ELSEVIER

Journal of Chromatography B, 694 (1997) 277–287

JOURNAL OF CHROMATOGRAPHY B

Simultaneous determination of eight lipid peroxidation degradation products in urine of rats treated with carbon tetrachloride using gas chromatography with electron-capture detection

Loeckie L. De Zwart[a], Jennifer Venhorst[a], Marjolein Groot[a], Jan N.M. Commandeur[a], Ralph C.A. Hermanns[b], John H.M. Meerman[b], Ben L.M. Van Baar[c], Nico P.E. Vermeulen[a,*]

[a]*Leiden–Amsterdam Center for Drug Research (LACDR), Division of Molecular Toxicology, Department of Pharmacochemistry, De Boelelaan 1083, 1081 HV Amsterdam, Netherlands*

[b]*Leiden–Amsterdam Center for Drug Research (LACDR), Division of Molecular Toxicology, Department of Organic Chemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, Netherlands*

[c]*Leiden–Amsterdam Center for Drug Research (LACDR), Division of Molecular Toxicology, Division of Toxicology, University of Leiden, Leiden, Netherlands*

Received 13 November 1996; revised 17 February 1997; accepted 25 February 1997

Abstract

One of the major processes that occur as a result of radical-induced oxidative stress is lipid peroxidation (LPO). Degradation of lipid peroxides results in various products, including a variety of carbonyl compounds. In the present study eight different lipid degradation products, i.e., formaldehyde, acetaldehyde, acetone, propanal, butanal, pentanal, hexanal and malondialdehyde were identified and measured simultaneously and quantitatively in rat urine after derivatization with O-(2,3,4,5,6-pentafluorbenzyl)hydroxylamine hydrochloride, extraction with heptane and using gas chromatography–electron-capture detection (GC–ECD). The identity of the respective oximes in urine was confirmed by gas chromatography–negative ion chemical ionization mass spectrometry (GC–NCI-MS). Simultaneously measured standard curves were linear for all oxime-products and the detection limits were between 39.0±5.3 (n=9) and 500±23 (n=9) fmol per μl injected sample. Recoveries of all products from urine or water were 73.0±5.2% and higher. In urine of CCl_4-treated rats an increase in all eight lipid degradation products in urine was found 24 h following exposure. ACON showed the most distinct increase, followed by PROPA, BUTA and MDA. It is concluded that the rapid, selective and sensitive analytical method based on GC–ECD presented here is well suited for routine measurement of eight different lipid degradation products. These products appear to be useful as non-invasive biomarkers for in vivo oxidative stress induced in rats by CCl_4. © 1997 Elsevier Science B.V.

Keywords: Lipid peroxidation; Formaldehyde; Acetaldehyde; Acetone; Propanal; Butanal; Pentanal; Hexanal; Malondialdehyde

*Corresponding author.

0378-4347/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0378-4347(97)00144-8

1. Introduction

Free radicals play an important role in the pathogenesis of a wide variety of human diseases, for example atherosclerosis, rheumatoid arthritis and toxicological processes such as necrosis and carcinogenesis. The identification of specific biomarkers for these pathological processes, preferably measurable non-invasively and before irreversible damage has occurred, is therefore considered an important topic of recent investigations. Hageman et al. suggested that non-invasive in vivo biomarkers for radical-mediated oxidative damage are needed [1].

One of the major processes that occur as a result of radical induced oxidative stress is lipid peroxidation (LPO). This degenerative propagation reaction in lipid membranes is usually accompanied by the formation of a wide variety of products, including a variety of carbonyl compounds [2,3]. Some of these products have been shown to be cytotoxic or genotoxic [2,4], to react with biomolecules such as proteins and nucleic acid bases [5] and also to have an effect on receptors and signal transmission [6,7]. In contrast to free radicals, aldehydes are relatively stable and therefore able to diffuse within or out of cells and to attack targets distant from the site of original free radical-initiated events [2]. Aldehydes and their metabolites are therefore attractive parameters of LPO. The most widely used index of LPO in vitro is the measurement of malondialdehyde (MDA), with the thiobarbituric acid (TBA) assay. This method has several drawbacks, however, due to the lack of selectivity and the possible formation of artefacts [8,9].

Therefore methods other than the TBA assay for measuring LPO products are still needed. Several analytical methods for individual aldehydic compounds in biological samples, mainly involving liquid chromatography (LC) and gas chromatography (GC), have meanwhile been developed. The detection of urinary MDA using derivatization with 2,4-dinitrophenylhydrazine (DNPH) and analysis by HPLC with UV detection was reported [10]. More recently, this method was extended by identifying three other aldehydes in urine samples, namely acetone (ACON), formaldehyde (FOR), and acetaldehyde (ACET) [11]. GC methods developed for determining lipid degradation products amongst others include a GC-method for measuring urinary MDA using derivatization with pentafluorophenyl hydrazine (PFPH) with electron-capture detection (ECD) [12] and mass spectrometry with negative chemical ionization (MS-NCI) in plasma and tissue homogenates [13]. Luo et al. developed a GC–NCI-MS method involving derivatization with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFB), allowing the detection of a broad spectrum of aldehydes in spiked water, tissues and urine [14].

Apart from the four lipid degradation products measured by Shara et al., in principle several other aldehydes are formed upon LPO [15,16]. The latter products in urine might also be useful as indicators of cellular damage induced by radicals. The measurement of more than one aldehydic product might moreover result in a more selective and sensitive method for determining LPO. The relative amounts of aldehyde found in urine might also give more specific information about the organ or tissue selectivity of damage. Preferentially, the analytical method should be rapid, simple and relatively cheap to allow for routine applications. These criteria are not fully met by the MS method of Luo et al. [14].

Therefore, in the present study an alternative quantitative analytical method was developed for the analysis of acetone (ACON) and seven aldehydes in urine: formaldehyde (FOR), acetaldehyde (ACET), propanal (PROPA), butanal (BUTA), pentanal (PENTA), hexanal (HEXA) and malondialdehyde (MDA). To evaluate the practical feasibility of assaying of the above mentioned aldehydes as in vivo biomarkers for LPO, in this study all eight lipid degradation products were identified and measured quantitatively in rat urine by GC–NCI-MS and GC–ECD. The effect of free radical-induced liver injury by CCl_4 on the excretion of all eight biomarkers has been examined as well.

2. Experimental

2.1. Chemicals and reagents

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFB), 3-bromofluoro-benzene, acetal-

dehyde (ACET), propanal (PROPA), butanal (BUTA), pentanal (PENTA), hexanal (HEXA) were purchased from Fluka (Buchs, Switzerland). Acetone (ACON) and sulphuric acid (H_2SO_4) were obtained from Riedel-de Haën (Seelze, Germany). 1,1,3,3-Tetraethoxypropane (TEP) and sodium wolframate were purchased form Merck (Darmstadt, Germany). Sodium-acetate, *n*-heptane and carbon tetrachloride (CCl_4) were purchased from J.T. Baker (Deventer, Netherlands). Sodium sulphate was obtained from Janssen (Beerse, Belgium).

2.2. Preparation of MDA

Hydrolysis of TEP to produce MDA was accomplished by the method of Csalany et al. [17]. TEP (1 mmol) was dissolved in 100 ml of 0.01 *M* hydrochloric acid and left at room temperature for 6 h. The MDA stock solution was kept in the dark at 4°C until used. The absorbance at 245 nm ($\epsilon = 13\,700$) was used to determine the MDA concentration of the stock solution. A working solution was prepared by diluting the stock solution in urine or water.

2.3. Extraction and derivatization

In order to hydrolyse protein-bound aldehydes, 15 μl of 3.3 *M* H_2SO_4 was added to 0.5 ml of urine in 2-ml capped glass tubes. The mixture was vortexed and allowed to stand at room temperature for 10 min. The proteins were precipitated with 75 μl of 0.3 *M* sodium wolframate [13]. After vigorous shaking and centrifuging for 10 min at 4000 *g*, 0.5 ml of the supernatant was used for derivatization with 0.5 ml PFB reagent which consisted of a solution of 1 m*M* PFB in 1.5 *M* sodium acetate buffer (pH 5.0). To derivatise the sample, it was vortexed and maintained at room temperature for 1 h. The oximes formed were extracted from the aqueous samples by adding 0.5 ml *n*-heptane containing 10 μ*M* 3-bromofluorobenzene (internal standard) and vortexing vigorously for 30 s. The heptane layer was removed and washed once with 0.5 ml 0.1 *M* HCl to remove excess PFB-reagent. The heptane layer was subsequently dried over anhydrous sodium sulphate and finally 1 μl was injected onto the GC column.

2.4. Stability, quantification and reproducibility

Spiked water and urine samples containing 0, 0.05, 0.1, 0.5, 1.0 and 5.0 μ*M* concentrations of mixtures of the seven aldehydes and acetone were prepared for the determination of the stability of the aldehydes and the reproducibility of the method. The stability of the volatile aldehydes was determined by measuring the same urine samples after one day and one week of storage at −20°C. All experiments were repeated nine times. All urine samples were treated as described above and analysed by GC–ECD as described below.

Standard response curves in both water and urine were constructed for all seven aldehydes and acetone by plotting individually the ratios of peak area of the aldehydes and the internal standard (3-bromofluorobenzene) against the concentration of aldehydes with eight different levels of the aldehydes and acetone in the range 0.05–4 μ*M*.

2.5. Apparatus and conditions

A Hewlett–Packard 5890 series II gas chromatograph equipped with a 15 mCi ^{63}Ni electron-capture detector was used (GC–ECD). The GC was equipped with a 30-m HP 5 (CP Sil 8 CB) column (0.32 mm I.D, 0.25-μm film thickness, Hewlett–Packard, Amstelveen, The Netherlands). For data acquisition and processing the HP CHEMSTATION software implemented on a HP Vectra 486/33VL was used. The temperature of the GC oven was programmed from 50°C (1 min) to 150°C, at 7°C min^{-1} and then to 270°C, at 20°C min^{-1}, and then kept at 270°C for 5 min. The temperatures of the injector and detector were 200°C and 300°C, respectively. The helium column flow-rate was 2.8 ml min^{-1}.

GC–MS analysis under negative chemical ionization conditions (NCI) was performed on MAT90 magnetic sector instrument (Finnigan MAT, Bremen, Germany) equipped with a Varian 3400 GC (Varian, Houten, Netherlands). Ammonia was used as the reagent gas, at a source temperature of 120°C and at an indicated source pressure of $5 \cdot 10^{-4}$ Torr. The emission current was 0.2 mA and the instrument was scanned from 35 to 500 a.m.u at 1 s/decade. The GC was equipped with a 50 m CP Sil 5 CB column (0.2 mm I.D., 0.33 μm film thickness; Chrompack,

Middelburg, Netherlands). The injector and transfer line were both kept at 270°C, while the oven was programmed as described above.

The NCI-mass spectra (with ammonia as a reagent gas) of the PFB-oxime derivatives showed ions at m/z 225 ($M^{-\cdot}$, 7%) and m/z 205 ($M^{-\cdot}$−HF, 15%) for FOR (t_R 4.97 min); at m/z 239 ($M^{-\cdot}$, 10%) and m/z 219 ($M^{-\cdot}$−HF, 18%) for ACET (t_R 6.70 and 6.87 min); at m/z 253 ($M^{-\cdot}$, <1%) and m/z 233 ($M^{-\cdot}$−HF, 17%) for ACON (t_R 7.97 min); at m/z 253 ($M^{-\cdot}$, 18%) and m/z 233 ($M^{-\cdot}$−HF, 17%) for PROPA (t_R 8.27 an 8.43 min); at m/z 267 ($M^{-\cdot}$, 10%) and m/z 247 ($M^{-\cdot}$−HF, 17%) for BUTA (t_R 10.07 and 10.20 min); at m/z 281 ($M^{-\cdot}$, 22%) and m/z 261 ($M^{-\cdot}$−HF, 22%) for PENTA (t_R 11.77 and 11.90 min); at m/z 295 ($M^{-\cdot}$, 10%) and m/z 275 ($M^{-\cdot}$−HF, 26%) for HEXA (t_R 13.45 and 13.55 min); at m/z 462 ($M^{-\cdot}$, 5%) and m/z 442 ($M^{-\cdot}$−HF, 24%) for MDA (t_R 19.12 and 19.28 min).

2.6. Animal treatments

Male Wistar rats (200–220 g) were obtained from Charles River Wiga (Sulzfeld, Germany). Water and commercial laboratory chow (Hope Farms, Woerden, Netherlands) were available to the rats ad libitum. During the experiment, the rats were housed individually in metabolism cages designed for the separate collection of urine and faeces. Animal facilities were maintained at 22°C and on a 12 h light–dark cycle. Urine was collected at 12, 24, 36 and 48 h following oral administration of CCl_4. Urine-collecting vessels were positioned in containers filled with dry ice which permitted the collection of urine in the frozen state over the 12-h periods. Urine volumes were measured and urine was stored at −20°C until further analysis. Four rats were dosed orally with 1 ml kg^{-1} body weight CCl_4, diluted 1:3 in corn oil, at t=6 h (i.e., 6 h after lights were turned on and urine-collecting vessels were changed). Control animals received the corresponding corn oil vehicule at t=6 h.

2.7. Clinical parameters

Plasma alanine aminotransterase activity (ALT) was determined by using a commercially available reagent kit (J.T. Baker).

2.8. Statistical methods

Experimental results were evaluated statistically using the Student's t-test and were considered significant if $P<0.05$ for all analyses.

3. Results

3.1. Identification of urinary lipid degradation products

Identification of the eight different lipid degradation products was performed by GC–NCI-MS analysis of both commercial standards of the seven aldehydes and acetone in water and a number of representative urine samples of rats treated with CCl_4, all of which were derivatized with PFB and extracted with n-heptane. The PFB-oximes of ACET, PROPA, BUTA, PENTA and HEXA exist as two isomers, i.e., *syn*- and the *anti*-isomers, which were separated chromatographically and which showed significantly different relative ion abundances in the NCI-MS spectra. Usually one of the isomers showed the negative molecular ion [$M^{-\cdot}$], whereas the other isomer showed a negative pseudo molecular ion $[M-HF]^{-\cdot}$. It was not attempted to determine which of the two peaks represented the *syn*- or the *anti*-isomer. In Fig. 1, a GC–ECD chromatogram is shown of a typical PFB-derivatized urine sample spiked with the eight different lipid degradation products.

3.2. Stability, quantification, reproducibility and limit of detection of urinary lipid degradation products

FOR, ACET, ACON, PROPA, BUTA, PENTA, HEXA and MDA could all be measured in rat urine accurately as their PFB-oxime derivatives, in a concentration range 0.05–5 μM. Standard curves were linear for ACON, MDA and the other aldehydes with 3-bromofluorobenzene as an internal standard and are presented in Table 1. The limit of detection of these lipid degradation products in urine ranged from 39.0±5.3 to 500±23 (n=9) fmol per μl injected (see Table 1). The limit of detection is defined as the concentration of aldehydic product,

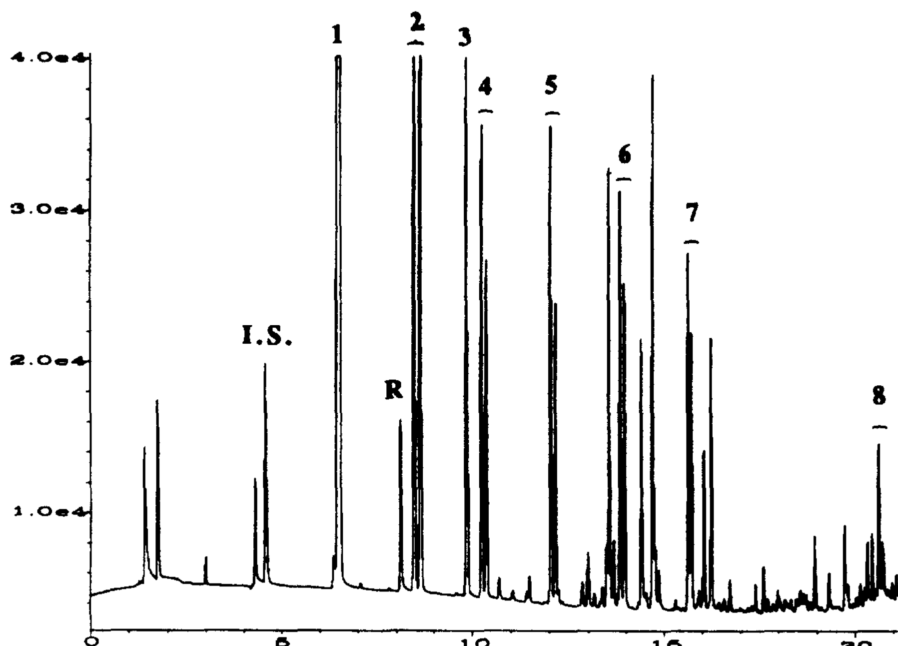

Fig. 1. GC–ECD chromatogram of a rat urine sample spiked with eight different lipid degradation products. I.S.=internal standard (3-bromofluorobenzene); 1=FOR; R=reagent (PFB); 2=ACET; 3=ACON; 4=PROPA; 5=BUTA; 6=PENTA; 7=HEXA; 8=MDA.

which results in a 3 times higher peak than the standard deviation of the average blank. Due to the higher background levels of FOR, ACET and ACON in biological samples, like urine, the limits of detection found for these products are relatively higher.

The extraction recovery of the derivatized compounds was determined by comparing the calibration curves in water and urine with the respective calibration curves in ethanol and ranged from 73.0±5.2% (ACON) to 99.0±8.2% (FOR) (n=9) (Table 2). Calibration curves were corrected for both non-biological background (water and ethanol) and for biological background (urine).

The coefficients of variation (C.V.) for the reproducibility of the overall assays are shown in Table 2

Table 1

Detection limits (mean±S.D.), coefficients of variation (C.V.; n=9) and calibration curves ($y=b+ax$) and correlation coefficients (r^2) of PFB-oximes of eight lipid degradation products in spiked rat urine samples by GC–ECD

Compound	Detection limit (fmol)	C.V. (%)		Calibration curve	
		0.05 μM	0.5 μM	$y=b+ax$	r^2
FOR[a]	500±23.0	4.6	1.2	$52.6+1.47x$	0.978
ACET	147±15.0	7.1	5.5	$2.78+0.61x$	0.997
ACON	500±18.0	3.6	4.0	$1.99+0.19x$	0.997
PROPA	53±5.4	9.9	4.5	$0.11+0.65x$	0.999
BUTA	53±10.7	5.9	6.2	$0.11+0.61x$	0.999
PENTA	39±5.3	10.2	4.8	$0.06+0.62x$	1.000
HEXA	61±7.1	5.9	8.2	$0.11+0.47x$	1.000
MDA	50±4.1	3.1	8.1	$0.22+0.04x$	0.999

[a] FOR, formaldehyde; ACET, acetaldehyde; ACON, acetone; PROPA, propanal; BUTA, butanal; PENTA, pentanal; HEXA, hexanal; MDA, malondialdehyde.

Table 2
Extraction recovery from spiked water and urine samples ($n=10$, mean±S.D.) and the stability of eight lipid degradation product in spiked urine samples ($n=9$, mean±S.D.)

Compound	Extraction recovery (1 μM)		One day recovery (%)		One week recovery (%)	
	Water	Urine	0.05 μM	0.5 μM	0.05 μM	0.5 μM
FOR[a]	99.7±8.2	98.0±12	n.r.[b]	n.r.	n.r.	n.r.
ACET	86.8±9.8	93.0±15	97.9±8.5	94.3±4.8	102±4.5	105±7.7
ACON	89.0±13	73.0±5.2	100±3.2	99.4±3.2	100±2.1	110±2.3
PROPA	85.5±3.2	86.5±2.6	100±12.7	99.0±4.2	107±4.4	111±7.3
BUTA	85.9±3.5	84.1±4.9	93.8±5.6	97.2±3.5	101±3.1	109±6.2
PENTA	83.3±3.3	77.8±3.7	98.1±8.1	95.8±3.9	106±6.4	104±6.8
HEXA	83.5±4.3	80.6±1.7	95.3±4.9	94.9±4.7	98.3±3.9	106±6.0
MDA	98.6±3.6	83.4±1.9	98.6±3.6	106±5.9	100±1.8	109±2.9

[a] FOR, formaldehyde; ACET, acetaldehyde; ACON, acetone; PROPA, propanal; BUTA, butanal; PENTA, pentanal; HEXA, hexanal; MDA, malondialdehyde.
[b] Not recovered.

and ranged from 4.6% for FOR (0.05 μM) to 10.2% for PENTA (0.05 μM) ($n=9$).

The stability of the aldehydes and acetone in urine was determined by measuring spiked urine samples ($n=9$) after one day and one week of storage at −20°C. Except for FOR, no significant change in recovery was observed after one day or after one week of storage, indicating that acetone and the aldehydes in urine are stable at −20°C.

3.3. Urinary excretion of lipid degradation products by rats treated with CCl_4

Fig. 2 shows a representative GC–ECD chromatogram of typical urine samples of an untreated rat and a rat treated with CCl_4. All 7 aldehydes and acetone could be easily identified in all urine samples both by comparing retention times by GC–ECD and by GC–NCI-MS.

The urinary excretion profile of the eight different lipid degradation products in 12-h urine fractions of rats treated with 1 ml kg^{-1} CCl_4 and of untreated rats is shown in Fig. 3. The amounts of lipid degradation products are given as a mean value±S.E.M. of four rats. The excretion of ACON and all aldehydes, except PENTA and HEXA, in urine was significantly increased after treatment of rats with CCl_4. For all measured products, the largest increase was found in the 12–24 h urine fractions. Overall, ACON showed the most distinct increase, followed by FOR, whereas an increase, though not statistically significant was found as well for ACET, PROPA, BUTA, PENTA, HEXA and MDA. The increase in PROPA, BUTA and MDA was found to be statistically significant in the 24–36 h urine fractions. Urinary background levels of FOR, ACET and ACON were relatively high compared to those of other degradation products. The analytical method therefore, seems sensitive enough for in vivo measurements, since urinary lipid degradation background levels could also be easily measured.

The inter-individual differences between rats were quite large. The large inter-individual differences are, however, also reflected by the ALT levels in blood plasma, measured as indicator of the extent of liver damage induced by CCl_4 (Fig. 4).

4. Discussion

The results of the present study show that seven aldehydes and one ketone in rat urine can be determined simultaneously as their PFB-oxime-derivatives in a single GC–ECD run. PFB-derivatives of aldehydes [18,19] could be measured with high accuracy and sensitivity using GC–ECD. PFB–oxime formation is pH dependent, with an optimum of 4–5 and proceeds readily in both aqueous and organic solutions [20]. The GC–ECD method developed is sensitive and selective, easy to perform and makes it possible to routinely measure large numbers of urine samples. Complete derivatization of the aldehydes and ACON in urine was readily accomplished at room temperature under mild con-

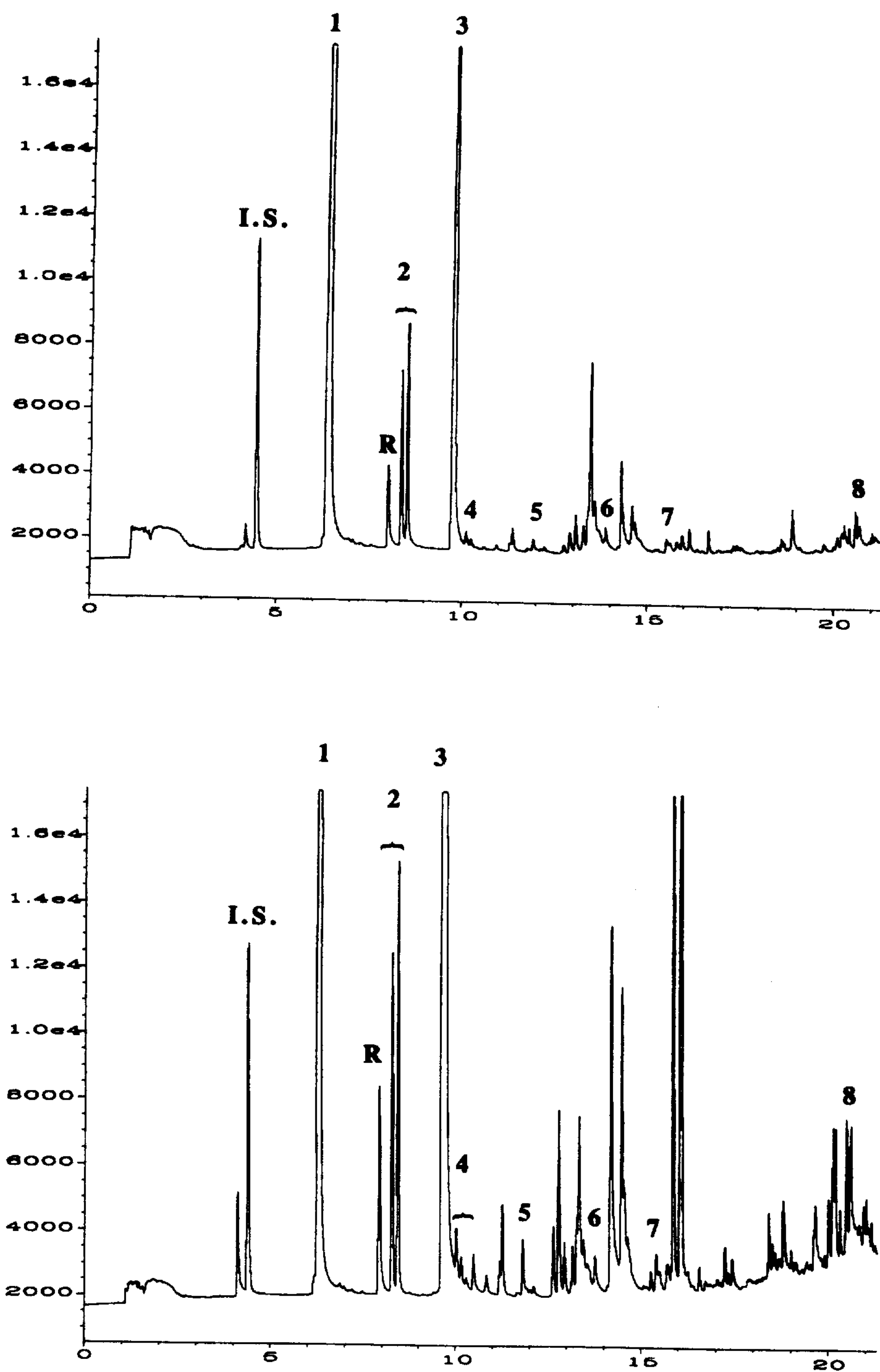

Fig. 2. (A) Representative GC–ECD chromatogram of a urine sample of an untreated rat. (B) Representative GC–ECD chromatogram of a urine sample of a CCl_4-treated rat. I.S.=internal standard (3-bromofluorobenzene); 1=FOR; R=reagent (PFB); 2=ACET; 3=ACON; 4=PROPA; 5=BUTA; 6=PENTA; 7=HEXA; 8=MDA.

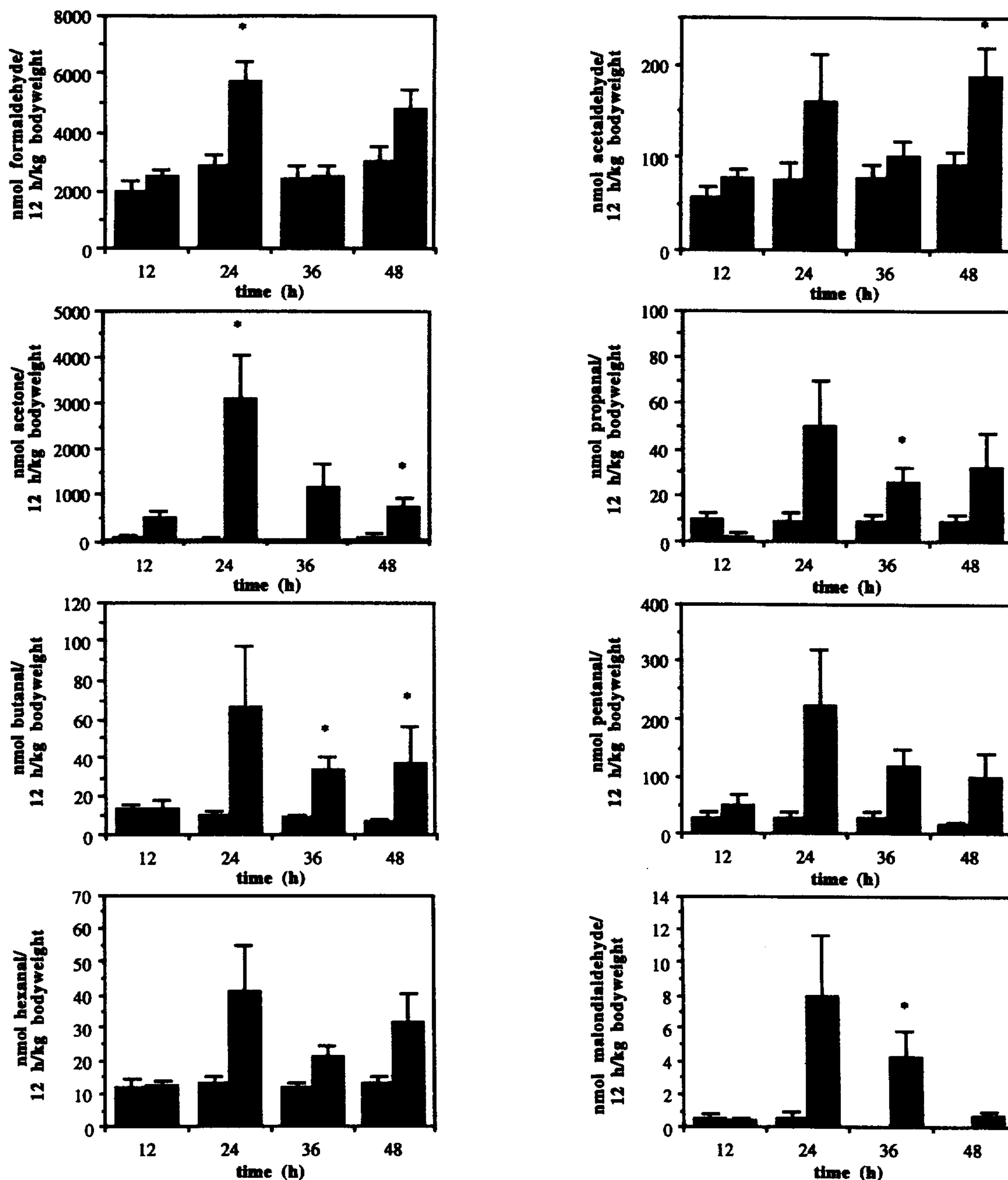

Fig. 3. Excretion of eight different lipid peroxidation products in urine of rats treated with CCl_4 (1 ml kg^{-1}). The amounts are given as a mean value±S.E.M. of four animals. The closed column represents the control group and the hatched column represents the treated group. Statistical analyses was done using the Student's *t*-test. A $P<0.05$ was considered significant and this is indicated with an *.

ditions. The identification of the PFB–oxime-derivatives of the aldehydes and ACON was performed by comparing GC–NCI-MS analysis of derivatized synthetical standards and urine samples of CCl_4-treated rats.

Previous studies have identified MDA and several

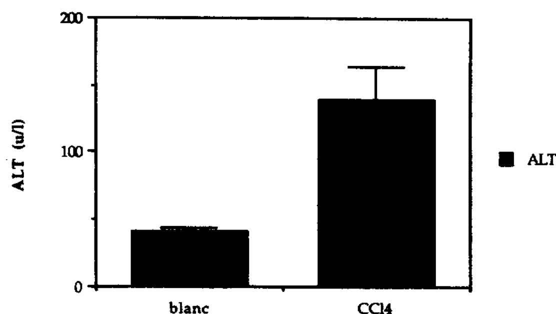

Fig. 4. ALT levels in plasma of both the control group and the group treated with CCl_4 48 h after treatment. The enzyme activities are given as a mean value±S.E.M. of four animals. This difference was found to be significant in the Student's *t*-test with $P<0.05$.

other aldehydes in various biological matrices by means of diverse spectroscopic and chromatographic techniques. The so-called spectroscopic TBA-assay is most widely used in in vitro systems but is not specific for MDA and neither does it give a complete view of the wide range of products formed from membrane lipids and polyunsaturated fatty acids [8,21]. In combination with HPLC, the selectivity of the TBA-assay was greatly improved and the detection limit in food and feed was about 3 pmol [22]. Draper et al. used this HPLC method to determine MDA in rat urine samples and found an increase of MDA excretion in urine of rats treated with the enolic sodium salt of MDA, high PUFA diet, vitamin E-deficient diet or with ferric nitrilotriacetate. They were not able, however, to detect an increase in MDA excretion in urine of rats dosed with up to 1 ml kg^{-1} CCl_4 [23]. The discrepancy between their results and the results of the present study is probably due to the significantly different detection limits for MDA, i.e., 3 pmol vs. 50 fmol, respectively.

Ekström et al. reported the quantitative determination of urinary MDA after derivatization with DNPH and separation of the derivatives by HPLC with UV detection as originally described by Selim [24,25]. A significant increase in urinary MDA excretion of phenobarbital-pretreated rats exposed to chloroform ($CHCl_3$) or CCl_4 was found with this assay [24]. The utility of DNPH as a derivatizing agent for MDA was further explored by Shara et al., who applied this derivatization to three other potential lipid degradation products, i.e., FOR, ACET and ACON. The limits of detection were 2 pmol, 0.5 pmol, 0.3 pmol and 0.2 pmol for MDA, FOR, ACET and ACON, respectively. Although the HPLC method of Shara et al. showed similar results it remains less sensitive than our GC–ECD method [11]. Moreover, with our method it was possible to measure at least eight lipid degradation products instead of four.

Previously, a GC method using electron capture detection (ECD) has been reported for the pentafluorophenylhydrazine (PFPH) derivative of MDA. The derivatization conditions were mild (pH 5 and room temperature), as is the case in the present method. The limit of detection in urine was 72.6 fmol [12]. Compared to our method which used a derivatization with PFB, PFPH derivatization was found to be less sensitive (data not shown) and therefore we preferred PFB derivatization.

4-Hydroxynonenal has been determined by GC–MS as well as its oxime derivative using the PFB-reagent in heart, liver, adrenal and testis of rats [26]. Both the *syn*- and *anti*-isomers of the synthetic standard were identified [27]. 4-Hydroxynonenal has not been identified in urine, however, probably because it is extensively metabolized in vivo [28,29]. More recently, PFB-derivatization was extended to other aldehydes as well. It was shown that in one run a broad spectrum of aldehydes could be detected using GC–MS with NCI detection. The detection limits for the analysis of the various aldehydes in pure methanol were between 50 and 100 fmol [14]. As yet, however, no attempt was made to apply this method for the measurement of aldehydes in vivo as has been done in the present study.

Although several useful methods already have been described in the past for measuring aldehydes in urine, the GC–ECD method described in this paper has several advantages. First of all, it is a very sensitive method for eight lipid peroxidation products with detection limits ranging from 39 (PENTA) to 500 fmol (FOR and ACON). Although this sensitivity is not as high as found with GC–NCI-MS for FOR, ACET and ACON, it is more than sufficiently sensitive to measure background and (patho)physiological levels of the aldehydes in urine. Furthermore, our GC–ECD method is relatively easily performed and easily automated, enabling the measurement of large quantities of samples and

especially when compared with GC–NCI-MS, it is far more easily applied routinely in most laboratories. Another advantage compared to methods measuring only MDA or even up to four lipid degradation products, is the simultaneous measurement of eight LPO products, which reduces the chance of interferences with other compounds. Finally, differences in urinary excretion patterns of the aldehydes might in principal also provide additional information about organ selectivity of radical-induced damage, because of differences in disposition, i.e., primary and secondary metabolism, of aldehydes generated in different organs. For example, it is known that MDA is extensively metabolized in vivo, which is one reason why only a low concentration of MDA is found in urine [10]. 4-Hydroxynonenal has been found to be completely metabolized and no 4-hydroxynonenal reaches the urine when administered i.v. or i.p. [28,29]. Secondary metabolism of the aldehydic products, however, is not necessarily the same in all organs, which could mean that oxidative damage in different organs will result in excretion in urine of different aldehydic products.

In order to determine if lipid degradation products could be used as an urinary indicator of radical-induced damage in vivo, four rats were treated orally with CCl_4. It was found that following CCl_4 treatment an increase in urinary excretion of all eight products occurred. MDA was excreted in urine in relatively small amounts compared to the other seven products. Ekström et al. also reported that the recovery of MDA following i.p. administration of MDA to rats was low (0.7%–2.6% in 24 h), indicating extensive metabolism of MDA [10]. Shara et al. reported increases in all four measured lipid degradation products in urine of rats treated with TCDD, paraquat, endrin and CCl_4 at high doses. Urinary levels of MDA, FOR, ACET and ACON were still increasing 48 h after exposure to a dose of CCl_4 of 2.5 $ml\ kg^{-1}$ [11]. In the present study a 2.5-times lower dose of CCl_4 was used and partly different results were obtained. The highest increase in urinary excretion of lipid degradation products was found 24 h after treatment of rats with CCl_4 and thereafter it declined again. This may indicate that in the present study the damage due to 1 $ml\ kg^{-1}$ CCl_4 is starting to be repaired after 24 h, whereas at a CCl_4 dose of 2.5 $ml\ kg^{-1}$ the animals may not be capable of repairing the damage. In both studies it was found that the increase in urinary excretion of ACON was most pronounced.

In conclusion, a sensitive, rapid and more or less universal routine method to measure aldehydic lipid peroxidation products in urine has been developed. The seven aldehydic products and acetone were shown to be potentially useful as in vivo urinary biomarkers for oxidative stress induced by CCl_4. Simultaneous measurement of different products might give a fingerprint of organ-specific damage. It still needs to be determined, however, whether the urinary parameters also reflect damage induced by other compounds and whether they are sensitive enough to be measured before significant damage has occurred.

Acknowledgments

This study was supported financially by the Dutch Technology Foundation, STW-NWO, grant LBI22.2822.

References

[1] J.J. Hageman, A. Bast, N.P.E. Vermeulen, Chem.-Biol. Interact. 82 (1992) 243.
[2] A. Benedetti, A.F. Casini, M. Ferrali, M. Comporti, Biochem. J. 180 (1979) 303.
[3] H. Esterbauer, in D.C.H. McBrien and T.F. Slater (Editors), Free Radicals, Lipid Peroxidation and Cancer, Academic Press, London, 1982, p. 101.
[4] M. Ferrali, R. Fulceri, A. Benedetti, M. Comporti, Res. Commun. Chem. Pathol. Pharmacol. 30 (1980) 99.
[5] A. Kautiainen, C.E. Vaca, F. Granath, Carcinogenesis. 14 (1992) 705.
[6] A. Bast and G.R.M.M. Haenen, in V. Claassen (Editor), Trends in Drug Research, Elsevier Science, Amsterdam, 1990, p. 273.
[7] A. Van der Vliet, A. Bast, Chem.-Biol. Interact. 85 (1992) 95.
[8] H.H. Draper, E.J. Squires, H. Mahmooch, J. Wu, S. Agarwal, M. Hadley, Free Rad. Biol. Med. 15 (1993) 353.
[9] H.H. Draper, M. Hadley, Methods Enzymol. 186 (1990) 421.
[10] T. Ekström, P. Garberg, B. Egestad, J. Högberg, Chem.-Biol Interact. 66 (1988) 177.
[11] M.A. Shara, P.H. Dickson, D. Bagchi, S.J. Stohs, J. Chromatogr. B. 576 (1992) 221.
[12] M. Tomita, T. Okuyama, Y. Hatta, S. Kawai, J. Chromatogr. 526 (1990) 174.

[13] H.C. Yeo, H.J. Helbock, D.W. Chyu, B.N. Ames, Anal. Biochem. 220 (1994) 391.
[14] X.P. Luo, M. Yazdanpanah, N. Bhooi, D.C. Lehotay, Anal. Biochem. 228 (1995) 294.
[15] H. Esterbauer, K.H. Cheeseman, M.U. Dianzani, G. Poli, T.F. Slater, Biochem. J. 208 (1982) 129.
[16] G. Poli, M.U. Dianzani, K.H. Cheeseman, T.F. Slater, J. Lang, H. Esterbauer, Biochem. J. 227 (1985) 629.
[17] A.S. Csalany, M.D. Guan, J.D. Manwaring, P.B. Addis, Anal. Biochem. 142 (1984) 277.
[18] K.T. Koshy, D.G. Kaiser, A.L. Vanderslik, J. Chromatogr. Sci. 13 (1975) 97.
[19] T. Nambara, K. Kigasawa, T. Iwata, M. Ibuki, J. Chromatogr. 114 (1975) 81.
[20] D.A. Cancilla, S.S. Que Hee, J. Chromatogr. 627 (1992) 1.
[21] D.R. Janero, Free Rad. Biol. Med. 9 (1990) 515.
[22] R.P. Bird, S. Silas, O. Hung, M. Hadley, H.H. Draper, Anal. Biochem. 128 (1983) 240.
[23] H.H. Draper, L. Polensek, M. Hadley, L.G. McGirr, Lipids. 19 (1984) 836.
[24] T. Ekström, A. Stahl, K. Sigvardsson, J. Högberg, Acta Pharmacol. Toxicol. 58 (1986) 289.
[25] S. Selim, J. Chromatogr. 136 (1977) 271.
[26] F.J.G.M. Van Kuijk, D.W. Thomas, R.J. Stephens, E.A. Dratz, Biochem. Biophys. Res. Commun. 139 (1986) 144.
[27] M.L. Selley, M.R. Bartlett, J.A. McGuiness, A.J. Hapel, N.G. Ardlie, J. Chromatogr. 488 (1989) 329.
[28] J. Alary, F. Bravais, J.-P. Cravedi, G. Bories, Chem. Res. Toxicol. 8 (1995) 34.
[29] L.L. De Zwart, R.C.A. Hermanns, J.H.N. Meerman, J.N.M. Commandeur, N.P.E. Vermeulen, Xenobiotica. 26 (1996) 1087.