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High-performance liquid chromatographic peak identification of 2,4-dinitrophenylhydrazine derivatives of lipid peroxidation aldehydes by photodiode array detection

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Abstract

Malonaldehyde (MDA), a product of lipid peroxidation, is a presumptive marker for the development of oxidative stress in tissues and plasmas. In this study we report the photodiode array detection of the 2,4-dinitrophenylhydrazine (DNPH) derivatives of MDA using HPLC. Oxidative stress was produced by injecting (i.p.) bacterial lipopolysaccharide (LPS) into rats at a dose of 100 μg/kg, or i.v. into rabbits (1 μg/kg), or added to freshly drawn human blood (200 ng/ml). Blood was collected at several time points up to 5 h, centrifuged, and equal volumes of 20% TCA were used to precipitate proteins from the plasma. The supernatants were derivatized with DNPH, and the aldehyde–DNPHs were extracted with pentane. After evaporation, aliquots of 10 μl in acetonitrile were injected onto a Beckman Ultrasphere C_{18} (3 μm) column, chromatographed with an acetonitrile–water–acetic acid gradient mobile phase and scanned using Waters 996 photodiode array detector. Peak identification and homogeneity was determined by comparing the experimental peaks and UV scans with those of authentic standards. A significant increase in the DNPH derivative of malonaldehyde (MDA–DNPH), but not of the other aldehyde–DNPH derivatives of formaldehyde (FDA), acetaldehyde (ADA), acetone and propionaldehyde (PDA) was seen over the first hour after LPS administration in anesthetized rats, while in conscious rabbits this trend lasted up to 3 h. The retention times as well as the UV scans of the derivatized aldehydes matched the authentic standards. Thus, photodiode array detection has proved valuable in establishing this HPLC method for estimating oxidative stress. This technique could accurately measure pmol amounts of MDA–DNPH indicating the usefulness of photodiode array detection method for estimating small changes in the oxidative stress. © 1998 Elsevier Science B.V.

Keywords: Lipid peroxidation; Dinitrophenylhydrazines; Aldehydes; Malonaldehyde; Lipopolysaccharide

1. Introduction

Estimation of malonaldehyde (MDA) is the most common approach for the detection of lipid peroxidation. The most frequently used method to determine MDA formation is the spectrophotometric assay of MDA following its reaction with thiobarbituric acid (TBA) [1]. However, TBA reacts not only with MDA but also with many other compounds including ribose, biliverdin, amino pyridines and sialic acid [2]. Therefore, derivatization of MDA and other carbonyls with 2,4-dinitrophenylhydrazine (DNPH) and conversion into pyrazole and hydrazine derivatives has since found to allow a more specific estimation of these compounds, especially if combined with their separation using HPLC [3–5]. Indeed, this method combined with mass spectrometry has conclusively discriminated MDA from

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many other compounds produced under oxidative stress conditions in biological samples [6]. In the present study we describe a rapid and simple method to monitor the development of oxidative stress by identifying HPLC peaks of DNPH derivatives of lipid peroxidation aldehydes by photodiode array detection.

2. Experimental

2.1. Materials

LPS from *Escherichia coli* (serotype 0113, Ass. Cape Cod, MA, obtained from Dr. Weidner, Walldorf, Germany) was dissolved to 5 μg/ml in saline for i.v. injections. Malonaldehyde-bis-dimethyl acetal and malonaldehyde-bis-diethyl acetal, formaldehyde (FDA), acetaldehyde (ADA), acetone and propionaldehyde (PDA) were purchased from Sigma-Aldrich, Deisenhofen, Germany. DNPH was obtained from Fluka (Buchs, Switzerland). High-purity DNPH standards were prepared by stoichiometric derivatization and crystallization from ethanol [4]. Acetonitrile, for HPLC, was from Acros (Neuss, Germany).

2.2. Methods

Male Wistar rats weighing about 230–250 g were anesthetized with intraperitonal (i.p.) pentobarbital (70 mg/kg), and the femoral artery was cannulated to monitor blood pressure and for blood sampling. Blood samples from female Chinchulla rabbits (Charles River, Kisslegg, Germany) weighing 3.2–4.5 kg were taken from a central ear artery cannulated under local anesthia using 1% xylocaine (Astra, Wedel, Germany). Human blood was taken from a cubal vein. Rabbits received 1 μg/kg LPS i.v., rats were injected with 200 μg/kg i.p. and human blood was incubated with LPS at a dose of 1 μg/ml at 37°C. For quantitative evaluation of aldehydes, blood was collected in chilled tubes containing 28.3 m*M* EDTA and 5.5 m*M* sodium bisulfite (final concentration in mixed, collected blood) and immediately centrifuged at 4°C at 5000 *g*. Deproteinization of plasma was performed with equal volumes of 20% TCA in 5.5 m*M* sodium bisulfite. After vortexing for 10 min at 4°C, the supernatant was derivatized with DNPH.

2.3. Derivatization and extraction of lipid metabolites

For derivatization, 31 mg of DNPH were dissolved in 10 ml of 2 *M* HCl, and 100 μl of this DNPH reagent (1.56 m*M*) was added to 2 ml of TCA supernatant in a 12 ml PTFE-lined screw-capped test tube. After mixing for 15 min at 30°C, 8 ml of pentane was added. The tubes were intermittently shaken for 30 min, the pentane was taken off, and the procedure was repeated two more times. Combined pentane extracts were dried under nitrogen at 30°C and reconstituted in 200 μl of acetonitrile.

2.4. HPLC procedure

A 10-μl volume of the acetonitrile extract was directly injected onto a Beckman Ultrasphere ODS C_{18} (3-μm particle size, 7.5 cm×4.6 mm I.D.) HPLC column kept at 30°C.

Chromatography was performed using a Waters Alliance 2690 Separations Module (Milford, MA, USA) controlled by a Waters 2010 MILLENNIUM chromatography manager. The DNPH derivatives of aldehydes were detected with a Waters 996 photodiode array detector at either 307 or 356 nm at a flow-rate of 1 ml/min with an isocratic gradient of acetonitrile–water–acetic acid (34:66:0.1, v/v/v) for 15 min, a linear gradient to acetonitrile–water–acetic acid (50:50:0.1, v/v/v) for 10 min, an isocratic gradient of acetonitrile–water–acetic acid (50:50:0.1, v/v/v) for 5 min, then acetonitrile–water–acetic acid (95:5:0.1, v/v/v) for 10 min with a 20-min reequilibration with the initial mobile phase. Wavelength scans were determined with starting and ending wavelengths of 195 and 500 nm, respectively. Spectral resolution was set at 2.4 nm. The retention times were confirmed by cochromatography and comparison of wavelength scans with authentic standards. The amounts of the various aldehydes were quantitated by performing peak area analysis using Waters MILLENNIUM software program.

2.5. Statistical analysis

All data are reported as means±S.E.M. Statistical analysis was carried out by the use of standard one- and two-way parametric analysis of variance with repeated measures design coupled with paired Student's *t*-test. A value of $P<0.05$ was taken to indicate a significant difference.

3. Results

3.1. Separation and identification of lipid metabolites

The DNPH derivatives of authentic standards of MDA, FDA, ADA, acetone and PDA were separated and analyzed using various different wavelengths. As shown in Fig. 1, MDA–DNPH gives an absorption maximum at 307 nm, whereas the absorption maxima for FDA–DNPH, ADA–DNPH, acetone–DNPH and PDA–DNPH is 356 nm. The retention times of MDA–DNPH, FDA–DNPH, ADA–DNPH, acetone–DNPH and PDA–DNPH were 5.9, 7.5, 12.7, 20.4, and 22.9 min, respectively. As shown by the data in Table 1, standard curves of all five lipid metabolites were linear in the range from 10 pmol to 5 μmol injected. A peak regularly detected using an isocratic gradient of acetonitrile–water–acetic acid (34:66:0.1, v/v/v) at 6.6 min could be clearly matched by wavelength scan with a 10% component of added DNPH dissolved in 2 *M* HCl (most likely the acidic form of DNPH, DNPA) and showed a significantly different wavelength scan from MDA–DNPH (Fig. 1).

3.2. Quantitative estimation of lipid metabolites in plasma of humans, rats and rabbits

A typical chromatogram of lipid metabolites detected at a wavelength of 307 nm of a LPS-treated rat is shown in Fig. 2. Fig. 3 shows the chromatogram detected at a wavelength of 356 nm. It proved to be essential for the detection of free MDA that the plasma samples were deproteinized with 10% TCA at a temperature of 4°C. Heating pure MDA standard solutions for 30 min at 60°C in 0.5 *M* NaOH [7] caused a total loss of absorbance at the specific wavelength of 307 nm. After heating plasma samples in the same manner in order to release bound MDA, a substantially higher peak with the retention time of MDA–DNPH at 5.9 min was observed, however, with a totally different wavelength scan.

Control plasma values of MDA were in the rat 25.6±5.4 nmol/ml, 30.8±2.7 nmol/ml in the rabbit, and 9.0±1.0 nmol/ml in human blood Table 2. LPS caused within 15 min an increase of MDA in all three species (data not given), with peak values of 120.6±21.5 nmol/ml MDA in the rat 1 h after i.p. injection of LPS, with a continuous rise of MDA within the next 2 h. Adding 1 μg/ml LPS to human blood caused an increase of MDA within 10 min, with peak MDA values of 132.5±10.5 nmol/ml after 60 min. LPS caused no significant changes of FDA, ADA, acetone and PDA.

4. Discussion

MDA and several other aldehydes and ketones, such as formaldehyde, acetaldehyde, acetone and propionaldehyde, are the breakdown products of spontaneous fragmentation (β-cleavage) of peroxides derived from interaction of free oxygen radicals with polyunsaturated fatty acids [8]. The results of the present study demonstrate that at least five different lipid breakdown products (MDA, FDA, ADA, acetone and PDA) can be clearly separated; however, following LPS, only MDA rises significantly within an observation time of 3 h. Since LPS caused a similar rise of plasma levels of prostaglandins in rabbits [9], it seems likely that MDA reflects oxidative decomposition of arachidonic acid metabolites [10].

Development of oxidative stress by endotoxin is an established method. A number of reports of various laboratories including our own have demonstrated development of oxidative stress in tissue within 1 h of endotoxin treatment [11].

Several methods are available for the quantitation of MDA in biological tissues. Among those, the measurement of TBA-reactive products has been frequently used because of its simplicity, although the method lacks specificity. To best of our knowledge the present study is the first showing that MDA can be detected highly specifically by using HPLC

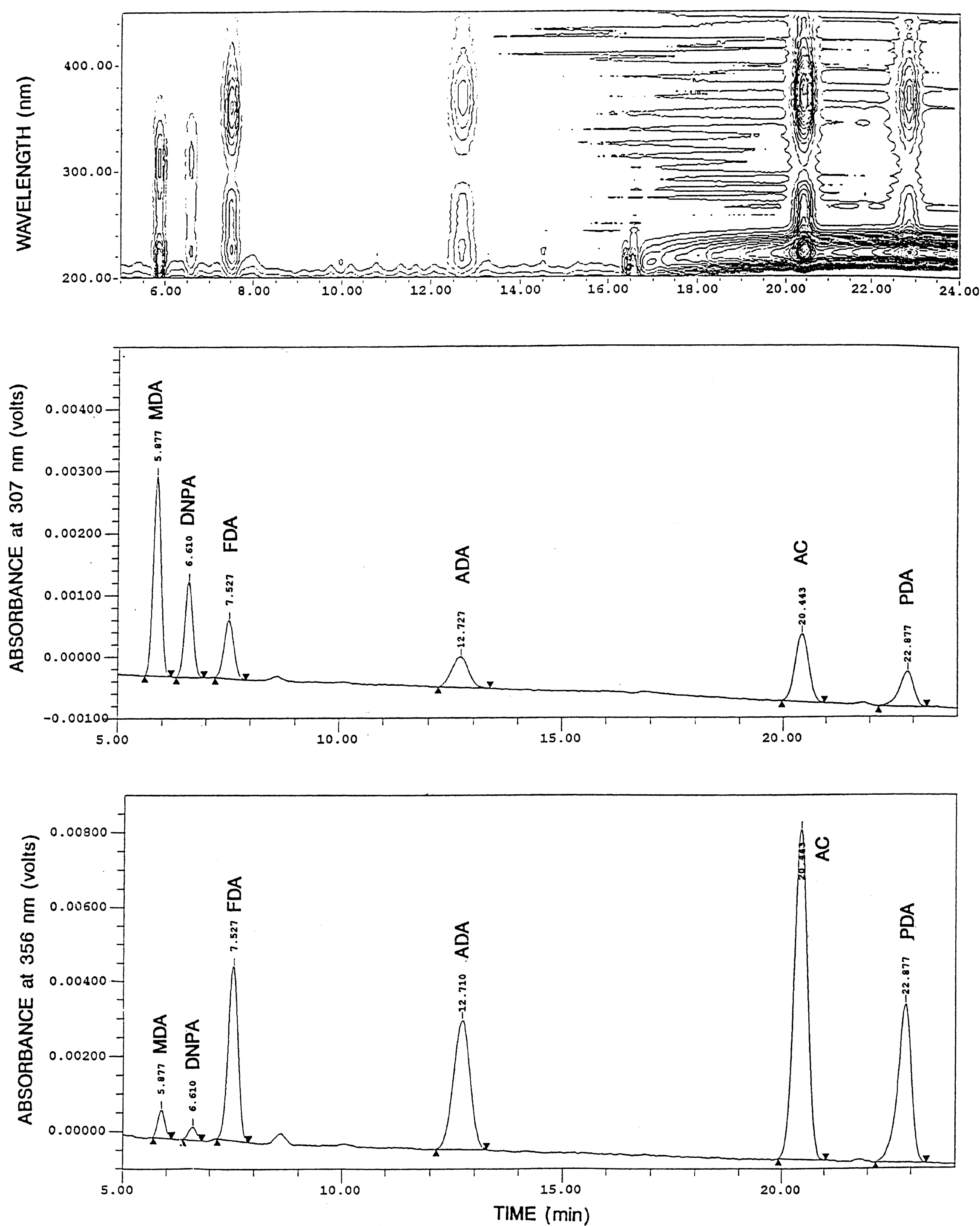

Fig. 1. Photodiode array detection of 100 pmol injected standards of DNPH derivatives of MDA, FDA, ADA, acetone (AC) and PDA. Upper part: contour plot. Middle part: absorbance at 307 nm. Lower part: absorbance at 356 nm. DNPA peak is caused by addition of stock DNPH–HCl solution.

Table 1
Retention times (t_R), linear regression equations (Regr. Equ.), coefficient of determination (r^2) and wavelength maximums (λ_{max}) of 307 or 356 nm for DNPH derivatives of authentic standards

	t_R (min)	Regr. Equ.	r^2	λ_{max} (nm)
MDA–DNPH	5.868	$y=4010x+34$	0.99999	307
FDA–DNPH	7.528	$y=6610x+31$	0.99995	356
ADA–DNPH	12.745	$y=8860x+42$	0.99999	356
Acetone–DNPH	20.495	$y=5360x+50$	0.97412	356
PDA–DNPH	22.928	$y=9300x+37$	0.99996	356

Range of standard injected was 10 pmol to 5 μmol.

and scanning the wavelength pattern. This is particularly important as there is an unknown peak which coelutes with MDA–DNPH when using a concentration of higher than 40% acetonitrile. Lowering the acetonitrile concentration to 34% separates the two peaks. This peak apparently is not an isomer of MDA–DNPH because retention time and wavelength spectrum differ significantly, although both peaks have an absorption maximum at 307 nm (Figs. 1 and 2). This peak also appears upon the addition of DNPH to 2 *M* HCl reagent (Fig. 1) and therefore is a product of this reagent (most likely DNPA). The study shows the reliability of measuring at least five lipid peroxidation products in biological fluids, be-

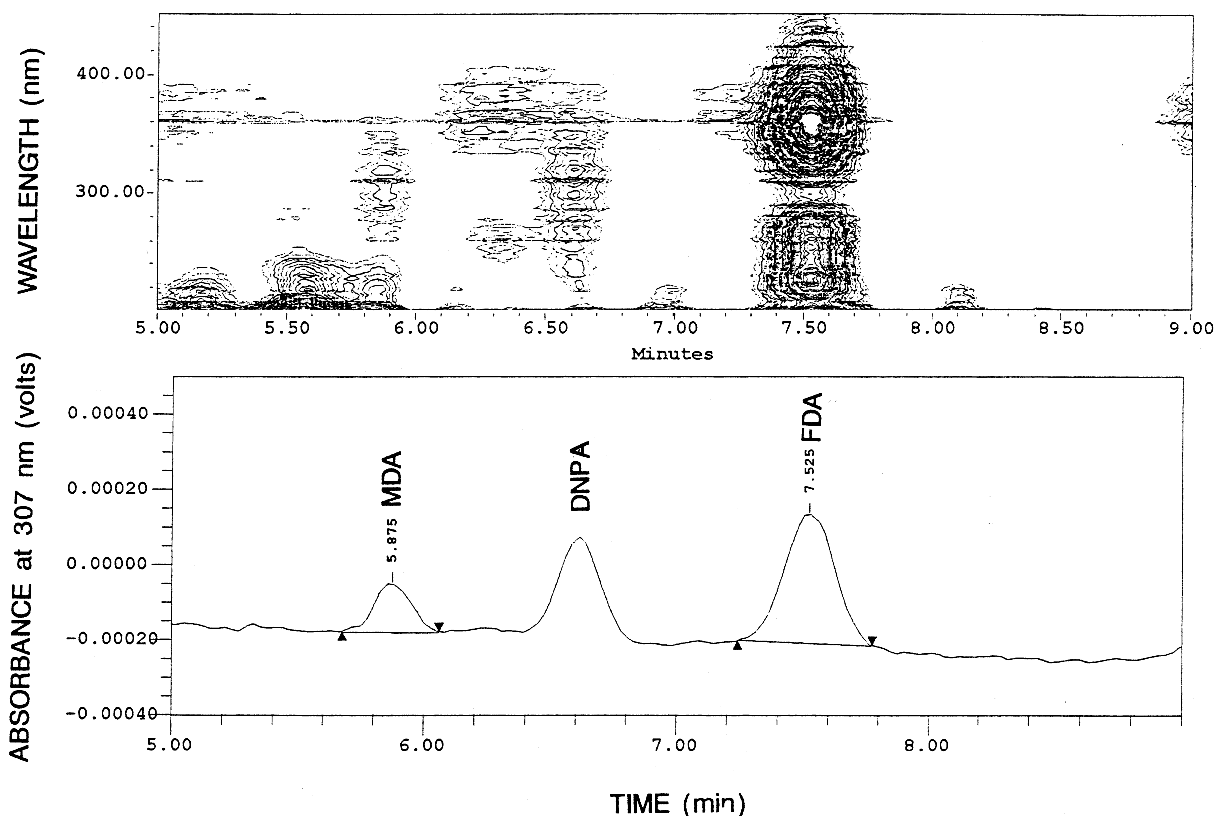

Fig. 2. Photodiode array detection of extracted rat plasma after LPS stimulation. Upper part: contour plot of DNPH derivatives of MDA and FDA. Lower part: absorbance at 307 nm.

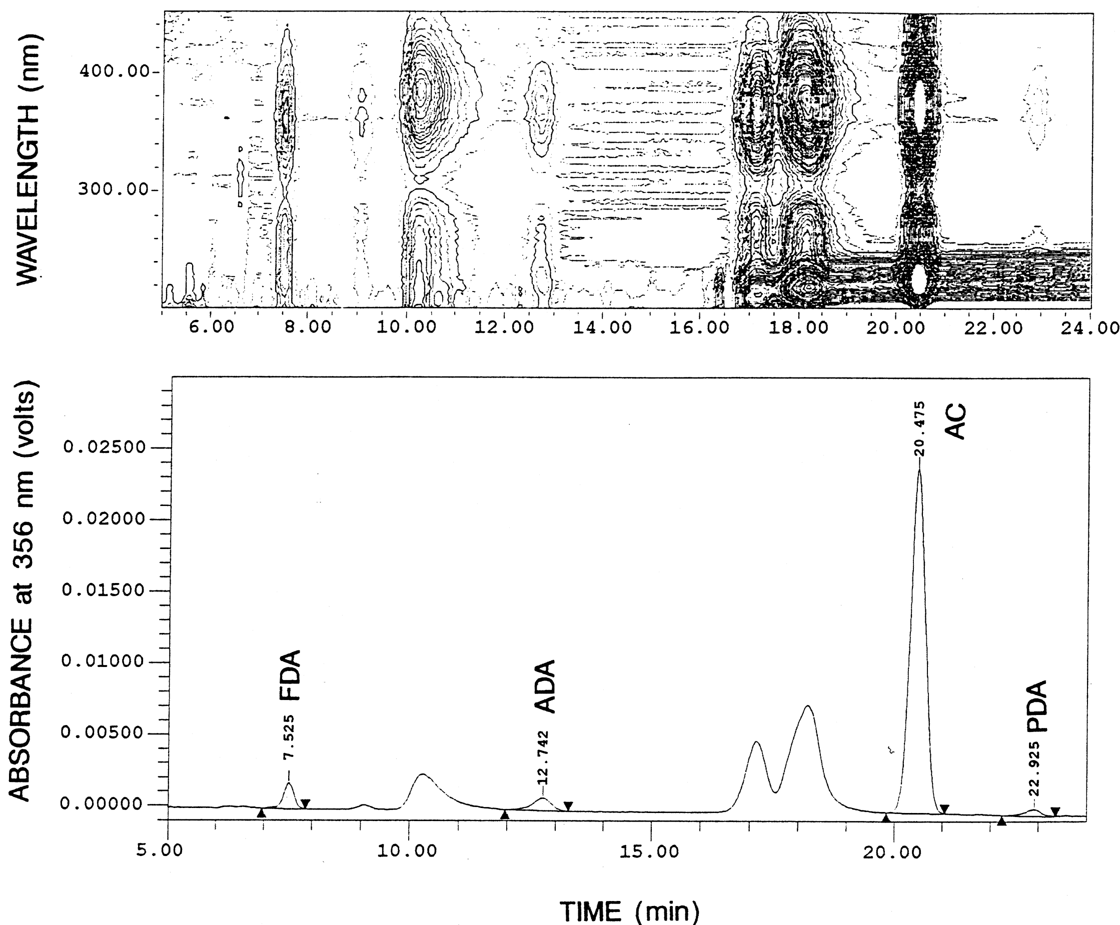

Fig. 3. Photodiode array detection of extracted rat plasma after LPS stimulation. Upper part: contour plot of DNPH derivatives of FDA, ADA, AC and PDA. Lower part: absorbance at 356 nm.

Table 2
Levels of DNPH-derivatives of lipid peroxidation aldehydes and ketones in rat, rabbit and human plasma before and 1 h after application of LPS

	MDA–DNPH (nmol/ml)	FDA–DNPH (nmol/ml)	ADA–DNPH (μmol/ml)	Acetone–DNPH (μmol/ml)	PDA–DNPH (μmol/ml)
Rat (n=9)					
Control	25.6±5.4	5.6±0.5	4.1±0.7	317.7±57.9	2.3±0.2
LPS	120.6±21.5[a]	5.6±0.5	5.4±0.5	431.7±133.4	2.1±0.1
Rabbit (n=6)					
Control	41.5±4.5	7.±3.3	5.9±3.5	207.8±32.1	1.9±1.0
LPS	111.6±9.5[a]	5.2±.28	4.8±3.1	68.2±16.3	1.7±1.1
Human (n=6)					
Control	8.0±1.9	4.5±1.3	7.0±2.0	42.9±10.5	1.9±0.8
LPS	122.5±12.1[a]	3.4±1.5	7.1±2.0	41.1±9.9	1.9±0.8

Means with standard error.

[a] Denotes significant difference from control values; $P<0.5$.

cause the scans of the biological peaks exactly matched the authentic standards. Thus, photodiode array detection has proved a valuable tool in establishing this HPLC method.

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