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Journal of Chromatography B, 826 (2005) 31–40
JOURNAL OF CHROMATOGRAPHY B
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A validated LC/MS/MS method for the quantification of pyrrole-2,3,5-tricarboxylic acid (PTCA), a eumelanin specific biomarker, in human skin punch biopsies

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Received 21 March 2005; accepted 4 August 2005
Available online 31 August 2005

Abstract

A novel skin tissue extraction method coupled with liquid chromatography-tandem mass spectrometry (LC/MS/MS) detection was developed and validated for the analysis of endogenous pyrrole-2,3,5-tricarboxylic acid (PTCA), a eumelanin specific biomarker, in human skin punch biopsies. The analyte is extracted from the matrix (2 mm skin punch biopsies) using a simple oxidative degradation procedure. The extract supernatants are evaporated, reconstituted in mobile phase solvent, and injected into the LC/MS/MS system without further derivatization. The chromatographic separation is achieved on a reverse phase high performance liquid chromatography (HPLC) column. The accuracy and precision of the method was determined over the concentration range of 1–1000 ng/mL PTCA from human skin extracts in three validation batch runs. Inter-assay precision (%CV) and accuracy (%R.E.) of the quality control samples were $\leq$18.5% (at lower limit quality control, LLQC) and $\leq$5.25%, respectively. The sensitivity and throughput of this assay is significantly improved relative to previously published methods resulting in much smaller tissue requirements and analysis time. This procedure could potentially be used in the investigation of therapies associated with skin pigmentation.

Keywords: Pyrrole-2,3,5-tricarboxylic acid; PTCA; Mass spectrometry; LC/MS/MS; Human skin; Pigmentation; Eumelanin; Melanin; Biomarker

1. Introduction

Melanins have attracted considerable clinical interest in dermatology because of their involvement in human pigmentation and related pathological and cosmetic conditions [1]. There are two major chemically distinct types of human melanin pigments, brown-black eumelanin and red-yellow pheomelanin [2–4]. Eumelanin is composed mainly of the monomer units 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, while pheomelanin is composed of benzothiazine units derived from cysteinyldopas [3]. Although these pigments are nominally classified by color, they are chemically distinct. Eumelanin is insoluble in both acidic and alkaline solutions, and contains nitrogen but no sulfur, while pheomelanin is soluble in alkaline solution, and possesses both nitrogen and sulfur [3]. Most melanins present in pigmented tissues appear as mixtures or copolymers of eumelanin and pheomelanin that combine in different proportions which tend to vary with ethnicity, photoexposure, or dermatological disorders [4,5].

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doi:10.1016/j.jchromb.2005.08.002

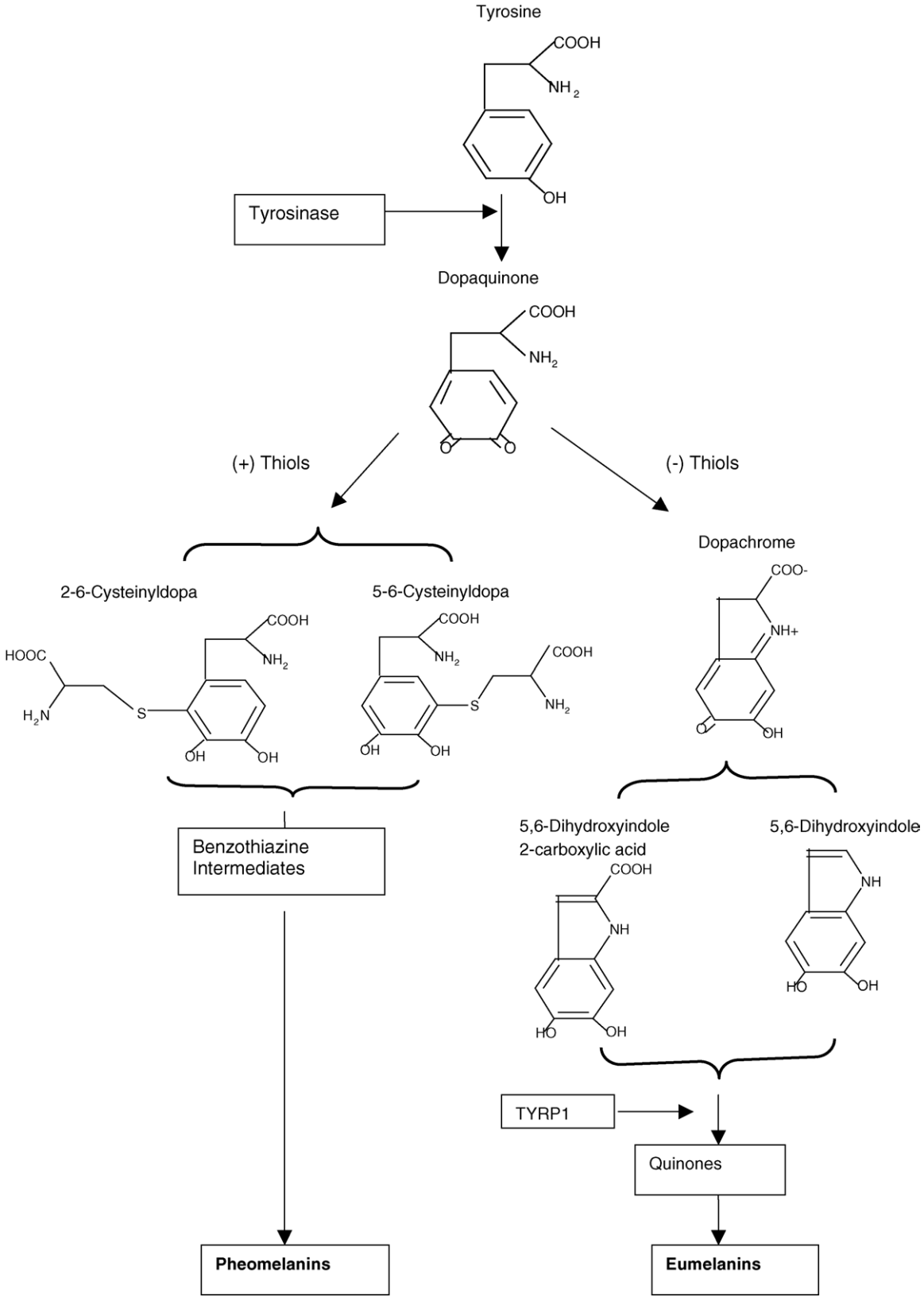

Fig. 1. Biosynthetic pathway for the formation of eumelanins and pheomelanins.

In human skin, eumelanin and pheomelanin are produced in the basal layer of the epidermis by neural crest derived melanocytes, packed in cytoplasmic melanosomes, and transferred to neighboring epithelial keratinocytes [2,3,6–9]. Melanogenesis of these two pigments is initiated with the common rate limiting step of L-tyrosine hydroxylation to dopaquinone catalyzed by the copper containing enzyme tyrosinase [2,4,9–12]. Following this step, however, their synthetic pathways diverge (Fig. 1). Dopaquinone, in the absence of thiols, undergoes intermolecular cyclization to form dopachrome and the eumelanin intermediates 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). These dihydroxyindoles are then converted to their respective quinone derivatives and undergo oxidative polymerization to eumelanin via tyrosinase related protein 1 (Tyrp1). However, with the intervention of thiols such as glutathione and cysteine, and the activation of glutathione reductase and gamma-glutamyl transpeptidase, further oxidation leads to pheomelanin production via cysteinyldopa and benzothiazine intermediates [4–6,12].

While clinical trials for pigmentation disorders have historically been evaluated by visual assessment, inter-observer bias limits accuracy and reproducibility [13]. Portable instruments that measure the ratio of reflected light to incidental light, such as reflectance spectrometers and tristimulus colorimeters, are increasingly being used by dermatologists for the quantitative measurement of melanin [14]. These instruments can provide non-invasive information for the melanin content of human skin in vivo [7]. However, there are several factors that can potentially influence the accuracy of these measurements such as skin irritation, redness and changes in skin blood flow. Also, since measured values for reflectance and skin color do not exist in absolute terms, but are conditional upon the unique characteristics of a particular instrument's aperture, different instruments have been reported to give different measurements from the same skin region resulting in poor inter-assay accuracy. In addition, these direct measures of skin pigmentation are used to define clinical outcome but do not quantitatively describe modulation of the proposed mechanistic pathway.

Previous attempts to quantify melanin in human skin by direct analysis have met with little success owing to melanin's relative insolubility over a broad pH range, lack of well defined physio-chemical properties, and susceptibility to structural alteration via radical isolation procedures [1,3–5]. Another obstacle to the quantitative isolation of melanin from natural matrices is its strong binding affinity to protein components [11]. Various histological methods used for melanin determination, such as the Fontana-Masson staining method, or immunohistochemical staining for melanocytes, provide relatively subjective quantification due to the difficulty in discriminating small differences [15].

Techniques to quantify the degradation products of melanin in biological samples after chemical oxidation, like those developed by Ito et al, have become popular with researchers because they can be used as a measurement of melanin content while making its isolation unnecessary [2–4,12]. These methods are based on the formation of monomers such as pyrrole-2,3,5-tricarboxylic acid (PTCA), a quantitatively significant indicator of eumelanin, followed by HPLC or spectrophotometric analysis. Typical oxidizing reagents employed in these procedures are potassium permanganate or alkaline hydrogen peroxide solutions. These methods, however, possess several disadvantages from an analytical and clinical perspective. First, they have relatively high limits of detection, up to 2 μg, impacting required clinical skin biopsy size. Second, the selectivity of these methods is based only on the HPLC retention time and UV absorption of PTCA. In a complex biological sample, such as a skin extract, accurate quantification and reproducibility are limited. Finally, the throughput of some published assays, as low as 20 samples in 3 working days, limits their application to smaller clinical studies.

To improve upon these analytical limitations, we have developed and validated a novel and objective method for quantitatively analyzing eumelanin in biological samples by LC/MS/MS. This assay comprises the oxidation of eumelanin in human skin punch biopsies using ammonium hydroxide and hydrogen peroxide, followed by tandem mass spectrometry analysis in the selected reaction monitoring (SRM) mode with electrospray ionization, to measure the concentration of PTCA. Eumelanin content can then be calculated based upon the corresponding PTCA yield of the extraction method [3]. The limit of detection has been improved by up to three orders of magnitude compared to other published methodologies, significantly decreasing the amount of tissue required for analysis. This clinically applicable, rapid, and reliable method provides a high degree of accuracy and precision over a linear quantification range from 1 to 1000 ng of PTCA per milliliter of sample extract.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade water, methanol (anhydrous), and ammonium hydroxide were obtained from Mallinkrodt (Paris, Kentucky). Ethanol was from AAPER (Shelbyville, Kentucky). Ammonium acetate (F.W. 77.09) was from EM Science (Gibbstown, New Jersey). Hydrogen sulfite was from Riedel-de Haen (Seelze, Germany). The internal standard (3-nitro-tyrosine) and hydrogen peroxide (30%) were from Sigma–Aldrich (St. Louis, Missouri). Pyrrole-2,3,5-tricarboxylic acid (PTCA) was synthesized at Pfizer Global R&D (Ann Arbor, Michigan). The purity of the synthesized PTCA was verified by HPLC, MS, and NMR elemental analysis. Results indicated that the compound was at least 98% pure. A stock solution prepared in water was determined to be stable at least 4 h at room temperature. Long term storage

stability of at least 3 months when stored at −20 °C was also confirmed.

All other solvents were ordered from commercial sources with the highest purity grades and used without further processing. A standard amino acid mixture for assay selectivity evaluation was obtained from Aldrich (St. Louis, Missouri) (L-amino-acids and Glycine, kit, cat. #29,846-8, contains: proline, tryptophan, cystine, serine, hydroxy proline, isoleucine, lysine, leucine, arginine, phenylalanine, methionine, glycine, tyrosine, glutamate, threonine, glutamine, cysteine, and valine).

2.2. Mass spectral instrumentation and optimization

Quantification was performed by electrospray LC/MS/MS in the selected reaction monitoring (SRM) mode on an Applied Biosystems (Foster City, California) Sciex API-4000 tandem quadrupole mass spectrometer with Analyst version 1.2 controlling software. Mass spectrometer conditions were typically as follows: source temperature 500 °C, negative ionization polarity with −4500 capillary voltage, unit resolution on Q1 and Q3, curtain gas 15 L/h, desolvation and nebulizing gases both set to 40 L/h, and exit potential −10 V. Dwell times for PTCA and 3-nitro-tyrosine (internal standard) were set to 250 and 50 ms, respectively.

For the analytes of interest, precursor-to-product ion transitions were established through direct infusion of each compound into the mass spectrometer. The following ion transitions were obtained: PTCA mass-to-charge ratio value (m/z) 198 → 110 and 3-nitro-tyrosine m/z 225 → 163. Sensitivity was optimized for each compound by varying the declustering potential (DP), collision energy (CE), and collision exit potential (CXP) in SRM mode and maximizing ion intensity. For these experiments, using a collisionally activated dissociation (CAD) gas setting of 12 L/h nitrogen, the DP, CE, and CXP voltages were as follows: PTCA (−60 V, −25 eV, −5 V) and 3-nitro-tyrosine (−55 V, −25 eV, −10 V).

2.3. HPLC separation method

Chromatographic conditions for the method consisted of two Shimadzu (Columbia, Maryland) LC-10ADvp pumps with an SCL-10Avp controller, a LEAP Technologies (Carrboro, North Carolina) HTS PAL autosampler, and a Varian (Torrance, California) MetaSil AQ C-18, 150 mm × 4.6 mm (3 μ), HPLC column. The mobile phase for Pump A consisted of 10mM ammonium acetate with 0.05% ammonium hydroxide, while the mobile phase for Pump B was methanol. The HPLC pumps were programmed to isocratically deliver 95% Pump A and 5% Pump B at a flow rate of 0.3 mL/min. The sample injection volume was 2 μL and the cycle time was 7 min per injection. Wash solvents for the autosampler were ethanol (wash solvent 1) and 2% ammonium hydroxide (wash solvent 2) with three syringe and three valve washes each (post injection).

2.4. Sample preparation

For method development and validation, individual Fitzpatrick Type I (lightly pigmented, Caucasian) and Type IV (darkly pigmented, African American) skin samples were chosen [16]. The tissue samples were collected with 2 mm stainless steel dermal biopsy punches and placed in separate 5 mL glass vials (screw-cap type). To each vial was added 0.5 mL of 30% hydrogen peroxide and 0.5 mL of 2 M ammonium hydroxide. Each tube was capped, vortexed vigorously for 1 min, and incubated on the bench at room temperature for 8 h. Following the incubation period, 0.2 mL of a 10% hydrogen sulfite solution was added to each vial and vortexed vigorously for 5 s to quench the reaction. Samples were then transferred to 2 mL glass tubes (96-well format) and evaporated to dryness under nitrogen at 37 °C. Finally, the samples were reconstituted in 1 mL of 10 mM ammonium acetate with 0.05% ammonium hydroxide, capped, and vortexed vigorously for 2 min. Several blank samples (i.e., without skin) were also prepared and extracted in parallel with the skin samples to make standard curve and blank sample matrices. Accuracy and precision of the assay was validated with quality control samples prepared via standard addition of spiked Caucasian skin extracts.

2.5. Standard curve and internal standard preparation

Validating a quantitative analytical method for an endogenous substance can pose additional challenges for the analytical chemist, mainly due to the lack of control biological matrix that is free of the analyte and can be used for the preparation of spiked calibration standards and quality control samples. Assay accuracy measurements must take into account the endogenous basal analyte concentration. Several approaches have been published to date to accommodate this, including the use of stripped and substitute matrices, standard addition to pooled matrix, or use of stable isotope labeled standards. Representative examples for some of these approaches to endogenous analyte quantification can be found in recent literature [17–19].

In developing the present assay, all available control skin matrix had detectable concentrations of PTCA and the clinical range required analyte sensitivity approaching the lower limit of quantification (LLOQ). Therefore, the calibrating standards for the assay were prepared in a substitute matrix solution of extracted blank samples as described above in the sample preparation section. A PTCA stock solution, containing 1 mg/mL PTCA in water, was serially diluted in standard curve matrix to prepare a 10-point calibration curve ranging from 1 to 1000 ng/mL. A 10 μg/mL working solution of 3-nitro-tyrosine (internal standard) was prepared in 10mM ammonium acetate with 0.05% ammonium hydroxide. The applicability of this substitute matrix to accurately and precisely quantify PTCA in biological matrix was established by using quality control (QC) samples. These were prepared by pooling and spiking Caucasian skin sample extracts with

known amounts of PTCA to prepare QC samples at five levels across the assay calibration range.

2.6. Quality control sample preparation

Caucasian skin extracts were pooled together to prepare the quality control (QC) matrix containing an endogenous baseline (EB) level of PTCA. The 1 mg/mL stock solution of PTCA in water was diluted to 15 μg/mL with water, and then diluted 20-fold in quality control matrix to prepare a QC sample containing EB + 750 ng/mL of PTCA (designated as high quality control 2 [HQC2]). This HQC2 sample was serially diluted in the quality control matrix to create the following QC samples that covered the calibration range: EB + 250 ng/mL (=high quality control 1 [HQC1]); EB + 50 ng/mL (=middle quality control [MQC]); EB + 10 ng/mL (=low quality control [LQC]); and no addition of stock solution to the matrix (i.e., EB in pooled matrix, =lower limit quality control [LLQC]).

2.7. Assay method

A typical quantitative assay for PTCA consisted of a single 10-point standard curve, blank matrix and blank matrix with internal standard samples in singlet, a quality control matrix sample without internal standard (matrix blank), as well as study samples and QCs. Samples, standards, blanks, and QCs were interspersed throughout the assay sequence. Assay runs were initiated by transferring 100 μL aliquots of the working standards, study samples, quality control samples, and blank samples into separate wells of a 96-well polypropylene autosampler plate. Subsequently, 20 μL of internal standard working solution (or 20 μL of 10mM ammonium acetate with 0.05% ammonium hydroxide for the blank samples) was added to all plate wells. The plate was covered, vortexed, and 2 μL was injected into the LC/MS/MS.

3. Results and discussion

3.1. LC/MS/MS optimization

To optimize the mass spectrometer ionization conditions, an aqueous solution of PTCA was infused into the ion source. Negative ion mode yielded a much higher signal-to-noise ratio for the pseudo-molecular ion than positive ionization mode due to the relative acidity the molecule. Therefore, optimization of the LC conditions and the sample preparation protocol for PTCA were carried out by monitoring the $[M-H]^-$ pseudo-molecular ion at mass-to-charge ratio of *m*/*z* 198.

The most sensitive and specific MS/MS transition for PTCA was obtained by examining its product ion spectra (Fig. 2). The CAD fragmentation of the PTCA precursor ion at mass-to-charge ratio (*m*/*z*) 198 produces abundant product ions at *m*/*z* 154 and 110. These product fragments are formed

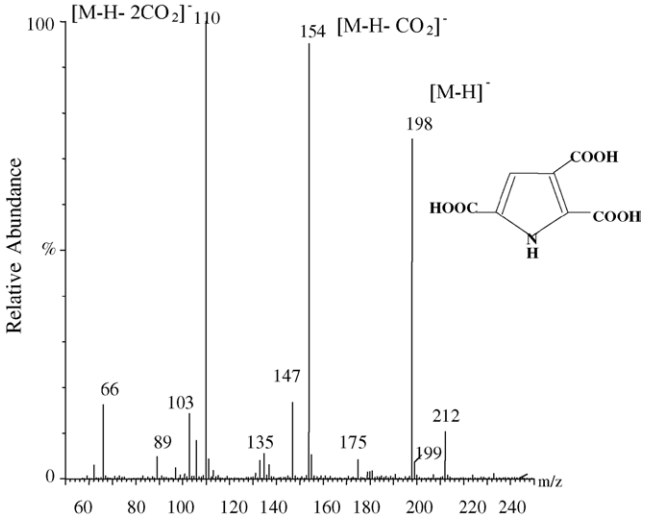

Fig. 2. The negative ion product spectra of the pseudo-molecular ion $[M-H]^-$ of PTCA at *m*/*z* 198.

by the loss of either one or two carboxylic acid functional groups from the PTCA molecule in the mass spectrometer collision cell. Of these precursor-to-product transitions, the *m*/*z* 198 → 110 transition produced the highest ion current with the best signal-to-noise ratio. Custom-synthesized stable label PTCA was not available for an internal standard at the time of method validation. Therefore, 3-nitro-tyrosine was chosen as a chemical analog since it exhibited desirable sensitivity and peak shape under the HPLC conditions optimized for PTCA in electrospray negative ionization mode. As part of the validation testing, skin sample extracts were analyzed without the addition of 3-nitro-tyrosine internal standard and no detectable amounts of 3-nitro-tyrosine were observed in the SRM channel of interest. This indicated that the endogenous level of 3-nitrotyrosine in skin punches was insignificant and excluded the possibility of artifactual formation of this amino acid in the samples during extraction. Comparison of internal standard peak areas measured between the standards, quality control samples and study samples showed a high degree of consistency indicating that no significant matrix effects were present, which was also supported by the QC accuracy and precision data. The transition *m*/*z* 225 → 163 was the chosen SRM channel for 3-nitro-tyrosine since it produced the maximum ion intensity with corresponding assay selectivity.

To increase throughput, limit tediousness, and decrease sources of variability in the assay, the extracted skin sample supernatants were directly injected into the LC/MS/MS instrument without further processing. This approach necessitated the use of extraction reagents amenable to electrospray mass spectrometry. Ito et al. and other researchers have reported that acid hydrolysis of melanins (e.g., with permanganate, HCl, HI, etc.) resulted in a sharp decrease in the yield of PTCA due to possible alterations in their structural properties [1,3]. While there seems to be a consensus that oxidation of eumelanin with alkaline hydrogen peroxide solu-

tions offers the best recovery of PTCA from natural sources, the solutions typically used were composed of nonvolatile reagents (e.g., NaOH, K_2CO_3, etc.) which are incompatible with the mass spectrometer source in the absence of further cleanup procedures [1,4]. This obstacle was overcome via use of ammonium hydroxide, which is volatile and produces adequate yields of PTCA from skin samples when used as the alkalizing agent. Variables involved in optimizing the extraction efficiency of PTCA from the skin punches included: (1) concentrations, as well as the ratio, of ammonium hydroxide and hydrogen peroxide in the oxidation solvent; (2) volume of extraction solvent per sample; (3) duration of extraction; and (4) concentration of the hydrogen sulfite solution to quench the oxidative reaction. These variables presented a unique challenge to improving recovery because the PTCA monomers are not only formed from eumelanin and extracted from the tissue, they are also simultaneously degraded by the oxidative reaction. In addition, an adjustment to any one of these variables during method optimization had a profound, and often deleterious, impact on the quality of the chromatography for both the analyte and internal standard when directly injected in the LC/MS/MS. After each experiment to optimize the PTCA extraction efficiency, iterative changes in HPLC column selection and mobile phase conditions were required to restore adequate peak shape and retention time of the analytes. However, by evaporating the samples following the addition of hydrogen sulfite quenching reagent, and reconstituting them in the aqueous mobile phase, consistent chromatographic conditions could be established for optimizing the sample preparation protocol.

It is important to note that the extraction procedure described here not only made the method less labor intensive and more compatible with LC/MS/MS, but also increased the extraction efficiency 3–10-fold when compared to the previously published method by Ito and Jimbow [3]. Therefore, the required amount of tissue sample necessary to obtain a detectable signal was reduced. This is demonstrated in Table 1 via a direct comparison between published data and PTCA yields obtained by the present method for similar samples. The improved efficiency in the chemical degradation of melanin to PTCA monomers by the current extraction procedure increased the amount of PTCA obtained per milligram of tissue or melanin standard.

Table 1
Comparison of the yields of PTCA by chemical degradation of synthetic melanin and mouse hair tissue using Ito and Jimbow's original method (hydrogen peroxide, sodium hydroxide) (published data in ref. [3]) and the current method (hydrogen peroxide, ammonium hydroxide degradation)

Material	Material (μg/mg)	
	Ito's method (published data from ref. [3])	Present method
Dopa-melanin	1.6[a]	4.7[b]
Mouse hair Black (C57BL)	1.09[b]	13.8[b]

[a] Average of two or three determinations.
[b] Average of three determinations.

Chromatographic resolution of the analytes from the solvent front and other potentially interfering components was evaluated on a variety of HPLC columns. In general, columns that possessed bound polar end-capping groups in their stationary phase offered an increase in the capacity factor ($k' = (t_r - t_0)/t_0$) of PTCA, while columns that contained only alkyl groups provided very little retention. This greater capacity factor decreased the likelihood of matrix or ion suppression effects and facilitated elution conditions that provided the best sensitivity on the mass spectrometer. Stationary phases containing amide functional groups in the columns provided the greatest analyte retention. These included both MetaChem® Polaris NH_2 [5 μm, 100 mm × 2 mm] and Phenomenex Luna® NH_2 [3 μm, 100 mm × 2 mm] columns. However, acceptable peak shape could not be established without the use of mobile phase conditions that were not conducive to efficient nebulization in electrospray mass spectrometry. The Metachem® MetaSil AQ was chosen since its polar end-capped stationary phase allowed a suitable peak shape for PTCA and 3-nitro-tyrosine while affording a retention time away from the solvent front.

3.2. Assay specificity

Because human skin samples that did not have significant concentrations of endogenous PTCA following extraction were unavailable, experiments to confirm assay specificity were necessary. Chromatographic selectivity was first demonstrated by comparing the peak shape and retention time of PTCA following the injection of spiked blank samples and extracted skin samples (both Caucasian and African American). The chromatograms appeared identical in the m/z 198 → 110 SRM channel for PTCA providing an early indication of assay selectivity. Spiked blank sample and skin sample chromatograms were also found to be identical in an SRM channel optimized to monitor the m/z 198 → 154 precursor-to-product transition. After investigating matrix interference with 3-nitro-tyrosine using Caucasian and African American skin sample extracts, specificity for the internal standard was also confirmed. In the second specificity experiment, a mixture of 18 endogenous amino acids (proline, tryptophan, cystine, serine, hydroxy proline, isoleucine, lysine, leucine, arginine, phenylalanine, methionine, glycine, tyrosine, glutamate, threonine, glutamine, cysteine, and valine) prepared in blank standard curve matrix at a concentration of 10 μg/mL each, were injected into the LC/MS/MS while monitoring the SRM channel m/z 198 → 110. This channel remained clear of interference from the amino acids at the retention time of interest. For the final specificity experiment, a precursor scan for the m/z 110 fragment ion was performed with the mass spectrometer. A comparison was made between spectra following the injection of extracted skin samples and neat samples. At the retention time of interest, m/z 198 was found to be the dominant pseudo molecular ion responsible for the m/z 110 and 154 product fragments, thus supporting assay specificity for PTCA.

A full validation was carried out for the method utilizing the FDA Guidance For Industry as a reference with additional considerations regarding the endogenous nature of the analyte, particularly assay selectivity, as described above [20]. Among the parameters included in the PTCA assay validation were assessments of short- and long-term storage stability in tissue punches, inter- and intra-assay precision and accuracy, short-term and freeze/thaw stability of samples following extraction, and analyte recovery.

3.3. Linear range, precision and accuracy

The limit of detection for this assay (minimum signal-to-noise ≥3) was 0.5 ng/mL. The assay quantification range for PTCA was 1–1000 ng/mL. Fig. 3 shows the SRM chromatograms obtained for PTCA at a concentration of 1 ng/mL (lower limit of quantification [LLOQ]), a pooled Caucasian skin extract (i.e., LLQC), and an African American skin sample. The data demonstrated that the signal-to-noise ratio was approximately 5 at the LLOQ concentration. A 10 point standard curve was prepared to establish the calibration range, and linear regression analysis with 1/concentration^2 weighting was applied. The inter-assay precision and accuracy were evaluated in three consecutive assay runs. Table 2 summarizes the back-calculated PTCA concentrations of calibration standards assayed in these three batch runs and the corresponding calibration curve parameters. The precision of the back-calculated concentrations of the calibration standards (%CV) was between 3.53 and 14.3%, while the accuracy of the assay (%R.E.) ranged between −7.67 and 10.3%. The regression coefficient was 0.9939 ± 0.0025.

The precision and accuracy for intra- and inter-assay runs were also evaluated using the tissue matrix based quality control samples prepared with $n = 5$ at five concentration levels. Since no tissue matrix could be obtained that was free of endogenous analyte, a pooled Caucasian skin homogenate matrix was used to prepare the QCs. Because of the relatively low eumelanin content of Caucasian skin, the baseline concentration in the pooled and un-spiked matrix was used for the LLQC concentration. Back-calculated PTCA concentrations of the quality control samples assayed in three separate runs are shown in Table 3. The intra-run precision and accuracy ranged from 2.34 to 22.7% (%CV) and −10.6 to 8.4% (%R.E.), respectively. Inter-run precision and accuracy ranged between 7.87 to 18.5% (%CV) and −0.244 to 5.25% (%R.E.). These assessments demonstrated that a calibration curve prepared in blank extract devoid of skin could be used to accurately quantify the analyte in a tissue matrix based QC sample. The LLOQ as determined by suitable accuracy and precision was 1 ng/mL based on an injection volume of 2 μL.

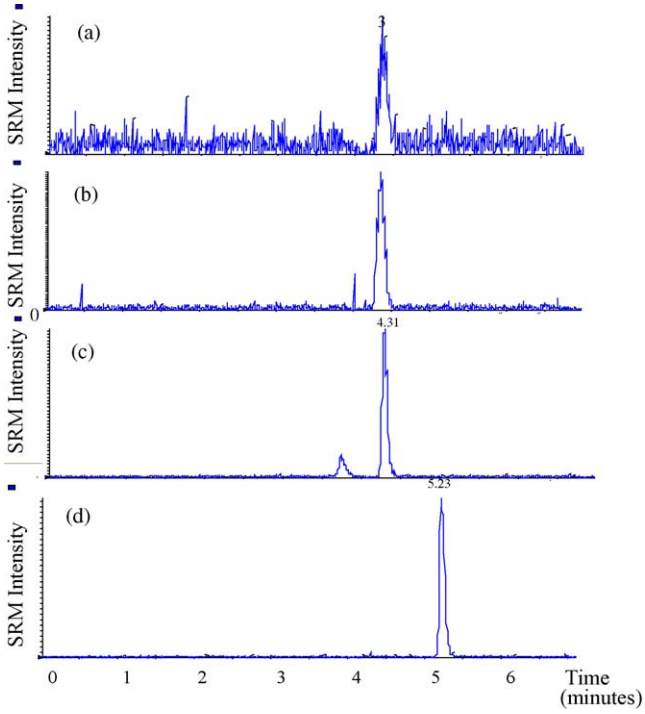

Fig. 3. Representative chromatograms of PTCA following the injection of a 1 ng/mL standard (a), a Caucasian skin sample calculated to be 2.80 ng/mL PTCA (b), and an African American skin sample calculated to be 38.7 ng/mL PTCA (c). A chromatogram of the internal standard (3-nitro-tyrosine) is also shown (d).

3.4. Assay suitability for intended purpose

The suitability of the assay method to reproducibly determine the concentration of PTCA in 2 mm punch biopsies from different human skin types was evaluated. For this

Table 2
Back-calculated PTCA concentrations of calibration standards assayed in three separate batch runs

Concentration (ng/mL)	S.D. A 1	S.D. B 2.5	S.D. C 5	S.D. D 10	S.D. E 25	S.D. F 50	S.D. G 100	S.D. H 250	S.D. I 500	S.D. J 1000	*X* slope	Intercept	R^2
Batch 1	0.884	2.41	5.17	9.69	28.1	49.5	106	230	510	1010	2.69E−3	1.20E−3	0.9963
Batch 2	1.00	2.27	5.40	12.4	22.9	41.8	102	224	520	913	2.15E−3	3.54E−4	0.9913
Batch 3	0.894	2.96	5.68	11.0	23.3	47.2	98.8	246	464	1030	7.27E−4	4.96E−4	0.9940
n	3	3	3	3	3	3	3	3	3	3	3	3	3
Overall mean	0.926	2.55	5.42	11.0	24.8	46.2	102	233	498	984	1.86E−03	6.83E−04	0.9939
S.D.	0.064	0.365	0.255	1.36	2.89	3.95	3.61	11.4	29.9	62.6	1.01E−03	4.53E−04	2.50e−03
%CV	6.94	14.3	4.72	12.3	11.7	8.56	3.53	4.87	6.00	6.36	54.6	66.3	0.252
%R.E.	−7.40	1.87	8.33	10.3	−0.933	−7.67	2.27	−6.67	−0.400	−1.57	NA	NA	NA

S.D., standard deviation; %R.E., percent relative error; %CV, percent coefficient of variation; NA, not applicable.

Table 3
Back-calculated PTCA concentrations of quality control samples assayed in three separate batch runs

	Batch Number	LLQC QC + 0 ng/mL	LQC QC + 10 ng/mL	MQC QC + 50 ng/mL	HQC1 QC + 250 ng/mL	HQC2 QC + 750 ng/mL
	1	2.02	13.8	51.8	251	722
		2.63	11.6	51.0	249	826
		2.20	12.6	50.1	285	713
		1.90	14.3	55.9	259	804
		2.06	12.9	50.8	255	753
Intrarun mean		2.16	13.0	51.9	260	764
Intrarun S.D.		0.283	1.05	2.31	14.6	49.8
Intrarun %CV		13.1	8.09	4.44	5.62	6.52
Intrarun %R.E.		[a]	7.22	−0.464	3.03	1.52
n		5	5	5	5	5
	2	1.74	10.5	42.8	204	665
		2.47	14.3	51.0	242	685
		2.42	12.5	48.0	285	648
		3.34	11.5	57.6	297	682
		2.72	14.3	55.1	265	682
Intrarun mean		2.54	12.6	50.9	259	672
Intrarun S.D.		0.577	1.69	5.84	37.0	15.8
Intrarun %CV		22.7	13.4	11.5	14.3	2.34
Intrarun %R.E.		[a]	0.638	−3.12	2.40	−10.6
n		5	5	5	5	5
	3	2.26	12.4	49.2	291	780
		2.79	15.7	57.4	277	963
		2.92	12.3	57.8	287	895
		2.17	13.1	53.1	258	736
		1.97	13.5	54.0	242	704
Intrarun mean		2.42	13.4	54.3	271	816
Intrarun S.D.		0.412	1.38	3.51	20.6	110
Intrarun %CV		17.0	10.3	6.47	7.61	13.4
Intrarun %R.E.		[a]	7.89	3.59	7.36	8.40
n		5	5	5	5	5
Mean concentration (ng/mL)		2.37	13.0	52.4	263	751
Inter-run S.D.		0.439	1.34	4.12	24.6	89.3
Inter-run %CV		18.5	10.3	7.87	9.36	11.9
Inter-run %R.E.		[a]	5.25	0.006	4.26	−0.244
n		15	15	15	15	15

LLQC, lower limit quality control; LQC, low quality control; MQC, medium quality control; HQC, high quality control; S.D., standard deviation; %CV, percent coefficient of variation; %R.E., percent relative error.
[a] %R.E. calculations based on mean endogenous back-calculated concentration.

assessment, African American and Caucasian skin biopsy samples ($n = 25$ each) were prepared and assayed using the described validated method. Punches were obtained from the same skin sections and care was taken to optimize their consistency. The results (Table 4) indicate that the sample PTCA concentrations from each skin type are within the quantification limits of assay (i.e., 1–1000 ng/mL). The African American skin samples were determined to be several fold higher in PTCA concentration compared to the Caucasian skin samples. This observation is supported by the findings of other researchers who have reported that the lowest concentrations of eumelanin were found in subjects with Fitzpatrick Type I skin (i.e., Caucasian) [21]. Investigations by Alaluf et al. have also suggested that darkly pigmented skin contains more melanin, compared to lightly pigmented skin, due to the significantly larger size of melanosomes and higher level of melanogenic tyrosinase activity [5,22].

The results in Table 4 also reveal a significant amount of inter-sample variability in PTCA concentration for each skin type. The coefficient of variance (%CV) for African American and Caucasian skin was determined to be 18.9 and 55.7%, respectively ($n = 25$). This amount of variability did not significantly improve when sample concentrations were normalized for their tissue weights. Reasons for this variability can be speculated. First, the amount of eumelanin within each biopsy could vary due to subtle differences in their composition (e.g., freckling patterns, hair follicle composition, etc.), even though efforts were made to punch from

Table 4
Inter-sample reproducibility for PTCA in Caucasian and African American skin punch biopsies ($n = 25$ each)

African American skin				Caucasian skin			
Sample#	PTCA conc. (ng/mL)	Tissue weight (mg)	PTCA (ng/mg)	Sample#	PTCA conc. (ng/mL)	Tissue weight (mg)	PTCA (ng/mg)
1	61.1	1.96	31235	1	1.95	3.78	518
2	51.0	1.26	40527	2	1.86	3.76	497
3	81.2	2.27	35852	3	1.83	3.43	535
4	72.1	1.91	37821	4	2.10	3.5	602
5	53.7	1.45	37088	5	1.55	2.86	544
6	63.7	2.25	28375	6	1.69	3.49	486
7	59.9	1.27	47225	7	2.49	3.42	731
8	64.8	1.35	48065	8	2.30	4.08	566
9	65.0	1.81	35977	9	1.48	3.37	441
10	67.1	2.43	27680	10	1.59	2.71	588
11	66.1	1.52	43553	11	1.40	2.63	534
12	68.7	1.86	37004	12	2.49	4.64	539
13	62.1	2.10	29634	13	7.81	5.66	1388
14	85.8	1.86	46215	14	1.62	2.55	637
15	65.4	2.23	29393	15	1.64	4.12	400
16	78.7	2.02	39039	16	1.85	4.34	428
17	113	1.87	60541	17	1.72	3.97	435
18	53.9	1.06	50903	18	1.61	3.15	513
19	63.1	1.12	56402	19	2.23	4.63	484
20	53.1	1.05	50625	20	2.31	3.99	581
21	72.9	2.43	30073	21	4.03	3.68	1099
22	61.9	2.2	28198	22	2.62	4.00	658
23	71.0	1.95	36481	23	2.08	4.40	475
24	78.6	1.89	41666	24	2.49	4.10	610
25	68.6	2.08	33049	25	2.49	4.10	610
Mean	68.1		39305	Mean	2.29		596
S.D.	12.9		9176	S.D.	1.28		214
%CV	18.9		23.3	%CV	55.7		35.9

Conc.: concentration; S.D., standard deviation; %CV: percent coefficient of variation.

adjacent skin sections. Second, since melanin is confined to the top surface of the skin punch, the majority of the biopsy mass is inconsequential to the concentration of the pigment. Therefore, alternative methods to tissue weight for PTCA normalization, such as tissue surface area, were investigated prior to the clinical application of the present method. A digital surface analysis measurement using a dissection microscope equipped with a digital camera was validated in house for this measurement. The normalization by surface area decreased the inter-sample variability in PTCA concentration (data not shown). Despite the well characterized variability in PTCA concentrations from samples collected from the same skin region, the PTCA results obtained from the different skin types sufficiently demonstrates that the assay can accurately measure pigment content and would be suitable to measure modulation of eumelanin derived PTCA in skin.

3.5. Recovery and stability assessments

Once eumelanin is removed from the skin punch biopsies in the form of its PTCA monomers, there is the potential liability of further degradation of PTCA before the extraction is completed. Therefore, recovery of PTCA was also evaluated in the sample extraction process. For this experiment, PTCA was spiked into sample vials at known concentrations (50 and 250 ng/mL, $n = 5$ each). Following extraction with the validated assay procedure, the sample concentrations were determined and compared to their nominal values. Results indicated a loss in the recovery of PTCA by approximately 30% at each concentration. However, since the precision (i.e., inter-sample reproducibility) was within acceptable limits (%CV < 10%), this loss in recovery appeared to be a systematic error and was not considered to negatively impact the accurate and reproducible quantification of assay samples.

The stability of PTCA in biological matrix was evaluated in both skin sample extracts directly after the evaporation step and in final reconstituted sample solutions. Following the extraction and evaporation of skin samples, PTCA was determined to be stable at room temperature for at least 4 h, as well as after three freeze–thaw cycles, compared to freshly prepared samples. Repeat injection of an assay validation batch run found the analyte to be stable for at least 21.5 h in reconstitution solvent in a 96-well polypropylene assay plate in the autosampler at room temperature. Long-term storage stability of eumelanin in tissue was assessed by preparing and assaying several African-American skin punch biopsies (at least 15 replicates), storing the remaining skin at −70 °C, and then repeating the analysis 12 weeks later. A comparison of

the assay results indicated that the average skin sample PTCA concentration fell by approximately 27% (from 68.1 ng/mL [%CV 18.9%, $n = 25$] to 50.0 ng/mL [%CV 24.4%, $n = 15$]). However, it was determined that the recovery of PTCA in the later assay was approximately 20% lower than that of the earlier assay possibly indicating a higher amount of PTCA degradation during the extraction. However, the LC/MS/MS method has an inherent variability in accuracy of up to 15%, and both assays used to assess long-term sample storage stability each had acceptable error (i.e., $\leq$15%) in their quality control sample precision and accuracy at each concentration level. When the inter-sample variability of PTCA concentrations from the skin samples within each assay was also taken into account, long-term storage stability of PTCA in the skin punch biopsies up to 12 weeks was deduced.

4. Conclusion

Human melanin is a complex polymer whose structure and quantification is still largely speculative primarily because of the difficulties connected with its isolation and insolubility. We have developed and validated a clinically translatable, robust, precise and accurate LC/MS/MS assay to quantify PTCA, a monomer specific to eumelanin, in human skin punch biopsies. Advantages over other common analytical techniques also include the selectivity for PTCA, with adequate sensitivity for the routine analysis of clinical samples including those from lightly pigmented skin. This method could potentially be used for the development of therapies related to human skin pigmentation.

Acknowledgements

We would like to acknowledge Jack Jie Li (Chemistry Department, Pfizer Global R&D, Ann Arbor Laboratories) for synthesizing the PTCA used for our assay development and validation, as well as Angel Diaz (Department of Analytical Research, Pfizer Global R&D, Ann Arbor Laboratories) for assessing the compound purity. In addition, we would like to extend our appreciation to Jane Harness (Department of Scientific Development, Pfizer Global R&D, Ann Arbor Laboratories) and Arthur Bertolino (Department of Experimental Medicine, Pfizer Global R&D, Ann Arbor Laboratories) for their guidance in developing this method.

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