-OH from the controls were the highest in liver of the three tissues.

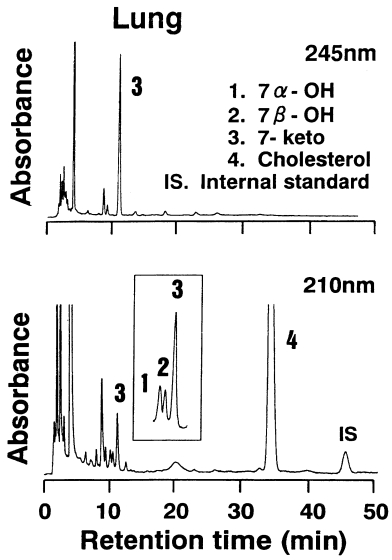

FIGURE 4 HPLC analysis of cholesterol oxidation products in human lung with UV detection at 210 and 245 nm. 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-keto, 7-ketocholesterol.

TABLE III Oxysterol concentrations in human lung, kidney, and liver from paraquat and control groups

Tissue		7-keto (nmol/g)	7β-OH (nmol/g)	7α-OH (nmol/g)
Lung	Paraquat	326 ± 134	127 ± 49	105 ± 30
	Control	162 ± 59	79 ± 21	85 ± 33
	p	0.01	0.03	0.32
Kidney	Paraquat	236 ± 63	113 ± 43	88 ± 39
	Control	158 ± 53	111 ± 40	78 ± 30
	p	0.04	0.91	0.66
Liver	Paraquat	621 ± 372	248 ± 119	369 ± 257
	Control	378 ± 88	212 ± 21	172 ± 55
	p	0.14	0.45	0.22

Values are mean ± SD ($n = 7$). 7β-OH, 7β-hydroxycholesterol; 7-keto, 7-ketocholesterol; 7α-OH, 7α-hydroxycholesterol.

In lung from the paraquat group, both 7-keto and 7β-OH were significantly higher than those from the controls, whereas in kidney from the paraquat group 7-keto had the significantly higher value. In neither lung nor kidney from the paraquat group, 7α-OH was higher than that from the controls. In liver, none of 7-keto, 7β-OH and 7α-OH from the paraquat group was significantly higher than those from the controls.

DISCUSSION

The major findings of the present study are: (1) Oxysterols (detected as 7-OH and 7-keto) were identified by LC–MS in the human lung and kidney, (2) Oxysterols were increased in response to paraquat ingestion indicative of oxidative stress and/or enhanced lipid peroxidation.

In a previous study, we showed augmented lipid peroxidation with 7α-OOH and 7β-OOH (by 70 and 147%, respectively) above levels in the kidney of rats at 2 h after paraquat administration.[8] In contrast, in the present study, both 7α-OOH and 7β-OOH in lung and kidney of human paraquat intoxication were as low as controls.

When LDL was exposed to four different oxidizing systems *in vitro*, 7α-OOH and 7β-OOH were formed at an early phase (8 h), while 7-keto and 7-OH were generated at a later phase of oxidation ($\geqq$ 24 h).[11] In addition, accumulation of 7-OH in the cells shows efficient reduction of 7-OOH.[11] 7-OOH is reduced to 7-OH by phospholipid hydroperoxide glutathione peroxidase (EC 1.11.1.12).[12] Consequently, 7-keto, as well as 7α-OH and 7β-OH, is formed from 7-OOH *in vivo*, when oxidative stress occurs repeatedly. As all of the subjects died more than 2 days after paraquat ingestion, the present findings that 7-keto and 7β-OH were significantly increased, whereas 7α-OOH and 7β-OOH did not alter, may be consistent with previous observations.[11]

Next, we discuss a pathohistological aspect and lipid peroxidation. Pulmonary fibrotic changes were observed in the three patients over 2 week postadmission to the hospital, while pulmonary edema or pulmonary hemorrhage was seen in the four patients who died within 2 weeks in hospital. The histological changes were consistent with a previous report.[6]

When Ogata and Manabe examined the relationship between pulmonary fibrotic change and lipid peroxidation detected as MDA, they showed a correlation between lipid peroxidation and morphological manifestation of paraquat-induced lung injury in rats.[13] In addition, increased lipid peroxidation was shown in four patients at 4.5–36 h after paraquat ingestion by measuring phospholipid diene-conjugated 18:2Δ9,11-linoleic acid in plasma.[5] In contrast, serum MDA was higher in three cases of subacute toxic phase of paraquat (5–11 days after ingestion) than controls before the appearance of pulmonary fibrosis, whereas MDA did not show any change in the three cases of acute toxic phase (death within 1–3 days).[6]

In the present study, we had two cases of acute toxic (death 2 days), two cases of subacute toxic phase (5 and 6 days after ingestion), and three cases (over 2 weeks after ingestion) in which pulmonary fibrosis appeared as shown in Table I. We failed to observe any correlation between the severity of lung injury and lipid peroxidation. The reason why inconsistent findings were obtained may be that the number of human subjects was fairly limited in each report, and that the marker of lipid peroxidation (MDA or conjugated diene) in their literature was different from ours (oxysterol).

Finally, we discuss oxysterol, the marker of oxidative stress. Oxysterol concentrations were determined in plasma from both healthy subjects[14–16] and cancer patients.[15] 7-Keto in cancer patients was 2–3 times higher than in the pooled normal sera.[17] Human cataratous lenses contained 7-keto and 7β-OH, whereas clear lenses did not. In human atheroma, 7β-OH was detected by GC,[18,19] and 7-keto, 7α-OH, and 7β-OH were determined by HPLC–UV,[11] whereas normal aorta did not contain them. As free radical-induced oxidative stress is one of the contributors of cataracts and atherosclerotic plaque, the accumulation of cholesterol oxidation products in these tissues must be involved in free radical-induced oxidative stress.

Although cholesterol is less reactive than polyunsaturated fatty acid, cholesterol hydroperoxide is considered to be more stable than phospholipid hydroperoxide and cholesteryl ester hydroperoxide. In addition, we found that 7-keto concentration in both lung and kidney in the present study was more than 300 times higher than the sum of 7α-OOH and 7β-OOH, while 7β-OH was about 200 times as high

as the sum of 7α-OOH and 7β-OOH. Consequently, when we evaluate oxidative stress in some tissues, we can analyze oxysterols more easily and accurately than hydroperoxides of phospholipid and cholesteryl ester as an index of lipid marker. We were able to separate 5α-OOH, 7α-OOH, and 7β-OOH using C8 column, as well as 7α-OH, 7β-OH, and 7-keto using C18 column. Therefore, we quantified successfully for the first time 7-OOH as well as oxysterols in lipid extracts using HPLC with chemiluminescent and UV detections under different chromatographic conditions.

In conclusion, we were able to demonstrate for the first time the evidence of enhanced lipid peroxidation by analyzing 7β-OH and 7-keto in human lung and 7-keto in kidney after paraquat ingestion. Furthermore, we have confirmed that oxysterols are suitable lipid markers of oxidative stress in man.

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References

[1] Autor, A.P. (1974) "Reduction of paraquat toxicity by superoxide dismutase", *Life Sci.* **14**, 1309–1319.
[2] Winterbourn, C.C. (1981) "Production of hydroxyl radicals from paraquat radicals and H_2O_2", *FEBS Lett.* **128**, 339–342.
[3] Youngman, R.J. and Elstner, E.F. (1981) "Oxygen species in paraquat toxicity: the crypto-OH radical", *FEBS Lett.* **129**, 265–268.
[4] Yasaka, T., Okudaira, K., Fujito, H., Matsumoto, J., Ohya, I. and Miyamoto, Y. (1986) "Further studies of lipid peroxidation in human paraquat poisoning", *Arch. Intern. Med.* **146**, 681–685.
[5] Situnayake, R.D., Crump, B.J., Thurnham, D.I., Davies, J.A. and Davis, M. (1987) "Evidence for lipid peroxidation in man following paraquat ingestion", *Hum. Toxicol.* **6**, 94–98.
[6] Yasaka, T., Ohya, I., Matsumoto, J., Shiramizu, T. and Sasaguri, Y. (1981) "Acceleration of lipid peroxidation in human paraquat poisoning", *Arch. Intern. Med.* **141**, 1169–1171.
[7] Kurisaki, E. (1985) "Lipid peroxidation in human paraquat poisoning", *J. Toxicol. Sci.* **10**, 29–33.
[8] Adachi, J., Tomita, M., Yamakawa, S., Asano, M., Naito, T. and Ueno, Y. (2000) "7-Hydroperoxycholesterol as a marker of oxidative stress in rat kidney induced by paraquat", *Free Radic. Res.* **33**, 321–327.
[9] Kulig, M.J. and Smith, L.L. (1973) "Sterol metabolism 25. Cholesterol oxidation by singlet molecular oxygen", *J. Org. Chem.* **38**, 3639–3642.
[10] Adachi, J., Asano, M., Naito, T., Ueno, Y. and Tatsuno, Y. (1998) "Chemiluminescent determination of cholesterol hydroperoxides in human erythrocyte membrane", *Lipids* **33**, 1235–1240.
[11] Brown, A.J., Leong, S.-L., Dean, R.T. and Jessup, W. (1997) "7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plague", *J. Lipid Res.* **38**, 1730–1745.
[12] Korytowski, W., Geiger, P.G. and Girotti, A.W. (1996) "Enzymatic reduction in relation to cytotoxicity for various cholesterol hydroperoxides", *Biochemistry* **35**, 8670–8676.
[13] Ogata, T. and Manabe, S. (1990) "Correlation between lipid peroxidation and morphological manifestation of paraquat-induced lung injury in rats", *Arch. Toxicol.* **64**, 7–13.
[14] Kudo, K., Emmos, G.T., Lasserly, E.W., Via, D.P., Smith, L.G., St. Pyrek, J. and Schroepfer, Jr., G.J. (1989) "Inhibition of sterol synthesis, chromatography of acetate derivatives of oxygenated sterols", *J. Lipid Res.* **30**, 1097–1111.
[15] Breuer, O. and Bjorkhem, I. (1990) "Simultaneous quantification of several cholesterol autoxidation and monohydroxylation products by isotopedilution mass spectrometry", *Steroids* **55**, 185–192.
[16] Sevanian, A., Seraglia, R., Traldi, P., Rossato, P., Ursini, F. and Hodis, H. (1994) "Analysis of plasma cholesterol oxidation products using gas- and high-performance liquid chromatography/mass spectrometry", *Free Radic. Biol. Med.* **17**, 397–409.
[17] Saito, H., Kitame, F., Uemura, Y. and Ishida, N. (1983) "The regulating effect of cholesterol derivatives isolated from human sera on lymphocyte response to phytohemagglutinin", *Tohoku J. Exp. Med.* **140**, 245–258.
[18] Carpenter, K.L.H., Taylor, S.E., Ballantine, J.A., Fussel, B., Halliwell, B. and Mitchinson, M.J. (1993) "Lipids and oxidized lipids in human atheroma and normal aorta", *Biochim. Biophys. Acta* **1167**, 121–130.
[19] Garcia-cruset, S., Carpenter, K.L.H., Guardiola, F. and Mitchinson, M.J. (1999) "Oxysterols in cap and core of human advanced atherosclerotic lesion", *Free Radic. Res.* **30**, 341–350.