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Determination of p-phenylenediamine and its metabolites MAPPD and DAPPD in biological samples using HPLC-DAD and amperometric detection

Axel Meyer[a,*], Brunhilde Blömeke[b], Klaus Fischer[a]

[a] Department of Analytical and Ecological Chemistry, Faculty VI – Geography/Geosciences, University of Trier, Campus II, Behringstrasse 21, D-54296 Trier, Germany
[b] Department of Environmental Toxicology, Faculty VI – Geography/Geosciences, University of Trier, Wissenschaftspark Trier-Petrisberg (WIP), Am Wissenschaftspark 25-27, Building 024, D-54296 Trier, Germany

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ABSTRACT

A sensitive and selective HPLC method using a diode array detector (DAD) and an electrochemical detector (ECD) in series has been developed and validated for the quantitative measurement of p-phenylenediamine and its acetylated metabolites N-acetyl-p-phenylenediamine (MAPPD) and N,N′-diacetyl-p-phenylenediamine (DAPPD) in biological samples. The separation was carried out on a hydrophilic modified AQUA C_{18} column and the mobile phase was composed of acetonitrile: ammonium acetate solution (5:95, 25 mM, v/v). Spectrophotometric detection was performed at 240 or 255 nm and amperometric detection was carried out using a positive oxidation potential of 400 mV. The quantification of the three analytes was validated in the range of 0.05–50 μM and the established limits of determination were 0.5 μM for PPD and MAPPD and 1 μM for DAPPD. The standard deviations ($N = 9$) were lower than 7.5% at a concentration of 1 μM. The samples were stabilised with ascorbic acid to prevent PPD from oxidizing. Pretreatment of samples or analyte enrichment before sample injection is not required. The method proved to be accurate, sensitive and sufficiently specific. It was applied to the ecotoxicological study of the kinetics of the PPD N-acetylation in cell lysates in two different media.

1. Introduction

The application of synthetic chemicals for hair dying has a long tradition. In 1856 the first synthetic hair dye was created and permanent hair colorants have been in commercial use for nearly 100 years [1]. Nowadays, the permanent oxidative hair dyes represent the largest group of hair colorants consisting of a colourless developer, a coupler and an oxidizing agent which mostly is hydrogen peroxide [2,3]. Mainly, p-phenylenediamine (PPD), p-aminophenol, toluone-2,5-diamine or other arylamines are used as developers.

Apart from being used in hair formulations PPD and its derivatives serve as starting materials for the synthesis of azo dyes, antioxidants, tattoo colours and pharmaceuticals. In addition, they are applied for colour photography and as accelerators for the synthesis of polymer fibres [4,5].

PPD is a strong contact allergen which might generate sensitizations and allergies, in particular with hairdressers [6–10]. Due to its small molecular size and its good water solubility it can penetrate the skin causing interactions in the human body. The biotransformation of PPD takes place via auto-oxidation, catalyzing enzymes or via acetylation [11]. During the acetylation process in the skin or the liver an acetyl group is transferred to the nitrogen atom of the amino group by N-acetyl transferases (NAT-1, NAT-2). Thus, monoacetylated (MAPPD) and diacetylated (DAPPD) p-phenylenediamine metabolites are formed [12–15].

Using ^{14}C-labeled PPD and HPLC–MS–MS Stanley et al. [16] could provide evidence that PPD is acetylated by human hepatic cytochrome P450 enzymes to form N-acetylated metabolites, but no indications were found for the formation of N-mono-oxygenated metabolites. In contrast, other aromatic amines underwent CYP-mediated metabolism to be transformed into different hydroxylated metabolites and to N-acetylated compounds.

Therefore, it is obligatory for dermatological, clinical, eco- and toxicological questions to quantify the transformation of PPD and the formation of both metabolites in different biomatrices.

Besides GC, HPLC is mostly applied to determine PPD in different biological samples and in formulations of various hair dyes. By reason of PPD's low volatility and high polarity a pre-column derivatization is necessary for the gas chromatographic determination. Using derivatization with benzaldehyde and subsequent GC–MS in SIM mode a limit of detection of 50 μM was obtained for PPD in

* Corresponding author. Tel.: +49 651 201 2293; fax: +49 651 201 2293.
E-mail address: meyerax@uni-trier.de (A. Meyer).

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hair dyes [17]. Stambouli et al. [18] derivatized PPD with methyl iodide or trifluoroacetic acid anhydride (TFA) for the quantification using GC–MS in urine, blood and bile acid. A preceding liquid–liquid extraction of the samples with chloroform considerably improves the limit of determination to 1 μM using the more sensitive method with TFA.

The HPLC-determination of PPD and other dyes in different matrices, like permanent hair colorants, washer fluid, blood etc., is usually carried out with RP-chromatography. Mostly, an eluent containing methanol and ammonium acetate or a buffer is employed. UV-absorption and diode array detection (DAD) are mainly used for the quantification of PPD and limits of detection in the range of 0.05–2 μM are achieved [19-21]. Apart from this, a post-column on-line photochemical derivatization [22] and MS-detection were reported [23]. Using HPLC–MS, PPD in black henna tattoos was determined with a limit of detection of 10 μM which corresponds to a limit of determination lower than 0.01% in commercial products [24]. DAD and MS-detection are primarily applied to structurally elucidate the chromatographic signals of PPD. Employing amperometric detection after the separation of different aminophenols, resorcinol and PPD via HPLC, a limit of determination of 0.5 μM of PPD was determined for hair dyes [25]. Dermatological and medical publications very often lack information about important methodical features of the analytical procedures applied such as the limit of determination, linearity, reproducibility, the influence of the matrix on analyte determination etc. [12,26–28].

PPD and its metabolites MAPPD and DAPPD, solved in biological fluids, can also be separated on a C_{18}-column using eluents, containing ammonium acetate and methanol or acetonitrile. The quantification of the separated analytes is then carried out with UV-detection [12]. The formation of both N-acetylated PPD metabolites was verified by means of ^{14}C-labeled PPD using HPLC and subsequent radiotracer-detection [29]. Besides, MAPPD and DAPPD could be detected in the presence of human hepatic cytochrome P450 enzymes using LC–MS–MS [16]. Here an approximate limit of determination of 1% of PPD is given for the metabolites. By means of HPLC Nohynek et al. [8] detected approximately 0.5% of the applied ^{14}C-labeled PPD in the urine of test persons after colouration of their hair with PPD-containing dyes.

In addition to MAPPD and DAPPD five further metabolites were found. The major urinary metabolites were concluded to be MAPPD and DAPPD. They were present in all urine samples and amounted to 80–95% of all metabolites. Using amperometric detection with a glassy carbon electrode at a potential of 1000 mV the limits of quantification for PPD, MAPPD and DAPPD were 1 μM in humane urine and rabbit blood, urine and faeces after extraction with chloroform [30].

The toxicological and dermatological studies lack both information about analytical features and information about the necessary sample preparation. Merely some authors [18,30,31] described a liquid–liquid extraction with chloroform to remove the biological sample matrix prior to the analytical determination of PPD. Solid-phase extraction with C_{18}-cartridges was performed to enrich the analytes which were fortified by ^{14}C-labeled PPD and to remove the matrix [32]. The recovery rates exceeded 80%.

In our preliminary investigations a direct and exact determination of PPD and its metabolites was not possible in cell lysates and other biological matrices, when the concentrations of PPD and MAPPD in the samples were lower than 2.5 μM. The aim of this paper is to present a new analytical method suited for this task. The new method should provide the required high analytical sensitivity and reproducibility. This rugged method is dedicated for advanced toxicological investigations, for instance the study of the kinetics of the PPD metabolism in various cell lines. Results of this examination will be reported in an additional paper.

2. Experimental

2.1. Chemicals and solvents

p-Phenylenediamine (PPD) from Acros Organics (99%) and N,N′-diacetyl-p-phenylenediamine (DAPPD) from ABCR (>98%) were used as analytical standards. N-acetyl-p-phenylenediamine (MAPPD) was synthesized in our laboratory [12]. The other chemicals, ascorbic acid (Merck), ammonium acetate (Roth), n-butanol (Merck), chloroform (Merck), diethylether (Roth), methylisobutylketone (MIBK) (Merck), propylencarbonate (Fluka), dichloromethane (Roth) and ethylacetate (Merck) including the ion-pair reagents heptanesulphonic acid (Merck), octanesulphonic acid (Fluka), and dodecylsulphate (Merck) were of analytical grade. Dulbecco's modified eagle medium (DMEM-Medium) (high glucose 4.5 g/l), foetal bovine serum (FCS), L-glutamine solution (200 mM) and antibiotic/antimycotic (AA) solution were obtained from PAA Laboratories GmbH. The solvents (methanol, acetonitrile) for preparing eluents were of HPLC quality. Water was purified by inverse osmosis and then passed through a Membrapure unit (Astacus Analytical).

The matrix for ecotoxicological studies was composed of the DMEM-Medium containing 10% FCS and 1% AA.

2.2. Stock solutions

PPD stock solutions were prepared by weighing of 10.81 mg of the compound in a 100 ml volumetric flask followed by dissolution in acetonitrile:water (10:90; v/v). Stock solutions of the N-acetylated metabolites were prepared by weighing of 15.02 mg MAPPD or 19.22 mg DAPPD in a 100 ml volumetric flask followed by dissolution in acetonitrile:water (50:50, v/v). The stock solution of PPD was freshly prepared every day. These solutions were stored at 4 °C and used for further dilutions. Lower concentrations (<20 μM) of the analytes were freshly prepared every day and stabilised with ascorbic acid (addition of 50 μl of 1 M ascorbic acid solution to 10 ml of analyte solution).

2.3. Equipment

The study was carried out utilizing two HPLC systems. Chromatographic separations on a Nucleosil column (Machery & Nagel) were conducted with a Shimadzu HPLC system consisting of an Autosampler SIL 10A, a controller SCL-10AVP, a gradient pump LC-10 ADVP, a DGU-14A on-line degasser, and a diode array detector SPD-M 10 AVP (DAD). Data acquisition and processing were accomplished with the Shimadzu CLASS VP 6.12 software. The second LC unit, a Dionex IC system, consisted in an AQUA separation column (Phenomenex), a GP50 gradient pump, an AS 50 autosampler, a TCC column thermostat, and an UCI 50 universal chromatography interface, which was connected with an SPD-10 AVP UV/vis-detector (Shimadzu) and with an electrochemical detector (Metrohm 656). After the column the eluent first passed the UV-detector and then the electrochemical detector. The latter consisted of an amperometric wall jet detector cell with a glassy carbon electrode (GCE) as working electrode and an electronic controller (Metrohm 641 VA detector). As reference electrode an Ag/AgCl/3 M KCl electrode was used and all potentials given in this paper refer to it. The counter electrode was a Pt-electrode. Data acquisition, processing, and system control were accomplished with the Chromeleon 6.50 software.

The following separation columns packed with C_{18}-RP resins were used: (a) Nucleosil 100-5C_{18}; 250 mm × 4 mm (Macherey & Nagel) and (b) AQUA 5C_{18}; 250 mm × 3 mm (Phenomenex); in each case in combination with the corresponding guard column.

2.4. Chromatographic conditions

At first, the HPLC method development was conducted with the Nucleosil column (Sections 3.1–3.4) followed by the AQUA column (Sections 3.5 and 3.6). The isocratic separation was carried out at 22 °C with column (a) and 15 °C with column (b) respectively. Acetonitrile ammonium acetate (25 mM), containing various ion-pair reagents, or a 0.1 M ammonium acetate buffer (pH 8) for electrochemical detection served as eluents. All mobile phases were degassed with an on-line degasser and filtered through a 0.45 μm PTFE filter prior to use. The flow rate of the eluent was 0.7 (AQUA) respectively 1.1 ml/min (Nucleosil) and the injection volume was 50 μl. The UV-detector was set at 255 nm and the potential of the GCE at +400 mV. The DAD data were recorded at 240 nm for PPD and 255 nm for MAPPD and DAPPD. For quantification with the DAD, wavelengths of 240 nm for PPD and of 255 nm for MAPPD and DAPPD were chosen, respectively.

2.5. Extraction procedure

2 ml of the organic solvent were added to 2 ml of a standard solution or a sample (standard + matrix) and the mixture was shaken for 3 min. Afterwards, 1.5 ml of the organic solvent were collected and dried in a N_2 stream. Then, the solid residue was solved in 1.5 ml of the eluent.

2.6. Preparation of samples

The immortalized human keratinocyte cell line HaCaT was kindly provided by Prof. Dr. N.E. Fusenig (DKFZ Heidelberg, Germany) and cultured in DMEM, supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS and 1% antibiotic/antimycotic solution. Cells were maintained under standard culture conditions at a temperature of 37 °C and an atmosphere of 5% CO_2. Subculture was routinely performed two times a week, and cells were grown up to a maximum of 70% confluence. For experiments, cells were seeded ($0.55 \times 105\,cm^{-2}$) in six-well plates (Sarstedt AG & Co.; Nümbrecht, Germany) and grown overnight up to 95% confluence. Cells were washed twice with phosphate buffer, once with DMEM and incubated for another 24 h in DMEM without FCS (2 ml/well) and then stimulated with PPD for 24 h in serum-free culture medium. For stimulation, PPD was freshly dissolved (10 mM) in DMEM immediately prior to stimulation. After incubation, cell culture supernatants were collected and stored at −80 °C till HPLC analyzation for PPD, MAPPD and DAPPD.

3. Results and discussion

3.1. Stabilization of samples

In oxidation tests of PPD Regnery [33] and Elsenberg [34] showed that by addition of different oxidizing agents PPD is rapidly oxidized with the process of degradation depending on the pH-value, the temperature, and the oxidant. But even in the absence of hydrogen peroxide or other oxidizing agents, both in pure solution [water/acetonitrile (90:10)] and in the biological matrix used the peak area of the PPD gradually decreases in contrast to the stable metabolites. As a countermeasure the samples were then stored in brown vials and ascorbic acid (5 mM) was added to keep them stable overnight in a non-cooled autosampler [35]. There is evidence that the addition of ascorbic acid prevented PPD from being transformed during a period of 4 days, whereas after 24 h in water/acetonitrile solution without addition of ascorbic acid the peak area of the PPD is reduced to 86% of the original value and in the matrix it is even reduced to 64%.

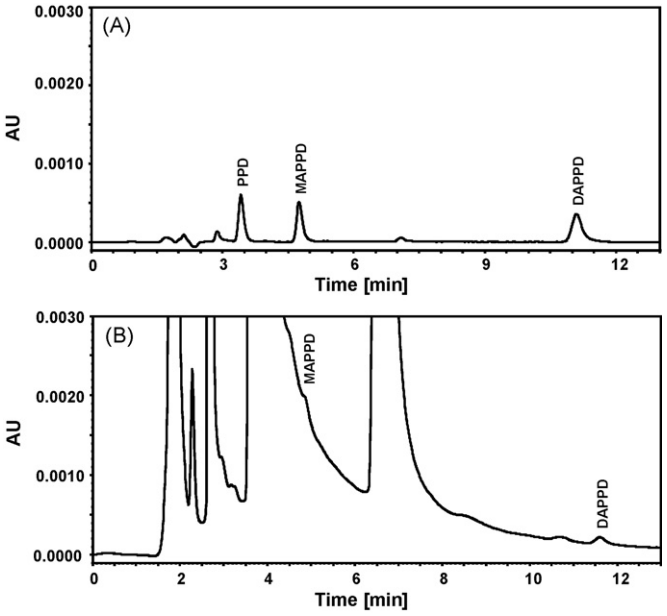

Fig. 1. Determination of PPD, MAPPD and DAPPD (0.1 μM each) without (A) and with matrix (B). Nucleosil column; eluent: 25 mM ammonium acetate/acetonitrile (92:8); flow: 1.1 ml/min; UV-detection: 255 nm.

3.2. Influence of the matrix

The chromatogram with and without matrix in Fig. 1 illustrates that the matrix considerably interferes with the determination of PPD and MAPPD when a Nucleosil column is applied. The matrix has a strong impact on the determination of PPD and MAPPD, as in the retention time period of both analytes large interfering peaks partially superpose the analyte peaks. The interfering peaks result from very polar components of the matrix which are supposed to be amino acids, short-chain peptides, carbohydrates etc. eluting early, too. Table 1 shows that in particular the sensitivity for PPD and MAPPD drastically decreases. The matrix affects the limits of detection and determination which increase by the factor 10 for PPD and by the factor 2 for MAPPD. The limits of detection and determination are nearly identical for DAPPD which is retarded most and its elution is not interfered with matrix compounds. Besides, in contrast to the pure aqueous solution, the reproducibility of the peak

Table 1
Limits of detection and of determination for PPD, MAPPD und DAPPD with and without matrix, separated on the Nucleosil column.

	Without matrix			With matrix		
	PPD	MAPPD	DAPPD	PPD	MAPPD	DAPPD
Regression coefficient R^2	0.9978	0.9995	0.9990	0.9943	0.9968	0.9985
Limit of detection [μM]	0.31	0.22	0.35	3.02	0.49	0.38
Limit of determination [μM]	0.78	0.67	0.73	8.21	1.06	0.94
Standard deviation [%]; 5 μM ($N = 9$)	4.52	4.04	3.87	12.8	8.72	7.93

Chromatographic conditions: Nucleosil column; eluent: 25 mM ammonium acetate/acetonitrile (92:8); flow: 1.1 ml/min; UV-detection: 255 nm; linear range: 0.2–50 μM.

Table 2
Recovery rates of PPD, MAPPD und DAPPD from aqueous solutions with different organic solvents.

Extractant	Concentration [μmol/l]	Recovery rate [%]		
		PPD	MAPPD	DAPPD
Ethyl acetate	50	10	35	39
	0.5	7	25	42
Dichloromethane	50	7	18	13
Propylene carbonate[a]	50	27	53	22
MIBK	50	1	26	51
	0.5	19	36	68
n-Butanol	50	29	35	40
Chloroform	50	40	12	6
Diethylether	50	1	2	5

Chromatographic conditions: cf. Table 1.
[a] Heated during evaporation.

areas and of the retention times is additionally impaired by the matrix. Varying the eluent composition, the pH-value of the eluent and the column temperature did not improve the separation on this column.

3.3. Extraction

Wang and Tsai [30] apply an extraction with chloroform to isolate the interfering biological matrix (urine, blood, faeces). Then the chloroform extract is evaporated and the residue is solved in the eluent. After the extraction however, interfering peaks still exist both with the UV- and the ECD-determination. Moreover, there is no data about the extraction yield of PPD and its metabolites. Stambouli et al. [18] also apply chloroform extraction as a sample preparation step for the GC–MS analysis and gain a recovery rate of 85% for PPD (0.1 mg/l). According to these examinations various polar organic solvents have been tested with respect to the isolation of the matrix and the determination of the extraction yield of the analytes. Since an injection of the organic solvent in the aqueous HPLC eluent is impossible, an aliquot of the organic solvent was evaporated, the residue was dissolved in the eluent and quantified by means of HPLC using the Nucleosil column. Table 2 represents the extraction yields for PPD, MAPPD, and DAPPD with different solvents. The recovery rates are below 50% for all 3 substances with all solvents except MIBK. Relatively high recovery rates (51% and 68% respectively) were achieved with MIBK for DAPPD. Except for chloroform, for nearly all extractants used the recovery rates increase from PPD via MAPPD to DAPPD which reflects the decreasing polarity of the molecules. The best recovery rates are gained with chloroform for PPD and with MIBK for MAPPD and DAPPD. Nevertheless, the interfering components are not completely cut off by the extraction. Due to the combination of errors belonging to several sample preparation steps, i.e. analyte extraction and volume reduction of the extracts, with chromatographic interferences, the standard deviations for the quantification of PPD and MAPPD regularly exceed 10% and mount up to 30% partly. Because of the described drawbacks of this analytical procedure it does not meet the required analytical sensitivity and accuracy.

3.4. Ion-pair reagents

Wang and Tsai [30] add the ion-pair reagent octyl ammonium phosphate to the eluent thus improving the separation of the analytes among themselves and from the matrix. Since octyl ammonium phosphate was proven not to be suited for our analytical task, we selected dodecylsulphate as ion-pair reagent. The addition of longer chain ion-pair reagents changes the elution sequence of the analytes and increases the PPD retention specifically (Fig. 2). The

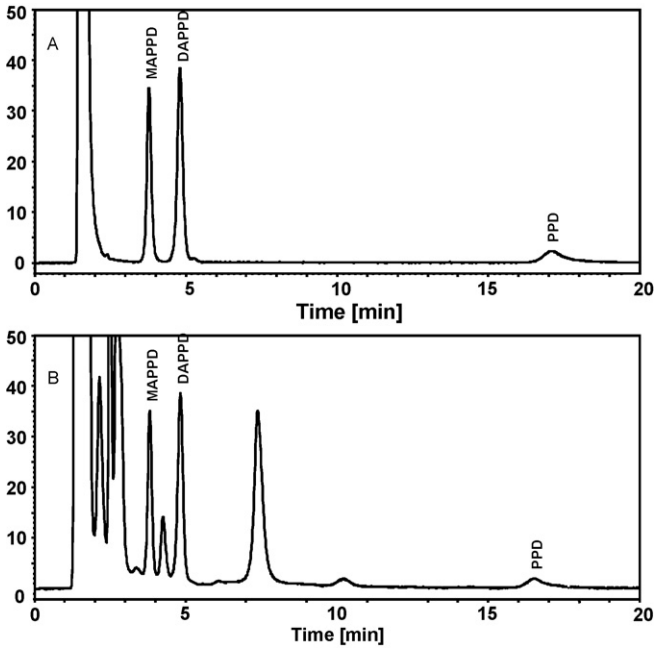

Fig. 2. Separation of PPD, MAPPD and DAPPD (10 μM each) using ion-pair reagents without (A) and with matrix (B). Nucleosil column; eluent: 25 mM ammonium acetate/acetonitrile (90:10) + 1 mM dodecylsulphate; flow: 0.7 ml/min; UV-detection: 255 nm.

retention time of DAPPD is reduced with the concentration of the ion-pair reagent rising. On the contrary, MAPPD elutes from the column only a little sooner (Table 3). Heptane- and octanesulphonic acid are inappropriate because they yield asymmetric peaks under the chosen chromatographic conditions. Fig. 2 illustrates that the peaks of the analytes are symmetric and easily integrable when dodecylsulphate is used. An optimal separation is achieved with a concentration of 1 mM of dodecylsulphate. However, the simultaneous prolongation of the retention times of the interfering peaks as a result of the addition of ion-pair reagents is unfavourable and might be based upon the ionic features of these compounds and upon an ion-pair formation with the reagent. This leads to interferences with the analytes. As a consequence, a baseline resolved separation between DAPPD and MAPPD and the matrix components was not attained, although the type of ion-pair reagent, its concentration, the composition of the eluents and the column temperature were varied. Furthermore, the equilibration and reconditioning of the separation system after every turn is time consuming. The interaction of the biological matrix and the ion-pair reagent causes considerably varying retention times of the analytes since the ion-pair concentration is decreased by interfering substances (>10% standard deviation for PPD). As shown in Fig. 2B, the retention time of PPD is reduced by 1 min compared to the absence of matrix compounds. The retention time varies and depends on the concentration of the analyte and the matrix components in the sample.

Table 3
Retention times of PPD, MAPPD and DAPPD depending on the dodecylsulphate concentration.

Dodecylsulphate concentration [mM]	Retention time [min]		
	PPD	MAPPD	DAPPD
0	4.1	8.4	31.5
0.2	10.9	7.6	23.6
1.0	28.7	5.1	7.2
5.0	24.4	3.7	4.2

Chromatographic conditions: Nucleosil column; eluent: 25 mM ammonium acetate/acetonitrile (95:5); flow: 1.1 ml/min; UV-detection: 255 nm.

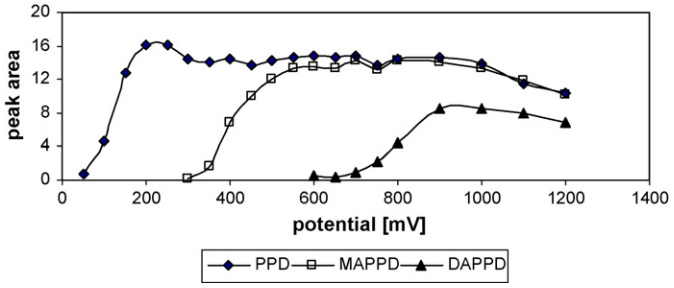

Fig. 3. Hydrodynamic voltammograms of PPD, MAPPD and DAPPD. AQUA column; eluent: 25 mM ammonium acetate (pH 8)/acetonitrile (95:5); flow: 0.7 ml/min; 10 μM analyte respectively.

3.5. AQUA-column in combination with DAD and ECD

In order to improve the separation the AQUA column is used for further investigations. It achieves a better separation for very polar compounds in combination with predominantly aqueous eluents [36]. Varying the composition of the eluent, the pH-value of the eluent, and the column temperature leads to a complete chromatographic resolution of the sample components. Due to the elevation of the pH-value from 6.8 to 8.0 the retention times of the three analytes rise a little. Thus, the separation is slightly improved in comparison to the Nucleosil column. In contrast to the use of the Nucleosil column the use of the AQUA column in this application also improves the separation of the polar components PPD and MAPPD from the matrix. The separation of MAPPD and the matrix peak makes this obvious (Fig. 1B, Fig. 4C). What is more, both chromatograms show a better separation of PPD from the matrix using the AQUA column. With falling column temperatures PPD elutes later. Compared to the Nucleosil column, the better separation from the matrix improves the limit of determination to 1 μM for PPD.

PPD and both metabolites can be oxidized on solid electrodes in the range from 0 to 1200 mV [30,37] representing the basis for the use of an amperometric detector. In comparison to UV detection the limits of determination for PPD could be improved by the application of this detector [25,30]. Additionally, Wang and Tsai [30] used an ion-pair reagent to improve the separation of PPD and of its metabolites. Taking into consideration the best conditions of separation for the analytes achieved so far, the potential advantage of the use of an amperometric detector was investigated. Fig. 3 shows the hydrodynamic voltammogram of the three analytes. All analytes can be sensitively detected at potentials of more than 1000 mV. A potential of 250 mV is sufficient to determine PPD, and a potential of 400 mV is needed to simultaneously determine PPD and MAPPD. A decreasing potential also entails less influence of the matrix, as some of the matrix peaks cannot be detected under these conditions due to their lower oxidation potential. The comparison of the matrix peaks recorded with UV- and ECD-detection in Fig. 4A and B reveals the advantage of the electrochemical detection. Under that aspect a potential of 400 mV seems to be optimal for the detection. Here, PPD and MAPPD are still oxidized and the interfering influence of the matrix is marginal. By means of the combination of both detectors DAPPD, which is not influenced by the matrix, can be determined with the more insensitive UV detection. The chromatograms in Fig. 4 illustrate this. Monitored with UV detection, matrix peaks interfere with PPD and MAPPD as shown in Fig. 4C. An interfering peak elutes together with the PPD peak independently from the chosen chromatographic conditions. Due to its electrochemical features this interfering peak is missing in the ECD-chromatogram (Fig. 4B and D). The same applies to the matrix peak coeluting with MAPPD. The combination of both detection systems enables the sensitive and simultaneous determination of PPD and MAPPD by way of amperometric detection at 400 mV and of DAPPD by way of UV detection at 240 nm (Fig. 5).

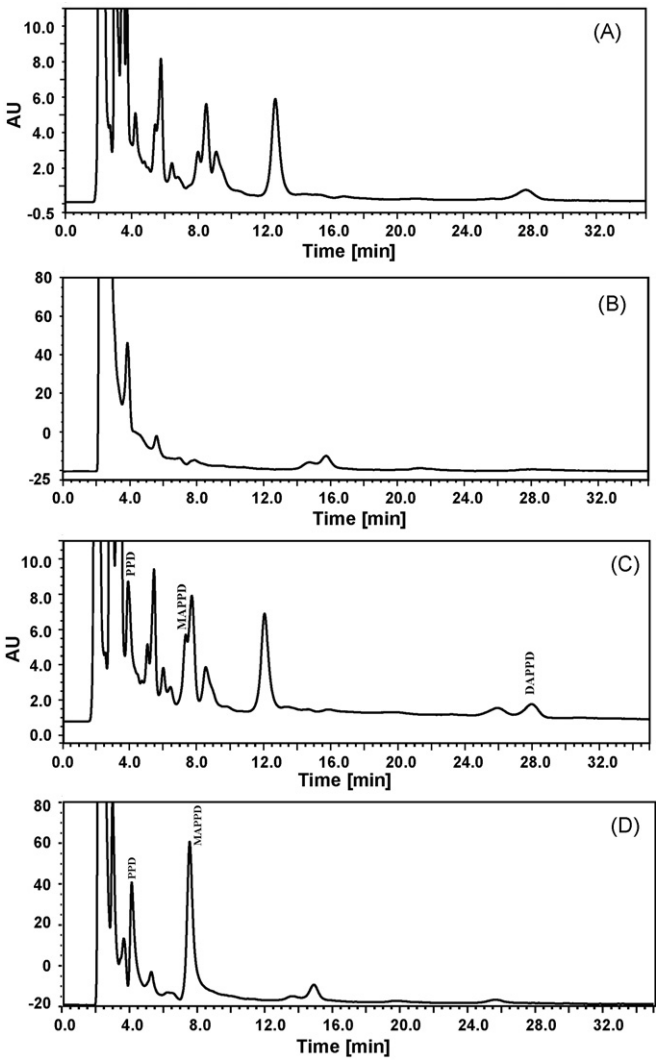

Fig. 4. Comparison of UV- and ECD-detection. (A) Matrix: UV detection at 240 nm, (B) matrix: ECD at +400 mV, (C) matrix + 5 μM PPD, MAPPD and DAPPD each; UV-detection at 240 nm, and (D) matrix + 5 μM PPD, MAPPD and DAPPD each; ECD at + 400 mV. AQUA column; eluent: 25 mM ammonium acetate (pH 8)/acetonitrile (95:5); flow: 0.7 ml/min.

With analyte concentrations ranging from 0.05 to 50 μM, limits of detection and of determination were determined for the elaborated analytical method. Limits of determination of 0.5 μM for PPD and MAPPD and 1 μM for DAPPD respectively were achieved with the isocratic elution technique. Standard deviations lower than 7.5% ($N = 9$; 1 μM) indicate a good data reproducibility (Table 4).

Table 4
Limits of matrix-specific detection and of determination for PPD, MAPPD and DAPPD, separated on the AQUA-column.

	ECD		UV
	PPD	MAPPD	DAPPD
Regression coefficient R^2	0.9987	0.9998	0.9993
Limit of detection [μM]	0.17	0.17	0.28
Limit of determination [μM]	0.61	0.64	0.99
Standard deviation [%]; 1 μM ($N = 9$)	7.4	4.4	3.1

Chromatographic conditions: AQUA column; eluent: 100 mM ammonium acetate/acetonitrile (92:8); flow: 0.7 ml/min; UV-detection: 240 nm (DAPPD); ECD: +400 mV; linear range: 0.05–20 μM.

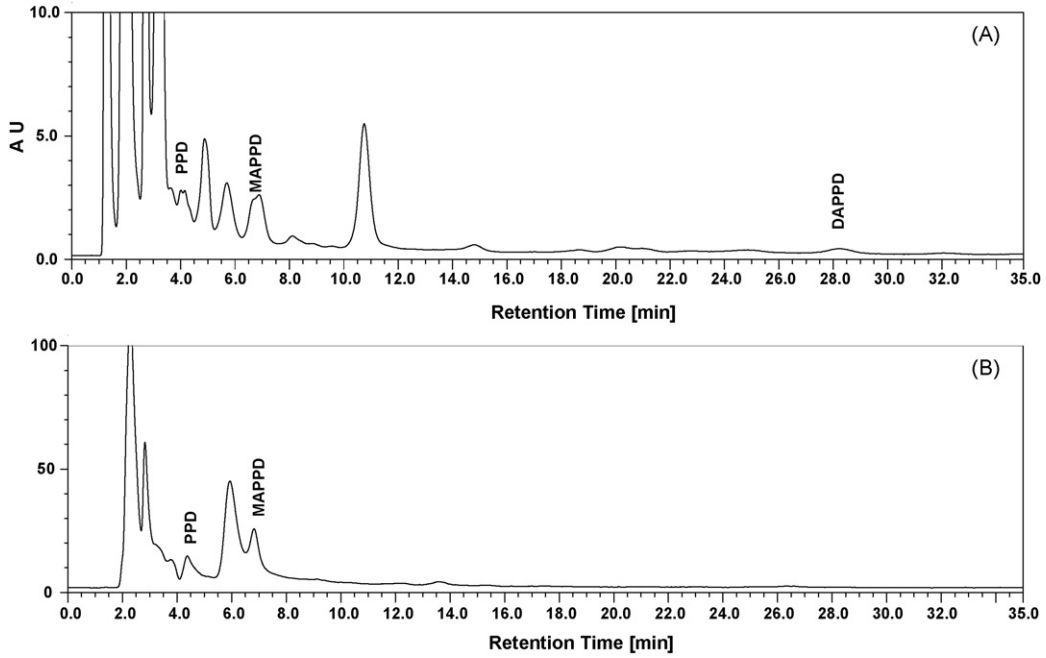

Fig. 5. Determination of PPD (1.2 μM), MAPPD (1.7 μM) and DAPPD (0.72 μM) in fresh peripheral mononuclear cells after addition of 50 μM PPD and 6 days incubation [38]. AQUA column; eluent: 25 mM ammonium acetate (pH 8)/acetonitrile (95:5); flow: 0.7 ml/min; UV-detection: 240 nm (A); ECD: +400 mV (B).

3.6. Determination of PPD and its metabolites in real samples

The method presented above is supposed to serve for the quantitative determination of PPD and both of its acetylated metabolites in biological media. Our investigations are orientated on toxicological questions. In different cell media different PPD concentrations are exposed to different cell lines and the time-dependent formation of acetylated products as well as the transformation degree of PPD have to be determined. For this purpose, some preliminary tests were made in the medium DMEM to document the applicability of the method defined above especially. Over different periods different PPD concentrations (20–100 μM) were exposed to different cell lines. Afterwards, the contents of PPD and its metabolites are determined in the different batches. The contents of PPD in the different samples ranged between 0.25 and 20 μM. For MAPPD and DAPPD amounts between 0.5 and 5 μM could be determined. Due to the small sample volume of 0.5 ml the samples could not be injected more often than three times. Calculated on the basis of triplicate injections, the standard deviation of the peak areas was less than 10% for PPD (analytical concentration about 0.5 μM) and less than 5% for MAPPD (about 1 μM). This shows clearly that the developed isocratic chromatographic method applied to simultaneously analyze PPD and its acetylated metabolites has the acquired accuracy, provided by the serial hyphenation of an UV/vis- with an ECD detection system.

A few tests were exemplarily carried out in a different biological medium. Even in this medium the advantage of the amperometric detection is obvious. Again matrix signals impede an accurate integration of the PPD UV-absorption signal at 4 min. At 7 min MAPPD is not separated either. On the contrary, in the ECD chromatogram both analytes can be quantified without interferences. DAPPD can be determined via UV detection. Even in these samples limits of determination lower than 0.75 μM for PPD and MAPPD and 1 μM for DAPPD could be established. As for the reproducibility, there are no differences between the media. These tests confirm that the elaborated method is suited to sensitively and selectively determine PPD and its acetylated metabolites in various biological media and experimental set-ups which are of great importance for toxicological studies.

4. Conclusion

The study presented above describes and validates a HPLC-UV-ECD-method for the simultaneous determination of PPD, MAPPD and DAPPD in different cell lysates. The method proved to be linear in the concentration range studied as well as accurate, precise and selective using ECD detection. The sensitivity of the described analytical procedure is better than the published results up to now and the method can be used without sample preparation. The sensitivity and the direct injection of the samples without sample pretreatment and enrichment step are advantageous because in most of the investigated assays only a sampling volume of 0.5 ml or less is available. The interfering influence of the different lysate compositions can be eliminated using the amperometric detector, which is connected in series to the UV-detector.

The presented method can be utilized for the determination of PPD, MAPPD and DAPPD in different samples to study the influence of various cell lines in different biological media for the time-dependent acetylation of PPD.

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