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High-performance liquid chromatographic determination of cortolic and cortolonic acids as pyrenyl ester derivatives

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ABSTRACT

A new procedure is described for the detection of the acidic metabolites of cortisol (cortoic acids) as the pyrenylmethyl-21-oic esters. The derivatizing reagent, diazomethylpyrene, was prepared by an improved procedure. The reagent was used at room temperature, required no catalyst, and was not restricted by stoichiometric requirements. The steroid esters were separated by reversed-phase high-performance liquid chromatography and analyzed simultaneously by their ultraviolet absorbance and fluorescence characteristics. Identities of the products were confirmed using the photodiode array detector to determine spectral profiles, absorbance maxima, and absorbance ratios. Further confirmation of identity of the cortoic acid esters used mass spectrometry under normal and collision-activated dissociation conditions. With the method described, a linear spectral response was obtained between 8 and 1680 fmol. Application of the technique to the analysis of steroid acids in human urine indicated the presence of cortoic acids.

INTRODUCTION[a]

Cortolic and cortolonic acids are quantitatively important end-products of cortisol metabolism [1]. Because they are excreted into the urine in variable quantities, their quantitative measurement requires a versatile and sensitive method, capable of accurately measuring them in biological fluids over a wide concentration range. The lack of a chromophore in these steroid metabolites limits the sensitivity of measurement by spectrometric procedures since it is only possible to

The following trivial names for steroid metabolites have been used: cortoic acids, cortisol metabolites characterized by a 21-oic acid group; α-cortolonic acid, 3α,17α,20α-trihydroxy-11-oxo-5β-pregnan-21-oic acid; β-cortolonic acid, 3α,17α,20β-trihydroxy-11-oxo-5β-pregnan-21-oic acid; α-cortolic acid, 3α,11β,17α,20α-tetrahydroxy-5β-pregnan-21-oic acid; β-cortolic acid, 3α,11β,17α,20β-tetrahydroxy-5β-pregnan-21-oic acid.

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measure their non-specific optical absorption at short wavelengths (190–210 nm). Cortoic acids share this limitation with most fatty acids.

Application of high-performance liquid chromatography (HPLC) for separation and identification of cortoic acids using spectrophotometric methods requires the introduction of a chromophore with a strong absorbance. Several general reagents have been developed for converting carboxylic acids to amide- or ester-containing chromophores capable of absorbing in the ultraviolet region. These include aryl triazines [2–4], nitrophenylhydrazine [5], substituted diisopropylisoureas [6,7], arylacyl halides [7–9], N-(9-acridinyl)bromoacetamide [10], and methoxyaniline [11]. In recent years, analogous reagents have been developed containing fluorogenic polycyclic aromatic chromophores [4,12–17] including quinoxaline, coumarin, naphthalene, phenanthrene, and pyrene moieties. In order to avoid the major drawbacks to use of these reagents, namely, the requirement for elevated temperature under alkaline conditions during derivatization and careful management of relative reagent concentrations, substituted diazomethanes have been developed [18–24]. These reagents are used at room temperature, do not require a catalyst, and are not restricted by stoichiometric requirements.

In this paper, we investigate the utilization of diazomethylpyrene for the simultaneous fluorimetric and UV absorbance analysis of cortoic acids. We describe an improved synthesis of the reagent and a procedure that permits the analysis of these steroids in the femtomole range.

EXPERIMENTAL

Chemicals

All solvents used were of HPLC grade (EM, Cherry Hill, NJ, U.S.A.). Other agents were obtained from a variety of commercial sources. Chemicals used for the synthesis described were purchased from Aldrich (Milwaukee, WI, U.S.A.).

Steroids

20α- and 20β-cortolic and cortolonic acids were synthesized as previously described [25].

Chromatography

Thin-layer chromatography (TLC) was performed using silica gel (Polygram SIL G/UV$_{254}$, Macherey-Nagel, Düren, F.R.G.; 0.25 mm thick) precoated plastic sheets.

HPLC was performed with a Perkin-Elmer liquid chromatographic system coupled with a 410 quaternary LC pump or a Hewlett-Packard 1090 liquid chromatograph. Both instruments were operated in the isocratic mode or gradient mode as indicated. Several detectors were used in series with the Perkin-Elmer instrument: (a) a programmable variable-wavelength detector; (b) an LC-40 pro-

grammable luminescence detector with two monochromators and a 4-μl flow cell. A spectrum filter–amplifier was installed before the detector, enabling the signal to be amplified ten-fold. Both signals were simultaneously displayed on the video monitor of the Omega-2 Analytical Workstation. The Hewlett-Packard instrument was equipped with a diode array detector and a Chemstation HP Model 310 computer with an HP 7440 Colorpro plotter.

Mass spectrometry

Mass spectrometry (MS) was performed on a VG 7070E double-focusing mass spectrometer. For the diazomethylpyrene reagent, both normal electron-impact (EI) and collision-activated dissociation (CAD) [26] mass spectra were obtained. A benzene solution of the reagent was deposited onto a direct insertion probe filament and the probe was introduced into the EI source through a vacuum interlock. The source was maintained at 200°C and the samples were volatilized directly without additional heating of the probe. Ions were formed by 50-eV electron ionization for both normal spectra and CAD analysis. For CAD analysis the instrument was operated in a B/E linked scan mode [26]. Argon was used as the collision gas, attenuating the parent ion intensity by 65%. The collision energy was set at 5 kV. Eleven CAD scans were signal-averaged and smoothed.

The pyrenyl esters were analyzed by both positive EI and negative chemical ionization (NCI). In NCI mode the OH^- ion was the chemical ionization reagent and was formed in the source using a 4:1 mixture of methane and nitrous oxide. Rapid heating of the probe sample filament was required for both EI and NCI mass spectral analysis.

Conditions for analytical or preparative separation of steroid acids

Chromatographic separation of cortoic acids used a Little Champ reversible fully end-capped ODS high efficiency column, 50 mm × 4.6 mm I.D., containing 3-μm spheres (Regis, Morton Grove, IL, U.S.A.). A Sphere 5 μm RP-18 guard column, 30 mm × 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.), was placed in tandem with the resolving column. Mobile phase was (acetonitrile–methanol, 1:1, v/v)–0.03% aqueous phosphoric acid (34:66). Flow-rate was 1.3 ml/min.

An alternative procedure for the analytical separation and analysis of cortoic acids utilized a Hypersil C_{18} monomeric fully end-capped column, 150 mm × 4.6 mm I.D., containing 3-μm spheres (Alltech Assoc., Deerfield, IL, U.S.A.). Mobile phase was (acetonitrile–methanol, 1:1, v/v)–0.03% aqueous phosphoric acid (46:54). Flow-rate was 1.0 ml/min.

For the preparative isolation of steroid acids, the stationary phase was a Partisil 5 ODS-3 RAC end-capped column, 100 mm × 9.4 mm I.D., containing 5-μm spheres (Whatman, Clifton, NJ, U.S.A.). Mobile phase was acetonitrile–methanol–aqueous phosphoric acid (0.03%) (1:1:3, v/v). Flow-rate was 3.0 ml/min.

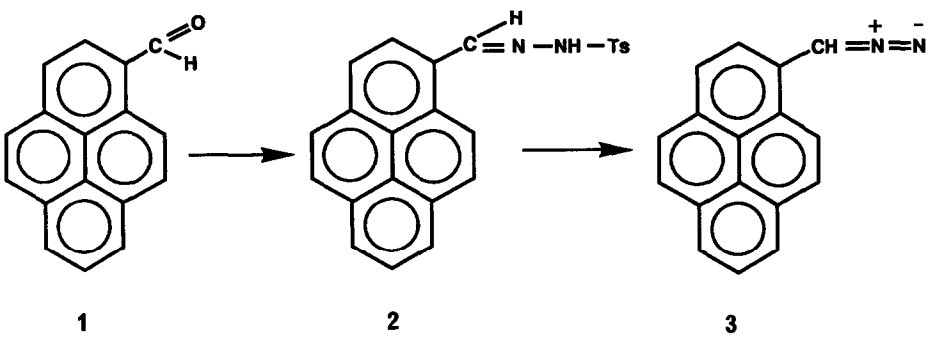

Fig. 1. Preparation of diazomethylpyrene.

Synthesis of diazomethylpyrene (Fig. 1)

Preparation of pyrenylmethyltosylhydrazone. The method is a modification of the general procedure described by Shapiro [27]. To a hot solution of *p*-toluenesulfonylmethyl hydrazide (0.33 g, 3.5 mmol) in ethanol (5 ml), pyrenecarboxaldehyde (**1**) (0.5 g, 4 mmol) dissolved in a hot mixture of ethanol (5 ml) and acetic acid (1 ml) was added. The yellow solution was kept at 80°C. After 2 min, separation of solid material started. After 5 min the hot suspension was filtered and washed with hot ethanol; 0.59 g of yellow crystals (68%) was collected, m.p. 186–193°C (decompose; sinter at 150°C). R_F = 0.62; R_F pyrenecarboxaldehyde = 0.65 (2% methanol in benzene).

*Preparation of diazomethylpyrene (**3**).* Pyrenylmethyltosylhydrazone (**2**) (0.1 g) was suspended in benzene (10 ml) and a 7% aqueous sodium hydroxide solution (5 ml) containing benzyltriethylammonium chloride (5 mg) was added (procedure modified from ref 28). The two-phase system was vigorously stirred in a 55–60°C bath for 30 min. After separating the two phases, the benzene solution was washed with water, dried (sodium sulfate), filtered, and placed in the refrigerator. TLC of the red solution on silica gel with 1% methanol in benzene as mobile phase did not show the spot of diazomethylpyrene (**3**) itself, but showed decomposition products (R_F = 0.45, 0.49) and several smaller, more polar spots. The R_F of the pyrenyltosylhydrazone in the same system was 0.2. Mass spectrum (EI): *m/z* 242 (M^+, 7%), 230 (pyrenecarboxaldehyde, 12%), 214 ($M^+ - N_2$, 64%), 201 ($M^+ - CHN_2$, 12%), 187 (9%), 149 (15%), 107 (19%), 78 (100%). CAD mass spectral fragmentation ions: *m/z* 242 (parent ion), 214, 201 (principal fragment ions).

In the EI source, 8 μg of the diazomethylpyrene, deposited on the probe, yielded a parent ion at *m/z* 242 which persisted for approximately 2 min. The signals from other ions (*e.g. m/z* 214) from the same sample remained intense for over 5 min. This suggests that thermal decomposition of the parent ion dominates after 2 min in the 200°C source. The CAD mass spectrum of the unstable parent ion (*m/z* 242) (Fig. 2) showed fragment ions derived exclusively from the uni-

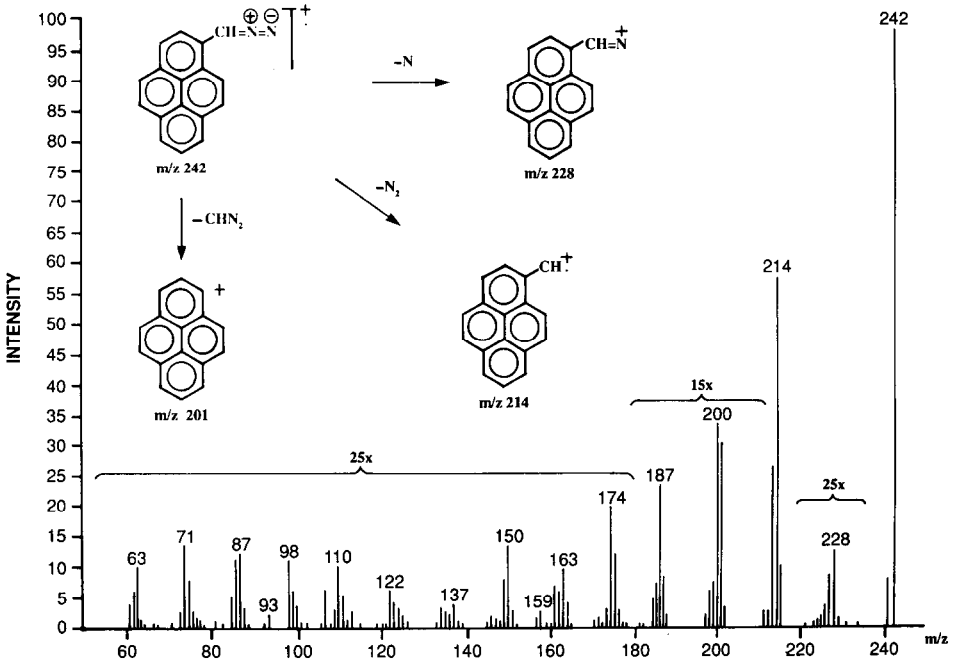

Fig. 2. CAD mass spectrum of diazomethylpyrene.

molecular fragmentations of the parent ion. The principle CAD fragment (*m/z* 214, facile loss of N_2) correlates strongly with the structure of the molecule. In addition, the series of low-intensity ions observed below *m/z* 200 are highly characteristic of the pyrene structure [29]. The reagent in benzene, stored at 3°C, is stable for at least four months. In the dry state diazomethylpyrene can be kept at −20°C for longer than one year.

Our method for the preparation of diazomethylpyrene was developed because we were unable to replicate the synthesis described by Nimura *et al.* [21]. They used a unique preparation of charcoal for the oxidation of the pyrenylmethyl hydrazone. We were unable to duplicate this step with any charcoals available to us.

Equivalence titration of diazomethylpyrene activity

The volume of diazomethylpyrene to be used in the derivatization procedure was estimated. Aliquots (10 μl) of 0.01 m*M* glycyrrhetinic acid in methanol were mixed with increasing amounts of diazomethylpyrene in benzene (3, 6, 9, and 12 μl). After 2 h at room temperature, 2-μl aliquots were spotted on silica gel-coated thin-layer plates and developed in methanol–benzene (5:95, v/v). R_F (acid) = 0.06; R_F (ester) = 0.35. On the lanes in which reagent equalled or exceeded the equivalence point, no acid was detected under UV light (254 nm).

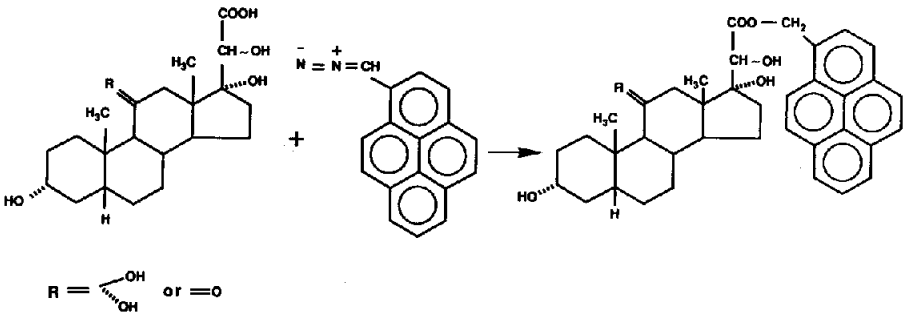

Fig. 3. Pyrenylmethylation of cortoic acids.

Derivatization procedure (Fig. 3)

A solution of the 21-oic acid in acetronitrile in the concentration range 0.01–100 μg/ml was mixed with four equivalents of diazomethylpyrene dissolved in benzene (10 mg/ml). The volume of acid was 100 μl or less. The mixture was maintained at room temperature for 2 h, filtered through a 0.2 μm syringe filter (Millipore, Bedford, MA, U.S.A.), evaporated to dryness under nitrogen and redissolved in acetonitrile. The solution was directly injected into the HPLC system. Esters may be stored in the dark at 4°C for at least seven days without decomposition for concentrations up to 200 fmol, and one month for higher concentrations. In the dried state the esters were stable for at least six months at −20°C.

Conditions for the analytical and preparative separation of 1-pyrenylmethyl 21-oic acid esters

For HPLC of pyrenyl esters of cortoic acids, a 3-μm CR-C_{18} Pecosphere deactivated and end-capped column, 30 mm × 4.6 mm I.D., with an efficiency of 150 000 plates/m (Perkin-Elmer, Norwalk, CT, U.S.A.) was used. Mobile phase was a 68% mixture of acetonitrile–methanol (1:1, v/v) in water, at 1.5 ml/min flow-rate, or a step gradient mode for urine samples consisting of 60–80% organic phase in water over 30-min interval, then 100% organic phase for 5 min. The same conditions were utilized for the analysis of pyrenyl esters with the Hewlett-Packard diode-array detector. Data were simultaneously recorded at 242, 272, and 340 nm, or by following fluorescence and UV absorbance simultaneously on a video monitor. The exitation wavelength was 340 nm, emission wavelength was 390 nm, and UV absorbance was fixed at 242 nm.

RESULTS AND DISCUSSION

Spectrophotometric analysis of the cortoic acids

Baseline separation of the four cortoic acids, α-cortolic and cortolonic acids,

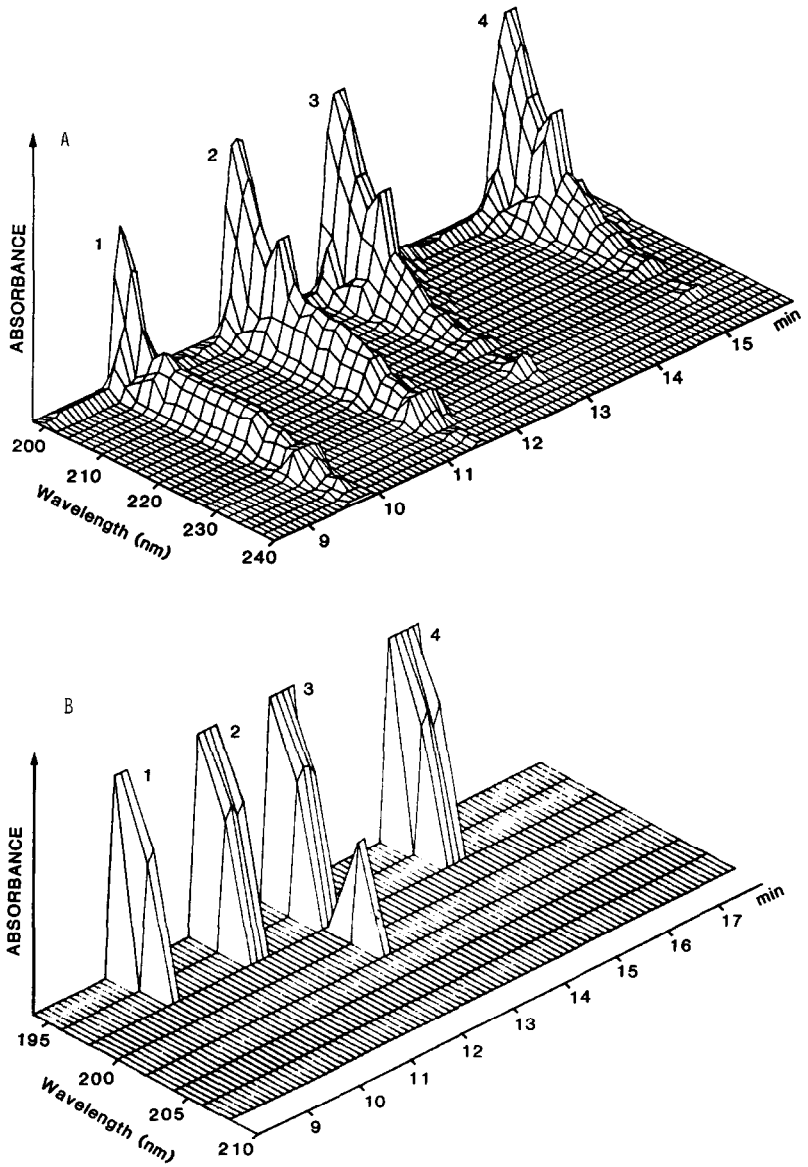

Fig. 4. (A) HPLC separation of cortoic acids. Three-dimensional profile was obtained by computer-generated integration of scans obtained with a Hewlett-Packard 1040 diode array detector, using a Hypersil C_{18} column, 50 mm × 4.6 mm I.D., 3-μm spheres. Mobile phase was acetonitrile–methanol (1:1, v/v)–0.03% aqueous phosphoric acid (46:54). Flow-rate was 1.0 ml/min. A mixture containing 4 μg of each acid was injected. Peaks: 1 = α-cortolic acid; 2 = β-cortolic acid; 3 = α-cortolonic acid; 4 = β-cortolonic acid. (B) Ratiogram of cortoic acids **1–4** (conditions as in A).

TABLE I

CHROMATOGRAPHIC AND OPTICAL CHARACTERISTICS OF FREE CORTOIC ACIDS AND THEIR 21-PYRENYL ESTERS

Cortoic acid	Retention time (min)		Molar absorptivity		Molar fluorescence pyrenyl ester[e] (mol^{-1} 0.7 cm^{-1} × 10^{12})
	Free acid[a]	Pyrenyl ester[b]	Free acid[c] (mol^{-1} cm^{-1} × 10^{2})	Pyrenyl ester[d] (mol^{-1} cm^{-1} × 10^{5})	
α-Cortolic acid	9.86	5.59	2.95 ± 0.16	6.26 ± 0.43	3.1 ± 0.13
α-Cortolonic acid	13.03	7.89	9.22 ± 0.46	7.22 ± 0.31	4.14 ± 0.21
β-Cortolic acid	11.55	12.56	5.49 ± 0.22	5.74 ± 0.37	2.25 ± 0.09
β-Cortolonic acid	15.53	14.19	9.24 ± 0.43	5.52 ± 0.18	1.82 ± 0.08

[a] Hypersil C_{18} column, 50 mm × 4.6 mm I.D., 3 μm spheres. Mobile phase, (acetonitrile–methanol, 1:1, v/v)–0.03% aqueous phosphoric acid (46:54). Flow-rate, 1.0 ml/min.

[b] Pecosphere CR-C_{18} column, 33 mm × 4.6 mm I.D., 3-mm spheres. Mobile phase, acetonitrile–methanol–water (34:34:32). Flow-rate, 15 ml/min.

[c] Mean ± S.D. for n = 5 at 209 nm. Measurement range, 0.7–26 nmol, 20°C.

[d] Mean ± S.D. for n = 12 at 242 nm. Measurement range, 1.68–3367 pmol, 20°C.

[e] Mean ± S.D. for n = 9. Exitation 340 nm, emission 390 nm. Measurement range, 337 fmol to 168 pmol, 20°C.

and β-cortolic and cortolonic acids, was achieved using the chromatographic conditions described in the Experimental section. The β-isomers were more hydrophobic than their α-counterparts.

Analysis of their three-dimensional profiles (Fig. 4A) at equivalent molarities computed from data obtained from photodiode array detector scans showed differences in the intensities of their optical absorbancies in the UV range. The former have greater detector response than the latter due to the influence of the stronger 11-oxo chromophore. The retention times and molar extinction coefficients are summarized in Table I. The lack of chromophoric groups in these acids resulted in low sensitivity, even at the lowest useable range of the UV spectrum. Determination of peak homogeneity using ratiogram analysis, showed that the four steroid acids were homogeneous (Fig. 4B). Fluorescence analysis of the cortoic acids was performed after reaction with diazomethylpyrene (Fig. 3). The derivatives were simultaneously detected by their absorption and their fluorescence. This technique combines the advantages of both methods and enables us to analyze the acids in a very large range of concentrations after a single injection. We studied the UV absorbance maxima and ratios, spectral profiles, detector responses, and minimum detection limits with the high-resolution diode array detector.

As illustrated in Fig. 5, excellent baseline separations were obtained with large differences in retention time between each of the four isomers. The free acids emerged in α, β sequence with cortolic acids preceding cortolonic acids. The emergence of the pyrenyl esters was determined by the chirality at C-20 with 20α-steroids preceding 20β-steroids. Excess derivatizing reagent did not interfere with the spectral analysis. Three-dimensional profiles of pyrenyl derivatives were obtained in the range of 200–400 nm. These profiles (Fig. 6) showed the similarity of the spectral shapes for the four compounds, and the differences in the detector responses. The high-resolution profile of the photodiode array detector response enabled us to distinguish three groups of UV absorbance maxima for the derivatives and for the reagent: (i) at 242 nm (most intense), 234, and 224 nm; (ii) at 274, 266, and 254 nm; and (iii) at 342, 326, and 314 nm. The intensities of the UV absorbances measured for the α-epimers of both acids were higher than those of the corresponding β-epimers. These differences may have been caused by quenching phenomena due to conformational differences of the side-chains.

The spectral profiles of each of the four isomers (Fig. 7A) and their first and second derivatives (Fig. 7B) have the same shapes, though with different intensities. The purity of the peaks was demonstrated by the perfectly superimposed profiles, measured at the upslope, apex, and downslope at 242 and 340 nm (Fig. 7A), and by the first derivative (Fig. 7B, profile a) and second derivative (Fig. 7B, profile b) of their spectra, similarly perfectly superimposable. Calibration plots using authentic pyrenyl ester standards gave excellent linearity with correlation coefficients exceeding 0.999 over the concentration range 1.0 ng (1.68 pmol) to 2 μg (3.37 nmol). The lower limit of detectability was 1.68 pmol. The

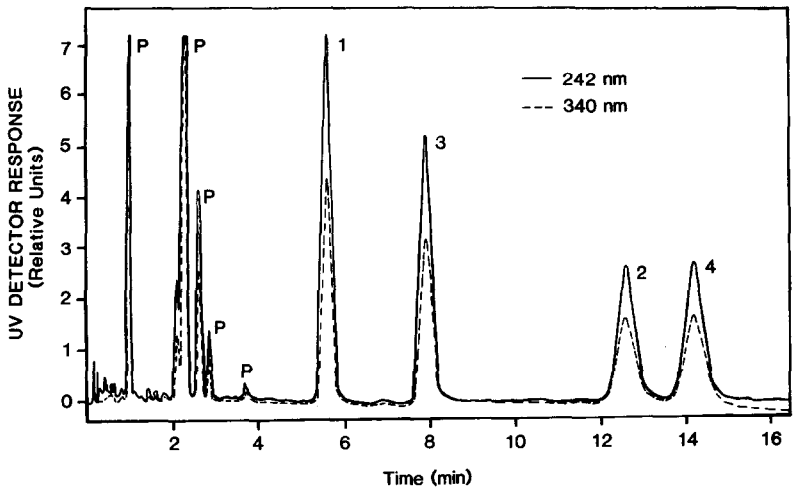

Fig. 5. HPLC separation of pyrenylmethyl cortoates. P = reagent and its degradation products. Steroid acids are identified in Fig. 4. Separations were performed using a Pecosphere CR-C_{18} column, 33 mm × 4.6 mm I.D., 3-μm spheres. Mobile phase was acetonitrile–methanol–water (34:34:32, v/v). A mixture containing 1.68 pmol of each acid was injected.

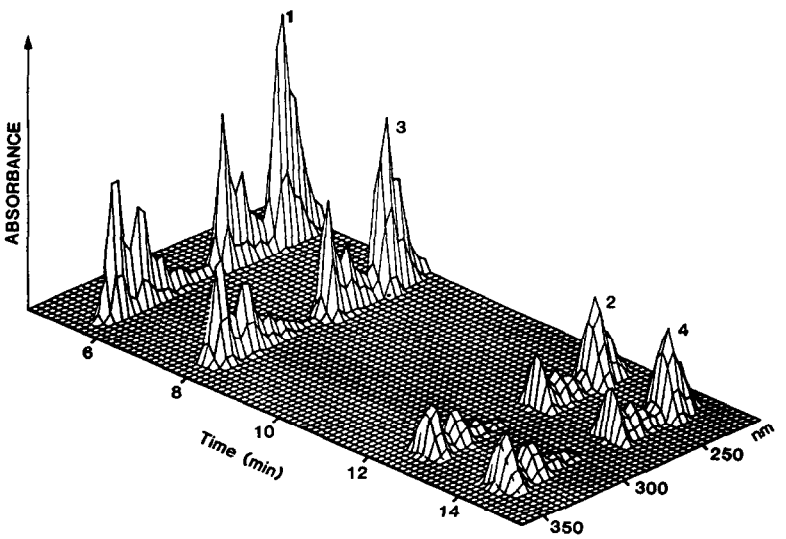

Fig. 6. Three-dimensional spectral profile depicting pattern of chromatographic separation of pyrenylmethyl cortoates.

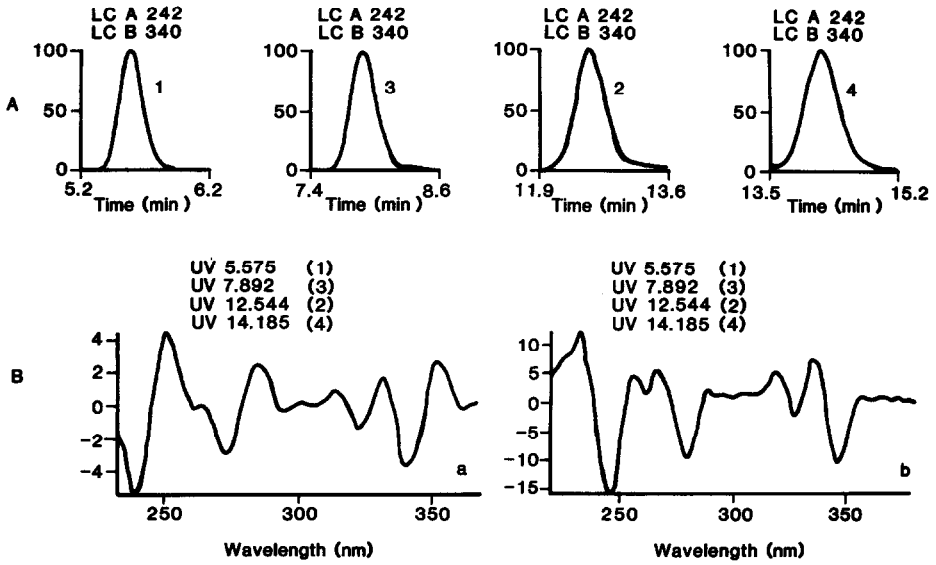

Fig. 7. Proof of peak purity of pyrenylmethyl cortoates. (A) Plots of dual-wavelength absorbance ratios of pyrenylmethyl esters **1–4**, measured across each elution peak (superimposed profiles; apparatus and conditions as in Fig. 4). LC A and LC B, wavelengths selected for measurement. (B) First derivative (a) and second derivative (b) spectra of steroids **1–4**. The displays show perfectly superimposed profiles. Values above profiles indicate retention times selected for indicated steroids.

molar absorptivities of the four esters in UV are summarized in Table I. The esterification increased the sensitivity of the determination by three orders of magnitude to the picomole level.

Mass spectral analysis of the pyrenyl esters

The HPLC effluents of 1-pyrenylmethyl derivatives of cortoic acids were collected, the organic phase was evaporated by nitrogen and further lyophilized or extracted with ethyl acetate. The structures of the isolated compounds were confirmed by MS.

In EI mode, molecular ions were observed at m/z 596 and 594 for cortolic and cortolonic esters, respectively. The base peak was the stable pyrenylmethyl ion at m/z 215 (M − 381, cortolic; M − 379, cortolonic) formed by ester cleavage. An intense peak at m/z 149 resulted from fission of ring D.

Under NCI conditions a small $[M-H]^-$ molecular ion was observed for both esters (m/z 595 for cortolic and m/z 593 for cortolonic esters). The NCI spectra were distinctly informative. The base peak for both esters was m/z 230, corresponding to the pyrene moiety. More significant were the prominent ions m/z 381 and 379 (from cortolic and cortolonic esters, respectively) which were highly characteristic of the intact steroid portion of the molecule.

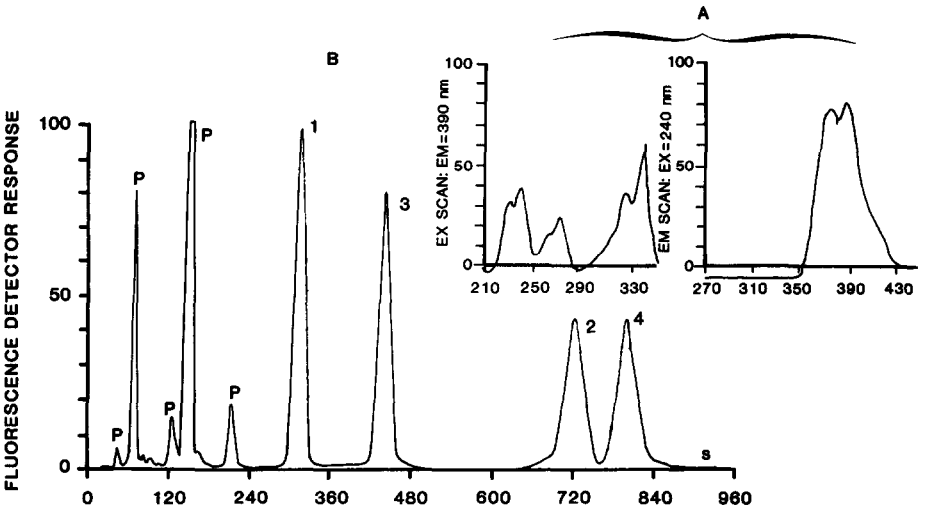

Fig. 8. (A) Excitation and emission spectra of pyrenylmethyl esters **1–4**. (B) Baseline separation of esters **1–4**, using fluorescence detection. Excitation, 340 nm; emission, 390 nm; amount injected, 16.8 pmol of each ester; conditions as in Fig. 4; detector response is normalized for peak 1. P = Reagent and its degradation products.

Fluorimetry of cortoic pyrenyl esters

Sensitivity of detection was increased by three orders of magnitude to the femtomole level by utilizing the fluorescent properties of the pyrenyl esters. The excitation and emission scan profiles are illustrated in Fig. 8. All four isomers had the same profile both in emission and excitation (excitation range 210–350, emission range 240–500 nm). Similarly to the UV absorbance maxima, three groups of

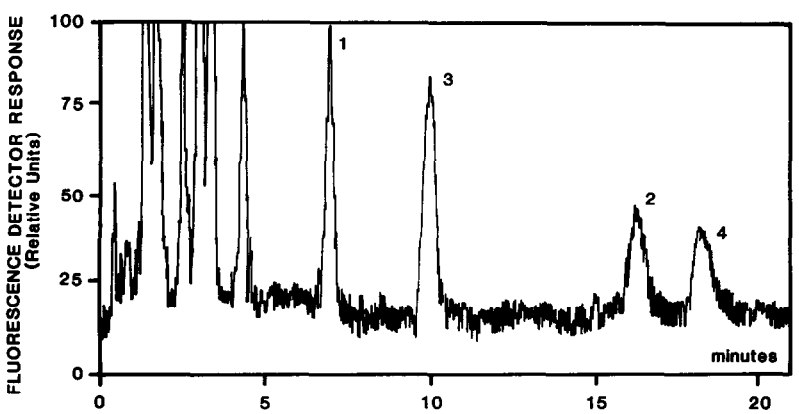

Fig. 9. Separation of 8.4-fmol amounts each of pyrenylmethyl cortoates **1–4**. Conditions as in Fig. 4, but with a flow-rate of 1.4 ml/min; detector response is normalized for peak 1.

two excitation maxima are found: (i) 340 (the most intense) and 325 nm; (ii) 275 and 262 nm and (iii) 240 and 232 nm. The order of decreasing maxima is reversed compared to UV absorbance. There are two fluorescence emission maxima, of almost equal intensities: one at 390 nm (the more intense) and the other one at 375 nm. In the gradient mode the latter maximum is slightly more intense than the former one. The chromatographic fluorescence profile of a mixture of 16.8 pmol each of the four authentic derivatized acids is illustrated in Fig. 8B. The sensitivity of the fluorescence method for the pyrenyl esters was 4 fmol for the α-isomers and 8.4 fmol for the β-isomers, based on a signal-to-noise ratio of 4 and 2, respectively. Fig. 9 shows the fluorescence profile of a mixture of four cortoic acids at 8.4 fmol each.

Concentration dependence of fluorescence response

Calibration plots were constructed for the pyrenylmethyl derivatives of the authentic acid standards. The overall linearity of the fluorescence response was demonstrated over the concentration range 10 pg (16.8 fmol) to 100 ng (168

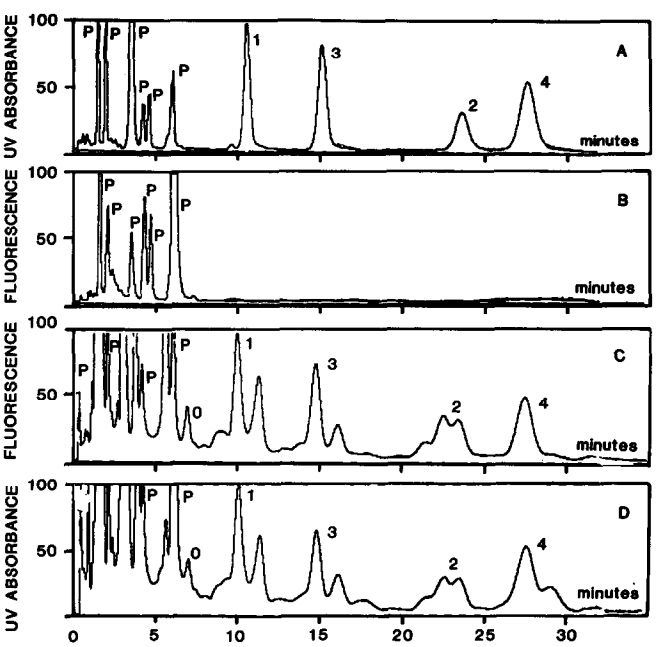

Fig. 10. Chromatographic resolution of a pyrenylmethylated urine sample. (A) Pyrenylmethyl cortoate standards; (B) diazomethylpyrene blank; (C and D) profiles of pyrenylmethylated urine samples. Conditions were simultaneous two-channel programmable UV and fluorescence detector; stationary phase as in Table I, footnote *b*, mobile phase, gradient as described in the text; A, C and D detector responses are normalized for peak 1; peak 0 was tentatively identified as 11β,20α-dihydroxy-3-keto-pregn-4-en-21-oic acid.

pmol). Correlation coefficients exceeded 0.999. Their molar fluorescence values are summarized in Table I. The reproducibility of the derivatization and fluorescence detector response was studied by duplicate injections of three separate derivatization reactions ($n = 6$). The reproducibility for all four isomers (Table I) was within $\pm 2.66\%$.

Application to urine analysis

Cortoic acids in human urine were separated from neutral steroids and urinary pigments by passing the mixture through a column of polyethylenimine cellulose [30]. Fig. 10 presents a study designed to determine the feasibility of measuring cortoic acids in urine by the pyrenyl ester fluorescence and UV absorbance techniques. The urine profile was compared to the profile of a mixture of pyrenyl cortoic acids that we had previously synthesized. Components emerged that corresponded to those of acids that we had previously isolated and identified [31,32], including 11β, 20α-dihydroxy-3-oxo-5β-pregn-4-en-21-oic acid, α-cortolic acid, β-cortolic acid, α-cortolonic acid, and β-cortolonic acid. The reagent blank (Fig. 10B) and the underivatized urine showed no UV absorbance or fluorescence in the range where pyrenyl steroid esters emerge. Fig. 11 shows the chromatographic profile of the pyrenylmethyl esters from two different human urine samples.

In summary, the pyrenyldiazomethane reagent offers the advantage of a sim-

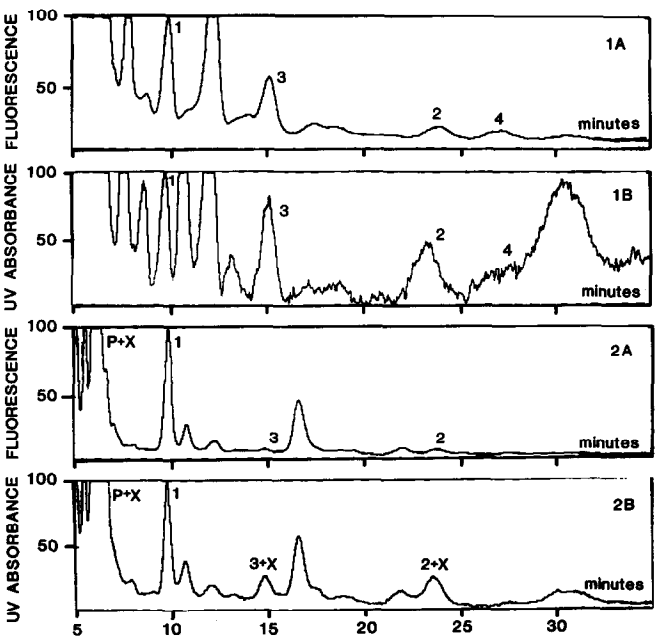

Fig. 11. Chromatographic profiles of pyrenylmethylated urine samples. Conditions as in Fig. 10; X = unidentified pyrenylmethyl esters.

ple, fast, selective, and sensitive method for the chromatographic analysis of cortoic acids. The sensitivity is in the femtomole range, well within the expected sensivity range of radioimmunoassay. It is probable that the stability characteristics and volatility of the ester would make it suitable for post-column MS detection, thus increasing its selectivity and sensitivity even further.

ACKNOWLEDGEMENTS

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