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DETERMINATION OF TRACE LEVELS OF STEROIDS IN BLOOD PLASMA BY LIQUID CHROMATOGRAPHY WITH PEROXYOXALATE CHEMILUMINESCENCE DETECTION

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SUMMARY

A highly sensitive and specific liquid chromatographic procedure with peroxyoxalate chemiluminescence detection has been developed for the determination of fluocortin butyl, a 3α-ketocorticosteroid, in blood plasma. The technique employs dansylation of the steriod to provide a highly chemiluminescent derivative. After separation by reversed-phase liquid chromatography, reagents necessary for chemiluminescence are added, followed by detection in a conventional fluorimetric detector in which the excitation source is deactivated. The precision is 2.5% relative standard deviation at the 10 ng/ml level, and the response is linear up to at least 4 ng injected steroid. The procedure requires only 1 ml blood plasma and has a limit of detection of 100 pg/ml or 7.5 pg injected steroid. The system is reliably used for routine pharmacokinetic studies and with modifications, is applicable to other steroids as well.

INTRODUCTION

While highly regarded for their impressive selectivity, the sensitivity of liquid chromatography (LC) detectors remains inadequate for ultratrace analysis, particularly when a solute is a poor fluorophor or electrophor. In fluorescence detection, stray light from Rayleigh scattering, Raman scattering, second order grating effects, and impurity emission, coupled with fluctuations in the excitation light source are the limiting factors in detectability. The recent developments in chemically excited fluorescence detection for LC, *i.e.* chemiluminescence (CL)[1–8], do not employ an excitation source and, as a result of the reduction in background[5], the fluorometer can be operated at very high sensitivity. The probable mechanism for peroxyoxalate CL is as shown in Scheme 1. Following attack by hydrogen peroxide, the chemical excitor precursor, in this case, bis(2,4,6-trichlorophenyl)oxalate (TCPO) is converted to diox-

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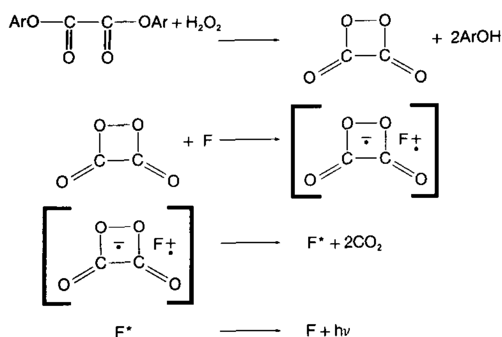
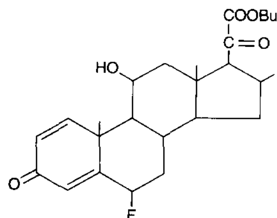


Scheme 1.

ethanedione. This species forms a donor–acceptor complex with a fluorophor and accepts an electron from the ground state of that fluorophor. The complex then destructively dissociates, but the electron is returned to the fluorophor generating its excited state, and finally, a photon is emitted as in conventional photolytic fluorescence. Thus, it follows that the ease of oxidation of the fluorophor can be correlated with the ability of a solute to be chemically excited[7] and this appears to be related to the energy level gap between the ground and excited single states. In general, amino polycyclic aromatic hydrocarbons are superior CL energy acceptors[3,5,7] and attomole (10^{-18} g/l) detectability has been achieved[5] for optimal solutes. The reagents necessary for CL production are added with a post-column reaction system to be described later.

Adrenal corticosterioids are used therapeutically as immunosupressive and anti-inflammatory agents. When administered orally or by injection, significant side effects leading to adrenal supression, osteoporosis and Cushings syndrome are major liabilities. The goal of topical steroid therapy is to employ the drug's anti-inflammatory effects to local conditions while minimizing systemic absorption.

New topical corticosteroids are designed to avoid systemic steroid effects. The degree of systemic effects is determined by the pharmacokinetic profile of the drugs, specifically by the degree of systemic bioavailability of unchanged compound. Fluocortin butyl (FCB) is currently being investigated for its topical use in rhinitis.



FCB has been shown to possess a high degree of topical activity with no systemic corticosteroid effects, even when administered in high doses[9]. This lack of systemic effect is due to the special kinetics and metabolism of the drug. FCB is a C_{21} butylester corticoid which is rapidly degraded after absorption to a C_{21} carbox-

ylic acid metabolite which has no systemic steroid activity because of its failure to bind to the steroid receptor. Dns chloride, a fluorogenic derivatizing agent has been successfully employed for CL detection of amino acids[1,4]. The Dns group contains an aminonaphthalene moiety which provides for its useful CL properties. Since FCB contains a 3-keto group, Dns hydrazine, a tag which labels aldehydes or ketones, was selected to derivatize FCB.

In this paper, we report a highly sensitive LC–CL method for the determination of FCB in blood plasma. This method has been refined to the point where it is now being used routinely for pharmacokinetic studies. While devoted to a single drug, it is believed that the approach described here will have general applicability to many steroid analytical problems.

EXPERIMENTAL

Chemicals

Fluocortin butyl (FCB) was obtained from Schering (Berlin, F.R.G.). Absolute ethyl alcohol (200 proof) was purchased from Publicker Chemical (Philadelphia, PA, U.S.A.). TCPO was synthesized following the procedure of Mohan and Turro[10]. All other reagents and chemicals were of analytical grade quality.

Chromatographic system

The chromatographic system shown in Fig. 1 consisted of a M6000A solvent delivery system (Waters Assoc.), solvent selector valve (Autochrom), WISP 710B autoinjector (Waters Assoc.), and a column block heater (Jones Chromatography). The pre-column was a 5-μm 10 cm × 4.6 mm I.D. ODS-3 RAC II (Whatman) and the analytical column was a 25 cm × 4.6 mm I.D. Partisil-5 ODS-3 (Whatman). The analytical column was thermostated to 30°C. All quantitation was carried out on an HP-3357 Lab Automation System (Hewlett Packard). Both the solvent selector valve and WISP were controlled by the HP-3357.

The mobile phase consisted of acetonitrile–water (4:1, v/v) containing 1.2 g of Tris buffer per litre. The pH of this solution was adjusted to 7.35 with nitric acid. A

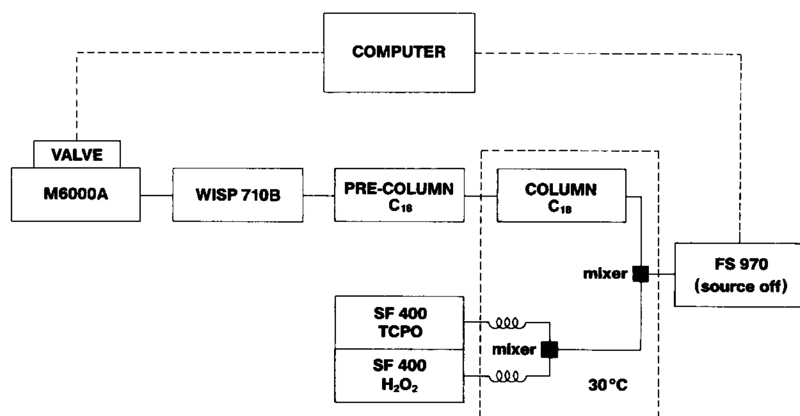


Fig. 1. Block diagram of the analytical system.

column wash solution was prepared exactly like the mobile phase, except with a 9:1 (v/v) ratio of acetonitrile–water. Both solutions were filtered through 0.45-μm PTFE filters. During a typical chromatographic analysis the mobile phase would flow at 2 ml/min for 48 min, followed by a flow of wash solution for a 10-min period. The overall analysis time per injection, including equilibration time, was 65 min. The injection volume was 25 μl.

Chemiluminescence system

The post-column CL system, connected as shown in Fig. 1, consisted of 2 Kratos Spectroflow 400 pumps and a Kratos FS 970 fluorescence detector. The output from each pump was pulsedampened with an SSI membrane pulse dampener. The effluents were passed through a second pulse dampener (Waters Assoc.) immersed in a 30°C water bath. These Bourdon tubes served to produce back pressure to increase the effectiveness of the membrane dampeners and also to assure that the solutions were equilibrated to 30°C. The Bourdon tubes contained 0.007 in. I.D. stainless-steel tubing and had a 2-ml dead volume.

The TCPO was dissolved in isopropanol–ethyl acetate (1:4, v/v) at a concentration of 3 g/l. The flow-rate of that reagent was 0.6 ml/min. The second post-column reagent was prepared by mixing 1 part of hydrogen peroxide (30%) with 4 parts of 2-propanol and 5 parts of ethyl acetate. The peroxide reagent was pumped at 1.2 ml/min.

The two post-column reagents were combined with a flow-induced vortex mixer (Kratos). The effluent was connected with about 3 in. of 0.01 in. I.D. stainless-steel tubing to a second mixer and combined with the chromatographic column effluent. Both mixers were immersed in a 30°C water bath. The blended reagent stream was connected with 12 in. of 0.01 in. I.D. stainless-steel tubing to the FS 970 fluorescence detector.

The fluorescence detector, which contained a 25-μl 2π steridian (hemispheric light collecting) flow cell, was operated with the light source deactivated. The photomultiplier tube was set at 920 V and the range was 0.2 μA full scale. The detector time constant was 4 sec and the emission wavelengths were selected with a 470-nm long wave-pass filter. A Kratos overload reset accessory, set at 3 min was used to reactivate the photomultiplier tube, which routinely shut down due to the intense light levels of excess Dns hydrazine reagent that was eluted early in the chromatographic experiment. The output from the detector was connected to a HP 3357 laboratory automation system.

Derivatization solutions

Dns hydrazine solution. A 0.06% (w/v) solution was prepared by dissolving 6 mg of Dns hydrazine in 10 ml of a 10% (v/v) ethanol in benzene solution. This solution was stored in a refrigerator until used. A new solution was prepared about every three days.

Trifluoroacetic acid solution. A solution was prepared by transferring 250 μl of trifluoroacetic acid to 10 ml of benzene. This solution was stored in a refrigerator until used and is stable for weeks.

FCB standard solution (1 ng/μl). An FCB stock solution was prepared in benzene and was made fresh daily.

Preparation of plasma standards

Prior to dosing, a 10-ml sample of blood was collected from each dog using EDTA as the anticoagulant. The blood was centrifuged for 10 minutes at 700 *g*. One-ml aliquots of plasma were placed into five separate 150 × 16 mm screw-cap culture tubes. The plasma was spiked with 0, 2, 5, 10 and 20 μl of FCB standard solution to yield a five-point standard curve, ranging from 2 to 20 ng/ml.

Note: Once the plasma is spiked with the steroid, the plasma must *immediately* be extracted as outlined below, since FCB has been found to have an *in vitro* plasma half-life of 10–30 min at 25°C.

Treating of plasma samples

Due to instability of FCB in blood or plasma, unusual sample treatment procedures must be followed. Routinely, a 5-ml sample of blood is drawn from a dog, using EDTA as the anticoagulent. The sample is *immediately* centrifuged for 10 min at 700 *g* and a 1-ml aliquot of plasma is transferred to a 150 × 16 mm disposable culture tube. The sample is *immediately* extracted as outlined below.

Extraction procedure

To each standard or sample tube, 10 ml of a 5% methyl *tert.*-butyl ether in isooctane solution was added. After capping each tube, the samples were transferred to a reciprocating shaker and shaken for 10 min at a rate of 280 excusions per min. The samples were centrifuged for 5 min at 700 *g*. The organic layer from each tube was transferred to separate 100 × 16 mm culture tubes. The samples were evaporated to dryness under dry nitrogen with the aid of mild heat. Samples and standards are fairly stable at this point and can be saved for 2–3 h prior to derivatization without significant loss of drug

Derivatization procedure

To each tube, 100 μl of Dns hydrazine (DnH) solution and 100 μl of trifluoroacetic acid solution was added. The tubes were transferred to a vortex evaporator (Buchler) fitted with a 16-mm, 48 tube block. The samples were evaporated for 10 min at a vortex speed of 4, 60°C block temperature, and 25–30 in. Hg vacuum. Samples are stable after derivatization and can be frozen at −20°C or reconstituted with 100 μl of mobile phase prior to injection.

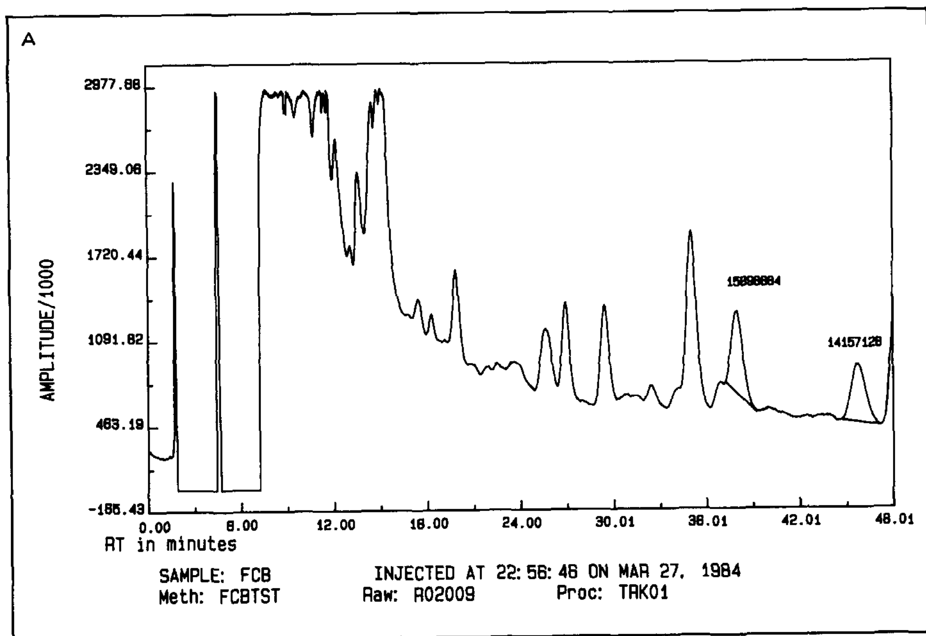
Quantitation

Peak 2 of the $FCB(DnH)_2$ derivative was integrated on a HP-2648 graphics terminal of the HP-3357 lab automation system. Using this terminal, the peak of interest could be expanded 10–20-fold and accurate baselines obtained. The areas of each sample and standard were recorded and a standard curve was constructed using linear regression with the *y*-intercept forced through zero ($y = bx$). Samples were quantitated by reference to a standard curve prepared with plasma from each dog.

RESULTS

Selectivity

Fig. 2a and b show chromatograms of a plasma sample containing 10 ng/ml



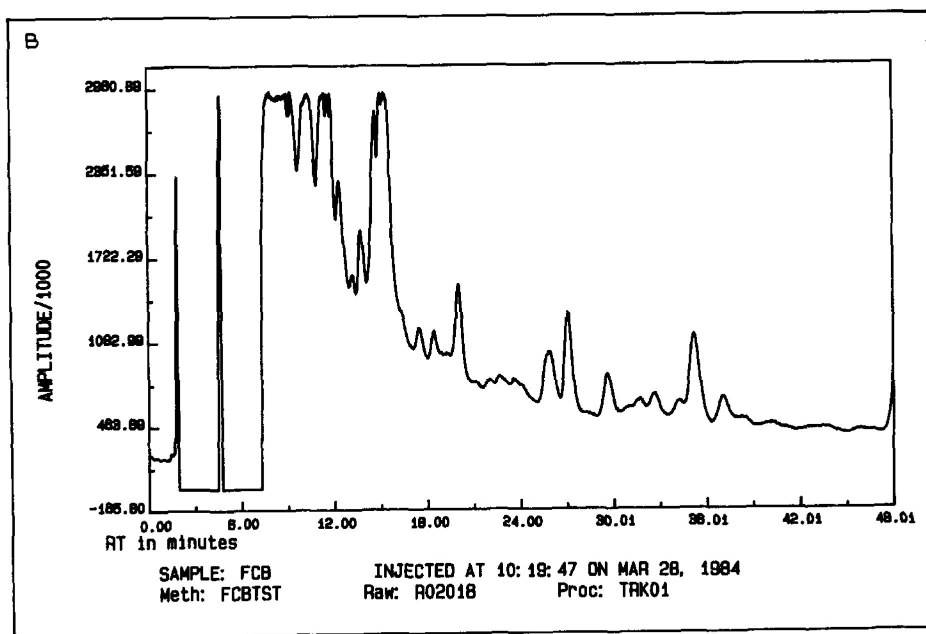


Fig. 2. Chromatograms of (A) 10 ng/ml FCB extracted from dog plasma and (B) dog plasma control sample containing no drug.

FCB, and a plasma sample taken prior to dosing with FCB, respectively. The second FCB dimer peak is free of interference from derivatized endogenous plasma excipients, while dimer peak 1 shows a small interference in approximately 50% of all dogs tested. For this reason, dimer peak 2 was always used for quantitation.

There is no interference from any of the FCB metabolites. The two major metabolites, the 17-acid and 21-acid, are not extracted by the solvents used.

Linearity

The linearity of FCB was calculated by using standard curve data from ten 5-point curves, prepared on ten different days over a one-month period. The average line of best fit was $y = 6.99x$ with an average coefficient of regression of 0.9968 over the concentration range of 2 to 40 ng FCB/ml in plasma.

Precision

Eight plasma samples of 1 ml were spiked with 10 ng of FCB and extracted according to the method outlined above. One injection of each sample was made and dimer peaks 1 and 2 were quantitated. The area counts for either peak differed by ±2.5% over the 10-h period necessary for these injections.

Accuracy

Using standard curve data over a one-month period, the residual error at various drug concentrations was calculated over ten separate standard curves. At plasma concentrations of 40, 20, 10, 5 and 2 ng/ml the % residual errors were 1.2 ($n = 2$), 1.7 ($n = 10$), 9.6 ($n = 10$), 14.6 ($n = 8$), and 15.9% ($n = 8$), respectively.

Plasma extraction efficiency

A comparison of two 5-point plasma standard curves with two benzene standard curves showed an average recovery of 74% over the range 2–40 ng/ml.

Derivatization efficiency

FCB is derivatized consistently to give two dimer peaks of equivalent area. The overall efficiency of derivatization has been calculated to be 80–85%.

Minimum quantitable level (MQL)

Using only dimer peak 2 with a retention of approximately 45 min, the MQL was 1 ng/ml (S/N = 5) or 75 pg on column. When the plasma was free of endogenous interference, the MQL was one order of magnitude lower (using dimer peak 1).

Bioavailability profiles

Fig. 3 illustrates the pharmacokinetic profile of FCB after intravenous and nasal dosing of a male beagle dog with 0.2 mg/kg drug. As can be seen, the absolute bioavailability of the drug by the nasal route is minimal.

Ruggedness of system

The system described has routinely run 24 h a day, four days a week for two months with little or no problem.

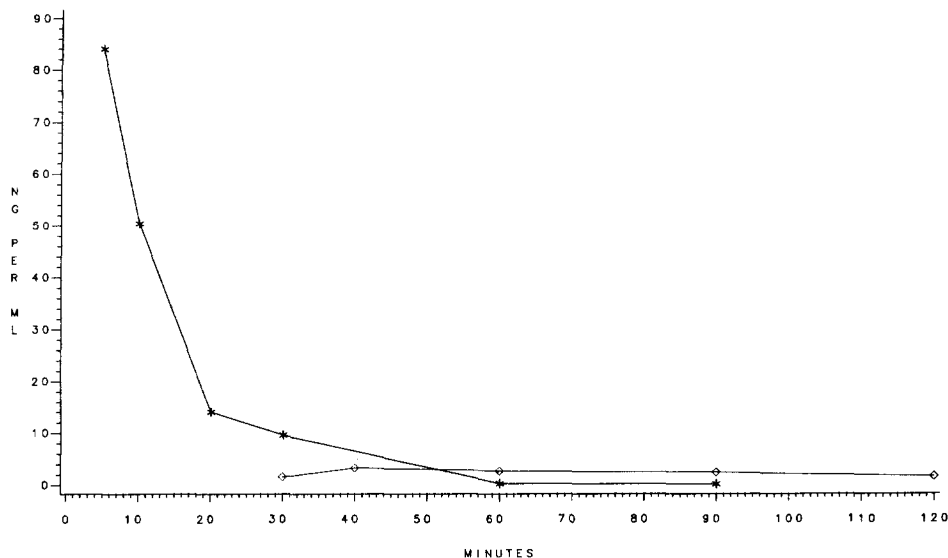


Fig. 3. Pharmacokinetic profile of FCB after intravenous (*) and nasal (◇) dosing of a male beagle dog with 0.2 mg/kg drug.

DISCUSSION

Derivatization

Most steroids exhibit little or no native fluorescence and some of those that do (estrogens) are not chemically excitable with the peroxyoxalate reaction. Dns chloride was shown to be a suitable CL energy acceptor[1] and, therefore, Dns hydrazine was selected to tag the 3-keto group of the steroid, as previously reported for hydrocortisone[11]. Following the procedure of Goehl *et al.*[11] in which an ethanolic hydrochloric acid derivatizing solution was employed, excessive decomposition of FCB, presumably though cleavage of the ester group at the 21-position, was observed. According to these authors, dansyl attack at the 3-position with corresponding *syn–anti* conformers occurs, and this was confirmed. There was no sign of D-ring keto Dns derivatization in protic media.

Because of excessive decomposition, other solvent blends were investigated. Kawasaki *et al.*[12] used benzene–ethanol blends with trichloroacetic acid as the catalyst. It was found early in our studies that trifluoroacetic acid provided a two-fold increase in the rate of reaction and, being volatile, had the added advantage of being removable during subsequent evaporation steps. with a solvent blend of 10% ethanol in benzene, two late-eluted peaks were noted that never appeared when more protic solvent blends were utilized.

Fig. 4 illustrates the products from the derivatization reaction in low protic media. Based on the known decomposition pathways of FCB, the following assignments (Table I) were made. The characterization of the so-called dimer peaks was confirmed in several ways: (i) retention times were significantly longer than would be expected for a mono-derivative; (ii) collection of one of the dimer peaks followed by reinjection into the chromatograph showed the reappearance of the second peak.

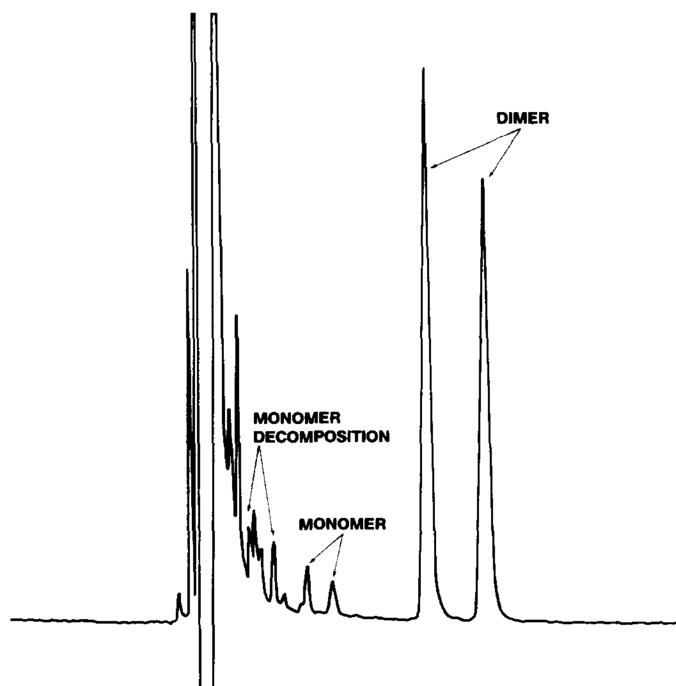


Fig. 4. Chromatogram of 500 ng of FCB, derivatized and chromatographed as described, but without the pre-column. A 1-μl injection volume was used. The peaks are identified in Table I.

This is consistent with the thermodynamic equilibrium that is expected for conformers of this type; and (iii) high-field 300 MHz NMR comparisons between derivatized and underivatized FCB are consistent with 3,20-Dns derivatization.

There are several significant features of dimer derivatization that have an impact on the analytical procedure: (i) FCB is a labile molecule. The mono-derivatives are also unstable owing to decomposition of the 17-side chain. The dimer derivative is stable for at least 1 week. (ii) The dimer derivative is very hydrophobic and therefore, in reversed-phase chromatography, the retention time is increased relative to the mono-derivative. This enhanced retention means that the derivative will be less

TABLE I

PEAK ASSIGNMENTS FOR DERIVATIZED FCB SAMPLE IN FIG. 4

Two chromatographically separable components are found for each peak so identified which reflects the conformer stereochemistry of the 3-Dns position.

Peaks	*Assignment*
FCB monomer decomposition*	3-Dns-17-COOH and 3-Dns-21-COOH
FCB monomers	3-Dns-D-ring intact
FCB dimers	3-Dns-20-Dns

* Structures not positively identified.

subject to interference from endogenous plasma components. (iii) By containing two Dns moietes, the derivative should double detector sensitivity, since there are two chemically excitable groups.

From the work on hydrazone formation[11] the position dependence on the rate of reaction with hydrazines was established. It was shown that the 3-position reacted more rapidly than the 17- or 20-position and that the 11-position did not react at all. Thus, dimer formation with FCB is not consistent with these results, but FCB is an uncommon steroid containing a "reversed ester" side chain.

In protic media, it is suspected that the D-ring keto groups are highly solvated and that attack by Dns hydrazine is sterically hindered. In low protic media, it appears that the D-ring keto group becomes labile to attack. At present, the D-ring reactivity of other steroids is unknown but, as studies are completed, they will be reported elsewhere.

Completeness of derivatization was assessed in an indirect fashion. The loss of parent compound was followed in a homogeneous benzene–Dns hydrazine–TFA solution maintained at 60°C. The reaction neared 100% completion after 2 h under these conditions. This experiment was repeated, but at concentrations that were in the working range of the assay. This time, the Dns derivative was measured. The procedure described in the Experimental section was used at the same low steroid level. It was shown that the $FCB(DnH)_2$ peak heights were a few percent higher than found in the homogeneous test blend. Based on these studies, it appears that completeness of dimerization of parent FCB approaches 85%. The slightly higher values obtained with our method probably reflect the short reaction time, which minimizes decomposition. The enhanced rate of reaction (10 min to completion) is probably due to the high reagent concentrations that exist as the derivatization–evaporation sequence proceeds to completion. This indirect method of testing the reaction was necessary, since there was no way to detect underivatized steroid at these levels. The radiolabeled steroid was unavailable. It should be noted that failure to add 10% ethanol to the benzene reaction blend results in precipitation and coating on the glass of both the dimer derivative and some Dns hydrazine.

NMR

Comparison of the 300 MHz proton NMR spectra of FCB and the collected derivatized material indicate that the bis-Dns hydrazone derivative is formed.

Reactions appear to have occurred at the carbonyl groups on the A-ring, 3-position and D-ring, 20-position. For FCB, the chemical shift for the 1-, 2- and 4-vinyl protons appear at $\delta = 7.18$, 6.29 and 6.32, respectively. Upon derivatization, the protons at the 1 and 2-position are shifted to more shielded positions, $\delta = 6.22$ and 6.12. Evidence for 20-Dns derivatization is indicated by a shift of a 16-methyl group doublet from $\delta = 0.95$ to 0.65, again due to increased shielding.

Surprisingly, NMR indicates an intact butyl ester side chain for the Dns derivative. This is evidenced by a broadened signal at $\delta = 4.1$ (from protons to CO_2^- and the proton of COH at position 11) and a triplet at $\delta = 0.89$, due to the terminal methyl group of the butyl chain.

The Dns derivative was isolated as the bis-salt (amine HCl?) and the signal from R_3NH^+ appears at $\delta = 11.82$. The signals from the Dns hydrazone protons are broadened due to the existence of the *syn–anti* conformers.

Chromatography and detection

Chromatograms of a blood plasma sample, spiked with 10 ng/ml FCB and that of blank plasma are shown in Fig. 2a and b. The presence of Dns derivatized endogenous plasma conponents necessitated relatively long (40-min) chromatography and explains why it was unnecessary to remove excess Dns reagent. In the interest of saving time, if there is no endogenous interference, it is desirable and simple to remove the excess Dns reagent with a C_{18} Bondelute (Analytichem) extraction column. But since over 10^4 theoretical plates are obtained with the column configuration shown, the Dns reagent was completely separated and removal was considered an unnecessary extra step. For less retained steroids, it is expected that cleanup will prove beneficial.

The mobile phase solvent, buffer and pH were selected for the goodness of separation and compatibility with CL detection. Solvent and pH effects on CL were reported elsewhere[8] and it was fortunate that no gross incompatibilities were encountered. The CL reagents and solvents were all based on previous work[5] and functioned as expected. One significant difference from prior studies was the thermostating of the column and post-column reagents. This system was designed to run unattended and overnight. It was found that temperature changes in the laboratory severely influence the baseline and overall CL response. The reaction temperature naturally influences CL kinetics and, thus, system response and background. Thermostating the column, reagents, and mixers effectively removed the ambient temperature effects on the system.

Experiments were performed to find an appropriate internal standard, but none were found, owing to the long retention time of the dimer derivative and the lack of suitable chromatographic windows. The absence of an internal standard did not impair the analytical method, and, in fact, it might have been difficult to use over the entire working range of the assay.

After the end of each chromatographic experiment, the solvent was automatically switched to 90% acetonitrile with identical buffers and pH as the mobile phase. This solvent switch served to wash the column and removed some late-eluted material that would have interfered with the peaks of interest in the next experiment. With attention to some of the subtleties of this method, the system has been found to be reliable over many months of operation and hundreds of determinations.

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