

Real-time detection of *p*-phenylenediamine penetration into human skin by *in vivo* Raman spectroscopy

Laura Marjolijn Pot¹, Pieter-Jan Coenraads¹, Brunhilde Blömeke², Gerwin J. Puppels^{3,4} and Peter J. Caspers^{3,4}

¹Department of Dermatology, University Medical Centre Groningen, University of Groningen, 9700 RB, Groningen, The Netherlands, ²Department of Environmental Toxicology, University of Trier, D-54296, Trier, Germany, ³Department of Dermatology, Centre for Optical Diagnostics and Therapy, Erasmus MC, 3015 CE, Rotterdam, The Netherlands, and ⁴RiverD International B.V., 3029 AK, Rotterdam, The Netherlands

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Summary

Background. Penetration, autoxidation and *N*-acetylation of *p*-phenylenediamine (PPD) have been studied *in vitro* and *ex vivo*. However, a clear understanding of *in vivo* PPD penetration and the formation of PPD derivatives is lacking.

Objectives. To obtain insights into the *in vivo* penetration, clearance and formation of PPD derivatives in human skin.

Methods. Patch test chambers containing PPD 1% pet. were applied to the forearms of two human volunteers, with increasing application times. Non-invasive Raman microspectroscopy was used for detection of PPD (derivatives) in skin at several follow-up times.

Results. Application of a PPD 1% pet. patch for 30 min resulted in substantial amounts of PPD in the stratum corneum of 90 mg PPD/g keratin. PPD contents were highest after three applications for 1 h each (330 mg PPD/g keratin), followed by single applications for 2 h 40 min, 2 h, and 23 h. The PPD half-time in the skin was 3 h. No spectral contributions of Bandrowski's base, monoacetyl-PPD and diacetyl-PPD were detected.

Conclusions. We have gained insights into the *in vivo* penetration of PPD in human skin by using non-invasive Raman spectroscopy. Penetration into the skin was fast, and the PPD concentrations detected in the stratum corneum were high. PPD was detected in both the stratum corneum and the viable epidermis. Oxidized or acetylated PPD derivatives could not be detected.

Key words: *in vivo*; *p*-phenylenediamine (PPD); penetration; Raman spectroscopy; skin.

p-Phenylenediamine (PPD) is intrinsically unstable, and under air exposure or in solution it is susceptible to autoxidation and conjugation reactions. The

highly reactive primary oxidation product of PPD, *p*-benzoquinonediimine (PBQD), is proposed to undergo oxido-conjugation reactions, to form dimers, the trimer Bandrowski's base (BB), or even pentamers (1). In addition, several studies have shown that PPD is metabolized within the skin into monoacetyl-PPD (MAPPD) and diacetyl-PPD (DAPPD) by the enzyme *N*-acetyltransferase 1 (2). In the local lymph node assay, these acetylation products have been shown to be non-sensitizing (3) and are therefore considered to be detoxified products of PPD, whereas BB is classified as an extreme sensitizer (2, 4). Nevertheless, an *in vivo* human elicitation study (4) has suggested that BB may not play an important role in

Correspondence: Laura Marjolijn Pot, Department of Dermatology, University Medical Centre Groningen, University of Groningen, Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands. Tel: +31 50 361 1481; Fax: +31 50 361 9247. E-mail: l.m.pot@umcg.nl

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human sensitization to hair dyes. Furthermore, probably not PPD itself, but its reactive primary oxidation product PBQD, which is able to covalently bind to proteins and thereby to serve as hapten–protein complex, is the key player (5–7).

Despite all the experimental knowledge about PPD and the formation of its oxidation and acetylation products, a clear *in vivo* picture of how fast and in what concentrations PPD penetrates into the skin, and of where and when these products are formed, is lacking. Although *ex vivo* penetration experiments have quantitatively studied the recovery of topically applied [^{14}C]PPD in a hair dye formulation in the different skin layers, no distinction was made between PPD, MAPPD, DAPPD, BB, or other oxidation products (8–11).

Non-invasive confocal Raman microspectroscopy (CRM) can be used to study the real-time penetration of a topically applied chemical *in vivo* (12). Briefly, CRM is an optical method in which a monochromatic laser light is focused onto the sample of interest, thereby exciting molecular vibrations and causing inelastic scattering of the light, called Raman scattering. This scattered light has frequencies different from that of the incident light. The frequency shift is dependent on atomic mass, chemical bonds, and molecular structure, and the Raman spectrum is therefore highly molecule-specific. Consequently, a Raman spectrum represents a 'fingerprint' or signature by which a molecule can be both identified and quantified. The Raman spectrum of skin represents the sum of all Raman signals from the molecules present in the skin, and thus provides a detailed picture of its molecular composition (12). Once the Raman signature of a particular chemical has been obtained, and a sufficient amount has penetrated the skin after topical application, the chemical can be identified and traced *in vivo* within the skin. This method has been used to study the epidermal penetration of topical drugs (metronidazole and *trans*-retinol) and various other chemicals, including 2-butoxyethanol and toluene (13–15).

This study was designed to determine: (i) the penetration characteristics of topically applied PPD as a function of depth in the skin and as a function of time; and (ii) whether oxidation (BB) or acetylation (MAPPD or DAPPD) products can be detected within the skin after topical application of PPD.

Materials and Methods

Sample preparation

PPD (CAS no. 160-50-3), MAPPD (CAS no. 122-80-5), DAPPD (CAS no. 140-50-1), dimethyl sulfoxide (DMSO)

(CAS no. 67-68-5) and bovine serum albumin (BSA) (CAS no. 9048-46-8) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). BB (CAS no. 20048-27-5) was purchased from Apollo Scientific Ltd (Stockport, UK). For determination of their reference spectra in solution, PPD (1%) and MAPPD (1%) were dissolved in water, and DAPPD (2%) and BB (1%) were dissolved in DMSO. For spectral analysis, the spectra of the solvents were subtracted. For penetration studies, PPD 1% pet. and blank petrolatum were used (Trolab-Almirall Hermal, Reinbeck, Germany). Difference spectra were created by subtracting a spectrum of untreated skin from that of skin after PPD patch application, to remove the Raman signals that originate from the untreated skin and uncover the signals of PPD and PPD derivatives, if present. For quantitative calibration of the PPD concentration, solutions were prepared of 0.5–2.0% wt/wt PPD and of 0.5–5% wt/wt BSA in demineralized water.

Volunteers

In vivo Raman spectra were obtained from two volunteers (one 30-year-old female and one 43-year-old male, without a history of PPD sensitization). The study was approved by the local ethics committee of the University Medical Centre Groningen, protocol number NL36149.042.11. The volunteers gave informed consent prior to participation. Experiments were conducted at the Department of Dermatology of the Erasmus MC, Rotterdam, The Netherlands.

Raman experiments

Experiments were performed with the Model 3510 Skin Composition Analyser (RiverD International B.V., Rotterdam, The Netherlands) with 785-nm laser excitation, 25-mW laser power on the skin, and a spatial depth resolution of 5 μm . For reference spectra, $\sim 20\ \mu\text{l}$ of the sample solution was pipetted into the cavity of a socket head cap screw and placed on the window. Pet. was directly applied onto the fused silica window. Signal collection times (up to 300 seconds) were chosen to obtain optimal signal quality (16). For *in vivo* experiments, $\sim 20\ \text{mg}$ of the PPD 1% pet. formulation (Trolab[®]) was spread evenly on a van der Bend[™] square patch test chamber covering a surface area of 0.64 cm^2 (van der Bend, Brielle, The Netherlands), and applied to the volar forearm of the subject with the associated tape. For each experiment, a patch was freshly prepared as described. These patches will be referred to as 'PPD 1% pet. patches'. A patch containing $\sim 20\ \text{mg}$ pet. served as a control. After removal of the patch, the skin was wiped once with a tissue, and the imprint of the

Table 1. Overview of Raman measurements performed as shown in Figs 3 and 4

Figure	3	4
Patch application time	0.5 h	3 × 1 h
	2 h	
	2 h 40 min	
	3 × 1 h	
	23 h	
Number of volunteers	1–2	1–2
Start of measurements per experiment at time after removal of the patch	Immediately (< 5 min)	0–0.5 h
		0.5–1 h
		3–3.5 h
		6–6.5 h
		27 h
Depth (μm)	0–32	0–36

patch test square was marked at the borders. The marked location was placed on the fused silica window of the Raman device. By automatically moving the objective in the vertical position, the depth of the laser focus in the skin (Z) could be varied accurately. Raman profiles were recorded from Z = 0 μm (skin surface) to a maximum of Z = 36 μm (viable epidermis), with increments of 2–4 μm and a signal collection time of 5 seconds per single spectrum.

Experiment 1: penetration of PPD as a function of application time. PPD 1% pet. patches were applied on the forearm, and left on for different application times, ranging from 30 min to 23 h. To supply an infinite dose of PPD, a PPD 1% pet. patch was applied on the forearm for 3 h, during which the patch was replaced by a fresh patch every hour (indicated as '3 × 1 h'), without wiping in between. Directly after removal of the patch, the skin was wiped clean, and the distribution of PPD across the stratum corneum was measured by Raman spectroscopy. For all other application times, a single patch was applied (Table 1).

Experiment 2: clearance of PPD as a function of time. Clearance was assessed by repeated Raman measurements after removal of the PPD 1% pet. patch, which had been applied for 3 × 1 h. Raman measurements were performed 0–6.5 h after removal of the patch, and once more after 27 h (Table 1). For each time interval, all concentration profiles of PPD content versus depth were averaged, and the area under the curve (AUC) from 0 to 20 μm below the skin surface was calculated with a standard trapezoid integration. The PPD clearance time (half-time) was determined as the time point at which the AUC had decreased by 50%.

Experiment 3: detection of oxidation and/or acetylation products of PPD. In a separate experiment, Raman spectra were recorded for a prolonged time (up to 60 min) at a fixed depth of 30 μm below the skin surface to maximize the total signal collection time per spectrum at a given depth. This depth is in the viable epidermis, which is the expected location of the enzymatic acetylation of PPD (16). In addition to these fixed-depth measurements, Raman spectra obtained in experiment 2 were examined for the presence of BB, MAPPD or DAPPD and pet.

Data analysis

The Raman instrument was calibrated as described in the User Manual of model 3510 SCA. Briefly, the spectrum of a neon–argon lamp in combination with an internal Raman standard was used to calibrate the spectrometer wave-number axis, and the NIST SRM 2241 Reference Material was used for the relative intensity correction of the spectrometer. Instrument background signals were subtracted from each measured spectrum. All spectra collected were analysed with SKINTOOLS™ 2.0 analysis software (RiverD International BV). The analysis consisted of fitting each spectrum collected from the skin with a fitting model based on the endogenous components of the skin (12). Briefly, this model uses a classic least squares fit to determine the contribution of each of the reference skin components to the *in vivo* spectrum collected from the skin. Reference spectra of PPD, blank pet., BB, MAPPD and DAPPD were added to this fit model. This allows the generation of depth profiles of each added component and the reference skin component to be obtained. In CRM, the collected signal decreases with deeper probing into the tissue, owing to the loss of light by scattering. To compensate for this effect, all fit coefficients were normalized on the keratin signal as an intrinsic standard, as keratin is the dominant protein and represents the majority of the dry weight of the stratum corneum. The intensity of the PPD Raman signal relative to the intensity of the keratin Raman signal was calibrated in order to determine the PPD content in the skin in mg/g keratin. For this calibration, Raman spectra of aqueous solutions of BSA (0.5–5% wt/wt) and of PPD (0.5–2%) were measured and normalized on the intensity of the water signal. BSA was used because it is highly water-soluble and because the Raman spectrum of BSA and the intensity of the Raman signal are very similar to those of keratin (12). The normalized Raman spectra from the PPD and BSA solutions were then used to calculate the scale factors of the fit model spectra of PPD and of keratin, such that the ratio between the fit coefficients of PPD and keratin represents the mass ratio of PPD to keratin. In this way, the

PPD content, as calculated from the classic least squares fit, was expressed in mg PPD/g keratin. The limit of detection for PPD was defined as the calculated PPD content after application of a pet. patch without PPD. This residual value represents the error of the classic least squares fit model.

Results

The reference spectra of PPD, MAPPD, DAPPD and BB after subtraction of the Raman spectrum of the respective solvent are shown in Fig. 1. Spectra for pet. and untreated stratum corneum are also shown. PPD shows four characteristic Raman bands that clearly separate PPD from the endogenous skin constituents (Fig. 1, a–d).

Detection of PPD in the stratum corneum

A PPD 1% pet. patch was applied to the skin of the volar forearm for 2 h 40 min. The untreated adjacent skin served as a control. The PPD signal can be clearly distinguished in the *in vivo* Raman spectrum of skin (Fig. 2). Shown are a spectrum of untreated control skin and a spectrum of skin after application of a PPD 1% pet. patch. The four characteristic Raman bands of PPD were clearly discernible, indicating the presence of PPD within the stratum corneum. No signals for the autoxidation product BB or other PPD derivatives were detected within the stratum corneum.

Detection of PPD and its derivatives in the living epidermis

Different application times were used to investigate the influence of application time on the identified signals of PPD and the subsequent formation of PPD derivatives in the living epidermis. In Fig. 3, the amount of PPD (mg PPD/g keratin) is plotted against depth. The coloured graphs represent the signals for different application times (30 min, 2 h, 2 h 40 min, 3 × 1 h, and 23 h) of the PPD 1% pet. patch. Approximately 5–10 repeated profiles were measured in one volunteer. Directly after removal of the patch, the relative amounts of PPD in the skin were highest after repeated application of 3 × 1 h (330 mg PPD/g keratin), followed by single applications for 2 h 40 min and 2 h; the lowest amount was found after an application time of 23 h. These differences became less apparent deeper in the skin. There was no clear indication that PPD also penetrated deeper into the skin after a longer application time. After the 30-min application, substantial amounts of PPD (~70 mg PPD/g keratin at a depth of 4 µm) were already detectable in the skin, and remained detectable to ~20 µm below the skin surface, where the

PPD content reached its lower detection limit of 13 mg PPD/g keratin (1.3 mass %). For readability purposes, the combined standard deviation (SD) (in one direction) is shown only for the upper graph. The SD of the remaining curves was similar.

PPD clearance as a function of time

PPD clearance was monitored after application of the 3 × 1 h patch. Follow-up measurements were performed at different times after removal of the patch (0–6.5 h). Figure 4 shows the PPD content (mg PPD/g keratin) as a function of distance to the skin surface. Each curve is the average of 5–20 profiles per volunteer. Evidently, the PPD content in the skin decreases with time, most clearly between depths of 0 and 10 µm. The PPD content approaches the baseline reading (black dashed line in Fig. 4) at ~20 µm below the skin surface. The SD is shown for the 0–0.5-h graph only. SDs for the other curves were comparable. None of the measurements detected pet. penetrating beyond the top few micrometres of the skin. On the basis of a 50% decrease in the AUC after application of the 3 × 1 h patch (AUC calculated from Fig. 4), the half-time of PPD was estimated to be 3 h.

Detection of *N*-acetylated PPD and its oxidation products

All spectra collected during the experiments were inspected for signals of BB, MAPPD, and DAPPD. Although PPD was clearly present, no characteristic Raman signals of these PPD derivatives could be detected in the skin. The exact location(s) of *N*-acetylation within the epidermis is (are) not known. On the basis of *in vitro* results obtained with non-differentiated keratinocytes (17), we expected to find *N*-acetylation in deeper layers of the viable epidermis. Measurements specifically aimed at the upper viable epidermis 30 µm below the skin surface showed small amounts of PPD. However, none of the reference spectra of BB, MAPPD and DAPPD could be distinguished. Thus, neither the stratum corneum nor the viable epidermis showed detectable amounts of BB, MAPPD, or DAPPD.

Discussion

In this study, we primarily aimed to obtain insights into the *in vivo* penetration and clearance of PPD in the human skin, as a function of time. As this is the first time in the literature that *in vivo* PPD penetration has been studied with CRM, we did not focus on interindividual differences or on differences between sensitized and non-sensitized subjects. Consequently, many measurements were performed

Fig. 1. Raman reference spectra of *p*-phenylenediamine (PPD), monoacetyl-PPD (MAPPD), diacetyl-PPD (DAPPD), Bandrowski's base (BB), petrolatum, and (one representative example of) the skin. The peaks characteristic of PPD that are distinguishable from skin signals can be found at (a) 470 cm^{-1} , (b) 648 cm^{-1} , (c) 844 cm^{-1} , and (d) 1622 cm^{-1} . The most distinguishing peaks of MAPPD [(e) 1180 cm^{-1} , (f) 1238 cm^{-1} , (g) 1330 cm^{-1} , and (h) 1616 cm^{-1}], DAPPD [(i) 1320 cm^{-1} , (j) 1615 cm^{-1} , and (k) 1686 cm^{-1}] and BB [(l) 1520 cm^{-1}] are also assigned. The asterixes around 650 cm^{-1} in the spectra of BB and DAPPD indicate an artefact resulting from the subtraction of the solvent spectrum (dimethyl sulfoxide).

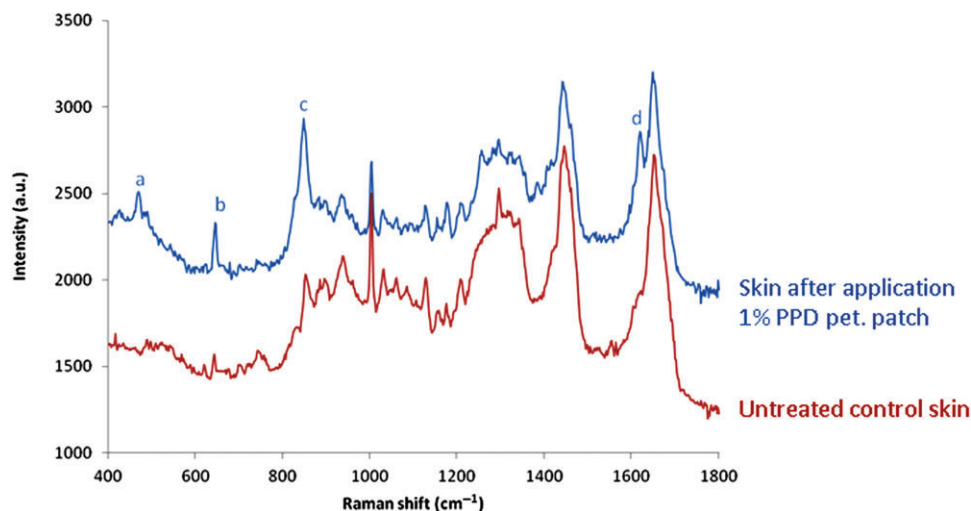
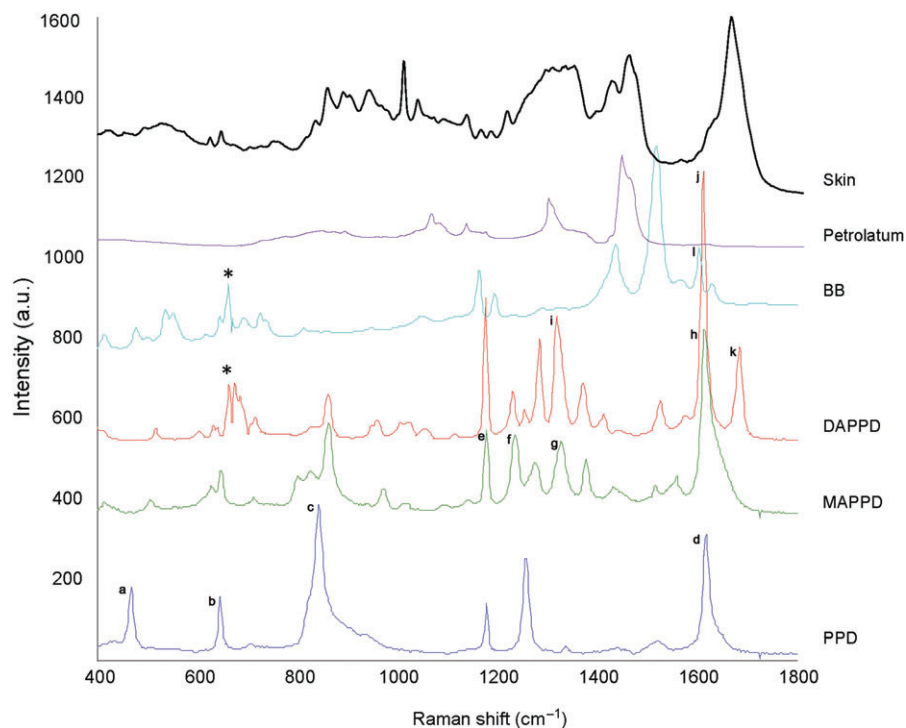


Fig. 2. Examples of Raman spectra of untreated control skin and skin treated with *p*-phenylenediamine (PPD) 1% pet. patch for 2 h 40 min, recorded at a depth of 8 μm . In comparison with the untreated skin, the spectrum of the treated skin contained peaks characteristic of PPD (a, b, c, and d). No peaks of pet. or the PPD derivatives were observed.

on many occasions, but in only a limited number of subjects (one or two volunteers). We have shown that measuring the penetration of PPD into living human skin is possible. Application of a PPD 1% pet. patch for 30 min showed that PPD penetrates the stratum corneum relatively quickly and in substantial amounts. This is consistent with data from dose–time relationship studies, in which even shorter application times (5 and 15 min) of a

PPD 1% pet. patch were sufficient to elicit allergic reactions in 16% and 38% of the PPD-sensitized subjects (18, 19), indicating that PPD had penetrated at least into the viable epidermis. Interestingly, application with a single patch for 23 h resulted in the lowest detected PPD content in the skin. This implies that the dose supplied with this single PPD patch was, in fact, finite, and that, after several hours (between 2 h 40 min and 23 h), clearance of

Fig. 3. PPD content in skin (mg/g keratin) immediately after removal (0–5 min) of *p*-phenylenediamine (PPD) 1% pet. patch, after different application times. *Measured in one volunteer. Each data point is an average of 5–10 repeated measurements on adjacent locations on the skin.

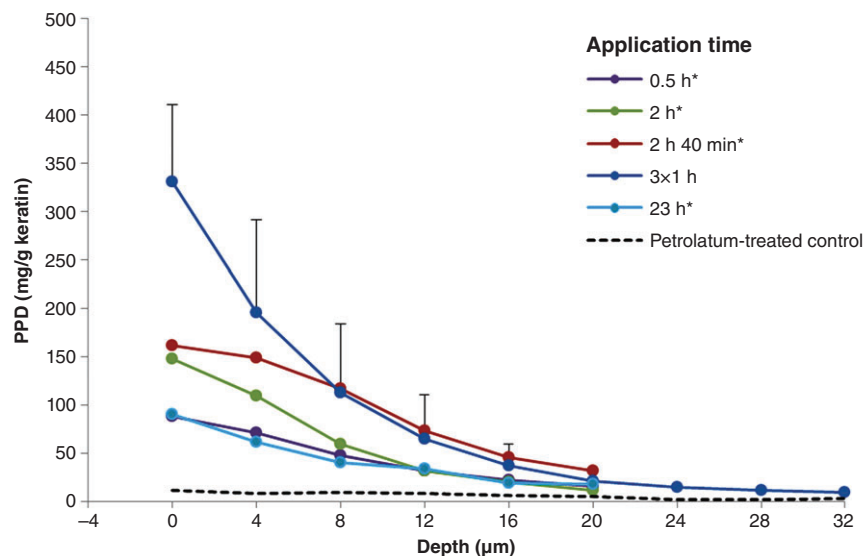
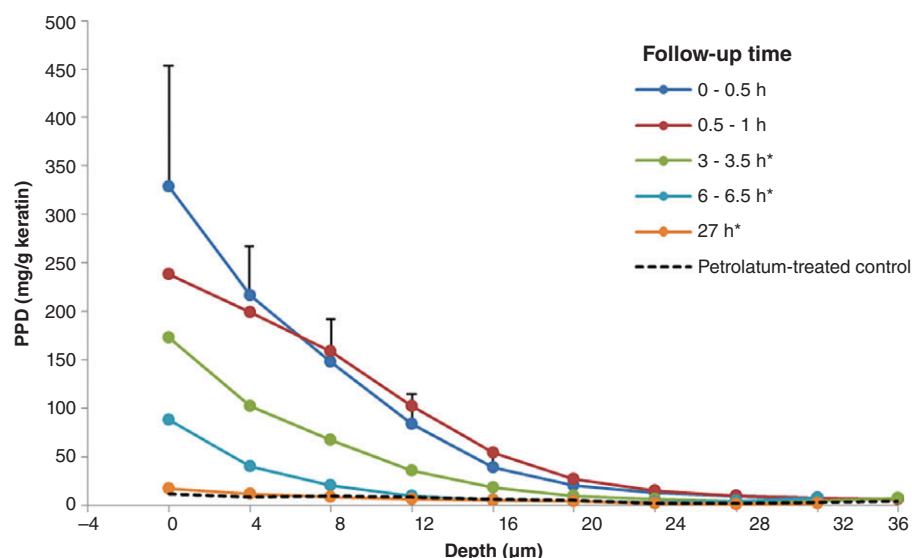


Fig. 4. *p*-Phenylenediamine (PPD) content in skin (mg/g keratin) at several time intervals after removal of PPD 1% pet. patch applied for 3 × 1 h. *Measured in one volunteer. For depth, 5–20 measurements were averaged.



PPD from the skin had become dominant over the supply of additional PPD from the patch. In terms of supply, this may call into question the gold standard of 48 h of application in diagnostic patch testing (20), although other general factors, such as the practicability and standardization of application time, may be much more decisive. Although the formation of possible sensitizing oxidized PPD derivatives in the patch and/or the skin over time is another interesting issue, this is beyond the scope of this study.

Figure 4 shows how the PPD content in the stratum corneum consistently decreases over time. Clearance of PPD from the stratum corneum by passive diffusion into the viable epidermis and deeper layers might be accompanied by disappearance of PPD by conversion

into the aforementioned PPD derivatives BB, MAPPD, and DAPPD. However, in this pilot study, we were not able to confirm this second route of clearance by conversion, as none of these PPD derivatives was detected in the skin. Various studies have shown the formation and presence of acetylated PPD derivatives in *in vitro* skin samples (reviewed by Pot et al.) (2). A possible explanation for the absence of these compounds in the current study is that formation of derivatives did not occur in concentrations above the limit of detection of the experimental design. In a study by Hu et al. (21), using human reconstructed epidermis, the concentration of PPD derivatives in skin (tissue homogenates) detected by liquid chromatography–mass spectrometry was also below the limit of detection. Hu et al. used an application

time of 30 min, a dose of 36.7 µg/cm² PPD, and a 23.5-h follow-up period.

Conclusions

We have monitored the *in vivo* penetration of PPD into the human skin by using Raman spectroscopy. A clear time-dependent and depth-dependent decrease in the local content of PPD in the stratum corneum could be shown. With a standard patch test application, the

detected PPD concentrations in the stratum corneum were remarkably high. PPD was detected both within the stratum corneum and in the viable epidermis. Within the design of our pilot study, oxidized or acetylated PPD derivatives were not detectable in the skin.

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