PREPARATION OF 1-ANTHROYL NITRILE AS A STRONG FLUOROPHORE FOR PRE-COLUMN LABELING OF HYDROXYSTEROIDS

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ABSTRACT

The preparation of 1-anthroyl nitrile as a useful labeling reagent for derivatization of hydroxysteroids is reported. This compound was prepared by some modifications in the literature procedure including the usage of copper cyanide instead of the expensive trimethylsilyl cyanide for conversion of 1-anthroyl chloride to 1-anthroyl nitrile and conducting the conversion of anthracene-1-carboxylic acid to 1-anthroyl chloride at 40 °C instead of 110 °C to avoid the formation of impurities. Derivatization of cortisol and prednisolone by 1-anthroyl nitrile resulted in effective separation of these compounds by HPLC with a detection limit of about 20 pg.

Key words: 1-Anthroyl nitrile, Hydroxysteroids, Derivatization, HPLC.

INTRODUCTION

In recent years, considerable attentions have been directed toward the trace analysis of medicines and physiologically active substances in biological fluids. The most favorable procedures have been the chromatographic and immunological methods. The high performance liquid chromatography (HPLC) is particularly useful for the separation and determination of the biologically active substances and medicines, because of its high applicability to a variety of compounds which lack volatility and thermo-stability (1-3). Since Kirkland developed HPLC in 1969 (4), the sensitivity of the detection system has always been of major concern. Although excellent detectors and skillful techniques have already been available, their sensitivities are not yet sufficient for the analysis of some medicines and the endogenous steroid hormones in biological fluids (5-7). Of various techniques, the fluorescence labeling appears to be the most promising to fulfill this requirement. Alcohols and phenols are readily transformed to UV absorbing derivatives by treatment with acyl chlorides or anhydrides (5). The use of the benzoates esters for HPLC analysis of steroids on Corasil C18 with a detection limit of about 1 ng for the p-nitrobenzoate derivatives is reported.

In another study, phenols were derivatized to dansyl esters for HPLC analysis with UV and fluorimeter detectors (6) and the dansyl derivative of p-hydroxybiphenyl was determined with 0.1 ng detection limit with fluorimetric detector. Later, Takedate *et al.* developed 4-diazomethyl-7-methoxycoumarin (7) and 2-dansylethyl chloroformate (8) as fluorescence labeling reagents for the alcoholic groups. The other known fluorescence labeling reagents for the hydroxysteroids are not satisfactory with respect to selectivity, stability or preparative procedures (9-11). Generally, a promising labeling reagent for HPLC separation and determination requires two structural features: a functional group moderately reactive toward the hydroxyl group and a highly responsive fluorophore for detection (12). Carbonyl chloride and anthroyl nitrile type reagents have these properties, however the later has more stability toward moisture (13,14). In this study, the total synthesis of 1-anthroyl nitrile from anthraxquinone by a modification in the literature procedure, the preliminary experiments for labeling of cortisol and prednisolone and their separation by HPLC are reported.

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MATERIALS AND METHODS

The starting material, anthraquinone (Merck) was of synthetic grade (99%). Copper cyanide (15,16), lithium iodide (17), benzanthrone (18), anthraquinone 1-carboxylic acid (19) and anthracene 1-carboxylic acid (20, 21) were prepared according to the literature procedures. With the exception of solvents used for HPLC analyses which were HPLC grade, all other reagents were of commercial grade (Merck).

Melting points were taken on Electrothermal IA9000 Series, Digital Melting Point Apparatus and are uncorrected. The IR Spectra were obtained using a Nicolat FT-IR Magna 550. The mass spectra were run on a Finigan Model MAT MS-311 spectrometer at 70 eV. The HPLC was a Shimadzu LC-6A Liquid chromatograph equipped with Shimadzu RF-535 Fluorescence detector (Shimadzu Ltd., Osaka, Japan). The Nova-Pak silica-60A (150 x 3.9 mm i.d., particle size 4μm, Waters Assoc., Milford, USA) was used under ambient conditions. The samples were applied by a Rheodyne 2791 sample loop injector with an effective volume of 50 μl.

Aanthroyl-1-chloride (IV): To a solution of the carboxylic acid III (2 g, 0.009 mole) in CH_2Cl_2 (15 ml) was added, $SOCl_2$ (3 ml) and the solution was refluxed for 1 h at 40 °C under dry conditions. After removal of the solvent under N_2 stream, the residue was dissolved in CH_2Cl_2 (3 ml) and by addition of n-hexane, yellow needles of IV (1.94 g, 90%), mp 90 °C (lit. 89-90 °C) precipitated. MS: m/z (%) 204.2 (M-Cl, 12), 176.4 (M-COCl, 100). IR (film) ν_{max} 1750 cm^{-1}(-C=O)

1-Anthroyl nitrile (V): To a dry ether solution (20 ml) of IV (1.16 g, 0.0057 mole) under N_2 atmosphere, copper cyanide (0.9 g) and LiI (1.34 g) were added and the mixture was refluxed for 3 h. The color of the solution turned to red indicating the formation of V. The ethereal layer was evaporated under N_2 stream and the residue was extracted by CH_2Cl_2 (20 ml x 3). After evaporation of the solvent, the residue was purified by column chromatography (50 cm column packed with silica gel 60 (70-230 μm)) using n-hexane/ethyl acetate (1:1) as a solvent system. By concentrating the eluted solutions, compound V (0.76 g, 70%), mp163 °C (lit. 164-165 °C) crystallized. MS: m/z(%) 231.4 (M+, 60), 203.3 (M-CN, 100), 176.3 (M-COCN, 30), IR (film) νmax 2217 (-C≡N), 1656 cm^{-1} (-C=O).

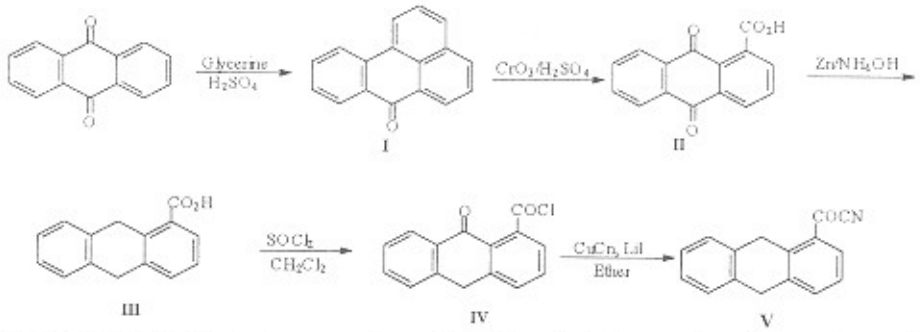
Labeling of cortisol and prednisolone with V: In a small test tube, cortisol or prednisolone (5 mg), compound V (10 mg) and a 10% solution of pyridine solution in CH_3CN (0.5 ml), were mixed and left at room temperature for 1 h. The solvent was evaporated under N_2 stream and the solution of the residue in acetone (0.5 ml) was analysed by TLC using n-hexane/ethyl acetate (1:1) as solvent system. TLC plate under UV light showed new fluorescent spots that gave a positive test with H_2SO_4 spray indicating formation of products containing the steroid molecule as a fluorescent compound. The labeled prednisolone and cortisol had R_f values of 0.2 and 0.25 respectively. A mixture of the two labeled steroids analyzed by HPLC with Nova pak column, showed efficient separation of the steroids (fig 1) with detection limit of about 20 pg (S/N=4). MS: m/z (%) 565.6 (M+, 5), 199.4 (20), 167.3 (100) for prednisolone-anthroyl derivative.

RESULTS AND DISCUSSION

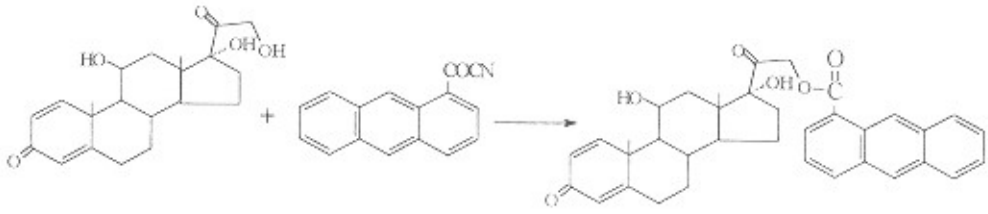
The fluorescent labeling reagent V could be prepared in 5 steps starting from the commercially available anthraquinone (Scheme 1), in 24.5% overall yield. The intermediate compounds were prepared by the reported procedures with some modifications. The preparation of compound IV according to the previously reported procedure (22) by refluxing of III with $SOCl_2$ at 110 °C was found unsatisfactory due to the formation of impurities. However conducting the reaction at 40 °C in CH_2Cl_2 eliminated this problem. Also, the preparation of V from IV was modified by the use of copper cyanide in the presence of LiI instead of the expensive reagent trimethylsilyl cyanide (13). Although the yield of the last 2 steps in the present procedure is 63% which is less than the previously reported procedure (80%), the low cost and availability of the reagents will compensate the difference in yield.

Cortisol and prednisolone were derivatized by reaction with compound V in acetonitrile in the presence of pyridine. Compound V reacts with the hydroxyl group at C_{21} of cortisol and prednisolone to form fluorescent derivatives (Scheme 2) suitable for determination of these steroids in biological fluids after special processing (14). The secondary hydroxyl group of C_{11} and the tertiary hydroxyl group of C_{17} do not react with compound V because of the steric hindrance around these groups. The formation of only one

product on TLC and its M+ value confirmed this point (Scheme 2). This is an initial step to utilize this reagent for determination of the endogenous cortisol in the presence of prednisolone as internal standard. This work will be reported in more details in future.



Scheme 1. synthesis steps of preparation of 1-anthroyl nitrile starting from anthraquinone.



Scheme 2. Labeling of prednisolone with 1-anthroyl nitrile

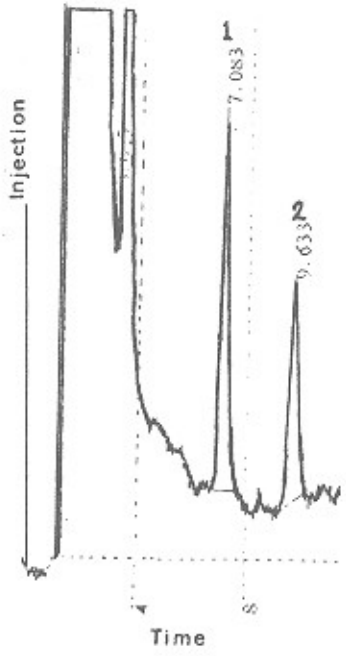


Figure 1. Chromatogram of Cortisol (1) and Prednisolone (2) derivatives with 1-anthroyl nitrile by HPLC. Conditions; column: Novapak silica, Mobile phase: n-hexane/ethylacetate (7:3), Flow rate: 1 ml/min, Detection: excitation at 360 nm and emission at 460 nm.

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