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p-Dimethylaminocinnamaldehyde derivatization for colorimetric detection and HPLC–UV/vis–MS/MS identification of indoles

Patrick R. Porubsky [a], Emily E. Scott [a,*], Todd D. Williams [b]

[a] *Department of Medicinal Chemistry, University of Kansas, 1251 Wescoe Hall Dr., Lawrence, KS 66045, USA*
[b] *Mass Spectrometry Laboratory, University of Kansas, Lawrence, KS 66045, USA*

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ABSTRACT

Cytochrome P450 2A13 (CYP2A13) is a lung specific enzyme known to activate the potent tobacco procarcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) into two carcinogenic metabolites. CYP2A13 has been crystallized and X-ray diffraction experiments illuminated the structure of this enzyme, but with an unknown ligand present in the enzyme active site. This unknown ligand was suspected to be indole but a selective method had to be developed to differentiate among indole and its metabolites in the protein sample. We successfully modified a microbiological colorimetric assay to spectrophotometrically differentiate between indole and a number of possible indole metabolites in nanomolar concentrations by derivatization with *p*-dimethylaminocinnamaldehyde (DMACA). Further differentiation of indoles was made by mass spectrometry (HPLC–UV/vis–MS/MS) utilizing the chromophore generated in the DMACA conjugation as a UV signature for HPLC detection. The ligand in the crystallized protein was identified as unsubstituted indole, which facilitated refinement of two alternate conformations in the CYP2A13 crystal structure active site.

Cytochromes P450 (CYP)[1] are xenobiotic metabolizing enzymes. The lung CYP2A13 activates the potent tobacco procarcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) into two possible DNA alkylators [1–3]. In an effort to more fully understand this enzyme's structure and function, CYP2A13 has been crystallized and X-ray diffraction experiments performed. These experiments illuminated the structure of this enzyme, but an unknown ligand was present in the enzyme active site. This ligand was either co-purified with the protein or added adventitiously. The three following facts suggested that the identity of the unknown ligand was indole or an indole metabolite. First, a 94% identical enzyme, CYP2A6, is known to catalyze the oxidation of indole to several different oxidized products and pigments [4]. Some of these pigments were also visible during the *Escherichia coli* expression of CYP2A13. Second, indole is present in the *E. coli* expression system due to the inherent tryptophanase activity of the bacteria. Third, the electron density of the ligand in the crystal structure appeared to indicate a planar bicyclic compound, but at 2.35 Å, the density was not distinct enough to definitively identify indole or to rule out possible indole metabolites. In order to determine the identity of this ligand and to subsequently differentiate between indole and indole metabolites that could have been produced by CYP2A13 metabolism, we developed a sensitive HPLC–UV/vis–MS/MS assay [5].

Several different aldehyde-containing reagents have been used in microbiology as tools to differentiate certain genera and species of bacteria. A positive colorimetric result demonstrates that the specific bacterium produces tryptophanase, an indole lyase. Some of the reagents used for indole detection are the Ehrlich indole reagent, the Kovacs indole reagent (both utilizing *p*-dimethylaminobenzaldehyde), and *p*-dimethylaminocinnamaldehyde (DMACA). DMACA is both more rapid and long lasting, yielding results that are easier to interpret in bacterial assays [6]. Two advantages for our purposes were the superior sensitivity of the DMACA reagent and its ability to detect indole derivatives [6,7]. In fact, DMACA has previously been utilized for the quantitative measurement of indole-3-methanol, also known as indole-3-carbinol, in purified plant protein samples [8]. A modified method of indole conjugate formation, discussed herein, was developed to distinguish the identities of indole and indole-like compounds by spectrophotometric and HPLC–UV/vis–MS/MS methods with increased sensitivity over previously reported techniques. The data from a set of known compounds was then utilized to determine the identity of an unknown ligand in the purified protein solution used to generate the CYP2A13/ligand X-ray crystallographic structure [5].

* Corresponding author. Fax: +1 785 864 5326.
E-mail address: eescott@ku.edu (E.E. Scott).

[1] *Abbreviations used:* CYP, cytochromes P450; DMACA, *p*-dimethylaminocinnamaldehyde.

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Materials and methods

Materials

Compounds 6-hydroxyindole, 5-hydroxyindole, 4-hydroxyindole, 3-hydroxyindole, isatin and tryptophan were obtained from Acros (Geel, Belgium), indole from Aldrich (St. Louis, MO), oxindole (indole-2-one) from Maybridge (Trevillett, UK) and *p*-dimethylaminocinnamaldehyde (DMACA) from Fluka (Japan). All other materials were obtained from Fisher Scientific.

Spot tests and λ_{max} determinations

Initial spot tests were performed by saturating filter paper with DMACA solution (DMACA solution: 0.117 g of DMACA, 39 mL of ethanol, 5 mL of concentrated aqueous HCl and diluted to 50 mL with water), then spotting a solution containing indole or an indole derivative on the paper with a capillary. To quantify these observations, this derivatization was modified to a spectrophotometric assay. In each coupling reaction, 500 μL of indole-containing solution (concentration between 10 ng/mL and 10 μg/mL) was combined with 250 μL of DMACA solution. The absorbance was then measured from 400 to 750 nm. When purified protein samples were used, the protein (500 μL of 65 μM) was combined with DMACA reagent. This precipitated the protein and thus the mixture was centrifuged at 10,000 rpm for 2 min to clarify the solution before acquisition of a spectrum. Consistent λ_{max} values (±1 nm) were observed over the concentration ranges tested.

LC–UV/vis–MS/MS analysis of derivatives

Identical conjugation conditions were used for LC–UV/vis–MS/MS detection with indole concentrations of 0.2 pmol/μL in standards and 65 μM protein for ligand analysis. HPLC was performed with the mobile phases (Solvent A) 99% H_2O, 1% methanol, 0.06% formic acid, 10 mM ammonium formate and (Solvent B) 99% methanol, 1% H_2O, 0.06% formic acid, 10 mM ammonium formate. The solvent gradient was run to 1% B by 1 min, then to 50% B by 2 min, and then to 80% B by 15 min. Separation was accomplished on a 50 × 2.1 mm Alltech Altima C18 column with 3.0 μm particle size running at 0.3 mL/min. The UV/vis detector was set at 625 nm for all LC–UV/vis–MS/MS experiments. A Waters 2695 chromatograph was used to develop gradients and present peaks to a Micromass Quattro Ultima "triple" quadrupole mass spectrometer with an electrospray source.

Results and discussion

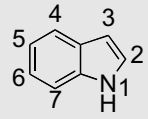
A set of indoles was initially screened by a spot test on filter paper saturated with DMACA solution. The compounds yielding a blue result from the spot test became candidates for the unknown ligand because the reaction between the unknown ligand from CYP2A13 and DMACA generated a conjugate that was blue (λ_{max} at 625 nm). Indoles were tested at concentrations between 10 μg/mL and 10 ng/mL (85 μM to 85 nM for indole, concentrations vary with molecular weight with respect to the other indoles).

From the initial UV/vis analysis, most of the oxidized indoles could be ruled out as the CYP2A13 ligand (Table 1). The λ_{max} of the UV/vis data suggested that the ligand (derivatized λ_{max} = 625 nm) was consistent with corresponding results from either indole (derivatized λ_{max} = 623 nm) or 5-hydroxyindole (derivatized λ_{max} = 627 nm). Although the purely spectrophotometric method of narrowing the identity of the CYP2A13 ligand yielded ambiguous results, it demonstrated that a highly absorbing conjugate of the CYP2A13 ligand could be formed and this characteristic was subsequently used for identification of the ligand conjugate on the HPLC phase of HPLC tandem MS.

From previous literature and the results of the spot test we hypothesized that the reaction that resulted in the conjugates absorbing in the 600–700 nm wavelength region was a different regiochemical reaction than the reaction of indole-3-methanol (indole-3-carbinol) reported by Muñoz and coworkers [8]. Muñoz reported the generation of an azafulvenium salt from the reaction of indole-3-methanol with DMACA. This reaction occurred at the 2 position on the indole nucleus because the 3 position was blocked by the methanol substituent (Fig. 1). It has been reported that in the reaction of indole and retinal, the 3 position of indole is the most nucleophilic when not blocked [9]. The compounds from our set of indoles that reacted with DMACA most rapidly and gave spectrophotometric responses at the lowest concentrations were those with the unhindered 3 position. It may be that the free 3 position of the indoles examined herein was the reason for the increased sensitivity.

Standards of DMACA conjugates for 4-hydroxyindole, 5-hydroxyindole, 6-hydroxyindole, and indole were tested to determine retention times and separability with injection concentrations of indoles at 0.2 pmol/μL. UV/vis detection was at 625 nm, λ_{max} of the DMACA conjugate from the crystallized protein. Absorption of DMACA conjugates of 4-hydroxyindole and 6-hydroxyindole were significantly lower than that of the other two conjugates due to their lower molar absorptivity at the 625 nm detection wavelength. It was also observed that the sample analysis was optimal when analyzed the same day. Samples that were exposed to ambient conditions overnight showed significant decomposition of the DMACA conjugate. The predicted products of DMACA and indole were detected as MH^+ coinciding with the 625 nm HPLC–UV/vis peak. In subsequent injections, product ion scans revealed a common fragment at m/z = 158 (Fig. 2) in all derivatives tested. Utilizing MRM targeted MS/MS detection with two transitions of 274 to 158 and 291 to 158, the HPLC chromatographic resolution was preserved with a fast detection cycle time (<1 s). Fig. 2 illustrates the MRM (A) and HPLC–UV/vis detection (B) of a standard mixture of the DMACA conjugates of 5-hydroxyindole and indole. Adjacent are the MRM and HPLC–UV/vis traces of the derivative from the CYP2A13/ligand mixture (C and D, respectively). The MS/MS scan of conjugated indole in the standard mixture is presented in E. DMACA conjugation and extraction of the protein/li-

Table 1
Comparison of results gathered from indoles tested (ND, not determined)

 (Indole Numbering System)	Spot test	λ_{max} (nm) of derivative	Retention time (min)	MW of derivative (experimental $M+H^+$)
Indole	Blue	623	8.6	275
3-Hydroxyindole	Purple	ND	ND	ND
4-Hydroxyindole	Blue	652	8.0	291
5-Hydroxyindole	Blue	627	7.3	291
6-Hydroxyindole	Blue	632	7.2	291
Tryptophan	Red	578, 480	ND	ND
Oxindole (indole-2-one)	Red	ND	ND	ND
Isatin (1*H*-indole-2,3-dione)	Orange	ND	ND	ND