BIOTRANSFORMATION STUDIES OF PREDNISONE USING HUMAN INTESTINAL BACTERIA; PART I: AEROBIC INCUBATION

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في حالة تقرح القولون وأعراض التهاب الأمعاء الذي يعالج بالكورتيزون ومشتقاته يتم في بعض الحالات تناول العلاج عن طريق القولون حيث يتعرض الدواء لخليط من ميكروبات الأمعاء لذلك تناول هذا الجزء من الدراسة تأثير هذا الخليط على البريدنيزون كنموذج تحت ظروف هوائية في الأمبوب. وقد وجد أنه بعد ٩٦ ساعة قد تحول معظم البريدنيزون إلى نواتج أيض متعددة أمكن فصل جزأين أساسيين بواسطة كروماتوجرافيا العمود الســـائل. من الجزء الأول تم فصل والتعرف على ناتج الايض **m1** : androst-4-ene-3,11,17-trione (adrenosterone). بينما من الجزء الثاني تم فصل والتعرف على ناتج الايض **m2** : -androst-1,4-diene 3,11,17-trione وقد قد تم التعرف على هذا الاخير كخليط من المتشابهات عديمة التناظر المرآتى بواسطة كروماتوجرافيا الأداء العالي السائلة. وقد تم تحديد البنية الجزيئية لنواتج الأيض باستعمال القياسات الطيفية المختلفة.

*Several corticosteroids are commonly used for treatment of conditions associated with inflammatory disorders and as immune modulators. Specifically in cases of ulcerative colitis and the related inflammatory bowel syndromes, these therapeutic agents are administered rectally and are subjected to several intestinal micro floras. The present study aimed to investigate the effect of human intestinal bacterial (HIB) aerobic incubation on prednisone **1** as a model for corticosteroids. Within 96 hours, **1** was mostly transformed by HIB in vitro to various metabolites, which could be separated by column chromatography into two fractions. The first eluted fraction A was found to contain the major metabolite adrenosterone, androst-4-ene-3,11,17-trione m_1, whereas fraction B contains metabolite m_2, which was identified by chiral HPLC as a mixture of androst-1,4-diene-3,11,17-trione enantiomers. The structures of metabolites m_1 and m_2 were identified and characterized by spectroscopic techniques, including 2D-NMR and*

mass spectrometry. Time course of biotransformation of 1 by HIB was also studied.
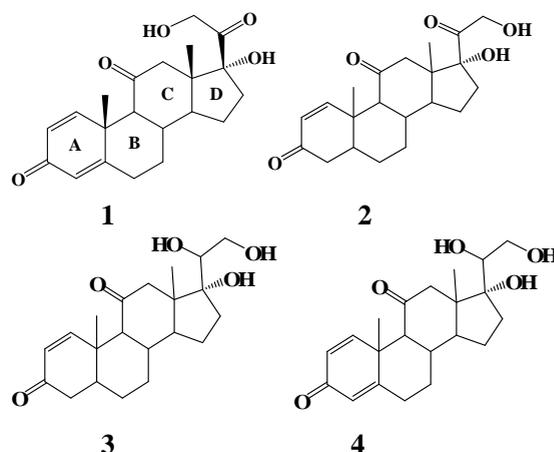
INTRODUCTION

The biotransformation studies represent one of the valuable tools at every stage of the drug discovery, from library construction, lead optimization to the development stage. In the initial lead expansion phase, biotransformation reactions are used as a tool in medicinal chemistry, leading to substitutions that are also at positions difficult to access by synthetic approaches[1]. These derivatives help in refining the structure-activity relationship, that potentially leading to new ideas of compounds to be synthesized. Additionally, they can be favorably combined with synthetic work applied to related structures, thus multiplying the number of accessible compounds for screening.

Several glucocorticoids are used per rectal administration in the treatment and management of inflammatory bowel syndromes (IBS) such as ulcerative colitis and Crohn's diseases[2]. Prednisone **1,** in order to be effective, must be bioactivated to the corresponding 11-hydroxy analogue, prednisolone, by liver enzymes in the body. The latter is then converted to less active hydrophilic metabolites such as 6 -hydroxyprednisolone, 20 / -hydroxy-prednisolone and 16 -hydroxyprednisolone, which are primarily excreted as glucuronide conjugates in the urine[3].

Microbial transformation of prednisone **1** using *Cunninghamella elegans* afforded two metabolites, 17 ,21-dihydroxypregn-1-ene-3,11,20-trione **2** and 17 ,20S,21-trihydroxypregn-1-ene-3,11-dione **3.** Meanwhile the fermentation with *Fusarium lini, Rhizopus stolonifer* and *Curvularia lunata* afforded one metabolite, 17 , 20S, 21-trihydroxypregna-1,4-diene-3,11-dione **4,** which elicits more inhibitory activity against lipoxygenase enzyme than prednisone[4].

Heretofore, the metabolic studies of **1** involving human intestinal flora have not been reported. Consequently, in the scope of the aforementioned therapeutic applications of corticosteroids, it is

216

worthy to investigate the microbial biotransformation of corticosteroid using human intestinal flora. In the present study, we report the isolation and structural determination of two metabolites obtained after aerobic incubation of prednisone **1** with the fecal bacterial mixture from a human subject.

EXPERIMENTAL

Melting points were determined on an electrothermal melting point apparatus (Stuart Scientific Co.) in capillary tubes and were uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded using either JEOL JNM ECA 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer at the NMR Laboratory Center, Faculty of Science, Alexandrian University, Alexandria/ Egypt; or Varian JEOL JNM-EX 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrophotometer, NMR Laboratory Center, Assiut University, Assiut/Egypt. Chemical shifts were given in (ppm) relative to TMS. ^{1}H-Heteronuclear Multiple Quantum Coherence (HMQC) experiments were performed with the usual pulse sequence; and data processing was obtained with standard Varian software. Electron impact (EI) mass spectra were recorded with a JEOL, JMS-600 spectrometer at an ionization voltage of 70 eV (JEOL, Tokyo, Japan) at Central lab, Assiut University, and the Micro analytical center, Faculty of Science, Cairo University, Cairo/Egypt. Binder

aerobic incubator was used for aerobic incubation**.**

Thin layer chromatography was carried out on pre-coated silica gel 60 GF$_{254}$ plates (0.25 mm thickness, Merck, Darmstadt, Germany) and the spots were detected under UV at 254 nm. Column chromatography was carried out using silica gel 60 (0.063-0.200 mm) (E. Merck, Germany). The HPLC system consisted of a Knauer model 64, solvent delivery module (Knauer, Germany), Knauer variable wavelength UV detector, 20-µl sample loop, and a Shimadzu CR-6A chromatopac integrator (Shimadzu, Tokyo, Japan), and the retention time (R$_t$) was recorded in minutes. The column used was Phenomenex LUNA C18 column (150 mm x 4.6 mm i.d., 5 µm). The effluent was monitored at 240 nm at a flow rate of 1 ml/min. Mobile phase consisting of acetonitrile/water (2:3). The chiral column used was Daicel Chiralcel OD-H column (150 mm x 4.6 mm i.d., 5 µm). The effluent was monitored at 240 nm at a flow rate of 1 ml/min. Mobile phase consisting of isopropanol/*n*-hexane (1:50).

Prednisone **1** was extracted from Hostacortin$^{®}$ tablets according to the procedure described in USP XXII. Two hundred tablets, equivalent to about 1 g of prednisone were pulverized and extracted with 1L of solvent hexane, with frequent agitation and slight warming, for 15 minutes. The supernatant liquid was decanted and discarded. The residue was then extracted with 400 ml of chloroform for 15 minutes, and the

mixture was filtered. Methanol (400 ml) was added to the filtrate, mixed, and evaporated to dryness on a steam bath with the aid of a current of air. The residue was dried at 105° for 30 minutes and used without further purification as working reference material[5]. All other chemicals and solvents were of analytical grade.

Stock solutions and mixtures

a) HIB suspension: Fresh fecal sample provided from healthy volunteer (150 g) was homogenized at 37°C with 1500 ml potassium phosphate buffer (50 mmol, pH 7.3) in a sterilized 2 L flask and the supernatant was decanted and used immediately.

b) Prednisone solution: One-gram prednisone was dissolved in 25 ml dioxane: methanol mixture (3:1).

c) Test suspension: Prepared by adding prednisone solution (25 ml) to HIB suspension (975 ml) to give a final concentration of 1 mg/ml and then mixed well using magnetic stirrer.

d) Substrate control: An aliquot of prednisone solution was added to potassium phosphate buffer (50 mmol, pH 7.3) to give a final dilution of 1 mg/ml and used as control solution.

e) HIB culture control: An aliquot of HIB suspension was used in the control experiments.

Procedure

The test suspension, the substrate control solution, and the HIB culture control were aerobically incubated at 37°C for 4 days. At 24 h intervals the reaction mixture was mixed using vortex mixer three times each for one minute and samples (2 ml) were withdrawn from the solutions as well as suspensions and transferred to test tubes. Ethyl acetate (2 ml) was added to each tube, and mixed by vortex three times each for one minute. Ethyl acetate extracts were applied on TLC plate then developed using n-hexane: ethyl acetate (3:1) and spots of metabolites were located under UV lamp. At the end of the incubation period, the test suspension was extracted with ethyl acetate (500 ml x 4). The extract was dried over anhydrous sodium sulphate, filtered and the solvent was removed under reduced pressure. The residue was chromatographed over silica gel column (25 cm length x 1.5 cm internal diameter) with hexane: ethyl acetate using gradient elution method starting with 100% hexane and ended to 70% hexane in ethyl acetate. Fraction A was collected and evaporated *in vacuo,* dried using vacuum desiccator to yield metabolite m_1 (60 mg). Fraction B was collected and concentrated then dried using vacuum desiccator to yield metabolite m_2 (21 mg).

Microbial biotransformation time course

Five aliquots of 10 ml each were pipetted from the stock solutions and suspensions c, d and e and transferred into 5 test tubes then aerobically incubated at 37°C for 4 days.

At 0, 24, 48, 72 and 96 h time intervals ethyl acetate (3 ml) was

added to each tube and mixed gently by vortex for 1 minute. Aliquots of 300 µl of ethyl acetate extract were transferred into 50 ml volumetric flask and completed to the mark with mobile phase CH_3CN: H_2O (2:3) and analyzed by injection into HPLC using CH_3CN : H_2O (2:3) as mobile phase.

Androst-4-ene-3, 11, 17-trione (m_1)[6]: Colorless crystalline solid; m.p. 222-224; UV (MeOH) $_{max}$ 240 nm; R_t = 7.87 min (CH_3CN:H_2O, 2:3); ^{1}H-NMR ($CDCl_3$, 500 MHz), and ^{13}C-NMR ($CDCl_3$, 125 MHz) [Tables 1 and 2, respectively]. EI-MS m/z (rel. int.) 300 $[M]^+$ (39), 282 (2), 256 (10), 228 (7), 122 (100); CAS 382-45-6.

Androsta-1,4-diene-3,11,17-trione (m_2)[6]: Colorless crystalline solid; m.p. 197-198°C; UV (MeOH) $_{max}$ 240 nm; R_t=7.1 min. (CH_3CN:H_2O, 2:3); ^{1}H-NMR ($CDCl_3$, 400 MHz), and ^{13}C-NMR ($CDCl_3$, 100 MHz) [Tables 1 and 2, respectively]. EI-MS m/z (rel. int.) 298.20 $[M]^+$ (100), 280 (7),270 (37), 254 (37); CAS 7738-93-4.

RESULTS AND DISCUSSION

Prednisone **1** was extracted from marketed Hostacortin® tablets following the procedure described by USP XXII[5]. ^{1}H-NMR and ^{13}C-NMR spectral data were carried out and listed in Tables 1 and 2 guided by the available reported figures[4]. Matching the found spectral data of prednisone **1** with that of the metabolites might assist in the elucidation of the structures of the isolated products.

Aerobic incubation of **1** with HIB was carried out parallel with substrate control and HIB culture control experiments. Each run of the three experiments was monitored by TLC at 24 h time intervals. During the total period of incubation (96 h), control experiments did not show any new profile of developed spots other than that elicited in the test experiment at incubation zero time. This demonstrated the fitness of viable aerobic bacteria from human feces as the unique source of the developed spots for the span of the test experiments. At the end of incubation period the test experiments were processed and the ethyl acetate extract residue was chromatographed by silica gel column. Two fractions A and B were collected from the open silica column.

Fraction A (R_f = 0.77) that eluted firstly was suitably purified to give a relatively high yield of the metabolite m_1. Mass spectrum of m_1 revealed the molecular ion peak at m/z 300 $[M^+]$ demonstrating 58 mass units less than the parent compound **1**, which most probably indicates cleavage of the -ketol group at C-17. The ^{1}H-NMR spectral data (Table 1), showed only one downfield olefinic proton signal at 5.67 (1H, s, H-4) in addition to two pairs of multiplets integrated by two protons each; one pair at 1.62, 2.05, and second at 2.4 and 2.5 ppm, that was assigned to the ethylenic protons on C_1 and C_2 respectively. Furtherly, the observed absence of

219

Table 1: ^{1}H-NMR spectral data of prednisone[6] and its aerobic HIB biotransformation products **m₁** and **m₂**.

Proton No	1 [a]	m₁ [b]	m₂ [c]
H-1	7.55 (1H, d, $J = 10.7$)	1.62 (1H, m), 2.05 (1H, m)	7.59 (1H, d, $J = 10.28$)
H-2	6.06 (1H, dd, $J = 10.7$, 1.9)	2.4 (1H, m), 2.5 (1H, m)	6.15 (1H, dd, $J = 10.28$, 1.9)
H-4	5.97 (1H, d, $J = 1.9$)	5.67 (1H, s)	6.04 (1H,d, J=1.9)
H-6	1.9 (2H, m)	2.4 (1H, m), 2.2 (1H, m)	2.4 (1H, m), 2.2 (1H, m)
H-7	1.65 (2H, m)	2.1 (1H, m), 1.8 (1H, m)	2.1 (1H, m), 1.8 (1H, m)
H-8	1.9 (1H, m)	1.9 (1H, m)	1.9 (1H, m)
H-9	2.13 (1H, m)	2.5 (1H, m)	2.5 (1H, m)
H-12	2.22 (1H, m) and 2.31 (1H, m)	2.4 (1H, m), 2.1 (1H, m)	2.4 (1H, m), 2.1 (1H, m)
H-14	1.9 (1H, m)	1.6 (1H, m)	1.6 (1H, m)
H-15	1.9 (2H, m)	1.8 (2H, m)	1.8 (1H, m)
H-16	2.84 (1H, m) and 1.9 (1H, m)	1.8 (1H, m), 1.6 (1H, m)	1.8 (1H, m), 1.6 (1H, m)
OH-17	5.58 s	-------------------	----------------------
H-18	0.46 (3H, s)	0.81 (3H, s)	0.85 (3H, s)
H-19	1.32 (3H, s)	1.36 (3H, s)	1.41 (3H, s)
OH-21	4.68 (1H, t, J= 6.1)	-------------------	----------------------
H-21	4.39 (1H, dd, J= 19, 6.1), 4.03 (1H, dd, J= 19, 6.1)	--------------------	----------------------

[a] ^{1}H-NMR spectrum recorded in DMSO-d₆ by 500 MHz spectrometer.
[b] ^{1}H-NMR spectrum recorded in CDCl₃ by 500 MHz spectrometer.
[c] ^{1}H-NMR spectrum recorded in CDCl₃ by 400 MHz spectrometer.

220

Table 2: ^{13}C-NMR assignments of prednisone[6] and its aerobic HIB biotransformation products **m$_1$** and **m$_2$**.

Carbon No.	1^a	*m$_1$* ^{b}	*m$_2$* ^{c}
1	155.6 (CH)	34.6 (CH$_2$)	153.8&155.5 (CH)
2	127.5 (CH)	33.7 (CH$_2$)	128.4&126.8 (CH)
3	185.6 (C)	199.7 (C)	186.1&186.0 (C)
4	124.3 (CH)	124.7 (CH)	125.4&123.8 (CH)
5	167.7 (C)	168.2 (C)	165.8 (C)
6	33.6 (CH$_2$)	32.0 (CH$_2$)	31.7&32.0 (CH$_2$)
7	32.1 (CH$_2$)	30.9 (CH$_2$)	30.5&30.7 (CH$_2$)
8	36.1 (CH)	36.3 (CH)	36.5&37.1 (CH)
9	59.3 (CH)	63.2 (CH)	60.0&61.2 (CH)
10	42.4 (C)	38.3 (C)	42.18 (C)
11	210.7 (C)	207.7 (C)	207.3&207.3 (C)
12	50.3 (CH$_2$)	50.4 (CH$_2$)	48.8&50.3 (CH$_2$)
13	51.0 (C)	49.8 (C)	49.9 (C)
14	49.3 (CH)	49.7 (CH)	48.6&51.2 (CH)
15	23.4 (CH$_2$)	21.6 (CH$_2$)	21.6&22.8 (CH$_2$)
16	34.1 (CH$_2$)	36.0 (CH$_2$)	35.2&35.8 (CH$_2$)
17	87.9 (C)	217.0 (C)	216.3 (C)
18	16.0 (CH$_3$)	14.7 (CH$_3$)	13.9&15.3 (CH$_3$)
19	19.3 (CH$_3$)	17.3 (CH$_3$)	18.2&19.5 (CH$_3$)
20	211.9 (C)	---------------	-----------------------
21	66.6 (CH$_2$)	---------------	-----------------------

^{a} Spectrum recorded in DMSO-d$_6$ by 125 MHz spectrometer.
^{b} Spectrum recorded in CDCl$_3$ by 125 MHz spectrometer.
^{c} Spectrum recorded in CDCl$_3$ by 100 MHz spectrometer.

-ketol group proton signals at 4.03, 4.39 and 4.68 which pointed to its cleavage in addition to the absence of singlet at 5.58 assigned for C_{17}-OH in **1** led us to primarily designate the structure of m_1 as androst-4- ene-3,11,17-trione.

Further confirmation of the suggested structure of m_1 was achieved by analysis of ^{13}C-NMR data (Fig. 1a). Table 2 displays a total of 19 carbon signals, two carbons less than the substrate **1**. Several DEPT and HMQC experiments were done to aid the assignment of the different ^{13}C-NMR signals to their respective C-atoms. DEPT spectrum at 45° (Fig. 1b) displayed six carbon signals less than those displayed by ^{13}C-NMR. The missing signals might be attributed to the quaternary carbons number 3, 5, 10, 11, 13, and 17 in the nineteen carbon steroidal skeleton. DEPT spectrum at 90° (Fig. 1c) showed four signals that are characteristic for the methine carbons C_4, C_8, C_9 and C_{14}, while DEPT spectrum at 135° (Fig. 1d) revealed two methyl groups and seven methylenes in addition to the four signals of the methine carbon atoms (Table 2). The HMQC spectrum (Fig. 2) showed coupling between C_4 at 124.75 and H-4 at 5.669 while there were no detected couplings between carbons number 3, 5, 10, 11, 13, and 17 at 199.7, 168.2, 38.3, 207.7, 49.8 and 217 respectively and any protons confirming their quaternary nature.

Structure of metabolite m_1 was therefore recognized as, androst-4-ene-3,11,17-trione, which

corresponds to adrenosterone, a natural hormone secreted from adrenal cortex and has mild androgenic activity[7].

Fraction B (R_f = 0.64) eluted after fraction A yielded metabolite m_2 after suitable purification procedure. An HPLC run using Rp-C_{18} column revealed a single peak at R_t 6.8 min. At the same time the sample in hands displayed ^{13}C-NMR spectrum with duplicated signals for each carbon atom that indicated the population of a mixture of two closely related components. On the other hand a run on chiral HPLC column, disclosed two peaks with R_t = 3.4 and 4.1 minutes in a ratio of 4:1 respectively (Fig. 3) most probably indicating that the fraction in hands was a mixture of enantiomers, which was reproducibly obtained after aerobic incubation for 48 h.

The mass spectrum of m_2 revealed a molecular ion peak at m/z 298 [M^+] (100%) with 60 m/z less than [M^+] of **1** suggesting loss of one m/z unit in addition to 59 m/z of the -ketol group. The ^{1}H-NMR spectral data (Table 1), demonstrated three downfield olefinic proton signals at 7.59, 6.15 and 6.04 comparable to the values showed by the parent compound **1**. The doublet centered at 7.59 was assigned to H-1, being vicinally coupled to H-2 methine proton doublet at 6.15. Vicinal protons with *J* value of 10.3 Hz illustrated cis coplanar arrangement. The olefinic proton H-2 appeared as a double doublet centered at 6.15 due to vicinal coupling with H-1 and long
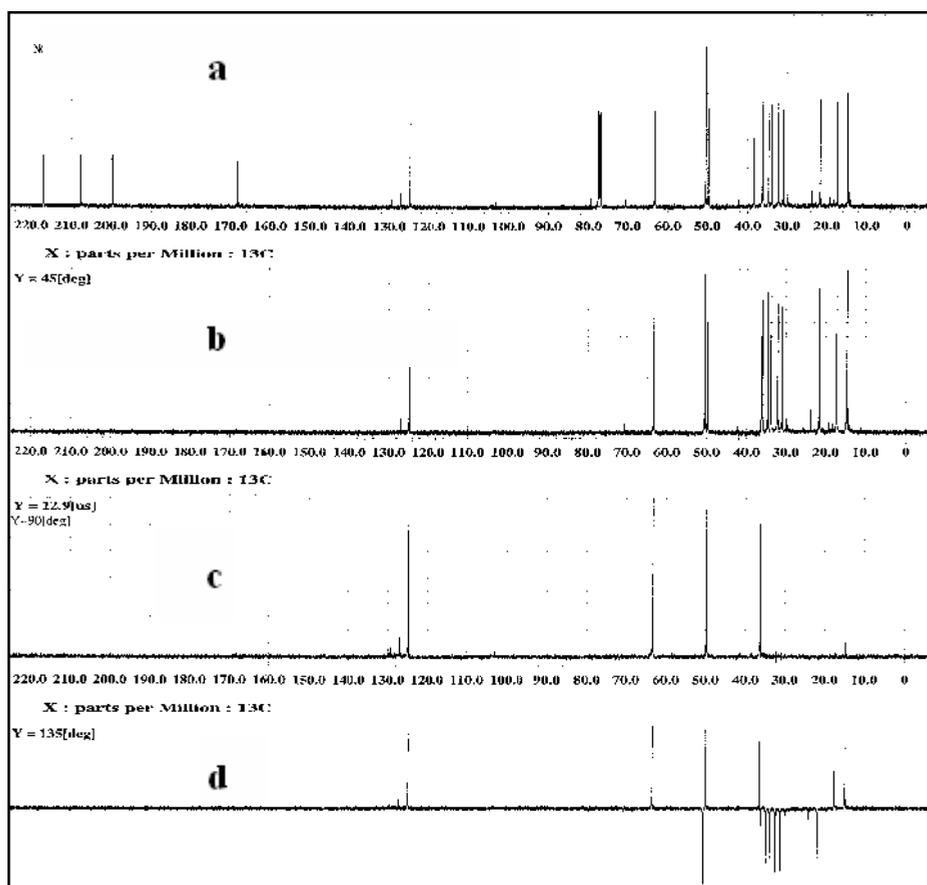
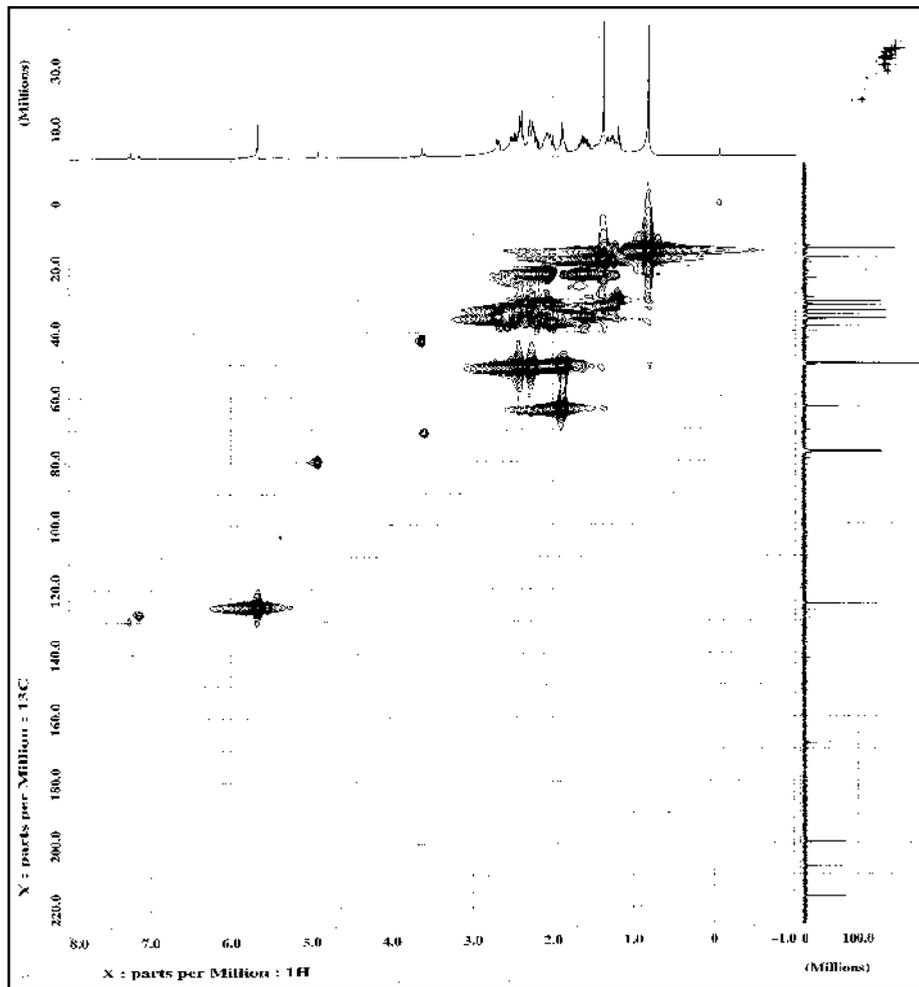**Fig. 1:** ^{13}C-NMR spectrum (a) DEPT spectra at 45° (b), 90° (c) and at 135° (d) of **m₁**.
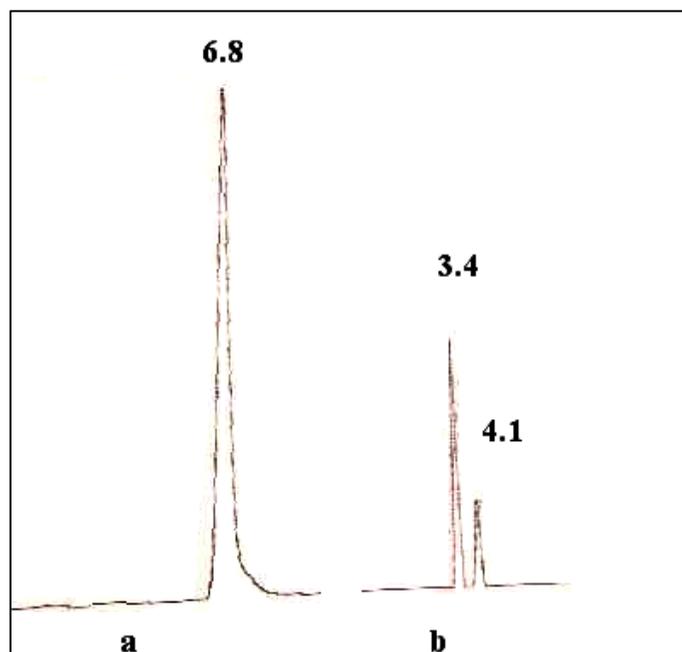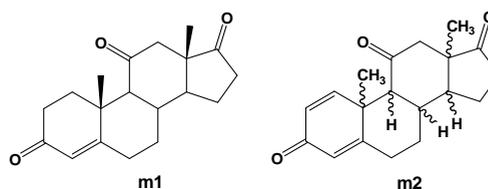
Fig. 2: HMQC spectrum of **m₁**.

Fig. 3: Analytical HPLC profiles of metabolites m_2: (a) Rp C-18 column, CH_3CN: H_2O (2:3); (b) chiral column isopropanol: *n*-hexane (1:50).

range "*W*" coupling ($^4J=1.9$) with H-4 methine proton which appeared at 6.04 ppm. Such ^{1}H-NMR pattern of m_2 indicated the preservation of 1,4-diene unsaturation like that in prednisone **1**. As previously observed in the spectrum of metabolite m_1, the absence of the characteristic pattern of -ketol group at 4.03, 4.39, and 4.68 ppm in addition to the signal corresponding to C_{17}-OH at 5.58 ppm, led us to propose that the structure of m_2 may exist as a mixture of enatiomers of androsta-1,4-diene derivative.

The ^{13}C-NMR spectral data, (Table 2), showed signals characteristic of three carbonyl carbons at 216.33, 207.34 (207.28) and 186.11 (186.01) assigned to C_{17}, C_{11} and C_3 respectively and three olefinic carbons at 155.51 (153.86), 128.46 (126.83) and 125.49 (123.89) assigned to C_1, C_2 and C_4 respectively. The ^{13}C-NMR chemical shifts of the other C-atoms of the enantiomers m_2 are listed in (Table 2). Metabolite m_2 was therefore recognized as mixture of androsta-1,4-diene-3,11,17-trione enantiomers.

225

Microbial biotransformation time course study of prednisone 1

The biotransformation of prednisone **1**, by HIB mixture under aerobic incubation for 96 h was monitored by HPLC. A representative HPLC chromatogram, Figure 4, illustrates the retention times of prednisone **1** and its metabolites **m₁** and **m₂** after incubation for 48 h. Figure 5 displays the change of the relative concentrations of the three components at 24 h intervals. It can be observed that after 48 h incubation, the compounds **1**, **m₁** and **m₂** showed common reluctant rate pattern of transformation. This may be attributed to gradual depletion of essential environmental elements to maintain the HIB culture vitality leading to its decreased biochemical transformation efficiency.

Matching the patterns of transformation in (Fig. 5), one can observe two phases for accumulation of **m₁** and **m₂.** At the first phase from zero time to 48 h, a steady increase of concentrations took place that apparently occurs at the expense of biotransformation of **1**. The second phase after 48 h, transformation of prednisone practically remained constant, however, the relative concentration of **m₁** increased significantly and relative concentration of **m₂** decreased approaching zero at the end time of incubation period. Increased **m₁** concentration that went in parallel

with decreased **m₂** concentration may be rationalized by assuming that **m₂** behaved as substrate for certain strains in HIB mixture having ^{1}-NDH (nuclear dehydrogenase) activity that reduced ^{1}-bond leading to accumulation of **m₁** on expense of **m₂**[8,9]. Musharraf _et al._ isolated the metabolites of adrenosterone **m₁** using three different micro-organisms[6]. Three metabolites of **m₁** were identified namely, **m₂**; 17 -hydroxyandrost-4-ene-3,11-dione **m₃**, and 17 -hydroxyandrosta-1,4-diene-3,11-dione **m₄**, (Fig. 6). The formation of **m₂** from **m₁** seems to go opposite to our above interpretation[6]. This may be explained in terms of the difference in biocatalyst used in experiments.

The catalytic enzymes involved in aerobic biotransformation of prednisone **1** that yielded the metabolites **m₁** and **m₂** can be summarized as: desmolases (17-side chain cleavage), oxidases (17 -OH oxidation) and reductases (^{1}-reduction).

Initial anaerobic metabolic investigations of **1** by HIB are in progress in our laboratory.
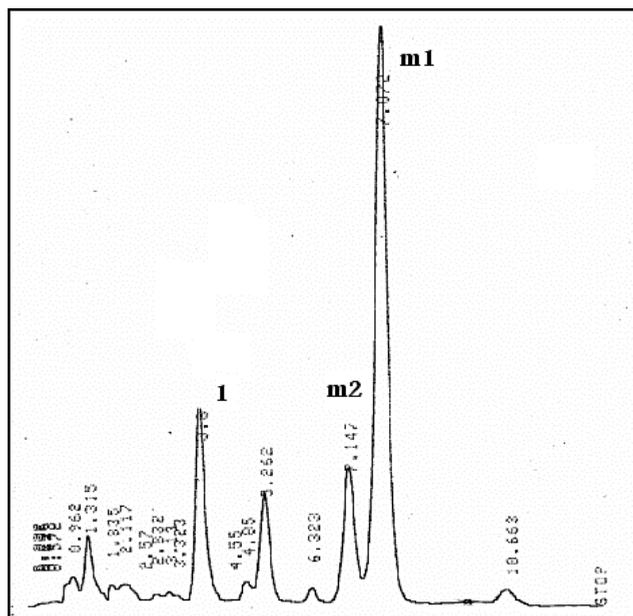
Acknowledgment

Fig. 4: HPLC chromatogram of the reaction mixture illustrating the relative concentration of **1** and its metabolites **m₁** and **m₂** after 48 h.
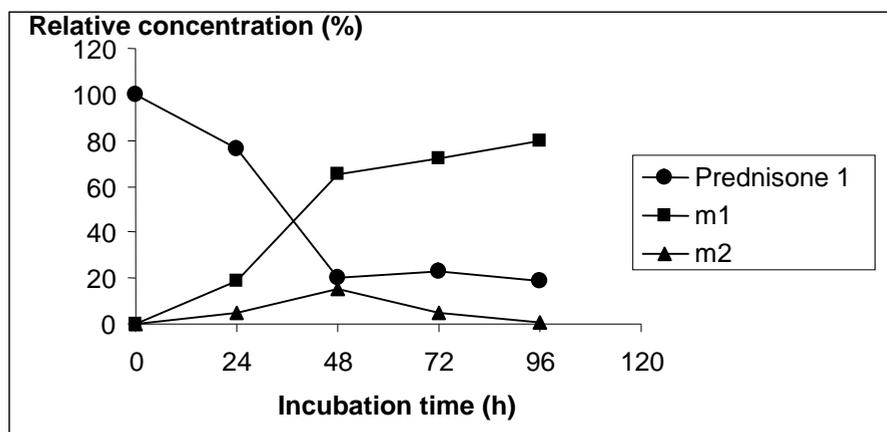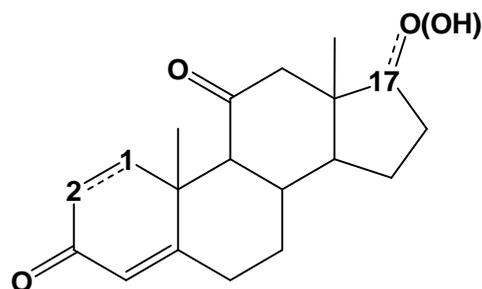

Fig. 5: Time course of biotransformation of prednisone and its metabolites **m₁** and **m₂** by HIB under aerobic conditions.

Compound No.	C1-C2	C17	Microorganism
m₁	------	-one	
m₂	-ene	-one	*Cephalosporium aphidicola*, *Fusarium lini*
m₃	------	-ol	*Cephalosporium aphidicola*, *Trichothecium roseum*
m₄	-ene	-ol	*Cephalosporium aphidicola*, *Fusarium lini*

Fig. 6: Adrenosterone m₁ and its metabolites **m₂**, **m₃**, **m₄** using three different fungal strains[9].

REFERENCES

1- K. Kieslich, Arzneimittelforschung, 36, 774 (1986).

2- K. Parfitt; "Martindale", 32nd Edn., USA, Pharmaceutical Press, 1999, pp. 1048-1049.

3- D. D. Miller, R. W. Brueggemeier and J. T. Daiton; "Foye's Principles of Medicinal Chemistry", D. A. Williams, and T. L. Lemke (Editors), 5th Edn., London, Lippincott and Wilkins, 2002, pp. 664-665.

4- M. I. Choudhary, Z. A. Siddiqui, S. G. Musharraf, S. A. Nawaz and Atta-Ur-Rahman, Nat. Prod. Res., 19, 311 (2005).

5- USP Pharmacopoeia XXII. USA, United States Pharmacopoeia Convention Inc, 1999, pp. 1387-1390.

6- S. G. Musharraf, Atta-Ur-Rahman, M. I. Choudhary and S. Sultan Nat. Prod. Res., 16, 345 (2002).

7- I. S. Kleiner, and J. M. Orten, "Biochemistry", 7th Edn., New York: C.V. Mosby Company, 1966, pp. 799-800.

8- V. D. Bokkenheuser, J. B. Suzuki, S. T. Polovsky and J. Winter, Appl. Microbiol., 30, 82 (1975).

9- V. D. Bokkenheuser and J. Winter, Am. J. Clin. Nutr., 33, 2502 (1980).